Transcription factor E2F4 facilitates SUMOylation to promote HCC progression through interaction with LIN9

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Abstract. SUMOylation plays a crucial role in numerous cellular biological and pathophysiological processes associated with human disease; however, the mechanisms regulating the genes involved in SUMOylation remain unclear. In the present study, E2F transcription factor 4 (E2F4) was identified as an E2F member related to hepatocellular carcinoma (HCC) progression by public database analysis. It was found that E2F4 promoted the proliferation and invasiveness of HCC cells via SUMOylation using Soft agar and Transwell migration assays. Mechanistically, it was demonstrated that E2F4 upregulated the transcript and protein expression levels of baculoviral IAP repeat containing 5, cell division cycle associated 8 and DNA topoisomerase II α using western blotting. Furthermore, the interaction between E2F4 with lin-9 DREAM multi-vulva class B core complex component (LIN9) was explored by co-immunoprecipitation, immunofluorescence co-localization and bimolecular fluorescence complementation assays. Moreover, it was demonstrated that E2F4 promoted the progression of HCC cells via LIN9. Rescue experiments revealed that LIN9 facilitated the SUMOylation and proliferation of HCC cells, which was prevented by knocking down E2F4 expression. In conclusion, the findings of the present study indicated that E2F4 plays a major role in the proliferation of HCC cells and may be a potential therapeutic target in the future.

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Introduction

Hepatocellular carcinoma (HCC) is the most prevalent type of liver cancer, accounting for 70-85% of all primary liver cancer cases worldwide. This severe disease, with high morbidity and mortality rates, is the third most common cause of cancer-related death worldwide (1). Despite the continuous development of therapeutic methods, the rate of recurrence and metastasis in HCC is also high (2,3). However, the precise mechanism underlying the onset and progression of HCC remains elusive. A number of studies have demonstrated the involvement of various cellular pathways, such as the nuclear factor κB pathway (4,5), ubiquitin-proteasome system (6) and autophagy pathway (7), in HCC pathogenesis. SUMOylation is an essential protein modification pathway and plays a pivotal role in the regulation of diverse biological processes, such as cellular localization, protein activity, protein stability or protein degradation (8). Upregulated expression of SUMOylated proteins in tumor tissues has been observed, and the activation of SUMOylation is closely linked to the development of tumors (9,10).

E2F transcription factor 4 (E2F4), a member of the E2F family, contributes to tumor progression (11-13). E2F4 influences the expression of toll-like receptor 8 and CD14, as well as the downstream activation of the signal transducer and activator of the transcription (STAT1) pathway (14). Studies have demonstrated that elevated levels of E2F4 are observed in the development of various types of cancer, including bladder cancer (15), Burkitt lymphoma (16), breast cancer (17), gastric cancer (18), cervical cancer (19), colorectal cancer (20) and acute myeloid leukemia (21). Consistently, there is a decrease in the G1-S phase transition and the proliferation of colon cancer cells with stable silencing of E2F4 (22). However, the precise regulatory role of E2F4 in HCC has yet to be elucidated, particularly in terms of regulating the SUMOylation pathway.

In the present study, the potential role of E2F4 in HCC was investigated through public database mining. To determine the changes in target gene expression, western blotting and reverse transcription-quantitative PCR (RT-qPCR) assays were used. Furthermore, co-immunoprecipitation (Co-IP), immunofluorescence co-localization and bimolecular fluorescence

complementation (BiFC) assays were used to observe the interactions between E2F4 and a copartner. Subsequently, soft agar and Transwell migration assays were used to detect the effect of E2F4 on the proliferation and invasion in HCC cells.

Materials and methods

Bioinformatics. Public RNA-sequencing data were obtained from the Gene Expression Omnibus (GEO) database (https://www. ncbi.nlm.nih.gov/geo/; accession no. GSE112221) (23), which included 10 samples. The data used in the present study were derived from 4 patients. 'N' represents cirrhotic tissue samples, while 'T' denotes HCC samples. Both the cirrhosis and HCC tissues originated from the same patients. A comprehensive analysis identified 105 common differentially expressed genes (DEGs) across these 4 patients. Enrichment analysis of these genes was conducted utilizing Metascape (https://www.metascape.org). Then, ChIP-X (https://www.maayanlab.net/X2K/; version 1.6) software prediction was used to obtain candidate TFs that regulate these target genes. Survival analysis and differential expression were analyzed using the GEPIA2 database (http://gepia2.cancer-pku.cn/#index), which included gene expression data from The Cancer Genome Atlas (https://portal.gdc.cancer.gov/; TCGA-LIHC) (24). Correlation analysis was carried out using the TIMER database (https://cistrome.shinyapps.io/timer/). The protein E2F4 co-partners were obtained using GENEMANIA (http://genemania.org), HINT (http://hint.yulab.org/) and STRING (https://string-db.org/) databases. E2F4 and DREAM multi-vulva class B core complex component (LIN9) protein docking studies were performed using the HDOCK Server (http://hdock.phys.hust.edu.cn/). A cut-off of $P<0.05$ was used to define a significant result.

Cell lines and culture. The human liver cancer cell lines, HepG2 (cat. no. CL-0103), HCC-LM3 (cat. no. CL-0278), Huh7 (cat. no. CL-0120), Huh6 (cat. no. CL-0119), and PLC (cat. no. CL-0415) were obtained from Procell Life Science & Technology Co., Ltd. MHCC 97H cells (cat. no. C6585) was purchased from Beyotime Institute of Biotechnology. The cell lines were cultured in 5% CO_2 at 37˚C, with DMEM containing 10% fetal bovine serum (Thermo Fisher Scientific, Inc.; cat. no. 12483020) and 100 U penicillin/streptomycin (Thermo Fisher Scientific, Inc.; cat. no. 15140122).

Cell transfection. E2F4 and LIN9 overexpression vectors were constructed using the CV186 lentiviral vector (Shanghai GeneChem Co., Ltd.). Short hairpin RNA (shRNA) against E2F4 or SUMO2/3 (Table SI) for knockdown were inserted into the GV298 lentiviral vector (Shanghai GeneChem Co., Ltd.; cat. no. GCD0316554). For transfection, the Huh7 ($1x10^6$ cells/well) and PLC ($1x10^6$ cells/well) cells were cultured in 6-well plates for 12 h at 37˚C, followed by replacement with fresh medium. Subsequently, 2 μg E2F4-CV186 plasmid was transfected into Huh7 cells or the E2F4-GV298 vector was transfected into PLC cells using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.; cat. no. L3000008) in a biological safety cabinet, based on the kit's instructions. Following transfection, the cell cultures were maintained at 37˚C for an additional 48 h. Stable cell lines were established with 2 μg/ml puromycin selection for 2 weeks. Then, the polyclonal stable cell lines were cultured in fresh medium with 1 μg/ml puromycin for maintenance.

RT-qPCR. Total RNA samples from Huh7 or PLC cells were extracted by the RNeasy Mini Kit (Qiagen China Co., Ltd.; cat. no. 74104). RT was conducted using the Transcriptor First Strand cDNA Synthesis Kit (Takara Biotechnology Co., Ltd.; cat. no. 6110A) according to the manufacturer's instructions. In the qPCR analysis, the SYBR Premix Ex Taq II (Takara Biotechnology Co., Ltd.; cat. no. RR820A) and primer sets (Table SII) were applied to determine the transcript levels via the $2^{-\Delta\Delta Cq}$ method (25), using β-actin (ACTB) as the internal control. The thermocycling protocol was as follows: Holding stage at 95˚C for 30 sec, cycling stage for 40 cycles at 95˚C for 5 sec and 60˚C for 30 sec, the melting curve stage at 95˚C for 15 sec, 60˚C for 1 min and 95˚C for 15 sec.

Western blotting. Protein from the liver cancer cells was isolated by cell lysis buffer (Beyotime Institute of Biotechnology; cat. no. P0013). The quantification of protein concentrations was measured with the BCA Protein Assay Kit (Beyotime Institute of Biotechnology; cat. no. P0012). Proteins (30 μg) were subjected to separation on a 10% SDS-polyacrylamide gel, followed by transfer to PVDF membranes (MilliporeSigma; cat. no. IPVH00010). The membranes were then blocked with 5% skimmed milk (Beyotime Institute of Biotechnology; cat. no. P0216) in Tris-buffered saline containing 0.1% Tween-20 (TBST; Beyotime Institute of Biotechnology; cat. no. ST671) at room temperature for 1 h and washed three times with TBST. Then, the membranes were incubated at 4˚C overnight with primary antibodies. After washing three times with TBST, the membranes were incubated with HRP-conjugated Goat anti-Rabbit IgG (ABclonal Biotech Co., Ltd.; cat. no. AS014, 1:5,000) or HRP-conjugated Goat anti-Mouse IgG (ABclonal Biotech Co., Ltd.; cat. no. AS003, 1:5,000) at room temperature for 1 h. Following incubation, the membranes were again washed three times with TBST. Then, the membranes were incubated with ECL reagent (MedChemExpress; cat. no. HY-K1005). Band visualization was performed using a ChemiDoc Imaging System (Bio-Rad Laboratories, Inc.) for 1 min. Densitometric analysis was conducted using ImageJ software (National Institutes of Health; version 1.53m). Western blotting was undertaken using antibodies specific for E2F4, LIN9, baculoviral IAP repeat containing 5 (BIRC5), cell division cycle associated 8 (CDCA8), DNA topoisomerase II α (TOP2A), small ubiquitin like modifier (SUMO)1, SUMO2/3, SUMO1/sentrin specific peptidase (SENP)1, SENP2, SENP3, SENP5, SENP6, SENP7, SENP8, LIN54, RBBP4, LIN52, LIN37 and ACTB (Table SIII).

Co-IP. The Co-IP assay was carried out as previously reported (26-29). Huh7 and PLC cells were lysed in 300 μl RIPA lysis buffer (Beyotime Institute of Biotechnology; cat. no. P0013D). Cell lysates were incubated with 10 μg of antibodies specific for E2F4 (Proteintech Group, Inc.; cat. no. 67812-1-Ig), LIN9 (Proteintech Group, Inc.; cat. no. 17882-1-AP), SUMO2/3 (Proteintech Group, Inc.; cat. no. 11251-1-AP), negative control rabbit IgG (Beyotime Institute of Biotechnology; cat. no. A7016) or negative

control mouse IgG (Beyotime Institute of Biotechnology; cat. no. A7028) overnight at 4˚C. Subsequently, the samples were incubated with 30 μl Pierce Protein A/G magnetic beads (MedChemExpress; cat. no. HY-K0202) at 4˚C for another 3 h. Following centrifugation at 1,000 x g at 4˚C for 10 min, the beads were washed with 1 ml lysis buffer three times and boiled for 10 min at 100˚C. The proteins were subsequently isolated and identified using western blotting analysis.

Immunofluorescence co-localization assay. Huh7 and PLC cells ($1x10^4$ cells/well) were grown on coverslips in 24-well plates at 37˚C for 24 h. Cells were fixed with 4% paraformaldehyde for 30 min at room temperature (20-25˚C) and washed three times with PBS. The glass coverslips were incubated with 5% milk for 1 h at room temperature, then treated with antibodies specific for E2F4 (Proteintech Group, Inc.; cat. no. 67812-1-Ig; 1:100 dilution) and LIN9 (Proteintech Group, Inc.; cat. no. 17882-1-AP; 1:100 dilution) at 4˚C overnight. Next, the coverslips were treated with Alexa Fluor 488 goat anti-mouse IgG (Abcam; cat. no. ab150113; 1:1,000 dilution) or Alexa Fluor 594 goat anti-rabbit IgG (Abcam; cat. no. ab150160; 1:1,000 dilution) at room temperature for 1 h in the dark. Cells were washed and stained with 4',6-diamidino-2-phenylindole (DAPI; 300 nmol/l) for 10 min at room temperature. The images were imaged under a confocal microscope. For EdU staining, Huh7 cells were cultured in a 12-well plate at a density of $1x10^5$ cells/well. Following corresponding treatment, cell proliferation was detected with the EdU Cell Proliferation Kit (Beyotime Institute of Biotechnology; cat. no. C0081S) according to the manufacturer's instructions. Finally, cells were observed and captured under a fluorescence microscope.

BiFC assay. Human LIN9 cDNA (1,473 bp) or E2F4 cDNA (1,241 bp) were respectively subcloned into BiFC vectors pBiFC-VC155 (Addgene, Inc.; cat. no. 22011) and pBiFC-VN173 (Addgene, Inc.; cat. no. 22010). Co-transfection of the recombinant vectors (1 μg LIN9-VC155 and 1 μg E2F4-VN173) into Huh7 cells was performed using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 24 h. Next, the cells were fixed with 4% paraformaldehyde at room temperature for 30 min and incubated with DAPI for 5 min at room temperature. Fluorescence emission was observed under a confocal microscope, with excitation and emission wavelengths set at 488 and 500 nm, respectively.

Soft agar assay. Huh7 cells ($5x10^3$ per well) were mixed with a solution of 0.05% Noble agar. The mixture was then incubated on 6-well plates containing solidified 0.1% Noble agar for 3 weeks. After the incubation period, the cellular colonies that had formed were stained using 0.5% crystal violet dye and subsequently observed and manually counted using a light microscope.

Matrigel invasion assay. The invasiveness of Huh7 cells was assessed using a 12-well Transwell insert with 8.0-μm pores (Corning, Inc.; cat. no. 3422). The upper wells of the inserts were coated with 100 μl Matrigel (Corning, Inc.; cat. no. 354262; 1 mg/ml) at 37˚C for 3 h. Serum-starved cells ($1x10^5$ per well) were seeded into the upper chamber of the Transwell insert with 200 μl serum-free DMEM, and 500 μl DMEM containing 10% FBS was added to the lower chamber. After cell culture for 24 h, the invaded cells were fixed with 4% paraformaldehyde at room temperature for 15 min, stained with 0.1% crystal violet for 30 min at room temperature, and subsequently quantified manually using a light microscope.

SUMOylation inhibitor. To assess the impact of SUMOylation on the proliferation and invasiveness of Huh7 cells, the SUMOylation inhibitor, TAK-981 (10 nM; MedChemExpress; cat. no. HY-111789), was added to the cell medium after E2F4-overexpression vector transfection. Following treatment, the cell cultures were maintained at 37˚C for 24 h, The soft agar assay was then used to detect cell proliferation and the Matrigel invasion assay was used to detect invasiveness of Huh7 cells (according to the aforementioned protocols).

Statistical analysis. In the present study, the data are presented as the mean ± standard deviation. To compare differences between two datasets, a two-tailed unpaired Student's t-test was applied. The differences between multiple groups were calculated using one-way ANOVA followed by Dunnett's post hoc test. Spearman's correlation analysis was used to determine the expression correlation. Survival curves were constructed using the Kaplan-Meier method. High- and low-expression levels were separated by a suitable adjustable cut-off value. The log-rank test was utilized to assess differences in survival. All statistical analyses conducted in the present study were two-sided. $P<0.05$ was considered to indicate a statistically significant difference.

Results

E2F4 is a potential TF in regulating the progression of HCC. To investigate potential genes involved in the outcomes of patients with HCC, a comprehensive analysis was performed using HCC and cirrhosis tissue gene expression profiles available from the GEO. A total of 105 DEGs in HCC tissues compared with cirrhosis tissues were identified [fold change (FC) >2; Fig. 1A]. Furthermore, 76 genes were upregulated whereas 29 genes were downregulated in patients with HCC (Fig. 1A). To analyze the crucial pathways affected by potential regulators, a REACTOME pathway analysis for 105 DEGs was performed using Metascape (30). The results revealed that SUMOylation was a significantly enriched pathway including 5 target genes (aurora kinase A, BIRC5, chromobox homolog, CDCA8 and TOP2A; Fig. 1B). To investigate the crucial TFs that regulate the SUMOylation-associated genes, ChIP-X software was used to reveal that the top 5 TFs were E2F4, ETS proto-oncogene 1 (ETS1), interferon regulatory factor 1 (IRF1), MYCN proto-oncogene and lysine demethylase 6A (KDM6A). Notably, E2F4 ranked first among the identified TFs by the number of target genes, including BIRCA, CDCA8 and TOP2A, which were more enriched in HCC tissues (Fig. 1C and E). Meanwhile, comprehensive analysis using GEPIA2 (31) showed that upregulation of E2F4 ($P=0.016$), BIRC5 ($P<0.001$), CDCA8 ($P<0.001$) and TOP2A ($P=0.01$) were associated with a poorer overall survival (OS) rate in patients with HCC (Figs. 1D and S1). A positive correlation between E2F4 and BIRC5 ($\rho=0.395$; $P<0.001$), CDCA8 ($\rho=0.58$; $P<0.001$) or TOP2A ($\rho=0.552$; $P<0.001$; Fig. 1F)

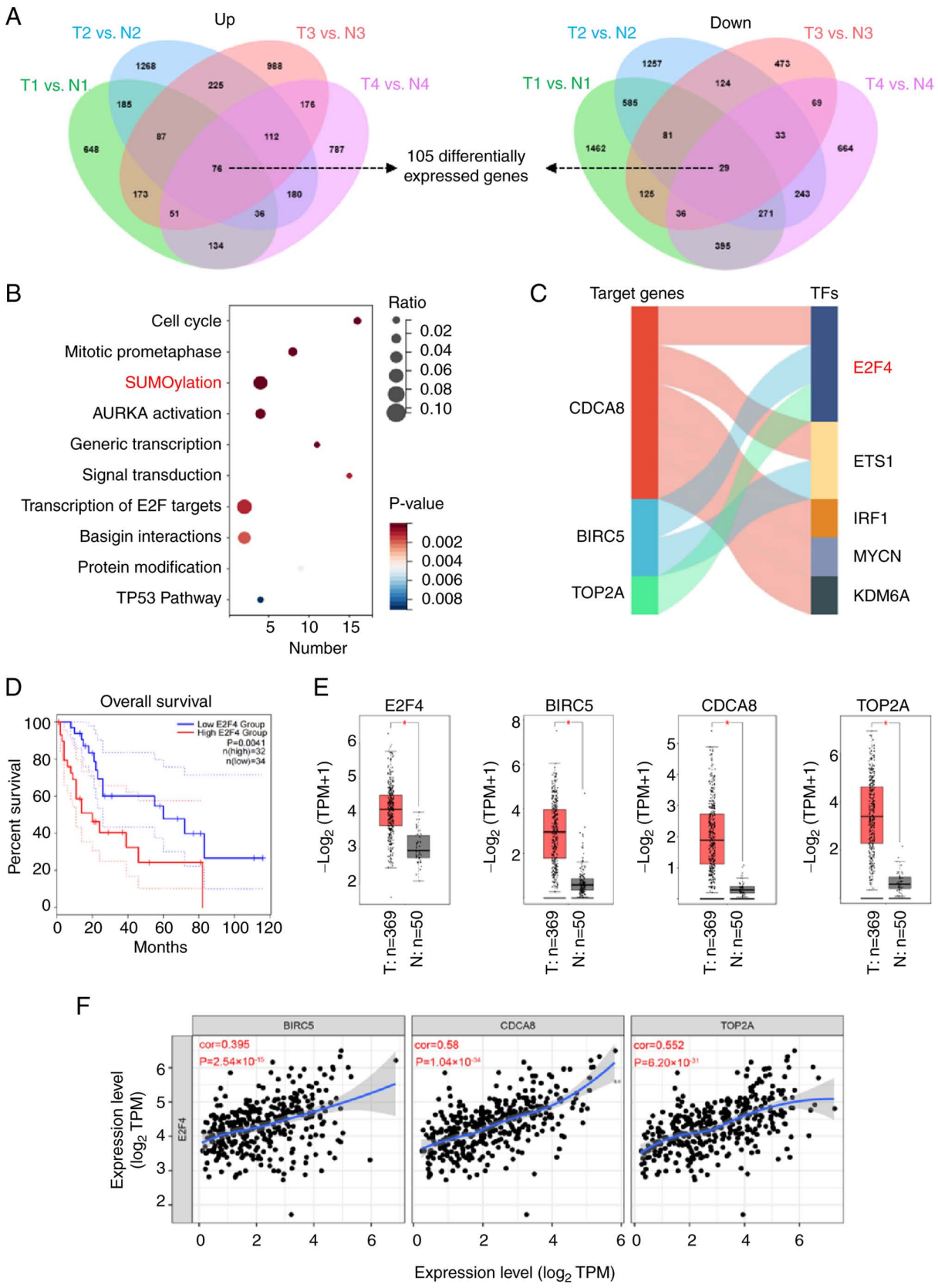

Figure 1. E2F4 is the potential transcription factor in regulating the progression of HCC. (A) Venn diagram revealing the DEGs (P<0.05, fold change >2) in the public dataset (accession no. GSE112221). 'N' represents cirrhotic tissue samples, while 'T' denotes HCC samples. (B) Gene set enrichment analysis was conducted on the DEGs obtained from the GSE112221 dataset. (C) The ChIP-X software was used to predict the top 5 TFs that regulate the expression of SUMOylation-associated genes. (D) Kaplan-Meier analysis was used to assess the association of E2F4 expression with overall survival, using data from the GEPIA2 database. (E) Relative expression levels of E2F4, BIRC5, CDCA8 and TOP2A in normal and HCC tissues in TCGA-LIHC dataset. 'N' represents normal samples, while 'T' represents HCC tissue. (F) Correlation analysis indicating the relationship between E2F4 and BIRC5, CDCA8 or TOP2A. *P<0.05. BIRC5, baculoviral IAP repeat containing 5; CDCA8, cell division cycle associated 8; DEGs, differentially expressed genes; E2F4, E2F transcription factor 4; ETS1, ETS proto-oncogene 1; HCC, hepatocellular carcinoma; IRF1, interferon regulatory factor 1; KDM6A, lysine demethylase 6A; MYCN, MYCN proto-oncogene; TFs, transcription factors; TOP2A, DNA topoisomerase II α; TPM, transcripts per million.

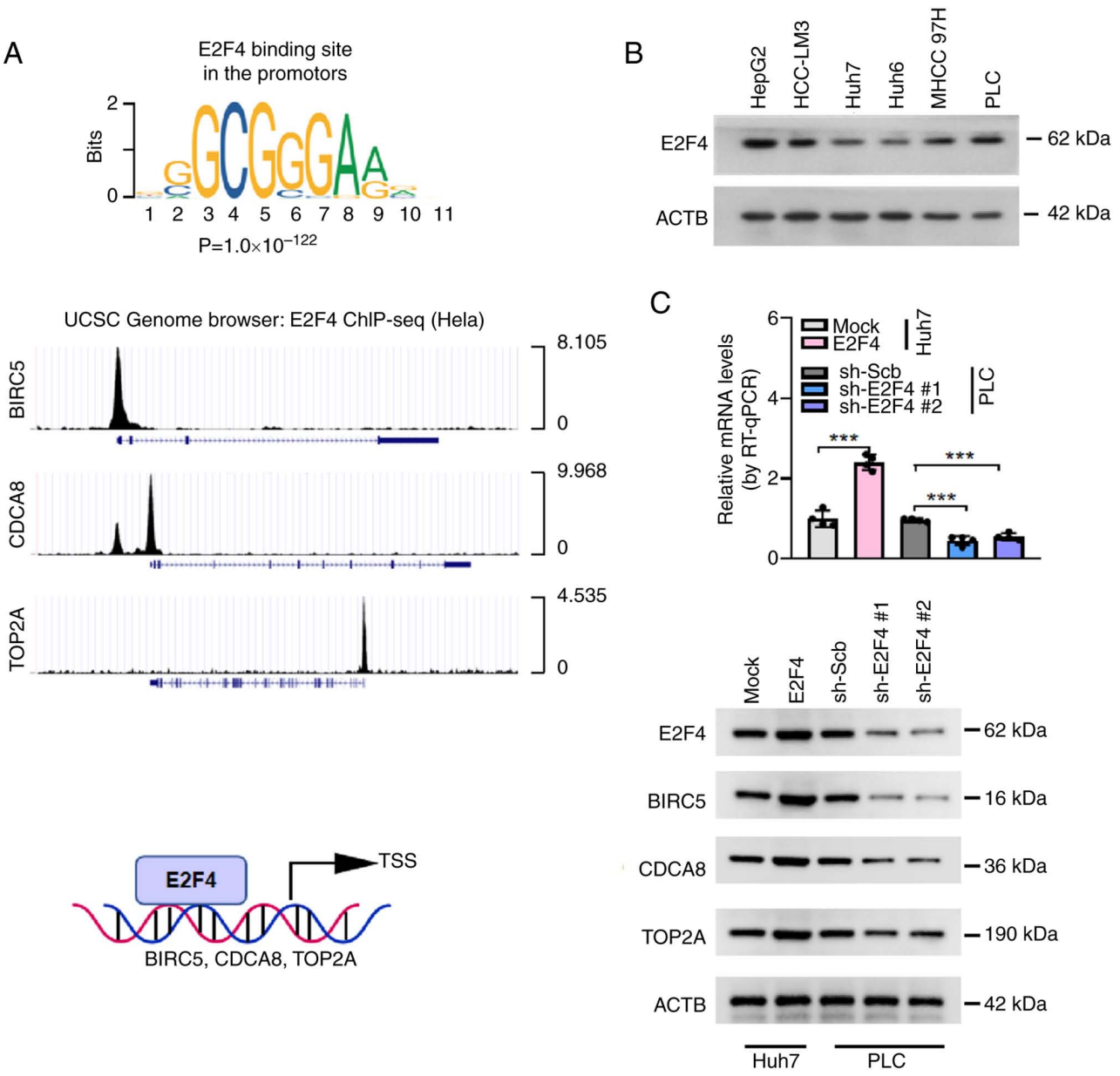

Figure 2. E2F4 promotes the expression of SUMOylation-associated target genes in HCC cells. (A) E2F4 binding site in the promotors identified using the JASPAR database, and publicly available ChIP-seq dataset from the UCSC database was used to reveal the direct binding of E2F4 to the promoters of BIRC5, CDCA8 and TOP2A. (B) Western blotting was used to show the relative protein expression levels of E2F4 in several types of liver cancer cells. (C) RT-qPCR analysis (n=4 per group) used to show the transcript levels of E2F4 and western blotting was used to show the protein expression levels of E2F4, BIRC5, CDCA8 and TOP2A in HCC cells following empty vector (mock), E2F4 overexpression, sh-Scb, sh-E2F4 #1 and sh-E2F4 #2 transfections. ***P<0.001. ACTB, β-actin; BIRC5, baculoviral IAP repeat containing 5; CDCA8, cell division cycle associated 8; ChIP-seq, chromatin immunoprecipitation-sequencing; E2F4, E2F transcription factor 4; HCC, hepatocellular carcinoma; LIN9, lin-9 DREAM MuvB core complex component; RT-qPCR, reverse transcription-quantitative PCR; Scb, scramble; sh, short hairpin; TOP2A, DNA topoisomerase II α; TSS, transcription start site.

in HCC specimens was observed using TIMER (32). The results indicated that E2F4 may be a potential regulator in the progression of HCC.

E2F4 promotes the expression of SUMOylation-associated target genes. To understand the direct effects of E2F4 on BIRC5, CDCA8 and TOP2A transcription and expression in HCC cell lines, JASPAR (http://jaspar.genereg.net) was used to identify the E2F4 binding site in the promotors (Fig. 2A). Moreover, analysis of E2F4 chromatin immunoprecipitation-sequencing datasets (UCSC browser) also revealed that E2F4 bound at the promoters of BICR5, CDCA8 and TOP2A in HeLa cells (Fig. 2A). Western blotting revealed higher E2F4 expression levels in HepG2 and PLC cells and lower E2F4 expression levels in Huh7 cells (Fig. 2B). Since the present study focused on HCC, PLC and Huh7 were chosen as the HCC cell line models. To explore the regulation of SUMOylation-associated target genes by E2F4, western blotting was performed, which revealed that stable overexpression of E2F4 increased the protein levels of BIRC5, CDCA8 and TOP2A in Huh7 cells (Fig. 2C). However, knocking down E2F4 expression led to a decrease in the BIRC5, CDCA5, and TOP2A levels in PLC cells (Fig. 2C). These findings demonstrated that E2F4 regulated the expression of SUMOylation-associated genes in HCC cells.

E2F4 facilitates the proliferation of HCC cells via SUMOylation. The effects of E2F4 on SUMOylation in HCC cells was further determined by western blotting. In PLC and Huh7 cells, stable overexpression or knockdown of E2F4 enhanced and reduced the whole cell SUMOylation levels, respectively (Fig. 3A). The SUMOylation process involves numerous crucial proteins and specific proteases. Comprehensive analysis using the GEPIA2 database revealed higher expression levels of SUMO2/3 and SENP3 in HCC (T) tissues compared with the normal control (N) group ($P<0.05$, FC>1.5; Fig. S2A). Consistently, western blotting analysis following E2F4 overexpression in Huh7 cells revealed an increase in SUMO2/3 protein levels, but not the levels of SUMO1 or other SUMO-specific proteases (Fig. S2B). The results of a SUMOylation assay indicated that the SUMOylation levels of target genes were elevated in cells overexpressing E2F4 (Fig. S3A and B), which were decreased by stable knockdown of SUMO2/3 (Fig. S3B). In addition, the results of an EdU assay revealed that stable overexpression or knockdown of E2F4 increased and decreased the number of EdU^+ cells compared with the controls, respectively (Fig. S4A). Subsequently, soft agar colony formation and Transwell Matrigel assays were performed to demonstrate that E2F4 significantly promoted the proliferation and invasion of HCC cells (Fig. S4B and C). To investigate the involvement of SUMOylation in E2F4-promoted proliferation and invasion, a SUMOylation inhibitor (TAK981) was applied to HCC cells stably overexpressing E2F4. Subsequent western blotting indicated that the SUMOylation levels in Huh7 cells were decreased by TAK-981 treatment (Fig. 3B). The results of EdU, soft agar colony formation and Transwell Matrigel assays showed that TAK-981 prevented the increase in proliferation and invasion levels of HCC cells stably overexpressing E2F4 (Fig. 3C-E). Similarly, the proliferation and invasion of HCC cells were inhibited by knocking down SUMO2/3 (Fig. S3C). Taken together, these results suggested that E2F4 elevated the proliferation and invasion of HCC cells by promoting SUMOylation.

LIN9 is an E2F4 potential protein partner. To determine potential protein partners that interact with E2F4, a E2F4 protein-protein interaction network was obtained using GENEMANIA (http://genemania.org), HINT (http://hint.yulab.org/) and STRING (https://string-db.org/) databases (Fig. 4A-C). Overlapping the results of the analyses derived from the aforementioned databases, LIN9, RB transcriptional corepressor like (RBL) 1, RBL2 and transcription factor Dp-1 (TFDP1) were identified as potential E2F4 binding partners (Fig. 4D). Subsequently, higher LIN9 levels were observed to be associated with a poorer OS rate in patients with HCC ($P=0.0028$), whereas the OS curves were not significantly different between the high and low RBL1 ($P=0.17$), RBL2 ($P=0.80$) and TFDP1 ($P=0.22$) (Fig. 4E) expression groups. Therefore, LIN9 was identified as a potential E2F4 co-partner in HCC cells. In addition, high expression of LIN9 was positively associated with MKI67 (Ki-67, a biomarker of proliferation) and MCAM (CD136, a potential biomarker of angiogenesis) in patients with HCC (Fig. 4F). According to the TCGA database, higher expression levels of E2F4, LIN9, BIRCA5, CDCA8 and TOP2A were observed in multiple cancer types (Fig. S5). Taken together, these results indicated that LIN9 likely interacts with E2F4 in HCC cells.

LIN9 physically interacts with E2F4 in HCC cells. To investigate the interaction between E2F4 with LIN9, protein docking studies were performed. The binding energy (-10.2 kcal/mol) revealed that E2F4 directly binds to LIN9 (Fig. 5A). Subsequently, a co-IP assay was used to demonstrated the endogenous interaction between E2F4 and LIN9 protein in PLC and Huh7 cells (Fig. 5B). Consistently, the results of an immunofluorescence staining assay demonstrated that E2F4 co-localized with LIN9 in the nucleus of PLC and Huh7 cells (Fig. 5C). To further determine the physical interaction between E2F4 and LIN9, a BiFC assay was performed to reveal that notable fluorescence occurred in Huh7 cells co-transfected with E2F4 and LIN9 plasmids (Fig. 5D). These findings suggested that E2F4 physically interacted with LIN9 protein in HCC cells.

LIN9 promotes the proliferation and invasion of HCC cells via E2F4. The cooperative roles of E2F4 and LIN9 in SUMOylation in HCC cells were further investigated. The results of a SUMOylation assay indicated that elevated SUMOylation of LIN9 was observed in cells stably overexpressing E2F4 (Fig. S3A and B). Unexpectedly, the interaction between E2F4 and other components (LIN54, LIN52, RBBP4 and LIN37) of the multi-vulva class B (MuvB) complex was prevented by overexpression of E2F4 (Fig. S6). The results suggested that E2F4 might inhibit DREAM complex stability following SUMOylation of LIN9. Western blotting and RT-qPCR revealed that stable overexpression of LIN9 increased the transcript and protein expression levels of BIRC5, CDCA8 and TOP2A, which was prevented by knocking down E2F4 (Fig. 6A and B). Consistently, there was an increase in the SUMOylation levels and proliferation of Huh7 cells stably overexpressing LIN9, which was rescued by knocking down E2F4 (Fig. 6C and D). The results of the soft agar colony formation and Matrigel invasion assays revealed that overexpression of LIN9 facilitated the anchor-independent proliferation and invasiveness of Huh7 cells, which was prevented by knocking down E2F4 (Fig. 6E and F). The aforementioned findings suggested that E2F4 coordinated with LIN9 to promote SUMOylation and the progression of HCC. Meanwhile, the identified SUMOylation-related proteins were significantly highly expressed in a variety of tumor types, further indicating that SUMOylation plays a critical role in the progression of cancer (Fig. S5).

Discussion

HCC ranks as the sixth most prevalent malignancy globally and emerges as the fourth primary cause of mortality in cancer-related deaths (1). The morbidity and mortality of HCC is contingent upon the disease stage at initial diagnosis. HCC fatalities are primarily caused by metastasis and postoperative recurrence (3), with an estimated recurrence rate of 60-70% (33). The dysregulation of gene expression, encompassing inactivation of tumor suppressor genes and activation of oncogenes, is widely recognized as a catalyst for tumor metastasis (24,34,35). Therefore, it is crucial to explore the

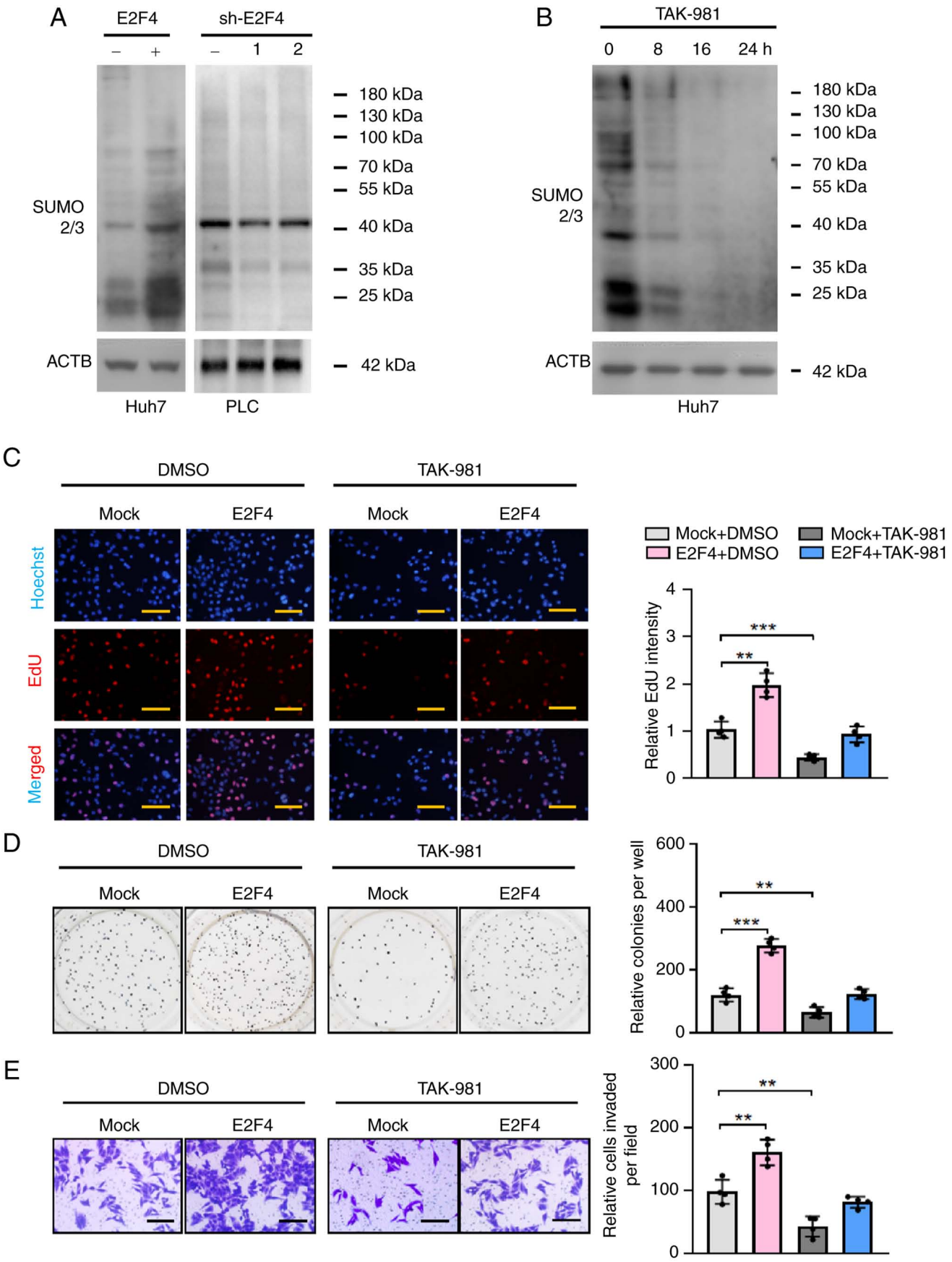

Figure 3. E2F4 facilitates the proliferation of hepatocellular carcinoma cells via SUMOylation. (A and B) Western blots revealing the levels of SUMOylation in Huh7 and PLC cells. (C) EdU staining assays (n=4 per group) showing the proliferation of Huh7 cells stably transfected with empty vector (mock) or E2F4, then treated with an inhibitor of SUMOylation (TAK-981, 10 nM). (D) Representative images (left panel) and quantification (right panel) of soft agar (n=4 per group) and (E) Transwell Matrigel invasion (n=4 per group) assays indicating the anchorage-independent proliferation and invasiveness of Huh7 cells stably transfected with empty vector (mock) or E2F4, then treated with an inhibitor of SUMOylation (TAK-981, 10 nM). **P<0.01, ***P<0.001. Scale bars, 100 μm. ACTB, actin-b; E2F4, E2F transcription factor 4; sh, short hairpin; SUMO2/3, small ubiquitin like modifier 2/3.

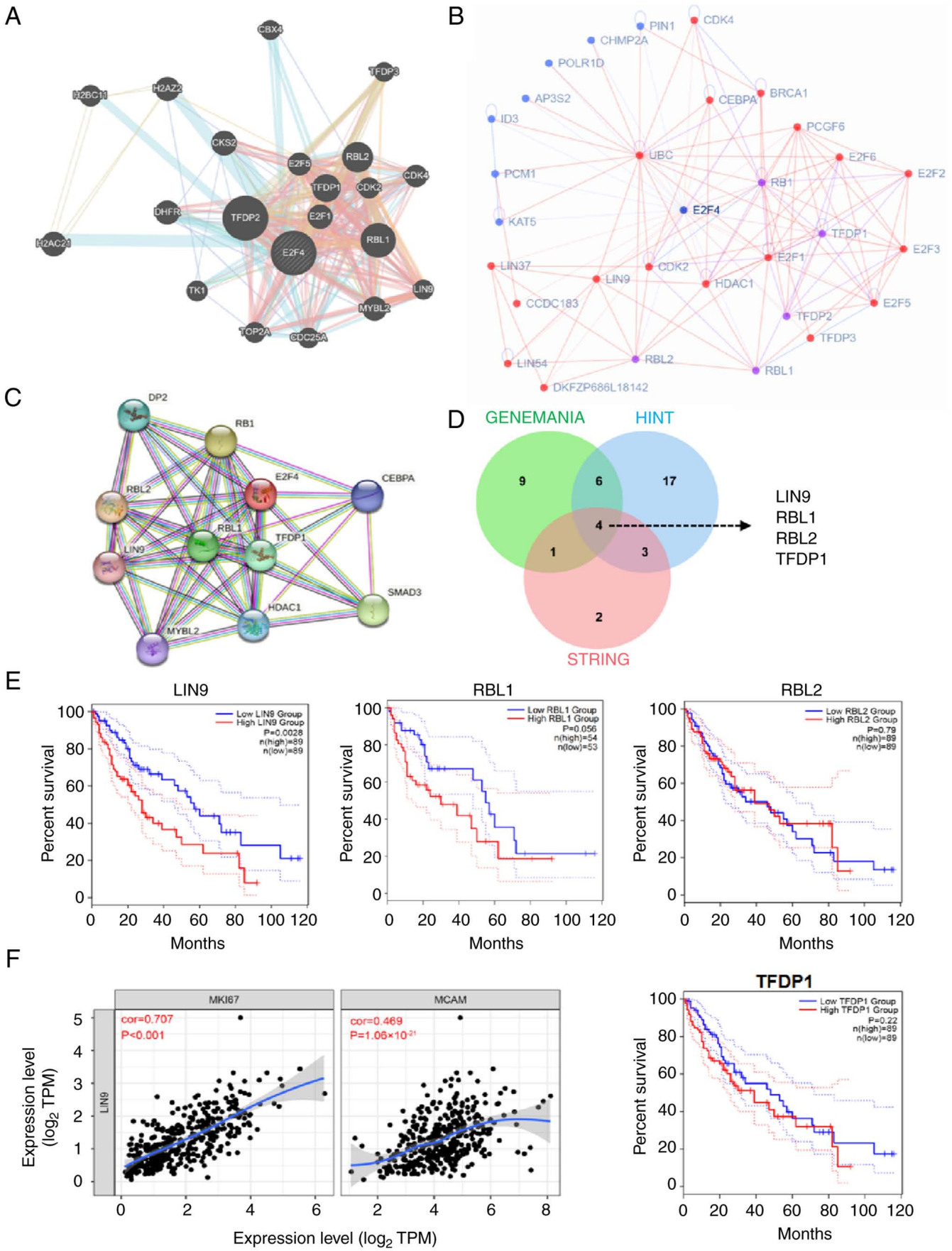

Figure 4. LIN9 is identified as a potential protein partner of E2F4. Network of E2F4 and E2F4 co-partners in the public (A) GENEMANIA, (B) HINT and (C) STRING websites. (D) Venn diagram revealing the four potential proteins that interact with E2F4, including LIN9, RBL1, RBL2 and TFDP1. (E) Kaplan-Meier tests showing the overall survival of LIN9, RBL1, RBL2 and TFDP1. (F) Correlation curves indicating that LIN9 is positively correlated with proliferation and angiogenesis in hepatocellular carcinoma. E2F4, E2F transcription factor 4; LIN9, lin-9 DREAM MuvB core complex component; RBL, RB transcriptional corepressor like; TFDP1, transcription factor Dp-1; TPM, transcripts per million.

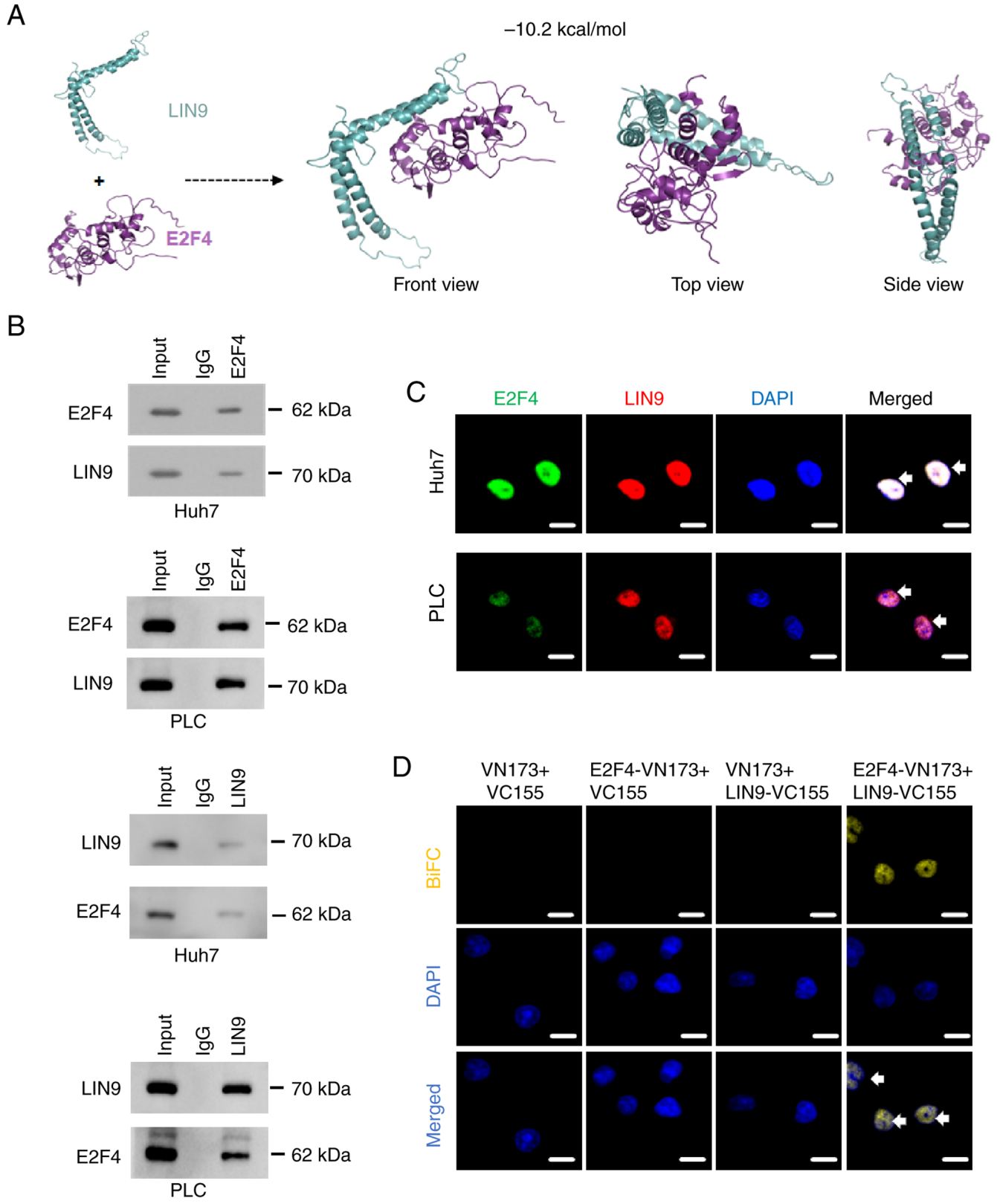

Figure 5. LIN9 physically interacts with E2F4 in hepatocellular carcinoma cells. (A) Protein docking analysis revealing the predicted physical interaction of E2F4 and LIN9. (B) Co-immunoprecipitation and western blot assays showing the interaction between E2F4 and LIN9 in Huh7 and PLC cells. (C) Immunofluorescence confocal images indicating the co-localization of E2F4 and LIN9 in Huh7 and PLC cells. Scale bars, 10 μm. (D) BiFC assay showing the physical interaction of E2F4 and LIN9 in Huh7 stably co-transfected with VN173-E2F4 and VC155-LIN9. Scale bars, 10 μm. BiFC, bimolecular fluorescence complementation; E2F4, E2F transcription factor 4; LIN9, lin-9 DREAM MuvB core complex component.

mechanism of gene expression in patients with HCC. In the present study, the identified DEGs in HCC were significantly enriched in the SUMOylation pathway. Several studies have demonstrated that SUMOylation is closely associated

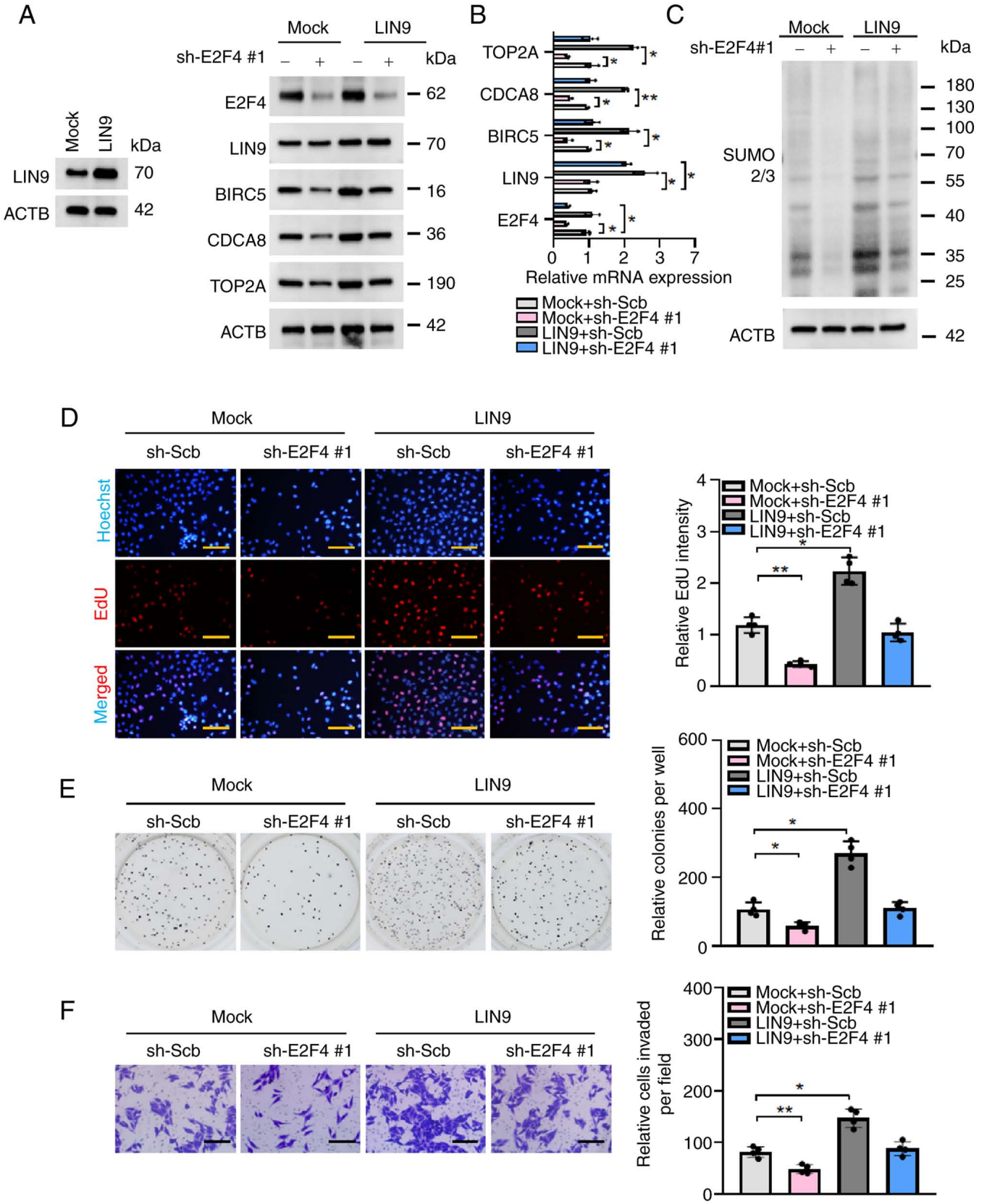

Figure 6. LIN9 promotes the proliferation and invasiveness of hepatocellular carcinoma cells via E2F4. (A) Western blotting and (B) reverse transcription-quantitative PCR (n=3 per group) assays revealing the protein expression and transcript levels of target genes in Huh7 transfected with sh-Scb or sh-E2F4 #1 and co-transfected with empty vector (mock) or LIN9. (C) Western blot revealing the levels of SUMOylation in Huh7 transfected with sh-Scb or sh-E2F4 #1 and co-transfected with empty vector (mock) or LIN9. (D) EdU staining assays (n=4 per group) showing the proliferation of Huh7 cells stably transfected with sh-Scb or sh-E2F4 #1, and co-transfected with mock or LIN9. (E) Representative images (left panel) and quantification (right panel) of soft agar (n=4 per group) and (F) Transwell Matrigel invasion (n=4 per group) assays indicating the anchorage-independent proliferation and invasiveness of Huh7 cells stably transfected with sh-Scb or sh-E2F4 #1, and co-transfected with mock or LIN9. *P<0.05, **P<0.01. Scale bars, 100 μm. ACTB, actin-b; BIRC5, baculoviral IAP repeat containing 5; CDCA8, cell division cycle associated 8; E2F4, E2F transcription factor 4; LIN9, lin-9 DREAM MuvB core complex component; sh, short hairpin; Scb, scramble (shRNA); SUMO2/3, small ubiquitin like modifier 2/3; TOP2A, DNA topoisomerase II α.

with tumorigenesis, proliferation (36) and metastasis (37), and higher expression is observed in the majority of tumor cases (37,38). However, the effects of SUMOylation in HCC still remain largely unclear.

SUMOylation, a posttranslational modification (PTM) marked by the attachment of a small ubiquitin-like modifier peptide to a lysine residue, significantly influences cellular biological processes and the progression of cancer (39). Comparable to other types of PTMs, such as phosphorylation, ubiquitination and acetylation, SUMOylation is a highly dynamic and reversible mechanism that regulates the translation, subcellular localization, stability and protein-protein interactions of target proteins (40,41). Proteins such as STAT1, when modulated via both SUMOylation and de-SUMOylation, have been linked to the pathogenesis of cancer (42,43). A study has revealed that higher levels of SUMOylation can be observed in HCC tissues, and inhibitors of SUMOylation (such as TAK-981 and ML-792) suppress tumor progression (44). Notably, the results of the present study further supported these findings and revealed that TAK-981 prevented the proliferation and invasion of HCC cells. The higher transcript and protein levels of SUMOylation-related genes (BIRC5, CDCA8 and TOP2A) in HCC cells was also observed in the present study. Nevertheless, the specific function of STAT1 SUMOylation in HCC remains unclear, warranting future investigative efforts.

Some studies have highlighted the critical role of BIRC5 in oncogenesis, demonstrating that its overexpression not only suppresses apoptosis but also endows cells with tumorigenic capabilities (45,46). Specifically, the dysregulation of BIRC5-related genes has been tightly linked to the malignant advancement of HCC (47). Moreover, BIRC5 has been identified as a downstream target of microRNA (miR)-497-5p, which notably exhibits diminished transcription levels in HCC tissues compared with healthy tissues (48). Circular (circ)ANKRD52 facilitates the proliferation of HCC by sponging miR-497-5p and increasing BIRC5 expression (49). CDCA8 serves as an essential component of the chromosomal passenger complex, indispensable for accurate chromosomal distribution during mitosis (50). Furthermore, abnormal expression of CDCA8 leads to disruption of cellular homeostasis (51). Targeted suppression of CDCA8 inhibits the MEK/ERK pathway and impedes colony formation by regulating the CDK1/cyclin B1 signaling axis (52). TOP2A maintains mitotic chromosome structure and genome stability by resolving DNA topological strains and controlling genome dynamics (53). Abnormal levels of TOP2A are related to tumor progression (54), and bioinformatic analyses have identified TOP2A as a driving factor of HCC progression (55,56). Notably, TOP2A is upregulated and associated with an unfavorable prognosis in HCC (57), while miR-139-5p inhibits TOP2A expression, triggering cellular senescence and inhibiting the proliferation of HCC cells (58). Furthermore, the interaction between TOP2A and β-catenin facilitates the detachment of β-catenin from Yes1 associated transcriptional regulator, which enhances the excessive proliferation of HCC cells (59). Taken together, these findings underscore the notable influence of BIRC5, CDCA8 and TOP2A on the pathogenesis and progression of HCC, marking them as significant players and potential therapeutic targets. Therefore, it is critical to identify the key factors that regulate these target genes.

E2F4, a member of the E2F TF family, markedly influences tumor progression via the regulation of various gene expression and cell cycle signaling pathways, such apoptosis, differentiation and G0 to S phase transition (60-63). The E2F family can be generally divided into canonical activators (E2F1, E2F2 and E2F3a), canonical repressors (E2F3b, E2F4, E2F5 and E2F6) and atypical repressors (E2F7 and E2F8) (64). Notably, the mRNA expression levels of E2F have been associated with the progression stage and histopathological grades in HCC, and E2F4 has emerged as a key regulator of HCC (65,66). E2F4 interacts with the CDCA3 promoter region, leading to increased CDCA3 expression and facilitating both cell cycle progression and the proliferation of HCC cells (67). Additionally, E2F4 has been shown to directly bind to viral covalently closed circDNA, activating the hepatitis B virus (HBV) core promoter and upregulating transcription levels (68). Therefore, further exploration of the epigenetic regulatory roles of E2F4 in HCC is warranted in the future. Moreover, E2F4 is associated with immune infiltration in HCC (65). In the present study, higher expression levels of E2F4 protein compared with the controls were observed in patients with HCC. Kaplan-Meier analyses also suggested that E2F4 was associated with poorer survival in patients with HCC. Therefore, the E2F4 protein may serve as a promising prognostic indicator for HCC.

The findings of the present study also indicated that E2F4 directly interacted with LIN9 protein in the nucleus of HCC cells. In addition, elevated levels of LIN9 were associated with poorer outcomes in patients with HCC. LIN9 is a part of the MuvB complex, including LIN9, LIN37, LIN52, RB binding protein 4 and LIN54 (69,70). During the G0 and early G1 phases, the MuvB core complex engages with E2F4/5, DP1/2 and RB-like proteins, p130 or p107, to form the DREAM complex, thus inhibiting gene transcription. In the late phase of G1, the MuvB core is separated from the DREAM complex and then binds to B-MYB to form the MMB complex in the S phase, activating cell-cycle genes related to the S/G2/M phases (71-74). Deregulation of the DREAM complex has been implicated in multiple cancer types (such as myeloid leukemia, glioblastoma and prostate, breast, lung, esophagus, ovarian and pancreas cancer) and plays a critical role in cell cycle dynamics (75,76). It has been observed that aberrant expression of the DREAM complex occurs in HCC (77). Similarly, the findings of the present study suggested that higher expression of LIN9 was related to a worse OS. Notably, the results of the rescue studies revealed that overexpression of LIN9 reduced whole cell levels of SUMOylation and the proliferation and invasion of HCC cells following stable knockdown of E2F4 expression. An elevation in the SUMOylation of LIN9 was also noted in Huh7 cells overexpressing E2F4, which may potentially disrupt the stability of the DREAM complex. These findings suggested that the LIN9-E2F4 axis promotes progression in HCC via activation of the SUMOylation pathway. However, the intricacies of these mechanisms warrant further in-depth research.

In addition, other major TFs, such as E2F1 (78), ETS1 (79), IRF1 (80) and KDM6A (81), have been identified as prognostic biomarkers in HCC. E2F1, the classical E2F member, has been extensively studied. Notably, higher expression of E2F1 is significantly related to unfavorable prognosis in HCC (78).

E2F1 can stimulate the proliferation of HCC cells by driving the expression of genes such as B-MYB (82), BRCA1 (83), DNA-binding protein A (84) and stathmin 1 (85), which enhance the development or progression of HCC. Additionally, mutations in the HBV core promoter lead to upregulated transcription of E2F1, resulting in HCC cell proliferation (86). Notably, nuclear E2F1 expression is positively correlated with the HCC tumor apoptotic index (87,88). E2F1 may disrupt the regulation of hepatitis B viral X protein on the p53 promoter and directly activate the p53 promoter via its specific binding sites, thereby blocking the HBV life cycle and HBV-associated HCC (89). As such, the intricate functions of E2F1 in HCC merit further investigation. ETS1, a member of the ETS family, is a crucial TF in regulating cell proliferation, invasion and metastasis. The interaction between murine double minute binding protein and ETS1 activates ETS1, consequently leading to the progression of HCC cells (90). Moreover, ETS1 also acts as a co-activator of pregnane X receptor to stimulate drug resistance in HCC (91,92). miR-338-3p inhibits progression of HCC through the downregulation of EST1 (93), while small molecule inhibitors targeting ETS1 have been shown to suppress proliferation or invasion of HCC cells (94). IRF1 is the main transcription regulator in the interferon-γ signaling pathway (95). IRF1 promotes autophagy in HCC cells, thus inhibiting their proliferation (96). Nevertheless, IRF1 increases the expression of programmed death-ligand 1, which may facilitate HCC cells in evading the antitumor immune response of the host (80). KDM6A plays different functions at different stages of HCC. On one hand, KDM6A promotes the progression of HCC through the upregulation of FGFR4 expression (97). On the other hand, KDM6A may hinder the proliferation of HCC cells through the negative regulation of the TGF-β/SMAD signaling pathway (98). Hence, the complexity of these TFs underscores the necessity to delve deeper into HCC development and new molecular therapeutic targets.

In summary, the present study demonstrated that E2F4 protein was highly expressed and related to poorer outcomes in patients with HCC. Stable expression of E2F4 promoted the whole cell SUMOylation of proteins by upregulating the transcription and protein expression levels of SUMOylation-associated genes including BIRC5, CDCA8 and TOP2A protein. Following treatment with the inhibitor of SUMOylation, TAK-981, the proliferation and invasion of HCC cells were downregulated. Notably, it was demonstrated that E2F4 directly bound to LIN9 protein, leading to the promotion of E2F4-mediated SUMOylation associated with HCC progression. Therefore, the results of the present study suggested that E2F4 and LIN9 play crucial roles in the progression of HCC and indicated that the E2F4-LIN9-SUMOylation axis may be a valuable potential therapeutic target in HCC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

ZD and QL conceived and designed the present study. ZM, QL and WW performed most of the experiments, analyzed the data and wrote the manuscript. ZD and WW supervised the entire project. ZM, QL, WW and ZD confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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