Am J Cancer Res 2019;9(12):2679-2692
www.ajcr.us /ISSN:2156-6976/ajcr0103198

Original Article

YY1 promotes colorectal cancer proliferation through the *miR-526b-3p*/E2F1 axis

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Received October 6, 2019; Accepted November 11, 2019; Epub December 1, 2019; Published December 15, 2019

Abstract: We previously reported that E2F1 expression is up-regulated and positively correlated with the malignant phenotypes of colorectal cancer (CRC). However, the underlying mechanisms leading to the aberrant up-regulation of E2F1 in CRC have not been clarified. In this study, we observed that *miR-526b-3p* directly targets the 3'UTR of *E2f1* mRNA, leading to reduced E2F1 expression. Overexpression of *miR-526b-3p* inhibited the proliferation of CRC cells by decreasing the level of E2F1. We also found that the Ying Yang 1 (YY1)-dependent transcriptional suppression of *miR-526b-3p* is responsible for the up-regulation of E2F1 in CRC, in which YY1 binds to the promoter of *miR-526b* gene and recruits histone deacetylase (HDAC). Knockdown of YY1 led to cell cycle arrest and diminished colony formation in CRC cells partly through relieving the *miR-526b-3p* suppression. Clinical analysis showed that YY1 and E2F1 were negatively correlated with *miR-526b-3p* in CRC tissues. Moreover, a high level of YY1 and E2F1, or a low level of *miR-526b-3p*, predicted poor survival of CRC patients. In conclusion, our findings highlight the dysregulation of the YY1/*miR-526b-3p*/E2F1 axis in CRC development, implicating a novel regulatory pathway for E2F1 as a potential therapeutic target in CRC.

Keywords: E2F1, *miR-526b-3p*, Ying Yang 1, colorectal cancer, proliferation

Introduction

Colorectal cancer (CRC) is one of the most common malignant and lethal cancers worldwide with high incidence [1]. It has been well-recognized that dysregulation of tumor suppressors and promoters is involved in CRC tumorigenesis and progression. As a well-characterized E2F family member, E2F1 exerts a complex role in cancer. Although E2F1 has been recognized as a strong inducer of apoptosis, especially after DNA damage, increased E2F1 expression was detected in cancers and promoted the cell cycle or proliferation through its transcriptional products [2]. We previously reported that E2F1 is involved in the regulation of invasion, migration, and proliferation of CRC cells, and a high E2F1 level indicated a poor prognosis of CRC patients [3, 4]. We also found that overexpression of E2F1 contributed to the oxaliplatin-resistance of CRC cells [5]. However, the mechanisms leading to the abnormally high expression of E2F1 in CRC remain elusive.

As well-documented tumor suppressors, microRNAs (miRNAs) inhibit various oncogenes by inducing translational repression or transcript degradation. A growing body of evidence suggests that miRNAs involved in the dysregulation of E2F1 play a crucial role in cancer development, especially in proliferation [6]. Recently, Yan *et al.* found that miR-1205 suppressed non-small cell lung cancer growth by directly binding to the coding sequence of E2F1 [7].

Additionally, both *miR-342-3p* and miR-377 target the 3'UTR of *E2f1* to suppress glioma proliferation [8]. *miR-362-3p*, which is mainly expressed in CRC with no recurrence, when compared with CRC with recurrence, decreased E2F1 expression and caused cell cycle arrest [9].

Overexpression of transcription factor Yin Yang 1 (YY1) is frequently correlated with tumor growth, metastasis, and chemotherapy resistance [10]. YY1 plays a role in regulating gene transcription depending upon the context in which it binds to DNA. Many YY1-inhibited genes, including several miRNAs, exhibit tumor suppressive potential [11-13]. High YY1 expression was detected in CRC cell lines or tissue samples, and poorly differentiated CRC tumors showed even higher YY1 expression than those in moderately and well-differentiated tumors [14]. Mechanistically, YY1 was reported to promote CRC growth and reduce apoptosis through the inhibition of p53 and the activation of Wnt signaling pathways [15, 16]. Other evidence revealed a close association between YY1 and sex-determining region Y-box 2 (SOX2) expressions in CRC, suggesting a potential involvement of YY1 in cancer stem cells [17]. However, it is not currently known how YY1 drives the proliferation of CRC cells.

In the present study, we demonstrated that the highly expressed YY1 in CRC impeded the transcription of miR-526b-3p, functioning as a tumor suppressor by directly targeting E2F1. Increased E2F1 then enhanced CRC growth and tumorigenicity. Clinical analysis also confirmed the existence of a YY1/*miR-526-3p*/E2F1 axis in CRC tissues and a relationship between this axis and CRC patient prognosis.

Methods

Cell cultures and reagents

HIEC, HCT116, SW620, and RKO cells were maintained in the RPMI 1640 medium supplemented with 10% of fetal bovine serum (Gibco, Carlsbad, CA) in a humidified 5% CO_2 atmosphere at 37°C. The antibodies against E2F1, YY1, and GAPDH were from Santa Cruz Biotechnology (Santa Cruz, CA). The small interfering RNA (siRNA) for E2F1 or YY1 and the short hairpin RNA (shRNA) for YY1 or miR-526b-3p were provided by GenePharma (Shanghai, China) and were transfected using Lipofectamine™ RNAiMAX (Invitrogen, Grand Island, NY). The sequences of siRNA and shRNA are shown in Table S1. The HCT116 cells stably transfected with shYY1 or shmiR-526b-3p were constructed using G418 (Sigma-Aldrich, Darmstadt, Germany).

Tissue samples

A total of 218 CRC samples (55 cases with frozen CRC and adjacent non-tumor normal tissues) were collected from the Sanmen People's Hospital of Zhejiang Province after all patients signed the consent inform. The protocol of this study was approved by the Ethics Committee of the Sanmen People's Hospital of Zhejiang Province. The 218 CRC samples were analyzed by immunohistochemistry, and the 55 frozen tissues were used to quantify *miR-526b-3p*, *Yy1* mRNA, and *E2f1* mRNA.

RNA extraction and quantitative PCR

Total RNA was extracted from the cells and tissues using the RNAiso Plus Kit (Takara Bio Inc., Otsu, Japan). For mRNA, cDNA was obtained by reverse transcription using the PrimeScript RT reagent Kit (Takara Bio, Inc), and qPCR was performed using the SYBR Green PCR Master Mix (Takara Bio Inc). For miRNA, reverse transcription was performed using a TaqMan microRNA RT Kit (Applied Biosystems, Foster City, CA), and the *miR-526b-3p* expression level was measured by qPCR using a TaqMan miRNA assay (Applied Biosystems). β-actin and U6 served as an internal control to normalize the mRNA and miRNA levels. The relative expression levels were calculated through the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

The Bradford method was applied to measure the protein concentrations in whole-cell lysates. After electrophoresis, the proteins on SDS-PAGE were transferred to nitrocellulose membranes (Whatman, Maidstone, UK). The membranes were incubated using primary antibodies and the corresponding secondary antibodies. The fluorescence intensities were measured by the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

CCK-8 assay

We transfected the cells in logarithmic growth with the indicated siRNA, overexpression plas-

mid, or shRNA. At the indicated time after transfection, we added the cell counting kit-8 (CCK-8) solution (Dojindo, Gaithersburg, MD) and measured the OD450 by an automatic plate reader.

EdU incorporation assay

The 5-ethynyl-2-deoxyuridine (EdU) incorporation assay indicates DNA synthesis in cells. After transfection of the mentioned siRNA, overexpression plasmid, or shRNA, the cells were cultured in serum-free RPMI 1640 medium with 10 mM EdU for 2 h. The cells were then washed with PBS extensively and blocked with 10% of FBS in PBS for 30 min. The incorporated EdU was measured using the fluorescent azide coupling reaction (Invitrogen). We used a fluorescence microscope (Nikon, Tokyo, Japan) to capture cell images and the EdU incorporation rates were analyzed using ImageJ (NIH, Bethesda, MD).

Reporter gene assay

The 3'UTR of the *E2f1* gene and the promoter of the *miR-526b* gene were amplified based on cDNA or genome DNA and then cloned into the pGL3 luciferase reporter vector (Promega, Madison, WI). Deletion or mutation reporter vectors were constructed by subcloning. The pre-plated cells were co-transfected with luciferase reporter plasmids, and the luciferase activity was determined by a dual-luciferase reporter assay system (Promega).

Chromatin immunoprecipitation

Formaldehyde at 1% for 10 min was used to cross-link chromatin and the cross-linked chromatin was sonicated by ultrasonic cell disruptor. After centrifugation, we added protein A/G PLUS-Agarose (Santa Cruz) into the supernatants for overnight immunoprecipitation at 4°C with an antibody against YY1 or normal IgG. After reversing the cross-linking, we purified and amplified the precipitated DNA fragments by qPCR using the following primers for *miR-526b*: TTTACATCCTAGCCTGTGATC (forward), and CAGCAATGGATTTTAAGCCAAG (reverse).

Flow cytometry

Approximately 1×10^6 HCT116 cells stably transfected with shYY1 or *shmiR-526b-3p* were re-suspended and fixed with PBS containing 70% ethanol at -20°C for 1 h. Before analysis, cells were re-suspended in PBS containing 100 mg/ml RNaseA (Roche, Basel, Switzerland) and 50 mg/ml propidium iodide (PI) (Sigma-Aldrich) for 30 min. We analyzed the cells immediately using a FACSCalibur flow cytometer with the CellQuest 3.0 software system (BD, San Jose, CA).

Colony formation in soft agar

The HCT116 cells stably transfected with shYY1 or *shmiR-526b-3p* were suspended in 0.3% agar and plated into 6-well plates, which were pre-coated with 1.0 ml of 0.6% agar. We replaced the culture medium every four days for three weeks, stained the colonies using crystal violet, and quantified the numbers of colonies by using Image J software.

Immunohistochemistry

Immunohistochemistry was carried out and analyzed as shown in our previous study [5]. To evaluate the score for CRC tissues, at least eight individual fields of each slide were included for counting 100 cancer cells/field at 200×. The immunohistochemistry score for each tissue was evaluated and calculated as described previously [5].

Statistical analysis

The statistical analysis was performed using SPSS 22.0 and GraphPad Prism 5.0. The results are presented as the mean ± S.E.M. of three separate experiments. Statistical data analysis included the two-tailed Student's *t* test, Mann-Whitney *U* test, chi-square, and ANOVA. The Wilcoxon matched-pairs test was used for the analysis of CRC and corresponding para-CRC tissues. The Spearman and Pearson tests were used for correlation analysis. Overall survival was analyzed by Kaplan-Meier plots and log-rank tests. A *P*-value <0.05 was considered to be statistically significant.

Results

Knockdown of E2F1 reduces the proliferation of CRC cells

E2F1 expression was analyzed using the Cancer Genome Atlas (TCGA) database, which indi-

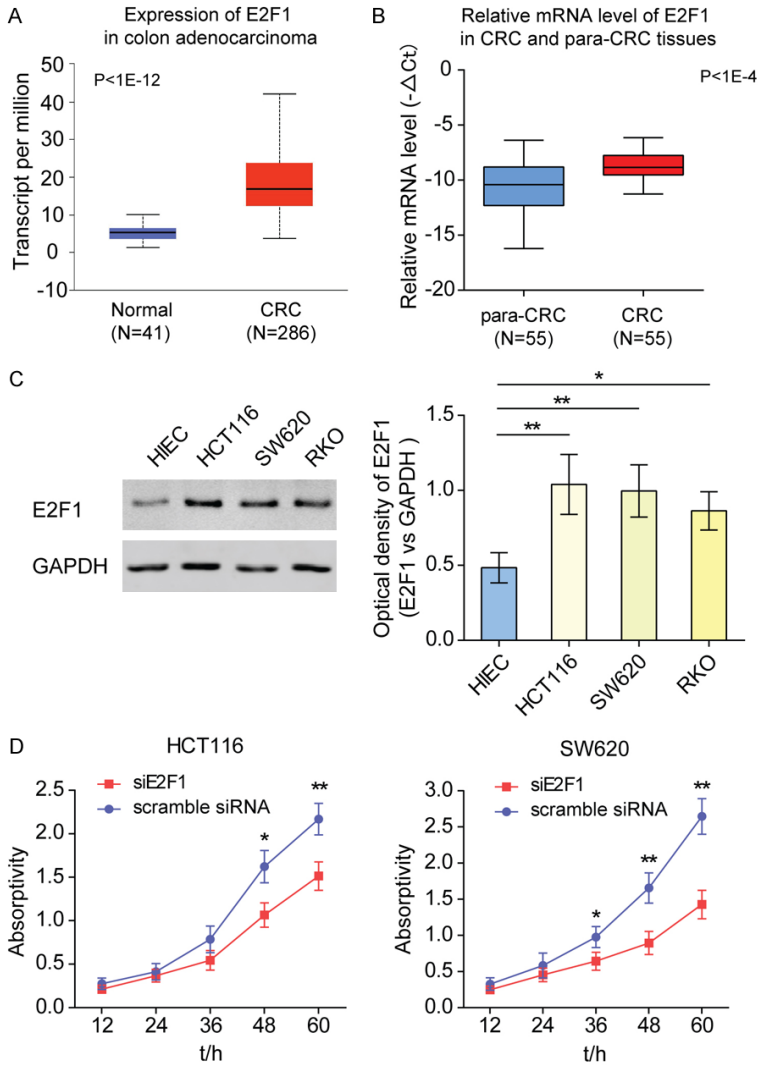


Figure 1. The knockdown of *E2F1* reduces the proliferation of CRC cells. A. The mRNA level of E2F1 in CRC and normal intestinal tissues based on TCGA database, N=327. A Mann-Whitney test was performed to compare the difference between the normal and cancer groups. B. The mRNA levels of E2F1 in 55 paired CRC and corresponding normal tissues were measured by qPCR, using β-actin as an internal control. -ΔCt (ΔCt: Ct (E2F1)-Ct (β-actin)) represented the relative expression level. A Wilcoxon matched pairs test was performed to compare the difference between the normal and cancer groups. C. Left panel: western blot analyses of E2F1 in HIEC, HCT116, SW620, and RKO cells; Right panel: the band density in the left panel was calculated by Image J software. D. Cell viability was examined in HCT116 and SW620 cells with E2F1 knockdown. **P*<0.05; ***P*<0.01.

cated that the E2F1 level was higher in CRC tissues than in the normal (**Figure 1A**). Consistently, the mRNA level of E2F1 was significantly higher in CRC than in adjacent tissues (**Figure 1B**). Then, we examined the protein expression of E2F1 in three CRC cell lines and a colonic epithelial cell line. The results showed that E2F1 expression was significantly increased in CRC cells, especially in HCT116 (**Figure 1C**). Consistent with our previous findings, E2F1 knockdown caused an obvious reduction in cell viability in both HCT116 and SW620 cells (**Figure 1D**).

miR-526b-3p inhibits E2F1 expression by targeting the 3'UTR of E2f1 mRNA

To explore the reason behind the high expression of E2F1 in CRC, we predicted the potential miRNA binding site at the 3'UTR of *E2f1* mRNA by two different prediction algorithms, TargetScan and miRanda. The seed sequence of miR-526b-3p is complementary to the 3'UTR and is highly conserved among nine different species (**Figure 2A**). We then determined the *miR-526b-3p* level in CRC cell lines and normal colonic epithelial cells, which indicated that CRC cells had significantly lower *miR-526b-3p* expression levels than normal epithelial cells (**Figure 2B**). The E2F1 expression levels were both reduced by *miR-526b-3p* mimics in HCT116 and SW620 cells (**Figure 2C** and **2D**). Furthermore, luciferase assay showed that *miR-526b-3p* repressed the activity of the reporter with wild-type 3'UTR of *E2f1* mRNA. Notably, *miR-526b-3p* was not able to induce changes in the levels of the reporter when the miR-526b-3p binding site was mutated (**Figure 2E**). These results reveal a specific inhibitory effect of *miR-526b-3p* on E2F1 expression via direct interaction with 3'UTR.

miR-526b-3p attenuates the proliferation of CRC cells by inhibiting E2F1

To clarify the role of *miR-526b-3p* in the proliferation of CRC cells, HCT116 and SW620 cells

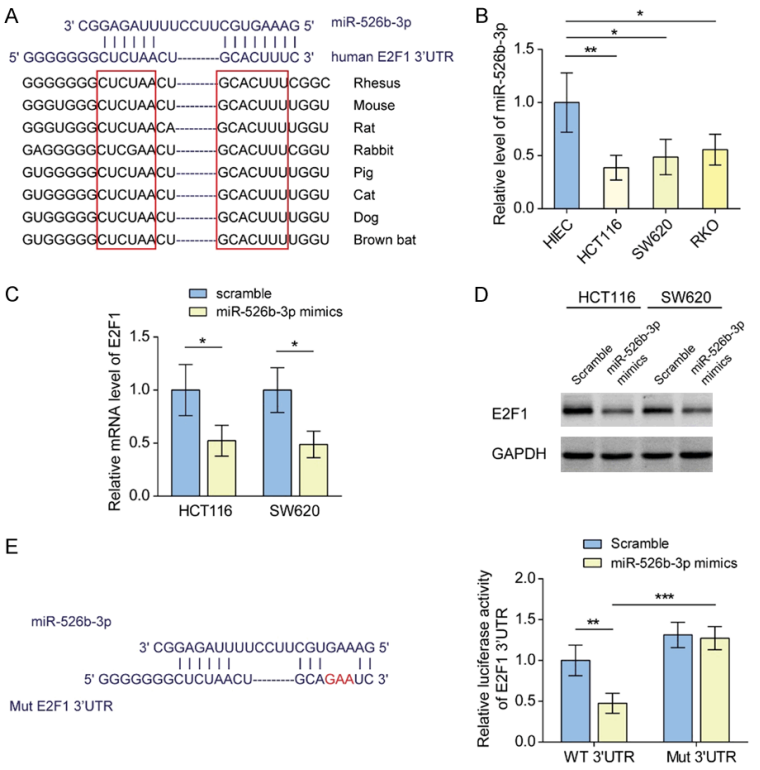


Figure 2. *miR-526b-3p* inhibits *E2F1* expression by targeting the 3'UTR of *E2f1* mRNA. A. The *miR-526b-3p* seed sequence complementary to the 3'UTR of *E2f1* mRNA was predicted by TargetScan Human 7.2 and miRanda. B. The level of *miR-526b-3p* in HIEC, HCT116, SW620, and RKO cells was analyzed by qPCR. **P*<0.05; ***P*<0.01. C. The mRNA level of *E2F1* in HCT116 and SW620 cells transfected with scramble or *miR-526b-3p* mimics was analyzed by qPCR. **P*<0.05. D. Western blot analyses of *E2F1* in HCT116 and SW620 cells transfected with scramble or *miR-526b-3p* mimics. E. Left panel: the potential sequences for *miR-526b-3p* binding were mutated as indicated. Right panel: the function of the wild-type or mutated *E2F1* 3'UTR was analyzed by luciferase reporter assay in HCT116 cells transfected with scramble or *miR-526b-3p* mimics. ***P*<0.01; ****P*<0.001.

were transfected with *miR-526b-3p* mimics, and then the cell viability and DNA synthesis were examined. The results showed that *miR-526-3p* inhibited cell proliferation and interrupted EdU incorporation, whereas further overexpression of E2F1 rescued cell growth and DNA synthesis (**Figure 3A** and **3B**), suggesting that E2F1 mediates the inhibitory effect of *miR-526b-3p* on CRC cell proliferation.

YY1 represses the transcription of the miR-526b gene

To understand why *miR-526b-3p* was silenced in CRC, the promoter of the *miR-526b* gene was deeply analyzed. Serial deletion constructs of the promoter were studied by a luciferase reporter assay, and it was shown that the transcriptional activity of the *miR-526b* promoter was remarkably increased when deleting the region between -600 and -300, suggesting a critical regulatory element located at this region (**Figure 4A**). Using the 'JASPAR' database, two potential YY1 binding sites (-582/-570; -365/-353) were found in the promoter sequence (**Figure 4B**). However, only the mutation at the -365/-353 site significantly increased the reporter activity (**Figure 4C**). The expression of YY1 was also found to increase in CRC tissues compared with adjacent tissues (Figure S1A and S1B). In line with the above findings, knockdown of YY1 increased the *miR-526b-3p* level and meanwhile reduced E2F1 expression in HCT116 and SW620 cells (**Figure 4D** and **4E**). ChIP-PCR and ChIP-qPCR analysis further verified that YY1 interacted with the *miR-526b* promoter around the -365/-353 site (**Figure 4F**). Previous studies reported that HDACs served as epigenetic co-repressors with YY1 [18, 19]. Accordingly, we addressed the role of HDACs in the expressions of *miR-526b-3p* and E2F1. We found that trichostatin A (TSA), a classical HDAC inhibitor, significantly increased the level of *miR-526b-3p* and silenced the E2F1 expression even when overexpressing YY1 (**Figure 4G** and **4H**), suggesting that HDACs participate in YY1-mediated transcription silence of *miR-526b-3p*. These findings indicate that the highly expressed YY1 in CRC suppresses the transcription of *miR-526b-3p*, eventually promoting E2F1 expression.

YY1 promotes CRC cell proliferation by suppressing miR-526b-3p

HCT116 cells with a stable knockdown of YY1 or *miR-526b-3p* were generated. As expected, the knocking down of YY1 induced significant up-regulation of *miR-526b-3p* but a down-regu-

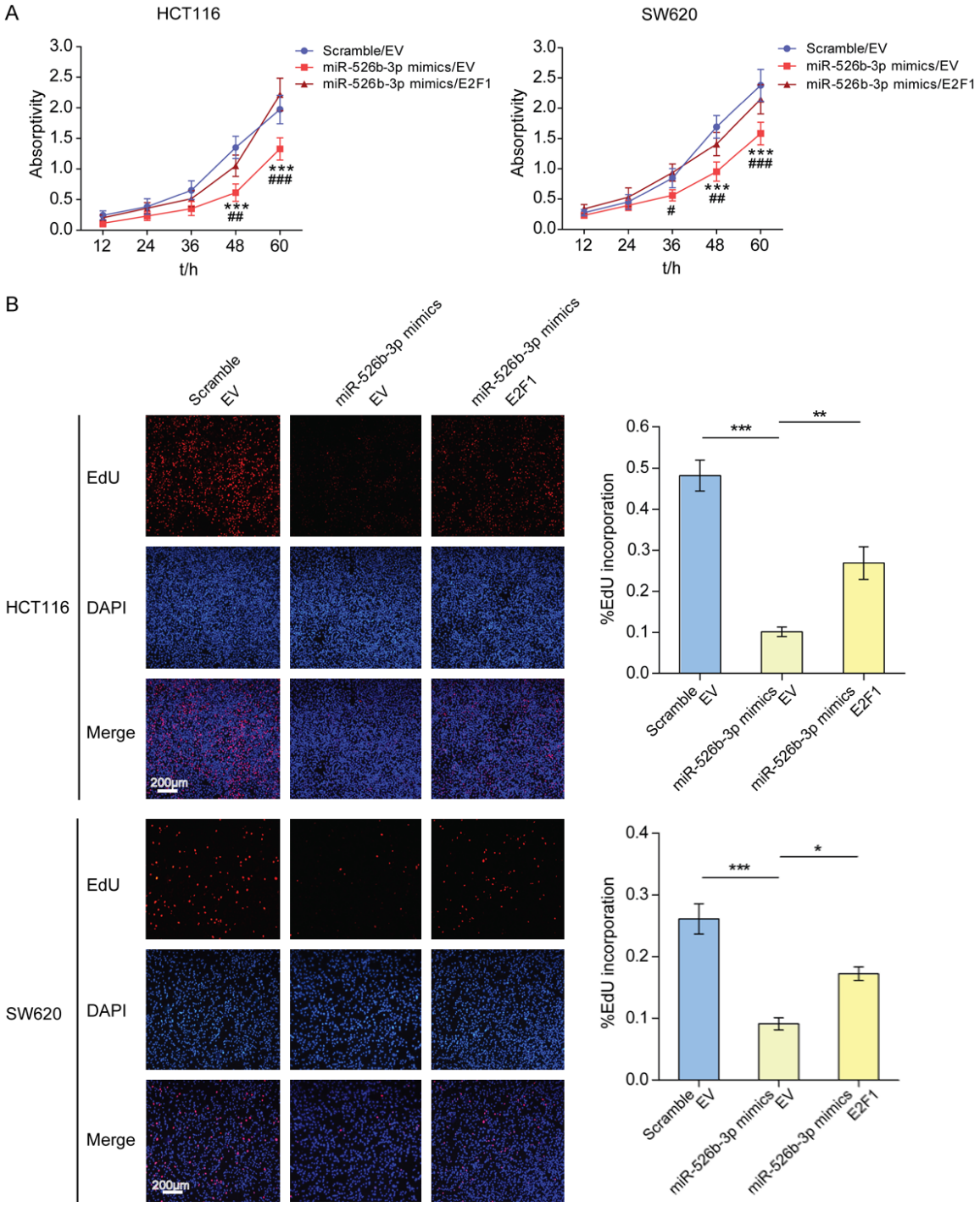


Figure 3. *miR-526b-3p* attenuates the proliferation of CRC cells by inhibiting *E2F1*. A. Cell viability was measured in HCT116 and SW620 cells co-transfected with *miR-526b-3p* mimics or *E2F1* overexpression plasmids vs scramble/EV: ***P<0.001; vs miR-526b-3p/E2F1: #P<0.05; ##P<0.01; ###P<0.001. B. DNA synthesis of HCT116 or SW620 cells was measured by EdU incorporation assay after co-transfection with *miR-526b-3p* mimics or *E2F1* overexpression plasmids. Left panel: representative images; Right panel: percentage of cells with EdU incorporation was measured using image-J.

lation of E2F1 in HCT116 cells, and depriving *miR-526b-3p* completely restored E2F1 expression (**Figure 5A** and **5B**). Compared with the control, a significant inhibition of proliferation

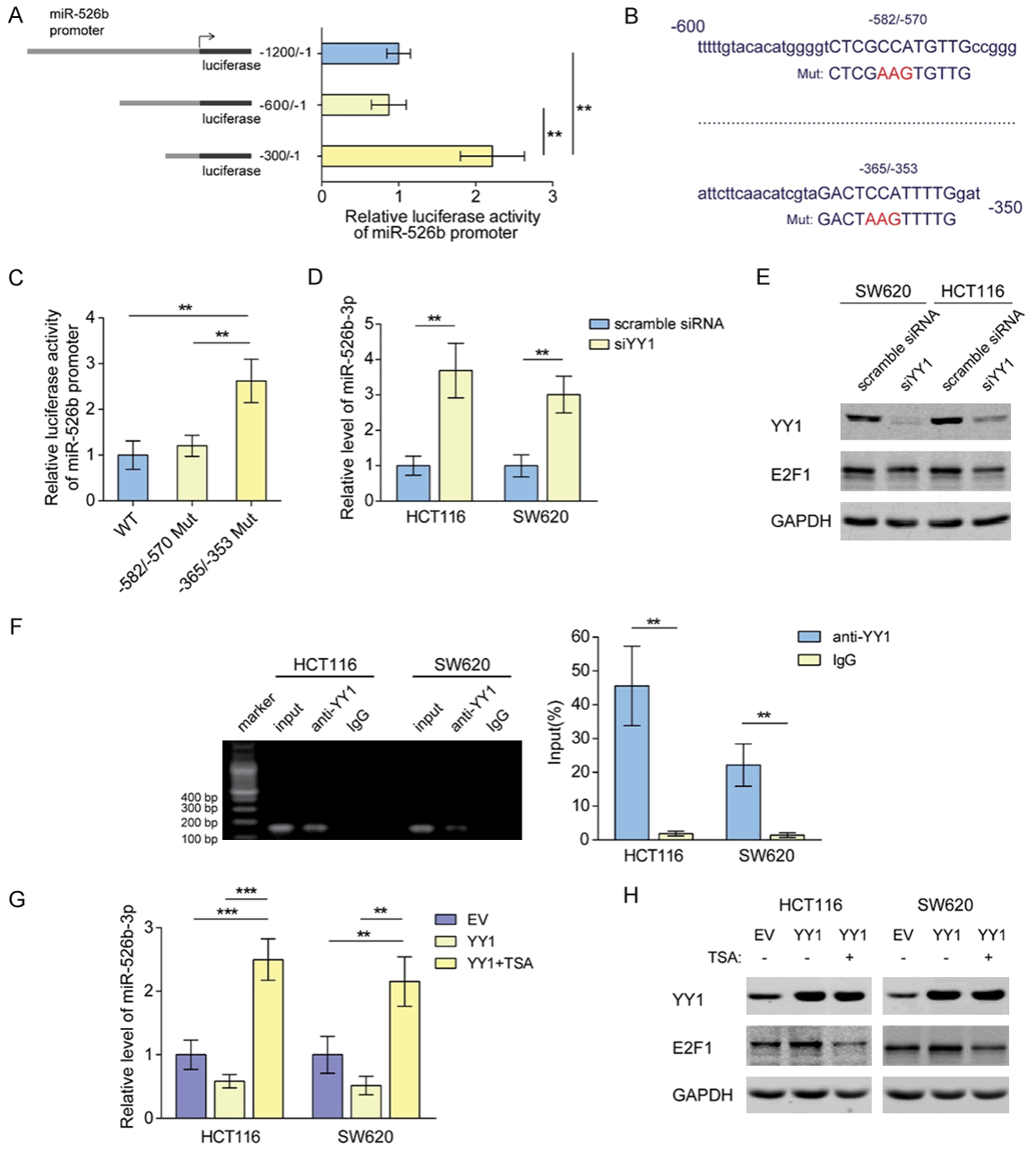
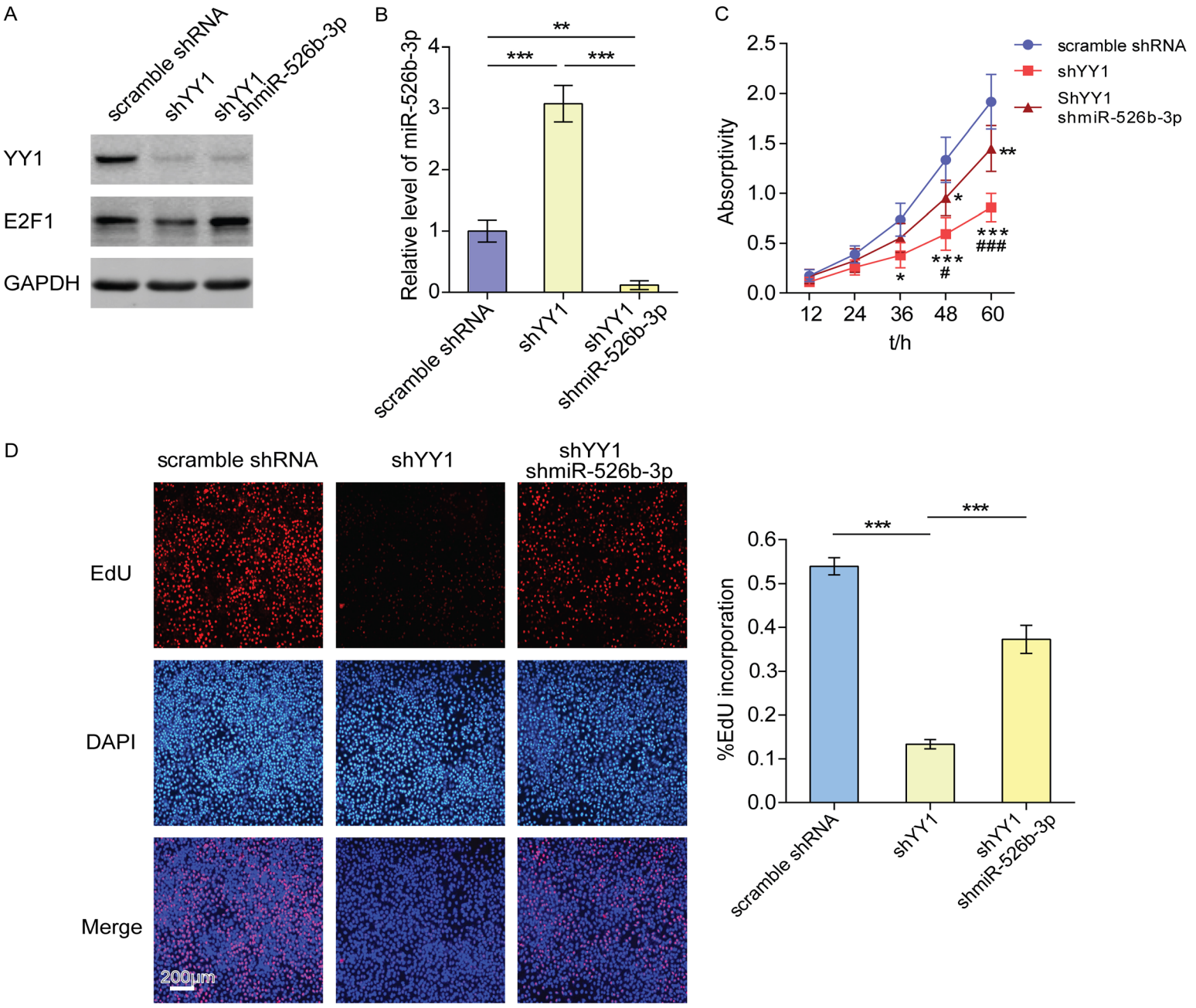


Figure 4. YY1 represses the transcription of the *miR-526b* gene. A. Transcription activity of the truncated *miR-526b* promoter was measured by luciferase reporter assay in HCT116 cells. ***P*<0.01. B. The potential sequences for YY1 binding were mutated as indicated. C. Transcription activity of the wild-type or mutated *miR-526b* promoter was measured by luciferase reporter assay in HCT116. ***P*<0.01. D. The level of *miR-526b-3p* in HCT116 and SW620 cells with knockdown of *YY1*. ***P*<0.01. E. Western blot analyses of E2F1 in HCT116 and SW620 cells with knockdown of *YY1*. F. The binding of YY1 to *miR-526b* promoter was examined by ChIP-PCR. Left panel: ChIP-PCR products in the input, ChIP and IgG groups were analyzed by agarose gel electrophoresis; Right panel: quantitative PCR analysis. G. The level of *miR-526b-3p* in HCT116 and SW620 cells transfected with *YY1* overexpression plasmids or treated with 100 ng/mL TSA. ***P*<0.01; ****P*<0.001. H. Western blot analyses of E2F1 in HCT116 and SW620 cells transfected with *YY1* overexpression plasmids or treated with 100 ng/mL TSA.

and DNA synthesis appeared in HCT116 cells with a stable knockdown of YY1, while silencing *miR-526b-3p* partially rescued the proliferation and DNA synthesis (**Figure 5C** and **5D**). Cell cycle analysis revealed that YY1-silenced HCT116 cells underwent arrest in G1 phases, with a significant depletion of cells entering the S phase. However, HCT116 cells with a stable


A
scramble shRNA
shYY1
shYY1 shmiR-526b-3p
YY1
E2F1
GAPDH
B
Relative level of miR-526b-3p
scramble shRNA
shYY1
shYY1 shmiR-526b-3p
C
Absorptivity
t/h
scramble shRNA
shYY1
ShYY1 shmiR-526b-3p
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DAPI
Merge
200μm
%EdU incorporation

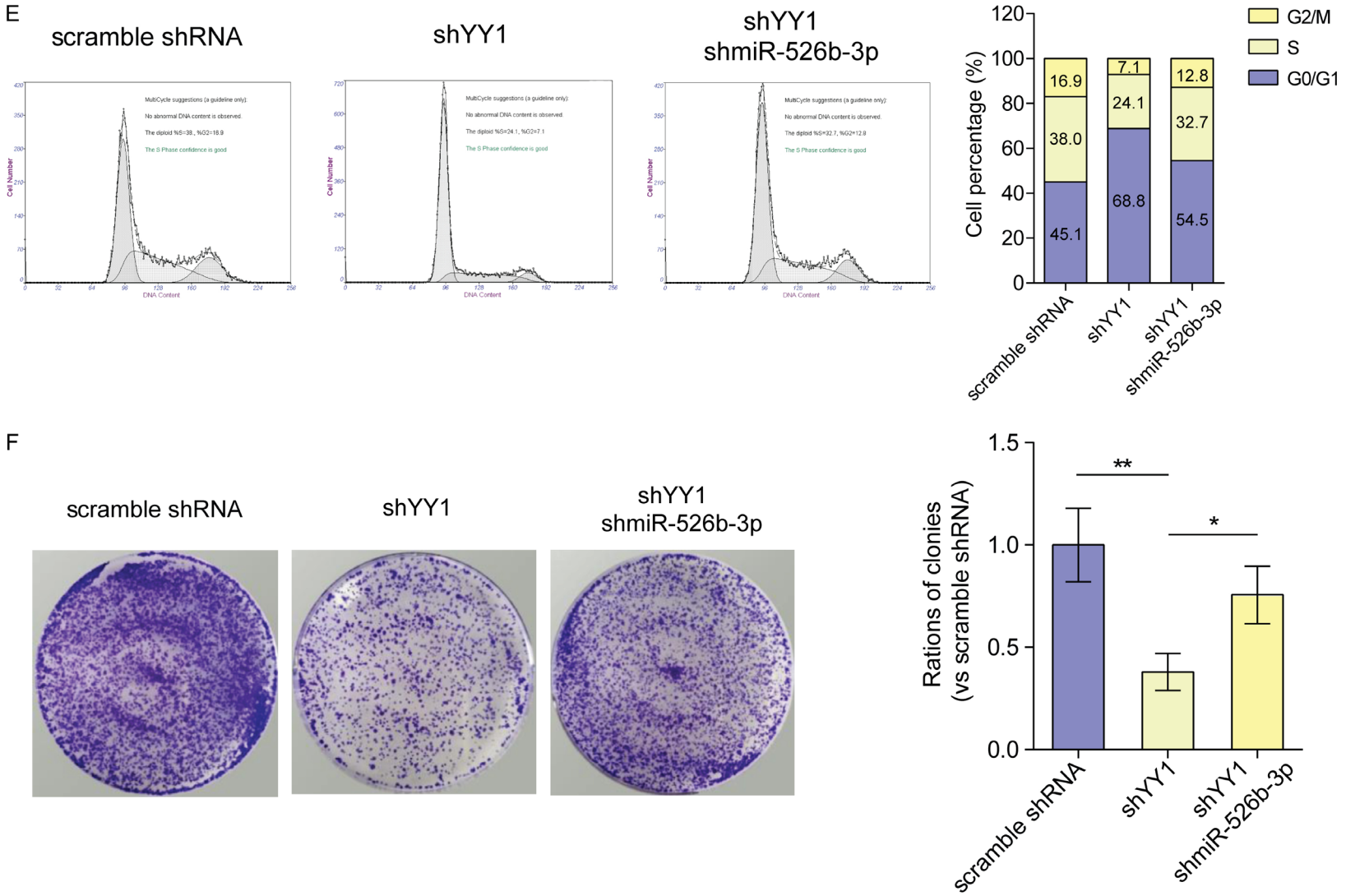

Figure 5. YY1 promotes CRC cell proliferation by suppressing *miR-526b-3p*. A. Western blot analyses of E2F1 in HCT116 cells with stable knockdown of *YY1* or *miR-526b-3p*. B. The level of *miR-526b-3p* in HCT116 cells with stable knockdown of *YY1* or *miR-526b-3p*. ***P*<0.01; ****P*<0.001. C. Cell viability was measured in HCT116 cells with stable knockdown of *YY1* or *miR-526b-3p*. vs shCtrl: **P*<0.05; ***P*<0.01; ****P*<0.001. vs sh*YY1*/*shmiR-526b-3p*: #*P*<0.05; ###*P*<0.001. D. DNA synthesis of HCT116 with stable knockdown of *YY1* or *miR-526b-3p* was measured by EdU incorporation assay. Left panel: representative images; Right panel: percentage of cells with EdU incorporation was measured using image-J. E. Cell cycle profiles of HCT116 cells with stable knockdown of *YY1* or *miR-526b-3p* were analyzed by flow cytometry. Left panel: representative images; Right panel: percentage of cells at different phases. F. Surviving colonies of HCT116 cells with stable knockdown of *YY1* or *miR-526b-3p*. Left panel: representative images; Right panel: ratios of colony numbers relative to control.

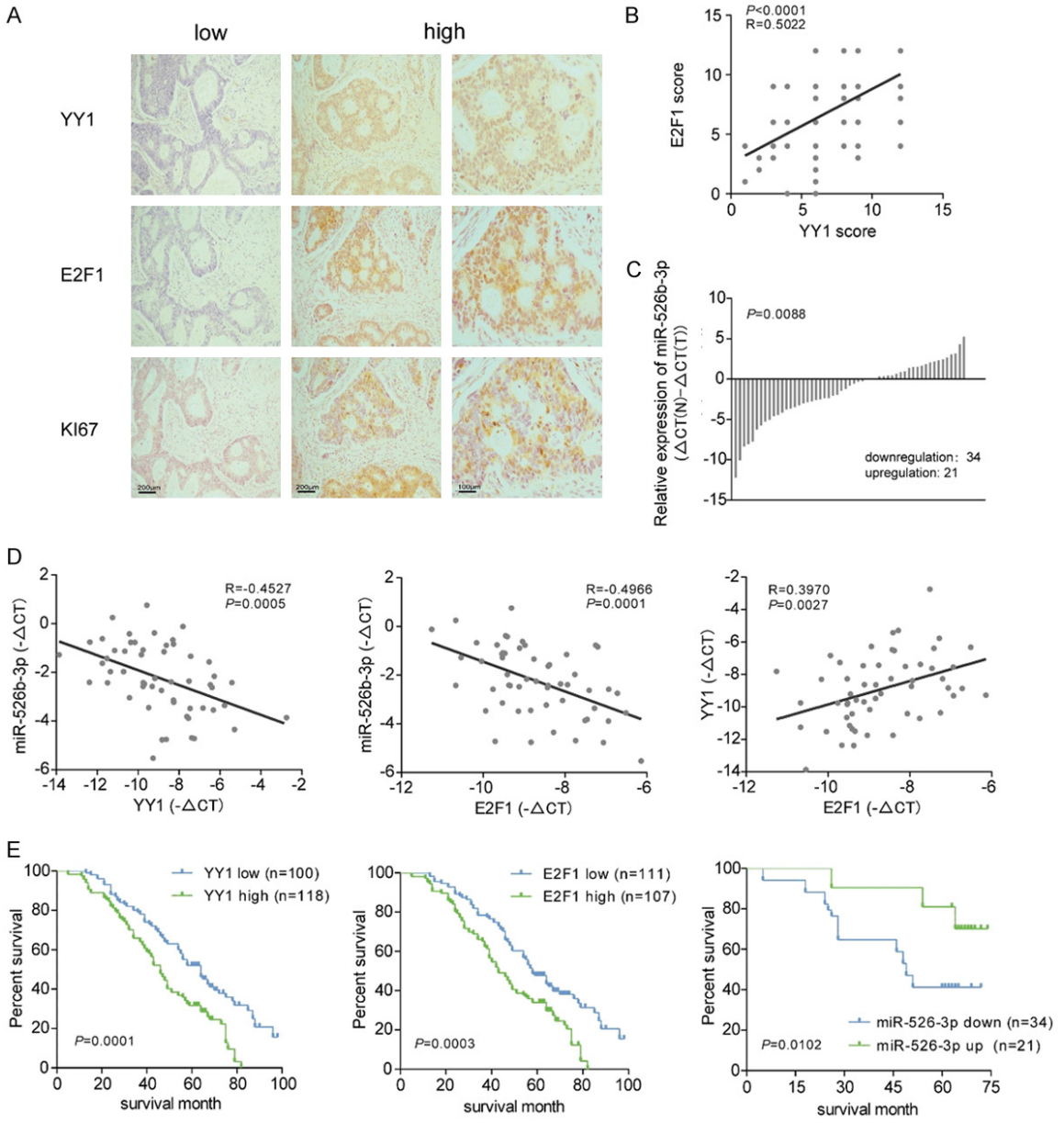

Figure 6. Activation of YY1/miR-526b-3p/E2F1 axis is related with a poor prognosis in CRC patients. A. Representative images of YY1, E2F1, and KI67 staining in CRC and adjacent normal tissues. B. The correlation between the immunostaining scores of YY1 and E2F1 in CRC tissues. Nonparametric spearman test was applied in analyzing the correlation. C. The miR-526b-3p levels in 55 paired CRC and corresponding normal tissues were measured by qPCR, using U6 as an internal control. The expression value (ΔCt (N)-ΔCt (T)) represented the difference in the miR-526b-3p level between normal tissue and tumor. An expression value >0 indicated that miR-526b-3p level was increased in tumors. An expression value <0 indicated that miR-526b-3p level was decreased in tumors. Wilcoxon matched pairs test was applied to compare the difference between the normal and cancer groups. D. The correlation among the levels of *Yy1* mRNA, *E2f1* mRNA, and miR-526b-3p in 55 CRC tissues. A Pearson test was applied in the correlation analysis. E. The overall survival of CRC patients with low and high expression of *Yy1* protein, *E2f1* protein, or miR-526b-3p was illustrated.

knockdown of both YY1 and *miR-526b-3p* went through a relatively slight S phase arrest (**Figure 5E**). Consistently, knockdown of YY1 decreased the colony formation numbers of HCT116 cells, and eliminating *miR-526b-3p* rescued the attenuated tumorigenesis to a certain extent

Table 1. Association of the expression of E2F1 and YY1 with clinicopathological features in CRC

	Cases	E2F1 expression			YY1 expression		
		Low cases	High cases	*P* value	Low cases	High cases	*P* value
Tumor location				0.7549			0.3785
Colon	90	44	46		41	49	
Rectum	128	67	61		59	69	
Gender				0.3220			0.1898
Male	116	54	62		48	68	
Female	102	57	45		52	50	
Age				0.7756			0.1206
≤65	88	44	44		47	41	
>65	130	67	63		53	77	
Differentiation status				0.1521			0.9288
Well	46	22	24		23	23	
Moderate	147	82	65		68	79	
Poor	25	7	18		9	16	
Tumor size				0.3672			0.3019
<5 cm	105	55	50		45	60	
≥5 cm	113	56	57		55	58	
LNM				0.0957			0.1565
N0	125	70	55		61	64	
N1	69	33	36		31	38	
N2	24	8	16		8	16	
TNM				0.0126			0.0515
I	35	19	16		18	17	
II	83	43	40		38	45	
III	83	45	38		41	42	
IV	17	4	13		3	14	
Distant metastasis				0.0010			0.0055
M0	201	107	94		97	104	
M1	17	4	13		3	14	

(**Figure 5F**). The above findings indicate that *miR-526b-3p* is essential for YY1-driven CRC proliferation.

Activation of the YY1/miR-526b-3p/E2F1 axis is correlated with a poor prognosis in CRC patients

Immunohistochemical staining was applied to evaluate YY1 and E2F1 expressions in CRC tissues. As shown in **Figure 6A**, the case with high expression of YY1 and E2F1 had a stronger KI67 staining than that with low expression of both proteins. Moreover, the YY1 expression was significantly associated with the E2F1 expression in the CRC cases (**Figure 6B**). Additionally, the expression of *miR-526b-3p* was significantly lower in CRC than in adjacent tissues (**Figure 6C**; Table S2). Further analysis showed that the *miR-526b-3p* level was negatively correlated with the mRNA levels of YY1 and E2F1 in CRC tissues, and a positive correlation between YY1 and *E2F1* was determined (**Figure 6D**; Table S2). We also found that both YY1 and E2F1 levels were associated with distant metastasis and TNM stage (**Table 1**). Kaplan-Meier analysis indicated that patients with strong staining of YY1 or E2F1 had worse overall survival than those with weak staining. It is noteworthy that patients with high *miR-526b-3p* levels had a better prognosis than those with low levels (**Figure 6E**). These results suggest that the YY1/miR-526b-3p/E2F1 axis has a close association with CRC development.

Discussion

In this study, we found that high expression of YY1 in CRC suppressed the transcription of *miR-526b-3p*, which decreased the expression of E2F1 by targeting *E2f1* mRNA. Consequently, the YY1-induced upregulation of E2F1 accelerated cell cycle progression and promoted CRC cell proliferation (**Figure 7**). Clinical analyses showed that YY1 was positively correlated with E2F1, and *miR-526b-3p* was negatively correlated with YY1 or E2F1 in CRC tissues. The CRC patients with high expression of YY1 and E2F1 or low expression of *miR-526b-3p* had a poor survival rate.

Recently, we found that overexpressed E2F1 transactivated the IQ motif-containing GTPase activating protein 3 (IQGAP3) and promoted the proliferation of hepatocellular carcinoma

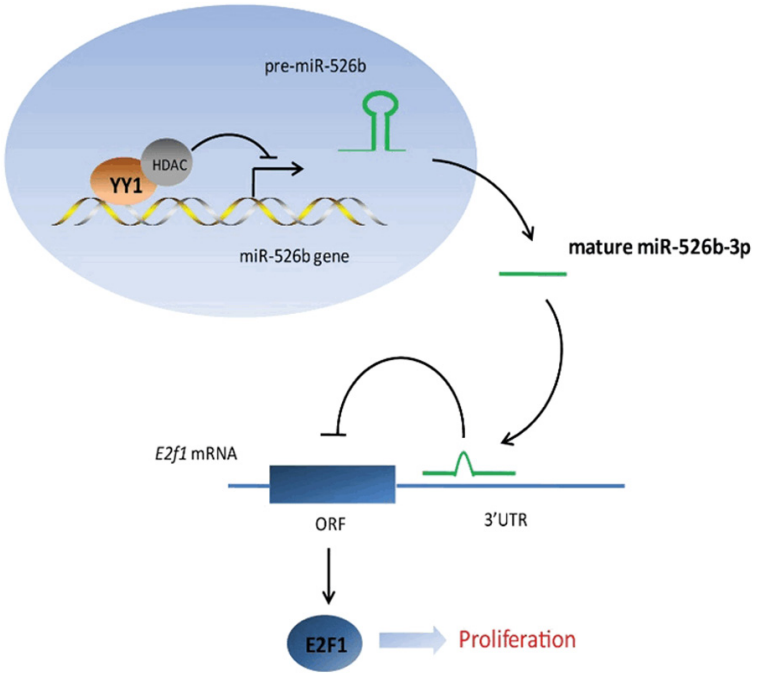

Figure 7. Schema indicating the promotive effect of YY1/*miR-526b-3p*/E2F1 axis on CRC proliferation. In CRC cells, highly expressed YY1 recruits HDAC to silence the transcription of the *miR-526b* gene, leading to the downregulation of *miR-526b-3p*, which targets the 3'UTR of *E2f1* mRNA. The resulting increased E2F1 level promotes CRC proliferation.

through IQGAP3-mediated PKC-alpha activation [20]. In colon cancer, the target genes of E2F1, thymidylate synthase (TS) and ribonucleotide reductase subunit M2 (RRM2) were elevated, effectively promoting cell cycle misregulation and oncogenesis [3, 21]. Consistently, the present study indicated that knockdown of E2F1 inhibited DNA synthesis and blocked cells from the G0 phase to the S phase, thereby suppressing CRC cell proliferation. Besides, the E2F1 level increased with tumor progression, and highly expressed E2F1 reduced the survival probability of CRC patients. TCGA database analysis showed that the *E2f1* mRNA levels were significantly up-regulated in CRC tissues compared with normal intestinal tissues, suggesting that E2F1 overexpression in CRC results from increased *E2f1* mRNA. Our previous work reported that the nuclear transcription factor Y subunit β (NFYB) transactivates the *E2F1* gene in oxaliplatin-resistant CRC cells, however NFYB was not highly expressed in nonresistant cells-perhaps not enough to explain the high expression of E2F1 in CRC cells [5]. Here, we showed that the loss of *miR-526b-3p* in CRC increased both *E2f1* mRNA and protein levels.

miR-526b-3p has been reported to be abnormally expressed in various tumors and exhibits a tumor-suppressive role in the progression of these diseases. Recent studies showed that *miR-526b-3p* was generally down-regulated in tumor tissues and can inhibit cell growth. In hepatocellular carcinoma, *miR-526b-3p* inhibited the growth of hepatocellular carcinoma cells *in vitro* and *in vivo*, whereas *miR-526b-3p* under-expression independently predicted a poor prognosis of hepatocellular carcinoma patients [22]. In addition, overexpression of *miR-526b-3p* suppressed glioma cell proliferation and down-regulation of *miR-526b-3p* was significantly associated with advanced WHO grade [23]. Zhang *et al.* reported that CRC tissues and cell lines showed lower miR-526b-3p expression levels than their normal controls [24]. In this study, we identified *miR-526b-3p* as a negative regulator of E2F1 via direct interaction with the 3'UTR of *E2f1* mRNA. Recovering *miR-526b-3p* expression inhibited the proliferation and tumorigenicity of CRC cells by decreasing *E2F1* expression. A negative correlation between *miR-526b-3p* and *E2F1* was further verified in CRC tissues and lower *miR-526b-3p* expression indicated a worse prognosis in CRC patients than the higher expression of this miRNA. Notably, we found that YY1 repressed the transcription of the *miR-526b* gene in CRC cells, suggesting that *miR-526b-5p* may also be decreased in CRC. It has been reported that *miR-526b-5p* could inhibit the proliferation of non-small cell lung cancer and gastric cancer [25, 26]. Thus, we speculated that the dysregulation of *miR-526b-5p* also promoted CRC growth.

In the present study, we found that YY1 exerted an oncogenic function by inactivating the transcription of the *miR-526b* gene in CRC. The *YY1* knockdown suppressed the proliferation and tumorigenicity of CRC cells in a *miR-526b-3p*-dependent manner. As a crucial epimodulator, YY1 recruits various epi-modifiers to regulate

target gene expression, in which histone deacetylases (HDACs) are responsible for transcription silence. Here, we found that YY1 directly bound to the *miR-526b* promoter but failed to block the transcription of *miR-526b-3p* when the HDAC inhibitor trichostatin A was added, suggesting that YY1 may silence the transcription of the *miR-526b* gene by recruiting HDACs. Several class I and II HDACs have been reported to act as co-repressors of YY1 [18, 19, 27, 28]. So, it is worthwhile to determine further which HDAC supports the YY1/HDAC co-repressor complex for the *miR-526b* gene silencing in CRC.

In conclusion, our study highlights the YY1/miR-526b-3p/E2F1 axis as a novel pathway for abnormal *E2F1* expression in CRC. Our findings may provide a novel prognostic marker and a potential therapeutic target for CRC patients.

Acknowledgements

This work was supported by The National Natural Science Foundation of China (81702401), the Zhejiang Medical and Health Science and Technology Plan (2018KY920 and 2018KY184), Taizhou Science and Technology Plan (1902KY191 and 1902KY192), and the Science and Technology Program of Sanmen County Public Technology Social Development Project (16302, 16303, 16304 and 18304).

Disclosure of conflict of interest

None.

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Table S1. The sequences of siRNA and shRNA

siRNA	sequence (5'-3')	shRNA	DNA sequence (5'-3')
scramble	UUCUCCGAACGUGUCACGUTT	scramble	TTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTTT
siE2F1	CUGCAGAGCAGAUGGUUAUTT	shYY1	GCTCCAAGAACAATAGCTTGCTTCAAGAGAGCAAGCTATTGTTCTTGGAGCTTTTTT
siYY1	GCUCCAAGAACAAUAGCUUGCTT	shmiR-526b-3p	GAAAGTGCTTCCTTTTAGAGGCTTCAAGAGAGCCTCTAAAAGGAAGCACTTTCTTTTTT

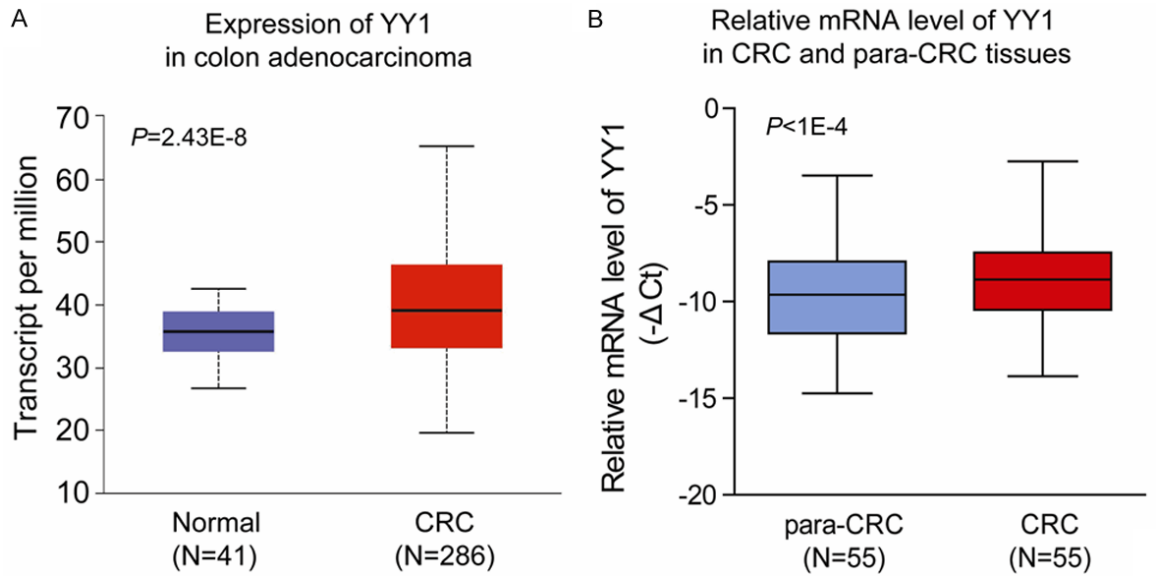

Figure S1. The expression of YY1 in CRC and normal tissues. A. The mRNA level of YY1 in CRC and normal intestinal tissues based on TCGA database, N=327. Mann-Whitney test was applied to compare the difference between the normal and cancer groups. B. The mRNA levels of YY1 in 55 paired CRC and corresponding normal tissues were measured by qPCR, using β-actin as an internal control. -ΔCt (ΔCt: Ct (YY1)-Ct (β-actin)) represented the relative expression level. Wilcoxon matched pairs test was applied to compare the difference between the normal and cancer groups.

Table S2. The relative expression levels of E2F1 mRNA, YY1 mRNA and miR-526-3p (ΔCt) CRC and para-CRC tissues

Case	E2F1 (T)	E2F1 (N)	YY1 (T)	YY1 (N)	miR-526b-3p (T)	miR-526-3p (N)
1	-11.26	-12.31	-7.81	-7.53	-0.12	12.19
2	-10.67	-11.25	-11.25	-12.38	0.25	10.42
3	-10.54	-10.24	-13.86	-14.56	-1.28	7.17
4	-10.16	-14.45	-8.30	-7.46	-0.67	7.49
5	-10.67	-13.30	-9.75	-10.73	-2.42	5.41
6	-10.04	-9.51	-11.53	-11.72	-1.43	4.91
7	-6.93	-12.20	-9.31	-12.82	-2.58	3.25
8	-9.94	-9.28	-6.83	-7.47	-3.47	1.89
9	-9.71	-12.15	-7.26	-7.92	-4.72	0.43
10	-9.53	-16.21	-8.27	-8.85	-3.14	1.57
11	-9.41	-8.25	-11.36	-13.37	-1.97	2.56
12	-8.83	-10.17	-8.63	-9.35	-4.76	-0.51
13	-8.28	-11.20	-5.29	-9.63	-4.35	-0.49
14	-7.74	-8.81	-7.38	-7.42	-4.69	-0.95
15	-7.51	-7.65	-2.75	-3.47	-3.86	-0.28
16	-7.46	-13.17	-7.62	-8.87	-3.82	-0.47
17	-6.14	-9.16	-9.27	-10.25	-5.53	-2.43

18	-7.06	-13.21	-8.83	-7.48	-4.77	-1.81
19	-6.94	-8.66	-7.54	-6.46	-3.89	-1.03
20	-6.51	-7.18	-6.32	-9.24	-3.55	-0.87
21	-9.31	-13.04	-9.57	-13.48	0.76	3.38
22	-7.25	-9.11	-10.36	-11.03	-0.77	1.68
23	-7.20	-7.71	-8.27	-10.07	-0.84	1.59
24	-7.96	-10.27	-8.73	-7.48	-1.15	1.00
25	-7.76	-12.25	-10.71	-10.75	-1.97	0.07
26	-7.41	-9.07	-6.39	-7.37	-2.62	-1.05
27	-7.28	-13.31	-5.77	-8.52	-3.36	-2.05
28	-9.45	-7.41	-9.21	-9.62	-0.38	0.54
29	-9.41	-10.38	-10.43	-8.41	-0.42	0.24
30	-9.66	-11.82	-12.36	-13.66	-0.76	-0.35
31	-9.53	-13.80	-9.84	-11.42	-1.28	-0.93
32	-9.37	-10.41	-12.38	-11.63	-2.41	-2.26
33	-9.34	-12.03	-11.50	-13.47	-2.43	-2.35
34	-9.14	-8.71	-7.46	-10.49	-3.48	-3.42
35	-8.84	-12.26	-9.48	-9.59	-3.45	-3.88
36	-8.46	-13.74	-8.26	-6.26	-3.23	-3.69
37	-9.04	-11.18	-11.75	-12.46	-0.62	-1.14
38	-9.11	-9.28	-8.62	-12.37	-0.89	-1.45
39	-9.52	-7.07	-9.76	-11.27	-1.08	-1.82
40	-9.47	-10.18	-11.15	-11.04	-1.10	-2.06
41	-9.54	-13.13	-10.65	-14.73	-1.13	-2.18
42	-8.98	-9.21	-10.16	-9.95	-0.77	-2.20
43	-8.76	-6.37	-7.43	-9.22	-1.34	-2.92
44	-8.94	-13.26	-6.28	-6.79	-2.16	-3.78
45	-8.71	-10.71	-9.18	-12.82	-2.25	-4.00
46	-8.41	-11.05	-5.42	-5.38	-2.42	-4.35
47	-8.43	-14.00	-6.47	-7.81	-2.51	-4.66
48	-8.38	-7.74	-6.53	-8.34	-1.57	-3.84
49	-8.41	-11.05	-11.75	-11.47	-1.63	-4.04
50	-8.11	-9.16	-7.51	-10.93	-2.36	-4.89
51	-7.94	-8.51	-8.38	-9.44	-2.39	-5.14
52	-8.06	-12.54	-10.42	-9.52	-3.04	-6.19
53	-9.12	-11.48	-9.69	-14.63	-2.47	-5.73
54	-9.76	-8.36	-9.76	-9.02	-2.58	-6.99
55	-6.72	-7.06	-8.86	-9.88	-2.74	-8.06