

## ORIGINAL ARTICLE

# c-KIT-ERK1/2 signaling activated ELK1 and upregulated carcinoembryonic antigen expression to promote colorectal cancer progression

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## Abstract

Carcinoembryonic antigen (CEA) is highly expressed in embryo and colorectal cancer (CRC) and has been widely used as a marker for CRC. Emerging evidence has demonstrated that elevated CEA levels promote CRC progression. However, the mechanism of the increased CEA expression in patients with primary and recurrent CRC is still an open question. In this study, we showed that c-KIT, ELK1, and CEA were hyperexpressed in patients with CRC, especially patients with recurrent disease. From bioinformatics analysis, we picked ELK1 as a candidate transcription factor (TF) for CEA; the binding site of ELK1 within the CEA promoter was confirmed by chromatin immunoprecipitation and dual luciferase reporter assays. Overexpression of ELK1 increased CEA expression in vitro, while knockdown of ELK1 decreased CEA. Upregulated ELK1 promoted the adhesion, migration, and invasion of CRC cells, however knockdown of CEA blocked the activities of ELK1-overexpressed CRC cells. Furthermore, we explored the role of c-KIT-ERK1/2 signaling in activation of ELK1. Blocking c-KIT signaling using Imatinib or ISCK03 reduced p-ELK1 expression and consequently decreased CEA levels in CRC cells, as did blocking the ERK1/2 pathway by U0126. Compared with wild type littermates, the *c-kit* loss-of-functional *Wads<sup>m/m</sup>* mice showed lowered c-KIT, ELK1, and CEA expression. In conclusion, our study revealed that ELK1, which was activated by c-KIT-ERK1/2 signaling, was a key TF for CEA expression. Blocking ELK1 or its upstream signaling could be an alternative way to decelerate CRC progression. Besides being a biomarker for CRC, CEA could be used for guiding targeted therapy.

## KEYWORDS

CEA, c-KIT, colorectal cancer, ELK1, targeted therapy

**Abbreviations:** CCK-8, cell counting kit-8; CEA, carcinoembryonic antigen; CRC, colorectal cancer; FCM, flow cytometry; GIST, gastrointestinal stromal tumor; MUT, mutant; PI, propidium iodide; qRT-PCR, quantitative RT-PCR; RTCA, real-time cellular analyzer; s-CEA, serum CEA; SCF, stem cell factor; TCF, ternary complex factor; TF, transcription factor; TSS, transcription start site.

Jian Ma and Xiaohui Liu contributed equally to this work.

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## 1 | INTRODUCTION

Colorectal cancer (CRC) ranks third and second, respectively, in terms of incidence and mortality worldwide.<sup>1</sup> There has been great advancement in the diagnosis and treatment of CRC, unfortunately many patients with CRC still suffer from tumor recurrence or metastasis after standardized treatment, resulting in a poor prognosis.<sup>2</sup> In recent years, a series of targeted therapeutic drugs has been approved for oncotherapy and these have shown promising effects. Targeted drugs aim to specifically inhibit or block precise oncogenes, therefore the aim in applying targeted drugs is to find out the precise oncogene(s) in a particular cancer.

Adhesion molecules play an important role in tumor progression and metastasis of CRC.<sup>3</sup> Carcinoembryonic antigen (CEA; also known as CEACAM5 or CD66e) is an intercellular adhesion molecule and a founding member of the CEACAM family, which is highly expressed in embryonic gut and CRC tissues. The pivotal roles of CEA in cellular biological events have been reviewed thoroughly, including inhibition of differentiation programs, anoikis and apoptosis, the disruption of cell polarization and tissue architecture, and facilitation of invasiveness by CRC.<sup>4</sup> In addition, CEA acts in the liver through its interaction with its receptor on Kupffer cells and causes activation and production of pro-inflammatory and anti-inflammatory cytokines, including IL-1, IL-10, IL-6, and TNF- $\alpha$ . These cytokines protect CRC cells from cytotoxicity by nitric oxide (NO) and other reactive oxygen radicals, and thus increase the survival of metastatic cells, which exhibit enhanced metastasis.<sup>5</sup> Although many studies have shown that CEA can promote CRC progression, the upstream regulatory signaling of CEA has not been well demonstrated. Due to their tumor-promoting effects, antibodies and vaccines that target CEA have been developed, but there is still much debate as to their sensitivity and effectiveness.<sup>6-9</sup> Here, we propose that, instead of targeting CEA itself, blocking upstream signaling that regulates CEA expression could be an alternative in order to decrease CEA and slow down CRC progression.

Transcriptional regulation is a fundamental step in gene expression and involves TF binding to specific promoter regions of target genes. Based on a TF binding site algorithm, ELK1, a member of the TCF subfamily of ETS-domain TFs, was selected as a candidate for CEA expression in this study. ELK1 functions through phosphorylation and can be regulated by the mitogen-activated protein kinase (MAPK) pathway.<sup>10,11</sup> Interestingly, ELK1 is highly expressed and hyperactivated in some cancers, including gastric cancer,<sup>12</sup> urothelial carcinoma,<sup>13</sup> hepatocarcinoma,<sup>14</sup> and cervical and endometrial carcinomas.<sup>15</sup> A global analysis of TFs suggested that ELK1 is an important TF, involved in CRC progression, and might be a potential biomarker for CRC treatment.<sup>16</sup> Therefore, special interest was paid to the function of ELK1 in CRC development and whether it was able to transcriptionally regulate its potential target CEA.

## 2 | MATERIALS AND METHODS

### 2.1 | Bioinformatics analysis

The University of California, Santa Cruz (UCSC) database was used to search for sites 2000 bp upstream and 100 bp downstream of the CEA TSS.<sup>17</sup> Potential TFs able to bind to the CEA promoter were predicted by PROMO software.<sup>18,19</sup> CEA and ELK1 expression levels in patients with CRC with or without recurrence were analyzed using The Cancer Genome Atlas (TCGA) data from cBioPortal.<sup>20,21</sup> mRNA expression in CEA and ELK1 in CRC and normal tissues, as well as the correlation between these 2 genes was analyzed using the GEPIA2 server.<sup>22</sup>

### 2.2 | Tissue samples and clinicopathological information

In total, samples from 30 patients with CRC and the corresponding adjacent normal tissues were obtained from Beijing Friendship Hospital, Capital Medical University. All patient cases were reviewed by a pathologist and diagnosed as CRC based on histopathological evaluation. Clinicopathological information for 349 patients from January, 2005 to December, 2012 was obtained from the Beijing Friendship Hospital.

### 2.3 | Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using TRIzol (Sigma-Aldrich, USA), in accordance with the manufacturer's instructions. qRT-PCR was performed on an RT-PCR 7500 system (ABI Company, USA) using the Powerup SYBR Master Mix (Thermo Scientific, USA). The oligonucleotide sequences of the qRT-PCR primers are listed in Table 1.

**TABLE 1** Primers

Gene	qRT-PCR primer sequences
CEA	Forward: 5'-TTACCTTTCGGGAGCGAACC-3' Reverse: 5'-TGTTGCTGCGGTATCCCATT-3'
ELK1	Forward: 5'-ACGCTACACAGGTACCCC-3' Reverse: 5'-TCTTGTCTTGCGTAGCCCC-3'
c-KIT	Forward: 5'-CAGGCAACGTTGACTATCAGT-3' Reverse: 5'-ATTCTCAGACTTGGGATAATC-3'
GAPDH	Forward: 5'-CCTGCACCACCAACTGCTTA-3' Reverse: 5'-AGTGATGGCATGGACTGTGG-3'
ChIP-qPCR primer sequences	
CEA promoter	Forward: 5'-TGACCCACGTGATGCTGAGA-3' Reverse: 5'-GTCAAGGCTGCTGTGACTGT-3'

## 2.4 | Western blot

Total protein was extracted from radioimmunoprecipitation assay (RIPA) lysate (Appligen, Beijing, China). Primary antibodies included mouse monoclonal anti-CEA (#2383, CST, USA), ELK1 (sc-365876, Santa Cruz, USA) and p-ELK1 (sc-8406, Santa Cruz), rabbit monoclonal anti-c-KIT (#3074, CST), p-KIT (#3391, CST), ERK1/2 (#9102, CST) and p-ERK1/2 (#9101, CST). Anti- $\beta$ -actin (sc-47778, Santa Cruz) was used as the internal control. Immunoblotting bands were detected using ECL chemiluminescence (Thermo Scientific) and viewed under a Fusion FX Vilber Lourmat imager (France).

## 2.5 | Immunohistochemical staining

Paraffin sections were provided by the Beijing Friendship Hospital, Capital Medical University. Sections were incubated with mouse monoclonal anti-ELK1 (sc-365876, Santa Cruz) or CEA (#2383, CST) at 4°C overnight. Staining detection was performed using the UltraSensitive™ SP (Mouse/Rabbit) IHC Kit (KIT-9710, MXB, Fuzhou, China) in accordance with the manufacturer's instructions; 0.1% BSA was used as the negative control throughout.

## 2.6 | Cell culture and treatment

CRC cell lines (HT-29, LS174T and Caco-2) and the HEK293T cell line were used in this study. All cells were cultured in DMEM (Gibco, USA) and supplemented with 10% FBS, (Biological Industries, Israel), 100 U/mL of penicillin-streptomycin (Gibco) and 1% of mycoplasma removal agent Myco-3 (AppliChem, German). Cells were grown at 37°C in the presence of 5% CO<sub>2</sub> in air.

Reagents included 50 ng/mL recombinant human stem cell factor (rhSCF, 255-SC, R&D Systems, USA) added for 15 min, 1  $\mu$ mol/L 12-O-tetradecanoylphorbol 13-acetate (TPA) (P8139, Sigma-Aldrich) added for 2.5 h, 10  $\mu$ mol/L U0126 (#9903, CST) added 2.5 h, 2  $\mu$ mol/L Imatinib (STI571, S1026, Selleck, Shanghai, China) added for 2.5 h and 30  $\mu$ mol/L ISCK03 (ab145066, Abcam) added for 2.5 h

## 2.7 | Chromatin immunoprecipitation

ChIP assay was performed in accordance with the manufacturer's instructions (EZ-Magna ChIP™ A Kit, #17-408, Merck-Millipore, USA). Chromatin was immunoprecipitated for 24 h at 4°C using rabbit monoclonal anti-ELK1 (ab-32106, Abcam). Primers are shown in Table 1.

## 2.8 | Dual luciferase reporter assay

CEA promoter luciferase reporter plasmids containing a predicted WT ELK1 binding site (5'-CTCAGGGCAGAGGGAGGAAGGACAGCA GACCA-3') and its mutant (MUT, 5'-CTCAGGGCAGAATACATCGAGA

CAGCAGACCA-3') were constructed by support from Genechem (Shanghai, China). The reporter plasmids were co-transfected with ELK1 overexpression plasmid (Genechem) into HEK293T cells using Lipofectamine™ 2000 (Thermo Scientific). At 48 h after transfection, firefly and Renilla luciferase activities were measured using the Dual Luciferase Assay System (72050, Promega, USA) in a Multiskan FC photometer (Thermo Scientific). The ratio of firefly/Renilla activity was calculated.

## 2.9 | Upregulation and downregulation assays in vitro

An ELK1 overexpression plasmid was constructed by Genechem and transfected into CRC cells using Lipofectamine™ 2000. Overexpression of c-KIT was achieved by the introduction of lentivirus and constructed by Genechem. For knockdown of ELK1, the shELK1 lentivirus was introduced into CRC cells by support from Genechem. The vector structures are shown in Figure S1.

## 2.10 | Cell adhesion evaluation by crystal violet staining

Cells were seeded into a 96-well plate, with the bottom of each well covered by 1% Matrigel (BD, USA) prepared in serum-free medium. After a 40-min incubation at 37°C in the presence of 5% CO<sub>2</sub> in air, the medium was removed and the cells were fixed in 4% para-formaldehyde for 30 min on a shaker. Cells were stained with crystal violet for 15 min. Photographs were taken and viewed under a microscope (Leica BME, Germany). Adhesive cells were counted using ImageJ software (NIH, USA).

## 2.11 | Cell migration and invasion detection by RTCA

RTCA (ACEA Biosciences, USA) was used to monitor cellular migration and invasion as previously described.<sup>23</sup>

## 2.12 | Wound healing assay

Cell monolayers were scratched in the center using a 10  $\mu$ L pipette tip to create a 1 mm cell-free area. Wound areas were visualized under a microscope (Nikon ECLIPSE 80i, Japan) at time points 0 and 24 h. Cell migratory capability was evaluated by measuring the gap closure and analyzed by ImageJ software.

## 2.13 | Cell proliferation detection by cell counting kit-8 (CCK-8) assay

Cell proliferation was detected by CCK-8 (Dojindo Laboratories, Japan) in accordance with the manufacturer's protocol. Absorbance was measured at 450 nm using a Multiskan FC photometer.

## 2.14 | Cell cycle detection by flow cytometry (FCM)

Cells were fixed in 70% ethanol for 2 h at 4°C, followed by RNase treatment (50 µg/mL) for 30 min at 37°C. Cells were stained with propidium iodide (PI; 500 µg/mL, Beyotime Biotechnology, China) for 30 s in the dark. Cell cycle was analyzed by measuring the DNA contents in Coulter EPLCS XL (Beckman Coulter, USA).

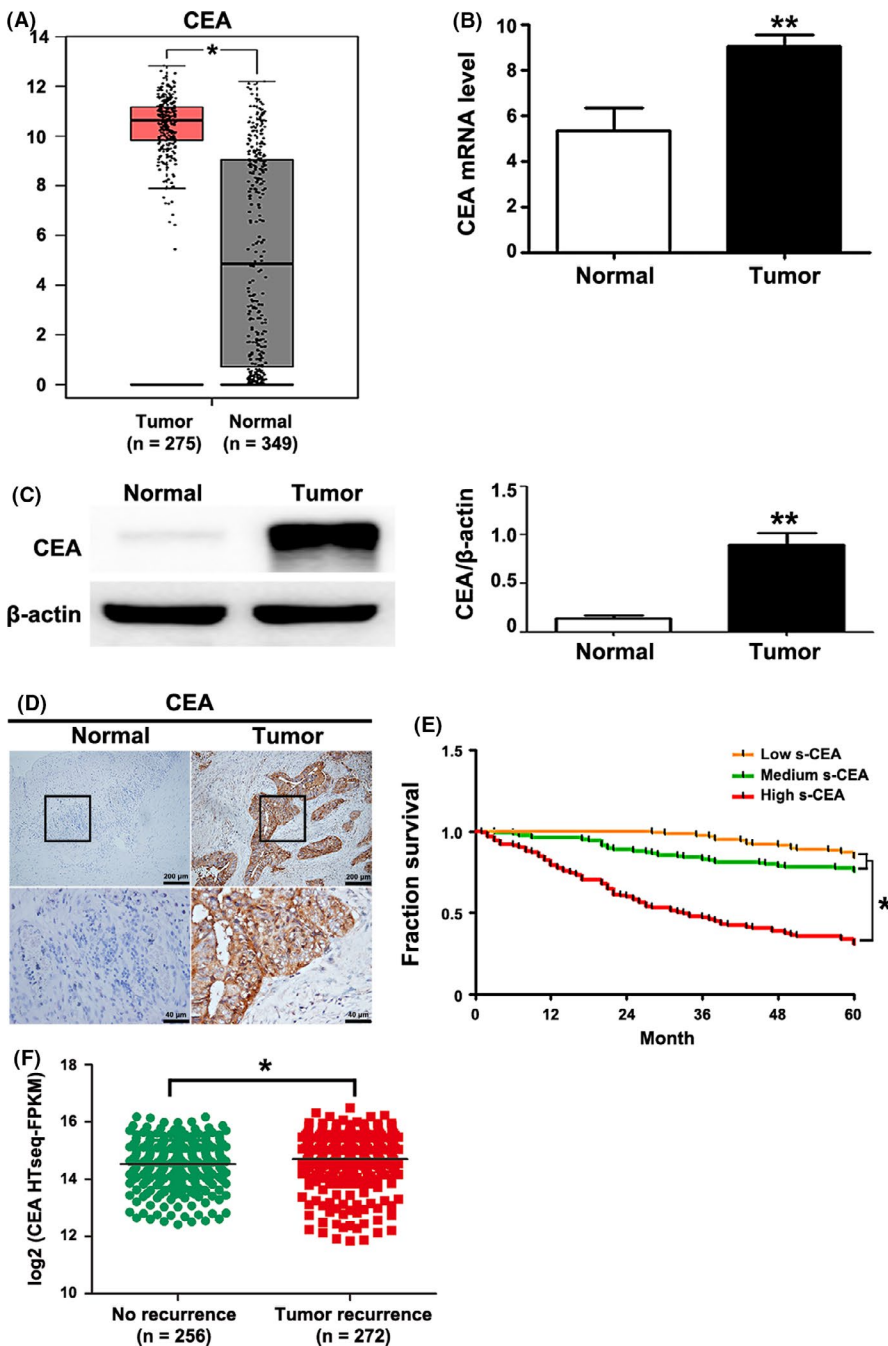
## 2.15 | Cell apoptosis detection by FCM

The PE Annexin V Apoptosis Detection Kit with 7-AAD (Biolegend, USA) was used in accordance with the manufacturer's

instruction. Cell apoptosis was analyzed on a Coulter EPICS XL spectrometer.

## 2.16 | c-kit loss-of-functional mutant mice (Wads<sup>m/m</sup>)

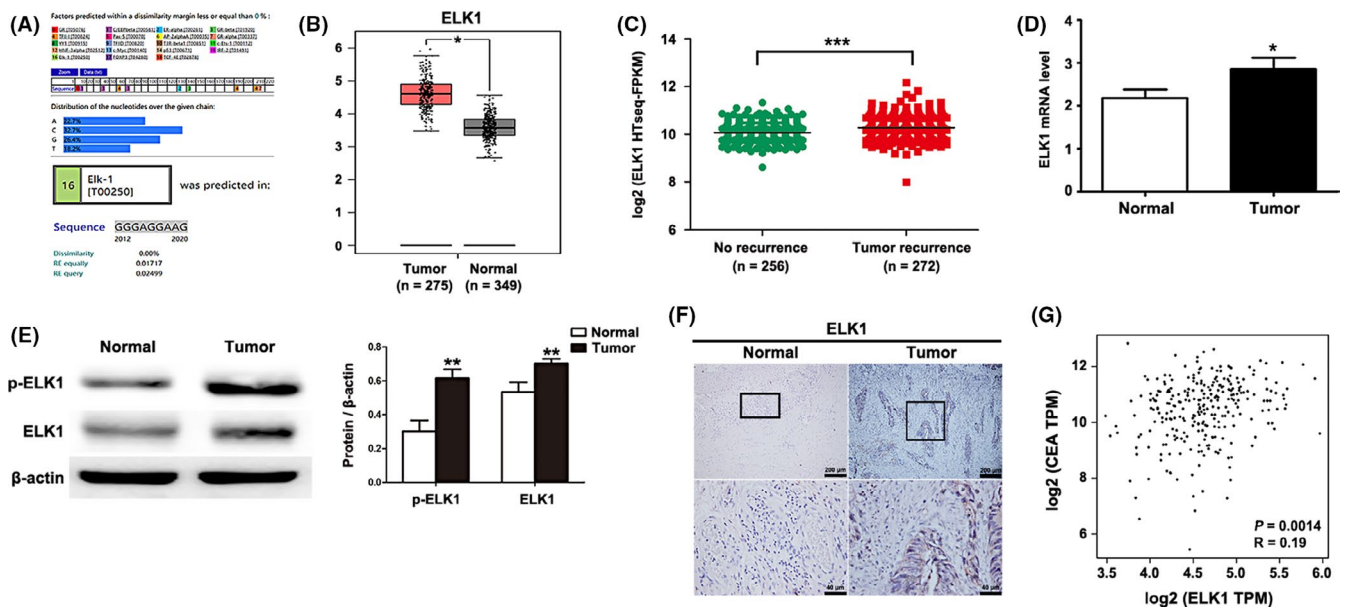
Five *c-kit* loss-of-functional mutant mice (Wads<sup>m/m</sup>) and 5 WT littermates were used, both of which were on the C57BL/6 background and used in a previous study.<sup>24</sup> Animal studies were carried out strictly under protocols approved by the Animal Care and Use Committee of Capital Medical University. Every effort was made to minimize the number of mice used as well as their suffering.



**FIGURE 1** CEA is highly expressed in patients with CRC and associated with a poor prognosis. A, CEA mRNA levels from CRC tissues (n = 275) were significantly higher compared with those from normal tissues (n = 349) obtained from the GEPIA2 database. B, CEA mRNA levels from samples from 30 patients with CRC, who were diagnosed at the Beijing Friendship Hospital, Capital Medical University, were elevated compared with the corresponding adjacent normal tissues. C, CEA protein levels were also markedly increased in the 30 CRC tissues compared with adjacent normal tissues. Left: Representative western blot bands for CEA with  $\beta$ -actin as control. Right: Statistical analysis on 30 samples. D, Representative immunohistochemical staining images of CEA in CRC tissues and adjacent normal tissues. CEA staining was markedly strong in CRC tissues, and was mainly located at the cell membrane. Framed views in the upper panel are shown magnified in the lower panel. E, Patients with high s-CEA expression have significantly shorter life spans compared with those with low or medium s-CEA expression, illustrated by the Kaplan-Meier survival curve. F, CEA mRNA levels were significantly higher in patients with recurrent CRC (n = 272) compared with that in patients with no recurrent disease (n = 256) after radical operation as illustrated by TCGA database. \* $P < .05$ , \*\* $P < .01$

**TABLE 2** Clinicopathological characteristics in patients with CRC with different s-CEA levels

Clinicopathological indexes		Low s-CEA	Medium s-CEA	High s-CEA	P
		(<5.00) ng/mL	(5.00-13.11) ng/mL	(13.11-325.06) ng/mL	
Age (y)	≤65	67	60	46	.029
	>65	58	65	78	
Sex	Male	61	67	69	.539
	Female	64	58	55	
Tumor size (cm)	<5	69	51	34	< .001
	≥5	56	74	90	
Tumor differentiation	II	74	39	22	< .001
	III	42	71	65	
Tumor location	Colon	70	89	92	.005
	Rectum	55	36	32	
Distant metastasis stage	M0	116	110	87	< .001
	M1	9	15	37	
Tumor recurrence	Yes	14	23	57	< .001
	No	111	102	67	



**FIGURE 2** ELK1 is highly expressed in patients with CRC especially patients with recurrent disease and positively correlated with CEA expression. A, Predicted transcription factors (TFs) that have the capability to bind to the CEA promoter, using bioinformatics analysis. The maximum matrix dissimilarity is set as zero, and with 18 output TFs. B, ELK1 mRNA levels in CRC tissues (n = 275) were significantly higher compared with normal tissues (n = 349) based on analysis from the GEPIA2 database. C, ELK1 mRNA in the recurrent patients with CRC (n = 272) maintains high compared with patients with no recurrent disease (n = 256) after radical operation as assessed by TCGA database. D, ELK1 mRNA expression in the diagnosed 30 CRC in Beijing Friendship Hospital, Capital Medical University is increased compared with corresponding adjacent normal tissues. E, ELK1 protein as well as its active form, the p-ELK1, is clearly increased in the 30 CRC tissues in comparison with adjacent normal tissues. Left: Representative western blot bands for ELK1 and p-ELK1 with  $\beta$ -actin as control. Right: Statistical analysis on the 30 samples. F, Representative immunohistochemical staining images of ELK1 in CRC tissues and adjacent normal tissues. ELK1 staining is more intensive in CRC tissues, and was mainly located in the cytoplasm and nucleus. The framed views in the upper panel are magnified in the lower panel. G, ELK1 mRNA expression is positively correlated with the CEA mRNA analyzed on GEPIA2 database. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$



## 2.17 | Statistics

Results were presented as the mean  $\pm$  SEM *T* test, ANOVA; chi-square test analyses were performed using GraphPad Prism 5 and SPSS23.0 software. A *P*-value of .05 or less was considered statistically significant.

## 3 | RESULTS

### 3.1 | CEA is highly expressed in patients with CRC especially those with recurrence

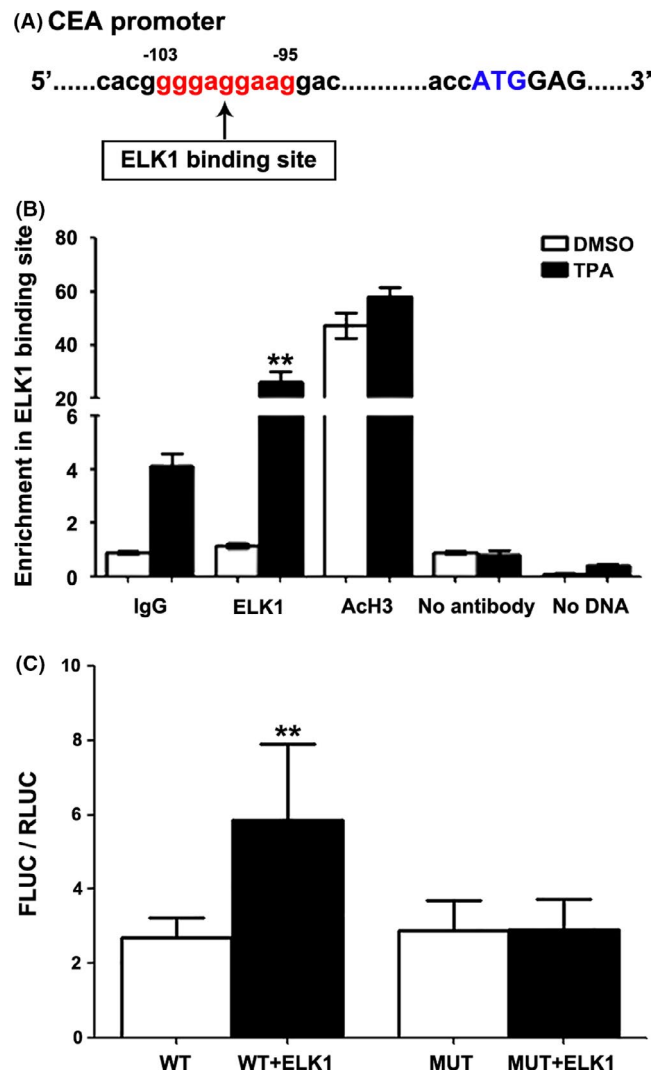
From analysis of the GEPIA2 data, we found that CEA was highly expressed in tumors compared with normal mucosa (Figure 1A). Consistent with the GEPIA2 analysis, the CEA mRNA and protein were highly expressed in CRC tissues compared with that in the corresponding adjacent normal tissues from 30 patients with CRC at the Beijing Friendship Hospital, Capital Medical University (Figure 1B-D). We further collected the clinical information of 349 patients with CRC from the Beijing Friendship Hospital, Capital Medical University. Based on their serum CEA (s-CEA), patients were divided into 3 groups: low s-CEA (<5 ng/mL, lower than the clinical diagnostic criterion), medium s-CEA (5.00-13.11 ng/mL) and high s-CEA (13.11-325.06 ng/mL). Compared with the low and the medium s-CEA groups, the high s-CEA group had larger tumor sizes and worse differentiation (Table 2). Significantly, patients with CRC with the high s-CEA were apt to suffer from metastasis and recurrence, but there was no difference between the low s-CEA and medium s-CEA groups (Table 2). A follow-up study suggested that the patients in the high s-CEA group had a shorter survival time compared with the other groups (Figure 1E). CEA mRNA levels were significantly higher in patients with CRC with recurrence (Figure 1F). These results indicated that higher expression of CEA might indicate CRC progression and relapse.

### 3.2 | ELK1 is highly expressed in CRC and associated with tumor recurrence

To find out the key TF that could promote CEA transcription, we performed bioinformatics analysis using the UCSC and PROMO databases, which predicted ELK1 as a potential TF for CEA transcription as ELK1 was likely to bind to the CEA promoter (Figure 2A). As for CEA, ELK1 expression prominently increased in patients with CRC (Figure 2B) and in those who had relapsed (Figure 2C), indicated by GEPIA2 and TCGA data. ELK1 mRNA and protein levels were hyperexpressed in the 30 CRC tissues compared with corresponding adjacent normal tissues collected from the Beijing Friendship Hospital, Capital Medical University (Figure 2D-F). Significantly, there was a positive correlation between ELK1 and CEA mRNA levels (Figure 2G).

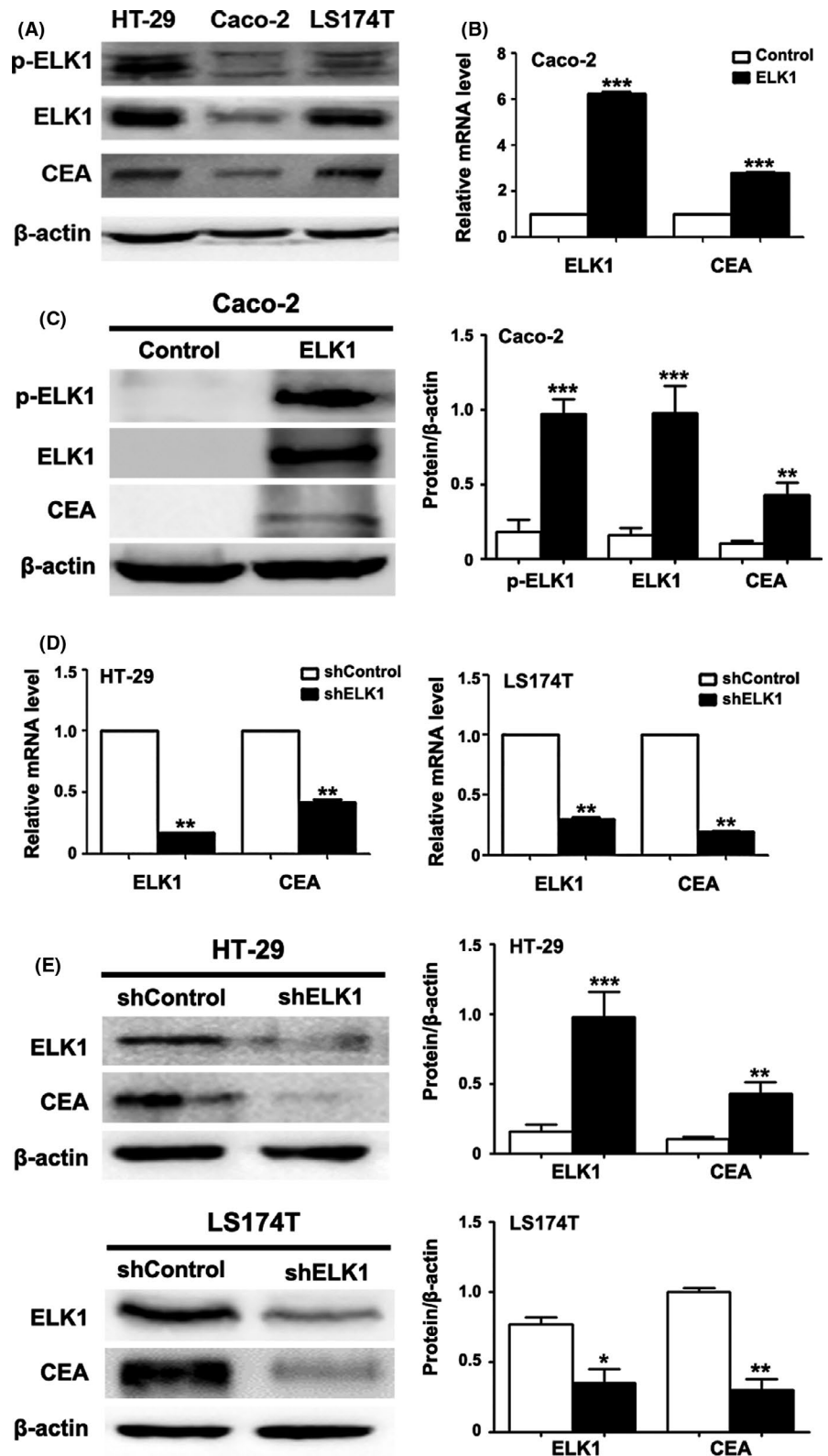
### 3.3 | ELK1 can bind to CEA promoter to raise CEA expression

The predicted binding site for ELK1 within the CEA promoter is located within the region 95-103 bp upstream of the TSS (Figure 3A). To confirm this binding, we performed ChIP assay following TPA (ERK1/2 agonist) treatment to activate ELK1. As expected, the result verified that ELK1 could bind to the CEA promoter (Figure 3B). Next, based on the sequence of the ELK1 binding site, we designed a mutated



**FIGURE 3** The binding site of ELK1 in CEA promoter was confirmed by ChIP and luciferase assays. A, Predicted binding site and sequence of ELK1 within the CEA promoter using UCSC and PROMO databases. The ATG sequence marked in blue indicates the transcription start site (TSS). B, ChIP assay was performed on the LS174T cells treated with DMSO or 1  $\mu$ mol/L of TPA for 3 h. Compared with DMSO group, the binding of ELK1 to the CEA promoter is evidently increased in the TPA-treated cells, in which ELK1 is hyperactivated. Ach3 is used as a positive control and IgG as a negative control. C, Luciferase activity of the WT CEA promoter-driven luciferase reporter is significantly increased by the co-transfected ELK1 plasmid. ELK1 plasmid transfection is unable to increase the luciferase activity of the MUT CEA promoter-driven luciferase reporter. \*\**P* < .01

**FIGURE 4** ELK1 upregulates CEA expression in vitro. A, Basic expression levels of p-ELK1, ELK1, and CEA in HT-29, Caco-2, and LS174T cells were detected. B, CEA mRNA is increased by the overexpression of ELK1 in Caco-2 cells. C, CEA protein is equally well increased in the ELK1 overexpressed Caco-2 cells. Left: Representative western blot bands for ELK1 and p-ELK1 and CEA with  $\beta$ -actin as the control. Right: Statistical analysis on 3 repeats. D, E, Specific ELK1 shRNA (shELK1) effectively knocks down ELK1 in HT-29 and LS174T cells, which leads to a decrease in CEA mRNA (D) and protein (E). Left: Representative western blot bands for ELK1 and CEA with  $\beta$ -actin as control. Right: Statistical analysis on 3 repeats. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$



sequence for use in a dual luciferase reporter assay, which also showed that ELK1 was able to bind to the WT CEA promoter and accelerate its transcription but not that of the MUT CEA promoter (Figure 3C).

Upon upregulation of ELK1 in Caco-2 cells, which have relatively low expression of ELK1 and CEA (Figure 4A), mRNA and protein levels for CEA both increased (Figure 4B,C). Conversely,

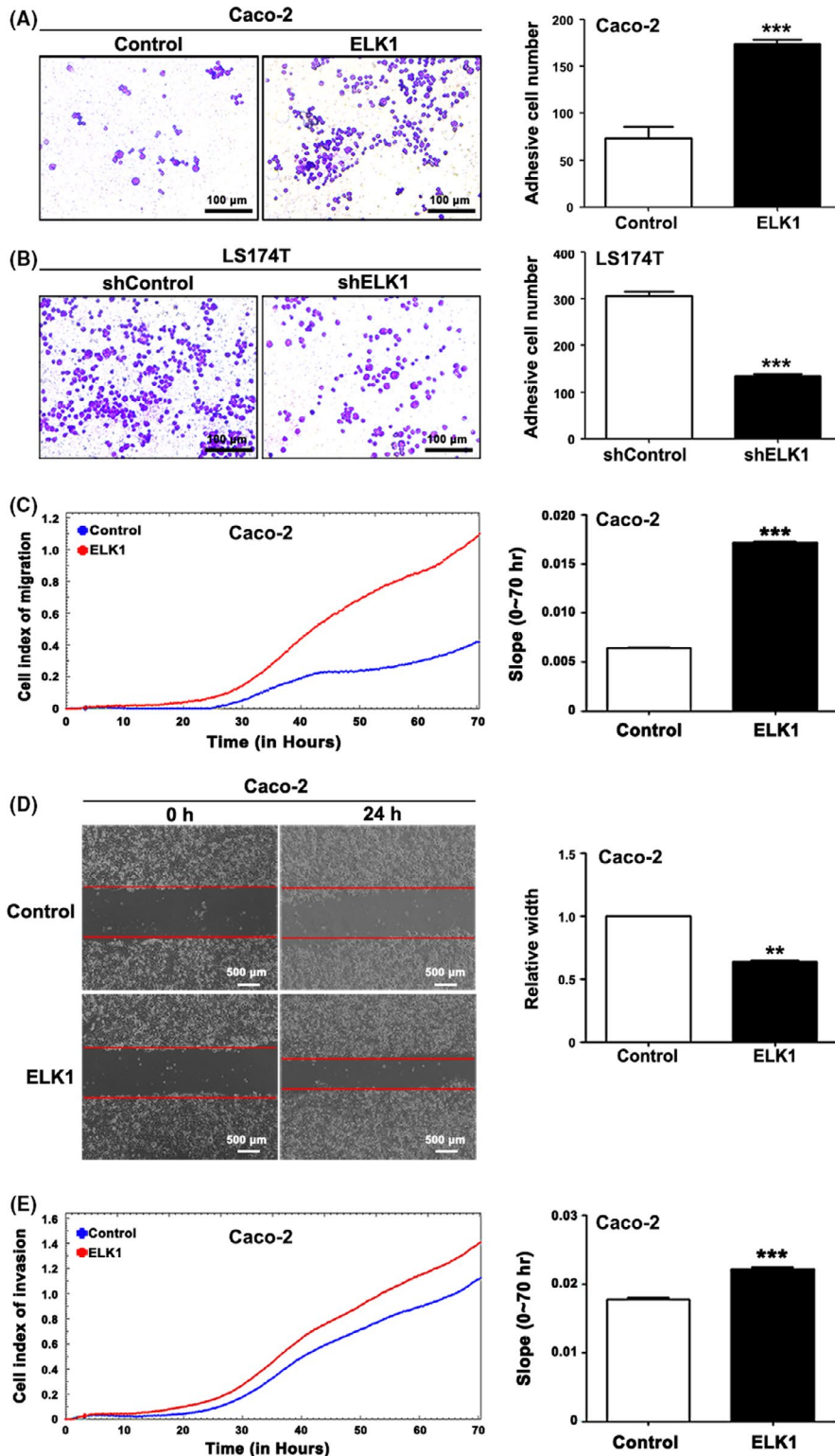
knockdown of ELK1 using a specific shRNA in HT-29 and LS174T cells, which have relatively high expression levels of ELK1 and CEA (Figure 4A), resulted in the decrease of CEA (Figures 4D, E and S2).

These results strongly indicated that ELK1 can bind to the CEA promoter and promote the expression of CEA in CRC cells.

### 3.4 | ELK1 could promote CRC progression by enhancing CRC cell adhesion, migration, and invasion by increasing CEA expression

Although it has been reported that ELK1 is hyperexpressed in CRC and could be a marker for prognosis of CRC, there has been very limited evidence demonstrating its role in CRC development. In this study, we used *in vitro* upregulation and downregulation

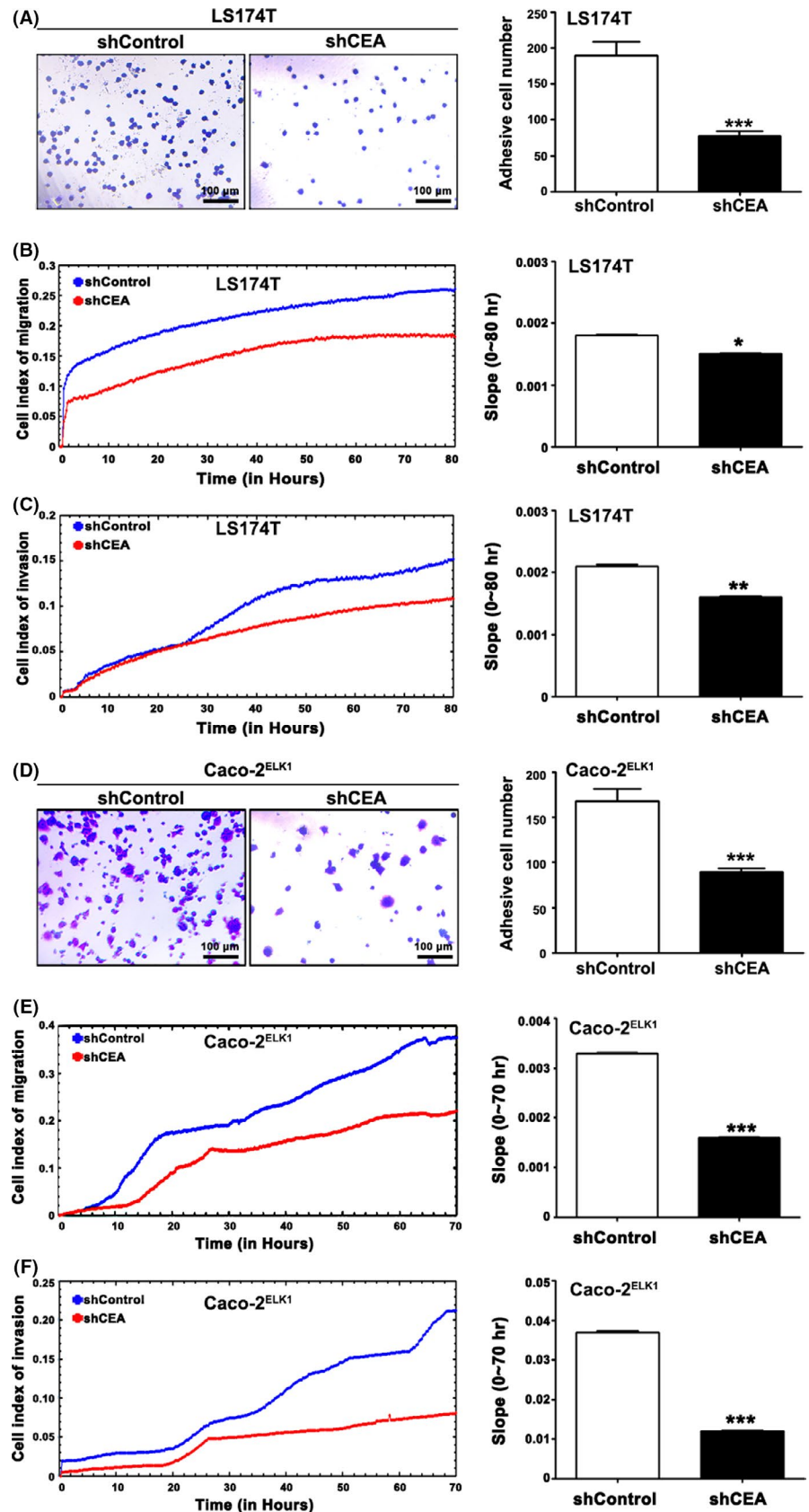
strategies to investigate the role of ELK1 in CRC cell activities. As shown in Figure 5, upregulation of ELK1 enhanced the adhesive ability of CRC cells, while downregulation of ELK1 reduced it (Figure 5A,B). Similarly, the abilities for migration and invasion increased in ELK1-overexpressed CRC cells (Figure 5C-E). However, we did not observe significant changes in cell proliferation, the cell cycle, or apoptosis when ELK1 was upregulated in CRC cells (Figure S3).



**FIGURE 5** ELK1 accelerates CRC cell adhesion, invasion, and migration. A, Overexpression of ELK1 clearly promotes the adhesive capacity of Caco-2 cells, indicated by more crystal violet-stained cells. Left: Representative images for crystal violet staining for 15 min. Right: Statistical analysis on 6 repeats. B, In contrast, knockdown of ELK1 decreased the adhesion capacity of LS174T cells, indicated by reduced numbers of crystal violet-stained cells. Left: Representative images for crystal violet staining for 15 min. Right: Statistical analysis on 6 repeats. C, Overexpression of ELK1 significantly promotes migration of Caco-2 cells monitored by RTCA for a successive 70 h period. Left: Recorded image. Right: Statistical analysis on 4 repeats. D, Wound healing migration assay further confirms that overexpression of ELK1 does promote cell migration, suggested by the decreased gap 24 h after the scratch. Left: Images visualized under an inverted microscope. Right: Statistical analysis on 4 repeats. E, Overexpression of ELK1 significantly promotes invasion of Caco-2 cells monitored by RTCA for a successive 70 h period. Left: Recorded image. Right: Statistical analysis on 4 repeats. \*\* $P < .01$ , \*\*\* $P < .001$



**FIGURE 6** ELK1 promotes CRC progression through increasing CEA expression. A, Knockdown of CEA in LS174T cells decreases cell adhesion, indicated by the reduced numbers of crystal violet-stained cells. Left: Representative images for crystal violet staining for 15 min. Right: Statistical analysis on 6 repeats. B, Migratory ability of LS174T cells is inhibited when CEA is knocked down. Left: Recorded image by RTCA for a 80 h period. Right: Statistical analysis on 4 repeats. C, The invasive ability is equally well inhibited in CEA knocked-down LS174T cells. Left: Recorded image by RTCA for 80 h. Right: Statistical analysis on 4 repeats. D, The number of adhesive cells stained by crystal violet is decreased in ELK1 overexpressed Caco-2 cells (Caco-2<sup>ELK1</sup>) upon introduction of shCEA. Left: Representative images for crystal violet staining for 15 min. Right: Statistical analysis on 6 repeats. E, F, CEA knocked-down Caco-2<sup>ELK1</sup> cells display impaired migratory (E) and invasive (F) abilities compared with Caco-2<sup>ELK1</sup> cells. Left: Recorded image by RTCA for 70 h. Right: Statistical analysis on 4 repeats. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$



To explore whether the tumor-promoting role of ELK1 was by induction of CEA expression, we detected activities in CRC cells that had been treated with shCEA. Results showed that cell adhesion,

migration, and invasion of LS174T cells were significantly inhibited upon knockdown of CEA (Figure 6A-C), confirming that CEA played a promoting role in CRC cell motility. Furthermore, ELK1-transfected

Caco-2 cells (Caco-2<sup>ELK1</sup>) displayed decreased adhesive, migratory, and invasive abilities when CEA was knocked down (Figure 6D-F).

These results suggested that highly ELK1 expression promoted the progression of CRC through increasing CEA expression.

### 3.5 | c-KIT-ERK1/2-ELK1 signaling upregulates CEA

In light of previous studies and Kyoto Encyclopedia of Genes and Genomes (KEGG) database analysis, we concentrated on the receptor tyrosine kinase (RTK)-RAS-MAPK signaling pathway that probably regulates ELK1 activation in CRC.<sup>25,26</sup> c-KIT (also known as KIT or CD117), a type III RTK receptor, is highly expressed and activated in most patients with CRC.<sup>27</sup> Activated c-KIT promotes CRC cell proliferation, survival, migration, and invasion by activating multiple downstream pathways including the MAPK pathway.<sup>19-21,28</sup> Here, we found that c-KIT mRNA and protein were highly expressed in CRC tissues (Figure 7A,B) which was consistent with previous reports. Activation of c-KIT by lentiviral mediation and addition of rhSCF, the ligand for c-KIT, induced upregulation and activation of ERK1/2 and ELK1 in CRC cells, as well as increased CEA expression (Figure 7C). Imatinib, an RTK inhibitor, and ISCK03, a specific c-KIT inhibitor that blocks SCF-induced c-KIT phosphorylation,<sup>29,30</sup> could inactivate c-KIT and subsequently inhibit phosphorylation of ERK1/2 and ELK1 and CEA expression (Figure 7D). Treatment with U0126, a MEK inhibitor, also decreased p-ERK1/2, p-ELK1 and CEA expression (Figure 7D). Furthermore, we used *c-kit* loss-of-functional mutant mice (Wads<sup>m/m</sup>) and their WT littermates to detect the activity of the downstream ERK1/2-ELK1 pathway. Wads<sup>m/m</sup> mice harbor a unique T-to-C transition mutation in the *c-kit* gene that results in a Phe-to-Ser substitution at amino acid 856 within a highly conserved tyrosine kinase domain and they displays the phenotype of white, anemia, deaf, and sterile and therefore, are an optimal model to investigate the function and downstream pathway of c-KIT signaling. The result showed that with loss of c-KIT activity in Wads<sup>m/m</sup> mice, the downstream ERK1/2-ELK1 pathway was significantly inhibited (Figure 7E). CEA bands were not detect in either mice strains, possibly because both were tumor free and adult, with minute CEA expression levels. These results suggested that c-KIT-ERK1/2 signaling

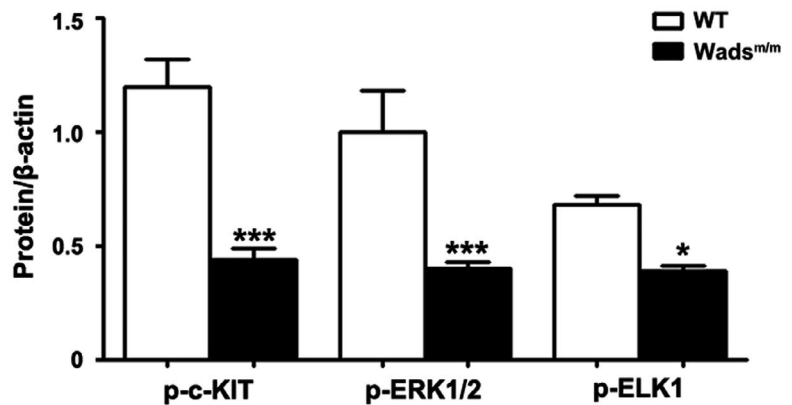
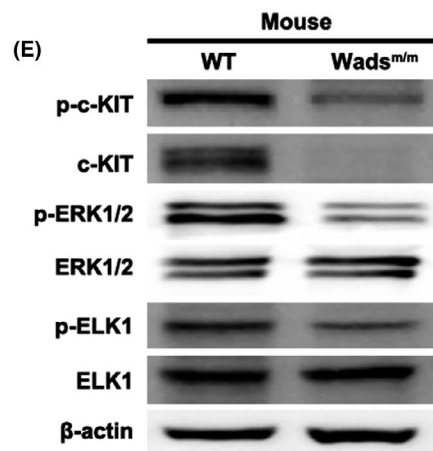
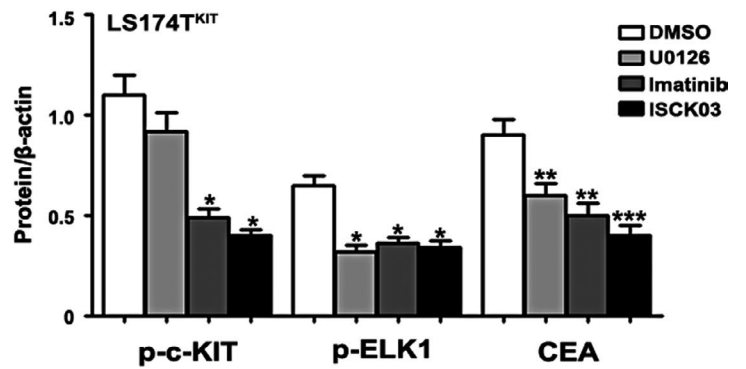
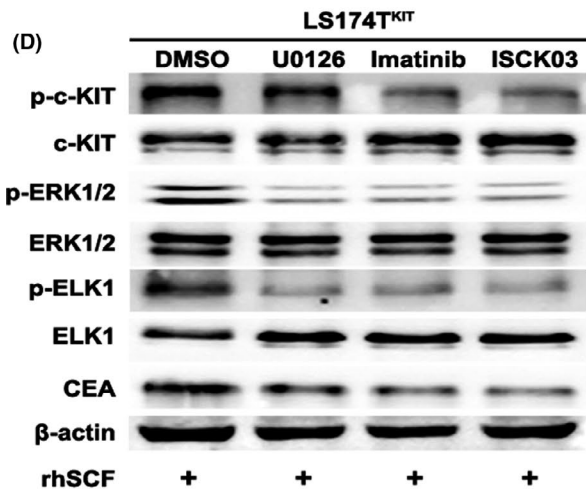
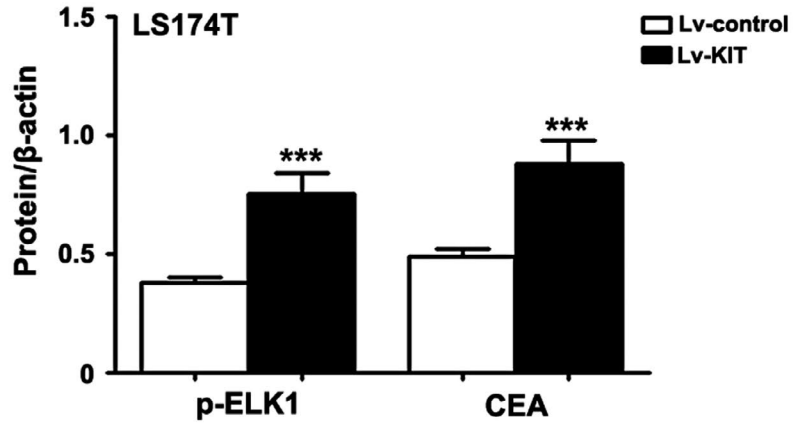
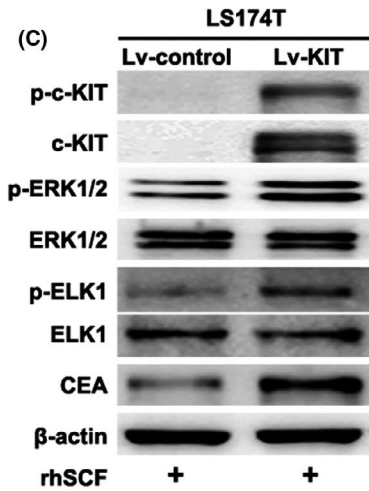
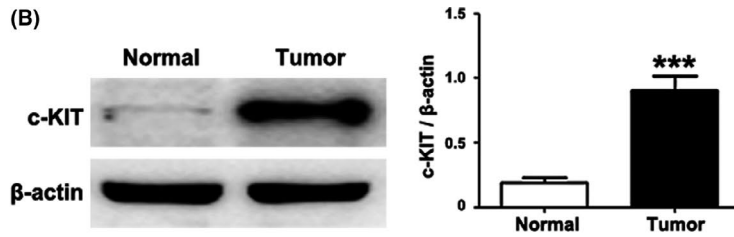
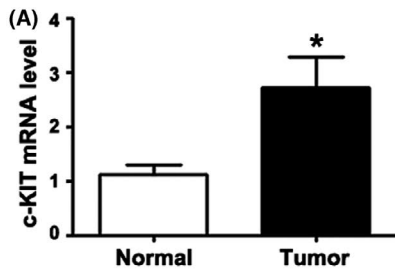
could activate ELK1 and elevate CEA expression in CRC cells and provided evidence that blocking this signaling could decrease activation of ELK and CEA expression in patients with CRC.

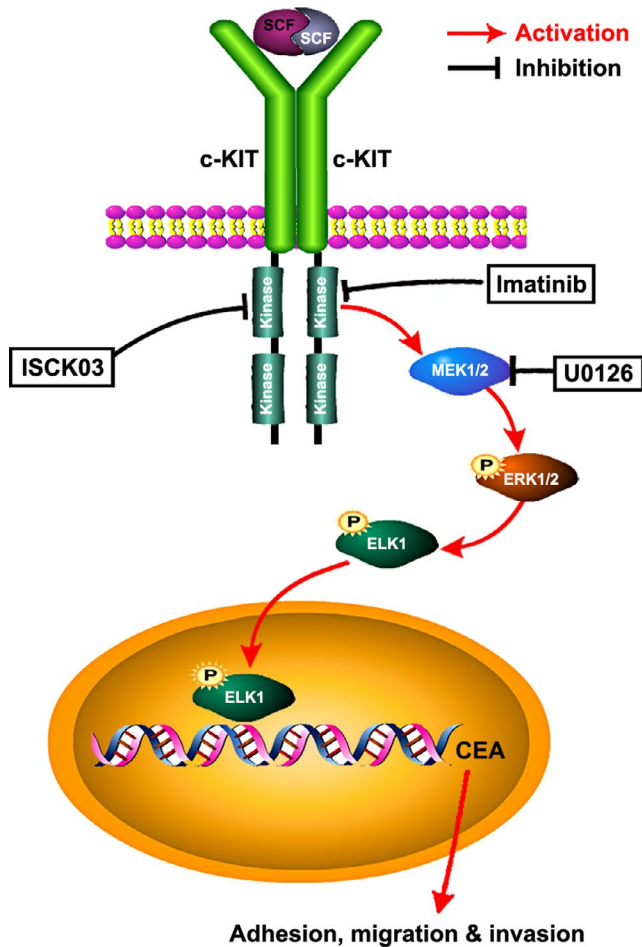
## 4 | DISCUSSION

In the present study, in addition to the implication that higher CEA levels were associated with CRC deterioration, we also explored if CEA expression was under the control of the c-KIT-ERK1/2-ELK1 axis.

CEA has been widely used as a CRC marker. Although, over recent years, researchers have paid attention to the biological roles of CEA in CRC development, there have been few studies on its transcriptional regulation. A few reports have implied that Upstream stimulatory factor 1 (USF), Sp1, Sp1-like, and other unidentified TFs, were probably responsible for CEA transcription.<sup>31</sup> With the rapid development of bioinformatics, it has become easier to screen TF candidates for genes with known promoter sequences. Here, we chose ELK1 based on the following reasons: (i) ELK1 is hyperexpressed in CRC and positively correlated with CEA mRNA levels; (ii) ELK1 is related to CRC differentiation, suggesting a potentially important transcriptional or biochemical role<sup>32</sup>; and (iii) the predicted ELK1 binding site is around 100 bp upstream of the TSS within the CEA promoter and which is covered by the core promoter region.<sup>33</sup> As expected, we confirmed binding of ELK1 to the CEA promoter using ChIP and dual luciferase reporter assays. ELK1 functions by stimulating its target genes. In bladder cancer, ELK1 promoted tumorigenesis by triggering its downstream molecules, including *c-Fos* and a proto-oncogene.<sup>34</sup> ELK1 also promoted clear cell renal cell carcinoma and breast cancer progression by upregulating *NDUFA4L2* and *PAI-1*, respectively.<sup>35,36</sup> However, the role of ELK1 in CRC has not been reported. Our results disclosed that hyperactivated ELK1 (p-ELK1) facilitated CRC cell adhesion, migration, and invasion. ELK1-driven activities was partly inhibited following knockdown of CEA in CRC cells, indicating that induction of CEA expression could be a critical way in which ELK1 elicited its CRC-promoting effect. Accordingly, blocking ELK1 activation might be a strategy for CRC treatment. One publication in 2018 in a murine model demonstrated that a transactivator of a transcription peptide (TAT-DEF-ELK1 peptide; TDE) that specifically impeded ERK and ELK1 molecular interaction and subsequent

**FIGURE 7** C-KIT-ERK1/2 signaling activates ELK1 and upregulates CEA expression in CRC. A, c-KIT mRNA levels from 30 patients diagnosed with CRC at the Beijing Friendship Hospital, Capital Medical University were increased compared with the corresponding adjacent normal tissues. B, c-KIT protein is also clearly increased in the tissue samples from 30 patients with CRC compared with adjacent normal tissues. Left: Representative western blot bands for c-KIT with  $\beta$ -actin as control. Right: Statistical analysis on the 30 samples. C, c-KIT is overexpressed in LS174T cells following lentiviral mediation. Upon additional treatment with 50 ng/mL of rhSCF for 15 min, c-KIT and its downstream ELK1/2 are hyperactivated in c-KIT overexpressed LS174T cells, with activation of ELK1 and increased expression of CEA. Left: Representative western blot bands with  $\beta$ -actin as control. Right: Statistical analysis on 3 repeats. D, c-KIT overexpressed LS174T cells (LS174T<sup>KIT</sup>) are treated with 10  $\mu$ mol/L U0126, 2  $\mu$ mol/L Imatinib or 30  $\mu$ mol/L ISCK03 for 2.5 h and 50 ng/mL of rhSCF for another 15 min. Compared with the DMSO control group, cells in the U0126 groups have decreased p-ERK1/2, p-ELK1, and CEA expression levels. In addition, both Imatinib and ISCK03 could significantly inhibit c-KIT phosphorylation which inhibits downstream p-ERK1/2 and p-ELK1 as well as CEA expression. Left: Representative western blot bands with  $\beta$ -actin as control. Right: Statistical analysis on 3 repeats. E, *c-kit* loss-of-functional Wads<sup>m/m</sup> mice are used to further verify the role of c-KIT signaling in ELK1 expression in vivo. Compared with their WT littermates, the Wads<sup>m/m</sup> mice had decreased c-KIT, ERK1/2 and ELK1 activities. Left: Representative western blot bands with  $\beta$ -actin as control. Right: Statistical analysis on 5 mice. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$





**FIGURE 8** Scheme of c-KIT-ERK1/2-ELK1 signaling pathway that upregulates CEA expression and promotes CRC. Upon activation of c-KIT signaling, downstream ERK1/2 and ELK1 are activated. P-ELK1 elevates CEA transcription, which promotes CRC cell adhesion, migration, and invasion

ELK1 activation by interfering with the DEF-docking domain, had the advantage of treating depression.<sup>37</sup> Future studies are necessary to explore whether this means is also efficient for treating CRC. Most patients with CRC have concomitant anxiety, and the anxiety may aggravate CRC progression.<sup>38</sup> We proposed that blocking ELK1 activation might alleviate anxiety, and would be beneficial for treatment of patients with CRC. As ELK1 is involved in multiple diseases, it should have many targets *in vivo* that deserve further investigations.

Nuclear ELK1 is mainly regulated and activated by the MAPK pathway,<sup>39</sup> which is a key pathway downstream of c-KIT. We have previously shown that hyperactivated c-KIT signaling promoted CRC cell proliferation, migration, and invasion.<sup>28</sup> Here, we revealed that c-KIT could increase CEA expression through the ERK1/2-ELK1 pathway to promote CRC progression. Some RTK or c-KIT targeted drugs have been approved for the clinic and have displayed marked effects. For example, Imatinib and its analogs were firstly used to treat CML and gastrointestinal stromal tumors (GIST) associated with constitutive activation of BCR-ABL and c-KIT, respectively.<sup>40</sup> Human CRCs hyperexpress c-KIT, especially in the most aggressive subtype, the consensus

molecular subtype 4 (CMS4), which has the highest chance of disease recurrence. A proof-of-concept study shed light on the biological effects of Imatinib intervention on CMS4 tumors and should form the basis for design of future trials with subtype-targeted therapy in patients with CRC.<sup>41</sup> According to the present results that c-KIT signaling upregulates CEA, we suggest that CEA might be an indicator for applying c-KIT and/or ELK1 inhibitors to CRC treatment.

In conclusion, we demonstrated that ELK1 was a key TF to elevate CEA expression in CRC, and was under c-KIT-ERK1/2 signaling regulation (Figure 8). The hyperactivated c-KIT-ERK1/2-ELK1-CEA axis accelerated CRC progression. Blocking ELK1 or its upstream signaling could be an alternative way to decelerate CRC progression. In addition to a biomarker for CRC diagnosis, CEA could also be a marker to guide targeted drug selection for CRC treatment.

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#### DISCLOSURE

We have no conflicts of interest to declare.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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