# lncRNA NEAT1 facilitates the progression of colorectal cancer via the KDM5A/Cul4A and Wnt signaling pathway

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**Abstract.** Colorectal cancer (CRC) is a major cause of cancer-related mortality. The aberrant expression of long non-coding RNAs (lncRNAs) is implicated in the pathogenesis of CRC. The present study investigated the role of lncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) in CRC. lncRNA NEAT1 expression was detected in CRC tissues and cell lines. HCT116 cells were transfected with si-NEAT1, and the malignant behavior of the cells was detected. The binding associations between NEAT1 and E2F1, as well as between E2F1 and KDM5A were verified. si-NEAT1-transfected cells were also transfected with si-KDM5A. H3K4me3 methylation and cullin 4A (Cul4A) expression in HCT116 cells were detected. The si-NEAT1-transfected cells were also transfected with pc-Cul4A. Proteins related to the Wnt pathway were detected. A xenograft model of CRC using nude mice was established and the mice were injected with si-NEAT1-transfected HCT116 cells. lncRNA NEAT1 was found to be upregulated in CRC tissues and cells. NEAT1 silencing inhibited the malignant behaviors of the HCT116 cells. lncRNA NEAT1 inhibited KDM5A expression by binding to E2F1. The downregulation of KDM5A reversed the inhibitory effects of NEAT1 silencing on the malignant behavior of the cells. KDM5A inhibited Cul4A expression via the demethylation of H3K4me3. The overexpression of Cul4A promoted the malignant behavior of the si-NEAT1-transfected HCT116 cells. lncRNA NEAT1 activated the Wnt pathway via KDM5A/Cul4A. *In vivo* experiments confirmed the role of NEAT1 in CRC. On the whole, the present study demonstrates that lncRNA NEAT1 facilitates the progression of CRC via the KDM5A/Cul4A/Wnt axis.

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## Introduction

Colorectal cancer (CRC) is the third most common type of cancer worldwide and the fourth leading cause of cancer-related mortality, exceeded by lung cancer, liver cancer and gastric cancer (1). The occurrence of CRC is the result of the gradual accumulation of genetic and epigenetic alterations, which lead to homeostasis dysfunction and neoplastic transformation (2). Age, genetic and environmental factors are widely involved in the initiation of CRC; other recognized risk factors include inflammatory bowel disease, obesity, a sedentary lifestyle, a history of abdominal radiation and acromegaly (3). Despite recent advancements being made in screening strategies and effective treatments, the prognosis of patients with advanced CRC remains poor. Furthermore, the latest molecular targeted agents seem to be active only for metastatic CRC, and they exponentially increase the cost of CRC treatment (4). Therefore, methods for the determination of potent diagnostic and prognostic biomarkers are urgently required for the effective intervention of CRC.

Long non-coding RNAs (lncRNAs) are a class of non-coding RNA transcripts that are >200 nucleotides in length and lack protein-coding ability (5). Dysregulated lncRNA expression is closely related to the pathogenesis of cancer, metabolic disorders and cardiovascular diseases (6). lncRNAs are implicated in tumorigenesis through different types of molecular mechanisms, and certain regulatory factors often lead to the abnormal expression of lncRNAs in CRC, thus causing malignant transformation (7). In addition, the association of abnormally regulated lncRNAs with clinical outcomes suggests the potential of lncRNAs as effective diagnostic and prognostic predictors and therapeutic targets for CRC (8). Nuclear paraspeckle assembly transcript 1 (NEAT1) is an emerging lncRNA located at nuclear paraspeckles (9). The aberrant overexpression of NEAT1 occurs in solid tumors, which is typically responsible for the poor survival of patients (10). NEAT1 drives carcinogenesis and progression by regulating the expression of genes involved in

cancer cell growth, migration and invasion, as well as epithelial-mesenchymal transition (EMT) and chemotherapeutic resistance (11). Notably, lncRNA NEAT1 knockdown has been shown to enhance 5-fluorouracil sensitivity in patients with CRC by attenuating autophagy (12). Although NEAT1 is generally viewed as a diagnostic and prognostic marker for CRC (13), the specific mechanisms of action of NEAT1 in CRC remain unclear.

Thus, the present study aimed to determine the lncRNA NEAT1 interacting partners and elucidate the molecular mechanisms underlying the oncogenic functions of NEAT1 in CRC. The findings presented herein may provide a novel theoretical basis for the management of CRC.

## Materials and methods

*Ethics statement*. The use of ovarian tissues was approved by the Ethics Committee of the Second Affiliated Hospital of Soochow University. Informed consent was signed by each eligible participant. All animal experiments are approved by the Ethics Committee of the Second Affiliated Hospital of Soochow University (S.No. 20190523b116). All experimental procedures were implemented on the Ethical Guidelines for the study of experimental pain in conscious animals.

*Bioinformatics analysis*. lncRNA NEAT1-related diseases were searched through the lncdisease database (14). The differential expression of NEAT1 and Cul4A in CRC samples (n=286) and normal samples (n=41) collected by TCGA were searched through the UALCAN cancer database (http://ualcan.path.uab.edu/analysis.html) (15). The differential expression of Cul4A in CRC samples (n=97) and normal samples (n=100) collected by CPTAC were searched through the UALCAN cancer database (http://ualcan.path.uab.edu/analysis.html) (15). The cellular localization of NEAT1 was predicted through LncMAP database (http://bio-bigdata.hrbmu.edu.cn/LncMAP/) (16). The NEAT1-related lncRNA-transcription factor-gene regulatory network in CRC was searched through LncMAP database (http://bio-bigdata.hrbmu.edu.cn/LncMAP/).

*Tissue samples*. The present study recruited 55 patients with CRC (30 males and 25 females) aged 27-49 years, from December, 2018 to December, 2019, at the Second Affiliated Hospital of Soochow University. All cancer tissues were collected via surgical tumor resection, and the adjacent non-cancerous tissues were used as negative controls (NCs). For the experiment, the cancer tissues and normal tissues of each patient were matched. The inclusion criteria were as follows: Confirmed CRC by pathology, compliance with surgical indications, no tumor-specific therapy before operation, complete clinicopathological data available and informed consent provided. The exclusion criteria were as follows: Patients with other tumors, gastrointestinal dysfunction, autoimmune diseases, or infectious diseases. The tissue fragments were refrigerated in liquid nitrogen immediately after dissection and stored at -80˚C.

*Cells and cell culture*. The human epithelial cell line, NCM460 (CC-YM02142, Shanghai Enzyme Research Biotechnology Co., Ltd.) and the HCT116 human CRC cell line (CCL-247™, American Type Culture Collection) were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin (100 U/ml)/streptomycin (0.1 mg/ml) (Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified incubator at 37˚C with 5% $CO_2$. The cells were used in subsequent experiments when they reached 60% confluency.

*Cell treatment*. Small interfering RNA (siRNA) targeting lncRNA NEAT1 (si-NEAT1-1, si-NEAT1-2 and si-NEAT1-3) and its NC (si-NC, Shanghai GenePharma Co., Ltd.) were transfected into the HCT116 cells, respectively at a final concentration of 50 nM. siRNA targeting E2F1 and its NC (Sangon Biotech, Co., Ltd.) termed si-E2F1 and si-NC, respectively, were transfected into the HCT116 cells. si-NEAT1-1 was transfected with si-KDM5A and its NC (Sangon Biotech, Co., Ltd.) or pcDNA-Cul4A and its pcDNA-NC (Sangon Biotech, Co., Ltd.) into HCT116 cells, and termed si-NEAT1 + si-KDM5A, si-NEAT1 + si-NC, si-NEAT1 + pc-Cul4A and si-NEAT1 + pc-NC, respectively. All transfections (2 $\mu$l siRNA or 1,000 ng plasmid) were conducted using Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.). Untreated HCT116 cells were used as the blank group. CPI-455 is a specific pan-KDM5A inhibitor, and the $IC_{50}$ of KDM5A is 10 nM (17). HCT116 cells were treated with 15 $\mu$mol/l CPI-455 (CAS 1628208-23-0, Topscience Co., Ltd.) or phosphate-buffered saline (PBS) for 48 h (18), and termed PBS and CPI-455. Subsequent experiments were carried out after 24 h.

*Colony formation assay*. The 2X RPMI-1640 medium containing 20% FBS (Gibco; Thermo Fisher Scientific, Inc.) and $2x10^3$ HCT116 cells were mixed with an equal volume of 0.7% agarose (Sigma-Aldrich, Merck KGaA). The mixture was immediately placed into six-well plates (Thermo Fisher Scientific Inc.) containing 0.5% agarose substrate made from 1X RPMI-1640 medium supplemented with 10% FBS and cultured at 37˚C with 5% $CO_2$ for 10 days. The medium was refreshed every three days. The medium was removed and the cells were washed with PBS and fixed with 4% paraformaldehyde for 20 min. The paraformaldehyde was then removed and the cells were stained with 0.2% crystal violet (Beyotime Institute of Biotechnology, Shanghai, China) at 37˚C for 5 min. The colonies were analyzed using ImageJ software v1.8.0 (National Institutes of Health).

*Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay*. TUNEL assay was performed using an apoptosis detection kit (KeyGEN Biotech Corp., Ltd.). The specific operations were as follows: The cells were fixed with 4% polyformaldehyde for 20 min, washed with PBS, and treated with 100 $\mu$l protease K (20 g/ml) was at room temperature for 20 min. The cells were then washed with PBS for 5 min, immersed in 4% paraformaldehyde for 5 min, and then supplemented with 100 $\mu$l DNase I buffer for 5 min. After the liquid was removed, the cells were detached with 100 $\mu$l DNase I (200 U/ml) for 10 min, washed with deionized water 4 times and PBS for 5 min, cultured with 10 $\mu$l equilibration solution in a wet box for 10 min, and supplemented with 100 $\mu$l TUNEL reaction mixture solution. After applying the sealing

film, the cells were reacted at 37˚C for 1 h in the dark in a wet box and immersed in 20X SSC for 15 min to terminate the reaction. TUNEL-positive HCT116 cells in five random fields of vision of each well were observed and counted under a fluorescence microscope (Olympus Corporation) to calculate the proportion of TUNEL-positive cells.

*Transwell assay*. HCT116 cells ($2x10^5$) were seeded into Matrigel-coated (for invasion detection) or uncoated (for migration detection) apical chambers. HCT116 cells were suspended in serum-free RPMI1640 medium and seeded into the apical chamber with $2x10^5$ cells per well. RPMI-1640 medium containing 10% FBS was added to the basolateral chamber. Following 24 h of incubation at 37˚C with 5% $CO_2$, the non-migrated or non-invasive cells were scraped off using a cotton swab. The cells at the bottom of the chamber were fixed with methanol (Sigma-Aldrich, Merck KGaA) for 10 min and stained with 0.5% crystal violet at room temperature for 20 min. Five visual fields were selected and randomly photographed using an inverted microscope (Nikon Corporation).

*RNA-fluorescence in situ hybridization (FISH)*. RNA-FISH was used to determine the localization of lncRNA NEAT1 in the cells. The DNA oligo probe of NEAT1 (FAM-labeled) was purchased from Shanghai GenePharma Co., Ltd.. Subsequently, $1x10^5$ HCT116 cells were seeded into 24-well plates, and the medium was removed after 24 h. Following three washes with PBS, the cells were fixed with paraformaldehyde and pre-hybridized with PBS containing 0.5% Triton X-100. The cells were then subjected to hybridization buffer with NEAT1 probe at 4˚C overnight. The nuclei were stained with 4',6-diamidino-2-phenylindole at room temperature for 5 min (Beyotime Institute of Biotechnology). Images were captured under a Leica SP5 confocal microscope (Leica Microsystems GmbH).

*Nuclear/cytosol fractionation assay*. The cytoplasmic and nuclear extracts were obtained using the NE-PER nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific, Inc.). NEAT1 expression in the nucleoplasm extract was then detected by reverse transcription-quantitative PCR (RT-qPCR) as described below.

*RNA immunoprecipitation (RIP)*. Anti-E2F1 (5 $\mu$g/mg, ab179445, Abcam) was used for RIP assay with immunoglobulin G (IgG) (ab172730, Abcam) as the control. RIP was performed in accordance with the instructions of the Magna RIP™ RNA-binding protein immunoprecipitation kit (Merck KGaA). The isolated RNAs were purified, and the co-precipitated RNAs were detected by RT-qPCR as described below.

*RNA pull-down assay*. Biotin-labeled lncRNA NEAT1 and NC (Sangon Biotech, Co., Ltd.) were incubated with HCT116 cell lysate then supplemented with streptavidin-coated magnetic beads (Life Technologies; Thermo Fisher Scientific, Inc.). The biotin-conjugated RNA complex was used for the RNA pull-down assay. The expression of E2F1 was detected by RT-qPCR as described below.

*Xenograft model of CRC using nude mice*. A xenograft model of CRC was established in nude mice aged four to six weeks. Nude mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. and kept in isolation cages. The humane endpoint used in the animal experiments was when the tumor growth burden was >10% of the body weight of the animal, the average tumor diameter was >20 mm, or the tumor metastasized or grew rapidly to ulcer, causing infection or necrosis. The mice were divided into two groups (12 mice per group) as follows: The si-NEAT1 group (0.2 ml of PBS containing si-NEAT1-1-treated $2x10^6$ HCT116 cells subcutaneously injected into the right armpit of the mice) and the si-NC group (0.2 ml of PBS containing si-NC-treated $2x10^6$ HCT116 cells subcutaneously injected into the right armpit of the mice). The mice were monitored every day. The tumor volume was measured every three days using the following formula: Volume = (length x $width^2$)/2. The nude mice were euthanized by an intraperitoneal injection of pentobarbital sodium (≥100 mg/kg) at 21 days after the injection. It was observed that the animals had no spontaneous breathing for 2-3 min without blinking reflex, which confirmed the death of the animals. The tumors of six mice in each group were excised for immunohistochemistry, and those from the other six mice were used for RT-qPCR.

*Immunohistochemistry*. After dewaxing, dehydration and antigen repair, the tissue sections were blocked with goat serum (Beyotime Institute of Biotechnology) for 20 min, and the sheep serum was discarded. The tissue sections were cultured overnight with the primary antibody (1:200, ab16667, Abcam) at 4˚C. The tissue sections were then cultured with the secondary antibody (1:2,000, ab205718, Abcam) and developed using DAB (ZSGB-Bio Co., Ltd.). The nuclei were counterstained with 15% hematoxylin (Beyotime Institute of Biotechnology), followed by observation under a microscope (CKX41, Olympus Corporation).

*RT-qPCR*. Total RNA was extracted from the cells and tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific Inc.). Subsequently, 1 $\mu$g RNA was reverse transcribed into cDNA using PrimerScript RT master mix (Takara Biotechnology Co., Ltd.). RT-qPCR was performed using the Quantitative SYBR-Green PCR kit (Qiagen GmbH) and the 7500 Fast Real Time PCR System (Applied Biosystems; Thermo Fisher Scientific Inc.). The reaction conditions were as follows: Pre-denaturation at 95˚C for 5 min, and then 40 cycles of denaturation at 95˚C for 10 sec, annealing at 65˚C for 20 sec, and extension at 72˚C for 30 sec. The primers used are presented in Table I. The data were analyzed using the $2^{-\Delta\Delta Cq}$ method (19).

*Western blot analysis*. Total protein was isolated from HCT116 cells using radio-immunoprecipitation assay buffer (Duanhuan Biotechnology Co., Ltd.), and the protein concentration was determined using the bicinchoninic acid (BCA) kit (Duanhuan Biotechnology Co., Ltd.). The protein was separated on 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with PBS containing 5% skim milk at room temperature for 2 h. The membranes were then incubated with the primary antibodies,

Table I. Primer sequences used for RT-qPCR.

| Primer | Sequence (5'-3') |
|---|---|
| NEAT1 (H) | F: ATGGAGCCCCGTGACCTCTCACCT |
| | R: CTAGACCTGCCATTTCTCACACAC |
| KDM5A (H) | F: ATGGCGGGCGTGGGGCCGGGGGG |
| | R: CTAACTGGTCTCTTTAAGATCCTC |
| E2F1 (H) | F: ATGGCCTTGGCCGGGGCCCCTGCG |
| | R: TCAGAAATCCAGGGGGGTGAGGTC |
| Cul4A (H) | F: ATGGCGGACGAGGCCCCGCGGAA |
| | R: TCAGGCCACGTAGTGGTACTGA |
| NEAT1 (M) | F: ATGGGGGTAGAGGCGTTCGACTGC |
| | R: CATATCTGGTGCCAAAAGTATTA |
| KDM5A (M) | F: ATGGCGTCCGTGGGCCCGGGGGGCT |
| | R: CTAACTGGTCTCTTTAAGATCCT |
| Cul4A (M) | F: ATGGCGGACGAGGGCCCTCGGA |
| | R: TCATGCCACGTAGTGGTACTGA |
| U6 (H) | F: CGCTTCGGCAGCACATATAC |
| | R: AATATGGAACGCTTCACGA |
| GAPDH (H) | F: ATGGTTTACATGTTCCAATATG |
| | R: TTACTCCTTGGAGGCCATGTGG |
| U6 (M) | F: GTGCTCGCTTCGGCAGCACATATA |
| | R: AATATGGAACGCTTCACGAATT |
| GAPDH (M) | F: ATGCTGCCCTTACCCCGGGGTCC |
| | R: TTACTCCTTGGAGGCCATGTAGGC |

NEAT1, nuclear paraspeckle assembly transcript 1; Cul4A, cullin 4A; H, human; M, mouse; F, forward; R, reverse.

tubulin (0.5 $\mu$g/ml, ab59680), GAPDH (1:2,500, ab9485), Wnt3a (1:1,000, ab219412), β-catenin (1:500, ab68183) and H3K4me3 (1:1,000, ab213224) (all from Abcam) at 4˚C overnight. The membranes were then incubated with the secondary antibody (1:2,000, ab205718, Abcam) at room temperature for 2 h and visualized using a chemiluminescence reagent (EMD Millipore). The gray value of each band was quantified using ImageJ software v1.8.0 (National Institutes of Health), with β-actin as the internal control.

*Statistical analysis*. Data analysis was performed using SPSS 21.0 (IBM, Inc.). Data are expressed as the mean ± standard deviation. An unpaired t-test was adopted for comparisons between two groups. One-way or two-way analysis of variance was employed for comparisons among multiple groups, following Sidak's multiple comparisons test or Tukey's multiple comparisons test. P<0.05 was considered to indicate a statistically significant difference.

## Results

*lncRNA NEAT1 is highly expressed in CRC*. The pathological mechanisms of CRC are complex with respect to an abnormal lncRNA expression (20-22). The implication of lncRNAs in CRC has become increasingly clear (23). The lncRNA disease database (http://www.cuilab.cn/lncRNAdisease) (14) revealed that lncRNA NEAT1 is associated with CRC (Fig. 1A). lncRNA NEAT1 expression in CRC was predicted through TCGA analysis (http://ualcan.path.uab.edu/analysis.html) (15), and the results revealed that the mRNA expression of NEAT1 was upregulated in CRC (Fig. 1B). Additionally, NEAT1 was overexpressed in CRC tissues and cells (Fig. 1C and D). The median relative expression of NEAT1 (3.6) in 55 patients with CRC was used as the cut-off point. The patients were divided into the NEAT1 low expression group (27 cases) and high expression group (28 cases). A high expression of NEAT1 was positively associated with the CEA level (P=0.015), tumor size (P=0.031) and tumor-node-metastasis (TNM) stage (P=0.029), as shown in Table II. Taken together, these findings demonstrated that lncRNA NEAT1 was highly expressed in CRC, and may thus be related to the occurrence and development of CRC.

*Silencing of lncRNA NEAT1 suppresses the malignant behaviors of HCT116 cells*. To determine the role of lncRNA NEAT1 in CRC, si-NEAT1-1, si-NEAT1-2 and si-NEAT1-3 were transfected into HCT116 cells, and the transfection efficiency was verified by RT-qPCR (Fig. 2A). si-NEAT1-1 exhibited the highest transfection efficiency (P<0.001); thus, si-NEAT1-1 was used in subsequent experiments. Subsequently, the malignant behaviors of the HCT116 cells transfected with si-NEAT1 were evaluated. The si-NEAT1-transfected cells exhibited a suppressed colony formation ability (P<0.01; Fig. 2B) and an increased apoptosis (P<0.01; Fig. 2C), compared with the si-NC-transfected cells. Additionally, si-NEAT1 transfection suppressed cell migration and invasion (all P<0.01; Fig. 2D and E). In brief, these findings demonstrated that the downregulation of NEAT1 suppressed the malignant behaviors of HCT116 cells.

*lncRNA NEAT1 inhibits KDM5A expression by binding to E2F1*. The aforementioned experiments confirmed that si-NEAT1 inhibited the malignant behaviors of HCT116 cells. The present study then explored the downstream molecular mechanisms of NEAT1 in CRC. First, NEAT1 was predicted to be mainly located in the nucleus of HCT116 cells, through LncATLAS database (http://lncatlas.crg.eu/) (24) (Fig. 3A). RNA-FISH and nuclear/cytosol fractionation assay confirmed the localization of lncRNA NEAT1 (Fig. 3B and C). Emerging evidence suggests that NEAT1 may bind to transcription factors to regulate the expression of downstream genes (25,26). Hence, the present study predicted the transcription factors and downstream genes of NEAT1 by using LncMAP database (http://bio-bigdata.hrbmu.edu.cn/LncMAP/) (16). The results demonstrated that NEAT1 bound to the transcription factor E2F1 (Fig. 3D), and E2F1 bound to the downstream gene, KDM5A (Fig. 3D). The results of the RIP assay revealed that the enrichment of the NEAT1 co-precipitation group was notably increased compared with that of the IgG co-precipitation group (P<0.01; Fig. 3E). The results of the RNA pull-down assay revealed that the enrichment of the biotin-NEAT1 group was higher than that of the biotin-NC group (P<0.01; Fig. 3F). These results suggested that there was a binding association between NEAT1 and E2F1. To verify the role of E2F1 in the regulation of KDM5A by NEAT1, the HCT116 cells were transfected with si-E2F1 (P<0.001; Fig. 3G), and the results revealed that KDM5A expression was notably promoted in the

A

| LncRNA name | Disease name | Dysfunction type | Description | Chr | Start | End | Strand | Species | Alias | Genbank |
|---|---|---|---|---|---|---|---|---|---|---|
| NEAT1 | colorectal cancer | Expression | Whole blood NEAT1 expression is a novel diagnostic and prognostic biomarker of overall survival in colorectal cancer. | chr11 | 65422798 | 65445540 | + | Human | LINC00084; NCRNA00084; TncRNA; VINC | NR_131012 |

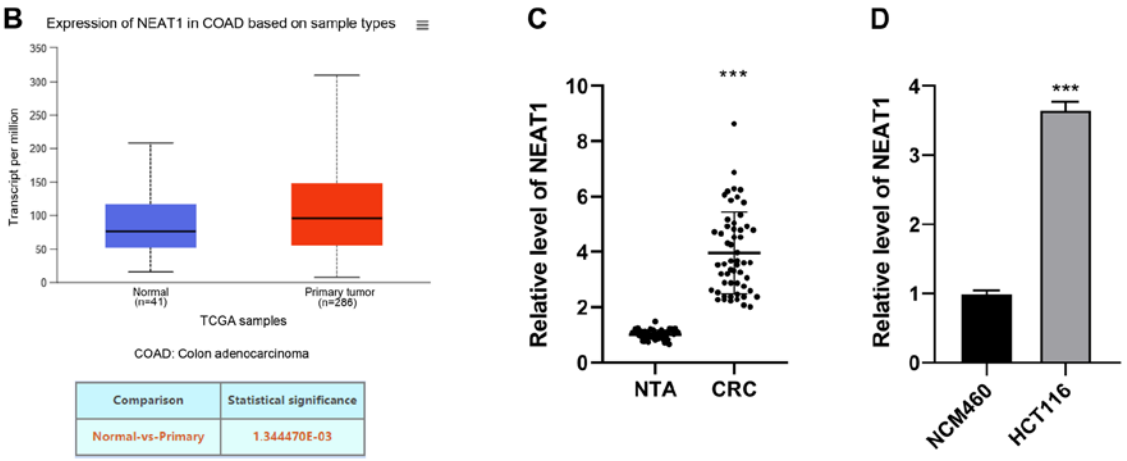


Figure 1. lncRNA NEAT1 is highly expressed in CRC. (A) lncRNA NEAT1 was predicted to be associated with CRC through the LncRNA Disease database. (B) lncRNA NEAT1 mRNA expression was predicted through TCGA. (C and D) lncRNA NEAT1 expression in cancer tissues or normal tissues and NCM460 cells or HCT116 cells was detected by RT-qPCR, n=55. The cell experiment was repeated three times independently. Data are expressed as the mean ± standard deviation and were analyzed using an unpaired t-test; ***P<0.001 vs. respective control. lncRNA, long non-coding RNA; NEAT1, nuclear paraspeckle assembly transcript 1; CRC, colorectal cancer.

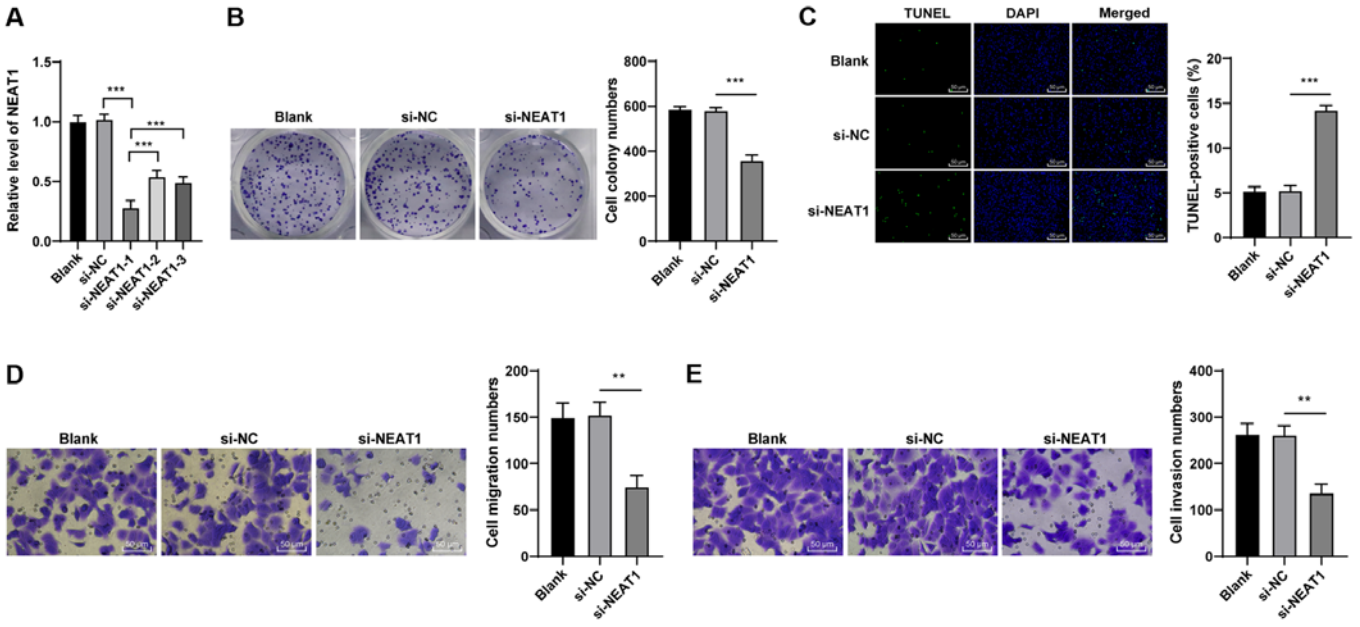


Figure 2. Downregulation of lncRNA NEAT1 inhibits the malignant behaviors of HCT116 cells. HCT116 cells were transfected with three siRNAs for NEAT1, with si-NC as the control and untreated HCT116 as the blank. (A) The transfection efficiency of lncRNA NEAT1 siRNA was confirmed by RT-qPCR. (B) HCT116 cell proliferation was detected using a colony formation assay. (C) HCT116 cell apoptosis was measured using a TUNEL assay. (D and E) Migration and invasion of HCT116 cells were determined using a Transwell assay. The cell experiment was repeated three times independently. Data are expressed as the mean ± standard deviation and were analyzed using one-way ANOVA, followed by Tukey's multiple comparisons test; **P<0.01, ***P<0.001. lncRNA, long non-coding RNA; NEAT1, nuclear paraspeckle assembly transcript 1.

si-E2F1-transfected HCT116 cells (P<0.01; Fig. 3H). Moreover, KDM5A expression was elevated in the si-NEAT1-transfected HCT116 cells (P<0.01; Fig. 3I). Thus, these data indicated that lncRNA NEAT1 could bind to E2F1 to inhibit KDM5A expression.

*Downregulation of KDM5A reverses the inhibitory effects of NEAT1 silencing on the malignant behaviors of HCT116 cells*. To explore the role of KDM5A in CRC, KDM5A expression was silenced in si-NEAT1-transfected cells (P<0.001; Fig. 4A) and observed that colony formation was enhanced

Table II. Association between NEAT1 expression and clinicopathological features of patients with colorectal cancer.

| Clinicopathological feature | No. of patients (n=55) | Expression of NEAT1 | | $\chi^2$ | P-value |
|---|---|---|---|---|---|
| | | Low (n=27) | High (n=28) | | |
| Age (years) | | | | | |
| <36 | 27 | 12 | 15 | 0.458 | 0.593 |
| ≥36 | 28 | 15 | 13 | | |
| Sex | | | | | |
| Male | 30 | 16 | 14 | 0.475 | 0.591 |
| Female | 25 | 11 | 14 | | |
| Tumor location | | | | | |
| Rectum | 25 | 13 | 12 | 0.155 | 0.798 |
| Colon | 30 | 14 | 16 | | |
| CEA level | | | | | |
| ≤5 ng/ml | 25 | 17 | 8 | 6.557 | 0.015 |
| >5 ng/ml | 30 | 10 | 20 | | |
| Lymph node metastasis | | | | | |
| Absent | 29 | 15 | 14 | 0.170 | 0.789 |
| Present | 26 | 12 | 14 | | |
| Tumor size | | | | | |
| ≤5 cm | 28 | 18 | 10 | 5.269 | 0.031 |
| >5 cm | 27 | 9 | 18 | | |
| Distant metastasis | | | | | |
| Absent | 34 | 20 | 14 | 3.375 | 0.097 |
| Present | 21 | 7 | 14 | | |
| TNM stage | | | | | |
| Stage I-II | 22 | 15 | 7 | 5.347 | 0.029 |
| Stage III-IV | 33 | 12 | 21 | | |

The data between the two groups were analyzed using a Chi-squared test, and P<0.05 indicates a statistically significant difference. NEAT1, nuclear paraspeckle assembly transcript 1; CEA, carcinoembryonic antigen; TNM, tumor-node-metastasis.

(P<0.01; Fig. 4B) and apoptosis was inhibited (P<0.01; Fig. 4C). In addition, cell migration and invasion were enhanced in the si-NEAT1 + si-KDM5A-transfected cells (P<0.05; Fig. 4D and E). Taken together, these findings demonstrated that the downregulation of KDM5A reversed the inhibitory effects of NEAT1 silencing on the malignant behaviors of HCT116 cells.

*KDM5A inhibits Cul4A expression via the demethylation of H3K4me3*. KDM5A is associated with transcriptional regulation due to its capacity to catalyze the removal of methyl groups from H3K4me3 (27). KDM5A regulates demethylation in the promoter of Cul4A (28). TCGA analysis (http://ualcan.path.uab.edu/analysis.html) (15) revealed that Cul4A was highly expressed in CRC (Fig. 5A). It was hypothesized that KDM5A may affect Cul4A expression by regulating H3K4me3; thus, H3K4me3 methylation and Cul4A expression were detected in the HCT116 cells. Compared with those of the si-NC-transfected cells, the methylation level of H3K4me3 and Cul4A expression were decreased in the si-NEAT1-transfected cells, but were increased in the si-NEAT1 + si-KDM5A-transfected cells (P<0.001; Fig. 5B and C), suggesting that KDM5A regulated Cul4A expression via H3K4me3. Subsequently, the level of H3K4me3 in HCT116 cells was enhanced by CPI-455 treatment (P<0.01; Fig. 5D) and it was found that Cul4A expression was promoted with the increase in the H3K4me3 levels (P<0.001; Fig. 5E). These results suggest that KDM5A inhibits H3K4me3 methylation in the promoter of Cul4A, thus inhibiting Cul4A expression.

*Overexpression of Cul4A promotes the proliferation and migration of si-NEAT1-transfected HCT116 cells*. To verify the role of Cul4A in CRC, joint experiments were designed. The si-NEAT1-transfected HCT116 cells were transfected with pc-Cul4A (P<0.001; Fig. 6A), and the malignant behaviors of the HCT116 cells were examined. Compared with the si-NEAT1 + pc-NC cells, the si-NEAT1 + Cul4A cells exhibited a notably increased colony formation ability (P<0.01; Fig. 6B) and a reduced apoptosis (P<0.01; Fig. 6C). In addition, an enhanced Cul4A expression promoted cell migration and invasion (all P<0.01; Fig. 6D and E). These results suggested that the overexpression of Cul4A facilitated the malignant behavior of si-NEAT1-transfected HCT116 cells.

*lncRNA NEAT1 activates the Wnt pathway via KDM5A/Cul4A*. The Wnt pathway plays a vital role in CRC (29,30), and there

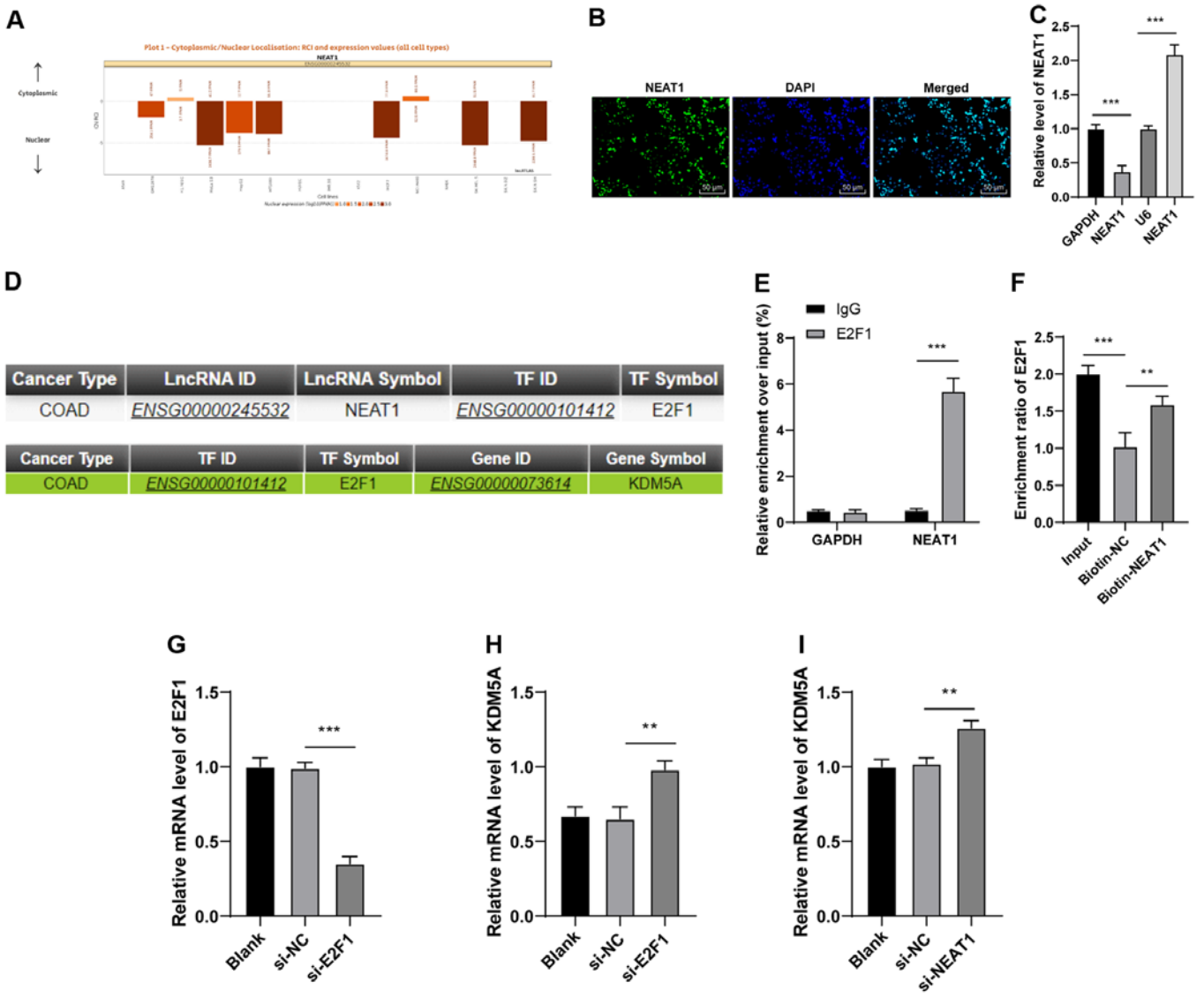


Figure 3. lncRNA NEAT1 inhibits KDM5A expression by binding to E2F1. (A) The localization of NEAT1 in cells was predicted through the lncATLAS database. (B) The fluorescence localization of lncRNA NEAT1 in HCT116 cells was detected using RNA-FISH. (C) lncRNA NEAT1 expression was detected by RT-qPCR following nuclear/cytosol fractionation assay. (D) lncRNA NEAT1 binding transcription factors and downstream genes were predicted by the LncMAP database. (E and F) The binding association between lncRNA NEAT1 and E2F1 was confirmed using RIP and RNA pull-down assays. (G) The transfection efficiency of E2F1 siRNA was confirmed by RT-qPCR. (H and I) KDM5A expression in HCT116 cells was detected by RT-qPCR. The cell experiment was repeated three times independently. Data are expressed as the mean ± standard deviation and were analyzed using one-way or two-way ANOVA followed by Tukey's multiple comparisons test or Sidak's multiple comparisons test; $^{**}$P<0.01, $^{***}$P<0.001. lncRNA, long non-coding RNA; NEAT1, nuclear paraspeckle assembly transcript 1.

exists a regulatory association between Cul4A and this pathway (31). Hence, the present study detected proteins related to the Wnt pathway. Compared with the si-NC-transfected cells, the si-NEAT1-transfected cells exhibited a decreased expression of Wnt3a and β-catenin (P<0.001; Fig. 7), while si-KDM5A transfection partially increased the expression of Wnt3a and β-catenin (all P<0.01; Fig. 7). Thus, lncRNA NEAT1 activated the Wnt pathway via KDM5A/Cul4A.

*lncRNA NEAT1 promotes CRC via KDM5A/Cul4A in vivo*. To verify the effects of lncRNA NEAT1 *in vivo*, a xenograft model of CRC was established by injecting HCT116 cells into the right armpits of mice (Fig. 8A). Tumor growth was inhibited (P<0.01; Fig. 8B and Table SI) and tumor weight was reduced in the mice injected with si-NEAT1-transfected cells (P<0.001; Fig. 8C). Ki67 is a marker of reactive proliferation (32). Immunohistochemistry revealed that the injection of si-NEAT1-transfected cells led to a decrease in the Ki67-positive rate (P<0.001; Fig. 8D). Compared with the si-NC group, the si-NEAT1 group exhibited a notably decreased NEAT1 expression, an increased KDM5A expression, and decreased H3K4me3 levels and Cul4A expression (all P<0.001; Fig. 8E-H). Thus, the silencing of lncRNA NEAT1 expression promoted KDM5A expression and inhibited Cul4A expression, thus, supprssing the development of CRC *in vivo*.

## Discussion

CRC mostly develops from benign polyps to distant metastasis and, consequently, early diagnosis and intervention are essential for the long-term survival of patients (33). Recently, the

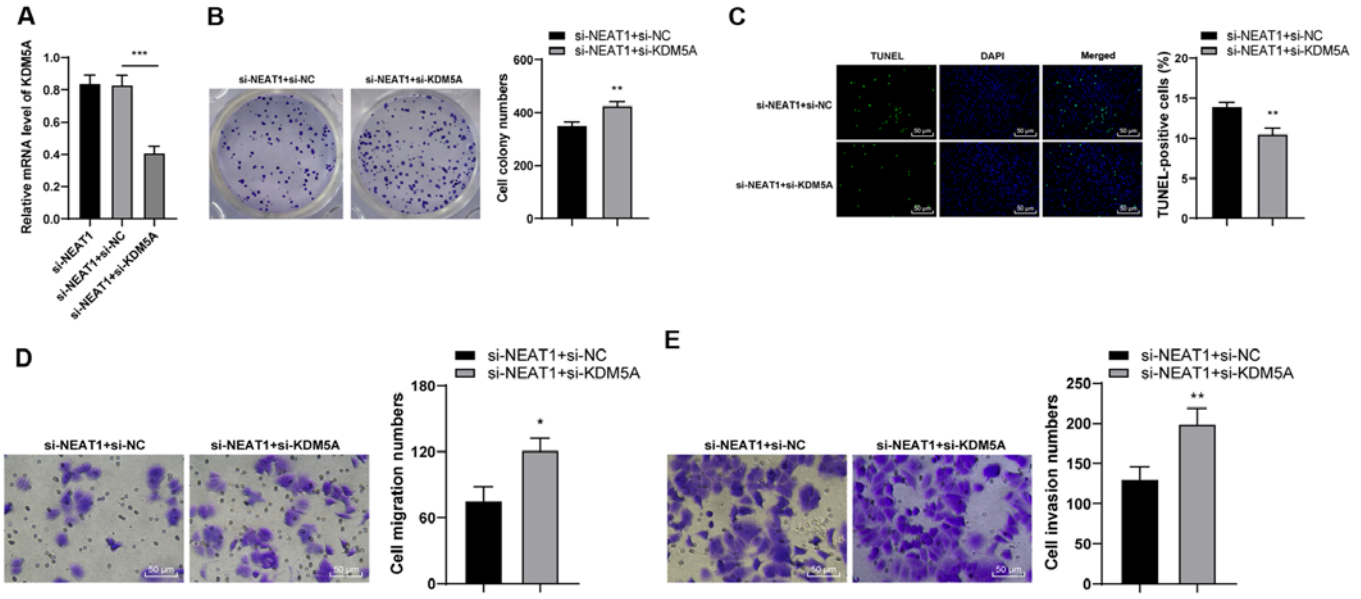


Figure 4. Downregulation of KDM5A reverses the inhibitory effects of NEAT1 silencing on the malignant behaviors of HCT116 cells. HCT116 cells were transfected with si-NEAT1 and si-KDM5A, with si-NC of KDM5A as the control. (A) The transfection efficiency of KDM5A siRNA was confirmed by RT-qPCR. (B) HCT116 cell proliferation was detected using colony formation assay. (C) HCT116 cell apoptosis was measured using a TUNEL assay. (D and E) Migration and invasion of HCT116 cells were measured using Transwell assays. The cell experiment was repeated three times independently. Data are expressed as the mean ± standard deviation. The t-test was employed for comparisons between two groups, and one-way ANOVA was employed for comparisons among multiple groups, followed by Tukey's multiple comparison test; $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001. lncRNA, long non-coding RNA; NEAT1, nuclear paraspeckle assembly transcript 1.

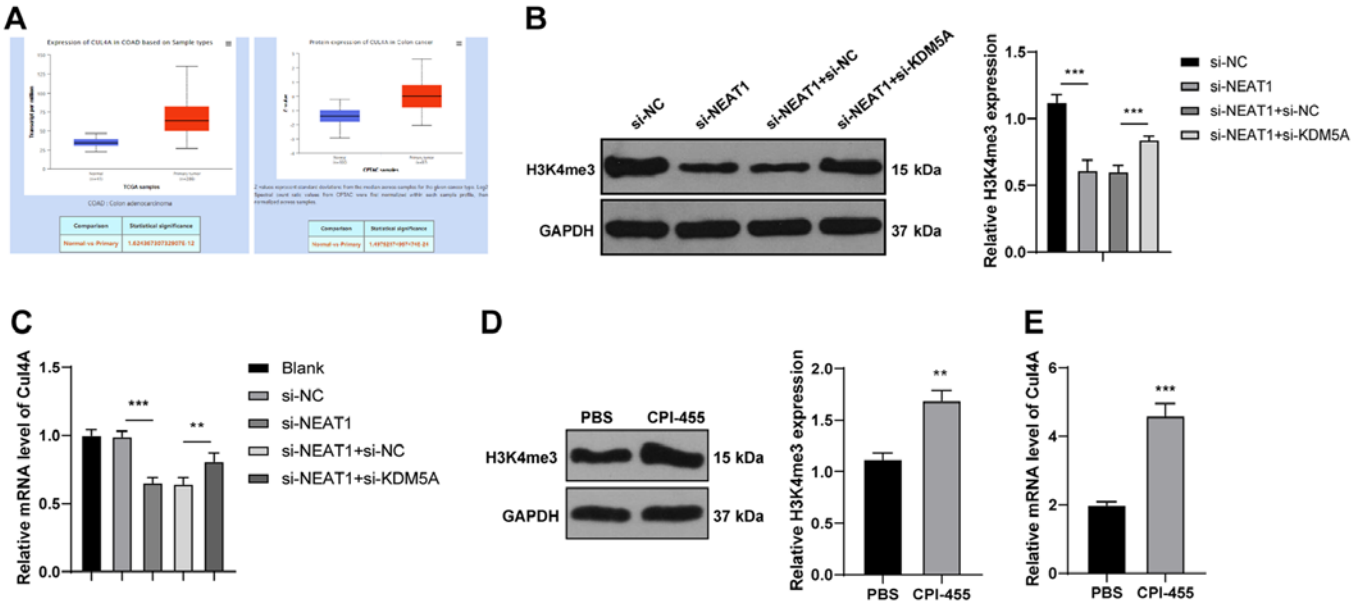


Figure 5. KDM5A inhibits Cul4A expression via the demethylation of H3K4me3. (A) Cul4A expression in CRC was analyzed using TCGA database. (B) The H3K4me3 level in HCT116 cells was detected by western blot analysis. (C) Cul4A expression in HCT116 cells was detected by RT-qPCR. (D) The H3K4me3 level was detected by western blot analysis. (E) Cul4A expression in CPI-455 or PBS-treated HCT116 cells was detected by RT-qPCR. The cell experiment was repeated three times independently. Data are expressed as the mean ± standard deviation. An unpaired t-test was employed for comparisons between two groups and one-way ANOVA was employed for comparisons among multiple groups, followed by Tukey's multiple comparison test; $^{**}$P<0.01, $^{***}$P<0.001. lncRNA, long non-coding RNA; NEAT1, nuclear paraspeckle assembly transcript 1.

biological characteristics of lncRNAs and novel gene therapies of CRC have attracted considerable attention (34). The present study elucidated the potential mechanisms of action of lncRNA NEAT1 in facilitating CRC progression.

With the advancement of tumor molecular biology, the role of lncRNAs in the progression of CRC has been increasingly investigated (35). lncRNA NEAT1 was found to be associated with CRC through the LncRNADisease database (14). The aberrant NEAT1 expression has been documented in CRC, in which its elevated expression is related to poor outcomes (10). In the present study, NEAT1 was predicted expression by TCGA analysis and it was found that NEAT1

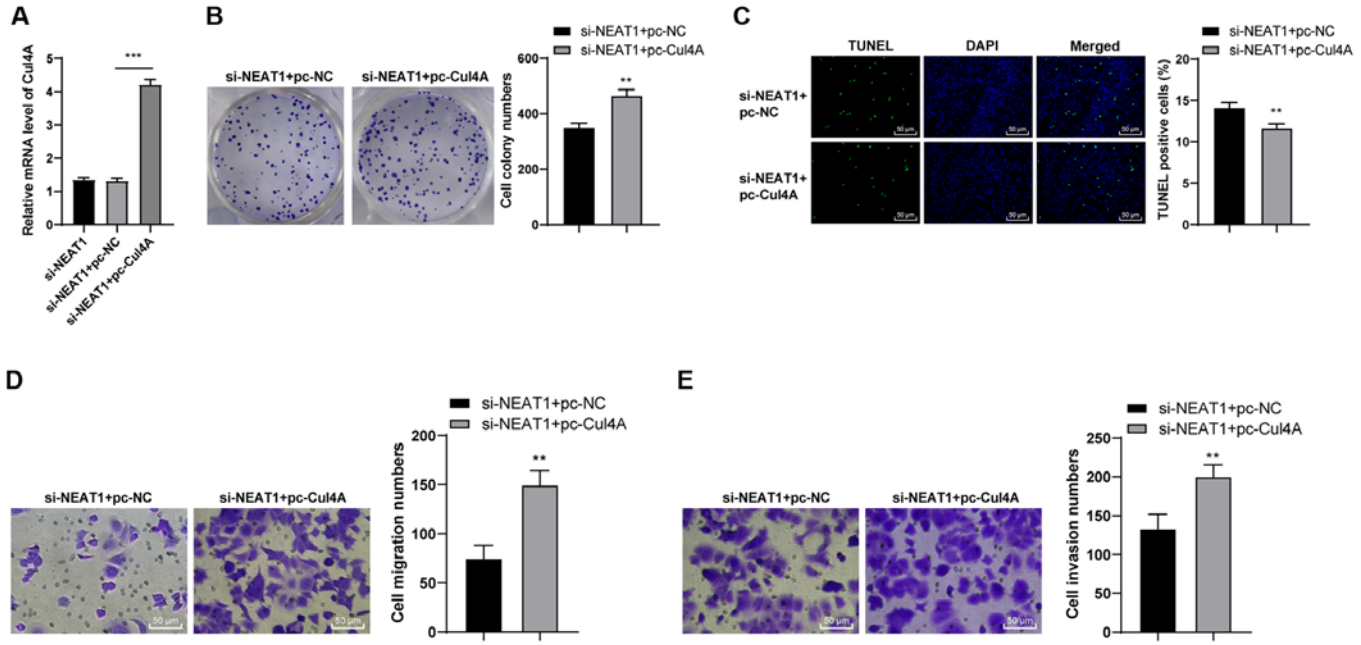


Figure 6. Upregulation of Cul4A promotes the proliferation and migration of si-NEAT1-treated HCT116 cells. HCT116 cells were co-treated with si-NEAT1 and pcDNA-Cul4A, with pcDNA-NC as the control. (A) The transfection efficiency of pcDNA-Cul4A was confirmed by RT-qPCR. (B) HCT116 cell proliferation was detected using a colony formation assay. (C) HCT116 cell apoptosis was measured using a TUNEL assay. (D and E) Migration and invasion of HCT116 cells were determined using Transwell assays. The cell experiment was repeated three times independently. Data are expressed as the mean ± standard deviation. An unpaired t-test was employed for comparisons between two groups and one-way ANOVA was employed for comparisons among multiple groups followed by Tukey's multiple comparison test; $^{**}$P<0.01, $^{***}$P<0.001, vs. control. lncRNA, long non-coding RNA; NEAT1, nuclear paraspeckle assembly transcript 1.

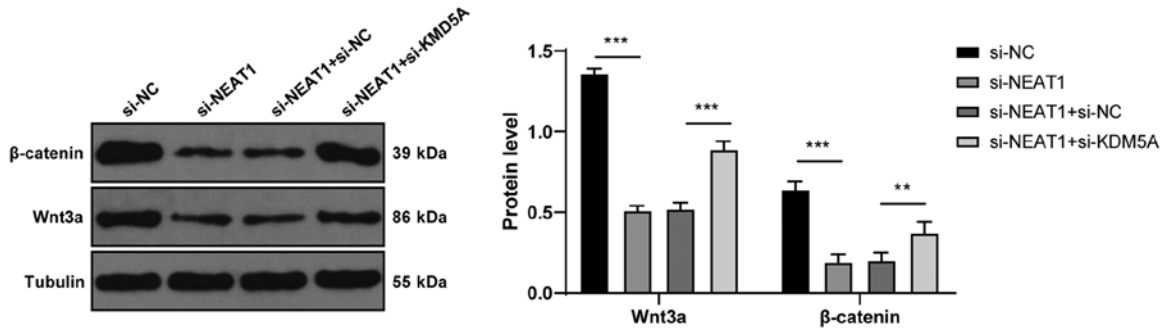


Figure 7. lncRNA NEAT1 activated the Wnt pathway via KDM5A/Cul4A. The expression levels of Wnt3a and β-catenin in HCT116 cells were detected by western blot analysis. The cell experiment was repeated three times independently. Data are expressed as the mean ± standard deviation and were analyzed using two-way ANOVA, followed by Tukey's multiple comparisons test; $^{**}$P<0.01, $^{***}$P<0.001. lncRNA, long non-coding RNA; NEAT1, nuclear paraspeckle assembly transcript 1.

was upregulated in CRC. Additionally, NEAT1 was highly expressed in cancer tissues and cells. An elevated NEAT1 expression contributes to poor differentiation, metastasis, and TNM stage (36). In the present study, HCT116 cells were then transfected with si-NEAT1 to further determine the role of NEAT1 in CRC. The si-NEAT1-transfected cells exhibited a suppressed colony formation ability, increased apoptosis, and a suppressed cell migration and invasion. The knockdown of NEAT1 impaired the proliferation and migration of CRC cells, thus suppressing CRC progression (37). Thus, the silencing of NEAT1 suppressed the malignant behaviors of HCT116 cells.

The potential mechanisms of lncRNA NEAT1 in CRC were then explored. The mechanisms of lncRNAs may be related to the extensive subcellular localization in cells (38). NEAT1 was found to be mainly located in the nucleus of HCT116 cells. NEAT1 may regulate the expression of downstream genes by binding to transcription factors. The database predicted that NEAT1 binds to transcription factor E2F1, and E2F1 binds to KDM5A. E2F1, a crucial molecule implicated in cell proliferation and apoptosis, is overexpressed in a broad range of human cancers, including CRC (39,40). An elevated E2F1 expression in cancer cells can induce metastasis and invasion (41), as well as enhance the aggressiveness of CRC (42). KDMs are enzymes of dimethyl-lysine residues in histones, and KDM5 specifically removes methylation from H3K4me1/2/3, which is a hallmark associated with the action of transcription (27). The dysregulation of KDM5A contributes to the pathogenesis

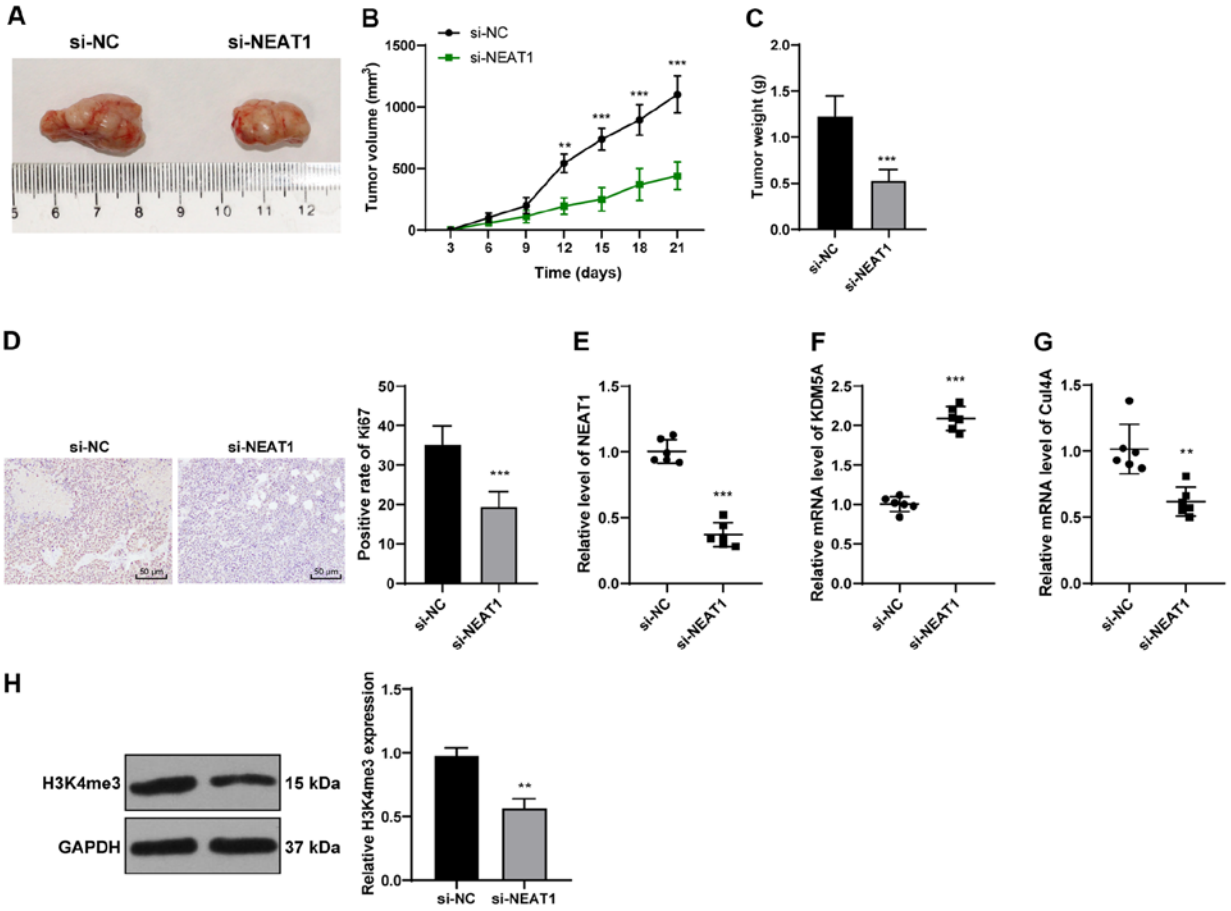


Figure 8. lncRNA NEAT1 promotes CRC via KDM5A/Cul4A *in vivo*. (A) Representative images of the xenograft tumor model. (B) Tumor volume. (C) Tumor weight. (D) The Ki67-positive rate was detected using immunohistochemistry. (E and F) The expression levels of lncRNA NEAT1 and KDM5A were detected by RT-qPCR. (G) The H3K4me3 level was detected by western blot analysis. (H) Cul4A expression was detected by RT-qPCR, n=6. Data are expressed as the mean ± standard deviation. An unpaired t-test was employed for comparisons between two groups and two-way ANOVA was employed for comparisons among multiple groups followed by Sidak's multiple comparisons test; $^{**}$P<0.01, $^{***}$P<0.001 vs. negative control. lncRNA, long non-coding RNA; NEAT1, nuclear paraspeckle assembly transcript 1; CRC, colorectal cancer.

of lung and gastric cancers (43). However, currently, knowledge regarding the specific role of KDM5A in CRC is limited. The present study confirmed the binding association between NEAT1 and E2F1. KDM5A expression was notably promoted in the si-E2F1- and si-NEAT1-transfected HCT116 cells. The present study is the first, to the best of our knowledge, to reveal that NEAT1 binds to E2F1 to inhibit KDM5A expression. To verify the role of KDM5A in CRC, KDM5A expression was silenced in si-NEAT1-transfected cells. The results revealed that the downregulation of KDM5A reversed the inhibitory effects of NEAT1 silencing on the malignant behaviors of HCT116 cells.

Subsequently, the downstream mechanism of KDM5D in CRC was investigated. KDM5A can catalyze the removal of methyl groups from H3K4me3 (27). KDM5D has been shown to suppress EMT in gastric cancer through the demethylation of the Cul4A promoter (28). Accordingly, it was hypothesized that KDM5A may affect Cul4A expression by regulating H3K4me3. Cul4A participates in a variety of critical cell functions, including apoptosis, cell cycle progression, genomic stability and histone modification (44). Cul4A amplification or overexpression can be observed in several human malignancies, including breast, prostate and lung cancer (45-47). Importantly, Cul4A facilitates the proliferation and metastasis of CRC cells by modulating H3K4 trimethylation in EMT (48). The present study demonstrated that H3K4me3 methylation and Cul4A expression were inhibited in the si-NEAT1-transfected cells, whereas they were enhanced in the si-NEAT1 + si-KDM5A-transfected cells, suggesting that KDM5A regulated Cul4A expression via H3K4me3. H3K4me3 was then overexpressed in HCT116 cells by CPI-455 treatment and it was found that Cul4A expression was promoted with an increase in the H3K4me3 levels. These results suggest that KDM5A inhibits H3K4me3 methylation in the promoter of Cul4A, thus inhibiting Cul4A expression. Joint experiments were conducted to verify the role of Cul4A in CRC. The upregulation of Cul4A facilitated the malignant behavior of si-NEAT1-treated HCT116 cells. Consistently, Li *et al* demonstrated that the knockdown of Cul4A notably suppressed the progression of EMT and metastasis of colon cancer cells *in vitro* (49).

Thereafter, the present study determined the downstream pathway regulated by Cul4A. The Wnt signaling pathway is a critical cascade closely related to cancer progression. In particular, the role of the Wnt pathway in tumorigenesis has been prominently described in CRC (50). The Wnt

pathway controls β-catenin levels for signal transduction through phosphorylation and ubiquitin-mediated degradation (51). Cul4A is a novel Wnt target gene that physically interacts with p27 in Wnt-responsive cells (52). Proteins related to the Wnt pathway were then detected. The expression of Wnt3a and β-catenin in the si-NEAT1-transfected cells was notably decreased, whereas it was partially increased following si-KDM5A transfection. Zhang *et al* demonstrated that NEAT1 activatesd the Wnt pathway to facilitate CRC progression and metastasis (38). Moreover, *in vivo* experiments confirmed that the silencing of lncRNA NEAT1 promoted KDM5A expression and inhibited Cul4A expression, thus suppressing the development of CRC. Briefly, lncRNA NEAT1 activated the Wnt pathway via KDM5A/Cul4A.

In conclusion, the present study demonstrated that lncRNA NEAT1 bound to E2F1 to inhibit KDM5A expression, promote Cul4A expression and activate the Wnt pathway, thereby facilitating the progression of CRC. However, the competing endogenous RNA mechanism of lncRNA NEAT1 in CRC remains to be elucidated. Additionally, the present study merely demonstrated that the Wnt pathway was activated; however, its function remains unclear. In future, the authors aim to verify the specific mechanisms of the Wnt pathway in CRC, and explore the feasibility and safety of NEAT1 as an entry point for CRC treatment.

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**Availability of data and materials**

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

**Authors' contributions**

XS and KZ were involved in the conceptualization. WW, YW, GC, ZYe, LX, LG and ZYa were involved in data validation, research, the provision of resources, data reviewing and manuscript writing. LX and LG were involved in the reviewing and editing of the manuscript. XS and ZYe confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

**Ethics approval and consent to participate**

The usage of ovarian tissues was approved by the Ethics Committee of the Second Affiliated Hospital of Soochow University. Informed consent was signed by each eligible participant. All animal experiments are approved by the Ethics Committee of the Second Affiliated Hospital of Soochow University (S.No. 20190523b116). All experimental procedures were implemented on the Ethical Guidelines for the study of experimental pain in conscious animals.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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