Received: 1 September 2022 | Revised: 11 November 2022 | Accepted: 26 November 2022

DOI: 10.1111/cas.15678

ORIGINAL ARTICLE

Cancer Science WILEY

# Let-7b-5p inhibits colon cancer progression by prohibiting APC ubiquitination degradation and the Wnt pathway by targeting NKD1

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**Funding information**
The funds of Changzhou High-Level Medical Talents Training Project, Grant/Award Number: 2022CZBJ110; National Natural Science Foundation of China, Grant/Award Number: 81872275,81972618; the funds of Changzhou Sci & Tech Program, Grant/Award Number: CJ20220007, CJ20220006, CJ20210015; the innovation team project of Guangdong Provincial Department of Education, Grant/Award Number: 2021KCXTD039; the Medical Research Project of Jiangsu Health Commission, Grant/Award Number: M2020002

## Abstract

Naked cuticle homolog 1 (NKD1), which is expressed at low levels in many tumors, is considered an inhibitor of the Wnt/β-catenin pathway, but it is highly expressed in colon cancer and can promote colon cancer cell proliferation. miRNAs are involved in the occurrence and progression of many tumors. However, miRNAs that can regulate NKD1 and the mechanisms by which NKD1 regulates tumor progression remain ambiguous. This research aims to reveal the potential regulatory network of NKD1 in colon cancer. miRNA data downloaded from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases were analyzed by bioinformatics to screen for potential miRNAs targeting NKD1. Let-7b-5p was found to inhibit proliferation, migration, and invasion of colon cancer cells targeting NKD1. Further studies suggested that let-7b-5p can modulate Wnt signaling activity, and the nuclear accumulation of β-catenin was significantly restrained by let-7b-5p through targeting NKD1. Moreover, NKD1 could prohibit the expression of the APC protein. Further studies manifested that NKD1 bound to APC and promoted the ubiquitination degradation of APC through restraining the expression of the deubiquitinating enzyme USP15 and blocking the combination between USP15 and APC. Functionally, NKD1 enhanced the proliferation and migration of colon cancer cells by inhibiting APC expression. This

**Abbreviations:** APC, adenomatous polyposis coli protein; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; NKD1, naked cuticle homolog 1; WGCNA, weighted gene coexpression network analysis.

research revealed a novel mechanism by which the let-7b-5p-NKD1-APC-β-catenin signaling pathway inhibited colon cancer cell progression.

**KEYWORDS**
APC, colon cancer, let-7b-5p, NKD1, Wnt/β-catenin

## 1 | INTRODUCTION

Colon cancer has the third-highest incidence and second-highest mortality rate.[1] Because of its easy recurrence, drug resistance, and other reasons, colorectal cancer patients have poor overall survival.[2,3] NKD1, considered being an inhibitor of the Wnt pathway,[4] is expressed at low levels in various tumors and binds DVL1 to inhibit the Wnt/β-catenin signaling pathway. However, NKD1 is highly expressed in colorectal carcinoma. Our previous study demonstrated that NKD1 could promote colon cancer cell proliferation.[5] The molecular mechanism by which NKD1 regulates colon cancer progression remains unclear.

The Let-7 family is a family of miRNAs that exert tumor suppressor effects.[6] We identified let-7b-5p, which regulates NKD1, by bioinformatics analysis. Initially, Johnson et al reported that let-7 was lowly expressed in lung cancer patients and could regulate the oncogene Ras.[7] In addition, studies reported let-7b-5p could hinder glioma cell migration and proliferation.[8] However, two studies showed that let-7b-5p was lowly expressed in colon cancer tissues,[9,10] while its function and related mechanism in colon carcinoma have not yet been reported.

In this study, we screened miRNAs targeting NKD1 through bioinformatics analysis and identified let-7b-5p. Quantitative real-time PCR, dual fluorescein reporter assays, and Western blot demonstrated that let-7b-5p targeted NKD1. Further analysis revealed that let-7b-5p inhibited colon cancer cell proliferation, migration, and invasion, suppressed APC ubiquitination degradation, and reduced the accumulation of β-catenin in the nucleus by targeting NKD1. Mechanically, NKD1 boosted the APC ubiquitination degradation by repressing the expression of USP15 and inhibiting the binding of USP5 to APC. Generally, this study elucidates the mechanism of the let-7b-5p/NKD1 axis in regulating colon cancer progression, indicating that NKD1 might act as a tumor marker for colon cancer and is anticipated to become a novel therapeutic target for colon carcinoma.

## 2 | MATERIALS AND METHODS

### 2.1 | Collection and processing of data

TCGA-COAD (colon adenocarcinoma) miRNA data and RNA-seq data were collected from the TCGA database[11] (https://portal.gdc.cancer.gov/). The GSE135918 dataset was captured from the GEO database[12] (https://www.ncbi.nlm.nih.gov/geo/). TCGA pan-cancer data were downloaded from UCSC[13] (http://xena.ucsc.edu/). Ten colon cancer tissue samples were obtained from Wujin People's Hospital's tumor specimen bank, and the Ethics Committee of Wujin Hospital Affiliated with Jiangsu University approved all operations (IRB number: 2022-SR-089). Sample information is provided in Table S1.

### 2.2 | Weighted gene coexpression network analysis (WGCNA)

Screening for miRNAs linked with colon cancer phenotype, WGCNA was performed on TCGA-miRNA and GSE195918 datasets. The minimum power value of the scale-free network map structure $R^2$ reaching 0.9 was considered the best soft threshold to establish a coexpression network.

### 2.3 | Gene set enrichment analysis (GSEA) and single-sample gene set enrichment analysis (ssGSEA)

The TCGA pan-cancer samples were ranked according to the NKD1 expression level; the first 25% of the samples were defined as the NKD1 low-expression group, and the last 75% were defined as the NKD1 high-expression group. Subsequently, GSEA was performed on the two groups of samples. GSEA[14,15] and ssGSEA[16] were used to analyze the enrichment of the Wnt/β-catenin pathway in TCGA pan-cancer and colon cancer samples, respectively.

### 2.4 | Identifying miRNAs that potentially regulate NKD1

The "limma" package was employed to select differentially expressed miRNAs,[17] with |LogFC>1| and FDR<0.05 as the screening criteria. miRWak (http://mirwalk.umm.uni-heidelberg.de/) for prediction of potential miRNAs targeting NKD1.[18]

### 2.5 | Cell culture

The human NCM460 normal colon epithelial cell lines, the human colon cancer HCT116, RKO, LOVO, SW480, SW620 cell lines, and HEK293 cell lines were purchased from the Cell Bank at the Shanghai Institute of Cells, Chinese Academy of Sciences. All the cells were cultured at 37°C in 5% $CO_2$.[19]

### 2.6 | RNA extraction, cDNA reverse transcription, and RT-qPCR

In accordance with the procedure of the RNA extraction kit (ES Science), total RNA was extracted. RNA reverse transcription was accomplished with an M-MuLV first-strand cDNA synthesis kit (Shanghai Sangong). A stem-loop miRNA reverse transcription kit (Shanghai Sangong) was utilized to carry out the reverse transcription of miRNA. GAPDH and U6 were used as internal controls for mRNA and miRNA, respectively.[5] The primer sequences are shown in Table S2.

### 2.7 | Transfection of oligonucleotides

The let-7b-5p mimics and inhibitor were purchased from RiboBio, and NKD1 siRNA was obtained from General Biosystems.[5] According to the manufacturer's guidelines, oligonucleotides were transfected into cells with Tuborfect (Thermo). All the oligonucleotide sequences used are recorded in Table S3.

### 2.8 | Construction and transfection of recombinant plasmids

The pcDNA3.0-FLAG-NKD1 and pCMV-HA-APC plasmids were constructed by Genechem and Miaolingbio, respectively. The empty plasmids were adopted as negative controls (NCs). Plasmid transfection was performed according to the instructions of Tuborfect.

### 2.9 | Cell proliferation experiments

For MTT assays, transfected cells were cultured for different times in 96-well plates. The cell proliferation activity was detected according to the MTT cell proliferation and cytotoxicity assay kit (Shanghai Sangon). For EdU, in accordance with the procedures of the EdU cell proliferation kit (Shanghai Sangong), the experimental steps were carried out, and the percentage of EdU-stained cells was determined. For plate colony formation, transfected cells were seeded in six-well plates and cultured for 1 week to calculate the number of clones formed.[20]

### 2.10 | Cell migration and invasion analysis

Wound-healing experiment: Horizontal lines were drawn in six-well plates, and cells were cultured for 48 hours. Finally, the percentage of the wound-healing area was counted. Migration and invasion assays: Transfected cells were spread in Transwell chambers (Corning), and the number of migrating and invading cells was counted after 24 or 48 hours of culture.

### 2.11 | Immunofluorescence

Transfected cells were spread on cell slides, incubated with primary antibody overnight, incubated with secondary antibody and DAPI in the dark, and finally photographed under a microscope.[20] The mean fluorescence intensity was calculated using ImageJ. Materials and antibodies used are listed in Table S4.

### 2.12 | Western blot

The primary and secondary antibodies were incubated on the membrane (Table S4), and target proteins were manifested utilizing ECL chemiluminescence kits (Biosharp). For coimmunoprecipitation (Co-IP) assays, the lysate was added to transfected cells. The target antibody was added to the total protein lysate and incubated for 6 hours at 4°C. After that, Protein A/G agarose (Thermo) were added and incubated overnight at 4°C. The quantitative results of Western blotting assays were all shown in Figure S1.

### 2.13 | Luciferase reporter assay

In a 24-well plate, HEK293 cells were seeded and cotransfected with mimics NC with pmirGLO-WT-NKD1, mimics NC with pmirGLO-MUT-NKD1, mimics with pmirGLO-WT-NKD1, and mimics with pmirGLO-MUT-NKD1. Then, the luciferase activity was evaluated using the Nano-Glo® Dual-Luciferase® reporter gene assay system (Promega).[21]

### 2.14 | Statistical analysis

GraphPad Prism 8.0 and SPSS 26.0 were utilized for statistical analysis. The *t* test (two-tailed) was used to assess the statistical significance between the two groups. Bonferroni test was used for comparison among multiple groups. The data are presented as the mean ± standard error of the mean. $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) were considered statistically significant.

## 3 | RESULTS

### 3.1 | Identification of miRNAs that target NKD1 via bioinformatics analysis

Weighted gene co-expression network analysis was performed on the TCGA-miRNA dataset and GSE135918 to identify miRNAs that are associated with colon cancer phenotypes.[22] Based on average hierarchical clustering and dynamic tree clipping (Figure 1A, C), we obtained five modules in TCGA (Figure 1B) and four in GEO (Figure 1D). TCGA-ME turquoise and GEO-ME blue were the

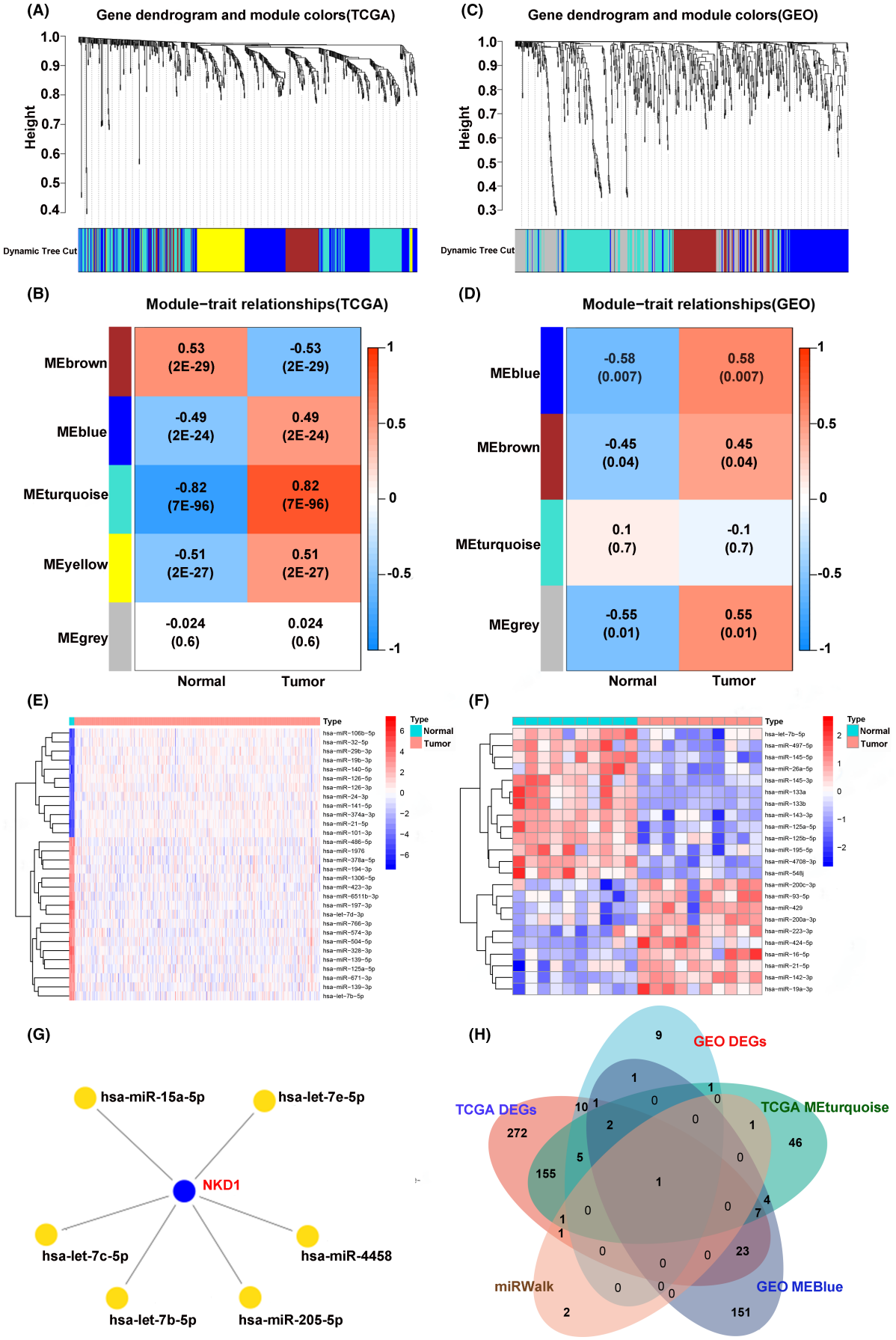

(A)
Gene dendrogram and module colors(TCGA)
Height
Dynamic Tree Cut
(C)
Gene dendrogram and module colors(GEO)
Height
Dynamic Tree Cut
(B)
Module−trait relationships(TCGA)
MEbrown
MEblue
MEturquoise
MEyellow
MEgrey
0.53 (2E-29)
-0.53 (2E-29)
-0.49 (2E-24)
0.49 (2E-24)
-0.82 (7E-96)
0.82 (7E-96)
-0.51 (2E-27)
0.51 (2E-27)
-0.024 (0.6)
0.024 (0.6)
Normal
Tumor
(D)
Module−trait relationships(GEO)
MEblue
MEbrown
MEturquoise
MEgrey
-0.58 (0.007)
0.58 (0.007)
-0.45 (0.04)
0.45 (0.04)
0.1 (0.7)
-0.1 (0.7)
-0.55 (0.01)
0.55 (0.01)
Normal
Tumor
(E)
Type
Normal
Tumor
(F)
Type
Normal
Tumor
(G)
hsa-miR-15a-5p
hsa-let-7e-5p
NKD1
hsa-let-7c-5p
hsa-miR-4458
hsa-let-7b-5p
hsa-miR-205-5p
(H)
GEO DEGs
TCGA DEGs
TCGA MEturquoise
miRWalk
GEO MEBlue

**FIGURE 1** Identification of let-7b-5p targeting NKD1 by bioinformatics analysis. The dynamic tree cut and merged dynamic method was performed to merge similar gene modules in TCGA (A) and GEO (C) datasets. The average correlation between modules and colon cancer phenotypes in TCGA (B) and GEO (D) and correlation *P* values are presented in parentheses. Heatmap of differential miRNA expression based on TCGA (E) and GEO (F) datasets. G, miRNAs targeting NKD1 were screened through the miRWalk website. Screening criteria: score ≥0.95. H, Let-7b-5p was identified by intersecting TCGA and GEO differentially expressed miRNAs, TCGA ME turquoise module miRNAs, GEO ME blue module miRNAs, and miRWalk-predicted miRNAs.

modules that had the highest correlation with the colon cancer phenotype. Moreover, TCGA-miRNA data and GSE135918 were also evaluated by differential analysis. 478 differentially expressed miRNAs were obtained from the TCGA-miRNA dataset, and 30 differentially expressed miRNAs were screened from GSE135918. Furthermore, six miRNAs that potentially target NKD1 were identified with miRWalk (Figure 1G). To obtain a more reliable miRNA targeting NKD1, Venn analysis was performed on the five groups of miRNAs, including the TCGA-ME turquoise and GEO-ME blue modules, six miRWalk miRNAs, and differentially expressed miRNAs from TCGA and GEO (Table S5) obtained above, and only let-7b-5p was identified (Figure 1H).

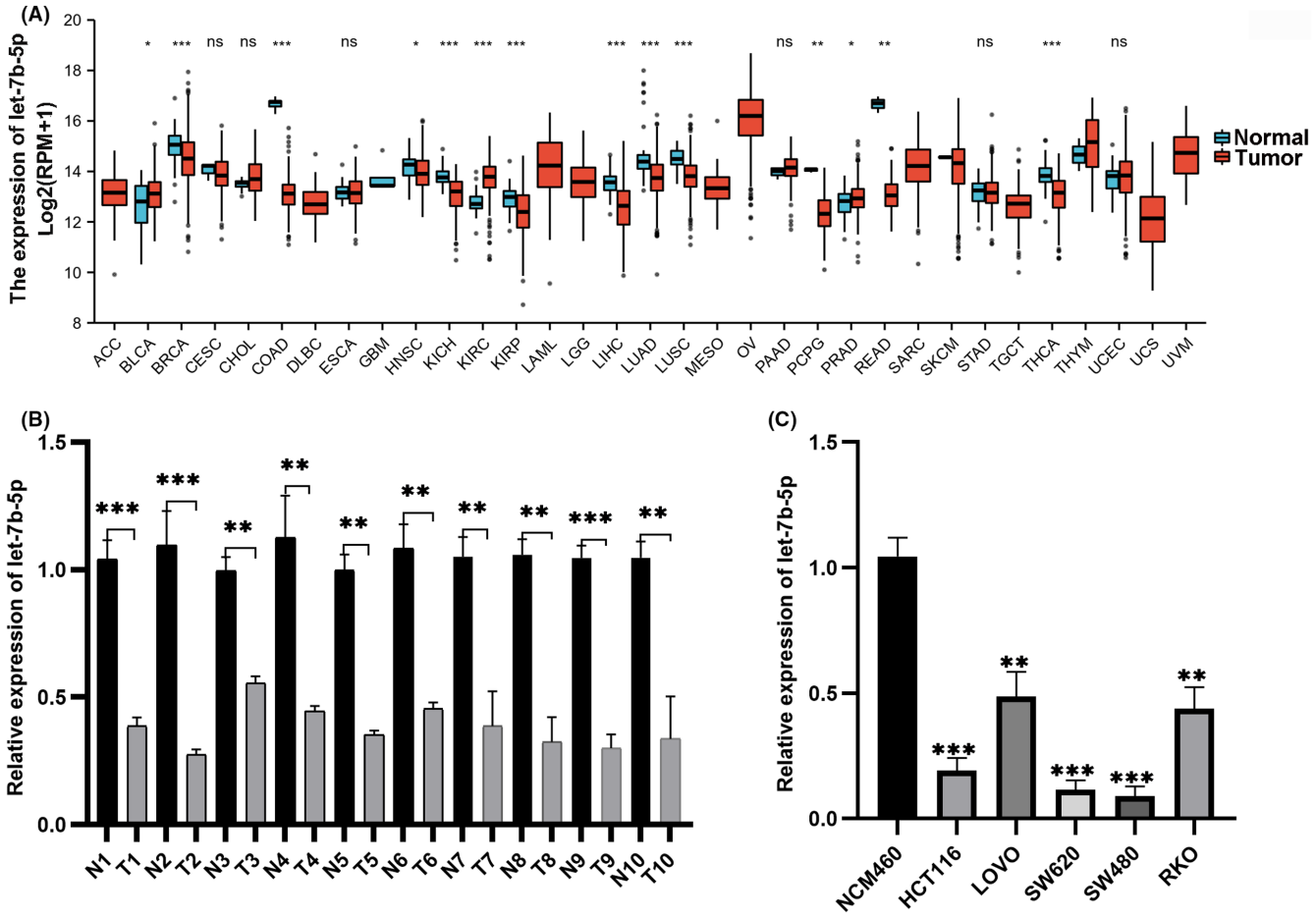



**FIGURE 2** Let-7b-5p is expressed at low levels in colon carcinoma tissues and cells. A, Pan-cancer analysis of let-7b-5p was performed based on the TCGA database. ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; READ, rectum adenocarcinoma; DLBC, lymphoid neoplasm diffuse barge B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower-grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; OV, ovarian serous cystadenocarcinoma; MESO, mesothelioma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; SARC, uterine carcinosarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma. qPCR was used to test the expression levels of let-7b-5p in colon cancer tissues (B) and adjacent normal tissues. C, Expression levels of let-7b-5p in the normal colon epithelial cell line NCM460 and different colon cancer cell lines HCT116, LOVO, SW620, SW480, and RKO were measured by qPCR.

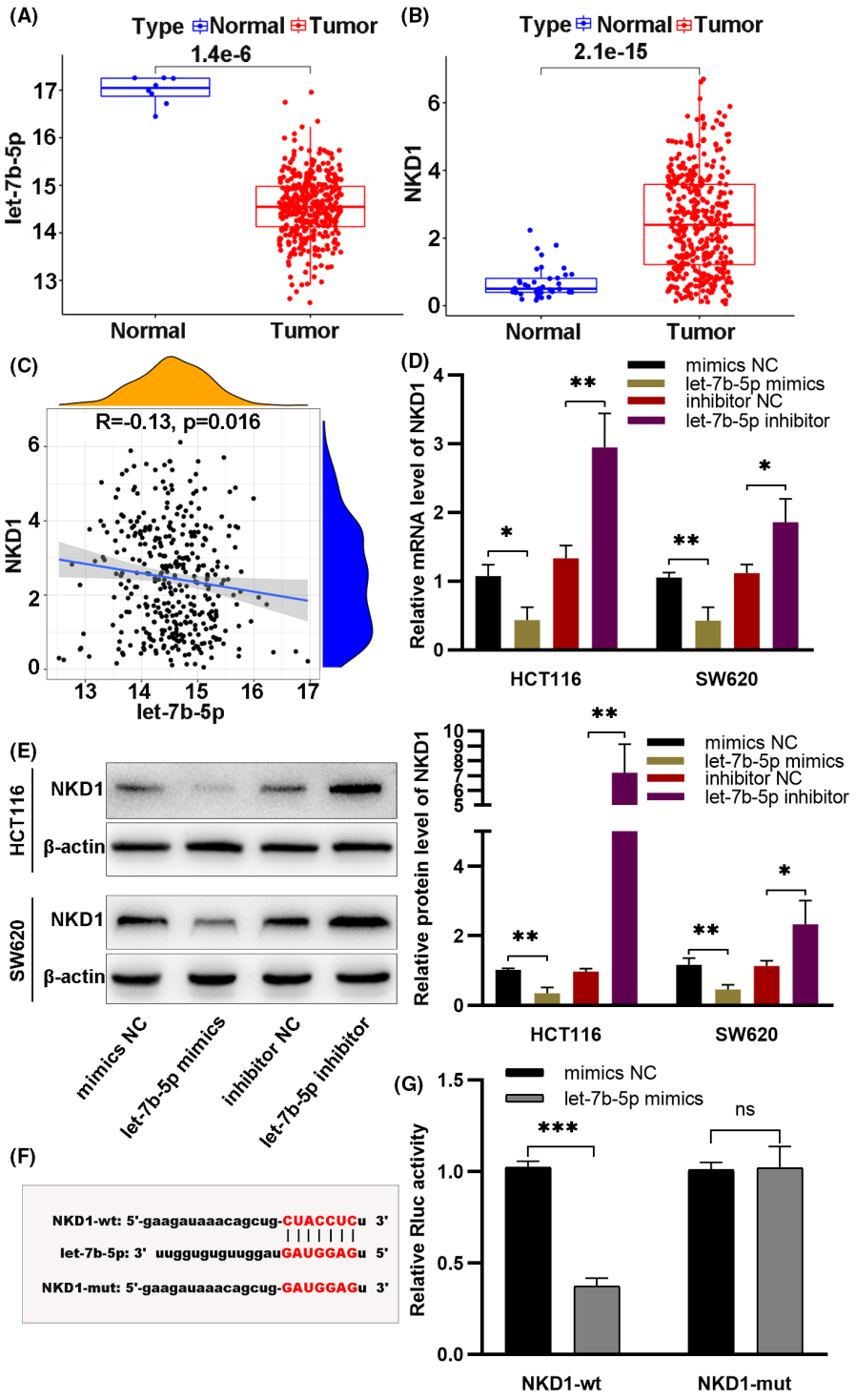



**FIGURE 3** Let-7b-5p targets the NKD1 gene in colon cancer cells. Expression levels of let-7b-5p (A) and NKD1 (B) in colon cancer tissues and normal colon samples obtained from the TCGA database. C, The Pearson correlation coefficient was used to analyze the expression correlation between NKD1 and let-7b-5p. The mRNA levels (D) and protein levels (E) of NKD1 in HCT116 and SW620 cells transfected with mimics negative control (NC) (50 nM), let-7b-5p mimics (50 nM), inhibitor NC (100 nM), or let-7b-5p inhibitor (100 nM) for 48 h were detected by qPCR and Western blot. F, Schematic diagram of let-7b-5p and NKD1 (wild-type, WT) targeting the complementary sequence and NKD1 sequence mutation (Mut). G, The relative Rluc activity in HEK293 cells transfected with Wt-NKD1 (1 μg) + mimics NC (50 nM), Wt-NKD1 (1 μg) + let-7b-5p mimics (50 nM), Mut-NKD1 (1 μg) + mimic NC (50 nM), or Mut-NKD1 (1 μg) + let-7b-5p mimics (50 nM) for 48 h was then measured by the dual-luciferase reporter system.

## 3.2 | Low let-7b-5p expression in colon cancer

A pan-cancer analysis was performed to examine the expression of let-7b-5p in various cancer and normal tissues, and the results manifested that let-7b-5p was expressed at relatively low levels in BRCA, COAD, HNSC, KICH, KIRP, LIHC, LUAD, LUSC, PCPG, and READ (Figure 2A). In addition, qPCR detection of let-7b-5p expression and the findings showed that let-7b-5p was lowly expressed in colon carcinoma tissues (Figure 2B) and cells (Figure 2C).

## 3.3 | Let-7b-5p targets NKD1

To verify the correlation between NKD1 and let-7b-5p, TCGA data were applied to identify the differential expression of NKD1 and let-7b-5p in colon carcinoma. The results demonstrated that Let-7b-5p is lowly expressed in normal colon tissue, and NKD1 is highly expressed in colon cancer tissue (Figure 3A, B). Furthermore, we found that the expression of NKD1 was inversely correlated with let-7b-5p (Figure 3C). Let-7b-5p mimics

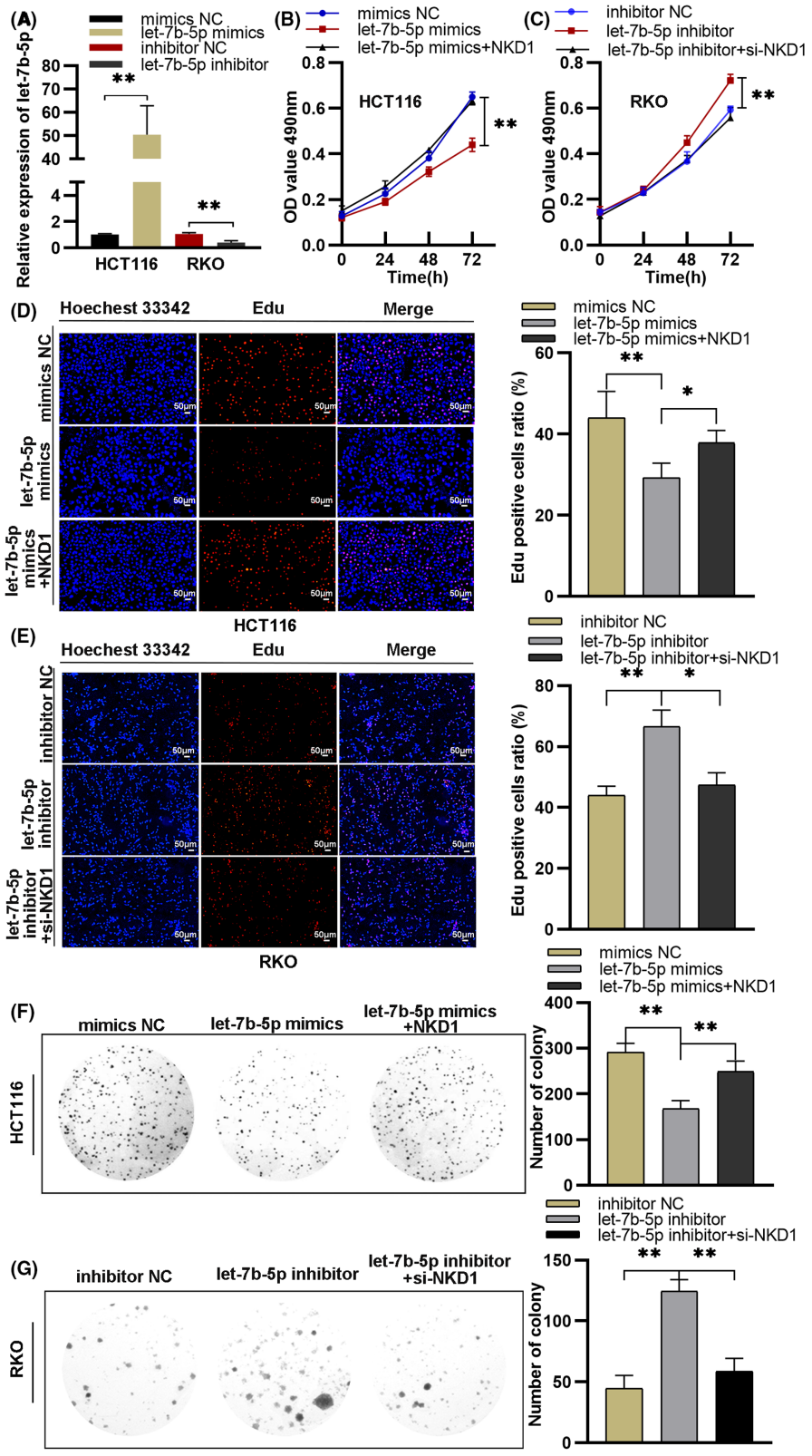



**FIGURE 4** Let-7b-5p inhibited colon cancer cell proliferation by targeting NKD1. A, qPCR was performed to measure transfection efficiencies. HCT116 cells were transfected with mimics negative control (NC) (50 nM), let-7b-5p mimics (50 nM), or let-7b-5p mimics (50 nM) + NKD1 (1 μg) for 48 h. RKO cells were transfected with inhibitor NC (100 nM), let-7b-5p inhibitor (100 nM), or let-7b-5p inhibitor (100 nM) + si-NKD1 (100 nM) for 48 h. The effects of let-7b-5p targeting NKD1 on proliferation in HCT116 and RKO cells were analyzed by MTT assays (B, C), EdU experiments (D, E), and plate clone formation assays (F, G). The scale bar length is 50 μm in the EdU microscope image.

or let-7b-5p inhibitors were transfected into HCT116 or SW620 cells, respectively, and then the mRNA or protein expression levels of NKD1 were measured. Let-7b-5p was found to inhibit the mRNA (Figure 3D) and protein (Figure 3E) expression of NKD1. To deeply analyze their interactions, their binding sites were predicted through the TargetScan website, and let-7b-5p was predicted to bind to the NKD1 3'UTR sequence (Figure 3F). Double luciferase reporter plasmids containing binding sites or binding site mutations were constructed (Figure 3F), and dual luciferase experiments were performed. The relative luciferase activity

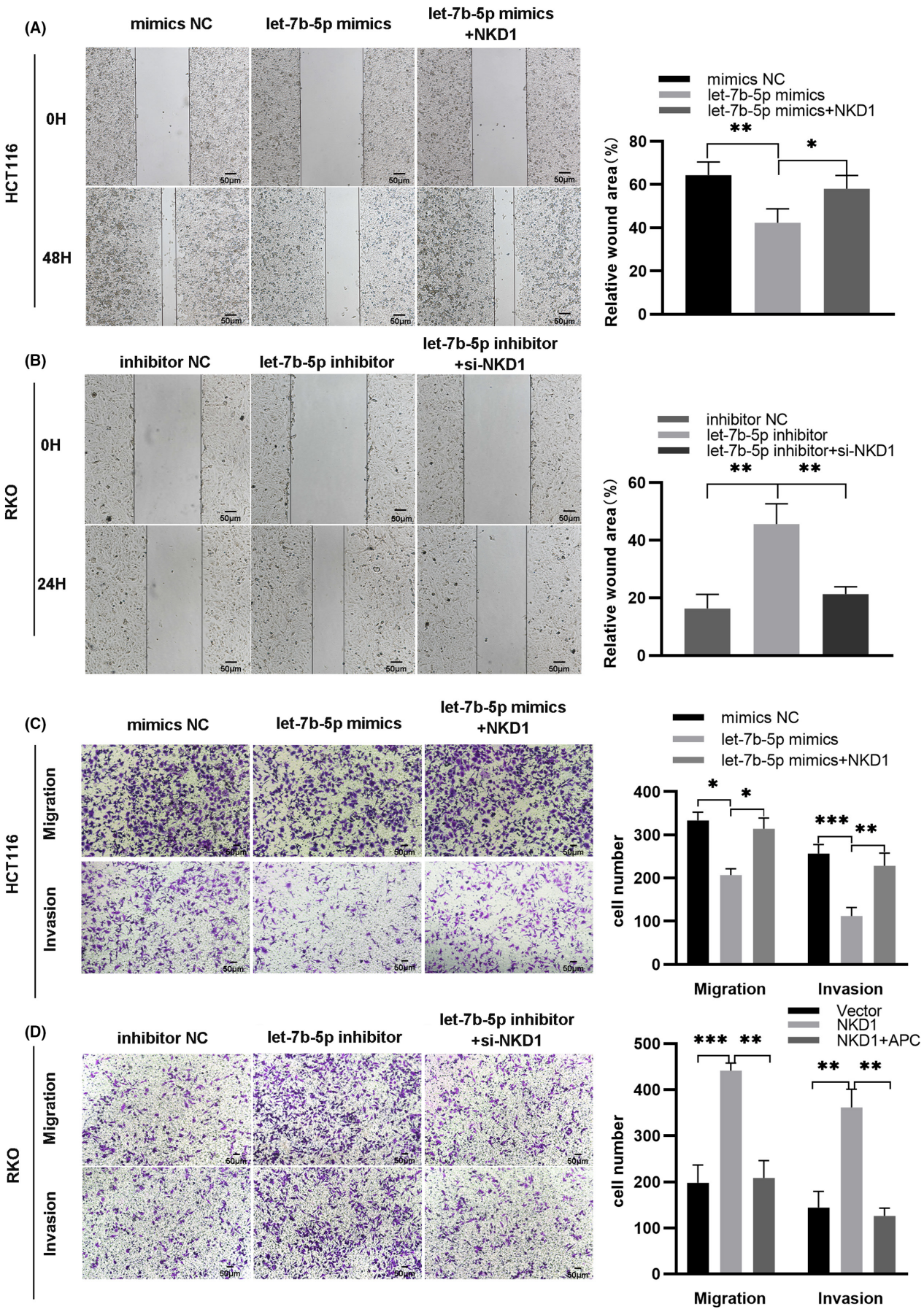

(A)
mimics NC
let-7b-5p mimics
let-7b-5p mimics +NKD1
HCT116
0H
48H
50μm
mimics NC
let-7b-5p mimics
let-7b-5p mimics+NKD1
Relative wound area (%)
80
60
40
20
0
**
*
(B)
inhibitor NC
let-7b-5p inhibitor
let-7b-5p inhibitor +si-NKD1
RKO
0H
24H
50μm
inhibitor NC
let-7b-5p inhibitor
let-7b-5p inhibitor+si-NKD1
Relative wound area (%)
60
40
20
0
**
**
(C)
mimics NC
let-7b-5p mimics
let-7b-5p mimics +NKD1
HCT116
Migration
Invasion
50μm
mimics NC
let-7b-5p mimics
let-7b-5p mimics+NKD1
cell number
400
300
200
100
0
*
*
***
**
Migration
Invasion
(D)
inhibitor NC
let-7b-5p inhibitor
let-7b-5p inhibitor +si-NKD1
RKO
Migration
Invasion
50μm
Vector
NKD1
NKD1+APC
cell number
500
400
300
200
100
0
*** **
** **
Migration
Invasion

**FIGURE 5** Let-7b-5p inhibited the migration and invasion of colon cancer cells by targeting NKD1. A wound-healing assay was performed to analyze the effect of let-7b-5p targeting NKD1 on the migration of HCT116 cells (A) transfected with mimics negative control (NC) (50 nM), let-7b-5p mimics (50 nM), or let-7b-5p mimics (50 nM) + NKD1 (1 μg) for 48 h or RKO cells (B) transfected with inhibitor NC (100 nM), let-7b-5p inhibitor (100 nM), or let-7b-5p inhibitor (100 nM) + si-NKD1 (100 nM) for 48 h. Transwell migration and Matrigel invasion assays were conducted to evaluate the effect of let-7b-5p targeting NKD1 on the migration and invasion of HCT116 (C) and RKO (D) cells. The microscope scale bar is 50 μm.

decreased in the cotransfected let-7b-5p mimics and pmirGLO-WT-NKD1 groups (Figure 3G). The above results suggest that let-7b-5p targets NKD1.

### 3.4 | Let-7b-5p constrained colon carcinoma cell proliferation by targeting NKD1

To examine whether let-7b-5p affects colon carcinoma cell proliferation, let-7b-5p mimic and inhibitor were transfected into colon cancer HCT116 or RKO cells, respectively. The overexpression and inhibition efficiency were tested by qPCR (Figure 4A), and the mimics NC, let-7b-5p mimics, and let-7b-5p mimics + pcDNA3.0-NKD1 were transfected into HCT116 cells. MTT (Figure 4B), EdU (Figure 4D), and clone formation experiment (Figure 4F) proved that overexpression of let-7b-5p remarkably suppressed colon cancer cell proliferation. This inhibition was abolished by overexpression of NKD1. Additionally, inhibitor NC, let-7b-5p inhibitor, and let-7b-5p + NKD1 siRNA (si-NKD1) were transfected into RKO cells, and the MTT (Figure 4C), EdU (Figure 4E), and clone formation assays (Figure 4G) demonstrated that let-7b-5p inhibitor significantly enhanced colon cancer cell proliferation, and the enhancement could be abolished by knockdown of NKD1. In summary, these results proved that let-7b-5p could constrain colon cancer cell proliferation by targeting NKD1.

### 3.5 | Let-7b-5p suppresses colon cancer cell migration and invasion by targeting NKD1

The mimics NC, let-7b-5p mimics, and let-7b-5p mimics + pcDNA3.0-NKD1 were transfected into HCT116 cells, and wound-healing and transwell assays were then used to analyze the effects of let-7b-5p on colon cancer cells migration and invasion. The results demonstrated that let-7b-5p notably repressed the migration (Figure 5A) and invasion (Figure 5C) of colon cancer cells, and the repression was reversed by the addition of NKD1 to the cells. Moreover, RKO cells were transfected with inhibitor NC, let-7b-5p inhibitor, and let-7b-5p inhibitor + si-NKD1, and wound-healing and transwell experiments revealed that the let-7b-5p inhibitor significantly enhanced the migration (Figure 5B) and invasion (Figure 5D) of colon cancer cells, and the enhancement was dissolved with the knockdown of NKD1. Therefore, let-7b-5p could remarkably prohibit colon carcinoma cell migration and invasion.

### 3.6 | Let-7b-5p restrained the Wnt pathway and inhibited β-catenin nuclear entry by targeting NKD1

Our previous study showed that NKD1 could maintain the stability of β-catenin proteins and promote its nuclear entry in colon carcinoma cells.[5] In addition, GSEA analysis of TCGA-COAD showed that the Wnt/β-catenin pathway was negatively correlated with NKD1 (Figure 6A). We wondered whether let-7b-5p could regulate the canonical Wnt/β-catenin pathway by targeting NKD1. HCT116 cells were transfected with mimics NC, let-7b-5p mimics, let-7b-5p mimics + pcDNA3-NKD1, pcDNA3, or pcDNA3-NKD1. Western blot showed that let-7b-5p could increase the expression of APC, GSK-3β, and Axin1 and decrease the expression of Dvl1, β-catenin, and the Wnt downstream target genes c-Myc and Axin2, which were reversed by the addition of NKD1 (Figure 6B, D). Additionally, RKO cells were transfected with the inhibitor NC, let-7b-5p inhibitor, let-7b-5p inhibitor + si-NKD1, si-NC, or si-NKD1. The Western blot results exhibited that inhibition of let-7b-5p concealed the expression of APC, GSK-3β, and Axin1 and raised the expression of Dvl1, β-catenin, Axin2, and c-Myc, while the changes were abrogated by knockdown of NKD1 expression (Figure 6C, E). Our previous research showed that NKD1 could promote the nuclear entry of β-catenin proteins in colon cancer cells.[5] We further speculated that let-7b-5p could affect β-catenin nuclear entry. Cytoplasmic and nuclear proteins were extracted from colon cancer cells and measured by Western blotting. The results proved that let-7b-5p could restrain the expression of β-catenin in the cytoplasm and nucleus, and overexpression of NKD1 reversed this phenomenon (Figure 6F). In addition, immunofluorescence experiments further confirmed that let-7b-5p notably lessened the protein levels of β-catenin in the cytoplasm and nucleus, and the overexpression of NKD1 prohibited the reduction (Figure 6G). In conclusion, let-7b-5p could regulate the Wnt pathway and inhibit the nuclear cumulation of β-catenin proteins by targeting NKD1.

### 3.7 | NKD1 constrained APC expression at the protein level

APC is a tumor suppressor protein that antagonizes the Wnt signaling pathway and can promote the rapid degradation of β-catenin.[23] Our study demonstrated that let-7b-5p regulates APC expression by targeting NKD1 (Figure 6B, C). In addition, NKD1 restrained APC protein expression in colon cancer cells (Figure 7A, B). To further study the molecular mechanism by

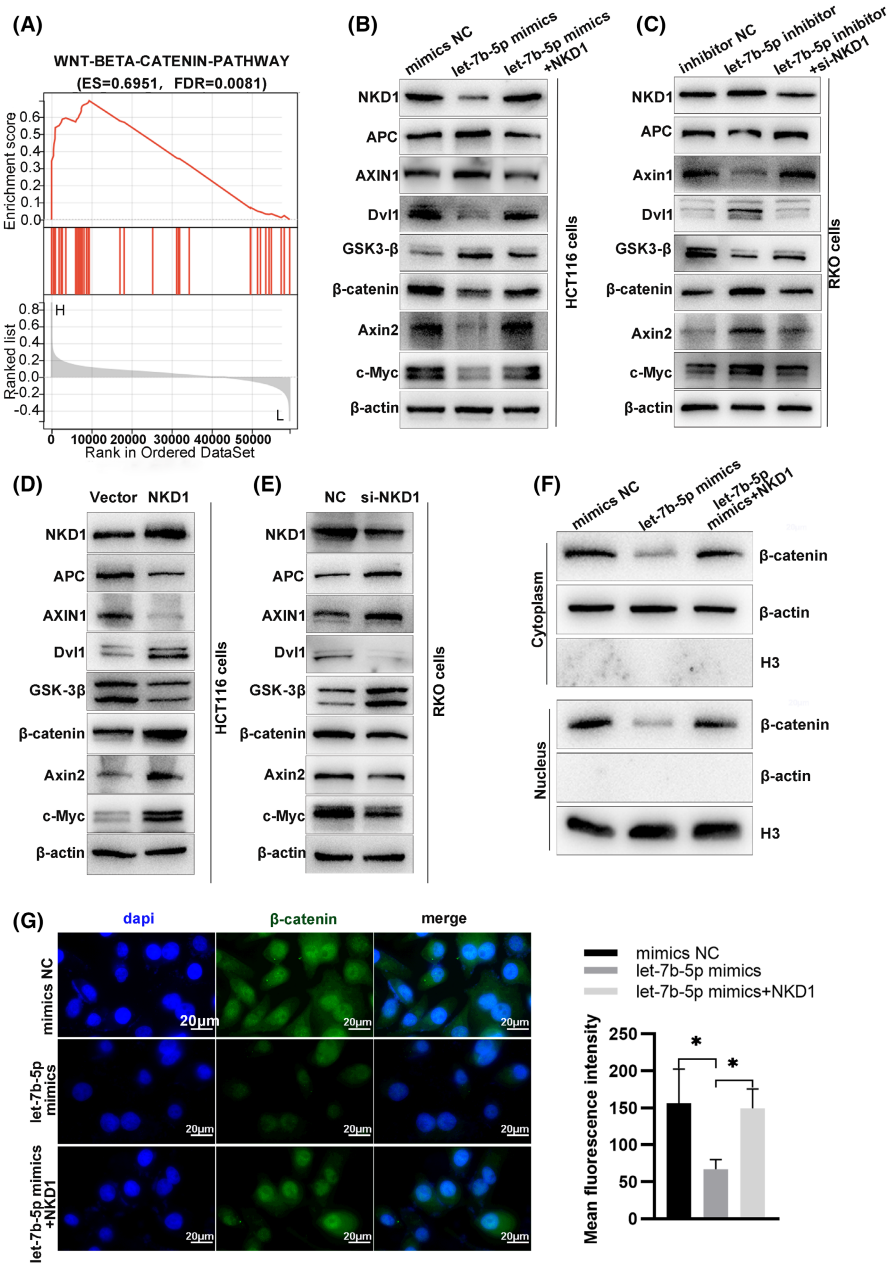



**FIGURE 6** Let-7b-5p inhibited the Wnt signaling pathway by targeting NKD1. A, Gene set enrichment analysis (GSEA) analysis of the Wnt signaling pathway was performed based on TCGA-COAD. $P < 0.05$ and $FDR < 0.25$ were considered to be statistically significant. B, D, The effect of let-7b-5p and NKD1 on the expression of important proteins in the Wnt pathway in HCT116 cells transfected with mimics negative control (NC) (50 nM), let-7b-5p mimics (50 nM), let-7b-5p mimics (50 nM) + NKD1 (1 μg), or NKD1 (1 μg) for 48 h and in RKO (C, E) cells transfected with inhibitor NC (100 nM), let-7b-5p inhibitor (100 nM), let-7b-5p inhibitor (100 nM) + si-NKD1 (100 nM), or si-NKD1 (100 nM) for 48 h was detected by Western blot. The nuclear accumulation of β-catenin proteins was detected by Western blotting (F) and immunofluorescence (G) in HCT116 cells. The microscope scale bar in the immunofluorescence pictures is 20 μm.

which NKD1 regulates APC, qPCR was performed, and the results demonstrated that NKD1 could not regulate the expression of APC at the transcriptional level (Figure 7C). To further determine whether these two proteins interact with each other, pcDNA3.0-Flag-NKD1 and PCMV-HA-APC were transfected into HEK293 cells. Co-IP experiments were implemented, and the results showed that NKD1 and APC proteins interacted with each other (Figure 7D, E). Moreover, immunofluorescence was also conducted, and the results showed that NKD1 and APC had similar intracellular localization distributions (Figure 7F). Taken together, NKD1 inhibited APC expression at the protein level, and they could bind to each other.

### 3.8 | Let-7b-5p suppresses APC ubiquitination degradation by targeting NKD1

To further investigate whether NKD1 affected the degradation of the APC protein, cycloheximide (CHX) was used to treat HCT116 cells and HCT116-NKD1 cells at different times, and Western blot showed that NKD1 notably promoted APC protein degradation (Figure 8A). Previous research has reported that APC is degraded through the proteasomal ubiquitination pathway.[24] HCT116 cells transfected with mimics NC, let-7b-5p, and let-7b-5p + pcDNA3.0-NKD1 were treated with MG132. Immunoprecipitation and Western blotting demonstrated that after protein synthesis had

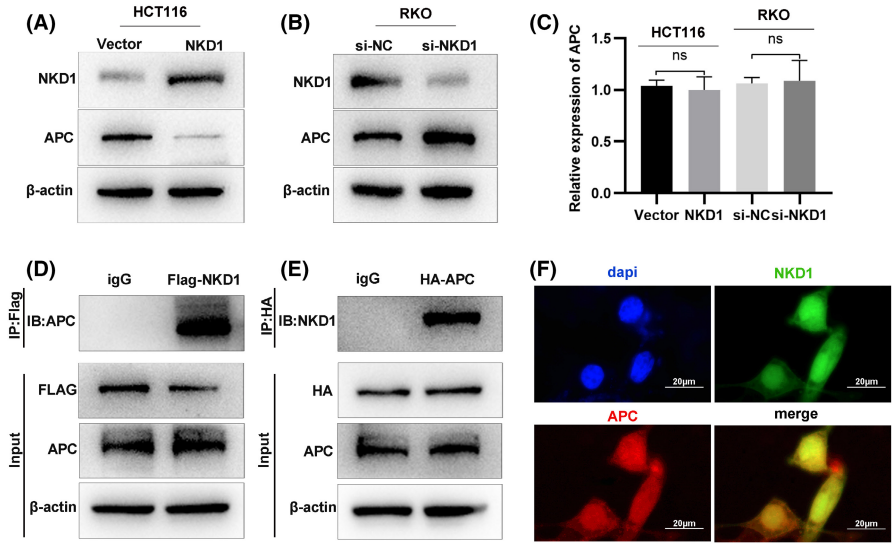



FIGURE 7 NKD1 inhibited APC expression at the protein level in colon cancer cells. APC protein expression in HCT116 cells (A) transfected with vector (1 μg) or NKD1 (1 μg) for 48 h or in RKO (B) cells transfected with si-NC (100 nM) or si-NKD1 (100 nM) for 48 h was then detected by Western blot. C, qPCR assays also measured the mRNA expression levels of APC in HCT116 or RKO cells. Immunoprecipitation of NKD1 and APC proteins in HEK293 cells transiently transfected with pcDNA-3.0-FLAG-NKD1 (D) (1 μg) or pCMV-HA-APC (1 μg) (E) for 48 h was then measured by Western blot assays. F, Immunofluorescence colocalization was used to observe the intracellular localization of NKD1 and APC proteins; the microscope scale bar is 20 μm.

been inhibited, let-7b-5p obviously constrained the ubiquitination of the APC protein. In addition, the inhibition was abolished by the addition of NKD1 (Figure 8B), which demonstrated that let-7b-5p suppressed APC protein ubiquitination and degradation by targeting NKD1 in colon cancer cells. Additionally, RKO cells transfected with the inhibitor NC, let-7b-5p inhibitor, or let-7b-5p inhibitor + si-NKD1 were treated with MG132. Immunoprecipitation and Western blot proved that the let-7b-5p inhibitor obviously enhanced APC protein ubiquitination and degradation, and the enhancement was inhibited by knocking down NKD1 expression (Figure 8C). Overall, let-7b-5p suppressed APC ubiquitination degradation, thereby enhancing APC protein stability by targeting NKD1.

## 3.9 | NKD1 suppressed the combination between deubiquitinating enzyme USP15 and APC

Huang et al reported that USP15, a deubiquitinating enzyme, protected APC from ubiquitination degradation.[25] We analyzed the correlation between NKD1, APC, and USP15 in colon cancer by GEPIA[26] (http://gepia.cancer-pku.cn/index.html), and the results proved that NKD1 was negatively correlated with USP15 ($R = -0.25$) (Figure 9A), while APC was positively correlated with USP15 ($R = 0.27$) (Figure 9B). Furthermore, Western blot demonstrated that overexpression of NKD1 in colon cancer HCT116 cells significantly reduced the expression of USP15, while knockdown of NKD1 in RKO cells evidently increased USP15 expression (Figure 9C), which implied that NKD1 prohibited the expression of USP15 in colon cancer cells. In addition, immunoprecipitation assays demonstrated that the binding of USP15 to APC was remarkably blocked by the NKD1 overexpression, while the combination between APC and USP15 was notably enhanced through knocking down NKD1 (Figure 9D). Altogether, these results present that NKD1 not only represses the expression of the USP15 protein but also blocks the binding between USP15 and APC.

## 3.10 | NKD1 promoted colon cancer cell proliferation and migration by inhibiting APC

The above experiments showed that NKD1 notably inhibited APC expression in colon cancer cells. Therefore, we speculated that NKD1 could promote the proliferation and migration of colon cancer cells by suppressing APC expression. HCT116 cells were transfected with pcDNA3.0 (vector), pcDNA3.0-NKD1, and pCMV-APC, and Western blotting was performed to detect transfection efficiency (Figure 10A). MTT (Figure 10B), EdU (Figure 10C), clone formation (Figure 10D), scratch (Figure 10E), and Transwell assays (Figure 10F) showed that NKD1 promoted the proliferation and migration of colon cancer cells, and this proliferative effect could be reversed by APC. Collectively, NKD1 notably boosted colon cancer cell proliferation and migration by restraining APC expression.

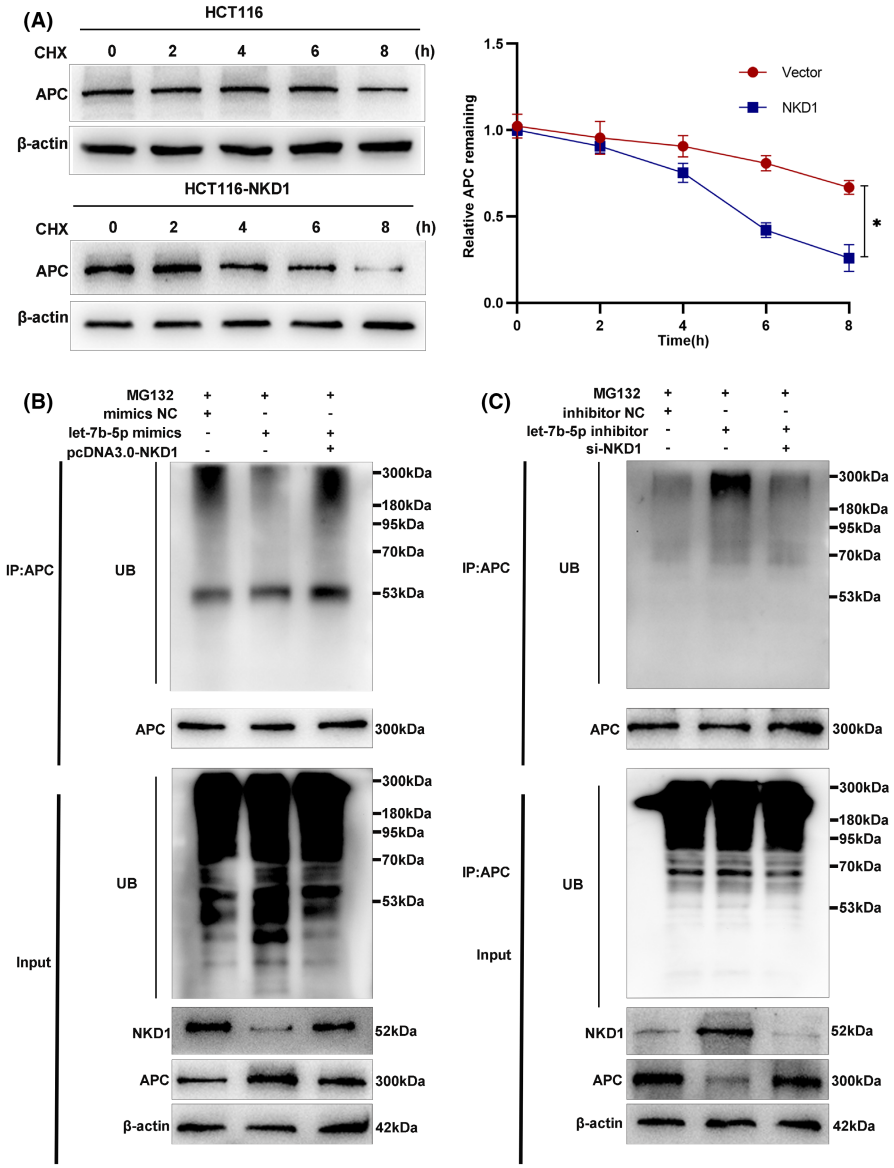



**FIGURE 8** Let-7b-5p inhibited the ubiquitination degradation of APC proteins by suppressing NKD1. A, The effect of NKD1 on the half-life of APC proteins in colon cancer HCT116 and HCT116-NKD1 cells treated with cycloheximide (30 μg/ml) for different periods (0, 2, 4, 6, 8 h). B, HCT116 cells transfected with mimics negative control (NC) (50 nM), let-7b-5p mimics (50 nM), or let-7b-5p mimics (50 nM) + NKD1 (1 μg) for 48 h blot and (C) RKO cells transfected with inhibitor NC (100 nM), let-7b-5p inhibitor (100 nM), or let-7b-5p inhibitor (100 nM) + si-NKD1 (100 nM). Then, transfected cells treated with MG132 (40 μM) for 6 h were detected with Western blotting.

## 4 | DISCUSSION

Among sporadic colorectal cancer patients, >80% have mutations in APC, most of which eventually produce truncated APC proteins.[27] Compared with the wild-type APC protein, truncated APC lacks the AXIN- and β-catenin–binding domain and thus cannot inhibit the Wnt signaling pathway; such mutation is thought to be an early event in colon carcinogenesis.[23,28] The HCT116 and RKO cells used in this study were not mutated,[29,30] which is why the two cell lines were used in this research.

Although NKD1 is considered an inhibitor of the Wnt pathway, its high expression and pro-proliferation effect demonstrate its unique function in colon cancer. Stancikova et al reported that NKD1 acts as a marker for intestinal and liver tumors with aberrant activation of the WNT/β-catenin pathway.[4] They believed that the presence of NKD1 might create an optimal growth environment for tumor growth because excessive Wnt signaling activity is not optimal for tumor growth.[31,32] Moreover, we conducted the GSEA analysis, and the results manifested that NKD1 was negatively correlated with the Wnt pathway activity in the NKD1 low-expression group, while NKD1 and Wnt pathway activity were positively correlated in the NKD1 high-expression group (Figure S2A, B), which well explained why NKD1 activated the Wnt signaling pathway in colon carcinoma and liver carcinoma with high expression of NKD1 and played the role of Wnt signaling pathway antagonist in other tumor tissues with low expression of NKD1. Furthermore, we also conducted ssGSEA analysis with colon carcinoma samples, and the data exposed a positive correlation between NKD1 and the Wnt signaling pathway ($R = 0.56$) (Figure S2C), which implied that NKD1 was a marker of Wnt signaling activation in colon carcinoma tissues. These results were consistent with the reported articles.[4,33]

Regarding the mechanism of NKD1 promoting the ubiquitination and degradation of APC, we found that NKD1 promoted the

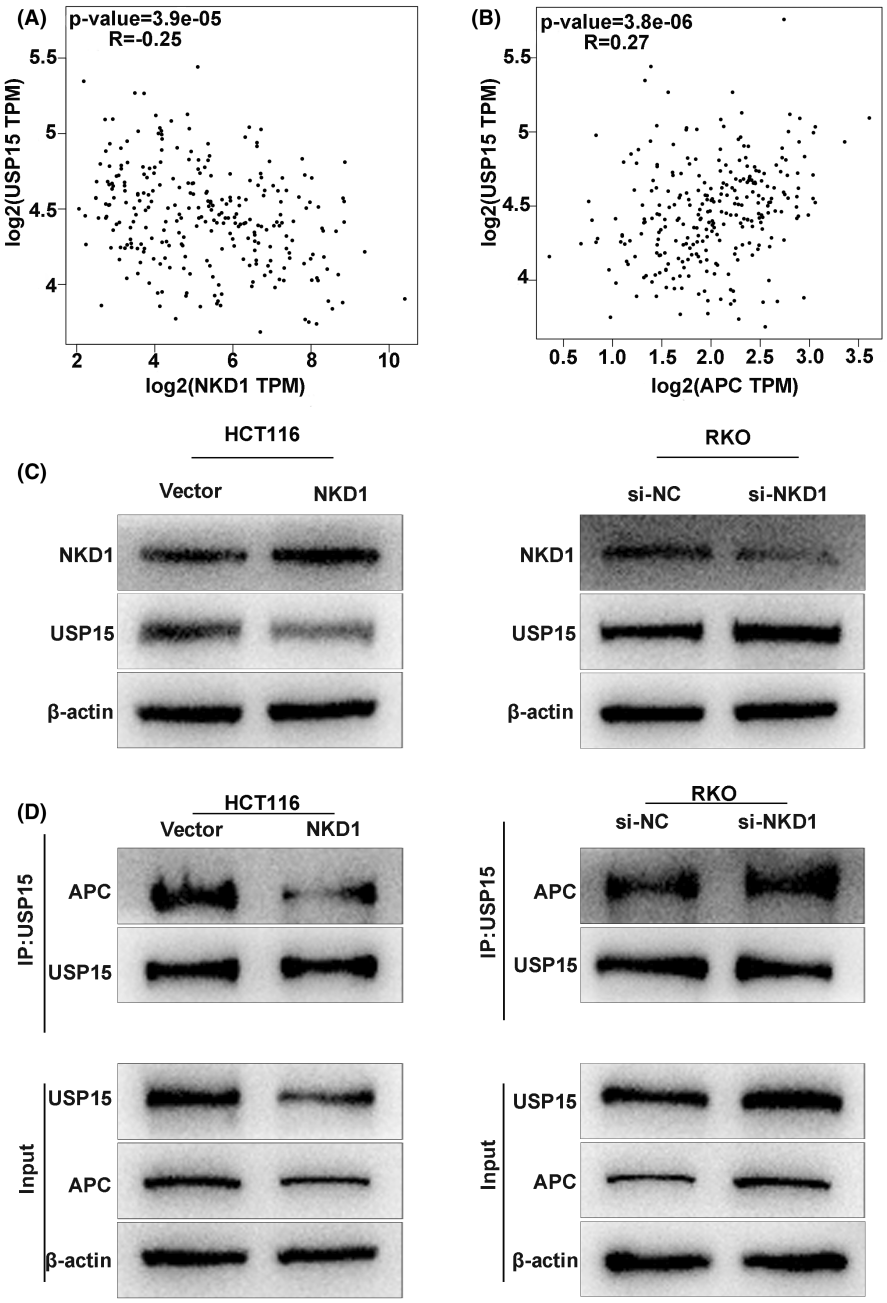
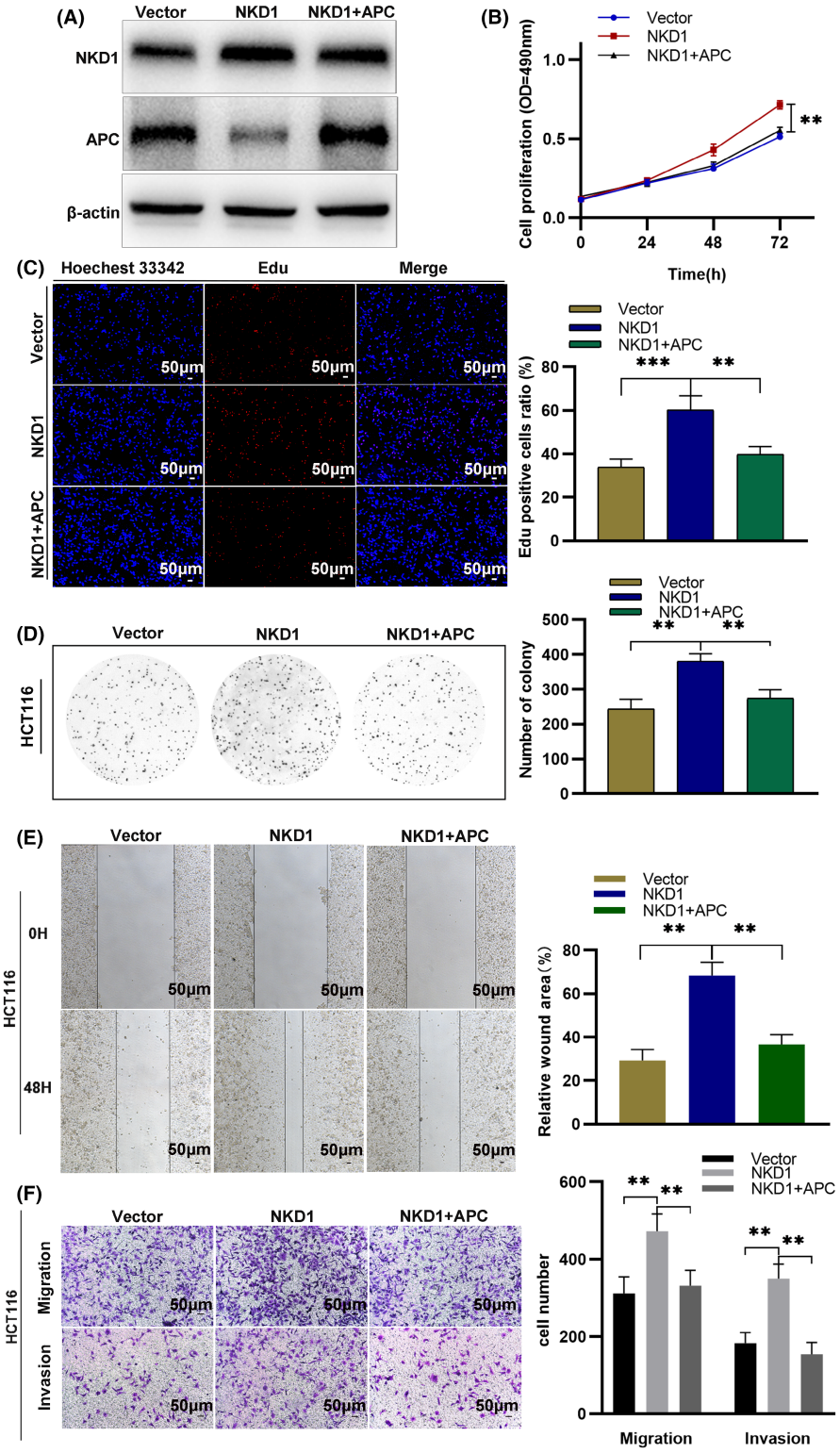



**FIGURE 9** NKD1 suppressed the combination between deubiquitinating enzyme USP15 and APC. Pearson correlations between USP15 and NKD1 (A) and between USP15 and APC (B) were analyzed by GEPIA in colon cancer. B, After transfecting of NKD1 (1 μg) in HCT116 cells or si-NKD1 (100 nM) in RKO cells (C), the protein expression of USP15 was detected by Western blot. (D) In the total protein lysate overexpressing or knockdown NKD1, USP15 antibody (1:100) and Protein A/G agarose beads (100 μl) were added, and then the amount of APC protein bound to USP15 was measured by Western blot.

ubiquitination and degradation of APC by inhibiting the expression of deubiquitination enzyme USP15 and blocking the interaction between SUP15 and APC. Besides, Huang et al[25] reported that inhibition of GSK-3β led to the disassembly of the CSN-based super-complex, the accumulation of β-catenin, and the ubiquitination of APC in an Axin1-dependent manner. Our experiments presented that NKD1 regulated several key proteins of this super-complex, including GSK-3β and Axin1 (Figures 6 and 9). Therefore, we speculated that NKD1 boosted the ubiquitination and degradation of APC through regulating the depolymerization of the super-complex.

In conclusion, bioinformatics identified let-7b-5p as a target of NKD1. In colon cancer cells, let-7b-5p and NKD1 upregulated the expression of positively regulated Wnt signaling and Wnt downstream target genes and inhibited the key proteins of the negatively regulated Wnt pathway. NKD1 promotes the ubiquitination degradation of APC by prohibiting the expression of USP15 and blocking binding of the deubiquitinating enzyme USP15 to APC and leads to the entry of β-catenin into the nucleus to promote colon cancer cell proliferation, migration, and invasion, while let-7b-5p inhibits this process by targeting NKD1 (Figure S3). These results suggest that let-7b-5p and NKD1 may be tissue-specific




markers for colon cancer and may serve as new therapeutic targets for colon cancer.

## ACKNOWLEDGEMENTS

Thanks to The Cancer Genome Atlas Program for providing a wealth of analyzable public data.

## FUNDING INFORMATION

This research project was funded by the National Natural Science Foundation of China [81872275, 81972618]; by the Changzhou High-Level Medical Talents Training Project [2022CZBJ110]; by the Medical Research Project of Jiangsu Health Commission [M2020002]; by the funds of Changzhou Sci & Tech Program

[CJ20220007, CJ20220006, CJ20210015]; and by the innovation team project of Guangdong Provincial Department of Education [2021KCXTD039].

## ETHICS STATEMENT

This study was approved by the Ethics Committee of Wujin Hospital affiliated to Jiangsu University. All participants were notified, and their consent was obtained prior to the start of the research.

Approval of the research protocol by an Institutional Review Board: Yes.

Informed Consent: N/A.

Registry and Registration No. of the study/trial: N/A.

Animal Studies: N/A.

## DISCLOSURE

The authors have no conflicts of interest.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Dai Y, Liu J, Li X, et al. Let-7b-5p inhibits colon cancer progression by prohibiting APC ubiquitination degradation and the Wnt pathway by targeting NKD1. *Cancer Sci*. 2023;114:1882-1897. doi:10.1111/cas.15678