Supplementary Materials and Methods

RNA extraction and quantitative reverse-transcribe PCR (qPCR)

The cells were lysed with Trizol (Invitrogen Life Technologies) to extract total mRNA according to the manufacturer's instruction. Total RNA (1 μg) was reverse-transcribed into cDNA using PrimeScript Reagent Kit with gDNA Eraser (Takara Bio). qPCR was done using SYBR Premix ExTaq (Takara Bio). The sequences of the primers used were listed in Supplementary Table S1.

Western blotting

Total cells were lysed with RIPA lysis buffer with protease inhibitor and phosphatase inhibitor cocktail (complete cocktail; Roche Applied Science, Mannheim, Germany). Equal amounts of the sample proteins were electrophoresed on sodium dodecyl sulphate polyacrylamide gel before being transferred to a polyvinylidene fluoride membrane with 0.45 μm pore (Millipore, Billerica, MA). The membrane was then incubated with first antibodies followed by second antibodies. The antibodies used are listed in Supplementary Table S2. Immunoblotting with anti-β-Actin antibody was conducted to ensure equal protein loading. The signals were measured using Super Signal West Femto Maximum Sensitivity Substrate detection system (Thermo Scientific, Waltham, MA).

Chromatin immunoprecipitation (ChIP) assay

Chromatin was immunoprecipitated using the ChIP Assay Kit (Beyotime) according to the manufacturer's instruction. Briefly, cells were lysed and chromatins were then

immunoprecipitated using protein A+G Agarose/Salmon Sperm DNA and anti-NeuroD1 antibody or normal rabbit IgG, de-crosslinked for 4 h at 65°C, and treated with 0.5M EDTA, 1M Tris pH6.5 and 20 mg/ml proteinase K. Immunoprecipitated chromatin was then subjected to PCR by using PrimeSTAR Max (Takara Bio). The primers for amplifying the p53 promoter region with predicted *NeuroD1* binding site were: 5'-TGA AAG CAC TGT GTT CCT TAG-3' (forward primer); and 5'-AAC TCC CAG CAG CCA CGA GGA-3' (reverse primer). The primers for amplifying *ALK* promoter region with *NeuroD1* binding site were: 5'-TGC ATA GGA GCC GAT CGA GC -3' (forward primer); and 5'- AGA GCC GCT GGA TCG CAT CT-3' (reverse primer). The primers for amplifying *p53* promoter region without predicted *NeuroD1* binding site were: 5'-CTG GGA GAA GGT GCG ATG AT-3' (forward primer); and 5'- CTT CCT AAG GGC AGG GCA GT-3' (reverse primer). The primers for amplifying intergenic region between *p53* and *ATP1B2* were: 5'-CAT TCT AGG CAC CGT GAG GAC-3' (forward primer); and 5'- TCC CTT TAA CGT GCC TGA CA-3' (reverse primer).

Cell cycle and apoptotic rate analysis

Cells were transfected with indicated shRNA expression vectors and selected with puromycin as described above. Selected cells were re-seeded in 6-well plate (3×10^5 cells/well), and 24 h later, the medium was changed with serum-free medium and cultured further for 24 h to synchronize the cell cycle phase. The cells were incubated further in normal medium for 24 h before being harvested. Cells were then stained with propidium iodide (KeyGen Biotech, Nanjing, China), and the percentages of the cells in each cell cycle phase were determined

using flow cytometry. For analyzing the apoptotic rate, after puromycin selection, cells were stained using Annexin V/PI, and the apoptotic rate were determined using flow cytometry.

Immunohistochemical analysis and TUNEL assay

Paraffin-embedded sections were obtained from fresh colorectal cancer and adjacent tissues or xenografted tumours at 4 μm thickness using a cryostat and subjected to immunohistochemistry. Briefly, the tissue sections were incubated with primary antibodies for 1 h followed by incubation with corresponding secondary antibodies conjugated with horse-radish peroxidase. Visualization was performed using a DAB Kit (DAKO, Beijing, China) under microscope. The nuclei were then counterstained with hematoxylin. Then the specimens were dehydrated and coverslip were mounted. The antibodies used were listed in Supplementary Table S2. For TUNEL assay, slides were permeabilized in 0.1% Triton X-100, and TUNEL positive cells were detected using Fluorescein in situ Cell Death Detection Kit (Roche Applied Science) according to manufacturer's instruction. Images were taken using Pannoramic Midi (3DHistech, Budapest, Hungary).

Dual luciferase assay

8×10^4 cells were seeded onto 24-well plates. 24 h later, the cells were co-transfected with the indicated shRNA expression vector or overexpression vector, reporter vector, and the *Renilla* luciferase expression vector (pRL-SV40, Promega) as internal control. 24 h after co-transfection, the luciferase activities were then measured with the Dual Luciferase Assay System (Promega).

Cell counting assay

Cells were transfected with indicated vectors, and 24 h after transfection, puromycin selection (final concentration: 1.2 μg/ml) was performed to eliminate the untransfected cells. The transfected cells were re-seeded into 96-well plates at the density of 5×10^3 cells/well. The cell numbers were measured by colorimetric assays with 3-(4,5-dimethylthiazol-2-yl)-5- (3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, Promega) in accordance with the manufacturer's instructions at indicated time points.

Colony formation assay

Cells were transfected with indicated shRNA expression vectors and selected using puromycin as indicated above. Cells were then re-seeded into 6-well plates at a density of 500 cells/well, and cultured for 8 days. Cells were then fixed with 30% paraformaldehyde and stained with methylene blue. The colonies were then counted. The investigator was blinded during the assessment.

Actinomycin D and cycloheximide treatment

1×10^6 cells were seeded in 6-well plate and cultured for 24 h. Proteins were collected after the cells were treated with actinomycin D (final concentration: 1 μM) or cycloheximide (final concentration: 30 μg/ml) for 1.5 h.

Caspase activity assay

Cells were transfected with indicated shRNA expression vectors, and puromycin selection was performed as described. After puromycin selection, cells were re-seeded onto 96-well plates for 24 h. Caspase activities were measured using Caspase-Glo 3/7 Assay (Promega) according to the manufacturer's instructions.

Animal experiments

For the *in vivo* tumor study, BALB/c-nu/nu mice (male, body weight: 18-22 g, 6 weeks old) were purchased from the Third Military Medical University (Chongqing, China; Permit Number SYXK-PLA-20120031). Animal studies were approved by the Laboratory Animal Welfare and Ethics Committee of the Third Military Medical University, and carried out in the Third Military Medical University. All animal experiments conformed to the approved guidelines of Animal Care and Use Committee of Third Military Medical University. All efforts were made to minimize suffering.

To generate an experimental subcutaneous tumor model, BALB/c-nu/nu mice were randomly divided into 3 groups (n = 6), and each group was injected subcutaneously with 3×10^6 indicated stable cell lines. Tumor size (V) was evaluated by a caliper every 2 days using the following equation: $V = a \times b^2/2$, where a and b are the major and minor axes of the tumor, respectively.[28] The investigator was blinded to the group allocation and during the assessment.

Table S1. Primer pairs used for quantitative RT-PCR

Genes	Refseq No.	Forward primer sequence (5′–3′)	Reverse primer sequence (3′–5′)
NeuroD1	NM_002500.4	ACCTGGTCTCCTTCGTTCAG	ACTGGTAGGAGTAGGGGTGT
p21	NM_000389.4	TCACTGTCTTGTACCCTTGTGC	GGCGTTTGGAGTGGTAGAAA
p53	NM_000546.5	AGGCCTTGGAACTCAAGGAT	GGTAGACTGACCCTTTTTGGAC
β–Actin	NM_001101.3	CGAGCGCGGCTACAGCTT	TCCTTAATGTCACGCACGATTT

Table S2. Antibodies used for western blotting, chromatin immunoprecipitation, and immunohistochemistry

Antibody	Product number	Maker	Experiment	Dilution
anti-NeuroD1	Proteintech	12081-1-AP	Western Blotting	1/1000
			Immunohistochemistry	1/200
			ChIP assay	3 μg/ml cell lysate
anti-p21	Proteintech	10355-1-AP	Western Blotting	1/1000
anti-cyclin B	Santa Cruz Biotechnology	sc-245	Western Blotting	1/500
anti-β-Actin	Proteintech	66009-I-Ig	Western Blotting	1/100000
anti-CDK1	Santa Cruz Biotechnology	sc-54	Western Blotting	1/500
anti-p53	Proteintech	10442-1-AP	Western Blotting	1/1000
Goat Anti-Rabbit IgG	ZSGB-BIO	ZB2301	Western Blotting	1/10000
Goat Anti-Mouse IgG	ZSGB-BIO	ZB2305	Western Blotting	1/10000
Cleaved-caspase 3	Servicebio	GB11009	Immunohistochemistry	1/200

Fig. S1

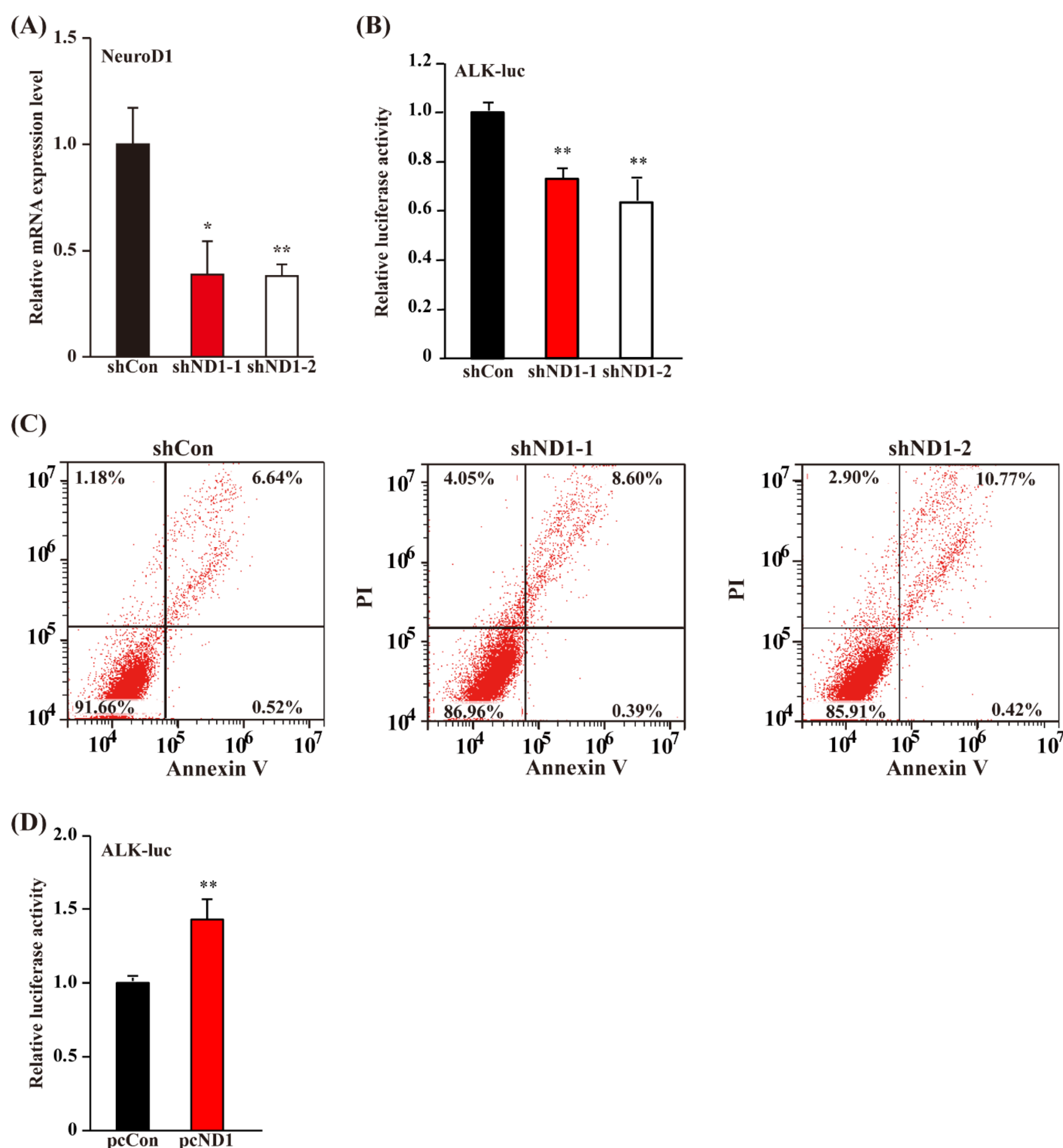

Fig. S1. The effect of NeuroD1 on the ALK transcription activity and apoptosis in HCT116 cells. A, The expression level of NeuroD1 mRNA in HCT116 cells transfected with two shRNA expression vectors targeting different sites of *NeuroD1*, as analyzed using quantitative RT-PCR (qPCR). **B,** Relative luciferase activity of ALK-luc in *NeuroD1*-silenced HCT116 cells, as measured using Dual Luciferase Assay. **C**, The effect of shNeuroD1 on HCT116 cells apoptosis as analyzed using Annexin V/PI staining and flow cytometry. **D,** Relative luciferase activity of ALK-luc in NeuroD1-overexpressed HCT16 cells, as measured using Dual Luciferase Assay. The activity of firefly luciferase was normalized with that of *Renilla* luciferase as inner control. β-Actin was used for qPCR normalization. Cells transfected with shCon were used as control. Quantitative data were shown as relative to control and expressed as mean ± S.D. (n = 3).

shND1: shRNA expression vector targeting *NeuroD1*; pcCon: pcDNA3.1(+); pcND1: *NeuroD1* overexpression vector, * $P < 0.05$; ** $P < 0.01$.

Fig. S2

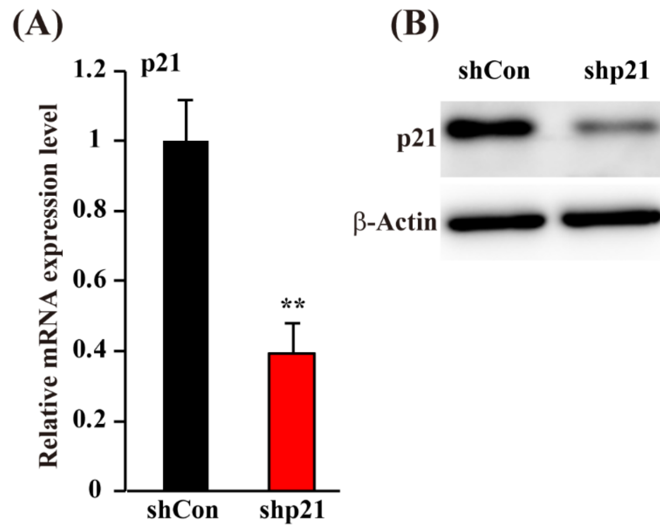

Fig. S2. The effect of shRNA vectors against *p21* in HCT116 cells. A, The expression level of p21 mRNA in *p21*-silenced HCT116 cells, as analyzed using qPCR. **B**, Protein expression level of p21 in *p21*-silenced HCT116 cells, as determined using western blotting. Cells transfected with shCon were used as control. Quantitative data were shown as relative to control and expressed as mean ± S.D. (n = 3). shp21: shRNA expression vector targeting *p21*; $^{**}P < 0.01$.

Fig. S3

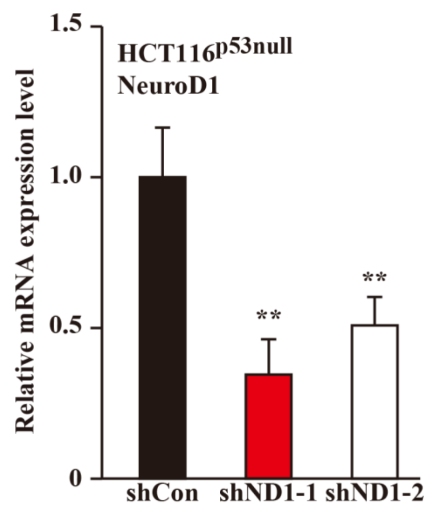

Fig. S3. The effect of shRNA vectors against *NeuroD1* in HCT116^{p53null} cells. A, The expression level of shNeuroD1 mRNA in HCT116^{p53null} cells, as analyzed using qPCR. Cells transfected with shCon were used as control. Quantitative data were shown as relative to control and expressed as mean ± S.D. from three independent experiments. shND1: shRNA expression vector targeting *NeuroD1*; $^{**}P < 0.01$.

Fig. S4

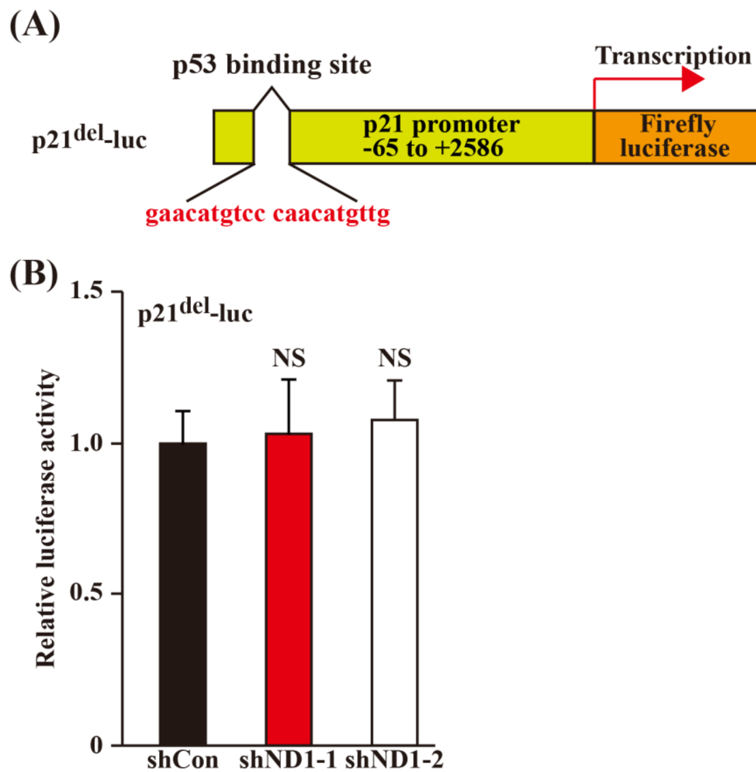

Fig. S4. The effect of shNeuroD1 on the transcriptional activity of luciferase reporter vector with *p21* promoter lacking p53 binding site ($p21^{del}$-luc). A, Schematic diagram of $p21^{del}$-luc. **B,** Relative luciferase activity of $p21^{del}$-luc in *NeuroD1*-silenced HCT116 cells, as measured using Dual Luciferase Assay. The activity of firefly luciferase was normalized with that of *Renilla* luciferase as inner control. Cells transfected with shCon were used as controls. Quantitative data were expressed as mean ± S.D. (n = 3). shND1: shRNA expression vector targeting *NeuroD1*; NS: not significant.

Fig. S5

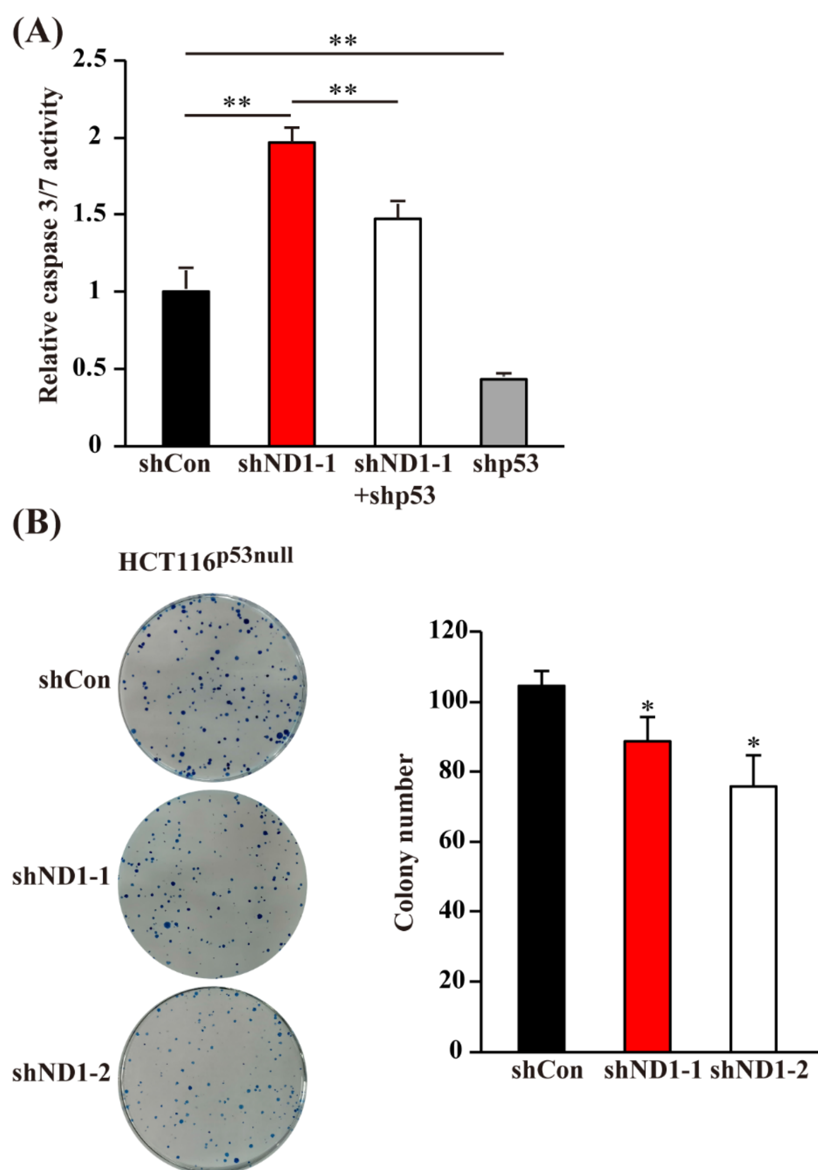

Fig. S5. The caspase3/7 activity in *NeuroD1, p53*-double silenced HCT116 cells and colony formation potential in HCT116^{p53null} cells. A, Caspase-3/7 activity was assessed by fluorometric assay. Cells transfected with shCon were used as controls. **B,** Colony formation potential of *NeuroD1*-silenced HCT116^{p53null} cells. The representative images (left) and quantification results (right) were shown. Quantitative data were expressed as mean ± S.D. (n = 3). shND1: shRNA expression vector targeting *NeuroD1*; NS: not significant. * $P < 0.05$; **$P < 0.01$.

Fig. S6

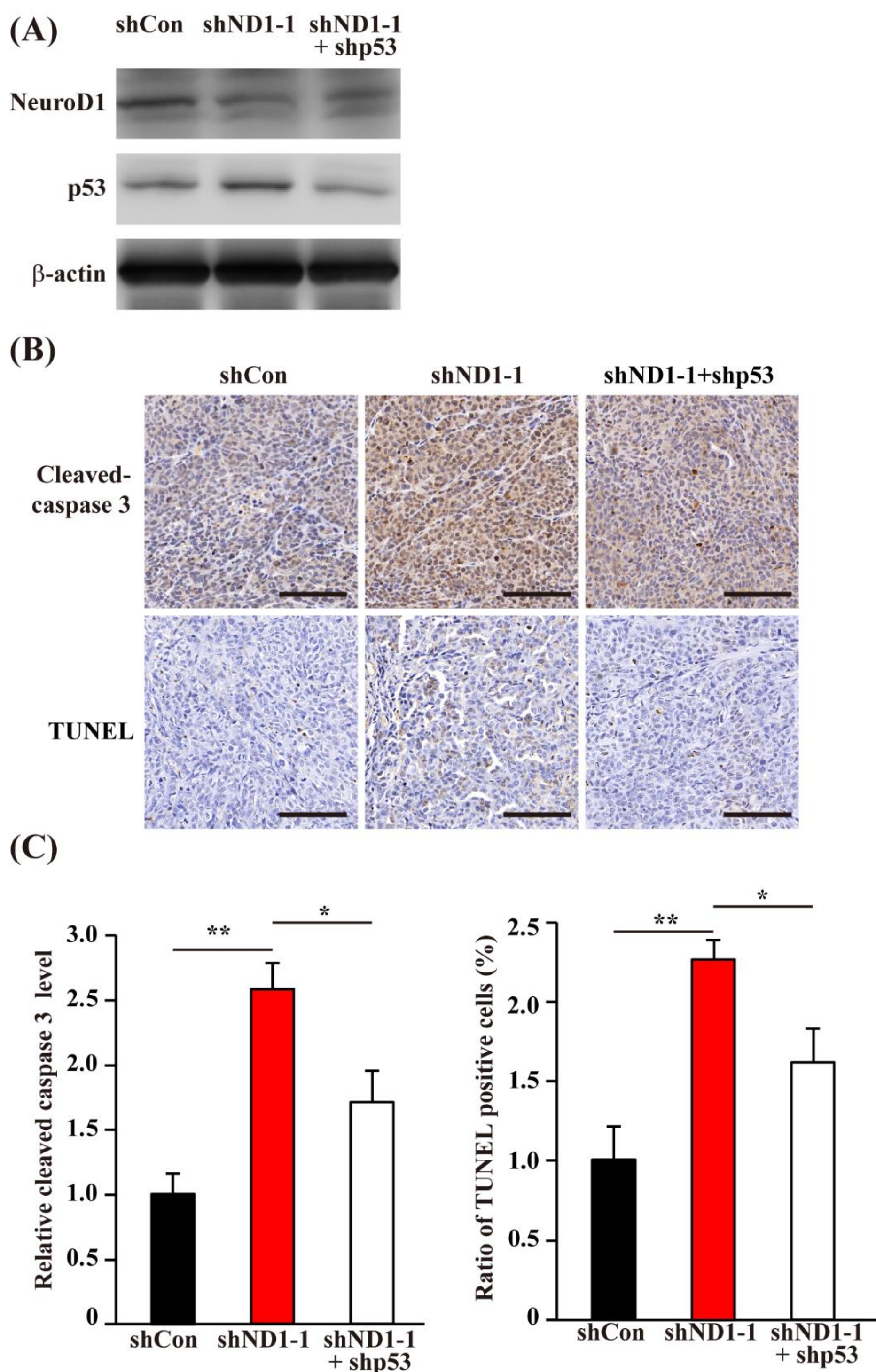

Fig. S6. The effect of NeuroD1 and p53 silencing on apoptosis in tumor xenografted mice. A, NeuroD1 and p53 protein expression levels in *NeuroD1*-silenced and *NeuroD1*, *p53*-double silenced HCT116 stable cell lines were determined using western blotting. β-Actin was used as loading control. **B–C**, Relative cleaved-caspase 3 level and the number of TUNEL positive cells in tissue sections of xenografted tumors in Balb/c-nu/nu mice injected with indicated cell lines. Representative images (B) and quantification results (C) were shown. Scale bars: 50 μm. Quantification was performed by counting the ratio of the positive cells to total cell number,

and the results are shown as relative to control. shND1: shRNA expression vector targeting *NeuroD1*. * $P < 0.05$; ** $P < 0.01$.