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Original Research

NELFCD Promotes Colon Cancer Progression by Regulating the DUSP2–p38 Axis

Weiwei Dai[1], Min Zhu[1], Yujing Sun[2], Baohong Xu[3], Guorong Ma[4], Haiyun Shi[1,*], Peng Li[1,*]

[1]Department of Gastroenterology, Beijing Friendship Hospital, Capital Medical University, State Key Laboratory of Digestive Health, National Clinical Research Center for Digestive Diseases, 100000, Beijing, China
[2]Department of Laboratory, Peking University International Hospital, 100000, Beijing, China
[3]Department of Gastroenterology, Beijing Luhe Hospital Affiliated to Capital Medical University, 100000, Beijing, China
[4]Department of Pathology, Beijing Luhe Hospital Affiliated to Capital Medical University, 100000, Beijing, China
*Correspondence: shihaiyun1016@gmail.com (Haiyun Shi); lipeng@ccmu.edu.cn (Peng Li)
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Abstract

Background: To investigate the significance of the negative elongation factor complex member C/D (NELFCD) in colon cancer progression. **Methods**: Immunohistochemistry staining, Western blot analysis, and real-time quantitative polymerase chain reaction (RT-qPCR) were used to quantify the protein/gene levels. NELFCD-protein arginine methyltransferase 5 (PRMT5) interaction was determined by co-immunoprecipitation assay. A chromatin immunoprecipitation (ChIP) assay was performed to determine the interaction between the promoter region of dual specificity phosphatase 2 (DUSP2), NELFCD, and PRMT5. Cell growth and cell cycle progression were assessed using the cell counting kit-8 proliferation assay, colony formation assay, and/or flow cytometry. **Results**: NELFCD was upregulated in colon cancer and promoted cancer cell growth. In colon cancer cells, the expression of NELFCD was negatively correlated with DUSP2 expression. The RNA sequencing results indicated that genes in the mitogen-activated protein kinase (MAPK) signaling pathway as well as DUSP2 were affected by NELFCD. The ChIP sequencing results revealed that DUSP2 and genes in the MAPK signaling pathway are direct targets of NELFCD. ChIP assay verified that PRMT5 is enriched at the promoter region of DUSP2 and that NELFCD overexpression promoted this enrichment. A co-immunoprecipitation assay demonstrated that NELFCD was bound to PRMT5, functioning as a macromolecular complex. **Conclusions**: This study suggests that NELFCD promotes the progression of colon cancer by recruiting PRMT5 to inhibit DUSP2 expression, which subsequently activates the p38 signaling pathway. Targeting the NELFCD–DUSP2–p38 signaling axis may be a promising therapeutic intervention for patients suffering from NELFCD-amplified tumors.

Keywords: colon cancer; DUSP2; NELFCD; PRMT5; p38

1. Introduction

Colon cancer is a prevalent gastrointestinal tumor that poses a severe threat to human health, with an annual cases number of 1.097 million and a worldwide death toll of 0.551 million [1]. Recent evidence suggests that impaired genetic stability plays critical roles in colon cancer. Chromosome instability, microsatellite instability, and the CpG island methylation phenotype are the three major pathways that contribute to genetic instability [2].

Chromosome instability, a landmark event in the occurrence of colon cancer, leads to copy number variation and is a key player in tumorigenesis. A variety of oncogenes in the q13 region of chromosome 20 are related to tumor development in colon cancer. Notably, over 65% of colon cancer patients have an increased copy number of the chromosome 20 long arm, with a high frequency involving the q13 region [3].

The negative elongation factor (NELF) complex member C/D gene (*NELFCD*) is located on the q13 region of chromosome 20 and encodes two highly correlated proteins, NELF-C and NELF-D [4]. A study has demonstrated a strong association between multiple oncogenes on chromosome 20q, such as *NELFCD*, and colon cancer development [5]. The NELF complex, consisting of NELF subunits A–E, has been documented to suppress RNA polymerase II (RNApolII)-mediated transcription [6]. Although the *NELFCD* gene is an important component of the NELF complex, it has been rarely studied in the context of colon cancer.

In the current study, our team explored the expression and function of NELFCD in colon cancer and interrogated the underlying mechanisms. Our goal was to evaluate the suitability of NELFCD as a biomarker for colon cancer early detection and intervention.

2. Materials and Methods

2.1 Bioinformatics Analysis

GEPIA2 (http://gepia2.cancer-pku.cn/#index) was employed to assess the mRNA levels of NELFCD and dual specificity phosphatase 2 (DUSP2) in colorectal

cancer (CRC). The sequencing data, containing 275 cases of cancerous and 349 cases of normal colon tissues, were retrieved from The Cancer Genome Atlas (TCGA; https://portal.gdc.cancer.gov/) database.

2.2 Patients and Tissue Microarrays

We obtained an array of continuous sections of human colon cancer tumor tissue from Xinchao Biotechnology (Shanghai, China). The study was approved by the medical ethics committee of the Shanghai Outdo Biotech Company, the ethical approval number are SHYJS-CP-1704002 and SHYJS-CP-1401003. The array consisted of sections from 90 patients with colon cancer, including 90 patients (47 males and 43 females, median age = 64.6 years old). These patients underwent surgery between January 2009 and December 2009, and they were followed up until August 2015. The clinical stage and pathological classification were determined according to the American Joint Committee on Cancer 8th TNM staging system (https://link.springer.com/book/9783319406176) classification. All patients were diagnosed with colon cancer through pathological examination and had not received any preoperative treatment. Additionally, non-tumor tissues were collected 1.5 cm away from the site where cancer tissues were harvested.

2.3 Cell Culture

Human colon cancer cell lines (RKO, DLD1, and HCT116) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HCT116 cells were cultured in McCoy 5A medium (16600082, Gibco, Carlsbad, CA, USA), while RKO and DLD1 cells were maintained in modified high-glucose Dulbecco's modified Eagle medium (11995065, Gibco, Carlsbad, CA, USA), and the cells were maintained in a humidified atmosphere at 37 °C with 5% CO_2. All culture media were supplemented with 10% fetal bovine serum (16000-044, Gibco, Carlsbad, CA, USA) and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin) (15140122, Gibco, Carlsbad, CA, USA). All three cell lines were authenticated using short tandem repeat profiling and validated to be free of mycoplasma contamination.

2.4 Cell Transfection

Lentivirus expressing *NELFCD* and *DUSP2* were produced by GenePharma (Shanghai, China) and used to transduce HCT116 and DLD1 cells as reported previously [7]. Forty-eight hours after the transduction, puromycin (5 µg/mL) (P8230, Solarbio, Beijing, China) was supplemented to the culture medium to establish stable cell lines overexpressing *NELFCD*/*DUSP2*.

To knockdown *NELFCD* or *DUSP2*, RKO cells were transfected with control (NC) or NELFCD- or DUSP2-targeting siRNAs using GP-Transfect-mate (GenePharma, Shanghai, China). GenePharma designed and produced the siRNAs: *si-NELFCD-1* (sense: 5′-GCCAGCAGCUAGAAGUGUUTT-3′, antisense: 5′-AACACUUCUAGCUGCUGGCTT-3′), *si-NELFCD-2* (sense: 5′-GGUUGAACUUAUCCGCGUUTT-3′, antisense: 5′-AACGCGGAUAAGUUCAACCTT-3′), *si-DUSP2-1* (sense: 5′-GCCGUGUACUUCCUGCGAGTT-3′, antisense: 5′-CUCGCAGGAAGUACACGGCTT-3′), *si-DUSP2-2* (sense: 5′-CCGCUACAAGAGUAUCCCUTT-3′, antisense: 5′-AGGGAUACUCUUGUAGCGGTT-3′), and si-NC (sense: 5′-UUCUCCGAACGUGUCACGUTT-3′, antisense: 5′-ACGUGACACGUUCGGAGAATT-3′).

2.5 Chromatin Immunoprecipitation (ChIP) Assay and ChIP Sequencing

DNA and proteins were cross-linked by incubating cells in formaldehyde (28906, Thermo Scientific, Waltham, MA, USA) for 10 min. The resulting DNA–protein complex was extracted using a Pierce™ Agarose ChIP kit (26156, Thermo Scientific, Waltham, MA, USA) and vortexed (3 times, 15-s pulse) to obtain 1000-bp fragments. Then, the supernatant was obtained after centrifugation (3000 *g*, 10 min at 4 °C). The chromatin solution was incubated overnight at 4 °C with rabbit IgG (#2729, Cell Signaling Technology (CST), Danvers, MA, USA) or anti-protein arginine methyltransferase 5 (PRMT5) (ab277792, Abcam, Cambridge, Cambridge, UK). The next day, protein A/G Plus agarose (#20423, Thermo Scientific, Waltham, MA, USA) was added to immobilize the recognized DNA-protein complexes, which were pelleted by centrifugation (3000 *g*, 5 min). Subsequently, the bound proteins were decrosslinked and the phenol/chloroform was used to elute the DNA fragments. The DUSP2 promoter region was amplified by real-time quantitative polymerase chain reaction (RT-qPCR). The sequences of the primers used are as follows: forward 5′-CAAGAGTATCCCTGTGGAGGAC-3′, reverse 5′-GAAACTGAAGTTGGGGGAGATG-3′.

For ChIP sequencing, RNA samples were fragmented, and the fragmented RNA was immobilized using an anti-m6A antibody containing an RNase inhibitor (M0253L, Promega, Madison, WI, USA). Subsequently, the template DNA was synthesized using M-MulV Reverse Transcriptase (New England Biolabs (NEB), Ipswich, MA, USA), and the second-strand DNA was generated using DNA polymerase I (M0210, NEB) supplemented with RNase H (M0297S, NEB). Blunt-end conversion was accomplished using Exonuclease T (M0265S, NEB, Ipswich, MA, USA) and T4 DNA polymerase (M0203S, NEB) simultaneously. Then, the library was prepared using NEBNext® Multiplex Oligos for Illumina (E7335L, NEB). After that, the library was cleaned with AMPure XP reagent (Beckman Coulter, Brea, CA, USA) and processed with USER Enzyme (M5505L, NEB) followed by PCR amplification. The resulting products were purified with the AMPure XP reagent (Beckman Coulter, Brea, CA, USA) and the library quality was evaluated on an Agilent 2100 Bioanalyzer platform (Agilent Technologies, Santa Clara, CA, USA). Clustering

and sequencing were performed on a HiSeq^{TM} sequencing platform (Illumina, San Diego, CA, USA) following the manufacturer's protocol.

2.6 RNA Sequencing

Total RNA was extracted from control (oe-NC) or NELFCD-overexpressing (oe-NELFCD) HCT116 or DLD1 cells using the TRIzol reagent (15596026, Invitrogen, Carlsbad, CA, USA). The RNA quality and integrity were evaluated using a NanoPhotometer® strephotometer (Implen, Westlake Village, CA, USA) and gel electrophoresis, respectively. Subsequently, mRNA purification, library construction, sequencing, and data analysis were conducted by Beijing Nuohe Zhiyuan Biotechnology Co., Ltd. (Beijing, China).

2.7 Colony Formation Assay

Colon cancer cells under the indicated conditions were subcultured into 6-cm plates (2000 cells/well) and maintained for 10 (DLD1 and HCT116 cells) or 12 (RKO cells) days. Upon the completion of the incubation period, the medium was removed and the cells were washed with phosphate-buffered saline (PBS) (10010023, Life Technologies, Carlsbad, CA, USA). Subsequently, the cells were fixed with ice-cold methanol (M813895, Macklin, Shanghai, China) for 20 min followed by staining with crystal violet (G1072, Solarbio, Beijing, China) (0.1% w/v, room temperature, 15–20 min). Then, the cells were washed with PBS and the number of colonies were recorded under a microscope. A cluster with less than 50 cells was not recognized as a colony.

2.8 Cell Proliferation Assay

Cell proliferation was determined using a cell counting kit-8 (CCK-8, CK04, Dojindo, Kumamoto, Japan). Cells under the indicated conditions were seeded into 96-well plates (3599, Corning, Corning, NY, USA) (1000 cells/well) in triplicate and were assessed at experiment initiation, and 24, 48, and 72 h afterward. The absorbance at 450 nm was determined by a spectrometer and used to represent the cell proliferation status. To activate p38 signaling, cells were treated with 1 µM dehydrocorydaline (HY-N0674, MedChemExpress, Monmouth Junction, NJ, USA) or DMSO (D2650-100ML, Sigma-Aldrich, St. Louis, MO, USA) vehicle control.

2.9 Cell Cycle Assay

The same amount of colon cancer cells under the indicated conditions were subjected to overnight fixation using 75% ethanol at 4 °C. Subsequently, the fixed cells were suspended in PBS containing propidium iodide (F10797, Thermo Scientific). Then, the cells were analyzed by a flow cytometer (Thermo Fisher Scientific), and the cell cycles were determined using Modfit LT software (Version 5.0, Verity Software House, Topsham, ME, USA).

2.10 RT-qPCR

Total RNA was isolated using TRIzol reagent (15596026, Invitrogen, Carlsbad, CA, USA), following the manufacturer's guidelines. Complementary DNA (cDNA) was synthesized using a High-Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems, Foster City, CA, USA). The samples were amplified in triplicate using FastStart Universal SYBR Green Master (ROX) (Roche, Basel, Switzerland) on a QuantStudio Absolute Q dPCR system (Applied Biosystem). The relative expression levels of the *NELFCD* and *DUSP2* were normalized to the level of *GAPDH* mRNA. The sequences of the primers used for RT-qPCR were as follows: *NELFCD_forward (F)*: 5′-CAGACAGGTGTTGAGCCAGT-3′, *NELFCD_reverse (R)*: 5′-GCCACGTGGTATGTGCAATC-3′; *GAPDH_F*: 5′-ACCCACTCCTCCACCTTTGA-3′, *GAPDH_R*: 5′-CTGTTGCTGTAGCCAAATTCGT-3′; *DUSP2_F*: 5′-CAAGAGTATCCCTGTGGAGGAC-3′, *DUSP2_R*: 5′-GAAACTGAAGTTGGGGGAGATG-3′.

2.11 Western Blot Analysis

Radioimmunoprecipitation assay buffer (KeyGen Biotech, Nanjing, China) containing protease inhibitors was used to solubilize the cells. Triton buffer containing a protease and phosphatase inhibitor cocktail (P0013, Beyotime, Beijing, China) was used for phosphorylation analysis. Protein concentration was determined using the bicinchoninic acid method. Equal amounts of proteins were separated and transferred to PVDF membranes (IPVH00010, Sigma-Aldrich, St. Louis, MO, USA). Then, the membranes were incubated with goat serum (5%, 10000C, ThermoFisher Scientific) at room temperature for 1 hour. Subsequently, the membranes were incubated overnight at 4 °C with primary antibodies: rabbit anti-NELFCD (#12265, CST, Danvers, MA, USA, 1:1000 dilution), rabbit anti-DUSP2 (ab137640, Abcam, Cambridge, UK, 1:1000 dilution), rabbit anti-phospho-p38 (ab4822, Abcam, Cambridge, UK, 1:1000 dilution), rabbit anti-phospho-p38 (ab178867, Abcam, Cambridge, UK, 1:1000 dilution), mouse anti-p38 (YM3513, Immunoway, Plano, TX, USA, 1:1000 dilution), rabbit anti-PRMT5 (ab277792, Abcam, Cambridge, UK, 1:1000 dilution), rabbit anti-PRMT5 (ab109451, Abcam, Cambridge, UK, 1:1000, dilution), rabbit anti-H4R4me2 (223292, USbio, Salem, MA, USA, 1:1000 dilution), rabbit anti-β-Actin (#4970, CST, Danvers, MA, USA, 1:1000 dilution). The next day, the membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (ab6721, Abcam, Cambridge, UK, 1:5000 dilution). Then, the membranes were washed developed by an Omni-ECL chemiluminescent kit (Epizyme, Shanghai, China), and the images were captured. The expression levels of proteins were normalized to that of β-actin and quantified by ImageJ (1.48V; National Institutes of Health, Bethesda, MD, USA).

2.12 Co-Immunoprecipitation (Co-IP)

NELFCD-PRMT5 interaction was determined using Co-IP as described previously [7]. Triton buffer supplemented with a protease and phosphatase inhibitor cocktail (P0013, Beyotime, Shanghai, China) was used to extract proteins from the cells. The cleaned cell lysates were then subjected to Co-IP using an antibody specific to NELFCD (#12265, CST, Danvers, MA, USA, 1:1000 dilution). Protein A/G beads (Beyotime, Shanghai, China) were added to immobilize the anti-NELFCD IgG, and the coprecipitated proteins were eluted with Laemmli sample buffer for subsequent western blotting. Finally, the blots were probed for the presence of PRMT5 protein.

2.13 Immunohistochemical (IHC) Assay

The IHC assay was performed following a previously documented protocol. In general, tissue sections were dewaxed, rehydrated, and endogenous peroxidase was eliminated by H_2O_2 (P0100A, Beyotime, Shanghai, China) (3%, 20 min, room temperature). Heat-induced antigen retrieval was conducted using sodium citrate buffer (C1010, Solarbio, Beijing, China) (10 mM, pH 6.0, 95 °C, 3 min). After cooling down, the tissues were incubated overnight at 4 °C with primary antibodies against NELFCD (#12265, CST, Danvers, MA, USA, 1:100 dilution), DUSP2 (Abcam, Cambridge, ab137640, 1:200 dilution), or phospho-p38 (Abcam, Cambridge, ab178867, 1:500 dilution). The target proteins were then detected using the EnVision complex (GK500705, DAKO, Glostrup, Denmark). Stained sections were examined in a blinded manner by two independent pathologists using a grading system ranging from 0 to 4 to evaluate the percentage of positive cells, with 0 indicating $<$5% positivity, 1 indicating 5–25% positivity, 2 indicating 26–50% positivity, 3 indicating 51–75% positivity, and 4 indicating $>$75% positivity. The staining intensity was graded on a 0–3 scale, with 0 for colorless, 1 for yellow, 2 for brown, and 3 for dark brown. The final staining score was calculated by multiplying the scores of positivity by the staining intensity grades. The expression level was interpreted as low when the score was 4 and as high when the score was equal to or greater than 5.

2.14 Statistical Analysis

The data were presented as the mean $\pm$ standard deviation (SD) for continuous data and percentages for categorical data. The graphs were plotted based on three or more independent experiments. Statistical analyses were performed using GraphPad Prism software (Version 8.0, GraphPad Software, Boston, MA, USA). To compare differences between two groups, the two-tailed Student's *t*-test was used to compare continuous data. For comparisons among multiple groups, one-way ANOVA and Tukey's post hoc analysis was used. The Chi-squared test was conducted to analyze categorical data. Survival analysis was conducted using the Kaplan–Meier method, and the log-rank test was used to evaluate survival differences among groups. Statistical significance was defined as $p < 0.05$.

3. Results

3.1 NELFCD was Upregulated in Colon Cancer and Correlated With the Clinicopathological Profiles of Colon Cancer Patients

The transcriptional level of NELFCD in colon cancer was evaluated and compared with non-tumor tissues using data obtained from the TCGA database. The results demonstrated that cancerous colon tissues expressed higher levels of NELFCD than the adjacent normal colon tissues (Fig. 1A). Meanwhile, our findings from the IHC analyses using patient samples confirmed elevation of NELFCD in the tumor (Fig. 1B,C). We also investigated whether NELFCD affects the overall survival of patients with colon cancer using data retrieved from the TCGA database. The results showed that the overall survival rates were comparable between patients with high and low NELFCD expression levels (Fig. 1A). However, we found that NELFCD expression was associated with poorer tumor differentiation (levels III–IV) ($p = 0.034$, Table 1). The results suggest that NELFCD might play critical roles in the colon cancer progression.

3.2 NELFCD Promoted Colon Cancer Cell Growth In Vitro

To determine the functional importance of NELFCD in colon cancer, we modulated its expression in colon cancer cells and evaluated its impact on cancer cell proliferation and cell cycle progression. NELFCD overexpression stimulated clonogenic growth (Fig. 2A) and viability (Fig. 2B,C) in colon cancer cells, whereas NELFCD knockdown had the opposite effects on these phenotypes (Fig. 2D,E). In addition, NELFCD overexpression promoted cell cycle progression (Fig. 2F,G) in colon cancer cells. Taken together, these results demonstrate that NELFCD is sufficient to drive and is required for colon cancer cell proliferation and cell cycle progression.

3.3 RNA Sequencing and Chip Sequencing Revealed the NELFCD Regulatory Gene Map

To reveal the mechanism that NELFCD implements to regulate the pathogenesis of colon cancer, RNA sequencing was performed on HCT116 and DLD1 cells with or without NELFCD overexpression. Gene Ontology (GO) functional analysis indicated that protein binding was the predominant enriched molecular function (Fig. 3A). Additionally, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis identified multiple signaling pathways that were affected by NELFCD overexpression, including the mitogen-activated protein kinase (MAPK) signaling, cellular metabolic pathway, and pathways in cancer (Fig. 3B). Moreover, the ChIP sequencing results showed substantial enrichment in NELFCD-overexpressing DLD1

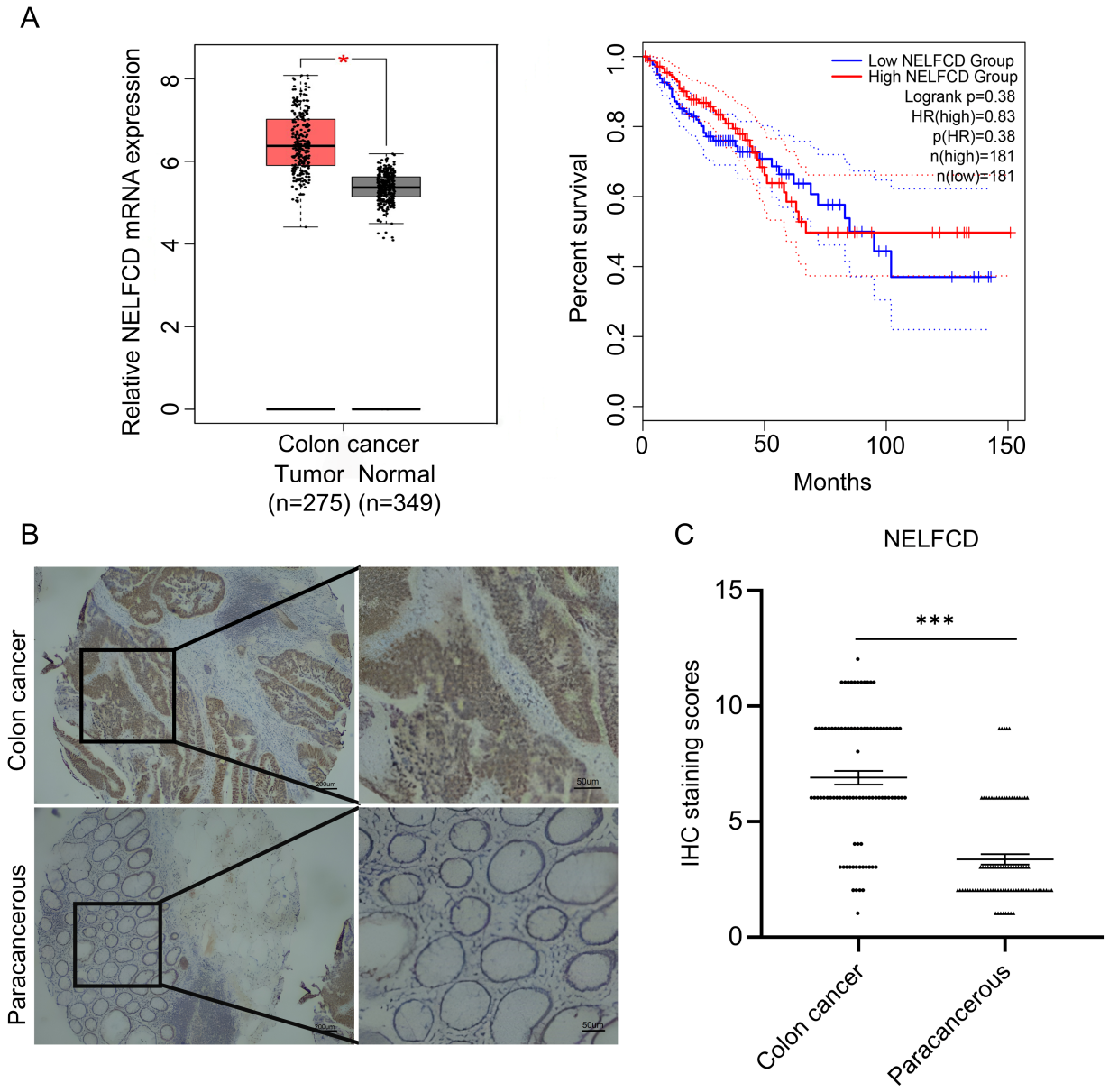

Fig. 1. Negative elongation factor complex member C/D (NELFCD) is overexpressed in colon cancer tissues and promotes *in vitro* tumor progression. (A) Left panel: RNA sequencing data from The Cancer Genome Atlas (TCGA) database showing NELFCD expression levels in normal and cancerous colon tissues, $*p < 0.05$ tumor vs. normal. Right panel: Comparison of survival rates in patients with high (red) vs. low (blue) NELFCD expression. (B,C) Representative immunohistochemical (IHC) staining images (B) and quantified data (C) from tissue microarray analysis showing increased NELFCD expression in colon cancer tissues. $***p < 0.001$ vs. paracancerous. Scale bars: 200 µm (left panels) and 50 µm (right panels).

and HCT116 cells, with 13,544 and 16,252 peaks, respectively (Fig. 3C). The main molecular function of these NELFCD-targeted genes is binding (Fig. 3D). Furthermore, KEGG pathway analysis highlighted that the target genes directly bound to NELFCD were involved in the MAPK signaling pathway (Fig. 3E).

Through RNA sequencing and ChIP sequencing, a long list of NELFCD targets was generated. The results revealed 30,697 genes, with 2266 significantly upregulated genes and 2346 significantly downregulated genes (|Log2fold-change (FC)| >1 and adjusted p value (p_{adj}) < 0.05). Among these differentially expressed genes (DEGs), DUSP2 attracted our attention (**Supplementary material 1**). Functioning as a phosphatase, DUSP2 negatively regulates the MAPK signaling pathway [8]. Considering the role of the MAPK signaling pathway in cell metabolism and tumorigenesis [9,10], we hypothesized that DUSP2 participates in NELFCD-mediated colon cancer progression [8,11].

Table 1. Association of *NELFCD* expression with clinical parameters in colon cancer.

Variable	*NELFCD* expression		*p*-value
	High (n = 70) n (%)	Low (n = 20) n (%)	
Age (years)			0.399
<60	21 (30.0)	8 (40.0)	
≥60	49 (70.0)	12 (60.0)	
Sex			0.463
Male	38 (54.3)	9 (45.0)	
Female	32 (45.7)	11 (55.0)	
Size			0.071
<5 cm	33 (47.1)	14 (70.0)	
≥5 cm	37 (52.9)	6 (30.0)	
Differentiation			0.034
I–II	56 (80.0)	20 (100.0)	
III–IV	14 (20.0)	0 (0)	
Tumor location			0.571
Left colon	30 (42.9)	10 (50.0)	
Right colon	40 (57.1)	10 (50.0)	
Invasive depth			0.858
T1–T2	8 (11.4)	2 (10.0)	
T3–T4	62 (88.6)	18 (90.0)	
Lymph node metastasis			0.810
N0	47 (67.1)	14 (70.0)	
N1–N3	23 (32.9)	6 (30.0)	
Peritoneal metastasis (M)			0.990
M0	67 (95.7)	20 (100.0)	
M1	3 (4.3)	0 (0)	
Stage (TNM)			0.521
I	9 (12.9)	1 (5.0)	
II	36 (51.4)	13 (65.0)	
III	22 (31.4)	6 (30.0)	
IV	3 (4.3)	0 (0)	

Notes: TNM, tumor–lymph node–metastasis.

3.4 NELFCD Promoted Colon Cancer Proliferation by Suppressing DUSP2 Expression

Next, we asked whether NELFCD affects DUSP2 in colon cancer. For this purpose, we determined DUSP2 expression in colon cancer cells with or without NELFCD overexpression. The findings indicated that NELFCD overexpression significantly decreased DUSP2 expression at both mRNA and protein levels (Fig. 4A–C, **Supplementary material 2**). We then evaluated the functional importance of DUSP2 in colon cancer growth and cell cycle progression. Our data demonstrated that DUSP2 overexpression could attenuate the promoting effects of NELFCD on growth and cell cycle progression (Fig. 4D–H). Specifically, NELFCD overexpression led to an increase in the S phase cell proportion and a reduction in the G0/G1 phase cell population. However, these effects could be compromised by DUSP2 overexpression (Fig. 4G,H).

Moreover, *NELFCD* knockdown (si-NELFCD) in colon cancer cells led to the upregulation of DUSP2 at both the transcriptional (Fig. 5A) and translational levels (Fig. 5B, **Supplementary material 3**). Additionally, our data revealed that silencing NELFCD suppressed colon cancer cell proliferation. However, this NELFCD depletion-mediated cell growth suppression was reversed by further knockdown of DUSP2, as demonstrated by increased colony formation (Fig. 5C) and accelerated cell growth (Fig. 5D). Furthermore, this is reflected by the increased cell population in the S phase and the reduced population in the G0/G1 phase (Fig. 5E). Thus, these observations suggest that NELFCD mediates colon cancer cell proliferation and cell cycle progression through modulating the expression of DUSP2.

3.5 NELFCD Regulated p38 Activation Through DUSP2 in Colon Cancer

The results from our RNA sequencing and ChIP sequencing revealed that NELFCD directly binds to genes in the MAPK signaling pathway and affects their transcription

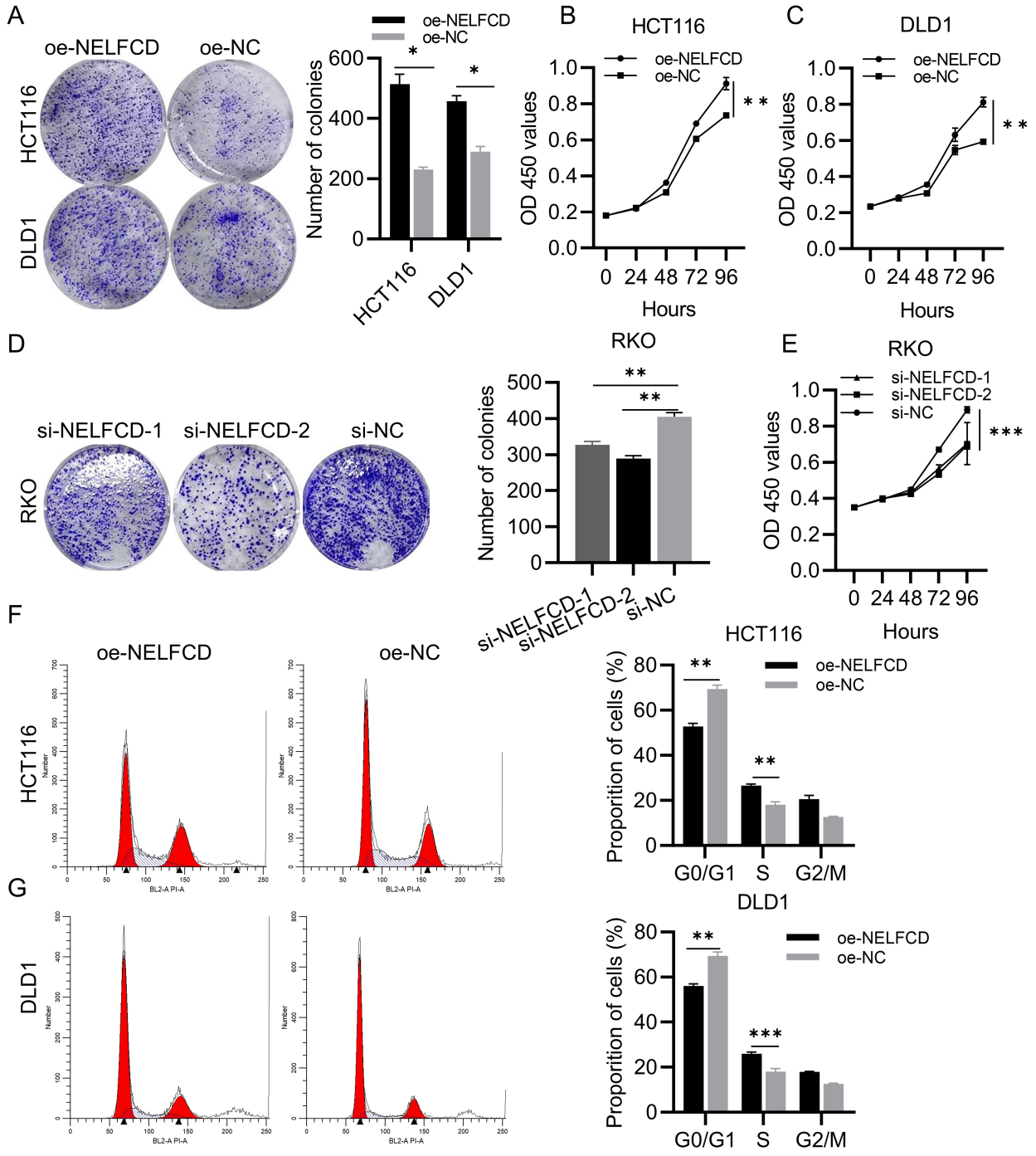

Fig. 2. NELFCD is upregulated in colon cancer tissues and promotes cancer progression *in vitro*. (A–C) Results of the colony formation (A) and cell proliferation (B,C) assays indicate that overexpression of NELFCD elevated colon cancer cell proliferation. *$p < 0.05$, **$p < 0.01$, vs. oe-NC. (D,E) Results of the colony formation (D) and proliferation (E) assays showing that silencing NELFCD inhibited colon cancer cell proliferation *in vitro*. **$p < 0.01$, ***$p < 0.001$, vs. si-NC. (F,G) Flow cytometry analysis of DLD1 and HCT116 cells (representative images and quantified results) showed that NELFCD overexpression increased the proportion of cells in the S phase, while reduced the cell population in the G0/G1 phase. **$p < 0.01$, ***$p < 0.001$, oe-NELFCD vs. oe-NC. NC, normal control; OD, optical density.

(Fig. 3). Meanwhile, our findings suggested that NELFCD overexpression resulted in a significantly downregulation of DUSP2 (log2FC = –1.14278, $p = 0.009545$), indicating NELFCD negatively regulates DUSP2 expression. Additionally, findings from our *in vitro* investigations demonstrated that NELFCD influences DUSP2 levels in colon cancer cells (Fig. 4A–D and Fig. 5B). Importantly, MAPK signaling pathway has been reported to be regulated by

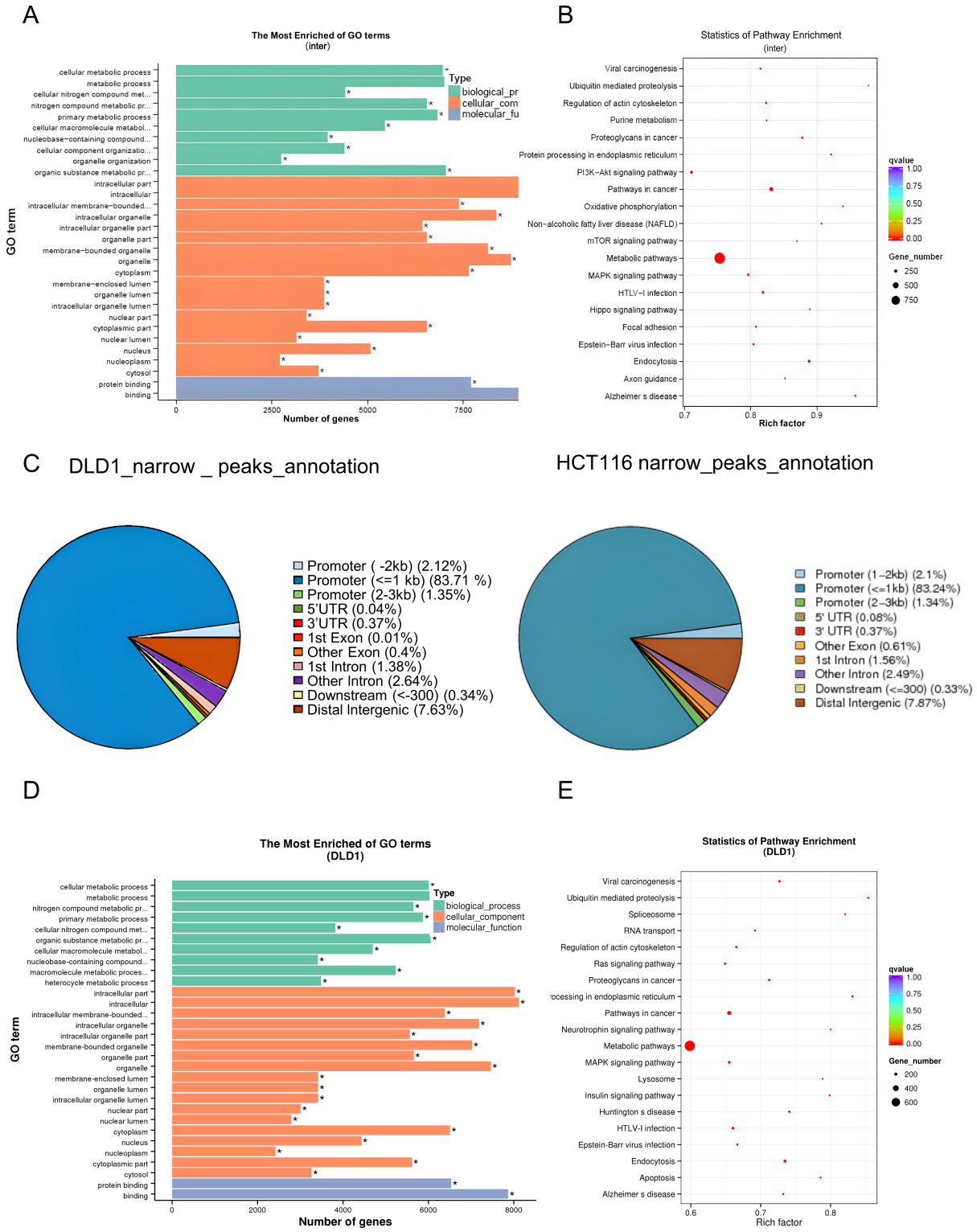

Fig. 3. Bioinformatics analysis of NELFCD gene. (A,B) Gene Ontology (GO) enrichment analysis results (A) and pathway enrichment analysis results (B) for differentially expressed genes (DEGs) in colon cancer cells with or without NELFCD overexpression. (C) Pie chart revealing the enrichment of NELFCD to the different regions of the genome. (D) Results of GO enrichment analysis of NELFCD-regulated genes in colon cancer cells. (E) Pathway enrichment analysis of NELFCD-regulated genes in colon cancer cells. p_{adj}, adjusted p-value; *$p_{adj} < 0.05$.

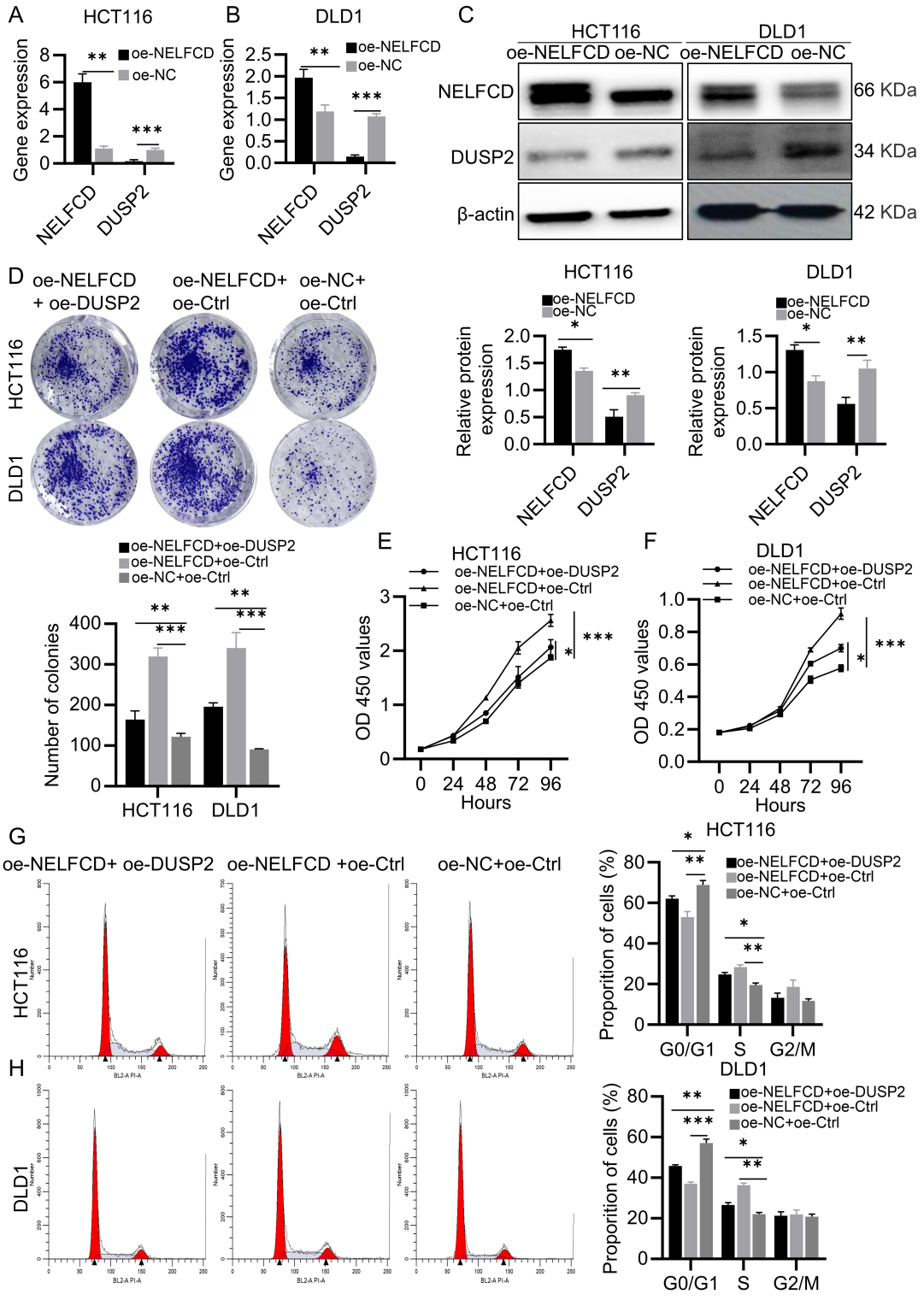

Fig. 4. Overexpressing dual specificity phosphatase 2 (DUSP2) suppresses the NELFCD-overexpression-promoted cell growth. (A–C) Results of real-time quantitative polymerase chain reaction (RT-qPCR) (A,B) and western blot (C) analyses showing the reduced expression of DUSP2 in NELFCD-overexpressed HCT116 and DLD1 cells compared to control cells (oe-NC, negative control for oe-NELFCD). $^{**}p < 0.01$, $^{***}p < 0.001$, vs. oe-NC. (D–H) Results of colony formation (D), proliferation (E,F), and cell cycle progression (G,H) analyses indicating that elevation of DUSP2 expression in NELFCD-overexpressed cells suppressed cell growth compared to cells with control cells (oe-NC + oe-ctrl, where oe-ctrl is the negative control for oe-DUSP2). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, vs. oe-NC+oe-ctrl.

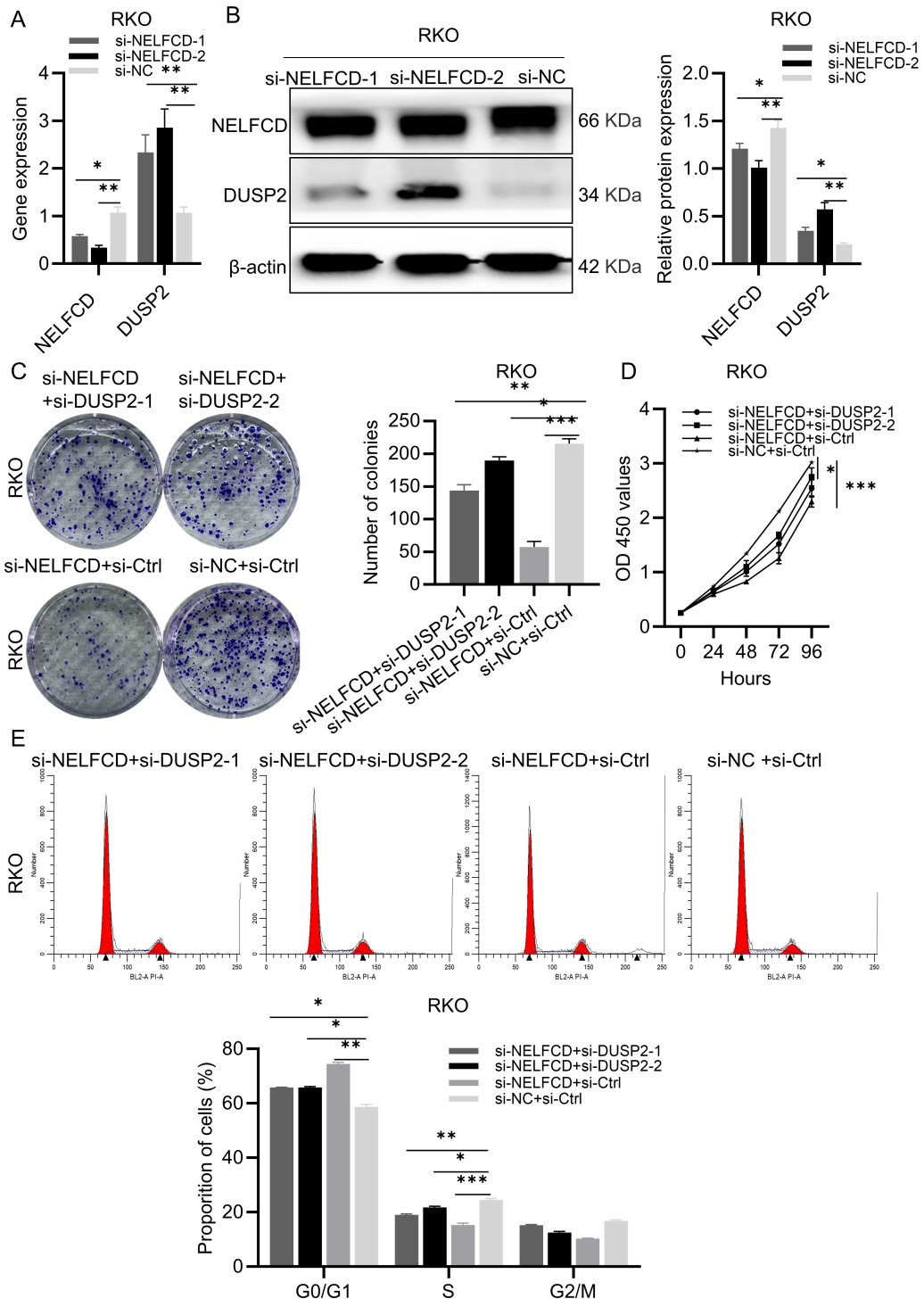

Fig. 5. Downregulating DUSP2 attenuates the suppressive effects of NELFCD depletion on cell proliferation *in vitro*. (A,B) RT-qPCR (A) and western blot (B) analyses demonstrated upregulated of DUSP2 in NELFCD-depleted cells. *$p < 0.05$, **$p < 0.01$, vs. si-NC. (C) Representative images (left) and analyzed data (right) from the colony formation assay indicated that reducing DUSP2 in NELFCD-silenced cells promoted cell growth. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, vs. si-NC+si-ctrl. (D) Results of the proliferation assay demonstrating that silencing DUSP2 in NELFCD-depleted cells promoted cell growth. *$p < 0.05$, ***$p < 0.001$, vs. si-NC+si-ctrl. (E) Flow cytometry analysis (representative images (top) and quantified data (bottom)) showed that downregulating DUSP2 in NELFCD-silenced cells increased the cell counts in the S phase, while it reduced the cell population in the G0/G1 phase. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, vs. si-NC+si-ctrl.

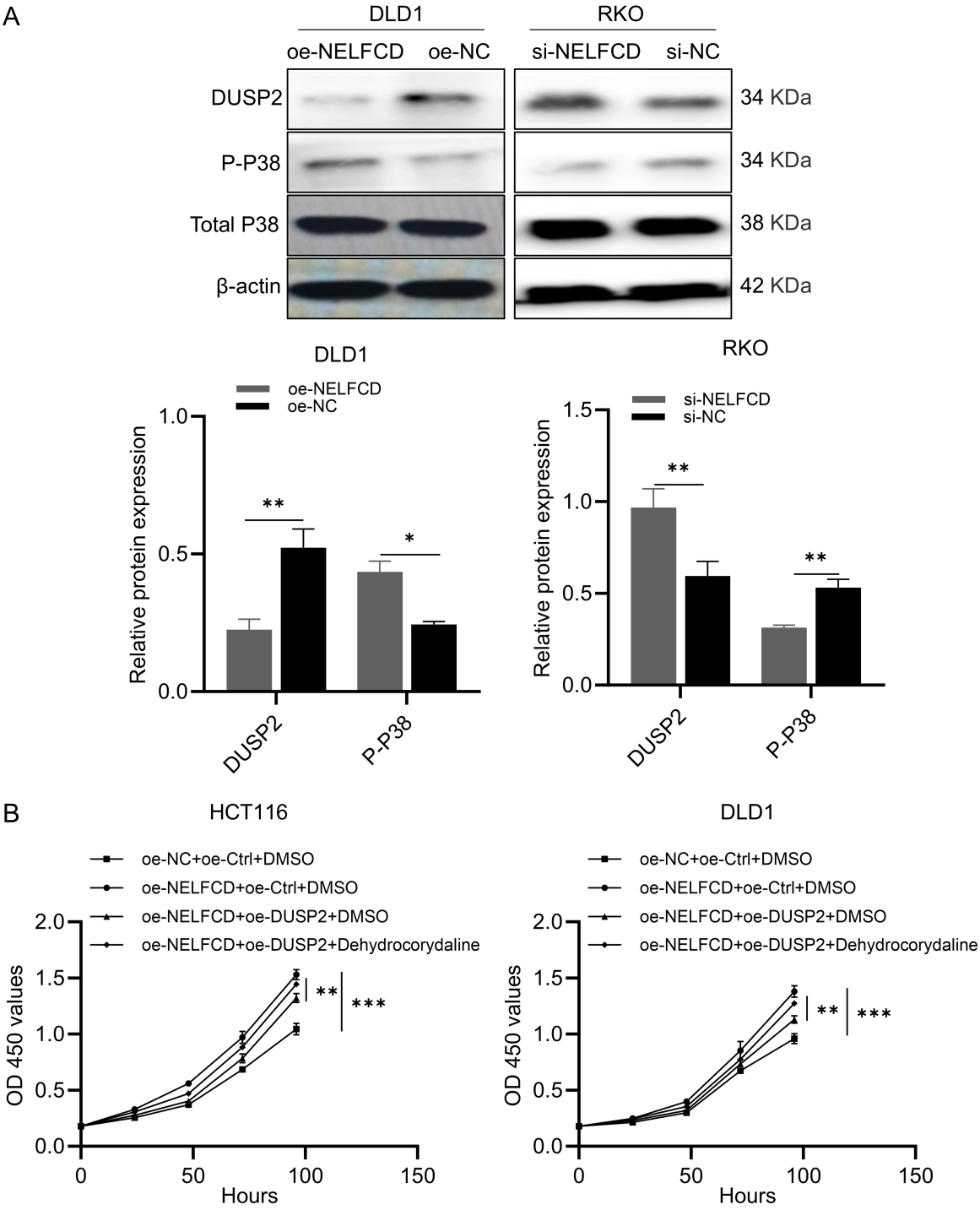

Fig. 6. NELFCD regulates p38 activation through DUSP2 in colon cancer. (A) Representative images (upper panel) and quantified data (lower panel) of western blot analysis demonstrating DUSP2 expression, total p38, and phosphorylated p38 in DLD1 cells with NELFCD overexpression and RKO cells with NELFCD knockdown compared to their representative controls. (B) Results of proliferation assays in HCT16 and DLD1 cells under indicated conditions. The p38 agonist (dehydrocorydaline) partially rescued the proliferation suppressed by DUSP2 overexpression *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, vs. oe-NC+oe-Ctrl+DMSO.

DUSP2, which inactivates p38 [8]. Therefore, we hypothesized that NELFCD may also participate in colon cancer tumorigenesis by regulating the DUSP2–p38 axis. To test this hypothesis, the status of p38 phosphorylation was determined through NELFCD overexpression or silencing in colon cancer cells. Our results from the western blot analy-

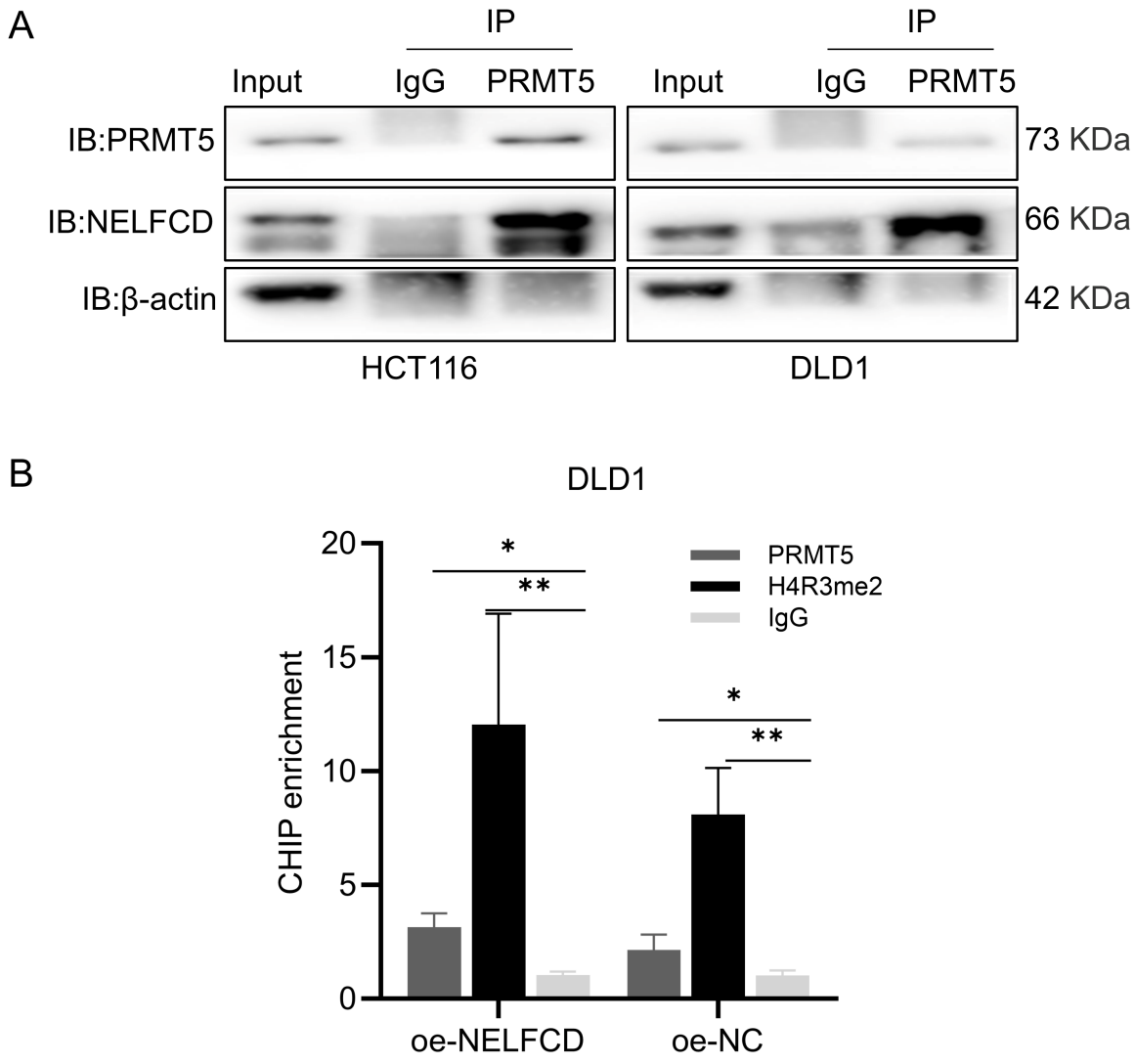

Fig. 7. NELFCD modulates the DUSP2/p38 pathway through protein arginine methyltransferase 5 (PRMT5) in colon cancer. (A) Representative images of Co-Immunoprecipitation (Co-IP) showing the NELFCD-PRMT5 interaction in HCT116 and DLD1 cells. (B) Results of the chromatin immunoprecipitation (ChIP) assay showing the enrichment of PRMT5 and H4R3me2 to the promoter region of DUSP2 in DLD1 cells and the promotive effect of NELFCD overexpression in this process, $*p < 0.05$, $**p < 0.01$, vs. IgG.

sis demonstrated that overexpression of NELFCD increased the phosphorylation level of p38, while NELFCD knockdown reduced p38 phosphorylation (Fig. 6A, **Supplementary material 4**). Notably, activated p38 has been demonstrated to promote cancer progression by facilitating cell proliferation in various types of tumors, including colon cancer [12]. Functionally, *in vitro* proliferation assays in HCT116 and DLD1 cells revealed that NELFCD overexpression enhanced cell proliferation, which was suppressed by DUSP2 overexpression. Moreover, the implementation of p38 agonist reversed the suppressive effect of DUSP2 on NELFCD-promoted cell proliferation (Fig. 6B). Together, these findings indicate the involvement of the p38 axis in NELFCD-regulated colon cancer development.

3.6 NELFCD Modulated the DUSP2-p38 Pathway Through PRMT5 in Colon Cancer

Subsequently, we explored how NELFCD regulates DUSP2 expression. The involvement of the PRMT family in cancer development has been well-established. Among the members, PRMT5 has attracted our attention due to its involvement in various signaling pathways, including the p38 MAPK pathway [13–15]. It has been documented that PRMT5 can catalyze the methylation of histone tails to activate/inhibit the expression of target genes [16]. NELFCD acts as a scaffold protein and a translational repressor. Therefore, it is possible that NELFCD binds to its target gene, *DUSP2*, and recruits gene modifiers such as *PRMT5* to suppress its expression. To test this speculation, Co-IP analyses were conducted, and we observed that *NELFCD* could be immunoprecipitated by *PRMT5* in colon cancer

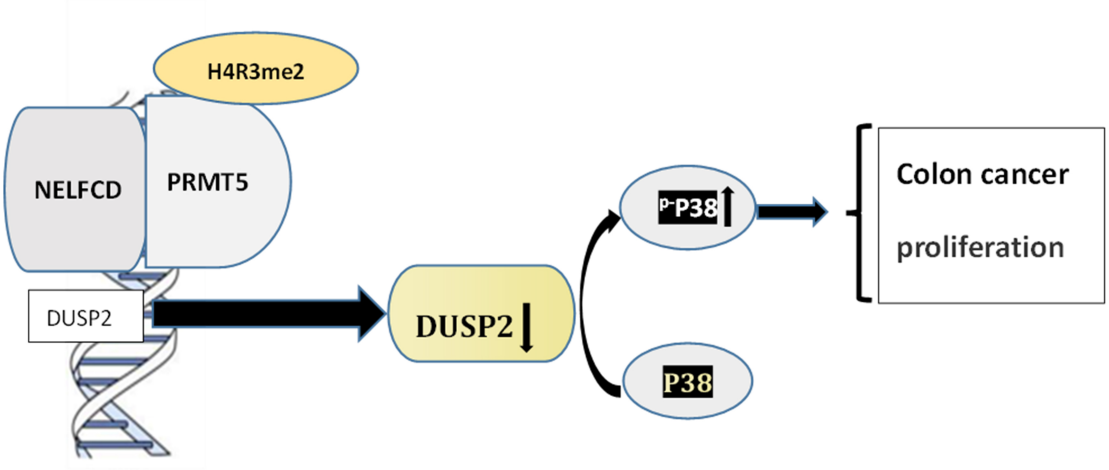

Fig. 8. A schematic model depicting the NELFCD–DUSP2–p-38 axis in regulating the progression of colon cancer. By recruiting PRMT5 to regulate the methylation status of the DUSP2 promoter region, NELFCD suppresses the expression of DUSP2, the p38 inhibitor, resulting in elevated p38 activation and enhanced cell growth and metabolism. The schematic diagram was created using Microsoft Word (Microsoft 365, Redmond, WA, USA).

cells (Fig. 7A, **Supplementary material 5**), suggesting that *PRMT5* and *NELFCD* can function as a complex. In addition, a ChIP assay was conducted in DLD1 cells with or without *NELFCD* overexpression, followed by qPCR quantification of the enriched *DUSP2* promoter region. The results revealed that compared to IgG, *PRMT5* and *H4R3me2* significantly precipitated the *DUSP2* promoter region in the oe-NC and oe-NELFCD groups (Fig. 7B). The data also suggest a trend of increased enrichment in the oe-NELFCD group for both PRMT5 and H4R3me2 when compared with the control group (oe-NC) ($p > 0.05$, Fig. 7B). It is possible that the temporal dynamics of histone modification could be critical and our study may not capture the optimal time point for these changes. Future time-course experiments and exploration alternative regulatory machinery will be essential to fully elucidate the NELFCD-DUSP2 relationship in tumorigenesis. Together, these observations indicate a mechanism in colon cancer cells where NELFCD recognizes and binds to the *DUSP2* gene promoter region, recruits *PRMT5* to catalyze the histone modification (H4R3me2), and consequently suppresses the transcription of DUSP2 and regulates the p38 signaling activity (Fig. 8).

4. Discussion

Colon cancer, a highly prevalent and lethal malignancy, ranks third globally in terms of both the number of cases and mortality rates [17]. One of the frequently observed genetic alterations in colon cancer is the gain of copy number on chromosome 20q, which leads to amplification of several genes, including DEAD-box helicase 27, transmembrane 9 superfamily member 4, polymorphic adenoma-like protein 2, and *NELFCD* [18–20]. Among these, NELFCD has been identified as being overexpressed in colon cancer. However, except for the observed overexpression, the molecular mechanisms by which NELFCD contributes to colon cancer development remain poorly understood, including its downstream targets and signaling pathways.

Dickinson and Keyse [21] have demonstrated that NELFCD functions as an inhibitor of PAK kinase activity and negatively regulates MAPK signaling. In our preliminary investigations, we conducted RNA sequencing and ChIP sequencing using colon cancer cell lines with overexpressed NELFCD. The results revealed that NELFCD binds to the promoters of various genes, indicating its ability to regulate gene expression at the transcriptional level. One notable target of NELFCD is DUSP2, which is regulated by multiple genes and signaling pathways and plays crucial roles in tumor development [22–25]. *SKA3*, for instance, has been found to activate the MAPK/ERK pathway by regulating DUSP2 [26]. Although DUSP2 expression varies among different tumor origins, it is generally expressed at low levels in many tumors, and its loss promotes cancer progression [27]. However, it is important to note that the upregulation of DUSP2 may be a result of negative feedback in response to colon cancer progression, rather than its direct causation [28]. Deeper research is needed to elucidate the importance of DUSP2 in colon cancer growth and advancement.

Mammals have four primary MAPK pathways: extracellular signal-regulated kinase (ERK), ERK5, c-Jun N-terminal kinase (JNK), and p38 pathway. JNK and p38 MAPK signal transduction have been implicated in human cancer and have been shown to regulate cancer progression [29–32]. Particularly, p38 has a dualistic role in mammals. In normal cells and some cancer cells, p38α acts as a negative regulator of proliferation. It primarily inhibits the cell cycle and induces apoptosis, thus suppressing tumors.

However, during tumor development, increased expression of p38α pathways promotes cell metabolism, invasion, angiogenesis, and other malignant effects. Numerous studies have explored the role of p38 MAPK in various cancers. For instance, p38 activation has been found to be reduced in lung cancer [33], while it is increased in hepatocellular tumors [34] and breast cancer [35]. Furthermore, dysregulated p38 activation has been linked to unfavorable outcomes in a variety of malignancies, such as breast, thyroid, colorectal, and ovarian cancer [35]. From this perspective, it is evident that *in vitro* and *in-vivo* colon cancer models may not fully depict the complex role of p38 in cancer progression and response to treatment [36].

Based on our results, PRMT5 is of critical importance in NELFCD–DUSP2–P-p38 axis. PRMTs are responsible for mediating the arginine methylation of histone tails and are classified into type I and type II based on the methylated arginine residues they introduced and the subsequent recruited readers. In addition, PRMT5, a type II PRMT, can catalyze the methylation and ubiquitination of arginine in the tail of various histones, including H4R3me2, which is primarily associated with transcriptional inhibition [16]. This result suggests that targeting PRMT5 could be a potential therapeutic approach for colon cancer. However, our study also has limitations. First, as a transcriptional suppressor, NELFCD regulates various genes/pathways in cancer cells. Therefore, it is reasonable to speculate that NELFCD promotes colon cancer growth via multiple pathways. Second, while we observed increased enrichment of H4R3me2 and PRMT5 to the promoter region of DUSP2 after NELFCD overexpression, the complex temporal dynamics of histone modifications requires deeper study at optimized time points. Third, the molecular mechanisms linking DUSP2 to p38 activation in colon cancer cells need to be further elucidated. Fourth, this study is a proof-of-concept investigation revealing the role of the NELFCD–DUSP–p38 axis in colon cancer development. However, *in-vivo* studies are needed to confirm the findings from the *in vitro* studies.

5. Conclusions

Our investigation revealed upregulation of NELFCD in colon cancer. We also have demonstrated the regulatory effects of NELFCD on NELFCD–DUSP2–P-p38 in the development of colon cancer. This intricate regulatory network involves various genes and proteins, such as PRMT5 and histones, that play pivotal roles in modulating tumor occurrence and progression. Gaining a comprehensive understanding of these underlying mechanisms has the potential to greatly impact the evaluation, diagnosis, and treatment strategies for colon cancer. Therefore, further investigations aimed at designing and testing drugs targeting this pathway are imperative for the development of effective colon cancer therapies.

Availability of Data and Materials

The raw data supporting the conclusions of this article will be made available by the authors upon reasonable request. Restrictions apply to the availability of these data due to privacy/ethical concerns. This study also analyzed the public data set. This dataset can be found here: https://portal.gdc.cancer.gov/.

Author Contributions

WD: Data curation and Formal analysis; HS: Funding acquisition and Investigation; Substantial contributions to the conception or design of the work; YS & GM: Methodology; BX: Resources; Software; PL: Supervision; Substantial contributions to the conception or design of the work; MZ was involved in data analysis and interpretation, making graphs and tables and searching for references; WD: Roles/Writing - original draft; MZ & HS: Writing - review & editing. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study was carried out in accordance with the guidelines of the Declaration of Helsinki. The study was approved by the medical ethics committee of the Shanghai Outdo Biotech Company (ethical approval numbers: SHYJS-CP-1704002 and SHYJS-CP-1401003). All tissue samples were obtained from the tissue bank, where patients or their families/legal guardians. informed consent was obtained during tissue collection and archiving according to institutional protocols. The informed consent documentation is maintained as confidential internal records by the biobank.

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Conflict of Interest

The authors declare no conflict of interest.

Declaration of AI and AI-assisted Technologies in the Writing Process

During the preparation of this manuscript, ChatGPT was used to improve the readability and language expression of the context. Following the use of this AI-based tool, the authors reviewed and edited the contents as needed. The

authors take all responsibility for the accuracy, integrity, and scientific rigor of the contents.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL25221.

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