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ORIGINAL RESEARCH

CYLD Promotes Apoptosis of Nasopharyngeal Carcinoma Cells by Regulating NDRG1

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Purpose: Nasopharyngeal carcinoma (NPC) is among the most common malignancies derived from the epithelium of the nasopharynx. To date, the regulatory networks involved in NPC have not been fully identified. Previous studies revealed multiple loss-of-function mutations in NPC and specifically in cylindromatosis lysine 63 deubiquitinase (*CYLD*); however, the exact role of CYLD in NPC progression and its potential mechanism remains unclear.

Methods: We performed immunohistochemical (IHC) staining and real-time quantitative polymerase chain reaction (qPCR) to measure *CYLD* expression in NPC tissues, and Western blot was conducted to determine CYLD levels in NPC cell lines. Cell proliferation was detected by CCK8 assay and colony formation analysis, and apoptosis was determined by Annexin V/propidium iodide staining. Potential targets of CYLD were verified by co-immunoprecipitation and mass spectrometry. Xenograft assay was conducted to confirm the role of *CYLD* in vivo.

Results: We found that CYLD levels were significantly decreased in both NPC tissues and cell lines, and that *CYLD* overexpression inhibited NPC cell proliferation and promoted apoptosis. Additionally, we revealed that CYLD bound and upregulated N-Myc downstream regulated 1 (NDRG1), and that silencing *NDRG1* abolished the tumor-suppressor effect of CYLD on NPC cells. Furthermore, CYLD suppressed tumor growth in xenograft mice models.

Conclusion: These results suggest CYLD as a tumor suppressor, potential biomarker for diagnosing NPC, and therapeutic target.

Keywords: NPC, CYLD, proliferation, apoptosis, NDRG1

Introduction

Nasopharyngeal carcinoma (NPC) is an epithelial carcinoma derived from the epithelium of the nasopharynx.[1] NPC has a unique geographical distribution, with >70% of cases found in East and Southeast Asia.[2] Although NPC incidence and mortality rates gradually declined during the previous decade, ~30% of NPC patients still eventually experience disease progression and therapy failure.[3,4] Previous studies reveal that genetic susceptibility increases the risk of NPC progression and therapeutic resistance; however, there is a lack of effective biomarkers capable of accurately predicting NPC progression and therapeutic outcomes. Therefore, novel NPC-specific biomarkers need to be identified to develop novel treatment strategies for patients.

Cylindromatosis lysine 63 deubiquitinase (*CYLD*) encodes a tumor suppressor and was initially identified as a mutated gene in familial cylindromatosis.[5] CYLD

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deconjugates Lys63-linked polyubiquitin chains from target proteins and protects them from proteasomal degradation.[6,7] Previous reports reveal that CYLD negatively regulates various signaling pathways, including nuclear factor kappaB (NF-κB), Wnt/β-catenin, c-Jun N-terminal kinase, Hippo, and Notch signaling.[8–14] Additionally, studies report significant downregulation of *CYLD* expression in different types of cancer, such as breast cancer, glioblastoma, and hepatocellular carcinoma.[15–17] In recent years, whole-exome sequencing and whole-genome sequencing have been performed in NPC patients, and especially in *CYLD*, identifying multiple loss-of-function mutations.[18–20] However, the molecular function and detailed mechanisms of CYLD in NPC remain elusive.

In this study, we demonstrated that *CYLD* expression is downregulated in NPC tissues, and that *CYLD* overexpression inhibits NPC cell proliferation and promotes apoptosis, whereas *CYLD* knockdown reverses these effects in NPC cells. Moreover, examination of the molecular mechanisms of CYLD revealed its direct interaction with N-Myc downstream regulated 1 (NDRG1). These findings suggest CYLD as a novel therapeutic target in NPC.

Materials and Methods

Clinical Specimens

We used 10 paraffin-embedded NPC specimens and 10 paraffin-embedded normal nasopharyngeal epithelium specimens for immunohistochemical (IHC) analysis of protein levels. Five primary, fresh NPC samples and five noncancerous, fresh nasopharyngeal samples were used for RNA extraction. All samples were obtained from Nanfang Hospital (Guangzhou, China) and before patients received therapy. Patients providing tissue samples for research purposes provided informed consent, and the experimental protocols were approval by the Ethics Committee of Nanfang Hospital.

Cell Culture

Epstein−Barr virus (EBV)-negative NPC cell lines (CNE2, HONE1, and 5–8F) were generously provided by Professor Musheng Zeng (Sun Yat-sen University Cancer Center, Guangzhou, China). The EBV-positive NPC cell line (HK1-EBV) and two immortalized normal human nasopharyngeal epithelial cell lines (NP460hTert-EBV and NP460hTert) were kindly provided by Professor George S. W. Tsao (University of Hong Kong). The cell lines were confirmed as negative for mycoplasma contamination (Qiagen, Hilden, Germany). All NPC cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% newborn cow serum (Gibco, Gaithersburg, MD, USA) at 37°C and 5% CO_2. NP460hTert-EBV and NP460hTert cells were cultured in defined keratinocyte serum-free medium (Invitrogen) at 37°C and 5% CO_2. All gifted cell lines were authenticated by STR profile.

IHC Staining

Paraffin sections underwent IHC staining for CYLD, as previously described.[21] All paraffin-embedded tissue specimens were deparaffinized in xylene and rehydrated with an ethanol gradient into distilled water. After treatment with proteinase K (Solarbio, Beijing, China), the tissue sections were preincubated with hydrogen peroxide and blocked with bovine serum albumin, followed by incubation with anti-CYLD (1:300; Proteintech, Wuhan, China), labeling with an avidin–biotin peroxidase complex, and diaminobenzidine development (Gene Tech, Shanghai, China). Sections were then counterstained with hematoxylin.

RNA Extraction and Quantitative Polymerase Chain Reaction (qPCR)

Total RNA in tissues and cell lines was extracted using TRIzol reagent (TaKaRa, Shiga, Japan), and complementary DNA (cDNA) was synthesized using the PrimeScript RT reagent kit (TaKaRa). qPCR was performed in triplicate using SYBR Premix ExTaq (TaKaRa), with *glyceraldehyde 3-phosphate dehydrogenase* used an endogenous control. Relative gene expression was evaluated as fold change using the $2^{-\Delta\Delta CT}$ method. All experiments were performed in triplicate, and primer sequences are shown in Supplementary Table 1.

Lentiviral Infection and RNA Interference (RNAi)

Lentiviral particles carrying the CYLD vector (Ubi-MCS -3FLAG-CBh-gcGFP-IRES-puromycin-CYLD) and their randomized flanking control sequence were constructed by GeneChem (Shanghai, China). Harvested virus was then used to infect NPC cells. Stable clones were selected using puromycin and confirmed using qPCR and Western blot assays. Small-interfering (si)RNA oligonucleotides targeting *CYLD* and *NDRG1* were purchased from GenePharma (Jiangsu, China), with the sequences shown

in Supplementary Table 2. siRNA transfection was performed using Lipofectamine 3000 (Invitrogen) for 48 h according to manufacturer instructions.

Protein Extraction and Western Blot Analysis

Proteins from NPC cells were extracted by radioimmunoprecipitation assay buffer (Fdbio Science, Guangzhou, China). Proteins were fractionated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The blots were probed with specific antibodies against human CYLD (Proteintech), NDRG1 (ZEN-BIO, Shanghai, China), or β-actin (Fdbio Science), followed by incubation with species-matched secondary antibodies (1:10,000; Fdbio Science). After developing the blots using an enhanced chemiluminescence Western blot kit (Wanleibio, Shenyang, China), they were visualized using an auto-exposure system (Tanon-5200; Tanon Science & Technology, Shanghai, China). The antibodies used for IHC and Western blot are shown in Supplementary Table 3.

Cell Viability Assay

A total of 1×10^3 NPC cells were inoculated per well in 96-well plates, and the viability was determined using a Cell Counting Kit-8 assay (CCK-8; Dojindo, Kumamoto, Japan) every 24 h for 4 consecutive days. Briefly, culture medium was replaced with 100 μL RPMI-1640 containing 10 μL CCK-8 solution, and absorbance was measured on a microplate reader (Bio-Rad) at 450 nm. Five replicates of each treatment were used, and experiments were performed in triplicate.

Colony Formation Assay

Transfected cells (200 cells/well) were plated in 6-well plates and cultured for 10 to 14 days. The colonies were subsequently washed twice with phosphate-buffered saline, fixed with 4% paraformaldehyde, and stained with 0.5% Crystal Violet. Cell colonies with diameters >1.5 mm were counted. The experiment was performed in triplicate.

Apoptosis Assay

Apoptosis of NPC cells following transfection was measured using a FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and an Annexin V-APC/propidium iodide (PI) apoptosis detection kit (KGA1030; Keygen Biotech, Nanjing, China) according to manufacturer instructions.

Co-Immunoprecipitation (Co-IP) and Mass Spectrometry (MS)

CNE2 cells stably expressing Flag-CYLD were lysed with immunoprecipitation lysis buffer (Beyotime Biotechnology, Beijing, China). Primary anti-Flag or anti-IgG (negative control) antibodies were incubated with the lysates overnight at 4° C. Protein A/G Sepharose beads (Santa Cruz Biotechnology, Dallas, TX, USA) were added to the immune complexes for recovery, followed by washing and collection. The immune complexes were then eluted with low-pH buffer and separated by SDS-PAGE, followed by silver staining. Candidate bands were subjected to MS analysis for protein identification.

In vivo Xenograft Tumor Models

The animal procedures in this study were approved by the Ethical Committee for Animal Research of Southern Medical University (Guangzhou, China) and were performed to minimize animal suffering. The 4-week-old BALB/C athymic nude mice were purchased from the Guangdong Medical Laboratory Animal Center (Guangzhou, China). CNE2 cells (1×10^7 cells in 100 μL PBS) that stably overexpressed the vector or CYLD were injected subcutaneously into the right flank of the mice. Tumor volume was measured using a caliper for every 2 days. Tumor volume was calculated using the formula: volume = [length *(width^2)]/2. Mice were sacrificed by cervical dislocation on 14th day, and the tumors were collected for hematoxylin-eosin (HE) and IHC staining.

Statistical Analysis

All statistical analyses were performed using SPSS software (v.19.0; IBM Corp., Armonk, NY, USA). Data are presented as the mean ± standard error of the mean (SEM) from at least three independent experiments. Differences were considered statistically significant at a $P < 0.05$ according to Student's *t*-tests for two groups or one-way analysis of variance for multiple groups.

Results

CYLD is Downregulated in Clinical NPC Tissues

To determine the role of CYLD in NPC development, we analyzed CYLD protein levels in NPC and normal tissues by IHC. We found that CYLD level was significantly

decreased in NPC tissue samples relative to that in normal tissue samples (Figure 1A and B). Additionally, qPCR analysis of *CYLD* mRNA levels in five paired, fresh-frozen NPC tissues (T) and normal tissues (N) from patients showed significantly downregulated expression in tumor tissues relative to that in normal tissues (Figure 1C). These findings suggest that CYLD might represent an NPC biomarker according to its downregulation in NPC tissues.

CYLD Overexpression Inhibits NPC Cell Proliferation

To investigate the biological function of CYLD, we examined CYLD transcript and protein levels in two normal nasopharyngeal epithelial cell lines and six NPC cell lines. The results showed that both CYLD mRNA (Supplementary Figure 1A) and protein (Figure 2A) levels were significantly downregulated in the NPC cell lines. We then established either

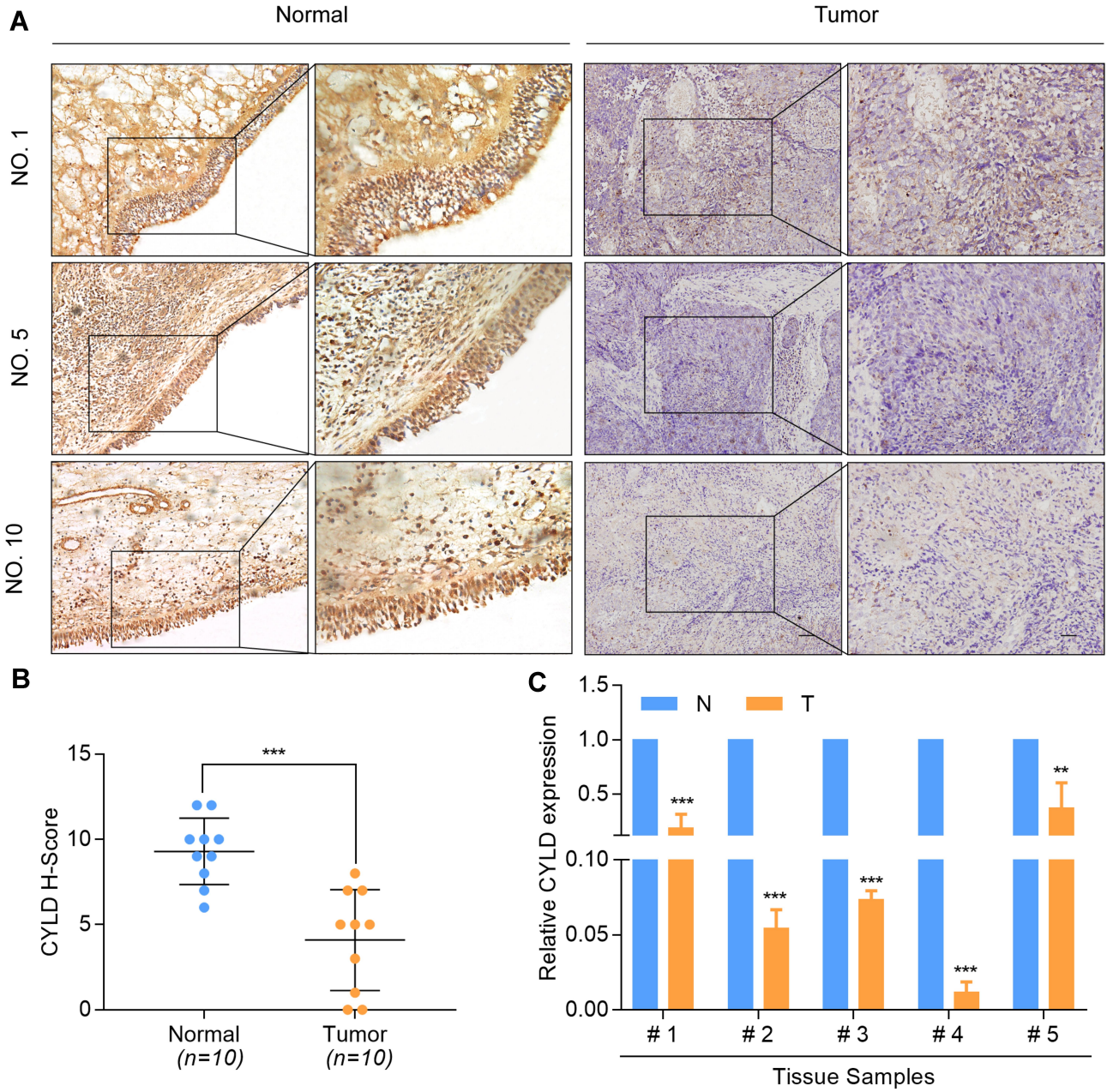

Figure 1 CYLD is downregulated in clinical NPC Tissues. (**A**) *CYLD* expression in 10 primary NPC tissues and 10 normal tissues and assessed by IHC. Images are representative of IHC staining results. Scale bar: 100 mm. (**B**) statistical analysis of CYLD levels. Data represent the mean ± SEM (n =10). ***$P < 0.001$. (**C**) qPCR analysis of relative *CYLD* expression in normal and NPC tissues. Data represent the mean ± SEM (n =3). **$P < 0.01$, ***$P < 0.001$.

control CNE2 and HK1-EBV cells or those stably expressing *CYLD*, as well as HONE1 and 5–8F cells transfected with CYLD-siRNAs or corresponding controls for *CYLD*-knockdown experiments (Figure 2B). The results of CCK-8 and colony formation assays used to evaluate the effects of CYLD on cell proliferation showed that forced expression of *CYLD* in NPC cell lines resulted in decreased cell viability. In contrast, *CYLD* silencing significantly promoted NPC cell proliferation relative to that observed in control cells ($P < 0.001$) (Figure 2C). Consistently, colony formation results showed that *CYLD* overexpression decreased the colony formation in both CNE2 and HK1-EBV cell lines relative to that observed in control cells, whereas colony formation by HONE1 and 5–8F cells increased following *CYLD* knockdown (Figure 2D and E). These results indicated that *CYLD* overexpression exerted an antitumor effect on NPC cells by inhibiting their proliferation.

CYLD Overexpression Promotes NPC Cell Apoptosis

To determine whether CYLD regulates NPC cell apoptosis, we performed Annexin V/PI staining and flow cytometric analysis. We found that *CYLD* overexpression increased both early and late apoptosis in CNE2 and HK1-EBV cells (Figure 3A and B), whereas *CYLD* knockdown in HONE1 and 5–8F cells inhibited apoptosis relative to that observed in control cells (Figure C and D). These results indicated that CYLD increased apoptosis in NPC cells, whereas CYLD downregulation inhibited this process.

CYLD Directly Interacts with NDRG1

To elucidate the molecular mechanism underlying the tumor-suppressive effect of CYLD, we co-immunoprecipitated Flag-tagged CYLD from CNE2 cells (Figure 4A) and performed MS analysis to identify potential interacting proteins. The data identified NDRG1 among the group of CYLD-interacting

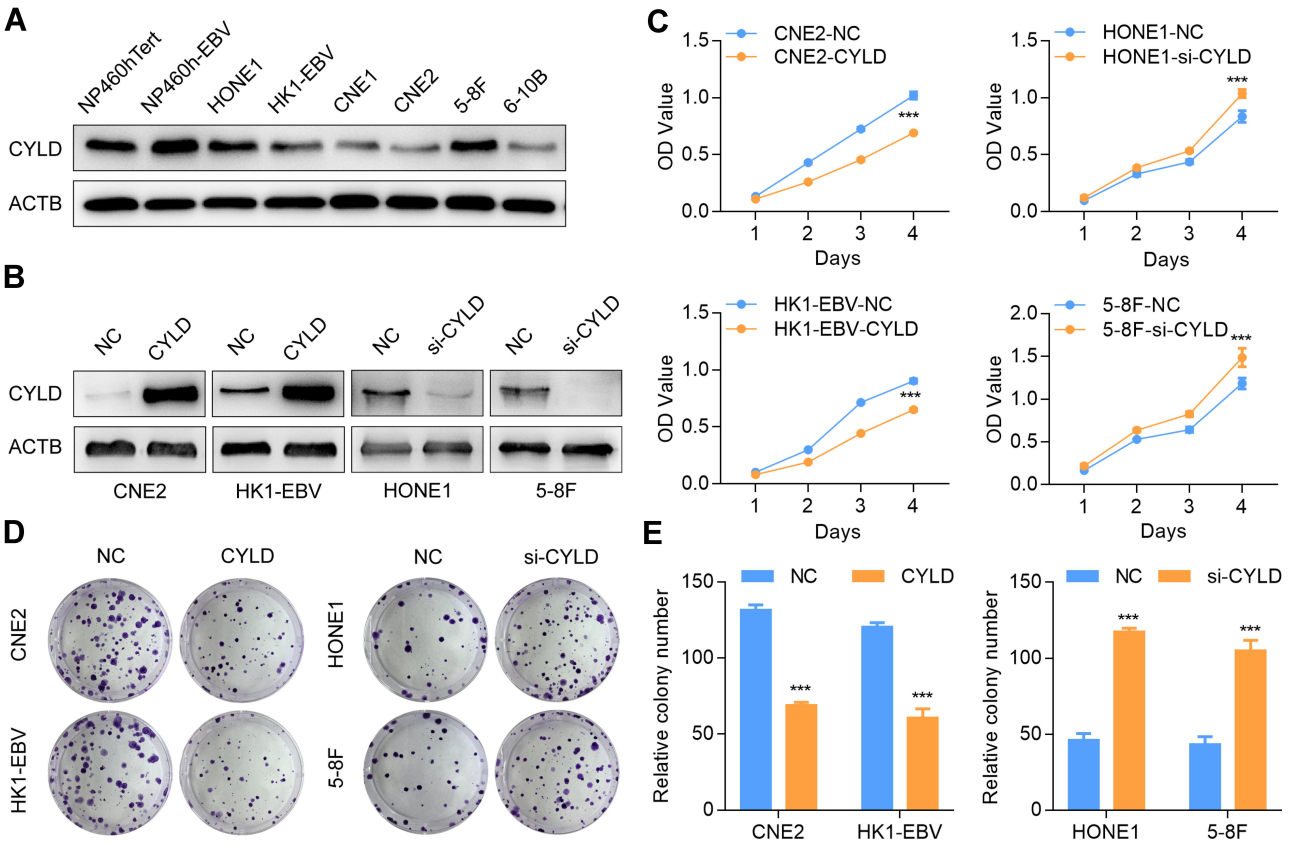

Figure 2 *CYLD* overexpression inhibits NPC cell proliferation. (**A**) CYLD protein levels measured by Western blot in two immortalized normal human nasopharyngeal epithelial cell lines and six NPC cell lines. β-actin (ACTB) was used as a loading control. (**B**) CNE2 and HK1-EBV cells stably overexpressed *CYLD*, whereas HONE1 and 5–8F cells were transfected with scrambled siRNA or CYLD-specific siRNA, and CYLD levels were detected by Western blot. (**C**) CCK-8 and (**D, E**) colony formation assays were used to evaluate cell growth and proliferation. Data represent the mean ± SEM (n =3). ***$P < 0.001$.

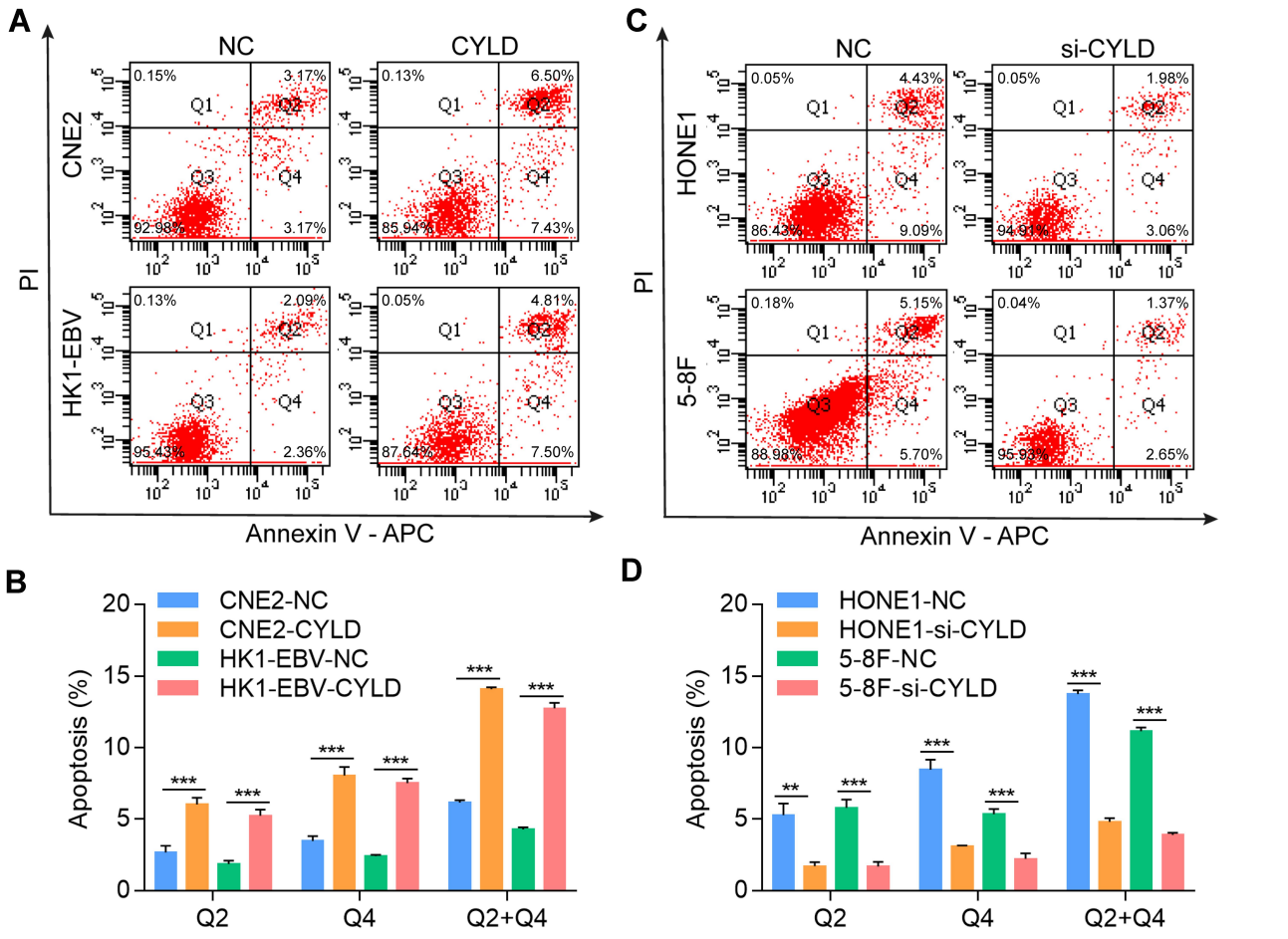

Figure 3 *CYLD* overexpression promotes NPC cell apoptosis. (**A, C**) Apoptosis rate was determined by Annexin V/PI staining following *CYLD* overexpression in CNE2 and HK1-EBV cells or knockdown in HONE1 and 5–8F cells. (**B, D**) Quantification of proportions of Q2, Q4, and Q2 + Q4 in cells represents cell apoptosis. Data represent the mean ± SEM (n =3). $^{**}P < 0.01$, $^{***}P < 0.001$.

proteins. To further study the CYLD–NDRG1 interaction, we performed co-IP experiments in CNE2 cells overexpressing *CYLD*. We found that the anti-Flag antibody, but not the anti-IgG antibody, pulled down Flag-CYLD, as well as NDRG1, from CNE2 cells. Consistently, the anti-NDRG1 antibody pulled down NDRG1, as well as CYLD, confirming an interaction between CYLD and NDRG1 in CNE2 cells (Figure 4B). We further evaluated the association between CYLD and NDRG1 in NPC cell lines. qPCR analysis confirmed that NDRG1 mRNA levels were not altered by either *CYLD* overexpression or knockdown in NPC cells (Supplementary Figure 1B). Interestingly, Western blot results indicated that *CYLD* overexpression increased NDRG1 protein levels, whereas *CYLD* knockdown decreased NDRG1 levels (Figure 4C). These results indicated that CYLD regulates NDRG1 at the posttranscriptional level. Given that CYLD is a deubiquitinase, we hypothesized that CYLD might inhibit NDRG1 ubiquitination and degradation via the ubiquitin–proteasome pathway.

NDRG1 is a Functional Target of CYLD in NPC

To determine whether CYLD-regulated NDRG1 levels are associated with the tumor-suppressive effect of CYLD, we knocked down *NDRG1* in NPC cells stably overexpressing *CYLD* (Figure 5A). The results showed that inhibitory effects of CYLD on NPC cell proliferation were significantly abrogated by *NDRG1* silencing (Figure 5B and C; Supplementary Figure 1C). Additionally, we observed that the CYLD-specific promotion of NPC cell apoptosis was reversed by *NDRG1* silencing (Figure 5D and E). These findings identified NDRG1 as a functional target of CYLD in NPC cells.

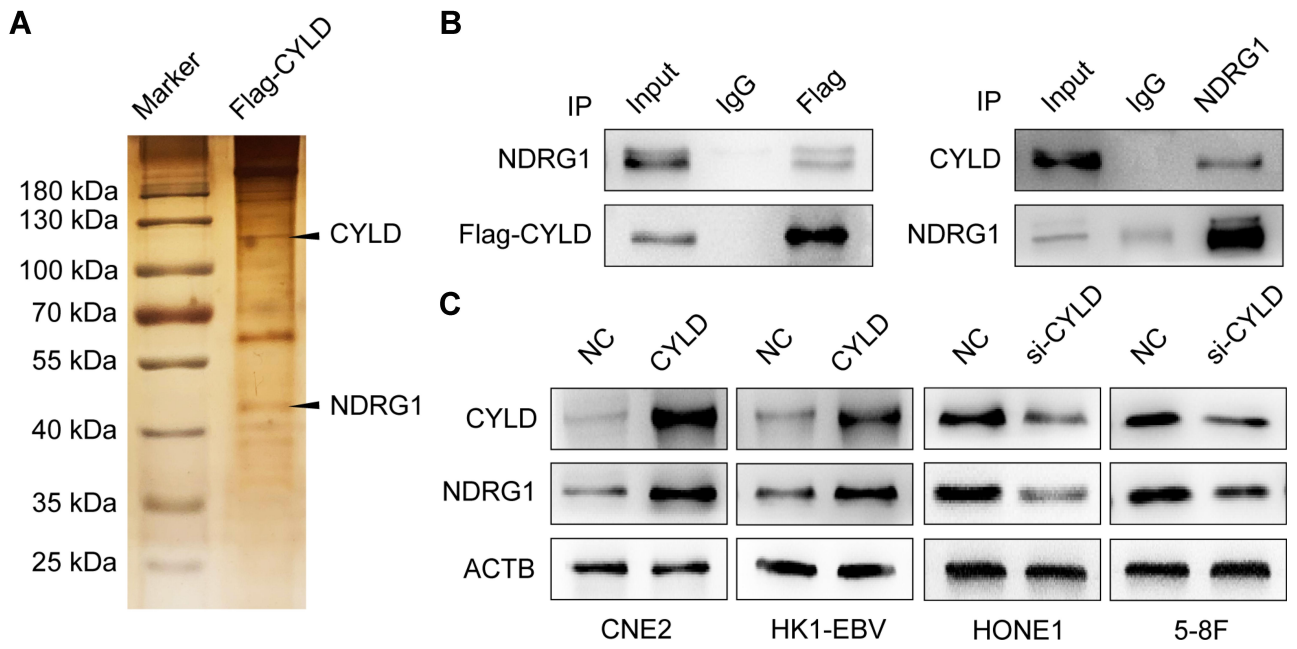

Figure 4 CYLD directly interacts with NDRG1. (**A**) Anti-Flag-CYLD proteins identified by co-IP and silver staining. Proteins captured by anti-Flag-CYLD were identified by MS (indicated by arrows). (**B**) Interaction between CYLD and NDRG1 confirmed by co-IP from CNE2 cells overexpressing *CYLD*. (**C**) NDRG1 protein levels measured by Western blot in *CYLD*-overexpressing CNE2 and HK1-EBV cells or *CYLD*-silenced HONE1 and 5–8F cells.

Overexpression of CYLD Suppressed Tumor Growth in vivo

To further explore the function of CYLD in vivo, xenograft mice models were established. In the subcutaneous model, the tumor volume and weight were significantly decreased in CYLD-overexpressing group when compared to the control group (Figure 6A and B). Furthermore, IHC staining results showed that CYLD overexpression increased the NDRG1 level in tumor tissue samples relative to that in control tissue samples, whereas Ki-67 and PCNA protein levels decreased following CYLD overexpression (Figure 6C).

Discussion

Despite the 5-year overall survival rate of NPC exceeding 80%, the efficacy of NPC treatments plateaued during the previous decade.[22] Genetic factors play an important role in NPC progression and provide a basis for developing novel therapeutic strategies. Here, we investigated the tumor-suppressive role of CYLD in NPC and revealed an underlying CYLD-specific mechanism according to analyses of results from NPC cell lines and clinical specimens.

IHC and qPCR analyses showed significantly reduced *CYLD* expression in NPC tissues, suggesting its possible role as a tumor suppressor. This was consistent with previous results identifying inactivating CYLD mutations in primary NPC tissue.[19] In addition to loss-of-function mutations, microRNA-mediated downregulation of *CYLD* expression has been reported in various types of human malignancies.[23–25] Future studies should focus on elucidating the underlying mechanism involved in CYLD downregulation in NPC to determine the involvement of epigenetic regulatory mechanisms.

We then determined the molecular function of CYLD in NPC cells, finding that CYLD inhibited NPC cell proliferation but promoted apoptosis according to *CYLD*-overexpression and -silencing analyses, respectively. A previous study reported that CYLD inhibits cell proliferation and apoptosis resistance in triple-negative breast cancer.[24] Additionally, Sanches et al[25] showed that CYLD suppresses cell migration and invasion in cervical cancer, and Suenaga et al[26] reported that CYLD downregulates induction of cisplatin resistance in oral squamous cell carcinoma. Because therapeutic resistance and distant metastasis are the main causes of therapeutic failure in NPX patients,[27,28] future studies should focus on determining additional CYLD-specific molecular functions in NPC.

Previous studies report that CYLD negatively regulates NF-κB signaling by removing K63- and M1-linked

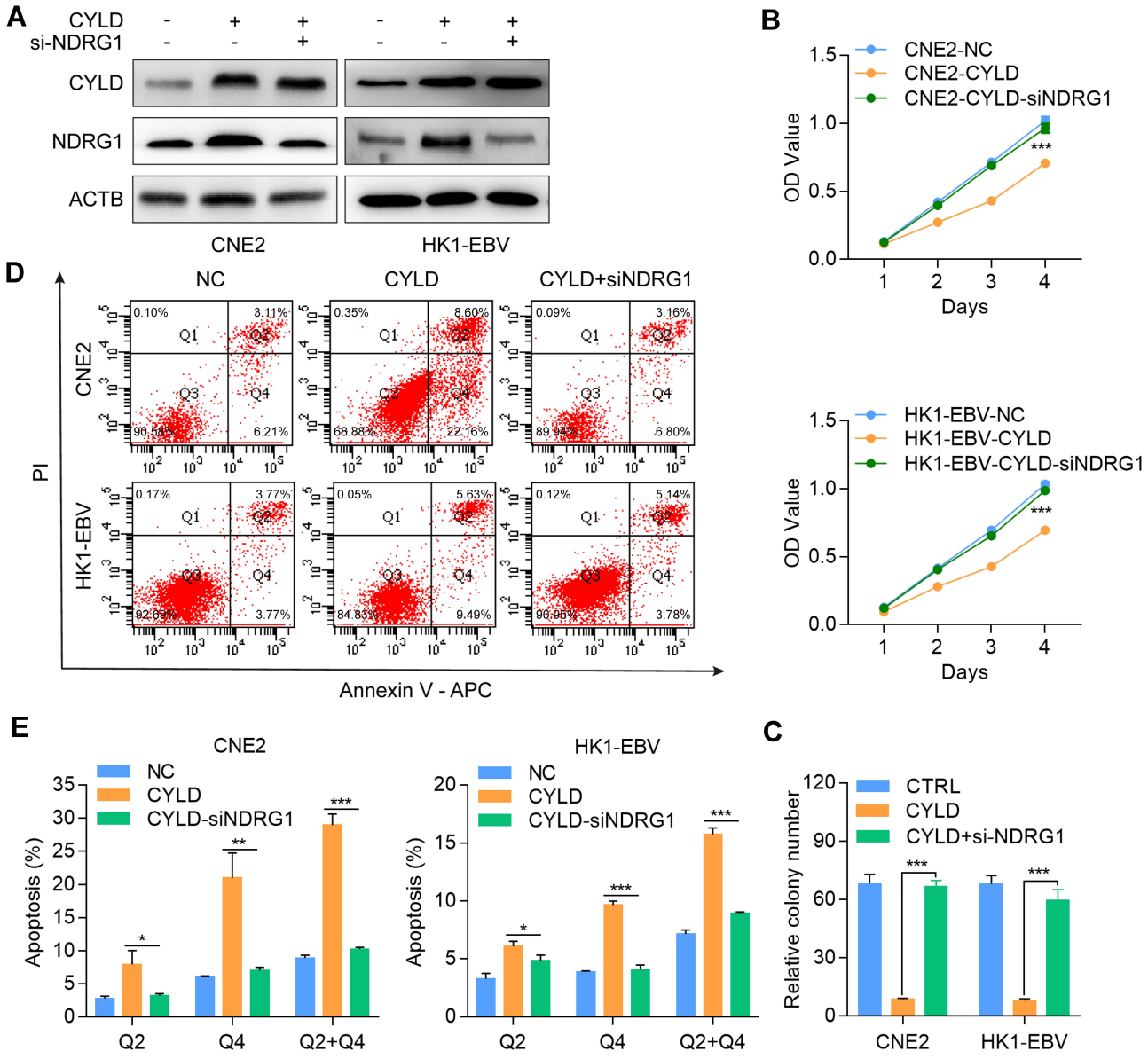

Figure 5 *NDRG1* silencing abolishes the tumor-suppressor effect of CYLD on NPC cells. (**A**) siRNA knockdown of *NDRG1* in CNE2 and HK1-EBV cells stably overexpressing *CYLD*. CYLD and NDRG1 protein levels were assessed by Western blot. (**B, C**) NPC cell proliferation evaluated by CCK-8 and colony formation assays. Data represent the mean ± SEM (n =3). ***$P < 0.001$. (**D, E**) NPC cell apoptosis evaluated by Annexin V/PI staining. Data represent the mean ± SEM (n =3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

polyubiquitin chains from key signaling molecules, including NF-κB essential modulator, tumor necrosis factor (TNF)-associated factor (TRAF) 2, TRAF6, and Receptor-interacting serine/threonine-protein kinase 1, in familial cylindromatosis tumors.[8–10] Additionally, Tauriello et al[11] demonstrated that CYLD inhibits the Wnt pathway by deubiquitinating disheveled in familial cylindromatosis tumors. Moreover, CYLD reportedly promotes TNF-α-Induced apoptosis and programmed necrosis in human lung cancer cells.[29,30] However, the detailed molecular mechanism of CYLD in NPC largely remains undetermined. To fully reveal the molecules potentially interacting with CYLD, we performed co-IP and MS analyses, with the results revealing for the first time that CYLD interacts with NDRG1. Furthermore, we found that *NDRG1* knockdown reversed CYLD-overexpression-mediated inhibition of cell proliferation and promotion of apoptosis. Further investigation is required to identify the signaling pathways involved in NDRG1-specific regulation of NPC cell proliferation and apoptosis.

NDRG1 is a member of the N-Myc downregulated-gene family and belongs to the α/β hydrolase superfamily.[31]

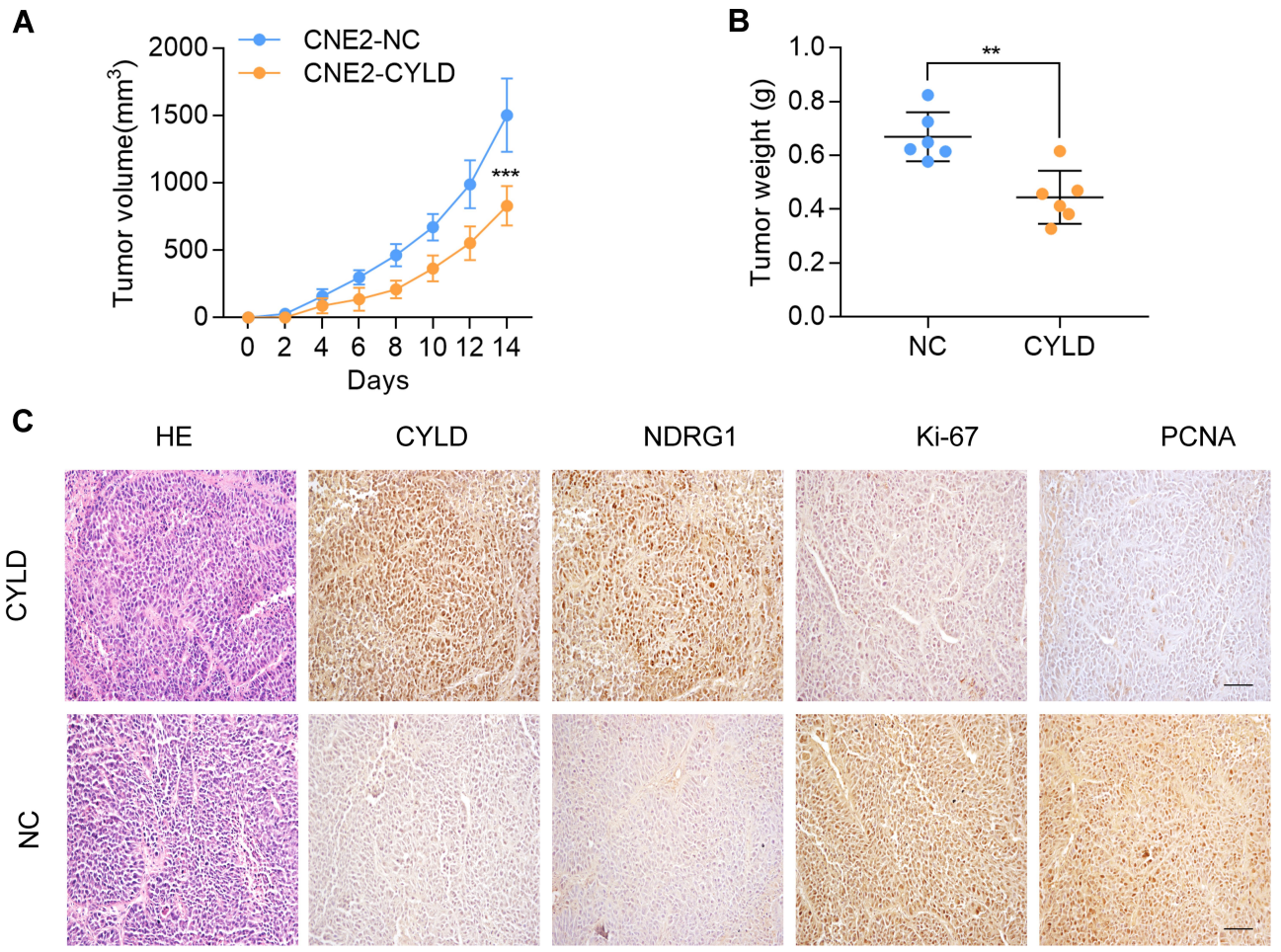

Figure 6 CYLD suppressed tumor growth in vivo. (**A**) Tumor growth curves. Data represent the mean ± SEM (n =6). ***$P < 0.001$. (**B**) Tumor weight was examined after mice were sacrificed. Data represent the mean ± SEM (n =6). **$P < 0.01$. (**C**) HE staining (left) and IHC staining for CYLD, NDRG1, Ki-67 and PCNA in control and CYLD-overexpressing tumors. Scale bar: 30 μm.

Previous studies reported that NDRG1 plays different roles in different types of human malignancies, and that NDRG1 works as a tumor suppressor in colorectal, gastric, and prostate cancers, as well as NPC,[32–36] while playing an oncogenic role in lung cancer and hepatocellular carcinomas.[37,38] There are only two previous reports concerning NDRG1 in NPC. Hu et al[35] reported that NDRG1 suppresses cell invasion and the epithelial–mesenchymal transition in NPC, and Kanda et al[36] demonstrated that *NDRG1* expression was decreased in NPC tissue. In the present study, we demonstrated for the first time a direct interaction between CYLD and NDRG1 in NPC cells along with upregulated NDRG1 protein levels but not mRNA levels. A previous study reported NDRG1 degradation via a proteasome-independent mechanism in pancreatic cancer cells.[39] Given that CYLD is a lysine deubiquitinase, we hypothesized that CYLD might remove Lys63-linked polyubiquitin chains from NDRG1 and protect it from proteasomal degradation. Future studies should focus on revealing the detailed mechanisms of CYLD in regulating NDRG1.

In conclusion, we demonstrated that CYLD downregulation promoted NPC cell proliferation and apoptosis resistance. Moreover, as a tumor suppressor, CYLD upregulated NDRG1 levels to subsequently suppress NPC progression. And This suppression effect of CYLD could partially release from the NDRG1 knockdown. These findings suggest CYLD as a potential therapeutic target for the treatment of patients with NPC.

Acknowledgments

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Disclosure

The authors report no conflicts of interest.

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