Ubiquitin ligase NEDD4 promotes the proliferation of hepatocellular carcinoma cells through targeting PCDH17 protein for ubiquitination and degradation

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Neural precursor cell expressed developmentally downregulated 4 (NEDD4), an E3 ubiquitin ligase, is commonly upregulated in human hepatocellular carcinoma (HCC) and functions as an oncogenic factor in the progression of HCC, but the molecular mechanism needs be further explored. In this study, we found that NEDD4 could facilitate the proliferation of HCC cells, which was associated with regulating the ERK signaling. Further investigation showed that protocadherin 17 (PCDH17) was a potential substrate of NEDD4, and restoration of PCDH17 could block the facilitation of ERK signaling and HCC cells proliferation induced by NEDD4 overexpression. Whereafter, we confirmed that NEDD4 interacted with PCDH17 and promoted the Lys33-linked polyubiquitination and degradation of it *via* the proteasome pathway. Finally, NEDD4 protein level was found to be inversely correlated with that of PCDH17 in human HCC tissues. In conclusion, these results suggest that NEDD4 acts as an E3 ubiquitin ligase for PCDH17 ubiquitination and degradation thereby promoting the proliferation of HCC cells through regulating the ERK signaling, which may provide novel evidence for NEDD4 to be a promising therapeutic target for HCC.

Hepatocellular carcinoma (HCC) is still the leading cause of tumor-related death worldwide up till now (1, 2). Although, in recent years, the remarkable progress in medicine could alleviate the pain of HCC patients in some extent, the prognosis is still poor (3, 4). Therefore, the underlying molecular mechanism of HCC requires further exploration to find more effective treatments for HCC patients to prolong their survival.

Neural precursor cell expressed developmentally downregulated 4 (NEDD4), a typical homologous to E6-APC terminus (HECT) E3 ubiquitin ligase, contains an N-terminal C2 domain, four WW domains and a C-terminal HECT domain (5). It has been reported that NEDD4 plays an important role in various biological processes, including cell proliferation, apoptosis, cell cycle, migration, and inflammation (6–8). In addition, studies have shown that NEDD4 involves in the development of many human cancers, such as lung cancer, bladder cancer, colorectal cancer, and breast cancer (9–11). In HCC, NEDD4 acts as a biomarker, and overexpression of NEDD4 could promote cell growth and motility through degrading tumor suppressor large tumor suppressor gene 1 (LATS1) (12). However, whereas the underlying mechanism of NEDD4 as an ubiquitin ligase in HCC remains to be studied.

Protocadherin 17 (PCDH17), a member of the protocadherin family, belongs to a group of cadherin superfamilies that mediate calcium-dependent cell-cell adhesion (13, 14). PCDH17 is localized on chromosome 13q21.1 in humans (15). Some evidence suggests that PCDH17 plays important roles in multiple biological activities, such as cell cycle, apoptosis, autophagy, and signal transduction (16–18). The function of PCDH17 in tumorigenesis also requires more attention. Multiple studies have shown that PCDH17 is downregulated and loss of PCDH17 can promote the proliferation and metastasis of tumor cells, such as gastric cancer, colorectal cancer, breast cancer, and HCC (19–21), thus it could be served as a biomarker in several human cancers. In addition, it has been reported that many factors can contribute to low PCDH17 expression, such as noncoding RNA (22), acetylation modification (23), and methylation modification (24); however, the regulatory mechanism of its posttranslational, such as ubiquitination, is still unclear.

In our study, we confirmed the positive effect of NEDD4 on the proliferation of HCC cells through regulating the ERK1/2 signaling. Importantly, we provided the evidence that PCDH17 is an ubiquitination substrate of NEDD4. We also revealed the relevance of NEDD4 and PCDH17 expression in clinical samples.

Results

Knock down of NEDD4 suppresses the proliferation of HCC cells

To verify the role of NEDD4 in the proliferation of HCC cells, we performed loss- and gain-of-function experiments.

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First, the stable cell lines with loss of NEDD4 were developed using three shRNA targets (shNEDD4#1, shNEDD4#2, and shNEDD4#3). Notably, shNEDD4#1 displayed a silencing efficiency of ∼55% and ∼60% in HepG2 and Huh7 cells, respectively (Fig. 1*A*). We then performed cell growth assays to determine whether NEDD4 silencing influences the proliferation of HCC cells. It was found that knock down of NEDD4 significantly decreased the number of EdU-positive cells (Fig. 1, *B* and *C*). Cell counting kit-8 (CCK-8) assay showed that NEDD4 silencing attenuated the cell viability of HepG2 and Huh7 cells (Fig. 1, *D* and *E*). In addition, the ability of colony formation was also obviously suppressed by silencing of NEDD4, comparing with the control group (Fig. 1*F*). Furthermore, to analyze the pathway NEDD4 regulated, the expressions of STAT3, AKT, and ERK1/2 signaling were determined, which are the classical signaling pathways that regulate cell proliferation. The results showed that NEDD4 knock down dramatically decreased both the protein levels of ERK1/2 and p-ERK1/2, while the expression of STAT3, p-STAT3, AKT, and p-AKT had no significant change (Fig. 1, *G* and *H*).

It has been reported that Heclin, a HECT E3 ubiquitin ligases inhibitor, could inhibit the ubiquitin ligase activity of NEDD4 (IC50 = 6.3) (25). To further analyze the regulatory of NEDD4 on HCC cell proliferation, we treated HCC cells using Heclin (6.3 μM) to simulate another inhibitory effect on NEDD4. The results showed that the protein level of ERK1/2 pathway was obviously decreased upon the ubiquitin ligase activity of NEDD4 was inhibited (Fig. S1, *A*–*C*). Meanwhile, the growth phenotype of HCC cells displayed that Heclin significantly decreased the cell proliferation (Fig. S1, *D*–*I*). These results suggest that knock down of NEDD4 suppresses the proliferation of HCC cells.

Overexpression of NEDD4 promotes the proliferation of HCC cells

To further determine the role of NEDD4 in the proliferation of HCC cells, we then transiently transfected Myc–tagged NEDD4 complementary DNA into HepG2 and Huh7 cells to achieve the gain-of-function. The expression efficiency of Myc-NEDD4 was confirmed by Western blotting and GFP images (Figs. 2*A* and S2). Therefore, 24 h after transfection, the cells were used to evaluate the cell growth. Briefly, overexpression of NEDD4 significantly increased the number of EdU-positive cells (Fig. 2, *B* and *C*). CCK-8 assay showed that NEDD4 upregulation increased the cell viability (Fig. 2, *D* and *E*). And the colony number of NEDD4 overexpression group was obviously increased, comparing with the control group (Fig. 2*F*). Correspondingly, overexpression of NEDD4 increased both the protein levels of ERK1/2 and p-ERK1/2, but not STAT3 or AKT pathways (Fig. 2, *G* and *H*). These results suggest that overexpression of NEDD4 promotes the proliferation of HCC cells.

NEDD4 accelerates the proliferation of HCC cells through regulating PCDH17

The results above have shown that NEDD4 is important for the proliferation of HCC cells. However, the underlined mechanisms should be addressed. It has been reported that NEDD4 could promote the ubiquitination level of many proteins thereby regulating the progression of several human cancers (26, 27). To explore the downstream proteins that involved in NEDD4 promoted HCC proliferation, we first analyzed the potential substrate using NEDD4 as query protein in the UbiBrowser database, and found that the confidence score of PCDH17 is 0.760, which was convincing (Fig. 3*A*, upper panel). In addition, we further screened potential upstream ubiquitin ligases using PCDH17 as query protein in the UbiBrowser database, and found that NEDD4 with the highest credibility coefficient is predicted as the most possible ubiquitin ligase (Fig. 3*A*, lower panel). More importantly, Kyoto Encyclopedia of Genes and Genomes (KEGG) gene enrichment analysis further determined that PCDH17 was enriched in ubiquitin-mediated proteolysis in HCC (normalized enrichment score = 1.97, $p < 0.05$, false discovery rate < 0.25; Fig. 3*B*). Thus, we asked whether PCDH17 was involved in NEDD4 regulated HCC cell proliferation. To address this question, we first verified the regulatory effect of NEDD4 on PCDH17 expression. It was found that knock down of NEDD4 significantly increased the protein level of PCDH17, while overexpression had the opposite effect (Fig. 3, *C* and *D*).

To confirm whether NEDD4 accelerates the proliferation of HCC cells through regulating PCDH17, we performed the rescue experiments by overexpressing 3×Flag-PCDH17 in NEDD4 up-regulated cells. The results showed that overexpression of PCDH17 effectively decreased the protein levels of ERK1/2 and p-ERK1/2 (Fig. 4, *A* and *B*), and also attenuated the cell proliferation induced by overexpressing of NEDD4 (Fig. 4*C*). In addition, we also performed the rescue experiments by knocking out PCDH17 using CRISPR-Cas9 (Fig. 4*D*), and then the above results were further verified. It was showed that KO PCDH17 obviously increased ERK1/2 pathway protein level, as well as restoring the cell proliferation induced by NEDD4 inactivation (Fig. 4, *E*–*I*). In conclusion, these results suggest that NEDD4 negatively regulates PCDH17 to promote the proliferation of HCC cells.

NEDD4 destabilizes PCDH17 through promoting its Lys33-linked polyubiquitination and degradation in the proteasome

NEDD4 has been reported to degrade several substrates mainly through proteasome pathway, such as VDAC2/3, SQSTM1, and FOXA1 (26, 28, 29). To determine whether the NEDD4-induced degradation of PCDH17 depends on the proteasome system, we treated HCC cells overexpressed NEDD4 using MG132 (10 μM), a kind of proteasome inhibitor. It was showed that MG132 can rescue the reduction of PCDH17 protein level induced by overexpressing of NEDD4 (Fig. 5, *A* and *B*), suggesting that NEDD4 promotes PCDH17 degradation *via* the proteasome pathway. We next examined whether NEDD4 affects the protein stability of PCDH17. The protein synthesis inhibitor cycloheximide (CHX) was used to treat HCC cells silencing NEDD4 for different time periods. The half-life of endogenous PCDH17 protein was substantially

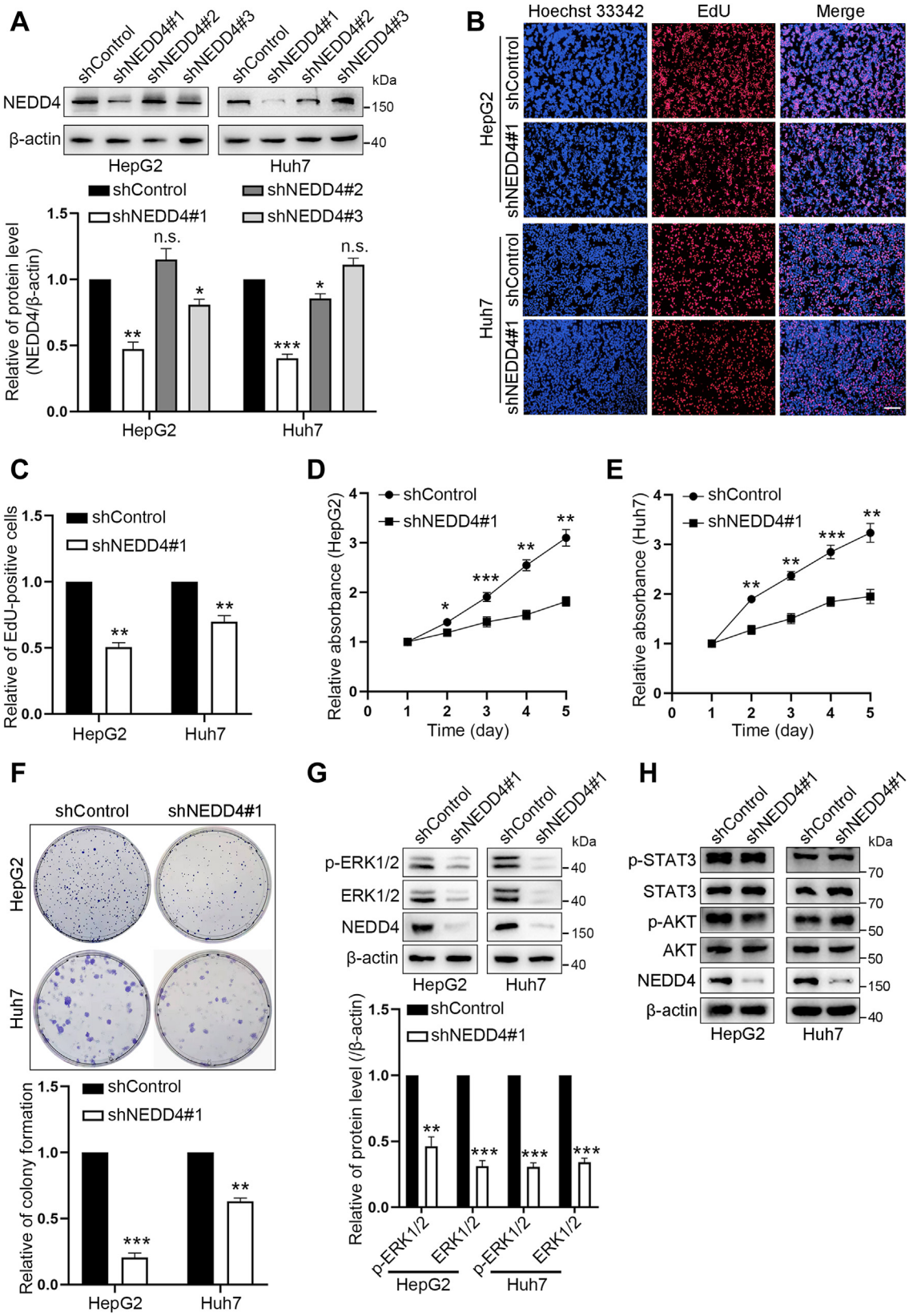

Figure 1. Knock down of NEDD4 suppressed the proliferation of HCC cells. *A*, representative blots and quantification of shNEDD4 efficiency. *B*, representative images of EdU assay performed in HepG2 and Huh7 cells upon knocking down of NEDD4, the scale bar represents 200 μm. *C*, quantification of EdU assay. *D* and *E*, CCK-8 assay results. *F*, representative images and quantification of the colony formation assay with NEDD4 silenced HepG2 and Huh7. *G*, representative blots and quantification showed the protein levels of ERK1/2 and p-ERK1/2 in NEDD4 silenced HepG2 and Huh7 cells. *H*, representative blots showed the protein levels of AKT, p-AKT, STAT3, and p-STAT3 in NEDD4 silenced HepG2 and Huh7 cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. CCK-8, cell counting kit-8; HCC, human hepatocellular carcinoma; NEDD4, neural precursor cell expressed developmentally downregulated 4.

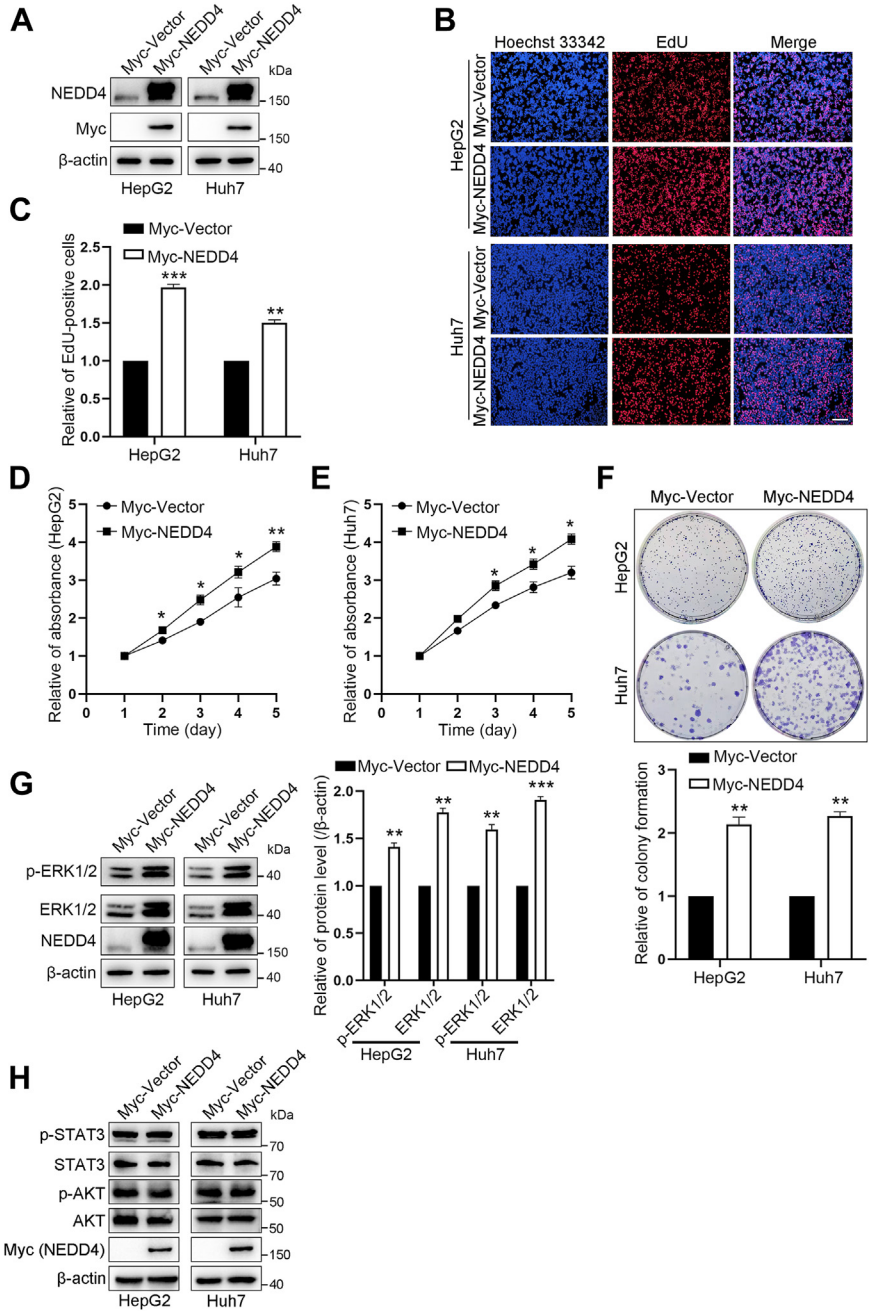

Figure 2. Overexpression of NEDD4 promotes the proliferation of HCC cells. *A*, representative blots of Myc-NEDD4 transfection efficiency. *B*, representative images of EdU assay performed in HepG2 and Huh7 cells upon overexpressing of NEDD4, the scale bar represents 200 μm. *C*, quantification of EdU assay. *D* and *E*, CCK-8 assay results. *F*, representative images and quantification of the colony formation assay with NEDD4 overexpressed HepG2 and Huh7. *G*, representative blots and quantification showed the protein levels of ERK1/2 and p-ERK1/2 in NEDD4 overexpressed HepG2 and Huh7 cells. *H*, representative blots showed the protein levels of AKT, p-AKT, STAT3, and p-STAT3 in NEDD4 overexpressed HepG2 and Huh7 cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. CCK-8, cell counting kit-8; HCC, human hepatocellular carcinoma; NEDD4, neural precursor cell expressed developmentally downregulated 4.

increased in NEDD4-silencing HCC cells compared with control (Fig. 5*C*). Conversely, overexpression of NEDD4 reduced the half-life of the endogenous PCDH17 protein in HCC cells (Fig. 5*D*).

To further study the regulatory relationship between NEDD4 and PCDH17, we cotransfected Myc-NEDD4 and 3×Flag-PCDH17 into HCC cells. It was found that Myc-tagged NEDD4 interacted with 3×Flag tagged PCDH17 in HCC cells (Fig. 6, *A* and *B*). In addition, we also found that the endogenous NEDD4 could interact with PCDH17 in HCC cells (Fig. 6*C*). Then, we performed intracellular ubiquitination experiment to examine the impact of NEDD4 on PCDH17 ubiquitination. Compared with cells transfected with the control vector, cells transfected with Myc-NEDD4 exhibited significantly increased ubiquitination of 3×Flag-PCDH17 in HEK293T cells (Fig. 6*D*). Next, we detected the effect of Heclin on the expression and ubiquitination level of PCDH17, and the results showed that Heclin could increase the expression of PCDH17 and reduce the ability of NEDD4 to ubiquitinate PCDH17 in HCC cells (Fig. S3). It has been reported that the

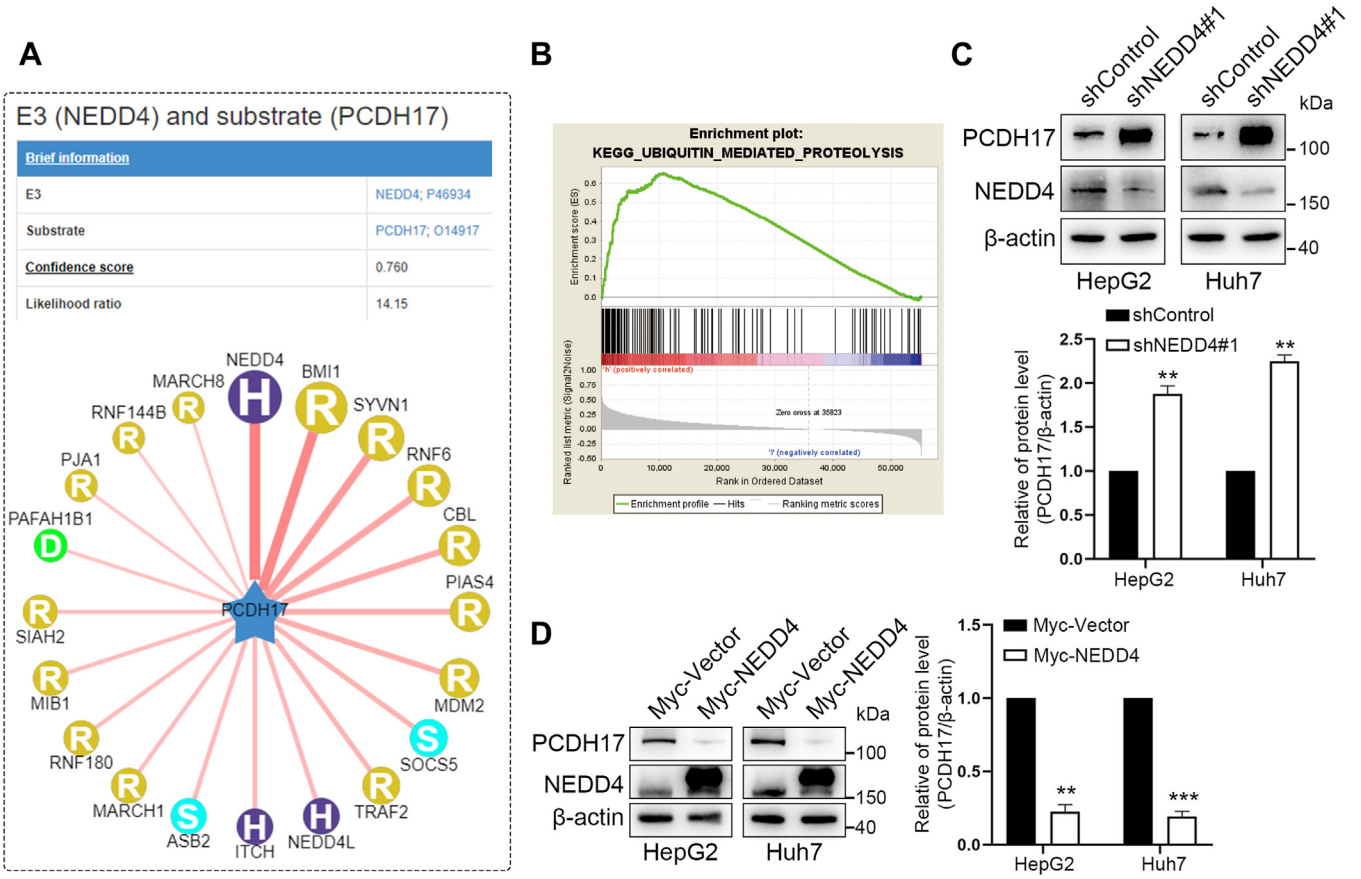

Figure 3. NEDD4 negatively regulates PCDH17 in HCC cells. *A*, NEDD4 is predicted as the most possible ubiquitin ligase of PCDH17 through the UbiBrowser database. *B*, KEGG gene enrichment analysis further determined that PCDH17 was enriched in ubiquitin-mediated proteolysis in HCC (NES = 1.97, $p < 0.05$, FDR <0.25). *C*, representative blots and quantification showed the protein levels of PCDH17 in NEDD4 silenced HepG2 and Huh7 cells. *D*, representative blots and quantification showed the protein levels of PCDH17 in NEDD4 overexpressed HepG2 and Huh7 cells. $^{**}p < 0.01$, $^{***}p < 0.001$. FDR, false discovery rate; HCC, human hepatocellular carcinoma; KEGG, Kyoto Encyclopedia of Genes and Genomes; NEDD4, neural precursor cell expressed developmentally downregulated 4; NES, normalized enrichment score; PCDH17, protocadherin 17.

HECT domain of NEDD4 is required for its ubiquitin ligase activity (30–32). To investigate whether NEDD4 E3 ligase activity is required for NEDD4-induced PCDH17 degradation and ubiquitination, we generated a NEDD4-HECT mutant in which Cys867 in the HECT domain were converted to serine (C867S). Previous studies revealed that this mutant has no E3 ligase activity (33, 34). Notably, the NEDD4-HECT mutant did not downregulate the PCDH17 protein, comparing with WT NEDD4 (NEDD4-WT) (Fig. 6*E*). Correspondingly, the NEDD4-HECT mutant markedly reduced the ability of NEDD4 to ubiquitinate PCDH17 in HCC cells (Fig. 6*F*).

There are seven lysine residues in ubiquitin, including K6, K11, K27, K29, K33, K48, and K63, therefore different types of polyubiquitin chains can be linked on substrates to perform diverse functions. To further determine the mechanism by which NEDD4 ubiquitinates PCDH17, HCC cells were cotransfected with WT or mutant HA-Ub vectors as well as the Myc-NEDD4 and 3×Flag-PCDH17 vectors, and the PCDH17 ubiquitination levels were analyzed by Western blotting using the indicated antibodies. It was apparent that K33R mutant dramatically reduced the ubiquitination level of PCDH17, which indicated that polyubiquitination of PCDH17 was mainly initiated by Lys33-linked chains in HCC cells (Fig. 6, *G* and *H*).

Furthermore, to get rid of the influence of ubiquitinated binding partners in our study, we performed an experiment to detect the ubiquitination level of PCDH17 under denaturing conditions, which can further confirm the above findings. In this experiment, an ubiquitin mutant vector K33 (K33-Ub) was used for transfection (K33-Ub contains arginine substitutions of all lysine residues except the one at positions K33). It was showed that, similarly to the results under the above nondenaturing conditions, NEDD4-HECT mutant could obviously reduce the ability of NEDD4 to ubiquitinate PCDH17 either after transfection of WT-Ub or K33-Ub, which was consistent with the above results (Fig. S4). In conclusion, these results suggest that NEDD4 destabilizes PCDH17 through promoting its Lys33-linked polyubiquitination and degradation in the proteasome.

NEDD4 expression is inversely correlated with PCDH17 expression in human HCC tissues

As mentioned above, we have demonstrated that NEDD4 downregulates PCDH17 protein levels in HCC cells *via* the proteasome pathway, preliminarily showing the regulatory relationships between them at the cellular level. Furthermore, we explored the clinical relevance of NEDD4 and PCDH17 and their relationship in clinical HCC patients. It was showed

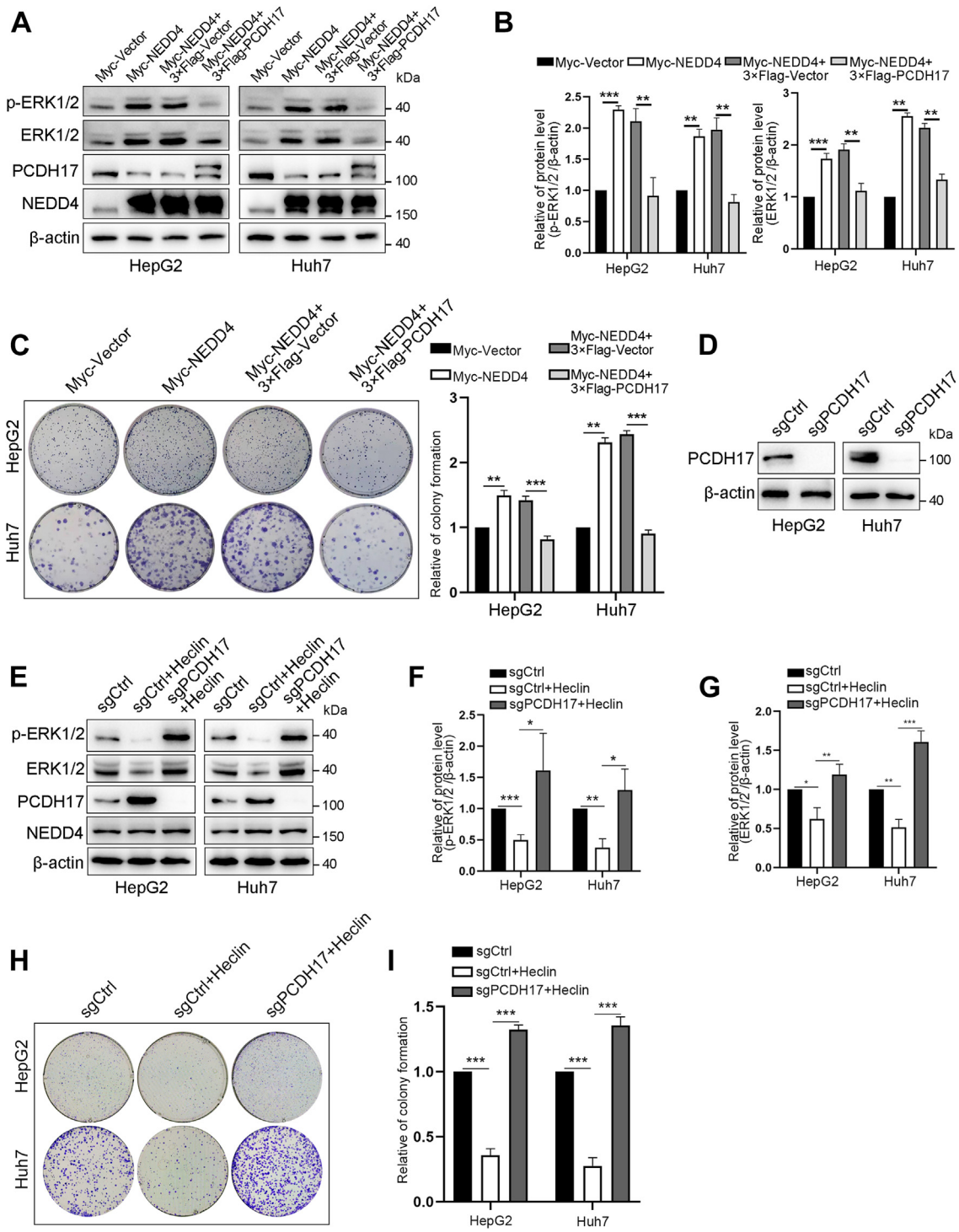

Figure 4. NEDD4 promotes the proliferation of HCC cells through regulating PCDH17-ERK1/2 pathway. *A*, representative bolts and *B*, quantification showed overexpression of PCDH17 effectively decreased the expressions of ERK1/2 and p-ERK1/2. *C*, overexpression of PCDH17 effectively attenuated the colony formation induced by overexpressing of NEDD4. *D*, representative bolts of sgPCDH17 efficiency. *E–G*, representative bolts and quantification showed knocked out PCDH17 effectively increased the expressions of ERK1/2 and p-ERK1/2 induced by Heclin. *H* and *I*, knocked out PCDH17 effectively rescued the colony formation induced by Heclin. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. HCC, human hepatocellular carcinoma; NEDD4, neural precursor cell expressed developmentally downregulated 4; PCDH17, protocadherin 17.

that the expression level of NEDD4 is much higher in HCC tissues than that in normal liver tissues, while the PCDH17 protein levels were much lower in HCC tissues than normal liver tissues (Fig. 7*A*), indicating that the expression levels of NEDD4 and PCDH17 maybe inversely correlated in human HCC tissues. Next, we analyzed the correlation between NEDD4 and PCDH17 and found that there was a directly negative correlation between NEDD4 and PCDH17 levels both in normal liver tissues and HCC tissues (r = −0.3954, p = 0.0373; Fig. 7*B*). These results suggest that NEDD4

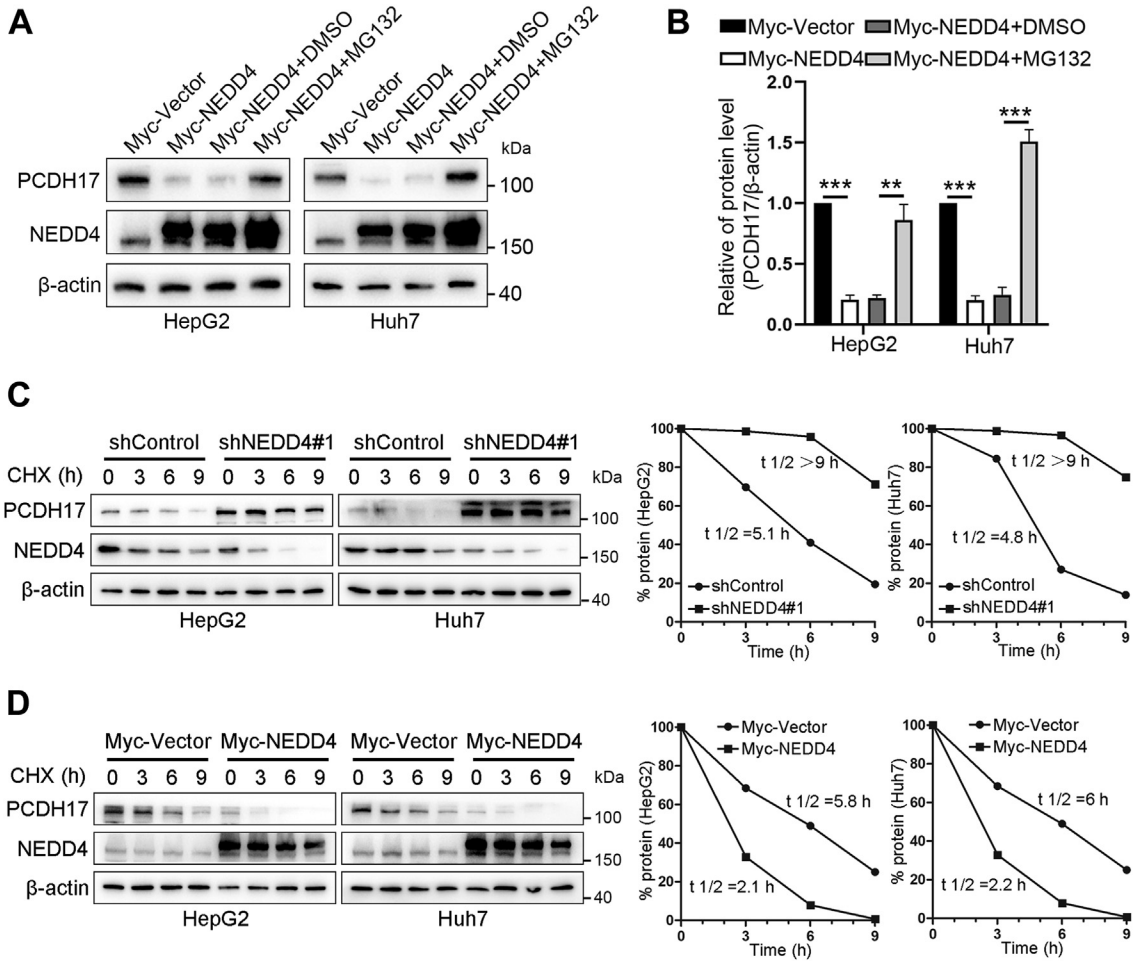

Figure 5. NEDD4 destabilizes PCDH17 through proteasome pathway. *A*, representative blots and *B*, quantification showed that MG132 could block the degradation of PCDH17 in NEDD4-upregulated cells. *C*, representative bolts and quantification showed knock down of NEDD4 increased the stability of PCDH17. *D*, representative bolts and quantification showed overexpression of NEDD4 decreased the stability of PCDH17. $^{**}p < 0.01$, $^{***}p < 0.001$. NEDD4, neural precursor cell expressed developmentally downregulated 4; PCDH17, protocadherin 17.

expression is inversely correlated with PCDH17 expression in human HCC tissues.

Discussion

NEDD4 has been confirmed to play several functions in cellular activities, such as proliferation, apoptosis, cell cycle, metastasis, and so on. (35). Recent studies have shown that upregulated NEDD4 plays an important role in the occurrence and development of some human cancers (34, 36–38), including HCC, which was also observed in this study (Figs. 1, 2, 7*A*, and S1). In addition, as an E3 ubiquitin ligase, NEDD4 has been reported to regulate multiple proteins by ubiquitination, for example, p-Akt (Ser473) (39), PD-L1 (9) and Robo1 (40). Therefore, it is reasonable to further explore the functional or mechanistic role of NEDD4 in ubiquitination modification.

PCDH17 is characterized as a tumor suppressor and downregulated in many tumor types, including HCC (19, 41, 42), which was also observed in this study (Fig. 7*A*). Downregulation of PCDH17 predicted a poor survival rate in patients (43–45). It is necessary to reveal the cause of PCDH17 low expression. Although some studies have been performed to investigate the regulation of PCDH17, the mechanisms by which PCDH17 acts as a tumor suppressor in certain types of human cancers have not been fully elucidated. It has been reported that many factors can contribute to low PCDH17 expression, such as noncoding RNA, acetylation modification, and methylation modification (22–24). However, whether there are other regulatory mechanisms for the abnormal expression of PCDH17 still needs to be further revealed.

In this study, we found that PCDH17 was associated with ubiquitination through bioinformatics analysis (Fig. 3*B*). We provided the evidence that NEDD4 was a potential upstream ligase of PCDH17. We demonstrated that NEDD4 promoted the proliferation of HCC cells through degrading PCDH17 (Fig. 4). Additionally, our follow-up studies have revealed that NEDD4 negatively regulates PCDH17 stability *via* the proteasome pathway (Fig. 5). Moreover, our study originally shows a novel type of PCDH17 regulation, concerning post-translational modifications. We found that NEDD4 could promote the polyubiquitination of PCDH17, upon NEDD4-HECT mutant had no effect on the ubiquitination and

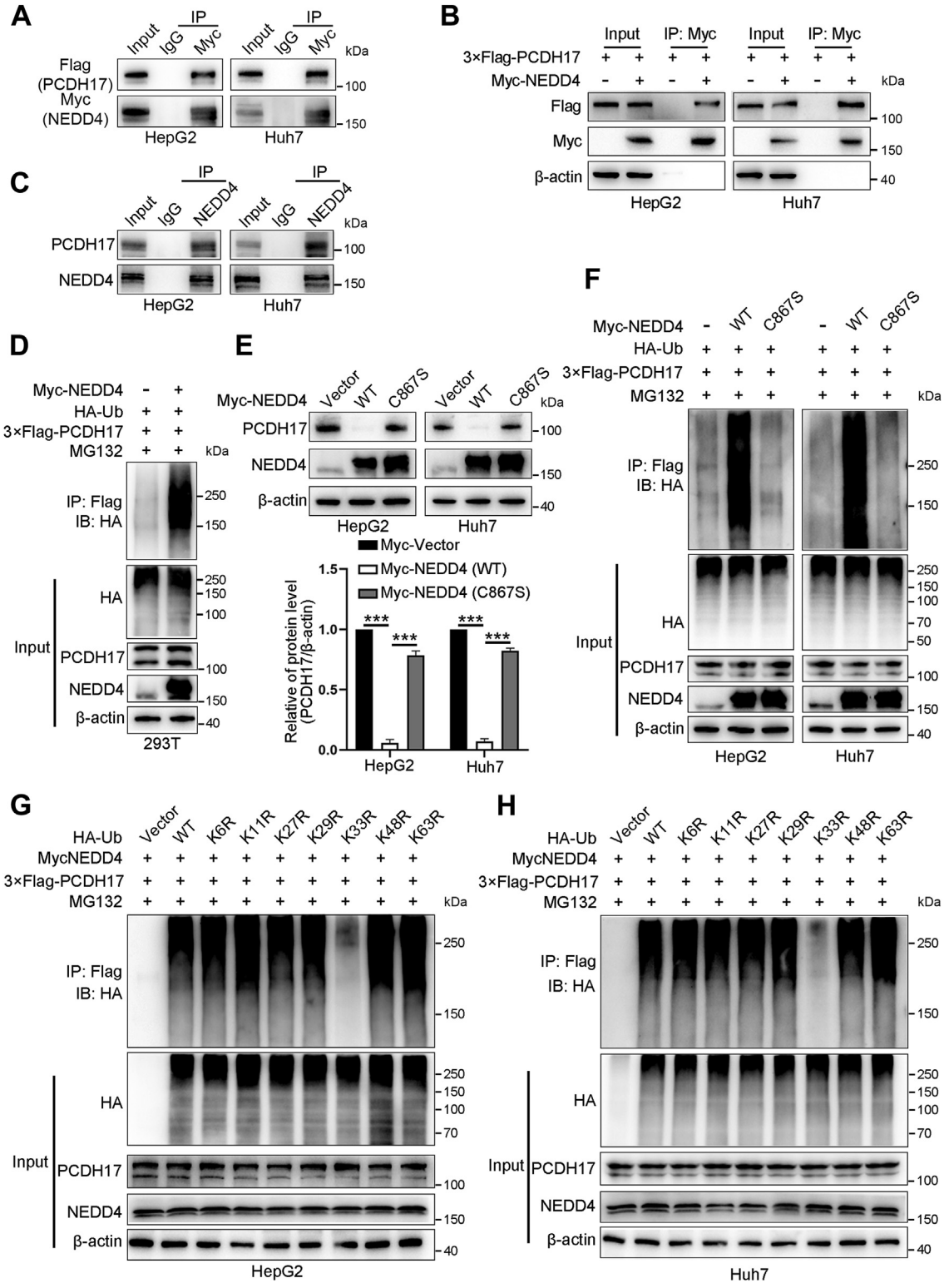

Figure 6. NEDD4 interacts with PCDH17 and promotes its Lys33-linked polyubiquitination and degradation. *A* and *B*, coimmunoprecipitation assay showed that Myc-tagged NEDD4 interacted with 3×Flag-tagged PCDH17 in HepG2 and Huh7. *C*, coimmunoprecipitation assay showed that endogenous NEDD4 interacted with PCDH17 in HepG2 and Huh7. *D*, representative blots of ubiquitination of PCDH17 by NEDD4 in HEK293T. *E*, representative blots and quantification of PCDH17 expression in HCC cells with a NEDD4 mutation. *F*, representative blots of ubiquitinated PCDH17 in HCC cells with a NEDD4 mutation. *G* and *H*, NEDD4 ubiquitinated PCDH17 through Lys33-linked ubiquitin chains in HCC cells. ***$p < 0.001$. HCC, human hepatocellular carcinoma; NEDD4, neural precursor cell expressed developmentally downregulated 4; PCDH17, protocadherin 17.

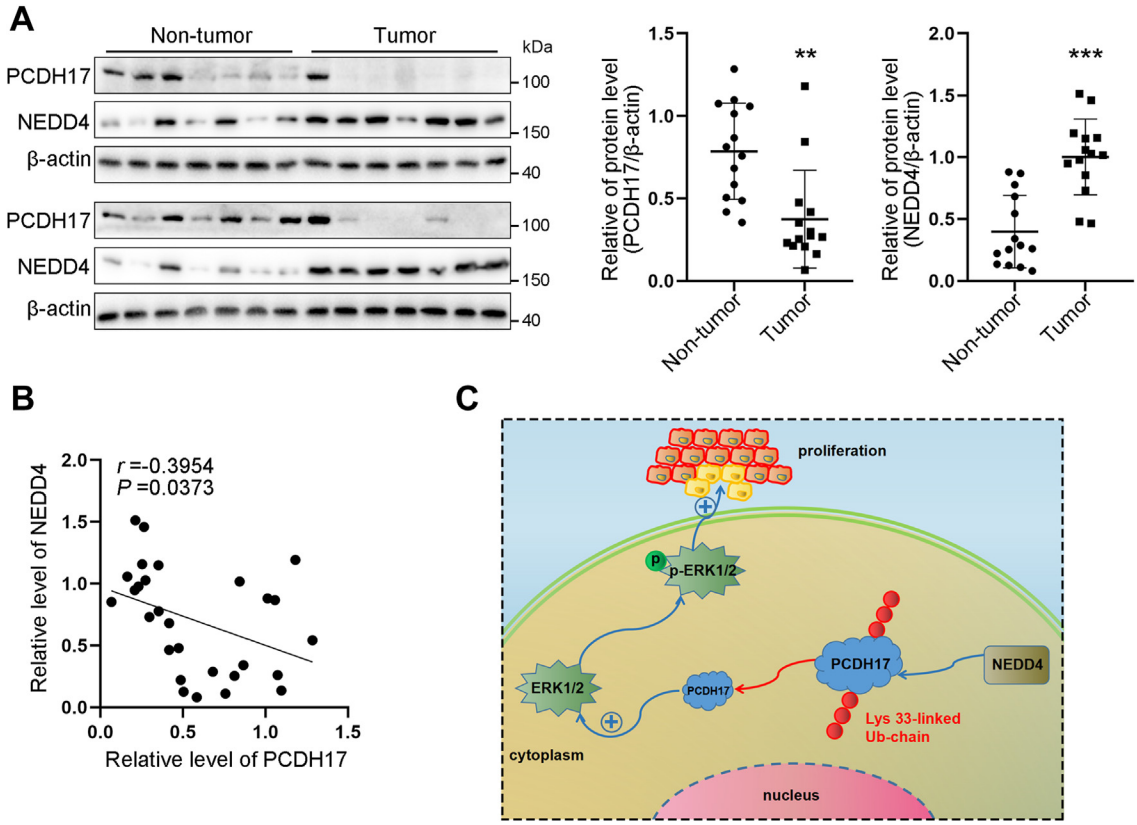

Figure 7. NEDD4 expression is negatively correlated with PCDH17 expression in human HCC tissues. *A*, protein levels of NEDD4 and PCDH17 in HCC tissues (n = 14) and normal liver tissues (n = 14). *B*, the correlation of NEDD4 expression with PCDH17 in normal liver tissues and HCC tissues. $R = -0.3954$; $p = 0.0373$. *C*, schematic illustration of this study. $^{**}p < 0.01$, $^{***}p < 0.001$. NEDD4, Neural precursor cell expressed developmentally downregulated 4; HCC, human hepatocellular carcinoma; PCDH17, protocadherin 17.

degradation of PCDH17, indicating that NEDD4 functions as an E3 ligase toward PCDH17 (Fig. 6, *E* and *F*).

Ubiquitination is mediated by seven lysine, including K6, K11, K27, K29, K33, K48, and K63. While K48- and K63-linked chains are broadly covered in the literature, the other types of chains assembled through K6, K11, K27, K29, and K33 residues deserve equal attention in light of the latest discoveries. Canonical K48-linked chains are widely believed to mediate proteasomal degradation and K63-linked chains involve in other processes, including protein trafficking, DNA repair, and immune response. Whereas, the roles of unconventional polyubiquitin chains linked through K6, K11, K27, K29, and K33 residues are equally important. K6-linked chains are verified to involve in DNA damage response and autophagy. K11-linked chains act in mitosis and regulate cell division. K27-linked chains and K29-linked chains play an important role in innate immunity. K33-linked chains involve in protein trafficking and degradation (46–48). In this study, we redefined the role of partial lysine residues in substrate degradation. We found that PCDH17 polyubiquitination by NEDD4 was initiated by Lys33-linked chains (Fig. 6, *G* and *H*). This could provide a basis for the study of canonical protein ubiquitination.

In conclusion, we identified PCDH17 as a novel substrate of NEDD4. We provide an original mechanism for PCDH17 regulation and infer that overexpression of NEDD4 may account for the frequent loss of PCDH17 expression thereby promoting the proliferation of HCC cells through regulating the ERK signaling (Fig. 7*C*). Our study provides new insights for further study of the molecular mechanism of PCDH17 and molecular targeted therapy for HCC. However, whether PCDH17 can also be regulated by other ubiquitin ligases or deubiquitinating enzymes needs to be further explored. Interestingly, it has been reported that most WW-containing HECT E3 ligases bind to their substrate through a WW-PY (LPxY/PPxY) interaction. However, there are multiple studies showed that NEDD4 could promote the ubiquitination of many substrates without PY motif (26, 49, 50). It is speculated that PY motif is not necessary for HECT E3 ligases to ubiquitinate the substrate. In addition, whether PCDH17 could be ubiquitinated by other WW domain containing HECT E3 ligases is not clear. It is of great significance to study the interaction between PCDH17 and WW domain containing HECT E3 ligases, which is what we are going to consider in the future projects.

Experimental procedures

Antibodies and plasmids

NEDD4 (Cat No. ET1611–42), ERK (Cat No. ET1601–29), p-ERK (Cat No. ET1603–22) antibodies were bought from Huabio. β-actin (Cat No. 66009-1-Ig), Flag (Cat No. 66008-4-Ig), HA (Cat No. 51064-2-AP), and Myc (Cat No. 60003-2-Ig)

antibodies were purchased from Proteintech. PCDH17 (Cat No. A10512) antibody was bought from ABclonal. Myc-Vector, 3×Flag-Vector, 3×Flag-PCDH17, Myc-NEDD4 (WT and C867S), and HA-Ub (WT, K6R, K11R, K27R, K29R, K33R, K48R, and K63R) plasmids were purchased from Changsha Youbio Technology Co.

Tissue samples

HCC tissue samples and normal liver tissue samples were provided by the Affiliated Hospital of Xuzhou Medical University. HCC tissue samples were taken from patients who had undergone surgical resection for HCC, and normal liver samples were taken from patients who had undergone partial hepatectomy for traumatic liver rupture. All tissue samples were immediately frozen in liquid nitrogen and then stored at −80 °C. This study was approved by the Ethics Research Committee of the Affiliated Hospital of Xuzhou Medical University (Approval No. XYFY2019-KL129–01), and all samples obtained informed consent from patients.

Cell culture

HEK293T, HepG2, and Huh7 were kindly provided by Stem Cell Bank, Chinese Academy of Sciences. Cells were cultured in Dulbecco's modified Eagle's medium or minimum essential medium (Yuanpei) supplemented with 10% fetal bovine serum (Gibco), in a 5% CO_2, 37 °C incubator.

Establishment of the stable cell lines

To produce the lentiviruses, HEK293T cells were cotransfected with the corresponding plasmids (shControl, shNEDD4, shPCDH17, sgControl, or sgPCDH17) and helper plasmids using Hieff Trans Liposomal Transfection Reagent (Yeasen). After 72 h, the lentiviruses were collected and subsequently used to infect HepG2 and Huh7 cells. Forty-eight hours after infection, the cells were continuously cultured in a medium containing 2.5 μg/ml puromycin (Beyotime). The surviving cells were cultured into cell lines stably expressing shControl, shNEDD4, shPCDH17, or sgPCDH17. The primer pairs used are as follow:

shNEDD4#1-F:
gatccGCACTAGCTCCGTTAATAAAGTTCAAGAGACTTTATTAACGGAGCTAGTGCTTTTTTg

shNEDD4#1-R:
aattcAAAAAAGCACTAGCTCCGTTAATAAAGTCTCTTGAACTTTATTAACGGAGCTAGTGCg

shNEDD4#2-F:
gatccGCACTAGTGCTAAAGGATTTCTTCAAGAGAGAAATCCTTTAGCACTAGTGCTTTTTTg

shNEDD4#2-R:
aattcAAAAAAGCACTAGTGCTAAAGGATTTCTCTCTTGAAGAAATCCTTTAGCACTAGTGCg

shNEDD4#3-F:
gatccGCATCGAGCTCAAATCATTCCTTCAAGAGAGGAATGATTTGAGCTCGATGCTTTTTTg

shNEDD4#3-R:
aattcAAAAAAGCATCGAGCTCAAATCATTCCTCTCTTGAAGGAATGATTTGAGCTCGATGCg

sgPCDH17 lentiCRISPR v2.
F:CACCgTACGAAACTGTGAATCCCAC; R:aaaacGTGGGATTCACAGTTTCGTAc

EdU assay

Cell proliferation was detected using 5-acetyney-20-deoxyuridine (EdU) kit (RiboBio). 1 × 10^4 cells/200 μl/well were seeded in 96-well plates and cultured for 24 h. Cells were then incubated with 50 μM EdU at 37 °C for 2 h, washed with PBS, and fixed with 4% paraformaldehyde at room temperature for 30 min. After permeabilization with 0.5% Triton X-100 for 10 min, cells were washed thrice with PBS and incubated with 1× Apollo reaction cocktail at room temperature for 30 min in dark. Finally, nuclei were stained with 5 μg/ml Hoechst 33342 at room temperature for 20 min and imaged using a fluorescence microscope (IX73; Olympus) in dark.

Cell viability assay

The cell viability was assessed *via* CCK-8 assay (APExBIO). Briefly, 3000 cells/200 μl/well were cultured in five replicate wells in a 96-well plate. At the designated time point, 20 μl of CCK-8 reagent was added to the cells. After incubation at 37 °C for additional 4 h, a Synergy Mx MultiMode Microplate reader was used to detect the absorbance at 450 nm. The cell viability was calculated based on the absorbance values.

Colony formation assay

A total of 500 cells/5 ml were inoculated into a 60-mm Petri dish for continuous culture until the visible clones appeared. Then the cells were fixed with 4% paraformaldehyde and stained with 0.3% crystal violet solution. After washing with PBS, the plates were photographed using a digital camera. Colony formation was confirmed by manual count, and colony number was defined as >50 cells.

Western blotting

The cells were lysed with radioimmunoprecipitation assay buffer and centrifuged at 12,000*g* and 4 °C for 10 min to collect the supernatant. Equal amounts of protein were separated by electrophoresis on a SDS-PAGE gel and then transferred onto a polyvinylidene fluoride membrane. After blocking with bovine serum albumin solution, membranes were incubated overnight at 4 °C with primary antibodies and secondary antibodies for 1 h at room temperature. Immunoreactive bands were detected using electrochemiluminescence Plus Western blotting substrate (Thermo Fisher Scientific) and visualized using a chemiluminescence detection system (Tanon Science & Technology Co). The band density was quantified using ImageJ software (v1.8.0.345, National Institutes of Health, https://imagej.net/ij/). Relative protein levels were determined by standardizing the optical density values of the target protein to β-actin.

Coimmunoprecipitation

The cells were lysed with ice-cold immunoprecipitation buffer (1% Triton-X-100, 150 mM NaCl, 20 mM Hepes, 2 mM

EDTA, 5 mM MgCl2, pH 7.4). The cell lysates containing the proteins were conjugated to the beads after being incubated overnight with the indicated antibodies. Subsequently, the beads were eluted and subjected to Western blotting assays using the indicated primary and corresponding secondary antibodies.

Ubiquitination assays

The HEK293T or HCC cells were successfully transfected with the desired plasmids and then lysed with ice-cold immunoprecipitation buffer. Similarly, the cell lysates containing the proteins were conjugated to the beads after being incubated overnight with the indicated antibodies. The beads were eluted and subjected to Western blotting assays using the anti-HA antibodies and corresponding secondary antibodies.

Public database analysis

The expression of NEDD4 in HCC and normal liver tissues was obtained from GEPIA (http://gepia.cancer-pku.cn/).

NEDD4 was used as query protein to analyze its potential substrate in the Ubibrowser database (http://ubibrowser.bio-it.cn/ubibrowser/), and found that the confidence score of PCDH17 is 0.760, which was convincing. Next, PCDH17 was used as substrate query protein to screen its potential upstream ubiquitin ligase in the UbiBrowser database again. A total of 37 potential upstream ubiquitin ligases of PCDH17 were identified. Then, the potential ubiquitin ligase was selected based on the credibility coefficient.

The expression information of PCDH17 was extract from The Cancer Genome Atlas (TCGA) (http://www.cancer.gov), and the enrichment information of PCDH17 was analyzed using GSEA software (GSEA-3.0.jar, Broad Institute, http://www.gsea-msigdb.org/gsea/index.jsp). The transcriptome sequencing data of liver hepatocellular carcinoma (LIHC) project was downloaded from the TCGA database, and the samples were divided into "high" and "low" groups according to the expression of PCDH17. Next, the enrichment analysis was conducted with GSEA software.

CHX chase

The transfected HCC cells were treated by CHX (50 μg/ml) for different time points (0, 3, 6, and 9 h). At the designated time, the cells were lysed and then subjected for Western blotting assay. The relative amount of proteins was determined by normalizing the densitometry value of interest to that of the loading control. In order to calculate the degradation rate of PCDH17, we normalized the respective zero-time point as a 100% standard. Then, the PCDH17 expression level of other time points divided by their corresponding zero time point and obtained two sets of ratios which were used for statistics. The slope of the two curves reflects the rate of protein degradation.

Ubiquitination assays under denaturing conditions

The ubiquitination assays under denaturing conditions was performed as described (51). HEK293T cells were transfected with the desired plasmids and then lysed with ice-cold ubiquitin buffer 1 (6 M guanidine-HCI, 0.1 M Na2HPO4, 6.8 mM NaH2PO4, 10 mM Tris–HCI (pH 8.0), and 0.2% Triton X-100, freshly supplemented with 10 mM β-mercaptoethanol). Similarly, the cell lysates containing the proteins were conjugated to the beads after being incubated overnight with the indicated antibodies. After incubation with ubiquitin buffer 1, ubiquitin buffer 2 (8 M urea, 0.1 M Na2HPO4, 6.8 mM NaH2PO4, 10 mM Tris–HCI (pH 8.0), and 0.2% Triton X-100) and PBS in sequence, the beads were eluted using 5×SDS loading buffer, and subjected to Western blotting assays using the anti-HA antibodies and corresponding secondary antibodies.

Statistical analysis

Data represent the results of experiments repeated at least three times, and all quantitative data are expressed as the mean ± SD. Statistical analysis was performed using SPSS (v.22.0; IBM Corp, http://www.spss.com.cn). Student's *t*-tests were used to compare samples with normality, homogeneity of variance, and independence. Nonparametric tests were used to analyze measurement or count data that did not meet these requirements. $p < 0.05$ was considered statistically significant.

Data availability

The data that support the findings of this study are available from the corresponding author, HL. S., upon request.

Supporting information—The article contains supporting information.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: CCK-8, cell counting kit-8; CHX, cycloheximide; HCC, human hepatocellular carcinoma; HECT, homologous to E6-APC terminus; NEDD4, neural precursor cell expressed developmentally downregulated 4; PCDH17, protocadherin 17.

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