The HECT Type Ubiquitin Ligase NEDL2 Is Degraded by Anaphase-promoting Complex/Cyclosome (APC/C)-Cdh1, and Its Tight Regulation Maintains the Metaphase to Anaphase Transition

Li Lu\(^1\), Shaohua Hu\(^1\), Rongfei Wei\(^1\), Xiao Qiu\(^1\), Kefeng Lu\(^1\), Yesheng Fu\(^1\), Hongchang Li\(^1\), Guichun Xing\(^1\), Dong Li\(^1\), Ruiyun Peng\(^1\), Fuchu He\(^1\)**, and Lingqiang Zhang\(^1\)**\(^1\)

From the \(^1\)State Key Laboratory of Proteomics, Beijing Proteome Research Center, Beijing Institute of Radiation Medicine, Beijing 100850, China, the \(^2\)Institute of Cancer Stem Cells, Dalian Medical University, Dalian, Liaoning Province 116023, China, the \(^3\)College of Life Science and Bio-Engineering, Beijing University of Technology, Beijing 100124, China, the \(^4\)Department of Experimental Pathology, Beijing Institute of Radiation Medicine, Beijing 100850, China, and the \(^5\)Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, China

**Background:** NEDL2 is a member of the HECT type ubiquitin ligase NEDD4 family, but its function remains largely unknown.

**Results:** NEDL2 is degraded by APC/C-Cdh1 during mitotic exit and regulates metaphase to anaphase transition.

**Conclusion:** NEDL2 appears to dynamically modulate regulation of mitosis.

**Significance:** Our data provide a novel substrate of APC/C-Cdh1 and reveal an additional protein by which HECT type ubiquitin ligase can regulate mitosis.

NEDD4-like ubiquitin ligase 2 (NEDL2) is a HECT type ubiquitin ligase. NEDL2 enhances p73 transcriptional activity and degrades ATR kinase in lamin misexpressed cells. Compared with the important functions of other HECT type ubiquitin ligase, there is less study concerning the function and regulation of NEDL2. Using primary antibody immunoprecipitation and mass spectrometry, we identify a list of potential proteins that of NEDL2. Using primary antibody immunoprecipitation and ligase, there is less study concerning the function and regulation with the important functions of other HECT type ubiquitin ligase. NEDL2 enhances p73 transcriptional activity and degrades ATR kinase in lamin misexpressed cells. Compared with the important functions of other HECT type ubiquitin ligase, there is less study concerning the function and regulation of NEDL2. Using primary antibody immunoprecipitation and mass spectrometry, we identify a list of potential proteins that

Ubiquitin-mediated proteasomal degradation represents the most critical pathway to control the stability and quality of cellular proteins in eukaryotes. Ubiquitin ligases are responsible for substrate recognition and are divided into two major classes: the RING (really interesting new gene) finger type and HECT (homologous to E6AP carboxyl terminus) domain type ligases. The anaphase-promoting complex/cyclosome (APC/C), a RING finger type ubiquitin ligase, is a key ubiquitin ligase that controls several transitions in the cell cycle (2, 3). The activity of the APC/C is tightly regulated in the cell cycle. One of the main regulatory mechanisms for the APC/C is through its association with accessory-activating factors, cell division cycle protein 20 (Cdc20/fizzy) and fizzy/cell division cycle 20-related 1 (Cdh1/Fzr1) (4, 5). Cdc20 associates with the APC/C from prometaphase to anaphase, whereas Cdh1 recognizes mitotic cyclins and additional substrates containing either a destruction box (D-box), composed of the sequence RXXL, or the KEN box for degradation in mitotic exit and G1 phase (6–8).

Among HECT type ligases, there are nine members in the Nedd4 (neural precursor cell-expressed developmentally down-regulated gene 4) family, all of which share a similar structure, including a C2 domain at the N terminus, 2–4 WW domains in the middle of the protein, and a HECT domain at the C terminus (9). Despite being similar in structure, each of the nine NEDD4 family members exhibits unique functions.

\(^{*\text{This work was supported by National Basic Research Programs 2012CB910304, 2011CB910602, and 2011CB910802 and National Natural Science Foundation Projects 31125010 and 81221004.}}\)

\(^{**\text{This article contains supplemental Tables S1 and S2 and Movies S1 and S2.}}\)

\(^{1\text{To whom correspondence may be addressed: Beijing Institute of Radiation Medicine, 27 Taiping Rd., Beijing 100850, China. Tel./Fax: 86-10-80705115; E-mail: hefc@nic.bmi.ac.cn.}}\)

\(^{2\text{To whom correspondence may be addressed: Beijing Institute of Radiation Medicine, 27 Taiping Rd., Beijing 100850, China. Tel./Fax: 8610-68177417; E-mail: zhanglq@nic.bmi.ac.cn.}}\)

\(^{3\text{The abbreviations used are: APC/C, anaphase-promoting complex/cyclosome; ANAPC, anaphase-promoting complex subunit; IGF, insulin-like growth factor; TRITC, tetramethylrhodamine isothiocyanate; IP, immunoprecipitation; SAC, spindle assembly checkpoint.}}\)
**NEDL2 Regulates Mitotic Progression**

Except for NEDD4-like ubiquitin ligase 1 (NEDL1) and NEDD4-like ubiquitin ligase 2 (NEDL2), also known as HECW1 and HECW2, the other seven members can regulate ubiquitin-mediated intracellular trafficking, proteosomal degradation, and nuclear translocation of multiple proteins. They have been found to be involved in many important signaling pathways, such as TGFβ-, EGF-, IGF-, VEGF-, and TNFα-mediated pathways (10). NEDL1 shares large homology with NEDL2. NEDL1 mRNA preferentially expresses in neuronal tissue and highly expresses in neuroblastomas with favorable prognosis (11). NEDL1 is proposed to ubiquitylate mutant SOD1 (superoxide dismutase 1) but not wild-type SOD1, which is found in patients with familial amyotrophic lateral sclerosis. It is thus suggested that mutant SOD1, Dvl1 (dishevelled-1), and TRAP-δ (translocon-associated protein-δ) accumulate as a ubiquitylated complex in neurons, potentially leading to neuronal death (11). Studies in cell lines suggest that NEDL1 stimulates p53-mediated apoptosis (12). Motor neuron degeneration and muscular atrophy occur in the NEDL1 transgenic mouse (13). NEDL2 regulates the stability of p73 (14) and is involved in the degradation of ATR kinase in lamin misexpressed cells (15). Compared with NEDL1 and the other seven members, little is known about NEDL2, and it will be interesting to explore it.

In this study, we first revealed that NEDL2 was localized specifically on mitotic spindles and that its protein level reached a maximum in mitosis and was degraded by APC/C-Cdh1 in a D-box dependent manner during mitotic exit. Moreover, NEDL2 depletion caused a marked delay of the metaphase-anaphase transition, and the overexpression of wild type or mutant NEDL2 containing the C2 domain caused earlier activation of APC/C, leading to chromosome lagging, which may induce tumorigenesis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—HEK293T cells, HeLa cells, and HeLa/GFP-H2B cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Cells were transfected with Lipofectamine 2000 following the manufacturer’s protocol (Invitrogen).

**Antibody and Reagents**—The proteasome inhibitors MG132, nocodazole, taxol, thymidine, and polyclonal antibody NEDL2 were purchased from Sigma-Aldrich. Anti-GAPDH, anti-Cdh1, anti-APC2, and secondary antibodies were purchased from Santa Cruz Biotechnology. Antibody against Cdc27 was purchased from BD Biosciences. Antibodies against α-tubulin, APC11, Cdc16, or Cdc23 were purchased from Abcam. Anti-HA was from Roche Applied Science. Anti-Myc and anti-FLAG antibodies were from MBL.

**Immunoprecipitation and Immunoblotting**—For general cell lysis, transfected cells were harvested and lysed in HEPES lysis buffer (20 mM HEPES, pH 7.2, 50 mM NaCl, 0.5% Triton X-100, 1 mM NaF, 1 mM dithiothreitol) and boiled with 2× SDS-PAGE loading buffer. For immunoprecipitation, cell lysates were prepared in 500 ml of HEPES buffer supplemented with protease inhibitor mixture (Roche Applied Science). Immunoprecipitation was performed using mouse anti-FLAG (2.5 mg) for 4 h at 4 °C followed by incubation with protein A/G-agarose beads (Santa Cruz Biotechnology) overnight at 4 °C. Beads were then washed three times in HEPES lysis buffer and examined by immunoblotting with the indicated primary antibodies and appropriate secondary antibody, followed by detection with the Super Signal chemiluminescence kit (Pierce).

**SDS-PAGE and Mass Spectrometry**—Immunoprecipitates were resolved on 12% Novex Tris/glycine gels. Gels were minimally stained with Coomassie Brilliant Blue to differentiate IgG bands. Each lane was then cut into three molecular weight regions. These bands were digested with 100 ng of trypsin overnight. LTQ Orbitrap Velos (Thermo Fisher Scientific) was run in a data-dependent mode, where each sample was eluted in a 1-h 5–30% acetonitrile gradient. Spectral data were then searched against the human protein RefSeq database in Proteome Discoverer1.3 Suites with Mascot software.

**RNA Interference**—The NEDL2 siRNA-1 (5′-GUGGGAUCUCCAGUUAAATT-3′), siRNA-2 (5′-CAGGGAGAUGUUAUTT-3′), siRNA-3 (5′-GGAGGCCUGAUCAUGUAAATT-3′), Cdh1 siRNA-1 (5′-GAGGGGTCTGTTCACGTATTCCCTT-3′), siRNA-2 (5′-GCAACGATGTGTCCTCCCTATT-3′), and non-targeting siRNAs (5′-UUCUCGGAACGUGUCAGCU-3′) were synthesized by Shanghai GenePharm. All siRNAs were transfected into the cells according to the manufacturer’s protocol.

**Immunofluorescence**—For subcellular localization analyses, cells were fixed with 4% paraformaldehyde and permeabilized in 0.2% Triton X-100 (PBS). Proteins were stained using the indicated antibodies and detected with a TRITC-conjugated or FITC-conjugated secondary antibody. The nuclei were stained with DAPI (Sigma), and images were visualized with a Zeiss LSM 510 Meta inverted confocal microscope.

**In Vitro Degradation and Ubiquitination Assays**—For in vitro degradation assays, extracts from HeLa cells were prepared. Then, extract was supplemented with degradation mixture (1.5 mg/ml ubiquitin, 7.5 mM creatine phosphate, 1 mM ATP, 1 mM MgCl2, 0.1 mg/ml cycloheximide) and 35S-labeled substrate at 30 °C. Aliquots were removed at the indicated times and resolved by SDS-PAGE and autoradiography. For in vitro ubiquitination, G1 extracts from HeLa cells were immunoprecipitated with anti-Cdc27 antibody-protein A beads for 2 h at 4 °C to purify APC/C. Ubiquitination reactions were initiated by mixing purified APC/C beads with 35S-labeled in vitro translated substrate, E1 (50 μg/ml), E2 (50 μg/ml), ubiquitin (1.25 mg/ml), and an energy regeneration mix. Samples from each time point were then analyzed by SDS-PAGE and autoradiography.

**Cell Synchronization and Time Lapse Imaging**—For double-thymidine arrest, cells were incubated in thymidine-containing (2 mM) medium for 18 h, released into fresh medium for 8 h, and incubated in thymidine-containing (2 mM) medium for 24 h. For thymidine-nocodazole arrest, cells were incubated in thymidine-containing (2 mM) medium for 18 h, released into fresh medium for 3 h, and treated with 100 ng/ml nocodazole for 11 h. G1/S border cells were obtained by releasing cells synchronized by double-thymidine block into fresh medium for 0 h, whereas S phase and G2 phase were collected at 4 and 8 h. For mitotic cells, cells were synchronized by thymidine-nocodazole arrest and shaken off. For G1 cells, nocodazole-arrested cells...
were released into fresh medium for 4 h. Cell cycle distributions were confirmed by flow cytometry.

For time-lapse imaging, HeLa/GFP-H2B stable cell lines were seeded in an eight-chambered cover glass (Lab-Tek Chambered 1.0 Borosilicate Cover Glass System, Nunc). Images were collected every 5 min using a 0.1-s exposure for 12 h using a 40 (or 20) lens objective on an inverted fluorescence microscope (Nikon Eclipse Ti-E) with an Ultra View spinning disc confocal scanner unit (PerkinElmer Life Sciences). The temperature of the imaging medium was kept at...
NEDL2 Regulates Mitotic Progression

A

Flag-NEDL2-WT  +  +  +  -  -
Flag-NEDL2-CA  -  -  -  +  +

HA-Cdh1  -  -  -  -  -
IB: Flag
IB: HA
IB: GFP
IB: GAPDH

B

Flag-NEDL2-WT  -  -  -  -  -
Flag-NEDL2-CA  -  -  -  +  +

HA-Cdh1  +  +  +  +  +
IB: Flag
IB: HA
IB: GFP
IB: GAPDH

C

Cdh1 siRNA  -  no.1  no.2

NEDL2
Cdh1
GAPDH

D

E

NEDL2 siRNA  -  no.1  no.2  no.3

Cdh1
NEDL2
GAPDH

F

NEDL2
Cdh1

G

H

Myc-Cdh1  -  WT  ΔC-box  ΔFuzzy
Flag-NEDL2  +  +  +  +

IB: Flag
IB: Myc
IB: GFP
IB: GAPDH

I

Myc-Cdh1  -  WT  ΔC-box  ΔFuzzy
Flag-NEDL2  +  +  +  +

IB: Cyclin B
IB: Myc
IB: GFP
IB: GAPDH

J

Flag-NEDL2  WT  1-324  324-1021  807-1572
HA-Cdh1  -  +  +  +  +

IB: Flag
IB: HA
IB: GFP
IB: GAPDH

K

HA-Cdh1  -  -  -  -  -
Flag-NEDL2 (2324-1021)  +  +  +  +  +

IB: Flag
IB: HA
IB: GFP
IB: GAPDH

L

Flag-NEDL2 (324-1021)  WT  ΔRGSL
HA-Cdh1  -  +  +  +

IB: Flag
IB: HA
IB: GFP
IB: GAPDH

M

Flag-NEDL2  WT  ΔRGSL
HA-Cdh1  -  +  +  +

IB: Flag
IB: HA
IB: GFP
IB: GAPDH

N
NEDL2 Regulates Mitotic Progression

Statistical Analysis—Statistical comparisons between only two groups were carried out by Student’s t test or the Mann-Whitney rank sum test when a normal distribution could not be assumed. One-way analysis of variance followed by Dennett’s post hoc test was used for multiple-group comparisons. Statistical calculations were carried out using SPSS version 19.0. We tested data for normality and variance and considered a p value of less than 0.05 significant.

RESULTS

An IP/MS Approach to Efficiently Identify Interactors of NEDL2—In a detailed study of the NEDL2 protein complex network, the IP protocol for protein complex isolation was performed. To maximize the number of protein identifications, we solved the immunocomplexes on SDS-PAGE and split each gel lane into three regions for subsequent sequencing in separate mass spectrometry runs. Interacting proteins of NEDL2 in HEK293T cells identified by mass spectrometry in two experiments are shown in supplemental Tables S1 and S2. High-confidence interactions identified in one IP/MS experiment were chosen to compare with another experiment. We concluded that potential proteins that could interact with NEDL2 were highly enriched with mitotic proteins (Table 1). Except for several cell cycle-related proteins, including PRC1, NUSAP1, PLK1, CENPF, CEP170, and CLASP2, there was an interesting list of APC/C proteins. ANAPC1, ANAPC2, CDC27, ANAPC4, ANAPC5, CDC16, ANAPC7, CDC23, and Cdh1 were included in the list. The first eight proteins are all subunits of APC/C, and Cdh1 directly binds to and activates the APC/C ligase. Cdh1 maintains the activity of APC/C from late anaphase through G1 phase, targeting cyclin B, Cdc20, and PLK1 for degradation. These results led us to examine whether the NEDL2 protein was involved in mitotic regulation.

NEDL2 Associates with Cdh1 in Vivo and in Vitro—We further confirmed the interaction of Cdh1 with NEDL2 in mammalian cells. Endogenous Cdh1 was co-immunoprecipitated with endogenous NEDL2, but not control IgG, from HeLa cells (Fig. 1A). To map the Cdh1-interacting region in NEDL2, a series of NEDL2 deletion mutants were generated (Fig. 1B). A co-IP assay was performed, and the result revealed that the C2 domain and the linker between the C2 and WW domain but not the WW domain and HECT domain of NEDL2 were required for Cdh1 binding (Fig. 1C). Additionally, a GST pull-down assay showed similar results (i.e. the N-terminal part of NEDL2 was co-precipitated with Cdh1).

FIGURE 2. NEDL2 is a substrate of APC/C-Cdh1 and one D-box is involved in NEDL2 degradation. A, HEK293T cells were transfected with FLAG-NEDL2 and together with increasing amounts of HA-Cdh1. Cells were harvested 36 h later and analyzed by Western blotting. B, HEK293T cells were transfected with HA-Cdh1 and together with increasing amounts of FLAG-WT-NEDL2 and FLAG-C1540A-NEDL2. Cells were harvested 36 h later and analyzed by Western blotting. C and D, Cdh1 depletion increased the endogenous NEDL2 protein level but had no effect on NEDL2 mRNA level. HeLa cells were transfected with non-targeted control or Cdh1-specific siRNA, and the endogenous NEDL2 and Cdh1 levels were analyzed by immunoblot or real-time PCR. Data are presented as mean ± S.D. (error bars) (n = 3). E and F, Cdh1 depletion had no effect on endogenous Cdh1 protein and mRNA level. HeLa cells were transfected with non-targeted control or Cdh1-specific siRNA, and the endogenous NEDL2 and Cdh1 level were analyzed by immunoblot or real-time PCR. Data are presented as mean ± S.D. (n = 3). G, schematic representation of the Cdh1 protein functional domains. H, immunoblot analysis of whole cell lysates derived from HEK293T cells transfected with FLAG-NEDL2 together with the indicated Myc-Cdh1 constructs. I, immunoblot analysis of whole cell lysates derived from HeLa cells transfected with the indicated Myc-Cdh1 constructs. J, immunoblot analysis of whole cell lysates derived from HeLa cells transfected with plasmids expressing either wild-type NEDL2 or mutants of NEDL2 together with or without a plasmid encoding HA-tagged wild-type Cdh1. K, HEK293T cells were transfected with NEDL2 mutants together with increasing amounts of HA-Cdh1. Cells were harvested 36 h later and analyzed by Western blotting. L, alignment of destruction box sequences in human, mouse, and rat NEDL2. M, HEK293T cells were transfected with plasmids expressing either NEDL2 324–1021 or NEDL2 324–1021-ΔRGSL together with or without HA-Cdh1. N, the experiments were similar to those in M, except the NEDL2 WT and ΔRGSL mutant were the full-length constructs.
mediated the interaction with Cdh1) (Fig. 1D). These results suggest that NEDL2 interacts with Cdh1 both in vivo and in vitro.

**NEDL2 Is a Substrate of APC/C-Cdh1—**Because APC/C and NEDL2 are both ubiquitin ligases, it is essential to investigate whether NEDL2 is a substrate of APC/C-Cdh1 or a ubiquitin ligase of Cdh1. Sequence analysis showed that NEDL2 contains a total of 11 putative D-boxes of the type RXXL (Fig. 1B), which might be recognized by APC/C-Cdh1. Therefore, we first examined the degradation of NEDL2 following ectopic overexpression of Cdh1 in HEK293T cells. The overexpression of Cdh1 resulted in a significant reduction in the protein level of WT-NEDL2 and ligase-defective C1540A-NEDL2 (Fig. 2A). It indicated that Cdh1 could mediate NEDL2 destruction independent of the ubiquitin ligase activity of NEDL2. By contrast, overexpression of NEDL2 could not induce the down-regulation of Cdh1 (Fig. 2B), suggesting that Cdh1 is not the substrate of NEDL2. To verify whether endogenous Cdh1 plays a role in controlling the NEDL2 level, Cdh1 was depleted by two independent siRNAs, and the mRNA and protein levels of NEDL2 were examined. Knockdown of Cdh1 increased the protein level but not the mRNA level of NEDL2 in vivo (Fig. 2, C and D). Conversely, knockdown of endogenous NEDL2 had no significant effects on Cdh1 levels (Figs. 2, E and F). Additionally, the degradation of NEDL2 could not be promoted by ectopic expression of ΔC-box-Cdh1 (Fig. 2, G and H), which is ubiquitin ligase-deficient due to its impaired ability to interact with the APC/C core complex. The result was similar to other well-characterized Cdh1 substrates, such as cyclin B (Fig. 2I), indicating that Cdh1-mediated NEDL2 destruction depends on APC/C. These data suggest that NEDL2 is a substrate for APC/C-Cdh1.

**Degradation of NEDL2 by APC/C-Cdh1 Is Dependent on Its RGS L Sequence—**To identify which D-box(es) is responsible for the recognition and degradation by Cdh1, different NEDL2 deletion mutants were expressed in HEK293T cells with HA-Cdh1. Degradation analysis clearly showed that the N-terminal mutant 1–324 containing the first three D-boxes and the C-terminal 807–1572 containing the last seven D-boxes could not be degraded by overexpressed Cdh1 (Fig. 2J). By contrast, the middle region 324–1021 was efficiently degraded by Cdh1 (Fig. 2, J and K), suggesting that the 324–807 region contains the critical degradation signal. Within this region, only one D-box exists (i.e. the fourth D-box R746GSL749). This D-box is conserved among humans, mice, and rats (Fig. 2L). Deletion of this D-box (residues 740–743) in NEDL2 resulted in the resistance against Cdh1-mediated degradation (Fig. 2, M and N). These data strongly indicate that R746GSL749 is the bona fide degradation box of NEDL2 recognized by Cdh1.

**NEDL2 Protein Level Reaches a Maximum in Mitosis and Is Degraded by APC/C-Cdh1 during Mitotic Exit—**The APC/C-Cdh1 has a well-established role in cell cycle control. To investigate the regulation of NEDL2 in vivo, we analyzed the protein levels of NEDL2 in G1, S, G2, and M phase. NEDL2 protein level was low in G1 phase and reached a maximum in mitosis (Fig. 3A). Next, NEDL2 protein was tested across the cell cycle. HeLa cells were synchronized by a double-thymidine protocol, and the cell cycle profile of released cells was analyzed by flow cytometry (Fig. 3C). Western blot analysis showed that NEDL2 started to increase when cells entered S phase, and the expression was higher at 8–12 h after release, which was the peak of cyclin B expression during mitosis, and then dropped between the mitosis and G1 phases (Fig. 3D). In order to ensure detailed kinetics of down-regulated NEDL2, cells were synchronized in M phase and then released from a thymidine/nocodazole block, and we observed that NEDL2 protein rapidly declined during the first 4 h after release as cells exited from mitosis and entered into G1 phase (Fig. 3F). On the other hand, NEDL2 disappearance was preceded by that of cyclin B (Fig. 3, D and F), the degradation of which was initiated during the transition from metaphase to anaphase, suggesting that the regulation of NEDL2 occurred during late mitosis and early G1 phases. The absence of NEDL2 could be explained by a down-regulation of NEDL2 transcription or a specific degradation of NEDL2 in G1 phase, so we investigated the mRNA levels of NEDL2 in the same samples harvested in Fig. 3, A, D, and F. As shown in Fig. 3, B, E, and G, NEDL2 mRNA was present throughout the cell cycle, and there was no correlation between NEDL2 protein and mRNA levels in the cell cycle. These results suggest that the low NEDL2 protein level in G1 phase results from protein degradation.

In order to investigate the mechanism of NEDL2 degradation, HeLa cells released from nocodazole into fresh medium were treated with the proteasome inhibitor MG132 for 4 h. MG132 treatment efficiently blocked the degradation of NEDL2 during mitotic exit independent of its mRNA abundance (Fig. 3, H and I), which revealed that NEDL2 was degraded by the proteasome. To confirm that the APC/C-Cdh1 is required for the destruction of NEDL2 during mitotic exit, Cdh1 was depleted by RNA interference. HeLa cells were transfected with two independent Cdh1-specific siRNAs or a control siRNA. NEDL2 protein remained at a high level 4 h postrelease.
in cells transfected with Cdh1 siRNA. By contrast, NEDL2 levels decreased substantially 4 h postrelease in cells transfected with control siRNA (Fig. 3J). Likewise, the regulation was not due to the change of NEDL2 mRNA abundance (Fig. 3K).

Next we performed in vitro ubiquitination assays to directly show that APC/C-Cdh1 is the E3 ligase of NEDL2. APC/C was purified by immunoprecipitation with a Cdc27 antibody from G_1 phase extracts, which were derived from HeLa cells transfected with control siRNA or Cdh1 siRNA. Silver-stained SDS-PAGE showed a comparison of purified APC/C (Fig. 3L). Cdc27 immunoprecipitates were also assayed by immunoblotting to determine the depletion of Cdh1 and the presence of Cdc27, the APC/C catalytic core subunits APC2 and APC11, and the tetra-tricopeptide repeat domain-containing subunits Cdc16 and Cdc23 (Fig. 3L). We then carried out in vitro ubiquitination assays using the purified APC/C and Cdh1 complex with 35S-labeled NEDL2 or cyclin B. As shown in Fig. 3M, in vitro translated cyclin B was efficiently ubiquitinated by APC/C, indicating that the purified APC/C complex harbors ubiquitin ligase activity. Then we examined the NEDL2 ubiquitination by APC/C. The results showed that NEDL2 324–1021 was efficiently ubiquitinated in vitro as early as at 30 min of incubation, and the ubiquitination was detectable throughout the 90-min course. This process was dependent on the presence of Cdh1 because the depletion of Cdh1 significantly attenuated the ubiquitination of NEDL2 (Fig. 3M, right). Taken together, these results demonstrate that NEDL2 protein level is cell cycle-regulated, and it is a novel substrate of the APC/C-Cdh1 complex by which it is targeted to proteasome-dependent degradation during mitotic exit.

**NEDL2 Localizes on the Mitotic Spindles throughout Mitosis**—The expression pattern of NEDL2 suggests that it may play a role during mitosis. To address this issue, we examined the subcellular localization of NEDL2, which ensured the absence of NEDL2 in G_1 phase. NEDL2 colocalized with a microtubule marker, α-tubulin in S and G_2 phase and mainly localized at mitotic spindles during mitosis (Fig. 4A). In untreated HeLa cells, NEDL2 and α-tubulin were arranged into a circular pattern around the nucleus. After a 4-h incubation in 100 ng/ml nocodazole, an MT depolymerization drug, both α-tubulin and NEDL2 were dispersed (Fig. 4B), indicating that NEDL2 was directly associated with the mitotic spindle. NEDL2, a new spindle-associated protein, may be involved in the organization and regulation of mitotic spindles during the M phases, and it is degraded during mitotic exit. However, it has not been studied in such fields.

**Depletion of NEDL2 Causes a Marked Delay in the Onset of Anaphase**—To investigate the function of NEDL2 in mitosis, we examined the effects of siRNA-mediated NEDL2 depletion on divisions of HeLa cells. Flow cytometry demonstrated that 24% of control cells were arrested with 4N DNA content 14 h after release from the thymidine block, whereas about 40% of NEDL2-depleted cells had 4N DNA (Fig. 5A). It suggested that NEDL2 silencing inhibited mitotic progression. We next monitored mitotic progression by time-lapse imaging of HeLa cells stably expressing green fluorescent protein (GFP)-tagged histone H2B. We found that NEDL2 knockdown caused a marked delay in the onset of anaphase, compared with the control siRNA (Fig. 5B and supplemental Movies S1 and S2). The average duration in mitosis increased from 56 min in control siRNA cells (n = 137) to 96 min in NEDL2 knockdown cells (n = 137; Fig. 5C). Interestingly, the NEDL2 knockdown-induced delay of anaphase onset was due to a significant elongation of metaphase, although the duration of prometaphase was almost
unchanged (Fig. 5, D and E). Data also showed that about 22% NEDL2-depleted cells took 2 h longer for the duration of metaphase, compared with the cells transfected with control siRNA (Fig. 5F). Taken together, different from another HECT type ubiquitin ligase Smurf2, depletion of which leads to increased misalignment and missegregation of chromosomes, premature
anaphase onset (16), NEDL2 silencing inhibits metaphase-anaphase transition.

To further determine the potential role of NEDL2 in metaphase-anaphase transition, we tested whether overexpression of NEDL2 could override taxol-induced mitotic arrest. Compared with empty vector, the percentage of mitotic cells was much lower in NEDL2-expressing cells after taxol treatment, but the percentage of cells with multilobed nuclei was higher (Fig. 6A). The result indicated that overexpression of NEDL2 may lead to earlier APC/C activation. We then found that the percentage of cells with lagging chromosomes, an important

**FIGURE 6. Overexpression of NEDL2 leads to chromosome missegregation.** A, HeLa/GFP-H2B cells overexpressed with NEDL2 or its mutants were treated with taxol. After 24 h, the mitotic index and multilobed nucleus index were determined. Data are representative of three independent experiments. Error bars, S.E. Representative images of mitotic cell and polykaryocytes are shown (right). Scale bar, 5 μm. B, analysis of chromosome segregation defects in HeLa cells expressing NEDL2 or its mutants. Data are representative of three independent experiments. Error bars, S.E. Representative images of normal and lagging chromosomes in HeLa cells are shown (right). Scale bar, 5 μm. C, in vitro degradation assays. Control or NEDL2-overexpressed extracts from nocodazole-arrested HeLa cells were supplemented with an energy-regenerating system, and reactions were initiated by the addition of 35S-labeled cyclin B or securin proteins. Samples were then taken at the indicated times and analyzed by SDS-PAGE and autoradiography. D, summary of the possibility of chromosome lagging and the stability of the NEDL2 mutants. The stability of the mutants was determined as described above.
NEDL2 Regulates Mitotic Progression

A. Expression of NEDL2 in various tissues.

B. NEDL2 scores in different tissues.

C. Comparison of NEDL2 scores in colon cancer vs. adjacent tissue.

D. Relative NEDL2 mRNA level in colon and cervical cancer.

E. Comparison of other proteins (Cdh1) in colon and cervical cancer.
NEDL2 regulates mitotic progression

index of chromosomal instability that might result from premature APC/C activation, increased in NEDL2-expressing cells (Fig. 6B). Next, we used the cell-free system to test the effects of NEDL2 on APC/C activity. The degradation of cyclin B and securin was markedly advanced in NEDL2 overexpression extracts (Fig. 6C), which directly verified that NEDL2 was required for the APC/C activation.

We also investigated whether the APC/C-Cdh1-regulated NEDL2 stability was implicated in mitotic progression. NEDL2-DRGSL and N-terminal 1–324 mutant (containing the C2 domain) behaved similarly to wild-type NEDL2. By contrast, 324–1021 and 807–1572 mutants could not override taxol-induced mitotic arrest (Fig. 6A). A similar conclusion was made in the chromosomal lagging assays (Fig. 6B). Because NEDL2-DRGSL, 1–324 and 807–1572 mutants were not degraded by Cdh1 (Fig. 2, I, M, and N), the recognition by Cdh1 seems not to be tightly related to the ability of NEDL2 to regulate cell cycle progression (Fig. 6D).

NEDL2 protein level is elevated in several types of human tumors—The majority of cancers involve chromosomal instability (17). Deregulation of cyclins and other cell cycle regulators can cause genomic instability, and aberrant expression of cell cycle regulators can be detected in various malignant tumors (18). Because overexpression of NEDL2 enhances chromosomal instability, it may induce the human tumors. We first screened the expression spectrum of NEDL2 in various tissues by immunohistochemistry staining. Consistent with the previous report that NEDL2 mRNA expresses predominantly in the adult brain, heart, and lung (14), NEDL2 protein was highly expressed in cerebral cortex, testis, heart, kidney, and adrenal but was weakly detected in colon, pancreas, skin, uterus, and rectum (Fig. 7, A and B).

Next, we screened several types of tumors, of which their normal tissues expressed little NEDL2, including colon cancer, rectal cancer, cervix cancer, pancreas cancer, lung cancer, and liver cancer. The results showed that expression of NEDL2 protein was significantly up-regulated in colon and cervix tumor tissues, compared with matched adjacent normal tissues (Fig. 7, C and E). Moreover, mRNA levels of NEDL2 in colon and cervix tumor tissues were significantly higher than those in matched adjacent normal tissues (Fig. 7D). A higher frequency of positive NEDL2 expression was observed in colon cancer tissue, whereas a lower frequency of Cdh1 expression was measured in the cancer area (Fig. 7E). Thus, NEDL2 and Cdh1 were inversely correlated in colon cancer. However, Cdh1 was expressed at comparable levels between the cervical cancer and the adjacent tissues. Similar expression profilings of Cdh1 have been reported in a previous study (19). Taken together, we propose that both the increase of mRNA synthesis and the decrease of protein degradation by Cdh1 might contribute to the elevated expression of NEDL2 in cancer tissues, at least in the colon cancer. This study is the first evidence to establish the relationship between the NEDL2 protein and tumors.

Discussion

Protein-protein interactions constitute the molecular backbone of cell biology, where select proteins assemble into metastable complexes to form bioactive units (20, 21). IP/MS has recently emerged as a preferred method in the analysis of protein complex components and cellular protein networks (22). In the case of NEDL2, a HECT type ubiquitin ligase stabilizing p73 and degrading ATR kinase, we performed an unbiased study of its protein complex networks. We analyzed the functional specificity of candidate proteins after using IP/MS, and fortunately this strategy has proved to be extremely successful. A list of mitotic proteins were identified by IP/MS, and the following evidence indicated that NEDL2 was indeed a regulator of mitosis and was degraded by APC/C-Cdh1, which was identified in the approach described above. On the other hand, NEDL2 was demonstrated to localize on mitotic spindles in the study. Microtubule-associated proteins and kinesins could also be found in the candidate proteins. Certainly, many other high confidence interactions are worthy of further study. Indeed, this is a generally applicable approach to identify protein complex networks important for many biological processes.

The APC/C E3 ubiquitin ligase consists of at least 13 proteins. The RING finger protein, APC11, and the Cull-related scaffold protein, APC2, form the catalytic core of the complex (23). APC substrate specificity is conferred by recruitment of a co-activator protein, Cdh1 or Cdc20. Both Cdh1 and Cdc20 target specific proteins for ubiquitination through the recognition of substrates containing a destruction box, or D-box, whereas Cdh1 recruits additional substrates containing other targeting motifs, including the KEN box, A-box, and CRY box (24, 25). Although NEDL2 contains 11 D-boxes, Cdh1 can specifically recognize only one of its D-boxes, which is located at amino acids 740–743 (RGSL) for degradation.

Cdh1 can also function in an APC complex-free mode. Recent exciting evidence has uncovered unexpected neurobiological, myogenesis, or other functions for Cdh1 (26–28). Another member of the Nedd4 family, Smad ubiquitination regulatory factor 1 (Smurf1), can be degraded by Cdh1 in its regulation of osteoblast function and axon growth in an APC/C-independent or -dependent manner, respectively (29, 30). NEDL2, whose mRNA was preferentially expressed in neuronal tissue, as previously reported (14), may function along with Cdh1 in regulation of neurobiological function or other physiological processes besides in cell cycle control. Cdh1 can ubiquitinate NEDL2 and degrade it in mitotic exit. Cdh1 deregulation may result in the increased expression of NEDL2, which may induce the colon tumor. But their physiological functions

FIGURE 7. The expression of NEDL2 is elevated in human tumor tissues. A, images of immunohistochemical staining for NEDL2 in 19 types of tissues. Scale bar, 50 μm. B, box plot of NEDL2 expression in 19 kinds of tissues. C, the tissue array of colon and cervix cancer was carried out by immunohistochemistry with anti-NEDL2 antibody. D, quantitative RT-PCR analysis of NEDL2 mRNA level in colon and cervix cancer tissues and matched adjacent normal control from 15 subjects. Data were analyzed using Student’s t test. Data are shown as means ± S.D. (error bars) of three independent experiments. E, NEDL2 and Cdh1 protein levels in colon and cervix cancer samples (left). Representative images of immunohistochemical staining for NEDL2 and Cdh1 are shown (right). Scale bar, 50 μm (×200) and 25 μm (×400).
are still needed to be fully understood in other cellular processes.

During metaphase-anaphase transition, APC/C mediates the ubiquitination of securin, mitotic cyclins, and other substrates for anaphase onset (31). The spindle assembly checkpoint (SAC) is a surveillance mechanism that ensures accurate chromosome segregation (32, 33). SAC is activated by improperly attached kinetochores and then inhibits the ability of APC/C to target the substrates for degradation to prevent anaphase onset until each kinetochore is stably bioriented on the spindle (34). Once the microtubule kinetochore attachment and tension are fully established, the SAC signals are turned off, and APC/C-Cdc20 is activated. Although it has been well established that SAC must be inactivated before anaphase onset, the molecular mechanisms of SAC inactivation remain obscure. NEDL2 is a novel regulator of anaphase onset. Inhibition of NEDL2 delays the metaphase to anaphase transition, whereas overexpression of NEDL2 leads to earlier release of APC/C and chromosome missegregation. Chromosome missegregation occurs when the SAC is defective and APC/C is prematurely activated. We speculated that NEDL2 might regulate SAC inactivation or the activation of APC/C.

It has been reported that dysregulation of SAC causes chromosome missegregation, chromosome instability, and aneuploidy (35, 36). It is clear that aneuploidy can promote tumorigenesis in certain contexts (37, 38). Ectopic expression of NEDL2 protein and mRNA was found in colon and cervix tumor tissues. It indicates that dysregulation of NEDL2 disturbs the metaphase to anaphase transition, which may lead to tumorigenesis. Consistent with NEDL2, the oncogenic potential of the Nedd4-like E3s is highlighted by the identification of a number of tumor suppressor molecules among their substrates. The assertion that Nedd4-like ubiquitin ligases play a role in cancer is supported by the overexpression of Smurf2 in esophageal squamous cell carcinoma, WWP1 in prostate and breast cancer, Nedd4 in prostate and bladder cancer, and Smurf1 in pancreatic cancer (10).

In conclusion, we found NEDL2 as a novel substrate of APC/C-Cdh1 during mitotic exit, and depletion of Cdh1 leads to significantly elevated levels of NEDL2, which may cause the colon cancer. It will be interesting to further investigate the physiological significance of the degradation of NEDL2 in cell cycle regulation or other physiological processes. This is the first time that the role of NEDL2 in the regulation of metaphase to anaphase transition has been shown, and it is worth investigating whether NEDL2 promotes the metaphase to anaphase transition by regulating SAC inactivation or the activation of APC/C. In addition, we uncovered the abnormal overexpression of NEDL2 in human tumors, and it will be important to study the relationship between the imbalance of NEDL2 in mitotic progression and tumorigenesis.

Acknowledgments—We thank Drs. Xue-Min Zhang, Hui-Yan Li, Teng Li, and Yan Chang for important suggestions and for providing materials.
NEDL2 Regulates Mitotic Progression

Kim, A. H., and Bonni, A. (2007) Thinking within the D box. Initial identification of Cdh1-APC substrates in the nervous system. Mol. Cell Neurosci. 34, 281–287

Reis, A., Levasseur, M., Chang, H. Y., Elliott, D. J., and Jones, K. T. (2006) The CRY box. A second APC-cdh1-dependent degron in mammalian cdc20. EMBO Rep. 7, 1040–1045

Littlepage, L. E., and Ruderman, J. V. (2002) Identification of a new APC/C recognition domain, the A box, which is required for the Cdh1-dependent destruction of the kinase Aurora-A during mitotic exit. Genes Dev. 16, 2274–2285

Gieffers, C., Peters, B. H., Kramer, E. R., Dotti, C. G., and Peters, J. M. (1999) Expression of the CDH1-associated form of the anaphase-promoting complex in postmitotic neurons. Proc. Natl. Acad. Sci. U.S.A. 96, 11317–11322

Konishi, Y., Stegmueller, J., Matsuda, T., Bonni, S., and Bonni, A. (2004) Cdh1-APC controls axonal growth and patterning in the mammalian brain. Science 303, 1026–1030

Li, W., Wu, G., and Wan, Y. (2007) The dual effects of Cdh1/APC in myogenesis. FASEB J. 21, 3606–3617

Kannan, M., Lee, S. J., Schwedhelm-Domeyer, N., and Stegmueller, J. (2012) The E3 ligase Cdh1-anaphase promoting complex operates upstream of the E3 ligase Smurfl in the control of axon growth. Development 139, 3600–3612

Kannan, M., Lee, S. J., Schwedhelm-Domeyer, N., Nakazawa, T., and Stegmueller, J. (2012) p250GAP is a novel player in the Cdh1-APC/Smurfl pathway of axon growth regulation. PLoS One 7, e50735

Yu, H. (2007) Cdc20. A WD40 activator for a cell cycle degradation machine. Mol. Cell 27, 3–16

Bharadwaj, R., and Yu, H. (2004) The spindle checkpoint, aneuploidy, and cancer. Oncogene 23, 1026–1027

Musacchio, A., and Salmon, E. D. (2007) The spindle-assembly checkpoint in space and time. Nat. Rev. Mol. Cell Biol. 8, 379–393

Pesin, J. A., and Orr-Weaver, T. L. (2008) Regulation of APC/C activators in mitosis and meiosis. Annu. Rev. Cell Dev. Biol. 24, 475–499

Michor, F. (2005) Chromosomal instability and human cancer. Philos. Trans. R. Soc. Lond. B Biol. Sci. 360, 631–635

Lengauer, C., and Wang, Z. (2004) From spindle checkpoint to cancer. Nat. Genet. 36, 1144–1145

Schwartzman, J. M., Sotillo, R., and Benezra, R. (2010) Mitotic chromosomal instability and cancer. Mouse modelling of the human disease. Nat. Rev. Cancer 10, 102–115

Weaver, B. A., and Cleveland, D. W. (2008) The aneuploidy paradox in cell growth and tumorigenesis. Cancer Cell 14, 431–433