Cell Cycle-dependent Expression of Centrosomal Ninein-like Protein in Human Cells Is Regulated by the Anaphase-promoting Complex*

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The recently identified centrosome protein Nlp (ninein-like protein) is a key regulator in centrosome maturation, which contributes to chromosome segregation and cytokinesis. However, the mechanism(s) controlling Nlp expression remains largely unknown. Here we have shown that Nlp expression is cell cycle-dependent with a peak at G2/M transition in human cells. Nlp is a short-lived protein and degraded by the proteasome via the anaphase-promoting cyclosome complex (APC/c) pathway. It interacts with the APC/c through the APC2 or Cdc27 subunits and is ubiquitinated. Following treatment with proteasome inhibitors, its protein level is elevated. Nlp binds in vivo to the degradation-targeting proteins Cdh1 and Cdc20, and overexpression of Cdh1 and Cdc20 enhances Nlp degradation. Using point mutations of the two putative degradation signals in Nlp, we have found that its degradation requires intact KEN-box and D-box. Interestingly, the Lys-Glu-Asn-D-box-mutated Nlp exhibits a much stronger capability of inducing anchorage-independent growth and multinuclearity compared with the wild type Nlp. Taken together, these findings indicate that Nlp expression is cell cycle-dependent and regulated by APC-mediated protein degradation.

Tumor cells always have unstable numbers of chromosomes. Several mechanisms have been involved in such chromosomal abnormality. One well characterized mechanism is improper centrosome duplication (1). In a normal cell, centrosomes start duplicating at early S phase, and by M phase, the cell has two mature centrosomes that form the bipolar spindle, an essential structure for proper separation of duplicated chromosomes between daughter cells. Because the centrosome is the organelle that organizes the spindle for separation of chromosomes during mitosis, centrosome stability is required for successful mitosis in mammalian cells. The presence of more than two centrosomes in a cell can result in lost or fragmented chromosomes after cell division and generation of aneuploidy, which are closely associated with cell transformation and tumorigenesis (2–4).

The regulation of centrosomes has been linked to several tumor suppressors and their targeted genes. Tumor suppressor p53 is thought to be required for the control of centrosome duplication, and mutations of p53 result in centrosome amplification (5). In addition, there is growing evidence that BRCA1 functions as a regulator of centrosome stability and function. BRCA1-conditional knock-outs exhibit increased centrosome copies and abnormal mitotic progression (6). The p53-regulated p21 plays vital roles in regulating centrosome replication in S phase through its inhibition of Cdk2/cyclin E kinase (7). In contrast, p53-regulated Gadd45 appears to regulate centrosome stability via its inhibition with Cdc2/cyclin B1 and Aurora-A (4). Also, several mitotic kinases have been involved in the control of centrosome maturation, including Plk1, Aurora A, and Nek2 kinases (8, 9). Plk1 contains a highly conserved polo box domain in the noncatalytic C-terminal half of the protein. This domain is required for Plk1 centrosome localization. It becomes localized to the centrosome in the late G2 and has peak activity at the G2/M transition. Plk1 is involved in the regulation of various cell cycle checkpoints that ensure the timing and order of cell cycle events, such as DNA repair, bipolar spindle formation, chromosome segregation, and mitotic exit (10). Aurora-A kinase is also implicated in centrosome separation, spindle assembly, and spindle maintenance. Deregulated expression of Aurora-A kinase results in centrosome hypertrophy and leads to cell transformation (10). Both of the two kinases have emerged as the key regulators of centrosome maturation in a variety of different organisms. Nek2A is a centrosomal kinase that participates in spindle-pole separation at the G2/M transition (10). Despite these findings, the machinery that controls centrosome stability and function still remains to be further defined.

Ninein-like protein (Nlp), a newly identified centrosome protein that is phosphorylated by Plk1, locates at chromosome 20. Nlp plays important roles in mitotic progression, including centrosome maturation and formation, bipolar spindles, and

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2 The abbreviations used are: Nlp, ninein-like protein; APC, anaphase-promoting complex; APC/c, anaphase-promoting cyclosome complex; PBS, phosphate-buffered saline; GFP, green fluorescent protein; EGFP, enhanced GFP; GST, glutathione S-transferase; TRITC, tetramethylrhodamine isothiocyanate; siRNA, small interfering RNA; Wt, wild type; KEN, Lys-Glu-Asn.
Regulation of Nlp Expression by APC

chromosome segregation (11–13). Plk1 can phosphorylate Nlp, and the phosphorylation disrupts the ability of Nlp to be associated with γ-tubulin as well as with the centrosome. As Nlp is an important component of the centrosome and essential for stimulating microtubule nucleation, it is proposed that, at the onset of mitosis, Plk1 phosphorylates Nlp and displaces it from the centrosome, thus establishing a condition for assembly of the mitotic spindle (11). Overexpression of Nlp has been shown to cause aberrant spindle formation, suggesting that the precise control of Nlp expression is required for proper centrosome maturation and spindle assembly. Interestingly, our most recent observations indicate that Nlp is deregulated in several types of human cancers and is able to promote cell transformation and tumor formation in nude mice.3 These findings suggest that the machinery controlling Nlp protein is important for maintaining cell cycle progression and genomic stability in mammalian cells, and disruption of such machinery would lead to tumorigenesis. However, the mechanism(s) that regulates Nlp protein expression is currently unclear.

The mechanism that controls mitosis is complex. The biological events that occur during late mitosis, from sister chromosome separation to exit from mitosis, are primarily governed by the ubiquitin-dependent degradation of key regulatory proteins. This ubiquitylation is mediated by the ubiquitin ligase, anaphase-promoting complex (APC) (14). Activation of the APC requires its association with tryptophan-aspartate-containing proteins. Two of these activators have been identified as Cdc20 and Cdh1 (15, 16). Although destruction of APC targets at the metaphase-anaphase transition is regulated by Cdc20, APC-dependent degradation during late mitosis and early G1 is controlled by Cdh1 (17, 18). The switch from Cdc20 to Cdh1 is induced by the inactivation of Cdk1, which in turn leads to destruction of Cdc20 itself by the Cdh1 (19, 20). The Cdc20 and Cdh1 forms of the APC present different substrate specificities. Cdc20 recognizes D-Box-containing proteins (21); meanwhile, Cdh1 recognizes proteins containing either D-Box or KEN-box sequences (17). The destruction box sequences appear to be quite variable except for the RXXLXXXN. Mutations in the destruction box and KEN-box stabilize substrates and severely reduce or abolish their ubiquitination.

In this report, we have shown that Nlp is expressed in a cell cycle-dependent manner and degraded by the ubiquitin-proteasome pathway via the APC/c. Nlp is physically associated with the APC/c through the APC2 or Cdc27 subunits and capable of binding in vivo to Cdh1 or Cdc20. A reduction of the Cdh1 and Cdc20 protein levels by RNA interference leads to stabilization of Nlp. We have also characterized the KEN-box and D-box sequences in Nlp as the important determinants of NLP degradation. Mutations of these boxes greatly enhance Nlp protein stability and its capability of inducing anchorage-independent growth and multinuclearity.

EXPERIMENTAL PROCEDURES

Cell Cultures and Synchronization Procedures—KYSE150 cells and HeLa cells were grown in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin, maintained at 37 °C in a humidified atmosphere of 5% carbon dioxide. 293T cells were grown in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum. To synchronize cells at G0/M phase, cells were treated with 400 ng/ml nocodazole (Sigma) for 16 h. To release the cells from drug-induced G0/M phase arrest, the cells were washed three times with phosphate-buffered saline (PBS) and switched into fresh medium for further incubation and collected every 2 h. For G0 arrest, exponentially growing cells were kept in 10% serum-containing medium for 24 h after they reached confluence and then were serum-starved (0.5% fetal calf serum) for 48 h. Thereafter, the cells were released into serum-containing medium and collected every 2 h. For cells arrested at the G1/S boundary, the cells were incubated with aphidicolin (6 µM, Sigma) for 14 h and then switched into fresh medium and collected every 2 h.

Reverse Transcription-PCR—Total RNA was isolated from diversely treated KYSE-150 cells with TRIZol reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA (6 µg) was reverse-transcribed as described in the instructions (Invitrogen). One microliter of cDNA solution, which is fifteen microliters in all, was then used as the template for PCR amplification. Sequences of the reverse transcription-PCR primers for Nlp were: forward, 5′-TGGAGCAGCTACGGAGA′-3′; reverse, 5′-ATCGGTGGCTCTGCA′-3′. The product is 399 bp in length. GAPDH were: forward, 5′-GCTGAGAGGGAGAGCAGTGCCTGCA′-3′; reverse, 5′-GCCAGGGTGCTAAGCAGC′-3′. The product is 299 bp in length.

Protein Stability Experiments—To determine the half-life of Nlp, 100 µg/ml cycloheximide (CHX) (Sigma) was added to the cell culture to inhibit protein synthesis, and cells were harvested in PBS containing 100 µg/ml phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, 2 µg/ml leupeptin, and 1% Nonidet P-40 (lysis buffer) at the indicated time intervals. To determine the effects of proteasome inhibitors on Nlp protein stability, the cells were preincubated with 20 µM MG132 (benzylxoy-carbonyl-Leu-Leu-Leu-aldehyde) for different times or with different concentrations of MG132 before the addition of cycloheximide. For each experiment, a corresponding volume of the vehicle dimethyl sulfoxide (Me_SO) was added as control.

Cell Extraction, Immunoprecipitation, and Immunoblotting—Cells were harvested, rinsed with PBS, and lysed in lysis buffer (composition given above). The lysates were collected by scraping and cleared by centrifugation at 4 °C. 100 µg of cellular protein were resolved by 10% SDS-PAGE and transferred to Protran membranes. The immunoblotting method was performed as described previously (4). For immunoprecipitation, cellular lysates were first incubated with 10 µl of the indicated antibodies for 2 h at room temperature and further incubated overnight at 4 °C after the addition of 20 µl of protein A/G-agarose beads (Santa Cruz Biotechnology). To pull down the immunocomplexes, the immunoprecipitated proteins were separated by SDS-PAGE. Western blot analysis was performed as described above. The following antibodies were used in this study. Nlp antibody was made by BioSource (Camarillo, CA). This polyclonal antibody was demonstrated to specifically hybridize against human KIAA0980 protein. Antibodies against actin, APC2, Cdc27, Cdc20, GFP, β-tubulin, cyclin A,

3 Y. Wang and Q. Zhan, unpublished data.
Regulation of Nlp Expression by APC

B1, D1, and E were commercially provided by Santa Cruz Biotechnology. Antibodies against ubiquitin (Cdh1) were commercially provided by Sigma.

In Vivo Ubiquitination Assay—Cells were cultured in RPMI 1640 medium with 10% fetal bovine serum, treated with 20 μM MG132 or a corresponding volume of the vehicle Me2SO, and lysed on ice with lysis buffer. The lysates were cleared by centrifugation in a microcentrifuge at 14,000 revolutions/min for 15 min. Five hundred micrograms of total cellular protein was used for each immunoprecipitation reaction with 15 μg of antibodies to actin or Nlp. The immunocomplexes were pulled down as described previously (4) and resolved by SDS-PAGE using a 10% gel and probed for the presence of polyubiquitin-Nlp protein complex using anti-ubiquitin antibody.

Plasmid Constructions, Mutagenesis, and Cell Transfection—pEGFP-Nlp was made by inserting human Nlp cDNA into Sall and Smal sites of the pEGFP-C3 vector. The construct expresses green fluorescent proteins (GFPs). pCS2-Cdc20 and pCS2-Cdh1 were constructed by inserting the corresponding Human cDNAs into the Fsd/Asd sites of pCS2 vector, respectively (a gift from Prof. Guowei Fang, Stanford University). Glutathione-S-transferase (GST)-Nlp deletions were made by cloning different lengths of Nlp cDNA into BamHI and NotI sites of the pGEX-5X-1 vector. To identify the sequences that potentially constitute degradation signals for Nlp, we generated a site-directed mutation construct. There are one putative D-box with a RXXXXRX motif and a KEN-box within the N-terminal domain of Nlp. KEN-D-box-mutated Nlp was generated using site-directed mutagenesis as previously described (22). Briefly, PCR primers were as follows: sense F1, 5′-CTGCTA-GTCGACGGTAGAAGAAGAAGACACTATGTC-3′; F2, 5′-GGGCTTGGGCGAGCCAGCTCGATACAGAAGGA-GATTG-3′; F3, 5′-AAGACCTCAGCAGCAATGAGCCAG-AACCATG-3′; antisense R1, 5′-CTGTAGGGCACTGTGCT-GCCGACCAGGGTCTCGGCGGAG-3′; R2, 5′-TACCTTGGTCTCCGGCTGCGGAGCTTGG-TAAGTG-3′; R3, 5′-CACATCTGGGGCGGCTGGCGGATC-GCGACAGGGTCGATTGCCC-3′. KEN-box and D-box were specifically changed with the consensus sequence

For cell transfection, 5 × 10^5 293T cells were seeded onto glass coverslips in a 6-well plate. Cells in each well were transfected with pEGFP-Nlp-Wt or pEGFP-Nlp-mutant, respectively. 96 h later, the cells were fixed with 2% paraformaldehyde in PBS for 10 min at room temperature and washed with PBS. The cells were treated with cold methanol at −20 °C followed by 0.5% Triton X-100 and 0.5% bovine serum albumin in PBS for 30 min. After incubation with antibody to β-tubulin, plates were washed and incubated with TRITC-conjugated goat anti-mouse IgG. DNA was stained with 4′,6-diamidino-2-phenylindole (Sigma). Following washing with PBS, cells were visualized using an Olympus fluorescence microscope, and photographs were generated using a Kodak digital camera.

RESULTS

Cell Cycle-dependent Expression of Nlp—To investigate whether Nlp expression is associated with cell cycle progression, we first employed the approach of cell cycle synchronization to examine the protein level of Nlp. KYSE150 cells were arrested at the G0 phase by serum starvation and released into the cell cycle synchronously. Following release, whole-cell extracts were prepared every 2 h, and Nlp protein levels were analyzed by Western blotting assays (Fig. 1A). To dissect the cell cycle progression, analyses of cyclin D1, E, A, and B1 were included. As shown in Fig. 1A, Nlp exhibited extremely low levels at the first 14 h but started to accumulate at the time point of 16 h and peaked at the points of 20–22 h post-release. According to the expression patterns of cyclins, Nlp expression appeared to be cell cycle-dependent. It started to express an abundant level at the late S phase and peaked at the mitotic phase. When cells moved out of mitotic phase and entered G1, Nlp levels decreased to a very low level (Fig. 1A). Notably, Nlp levels behaved similarly to that of cyclin B1 (Fig. 1A), one known mitotic substrate of APC/c. To further confirm the cell
cycle-dependent expression of Nlp, we next synchronized KYSE150 cells at the G1/S boundary using aphidicolin, a DNA polymerase inhibitor. This approach would allow cells to synchronously enter S phase. Similarly, Nlp displayed high abundant levels as cells entered G2/M transition but began to decrease when cells completed mitosis and entered G1 phase (Fig. 1B). We also examined the levels of NLP mRNA, and it did not show a significant change with the cell cycle progression (Fig. 1C). Further experiments were performed using nocodazole (a microtubule inhibitor) to synchronize cells at mitotic phase, and we got the similar results to Fig. 1, A and B (Fig. 1D). It should be noted here that the same patterns of Nlp expression were observed in other human cell lines, including HeLa and U2OS (results not shown). Taken together, these observations indicate that Nlp protein expression was done in a cell cycle-dependent manner.

**FIGURE 1. Levels of the Nlp protein fluctuate in the cell cycle.** A, KYSE150 cells were arrested at the G0 phase by a serum starvation treatment, released into fresh medium, and harvested every 2 h. Cell lysates were immunoblotted with antibodies to Nlp, cyclin D1, E, A, B1, and actin. Cell cycle stages are indicated at the bottoms of the panels. B and C, KYSE150 cells were arrested at the G1/S boundary using an aphidicolin treatment, released into fresh medium, and harvested at the indicated times. Cell lysates were immunoblotted with Nlp and actin antibodies. Total RNA (6 μg) was reverse-transcribed into cDNA to detect the mRNA level of Nlp and GAPDH. Cell cycle stages are indicated in the figure. D, KYSE150 cells were arrested at mitotic phase using a nocodazole treatment, released into fresh medium, and harvested at the indicated times. Cell lysates were immunoblotted with Nlp and actin antibodies. Cell cycle stages are indicated at the bottoms of the panels.

Nlp Protein Stability Is Regulated by Anaphase-promoting Complex

**FIGURE 2. Nlp has a short half-life and accumulates in the presence of proteasome inhibitors.** A, KYSE150 cells were treated with 100 μg/ml cycloheximide and collected at the indicated times for Western blotting analysis. Proteins (100 μg/lane) were loaded onto an SDS-polyacrylamide gel, transferred to a filter, and incubated with anti-Nlp antibody. The β-actin level was also blotted for a loading control. B, to quantitatively evaluate the half-life of Nlp, its protein level, as determined by Western blotting, was normalized to the β-actin level in three independent experiments. C and D, KYSE150 cells were treated for different lengths of time with a proteasome inhibitor MG132 (20 μM) or different concentrations with MG132. The cells were lysed and subjected to Western blot analysis with anti-Nlp and β-actin antibodies. Results shown are representatives of three independent experiments.

**FIGURE 3. Nlp is ubiquitinated and capable of binding in vivo to the APC component APC2.** A, KYSE150 cells were incubated with (+) or without (−) MG132 (20 μM) for 12 h, lysed, and collected for immunoprecipitation (IP) with anti-Nlp antibody or with anti-actin as a control. Equal amounts of immunoprotein complex were loaded and resolved by SDS-PAGE. Polyubiquitinated Nlp appears in multiple forms with a molecular mass range >156 kDa. The presence of Nlp in the immunocomplex was further verified by blotting the same membrane with anti-Nlp antibody, as shown in the lower panel. B, to test whether Nlp can bind directly to the APC/c, KYSE150 cells were arrested at M phase with nocodazole (400 ng/ml) for 16 h, lysed, and subjected to IP with anti-APC2 or anti-Nlp antibodies. IP with anti-actin antibody was also performed as a negative control. The same blot was reprobed with anti-APC2 to show the efficiency of APC2 immunoprecipitation. The data shown are representative of two experiments. The Nlp-APC2 immunocomplex was present in synchronized mitotic cells but was less in asynchronous cells, as examined by Western blotting and reaction with anti-Nlp. C, to further test whether Nlp can bind to the APC/c directly, we also arrested KYSE150 cells at the G1/S boundary with aphidicolin for 14 h and lysed and subjected them to IP with anti-Cdc27 or anti-Nlp antibodies. The data shown are representative of two experiments.
Regulation of Nlp Expression by APC

A short half-life. The half-life of Nlp was then determined in KYSE150 cells treated with the protein synthesis inhibitor CHX. After 6 h of post-treatment with cycloheximide, Nlp protein was reduced by >50% compared with that seen in untreated control cells (Fig. 2, A and B), suggesting that Nlp is a fast turnover protein and has a short half-life.

The APC/c E3 ligase, through its ability to tag specific proteins with polyubiquitin for targeted degradation, is believed to globally direct the progression of mitosis (23, 24). Because Nlp accumulates in G2/M phases, which is similar to APC/c-regulated substrates, it was suspected that Nlp degradation might involve in the APC/c ubiquitin-proteasome pathway. Therefore, KYSE150 cells were treated with cycloheximide in the presence of MG132, a selective inhibitor of the proteasome and followed by the analysis of Nlp protein levels. Apparently, the addition of MG132 resulted in an increased Nlp level (Fig. 2, C and D). This strongly suggests that degradation of Nlp is mediated by the APC ubiquitin-proteasome pathway. To further prove this, immunoprecipitation experiments with anti-Nlp antibody were utilized and followed by immunoblotting with anti-ubiquitin antibody. To our expectation, the ubiquitin was observed in the Nlp immunocomplex, and its amount was substantially increased in the presence of MG132 (Fig. 3A). Additionally, Nlp interactions with the APC/c subunits APC2 or Cdc27 were also examined (Fig. 3, B and C). Clearly, Nlp antibody was able to immunoprecipitate APC2 or Cdc27, and more abundant APC2 was detected in Nlp immunocomplex when cells were treated with nocodazole (Fig. 3B). On the other hand, we could also detect Cdc27 in Nlp complex when cells were treated with aphidicolin. It implied that Nlp began to decrease from anaphase till early G1 phase (Fig. 3C). Vise versa, Nlp protein were immunoprecipitated by APC2 or Cdc27 antibodies. Collectively, these results indicate that Nlp degradation is mediated by the APC/c-ubiquitin pathway.

Cdh1 or Cdc20 Can Stimulate Nlp Degradation—It has been demonstrated that Cdc20 (Fizzy in Drosophila) and a related protein Cdh1 (Fizzy-related in Drosophila) activate APC through giving temporal control and substrate specificity to the APC/c E3 ligase (25, 26). Each of these two activators physically associates with APC in a cell cycle-dependent manner and stimulates substrate degradation. We transiently transfected expression vectors for Cdh1 and Cdc20 into 293T cells and found that expression of these two proteins resulted in reduced protein levels of Nlp (Fig. 4A). To further determine whether Cdh1 or Cdc20 are required for the degradation of Nlp, we attempted to inhibit the endog—

FIGURE 4. Nlp protein level decreases in cells transiently transfected with the APC/c modulator protein Cdh1 or Cdc20. A, 293T cells were transiently transfected with different concentrations (1-4 μg of DNA) of hCdh1 or hCdc20 expression vectors. Mock transfections were also pursued with empty vector. Cells were lysed after 48 h of incubation and evaluated for Nlp, Cdh1, and Cdc20 protein levels by Western blot analysis with the indicated antibodies. The Western blot was also probed with anti-β-actin (43 kDa) for a loading control. B, 293T cells were transiently transfected with Cdh1 siRNA, Cdc20 siRNA, or mock siRNA. Cells were lysed after 72 h of incubation and evaluated for Nlp, Cdh1, and Cdc20 protein levels by Western blot analysis with the appropriate antibodies. The Western blot was also probed with anti-β-actin for a loading control. C, 293T cells were incubated with MG132 (20 μM) for 12 h, lysed, and collected for immunoprecipitation (IP) with anti-Nlp, anti-Cdc20, and anti-Cdh1 antibodies or with anti-actin as a control. Equal amounts of immunoprotein complex were loaded and resolved by SDS-PAGE.
enous expression of either Cdh1 or Cdc20 via small interfering double-stranded RNAs corresponding to different portions of either the Cdh1 or the Cdc20 gene (27). Transfection with two distinct small interfering RNAs (siRNAs) resulted in reduced amounts of endogenous Cdh1 and Cdc20, which were coupled with increased levels of endogenous Nlp (Fig. 4B). Furthermore, both Cdh1 and Cdc20 were identified to have physical interactions with Nlp, according to a series of immunoprecipitation assays (Fig. 4C). Therefore, Cdh1 and Cdc20 are likely required for Nlp degradation.

Identification of Domains in Nlp Required for Its Degradation—The next effort was taken to determine the APC/c recognition sites of Nlp. Generally, it is believed that Cdc20 is restricted to D-box (RXLXXLXXX) in substrates and Cdh1 targets at KEN-box-containing proteins. Cdc20- and Cdh1-activated APC/c play roles in mediating the ubiquitination-dependent destruction of several KEN-box- and D-box-containing proteins during the completion of mitotic exit. In agreement with these requirements, analysis of the Nlp protein sequence revealed that there are both KEN-box (495–497th amino acids) and D-box (633–641st amino acids) in the protein, and the illustration in Fig. 5A shows the putative destruction boxes in Nlp, including KEN- and D-box. We then mutated these conserved motifs as shown in Fig. 5B and tested their effects on Nlp protein stability. The mutations of the KEN- and D-box conserved sequences seemed to disrupt Nlp protein degradation (Fig. 5C), suggesting that the key sequences responsible for Nlp degradation might be Cdc20- and Cdh1-targeted motifs. To further prove it, 293T cells were co-transfected with Nlp (mutant) and different concentrations of hCdh1 or hCdc20 expression vectors. The results showed that Cdh1 and Cdc20 could no longer enhance the degradation of Nlp mutant, which is consistent with the observations in Fig. 5C. Given that Cdc20 and/or Cdh1 have been thought to interact and recruit targeted proteins to the APC/c E3 ligase for ubiquitination, we sought to evaluate this possibility in Nlp degradation. We expressed wild type GFP-Nlp, KEN-box- and D-box-mutated GFP-Nlp and extracted cellular proteins to perform immunoprecipitation assays with anti-GFP antibody (Fig. 5D). In such an experiment, mutations of KEN-box and D-box disrupted the binding of Nlp to either Cdc20 or Cdh1 and substantially affected ubiquitination of Nlp, as manifested by a lesser multiubiquitin chain in the Nlp mutant compared with that seen in wild type Nlp. Furthermore, deletion of KEN-box and D-box in Nlp abolished its binding to Cdh1 and Cdc20 (Fig. 5E).

Non-degradable Nlp Significantly Promotes Cell Growth, Anchorage-independent Growth, and Results in Multinucleated Cells—Our recent observations demonstrate that Nlp was an oncogenic protein, as reflected by its capabilities of allowing anchorage-independent growth and inducing tumorigenesis in nude mice. Additionally, Nlp was found to be overexpressed in human tumors. Thus, the further study was undertaken to examine whether non-degradable Nlp might be a more malignant oncprotein. First, colony formation assay was performed, and the results showed that Nlp with mutated KEN-D-box promoted cell growth much faster than the wild type Nlp (Fig. 6A), suggesting that the non-degradable Nlp has a stronger capability of accelerating cell growth. In addition, soft agar assays were conducted. As expected, the normal 293T cells did not form an appreciable number of colonies in soft agar, and some colonies were noted in the cell expressing wild type Nlp. However, a significantly greater number of colonies were observed in the cell expressing mutated Nlp (Fig. 6B). Interestingly, the sizes of the colonies with mutant Nlp were also substantially larger compared with that seen in cells expressing WT Nlp. We further examined the cellular consequence of overexpressing mutated Nlp and observed multinuclei in these cells, which was probably due to the defect of cytokinesis. There was a higher tendency in the cells expressing mutant Nlp to become multinucleated (9.7% multinuclei caused by Nlp mutants compared with 3.6% due to the defect of cytokinesis). Therefore, Cdh1 and Cdc20 are likely required for Nlp degradation.
DISCUSSION

During the cell cycle, centrosomes are duplicated and segregated in synchrony with the genome, and aberrations in the centrosome cycle result in abnormal spindle formation and aneuploidy (3). Multiple investigations have connected aneuploidy to cell malignant transformation. One of the important reasons for aneuploidy is an aberrant cytokinesis. The latter can be caused by deregulated expression of centrosome regulators, such as Aurora-A and Plk1 (10). Therefore, the expressions of mitotic proteins are under precise control to ensure successful mitosis. In this study, we demonstrated that centrosomal Nlp, whose expression is required for centrosome maturation and spindle formation, is a short-lived protein, and its degradation is regulated by the APC/c-mediated proteasome pathway.

Nlp is a centrosome protein and plays an important role in microtubule nucleation. It recruited two distinct subunits of γ-TuRC, γ-tubulin and hGCP4, to promote centrosome maturation and spindle formation. The centrosome association of Nlp is regulated during the cell cycle (12). Therefore, the expression of Nlp kinetic changes is precisely regulated through the cell cycle. It has been shown that overexpression of Nlp caused aberrant spindle formation, which often leads to missegregation and cell transformation (12), suggesting that the displacement of Nlp from the centrosome and its degradation are important for proper centrosome maturation and spindle assembly. It is worth noting that our recent data demonstrate that Nlp is an oncogenic protein and overexpressed in various types of tumors. Deregressed Nlp has been shown to induce cell transformation and spontaneous tumors.3 All of those observations suggest that the machinery controlling Nlp expression is critical in accomplishing mitotic progression, and disruption of this machinery would result in genomic instability and tumorigenesis.

Mitotic protein degradation plays vital roles in the control of the mitotic progression, and the deregulated levels of those proteins abrogate the fidelity of chromosome segregation during mitosis (14). Mammalian cells contain two distinct major proteolytic pathways. One important nonlysosomal machinery for the degradation of intracellular proteins is the ubiquitin-proteasome pathway (28). Several impor-
Regulation of Nlp Expression by APC

tant cell cycle regulators, including the cyclins, Cdk inhibitors, and p53, are all degraded by such a pathway (29, 30). It is thought that periodic degradation of these proteins is required for cell cycle progression. For example, APC/c ubiquinates proteins such as cyclin B, whose subsequent degradation by the 26 S proteasome is essential for the initiation of sister chromatid separation at the metaphase-anaphase transition and exit from mitosis, respectively (21). Similar to mitotic regulators, the expression of Nlp is cell cycle-dependent, with the peak expression at G2-M phases. Because there are almost no differences in mRNA levels of NLP throughout cell cycle, Nlp protein expression is mainly regulated via ubiquitin-mediated proteolysis. Nlp has a relatively short half-life and is ubiquitinated through its association with the APC/c subunit APC2 or Cdc27. In support of these findings, the levels of Nlp are elevated by inhibitors of the proteasome. All of this evidence indicates that Nlp degradation is regulated by the ubiquitin-proteasome pathway.

It has been shown that regulators of early mitosis are typically targeted to degradation by association with Cdc20 protein via D-box, whereas regulators of late mitosis are targeted by Cdh1 through KEN-box. Interestingly, there are both D-box and KEN-box sequences in Nlp (Fig. 5), suggesting that Nlp degradation might be mediated by Cdc20 and Cdh1. Consistently, we found that these two proteins were able to interact with Nlp in vivo, and their overexpression resulted in diminished Nlp levels. In agreement with these observations, cells silenced for Cdc20 and Cdh1 via siRNA exhibited stabilization of Nlp protein. Collectively, these findings further confirm that Nlp degradation is regulated by the APC/c-mediated ubiquitin-proteasome pathway.

However, the observations that Nlp expression is cell cycle-dependent and it is degraded by APC/c complex are different from the previous report by Rapley et al. (13), in which the total abundance of Xenopus Nlp is unchanged throughout the cell cycle, and the destruction assays confirmed that in vitro translated Nlp was stable in mitotic Xenopus extracts, which contained APC/c complex. Likely, the different experimental models (human cells versus Xenopus) could be the reasonable explanation regarding such discrepancy. We have found that there was no D-box in Xenopus Nlp that functions as a degradation signal in human Nlp. Similarly, it has been reported that Aurora-B protein expression is maintained unchanged in Xenopus extracts but is clearly cell cycle-dependent in mammalian cells (31). Likely, there is some difference between these two experimental systems. The findings that Nlp degradation is mediated through APC/c were further supported by mutations of D-box and KEN-box in Nlp. Disruption of their sequences significantly reduced the binding of Cdh1 and Cdc20 to Nlp and abolished Nlp degradation.

Degradation of Nlp after G2/M transition is thought to be a critical event for completion of mitosis. Nlp regulates centrosome maturation, chromosome segregation, and cytokinesis. Cell cycle-dependent degradation of Nlp by the APC/c pathway suggests that this degradation may be coupled with the initiation, progression, or completion of cytokinesis. Given that Nlp is one of the key regulators in the process of cytokinesis, destruction of Nlp may provide a temporal as well as a spatial control of cytokinesis. Consistent with this hypothesis, we have recently found that overexpression of Nlp leads to multinuclearity and chromosomal instability. Furthermore, expression Nlp (mutant) in cells can result in a higher tendency of multinuclearity compared with expression of wild type Nlp. In conclusion, APC/c-mediated destruction of Nlp provides a novel mechanism for precise control of Nlp in the cell cycle. The fact that Nlp is frequently overexpressed in various cancer cells may further emphasize the importance of this regulatory mechanism in the control of cell cycle progression.

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