A Tumor Suppressive Role of CYLD as a Novel Potential DUB of Aurora B in Cervical Cancer

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ABSTRACT

BACKGROUND: Cervical cancer is a common leading cause of cancer related to women death worldwide. Cylindromatosis (CYLD) is known as an important tumor suppressor in various human cancers, and a deubiquitination enzyme (DUB) as well. Previously, we identified Skp2 as an E3 ligase of Aurora B ubiquitination, but the DUB of Aurora B still remains unknown.

METHODS: Aurora B ubiquitination site is identified through in vivo ubiquitination assay. Activity of Aurora B and CENPA was detected by immunoblotting (IB) and immunofluorescence (IF) assay. Protein-to-protein interaction was investigated by immunoprecipitation (IP). Cell chromosome dynamics was monitored by live-cell time-lapse Imaging. Cancer cell proliferation, colony formation, apoptosis, and cell invasion and migration assays were also performed. Protein level was checked by immunohistochemical (IHC) staining in clinical cervical cancer samples.

RESULTS: We identified Lysine 115 (K115) as the main Aurora B ubiquitination site for Skp2. We could also detect an interaction of Aurora B with the DUB CYLD. We found that CYLD promoted deubiquitination of Aurora B, and regulated Aurora B activity and function as well. Compared with control, we found it took more time for the cells to finish cell mitosis with CYLD over-expression. Furthermore, we found that CYLD deficiency promoted cervical cancer cell proliferation, colony formation, cell migration and invasion, and inhibited apoptosis instead, whereas it is just opposite with CYLD over-expression. In clinical cervical cancer samples, we showed a negative correlation of CYLD expression with Aurora B activation and histological cancer cell invasion. Furthermore, there was less CYLD abundance and higher Aurora B activity in advanced cancer samples compared with early stage.

CONCLUSIONS: Our findings uncover CYLD as a novel potential DUB of Aurora B, which inhibits Aurora B activation and its subsequent function in cell mitosis, and also provide more evidence for its tumor suppressor function in cervical cancer.

KEYWORDS: Aurora B, CYLD, deubiquitination enzyme, tumor suppressor, cervical cancer

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Introduction

Cervical cancer is a common gynecological cancer, which is one of the main causes of women's death all over the world.[1,2] Human papillomavirus infection (HPV) is recognized as a major risk factor for cervical cancer, among which the HPV 16 and 18 subtypes are the most dangerous.[1,3,4] There are also some other causes involved.[5] As far as the current medicology developed, a considerable part of the early and medium-term cervical cancer can be cured by surgery and chemo-radiotherapy. However, there are still many cervical cancer patients with late stage are suffering.[6] Therefore, the pathogenesis study may have important guiding significance for the future diagnosis and treatment of cervical cancer.

Genomic instability caused by disorder of cell cycle progression is an important characteristic of cancer cells. Aurora B is a component of the CPC Complex, which also includes INCENP, Dasra, and Survivin. As a central kinase, Aurora B plays an important role in cell mitosis and spindle checkpoint, which also needs the precise assembly of the CPC Complex.[7-9] Our previous study discovered that Skp2 could interact with Aurora B and trigger its ubiquitination, which served as an important event for Aurora B activation but not protein degradation in cell mitosis and spindle checkpoint.[10]

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Cylindromatosis (CYLD) is an important tumor suppressor, and a deubiquitination enzyme (DUB) as well. CYLD was first discovered mutated in familial cylindromatosis, a kind of benign skin tumor developing from ectodermal appendages.[11] Later reduced expression or mutation of CYLD was found in various cancers such as colon cancer, hepatocellular carcinoma, melanoma or multiple myeloma, indicating that CYLD may have broad tumor suppressor function.[12-16] CYLD inhibited several tumor signal pathways such as nuclear factor kappa B (NF-κB), mitogen-activated protein kinase (MAPK), Wnt, and Akt pathways by removing Lysine (K)63-linked polyubiquitin chains from important effectors in these pathways like NEMO, TRAF2, TRAF6, RIPK1, Dvl, and Akt.[17-22]

In this study, we try to understand the new function of tumor suppressor CYLD as a potential DUB of Aurora B in cell mitosis. Here, we show that CYLD inhibits Aurora B activation through its DUB function in cell mitosis and then plays important roles in preventing cancer cell proliferation, invasion, and migration in cervical cancer.

Materials and Methods

Cell culture and reagents

293T, HeLa, and SiHa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). To clone Xp-Skp2, Skp2 was amplified by polymerase chain reaction (PCR) using pcDNA3-Skp2 as a template and inserted into a pcDNA4/HisMax-TOPO vector (Invitrogen, Waltham, MA, USA). $(His)_6$-ubiquitin, HA-Aurora B plasmids were previously described.[10] Flag-CYLD was a gift from Dr Hui-Kuan Lin. All the HA-Aurora B Lysine (K) to Arginine (R) site mutants used in Figure 1A were generated through PCR using HA-Aurora B as a template.

Immunoprecipitation and immunoblotting

Immunoprecipitation (IP) and immunoblotting (IB) were done essentially as described with mild modification.[23,24] The following antibodies were used for IP and IB: Xpress antibody (IB: 1:5000, Invitrogen), Flag antibody (M2) (IP: 1:200; IB: 1:3000, Sigma-Aldrich, St. Louis, MO, USA), HA antibody (IP: 1:200; IB: 1:5000, Cell Signal Technology, Danvers, MA, USA), Aurora B phospho T232 (IB: 1:1000, Abcam, England), p-CENPA (IB: 1:1000, Cell Signal Technology), Aurora B (IB: 1:1000, Cell Signal Technology), and CENPA (IB: 1:1000, Cell Signal Technology).

Immunofluorescence

HeLa cells were seeded in a special chamber used for immunofluorescence (IF). The following antibodies were used for IF: Flag antibody (M2) (1:200, Sigma-Aldrich), HA antibody (1:200 Cell Signal Technology), Aurora B phospho T232 (1:200, Abcam), and p-CENPA (1:200, Cell Signal Technology).

In vivo ubiquitination assay

In vivo ubiquitination assay was performed as described.[25] In brief, 293T cells were transfected with indicated plasmids for 48 h and harvested by denatured buffer (6 M guanidine-HCl, 0.1 M Na_2HPo_4/NaH_2Po_4, 10 mM imidazole). The cell extracts were then incubated with nickel beads (Ni-NTA) for 3 hours, washed, and subjected to western blot (WB) analysis.

Viral infection

For lentiviral short hairpin RNA (shRNA) infection, 293T cells were co-transfected with shRNAs against luciferase or CYLD along with packing plasmids (deltaVPR8.9) and envelope plasmid (VSV-G). Two days after transfection, virus particles containing CYLD and luciferase shRNAs were used to infect HeLa and SiHa cells. All the infected cells were cultured in the medium containing 1.5 μg/mL puromycin for 1 week. The following lentiviral shRNAs were used for transfection: CYLD-lentiviral shRNA#1 (5′-TACTTAGACTCAACCTTATTC-3′), CYLD-lentiviral shRNA #2 (5′-AAGAAGGTCGTGGTCAAGGTC-3′), and luciferase shRNA (5′-GCAAGCTGACCCTGAAGTTC-3′).

Cell proliferation detection

HeLa and SiHa cells were counted and seeded into 96-well plates. Cell viability was detected with a CCK8 kit (CCK8, Yeasen, Shanghai, China) for indicated days continuously following the instruction. Growth curves were then made according to the OD (observation density) values, which were repeated 4 times at each time point.

Apoptosis assay

HeLa and SiHa cells with control and CYLD knock-down were seeded into 6-well plates. At the density of 90%, cells were collected and labeled with annexin -FITC/PI apoptosis kit (A026, ABP Biosciences, Beltsville, MD, USA) according to the manufacturer's standard procedure, followed by Flow Cytometric analysis. All experiments were repeated at least 3 times.

Colony formation assay

HeLa and SiHa cells were trypsinized, resuspended in single-cell supernatant, seeded into 6-well plates for colony formation assay in common medium at a density of 1000 cells per well as reported.[26] After culturing for 10 to 15 days, the colonies were fixed with 4% paraformaldehyde and stained with crystal violet, colonies with more than 50 cells were counted.

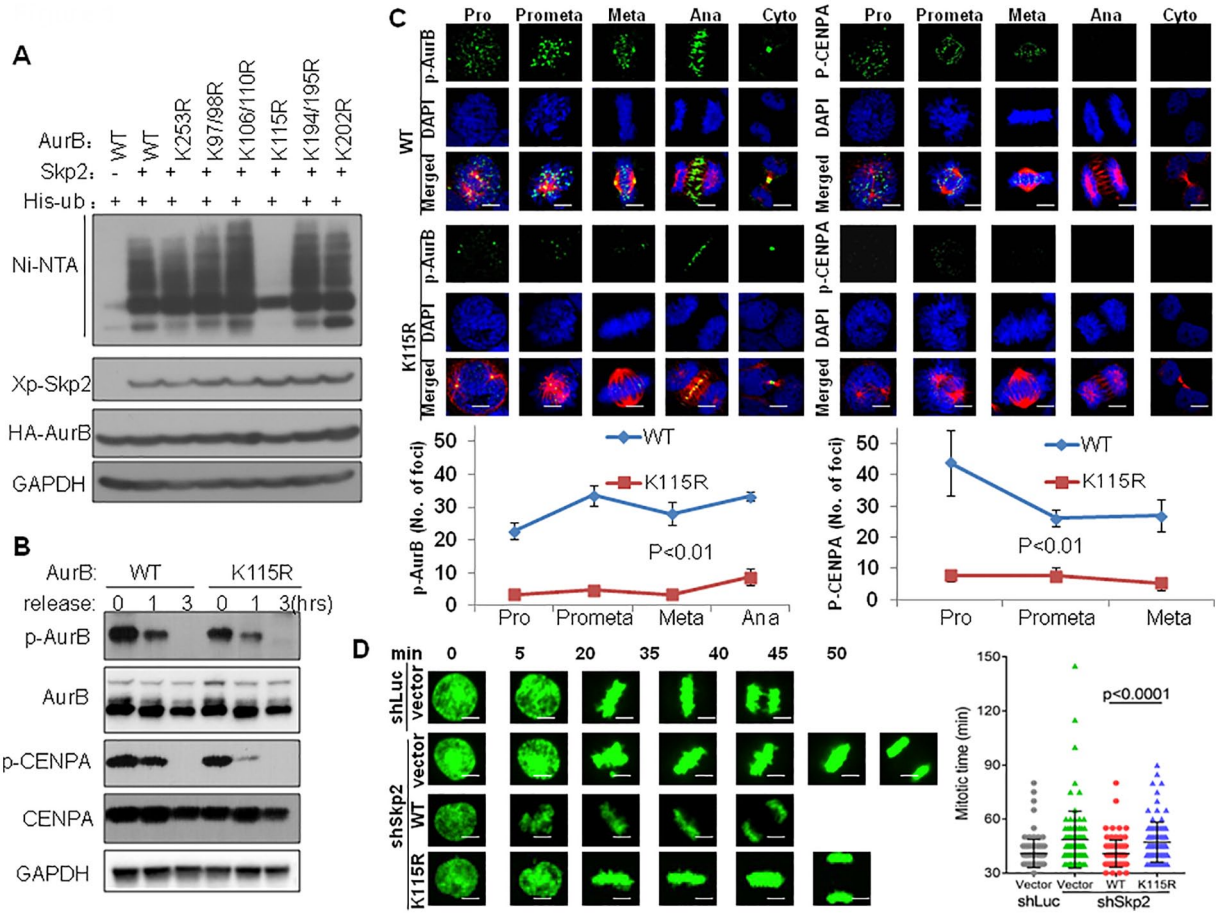

Figure 1. K115 is the major ubiquitination site in Aurora B triggered by Skp2. (A) 293T cells were transfected with indicated plasmids and harvested for in vivo ubiquitination assay 48 h later. (B and C) Expression of Aurora B WT in Skp2 knock-down cells could restore the activity of Aurora B and CENPA, but the K115R mutant could not. (B) HeLa Skp2 knock-down cells transfected with WT or K115R Aurora B were incubated for around 32 h and later synchronized by nocodazole for 18 h. Then, cells were changed with fresh medium and incubated for indicated time and harvested for WB assay. (C) HeLa Skp2 knock-down cells were transfected with WT or K115R Aurora B. Then, we detected expression of p-Aurora B and p-CENPA in these cells through IF assay in different mitosis stages. Green: p-Aurora B or p-CENPA, blue: DAPI, and red: tubulin. Scale bar represents 5 μm. The upper panel showed the representative image of each stage and the down panel displayed the quantitative analysis. The foci of each image were counted through the Image J software. (D) Skp2 knock-down HeLa cells expressing H2B-GFP were transfected with vector, WT, or K115R Aurora B; 36 h later, cells were imaged at every 5 min. Luciferase control knock-down HeLa cells were also included. Around 100 cells in each group were randomly chosen for statistics. The left panel showed the representative fluorescence video microscopy series from the onset of mitosis to monitor chromosome dynamics and the right panel displayed the statistical results. Scale bar represents 5 μm. Cell numbers were 109 (Control shRNA + Vector), 99 (Skp2 shRNA#1 + Vector), 97 (Skp2 shRNA + WT Aurora B), and 100 (Skp2 shRNA + K115R Aurora B). Error bars represent the standard deviation. *P* value was calculated using unpaired *T*-test.
Ana indicates anaphase; Cyto, cytokinesis; IF, immunofluorescence; K115, lysine 115; Meta, metaphase; Pro, prophase; Prometa, prometaphase.

Cell invasion and migration assay

A 24-well transwell plate with 8 μm porous membrane that was separated as the lower and upper culture chambers was used for the cell invasion and migration assay. Generally, 2.5×10^5 HeLa or SiHa cells with serum-free medium were plated in the upper chamber with (Invasion Assay) or without (Migration Assay) precoated diluted Matrigel (Matrigel: serum-free DMEM,1:10) prior to the assay. Dulbecco's Modified Eagle Medium with 10% FBS was added to the lower chamber. The plate was incubated at 37°C for 12 to 16 h (Migration Assay) or 20 to 24 h (Invasion Assay). After the invasion or migration period, cells on the upper side of the chamber were removed with a cotton swab. Then, cells in the lower chamber were fixed with 4% paraformaldehyde at room temperature for 30 min and later stained with 0.5% crystal violet for 10 min. Three random fields of each chamber were taken pictures under a microscope and then counted to get an average.

Patients, immunohistochemical staining, and evaluation

Cervical cancer specimens were obtained from Department of Gynecology at Affiliated Cancer Hospital of Guangzhou Medical University with written informed consent (n = 100) from 2015 to 2019, which was also approved by the Medical Ethical Review Committee from Affiliated Cancer Hospital of

Guangzhou Medical University (2014 Ethical Review no. 49). Patients confirmed as cervical cancer by histopathology could be included, while those patients without enough clinical information including tumor size and clinical stage were excluded. All including patients were classified by a pathologist following the International Federation of Gynecology and Obstetrics (FIGO) guidelines. According to the FIGO staging of cancer of the cervix uteri (2018), cervical cancer were tagged from Stage I to Stage IV. For Stage I, the carcinoma is strictly confined to the cervix, which we assigned to early stage, and the rest were assigned to late stage.[27] Immunohistochemical (IHC) staining was performed following a standard protocol.[26] Negative controls were corresponding non-immune serum immunoglobulin instead of CYLD antibody (ab137524, Abcam), anti-p-CENPA (af2330, Affinity, USA), anti-p-Aurora B (ab115793, Abcam). Two pathologists reviewed the slides independently. The principle that we evaluate the abundance of biomarkers in slides was referred to previous publications.[22,28] $P < .05$ was considered statistically significant. Statistical analysis was performed with Graph Pad Prism 5.0 (GraphPad Software, CA, San Diego, USA).

Live-cell time-lapse imaging

To monitor chromosome dynamics, green fluorescence protein (GFP)–tagged H2B was transfected into indicated HeLa cells. Twenty-four hours later, cells were imaged at 5 min interval for 24 h using live-cell imaging under a confocal microscope. Then, the mitotic time was calculated from the beginning of prophase to the end of cytokinesis.

Flow cytometry

For mitotic arrest, HeLa cells were synchronized by 200 ng/mL nocodazole for 18 h. For flow cytometry analysis, cells were then collected and permeabilized with 0.1% Triton X-100 after fixation with 75% ethanol, stained with Histone H3p antibody (Millipore, Burlington, MA, USA) and FITC-conjugated second antibody. For DNA content, cells were stained with propidium iodide (PI) (Sigma-Aldrich). After that, analysis was performed with an FACS flow cytometer (BD Biosciences, Mountain View, CA, USA).

Results

Lysine 115 is the major ubiquitination site in Aurora B triggered by Skp2

Previously, we reported that Skp2 triggered Aurora B ubiquitination and regulated its function in cell mitosis.[10] To identify the ubiquitination site, with single or double point mutation, we mutated the lysine (K) residues to arginine (R) in Aurora B, which cannot be ubiquitinated and did the ubiquitination assay with these mutants. As shown in Figure 1A, only the K115R mutant markedly inhibited Aurora B ubiquitination, indicating that K115 was the major ubiquitination site in Aurora B. Then, we wonder whether this K115R mutant has kinase activity. We synchronized the Skp2 knock-down HeLa cells that were expressed Aurora B WT or K115R mutant by nocodazole or released to cell cycle again by further incubating without nocodazole for different time periods. As p-Aurora B (phospho-Aurora B) and p-CENPA (phospho-CENPA) are the activated forms of Aurora B and its downstream protein CENPA, we examined the level of p-Aurora B and p-CENPA through both WB test and IF assay, and found that Aurora B WT could restore the activity of Aurora B and CENPA, but the Aurora B K115R mutant could not (Figure 1B and C). Furthermore, in both groups, the expression of Aurora B and CENPA in metaphase for the IF assay was shown with no difference in Supplementary Figure 1A. As a complement of Figure 1C, we also stained exogenous Aurora B with HA antibody and demonstrated that the p-Aurora B and p-CENPA expression was normal in cells which we transfected WT Aurora B but weak in the K115R Aurora B mutant group as shown in Supplementary Figure 1B. Then consistent as reported before,[10] the results showed that compared with 40.9 min of the luciferase control knock-down group, mitotic time was prolonged to about 48.6 min for the Skp2 knock-down group, and the expression of WT Aurora B restored the mitotic time to 40.8 min, while it was still approximately 47.1 min for the K115R Aurora B expressing group. Indicating that it took more time for Skp2 knock-down HeLa cells to progress into cytokinesis compared with control, and WT Aurora B re-expression could restore this phenomenon but the K115R mutant failed (Figure 1D). These data demonstrated that the Aurora B K115R mutant had defect to induce the activity of Aurora B and its downstream protein and prolonged cell mitosis progression, which further verified the importance of Aurora B ubiquitination in cell cycle regulation.

CYLD inhibits ubiquitination of Aurora B and interacts with Aurora B

Because before we reported that K63-linked ubiquitination may be responsible for Aurora B activation in cell mitosis progression, we speculate it must lose activity if the poly-ubiquitin chain of Aurora B is removed. However, the DUB of Aurora B still remains unknown. Interestingly, our colleagues previously found that as an important kinase in cell growth, metabolism, and tumorigenesis, Akt could be ubiquitinated and activated by Skp2.[29] Later, they identified CYLD as its DUB and inhibited its activity.[22] As Skp2 is also responsible for Aurora B ubiquitination and activation, we think CYLD may be involved in Aurora B deubiquitination as well. Luckily as shown in Figure 2A, we found that CYLD could effectively reduce Aurora B ubiquitination, and we also verified enhancement of Aurora B ubiquitination by Skp2. Having showing that CYLD promotes Aurora B deubiquitination, then we wonder whether CYLD

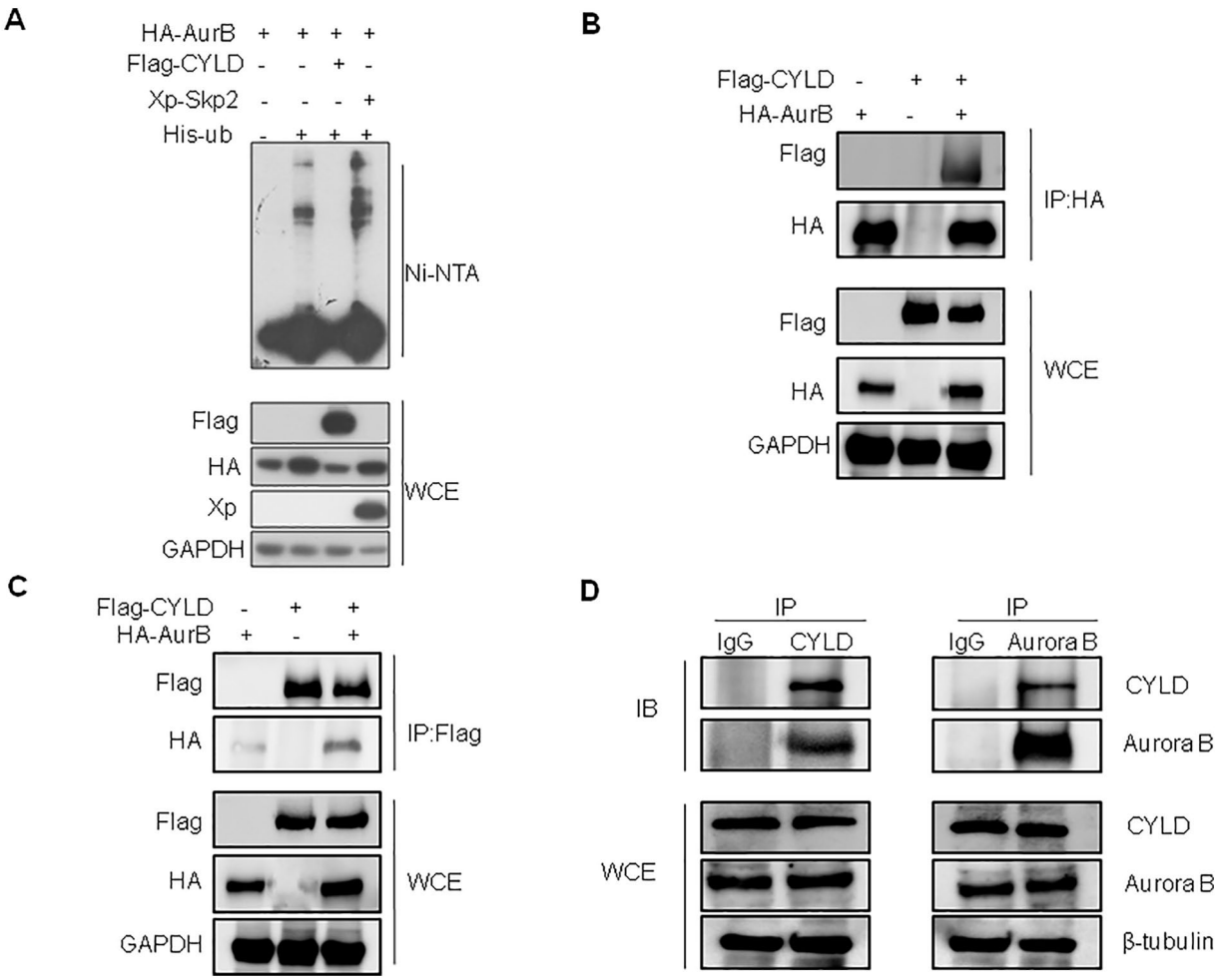

Figure 2. CYLD inhibits ubiquitination of Aurora B and interacts with Aurora B. (A) 293T cells were transfected with indicated plasmids and harvested for in vivo ubiquitination assay (see section "Materials and Methods" for details). (B and C) 293T cells were transfected with Flag-CYLD and/or HA-Aurora B, and cells were harvested for Co-IP assay with HA antibody (B) or Flag antibody (C), followed by western blot analysis with indicated antibodies. (D) HeLa cells were harvested for Co-IP assay with CYLD antibody (left panel) or Aurora B antibody (right panel), followed by western blot analysis with indicated antibodies.
Co-IP indicates coimmunoprecipitation; CYLD, cylindromatosis.

associates with Aurora B. To resolve this, we performed a series of coimmunoprecipitation (Co-IP) assay. As predicted, we found that CYLD and Aurora B interacted with each other exogenously by reciprocal IP as shown in Figure 2B and C. And we could also detect their endogenous interaction in HeLa cells (Figure 2D). These data suggest that CYLD interacts with Aurora B, which may give a chance for CYLD to remove poly-ubiquitin chains from Aurora B and act as its DUB.

CYLD regulates Aurora B activity and function

Our finding that CYLD interacts with Aurora B and inhibits its ubiquitination made us to wonder whether CYLD regulates Aurora B activity in cell mitosis. First, HeLa cells transfected with control or CYLD plasmid were synchronized by nocodazole and then released to cell cycle again by further incubating without nocodazole for different time periods. Before this experiment, we determined the effect of the cell cycle arrest by nocodazole treatment through Flow Cytometry analysis. From Supplementary Figure 2, we could see that most of the cells were arrested in G2/M phase after nocodazole treatment, from which we could also see phospho-H3 enhancement with nocodazole treatment. Later, we examined the level of p-Aurora B, which is the activated form. We discovered in Figure 3A that p-Aurora B was impaired when we transfected with CYLD compared with control. We also performed the IF assay, we found in Figure 3B the same impairment of p-Aurora B in synchronized cells with CYLD over-expression compared with control. Furthermore, we also checked the activity of CENPA, the downstream protein of Aurora B. As the activated form, we found the p-CENPA level decreased in CYLD over-expressed cells in both WB test and IF assay compared with control as shown in Figure 3A and B. In the other side, we also detected p-Aurora B and p-CENPA level in CYLD and control knock-down stable HeLa cells by WB and IF assay. As expected, we

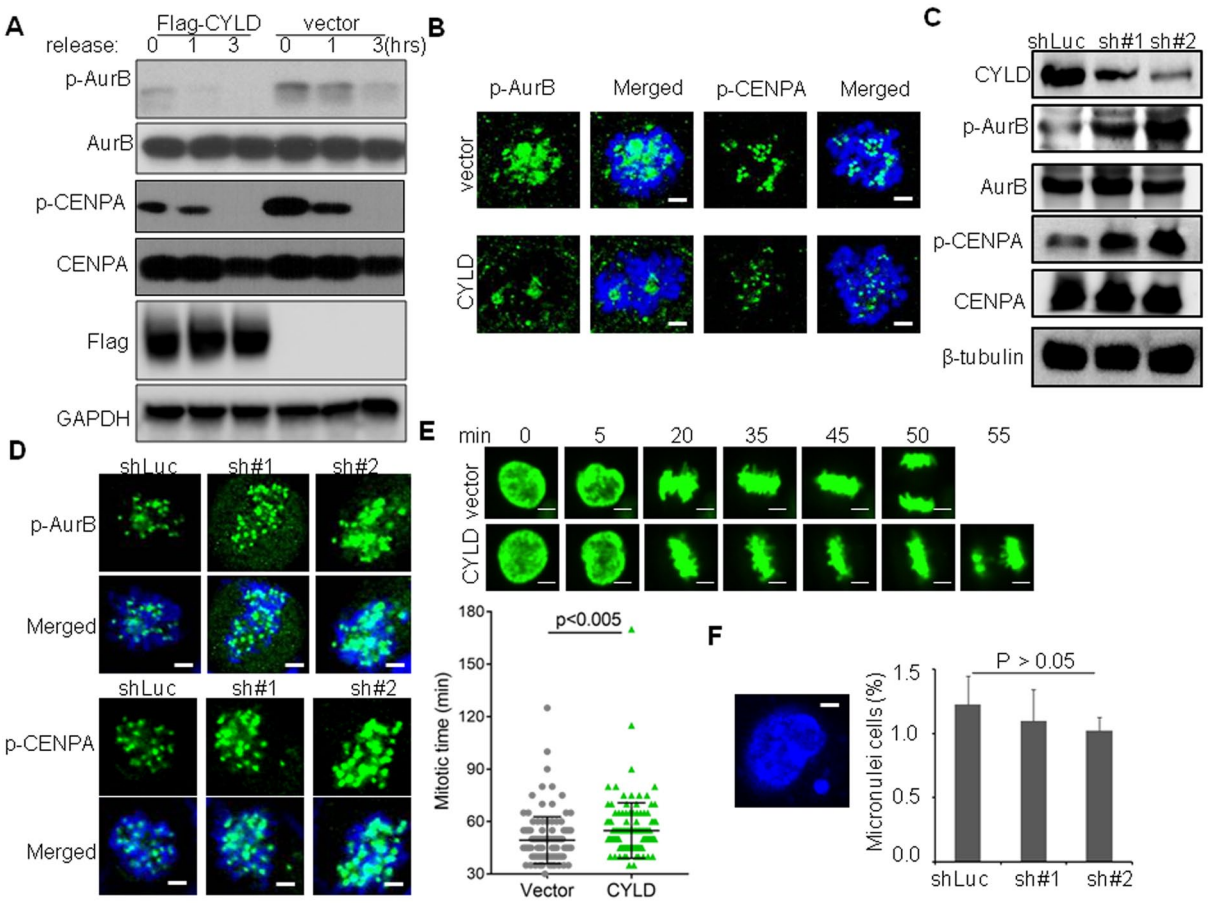

Figure 3. CYLD regulates Aurora B activity and function. (A) HeLa cells transfected with CYLD or control plasmid were synchronized by nocodazole for 18 h before harvested. Mitotic HeLa cells were collected by mitotic shake-off. The collected cells were then continued to incubate for the indicated time points (0, 1, 3 h) and following immunoblotted with indicated antibodies. (B) HeLa cells transfected with control or Flag-CYLD plasmid were synchronized by nocodazole for 18 h and then were fixed for IF analysis with indicated antibodies. Scale bar represents 2 μm. (C) Luciferase control and 2 clones of CYLD knock-down stable HeLa cells were generated and harvested for WB assay with indicated antibodies. (D) Luciferase control or CYLD knock-down stable HeLa cells generated above were synchronized by nocodazole for 18 h and then fixed for IF analysis with indicated antibodies. Scale bar represents 2 μm. (E) HeLa cells expressing H2B-GFP were transfected with control or CYLD plasmid; 36 h later, cells were imaged at every 5 min. Around 100 cells in each group were randomly chosen for statistics. The upper panel showed the representative fluorescence video microscopy series from the onset of mitosis to monitor chromosome dynamics and the lower panel displayed the statistical results. Cell numbers were 123 (Control) and 123 (CYLD). *P* value was calculated using unpaired *T*-test. Error bars represent the standard deviation. Scale bar represents 5 μm. (F) Control and CYLD stable knock-down SiHa cervical cancer cells were plated and cultured for 24 h; cells were then stained with Hoechst. Later, more than 30 random pictures of every group were captured under a confocal microscope. Finally, we counted and summed up the proportion of cells with micronuclei. The left panel showed a representative micronuclei microscopy of the cells and the right panel displayed the statistical results. For each group, the cell number is more than 1000. *P* value was calculated using unpaired *T*-test. Scale bar represents 2 μm.
CYLD indicates cylindromatosis; GFP, green fluorescence protein; IF, immunofluorescence; WB, western blot.

could see the enhancement of both p-Aurora B and p-CENPA with CYLD knock-down compared with control (Figure 3C and D). From another SiHa cervical cancer cell line, we could also find more p-Aurora B and p-CENPA expression in CYLD stable knock-down cells compared with control (Supplementary Figure 3A). As Aurora B is a key component of the CPC complex, we wonder whether CYLD affects the assembly of the CPC complex. To address this question, we over-expressed CYLD and performed immunoprecipitation assay. We found CYLD over-expression did not affect the binding of Aurora B with INCENP or Survivin (Supplementary Figure 3B). Aurora B is a central kinase in cell mitosis. As shown that CYLD interacts with Aurora B, induces Aurora B deubiquitination, and inhibits its activity, we speculate that CYLD may also take action in cell mitosis. To investigate the role of CYLD in cell mitosis, HeLa cells transfected with CYLD or control were also collected for live-cell time-lapse Imaging. We found in Figure 3E that it needed 49 min for the control cells to finish the cell mitosis process while it took around 54 min for the CYLD over-expression group. It indicated that CYLD expression delayed about 5 min for the cell mitosis. To test whether CYLD had a role in micronuclei formation, we generated a series of stable CYLD knock-down SiHa cervical cancer cell line. However, we did not find a significant difference of micronuclei formation between the control (1.23%) and the 2 CYLD knock-down (1.09% and 1.02%)

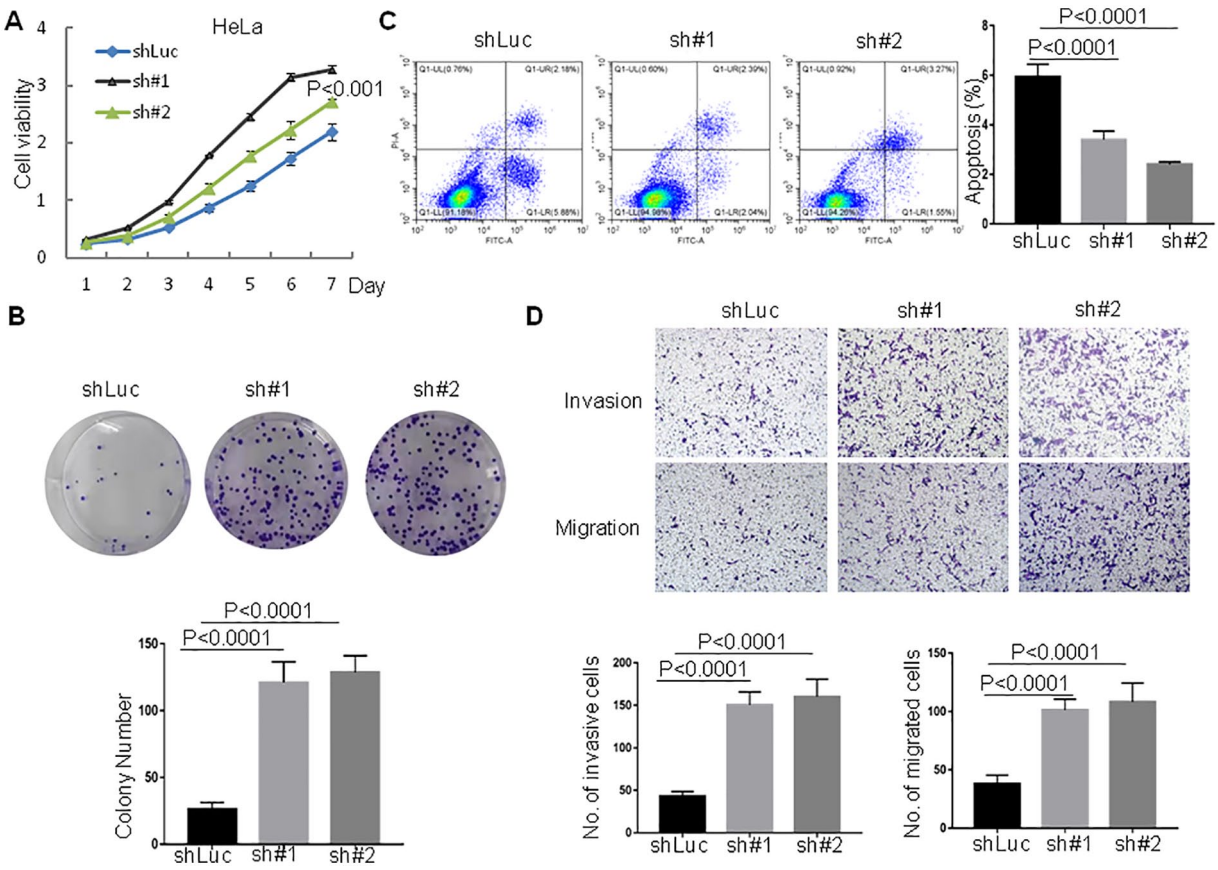

Figure 4. CYLD inhibits cancer cell growth, survival, invasion, and migration. (A) Cell proliferation in control and CYLD stable knock-down HeLa cells, results were presented as mean values ± SD from 4 biological replicates. *P* value was calculated using unpaired *T*-test. (B) Colony formation assay in control and CYLD stable knock-down HeLa cells, 3 biological replicates were included. *P* value was calculated using unpaired *T*-test. (C) Cell apoptosis as determined by annexin-staining and flow cytometric analysis in control or CYLD stable knock-down HeLa cells, presented as mean values ± SD from 4 biological replicates. *P* value was calculated using unpaired *T*-test. (D) Cell invasion and migration assay in HeLa cells with control or CYLD stable knock-down. *P* value was calculated using unpaired *T*-test. The upper panel showed the representative pictures. The lower panel displayed the statistical results, presented as mean values ± SD from 6 biological replicates.
CYLD indicates cylindromatosis; SD, standard deviation.

clones (Figure 3F). Taken together, this study demonstrates the inhibitory role of CYLD in cell mitosis.

CYLD inhibits cervical cancer cell growth, survival, invasion, and migration

Aurora B plays an important role in cell mitosis. As we found that CYLD inhibited Aurora B ubiquitination and its activity, we speculate CYLD may be also involved in cell growth and proliferation. Before we tested the function of CYLD, we first verified the role of Aurora B in cancer cell proliferation, migration, and apoptosis. As expected, we found that Aurora B promotes cell proliferation and cell migration and invasion, whereas suppresses apoptosis in SiHa cells (Supplementary Figure 4). Then, as we expected we found that HeLa cells with CYLD knock-down had stronger cell growth and colony formation ability compared with control knock-down cells as shown in Figure 4A and B. In the other side, we also checked its function in cell apoptosis. We showed that CYLD knock-down greatly diminished apoptosis compared with control in HeLa cells (Figure 4C). Furthermore, we found that loss of CYLD promoted HeLa cells invasion and migration (Figure 4D). We also found the same phenomenon in SiHa CYLD knock-down cells compared with control as shown in Supplementary Figure 5. These data suggested CYLD deficiency promoted cell proliferation, colony formation, and cell migration and invasion, whereas suppressed apoptosis in both HeLa and SiHa cells. However, we also did some experiments with CYLD over-expression. We found in Supplementary Figure 6A–C that CYLD over-expression suppressed cell proliferation and cell migration and invasion, whereas promoted apoptosis in SiHa cells. Our result shows the inhibitory role of CYLD in cervical cancer cell growth, survival, invasion, and migration. As HPV is known as a major risk factor for cervical cancer, we are interested to know whether HPV proteins have any influence on CYLD expression. To resolve this question, we over-expressed HPV16 E6 protein, which is a major oncoprotein to induce cervical cancer oncogenesis, and found that this E6 protein promoted CYLD expression (Supplementary Figure 6D). This interesting finding needs our further exploration in the future.

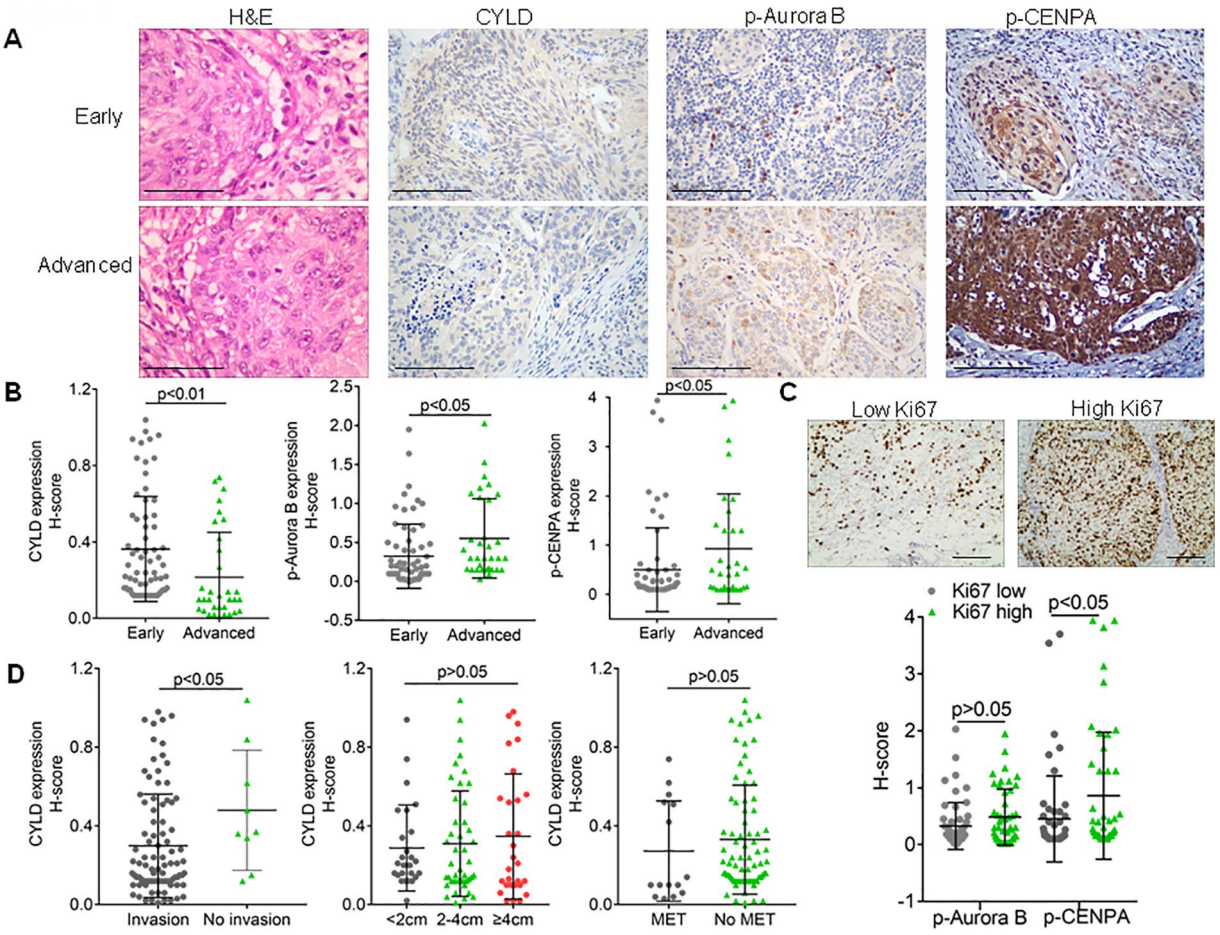

Figure 5. CYLD expression and Aurora B activity in clinical cervical cancer samples. (A) A panel of representative images showing H&E staining, CYLD, phospho-Aurora B and phospho-CENPA immunohistochemistry expression in early and advanced-staged cervical cancer samples. Scale bar represents 100 μm. (B) *H*-score evaluation of CYLD, phospho-Aurora B, and phospho-CENPA in early and advanced-staged cancer samples. Statistical results were presented as mean values ± SD. *P* value was calculated using Mann-Whitney *U*-test. (C) Patients were classified to 2 groups according with high and low Ki67 expression, and then phospho-Aurora B and phospho-CENPA level was analyzed. The upper panel shows the representative image of high and low Ki67 immunohistochemical staining and the down panel displays the statistical results, presented as mean values ± SD. *P* value was calculated using Mann-Whitney *U*-test. (D) Patients were classified to 2 or 3 groups according with local histological cancer cell invasion or not (left panel), with tumor diameter (middle panel), and with histological lymph node metastasis or not (right panel). And then, the CYLD level of each group was analyzed and displayed with statistical results, presented as mean values ± SD. *P* value was calculated using the Mann-Whitney *U*-test.
CYLD indicates cylindromatosis; H&E, hematoxylin and eosin.

CYLD expression and Aurora B activity in clinical cervical cancer samples

Because CYLD is found to play a tumor-suppressive role in various cancers such as melanoma, colon cancer, liver cancer, and prostate cancer,[22,30] we speculate it also takes an action in cervical cancer. Then, we investigated the relationship between CYLD and Aurora B activation in human cervical cancer samples. We analyzed 100 cancer samples whose clinical characteristics were summarized in Table 1 and found a reverse correlation between CYLD and p-Aurora B expression, between CYLD and p-CENPA expression as well (Figure 5A and B). Furthermore, there were more expression of p-Aurora B and p-CENPA and less expression of CYLD in late stage (n = 33) cancer samples than those in early stage (n = 67) (Figure 5A and B). It indicates higher Aurora B activity and less CYLD expression in late stage cervical cancer samples. As Ki67 is an important index represents cell proliferation, then we analyzed the correlation between Ki67 level and expression of p-Aurora B and p-CENPA in these samples. Actually, we found in Figure 5C a positive relationship between Ki67 (Ki67 low: n = 53, Ki67 high: n = 47) and p-CENPA. And there was also a positive tendency between Ki67 and p-Aurora B although without statistical significance. Maybe we will get a positive result with statistical significance if we expand the sample size. Furthermore, we also investigated the correlation between expression of CYLD and clinicopathological parameters of cervical cancer patients including local histological cancer cell invasion, tumor diameter, and lymph node metastasis confirmed by pathology after surgery. We found in Figure 5D that expression of CYLD was higher in non-invasive (n = 9) cancer samples than that in invasive (n = 91) ones. However, there was no difference of CYLD level in samples with different tumor

Table 1. Clinical characteristics of cervical cancer patients (n = 100).

Age (years)	
Median (range)	50 (30-68)
The pathology of the primary tumor	
Squamous carcinoma	70
Adenocarcinoma	14
Adenosquamous carcinoma	6
Others	10
Lymph node metastasis confirmed by pathology after surgery	
Yes	15
No	78
Without surgery	7
Tumor size	
Less than 2 cm	26
2-4 cm	45
Equal or more than 4 cm	29
Disease stage	
I	67
II	15
III	18

diameters (less than 2 cm: n = 27, 2-4 cm: n = 45, more than 4 cm: n = 28) and histopathologically confirmed lymph node metastatic state (with metastasis: n = 78, without metastasis: n = 15) (Figure 5D). Our data displayed that CYLD was less expressed and Aurora B activity was stronger in late staged cervical cancer samples compared with early staged samples, and CYLD expression was lower in invasive cancer samples than those in no-invasive ones.

Discussion

Traditionally, ubiquitination is always thought to target protein for degradation, but recently K63-linked ubiquitination is suggested to activate protein kinase activity and function in several studies of our team.[23,25,29] Aurora B is a key kinase in cell mitosis, whose activity depends on the phosphorylation of threonine at position 232.[31,32] And Aurora B expression is mainly regulated by K48-linked ubiquitination-mediated protein degradation.[33] Our previous research demonstrates that Skp2-triggered Aurora B ubiquitination is essential for Aurora B activation in cell mitosis and spindle checkpoint.[10] We speculate it is K63-linked ubiquitination-mediated protein kinase activation that needs our further validation. In this study, we further identify the major ubiquitination site of Aurora B that is also essential for its activity and function.

Ubiquitination modification is a reversible process. As a DUB CYLD is responsible for the removement of K63-linked poly-ubiquitin chain from Skp2-triggered Akt ubiquitination and further inhibit Akt activity, we doubt that CYLD is also a DUB for Aurora B. First, we discovered the interaction of CYLD and Aurora B that may provide the chance for CYLD to play its DUB function. We indeed show that CYLD can effectively reduce Aurora B ubiquitination and further inhibits its activity and function as well, indicating that CYLD may be a DUB for Aurora B. In this study, we provide novel insight into regulation of Aurora B deubiquitination, which further verifies our notion that cycles of Aurora B ubiquitination and deubiquitination may be responsible for its activity and function. In the future, we will focus on the more detailed mechanism of CYLD promoting Aurora B deubiquitination.

Cervical cancer greatly threats women's health especially in low-income countries. Research about cervical cancer tumorigenesis, invasion, and migration will bring great help to its prevention and treatment. Cylindromatosis is identified as an important tumor suppressor in various human cancers. Here, in this study, we discover that CYLD knock-down in cervical cancer cells promote cell proliferation, colony formation, cell migration and invasion, and inhibit apoptosis instead, whereas it is opposite with CYLD over-expression. It further verifies the tumor suppressor role of CYLD in cervical cancer cells.

Due to its negative regulation in NF-κB, MAPK, Wnt, and Akt pathways, CYLD has been established as an important tumor suppressor in various malignancies in recent years.[17-22] Here, we show that CYLD may also play a tumor-suppressive role in cervical cancer. However, the mechanism of CYLD as a tumor suppressor still remains largely unknown. Here, we reveal the new tumor suppressing function of CYLD as a negative regulator in cell mitosis progression. As an important regulator in cell cycle control, previously we discovered for the first time that Aurora B ubiquitination was essential for its kinase activity and identified Skp2 as its E3 ubiquitination ligase.[10] And here, we show for the first time again that the deubiquitinase CYLD is a novel potential DUB of Aurora B and plays a negative role in cell cycle progression, which may contribute to its tumor-suppressive function in cervical cancer. As an oncogene Aurora B is always found to be over-expressed or hyperactivated in several cancers. Meanwhile Aurora B expression positively correlates with cancer malignancy in prostate cancer and colorectal cancer.[34,35] Consistently, we show increased p-Aurora B expression in advanced-staged cervical cancer samples compared with those in early staged samples, accompanied with CYLD lower expression. As we propose that Aurora B activity is inhibited by CYLD, we think that following the progression of cervical cancer the gradual loss expression of CYLD leads to Aurora B out-of-control and activation. This study will bring great value to the mechanism study of cervical cancer tumorigenesis and migration and also provide new evidence for its prevention and treatment.

Conclusions

In this study, we demonstrated that CYLD interacted with Aurora B and promoted its deubiquitination and further regulated Aurora B activity and function. In early staged and no-invasive cervical cancer samples, CYLD expression was lower, and in late staged or invasive samples, p-Aurora B and p-CENPA expression was higher. Our data indicated that CYLD may play its tumor-suppressive role in cervical cancer through its potential DUB function. CYLD could be recognized as a prognostic marker and therapeutic target of cervical cancer in the future.

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Author Contributions

JW and JW contributed to the conception and design of the study. YH, WZ, ZY, and HS designed the study and performed most of the experiments. BZ and JP performed some of the immunohistochemical (IHC) staining, and JW and YH contributed to data analysis and writing of the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The study was approved by the Medical Ethical Reviewer Board of Affiliated Cancer Hospital of Guangzhou Medical University (2014 Ethical Review no. 49). Written informed consent was obtained from all patients.

Consent for Publication

Not applicable.

Availability of Data and Materials

The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding authors.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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