

KEN-Box-dependent Degradation of the Bub1 Spindle Checkpoint Kinase by the Anaphase-promoting Complex/Cyclosome*

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Wei Qi and Hongtao Yu¹

From the Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas 75390

The spindle checkpoint is a cell cycle surveillance mechanism that ensures the fidelity of chromosome segregation during mitosis and meiosis. Bub1 is a protein serine-threonine kinase that plays multiple roles in chromosome segregation and the spindle checkpoint. In response to misaligned chromosomes, Bub1 directly inhibits the ubiquitin ligase activity of the anaphase-promoting complex or cyclosome (APC/C) by phosphorylating its activator Cdc20. The protein level and the kinase activity of Bub1 are regulated during the cell cycle; they peak in mitosis and are low in G₁/S phase. Here we show that Bub1 is degraded during mitotic exit and that degradation of Bub1 is mediated by APC/C in complex with its activator Cdh1 (APC/C^{Cdh1}). Overexpression of Cdh1 reduces the protein levels of ectopically expressed Bub1, whereas depletion of Cdh1 by RNA interference increases the level of the endogenous Bub1 protein. Bub1 is ubiquitinated by immunopurified APC/C^{Cdh1} *in vitro*. We further identify two KEN-box motifs on Bub1 that are required for its degradation *in vivo* and ubiquitination *in vitro*. A Bub1 mutant protein with both KEN-boxes mutated is stable in cells but fails to elicit a cell cycle phenotype, indicating that degradation of Bub1 by APC/C^{Cdh1} is not required for mitotic exit. Nevertheless, our study clearly demonstrates that Bub1, an APC/C inhibitor, is also an APC/C substrate. The antagonistic relationship between Bub1 and APC/C may help to prevent the premature accumulation of Bub1 during G₁.

Ubiquitin- and proteasome-dependent proteolysis is one of the key mechanisms that ensure the uni-directional progression of the cell cycle (1, 2). The ubiquitin molecule is activated by the ubiquitin-activating enzyme, transferred to a ubiquitin-conjugating enzyme, and finally attached to a lysine residue in the substrate with the help of a ubiquitin ligase (E3).² These reactions can be repeated to allow the formation of ubiquitin

chains on substrates. Polyubiquitinated substrates are then recognized by proteasome for destruction (3). The efficiency and specificity of substrate ubiquitination are usually determined by the E3s (3).

The anaphase-promoting complex or cyclosome (APC/C) is a multisubunit E3 ubiquitin ligase and is required for the proper segregation of sister chromatids and for the exit from mitosis (4). Prior to anaphase, the sister chromatids are bound together by the cohesin protein complex that consists of Smc1, Smc3, a kleisin subunit (Scc1/Rad21/Mcd1), and Scc3 (known as SA1 and SA2 in vertebrates). At the metaphase-anaphase transition, APC/C together with its activator Cdc20 ubiquitinates securin and targets it for degradation (5, 6). Securin is an inhibitor of separase, the protease that cleaves the Scc1 subunit of cohesin to allow sister chromatid separation. Therefore, the activation of APC/C^{Cdc20} triggers the degradation of securin, activation of separase, cleavage of cohesin, and the onset of sister chromatid separation (7). The activity of APC/C is controlled by two related activators, Cdc20 and Cdh1. Both Cdc20 and Cdh1 contain a C-terminal WD40 repeat domain and are involved in recruiting substrates to APC/C (6). APC/C^{Cdc20} and APC/C^{Cdh1} perform distinct functions. APC/C^{Cdc20} is required for sister chromatid separation and the metaphase-anaphase transition by ubiquitinating securin and cyclin B1, whereas APC/C^{Cdh1} mediates the degradation of a broader spectrum of substrates in late anaphase and early G₁ (4). Many APC/C substrates contain cis-elements or degrons, called the destruction box (D-box) or KEN box, that are required for their ubiquitination and degradation by APC/C.

The spindle checkpoint is a cell cycle surveillance mechanism that ensures the accuracy of chromosome segregation and helps to maintain genetic stability (6, 7). For sister chromatids to separate correctly, the two opposing kinetochores of a pair of sister chromatids must be captured by microtubules emanating from the two opposite poles of the mitotic spindle (bi-orientation). Once the pair of sister chromatids achieves bi-orientation, the kinetochores are under tension, because sister chromatid cohesion resists the spindle pulling force and holds the two sisters together. The spindle checkpoint senses the lack of attachment and tension at the kinetochore and transduces the signal to prevent premature sister chromatid separation until the correct kinetochore-microtubule attachment is established for all sister chromatids (7). The spindle checkpoint selectively inhibits the activity of APC/C^{Cdc20}, stabilizes securin, delays the activation of separase, and prevents premature sister chromatid separation (7).

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¹ Michael Rosenberg Scholar in Biomedical Research. To whom correspondence should be addressed: Dept. of Pharmacology, University of Texas Southwestern Medical Center, 6001 Forest Park Rd., Dallas, TX 75390-9041. Tel.: 214-645-6161; Fax: 214-645-6156; E-mail: hongtao.yu@utsouthwestern.edu.

² The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; RNAi, RNA interference; APC/C, anaphase-promoting complex or cyclosome; CHX, cycloheximide; siRNA, small interfering RNA; FACS, fluorescence-activated cell sorter; Dox, doxycycline.

Bub1 is a key kinase involved in the spindle checkpoint signaling (8). Genetic studies in yeast have firmly established the requirement of Bub1 for proper spindle checkpoint function (9, 10). Bub1 localizes to kinetochores from early prophase to metaphase, and the kinetochore localization of Bub1 is diminished after anaphase onset (11). Bub1 is also required for the kinetochore localization of other components of the spindle checkpoint, such as BubR1 and Mad2 in vertebrates and yeast (12–14). Furthermore, Bub1 phosphorylates Cdc20 directly and therefore inhibits the activity of APC/C (15). Phosphorylation of Cdc20 by Bub1 is required for efficient checkpoint signaling (15). Consistently, the kinase activity of Bub1 is enhanced in mitosis (15). The mechanism by which Bub1 is regulated is still unknown. Bub1 is phosphorylated in mitosis in yeast (16), *Xenopus* (17, 18), and mammalian cells (15, 19). In *Xenopus* egg extract, the chromosome-associated Bub1 is hyperphosphorylated and exhibits higher autophosphorylation activity (17). However, it is unclear whether phosphorylation regulates the kinase activity of Bub1 and whether there are additional mechanisms that regulate Bub1.

Here we show that the Bub1 protein level oscillates during the cell cycle. Bub1 is degraded in late anaphase and G₁ by APC/C^{Cdh1}. A fragment of Bub1 is ubiquitinated *in vitro* by APC/C^{Cdh1}. We further identify two KEN-boxes in human Bub1 that are required for its degradation *in vivo* and for its APC/C-dependent ubiquitination *in vitro*. A stable, inducible cell line that expresses a Bub1 mutant containing mutations in these two KEN-boxes undergoes mitotic exit with regular kinetics during the normal cell cycle or during the recovery from nocodazole-mediated mitotic arrest. Therefore, APC/C^{Cdh1}-dependent degradation of Bub1 is not required for the normal progression through mitosis. Instead, it may safeguard cells from prematurely accumulating Bub1 during G₁ or quiescence.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies, and Immunoblotting—The production of the Bub1, APC3 (Cdc27), and APC2 antibodies was described previously (5, 15, 20). The cyclin B1 antibody was purchased from Santa Cruz Biotechnology. The Cdh1 antibody was from Neomarker. HA and Myc antibodies were from Roche Applied Science. For immunoblotting, the antibodies were used at 1:1000 dilution for crude sera or 1 μg/ml for purified IgG.

Tissue Culture, Drug Treatments, and Transfection—HeLa Tet-On (Clontech) and HCT116 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and 10 mM L-glutamine. To arrest cells at G₁/S, cells were incubated in the growth medium containing 2 mM thymidine (Sigma) for 18 h. To obtain mitotic cells, the cultured cells were treated with 100 ng/ml nocodazole (Sigma) for 16–18 h.

To analyze the stability of the Bub1 protein in late anaphase, the cells were treated with nocodazole for 16 h. Nocodazole was then washed out, and the cells were replated for 1 h before cycloheximide (50 μM) was added to the medium. Cells were harvested at different time points after cycloheximide addition.

To analyze the protein stability in metaphase, cycloheximide was added without washing out nocodazole.

Transfection was performed when cells reached about 50% confluence using the Effectene reagent (Qiagen) according to the manufacturer's instructions. The plasmids were derived from pCS2-Myc or pCS2-HA. For RNAi experiments, the siRNA oligonucleotides targeting human Cdh1 (GAAGGGU-CUGUUCACGUAUTT) was chemically synthesized by Dharmacon. HeLa cells were transfected as described (15) and analyzed 48 h after transfection.

To establish cell lines that stably express Myc-Bub1 and Myc-Bub1-KdM, HeLa Tet-On cells at 40% confluence were transfected with pTRE2-hygro-Myc-Bub1 and pTRE2-hygro-Myc-Bub1-KdM plasmids. The cells were then selected with 300 μg/ml hygromycin (Clontech). The surviving clones were expanded and screened for the induced expression of Myc-tagged Bub1 proteins in the absence or presence of 1 μg/ml doxycycline (Clontech).

FACS Analysis—HeLa Tet-on cells were harvested by trypsin digestion and then washed once with phosphate-buffered saline. The cells were fixed with 70% ethanol that had been pre-cooled to –20 °C. After overnight fixation on ice, the cells were stained with propidium iodide and analyzed by flow cytometry. The data were acquired and analyzed using the CellQuest software.

In Vitro Transcription and Ubiquitination Assays—The *in vitro* transcription and translation system was purchased from Promega, and the reactions were performed according to manufacturer's instructions. Briefly, 80 ng of plasmid DNA, nuclease-free water, 0.2 μl of [³⁵S]methionine (10 μCi/μl), and 4 μl of rabbit reticulocyte lysate were mixed to give a final volume of 5 μl. The reaction mixture was incubated at 30 °C for 90 min.

The expression and purification of human Cdc20 and Cdh1 proteins from Sf9 cells were described previously (20). A fragment of Bub1 containing residues 520–653 (Bub1^{520–653}) and the corresponding KEN-box mutants were expressed as His₆-tagged fusion proteins in bacteria and purified using Ni²⁺-nitrilotriacetic acid beads (Qiagen). The interphase APC/C was purified from interphase *Xenopus* egg extracts using anti-APC3/Cdc27 antibody coupled to protein A support. After washing with high salt XB buffer (10 mM HEPES, pH 7.7, 500 mM KCl, 0.1 mM CaCl₂, 1 mM MgCl₂, 50 mM sucrose, and 0.5% Nonidet P-40) five times and with XB buffer (10 mM HEPES pH 7.7, 100 mM KCl, 0.1 mM CaCl₂, 1 mM MgCl₂, and 50 mM sucrose) twice, the APC/C beads were incubated with recombinant Cdc20 or Cdh1 proteins for 1 h at room temperature. The APC/C beads were again washed twice with XB buffer and assayed for its ability to ubiquitinate an N-terminal fragment of human cyclin B1 or various Bub1 fragments. Each ubiquitination assay contained a 5-μl mixture of an energy-regenerating system, 150 μM ubiquitin, 5 μM recombinant ubiquitin-activating enzyme, 2 μM recombinant Ubch10, 1 μl of *in vitro* transcribed and translated substrates, and 3 μl of the APC/C beads. The reactions were incubated under constant shaking for 1 h at room temperature, quenched with SDS sample buffer, resolved by SDS-PAGE, and analyzed using a PhosphorImager.

Immunoprecipitation Kinase Assays—The His₆-tagged Cdc20 N-terminal fragment containing residues 1–170

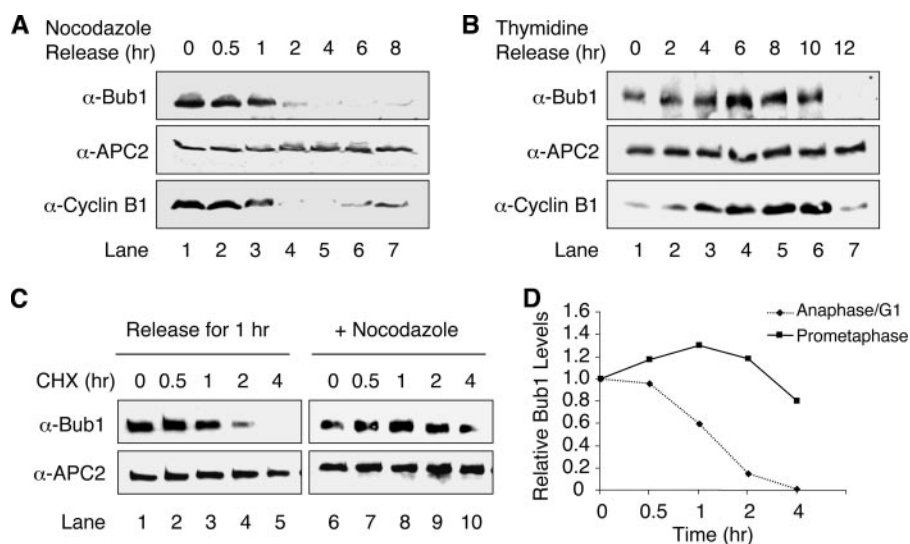


FIGURE 1. Bub1 is rapidly degraded during mitotic exit. *A*, HCT116 cells were arrested in mitosis by 18 h of nocodazole treatment, released into fresh growth medium, and collected at the indicated times for Western blot analysis with anti-Bub1 antibody. APC2 blot was used as a loading control. Cyclin B1 was also blotted to indicate proper mitotic exit. *B*, HeLa cells were blocked at the G₁/S boundary by thymidine, released into fresh medium, and harvested for Western blot with antibodies against Bub1, APC2, and cyclin B1 at the indicated times. *C*, HeLa cells arrested in mitosis were replated for 1 h in the presence or absence of nocodazole. CHX was then added. Cell lysates were prepared at the indicated times and subjected to immunoblotting with the indicated antibodies. *D*, quantification of the relative Bub1 protein levels is shown in *C*.

(Cdc20NT) was expressed in bacteria and purified using Ni²⁺-nitrilotriacetic acid beads (Qiagen). The anti-Myc monoclonal antibody (Roche Applied Science) was coupled to Affi-Prep protein A beads (Bio-Rad) at a concentration of 1 mg/ml. HeLa cells were transfected with plasmids encoding Myc-Bub1, Myc-Bub1-kinase dead (KD), or Myc-Bub1-KdM and treated with nocodazole. After 18 h, cells were lysed with the lysis buffer (50 mM Tris-HCl, pH 7.7, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM dithiothreitol, 0.5 μM okadaic acid, and 10 μg/ml each of leupeptin, pepstatin, and chymostatin). After clearing by centrifugation for 30 min at 4 °C at 13,000 rpm, the lysate was incubated with the antibody beads for 2 h at 4 °C. The beads were washed with the wash buffer (50 mM Tris-HCl, pH 7.7, 500 mM NaCl, 0.5% Nonidet P-40) three times and with kinase buffer two times. The proteins bound to the beads were then used directly in the kinase reactions. The kinase assays were performed in 20-μl reactions. Each reaction contains 100 μM cold ATP, 0.1 μCi/μl [³²P]ATP, 1 μM Cdc20NT protein in the kinase buffer (50 mM Tris-HCl pH 7.7, 100 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol). The reactions were incubated at room temperature for 30 min, quenched with SDS sample buffer, and analyzed by SDS-PAGE followed by autoradiography or immunoblotting with anti-Myc.

RESULTS

Bub1 Is Degraded in Late Anaphase and G₁—Previous studies showed that the Bub1 protein level is high in nocodazole-arrested mitotic cells but low in G₁/S cells enriched by a thymidine block (21). We hypothesized that Bub1 is regulated at the protein level and thus examined the steady state levels of Bub1 in synchronized human tissue culture cells. Cells were arrested at metaphase by nocodazole treatment, released into normal growth medium, and then harvested at the indicated time

points. The level of Bub1 protein was then analyzed by SDS-PAGE and Western blotting. As shown in Fig. 1*A*, the protein level of Bub1 decreased sharply as cells underwent mitotic exit, whereas the level of an APC/C subunit, APC2, remained constant (Fig. 1*A*, upper panel). The expression pattern of Bub1 was similar to that of cyclin B1, a well established APC/C substrate (Fig. 1*A*, bottom panel). The timing of Bub1 down-regulation was identical in HCT116 (Fig. 1*A*) and HeLa cells (data not shown). To rule out the possibility that the decrease of the Bub1 protein level only occurred during mitotic exit following the recovery from nocodazole-mediated mitotic arrest, we synchronized HeLa cells at the G₁/S boundary using a double-thymidine block. The cells were then released into fresh medium and harvested at different time points after release.

The Bub1 protein accumulated gradually during G₁ and S, peaked at G₂/M, and dropped dramatically after mitosis (Fig. 1*B*). The expression pattern of Bub1 protein again mirrored that of cyclin B1 during this experiment.

The reduction in the protein level of Bub1 during mitotic exit may be caused by increased protein degradation or decreased protein synthesis. To assess whether the reduction in the Bub1 protein level was because of protein degradation, we measured the half-life of Bub1 by cycloheximide (CHX) chase in nocodazole-arrested prometaphase cells and in late anaphase or early G₁ cells released from nocodazole block for 1 h. We found that Bub1 was stable in prometaphase cells, and it was degraded with a half-life of about 1 h in late anaphase/early G₁ cells (Fig. 1*C*). This result indicated that Bub1 was rapidly degraded during mitotic exit.

Cdh1 Overexpression Reduces the Levels of Bub1 in Cells—The oscillation pattern of the Bub1 protein during the cell cycle is very similar to those of known substrates of APC/C^{Cdh1}, such as cyclin B1 (22), Cdc20 (23), and Plk1 (24), suggesting that Bub1 may also be an APC/C^{Cdh1} substrate. We thus tested whether Cdh1 promoted the degradation of Bub1 in cells. HeLa cells were co-transfected with vectors encoding Myc-Bub1 and HA-Cdh1 and treated with cycloheximide. The protein level of Myc-Bub1 was examined by α-Myc blot. The protein level of Myc-Bub1 was decreased considerably when Cdh1 was co-expressed (Fig. 2*A*, compare lanes 1 and 4). Furthermore, Myc-Bub1 was relatively stable in the absence of HA-Cdh1, whereas co-expression of HA-Cdh1 reduced the half-life of Myc-Bub1 to about 30 min (Fig. 2*A*). These results suggested that Bub1 might be a substrate of APC/C^{Cdh1}.

The KEN-box Motifs of Bub1 Are Required for Its Cdh1-dependent Degradation in Vivo—Previous studies revealed that APC/C recognizes two types motifs in its substrates, the D-box

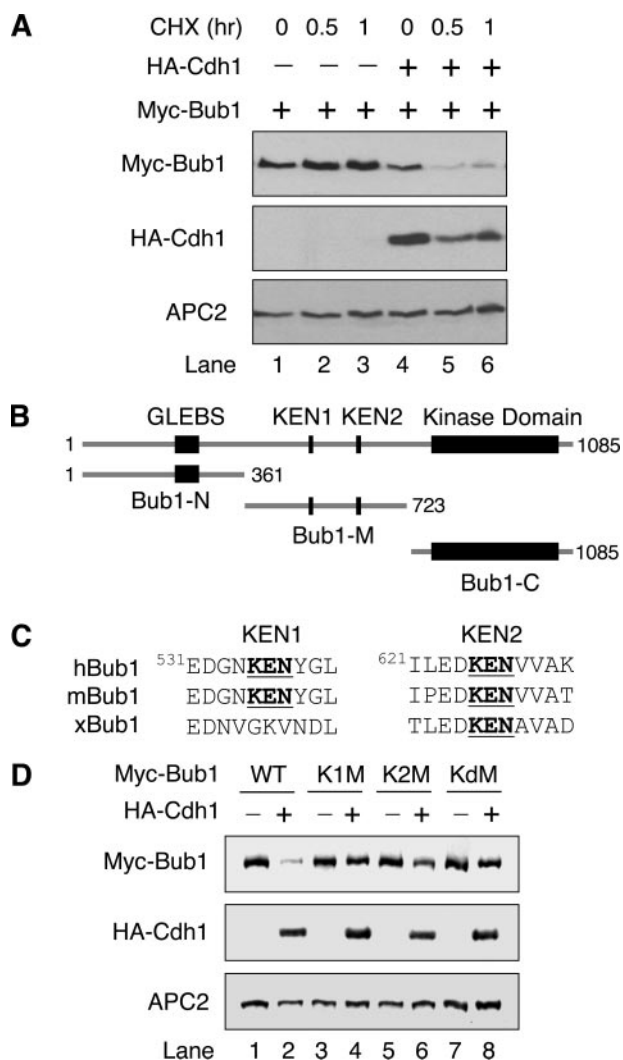


FIGURE 2. Ectopically expressed Cdh1 reduces the levels of Bub1 in a KEN-box-dependent manner. *A*, HeLa cells were co-transfected with Myc-Bub1 with or without HA-Cdh1 constructs. Twenty four hours after transfection, CHX was added for the indicated times. Cells were collected and subjected to Western blot with the indicated antibodies. *B*, domain structure of human Bub1. The GLEBS motif required for Bub3 binding, two putative KEN-boxes, and the kinase domain are indicated. Three Bub1 fragments used in later experiments are also shown. *C*, alignment of the sequences surrounding the two putative KEN-box motifs of the Bub1 orthologs. The two putative KEN-box motifs are in **boldface** and underlined. *D*, HeLa cells were transfected with Myc-Bub1 wild-type (WT) or mutant constructs (K1M, with Lys-535, Glu-536, and Asn-537 mutated to alanines; K2M, with Lys-625, Glu-626, and Asn-627 mutated to alanines; KdM, with both KEN-boxes mutated to alanines) together with or without HA-Cdh1, dissolved in SDS sample buffer, separated on SDS-PAGE, and blotted with the indicated antibodies.

(RXXLXXXXN) and the KEN-box (KEN) (25). By sequence analysis, no D-boxes were found in human Bub1. However, two putative KEN-box motifs were identified in the central region of Bub1 (Fig. 2, *B* and *C*). We thus constructed Bub1 mutants in which the first KEN-box (K1M), the second KEN-box (K2M), or both KEN-boxes (KdM) were mutated to alanine residues, and we tested whether the protein levels of these Bub1 mutants were reduced by Cdh1 overexpression. HeLa cells were transfected with plasmids encoding Myc-Bub1 or the three Myc-Bub1 KEN-box mutants with or without the HA-Cdh1 expression vector. The protein levels of Myc-Bub1 mutants were examined by α -Myc blot. The protein levels of Bub1-K1M and

Bub1-KdM were not significantly reduced in the presence of Cdh1 (Fig. 2*D*). Bub1-K2M also showed partial stabilization as compared with the wild-type Bub1 protein (Fig. 2*D*). These results indicated that both KEN-boxes are involved in the degradation of Bub1 and that the first KEN-box is the major degron of Bub1. The fact that degradation of Bub1 in the presence of excess amount of Cdh1 depended on KEN-boxes further suggested that Bub1 might be a substrate of APC/C^{Cdh1}.

Bub1 Is Ubiquitinated by APC/C^{Cdh1} in Vitro—To confirm that Bub1 was a substrate of APC/C, we tested whether immunopurified APC/C^{Cdh1} directly catalyzed the ubiquitination of Bub1 *in vitro*. Human Bub1 is a large protein with 1085 residues. It would be difficult to resolve ubiquitin conjugates of the full-length Bub1 protein on SDS-PAGE. We thus designed three fragments of Bub1 as follows: Bub1-N, Bub1-M, and Bub1-C, each containing one-third of the Bub1 protein (Fig. 2*B*). The fragments were translated *in vitro* in the presence of [³⁵S]methionine and used as substrates in the ubiquitination assays. The N-terminal fragment of human cyclin B1 (residues 1–102) was used as the positive control (20). Cyclin B1 was efficiently ubiquitinated by both APC/C^{Cdh1} and APC/C^{Cdc20} (Fig. 3*A*, left panel). Of the three Bub1 fragments, only Bub1-M was ubiquitinated by APC/C^{Cdh1} but not by APC/C^{Cdc20} (Fig. 3*A*). This indicates that Bub1 is a substrate of APC/C^{Cdh1} and that the APC/C recognition elements of Bub1 are located in the central region of the protein. To further narrow down the ubiquitination sites and obtain more efficient ubiquitination of Bub1, we expressed and purified a fragment of Bub1 containing residues 520–653 (named Bub1^{520–653}) in bacteria and tested it in the *in vitro* ubiquitination assay. The Bub1^{520–653} fragment was efficiently ubiquitinated by APC/C^{Cdh1} and to a lesser extent by APC/C^{Cdc20} (Fig. 3*B*).

Both KEN-boxes are contained in the central fragment of Bub1 and in Bub1^{520–653}. We therefore introduced the same KEN-box mutations into Bub1-M and Bub1^{520–653} and tested the ability of these mutants to be ubiquitinated by APC/C^{Cdh1} *in vitro*. Consistent with the results from the *in vivo* degradation assay, mutation of the first KEN-box in Bub1-M dramatically decreased the amount of the polyubiquitinated species (Fig. 3*C*, compare lanes 2 and 4). Mutation of the second KEN-box had a less dramatic effect (Fig. 3*C*, compare lanes 2 and 6). Mutation of both KEN-boxes completely eliminated the ubiquitination of Bub1-M by APC/C^{Cdh1} (Fig. 3*B*, lane 8). Similar results were obtained using recombinant purified Bub1^{520–653} and the corresponding KEN-box mutants as substrates (Fig. 3*D*). Mutation of either KEN-box decreased ubiquitination. Mutation of both KEN-boxes led to the loss of ubiquitination. Taken together, our results clearly establish that Bub1 is ubiquitinated by APC/C *in vitro* in a KEN-box-dependent manner.

Cdh1 Mediates the Degradation of Bub1 in Vivo—To further test whether Cdh1 was required for the degradation of the endogenous Bub1 *in vivo*, we used siRNA to deplete Cdh1, and we measured the levels of the endogenous Bub1 and other known APC/C substrates, such as cyclin B1, by immunoblotting (Fig. 4*A*). The Cdh1 siRNA efficiently knocked down the expression of Cdh1 (Fig. 4*A*). The steady state levels of Bub1 and cyclin B1 were significantly elevated in Cdh1-RNAi cells (Fig. 3*A*). This result suggested that APC/C^{Cdh1} was a major

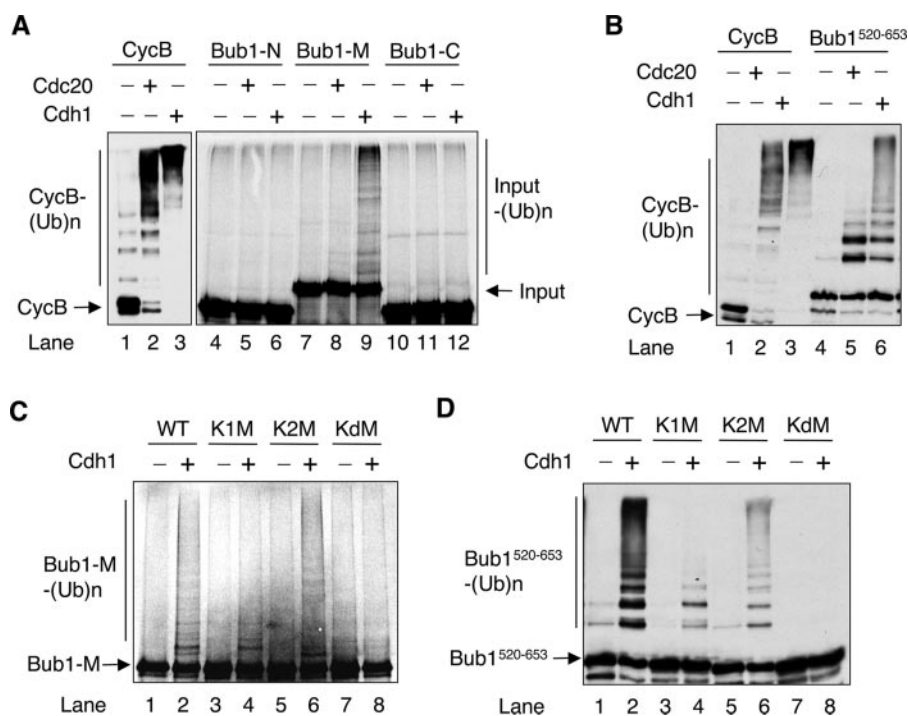


FIGURE 3. Bub1 is ubiquitinated by APC/C^{Cdh1} *in vitro*. *A*, APC/C was immunoprecipitated from *Xenopus* egg extracts and supplemented with recombinant Cdc20 or Cdh1. The recombinant Myc-tagged human cyclin B1 fragment (residues 1–102) or *in vitro* translated ³⁵S-labeled Myc-Bub1 fragments (Bub1-N, Bub1-M, and Bub1-C) were used as substrates. The reaction mixtures were separated on SDS-PAGE and analyzed by α -Myc blot in the case of cyclin B1 fragment or autoradiography in the case of Myc-Bub1 fragments. *B*, same as *A* except that the recombinant purified Myc-tagged human cyclin B1 fragment (residues 1–102) or Bub1 fragment (residues 520–653) was used as substrate for ubiquitination. *C*, same as *A* except that the wild-type (WT) and mutant Myc-Bub1-M fragments were used as substrates for ubiquitination. *D*, same as *A* except that the recombinant wild-type (WT) and mutant Bub1^{520–653} fragments were used as substrates for ubiquitination.

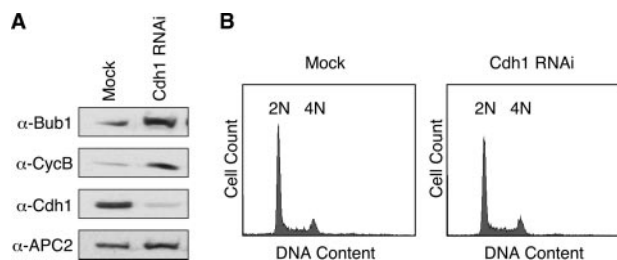


FIGURE 4. Depletion of Cdh1 from HeLa cells by RNAi causes accumulation of Bub1. *A*, HeLa cells transfected with control or Cdh1 siRNA were harvested. The total cell lysates were immunoblotted with the indicated antibodies. *B*, HeLa cells transfected with control or Cdh1 siRNA were fixed by ethanol and subjected to FACS analysis.

ubiquitin ligase for the degradation of Bub1 *in vivo*. Because the protein level of Bub1 was higher in mitosis, an alternative explanation of this result was that depletion of Cdh1 by RNAi caused a mitotic arrest/delay, which in turn caused the accumulation of Bub1 as a secondary effect. To rule out this possibility, we performed FACS analysis of mock-transfected and Cdh1-RNAi cells (Fig. 4*B*). No significant differences were observed between the cell cycle profiles of the mock-transfected and Cdh1-RNAi cells (Fig. 4*B*). Therefore, our results are consistent with the notion that APC/C^{Cdh1} mediates the degradation of Bub1 *in vivo*. We could not determine whether APC/C^{Cdc20} is involved in Bub1 degradation *in vivo*, because Cdc20-RNAi causes a mitotic arrest in human cells.

The KEN-box Mutant of Bub1 Is Stabilized during Mitotic Exit—We next tested whether the KEN-boxes of Bub1 are required for its degradation *in vivo* during mitotic exit. For this purpose, we established HeLa cell lines that stably expressed either Myc-Bub1 or Myc-Bub1-KdM driven by a tetracycline-inducible promoter. The stability of the Myc-Bub1 and Myc-Bub1-KdM proteins after nocodazole arrest-release was examined by blotting with anti-Myc antibody. As expected, Myc-Bub1 protein was degraded during mitotic exit after the recovery from the nocodazole-mediated mitotic arrest (Fig. 5*A*, lanes 1–6). In contrast, the Myc-Bub1-KdM protein was stable even at the 6-h time point after nocodazole release, when cells had already progressed to the G₁ phase (Fig. 5*A*, lanes 7–12). By using cycloheximide treatment, we also measured the half-lives of the Myc-Bub1 and Myc-Bub1-KdM proteins in the cells that were released from nocodazole arrest for 1 h. As shown in Fig. 5, *B* and *C*, Myc-Bub1 had a half-life of about 1.5 h, and Myc-Bub1-KdM was stable during the

course of the experiment. These results demonstrate that the KEN-box mutant of the Bub1 protein is not degraded during mitotic exit *in vivo*.

We noticed that securin and cyclin B1 were degraded with normal kinetics in Bub1-KdM-expressing cells (Fig. 5*A* and data not shown), suggesting that degradation of Bub1 is not required for the exit from mitosis following the recovery from a nocodazole-mediated mitotic arrest.

Degradation of Bub1 Is Not Required for Cellular Adaptation upon Prolonged Exposure to Nocodazole—We did not observe acute cell cycle arrest/delay as induced by Myc-Bub1-KdM. The protein level of Myc-Bub1-KdM in the presence of Dox was much higher than that of the endogenous Bub1, as the anti-Bub1 antibody only detected Myc-Bub1-KdM, not the endogenous Bub1, in this particular Western blot (Fig. 6*A*). Consistently, when the proliferative property of the Bub1-KdM-expressing clone was monitored, the growth curves of these cells in the absence or presence of doxycycline were very similar. Cells in both conditions divided with a doubling time of ~25 h (Fig. 6*B*). To exclude the possibility that mutation of the KEN-boxes decreased the kinase activity of Bub1, we compared the kinase activities of Bub1-WT and Bub1-KdM using immunoprecipitation kinase assays. Bub1-KdM immunoprecipitated from mitotic HeLa cells showed similar kinase activities toward itself (autophosphorylation) and the N-terminal fragment of Cdc20 (Cdc20NT) as did Bub1-WT (Fig. 6*C*). A kinase-dead mutant of Bub1 (Bub1-KD) did not exhibit detectable kinase

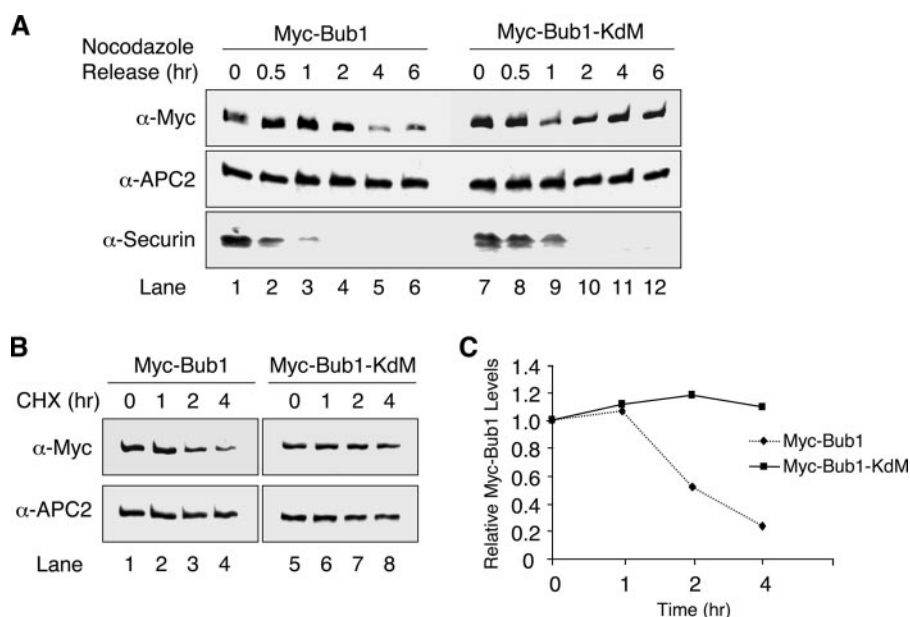


FIGURE 5. A KEN-box mutant of Bub1 is not degraded during mitotic exit. *A*, HeLa Tet-On cells stably transfected with Myc-Bub1 or Myc-Bub1-KdM were cultured in the presence of doxycycline, treated with nocodazole for 18 h, harvested, and analyzed by SDS-PAGE followed by immunoblotting with the indicated antibodies. *B*, HeLa Tet-On cells stably transfected with Myc-Bub1 or Myc-Bub1-KdM were cultured in the presence of doxycycline, treated with nocodazole for 18 h, and released into fresh medium for 1 h. CHX was then added into the medium for the indicated times. Cells were collected and analyzed by SDS-PAGE followed by immunoblotting with the indicated antibodies. *C*, quantification of the relative Myc-Bub1 protein levels in *B*.

activity and was used as the negative control (Fig. 6C). Similar amounts of Bub1-WT, Bub1-KdM, and Bub1-KD were present in the immunoprecipitates (Fig. 6C). This result indicates that Bub1-KdM retains its kinase activity.

It has been reported recently that another spindle checkpoint protein, BubR1, is degraded in polyploid cells that had adapted and survived a prolonged nocodazole treatment, and re-introduction of BubR1 causes apoptosis of these polyploid cells (26). Re-introduction of Bub1 into these polyploid cells also causes apoptosis, although the apoptosis-inducing effect of Bub1 was less dramatic than that of BubR1 (26). We therefore tested whether induction of the expression of Bub1-KdM caused apoptosis in polyploid HeLa cells that had adapted during prolonged nocodazole treatment. HeLa cells were treated with nocodazole for the indicated duration in the presence or absence of doxycycline and were then harvested for FACS analysis. As shown in Fig. 6C, after 18 h of treatment, most of the cells in both conditions had 4N DNA content, indicating they were arrested in G₂/M. By 54 h, almost all the cells were in the sub-G₁ population, which corresponded to apoptotic cells. A minor population of cells was able to adapt and possessed 8N DNA content (polyploid) (Fig. 6C). However, doxycycline-induced expression of Bub1-KdM did not significantly alter the percentage of polyploid cells (Fig. 6C). Therefore, degradation of Bub1 did not appear to be required for the adaptation process when cells were subjected to a prolonged mitotic arrest.

DISCUSSION

Bub1 Is a Substrate of APC/C^{Cdh1}—We have presented several lines of evidence to indicate that Bub1 is degraded during the exit from mitosis and that APC/C^{Cdh1} is the E3 ubiquitin

ligase for the ubiquitination of Bub1. First, the oscillation pattern of Bub1 closely resembles that of other APC/C^{Cdh1} substrates, such as cyclin B1 (22) and Plk1 (24). Second, overexpression of Cdh1 decreases the level of ectopically expressed Bub1 in cells. Third, APC/C^{Cdh1} ubiquitinates Bub1 *in vitro*. Fourth, mutation of KEN-boxes in Bub1 stabilizes Bub1 *in vivo* and abrogates Bub1 ubiquitination *in vitro*. Finally and most importantly, depletion of Cdh1 by RNAi leads to an accumulation of the Bub1 protein in living cells.

The two related activators of APC/C, Cdc20 and Cdh1, are involved in recruiting substrates to APC/C (4, 27). However, APC/C^{Cdc20} and APC/C^{Cdh1} perform distinct functions and are differentially regulated during the cell cycle (23, 27, 28). The functional difference between Cdc20 and Cdh1 is partly because of their different substrate specificities (6). Cdc20 recognizes

the D-box in substrates (29), whereas Cdh1 is less selective, recognizing a variety of elements, including the D-box, KEN-box, and other types of motifs (29–31). We have identified two KEN-boxes in Bub1 that are required for its ubiquitination and degradation, again consistent with APC/C^{Cdh1} being the major form of APC/C that mediates Bub1 degradation. On the other hand, the involvement of APC/C^{Cdc20} in Bub1 degradation *in vivo* cannot be formally excluded because Cdc20 RNAi is known to cause mitotic arrest.

Both KEN-boxes of human Bub1 are required for its efficient degradation, although the first KEN-box appears to be the major degron. Interestingly, the first KEN-box is not conserved in *Xenopus* Bub1. One possibility is that the second KEN-box in *Xenopus* Bub1 functions as the degron. Alternatively, *Xenopus* Bub1 is not efficiently degraded following mitotic exit.

Bub1 Degradation and Spindle Checkpoint Inactivation—Bub1 is required for the inhibition of APC/C when the spindle checkpoint is activated (15). Therefore, the degradation of Bub1 during mitotic exit is expected to contribute to the inactivation of the spindle checkpoint and may help to prevent the reactivation of the spindle checkpoint in late anaphase. However, expression of the nondegradable Bub1-KdM in cells does not result in a mitotic arrest. Cells expressing Bub1-KdM also undergo mitotic exit with normal kinetics during the recovery from a nocodazole-mediated mitotic arrest. These results suggest that degradation of Bub1 is not the sole mechanism to inactivate the spindle checkpoint in mammalian cells.

Recently, the yeast spindle checkpoint kinase, Mps1, has been shown to be degraded by APC/C (32). Forced expression of Mps1 in anaphase re-establishes the spindle checkpoint. It has been proposed that the mutual inhibition of Mps1 and

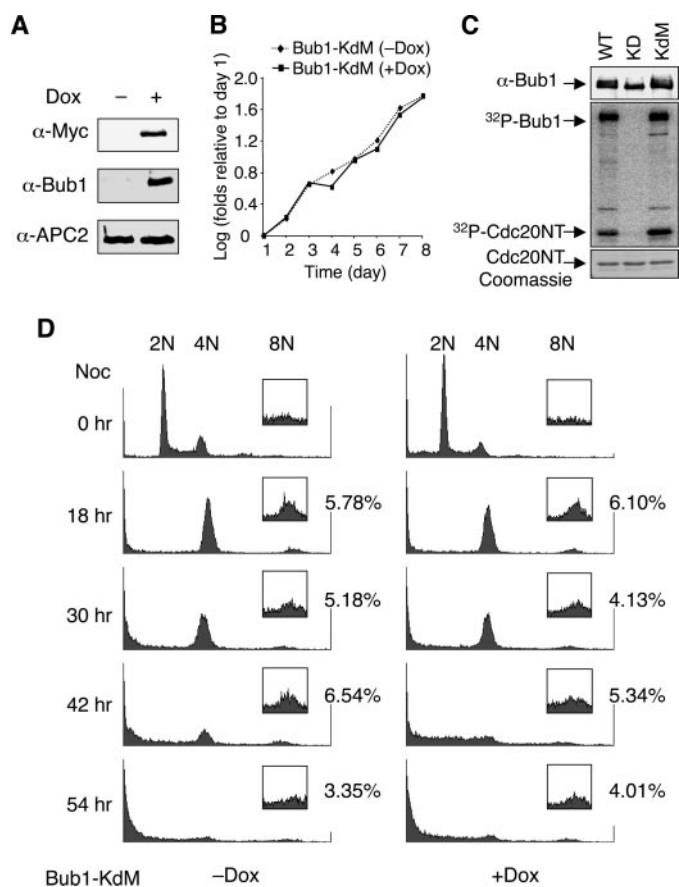


FIGURE 6. Overexpression of a nondegradable form of Bub1 does not cause mitotic arrest or prevent cellular adaptation after prolonged nocodazole treatment. *A*, HeLa cells stably transfected with Myc-Bub1-KdM construct were cultured in the absence (–Dox) or presence (+Dox) of doxycycline. The total cell lysates were blotted with the indicated antibodies. *B*, growth curves of HeLa cells stably transfected with Myc-Bub1-KdM construct in the absence (–Dox) or presence (+Dox) of doxycycline. *C*, recombinant N-terminal fragment of human Cdc20 containing residues 1–170 (Cdc20NT) was incubated with Myc-Bub1, Myc-Bub1-KD, or Myc-Bub1-KdM immunoprecipitated from mitotic HeLa cells in the presence of [³²P]ATP for 30 min at room temperature. The reaction mixture was quenched with SDS sample buffer, separated on SDS-PAGE, and analyzed by autoradiography. The positions of phosphorylated Bub1 and Cdc20NT are indicated. The top panel is an anti-Myc blot to show that similar amounts of Bub1 proteins were immunoprecipitated. The bottom panel is a Coomassie Blue-stained gel to show that similar amounts of Cdc20NT were present in each kinase reaction. *D*, HeLa cells stably transfected with Myc-Bub1-KdM construct were cultured in the nocodazole-containing medium with (+Dox) or without (–Dox) doxycycline for the indicated times and analyzed by FACS. The 8N peaks were enlarged with a different scale in the insets, and the percentage of 8N cells is indicated.

APC/C forms a feedback loop to control the inactivation of the spindle checkpoint in anaphase (32). Intriguingly, overexpression of Mps1 is sufficient to cause mitotic arrest that is dependent on the spindle checkpoint in yeast (33). However, overexpression of Mps1 does not result in mitotic arrest in mammalian cells³ (34). This important difference suggests that mechanisms regulating the activation and inactivation of the spindle checkpoint are more complex in mammalian cells than in yeast. Nevertheless, Mps1 is degraded during mitotic exit in mammalian cells.³ Degradation of Mps1 might contribute to checkpoint inactivation in mammalian cells. In addition, sev-

³ W. Qi and H. Yu, unpublished data.

eral other spindle checkpoint proteins, including Aurora B and Plk1, have been shown to be degraded in an APC/C-dependent manner. It is thus possible that degradation of multiple spindle checkpoint proteins by APC/C mediates spindle checkpoint inactivation and mitotic exit. On the other hand, Cdh1^{-/-} chicken DT40 cells are viable and proliferate with very similar rate as the wild-type DT40 cells (35). This argues against a major role of APC/C^{Cdh1} in mitotic exit and against a requirement for APC/C^{Cdh1}-mediated degradation of spindle checkpoint proteins in mitotic exit.

In addition to regulation of its protein levels, Bub1 is phosphorylated in mitosis by Cdk1 (19) and mitogen-activated protein kinase (MAPK) (17). The activity of Bub1 is regulated by phosphorylation in *Xenopus* egg extracts (17). Therefore, dephosphorylation of Bub1 may be another mechanism to inactivate the spindle checkpoint. Indeed, Bub1 is rapidly dephosphorylated when Cdk1 is inhibited by roscovitine (19). Recently, it has been shown that phosphorylation of Cdc6 protects it from APC/C^{Cdh1}-mediated degradation (36). It is conceivable that degradation of Bub1 might also be regulated by the phosphorylation state of Bub1. In support of this notion, the phosphorylation sites identified on Bub1 are in the vicinity of the KEN boxes (17, 19). In the future, it will be interesting to examine whether dephosphorylation of Bub1 is a key mechanism for its inactivation and whether dephosphorylation and degradation of Bub1 are two coupled events during mitotic exit.

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