Degradation of Human RAP80 is Cell Cycle Regulated by Cdc20 and Cdh1 Ubiquitin Ligases

Hyun Jung Cho, Eun Hee Lee, Seung Hun Han, Hee Jin Chung, Ji Hoon Jeong, Junhye Kwon*, and Hongtae Kim

Abstract
Receptor-associated protein 80 (RAP80) is a component of the BRCA1-A complex that recruits BRCA1 to DNA damage sites in the DNA damage-induced ubiquitin signaling pathway. RAP80-depleted cells showed defective G2–M phase checkpoint control. In this study, we show that RAP80 protein levels fluctuate during the cell cycle. Its expression level peaked in the G2 phase and declined during mitosis and progression into the G1 phase. Also, RAP80 is polyubiquitinated and degraded by the anaphase-promoting complex (APC/C)Cdc20 or (APC/C)Cdh1. Consistent with this, knockdown of Cdc20 or Cdh1 expression by transfecting with small interfering RNAs blocked RAP80 degradation during mitosis or the G1 phase, respectively. A conserved destruction box (D box) in RAP80 affected its stability and ubiquitination, which was dependent on APC/C-cyclosomeCdc20 (C(Cdc20)) or APC/C-cyclosomeCdh1 (C(Cdh1)). In addition, overexpression of RAP80 destruction box1 deletion mutant attenuated mitotic progression. Thus, APC/C-Cdc20 or APC/C-Cdh1 complexes regulate RAP80 stability during mitosis to the G1 phase, and these events are critical for a novel function of RAP80 in mitotic progression. Mol Cancer Res; 10(5); 615–25. ©2012 AACR.

Introduction
Successful cell division occurs as a result of a well-ordered serial series of events called the cell cycle, which include DNA replication, spindle assembly, nuclear division, and cytokinesis. The eukaryotic cell cycle is tightly regulated by cyclin and cyclin-dependent kinase (Cdk) complexes, which are key regulatory complexes in cell-cycle progression. The Cdk4-cyclin D complex promotes initiation of the cell cycle, and Cdk2-cyclin E promotes cell-cycle progression from the G1 to S phase (G1–S transition). The Cdk1-cyclin B complex initiates the G2–M transition (1). Another key regulatory mechanism of cell-cycle progression is ubiquitination/proteasome-mediated proteolysis of cell-cycle machinery. The Skp1/Cullin/F-box protein complex (SCF) and anaphase-promoting complex/cyclosome (APC/C) are 2 major E3 ligases involved in the regulation of cell-cycle progression. Although SCF mainly regulates the expression levels of many proteins during S phase, APC/C regulates the expression of proteins involved in the progression from mitosis to late G1 (2, 3). Two different activators, Cdc20 and Cdh1, are required for the activation of APC/C and have specificity for substrates regulating mitotic cell-cycle progression. In the early mitotic phase, APC/C is activated by binding to Cdc20, and in the late mitotic and G1 phase, Cdc20 is replaced by Cdh1 (4, 5).

Receptor-associated protein 80 (RAP80) plays an important role in signal transduction in the DNA damage response by recruiting the BRCA1-A complex to DNA damage sites in a lysine 63-mediated polyubiquitin-binding dependent manner (6–14). This recruitment is required for the activation of BRCA1 at the DNA damage checkpoint and for DNA repair.

In this study, we show that the expression levels of RAP80 peak in the G2 phase and early mitosis and decline during mitotic exit and progression into the G1 phase. Fluctuating RAP80 protein during the cell cycle is dependent on the APC/C-ubiquitin-proteasome pathway. We also show that D box1, conserved in several mammalian species and located in the central region of RAP80, is involved in regulating RAP80 stability through ubiquitination during mitosis. Thus, we can conclude that the RAP80 protein is a target of the APC/C-ubiquitin-proteasome pathway and undergoes proteolysis during anaphase.
through the G₁ phase to allow for proper cell-cycle progression.

**Material and Methods**

**Plasmids**

The SFB-RAP80 and Myc-ubiquitin expression vectors (Myc-Ubi) were previously described (6, 9). The small interfering RNA (siRNA) resistant RAP80 expression plasmid was generated by site-directed mutagenesis using the forward primer 5'-GGAACTGGAGAAAGAGCTTGAGACCCAGGTTCTCTGAGTG-3' and reverse primer 5'-CACTGACAGACACTCGTTCAAGCTCTTTTCTCCAGTGTC-3'. RAP80 Dbox1, 2, 3, or 4 deletion mutants were generated by site-directed mutagenesis and subcloned into a SFB-tagged mammalian expression vector. The HA-tagged Cdh1 expression plasmid was cloned into a HA-tagged mammalian expression vector. The Flag-Cdc20 expression vector was previously described (15). Antibodies, transfection, and immunoprecipitation

Anti-RAP80 antibody was previously described (9). Rabbit RAP80 polyclonal antibody was affinity purified using the Sulfolink Plus Immobilization and Purification Kit (Pierce). Anti-Flag, anti-HA, anti-Myc, and anti-β-actin antibodies were purchased from Sigma-Aldrich, and human cyclin E1, cyclin A2, cyclin B1, Cdc20, and Cdh1 antibodies were purchased from Cell Signaling Technology. Transient transfection was carried out using the FuGene 6 reagent (Roche Applied Science). For immunoprecipitation, cells were washed with ice-cold phosphate-buffered saline (PBS) and then lysed in NETN buffer (0.5% Nonidet P-40, 20 mmol/L Tris (pH 8.0), 50 mmol/L NaCl, 50 mmol/L NaF, 100 μmol/L Na₃VO₄, 1 mmol/L diithiothreitol, and 50 μg/mL phenylmethylsulfonyl fluoride) at 4°C for 10 minutes. Crude lysates were cleared by centrifugation at 14,000 rpm at 4°C for 5 minutes, and supernatants were incubated with protein G-agarose–conjugated primary antibodies. The immuno complexes were washed 3 times with NETN buffer and subjected to PAGE. Western blotting was conducted using the antibodies indicated in the figure legend.

**Establishment of stable cell lines**

The establishment of stable cell lines was previously described (9). To establish cell lines stably expressing epitope-tagged proteins, HeLa cells were transfected with plasmids encoding RAP80 wild type (WT), or RAP80D-box1 and puromycin-resistant protein. Forty-eight hours after transfection, the cells were split at a 10:1 ratio and cultured in medium containing puromycin (10 μg/mL) for 3 weeks. Individual puromycin-resistant colonies were isolated and screened by Western blotting for expression of the RAP80 protein.

**Cell synchronization**

Cells were synchronized at late G₁ phase with the thymidine double blocking method (18). Briefly, the cells were plated in 100 mm diameter Petri dishes and thymidine was added to a final concentration of 2 mmol/L after cell adherence. The cells were cultured for 16 hours. After removal of the thymidine and incubation for 10 hours in fresh medium, thymidine was added to a final concentration of 2 mmol/L for an additional 16 hours. After removal of thymidine, synchronized cells were cultured in fresh medium and collected at different times for cell-cycle analysis and Western blotting. Cells were synchronized in prometaphase with 17 hours of nocodazole treatment and then released into fresh medium for further incubation.

**Cell-cycle analysis using flow cytometry**

The double thymidine or nocodazole synchronized cells were collected at different times after release from a G₁–S boundary. After washing twice with PBS, cells were fixed with chilled 70% alcohol at 20°C for 24 hours. The fixed cells were collected by centrifugation (2,000 rpm; 5 minutes), washed twice with PBS, incubated with RNaseA (30 mg/mL) for 30 minutes at 37°C, stained with 50 μg/mL propidium iodide (Sigma-Aldrich) for 30 minutes at room temperature, and then analyzed by flow cytometry.

**Results**

**RAP80 stability is regulated during the cell cycle**

To investigate whether RAP80 expression level is cell cycle regulated, we synchronized human HeLa cells and MEFs at the G₁–S boundary with a double thymidine block; after release from this block, we harvested cells at the indicated time points (Fig. 1A and B). This analysis showed that the expression level of RAP80 was the highest in the G₂–M phase and also may indicate that the RAP80 protein is degraded during mitosis and progression into the G₁ phase. This may be consistent with a specific role of the RAP80 protein in the G₂–M phase and also may indicate that the RAP80 protein is degraded during mitosis and the G₁ phase. Cyclin B1 expression levels and flow cytometric analysis confirmed cell-cycle progression (Fig. 1A and B). We further confirmed the changes in RAP80 expression levels at different stages of the cell cycle. Low levels of RAP80 expression were observed when HeLa cells were in the G₁–S
boundary and mitotic (M) phase (Fig. 1C). Elevated cyclin E1 expression, elevated cyclin A2 expression, or Ser10 phosphorylation of histone H3 (a marker of chromosome condensation) was observed for each respective stage and flow cytometric analysis confirmed cell-cycle progression. RAP80 expression levels during mitotic progression were also examined. HeLa cells arrested in prometaphase with nocodazole (Fig. 1D) or taxol (data not shown) were collected by shake-off and replated to allow progress through mitosis, and RAP80 protein levels were checked at different time points. RAP80 protein levels gradually decreased over time during mitosis and the G1 phase.

RAP80 degradation is regulated by the ubiquitin-proteasome pathway

Given the above findings, we were prompted to investigate whether RAP80 degradation could be regulated by the ubiquitin-proteasome pathway. Overexpressed RAP80 was polyubiquitinated in HEK 293T cells (Fig. 2A and B). We also investigated whether the ubiquitin-proteasome system was involved in regulating RAP80 levels in HeLa cell lines. HeLa cells incubated in the presence of the proteasome inhibitor MG132 were collected at indicated times and endogenous RAP80 expression levels were assessed by immunoblot analysis. RAP80 expression levels steadily increased in the presence of MG132 (Fig. 2C).

Regulation of RAP80 expression levels by the APC/C-proteasome pathway

Many mitotic regulatory proteins and cyclins are degraded by the APC/cyclosome complex (Cdc20) and/or the APC/Cdh1 complexes during mitosis and the G1 phase. In addition, RAP80 downregulation in mitosis and the G1 phase led us to test whether RAP80 degradation is dependent on Cdc20 or Cdh1. To show whether Cdh1 and Cdc20 are required for RAP80 degradation in vivo, we used siRNAs to reduce their expression and the transfected cells were arrested at a mitotic phase with nocodazole. Following the removal of nocodazole, control or Cdc20 siRNA-transfected cells were collected at the indicated times to detect RAP80 protein levels and cell-cycle progression was confirmed by flow cytometric analysis. In control siRNA-transfected cells, RAP80 levels decreased by 1 hour following nocodazole removal, which may correspond to anaphase entry, and further decreased by 3 hours following nocodazole removal, which may correspond to anaphase entry.
correspond to mixed late mitotic and G1 phase (Fig. 3A). In contrast, RAP80 protein accumulated in mitotically synchronized HeLa cells transfected with Cdc20 siRNA up to 3 hours following nocodazole removal (Fig. 3A). Although this may seem to result in a delay into mitotic and G1 phase, flow cytometry analysis shows that Cdc20 knockdown cells were in mixed mitotic and G1 phase at 3 hours following nocodazole removal. Thus, our data suggest that Cdc20 is important for regulating the accumulation of RAP80 protein during mitotic progression. This accumulation in mitosis seems to be caused by RAP80 stabilization, as shown by measuring the RAP80 half-life (Fig. 3B). We also checked the effect of Cdh1 on RAP80 degradation. Control (Con) or Cdh1 siRNA-transfected HeLa cells were arrested in prometaphase with nocodazole, washed, and released. As Cdh1 is mainly activated in late mitosis and the G1 phase, we checked RAP80 expression and turnover after 1 hour postrelease. As shown in Fig. 3C, RAP80 protein accumulated in mitotically synchronized HeLa cells transfected with Cdh1 siRNA compared with cells transfected with control siRNA. This accumulation in mitosis and G1 phase seems to be caused by RAP80 stabilization, as shown by measuring the RAP80 half-life (Fig. 3B). We also observed RAP80 degradation upon Cdc20 or Cdh1 overexpression in HEK 293T cells. In addition, treatment with MG132 inhibited Cdc20- or Cdh1-mediated RAP80 degradation (Fig. 3E). Furthermore, overexpressed Cdc20 or Cdh1 increased RAP80 polyubiquitination in HEK 293T cells (Fig. 3F and G). Thus, the data indicate that Cdc20 and Cdh1 stimulate APC/C-mediated ubiquitination and degradation of RAP80.

Conserved D box1 is required for RAP80 degradation

Many proteins containing a destruction box (D box) are degraded by the APC/C/Cdc20 and APC/C/Cdh1 complexes. The D box consensus sequence consists of RxxLxxxxN/E/D (x, any amino acid), although most substrates only contain the minimal RxxL motif. The RAP80 protein has 4 putative minimal RxxL motifs (D box1-4) that potentially serve as D boxes (Fig. 4A and B), but does not have any APC/C/Cdc20 and APC/C/Cdh1 complex recognition motifs, such as the KEN box or A box. As only the putative D box1 among these 4 putative D boxes is conserved in most mammalian species, including human, chimpanzee, monkey, dog, rat, and mouse (Fig. 4C and data not shown), we investigated the importance of the putative D box1 on the regulation of RAP80 ubiquitination and degradation. As shown in Fig. 4D and E, ubiquitination of the D box1 deletion mutant of RAP80 (SFP-RAP80Dbox1) was reduced compared with the wild type (SFP-RAP80). In addition, we further showed the dependence of Cdc20- or Cdh1-mediated RAP80 ubiquitination on the D box1 (Fig. 4F and G). Next, we checked whether the D box1 deletion mutant was resistant to degradation during mitosis and the G1 phase. HeLa cells expressing wild-type SFP-RAP80, SFP-RAP80Dbox1, 2, 3, or 4 were arrested in prometaphase with nocodazole, collected by shake-off, and replated to allow progress through mitosis and G1 phase. Subsequently, RAP80 protein levels were checked at different time points (Fig. 4H). Similar to endogenous RAP80, wild-type RAP80, SFP-RAP80Dbox2, 3, and 4 were degraded after nocodazole release. In contrast, the RAP80D box1 deletion mutant remained stable.
indicated antibodies. Cell lysates were also immunoblotted (W) using the indicated antibodies. F and G, overexpression of Cdc20 or Cdh1 promotes ubiquitination of RAP80 in HEK 293T cells. HEK 293T cells were transfected with SFB-RAP80 plasmid alone and in the presence of Flag-Cdc20 or HA-Cdh1 plasmid at ratios of 1:4 (RAP80 and each ubiquitin ligase). Twenty-four hours after transfection, cells were treated with dimethylsulfoxide or MG132 (10 μM). At each time point (0, 0.5, 1, 1.5, or 2 hours) after incubation, cells were harvested for analysis. Cell lysates were immunoblotted (W) using the indicated antibodies.

Figure 3. Regulation of RAP80 expression levels during mitosis by the APC/C-proteasome pathway. A, knockdown of Cdc20 protein inhibits the degradation of RAP80 during mitosis. Control (Con) or Cdc20 siRNA-transfected HeLa cells were synchronized in mitosis by treatment with nocodazole (1 μg/mL) for 17 hours and released. At each time point (0, 1, or 3 hours) postrelease, cells were harvested for analysis. Cell lysates were immunoblotted (W) using the indicated antibodies. Cell-cycle distributions were analyzed by flow cytometry, and the results are summarized at the bottom. B, Knockdown of Cdc20 decreases RAP80 turnover during mitosis. Control (Con) or Cdc20 siRNA-transfected HeLa cells were synchronized in mitosis by treatment with nocodazole (1 μg/mL) for 17 hours. Cells were washed and released into fresh medium. Cells were then treated with cycloheximide (CHX). At each time point (0, 0.5, 1, 2 or 2 hours) after incubation, cells were harvested for analysis. Cell lysates were immunoblotted (W) using the indicated antibodies. C, Knockdown of Cdh1 protein inhibits the degradation of RAP80 during late mitosis and the G1 phase. Control (Con) or Cdh1 siRNA-transfected HeLa cells were synchronized in mitosis by treatment with nocodazole (1 μg/mL) for 17 hours and released. At each time point (0, 1, 2 or 2 hours) postrelease, the cells exist in the late mitotic phase or G1 phase, respectively, and cells were harvested for analysis. Cell lysates were immunoblotted (W) using the indicated antibodies. Cell-cycle distributions were analyzed by flow cytometry, and the results are summarized at the bottom. D, Control (Con) or Cdh1 siRNA-transfected HeLa cells were synchronized in mitosis by treatment with nocodazole (1 μg/mL) for 17 hours. Cells were washed and released into fresh medium. After incubation for 1 hour (the cells are in late mitosis), cells were then treated with cycloheximide (CHX). At each time point (0.5, 1, 1.5, or 2 hours) after incubation, cells were harvested for analysis. Cell lysates were immunoblotted (W) using the indicated antibodies. E, Cdc20 and Cdh1 reduce ectopic RAP80 expression levels. HEK 293T cells were transfected with SFB-RAP80 plasmid alone and in the presence of Flag-Cdc20 or HA-Cdh1 plasmid at ratios of 1:4 (RAP80 and each ubiquitin ligase). Twenty-four hours after transfection, cells were treated with dimethylsulfoxide or MG132 (10 μM) for 6 hours and harvested. Cell extracts were immunoblotted (W) using the indicated antibodies. F and G, overexpression of Cdc20 or Cdh1 promotes ubiquitination of RAP80 in HEK 293T cells. HEK 293T cells cotransfected with indicated expression plasmids were incubated in the presence of MG132 (10 μM) for 6 hours before harvesting. Cell lysates were then subjected to pull down with streptavidin beads (F) or immunoprecipitated (IP) with an antibody (G) and subjected to immunoblotting (W) using the indicated antibodies. Cell lysates were also immunoblotted (W) using the indicated antibodies.

Table 1. Mitotic Degradation of RAP80

<table>
<thead>
<tr>
<th>Time (h) after release</th>
<th>G0 (%)</th>
<th>G1 (%)</th>
<th>S (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>97</td>
<td>62</td>
<td>28</td>
</tr>
<tr>
<td>1</td>
<td>94</td>
<td>78</td>
<td>37</td>
</tr>
<tr>
<td>2</td>
<td>96</td>
<td>92</td>
<td>79</td>
</tr>
</tbody>
</table>

RAP80 binds to Cdc20 and Cdh1

The data in this article show that Cdc20 and Cdh1 are involved in mediating RAP80 ubiquitination and degradation. These data led us to check the possibility of an
association between RAP80 and Cdc20 or Cdh1. Overexpressed RAP80 protein associated with endogenous Cdc20 or Cdh1 protein (Fig. 5A and B). To further show the role of the D box1 in binding between RAP80 and Cdc20 or Cdh1, we checked the ability of the RAP80Dbox1 deletion mutant to bind to Cdc20 or Cdh1. Compared with wild-type RAP80, the D box1 deletion mutant did not coimmunoprecipitate with Cdc20 or Cdh1 using overexpressed HEK 293T cells (Fig. 5C and D). These data indicate that RAP80 is a direct target of Cdc20 and Cdh1 and is degraded by the ubiquitination/proteosome pathway in mitosis.

Overexpressed RAP80 causes a delay in mitotic progression

We then investigated the biological significance of RAP80 protein during cell-cycle progression. The downregulation of RAP80 in mitosis and the G1 phase of the cell cycle led us to examine the requirement of RAP80 for cell-cycle progression. We first generated a RAP80 wild-type expression plasmid resistant to the effects of RAP80 siRNA (R-RAP80) tagged with Flag and HA (Fig. 6A) to identify RAP80 functions in cell-cycle progression. Next, HeLa cell lines stably expressing control or R-RAP80 were

Figure 4. The D box1 is required for degradation and ubiquitination of RAP80. A, domain organization of RAP80. B, alignment of the amino acid regions corresponding to the putative destruction box motifs (D box1-4) in human RAP80 with the D box motifs of human cyclin A2, cyclin B1, securin, Mcl-1, and Nek2A. (X = any amino acid). C, sequence alignment of the RAP80 region containing the putative D box1 in mammalian species. The numbers indicate the region of RAP80 amino acids in each mammalian species. The D box consensus sequence consists of RxxLxxxxN/E/D (x, any amino acid). D and E, RAP80 ubiquitination is dependent on the D box1. HEK 293T cells cotransfected with wild-type RAP80 (SFB-RAP80) or the RAP80Dbox1 deletion mutant (SFB-RAP80Dbox1) expression plasmid with/without Myc-Ubi plasmids were incubated in the presence of MG132 (10 μmol/L) for 6 hours before harvesting. Cell lysates were then subjected to immunoprecipitation (IP) with anti-Flag (D) or anti-ubiquitin (E) antibody and immunoblotting (W) was carried out using the indicated antibodies. Cell lysates were immunoblotted (W) using the indicated antibody.

Overexpressed RAP80 causes a delay in mitotic progression

We then investigated the biological significance of RAP80 protein during cell-cycle progression. The downregulation of RAP80 in mitosis and the G1 phase of the cell cycle led us to examine the requirement of RAP80 for cell-cycle progression. We first generated a RAP80 wild-type expression plasmid resistant to the effects of RAP80 siRNA (R-RAP80) tagged with Flag and HA (Fig. 6A) to identify RAP80 functions in cell-cycle progression. Next, HeLa cell lines stably expressing control or R-RAP80 were
generated (Fig. 6B). Using these stable cell lines, we examined whether RAP80 overexpression is important for mitotic cell-cycle progression. The established stable HeLa cell lines expressing control or R-RAP80 (Fig. 6B) were transfected with RAP80 siRNA to reduce the effects of endogenous RAP80 protein. When mitotic progression was examined using synchronized cells after release from a double thymidine block, mitotic delay was apparent in R-RAP80-overexpressing HeLa cells compared with control-expressing HeLa cells (Fig. 6C). To check whether the RAP80Dbox1 deletion mutant has any effect on mitotic progression, we established 2 different new R-RAP80- or R-RAP80Dbox1-expressing HeLa cells, which showed different expression levels (Fig. 6D). When mitotic progression was examined using synchronized cells after release from a double thymidine block, mitotic delay was apparent in R-RAP80Dbox1-expressing HeLa cells compared with R-RAP80-expressing HeLa cells in an expression-dependent manner (Fig. 6E). These data suggest that preventing the degradation of RAP80 is sufficient for mitotic progression. In addition, the RAP80Dbox1 deletion mutant remained stable throughout the cell cycle, indicating the importance of the putative D box1 motif for regulating degradation of RAP80 during the cell cycle (Fig. 6F). Next, we checked whether this mitotic function of RAP80 is distinctly different from its role in DNA damage response. The amount of γ-H2AX was determined throughout cell cycle. R-RAP80-2 or R-RAP80Dbox1-2 cells were synchronized at G1 by double thymidine block and released into the cell cycle. At different times after the release from thymidine, the amount of γ-H2AX was determined throughout the cell cycle. R-RAP80-2 or R-RAP80Dbox1-2 cells were synchronized at G1−S by double thymidine block and released into the cell cycle. At different times after the release from thymidine, the amount of γ-H2AX was determined throughout the cell cycle. Because nucodazole has been reported to either cause DNA damage (19) or not to cause DNA damage (20), immunofluorescence microscopy staining for γH2AX foci (an index of DNA DSBs) was carried out.
out. Nocodazole did not induce DNA damage at the dose used (Fig. 6H). This data indicated that overexpression of wild type or RAP80Dbox1 deletion mutant does not induce DNA damage. Therefore, RAP80-mediated delay of mitotic progression is induced in the absence of DNA damage. Initiation of anaphase and mitotic exit are dependent on active APC/C E3 ligase complex. To determine whether RAP80-mediated inhibition of mitotic progression was due to the inactivation of APC/C, we compared the protein levels of known APC/C substrates (cyclin A, cyclin B1, and securin) using control, wild type (R-RAP80-2), or RAP80Dbox1 deletion mutant (R-RAP80Dbox1-2)-stable cell lines synchronized with nocodazole. As expected, nocodazole-synchronized wild type or RAP80Dbox1 deletion mutant-stable cell lines expressed high levels of cyclin A, cyclin B1, and securin compared with control-stable cell lines (Fig. 6I).

**Discussion**

In this study, we investigated the regulatory mechanism of RAP80 ubiquitination and degradation during cell-cycle progression and showed an unexpected role of RAP80 in the regulation of mitotic progression. Our results show that regulation of RAP80 expression is dependent on cell-cycle progression. RAP80 is a target molecule of APC/C<sup>Cdc20</sup> and APC/C<sup>Cdh1</sup> for degradation during mitosis and the G<sub>1</sub> phase.

A single conserved D box1 (RHCLTPLAD), in which deletion prevented RAP80 polyubiquitination and degradation, was identified in the middle region of RAP80 (254–262 amino acids). In addition, RAP80 bound to Cdc20 and Cdh1 through its D box1. Thus, this posttranslational modification of RAP80 seems to be a novel mechanism that regulates RAP80 stability and functions in mitotic cell-cycle progression. RAP80 D box1 is not required for translocation to DNA damage sites (data not shown) and no different level of phosphorylation of H2AX was detected throughout cell cycle using control, wild type, or RAP80Dbox1 deletion mutant-stable cell lines. These data indicate that overexpression of wild type or RAP80Dbox1 deletion mutant does not induce DNA damage and might regulate the mitotic progression independent of its involvement in the DNA damage response. Nocodazole-synchronized wild type or RAP80Dbox1 deletion mutant-stable cell lines expressed high levels of cyclin A, cyclin B1, and securin, which are the substrates of APC/C complexes, compared with control-stable cell lines (Fig. 6I).
Figure 6. Overexpressed RAP80 causes a delay in mitotic progression. A, construction of the Flag-HA–tagged RAP80 expression plasmid with siRNA resistance (R-RAP80). Wild-type RAP80 or R-RAP80 plasmids were individually transfected into HEK 293T cell lines with control or RAP80 siRNA. The transfected cell lysates were immunoblotted (W) using the indicated antibodies. GFP was used to show that the degradation of RAP80 was specific, and that the cells were transfected with an equal amount of plasmid. B, establishment of HeLa cell lines stably expressing the control or R-RAP80 plasmid. Each stable cell line was immunoblotted (W) using the indicated antibodies. C, overexpressed RAP80 causes a delay in mitotic progression. The stable cell lines expressing control or R-RAP80 plasmid were transfected with RAP80 siRNA, and cells were synchronized by a double thymidine block and released. Cells were harvested for flow cytometry analysis after release of the cell-cycle block at different time points (0, 8, 10, 12, or 16 hours). The arrow indicates the G2–M population in the HeLa cell line stably expressing control or R-RAP80. The number indicates the percentage of the G2–M population. “As” indicates asynchronous cells. D, establishment of HeLa cell lines stably expressing the R-RAP80 or R-RAP80Dbox1 plasmid. Two different RAP80 stable cell lines (R-RAP80-1 or -2) or 2 different R-RAP80Dbox1 stable cell lines (R-RAP80Dbox1-1 or -2) were immunoblotted (W) using the indicated antibodies. E, Nondegradable RAP80Dbox1 deletion mutants cause a delay in mitotic progression. The stable cell lines expressing R-RAP80 or R-RAP80Dbox1 were transfected with RAP80 siRNA, and cells were synchronized by a double thymidine block and released. Cells were harvested for flow cytometry analysis after release of the cell-cycle block at different time points (0, 8, 10, 12, or 16 hours). The arrow indicates the G2–M population in the HeLa cell line stably expressing R-RAP80 or R-RAP80Dbox1. The number indicates the percentage of G2–M population. “As” indicates asynchronous cells.
transition (21, 23). Recently, we obtained evidence that RAP80 binds to Cdk1 (unpublished data). It is possible that RAP80 may inhibit the Cdk1 activity to phosphorylate the substrate to be required for the metaphase–anaphase transition. However, the exact reason why RAP80 degradation is an essential step for mitotic cell-cycle progression is not clear. Presently, RAP80 protein levels fluctuated during the cell cycle. Its expression level peaked at the G2 phase and declined during mitosis and progression into the G1 phase. In addition, overexpressed and nondegradable RAP80 delays mitotic cell-cycle progression. These data may indicate that elevated RAP80 during the G2 phase functions to inhibit the G2–M transition and RAP80 inhibition of the G2–M transition may be blocked to resume progression through the cell cycle. The degradation of RAP80 by APC/C\(^{Cdc20}\) and APC/C\(^{Cdh1}\) may be the major mechanism to progress mitotic cell cycle.

In summary, the decline in human RAP80 expression levels following entry into mitosis is regulated by the APC-ubiquitin-proteosome pathway during mitosis and the G1 phase. Overexpressed RAP80 inhibits mitotic cell-cycle progression. It will be interesting to investigate whether other modifications of RAP80 are also important for regulating RAP80 stability throughout the cell cycle.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: J.H. Jeong, J. Kwon, H. Kim.
Writing, review, and/or revision of the manuscript: H.J. Cho, J.H. Jeong, J. Kwon, H. Kim.

Figure 6. (Continued) F, the stable cell lines expressing R-RAP80-2 or R-RAP80Dbox1-2 were synchronized by a double thymidine block and released. Cells were harvested for Western blotting analysis after release of the cell-cycle block at different time points (0, 8, 10, 12, or 16 hours). Cell lysates were immunoblotted (W) using the indicated antibodies. G, overexpression of wild type or RAP80Dbox1 deletion mutant does not induce DNA damage throughout cell cycle. R-RAP80-2 or R-RAP80Dbox1-2 cells were synchronized at G1–S by a double thymidine block and released into the cell cycle. At different time points after the release from double thymidine block, the amount of \( \gamma \)-H2AX was determined on Western blots. H, exposure to nocodazole does not induce DNA damage. Control (Con) RAP80-2 or R-RAP80Dbox1-2 cells were either irradiated (10 Gy) or treated with 1 \( \mu \)g/mL of nocodazole for 16 hours, and the cells were processed for immunofluorescence assay using the antibody specific for the \( \gamma \)-H2AX. I, overexpression of wild type or RAP80Dbox1 deletion mutant blocks the degradation of securin, cyclin A2, and cyclin B1 during mitosis. R-RAP80-2 or R-RAP80Dbox1-2 cells were synchronized at prometaphase by treatment with nocodazole (1 \( \mu \)g/mL) for 17 hours. Cells were washed and released into fresh medium. At each time point (2 or 4 hours) after incubation, cells were harvested for analysis. Cell lysates were immunoblotted (W) using the indicated antibodies. “As” indicates asynchronous cells.
Mitotic Degradation of RAP80

Acknowledgments
The authors thank members of Dr. Kim’s laboratory for helpful discussion and technical support.

Grant Support
This work was supported by a grant from the National R&D Program for Cancer Control, Ministry for Health, Welfare and Family Affairs, Republic of Korea (0920670), Korean Research Foundation grant funded by the Korean government (2009-0067028), and National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST; no. 20110030831).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 3, 2011; revised February 22, 2012; accepted February 27, 2012; published OnlineFirst March 16, 2012.

References

Degradation of Human RAP80 is Cell Cycle Regulated by Cdc20 and Cdh1 Ubiquitin Ligases

Hyun Jung Cho, Eun Hee Lee, Seung Hun Han, et al.


**Updated version**

Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-11-0481

**Cited articles**

This article cites 23 articles, 10 of which you can access for free at:
http://mcr.aacrjournals.org/content/10/5/615.full.html#ref-list-1

**Citing articles**

This article has been cited by 2 HighWire-hosted articles. Access the articles at:
/content/10/5/615.full.html#related-urls

**E-mail alerts**

Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.