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# APC/C$^{Cdc20}$ Controls the Ubiquitin-Mediated Degradation of p21 in Prometaphase

Virginia Amador,[1] Sheng Ge,[1] Patricia G. Santamaría,[1] Daniele Guardavaccaro,[1] and Michele Pagano[1,*]
[1] Department of Pathology, NYU Cancer Institute, New York University School of Medicine, 550 First Avenue MSB 599, New York, NY 10016, USA
*Correspondence: michele.pagano@med.nyu.edu
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## SUMMARY

**During the G1/S transition, p21 proteolysis is mediated by Skp2; however, p21 reaccumulates in G2 and is degraded again in prometaphase. How p21 degradation is controlled in mitosis remains unexplored. We found that Cdc20 (an activator of the ubiquitin ligase APC/C) binds p21 in cultured cells and identified a D box motif in p21 necessary for APC/C$^{Cdc20}$-mediated ubiquitylation of p21. Overexpression of Cdc20 or Skp2 destabilized wild-type p21; however, only Skp2, but not Cdc20, was able to destabilize a p21(D box) mutant. Silencing of Cdc20 induced an accumulation of p21, increased the fraction of p21 bound to Cdk1, and inhibited Cdk1 activity in *p21*$^{+/+}$ prometaphase cells, but not in *p21*$^{-/-}$ cells. Thus, in prometaphase Cdc20 positively regulates Cdk1 by mediating the degradation of p21. We propose that the APC/C$^{Cdc20}$-mediated degradation of p21 contributes to the full activation of Cdk1 necessary for mitotic events and prevents mitotic slippage during spindle checkpoint activation.**

## INTRODUCTION

The cyclin-dependent kinase (CDK) inhibitor p21 is a critical negative regulator of the cell division cycle (Bloom and Pagano, 2004; Pei and Xiong, 2005). Its N-terminal domain binds to and inhibits cyclin-CDK complexes, while the C-terminal domain binds to PCNA and inhibits the elongation of DNA synthesis. In agreement with these inhibitory functions, levels of p21 increase in response to stimuli that inhibit cell proliferation or induce differentiation and senescence.

During an unperturbed cell cycle, p21 plays a role in G1 and G2 by contributing to temporal pauses that facilitate the integration of critical checkpoint signals for regulated entry into S phase and mitosis, respectively. While p21 contributes to the maintenance of the G1 state by inhibiting the activity of Cdk2—and likely Cdk1 and Cdk3 as well (Bloom and Pagano, 2004; Pei and Xiong, 2005)—in G2, the major target of p21 is Cdk1 (Bates et al., 1998; Bunz et al., 1998; Chan et al., 2000; Dulic et al., 1998; Medema et al., 1998; Niculescu et al., 1998). Forced expression of p21 inhibits DNA synthesis and entry into mitosis. In addition to these two well-known inhibitory effects, p21 overexpression induces polyploidy, particularly when the mitotic spindle is disrupted with nocodazole (Bates et al., 1998; Chang et al., 2000; Niculescu et al., 1998). However, the reasons for this effect on ploidy are not well understood.

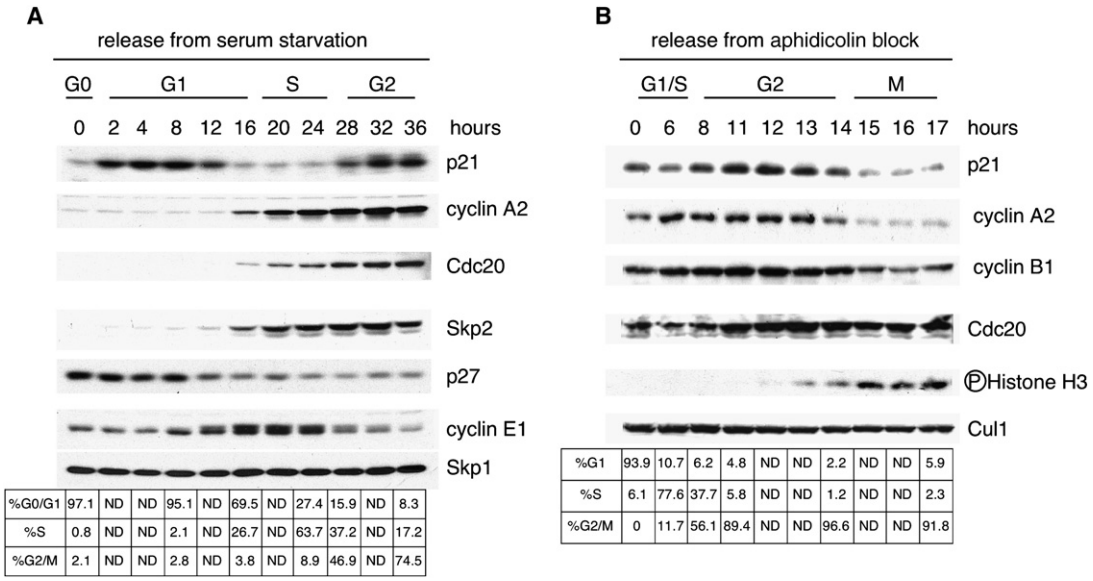
In nontransformed fibroblasts, p21 shows bimodal periodicity, with an initial peak during G1 and a second peak in G2, at both the mRNA and protein level (Dulic et al., 1998; Li et al., 1994). Cells in G2 (displaying visible nucleoli and no mitotic chromosome condensation) exhibit high levels of nuclear p21 associated with Cdk1 complexes. In the course of DNA condensation, p21 levels drop, and p21 remains absent during mitosis (Dulic et al., 1998). How p21 degradation is controlled in early mitosis has not been investigated. In the study presented herein, we investigated the regulation of p21 degradation in mitosis and found that the ubiquitin ligase APC/C$^{Cdc20}$ (anaphase-promoting complex/cyclosome and its activator Cdc20) controls this process.

## RESULTS

### Cdc20 Physically Interacts with p21

Initially, we confirmed that p21 protein levels show a bimodal distribution during the cell cycle (Dulic et al., 1998). Human T98G cells (Dorrello et al., 2006; Stein, 1979) were synchronized in G0 by serum deprivation and released from the arrest by the addition of serum. As expected, cyclin E1 levels rose in late G1, and the increase in the expression of Skp2, Cdc20, and cyclin A2 coincided with the G1/S transition, inversely correlating with p27 levels (Figure 1A). Levels of p21 were low in G0, increased in G1, and decreased again at G1/S in parallel with p27 degradation. However, whereas p27 expression remained low for the remainder of the cell cycle, levels of p21 increased 28 hr after serum addition, when cells were exiting S phase and approaching G2.

To pinpoint when p21 levels decrease again late in the cell cycle, we synchronized IMR90 cells in S phase using aphidicolin and then released them in fresh medium to allow synchronized progression through G2 and mitosis. Levels of p21 increased and reached a peak 12 hr after




| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| %G0/G1 | 97.1 | ND | ND | 95.1 | ND | 69.5 | ND | 27.4 | 15.9 | ND | 8.3 |
| %S | 0.8 | ND | ND | 2.1 | ND | 26.7 | ND | 63.7 | 37.2 | ND | 17.2 |
| %G2/M | 2.1 | ND | ND | 2.8 | ND | 3.8 | ND | 8.9 | 46.9 | ND | 74.5 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| %G1 | 93.9 | 10.7 | 6.2 | 4.8 | ND | ND | 2.2 | ND | ND | 5.9 | |
| %S | 6.1 | 77.6 | 37.7 | 5.8 | ND | ND | 1.2 | ND | ND | 2.3 | |
| %G2/M | 0 | 11.7 | 56.1 | 89.4 | ND | ND | 96.6 | ND | ND | 91.8 | |

**Figure 1. p21 Is Degraded in Early Mitosis**

(A) T98G cells were synchronized in G0 by 72 hr of serum starvation (indicated as time 0). Cells were then restimulated with serum and collected at the indicated time points. Protein extracts were analyzed by immunoblotting with antibodies against the indicated proteins. Cell-cycle phases were monitored by flow cytometry.

(B) IMR-90 fibroblasts were first synchronized in G0 by 72 hr of serum starvation and then at the G1/S boundary (indicated as time 0) by restimulating them with serum for 28 hr, adding aphidicolin for the last 18 hr. Cells were subsequently washed and allowed to progress through the cell cycle for the indicated times. Protein extracts were analyzed by immunoblotting with antibodies against the indicated proteins. Cell-cycle phases were monitored by flow cytometry.

aphidicolin release, then steadily decreased in coincidence with the appearance of the Ser10 phosphorylation of histone H3 (a marker of chromosome condensation) and the degradation of cyclin A2 (Figure 1B). Similar behavior of p21 levels was observed in multiple cell types, including T98G and U-2 OS (see Figure S1 in the Supplemental Data available with this article online).

The SCF$^{Skp2}$ ubiquitin ligase is involved in the degradation of p21 (Bornstein et al., 2003; Wang et al., 2005; Yu et al., 1998); therefore, we analyzed the levels of p21 in cells lacking Skp2. Both *Skp2*$^{-/-}$ and *Skp2*$^{-/-}$;*p27*$^{-/-}$ mouse embryonic fibroblasts (MEFs) displayed high levels of p21 when synchronized in S phase (as previously observed [Bornstein et al., 2003]), but not in G2 and prometaphase (Figure 2). Moreover, the physical interaction between Skp2 and p21 was significantly decreased in early mitotic cells compared to S phase cells (data not shown). Thus, to explore the possibility that p21 is targeted for degradation by a different ubiquitin ligase, we screened 21 human F box proteins, two activators of the APC/C, and the ubiquitin ligase Mdm2 for binding to p21. Constructs for the FLAG-tagged versions of these proteins were transfected into HEK293T cells and, after the addition of the proteasome inhibitor MG132 6 hr prior to lysis, immunoprecipitations for the ubiquitin ligases were performed to evaluate their interaction with endogenous p21. We observed that, in addition to Skp2, the only other protein able to coimmunoprecipitate endogenous p21 was Cdc20 (Figure 3A). In contrast, related proteins such as Cdh1, βTrcp1, βTrcp2, Fbxw2, Fbxw4, Fbxw5, Fbxw7α, Fbxw7γ, or Fbxw8 (all of which, like Cdc20, contain WD-40 repeats) did not bind p21 (Figure 3A). Similarly, additional F box proteins (Fbxl2, Fbxl3, Fbxl7, Fbxl10, Fbxl11, Fbxl15, Fbxl20, Fbxo4, Fbxo5, Fbxo7, Fbxo9, Fbxo17, and Fbxo21) or Mdm2 also did not coimmunoprecipitate p21 (Figure 3A and data not shown). Finally, the interaction of p21 with Cdc20, as well as Cdc27 (an integral subunit of APC/C), was also observed for the endogenous proteins (Figure 3B).

We then used seven Cdc20 mutants (Figure S2A) to map the p21-binding domain in Cdc20. FLAG-tagged wild-type Cdc20 and Cdc20 mutants were expressed in HEK293T cells and then immunoprecipitated to evaluate their interaction with endogenous p21 and other proteins. The only mutant that lost the ability to bind p21 was ΔC(471–499), which lacks the last 19 amino acids (Figure S2B). This mutant, as well as two different mutants with N-terminal deletions (ΔN133 and ΔN165), did not bind Cdc27 (Schwab et al., 2001; Thornton et al., 2006; Figure S2B]. However, in contrast to ΔC(471–499), ΔN133 and ΔN165 coimmunoprecipitated p21 much more efficiently than wild-type Cdc20 (Figure 3C and Figure S2B).

## The APC/C$^{Cdc20}$-Mediated Ubiquitylation and Degradation of p21 Requires an Intact D Box Motif in p21

Results from the immunoprecipitation experiments described above suggested that p21 may be a substrate of

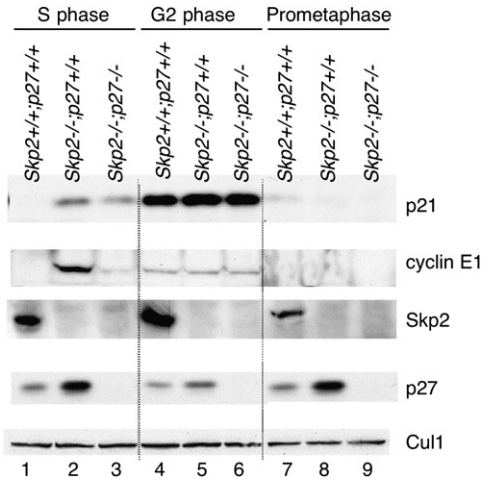



**Figure 2. Mitotic Degradation of p21 Is Not Dependent on Skp2**

MEFs from *Skp2*$^{-/-}$;*p27*$^{+/+}$, *Skp2*$^{-/-}$;*p27*$^{-/-}$, and *Skp2*$^{+/+}$;*p27*$^{+/+}$ mice were synchronized at G1/S by a double thymidine block (S phase). Cells were subsequently washed and allowed to progress through the cell cycle for 12 hr in the presence of nocodazole. The prometaphase, round cells were collected by gentle shake-off, and finally, the remaining attached cells (G2 phase) were also harvested. Protein extracts were analyzed by immunoblotting with antibodies to the indicated proteins.

APC/C$^{Cdc20}$ for the following reasons: (1) p21 interacted with the C terminus of Cdc20, similarly to other substrates of APC/C$^{Cdc20}$, such as cyclin A2 and Securin (Ohtoshi et al., 2000 and data not shown). (2) Similarly to cyclin A2, p21 bound more efficiently to ΔN165 than to wild-type Cdc20 (Figure 3C). If p21 is a substrate of APC/C$^{Cdc20}$, this difference could be explained by the differential binding of wild-type Cdc20 and ΔN165 to Cdc27. ΔN165 is unable to bind Cdc27 and, therefore, could sequester substrates without delivering them to the APC/C ligase for ubiquitylation and consequent degradation. In contrast, wild-type Cdc20 binds p21, but it may also induce p21 degradation. In favor of this hypothesis, the binding between wild-type Cdc20 and p21 is not immediately detectable without the addition of the proteasome inhibitor MG132 prior to harvesting the cells (Figure 3C). (3) Wild-type Cdc20 and ΔN165 also show a second differential behavior. Whereas wild-type Cdc20 interacted better with p21 and cyclin A2 in asynchronous cells than in prometaphase cells, the opposite situation was observed for ΔN165 (Figure 3C). This result suggests that in prometaphase cells, where APC/C$^{Cdc20}$ is more active, wild-type Cdc20 is more competent in promoting the degradation of p21 and cyclin A2, whereas ΔN165 is unable to do so, allowing increasing amounts of p21 and cyclin A2 to bind.

Based on these considerations, we investigated the involvement of APC/C$^{Cdc20}$ in controlling the degradation of p21. We transfected HEK293T cells with a construct expressing p21 and constructs expressing either Cdc20, Cdh1, or Skp2 (Figure 4A). Enforced expression of Cdc20 or Skp2 resulted in a considerable decrease in the steady-state amounts of p21 (Figure 4A, lanes 6 and 8), whereas overexpression of Cdh1 had no effect (Figure 4A, lane 4). Notably, both the Cdc20- and the Skp2-mediated decrease in p21 levels were due to the destabilization of the CDK inhibitor, as shown by the ability of the proteasome inhibitor MG132 to prevent this downregulation (Figure 4A, lanes 7 and 9) and by the decrease in p21 half-life (Figure 4C, lanes 1–12). In contrast to wild-type Cdc20, ΔN165 did not induce degradation of p21 (Figure 4D), supporting the hypothesis that this mutant is inactive and unable to target p21 for degradation, regardless of its ability to bind p21.

Substrates of APC/C are often characterized by the presence of a "destruction box" or D box, which was first identified in cyclin B as a stretch of nine amino acids (RxxLxxIxN) that when mutated stabilized the protein (Glotzer et al., 1991). However, most substrates contain only the minimal RxxL motif in their D boxes. We identified one conserved RxxL motif in p21 (positions 86–89 in human) that could potentially serve as a D box (Figure 4B), but no other potential APC/C recognition motifs (KEN box, A box, etc.) were identified. The RxxL domain of p21 is not present in p27, does not overlap with either the CDK- or the PCNA-binding domains, and is encompassed in a low complexity region (amino acids 81–96), similar to the D box motifs of A and B type cyclins. We mutated the Arg and Leu residues in the putative D box motif to Ala (Figure 4B) and determined the stability of the p21 D box (p21[D box]) mutant in HEK293T cells coexpressing either Cdc20 or Skp2. Notably, whereas the half-lives of both wild-type p21 and p21(D box) mutant were shortened by Skp2 (Figure 4C), p21(D box) mutant was refractory to the effect of Cdc20 (Figure 4C, lanes 17–20).

To test whether p21 is ubiquitylated via the APC/C$^{Cdc20}$ ubiquitin ligase and further investigate the importance of the D box motif, we reconstituted the ubiquitylation of p21 in vitro. p21 ubiquitylation was promoted by the addition of APC/C and was further promoted by addition of both APC/C and Cdc20 (Figure 4E). Methylated ubiquitin added to the in vitro reaction inhibited the formation of the highest molecular weight forms of p21 (Figure S3), formally demonstrating that the high molecular weight forms of p21 are indeed polyubiquitylated species. In contrast to wild-type p21, a p21 deleted in the D box motif was not efficiently ubiquitylated, even in the presence of both APC/C and Cdc20 (Figure 4E and Figure S3).

## Silencing of Cdc20 Stabilizes p21 in Early Mitosis and Inhibits Cdk1 Activity

To confirm that Cdc20 is required for the degradation of p21, we used the small interfering RNA (siRNA) technique to reduce the expression of Cdc20. When compared to cells transfected with control double-stranded RNA (dsRNA) oligos, cells transfected with two different dsRNA oligos targeting Cdc20 (including a previously validated dsRNA oligo [Donzelli et al., 2002; Elbashir et al., 2001]) showed an upregulation of p21 levels (Figure 5A). Because

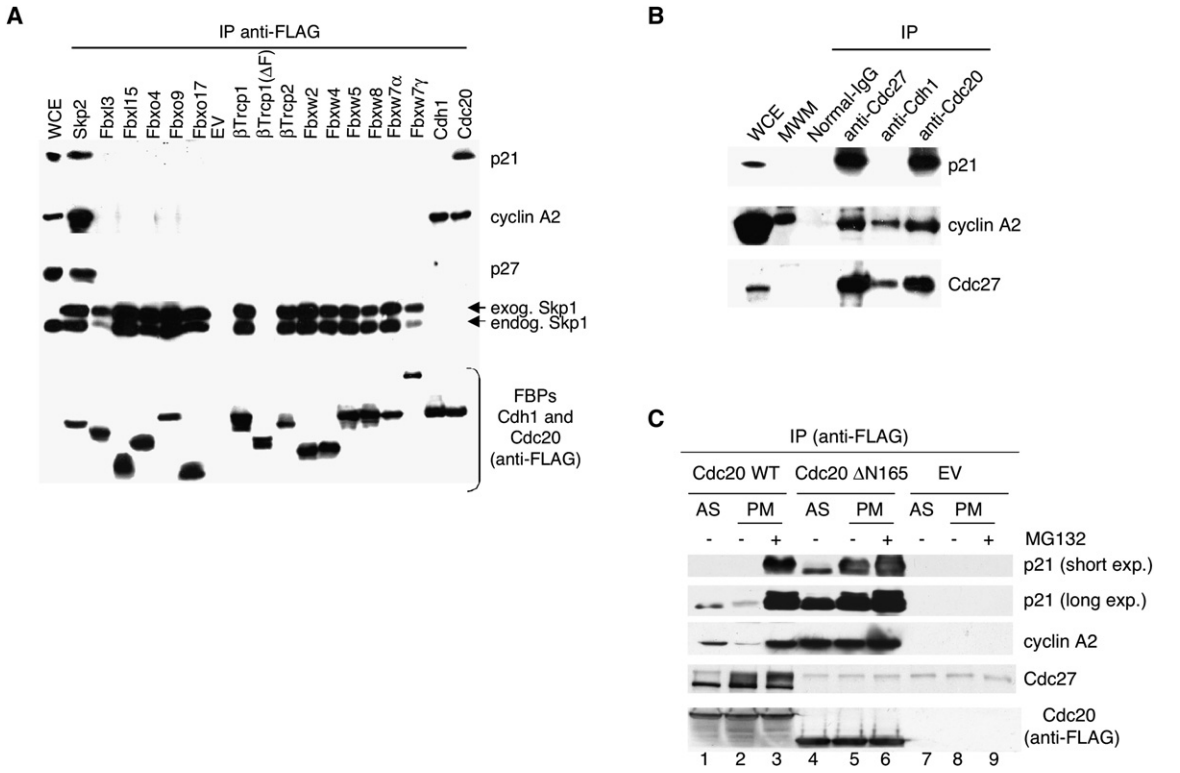



**Figure 3. p21 Interacts with Cdc20**

(A) HEK293T cells were transfected with an empty vector (EV) or constructs encoding the indicated FLAG-tagged F box proteins (FBPs), FLAG-Cdh1, or FLAG-Cdc20, all in the presence of a plasmid encoding His-tagged Skp1. During the last 6 hr before harvesting, cells were treated with the proteasome inhibitor MG132. Exogenous proteins were immunoprecipitated (IP) from whole-cell extracts (WCE) with an anti-FLAG resin, and immunocomplexes were probed with antibodies to the indicated proteins.

(B) Endogenous Cdc20 and Cdc27 coimmunoprecipitate endogenous p21. Whole-cell extracts (WCE) from HeLa cells were immunoprecipitated (IP) with normal IgG, anti-Cdc20, anti-Cdc27, or anti-Cdh1 antibodies. Immunocomplexes were probed with antibodies to the indicated proteins. The second lane contained molecular weight markers (MWM) that appear to crossreact with the anti-cyclin A2 antibody.

(C) HeLa cells were infected with an empty vector (EV) or constructs encoding FLAG-tagged Cdc20 or FLAG-tagged Cdc20 ΔN165 mutant. Where indicated, the proteasome inhibitor MG132 was added during the last 6 hr in culture. Exogenous proteins were immunoprecipitated (IP) from extracts obtained from asynchronous (AS) or prometaphase (PM) cells using an anti-FLAG resin. Immunocomplexes were then probed with antibodies to the indicated proteins.

Cdc20 is mostly active in mitosis, the accumulation of p21 was presumably due to the mitotic fraction present in asynchronous cells. In agreement with this hypothesis, silencing of Cdc20 induced an increase in p21 levels only in U-2 OS cells synchronized in prometaphase but not at G1/S (Figure 5B). We also performed a complementary experiment: U-2 OS cells arrested in prometaphase with nocodazole were collected by mitotic shake-off and then allowed to progress through mitosis and into the next cell cycle. The levels of p21 were found to be low in mitosis and to steadily increase during the G1 phase (concomitantly with an increase in p27 levels) (Figure 5C). However, when Cdc20 was knocked down by siRNA, a delay in the degradation of both p21 and cyclin A2 was observed (Figure 5C). In addition, a delay in the exit from mitosis was observed, as judged by flow cytometry and the levels of phosphorylated Cdc27 and phosphorylated histone H3.

The fact that knockdown of Cdc20 is ineffective in inducing an increase in p21 levels during G1 (Figure 5C), G1/S (Figure 5B), and G2 (not shown) demonstrates that Cdc20 promotes the degradation of p21 only in early mitosis. The accumulation of p21 in response to Cdc20 knockdown both in unperturbed cells (Figure 5A and data not shown) and in cells in which the spindle assembly checkpoint is activated and sustained by nocodazole (Figures 5B and 5C), indicates that Cdc20 promotes p21 degradation irrespective of the spindle checkpoint.

Finally, we investigated whether downregulation of Cdc20 in prometaphase affects the activity of Cdk1. After downregulating Cdc20 in prometaphase HCT116 cells, we observed an accumulation of p21 (Figure S4) and an increase of p21 bound to Cdk1, cyclin A2, and cyclin B1 (Figure 6). Accordingly, the kinase activities associated with these cyclin-CDK complexes were strongly reduced, as assayed using histone H1 as a substrate. However, when the

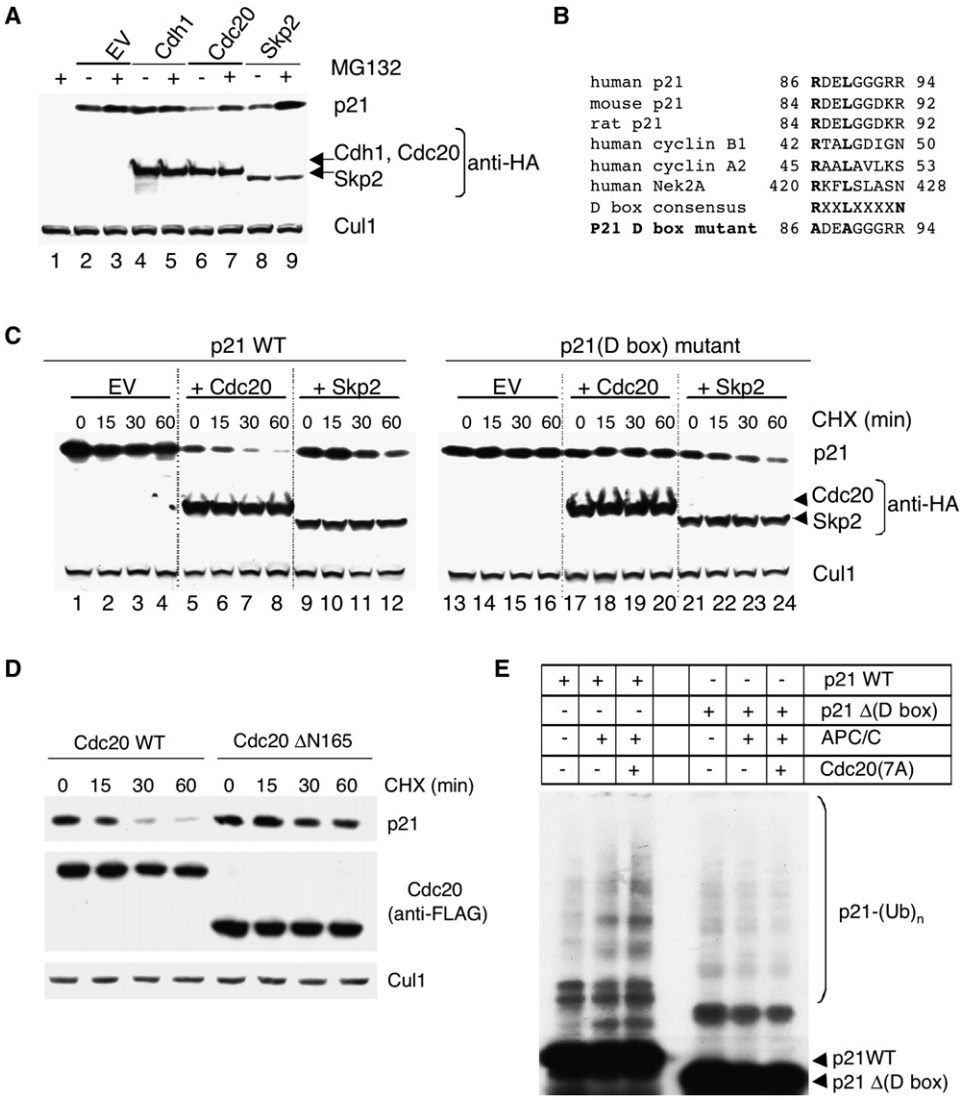



**Figure 4. Cdc20-Dependent Ubiquitylation and Degradation of p21 Depend on the D Box of p21**

(A) Whole-cell extracts were prepared from HEK293T cells transfected with p21 and either HA-tagged Cdc20, HA-tagged Cdh1, or HA-tagged Skp2. Levels of p21 were detected by immunoblotting with an anti-p21 antibody, whereas Cdc20, Cdh1, and Skp2 were analyzed using an anti-HA antibody. Cul1 was used as a loading control. Where indicated, the proteasome inhibitor MG132 was added during the last 6 hr in culture. No significant changes in the cell-cycle phases (as monitored by flow cytometry) of transfected HEK293T cells were observed compared to controls.

(B) Alignment of the amino acid regions corresponding to the putative Destruction box motif (D box) in human, mouse, and rat p21 with the D box motifs of human cyclin B1, cyclin A2, and Nek2A. A schematic representation of the Arg-to-Ala and Leu-to-Ala substitutions in the putative D box motif of human p21 is depicted at the bottom.

(C) HEK293T cells were transfected with wild-type p21 or the p21(D box) mutant in the presence of either an empty vector (EV), HA-tagged Cdc20, or HA-tagged Skp2. Twenty-four hours after transfection, cells were treated with cycloheximide (CHX), and the half-life of p21 was analyzed by immunoblotting.

(D) HEK293T cells were transfected with wild-type p21 in the presence of either FLAG-tagged Cdc20 or FLAG-tagged Cdc20 ΔN165 mutant. Twenty-four hours after transfection, cells were treated with cycloheximide (CHX), and the half-life of p21 was analyzed by immunoblotting.

(E) In vitro ubiquitin ligation assay of $^{35}$S-labeled in vitro-transcribed/translated p21 or p21Δ(D box) mutant was conducted in the absence or presence of immunopurified APC/C and constitutively active Cdc20(7A) mutant, as indicated. Samples were incubated at 30°C for 90 min. The bracket on the right side of the panels marks a ladder of bands corresponding to polyubiquitylated p21.

same experiments were repeated in HCT116 *p21*$^{-/-}$ cells (Waldman et al., 1996), the kinase activities associated with Cdk1 and cyclin B1 were unaffected. Cyclin A2 activity increased, because cyclin A2 is a substrate of APC/C$^{Cdc20}$. These experiments show that Cdc20 is involved in the activation of Cdk1 by mediating the proteolysis of p21.

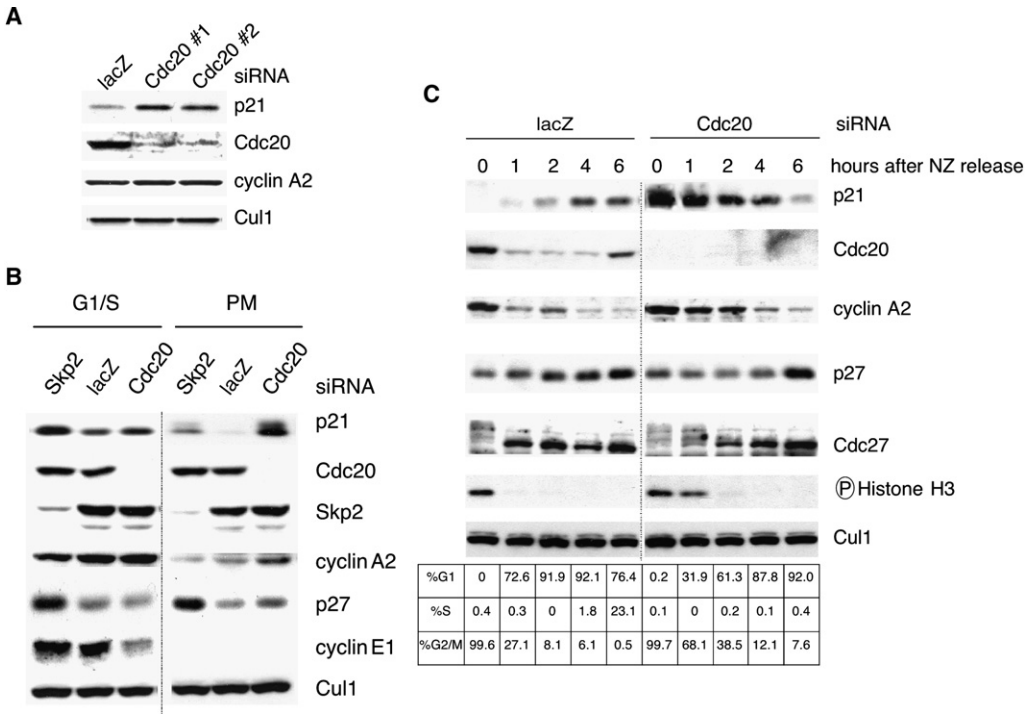



**Figure 5. Cdc20 Targets p21 for Degradation in Prometaphase**

(A) HeLa cells were transfected twice with siRNA molecules to a nonrelevant mRNA (lacZ) or to Cdc20 mRNA (the latter using two different siRNA oligos: #1 and #2). Protein extracts were probed with antibodies to the indicated proteins.

(B) U-2 OS cells were transfected twice with siRNA oligos to a nonrelevant mRNA (lacZ) or to Cdc20 mRNA. Cells were then treated with aphidicolin for 24 hr to arrest cells at G1/S (first three lanes). In a parallel experiment, aphidicolin was washed away to allow cells to progress through the cell cycle for an additional 18 hr in the presence of nocodazole (last three lanes). The prometaphase (PM), round cells were collected by gentle shake-off. Cell extracts were subjected to immunoblotting with antibodies to the indicated proteins.

(C) U-2 OS cells were transfected twice with siRNA oligos corresponding to a nonrelevant mRNA (lacZ) or to Cdc20 mRNA. Twelve hours after the last transfection, nocodazole (NZ) was added for an additional 16 hr. Round, prometaphase cells were then collected by gentle shake-off and replated in fresh medium for the indicated times. Cells were harvested, and cell extracts were analyzed by immunoblotting with antibodies to the indicated proteins. Cell-cycle phases were monitored by flow cytometry.

## DISCUSSION

### Control of p21 Ubiquitylation by APC/C$^{Cdc20}$ and SCF$^{Skp2}$ at Different Phases of the Cell Cycle

During cell-cycle progression, levels of p21 increase in G1 and then decrease at G1/S to free CDK and PCNA complexes. Genetic and biochemical evidence indicates that at G1/S the degradation of p21 (Bornstein et al., 2003; Wang et al., 2005; Yu et al., 1998) and p27 (Guardavaccaro and Pagano, 2006) is controlled by the SCF$^{Skp2}$ ubiquitin ligase. However, in contrast to p27, p21 levels rise again in G2 and then decline once more in early M (Dulic et al., 1998; Li et al., 1994; and Figures 1A and 1B). This final decrease contributes to the full activation of Cdk1 (Bates et al., 1998; Bunz et al., 1998; Chan et al., 2000; Dulic et al., 1998; Medema et al., 1998; Niculescu et al., 1998), which is necessary for mitotic events.

During G2 and M, the SCF$^{Skp2}$ ubiquitin ligase promotes the degradation of p27 and likely additional substrates. However, the mitotic phenotype present in *Skp2*$^{-/-}$ MEFs and *Skp2*$^{-/-}$ hepatocytes is not observed in *Skp2*$^{-/-}$;*p27*$^{-/-}$ cells (Nakayama et al., 2004), strongly advocating the idea that p27 is the major G2/M substrate of Skp2 and that other substrates are degraded via different or alternative routes. Accordingly, p21 accumulates in *Skp2*$^{-/-}$ and *Skp2*$^{-/-}$;*p27*$^{-/-}$ MEFs during S phase but not in M (Figure 2). Herein, we show that p21 degradation in mitosis is promoted by APC/C$^{Cdc20}$. This process requires a conserved D box present in p21 (Figure 4). In contrast to APC/C$^{Cdc20}$, APC/C$^{Cdh1}$ does not induce the degradation of p21. In fact, by promoting degradation of Skp2 and Cdc20 (Bashir et al., 2004; Reis et al., 2006; Wei et al., 2004) APC/C$^{Cdh1}$ induces the stabilization of p21. Thus, APC/C exerts dual control on p21 stability: during G1, it allows p21 stabilization (via the Cdh1-mediated degradation of Skp2 and Cdc20), while in M, it induces the proteolysis of p21 (via Cdc20). Moreover, APC/C$^{Cdc20}$ maintains dual control over Cdk1 activity: in early M, Cdc20 promotes Cdk1 activation (by inducing the elimination of p21), whereas in late M, it induces Cdk1 inactivation (by promoting the degradation of mitotic cyclins). Notably, the positive effect of Cdc20 in the activation of Cdk1

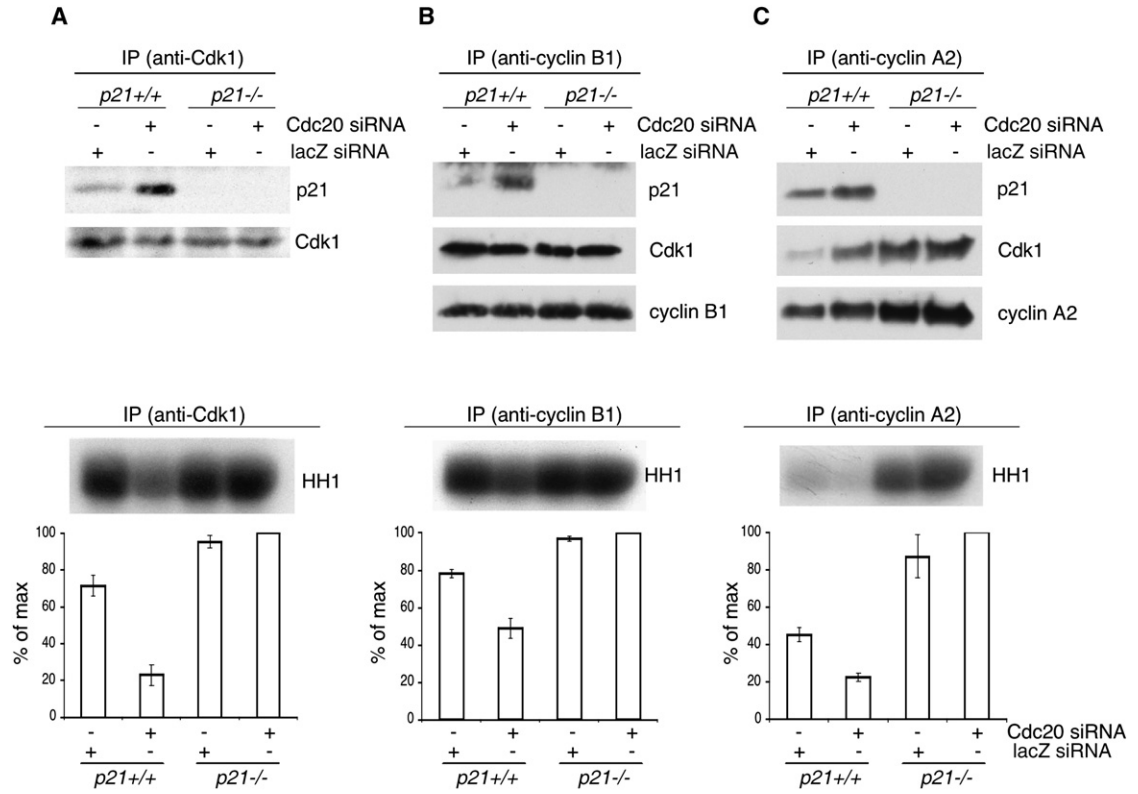



**Figure 6. Cdc20-Mediated Degradation of p21 Activates Cdk1 in Early Prometaphase**

Hct116 $p21^{+/+}$ and Hct116 $p21^{-/-}$ cells were transfected twice with siRNA oligos to a nonrelevant mRNA (lacZ) or to Cdc20 mRNA, as indicated. Cells were then synchronized at G1/S using a double thymidine block and then released to allow cells to progress through the cell cycle for 18 hr (adding nocodazole during the last 15 hr). Prometaphase cells were collected by gentle shake-off. Cell extracts were subjected to immunoprecipitation (IP) with antibodies against Cdk1 (A), cyclin B1 (B), or cyclin A2 (C). Immunocomplexes were probed with antibodies to the indicated proteins (upper panels) or used to perform kinase assays using histone H1 (HH1) as a substrate (bottom panels). The error bars represent ±SD from triplicate experiments quantified by measuring radioactivity in the HH1 band excised from the dried gel. A representative experiment showing an autoradiograph of $^{32}$P-labeled HH1 is presented on the top of the bottom panels.

during prometaphase is mediated by the induction of p21 proteolysis, as silencing of Cdc20 has no effect on Cdk1 activity in $p21^{-/-}$ cells (Figure 6). While both APC/C$^{Cdc20}$ and APC/C$^{Cdh1}$ are known to be involved in either the direct or indirect inactivation of CDKs (Peters, 2006), this study demonstrates that APC/C also positively controls the activity of a CDK, namely, Cdk1. Additionally, whereas, with the exception of Nek2A, all substrates of APC/C$^{Cdc20}$ identified so far in metazoans are also recognized by APC/C$^{Cdh1}$, APC/C$^{Cdh1}$ has many specific substrates (Acquaviva and Pines, 2006; Peters, 2006; Thornton and Toczyski, 2006). Thus, p21 represents a specific substrate of metazoan APC/C$^{Cdc20}$.

In mitosis, the spindle assembly checkpoint inhibits the separation of sister chromatids until the microtubules radiating from the spindle poles are correctly attached to the kinetochores. At a molecular level, the spindle checkpoint ensures the inhibition of the bulk of APC/C$^{Cdc20}$ activity to avoid the degradation of both Securin (to prevent the separation of sister chromatids) and cyclin B (to prevent a premature exit from mitosis). However, the reason for a subpopulation of APC/C$^{Cdc20}$ to remain active in the presence of spindle checkpoint activity is not well understood. Because Cdk1 inhibition overcomes the prometaphase arrest mediated by the spindle checkpoint (Brito and Rieder, 2006; D'Angiolella et al., 2003), our results suggest that APC/C$^{Cdc20}$-mediated degradation of p21 contributes to the maintenance of the high levels of Cdk1 activity necessary to sustain the spindle checkpoint. If p21 was not degraded at this time, Cdk1 would be inhibited with consequent, rapid adaptation to the spindle checkpoint, resulting in mitotic slippage without cytokinesis and consequent aneuploidy. Interestingly, forced expression of p21 has been reported to cause polyploidy, particularly after mitotic spindle disruption (Bates et al., 1998; Chang et al., 2000; Niculescu et al., 1998).

There is extensive evidence in favor of Skp2-mediated degradation of p21. First, in vitro ubiquitylation of p21 is mediated by SCF$^{Skp2}$ and its cofactor Cks1 and is promoted by the phosphorylation of p21 on Ser130 by the cyclin E-Cdk2 complex (Bornstein et al., 2003; Wang et al., 2005; Zhu et al., 2005). A conserved glutamic acid

at the –2 position of Ser130 is also involved in the binding of p21 to SCF$^{Skp2}$ (V.A. and M.P., unpublished data). The corresponding residues in p27 (Glu185 and Thr187) play a critical role in allowing p27 binding to the Skp2-Cks1 complex (Hao et al., 2005), suggesting that p21 and p27 are similarly recognized by SCF$^{Skp2}$. Second, p21 is stabilized in *Skp2*$^{-/-}$ cells (Figure 2 and Bornstein et al., 2003), when either Skp2 or Cul1 is downregulated (Figure 4; Sarmento et al., 2005; Yu et al., 1998), or when a Cul1 dominant-negative mutant is overexpressed (J. Bloom, R. Piva, and M.P., unpublished data). In addition, silencing of the SCF modulator Cand1 (Y. Xiong, personal communication) or the inactivation of a temperature-sensitive Nedd8-activating enzyme (Bloom et al., 2003), which is required for optimal SCF activity, also induces a significant accumulation of p21. Third, siRNA oligos targeting Cdh1 (a negative regulator of Skp2) or the forced expression of Skp2 induce the degradation of p21 (Figure 4A; Bashir et al., 2004; Wang et al., 2005). Despite this evidence, it has been suggested that the accumulation of p21 in *Skp2*$^{-/-}$ MEFs is not due to a direct role of Skp2 in mediating p21 degradation but rather is a result of an accumulation of cyclin E (Chen et al., 2004), which would stabilize p21 by a unknown mechanism. However, several data conflict with this explanation: (1) p21 stabilization is also observed under conditions in which cyclin E accumulation is not observed, such as in *Skp2*$^{-/-}$;*p27*$^{-/-}$ MEFs (Figure 4 of Bornstein et al. [2003]), Figure 3 of Kossatz et al. [2004], and Figure 2 of this paper) and after Skp2 silencing (Figure 5B). (2) p21 is destabilized in response to the forced expression of cyclin E (Wang et al., 2005). (3) A p21 mutant deficient for interaction with cyclin-CDK complexes displayed enhanced stability (Cayrol and Ducommun, 1998). (4) Phosphorylation of p21 by cyclin E-Cdk2 promotes its ubiquitylation and degradation (Bornstein et al., 2003; Wang et al., 2005; Zhu et al., 2005). (5) At G1/S, p21 is degraded in concomitance with an increase in cyclin E levels (see, for example, Figure 1A).

In conclusion, p21 is targeted by APC/C and SCF at different phases of the cell cycle, similar to other cell cycle regulatory proteins such as Cdc25A (Guardavaccaro and Pagano, 2006). Although our data indicate a role for Skp2 in the proteolysis of p21 during S phase, we cannot exclude that a minor fraction of p21 is also degraded via Skp2 in mitosis, because silencing of Skp2 induces a slight accumulation of p21 in prometaphase cells (Figure 5B). The lack of p21 accumulation in mitotic *Skp2*$^{-/-}$ MEFs (Figure 2) could be in fact due to compensation by related proteins during development, which cannot occur during acute silencing of Skp2 in somatic cells.

## Ubiquitin-Mediated and Ubiquitin-Independent Degradation of p21

p21 is a short-lived protein and is degraded by the proteasome. Ubiquitylated forms of both endogenously and exogenously expressed p21 have been detected in cultured cells, and these ubiquitylated forms increase after proteasome inhibition (Bloom et al., 2003; Cayrol and Ducommun, 1998; Coulombe et al., 2004; Fukuchi et al., 2002; Maki and Howley, 1997; Maki et al., 1996; Rousseau et al., 1999; Sheaff et al., 2000; Sugimoto et al., 2006), suggesting that the proteasome degrades p21 marked with ubiquitin chains. In spite of these reports, a ubiquitin-independent, proteasome-dependent mechanism for p21 degradation has been proposed as the sole route for proteolysis (Chen et al., 2004, 2007; Sheaff et al., 2000). Initially, this theory was based on the observation that a lysine-less p21 mutant (p21[K0]) is degraded with kinetics similar to those of p21 wild-type. Because ubiquitin chains are usually covalently bound to lysine residues of the substrate, there was a potential that this mutant could not be ubiquitylated. In contrast to this idea, two groups have independently shown that the p21(K0) mutant is indeed N-terminal ubiquitylated both in vitro and in vivo (Bloom et al., 2003; Coulombe et al., 2004). In addition, two temperature-sensitive cell lines carrying mutations in the ubiquitin- and Nedd8-activating enzymes, respectively, displayed stabilization of p21 at the restrictive temperature (Bloom et al., 2003; Coulombe et al., 2004; and F. Bernassola and G. Melino, personal communication). Inhibition of p21 ubiquitylation both in vitro (using methylated ubiquitin) and in vivo (using a lysine-less ubiquitin mutant) stabilized endogenous p21, as well as exogenous wild-type p21 and a p21(K0) mutant (Bloom et al., 2003; Coulombe et al., 2004). These experiments strongly indicated that the degradation of p21 largely requires its ubquitylation. The regulating function exerted by the APC/C$^{Cdc20}$ and SCF$^{Skp2}$ ubiquitin ligases on p21 stability further confirms the role of the ubiquitin system in this important biological process.

It is possible, however, that a fraction of cellular p21 is degraded in a ubiquitin-independent manner. For example, free recombinant p21, but not p21 bound to a cyclin-CDK complex, is degraded in vitro by the proteasome in the absence of ubiquitylation (Bloom et al., 2003; Liu et al., 2003). This effect is due to the fact that p21 is natively unfolded and, as such, can enter the proteasome pore by free diffusion. Although we have been unable to detect free p21 in extracts from human fibroblasts (Bloom et al., 2003), it is conceivable that some free p21 exists, at least transiently in certain cell types and under certain cellular conditions, and that this fraction is degraded in a ubiquitin-independent manner. Accordingly, a recent study showed that only unbound p21 is targeted by the proteasome in the absence of ubiquitylation, and this process is limited to only certain cell lines (Li et al., 2007). Together, these views would reconcile the ubiquitin-independent and the ubiquitin-dependent theories of p21 degradation. In contrast, a hypothesis proposing the ubiquitin-independent degradation of p21 as the only mechanism cannot explain how the proteasome degrades, in a temporally regulated fashion, p21 that is present in different protein complexes in the absence of ubiquitylation, and why, if p21 is ubiquitylated in vivo, its degradation should not require ubiquitylation.

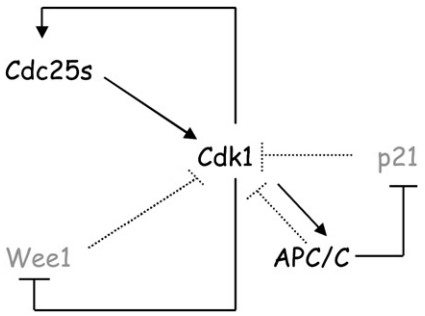



**Figure 7. Model of Cdk1 Autoactivation via Three Feedback Loops, Including the Activation of the APC/C$^{Cdc20}$-Mediated Degradation of p21 Occurring in Early Mitosis**

Black signifies activated forms of the respective proteins, gray indicates inactive forms or degraded proteins. Solid lines indicate activating (→) or inhibitory (–|) activities in prometaphase; dotted lines indicate activating or inhibitory activities in different phase of the cell cycle. See text for details.

## Ubiquitylation on the N Terminus Is Sufficient for p21 Degradation

It has been reported that endogenous p21 is acetylated at the N terminus (Figure 1 of Chen et al. [2004]). Based on the assumption that mass spectrometry can quantitatively attest that 100% of a protein is acetylated and that acetylation is irreversible, it has been inferred that endogenous p21 cannot be ubiquitylated at the N terminus as N-terminal acetylation would block N-terminal ubiquitylation. However, in this experiment the p21 band for the mass spectrometry analysis was excised from a gel, and therefore, slower-migrating, ubiquitylated species of p21 (which potentially may not be acetylated) were not accounted for. Moreover, N-terminal acetylation has been proposed as corroboration for the ubiquitin-independent degradation of p21, based on the belief that ubiquitylation of internal lysines, although present, is not necessary for p21 degradation. However, there is no evidence that ubiquitylation of internal lysines is not required to target endogenous p21 for degradation. Yet, when a p21(K0) mutant is expressed in mammalian cells, N-terminal ubiquitylation appears as one possible way of p21 degradation. Accordingly, a p21(K0) mutant tagged at the C terminus (and therefore not interfering with N-terminal acetylation) is robustly ubiquitylated in vivo (Figure 5 in Bloom et al. [2003]). Thus, ubiquitylation at the N terminus is sufficient for degradation, though it may not be essential for endogenous p21. Interestingly, Cdc20 and Skp2 are both able to induce the degradation of the p21(K0) mutant (Figure S5), suggesting that Cdc20 and Skp2 can use N-terminal ubiquitylation of p21 to induce its degradation, at least under these particular circumstances.

## Conclusions

p21 contributes to the maintenance of the G2 state by inhibiting Cdk1 activity (Bates et al., 1998; Bunz et al., 1998; Chan et al., 2000; Dulic et al., 1998; Medema et al., 1998; Niculescu et al., 1998). It is well established that Cdk1 promotes its own full activation through two amplification loops, one involving the activation or stabilization of its activators (the Cdc25 phosphatases) and a second promoting the degradation of its inhibitor (the Wee1 kinase) (Guardavaccaro and Pagano, 2006). Our results suggest a positive feedback model where Cdk1, by phosphorylating certain subunits of APC/C$^{Cdc20}$, promotes the activity of APC/C$^{Cdc20}$ (Peters, 2006), consequently triggering the degradation of p21, resulting in a further activation of Cdk1 (Figure 7). We propose that the degradation of p21 via APC/C$^{Cdc20}$ contributes to the full activation of Cdk1 in early M and prevents mitotic slippage during activation of the spindle assembly checkpoint.

## EXPERIMENTAL PROCEDURES

### Cell Culture, Synchronization, and Treatment with Drugs

T98G cells are revertants from T98 glioblastoma cells that acquired the ability to arrest in G0/G1 in low serum (Dorrello et al., 2006; Lisztwan et al., 1998; Stein, 1979). Normal human fetal lung fibroblasts (IMR-90) were obtained from the American Type Culture Collection. HEK293T, IMR90, U-2 OS, HeLa, and T98G cells and MEFs from *Skp2*$^{-/-}$;*p27*$^{+/+}$, *Skp2*$^{-/-}$;*p27*$^{-/-}$, and wild-type littermate control mice (*Skp2*$^{+/+}$;*p27*$^{+/+}$) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS). *Skp2*$^{-/-}$;*p27*$^{+/+}$, *Skp2*$^{-/-}$;*p27*$^{-/-}$, and wild-type MEFs were a gift from K. Nakayama (Kyushu University). HCT116 *p21*$^{+/+}$ and HCT116 *p21*$^{-/-}$ colon cancer cells (Waldman et al., 1996) were a gift from B. Vogelstein (Johns Hopkins University). T98G and IMR90 cells were serum starved in the presence of 0.02% and 0.2% FCS, respectively, as previously described (Dorrello et al., 2006; Lisztwan et al., 1998). Cells were synchronized at G1/S using aphidicolin (2 μg/ml) or a double thymidine block (2.5 mM), as described (Peschiaroli et al., 2006). Synchronization in prometaphase with nocodazole and mitotic shake-off was performed as described (Guardavaccaro et al., 2003). To test the in vivo interaction between different proteins and endogenous p21, the proteasome inhibitor MG132 (10 μM final concentration) was added for 6 hr prior to harvesting the cells. To measure protein half-lives, cells were incubated in the presence of 100 μg/ml cycloheximide (CHX) (Sigma-Aldrich) diluted in 100% ethanol. Cell-cycle phases were monitored by flow cytometry, as described (Carrano and Pagano, 2001).

### Biochemical Methods

Extract preparation, immunoprecipitation, and immunoblotting were previously described (Dorrello et al., 2006; Busino et al., 2007). Rabbit polyclonal antibodies were from Santa Cruz Biotechnology (against p21, Cdc20, and HA-tag). Mouse monoclonal antibodies were from Sigma-Aldrich (anti-Cdc27 and FLAG), Lab Vision (anti-Cdh1), Invitrogen (anti-Cul1, anti-Skp1, and anti-Skp2), Covance (anti-HA), and BD Transduction Laboratories (anti-p21 and anti-p27). The rabbit polyclonal antibodies against Cdk1, cyclin A2, cyclin B1, cyclin E1, and phospho-histone H3 were previously described (Carrano et al., 1999; Peschiaroli et al., 2006). Histone H1 kinase reactions were performed as described (Carrano and Pagano, 2001).

### Plasmids

Cdc20 and p21 point mutants were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Deletion mutants were generated by a PCR-based approach. All constructs were sequenced prior to use, and primer sequences are available upon request.

### Transient Transfections and Retroviral-Mediated Gene Transfer

HEK293T cells were transfected using the calcium phosphate method as described (Bashir et al., 2004). U-2 OS cells were transiently transfected using ExGen 500 reagent (Fermentas), according to the manufacturer's instructions. For experiments investigating the ability of Cdc20 to induce the degradation of p21 (Figure 4), Cdc20 and p21 were cotransfected in a 3:1 ratio. For retrovirus production, packaging GP-293 cells (Clontech) were transfected with FuGENE transfection reagent (Roche) according to the manufacturer's instructions. Forty-eight hours after transfection, the virus-containing medium was collected and supplemented with 8 μg/ml polybrene (Sigma). Cells were then infected by replacing the cell culture medium with the viral supernatant for 6 hr.

### In Vitro Ubiquitylation Assay

Ubiquitylation assays were carried out using a procedure based on a combination of previously described protocols (Fang et al., 1998; Kramer et al., 1998). Briefly, prometaphase HeLa cells were washed with PBS and lysed for 30 min on ice using Triton buffer (50 mM Tris [pH 7.5], 250 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 1 mM DTT, and phosphatase and protease inhibitors). An anti-Cdc27 antibody (15 μg) was added to 10 mg of cell extract and incubated for ~3 hr at 4°C. Protein G agarose was then added and incubated for 45 min at 4°C on a rotating wheel. The beads were washed four times in Triton buffer and four times in QA buffer (10 mM Tris-HCl [pH 7.5], 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 1 mM DTT). In vitro-transcribed/translated, unlabeled, constitutively active Cdc20(7A) mutant (Yudkovsky et al., 2000) was added and incubated 30 min at 37°C. The beads were subsequently washed three times with QA buffer. The resulting beads were used for two reactions of in vitro ubiquitylation. Ubiquitylation assays were performed in a volume of 10 μl containing 40 mM Tris (pH 7.6), 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 1.5 μg/ml E1 (Boston Biochem), 20 μg/ml UbcH10 (Boston Biochem), 2.5 mg/ml ubiquitin (Sigma), 1 μM ubiquitin aldehyde, ~0.25 μg/ml cyclin A2/Cdk2 complex, 20 μM staurosporine, and 1 μl of in vitro-transcribed/translated [$^{35}$S]p21 as a substrate. Methyl-ubiquitin (0.2 mg/ml) was added where indicated. The reactions were incubated at 30°C for the indicated times and analyzed by SDS-PAGE.

### Gene Silencing by Small Interfering RNA

siRNA duplexes were transfected into cells using oligofectamine (Invitrogen) according to manufacturer's instructions and as described (Dorrello et al., 2006; Peschiaroli et al., 2006). Forty-eight hours after transfection, cells were collected after further treatments. The siRNA oligonucleotide sequence for Cdh1 was 5′-AAUGAGAAGUCUCCCAG UCAGdTdT-3′ (corresponding to nt 199–219 of human Cdh1 cDNA). The siRNA oligonucleotide sequences for Cdc20 were 5′-AACGGCA GGACUCCGGGCCGAdTdT-3′ (oligo #1, corresponding to nt 156–170 of human Cdc20 cDNA) and 5′-AAUGGCCAGUGGUGGUAAU GAdTdT-3′ (oligo #2, corresponding to nt 969–989 of human Cdc20 cDNA). The siRNA oligonucleotide sequence for Skp2 was 5′- AAGGG AGUGACAAAGACUUUGdTdT-3′ (corresponding to nt 218–238 of human Skp2 cDNA). A 21 nt siRNA duplex corresponding to a nonrelevant gene (lacZ) was used as control.

### Supplemental Data

Supplemental Data include five figures and Supplemental References and can be found with this article online at http://www.molecule.org/cgi/content/full/27/3/462/DC1/.

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