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Article

Ubiquitination of Cdc20 by the APC Occurs through an Intramolecular Mechanism

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Summary

Background: Cells control progression through late mitosis by regulating Cdc20 and Cdh1, the two mitotic activators of the anaphase-promoting complex (APC). The control of Cdc20 protein levels during the cell cycle is not well understood.
Results: Here, we demonstrate that Cdc20 is degraded in budding yeast by multiple APC-dependent mechanisms. We find that the majority of Cdc20 turnover does not involve a second activator molecule but instead depends on in cis Cdc20 autoubiquitination while it is bound to its activator-binding site on the APC core. Unlike in trans ubiquitination of Cdc20 substrates, the APC ubiquitinates Cdc20 independent of APC activation by Cdc20's C box. Cdc20 turnover by this intramolecular mechanism is cell cycle regulated, contributing to the decline in Cdc20 levels that occurs after anaphase. Interestingly, high substrate levels in vitro significantly reduce Cdc20 autoubiquitination.
Conclusion: We show here that Cdc20 fluctuates through the cell cycle via a distinct form of APC-mediated ubiquitination. This in cis autoubiquitination may preferentially occur in early anaphase, following depletion of Cdc20 substrates. This suggests that distinct mechanisms are able to target Cdc20 for ubiquitination at different points during the cell cycle.

Introduction

Chromosome segregation is one of the most tightly regulated events in the dividing cell. Incorrect entry into anaphase can have catastrophic cellular consequences ranging from genomic instability to cell death. Anaphase is initiated by the anaphase-promoting complex/cyclosome (APC) [1, 2], an E3 ubiquitin ligase composed of at least 13 core subunits [3, 4]. APC function is regulated by association with one of two activator subunits, Cdc20 or Cdh1 (also known as Hct1) [5–7]. These proteins are thought to function both in the binding of substrates to the APC [8] and APC activation [9]. Cdc20 associates with the APC in early mitosis and triggers anaphase onset by promoting the destruction of a subset of mitotic cyclins and securin (also known as Pds1) [10, 11], resulting in the activation of Esp1 and the separation of sister chromatids through cleavage of cohesion [12]. In late mitosis and G1, Cdh1 associates with the APC, promoting mitotic exit and maintaining low Cdk activity.

Both activators contain well-conserved motifs involved in APC and substrate binding (Figure 1A). APC binding is mediated by both a C-box motif within the activator's N terminus [8] and a C-terminal isoleucine-arginine (IR) motif [13, 14] (Figure 1A). Substrate binding is mediated by a WD40 domain that is likely to interact directly with degradation signals found within substrates [15], the most common being the destruction box (D box, DB) [16] and KEN box [17]. Processive substrate ubiquitination has also been shown to require the core APC subunit Doc1 [14, 18], which is thought to function as a coreceptor for the D box in conjunction with the WD40 of Cdc20/Cdh1 [19, 20].

The two mitotic APC activators are thought to function analogously, but they are regulated in distinct ways. Whereas Cdh1 protein and transcript levels are constitutive, both Cdc20 transcription and protein levels oscillate throughout the cell cycle [21, 29]. Cdc20 is absent in G1 but begins to accumulate in late S phase, its peak coinciding with the initiation of anaphase. Cdh1 is thought to bind an N-terminal D box within Cdc20, leading to the destruction of Cdc20 in late mitosis and G1 [22–24]. However, although Cdh1-mediated turnover of Cdc20 is likely important, several studies have suggested that Cdc20 is also turned over by Cdh1-independent mechanisms [21, 25, 26]. Regulation of Cdc20 levels is very important, because high-level overexpression of Cdc20 is lethal [27] and as little as 3-fold overexpression of Cdc20 is sufficient to override the spindle assembly checkpoint [28].

Previously, we found that deletion of Cdc20's IR motif caused a strong accumulation of Cdc20 in vivo [25], which is inconsistent with Cdc20 simply being a passive Cdh1 substrate. Here, we show that Cdc20 turnover is fully APC-dependent but does not depend on a second activator molecule. Although Cdc20 can be targeted by the APC associated with either Cdh1 or, more poorly, by a second Cdc20 molecule (i.e., in trans turnover), we find that most turnover in vivo, and ubiquitination in vitro, is promoted by direct association with the APC (in cis turnover) (Figure 1B). Consistent with this model, we show that processive ubiquitination of Cdc20 does not require Doc1. Importantly, we find that Cdc20 levels oscillate independently of *CDC20* transcription and Cdh1 activity, implying that the in cis autoregulation of Cdc20 turnover changes during the cell cycle. Additionally this regulation can be influenced by the presence of APC^{Cdc20} substrates. These findings uncover another mechanism by which the activity of the APC is tightly controlled during the cell cycle.

Results

Cdc20 Turnover Depends on the APC

Cdc20 is thought to be destroyed by both APC-dependent mechanisms [21, 29, 30] and APC-independent mechanisms [30]. However, previous experiments suggesting APC-independent Cdc20 turnover were performed with temperature-sensitive APC mutants, which do not necessarily eliminate all APC function. Although the APC is normally essential, we have previously shown that deletion of genes encoding two Cdc20 substrates, Pds1 and Clb5, combined with 10-fold overexpression of the Cdk inhibitor Sic1 ($SIC1^{10x}$), allows cells to survive in the absence of the APC [31]. To determine whether Cdc20 turnover is dependent upon a functional APC, we examined Cdc20 turnover in an *apc11Δ pds1Δ*

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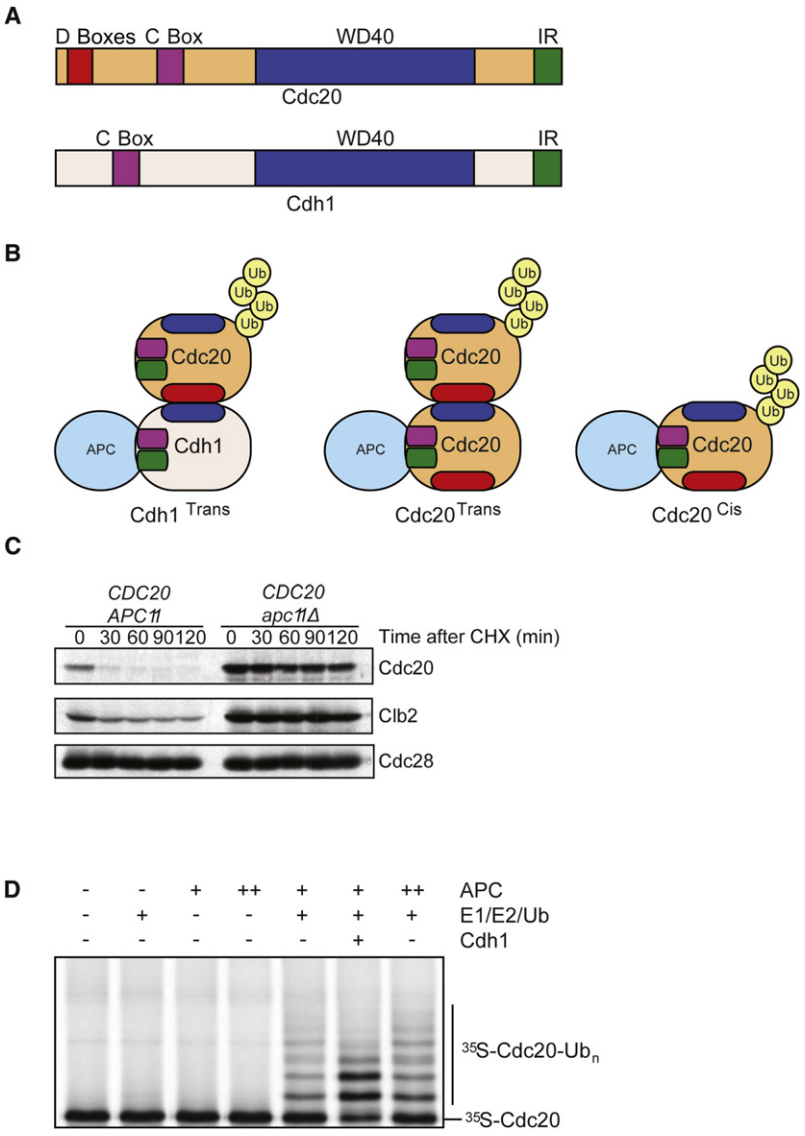

Figure 1. Cdc20 Is Turned Over by the Anaphase-Promoting Complex by Cdh1-Dependent and Cdh1-Independent Mechanisms

(A) Diagrams of Cdc20 and Cdh1. Red, purple, blue, and green boxes represent the D boxes, the C box (CB), the WD40, and the C-terminal isoleucine-arginine (IR), respectively.

(B) Three possible mechanisms of Cdc20 turnover: Cdh1^{trans}, Cdc20^{trans}, and Cdc20^{cis}.

(C) Asynchronous *pds1Δ clb5Δ SIC1^{10x}* cells were collected at indicated time points after cycloheximide addition. Blots were probed with antibodies against Cdc20, Clb2, and Cdc28, which served as a loading control.

(D) Anaphase-promoting complex (APC) immunopurified from tandem affinity purification (TAP)-Cdc16 lysates in a *cdh1Δ* background was used in ubiquitination reactions using in vitro translated (IVT) ZZ-tagged ^{35}S-methionine-Cdc20 purified from rabbit reticulocyte lysates using immunoglobin G beads. APC (++) and (+) are 5 nM and 1 nM final concentrations, respectively. Controls show the dependence on the presence of exogenous E1/E2(Ubc4)/methyl-ubiquitin mix and the APC.

clb5Δ SIC1^{10x} strain. Deletion of *APC11*, which encodes the essential RING finger subunit of the APC [32], abolishes APC activity in the cell. We found that, as with the known APC substrate Clb2 [33], turnover of Cdc20 was eliminated in the *apc11Δ* strain (Figure 1C). This strongly suggests that, under normal conditions, the majority of Cdc20 turnover depends on APC activity.

We postulated that there could be three modes of APC-dependent Cdc20 turnover (Figure 1B). First, as previously suggested, Cdh1 bound to the APC as an activator could recognize Cdc20 as a substrate through Cdc20's D box (Cdh1^{trans}) [22–24]. However, we found previously that whereas Cdc20 levels were slightly increased in *cdh1Δ* cells, they were more dramatically increased in Apc^{-} cells [25], suggesting that the APC targets Cdc20 by Cdh1-independent mechanisms as well. Consistent with this, we observed APC-dependent ubiquitination of Cdc20 both in the presence and absence of Cdh1 in vitro (Figure 1D).

There are two distinct mechanisms by which Cdh1-independent ubiquitination could occur. The first is similar to the Cdh1^{trans} mechanism. Here, one molecule of Cdc20 associates with the APC as an activator and this APC-Cdc20 complex binds a second Cdc20 molecule as a substrate through a WD40/D-box interaction (Cdc20^{trans}, Figure 1B). Alternatively, a single Cdc20 molecule bound to the APC as an activator could be ubiquitinated directly by the APC (Cdc20^{cis}, Figure 1B).

Contribution of the Cdh1-Dependent and Independent Mechanisms to Cdc20 Turnover

We found previously that mutation of Cdc20's IR motif increased steady-state Cdc20 levels [25], consistent with a Cdh1-independent mechanism for Cdc20 turnover. This increase in steady-state level is higher than that observed for wild-type Cdc20 in a *cdh1Δ* strain, suggesting that the Cdh1-independent mechanism is responsible for the majority of Cdc20 turnover (Figure 2A, lanes 5 and 9) [25]. The IRΔ and *cdh1Δ* double mutant was more stable than either single mutant, consistent with multiple mechanisms controlling Cdc20 stability (Figure 2A, lanes 5–16). Because mutation of the IR decreases Cdc20 binding to the APC (data not shown), both Cdc20^{trans} and Cdc20^{cis} could, in principle, be affected. Consistent with this idea, we found that mutation of the IR had no effect on Cdh1-dependent ubiquitination in vitro (see Figure S1A available online) but greatly inhibited autoubiquitination (Figure 2B, lanes 9–12).

To further assess the contribution of the Cdh1^{trans} mechanism in isolation, we sought to create a Cdc20 mutant that was defective in binding to the APC as an activator but could be bound as a substrate through its D boxes. The observation that mutation of Cdc20's IR motif has no obvious growth phenotypes is consistent with it only having a partial effect on Cdc20 binding to the APC. Mutation of C box, however, is lethal and decreases Cdc20 binding to the APC [8, 25], suggesting that C-box mutations greatly reduce interaction with the APC. Therefore, we expected a C-box mutation to eliminate Cdc20^{cis} and Cdc20^{trans} mediated turnover. The

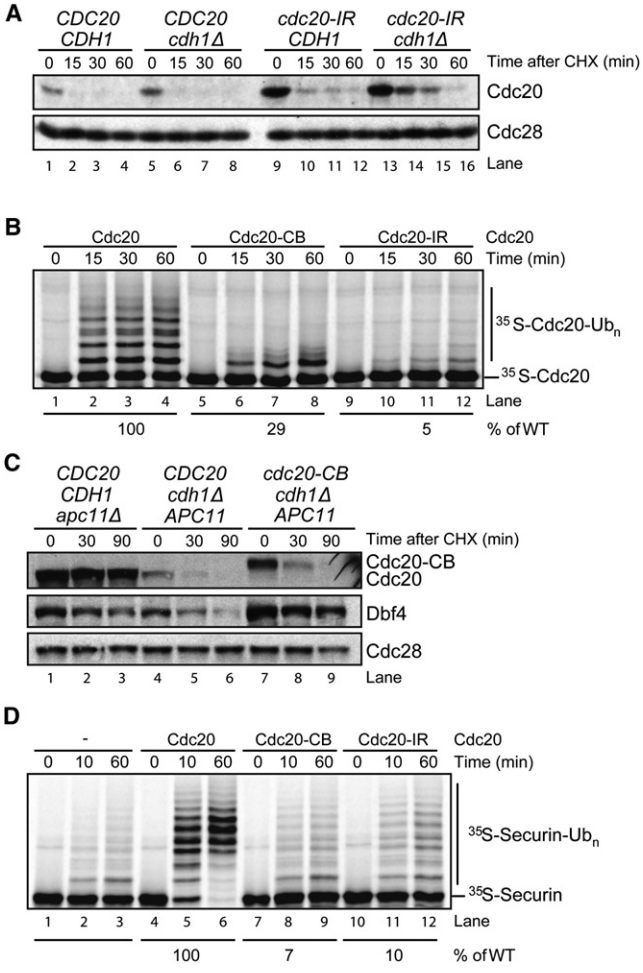

Figure 2. Cdc20 Ubiquitination and Turnover in *cdc20, cdh1* Mutants

(A) Asynchronous *PDS1 CLB5 SIC1* cells were treated with cycloheximide, and samples were analyzed as in Figure 1C. Cdc20-IR denotes the Cdc20-ΔIR allele.

(B) ZZ-tagged ^{35}S-Cdc20 wild-type, C-box mutant (I147A, P148A) or IR mutant (I609A, R610A) were generated by IVT and incubated with APC (5 nM) and E1/E2(Ubc4)/methyl-ubiquitin mix for the indicated times. Quantifications are shown below.

(C) Asynchronous *pds1Δ clb5Δ SIC1^{10x}* cells were treated with cycloheximide and analyzed as in Figure 1C. Cdc20-CB denotes the Cdc20-R145D allele. Cdc20-R145D (Cdc20-CB) protein migrates more slowly on an electrophoretic gel as compared to wild-type Cdc20.

(D) ZZ-tagged unlabeled Cdc20 wild-type, C-box mutant I147A, P148A (Cdc20-CB) or IR mutant I609A, R610A (Cdc20-IR), or a mock purification from IVT lysate with no Cdc20 (−) was preincubated with APC (5 nM) and ZZ-tagged ^{35}S-securin generated by IVT. After a 15 min preincubation, E1/E2(Ubc4)/methyl-ubiquitin mix was added and ubiquitination reactions were performed for the indicated times. See also Figure S1.

minimal conserved sequence of the C box in both Cdc20 and Cdh1 is DRYIP [8]. Previously, we characterized two C-box mutants, a weaker *cdc20-I147A,P148A* allele and a stronger *cdc20-R145D* allele (which did not translate well in vitro) [25]. We examined the turnover of Cdc20-R145D in a *cdh1Δ* strain. Surprisingly, whereas the known Cdc20 substrate Dbf4 was stabilized, the Cdc20-R145D protein was still turned over rapidly, although there was an increase in steady-state levels (Figure 2C, lanes 4–9; Figure S1B). It was possible that this mutation did not entirely eliminate C-box function, so we also analyzed a *cdc20-D144R, R145D* double mutant. This mutant turned over with similar kinetics to the *cdc20-R145D* allele (Figure S1C).

We also observed that the *I147A, P148A* C-box mutant had a larger effect than the IR mutation on securin ubiquitination in vitro (Figure 2D, lanes 7–12). Yet the defect observed with the same C-box mutation is less severe than that observed with the IR mutant in autoubiquitination activity (Figure 2B, lanes 5–12). Thus, although the C box is essential for APC function in vivo, considerable Cdc20 turnover occurs when the C box is mutated. Our results, together with previous evidence that the C box, but not the IR, is essential for viability, indicate that the C box is more important than the IR motif for substrate turnover and less critical for Cdc20 autoubiquitination.

Because neither the IR nor C-box mutation alone eliminated Cdh1-independent turnover, we generated a C box, IR double mutant. Cdc20-IR, R145D should not be able to interact with the APC as an activator and therefore should eliminate both the Cdc20^{trans} and Cdc20^{cis} mechanisms of turnover. Consistent with this, the Cdc20-IR, R145D mutant was strongly stabilized in a *cdh1Δ* strain but could be turned over in a *CDH1* strain (Figure 3A, lanes 7–9 and 13–15). Similarly, we detected ubiquitination of a Cdc20-C-box-IR mutant in the presence of Cdh1 in vitro, and this activity was entirely D-box dependent (Figure 3B, lanes 1–6). These results are consistent with previously suggested model that Cdh1 can target Cdc20 [22–24]. However, the dramatic increase in steady-state levels and the relatively slow rate of turnover in the Cdh1^{trans}-only strain suggests that Cdh1-dependent turnover likely contributes to a small portion of normal Cdc20 turnover (Figure 3A, lanes 7–9).

We next sought to investigate whether the Cdc20^{trans} mechanism makes any contribution to Cdh1-independent turnover. We generated a *cdh1Δ* strain containing a wild-type copy of *CDC20* and the *cdc20-IR, R145D* allele at a second locus. Turnover of Cdc20-IR, R145D should be defective in both the Cdh1^{trans} and Cdc20^{cis} mechanisms in this strain and should therefore be turned over exclusively by Cdc20^{trans}. This Cdc20-IR, R145D mutant was slightly more stable than that observed in the Cdh1^{trans}-only strain, suggesting that the Cdc20^{trans} mechanism does occur but likely contributes very little to Cdh1-independent turnover (Figure 3A, lanes 10–12). To further characterize the Cdc20^{trans} mechanism, we tested whether a wild-type copy of Cdc20 can ubiquitinate this double mutant in vitro. We detected very little ubiquitination of this mutant in the presence of a wild-type copy of Cdc20, and the little stimulation seen over background was D-box dependent (Figure 3C, lanes 4–6 and 10–12). Interestingly, although this D box appears Cdh1-specific in terms of targeting Cdc20 as a substrate in vitro, we did see a slight defect with this mutant both in direct binding to the APC and in targeting securin for ubiquitination in vitro, suggesting that Cdc20's D box may have an additional function (data not shown).

Given that total Cdc20 turnover appeared significantly faster than turnover via either Cdc20^{trans} or Cdh1^{trans}, we examined the contribution of the Cdc20^{cis} mechanism using an allele of Cdc20 that could only be bound to the APC as an activator and not as a substrate. We generated a *cdh1Δ* strain in which the only copy of Cdc20 is mutated at its first D box (*cdc20-DB*) and thus cannot function as a substrate in a Cdc20^{trans} reaction. In this strain, where only Cdc20^{cis} turnover occurs, Cdc20 turnover is quite fast, and steady-state Cdc20 levels are low, similar to those in a *cdh1Δ* strain where both Cdh1-independent mechanisms can occur (Figure 3D, lanes 1–8). These data suggest that Cdc20^{cis} is the dominant form of

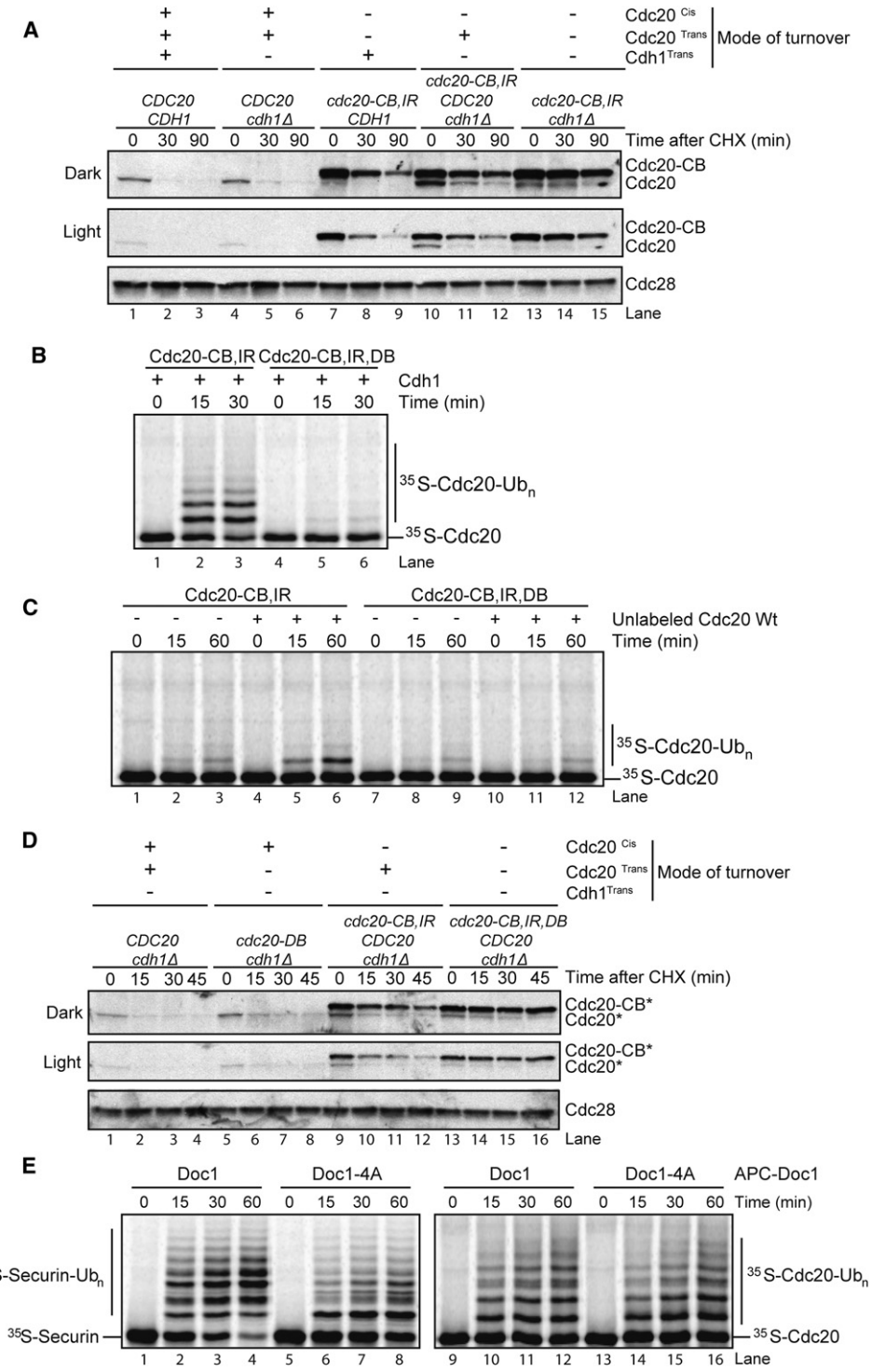

Figure 3. The Majority of Cdc20 Turnover Occurs by the Cdc20^{cis} Mechanism

(A) Asynchronous *pds1Δ clb5Δ SIC1^{10x}* cells were analyzed as in Figure 2C. Cdc20-CB denotes the Cdc20-R145D, IRΔ allele. Bands represented by Cdc20 and Cdc20-CB are indicated. Two exposures are shown.

(B) ZZ-tagged ^{35}S-Cdc20 C box, IR mutant (I147A, P148A, I609A, R610A) or ^{35}S-Cdc20 C box, IR, D-box mutant (I147A, P148A, I609A, R610A, R17A, L20A) was incubated with recombinant Cdh1, APC (1 nM), and E1/E2(Ubc4)/methyl-ubiquitin mix for the indicated times.

(C) IVT-generated ZZ-tagged ^{35}S-Cdc20 mutants, as in (B), were incubated with APC (5 nM), E1/E2(Ubc4)/methyl-ubiquitin mix, and with or without IVT-generated ZZ-tagged unlabeled Cdc20 for the indicated times.

(D) Asynchronous *pds1Δ clb5Δ SIC1^{10x}* cells were treated with cycloheximide and examined as in Figure 2C. Bands labeled Cdc20* are Cdc20 or Cdc20 D-box allele (cdc20-R17A, L20A), whereas Cdc20-CB* indicates the Cdc20-IRΔ, R145D allele or Cdc20-R145D, IRΔ, D-box (R17A, L20A) allele.

(E) Securin and Cdc20 ubiquitination assays as in Figure 2B, except that APC was purified from *DOC1 cdh1Δ* or *doc1-4A cdh1Δ* strains.

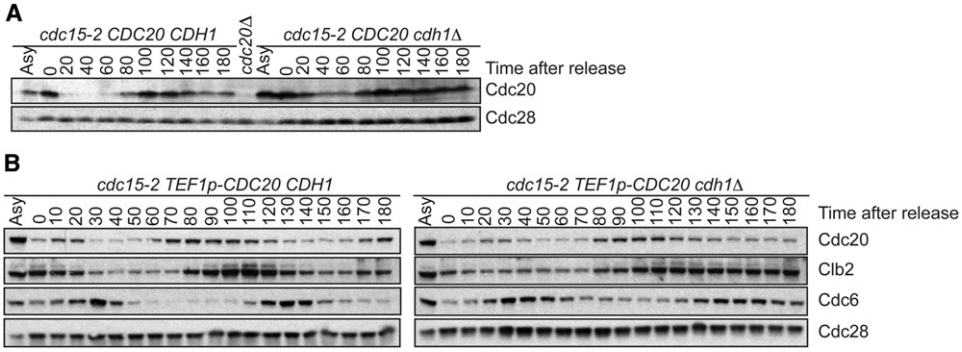

Figure 4. Cdc20 Levels Oscillate with the Cell Cycle in a Cdh1- and Transcription-Independent Manner

(A) Asynchronous *cdc15-2* or *cdc15-2 cdh1Δ* cells were arrested at 37°C and released into 23°C media. Time points were taken every 20 min. A sample of each asynchronous (Asy) culture and a *cdc20Δ* mutant are shown for reference.

(B) *cdc15-2 TEF1p-CDC20* or *cdc15-2 TEF1p-CDC20 cdh1Δ* strains were arrested and released as in (A). Time points were taken every 10 min. Western blots were performed with the indicated antibody. See also Figure S2.

Cdc20 turnover, with the contribution of $Cdc20^{trans}$ being very small (Figure 3D, lanes 9–12).

To determine the extent to which the first D-box mutation eliminates $Cdc20^{trans}$ turnover in vivo, we examined its effect in our strain that uses $Cdc20^{trans}$ exclusively (see Figure 3A, lanes 10–12). We found that Cdc20-IR, R145D, DB was extremely stable in a *CDC20 cdh1Δ* strain, although a very low level of turnover did occur (Figure 3D, lanes 13–16). Mutation of a second N-terminal D box had no additional effect (data not shown). Thus, the D-box mutation eliminated in trans turnover, consistent with previous reports [21, 29]. These data suggest that $Cdc20^{cis}$ is the dominant form of Cdc20 turnover, with the contribution of $Cdc20^{trans}$ being very small (Figure 3D, lanes 5–8 and 9–12).

The nonessential APC subunit Doc1 (APC10) is thought to interact directly with the D box of substrates and enhance processivity by limiting the dissociation rate of the substrate [14, 18, 19]. Deletion of this subunit or mutation of four residues (Doc1-4A) within its putative substrate binding site leads to a decrease in the number of ubiquitins conjugated to the substrate, as visualized by a significant decrease in higher molecular weight substrate-ubiquitin bands and accumulation of monoubiquitinated substrate (Figure 3E, lanes 1–8) [19]. Cdc20 contains a D box that has been shown to be important in Cdh1-dependent ubiquitination [21]. We tested whether a Doc1/D-box interaction was required for processive ubiquitination of Cdc20 in vitro in the absence of Cdh1. Unlike our results with all other substrates tested, mutation of Doc1 had no effect on the processivity of this reaction. Doc1 and Doc1-4A had nearly identical activity toward Cdc20 (Figure 3E, lanes 9–16), implying that Doc1 is not required for Cdh1-independent ubiquitination of Cdc20. These data strongly suggest that Cdc20 is not ubiquitinated by the APC as a canonical substrate and can best be explained by the $Cdc20^{cis}$ mechanism of autoubiquitination.

Cdc20 Levels Oscillate Independently of Cdh1 and Cdc20 Transcriptional Oscillation

Cdh1 activity is cell cycle regulated, which contributes to Cdc20 periodicity. We sought to determine whether Cdh1-independent mechanisms are also important for oscillations in Cdc20 levels. Because *cdh1Δ* cells do not arrest well in alpha factor, we examined Cdc20 levels through the cell cycle using *cdh1Δ cdc15-2* cells. Cells were arrested at the nonpermissive temperature in anaphase and released into the permissive temperature. Consistent with a recent report, we found that Cdh1 is not necessary for Cdc20 levels to fluctuate with the cell cycle [26] (Figure 4A; Figure S2A).

To examine the extent to which oscillations in *CDC20* transcription contribute to the fluctuation of Cdc20 levels, we generated a strain with *CDC20* under the control of a constitutive promoter (*TEF1p*). Cdc20 levels were still periodic in this strain. Moreover, Cdh1 was not required for this periodicity (Figure 4B; Figure S2B). Whereas Cdh1-dependent turnover of Cdc20 and cell cycle regulated transcription both contribute to Cdc20 cycling, Cdh1-independent turnover mechanisms appear to add significantly to Cdc20 oscillation.

Substrates Inhibit Autoubiquitination

If Cdc20 targets itself while bound to the APC as an activator, then how does the cell maintain Cdc20 levels sufficient to trigger anaphase? We tested the possibility that the binding of substrates to Cdc20 might inhibit autoubiquitination, maintaining Cdc20 stability until its targets are depleted in anaphase. We generated an N-terminal fragment (aa1–110) of budding yeast securin, containing the characterized destruction motif [11]. As expected for a competitive inhibitor, this fragment potently inhibited securin ubiquitination (IC_{50} ~200 nM) (Figure 5A). A 10 μM concentration of the securin fragment completely inhibited ubiquitination of securin (Figure 5A). This concentration of the fragment also inhibited the total activity and processivity of Cdc20 autoubiquitination (Figure 5B). These results support the notion that substrate blocks autoubiquitination, prolonging Cdc20 levels in the cell until substrates are depleted (Figure 6).

Discussion

One of the first APC substrates to be identified was its own activator, Cdc20, hinting at the existence of autoregulation [21, 29]. Initial reports suggested that Cdc20 behaved similarly to other APC substrates, being targeted in part via a different activator (Cdh1) through Cdc20's D box [22–24]. Interestingly, we show here that, unlike other APC substrates, Cdc20 is largely targeted for destruction by the APC through an autoubiquitination mechanism that occurs when Cdc20 is bound to the APC as an activator. Importantly, this mechanism appears to be regulated throughout the cell cycle and may be influenced by the presence or absence of substrates.

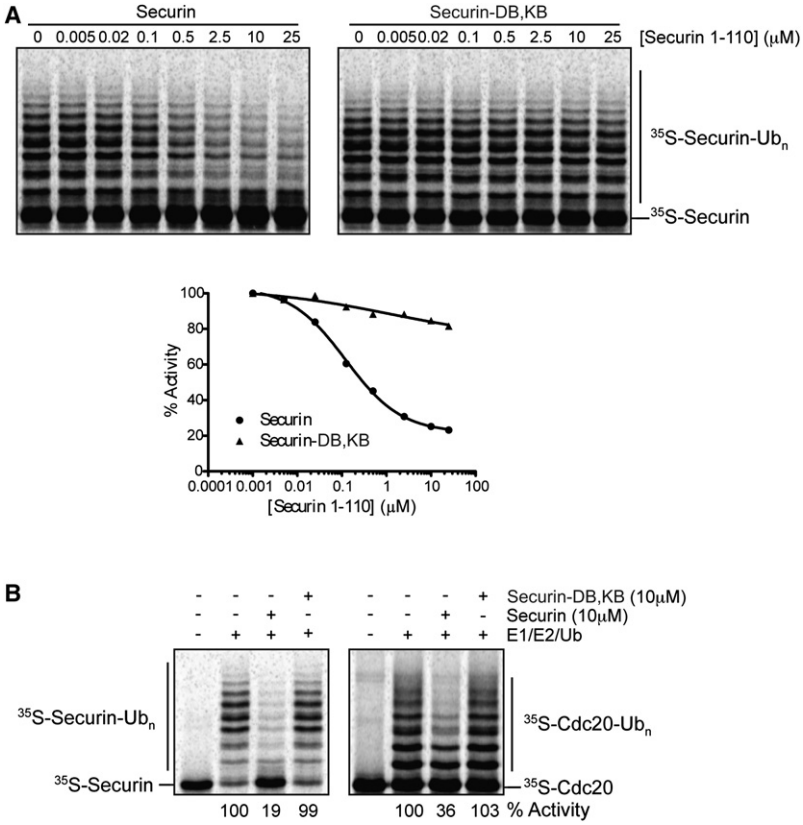

Figure 5. Cdc20^{cis} Mechanism Is Inhibited by High Substrate Concentrations

(A) ZZ-tagged unlabeled Cdc20 generated by IVT was preincubated with APC (5 nM) and the specified concentration of the securin/Pds1 fragment (referred to as securin 1–110; values represent the final assay concentrations). After a 15 min preincubation, E1/E2(Ubc4)/methyl-ubiquitin mix and ZZ-tagged ^{35}S-securin generated by IVT was added and ubiquitination reactions were performed for 10 min.

(B) ZZ-tagged unlabeled Cdc20 or ^{35}S-Cdc20 generated by IVT was preincubated for 15 min with APC (5 nM) and 10 μM securin 1–110. For securin ubiquitination, E1/E2(Ubc4)/methyl-ubiquitin mix and ZZ-tagged ^{35}S-securin generated by IVT was added for 10 min. For autoubiquitination, E1/E2(Ubc4)/methyl-ubiquitin mix was added for 10 min.

The observation that Cdc20 turnover was only partially reduced in conditional APC mutants led some authors to speculate that the residual turnover observed might be mediated by an APC-independent pathway. Our work in a strain that permits the deletion of the *APC11* gene shows that in unperturbed cells, Cdc20 is turned over solely by the APC. This discrepancy is likely due to the fact that conditional alleles may not be completely null for APC activity, whereas deletion of the gene encoding the catalytic subunit (*APC11*) eliminates activity completely.

APC^{Cdh1} has long been assumed to be the APC complex that targets Cdc20 for destruction (Cdh1^{trans}, Figure 1B) [17, 22–24]. However, deletion of *APC11* leads to much greater steady-state levels of Cdc20 than deletion of *CDH1*, suggesting the existence of other APC-mediated mechanisms [25]. This suggests two obvious models for turnover. First, Cdc20 bound to the APC as an activator could recognize another molecule of Cdc20 leading to ubiquitination of the substrate Cdc20 (Cdc20^{trans}, Figure 1B). In this case, the substrate Cdc20 should behave similarly to other Cdc20 substrates. Alternatively, Cdc20 may bind to the APC as an activator and this binding alone may be sufficient for autoubiquitination (Cdc20^{cis}, Figure 1B). To evaluate the relative contributions of the three possible modes of Cdc20 turnover, we generated strains in which only one mechanism of turnover was possible and performed in vitro experiments with similar perturbations. These experiments strongly suggested that Cdc20^{cis} is the predominant form of Cdc20 turnover.

Previous work showed that Cdc20 not only recruits substrates to the APC but also serves to activate the APC, because its presence was also required for the ubiquitination of the APC substrate Nek2A, which can bind the APC independently of an activator [9, 34]. Importantly, these results suggested that an N-terminal fragment of Cdc20 containing the C box was sufficient to activate the APC toward Nek2A and that the C box was required for this activation [9]. Interestingly, we find that a Cdc20 C-box mutant, which does not support viability and is unable to drive Dbf4 turnover in vivo [25](Figure 2C), is still targeted for turnover by the APC, although its turnover is compromised. This result suggests that the C box is not absolutely required for APC activity but is specifically required for stimulating APC activity toward other APC substrates, potentially by properly orientating either the substrate and or the catalytic arm of the APC so substrate ubiquitination can occur. Interestingly, deletion of the C-terminal IR domain, which does not result in a growth defect, has a significant effect on Cdc20 turnover, slightly greater than the defect seen for the lethal C-box mutant. The IR domain has been shown to interact with Cdc27, the terminal subunit of the tetratricopeptide repeats (TPR) arm of the APC [13, 25, 35]. The nonessential nature of the IR-Cdc27 interaction could suggest that it is an intermediate in the reaction mechanism when Cdc20 is particularly susceptible to autoubiquitination. Consistent with this observation, this interaction is not required for the processive ubiquitination of other APC substrates [35]. However, the lack of affinity provided by the Cdc27-IR interaction may be compensated by an interaction between the activator, substrate, and Doc1 on the APC core. Cdc20 autoubiquitination, however, does not require Doc1, potentially making the affinity provided by the Cdc27-IR interaction more important for Cdc20 autoubiquitination.

The discovery that Cdc20 is targeted for turnover by Cdh1, which is itself cell cycle regulated, suggested a mechanism by which Cdc20's cyclical expression could be achieved. Work from the Cross laboratory [26] and from experiments presented here suggests that Cdh1 may contribute to but is not necessary for Cdc20's cell cycle oscillation. However, previous work [21] suggested that oscillation in Cdc20 levels is also achieved by transcriptional regulation. *CDC20* is a member of the *CLB2* cluster of genes [36], whose transcription is under the control of Fkh2/Ndd1 [37, 38]. The observation that Cdc20 levels still oscillate in cells that express *CDC20* under a constitutive promoter (*TEF1p*) in the absence of Cdh1 implies an additional cell cycle regulated mechanism.

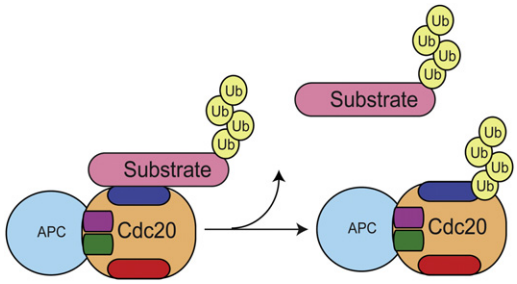

Figure 6. Model Demonstrating How Cdc20^{cis} May Be Regulated by Substrate

Red, purple, blue, and green boxes represent the D boxes, the C box, the WD40, and the C-terminal IR of Cdc20, respectively (see Figure 1A).

This is strong evidence that regulation of the Cdc20^{cis} mechanism we observe is sufficient to drive the oscillatory behavior of Cdc20 throughout the cell cycle.

Previous work has shown that phosphorylation of the TPR subunits (Cdc27, Cdc16, and Cdc23) by the cyclin-dependent kinase (CDK) increases the affinity of Cdc20 for the APC [39]. It is possible that these phosphorylations are regulating the Cdc20^{cis} mechanism. However, these phosphorylations promote Cdc20 binding to the APC and occur when CDK activity is high. If these phosphorylations are promoting the Cdc20^{cis} turnover during the cell cycle, we would expect to see the lowest Cdc20 levels when CDK activity is highest. However, we observe that the lowest Cdc20 levels occur during the G1 phase of the cell cycle, when CDK activity is lowest. Alternatively, phosphorylation of the TPR proteins may cause Cdc20 to bind in a slightly different position on the APC, which may inhibit the Cdc20^{cis} mechanism.

These data suggest the following model. The APC is hyperphosphorylated in early mitosis, which increases its affinity for Cdc20. As APC^{Cdc20} runs out of substrates, Cdc20 begins to autoubiquitinate, constituting the majority of the late mitotic turnover. This model for the regulation of Cdc20 stability by the presence of substrates (Figure 6) is similar to that put forth for the ubiquitin conjugase Ube2C [40]. As cells exit mitosis, APC becomes dephosphorylated and Cdh1 becomes active, thus removing residual Cdc20. Additionally, our model for substrate inhibition of Cdc20 turnover may explain why it is advantageous for the cell to have Cdc20 binding to the APC strongly enhanced by the presence of substrates [35, 41]. In this way, Cdc20 would be unlikely to be prematurely degraded when substrates are present.

Interestingly, Cdc20 turnover has been shown to increase in the presence of spindle poisons. This turnover is dependent on an intact spindle assembly checkpoint (SAC) [28]. The exact mechanism for this turnover is unknown, but it will be interesting to determine the mechanism for Cdc20 turnover during SAC activation.

Experimental Procedures

Yeast Methods

Yeast were grown in Ym-1 media [42] and 2% dextrose. All cells were grown at 23°C unless otherwise noted. Cdc20 integrating plasmid was created by cloning Cdc20 and its promoter into pRS306 using standard techniques. Mutations to pRS306-Cdc20 were accomplished using quick change mutagenesis. Cdc20 plasmids were integrated at the *URA3* locus into derivations of three strains: *pds1Δ clb5Δ SIC1^{10x} cdc20Δ cdh1Δ*, *pds1Δ clb5Δ SIC1^{10x} cdc20Δ CDH1*, or *pds1Δ clb5Δ SIC1^{10x} CDC20 cdh1Δ*. All strains created in this manner were checked for single integration by Southern blot. Replacement of the *CDC20* promoter with *TEF1p* was accomplished using standard PCR-based techniques, as was deletion of *CDH1* and mutation of Cdc20's IR motif in Figure 2A.

Half-Life Assays

Cells were grown to saturation, diluted, and allowed to grow for at least two doublings to an optical density (OD) between 0.6 and 1.0. Six ODs of cells were collected for the zero time point. Cell pellets were washed with 1 ml cold H_2O and frozen on dry ice. Cycloheximide was added to cultures for a final concentration of 50 μg/ml media. Six ODs of cells were collected for each time point as indicated. Cell pellets processed as described below.

cdc15-2 Arrest and Release

Cells were grown to saturation, then diluted to an OD of 0.3 and allowed to grow to an OD between 0.6 and 1.0. Six ODs of cells were collected as described above for an asynchronous sample. Cells were diluted to an OD of 0.5 and placed at 37°C for 3 hr. Cells were examined under a microscope to confirm anaphase arrest. Six ODs of cells were collected for the zero time point, as described above. Cells were then released into media at 23°C at an OD of 0.6, and six ODs of cells were collected at time points indicated. Cells were collected for flow cytometry at every time point and processed [43].

Western Blots

Cell pellets were processed as follows: cell pellets were thawed in boiling sample buffer (50 mM Tris pH 7.5, 5% SDS, 5 mM EDTA, 10% glycerol, 0.5% beta-mercaptoethanol, 0.1 μg/ml pepstatin A, 0.1 μg/ml leupeptin, 0.1 μg/ml bestatin, 0.1 mM benzamidine, 5 mM NaF, 0.5 mM Na_3VO_4, 40 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride). Cells were boiled for 5 min, followed by bead-beating three times, 30 s each, and then boiled again for 5 min for SDS-PAGE and transferred to nitrocellulose. Western blots were performed with low salt phosphate buffered saline with Tween 20 (PBST) (15 mM NaCl, 1.3 mM NaH_2PO_4, 5.4 mM Na_2HPO_4, 0.05% Tween pH 6.8). All primary antibody incubations were performed overnight in 5% milk and low salt PBST unless otherwise noted. Antibodies were used as follows: Cdc20 (yC-20) from Santa Cruz at 1:1000, Cdc28 from Santa Cruz (yC-20) at 1:1000, Dbf4 (yN-15) from Santa Cruz at 1:500, Clb2 (y180) from Santa Cruz 1:1000 (Figure 4B), and Cdc6 9H8/5 from Abcam at 1:2000.

APC Assays

APC was purified from a *TAP-CDC16, cdh1Δ* strain. E1, E2 (Ubc4), APC, and Cdh1 were expressed and purified as previously described [19, 44]. ZZ-tagged Cdc20 wild-type and mutants were transcribed and translated in vitro with TnT Quick Coupled Transcription/Translation Systems (Promega) either in the presence of ^{35}S-methionine or unlabeled methionine. Briefly, APC assays were performed by first charging the E2 in the presence of E1 (Uba1, 300 nM), E2 (Ubc4, 50 μM), methyl-ubiquitin (Boston Biochem, 150 μM), and ATP (1 mM) for 20 min. E1/E2(Ubc4)/methyl-ubiquitin mix was added to APC (1–5 nM), ZZ-Cdc20 purified from reticulocyte lysate using IgG beads and cleaved using TEV protease, and securin purified similarly from reticulocyte lysate. In Figure 2D, APC, Cdc20, and securin were preincubated to increase the amount of activity observed for the mutants. For Figures 5A and 5B, histidine (His)-tagged securin (aa1–110) was expressed in bacteria and purified using Ni-NTA resin. After tobacco etch virus protease cleavage to remove the His_6-tag, the protein was further purified using cation exchange and size exclusion chromatography. APC, Cdc20, and securin (aa1–110) were preincubated before adding in vitro translated securin and E1/E2(Ubc4)/methyl-ubiquitin mix or E1/E2(Ubc4)/methyl-ubiquitin mix alone. All reactions were stopped by the addition of sample buffer, separated by SDS-PAGE, and visualized and quantified with a Molecular Dynamics PhosphorImager and ImageQuant (Amersham Biosciences/GE Healthcare).

Supplemental Information

Supplemental Information includes two figures and can be found with this article online at doi:10.1016/j.cub.2011.09.051.

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