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Two different modes of cyclin Clb2 proteolysis during mitosis in *Saccharomyces cerevisiae*

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Abstract Sister chromatid separation and mitotic exit are triggered by the anaphase-promoting complex (APC/C) which is a multi-subunit ubiquitin ligase required for proteolytic degradation of various target proteins. Cdc20 and Cdh1 are substrate-specific activators of the APC/C. It was previously proposed that Cdh1 is essential for proteolysis of the yeast mitotic cyclin Clb2. We show that Clb2 proteolysis is triggered by two different modes during mitosis. A fraction of Clb2 is degraded during anaphase in the absence of Cdh1. However, a second fraction of Clb2 remains stable during anaphase and is degraded in a Cdh1-dependent manner as cells exit from mitosis. Most of cyclin Clb3 is degraded independently of Cdh1. Our data imply that degradation of mitotic cyclins is initiated by a Cdh1-independent mechanism.

Key words: Anaphase-promoting complex; Cyclin degradation; Cdh1; Cdc15; Mitotic exit; Ubiquitin-dependent proteolysis

1. Introduction

Proteolysis is an important mechanism for progression through the eukaryotic cell cycle. Major cell cycle transitions such as the initiation of DNA replication, the separation of sister chromatids and the exit from mitosis are all dependent on proteolytic degradation of specific proteins [1,2]. Chains of ubiquitin molecules are ligated to these target proteins which subsequently are rapidly degraded by the 26S proteasome.

The anaphase-promoting complex (APC/C), also called cyclosome, is an ubiquitin–protein ligase which is essential for ubiquitin-dependent proteolysis during mitosis. This large complex, consisting of 12 subunits in *Saccharomyces cerevisiae*, appears to be highly conserved in all eukaryotes [3–6]. At the metaphase to anaphase transition, the APC/C triggers proteolytic degradation of Pds1 or Cut2 which act as anaphase inhibitor proteins in budding or fission yeast, respectively [7,8]. Pds1 blocks the sister separating protein Esp1 and thereby prevents the dissociation of cohesins from chromosomes [9–11]. Pds1 degradation, initiated by APC/C activation, liberates Esp1 which then induces the cleavage of the cohesin subunit Scc1 and the separation of sister chromatids [12]. Activation of the APC/C prior to anaphase onset requires an additional activating subunit, the WD40 repeat protein Cdc20 or related proteins in vertebrate cells [13–16]. The activity of the APC/C^{Cdc20} complex is inhibited by the spindle assembly checkpoint which is activated upon defects in the mitotic spindle or by unattached kinetochores and thereby prevents the separation of sister chromatids [17–20].

Cdh1 (also termed Hct1), a protein related to Cdc20, binds to the APC/C in telophase and is needed for degradation of the mitotic cyclin Clb2 and other proteins such as the spindle-associated protein Ase1 and the polo-like protein kinase Cdc5 [13,21–23]. It is thought that Cdc20 and Cdh1 act as substrate-specific activators of the APC/C and thereby ensure that different target proteins of the APC/C are degraded in a proper temporal order during mitosis.

Cdc20 is an unstable protein whose abundance is tightly regulated during the cell cycle [16]. Cdc20 instability depends on APC/C activity, indicating that Cdc20 is also a substrate of this ubiquitin ligase. In contrast, Cdh1 protein levels do not significantly fluctuate during the cell cycle [24,25]. Instead, the association of Cdh1 and APC/C is cell cycle-regulated by phosphorylation and dephosphorylation of Cdh1. Cyclin-dependent kinases (CDKs) inhibit the interaction of Cdh1 with the APC/C [24,25], whereas the protein phosphatase Cdc14 reverses CDK-mediated phosphorylation and promotes Cdh1 binding [25,26]. A network of additional late mitotic proteins, including the protein kinases Cdc5 [27], Cdc15 [28], Dbf2 and Dbf20 [29], the small GTPase Tem1 [30] and the guanine-nucleotide exchange factor Lte1 [31] are also required for mitotic exit. The function of these factors and their regulation are poorly understood. Many of these proteins are presumably involved in the activation of Cdc14, which is sequestered and kept inactive in the nucleolus for most of the cell cycle [32,33]. Cdc14 phosphatase activity not only enables APC/C^{Cdh1} complex formation, but also stabilizes Sic1, a CDK inhibitor, and induces nuclear entry of Swi5, a transcription factor required for efficient *SIC1* expression [26]. Thus, Cdc14 promotes two different pathways leading to the inactivation of Cdk1 in late mitosis: cyclin proteolysis and Cdk1 inhibition. Inactivation of Cdk1 enables cells to disassemble the mitotic spindle, to undergo cytokinesis and to establish pre-replicative complexes in preparation for a new round of DNA replication.

Studies on the major mitotic cyclin Clb2 showed that proteolysis of this cyclin is fully dependent on Cdh1 upon a release from a telophase arrest and during a G1 arrest [22]. Previous experiments indicated that Clb2 destruction is initiated already early in anaphase [34]. Here, we analyzed the role of Cdh1 and proteins of the mitotic exit network in Clb2 proteolysis. We found that a first fraction of Clb2 is degraded during anaphase in the absence of the substrate-specific activator Cdh1, whereas proteolysis of a second fraction of Clb2

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Abbreviations: APC/C, anaphase-promoting complex/cyclosome; CDK, cyclin-dependent kinase; OD, optical density

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depends on Cdh1 and late mitotic proteins such as Cdc15. Clb3 destruction also occurred in the absence of Cdh1. We conclude that mitotic cyclins are degraded in a biphasic manner and by two different modes during mitosis.

2. Materials and methods

2.1. Yeast strains and plasmid

All yeast strains used in this study are derivatives of the *S. cerevisiae* W303 strain (*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,12 his3-11,15 ura3 GAL psi+*). All strains with *cdh1* deletions used in this study are derivatives of K7007 (*cdh1::HIS5, HIS5* gene from *Schizosaccharomyces pombe*), kindly provided by M. Shirayama. To confirm that *HIS+* segregants are indeed deleted in the *CDH1* gene, the absence of *CDH1* mRNA was verified by Northern hybridization. The *GAL-CLB3-HA3* construct was previously described [35]. The *GAL1-CLB1-ΔN* construct contains a truncated *CLB1* gene under the control of the *GAL1* promoter. The truncated *CLB1* gene lacking the first 120 N-terminal amino acids was fused to the *GAL1* promoter and the gene fusion construct was cloned into the integrative plasmid YIp211 [36]. The resulting plasmid was linearized with *Apa*I and integrated into the corresponding yeast strains.

2.2. Growth conditions and cell cycle arrests

Cells were grown in YEP medium (2% bactopeptone, 1% yeast extract, 0.005% adenine sulfate) supplemented either with 2% glucose (YEPD) or 2% raffinose (YEP+Raff) or appropriate minimal media [37]. Prior to cell cycle arrests, cultures were pre-grown to log phase (OD_{600} 0.3–0.6) at 25°C in YEPD. When a gene was expressed from the inducible *GAL1* promoter, cells were pre-grown in YEP+Raff. The *GAL1* promoter was induced by the addition of galactose (2% final concentration). To turn off the *GAL1* promoter, glucose was added (2% final concentration) or cells were filtered and resuspended in fresh medium containing 2% glucose.

To arrest cells in G1-phase, yeast cultures were incubated for 2.5 h in the presence of α-factor (5 μg/ml final concentration). To arrest cells with the microtubule depolymerizing drug nocodazole, cells were incubated for 2.5–3 h in the presence of 15 μg/ml nocodazole (added from a 1.5 mg/ml stock solution in dimethylsulfoxide). For releasing cells from a nocodazole block, cells were filtered and washed with five volumes of YEP medium containing 1% dimethylsulfoxide. 1% Dimethylsulfoxide was also added to the release medium.

2.3. Immunoblot analysis

Preparation of whole cell extracts and protein immunoblot analysis were performed as described [38]. After separation on SDS gels, proteins were transferred to nitrocellulose membranes (Amersham). The enhanced chemiluminescence detection system (ECL, Amersham) was used to detect specific proteins. Antibodies were used in 1:1000 (Clb2), 1:2000 (Cdc28), 1:10 000 (Swi6) and 1:100 (HA antibody, 12CA5) dilutions, respectively.

2.4. Other methods

RNA isolation and Northern hybridization were performed as described [39]. Polymerase chain reaction-generated fragments of the respective genes were radiolabelled by random primer labelling (Stratagene) and used as hybridization probes. For immunofluorescence microscopy, cells were fixed in 3.7% formaldehyde and spheroplasts were prepared as described in [40]. DAPI (4,6-diamidino-2-phenylindole) staining and anti-tubulin antibodies were used for visualization of nuclei and spindles, respectively.

3. Results

3.1. Mitotic exit mutants of S. cerevisiae arrest with reduced Clb2 levels

To analyze Clb2 stability during anaphase, we determined Clb2 levels in a synchronous culture of temperature-sensitive *cdc15-2* mutants released from an α-factor-induced G1 arrest. These cells were incubated at 37°C and therefore blocked cell cycle progression in late anaphase/telophase. We found that Clb2 accumulates to high levels during mitosis, but the amount of this mitotic cyclin subsequently decreases as cells arrest in late anaphase/telophase (Fig. 1A). These data confirm previous findings showing that Clb2 is partially degraded in *cdc15-2* mutants [34,38].

We next tested the role of several factors of the mitotic exit network in Clb2 degradation. Clb2 levels were determined in temperature-sensitive *cdc5 (msd2-1)*, *dbf2-2*, *cdc14-3, tem1-3* and *cdc15-2* yeast mutants. Each of these mutants is defective in mitotic exit and arrests at the non-permissive temperature with elongated mitotic spindles and chromosomes segregated to opposite poles [5,23]. These mutant strains were arrested either in metaphase using the spindle depolymerizing drug nocodazole or in late anaphase/telophase by a shift to the non-permissive temperature. In each of the mutants, Clb2 levels were lower in telophase cells than in metaphase cells (Fig. 1B). Thus, a partial decrease in Clb2 levels during anaphase seems to occur independently of proteins of the mitotic exit network. In contrast, *cdc20-3* and *cdc23-1* mutants which are impaired in anaphase onset contain similar amounts of Clb2 after nocodazole treatment or after a temperature shift, indicating that Clb2 is completely stable in these mutants.

cdc20-3 pds1 mutants are able to undergo anaphase because the anaphase inhibitor Pds1 is absent. Instead, this double mutant arrests in telophase [41]. To test whether *cdc20-3 pds1* cells contain similar amounts of Clb2-like mitotic exit mutants at their non-permissive temperature, protein levels were determined in these telophase-arrested cells. We found that *cdc20-3 pds1* cells arrest with Clb2 levels similar to metaphase-arrested *cdc20-3* cells. In contrast to other mitotic exit mutants, Clb2 levels are not significantly decreased in telophase-arrested cells lacking a functional Cdc20. Thus, Cdc20 is apparently required for the decrease of Clb2 levels during anaphase.

Remarkably, *cdc15-2* mutants lacking the *CDH1* gene also contain lower levels of Clb2 when compared to metaphase-arrested cells. Nocodazole-arrested *cdc15-2 cdh1* cells were also shifted to 37°C to ensure that the difference between metaphase- and telophase-arrested cells is not merely due to the temperature shift (Fig. 1C). In comparison to telophase cells, Clb2 remained at constantly higher levels in metaphase cells. These data indicate that a decrease of Clb2 during anaphase occurs independently of the substrate-specific activator Cdh1.

3.2. Destruction box-mediated proteolysis of Clb2 is independent of Cdh1 during anaphase

To test whether Clb2 proteolysis during anaphase occurs independently of Cdh1, Clb2 levels were analyzed during anaphase in cells lacking the *CDH1* gene. *cdc15-2 cdh1* mutant cells were first blocked in metaphase by nocodazole treatment. Clb2 was transiently expressed from the *GAL1* promoter and accumulated to high levels because Clb2 is stable in these arrested cells [42]. Cells were then released from the metaphase arrest and were subsequently blocked in late anaphase/telophase, as a consequence of the *cdc15-2* mutation. During anaphase, a large fraction of Clb2 was degraded despite the *cdh1* mutation, whereas a second fraction of Clb2 remained stable under these conditions (Fig. 2A). The decrease in Clb2 levels during anaphase was dependent on its cyclin destruction box, because a mutant Clb2 protein lacking its 9 amino acid destruction box, Clb2ΔDB [42], remained

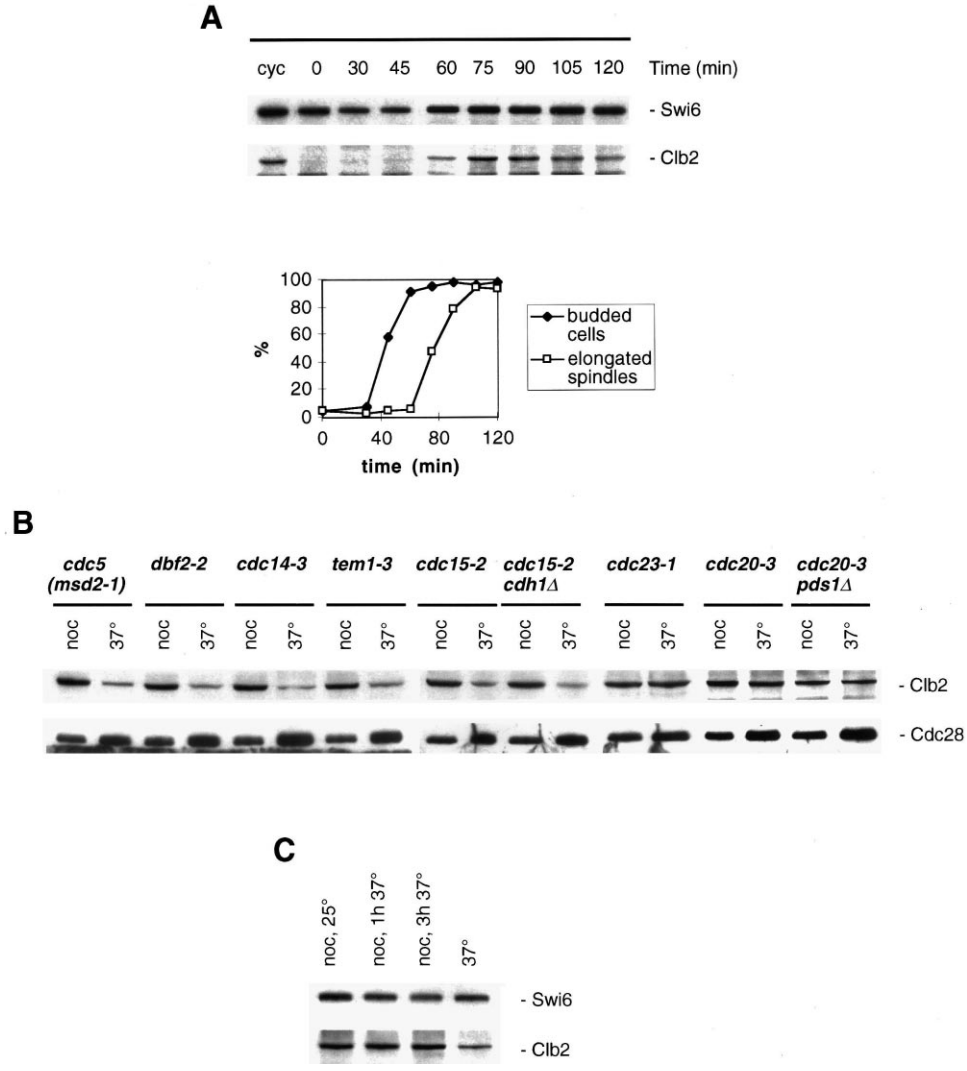

Fig. 1. Late mitotic mutants arrest with decreased Clb2 levels. (A) *cdc15-2* mutant cells (K1993) were arrested with α-factor in G1-phase for 2.5 h at 25°C. Subsequently, cells were released by removing the pheromone and incubated at 37°C. The synchrony of the culture was checked by counting the number of buds and elongated spindles. Clb2 protein levels were determined by immunoblotting with antibodies against Clb2. Swi6 protein was used as a loading control. (B) Various mutants defective in mitotic exit, *cdc5 (msd2-1*, K5998), *dbf2-2* (K4902), *cdc14-3* (K2887), *tem1-3* (K5850), *cdc15-2* (K1993) and a *cdc15-2 cdh1* double mutant (S212) were pre-grown at 25°C in YEPD medium. Cultures were split and incubated for 3 h either at 25°C in the presence of the spindle depolymerizing drug nocodazole or at 37°C. Cultures of two mutants defective in anaphase onset, *cdc23-1* (K2531) and *cdc20-3* (K7108), as well as of a *cdc20-3 pds1* double mutant (K7109), were also incubated under these conditions. Clb2 was visualized using Clb2 antibodies. Cdc28 was used as a loading control. (C) The *cdc15-2 cdh1* culture was treated as described in (B), but the nocodazole-treated culture was split after 2.5 h and incubation was continued in the presence of nocodazole either at 25°C for 3 h or at 37°C for 1 and 3 h. Swi6 was used as a loading control.

stable during anaphase. Furthermore, the decrease in Clb2 levels was dependent on the removal of nocodazole and therefore on anaphase entry, because Clb2 degradation did not occur when nocodazole was present at all times (Fig. 2B). These results indicate that proteolytic degradation of a large fraction of Clb2 during anaphase is dependent on APC/C activity, but occurs independently of Cdh1 and Cdc15. Partial degradation of Clb2 was similar in a *CDH1* wild-type strain and a *cdh1* deletion strain, indicating that Cdh1 has no important role in proteolysis of this fraction of Clb2 (Fig. 2C).

3.3. Proteolysis of a second pool of Clb2 requires Cdh1

Our findings showed that during anaphase a first pool of Clb2 is unstable, whereas a second pool remains stable in the absence of Cdh1 and Cdc15 function. Cdh1 was previously shown to be essential for Clb2 degradation as cells exit from mitosis [22]. We analyzed whether Cdh1 is exclusively needed for proteolysis of this second fraction of Clb2. To test this, *cdc15-2 CDH1* and *cdc15-2 cdh1* strains were pre-arrested in metaphase with nocodazole. Then, Clb2 expression was transiently induced and cells were subsequently released from this arrest at 37°C. The *cdc15-2* mutation then caused these cells to arrest in late anaphase/telophase. During the metaphase to telophase transition, Clb2 levels were distinctly reduced independently of Cdh1 (Fig. 3). Subsequently, cells were released from the *cdc15-2* arrest by a shift to the permissive temperature. Proteolysis of the remaining Clb2 protein was dependent on Cdh1, whereas no further decrease in Clb2 levels occurred in the *cdh1* mutant strain.

Taken together, degradation of the second pool of Clb2

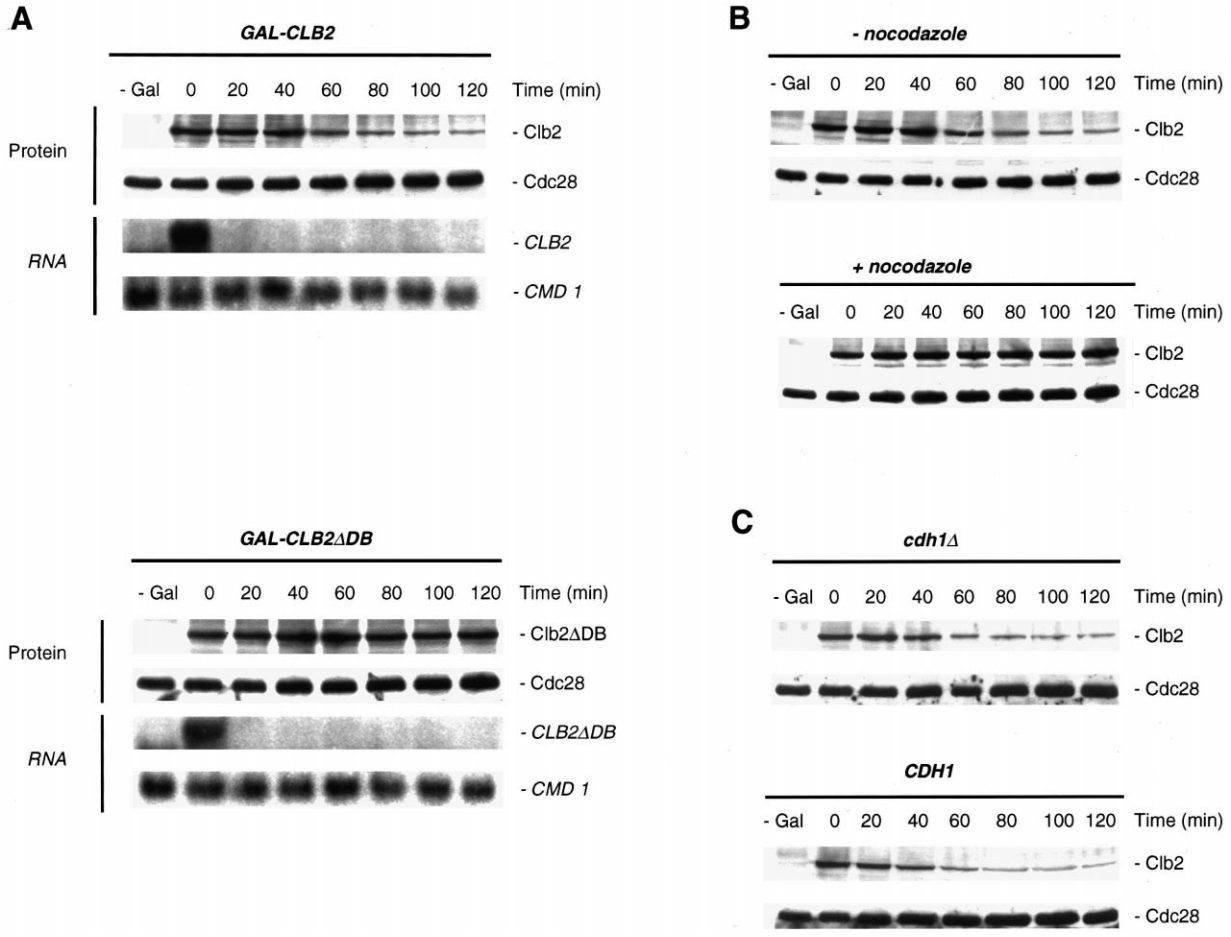

Fig. 2. Clb2 degradation during anaphase occurs independently of Cdh1. (A) *cdc15-2* strains, in which the endogenous *CDH1* and *CLB2* genes are deleted (*cdc15-2 cdh1 clb2*) and which contain either a *GAL-CLB2* (S283) or a *GAL-CLB2ΔDB* (CLB2 with a cyclin destruction box deletion, S284) construct were pre-grown in YEP+Raf medium at 25°C and subsequently treated with nocodazole for 2.5 h to arrest cells in metaphase. 2% Galactose was added to express the *CLB2* or *CLB2ΔDB* genes, respectively. After 30 min, the cultures were shifted to 35°C to inactivate the Cdc15 protein and incubation in the presence of nocodazole was continued for 30 min. Then cells were filtered, washed and released from the nocodazole arrest in YEPD medium at 37°C. The glucose medium repressed the *GAL1* promoter. Samples were collected at the indicated time points after the shift to YEPD medium (0 min time point). The *cdc15-2* mutation caused these cells to arrest in late anaphase/telophase. Clb2 and Clb2ΔDB levels were determined by immunoblotting using antibodies against Clb2. Cdc28 was used as a loading control. To confirm that expression of *CLB2* and *CLB2ΔDB* was turned off in glucose medium, RNA levels were analyzed by Northern hybridization. *CMD1* mRNA (calmodulin mRNA) was used as a loading control. To confirm that cells released from the nocodazole-induced metaphase arrest, the appearance of mitotic spindles was verified by immunofluorescence microscopy using antibodies against tubulin. In both strains, no tubulin staining was visible when cells were treated with nocodazole. Upon removal of the nocodazole, spindles appeared in more than 80% of the cells between the 20 min and 40 min time points after the release (data not shown). (B) The *cdc15-2 cdh1 clb2 GAL-CLB2* strain (S283) was arrested in metaphase with nocodazole and *CLB2* was expressed as described in (A). Prior to the release, the culture was split; the first half was incubated in YEPD medium containing nocodazole and the second half was incubated without nocodazole. (C) Strain S283 and an isogenic *CDH1* strain (S80) were arrested in metaphase with nocodazole. *CLB2* expression was transiently induced by galactose addition and cells were released from the nocodazole arrest as described in (A).

which occurs in the presence of functional Cdc15 is dependent on Cdh1, in contrast to the initial proteolysis of Clb2 during anaphase. These findings suggest that Clb2 is ubiquitinated during mitosis by two different modes which are Cdh1-independent during anaphase or Cdh1-dependent as cells exit from mitosis.

3.4. *Clb2 levels are reduced in the presence of highly active Cdk1*

Similar to a *cdc15-2* mutation, the expression of non-degradable mitotic cyclins blocks cells in late anaphase/telophase. The presence of large amounts of cyclins and highly active Cdk1 prevents these cells to exit from mitosis [38,43]. We analyzed the influence of highly active Cdk1 on Clb2 levels. For this purpose, a construct containing an N-terminally truncated Clb1 cyclin expressed from the *GAL1* promoter was integrated into *CDH1* and *cdh1* strains. This Clb1 mutant protein was apparently very stable because cells were inviable on galactose containing plates and the addition of galactose rapidly caused cells to arrest with elongated spindles (data not shown). Cells containing the *GAL1-CLB1-ΔN* construct were arrested either in metaphase by nocodazole treatment or in telophase by the addition of galactose. In the presence of stable Clb1 and highly active Cdk1, Clb2 levels were lower in telophase-arrested cells than in metaphase cells (Fig. 4). The decrease in Clb2 protein was independent of Cdh1, because Clb2 levels were similarly decreased in *CDH1* and *cdh1* strains.

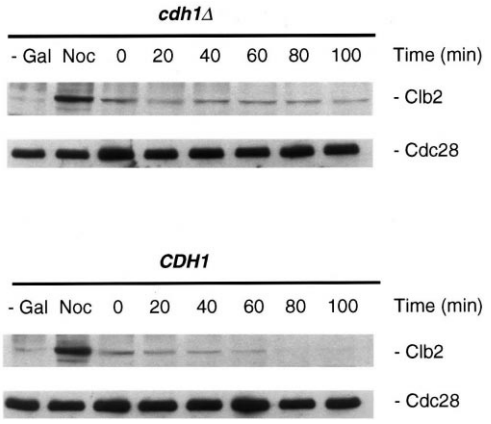

Fig. 3. Degradation of a second fraction of Clb2 is dependent on Cdh1. Strains *cdc15-2 CDH1 clb2 GAL-CLB2* (S80) and *cdc15-2 cdh1 clb2 GAL-CLB2* (S283) were arrested with nocodazole as described in Fig. 2A. *CLB2* was expressed by galactose addition (Noc sample) and cells were released in YEPD medium at 37°C. After 2 h at 37°C, an appropriate amount of cold YEPD medium was added to shift the temperature to 25°C, a permissive temperature for *cdc15-2* mutants. Samples were collected at the indicated time points after incubation at 25°C.

Thus, highly active Cdk1 apparently prevents proteolysis of the second pool of Clb2, which is Cdh1-dependent, but does not affect the Cdh1-independent mode of Clb2 degradation.

3.5. Most of Clb3 is degraded independently of Cdh1

It was previously shown that the B-type cyclin Clb5, an S-phase cyclin, is fully unstable during anaphase [34] and is destroyed exclusively by the APC/C^{Cdc20} [44]. To analyze proteolytic degradation of another mitotic cyclin, we determined the stability of cyclin Clb3 during anaphase. Previous experiments implied that degradation of this mitotic cyclin may be initiated by APC/C^{Cdc20} [45].

Cells containing a *GAL1-CLB3* construct were released from a nocodazole arrest under similar conditions as in the experiments described for Clb2 (Fig. 2B). Analysis of Clb3 levels revealed that most of this cyclin was degraded during

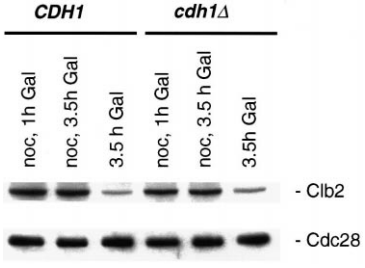

Fig. 4. Non-destructible cyclins do not inhibit Clb2 degradation during anaphase. A wild-type *CDH1* strain (S300) and a *cdh1* mutant strain (S316), both containing a *GAL-CLB1-ΔN* construct (expressing a truncated Clb1 protein lacking the N-terminal 120 amino acids including the cyclin destruction box) were pre-grown in YEP+Raf at 30°C. Cultures were split and either nocodazole or galactose was added. Therefore, cells arrested either in metaphase or telophase, respectively, which was confirmed by immunofluorescence microscopy (data not shown). After 2.5 h, galactose was also added to the nocodazole-arrested culture to confirm that expression of *CLB1-ΔN* did not affect Clb2 levels in metaphase-arrested cells. Samples were collected at the indicated time points after galactose addition. Clb2 protein levels were determined by immunoblotting, using Cdc28 as a loading control.

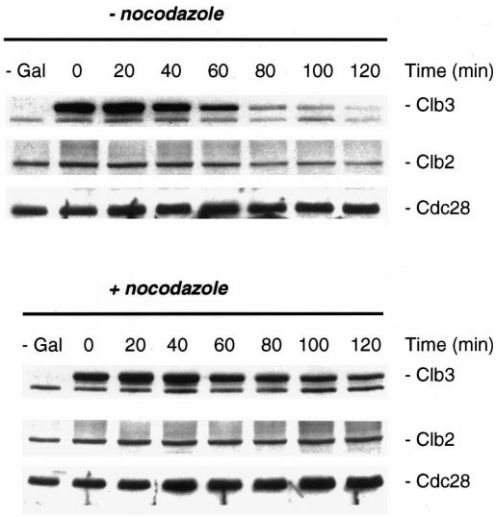

Fig. 5. Most of the mitotic cyclin Clb3 is degraded independently of Cdh1. *cdc15-2 cdh1 GAL-CLB3-HA3* (S315) cells were treated with nocodazole to arrest them in metaphase as described in Fig. 2A. Galactose was added to express the *CLB3* gene and cells were incubated for 30 min at 25°C. Subsequently, cells were shifted to 35°C and incubated for another 30 min. After filtering and washing, the cultures were split in two halves, which were incubated in YEPD medium at 37°C either in the presence or absence of nocodazole. Samples were collected at the indicated time points after filtration. Epitope-tagged Clb3 protein was detected with antibodies against HA (12CA5 antibodies). Clb2 was detected using Clb2 antibodies. Cdc28 was used as a loading control.

anaphase in the absence of Cdh1 and only a small fraction of Clb3 appeared to be stable in *cdc15-2* cells (Fig. 5). Thus, proteolysis of most of Clb3 protein occurs independently of Cdh1 and Cdc15 during anaphase.

4. Discussion

4.1. The regulation of cyclin proteolysis during mitosis

Precise regulation of the proteolytic destruction of different target proteins is a prerequisite to ensure a proper order of mitotic events. The anaphase inhibitor Pds1 and mitotic cyclins are apparently the most important targets of the APC/C during mitosis in yeast [6,46]. Pds1 blocks sister chromatid separation by keeping separins inactive, whereas mitotic cyclins maintain CDK activity and prevent mitotic exit. It is crucial for the eukaryotic cell cycle that sister chromatid separation is always triggered earlier than the exit from mitosis.

It is thought that the APC/C activators Cdc20 and Cdh1 are the major factors controlling ubiquitination of different substrates by the APC/C [13]. Cdc20 is essential for proteolysis of Pds1, whereas Cdh1 is needed for cyclin proteolysis as cells exit from mitosis. The APC/C^{Cdc20} complex is activated at metaphase, but Cdh1's association with the APC/C is blocked by Cdk1 activity until mitotic exit proteins such as Cdc14 and Cdc15 are activated during telophase. Thus, Cdc20-mediated activation of the APC/C always precedes Cdh1's activation during mitosis.

It is still unclear how the WD40 proteins Cdc20 and Cdh1 confer substrate-specific ubiquitination. The most simple model would propose that Cdc20 binds and targets Pds1 to the APC/C, but does not affect Clb2. Only the association of Cdh1 with the APC/C may then allow Clb2 recognition and ubiquitination.

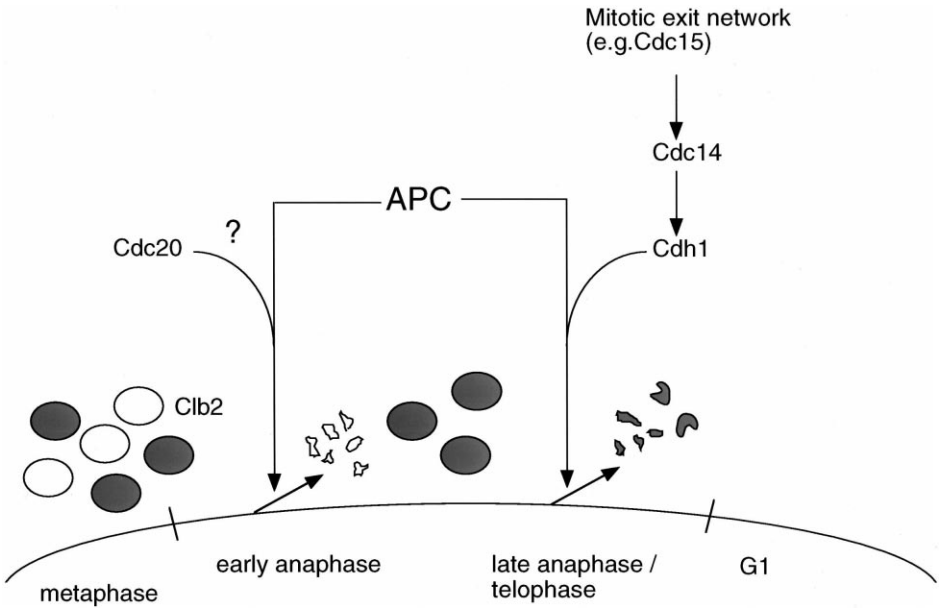

Fig. 6. Model for a biphasic mode of mitotic cyclin degradation during mitosis. At the metaphase to anaphase transition, APC/C^{Cdc20} may not only ubiquitinate the anaphase inhibitor Pds1 (and Clb5) but also a fraction of mitotic cyclins such as Clb2 and Clb3. This process occurs independently of Cdh1 and late mitotic proteins. A second fraction of Clb2 remains protected from the proteolytic machinery during anaphase. Ubiquitination of these cyclin proteins is dependent on Cdh1. Late mitotic proteins such as Cdc15 are apparently involved in the liberation of Cdc14 phosphatase which is kept inactive in the nucleolus until late mitotic proteins are activated in telophase.

We have shown here that this model is unlikely and that more complex processes are involved in the regulation of cyclin destruction. We found that proteolysis of mitotic cyclins is not initiated by Cdh1-mediated activation of the APC/C. Instead, Clb2 and Clb3 destruction is initiated in the absence of Cdh1 and a fraction of these mitotic cyclins is degraded during anaphase. Cdh1-independent proteolytic degradation is most likely mediated by the APC/C because degradation is dependent on the cyclin destruction box and on entry into anaphase. The pool of Clb2 which remains stable throughout anaphase is degraded prior to the mitotic exit by a mechanism which is fully dependent on Cdh1.

Furthermore, we found that many mutants defective in mitotic exit contain lower Clb2 levels compared to metaphase cells. Similarly, cells arrested in telophase due to the expression of a truncated, non-degradable Clb1 cyclin also arrest with decreased Clb2 levels. Thus, neither the absence of Cdh1 and mitotic exit proteins nor highly active CDKs inhibit partial Clb2 proteolysis during anaphase, but prevent the completion of cyclin proteolysis.

We conclude that Clb2 is degraded by two different modes during mitosis. Initially, Clb2 proteolysis occurs during anaphase independently of Cdh1 and mitotic exit proteins such as Cdc15. Later, when cells exit from mitosis, Clb2 degradation occurs in a Cdh1-dependent manner (Fig. 6).

Recent data showed that proteolysis of mitotic cyclins is activated early in anaphase also in other organisms. Degradation of human cyclin B1 was found to be regulated temporally and spatially [47]. Cyclin B1 disappeared from chromosomes and spindle poles immediately after the spindle checkpoint had been inactivated. A localized disappearance of cyclin B was also observed in *Drosophila* cells [48]. Thus, mitotic cyclin destruction seems to be initiated at defined locations, mainly at the spindle poles, in these cells.

Similarly, Clb2 proteolysis in yeast might be regulated by its subcellular localization. Some Clb2 molecules might get ubiquitinated by the APC/C even if Cdh1 is not present, whereas a second pool of Clb2 molecules might be present at specific locations in the cell where their access to the APC/C is strictly dependent on Cdh1. Alternatively, the access of Clb2 to the APC/C might be differentially regulated by post-translational modifications of some Clb2 molecules.

4.2. The role of Cdc20 in cyclin proteolysis

Another important question is how a pool of mitotic cyclins may be ubiquitinated by the APC/C during anaphase in the absence of the substrate-specific activator Cdh1. The observation that *cdc20-3* and *cdc20-3 pds1* cells arrest at their restrictive temperature with high levels of Clb2 indicates that Cdc20 is required to initiate Clb2 proteolysis (Fig. 1B; [16,44]). Cdc20 may directly act as a substrate-specific activator of Clb2 ubiquitination during early anaphase or, alternatively, APC/C^{Cdc20}-mediated proteolysis may indirectly activate a pathway leading to Clb2 proteolysis. It was earlier shown that in vitro ubiquitination of mitotic cyclins is fully dependent on Cdh1 in G1-arrested yeast cells [24]. Assuming that proteolysis of a fraction of Clb2 is directly dependent on Cdc20, then the failure to detect ubiquitination of mitotic cyclins in *cdh1* G1 extracts might be due to the absence of sufficient Cdc20 protein in G1 cells [16]. Ubiquitination assays using mammalian cell extracts demonstrated that APC/C^{Cdc20} complexes ubiquitinate mitotic cyclins in vitro [14,15]. Yeast Cdc20 may also target a pool of mitotic cyclins for ubiquitination by the APC/C. Indeed, Alexandru et al. [45] provided evidence that Clb3 is destroyed during anaphase by the APC/C^{Cdc20}, similarly as the S-phase cyclin Clb5 [44]. However, APC/C^{Cdc20} is not sufficient to trigger complete mitotic cyclin destruction in the absence of Cdh1.

4.3. Cyclin proteolysis and mitotic exit

What might be the purpose of biphasic Clb2 degradation during mitosis? Keeping Cdk1 at least partially active

throughout anaphase is needed to prevent cytokinesis and DNA re-replication prior to the completion of chromosome segregation. On the other hand, a partial decrease in mitotic cyclin levels and Cdk1 activity might be a prerequisite for the proper activation of the mitotic exit network. Highly active Cdk1 may inhibit the activity of these proteins required for mitotic exit. Indeed, elevated Clb2 levels have a drastic effect on many mitotic exit mutants even under permissive conditions [23].

Highly active Cdk1 might preliminarily interfere with Cdc14 phosphatase activity. Release of Cdc14 from the nucleolus is not affected by the expression of high levels of non-degradable cyclins [33], but the activity of Cdc14 might be insufficient to reverse phosphorylation of a fully active Cdk1. Then, Cdc14 phosphatase, upon release from the nucleolus, could only win the competition against Cdk1 if mitotic cyclins are partially destroyed, but never in the presence of very active kinase. Such a mechanism may help to ensure that Cdc14-dependent dephosphorylation of Cdh1, Sic1 and Swi5 never occurs before sister chromatid separation, which takes place in the presence of highly active Cdk1 [38,49].

In conclusion, degradation of a fraction of Clb2 and other mitotic cyclins might be necessary to activate the pathway leading to completion of Clb2 proteolysis, Cdk1 inactivation and cytokinesis.

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