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A Process Independent of the Anaphase-promoting Complex Contributes to Instability of the Yeast S Phase Cyclin Clb5*

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Proteolytic destruction of many cyclins is induced by a multisubunit ubiquitin ligase termed the anaphase promoting complex/cyclosome (APC/C). In the budding yeast *Saccharomyces cerevisiae*, the S phase cyclin Clb5 and the mitotic cyclins Clb1–4 are known as substrates of this complex. The relevance of APC/C in proteolysis of Clb5 is still under debate. Importantly, a deletion of the Clb5 destruction box has little influence on cell cycle progression. To understand Clb5 degradation in more detail, we applied *in vivo* pulse labeling to determine the half-life of Clb5 at different cell cycle stages and in the presence or absence of APC/C activity. Clb5 is significantly unstable, with a half-life of ~8–10 min, at cell cycle periods when APC/C is inactive and in mutants impaired in APC/C function. A Clb5 version lacking its cyclin destruction box is similarly unstable. The half-life of Clb5 is further decreased in a destruction box-dependent manner to 3–5 min in mitotic or G_1 cells with active APC/C. Clb5 instability is highly dependent on the function of the proteasome. We conclude that Clb5 proteolysis involves two different modes for targeting of Clb5 to the proteasome, an APC/C-dependent and an APC/C-independent mechanism. These different modes apparently have overlapping functions in restricting Clb5 levels in a normal cell cycle, but APC/C function is essential in the presence of abnormally high Clb5 levels.

Cyclin-dependent kinases (Cdks)[2] are pivotal regulators of the eukaryotic cell cycle. The activity of these enzymes depends on the interaction with regulatory subunits, the cyclins. In yeast, nine cyclins are known to associate with the cyclin-dependent kinase Cdk1, also known as Cdc28 (1, 2). Six of these cyclins, Clb1–6, are members of the family of B-type cyclins. Two of these, Clb5 and Clb6, are expressed early in S phase and are required for the timely initiation of DNA replication (3, 4). Activation of Cdk1 associated with S-cyclins requires the removal of the Sic1 protein (5, 6). Proteolytic destruction of this Cdk1 inhibitor is initiated by Sic1 phosphorylation, mediated by G_1-specific Cdk1 (7). The Clb1–4 cyclins are expressed later in the cell cycle and have important functions in the G_2 and M phases (8, 9).

Cyclins have important roles in the cell cycle regulation of Cdk1 activity and in establishing substrate specificity (2). Cyclins are unstable proteins, and their rapid destruction is important for inactivation of Cdk1. Cyclin ubiquitination is mediated by the anaphase promoting complex/cyclosome (APC/C). This ubiquitin ligase is a large complex consisting of 13 subunits in *Saccharomyces cerevisiae* and has a highly conserved function in eukaryotes (10). APC/C activity requires the association of coactivators, either Cdc20 or Cdh1, which are involved in recognition and recruitment of substrates to the APC/C core complex. Coactivators bind directly to destruction motifs, the D-box (K*XX*L*XXXX*N) or the KEN box of substrates (11, 12). Cdc20 activates APC/C in metaphase, when all chromosomes are properly attached to the mitotic spindle, whereas Cdh1 binds APC/C in late anaphase and keeps the ubiquitin-ligase active during G_1 phase (10).

Five of the six Clb cyclins are known to be substrates of the APC/C. The sole exception is Clb6, which recently was identified as a substrate of another ubiquitin ligase, the Skp1/Cullin/F-box (SCF) complex (13). Clb5, the second S phase cyclin, seems to be degraded by an APC/C-dependent process (14), but in contrast to the mitotic Clb cyclins, it was observed that Clb5 was unstable throughout the cell cycle (15, 16).

Different previous studies came to opposite conclusions concerning the importance of APC/C in controlling Clb5 levels. Initially, a *clb5* mutation was identified in a screening for suppressors of the lethality of a strain lacking the essential coactivator Cdc20 and the securin Pds1 (17). The viability of a *cdc20*Δ *pds1*Δ *clb5*Δ mutant suggested that Clb5 degradation is a key process of APC/C-Cdc20.

Subsequent experiments, however, demonstrated that a deletion of the D-box of Clb5 did not cause defects in mitosis, in contrast to a stabilized version cyclin Clb2 lacking the D-box that efficiently blocked exit from mitosis (18). The presence of Clb5 without destruction box did not significantly affect cell cycle progression, although protein levels of this version of Clb5 were deregulated compared with normal Clb5. These data implied that APC/C-mediated proteolysis of Clb5 is not essential for cell cycle progression.

Later data showed that cells lacking essential APC/C subunits were viable in the presence of very high levels of the Cdk1 inhibitor Sic1, but only in a strain containing deletions of the *PDS1* and *CLB5* genes (19). These experiments showing that cells can survive without APC/C under specific conditions raised again the model that Clb5 is a key target of APC/C.

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[2] The abbreviations used are: Cdk, Cyclin-dependent kinase; APC/C, anaphase promoting complex/cyclosome; FACS, fluorescence activated cell sorting; SCF, Skp1/Cullin F-box protein; HA, hemagglutinin.

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TABLE 1
***Saccharomyces cerevisiae* strains used in this study**

Strain	Relevant genotype
S001	W303-1A wild-type strain, *MATa, ade2-1, trp1-1, leu2-3,112, his3-11,15, ura3, can1-100, GAL, psi+*
S006	*MATa, bar1::HisG*
S026	*MATa, cdc16-123*
S028	*MATa, cdc16-123, GAL1-CLB2-HA3/URA3*
S030	*MATa, cdc16-123, GAL1-CLB5-HA3/URA3, bar1::HisG*
S039	*MATa, cdc23-1*
S040	*MATa, cdc23-1, GAL1-CLB2-HA3/URA3*
S056	*MATa, GAL1-CLB3-HA3/URA3*
S057	*MATa, GAL1-CLB2-HA3/URA3*
S095	*MATa, apc10-22*
S114	*MATa, cdc28-4, GAL1-CLB5-HA3/URA3, bar1::HisG*
S116	*MATa, GAL1-CLB5-HA3/URA3, bar1::HisG*
S117	*MATa, GAL1-CLB5ΔDB-HA3/URA3, bar1::HisG*
S119	*MATa, cdc23-1 cdc28-4, GAL1-CLB5-HA3/URA3, bar1::HisG*
S128	*MATa, apc10-22 cdc28-4, GAL1-CLB5-HA3/URA3, bar1::HisG*
S130	*MATa, cdc15-2, GAL1-CLB5-HA3/URA3*
S131	*MATa, cdc15-2, GAL1-CLB5ΔDB -HA3/URA3*
S132	*MATa, cdc23-1, GAL1-CLB5-HA3/URA3*
S148	*MATa, apc10-22, GAL1-CLB5-HA3/URA3, bar1::HisG*
S160	*MATa, cdc23-1, GAL1-CLB5-HA3/URA3, bar1::HisG*
S401	*MATa, cdc34-2, GAL1-CLB5-HA3/URA3*
S403	*MATa, cdc4-1, GAL1-CLB5-HA3/URA3*
S432	*MATa, cdc34-2 cdc23-1, GAL1-CLB5-HA3/URA3*
S474	*MATa, GALL-HA-CDH1/TRP1, bar1::HisG*
S583	*MATa CLB5ΔDB, bar1::HisG*
S596	*MATa GALL-HA-CDH1/TRP1, GAL1-CLB5-HA3/URA3, bar1::HisG*
S597	*MATa, GALL-HA-CDH1/TRP1, GAL1-CLB5ΔDB-HA3/URA3, bar1::HisG*
S598	*MATa, GALL-HA-CDH1/TRP1, CLB5ΔDB, bar1::HisG*
S711	*MATa, cdc53-1, GAL1-CLB5-HA3/URA3*
S713	*MATa, cim3-1, GAL1-CLB5-HA3/URA3*
S714	*MATa, cim5-1, GAL1-CLB5-HA3/URA3*

These conflicting data on the role of APC/C in Clb5 proteolysis prompted us to perform a comprehensive analysis of the cell cycle regulation of Clb5 stability by applying *in vivo* pulse labeling experiments. Our data on Clb5 stability imply that Clb5 degradation involves at least two different pathways, one APC/C-dependent and one APC/C-independent. We further found that Clb5 instability is highly dependent on the 26 S proteasome but independent of the SCF ubiquitin ligase.

EXPERIMENTAL PROCEDURES

Yeast Strains—All strains are derivatives of the *S. cerevisiae* W303 strain. The strains are listed in Table 1. Yeast strains were usually produced by crossing of strains from opposite mating types, subsequent sporulation and tetrad dissection with a micromanipulator. Strains carrying HA-tagged versions of Clb2 (20), Clb3 (14), Clb5 (5), and the strains carrying N-terminally HA-tagged *CDH1* expressed from the weak *GALL* promoter (20) have been previously described. Strains containing a destruction box deletion variant of *CLB5* (*CLB5ΔDB*) expressed from the *CLB5* promoter lacked a selectable marker (18) but were verified by PCR analysis.

Growth of Yeast Strains and Cell Cycle Arrests—Either complete medium (YP) or minimal medium was used for cultivation of yeast. The media were supplemented with either 2% glucose or 2% raffinose as carbon sources. Prior to gene expression from the *GAL1* promoter, the cells were pregrown in raffinose medium. The *GAL1* promoter was induced by the addition of galactose (final concentration, 2%). To turn off the *GAL1* promoter, the cells were filtered, washed, and resuspended in medium containing 2% glucose. To arrest cells in G_1 phase, α-factor pheromone was added to exponentially growing cultures, and the cells were incubated for 2.5 h in the presence of 5 μg/ml α-factor. For all pheromone arrests, strains hypersensitive to α-factor by *bar1* mutations were used. For cell cycle arrest in S phase, the cells were treated with 5 mg/ml hydroxyurea. To arrest cells in metaphase, the cultures were incubated for 2.5 h in the presence of 15 μg/ml of the microtubuli-depolymerizing drug nocodazole (added from a 1.5 mg/ml stock solution in dimethyl sulfoxide). To arrest cells by temperature-sensitive mutations, the cells were pregrown at 25 °C and then shifted for 2.5 h to 36 °C.

Pulse Labeling Experiments—To determine the half-life of proteins, pulse labeling experiments were performed as described (21). In brief, the cells were pregrown in minimal medium lacking methionine and containing raffinose as the sole carbon source. The *GAL1* promoter was induced by galactose addition (2% end concentration). For *in vivo* labeling, 0.5 mCi of [^{35}S]methionine was added, and the cells were incubated for 5 min (pulse), followed by the addition of 2 mM unlabeled methionine (chase). At the same time, glucose (final concentration, 2%) was added to turn off the promoter. The samples were collected at specific time points after the chase. The cells were broken with glass beads, and immunoprecipitations were performed with anti-hemagglutinin antibodies (anti-HA, 1:10 dilutions). Proteins were loaded on SDS-polyacrylamide gels, and the bands were quantified with a Fuji phosphorimager. To verify cell cycle arrests, DNA content was determined by FACS analysis. The cultures for FACS analysis were treated similarly to the main culture except that [^{35}S]methionine was omitted.

Immunoblot Analysis—Preparation of yeast cell extracts and protein immunoblot analysis were performed as described (22). The enhanced chemiluminescence detection system (Amersham Biosciences) was used for the detection of specific proteins. The antibodies were used in 1:1000 (HA) or 1:2000 (Cdc28) concentrations.

FACS Analysis and Immunofluorescence—To determine the DNA content of cells, FACS analysis was performed as previously described (3), using a Becton-Dickinson FACScan. The cells were fixed with 70% ethanol and stained with propidiumiodide. For indirect immunofluorescence, the cells were fixed in 3.7% formaldehyde. The spheroplasts were prepared as described (23). 4′,6-Diamidino-2-phenylindole staining and anti-tubulin antibodies (YOL37, 1:60 dilution) were used for visualization of nuclei and spindles, respectively.

RESULTS

Determination of the Half-lives of Clb5 and Clb5ΔDB at Different Cell Cycle Stages—Previously, Clb5 stability has been analyzed by promoter shut-off experiments with the glucose-repressible *GAL1* promoter (15, 16). A comprehensive analysis of Clb5 stability by pulse labeling experiments has not been performed up to present, and therefore the knowledge of the accurate half-life of this protein is unclear. We applied this method to determine the half-life of Clb5 at different stages of the cell cycle. The stability of a cyclin destruction

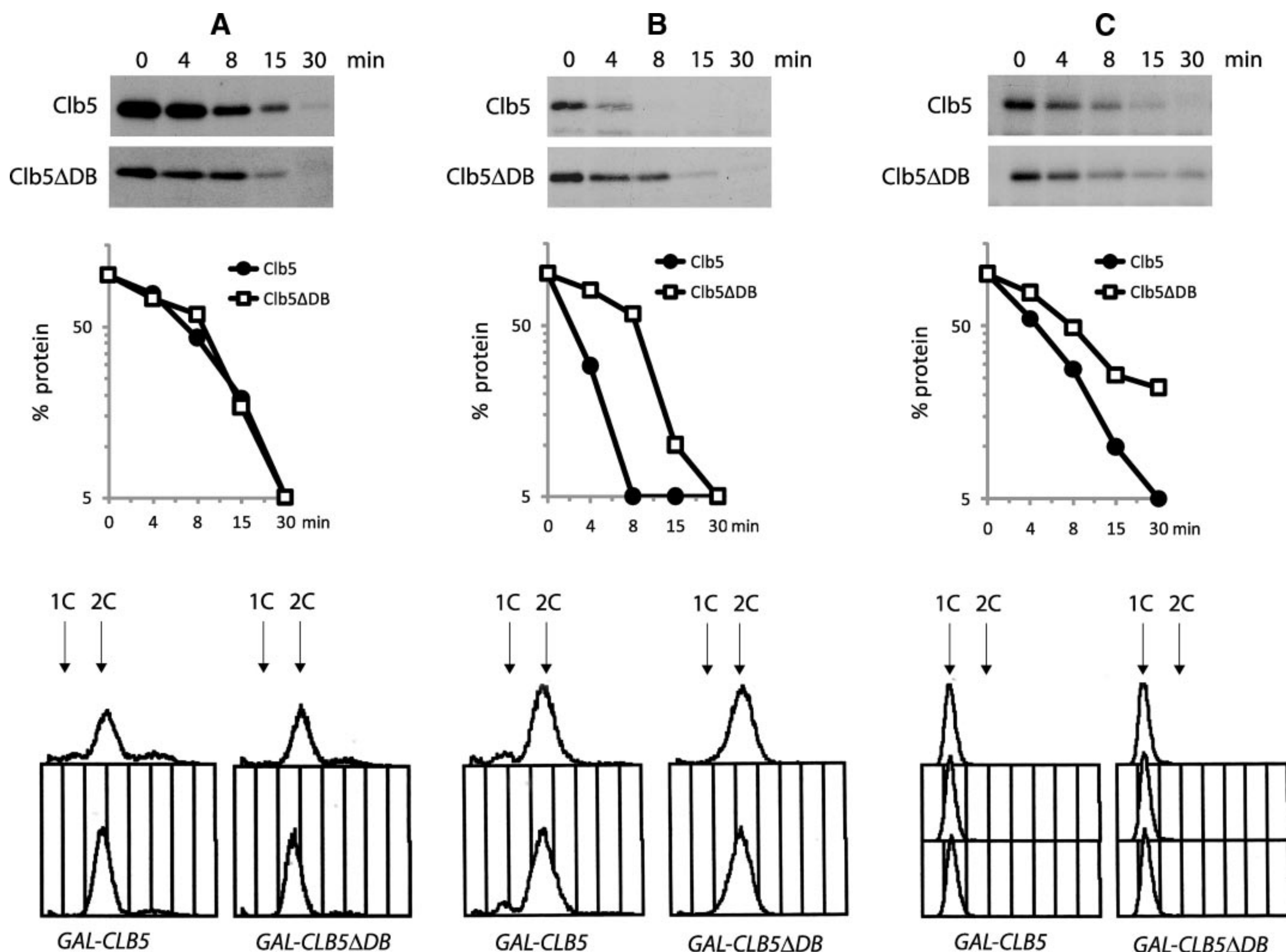

FIGURE 1. **Stability of Clb5 and Clb5ΔDB proteins at different cell cycle stages.** *A*, yeast strains containing either a *GAL1-CLB5-HA3* (S116) or a *GAL1-CLB5ΔDB-HA3* (S117) construct were grown at 30 °C in minimal medium lacking methionine with raffinose as carbon source and arrested in metaphase by nocodazole addition. Galactose was added for 30 min, followed by the addition of [^{35}S]methionine. After 5 min., glucose and an excess of unlabeled methionine (2 mM) were added (0 min time point). HA-tagged proteins were immunoprecipitated at the indicated time points. A quantification of the amount of protein levels is shown in graphs. Cell cycle arrests were verified by FACS analysis. *B*, *cdc15-2* mutants containing either a *GAL1-CLB5-HA3* (S130) or a *GAL1-CLB5ΔDB-HA3* (S131) construct were grown at 25 °C and then arrested at 36 °C for 2,5 h. Galactose was added, and the cells were then pulse-chased as described for *A*. Protein levels, quantification, and FACS analysis are shown. *C*, yeast strains S116 or S117, which both are *bar1* mutants, were grown at 30 °C and then arrested with the pheromone α-factor. The cells were pulse-chased in the presence of α-factor as in *A*. Protein levels, quantification, and FACS analysis are shown.

box mutant of Clb5, termed here Clb5ΔDB, was also measured.

Yeast strains carrying HA3-tagged versions of Clb5 or Clb5ΔDB expressed from the inducible *GAL1* promoter were arrested at specific periods of the cell cycle. First, we determined the half-life of Clb5 and Clb5ΔDB in the absence of APC/C activity, in cells arrested in metaphase with the spindle depolymerizing drug nocodazole. Upon cell cycle arrest, galactose was added to express the *GAL1-CLB5-HA3* or *GAL1-CLB5ΔDB-HA3* constructs. The cells were incubated for 5 min with radiolabeled [^{35}S]methionine and then chased with an excess of unlabeled methionine. Clb5 was immunoprecipitated from samples collected at different periods after the chase. Pulse labeling experiments showed that Clb5 and Clb5ΔDB were similarly unstable in metaphase cells (Fig. 1*A*). The half-life of both proteins was ~8–10 min. This instability indicates that Clb5 is subject to a degradation mechanism independent of the destruction box and of APC/C activity.

Destruction Box Further Destabilizes Clb5 in Late Mitosis and G_1 Phase—To test the degradation of Clb5 and Clb5ΔDB later in mitosis, we used *cdc15-2* mutants, impaired in a protein kinase essential for the exit from mitosis. *cdc15-2* mutants carrying *GAL1-CLB5-HA3* or *GAL1-CLB5ΔDB-HA3* constructs were arrested in telophase by a shift to a restrictive temperature, 36 °C. Pulse-chase experiments showed that the half-life of Clb5 is further reduced to less than 5 min, whereas the stability of Clb5ΔDB is only modestly different from metaphase-arrested cells (Fig. 1*B*). Thus, a further destabilization of Clb5 occurs during anaphase, consistent with the findings that Clb5 is a substrate of APC/C-Cdc20, which is highly active in telophase cells (24).

To compare the stability of Clb5 and Clb5ΔDB in G_1 phase, the cells were arrested in the G_1 phase with the pheromone α-factor. In this period of the cell cycle, APC/C-Cdh1 is active (25, 26). We found that Clb5 is more unstable than Clb5ΔDB in pheromone-arrested G_1 cells (Fig. 1*C*). The half-life of Clb5 in these cells is ~5 min. These findings support the model that

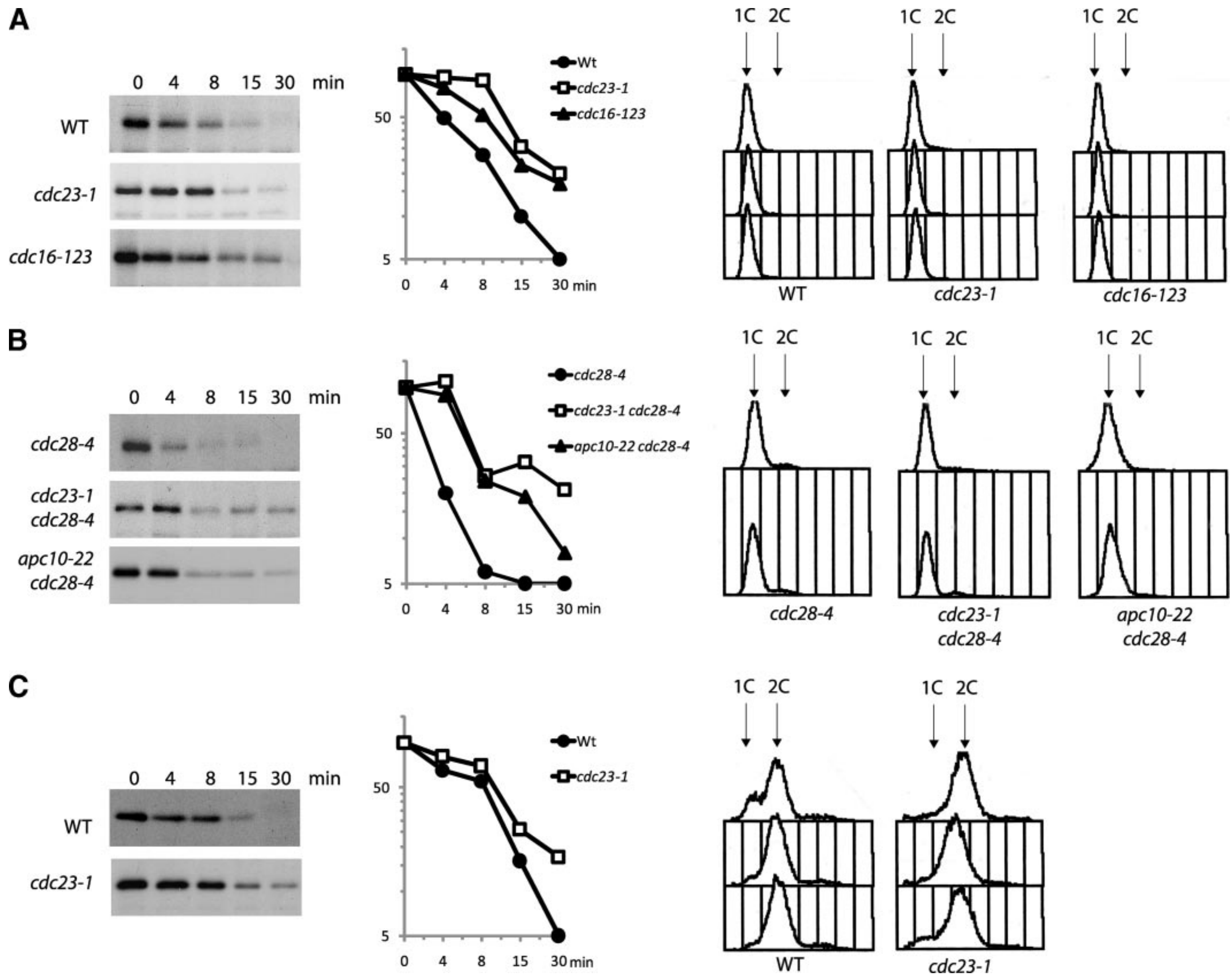

FIGURE 2. **Clb5 is unstable in mutants defective in APC/C function.** *A*, wild-type (*WT*) cells (S116), *cdc16-123* mutants (S030), and *cdc23-1* mutants (S160) containing *GAL1-CLB5-HA3*, all *bar1*, were pregrown at 25 °C and arrested in G_1 phase with α-factor. Then galactose was added, and the temperature was shifted to 36 °C. After 30 min of incubation, the cells were pulse-chased as described for Fig. 1*A* at 36 °C in the presence of α-factor. Cultures for FACS analysis were treated similarly except that [^{35}S]methionine was omitted. *B*, *cdc28-4* mutants (S114), *cdc23-1 cdc28-4* mutants (S119), and *apc10-22 cdc28-4* mutants (S128) containing *GAL1-CLB5-HA3*, all *bar*, were arrested and pulse-chased as in *A*. *C*, wild-type cells (S116) and *cdc23-1* mutants (S160) containing *GAL-CLB5-HA3* were pregrown at 25 °C in minimal medium lacking methionine containing raffinose as carbon source and were then arrested in nocodazole at 25 °C. Then galactose was added, and the temperature was shifted to 36 °C. After 30 min of incubation, the cells were pulse-chased at 36 °C in the presence of nocodazole.

Clb5 is unstable throughout the cell cycle and that a more rapid Clb5 turnover occurs during telophase and the G_1 phase in an APC/C-dependent manner.

Clb5 Is Degraded by an APC/C-independent Pathway—The instability of Clb5 and of Clb5ΔDB in metaphase (Fig. 1*A*) suggests that Clb5 is degraded by a process independent of APC/C. To underline this assumption, we determined Clb5 stability in temperature-sensitive mutants defective in APC/C function, *cdc16-123* and *cdc23-1*.

The half-life of Clb5 was measured in G_1-arrested mutant strains. The cells were first arrested at the permissive temperature in the G_1 phase by the addition of α-factor. Subsequently, the cells were shifted to the restrictive temperature, and then pulse-labeling experiments were performed in the presence of the pheromone. Clb5 was unstable in both mutants with a half-life of ~10 min but moderately stabilized compared with wild-type cells (Fig. 2*A*). FACS analysis confirmed that cells did not escape from the G_1 arrest during the course of the experiment.

To further exclude the possibility that cells lacking APC/C are not properly arrested in the G_1 phase, we used *cdc28-4* mutants combined with mutants in APC/C functions. The *cdc28-4* mutation prevents cells from entering S phase upon the temperature shift. We tested Clb5 stability in G_1-arrested *cdc28-4 cdc23-1* and *cdc28-4 apc10-22* double mutants. The cells were arrested in the G_1 phase at the permissive temperature, and then Clb5 stability was determined at the restrictive temperature. In both double mutants, Clb5 was more stable than in the *cdc28-4* single mutant but still significantly unstable (Fig. 2*B*).

It cannot be completely ruled out that residual APC/C activity contributes to Clb5 instability in G_1-arrested mutants. To further support the model that Clb5 proteolysis is independent of APC/C, we performed pulse labeling experiments with *cdc23-1* cells arrested in metaphase, when APC/C is inactive. Wild-type and

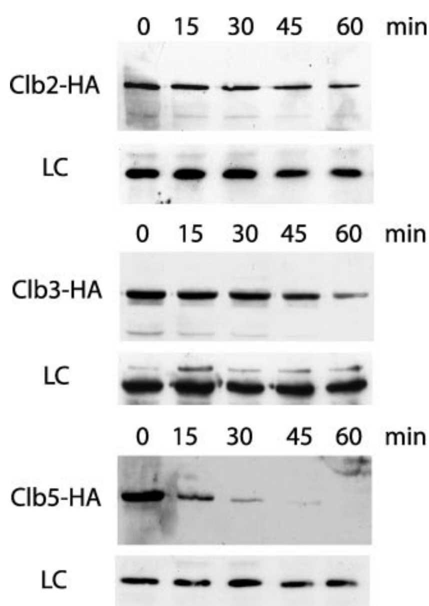

FIGURE 3. **Clb5 is more unstable than other Clb cyclins in the absence of APC/C activity.** Yeast strains carrying either *GAL1-CLB2-HA3* (S057), *GAL1-CLB3-HA3* (S056), or *GAL1-CLB5-HA3* (S116) were grown at 30 °C in YP medium containing raffinose as carbon source (YP+Raf). The cells were arrested in M phase by nocodazole addition for 2,5 h. The arrest was confirmed by light microscopy (not shown). Galactose was added for 60 min, and then cells were filtered and transferred to YP medium containing glucose (YP+Glu). The samples were collected at the indicated time points after galactose addition and analyzed by immunoblotting using the anti-HA-antibody. Cdc28 antibody was used as a loading control (*LC*).

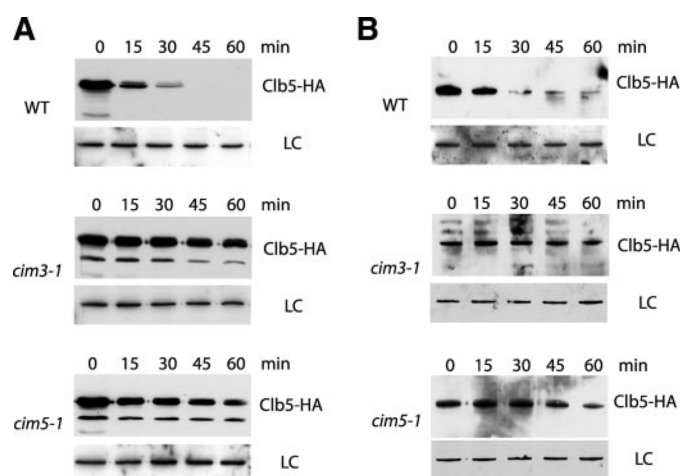

FIGURE 4. **Clb5 instability is mediated by the proteasome.** Wild-type (*WT*) cells (S116) and the proteasomal mutants *cim3-1* mutants (S713) and *cim5-1* mutants (S714), all containing *GAL1-CLB5-HA3,* were grown at 25 °C in YP+Raf medium. Promoter shut-off experiments were performed with either cycling cells (*A*) or cells arrested in M phase with nocodazole for 2.5 h (*B*). Galactose was added to induce the *GAL1* promoter. After 30 min, the temperature was shifted to 36 °C, and the cells were incubated for another 30 min. To turn off the *GAL1* promoter, the cells were filtered and transferred to YP+Glu medium (prewarmed to 36 °C) without (*A*) or with nocodazole (*B*). The samples collected at the indicated time points after transfer to glucose medium were analyzed by immunoblotting using the anti-HA antibody. Cdc28 antibody was used as a loading control (*LC*).

cdc23-1 mutants were arrested in metaphase with nocodazole at the permissive temperature. Upon a shift to 36 °C, the half-life of Clb5 was measured. In both strains, Clb5 stability was similar, with a half-life of ~10 min (Fig. 2*C*). Thus, even in *cdc23-1* mutants arrested in a period of the cell cycle when APC is inactive, Clb5 is still unstable. These data underline that an APC/C-independent mechanism contributes to Clb5 proteolysis.

Mitotic Cyclins Are More Stable than Clb5 in the Absence of APC/C Activity—After having determined the half-life of Clb5 by pulse labeling, we applied the more convenient promoter shut-off method for further experiments. Although not as precise in the determination of the half-lives of proteins, the application of this method provided results of sufficient clarity.

We next tested whether Clb5 is indeed more unstable than mitotic cyclins in the absence of APC/C activity. Therefore, we compared the stability of Clb5 with the stability of Clb2 and Clb3 in cells arrested in metaphase. Strains carrying HA-tagged versions of these cyclins expressed from the *GAL1* promoter were arrested with nocodazole. Then the expression of cyclin genes was induced by galactose addition, followed by a shift to glucose medium. In accordance with the pulse labeling experiments in Fig. 1*A*, Clb5 is rapidly degraded (Fig. 3). In contrast, Clb2 and Clb3 levels decreased slowly in these metaphase-arrested cells. These data underline that Clb5, but not Clb2 or Clb3, is subject to a distinct APC-independent mode of proteolysis.

Clb5 Instability Depends on the Proteasome—We next tested whether the persistent instability of Clb5 during the cell cycle requires the function of the proteasome. To this end, the stability of Clb5 was analyzed in mutants impaired in the function of the proteasome, the temperature-sensitive *cim3-1* and *cim5-1* mutants (27). Cim3, also known as Sug1/Rpt6, and Cim5, also known as Rpt1, are both ATPases of the regulatory 19 S particle of the 26 S proteasome and essential for viability (28). To determine the stability of Clb5, promoter shut-off experiments were performed at the restrictive temperature. We found that Clb5 was virtually stable during the course of the experiment (Fig. 4*A*). Similar experiments were performed with cell cycle-arrested cells by nocodazole treatment of cells prior to the promoter shut-off experiments (Fig. 4*B*). Also in these arrested cells, Clb5 appeared to be distinctly stabilized compared with wild-type cells. We conclude that Clb5 degradation is highly dependent on the function of the 26 S proteasome.

Clb5 Instability Does Not Involve the SCF Ubiquitin Ligase—The findings that Clb5 is targeted to the proteasome by an APC/C-independent process prompted us to test whether this alternative mode may be mediated by the activity of the SCF ubiquitin ligase. Remarkably, the S phase cyclin Clb6 was recently identified as a SCF-Cdc4 target (13). Involvement of SCF in Clb5 proteolysis had been suggested earlier by Bai *et al.* (29), whereas data from Wasch and Cross (18) resulted in opposite conclusions. These experiments, however, were performed in dividing cells and did not uncouple the APC/C-dependent and APC/C-independent degradation modes.

To exclude APC/C activity, we used S or M phase-arrested cells. To test whether SCF is involved in Clb5 degradation, we compared wild-type and *cdc34-2* mutants. *CDC34* encodes the essential E2 ubiquitin-conjugating enzyme of the yeast SCF complex (30). The cells were arrested in the S phase or metaphase with hydoxyurea or nocodazole, respectively. Then promoter shut-off experiments were performed. Clb5 and Clb5ΔDB were both unstable in these arrested mutant cells, similar to wild-type cells (Fig. 5, *A* and *B*).

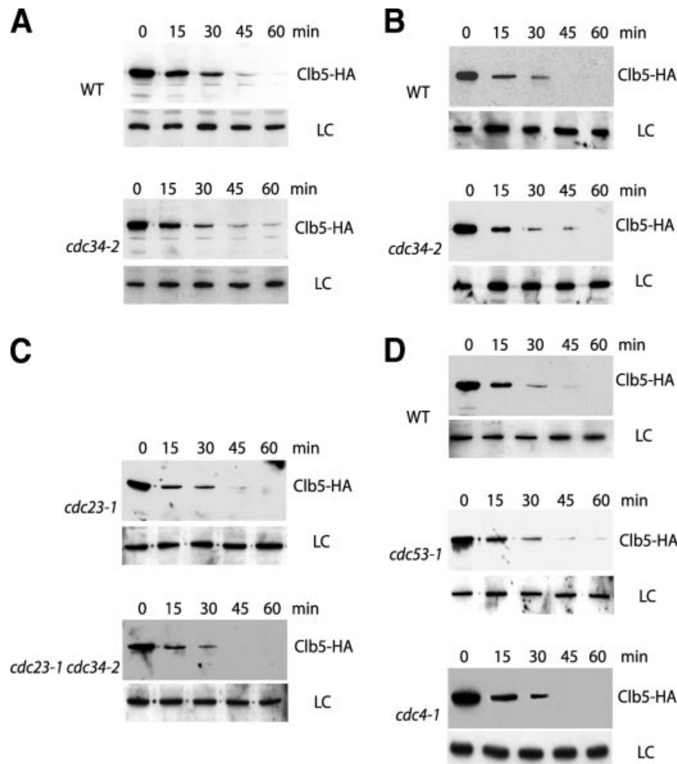

FIGURE 5. **Clb5 instability is independent of SCF activity.** *A* and *B*, wild-type (*WT*) cells (S116) and a *cdc34-2* mutant (S401) both containing *GAL1-CLB5-HA3* were grown at 25 °C in YP+Raf medium. Yeast strains were treated for 2.5 h with either nocodazole (*A*) or hydroxyurea (*B*) to arrest cells in the M or S phase, respectively. Galactose was added to induce the *GAL1* promoter. After 30 min, the temperature was shifted to 36 °C, and the cells were incubated for another 30 min. To turn off the *GAL1* promoter, the cells were filtered and transferred to YP+Glu medium (prewarmed to 36 °C) containing nocodazole (*A*) or hydroxyurea (*B*). Immunoblotting was performed using the anti-HA antibody to detect Clb5-HA and Cdc28 antibody as a loading control (*LC*). *C*, *cdc23-1* (S132) and *cdc23-1 cdc34-2* (S432) mutants containing *GAL1-CLB5-HA3* were grown at 25 °C. Galactose was added to induce the *GAL1* promoter. After 30 min, the temperature was shifted to 36 °C, and the cells were incubated for another 30 min. To turn off the *GAL1* promoter, the cells were filtered and transferred to YP+Glu medium (prewarmed to 36 °C). Immunoblotting was performed with anti-HA and Cdc28 (LC) antibodies. *D*, promoter shut-off experiments and immunoblotting with S116, a *cdc53-1* mutant (S711), and a *cdc4-1* mutant (S403) were performed with nocodazole-arrested cells as in *A*.

To further support the idea that Clb5 is degraded by a pathway independent of APC/C and SCF, we constructed yeast strains with temperature-sensitive mutations causing defects in both ubiquitin ligases, *cdc34-2 cdc23-1* double mutants. Promoter shut-off experiments showed that Clb5 are significantly unstable in this yeast strain, similar to a *cdc23-1* mutant (Fig. 5*C*). By performing similar experiments as described in Fig. 5*A*, we found that Clb5 was also unstable in *cdc53-1* and *cdc4-1* mutants (Fig. 5*D*). Cdc53 is the cullin subunit of SCF, and Cdc4 is an F-box protein (29, 31). Thus, targeting of Clb5 to the 26 S proteasome can apparently occur by a pathway independent of the two ubiquitin-ligases with known roles in cell cycle progression, APC/C and SCF.

APC/C-mediated Clb5 Degradation Is Essential under Conditions of Elevated CLB5 Expression Levels—It was previously shown that *CLB5ΔDB* expressed from its own promoter does not exhibit distinct cell cycle defects, whereas *GAL1-CLB5ΔDB* is toxic to yeast cells (16). The APC/C-independent pathway may be sufficient for Clb5 proteolysis in a normal cell cycle but not when *CLB5* is overexpressed.

To test the relevance of APC/C in the presence of abnormally high levels of Clb5, we analyzed the elevated *CLB5* expression in *cdc23-1* mutants. The viability of *cdc23-1* mutants containing a single copy of *GAL1-CLB5-HA3* was tested on galactose plates (Fig. 6*A*). *cdc23-1* mutants are viable at 25 °C, but the expression of elevated levels of *CLB5* was highly deleterious for this mutant. The toxicity of high levels of Clb5 to *cdc23-1* mutants was comparable with increased *CLB2* expression. The expression of *GAL1-CLB5* had no discernible effects on wild-type cells. In contrast, high levels of Clb5 affected the viability of additional temperature-sensitive mutants, *cdc16-123* and *apc10-22*. Both of these strains defective in APC/C subunit genes are viable at 28 °C, but the expression of *GAL1-CLB5* inhibited growth. The findings that cells impaired in APC/C function are sensitive to high levels of Clb5 even at permissive temperatures implies that the alternative degradation pathway is not sufficient to control Clb5 as soon as this cyclin is expressed to high levels. Under these conditions, APC/C gets essential for Clb5 degradation.

Remarkably, increased *CLB5* expression in *cdc23-1* mutants caused a cell cycle arrest, which differed from *CLB2* expression. *GAL1-CLB2* resulted in an uniform telophase arrest in *cdc23-1* mutants (Fig. 6*B*). In contrast, in *cdc23-1* mutants expressing *CLB5*, only ~30% of cells contained divided nuclei and elongated spindles. Most cells had short spindles and undivided nuclei, indicating that cells were arrested predominantly prior to anaphase. These data support the model that high Clb5 levels do not inhibit the exit from mitosis (18).

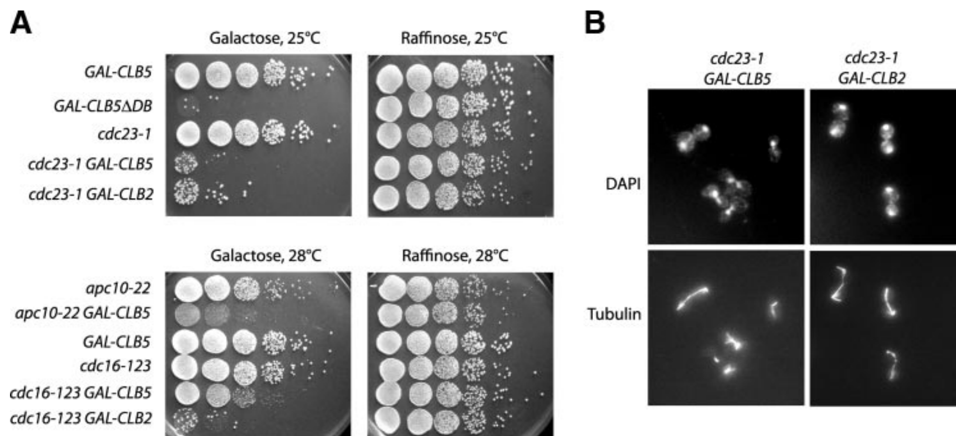

FIGURE 6. **Elevated Clb5 levels are lethal for mutants impaired in APC/C function.** *A*, serial dilutions (1:10) of the indicated strains were spotted on YP medium containing either galactose or raffinose as carbon source, respectively. *B*, immunofluorescence microscopy of cells grown for 4 h in the presence of galactose at 25 °C. *DAPI*, 4′,6-diamidino-2-phenylindole.

APC/C Is Required for Preventing S Phase Entry in Cells Expressing High Levels of CLB5—We also tested the relevance of APC/C-me-

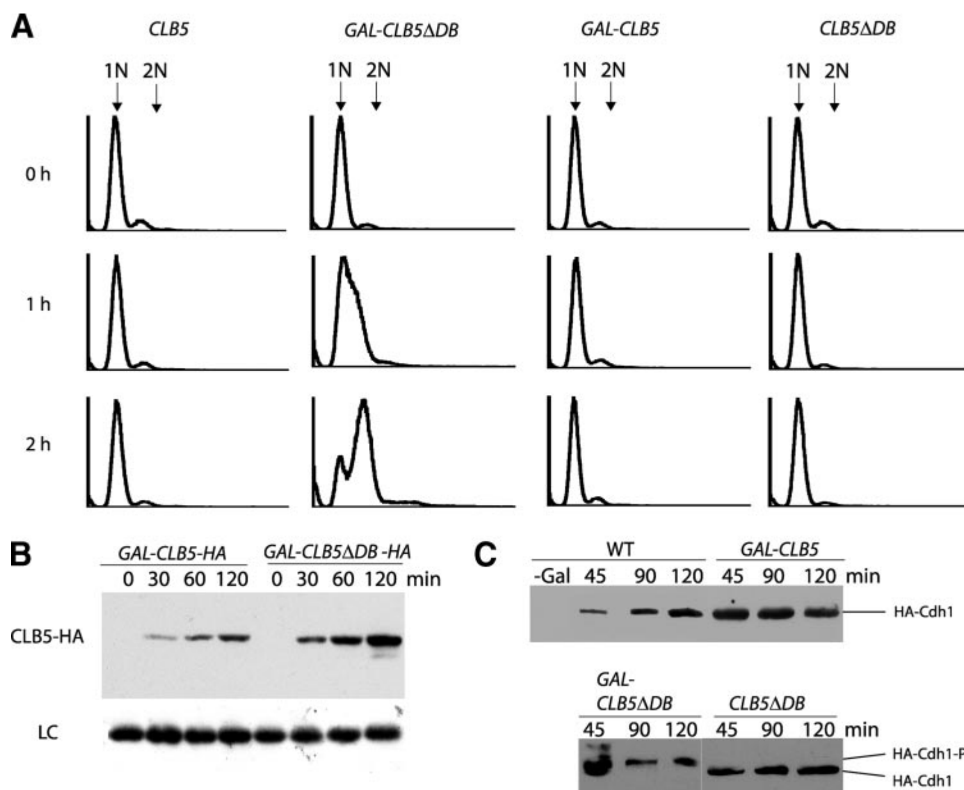

FIGURE 7. **APC/C is required in G_1 cells to restrict S phase entry under conditions of abnormal *CLB5* expression levels.** *A* and *B*, a wild-type strain (S006) and strains containing either *GAL1-CLB5-HA3* (S116), *GAL1-CLB5ΔDB-HA3* (S117), or *CLB5ΔDB-HA3* (S583) construct were arrested at 30 °C in YP+Raf medium with α-factor. 2% Galactose was added, and the cells were incubated further in the presence of pheromone. *A*, FACS analysis of cells collected at indicated time points after galactose addition. *B*, immunoblotting of samples collected from strains S116 and S117 at indicated time points after galactose addition. *C*, a strain carrying *GALL-HA-CDH1* (S474) and strains carrying *GALL-HA-CDH1* and in addition either *GAL1-CLB5-HA3* (S596), *GAL1-CLB5ΔDB-HA3* (S597), or *CLB5ΔDB-HA3* (S598) strains were arrested at 30 °C in YP+Raf medium with α-factor. 2% Galactose was added, and the cells were incubated further in the presence of pheromone. The samples for immunoblotting were collected at the indicated time points after galactose addition.

diated degradation of Clb5 in G_1-arrested cells. Strains expressing *CLB5* and *CLB5ΔDB* from its own promoter and strains expressing either *CLB5* or *CLB5ΔDB* from the *GAL1* promoter were arrested with α-factor in the G_1 phase. Galactose was added, and the incubation in the presence of pheromone was continued. We found that DNA replication did not occur in cells expressing high levels of *CLB5*, whereas the expression of *GAL1-CLB5ΔDB* efficiently triggered the initiation of DNA replication (Fig. 7*A*). This is most likely due to the appearance of the Clb5ΔDB protein, which accumulated to higher levels than normal Clb5 (Fig. 7*B*). Cells containing *CLB5ΔDB* expressed from its own promoter did not initiate DNA replication (Fig. 7*A*).

An important process for the initiation of DNA replication is the phosphorylation of Cdh1, which results in the inactivation of APC/C. We tested the potential of Clb5 and Clb5ΔDB to phosphorylate Cdh1 *in vivo*. Cdh1 phosphorylation results in a slower migrating form (20). We found that the expression of *GAL1-CLB5ΔDB* caused a distinct shift of most of Cdh1 to a slower migrating version (Fig. 7*C*). In contrast, the expression of *GAL1-CLB5* did not result in a significant modification of Cdh1. Cdh1 was also not affected in cells expressing *CLBΔDB5* from its own promoter.

These results show that under normal *CLB5* expression conditions, the destruction box is not required in G_1 cells to prevent DNA replication and Cdh1 phosphorylation. In contrast, upon expression of *CLB5* to abnormally high levels, destruction box-mediated proteolysis is essential, suggesting that the APC/C-dependent pathway is needed to prevent a precocious entry into S phase under these conditions. High Clb5 levels apparently exceed the potential of the APC/C-independent mode to control the levels of this cyclin. We conclude that the two different degradation modes have relevant roles in restricting Clb5 activity upon deregulated *CLB5* expression.

DISCUSSION

Cyclin proteolysis is an important process for the regulation of the activity of cyclin-dependent kinases during the cell cycle. APC/C has a crucial role in the cell cycle regulation of the stability of B-type cyclins. Various previous reports indicated that the yeast S phase cyclin Clb5 appears to be differently regulated than the mitotic Clb cyclins (14–16, 18). To better understand regulation of Clb5 stability, we have presented here the first in-depth analysis of the half-life of Clb5 by *in vivo* pulse labeling experiments. We have shown that Clb5 lacking its cyclin destruction box, Clb5ΔDB, is an unstable protein with a half-life of 8–10 min and that Clb5 is similarly unstable in the absence of APC/C activity. In cells with active APC/C, Clb5 is further destabilized. Our data underline the model that two distinct modes, an APC/C-dependent and an alternative APC/C-independent mode, both contribute to Clb5 instability.

The remarkable instability of Clb5 in the absence of APC/C activity indicates that the alternative Clb5 degradation pathway has a considerable role in restricting the activity of this cyclin during the cell cycle. The two modes may have overlapping functions in restricting Cdk1-Clb5 kinase activity. Indeed, destruction box-mediated proteolysis of Clb5 is dispensable for cell cycle progression, as manifested by the viability of a strain expressing *CLB5ΔDB* (18).

In contrast to this model, two previous studies have provided evidence that Clb5 is an essential target of APC/C. Shirayama *et al.* (17) identified that a deletion of *CLB5* suppressed the lethality of *cdc20 pds1* strains. This effect is probably indirect, because Cdh1 is abnormally active in such a strain background and probably substitutes Cdc20 function (32, 33). Thornton *et al.* (19) showed that yeast cells completely devoid of APC/C

activity are viable, when the *PDS1* and *CLB5* genes were deleted and when in addition *SIC1* was strongly overexpressed. These cells cannot survive if Clb5 is present, even if the Sic1 inhibitor protein is present in excess amounts. Thus, at least under these specific conditions, the failure of APC/C to degrade Clb5 results in lethality. One possible explanation suggested by the authors of this study was the idea that Cdk1-Clb5 counteracted the Sic1 protein, targeting it for efficient proteolysis. In these cells requiring vast amounts of Sic1 for viability, the absence of Cdk1-Clb5 is therefore of crucial importance. However, it is important to point out that this does not necessarily mean that this also applies to wild-type cells, which do not rely on the presence of excessive amounts of Sic1. The importance of APC/C for Clb5 proteolysis in a normal cell cycle is still not fully understood. Our data showing the continuous APC/C-independent instability of Clb5 rather support the idea that the different modes of Clb5 proteolysis have redundant roles in controlling this cyclin.

An interesting question is now whether Clb5 degradation by at least one of these mechanisms is important for a proper cell cycle. Substantial evidence for the importance of Clb5 instability was provided by experiments with an N-terminally tagged version of Clb5 lacking the destruction box (18). This N-terminal modification resulted accidentally in a completely stabilized Clb5 protein. Importantly, the expression of this stable Clb5 variant was lethal for cells. Therefore, it seems that at least one proteolytic mechanism is essential for restricting Clb5 activity.

How may Clb5 get degraded in the absence of APC/C? It was recently shown that the second S phase cyclin in yeast, Clb6, is degraded by the ubiquitin ligase SCF-Cdc4 (13). Previous studies on a possible role for SCF in Clb5 degradation revealed contradictory results. Whereas the analysis of *skp1-11* mutants revealed a stabilization of Clb5 (29), Clb5 was found to be highly unstable in the same mutant by others (18). It was not ruled out in this report that Clb5 instability is due to APC/C activity. Here, we have tested whether SCF is involved in the APC/C-independent degradation pathway by using cells arrested in periods of the cell cycle where APC/C is inactive. Therefore APC/C-dependent and APC/C-independent modes were uncoupled. We showed that Clb5 is unstable in mutants defective in SCF activity and also in *cdc23-1 cdc34-2* double mutants. Our data argue against a role for SCF in Clb5 proteolysis.

The significant stabilization of Clb5 in proteasomal mutants suggests that degradation of Clb5 is dependent on the activity of the proteasome. At present it is unknown how Clb5 is targeted to the proteasome in the absence of APC/C activity and whether this alternative pathway involves ubiquitination. Intriguingly, ubiquitin-independent targeting to the proteasome has been observed for different cell cycle regulatory proteins such as p53, c-Jun, and p21/Kip1 (34, 35). Each of these proteins is degraded by both ubiquitin-dependent and ubiquitin-independent processes. Examination of p53 degradation revealed that ubiquitin-independent degradation appeared to be mediated by the 20 S proteasome (35). Because Clb5 is stabilized in *cim3* und *cim5* strains, mutated in genes of the 19 S regulatory particle of the 26 S proteasome (27, 28), Clb5 degradation seems to be rather mediated by the 26 S proteasome.

Why is a persistent Clb5 instability important in the cell cycle, and why may this instability have evolved for Clb5 rather than for mitotic cyclins? *CLB5* expression is strongly induced at the G_1/S phase transition (4), and permanent Clb5 instability could be a process to prevent Clb5 accumulation to exceedingly high levels, which could then interfere with cell cycle events after completion of S phase. Clb5 instability in the G_2 and early M phases may be a process to lower Clb5 levels, before APC/C activation at the metaphase/anaphase transition occurs. Clb5 instability and dissociation from Cdk1 molecules may allow the efficient association of Cdk1 with mitotic cyclins Clb1-4. These cyclins are essential in early mitosis for spindle formation and other processes that need to occur before APC/C is activated. Clb5 instability may be required for reducing and/or reversing the phosphorylation of S phase-specific substrates, enabling the dephosphorylation of targets before the activation of APC/C. It is known that Cdk1-Clb5 exhibits much higher substrate specificity than Cdk1-Clb2 (36). Cdk1-Clb5 specifically phosphorylates various proteins required for DNA replication, whereas Cdk1-Clb2 has a higher kinase activity but phosphorylates Cdk1 targets with low specificity. Thus, Clb5 instability could be important for restricting the activity of the Cdk1-Clb5 kinase against its substrates.

Remarkably, cyclin A, the S phase cyclin in vertebrate cells, was found to be degraded earlier than cyclin B (37) during mitosis. In this case, however, this preceding degradation is mediated by APC/C-Cdc20 but occurs prior to the inactivation of the spindle assembly checkpoint.

Different degradation pathways for Clb5 may have further significant roles. It is conceivable that they are important for preventing a disastrous outcome because of an expression of Clb5 to exceedingly high levels. Although Clb5 expression is normally tightly regulated, failures in this process may result in elevated expression levels. In this regard, it is worth mentioning that such abnormalities in gene expression are often identified in cancer cells. Overexpression of cyclins, such as cyclin D1, is frequently found in human malignancies, resulting in up-regulated cyclin-dependent kinases (38).

It is important to point out that high levels of Clb5 kinase activity, as in strains expressing *GAL1-CLB5ΔDB*, do not cause a reversible cell cycle arrest but instead cause cell death (16). In contrast, a stabilized Clb2 induces a reversible cell cycle arrest in telophase and does not cause cell death (16). These findings highlight the importance of alternative processes for Clb5 degradation, acting in concert to ensure that toxic effects of a deregulated Cdk1-Clb5 activity are efficiently prevented.

We have provided evidence that this also applies to G_1 cells, to prevent an uncontrolled entry into S phase. In the G_1 phase, Clb5 is controlled by multiple mechanisms: repression of *CLB5* expression, the presence of Sic1, and Clb5 instability (2). Each process contributes to maintain a solid G_1 arrest. Control of Clb5 in G_1 phase is crucial because Cdk1-Clb5 phosphorylates many substrates required for S phase, including Cdh1, with high substrate specificity (36), and it is a potent inducer of DNA replication. We found that elevated levels of Clb5 lacking a destruction box, which is therefore inaccessible by APC/C, triggered efficient Cdh1 phosphorylation in G_1 cells and induced DNA replication. Thus, an increase in *CLB5* expression renders

APC/C activity essential for proteolysis of this cyclin. Under such conditions, the APC/C-independent mode is not any more sufficient, and destruction box-mediated degradation gets essential for preventing Cdh1 phosphorylation and S phase entry in the presence of pheromone.

The findings on Clb5 regulation are an example showing how different mechanisms with overlapping functions may help to prevent defects in the cell cycle, even if other processes, such as transcriptional regulation, fail. Thereby, death of dividing cells or abnormal cell cycle entry of G_1-arrested cells is avoided.

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