# Inhibition of APC-mediated proteolysis by the meiosis-specific protein kinase Ime2

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**Proteolysis triggered by the anaphase-promoting complex (APC) is needed for sister chromatid separation and the exit from mitosis. APC is a ubiquitin ligase whose activity is tightly controlled during the cell cycle. To identify factors involved in the regulation of APC-mediated proteolysis, a *Saccharomyces cerevisiae GAL-cDNA* library was screened for genes whose overexpression prevented degradation of an APC target protein, the mitotic cyclin Clb2. Genes encoding $G_1$, S, and mitotic cyclins were identified, consistent with previous data showing that the cyclin-dependent kinase Cdk1 associated with different cyclins is a key factor for inhibiting $APC^{Cdh1}$ activity from late-$G_1$ phase until mitosis. In addition, the meiosis-specific protein kinase Ime2 was identified as a negative regulator of APC-mediated proteolysis. Ectopic expression of *IME2* in $G_1$ arrested cells inhibited the degradation of mitotic cyclins and of other APC substrates. *IME2* expression resulted in the phosphorylation of Cdh1 in $G_1$ cells, indicating that Ime2 and Cdk1 regulate $APC^{Cdh1}$ in a similar manner. The expression of *IME2* in cycling cells inhibited bud formation and caused cells to arrest in mitosis. We show further that Ime2 itself is an unstable protein whose proteolysis occurs independently of the APC and SCF (Skp1/Cdc53/F-box) ubiquitin ligases. Our findings suggest that Ime2 represents an unstable, meiosis-specific regulator of $APC^{Cdh1}$.**

Crucial processes in the cell cycle, such as initiation of DNA replication, separation of sister chromatids, and exit from mitosis, depend on proteolytic degradation of critical regulatory proteins (1–3). Ubiquitin ligases play essential roles in these degradation processes. These enzymes catalyze the formation of chains of ubiquitin on their substrates, thereby targeting them for degradation by the 26S proteasome (4). The anaphase-promoting complex (APC), also known as cyclosome, is a multisubunit complex acting as ubiquitin ligase (5, 6). APC is essential for mitosis, and its activity is tightly cell cycle-regulated. Its activation at the metaphase/anaphase transition requires its association with the activator protein Cdc20. $APC^{Cdc20}$ triggers proteolytic degradation of the securin Pds1. Upon Pds1 proteolysis, the separase Esp1 is liberated, cleaves the cohesin subunit Scc1, and thereby triggers sister chromatid separation (7). $APC^{Cdc20}$ also initiates proteolysis of the S phase cyclin Clb5 and a fraction of mitotic cyclins (8–10). $APC^{Cdc20}$ activation is inhibited by the spindle assembly checkpoint, which prevents sister chromatid separation upon defects in the mitotic spindle or in the attachment of kinetochores (11).

Cdh1 (also termed Hct1) is related to Cdc20 and is needed for complete degradation of mitotic cyclins (12, 13). Cdh1's potential to associate with the APC is controlled by phosphorylation (14, 15). The cyclin-dependent kinase Cdk1 phosphorylates Cdh1, thereby preventing its interaction with APC. A Cdk1-antagonizing phosphatase, Cdc14, is kept inactive in the nucleolus for most of the cell cycle (16, 17). Its release during anaphase promotes $APC^{Cdh1}$ complex formation and also induces transcription and stabilization of the Cdk1 inhibitor Sic1 (14). Thus, Cdc14 activates two mechanisms, cyclin proteolysis and Cdk1 inhibition, both resulting in Cdk1 inactivation and exit from mitosis. $APC^{Cdh1}$ remains active in the subsequent $G_1$ phase and is turned off at the $G_1$/S transition by Cdk1 phosphorylating the Cdh1 protein (18, 19).

Recent data showed that APC is also important for meiotic cell divisions. During meiosis, two rounds of chromosome segregation follow one round of DNA duplication. Little is known about APC regulation during meiosis. Pds1 is degraded during meiosis I in an $APC^{Cdc20}$-dependent manner, reaccumulates, and disappears again in meiosis II (20). Furthermore, destruction of the cyclin Clb1 seems to be mediated by a meiosis-specific activator related to Cdc20/Cdh1, Ama1 (21).

We performed a genetic screening to identify factors involved in the APC regulation. We screened for cDNAs whose expression in $G_1$ phase inhibit proteolysis of a fusion protein of the mitotic cyclin Clb2 with lacZ. Various cyclins and the meiosis-specific protein kinase Ime2 were identified as proteins that stabilized Clb2-lacZ. Ectopic expression of *IME2* in $G_1$ cells stabilized mitotic cyclins and other APC substrates. Because Ime2 accumulation caused phosphorylation of Cdh1, Ime2 may be involved in $APC^{Cdh1}$ regulation during meiosis.

## Experimental Procedures

**Yeast Strains and Plasmids.** All strains are derivatives of the *Saccharomyces cerevisiae* W303 strain. Strains carrying hemagglutinin (HA)-tagged versions of Clb2 (19), Clb3 (22), Pds1 (23), and Cdh1 (19) have been described. A 2.8-kb fragment containing the *GAL-IME2* fusion was cloned from the *CEN* plasmid, isolated from the *GAL-cDNA* library, into integrative plasmids YIplac204 and YIplac211 (24). For integrations, plasmids were linearized by *Bsu*36I and transformed into yeast cells. To tag the *IME2* gene, a *Xba*I restriction site was introduced upstream of the stop codon of *IME2* in the plasmid YIplac204-*GAL-IME2*. Six copies of the HA fragment were cloned as an *Xba*I fragment into this site.

**Growth Conditions and Cell Cycle Arrests.** Before gene expression from the *GAL1* promoter, cells were grown in raffinose medium. The *GAL1* promoter was induced by the addition of galactose (2% final concentration). To turn off the *GAL1* promoter, cells were filtered and resuspended in medium containing 2% glucose.

Strains lacking the $G_1$ cyclins *CLN1*, *CLN2*, and *CLN3*, but containing *CLN2* expressed from the methionine-repressible *MET3* promoter, were cultivated in minimal medium lacking methionine. To arrest these cells in $G_1$, methionine was added to a final concentration of 2 mM. To arrest cells in $G_1$ with $\alpha$-factor pheromone, cultures were incubated for 2.5 h in the presence of 5 $\mu$g/ml $\alpha$-factor. To arrest cells in metaphase, cultures were incubated for 2 h in the presence of 15 $\mu$g/ml nocodazole.

**Screening for cDNAs Stabilizing Clb2-lacZ.** A strain lacking all $G_1$ cyclins (*cln1*, *cln2*, and *cln3*) containing *MET-CLN2* and *GAL-*

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Abbreviations: APC, anaphase-promoting complex; CDK, cyclin-dependent kinase; FACS, fluorescence-activated cell sorter; HA, hemagglutinin; SCF, Skp1/Cdc53/F-box.

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*CLB2-lacZ* gene fusions (S65) was transformed with a *GAL-cDNA* library (25) on a centromeric plasmid (*URA3* marker). Transformants were plated on minimal medium plates lacking methionine and uracil. Colonies were transferred to nylon filters and arrested in $G_1$ phase by placing filters on plates containing 2 mM methionine. After 3 h, filters were transferred to plates containing 2% galactose and 2 mM methionine. After 2.5 h, filters were frozen in liquid nitrogen and colonies were analyzed for $\beta$-galactosidase activity. Blue colonies were collected. Plasmids of these colonies were retested and analyzed by restriction and sequencing. Because the expression of cDNAs encoding APC inhibitors likely results in cell cycle defects, plasmids were transformed into wild-type cells and only plasmids producing cell cycle phenotypes on galactose plates were analyzed further.

**Immunoblot, Immunofluorescence, and Fluorescence-Activated Cell Sorter (FACS) Analysis.** Preparation of yeast cell extracts and protein immunoblot analysis were performed as described (26). The enhanced chemiluminescence detection system was used. For indirect immunofluorescence, cells were fixed in 3.7% formaldehyde. Spheroplasts were prepared as described (27). DAPI (4′,6-diamidino-2-phenylindole) staining and antitubulin antibodies were used for visualization of nuclei and spindles, respectively. FACS analysis was performed as described (28).

## Results

**A Screen for cDNAs Inhibiting Cyclin Degradation in *S. cerevisiae*.** To identify putative regulators of the APC, we screened for genes whose ectopic expression inhibited the degradation of a Clb2-lacZ fusion protein in $G_1$-arrested cells. To arrest cells in $G_1$ phase, we used a strain deleted of the $G_1$ cyclins *CLN1*, *CLN2*, and *CLN3* and containing a methionine-repressible *MET3-CLN2* gene fusion, which caused cells to arrest in $G_1$ upon methionine addition. This strain, also containing a *GAL-CLB2-lacZ* gene fusion (29), was transformed with a yeast cDNA library expressed from the *GAL1* promoter (25). About $10^5$ plasmid-carrying colonies were screened. Colonies were arrested on plates containing methionine and then transferred to galactose plates to express simultaneously the *GAL-CLB2-lacZ* and *GAL-cDNA* constructs. Cells impaired in cyclin proteolysis were expected to produce blue colonies on galactose medium, in contrast to white colonies produced by cells with normal APC activity.

Plasmids from blue colonies were retransformed into a wild-type strain, and those plasmids causing cell cycle defects on galactose plates were analyzed further. They contained the complete ORFs from the $G_1$ cyclin gene *CLN2*, the B-type cyclin genes *CLB1*, *CLB3*, and *CLB5*, and a truncated *CLB1* cDNA lacking the N-terminal 120 aa, *CLB1-dN*. In addition, we identified plasmids containing the entire ORF of *IME2*, encoding a meiosis-specific protein kinase with sequence similarities to CDKs (30, 31). Immunoblotting showed that the expression of these *cDNAs* caused the accumulation of Clb2-lacZ protein in $G_1$-arrested cells (Fig. 1*A*). Excepting the strain expressing *CLN2*, cells remained mostly arrested as unbudded cells (not shown).

The identification of *CLB1* and *CLB1-dN* cDNAs prompted us to compare the potential of full-length and truncated Clb1 protein to stabilize Clb2. In contrast to Clb1, Clb1-dN is thought to be stable in $G_1$ cells, because it lacks the region encompassing the cyclin destruction box. It therefore can efficiently accumulate and activate Cdk1. We coexpressed *CLB2* and either *CLB1* or *CLB1-dN* in $\alpha$-factor-arrested $G_1$ cells. Cells expressing full-length *CLB1* accumulated only low amounts of Clb2, but *CLB1-dN* caused Clb2 accumulation to high levels (Fig. 1*B*). Promoter shutoff experiments demonstrated that Clb2 was stabilized by Clb1-dN (Fig. 1*C*). These results show that Clb1-dN, but not Clb1, efficiently inhibits Clb2 degradation. Thus, high Cdk1 activity apparently correlates with a high capacity to inhibit Clb2 degradation.

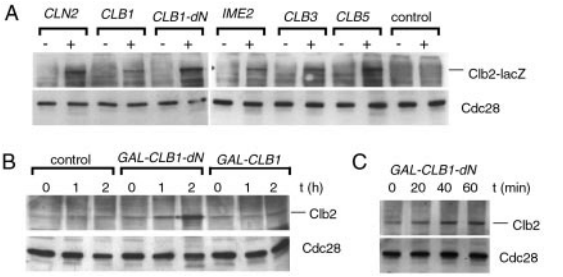


**Fig. 1.** Ectopic expression of various cyclin genes and *IME2* inhibits Clb2 proteolysis in $G_1$ cells. (*A*) A *S. cerevisiae* strain deleted for all $G_1$ cyclins (*cln1*, *cln2*, and *cln3*), containing *MET-CLN2* and *GAL-CLB2-lacZ* gene fusions (S65), was retransformed with centromeric plasmids isolated from the screening of the *GAL-cDNA* library. Plasmids contained cDNAs of either the *CLN2*, *CLB1*, *CLB1-dN* (N-terminal 120 aa deleted), *CLB3*, *CLB5*, or *IME2* genes. Cells were arrested in $G_1$ phase by methionine addition, and genes were expressed by galactose addition. Clb2-lacZ protein was detected by immunoblotting with Clb2 antibodies before (−) and 2.5 h after galactose addition (+). Cdc28 (Cdk1) was used as loading control. (*B*) A *bar1* deletion strain containing *GAL-CLB2* (S61) was transformed with centromeric plasmids containing either *GAL-CLB1*, *GAL-CLB1-dN*, or a control plasmid. Cells were arrested in $G_1$ with $\alpha$-factor. Galactose was added and cells were incubated for 2 h. (*C*) Cells containing the *GAL-CLB1-dN* plasmid were transferred to glucose medium 60 min after galactose addition to turn off the *GAL1* promoter (0 time point).

Our identification of $G_1$, S, and mitotic cyclins as negative regulators of Clb2 proteolysis is consistent with earlier data demonstrating that Cdk1 inhibits $APC^{Cdh1}$ from late $G_1$ until anaphase (32).

**Ectopic Expression of *IME2* Stabilizes Cyclins Clb2 and Clb3.** Our screening revealed that *IME2* expression displayed a phenotype similar to the expression of cyclins. Thus, Ime2, as Cdk1, may inhibit APC-mediated proteolysis. To analyze the effect of Ime2 in more detail, a *GAL-IME2* gene fusion was integrated into yeast cells. Cells containing a single integration of *GAL-IME2* were only modestly affected when grown on galactose medium, but cells carrying five copies of this construct displayed significant cell cycle defects (see below). Therefore, this strain was used for further experiments.

To test whether ectopic expression of *IME2* affects Clb2 proteolysis in $G_1$ phase, cells containing both *GAL-CLB2* and *GAL-IME2* constructs were arrested in $G_1$ by $\alpha$-factor pheromone. Then *CLB2* and *IME2* expression was induced by galactose. The simultaneous expression of both genes caused the accumulation and stabilization of Clb2, in contrast to cells containing only the *GAL-CLB2* construct (Fig. 2 *A* and *B*). *IME2* overexpression thereby resembles the phenotype of cells expressing *CLB1-dN* in $G_1$ phase (Fig. 1). It is known that the expression of stable cyclins induces $G_1$ cells to enter into S phase (18). The coexpression of *IME2* and *CLB2* caused many of the $G_1$-arrested cells to initiate DNA replication (Fig. 2*C*), but cells did not start budding (not shown). Thus, the activity of the meiosis-specific kinase *IME2* inhibits Clb2 proteolysis and causes entry into S phase.

We next tested whether Ime2 stabilizes other mitotic cyclins. Unlike *CLB2*, *CLB3* is a cyclin gene also expressed during meiosis (33, 34). *CLB3* and *IME2* were expressed in $G_1$-arrested cells. Clb3 accumulated to only low levels in the absence of Ime2, but the coexpression of both genes resulted in the accumulation and stabilization of Clb3 (Fig. 2*D*). The appearance of Clb3 caused cells to initiate DNA replication (Fig. 2*E*). These results show that Ime2 inhibits proteolysis of at least two mitotic cyclins.

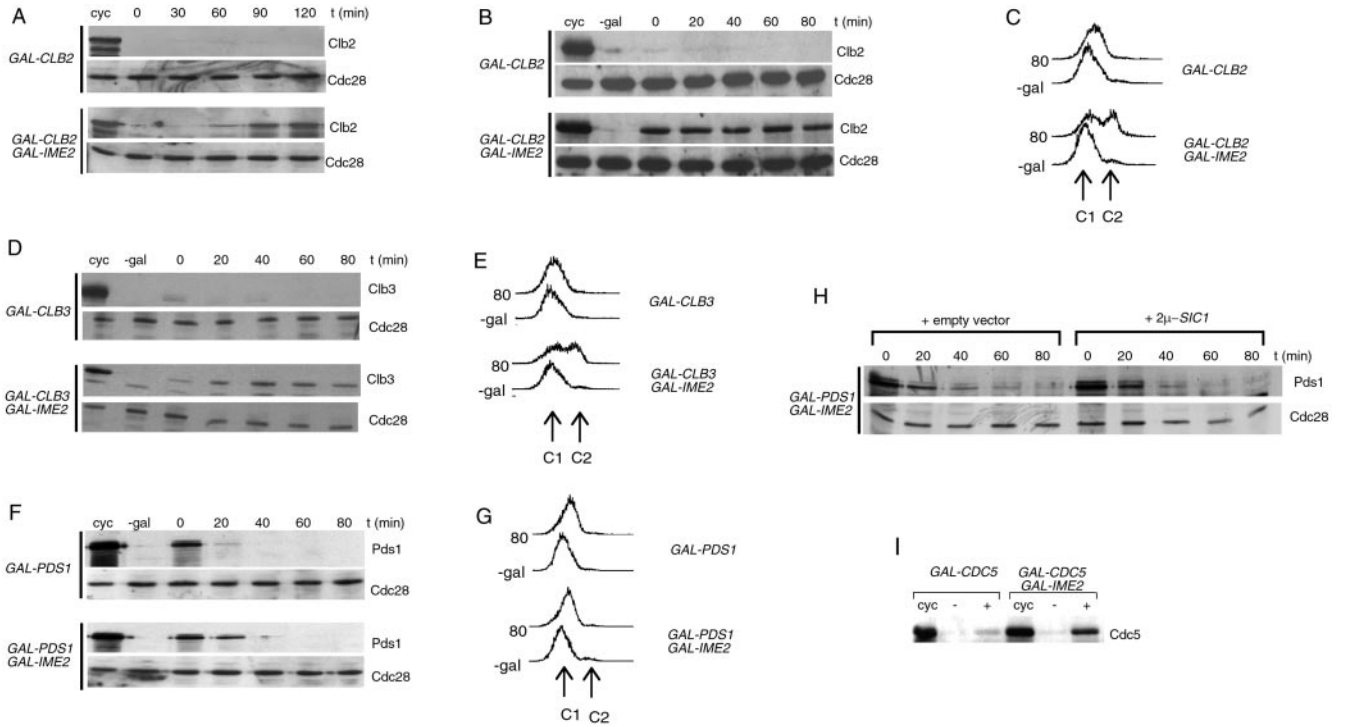


**Fig. 2.** Ectopic expression of *IME2* inhibits degradation of mitotic cyclins and securin Pds1. (*A*) Strains containing *bar1* deletions and either *GAL-CLB2* (S57) or *GAL-CLB2 GAL-IME2* (S381) constructs (*CLB2* C-terminally tagged with the HA epitope) were arrested in $G_1$ phase with $\alpha$-factor for 2.5 h. Galactose was added (0 time point), and cells were incubated for 120 min. HA-tagged Clb2 was detected by immunoblotting with the anti-HA antibody (12CA5). Cdc28 was used as loading control. (*B*) Strains S57 and S381 were arrested with $\alpha$-factor for 2.5 h. Galactose was added, and, after 1.5 h, cells were filtered and transferred to glucose medium containing $\alpha$-factor (0 time point). cyc, cycling cells treated with galactose for 1.5 h. (*C*) DNA content of samples collected either before galactose addition (-gal) or 80 min after glucose addition. (*D*) Strains containing *bar1* deletions and either *GAL-CLB3* (S56) or *GAL-CLB3 GAL-IME2* (S416) constructs (*CLB3* C-terminally HA-tagged) were treated as in *B*. (*E*) DNA content. (*F*) Strains containing *bar1* deletions and either *GAL-PDS1* (S206) or *GAL-PDS1 GAL-IME2* (S415) constructs (*PDS1* C-terminally HA-tagged) were treated as in *B*. (*G*) DNA content. (*H*) Strain S415 was transformed with either a high-copy plasmid carrying the *SIC1* gene or an empty plasmid. Transformed strains were treated as in *B* except that synthetic medium lacking leucine was used for plasmid selection. (*I*) Strains containing *bar1* deletions and either *GAL-CDC5* (S88) or *GAL-CDC5 GAL-IME2* (S417) constructs (*CDC5* C-terminally HA-tagged) were arrested with $\alpha$-factor and galactose was added. Samples before (−) or 2 h after (+) galactose addition were collected.

**Ime2 Inhibits Proteolysis of APC Substrates Pds1 and Cdc5.** We next tested whether *IME2* overexpression causes the stabilization of a noncyclin APC substrate, the anaphase inhibitor protein Pds1. *GAL-PDS1* and *GAL-IME2* gene fusions were transiently coexpressed in $G_1$-arrested cells. Whereas Pds1 rapidly disappeared in cells expressing only *PDS1*, the simultaneous expression of *PDS1* and *IME2* resulted in a partial but reproducible stabilization of Pds1 within 20 min after the promoter shutoff (Fig. 2*F*). This experiment shows that ectopic expression of *IME2* delays Pds1 degradation in $G_1$ cells.

The expression of *GAL-IME2* and *GAL-PDS1* did not induce cells to initiate DNA replication (Fig. 2*G*), indicating that Cdk1/Clb kinases are not activated under these conditions. However, it was shown previously that Ime2 induces activation of Cdk1/Clb kinases in meiosis (35). To distinguish whether the partial Pds1 stabilization is caused by Ime2 activity or, rather, residual Cdk1/Clb activity in these $G_1$ cells, we determined the effect of Ime2 on the half-life of Pds1 in cells containing the Cdk1/Clb kinase inhibitor *SIC1* on a high-copy plasmid. Pds1 was stabilized partially in the presence of high Sic1 levels, similar to the control strain (Fig. 2*H*).

To test whether Ime2 affected proteolysis of the polo-like kinase Cdc5, another APC substrate (36), we determined the accumulation of Cdc5 in $G_1$ cells. In wild-type cells, Cdc5

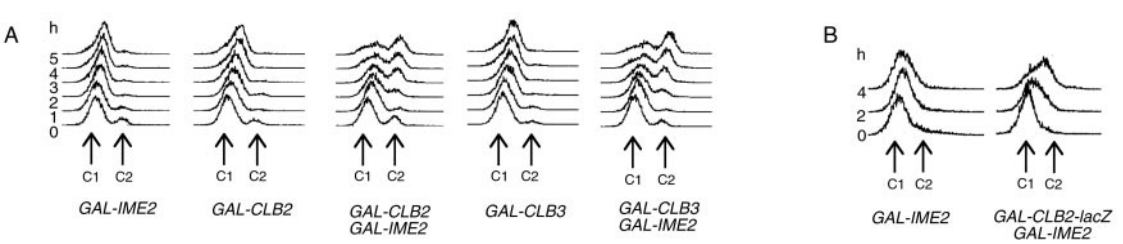


**Fig. 3.** High levels of Ime2 are not sufficient to trigger DNA replication in $G_1$-arrested cells. (*A*) A *bar1* deletion strain carrying *GAL-IME2* (S418) was arrested in $G_1$ with $\alpha$-factor. Galactose was added (0 time point), and cells were incubated in the presence of $\alpha$-factor. The DNA content was determined by FACS analysis. Similarly, strains expressing the indicated *GAL* constructs were analyzed. (*B*) A *cln1*, *cln2*, *cln3* deletion strain carrying a *MET-CLN2* construct (S66) and a similar strain containing, in addition, a *GAL-CLB2-lacZ* construct (S65) were transformed with a centromeric plasmid containing *GAL-IME2*. Transformed strains were arrested in $G_1$ with methionine, and galactose was added.

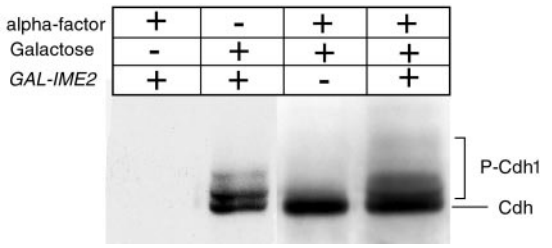


**Fig. 4.** Ectopic expression of *IME2* promotes phosphorylation of Cdh1 in $G_1$-arrested cells. A wild-type strain (S437) and a *GAL-IME2* strain (S457), both containing *bar1* deletions and N-terminally HA-tagged versions of *CDH1* expressed from the *GALL*-promoter (19), were arrested in $G_1$ with $\alpha$-factor. Galactose was added to express the *GAL* constructs. HA-tagged Cdh1 was analyzed by immunoblotting (lane 3, S437; lane 4, S457). As control, strain S457 either was not treated with galactose (lane 1) or not arrested with $\alpha$-factor (lane 2).

accumulates only to low levels when expressed from the *GAL* promoter, because of its instability (ref. 36; Fig. 2*I*). Upon expression of *IME2*, increased levels of Cdc5 accumulated, suggesting that Ime2 inhibits efficient Cdc5 degradation.

We conclude that high levels of *IME2* affect proteolysis of various APC substrates during $G_1$ phase.

**High Levels of *IME2* in $G_1$-Arrested Cells Are Not Sufficient to Trigger DNA Replication.** It was proposed earlier that Ime2 replaces the $G_1$-specific Cdk1, the Cdk1/Cln kinase, during the meiotic cell cycle (35, 37). If Ime2 was functionally equivalent to Cdk1/Cln, then ectopic expression of *IME2* should suppress the $G_1$ arrest of cells having functional Cdk1/Cln kinases. To determine whether high levels of *IME2* trigger DNA replication in the absence of Cdk1/Cln, *GAL-IME2* was expressed for a prolonged period in $\alpha$-factor-arrested cells. FACS analysis revealed that *IME2* expression alone did not trigger DNA replication within 5 h (Fig. 3*A*). In contrast, cells coexpressing *IME2* and *CLB2* or *CLB3* mostly replicated their DNA. Therefore, high levels of Ime2 are not sufficient to initiate DNA replication in $\alpha$-factor-arrested cells. The expression of a cyclin gene also is required.

Similarly, Ime2 did not trigger DNA replication in cells deleted for all $G_1$ cyclins (Fig. 3*B*), whereas cells additionally expressing a *CLB2-lacZ* gene frequently entered S phase.

Thus, high levels of Ime2 cannot replace Cdk1/Cln kinases in triggering DNA replication, implying that Ime2 is not capable to take over all of the functions of the $G_1$-specific Cdk1 at the $G_1$/S transition.

**Ectopic Expression of *IME2* Triggers Phosphorylation of Cdh1.** $APC^{Cdh1}$ inactivation in the mitotic cell cycle is meditated by phosphorylation of Cdh1, causing the dissociation of Cdh1 from APC. Both Cln- and Clb-associated Cdk1 kinases seem to contribute to $APC^{Cdh1}$ inactivation (38, 39).

To test whether the inhibitory effect of Ime2 on APC-mediated proteolysis also is caused by Cdh1 phosphorylation, we used strains containing an HA-tagged version of Cdh1 expressed from a weak *GAL* promoter, described earlier as *GALL-HA3-HCT1* (19). In $G_1$-arrested wild-type cells, Cdh1 is not phosphorylated (Fig. 4, lane 3), but $G_1$-arrested cells expressing the *GAL-IME2* construct produced slower migrating bands (Fig. 4, lane 4), which most likely correspond to phosphorylated forms of Cdh1 (19). Similar mobility shifts were observed in cycling cultures that contain active Cdk1 kinases (Fig. 4, lane 2).

These results suggest that ectopic expression of *IME2* promotes phosphorylation of Cdh1 during $G_1$ phase.

**High Levels of Ime2 Inhibit Bud Formation and Arrest Cells in Mitosis.** To analyze the phenotype of high levels of *IME2* expressed in dividing cells, strains containing five copies of *GAL-IME2* were shifted to galactose medium. The expression of *IME2* caused the

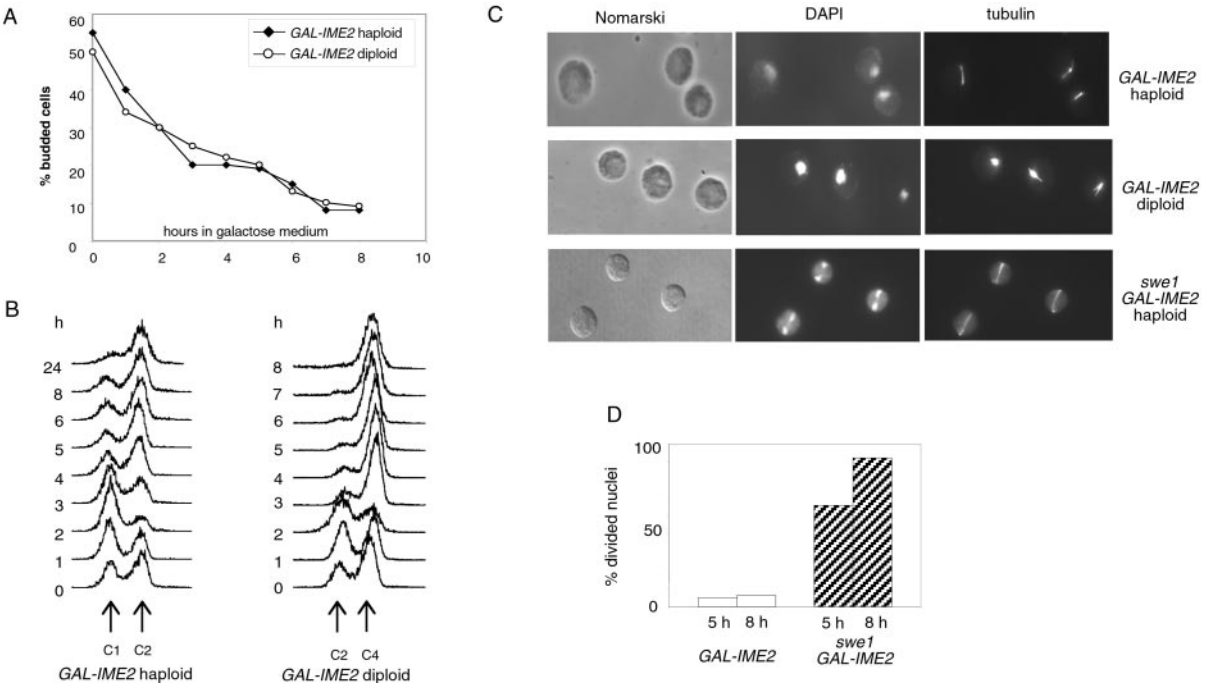


**Fig. 5.** High levels of Ime2 cause the accumulation of unbudded cells delayed in mitosis. Haploid (S379) and diploid (S424) strains containing the *GAL-IME2* construct were grown in raffinose medium. Galactose was added (0 time point) and samples were collected at the indicated time points for monitoring the percentage of budded cells (*A*) and for determining the DNA content (*B*). (*C*) Immunofluorescence microscopy of haploid and diploid cells and of a *swe1* deletion strain (*swe1*::*LEU2*, S454) containing *GAL-IME2*, 5 h after galactose addition. (*D*) Percentage of divided nuclei in haploid *SWE1* and *swe1* strains, 5 and 8 h after galactose addition.

accumulation of unbudded cells in both haploid and diploid cells (Fig. 5*A*). Initially, cells were delayed in $G_1$ phase (Fig. 5*B*, 2-h sample), but then they replicated their DNA. Nuclei and spindle staining revealed that these cells failed to segregate their DNA and arrest in $G_2$/M phase, sometimes containing short spindles (Fig. 5 *C* and *D*).

It was shown previously that cells defective in budding activate a control mechanism termed the morphogenesis checkpoint, resulting in a cell cycle delay in $G_2$/M phase (40). To test whether the arrest of *GAL-IME2* cells is caused by this control mechanism, we expressed *IME2* in *swe1* mutant cells defective in the morphogenesis checkpoint. Only few *swe1* cells had undivided nuclei, whereas most cells contained segregated chromosomes and elongated mitotic spindles (Fig. 5 *C* and *D*).

Thus, the expression of Ime2 to high levels inhibits budding and blocks progression through mitosis. The cell cycle block in late anaphase/telophase is consistent with the model that Ime2 specifically affects $APC^{Cdh1}$ rather than general APC function.

**Ime2 Is an Unstable Protein Whose Degradation Does Not Depend on APC or SCF (Skp1/Cdc53/F-box) Ubiquitin Ligases.** Because high levels of Ime2 have deleterious effects on cell cycle progression, it is tempting to speculate that Ime2 inactivation during meiosis may be important as CDK inactivation during mitosis. Ime2 contains two motifs (KxxL, KxxLxxxxxN) resembling the cyclin destruction box motif (KxxLxxxxN) and a PEST-rich region [region rich in proline (P), glutamate (E), serine (S), and threonine (T)] implicated in protein instability. This finding prompted us to analyze the stability of Ime2 by promoter shutoff experiments. HA-tagged Ime2 was degraded rapidly in normally dividing cells (Fig. 6*A*). Ime2's instability was not cell cycle-regulated, because Ime2 was degraded similarly in cells arrested in $G_1$ by $\alpha$-factor (Fig. 6*B*) or in M phase by the microtubule-depolymerizing drug nocodazole (Fig. 6*C*). Ime2 proteolysis was not delayed in *cdc23–1* mutants defective in APC function (Fig. 6*B*) or in *cdc4–1* and *cdc34–2* mutants impaired in the SCF complex (Fig. 6*C*).

These findings indicate that Ime2 is an unstable protein kinase whose proteolysis occurs independently of the conventional APC and SCF ubiquitin ligases.

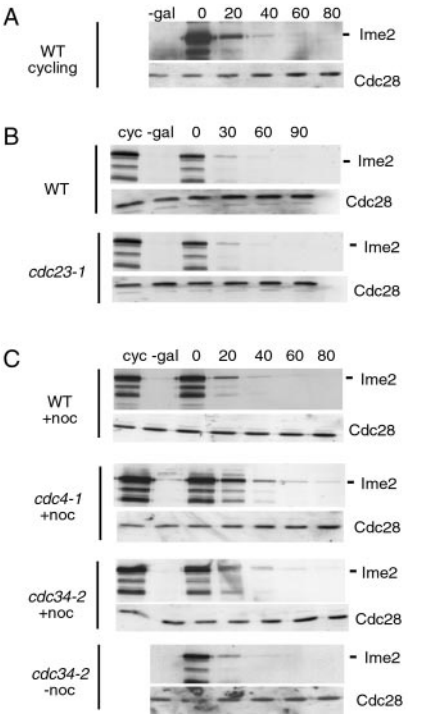


**Fig. 6.** Ime2 is an unstable protein whose degradation is independent of APC and SCF. (*A*) A strain (S396) containing a *GAL-IME2* construct (*IME2* C-terminally HA-tagged) was shifted for 2 h to galactose medium and then transferred to glucose medium (0 time point). Ime2 was analyzed by immunoblotting with HA-antibodies. cyc, cycling cells. (*B*) S396 and isogenic *cdc23–1* mutants (S397), both containing *bar1* deletions, were arrested in $G_1$ with $\alpha$-factor at 25°C. Galactose was added and cells were incubated for 45 min. Cells were shifted to 36°C, incubated for 45 min, then transferred to glucose medium containing $\alpha$-factor and incubated at 36°C. (*C*) S396 and isogenic *cdc4–1* (S407) and *cdc34–2* (S408) mutants were arrested in M phase with nocodazole at 25°C (+noc). Expression of *IME2* was induced by galactose for 45 min. Then, cells were shifted to 36°C, incubated for 45 min, transferred to glucose medium containing nocodazole, and incubated at 36°C. A *cdc34–2* culture was treated similarly, but nocodazole was omitted (−noc).

## Discussion

**Identification of Negative Regulators of APC-Mediated Proteolysis.** We aimed to identify regulators of the APC by screening a yeast cDNA library for genes whose expression to high levels prevents degradation of a Clb2-lacZ reporter protein in $G_1$-arrested cells. We identified various cyclins and the meiosis-specific protein kinase Ime2 as inhibitors of APC-mediated proteolysis.

The identification of $G_1$, S, and mitotic cyclins implies that Cdk1 associated with any of these cyclins is capable of inhibiting APC-mediated proteolysis. These findings are consistent with previous data showing that Cdk1 is a key factor for turning off $APC^{Cdh1}$ in late $G_1$ and for keeping it inactive during S, $G_2$, and most of M phase (6, 32). Cdk1 associated with different cyclin proteins was shown to directly phosphorylate the activator protein Cdh1 and trigger its dissociation from APC (15, 19). Our findings underline the crucial role of Cdk1 as an inhibitor of $APC^{Cdh1}$. The continuous presence of Cdk1 activated by different types of cyclins helps to ensure that $APC^{Cdh1}$ cannot be activated during the cell cycle before Cdc14 phosphatase is released from the nucleolus, a process that depends on proper spindle orientation and the mitotic exit network (41).

**Ime2, a Putative Meiosis-Specific Regulator of $APC^{Cdh1}$.** We have shown that ectopic expression of *IME2* results in the accumulation and stabilization of the mitotic cyclins Clb2 and Clb3 in $G_1$-arrested cells. We showed further that Ime2 triggers phosphorylation of Cdh1, suggesting that Ime2 inhibits APC activity by means of the Cdh1 activator protein. *IME2* expression also delayed proteolysis of Pds1 and Cdc5, two additional APC substrates. Pds1 was affected only slightly, possibly because it is degraded primarily by $APC^{Cdc20}$ during $G_1$ phase, but other data suggested that $APC^{Cdh1}$ is involved in Pds1 removal (12, 42). The rather weak influence of Ime2 on Pds1 also could be explained by Ime2's own instability. After turning off the *GAL* promoter in our test system, Ime2 gets degraded at about the same time as Pds1 (compare Figs. 2*F* and 6).

In summary, our results demonstrate that ectopic expression of *IME2* in $G_1$ cells inhibits APC-meditated proteolysis. Because *IME2* normally is expressed only in meiotic cells, we suggest that this protein kinase acts as a regulator of $APC^{Cdh1}$ during meiosis.

**Similarities and Differences of Ime2 with Cdk1/Cln Kinases.** There are several indications that Ime2 fulfills in meiosis the functions of the $G_1$-specific CDKs, the Cdk1/Cln kinases. First, Ime2 shares sequence similarities with CDKs (43). Second, deletion of all *CLN* genes does not affect sporulation, implying that Cdk1/Cln kinases are dispensable for meiosis (35). Third, degradation of the Cdk1 inhibitor Sic1 is triggered in the mitotic cell cycle by Cdk1/Cln and in meiosis by Ime2 (35).

The capacity of Ime2 to promote Cdh1 phosphorylation suggests that this kinase may replace Cdk1/Cln in triggering inactivation of $APC^{Cdh1}$ in late $G_1$ phase of the meiotic cell cycle. It was shown recently that $G_1$-specific kinases are not sufficient

for completely inactivating $APC^{Cdh1}$ in the mitotic cell cycle. This process also requires Cdk1/Clb kinases (38, 39). Similarly, the combined function of both Ime2 and accumulating Cdk1/Clb kinases may be needed for completely turning off $APC^{Cdh1}$ in meiosis.

Ime2 clearly displays some functional similarities with Cdk1/Cln, but there are significant differences between these kinases. High levels of Ime2 expressed in $\alpha$-factor-arrested cells or in cells lacking $G_1$ cyclins failed to promote DNA replication, implying that Ime2 is not sufficient to replace Cdk1/Cln in this process. Ime2 may not be sufficient to induce transcription of *CLB* genes. Indeed, upon coexpression of *IME2* with *CLB* genes, most cells replicated their DNA. We conclude that Ime2 has only partially functional similarities to Cdk1/Cln kinases and fulfills some but not all of their functions in promoting entry into S phase.

Another difference between Cdk1/Cln and Ime2 is striking. The $G_1$-specific kinase triggers budding, but Ime2 clearly inhibits this process. The expression of *IME2* in cycling cultures induced the accumulation of unbudded cells that replicated their DNA. The inhibition of budding in mitotic cells also suggests a role of Ime2 in preventing budding in meiosis.

**Is Ime2 Activity Regulated by Its Stability?** We have shown that Ime2 is unstable when expressed in normally dividing cells. Proteolysis of Ime2 appears to be independent of the APC or the SCF ubiquitin ligases, and the machinery responsible for Ime2 instability remains to be identified. The permanent instability of Ime2 in dividing cells may help to ensure that Ime2 never accumulates in the mitotic cell cycle, where it could interfere with processes such as budding or APC activity. It is unknown whether Ime2 also is permanently unstable during meiosis or whether it then gets periodically stabilized. Stabilization could be a mechanism to allow efficient accumulation of Ime2 in early meiosis, in addition to the transcriptional induction of the *IME2* gene (31, 43).

Accumulation as well as removal of Ime2 may be important during meiosis. We have shown that high levels of Ime2 blocked progression through mitosis. The continued presence of active Ime2 also may interfere with cell cycle progression in meiosis. Because Ime2 itself is an unstable protein, its inactivation during meiosis may be triggered by turning on its rapid proteolysis.

Little is known about APC regulation during meiosis. Pds1 was found to be degraded at anaphase onset in both meiosis I and II (20). Pds1 reaccumulation after its destruction in meiosis I implies that APC needs to be inactivated between meiosis I and II. Recently, Ama1, a meiosis-specific protein related to Cdc20/Cdh1, was identified (21). Ama1 seems to be required for Clb1 degradation in meiosis I, but it is not essential for meiosis II. *CDH1* expression also was found to be induced in meiosis (44), indicating that Cdh1 and Ama1 may have redundant roles in triggering cyclin proteolysis.

It seems that at least three different APC complexes need to be regulated during meiosis. The identification of Ime2 as an inhibitor of APC-mediated proteolysis suggests that this protein kinase represents an important player in APC regulation during meiosis.

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