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# A Novel Yeast Screen for Mitotic Arrest Mutants Identifies *DOC1*, a New Gene Involved in Cyclin Proteolysis

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B-type cyclins are rapidly degraded at the transition between metaphase and anaphase and their ubiquitin-mediated proteolysis is required for cells to exit mitosis. We used a novel enrichment to isolate new budding mutants that arrest the cell cycle in mitosis. Most of these mutants lie in the *CDC16*, *CDC23*, and *CDC27* genes, which have already been shown to play a role in cyclin proteolysis and encode components of a 20S complex (called the cyclosome or anaphase promoting complex) that ubiquitinates mitotic cyclins. We show that mutations in *CDC26* and a novel gene, *DOC1*, also prevent mitotic cyclin proteolysis. Mutants in either gene arrest as large budded cells with high levels of the major mitotic cyclin (Clb2) protein at 37°C and cannot degrade Clb2 in $G_1$-arrested cells. Cdc26 associates in vivo with Doc1, Cdc16, Cdc23, and Cdc27. In addition, the majority of Doc1 cosediments at 20S with Cdc27 in a sucrose gradient, indicating that Cdc26 and Doc1 are components of the anaphase promoting complex.

## INTRODUCTION

Oscillations in the activity of cyclin-dependent kinases (Cdk) control the eukaryotic cell cycle (reviewed in Morgan, 1995). Multicellular eukaryotes express a number of Cdks with different activities through the cell cycle. Yeasts, however, express a single Cdk, called Cdc28 in budding yeast (*Saccharomyces cerevisiae*) and Cdc2 in fission yeast (*Schizosaccharomyces pombe*). The oscillation of Cdk activity in all eukaryotes depends on phosphorylations of the kinase subunit and the cyclic expression and degradation of cyclins, the regulatory subunits of Cdks. Entry into mitosis requires the expression of B-type (mitotic) cyclins, and exit from mitosis requires their degradation (reviewed in Murray and Kirschner, 1989; Nurse, 1990). The Cdk activity associated with B-type cyclins is required for chromosome condensation and proper formation of the mitotic spindle. The degradation of B-type cyclins, which leads to inactivation of the mitotic Cdk, results in chromosome decondensation, breakdown of the mitotic spindle, and cell division.

A short N-terminal sequence found in A- and B-type cyclins, termed the destruction box, is required for their proteolysis (Glotzer *et al.*, 1991). Deletion or mutation of this sequence produces a nondegradable cyclin, and cells or extracts expressing these nondegradable B-type cyclins are unable to exit mitosis and remain arrested in anaphase (Murray *et al.*, 1989; Holloway *et al.*, 1993; Surana *et al.*, 1993; Sigrist *et al.*, 1995). In contrast, inhibiting the machinery that is required for cyclin proteolysis arrests cells in metaphase with unseparated sister chromatids (Holloway *et al.*, 1993). This observation suggests that additional proteins, whose destruction is required for the separation of sister chromatids, must also be targets of the machinery that degrades cyclins. Candidates for these regulators of sister cohesion have recently been identified (Funabiki *et al.*, 1996; Yamamoto *et al.*, 1996).

The degradation of cyclin is regulated by the assembly of multiubiquitin chains onto the cyclin substrate that targets it for proteolysis by the proteasome. In frog egg extracts, mutations in cyclins that block their degradation also block ubiquitination (Glotzer *et al.*,

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1991; King *et al.*, 1995), and methylated ubiquitin, an inhibitor of multiubiquitin chain formation, inhibits the degradation of cyclins in clam egg extracts (Hershko *et al.*, 1991). In yeast, a number of mutants that block cyclin degradation also block ubiquitination (Zachariae and Nasmyth, 1996).

Ubiquitination of proteins begins with the activation of ubiquitin by a ubiquitin-activating enzyme (E1). The E1 forms a thiol ester with ubiquitin and then transfers that ubiquitin onto a ubiquitin-carrier protein (E2). Some substrates are directly ubiquitinated by an E2. Other substrates, however, also require a ubiquitin-protein ligase (E3), which appears to provide a higher level of specificity to the degradation system. Finally, the 26S proteasome recognizes multiubiquitinated substrates and degrades them (reviewed in Ciechanover, 1994).

In budding yeast, clam, and frog, a nonspecific E1 is capable of activating ubiquitin. A large family of E2s (*UBC*s) exist in yeast, and the E2 required for Clb degradation has not yet been definitively identified. E2-C (clam), homologues of the budding yeast UBC4, and a novel UBC, UBCX (frog) are capable of acting as E2s for cyclin B in reconstituted cyclin ubiquitination systems derived from clam and frog egg extracts (King *et al.*, 1995; Sudakin *et al.*, 1995). The E3 for B-type cyclins was first identified as a 20S complex in clam and frog egg extracts, known as the cyclosome or anaphase-promoting complex (APC; King *et al.*, 1995; Sudakin *et al.*, 1995). In frog egg extracts, yeast homologues of *CDC16* and *CDC27* were identified as components of this E3 complex, which appears to be active only in mitosis in both clams and frogs.

*CDC16*, *CDC23,* and *CDC27* are all members of a family of tetratricopeptide repeat proteins and form a complex in yeast that is required for the ubiquitination and degradation of Clb2, the major mitotic cyclin (Lamb *et al.*, 1994; Irniger *et al.*, 1995; Zachariae and Nasmyth, 1996). Temperature-sensitive mutants in any of these genes cause a metaphase arrest with high levels of Clb2 and unseparated sister chromosomes. Another gene, *CSE1*, has also been implicated in cyclin ubiquitination (Irniger *et al.*, 1995). Mutations in *CDC16*, *CDC23*, and *CSE1* all result in increased chromosome loss, suggesting that proper regulation of the cyclin proteolysis machinery is required for faithful chromosome segregation (Hartwell and Smith, 1985; Xiao *et al.*, 1993).

We developed an enrichment for cell cycle arrest mutants in budding yeast and used this to screen for new mitotic arrest mutants. We isolated a large number of alleles of previously identified *cdc* (cell division cycle) mutants, and identified a novel mutant, *doc1* (*d*estruction *o*f *c*yclin B). *CDC26* and *DOC1* are involved in the degradation of Clb2 and the products of both genes associate with the yeast APC.

## MATERIALS AND METHODS

### Yeast Strains, Media, and Genetic Methods

All yeast strains are derivatives of W303, except those used for complementation analysis. All strains used in this work are listed in Table 1. Yeast media and genetic manipulations were as described (Sherman *et al.*, 1974). LH103 (*mec1–1*) was made by backcrossing AFS85 (*MAT***a**, *mec1–1*, *GAL-CLN3::URA3::cln3*Δ, *ssd1-v1::LEU2*, *leu2–3,112*, *ura3*, *his3–11,15*,*ade2–1*, *trp1–1)* four times into W303 and subsequently crossing in *mad1*Δ*::HIS3* (from KH123, Hardwick and Murray, 1995) and *bar1* (from AFS92, kindly provided by Aaron Straight, University of California, San Francisco). *Bgl*II-cut pLH17 (*CLB2-lacZ*) was integrated at *CLB2*, recombination and loss of the *URA3* marker were selected for on 5-fluoroorotic acid medium, and retention of *CLB2-lacZ* was screened for by using β-galactosidase (β-gal) plate assays. Strains containing *GAL-CLB2* were made by integrating pDK27 (kindly provided by Doug Kellogg, University of California, Santa Cruz) at *URA3*.

Hydroxyurea (Sigma, St. Louis, MO) was used at 10 mg/ml, final concentration, in medium. α-Factor (Bio-synthesis, Lewisville, TX) was used at 1 μg/ml for *bar1*$^-$ strains and 10 μg/ml for *BAR1* strains, from a stock solution at 10 mg/ml in dimethyl sulfoxide (Aldrich, Milwaukee, WI). Nocodazole (Sigma) was used at 15 μg/ml from a stock solution of 10 mg/ml in dimethyl sulfoxide. Cycloheximide (Sigma) was used at 10 μg/ml from a stock solution of 10 mg/ml. Nocodazole treatments were carried out at 23°C. The other treatments were performed at various temperatures.

### Plasmid Constructions

For *CLB2-lacZ* (pLH17), *CLB2* (including the open reading frame [ORF] and 301 bp upstream) was amplified by polymerase chain reaction (PCR) using oligomers containing *Bam*HI and *Sal*I sites at 5′ and 3′ ends, respectively. This was ligated into pRS304 (Sikorski and Hieter, 1989) and then cut with *Sal*I and *Kpn*I. The *Kpn*I site was blunted with T4 polymerase. *lacZ* was cut from pAFS35 (kindly provided by Aaron Straight) by using *Sal*I and *Bam*HI. The *Bam*HI site was blunted by using the Klenow fragment of DNA polymerase I. The *lacZ* fragment was then ligated into the *CLB2* construct. A second *Bam*HI site was created 3′ of *lacZ* by ligation of the blunted *Bam*HI and *Kpn*I sites. A 270-bp fragment of sequence immediately 3′ of the *CLB2* ORF was amplified by PCR with *Bam*HI and *Sal*I sites 5′ and 3′, respectively. This was ligated into pRS306 (Sikorski and Hieter, 1989). The *Bam*HI fragment containing *CLB2-lacZ* was then ligated into the pRS306 construct containing the 3′ flanking region of *CLB2.* The Clb2-LacZ protein is functional and capable of acting as the sole mitotic cyclin since pLH17 rescues the temperature sensitivity of a strain deleted for *CLB1, CLB3,* and *CLB4* with *CLB2* replaced with a temperature sensitive allele, K3080 (Amon *et al.*, 1993).

pLH25 was made by PCR amplifying the ORF of *DOC1* and 508 bp upstream with a *Sal*I site 5′ and an *Nco*I site 3′ to the ORF. The PCR product was ligated into pKH511 (kindly provided by Kevin Hardwick, University of Edinburgh), to place a single myc tag at the C terminus of the protein. To make pLH23, pLH25 was cut with *Eco*RV and *Bam*HI and the 1.0-kb fragment was ligated into pDK20 (kindly provided by Doug Kellogg) cut with *Sma*I and *Bam*HI, to place the *DOC1* ORF behind the GAL1–10 promoter. pLH24 was made by cutting pLH25 with *Sal*I and *Bam*HI and ligating the 1.5-kb fragment into YIplac211, cut with the same enzymes. pLH26–1B was made by cutting T1 (the original rescuing plasmid from the YCp50 library) with *Nde*I and blunting with Klenow. For pLH26–1A, *URA3* was cut from pLH3 with *Xho*I and *Xba*I and also blunted with Klenow. The *URA3* fragment was ligated into the blunted *Nde*I site in T1.

To make pLH32, the ORF of *DOC1* with 508 bp upstream was amplified by PCR with a *Sal*I site 5′ and a *Kpn*I 3′ to the ORF. The *Kpn*I site was designed so that the stop codon of *DOC1* was ex-

**Table 1.** Yeast strains

| Strain | Relevant genotype | Source |
|---|---|---|
| LH103 | *MAT***a** *mec1-1 mad1Δ::HIS3 clb2::CLB2-LacZ bar1* YCp50/*MAD1* | This work |
| AFS34 (W303-1a) | *MAT***a** *ade2-1 can1-100 ura3-1 leu2-3,112 his3-11,15 trp1-1* | R. Rothstein |
| YPH218 | *MATα cdc16-1* | P. Hieter |
| YPH221 | *MATα cdc23-1* | P. Hieter |
| LH127 | *MATα cdc20-1 ura3-1 leu2-3,112 trp1-1* | This work |
| H160-3-3 | *MATα cdc27-1* | L. Hartwell |
| H152-4-2 | *MATα cdc26-1* | L. Hartwell |
| ELW65-93 | *MAT***a** *cdc31-2* YCp50/*CDC31* | M. Winey |
| CMY763 | *MATα cim3-1* | C. Mann |
| CMY765 | *MATα cim5-1* | C. Mann |
| LH202 | *MAT***a** *cim5-1 bar1* | This work |
| LH103-15 | *MAT***a** *doc1-1 mec101 mad1Δ::HIS3 clb2::CLB2-lacZ bar1* YCp50/*MAD1* | This work |
| LH103-66 | *MAT***a** *cdc26-100 mec1-1 mad1Δ::HIS3 clb2::CLB2-lacZ bar1* YCp50/*MAD1* | This work |
| LH225 | *MAT***a** *cdc26-100 clb2::CLB2-lacZ bar1* | This work |
| LH226 | *MAT***a** *doc1-1 clb2::CLB2-lacZ bar1* | This work |
| ADR58 | *MAT***a** pDK27 *(GAL-CLB2)* | This work |
| ADR103 | *MAT***a** *cdc16-1* pDK27 | This work |
| LH227 | *MAT***a** *cdc26-100 bar1* pDK27 | This work |
| LH228 | *MAT***a** *doc1-1 bar1* pDK27 | This work |
| LH229 | *MAT***a** *cdc26-100 bar1 ura3-1::CDC26myc-URA3* pDK27 | This work |
| LH230 | *MAT***a** *doc1-1 bar1 ura3-1::DOC1myc-URA3* pDK27 | This work |
| LH209 | *MAT***a** *bar1* p*Ub-R-βgal* | This work |
| LH210 | *MAT***a** *bar1* p$Ub^{V76}$-*V*-$e^{\Delta K}$-*βgal* | This work |
| LH211 | *MAT***a** *doc1-1 bar1* p*Ub-R-βgal* | This work |
| LH212 | *MAT***a** *doc1-1 bar1* p$Ub^{V76}$-*V*-$e^{\Delta K}$-*βgal* | This work |
| LH213 | *MAT***a** *cdc26-100 bar1* p*Ub-R-βgal* | This work |
| LH214 | *MAT***a** *cdc26-100 bar1* p$Ub^{V76}$-*V*-$e^{\Delta K}$-*βgal* | This work |
| LH215 | *MAT***a** *cim3-1 bar1* p*Ub-R-βgal* | This work |
| LH216 | *MAT***a** *cim3-1 bar1* p$Ub^{V76}$-*V*-$e^{\Delta K}$-*βgal* | This work |
| LH231 | *MAT***a** *bar1* pWAM10 *(CDC16HA)* | This work |
| LH232 | *MAT***a** *bar1* pRS239 *(CDC23HA)* | This work |
| LH233 | *MAT***a** *bar1* pJL25 *(CDC27HA)* | This work |
| LH234 | *MAT***a** *bar1* pLH32 *(DOC1HA)* | This work |
| LH235 | *MAT***a** *bar1 cdc26Δ::URA3* pWAM10 | This work |
| LH236 | *MAT***a** *bar1 cdc26Δ::URA3* pRS239 | This work |
| LH237 | *MAT***a** *bar1 cdc26Δ::URA3* pJL25 | This work |
| LH238 | *MAT***a** *bar1 cdc26Δ::URA3* pLH32 | This work |
| LH297 | *MAT***a** *bar1 doc1Δ::URA3 leu2-3,112::DOC13XHA-LEU2* pJL25 | This work |

All strains from this work are W303 (R. Rothstein).

cluded and the 3′ end of *DOC1* would be in-frame with a triple hemaglutinin (3×HA) tag in YCplac111–3×HA. The *DOC1* PCR product was cut with *Sal*I, blunted with Klenow, and then cut with *Kpn*I. YCplac111–3×HA was cut with *Eco*RI, blunted with Klenow, and then cut with *Kpn*I. The PCR fragment was then ligated into YCplac111–3×HA. pLH59 (*DOC1–3×HA-LEU2*) was made by cutting pLH32 with *Sph*I and *Spe*I and ligating the 1.8-kb fragment into YIplac128 cut with *Sph*I and *Xba*I. All enzymes were from New England Biolabs (Beverly, MA) and used according to the manufacturer's specifications.

Plasmids expressing Ub-R-βgal and $Ub^{V76}$-V-$e^{\Delta K}$-βgal were kindly provided by Dr. Erica Johnson (Rockefeller University, NY). Plasmids containing HA-tagged *CDC16* (pWAM10), *CDC23* (pRS239), and *CDC27* (pRS248) were kindly provided by Dr. Phillip Heiter (Johns Hopkins University, MD).

## *Mutant Isolation*

Strain LH103 ($8 \times 10^6$ cells) was mutagenized with ethyl methanesulfonate (Sigma) to 50% killing. The mutagenized cells were diluted 1:25 and allowed to recover for 12 h at room temperature in YPD. They were shifted to 37°C, to prearrest potential $G_1$ and mitotic arrest mutants, for 2 h, then hydroxyurea was added to 10 mg/ml, and cells were incubated at 37°C for an additional 5 h. This culture was then plated on YPD plates and incubated at 23°C. Surviving colonies were replica plated onto YPD plates in duplicate with one set at 23°C and the other at 37°C to test for temperature sensitivity.

## *Clb2-LacZ and Visual Screen*

Temperature-sensitive mutants were patched onto YPD plates and allowed to grow overnight at 23°C. They were then replica plated to

YPD and placed at 37°C for 4 h, replica plated again to Whatman filters (VWR, San Francisco, CA) on YPD plates that contained 1 μg/ml α-factor, and returned to 37°C for 5 h. The filters were assayed for β-gal activity by freezing them in liquid nitrogen, thawing them, and incubating them on Whatman paper soaked in Z buffer plus 5-bromo-4-chloro-3-indolyl β-D-galactoside, US Biological, Swampscott, MA; 60 mM $Na_2HPO_4$, 40 mM $NaH_2PO_4$, 10 mM KCl, 1 mM $MgSO_4$, 0.03% 5-bromo-4-chloro-3-indolyl β-D-galactoside) at 30°C overnight.

Strains that produced bright blue patches were further analyzed by microscopy. The strains were grown in liquid medium to logarithmic phase at 23°C and then shifted to 37°C. Cells were taken and fixed in 3.7% formaldehyde (Fisher, Santa Clara, CA) after 3 and 6 h at 37°C. They were stained with 4,6-diamidino-2-phenylindole (1 μg/ml) and anti-tubulin (YOL1/34, Accurate Chemical and Scientific, Westbury, NY) diluted 1:200, and their arrest phenotype was determined by light microscopy.

Mitotic arrest mutants were tested for complementation against a collection of known mitotic mutants. Only two mutants were not identified as previously isolated mutants. One was cloned and named *DOC1*, and the other (two alleles) has not been cloned and appears to arrest in mitosis with pleiotropic defects. One allele of *cdc14* was also isolated; however, it was isolated as a double mutant with a *cdc16* mutant. *CDC14* codes for a phosphatase, and mutants in this gene arrest in anaphase of the cell cycle (Wan *et al.*, 1992).

### Cloning of DOC1

A YCp50 library (described in Hardwick and Murray, 1995) was transformed into *doc1–1* by lithium acetate transformation and plated on −Ura medium at 37°C. Plasmids that were capable of rescuing the temperature sensitivity were recovered and the inserts were sequenced at both 5′ and 3′ ends. These sequences were used to probe the yeast genome database.

### Preparation of Antibodies against Cdc26, Western Blot Analysis, and Immunoprecipitations

The ORF of *CDC26* was amplified by PCR with oligomers containing *Bam*HI and *Eco*RI sites 5′ and 3′ to the ORF, respectively. This fragment was cloned into pGEX-1 cut with *Bam*HI and *Eco*RI (Smith and Johnson, 1988), forming a glutathione *S*-transferase (GST) fusion construct (pLH29). pLH29 was transformed into *Escherichia coli* strain TG1 (Maniatis *et al.*, 1982) and induced for expression with 0.1 mM isopropyl β-D-thiogalactoside (US Biologicals) for 2 h at 37°C. Cells were pelleted, washed with PBS (140 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 138 mM NaCl, 2.7 mM KCl, pH 7.2), pelleted again, and frozen in liquid nitrogen. The frozen pellet was resuspended in five volumes of PBS containing 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 200 μg/ml lysozyme and then briefly sonicated. Triton X-100 was added to 0.5%, dithiothreitol was added to 15 mM, and cells were sonicated again. The lysate was spun at 15,000 rpm in an SS34 rotor (Sorvall, Burbank, CA) for 30 min, and then the supernatant was transferred to a new tube and spun again for 20 min. The cleared supernatant was then loaded onto a 5-ml glutathione-agarose column (Sigma) that was then washed with PBS containing 0.5 M NaCl, 1 mM EDTA, and 1 mM EGTA. The Cdc26-GST fusion protein was eluted with 5 mM reduced glutathione (Sigma) in 50 mM Tris(hydroxymethyl)aminomethane, pH 8.1. The peak fractions were pooled and dialyzed into 50 mM HEPES, pH 7.6, 50 mM KCl, and 30% glycerol. The protein was sent to Berkeley Antibody (Berkeley, CA) where it was used to immunize a rabbit. The rabbit serum was passed over a 50-ml column of GST protein coupled to Affi-Gel 10 (Bio-Rad Labs, Hercules, CA) to remove anti-GST antibodies and then affinity purified by using a 5-ml column of the Cdc26-GST fusion protein coupled to Affi-Gel 10 (Bio-Rad).

Yeast extracts for immunoblotting and immunoprecipitations were made by bead beating cells for two 90 s periods, in lysis buffer A (50 mM HEPES, pH 7.6, 75 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.1% Nonidet P-40, 50 mM NaF, 100 μM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and LPC (10 μg/ml each leupeptin, pepstatin, and chymostatin; Boehringer, Indianapolis, IN). The lysates were spun briefly to separate beads from the lysate and then cleared by centrifugation for 5 min in an Eppendorf microcentrifuge at 4°C. This step was repeated three times. Protein concentrations of extracts were determined with the Bradford assay using bovine serum albumin as a standard (Bradford, 1976). For immunoblotting, the extracts were adjusted to the same protein concentration and diluted with 2× SDS sample buffer (1× SDS sample buffer: 80 mM Tris(hydroxymethyl)aminomethane, pH 6.8, 2% SDS, 10% glycerol, 10 mM EDTA, 0.0013% bromophenol blue, 5% 2-mercaptoethanol). Standard methods were used for SDS-PAGE and protein transfer to nitrocellulose (Harlow and Lane, 1988). Blots were stained with Ponceau S (Fisher) to confirm transfer and equal protein loading and then blocked for 30 min with blotto (4% dried milk, PBS, 0.2% Tween 20). Antibodies were used at a 1:1200 dilution for anti-Clb2, a 1:1000 dilution for 12CA5 (BABCO, Berkeley, CA), a 1:1000 dilution for anti-β-gal (Cappel, Durham, NC), and a 1:1000 dilution for anti-Cdc26 at either room temperature for 1 h or 4°C overnight. Blots were washed three times 10 min in PBS with 0.2% Tween 20 (PBST) and then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (Amersham, Buckinghamshire, United Kingdom) at a 1:5000 dilution in PBST. They were washed again and developed by using Amersham ECL detection reagents following manufacturer's instructions. Cdc26 protein is not detectable by Western blot, likely due to an inability to bind to nitrocellulose.

For immunoprecipitations, antibodies were used at a 1:33 dilution for 12CA5 and a 1:50 dilution for Cdc26. Lysates with primary antibody were rotated for 1 h at 4°C, then transferred to a tube containing protein A-Sepharose beads (Pharmacia, Pleasant Hill, CA), and rotated at 4°C for an additional hour. The beads were washed twice with lysis buffer A, transferred to a new tube, washed again with PBST, and then resuspended in SDS sample buffer.

### Sucrose Gradient Analysis

Solutions with lysis buffer A were prepared containing either 5% or 40% sucrose. Additional solutions with 13.75%, 22.5%, and 31.25% sucrose were made by combining various amounts of the 5% and 40% solutions. Nine hundred fifty microliters of each solution were layered into Beckman Ultra-Clear Centrifuge tubes (Beckman, Palo Alto, CA), size 0.5 × 2 inches. The gradient was incubated 12–16 h at 4°C. Yeast extract (100 μl), made as described above, was layered onto the gradient and spun at 50,000 rpm in a SW-55 rotor (Sorvall) for 4 h at 4°C. Sixteen 300-μl fractions were taken, the fractions were precipitated with 10% trichloroacetic acid, loaded onto SDS-Page gels, and analyzed by immunoblotting. Molecular weight markers were analyzed by Coomassie blue staining (Fisher).

## RESULTS

### Screening for New Mitotic cdc Mutants

We designed a strategy that enabled us to enrich for temperature-sensitive budding yeast mutants that arrest in $G_1$ or mitosis (Figure 1). We used *mec1–1*, a cell cycle checkpoint mutant, to devise conditions under which cycling cells would die and arrested ones would survive. *mec1–1* mutants lack the checkpoint that detects unreplicated DNA and DNA damage and cannot arrest their cell cycle in the presence of unreplicated DNA (Weinert, 1992). As a result, treating cycling *mec1–1* cells with DNA replication inhibitors, such as hydroxyurea, induces rapid cell death. In contrast, treating $G_1$ or mitotically arrested *mec1–1* cells

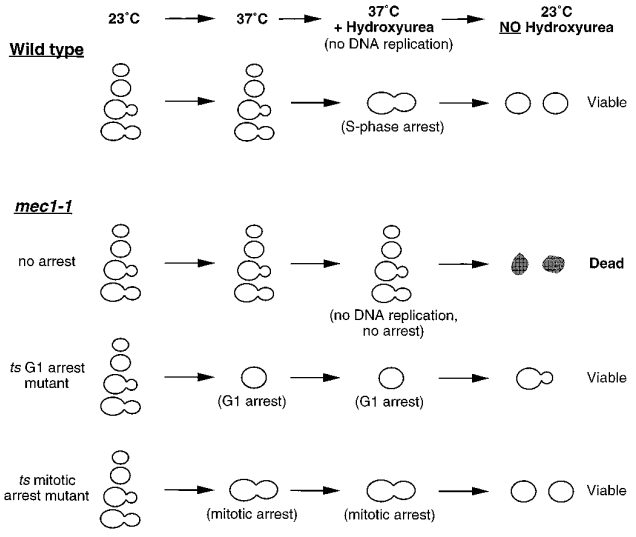


**Figure 1.** Schematic representation of the mutant enrichment strategy. Hydroxyurea, a drug that inhibits DNA synthesis, arrests wild-type cells in S phase. Mutants that are defective in the checkpoint gene *MEC1* are unable to arrest in response to unreplicated DNA and die in the presence of hydroxyurea. If *mec1–1* cells are prearrested in $G_1$ or mitosis, they remain viable after exposure to hydroxyurea.

with hydroxyurea will not kill them. We exploited this difference to enrich for mutants that caused a temperature-sensitive arrest in $G_1$ or mitosis. This protocol does not enrich for most mutants arrested in S or $G_2$ phase, because their arrest requires the *MEC1*-dependent checkpoint. A *mec1–1* strain was mutagenized and the mutagenized cells were shifted to 37°C to allow potential cell division cycle mutants to prearrest and then treated with hydroxyurea to kill the majority of cells that continued to progress through the cell cycle. Cells that arrest in either $G_1$ or mitosis remain viable during the hydroxyurea treatment and form colonies on plating at the permissive temperature of 23°C.

We mutagenized $8 \times 10^6$ cells to 50% survival with ethyl methanesulfonate, and 40,000 survived the *mec1–1* enrichment. Of those, 1968 (5%) were temperature sensitive. To distinguish mutants that arrested in mitosis from those that arrested in $G_1$, we exploited the observation that mitotic cyclins are strongly expressed in mitosis but rapidly degraded as cells exit mitosis and enter $G_1$ (Amon *et al.*, 1994). The temperature-sensitive mutants were tested for expression of Clb2-lacZ, a protein fusion between the major mitotic cyclin (Clb2) and $\beta$-gal, whose activity can be easily monitored by exposing cells to a chromogenic substrate. Cells from mutants that expressed Clb2-lacZ strongly at 37°C (see MATERIALS AND METHODS) were screened visually for their arrest phenotypes. We found 32 mutants that arrested with large budded cells at 37°C, a phenotype that is consistent with a mitotic arrest. These mutants fell into 11 complementation groups. Each group was tested against known mitotic mutants by complementation of temperature sensitivity and for rescue of temperature sensitivity by transformation with plasmids containing wild-type copies of genes known to function in mitosis.

We isolated mitotic arrest mutations in nine genes (Table 2). One, *CDC31* (one allele) is involved in spindle pole body duplication and *cdc31*$^{ts}$ mutants arrest in mitosis with only one spindle pole body (Byers, 1981; Baum *et al.*, 1986). Another, *PRP22* (two alleles) codes for an RNA helicase involved in mRNA splicing (Company *et al.*, 1991). The alleles of *PRP22* isolated in this screen arrests in mitosis with no microtubules (our unpublished results). *TUB1* and *TUB3*, the genes that code for $\alpha$-tubulin, each contain an intron. It is likely that the tubulin defect seen in these mutants is due to a failure to splice the mRNAs from these genes that leads to a deficiency in $\alpha$-tubulin.

**Table 2.** Mitotic-arrest mutants isolated by *mec1-1* enrichment

| Gene | No. of alleles isolated | Biochemical function | Reference |
|---|---|---|---|
| *CDC16* | 16 | APC component | Irniger *et al.* (1995), King *et al.* (1995) |
| *CDC23* | 3 | APC component | Irniger *et al.* (1995) |
| *CDC27* | 4 | APC component | King *et al.* (1995) |
| *CDC26* | 1 | APC component | This work |
| *DOC1* | 1 | APC associated | This work |
| *CIM3* | 1 | Proteasome component | Ghislain *et al.* (1993) |
| *CDC20* | 1 | Unknown | Sethi *et al.* (1991) |
| *CDC31* | 1 | SPB component | Baum *et al.* (1986) |
| *PRP22* | 2 | Splicing | Company *et al.* (1991) |
| Uncloned | 2 | Unknown | |

Mutants were identified based on complementation of temperature sensitivity with previously identified mutants. They were also tested for rescue of temperature sensitivity by wild-type copies of known genes on plasmids.

Five of the genes we identified are involved in the proteolysis of cyclin that triggers the exit from mitosis. *CDC16* (16 alleles), *CDC23* (3 alleles), and *CDC27* (4 alleles) encode proteins that are involved in cyclin proteolysis and have been shown to form a multiprotein complex in yeast, frogs, and clams (Lamb *et al.*, 1994; Irniger *et al.*, 1995; King *et al.*, 1995; Sudakin *et al.*, 1995). *CDC20* (1 allele) has also been implicated in cyclin proteolysis. Its homologue in *Drosophila*, fizzy, appears to be required for the proteolysis of cyclin A and B (Dawson *et al.*, 1995; Sigrist *et al.*, 1995). In yeast, *cdc20* mutants arrest in metaphase and appear to have microtubule abnormalities (Byers and Goetsch, 1974; Palmer *et al.*, 1989; Sethi *et al.*, 1991). *CIM3* (1 allele) is a subunit of the 26S proteasome, the multiprotein complex that degrades ubiquitinated proteins (Ghislain *et al.*, 1993).

Finally, we isolated one allele each of *CDC26* and a novel gene that we named *DOC1* (destruction of cyclin B). *CDC26* is a mitotic arrest mutant, identified in the original Hartwell screen for cdc mutants (Hartwell *et al.*, 1970). The *CDC26* gene is essential only at 37°C; *cdc26*Δ cells grow well at 23°C (Araki *et al.*., 1992), a finding that we confirmed in the W303 strain background.

### *CDC26 and DOC1 Are Required for Clb2 Proteolysis*

Because the majority of mutants isolated in our screen identify genes involved in mitotic cyclin proteolysis, we tested the *cdc26* and *doc1* mutants for defects in this process. To determine whether *CDC26* and *DOC1* were involved in cyclin proteolysis, we exploited the observation that cyclin proteolysis begins in late mitosis and persists well into $G_1$ (Amon *et al.*, 1994). Haploid yeast cells can be arrested in $G_1$ by treatment with mating pheromone. Thus in *MAT***a** cells arrested in $G_1$ by α-factor treatment, the B-type cyclin proteolysis machinery is active and ectopic expression of the *CLB2* gene does not lead to Clb2 protein accumulation. In a *cdc16–1* mutant, however, Clb2 protein does accumulate in $G_1$-arrested cells as a result of a defect in the ubiquitination of Clb2 (Irniger *et al.*, 1995; Zachariae and Nasmyth, 1996).

To determine the half-life of mitotic cyclins, we integrated a copy of *CLB2* under the control of the inducible GAL promoter into wild-type and mutant strains. Wild-type and the *doc1–1* and *cdc26–100* mutants isolated in our screen were arrested with α-factor and shifted to 37°C for 20 min, and *CLB2* expression was induced with galactose. Cycloheximide and glucose were added after a 30-min induction to stop Clb2 protein synthesis and *CLB2* transcription. Clb2 protein levels were monitored by Western blotting using anti-Clb2 antibodies. Figure 2A shows that the half-life of Clb2 in wild-type cells is less than 1 min. In *doc1–1* and *cdc26–100* mutants, the half-life of Clb2 protein increases to greater than 20 min. This defect is rescued in *doc1–1* and *cdc26–100* by the integration of a wild-type copy of the corresponding gene at the *LEU2* locus (our unpublished results). Others have also identified *CDC26* as playing a role in cyclin proteolysis (Zachariae *et al.*, 1996).

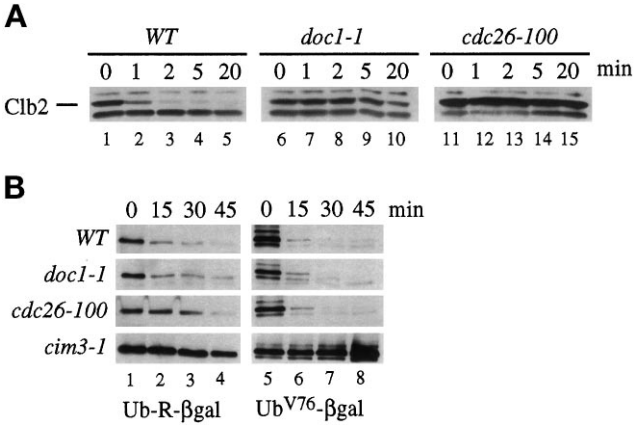


**Figure 2.** Phenotype of *cdc26* and *doc1* mutants. (A) Stability of Clb2 in proteolysis mutants. The indicated strains contained an integrated copy of *pGAL-CLB2*, were grown in YEP + 2% raffinose medium, and arrested in $G_1$ by exposure to α-factor for 3 h at 23°C. The cells were shifted to 37°C for 20 min before *CLB2* expression was induced by the addition of 2% galactose while maintaining the α-factor arrest. Cycloheximide (10 μg/ml) and glucose (2%) were added after a 30-min induction and time points were taken at 0, 1, 2, 5, and 20 min. An exposure of a Western blot probed with polyclonal anti-Clb2 antibodies is shown. Wild-type (lanes 1–5), *doc1–1* (lanes 6–10), and *cdc26–100* (lanes 11–15). (B) Stability of β-gal derivatives in proteolysis mutants. Wild-type, *doc1–1*, *cdc26–100*, and *cim3–1* strains containing plasmids expressing either Ub-R-β-gal (lanes 1–4) or $Ub^{V76}$-V-$e^{\Delta K}$-βgal ($Ub^{V76}$-βgal; lanes 5–8) under the control of the *GAL* promoter were grown in YEP + 2% raffinose and then arrested in $G_1$ with α-factor for 3 h. Expression from the plasmids was induced by the addition of 2% galactose for 2 h. At time zero, cycloheximide was added to 10 μg/ml and samples were taken at 15, 30, and 45 min. An exposure of a Western blot probed with anti-β-gal antibodies is shown.

The Clb2 destruction defect in *cdc26–100* and *doc1–1* appears to be specific to Clb2 and not a general defect in the proteolysis of ubiquitinated substrates. Figure 2B shows that *cdc26–100* and *doc1–1* cells have no defect in the degradation of two substrates that require ubiquitination for destruction: Ub-R-βgal, which generates β-gal with an N-terminal arginine, a substrate that is degraded by the N-end rule (Figure 2B, Ub-R-βgal; Bachmair *et al.*, 1986), and $Ub^{V76}$-V-$e^{\Delta K}$-βgal, a fusion with a noncleavable N-terminal ubiquitin (Figure 2B, $UB^{V76}$-βgal; Johnson *et al.*, 1992). Cycloheximide was added to cells expressing Ub-R-βgal or $Ub^{V76}$-V-$e^{\Delta K}$-βgal to inhibit protein synthesis, and stability of the substrates was determined by Western blotting with anti-β-gal antibodies at different times after inhibiting protein synthesis. *cim3–1*, a mutant in a subunit of the 26S proteasome (Ghislain *et al.*, 1993) that degrades all ubi-

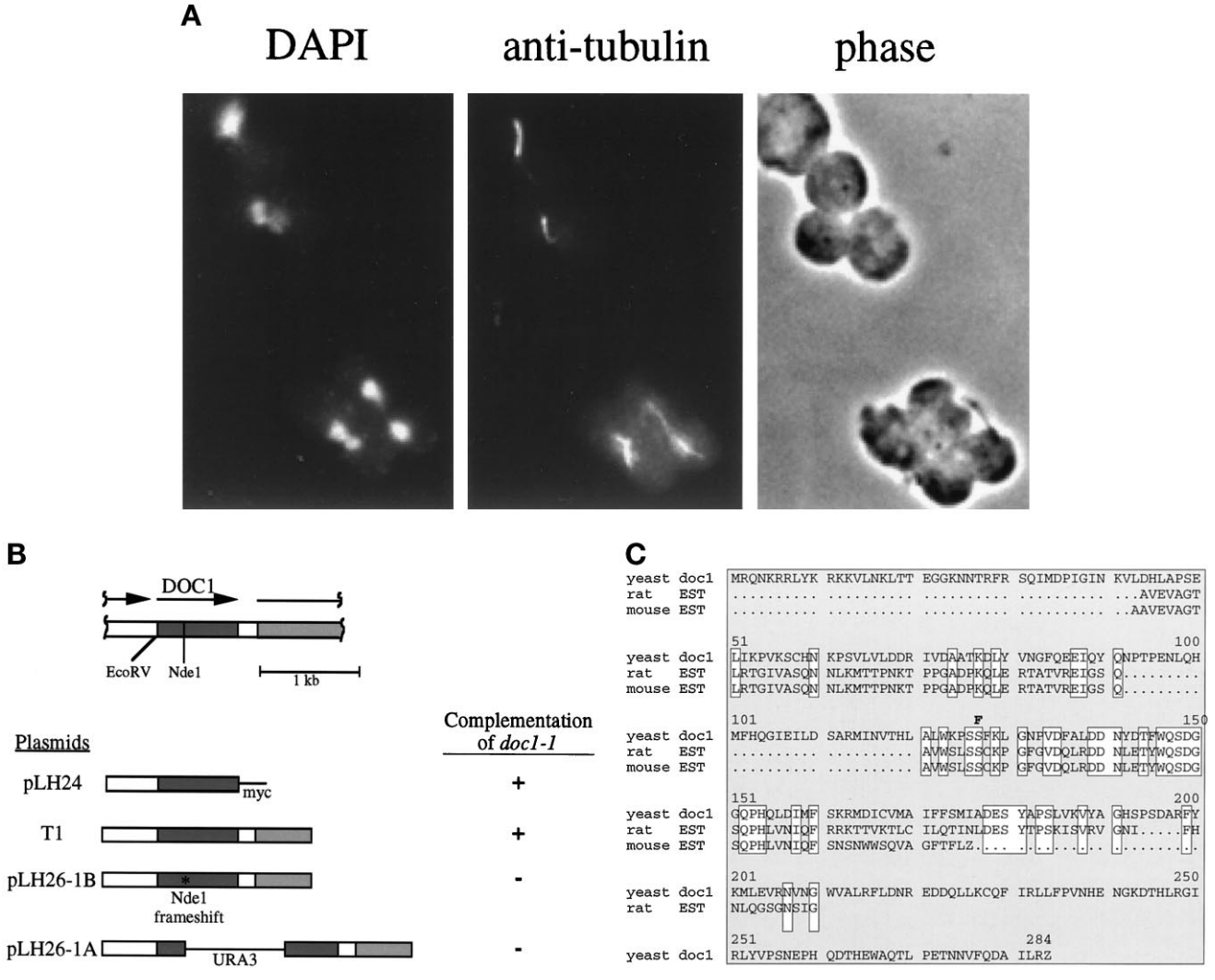


**Figure 3.** (A) *doc1–1* arrest phenotype. The top two large-budded cells arrested with short spindles and a single DNA mass. The bottom two adjacent cells are large-budded. One has arrested with a short spindle and single DNA mass, the other appears to have undergone anaphase. *doc1–1* cells were grown to logarithmic phase at room temperature and then shifted to 37°C for 3 h. The cells were fixed and stained with 4,6-diamidino-2-phenylindole and anti-tubulin. (B) Structure of the *DOC1* locus and complementation of *doc1–1* temperature sensitivity with various plasmids. The shaded region represents the *DOC1* ORF and the hatched region represents the 5′ end of the *CSE1* ORF. The arrows show the direction of transcription. The *DOC1* ORF with 508 bp upstream is sufficient to complement the temperature sensitivity of *doc1–1* at 37°C. TI, the rescuing plasmid from a YCp50-based library, contains approximately 640 bp upstream of the ORF of *DOC1* and approximately 1150 bp of the *CSE1* ORF. T1 was cut with *Nde*I and blunted with the Klenow fragment of DNA polymerase I (New England Biolabs), producing a frameshift in the *DOC1* ORF (pLH26–1B). The frameshifted construct was unable to rescue *doc1–1*. pLH26–1A, in which *URA3* is inserted into the *Nde*I-blunted site, was also unable to rescue *doc1–1*. (C) Amino acid sequence of *DOC1*. This sequence corresponds to ORF YGL240W in the yeast genome database (the GenBank accession number for *DOC1* is Z72762). The *DOC1* amino acid sequence is aligned with rat and mouse expressed sequence tag sequences (accession numbers H33761 and W49295, respectively). Identities are boxed. The *doc1–1* mutation is indicated above the sequence. The mutation in *doc1–1* (S137 → F137) is indicated in boldface type.

quitinated proteins, is defective in the degradation of both substrates (Figure 2B, lanes 1–8).

To see if the mutant phenotype of *cdc26–100* and *doc1–1* cells reflected defects in mitotic proteolysis, we overexpressed a mitotic cyclin in these mutants. *cdc26–100* and *doc1–1* strains containing an integrated copy of *GAL-CLB2* are unable to grow on galactose at their permissive temperature (data not shown). We suggest that these strains are already crippled in their Clb2 destruction machinery at the permissive temperature and that excess Clb2 overloads this machinery and results in a cell cycle arrest.

### *Cloning and Disruption of DOC1*

Like previously identified mitotic cyclin proteolysis mutants, *doc1–1* mutants arrest as large budded cells

**Table 3.** *doc1-1* arrest phenotype

| Strain | % of total | | | |
|---|---|---|---|---|
| |  |  |  |  |
| Wild type | 75 | 10 | 11 | 4 |
| *doc1-1* | 16 | 1 | 79 | 4 |

Wild-type and *doc1-1* strains were grown to logarithmic phase and then shifted to 37°C for 3 h. The cells were fixed and then stained for DNA using 4,6-diamino-2-phenylindole and tubulin by using immunofluorescence. Percentage of total cell morphologies of unbudded with a single nucleus and a $G_1$ microtubule array, small budded with a single nucleus and short spindle, large budded with a single nucleus and short spindle, and large budded with separated DNA masses and long spindle are indicated from left to right. Two hundred cells were counted for each strain.

at 37°C with a single nucleus and a short spindle (Figure 3A and Table 3).

DOC1 was cloned by complementing the temperature sensitivity of *doc1–1* with a genomic yeast library on a centromeric vector (Figure 3B, plasmid T1). The minimal complementing region contains a single ORF coding for a protein of 283 amino acids. A construct containing the ORF of *DOC1* and 508 bp upstream, with a single myc tag on the C terminus and no sequences 3′ to the ORF, complements the temperature sensitivity and Clb2 proteolysis defect of the *doc1–1* mutant (Figure 3B, plasmid pLH24). In addition, a construct containing a frameshift at an *Nde*I site 297 bp into the *DOC1* ORF, will not rescue the temperature sensitivity of *doc1–1* (Figure 3B, plasmid pLH26–1B). Disruption of *DOC1* by insertion of the *URA3* gene at the *Nde*I site (Figure 3B, plasmid pLH26–1A) results in viable cells that grow poorly at 23°C, forming colonies in which most of the cells have large buds and cannot grow at 37°C. Coincidentally, *DOC1* lies directly upstream of the *CSE1* gene, which is also implicated in cyclin proteolysis (Xiao *et al.*, 1993; Irniger *et al.*, 1995). These genes have distinct functions in proteolysis because plasmids that contain only the *CSE1* gene do not complement *doc1–1*. The Doc1 amino acid sequence is shown in Figure 3C. The Doc1 sequence lacks obvious motifs and shows no homology to proteins of known function; however, it does show homology to rat (Lee *et al.*, 1995) and mouse expressed sequence tag sequences (GenBank accession numbers H33761 and W49295, respectively). The *doc1–1* mutation was recovered and sequenced. The mutation changes a conserved serine (Ser-137) to phenylalanine.

### *Cdc26 and Doc1 Associate with APC Components (Cdc16, Cdc23, and Cdc27)*

Cdc16, Cdc23, and Cdc27 form a complex in yeast (Lamb *et al.*, 1994) and their homologues are components of a 20S complex in frog egg extracts (King *et al.*, 1995). This complex behaves as a ubiquitin ligase for B-type cyclins, and *CDC16*, *CDC23*, and *CDC27* are required for the ubiquitination of Clb2 in yeast (Zachariae and Nasmyth, 1996). We therefore tested whether Cdc26 or Doc1 associated with Cdc16, Cdc23, or Cdc27. Extracts were made from exponentially growing wild-type or *cdc26*Δ strains that had been transformed with centromeric plasmids containing HA-tagged versions of *CDC16*, *CDC23*, *CDC27*, and *DOC1*. Cdc26 was immunoprecipitated with polyclonal antibodies against Cdc26. The bound proteins were probed by Western blotting with mouse monoclonal antibodies against HA (12CA5) to test whether the tagged proteins coimmunoprecipitate with Cdc26.

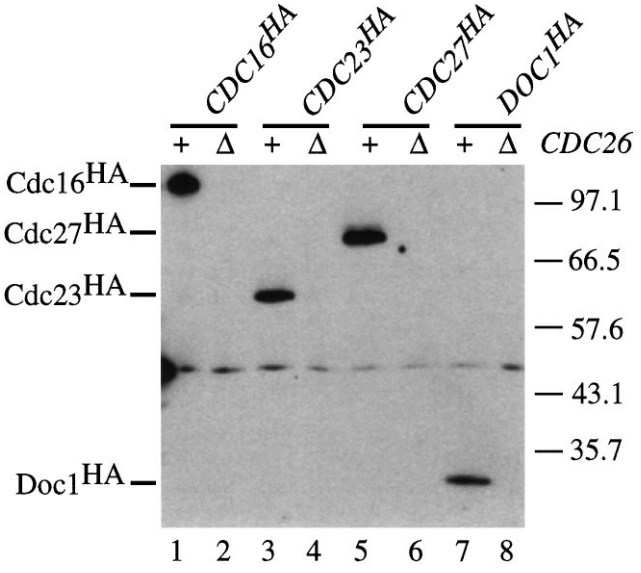


**Figure 4.** Coimmunoprecipitation of Cdc26 with epitope-tagged forms of Cdc16, Cdc23, Cdc27, and Doc1 ($Cdc16^{HA}$, $Cdc23^{HA}$, $Cdc27^{HA}$, and $Doc1^{HA}$). Wild-type (+) and *cdc26*Δ (Δ) strains containing centromeric plasmids expressing $Cdc16^{HA}$ (pWAM10; lanes 1 and 2), $Cdc23^{HA}$ (pRS239; lanes 3 and 4), $Cdc27^{HA}$ (pRS248; lanes 5 and 6), or $Doc1^{HA}$ (pLH32; lanes 7 and 8) were grown to logarithmic phase in selective medium. Cdc26 was immunoprecipitated from those cell lysates with polyclonal anti-Cdc26 antibodies. Immunoprecipitates were analyzed by immunoblotting with 12CA5. The positions of molecular mass markers and $Cdc16^{HA}$, $Cdc23^{HA}$, $Cdc27^{HA}$, and $Doc1^{HA}$ are indicated in kilodaltons.

Figure 4 shows that immunoprecipitation of Cdc26 in a wild-type strain results in the coimmunoprecipitation of HA-tagged Cdc16, Cdc23, Cdc27, and Doc1. No HA-tagged Cdc16, Cdc23, Cdc27, or Doc1 was detected when immunoprecipitations were performed on extracts made from a *cdc26*Δ strain. These experiments suggest that Cdc26 is a functional component of the APC, and this suggestion is strengthened by the observation that the *Xenopus* homologue of Cdc26 is a component of the purified APC (M. Kirschner, personal communication).

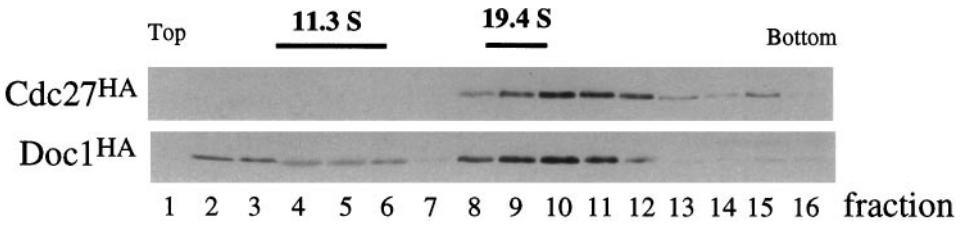


**Figure 5.** Cosedimentation of Doc1 with Cdc27. A lysate from exponentially growing cells expressing both Doc1 and Cdc27 tagged with the HA epitope ($Doc1^{HA}$ and $Cdc27^{HA}$) was separated through a 5–40% sucrose gradient. Fractions were analyzed by immunoblotting with 12CA5.

The association of Doc1 with Cdc26 could be interpreted in two ways. Either Doc1 is a component of the APC or there are two pools of Cdc26, one associated with the APC and one bound to Doc1. To distinguish these possibilities, we analyzed the sedimentation of Doc1 and Cdc27 in a sucrose gradient. Extracts from exponentially growing cells expressing both HA-tagged Doc1 and HA-tagged Cdc27 were separated on a 5–40% sucrose gradient (Figure 5). The majority of Doc1 sediments in a peak at approximately 20S, coinciding with the sedimentation of Cdc27. The sedimentation coefficient of the frog and clam APC is 20S (King *et al.*., 1995; Sudakin *et al.*, 1995).

### *Genetic Interactions among CDC26, DOC1, and Other Mitotic Mutants*

Genetic interactions between mutants often indicate that they function in the same pathway. We found that double mutants of *cdc26–100* and *doc1–1* are synthetically lethal (Table 4), suggesting that the functions of Cdc26 and Doc1 overlap. In addition, *cdc26–100* is synthetically lethal with *cdc16–1* and *cdc23–1*, and *cdc26–100 cdc27–1* double mutants have a lower permissive temperature than either single mutant. *doc1–1* double mutants with *cdc16–1* or *cdc23–1* have a lower permissive temperature than any of the single mutants. The interactions of *cdc26–100* and *doc1–1* with mutants involved in the ubiquitination of mitotic cyclins do not simply reflect synthetic interactions between any pair of mitotic mutants. Double mutants between *cdc26–100* or *doc1–1* and *cim5–1* (a subunit of the 26S proteasome, required for the destruction of ubiquitinated proteins; Ghislain *et al.*, 1993) have the same nonpermissive temperature as the *cdc26–100* or *doc1–1* single mutants.

## DISCUSSION

We have identified two new components of the cyclin proteolysis machinery, Cdc26 and Doc1, and show that Cdc26 and Doc1 are physically associated with the APC/cyclosome, the multiprotein complex that acts as an E3 for cyclin ubiquitination (King *et al.*, 1995; Sudakin *et al.*, 1995; Zachariae and Nasmyth, 1996). Similar results were recently obtained for *CDC26* by Zachariae *et al.* (1996).

We isolated temperature-sensitive mitotic mutants by using an enrichment that kills the majority of cells passing through S phase at the nonpermissive temperature. Of the 32 temperature-sensitive mitotic arrest mutations we isolated, 27 are in genes involved in the cyclin proteolysis machinery; 1 is in *CDC31*, a gene required for spindle pole body duplication; and 2 are in *PRP22*, which encodes a splicing function that is probably required to ensure adequate levels of α-tubulin synthesis. Why do proteolysis mutants dominate the metaphase arrest mutants isolated in both this screen and the original Hartwell screen for *cdc* mutants? We believe that three factors account for this observation. First, although defects in the mitotic spindle can arrest cells in mitosis by transiently activating the spindle assembly checkpoint, the checkpoint even-

**Table 4.** Genetic interactions between *cdc26, doc1,* and other mitotic mutants

| | *cdc26-100* | *doc1-1* | *cdc16-1* | *cdc23-1* | *cdc27-1* | *cim5-1* |
|---|---|---|---|---|---|---|
| Maximum permissive temperature (°C) | **33** | **33** | 30 | 30 | 33 | 37 |
| Maximum permissive temperature of double mutant with *cdc26-100* (°C) | **33** | Dead | Dead | Dead | 30 | 33 |
| Maximum permissive temperature of double mutant with *doc1-1* (°C) | Dead | **33** | 23 | 23 | 30 | 33 |

Synthetic interactions among *cdc26-100, doc1-1*, and various mitotic mutants. *cdc26-100* and *doc1-1* were mated to each other or to *cdc16-1, cdc23-1, cdc27-1,* or *cim5-1*. The resultant diploids were sporulated and tetrads were dissected and germinated at 23°C. They were then tested for growth at 23°C, 30°C, 33°C, 35°C, and 37°C. The genotype of viable spores was determined by complementation testing with appropriate mutant tester strains. The maximum permissive temperatures of the *cdc26-100* and *doc1-1* mutants are shown in boldface type.

tually adapts to persistent defects thus allowing cells to exit mitosis (Hardwick, Rudner, Wiess, Winey, and Murray, unpublished results). Because cells that adapt and leave mitosis with a defective spindle are likely to suffer lethal errors in chromosome segregation, mutants that cause spindle defects may have died during the prolonged incubation at 37°C that was used during the mutant enrichment. Second, there appears to be considerable functional overlap among the components that assemble the mitotic spindle, so that mutations that inactivate single components do not cause a mitotic arrest. For example, strains lacking the Kar3 microtubule motor are viable, although mitosis in these cells is clearly abnormal (Meluh and Rose, 1990). Finally, the integrity of some spindle functions, such as the protein kinase Mps1 (Hardwick *et al.*, 1996), are required for the spindle assembly checkpoint to detect defects in the spindle. As a result, lesions in these components would not arrest cells in mitosis.

*cdc26* and *doc1* mutants arrest in metaphase and cannot destroy mitotic cyclins that are expressed in $G_1$-arrested cells. Unlike mutations in the proteasome, *cdc26* and *doc1* mutants do not suffer general defects in ubiquitin-mediated proteolysis. A substrate that is recognized by a destabilizing N-terminal amino acid or a noncleavable N-terminal ubiquitin is degraded normally in *cdc26* and *doc1* mutants. Thus, these observations suggest that the mitotic arrest of *cdc26* and *doc1* reflects their inability to degrade mitotic cyclins and proteins, such as Cut2 and Pds1, whose destruction is required for sister chromatid separation (Funabiki *et al.*, 1996; Yamamoto *et al.*, 1996). This conclusion is supported by both genetic and biochemical evidence for interactions of Cdc26 and Doc1 with the APC. Immunoprecipitates prepared with anti-Cdc26 antibodies contain Cdc16, Cdc23, and Cdc27, all of which are characterized components of the APC, as well as Doc1. In addition, a population of Doc1 cosediments with Cdc27. This evidence suggests that both Cdc26 and Doc1 are associated with the APC. Because we cannot easily measure the amounts of Cdc26 and Doc1 relative to other components of the APC, it is unclear whether Cdc26 and Doc1 are stoichiometric components of the APC. The observation that neither Cdc26 or Doc1 show any homology to cloned subunits of the biochemically purified *Xenopus* APC suggests that Cdc26 and Doc1 cannot be tightly associated with all APC complexes (King *et al.*, 1995). The observation that *doc1–1* is synthetically lethal with *cdc26–100*, but not with mutants in other components of the APC, suggests that Doc1 and Cdc26 may play partially overlapping roles in cyclin proteolysis. Although *cdc26*Δ mutants grow well at 23°C, they are synthetically lethal with *cdc16–1* and *cdc23–1*, suggesting that Cdc26 may play a role in stabilizing the APC and that this role is dispensable under optimal conditions but becomes essential under environmental stress or in the presence of defects in other APC components.

Defects in the mitotic spindle activate a spindle assembly checkpoint that arrests eukaryotic cells in mitosis (Hoyt *et al.*, 1991; Li and Murray, 1991). Activation of the checkpoint prevents the destruction of mitotic cyclins and sister chromatid separation, suggesting that the principal action of the checkpoint is to inhibit the proteolysis of mitotic cyclins and proteins that play a role in maintaining the linkage between sisters. It is currently unclear whether this inhibition is due to inactivation of the APC itself or to reactions that protect specific substrates from a normally active APC in checkpoint-arrested cells. Further biochemical comparison between APC components in anaphase- and checkpoint-arrested extracts should help to resolve this issue.

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