

# Genetic and Biochemical Evaluation of the Importance of Cdc6 in Regulating Mitotic Exit<sup>□</sup>

Vincent Archambault, Caihong X. Li, Alan J. Tackett, Ralph Wäsch,\*  
Brian T. Chait, Michael P. Rout, and Frederick R. Cross<sup>†</sup>

The Rockefeller University, New York, New York 10021

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We evaluated the hypothesis that the N-terminal region of the replication control protein Cdc6 acts as an inhibitor of cyclin-dependent kinase (Cdk) activity, promoting mitotic exit. Cdc6 accumulation is restricted to the period from mid-cell cycle until the succeeding G1, due to proteolytic control that requires the Cdc6 N-terminal region. During late mitosis, Cdc6 is present at levels comparable with Sic1 and binds specifically to the mitotic cyclin Clb2. Moderate overexpression of Cdc6 promotes viability of *CLB2Δdb* strains, which otherwise arrest at mitotic exit, and rescue is dependent on the N-terminal putative Cdk-inhibitory domain. These observations support the potential for Cdc6 to inhibit Clb2-Cdk, thus promoting mitotic exit. Consistent with this idea, we observed a cytokinesis defect in *cdh1Δ sic1Δ cdc6Δ2–49* triple mutants. However, we were able to construct viable strains, in three different backgrounds, containing neither *SIC1* nor the Cdc6 Cdk-inhibitory domain, in contradiction to previous work. We conclude, therefore, that although both Cdc6 and Sic1 have the potential to facilitate mitotic exit by inhibiting Clb2-Cdk, mitotic exit nevertheless does not require any identified stoichiometric inhibitor of Cdk activity.

## INTRODUCTION

Exit from mitosis is a critical cell cycle transition (Zachariae and Nasmyth, 1999). Although DNA replication and spindle morphogenesis are under positive control by cyclin-dependent kinase (Cdk), mitotic exit requires at least partial Cdk inactivation to occur. Thus, an oscillation in Cdk activity is obligatory for a complete cycle, because DNA replication and initiation of mitotic division are tied to high Cdk activity, but the replicated chromosomes assembled on the spindle will not complete division into two daughter cells until low Cdk activity is attained. Buildup of Cdk activity in the next cell cycle is then required before DNA replication can occur. This simple mechanism, combined with an oscillatory mechanism for Cdk activity, will thus reproduce much of the essential biology of the cell cycle (Nasmyth, 1996; Stern and Nurse, 1996; Zachariae and Nasmyth, 1999). Therefore, it is important to understand the mechanisms of Cdk inactivation.

A critical conserved mechanism for Cdk inactivation at exit from mitosis is cyclin proteolysis, under the control of the anaphase-promoting complex (APC) activated by Cdc20 and Cdh1 (Evans *et al.*, 1983; Zachariae and Nasmyth, 1999; Morgan and Roberts, 2002; Wäsch and Cross, 2002). In budding yeast, two additional regulators have been proposed to inactivate Cdk to allow exit from mitosis: the Sic1 stoichiometric inhibitor, activated by the Cdc14 phosphatase (Visintin *et al.*, 1998), and the N terminus of the Cdc6 replication protein (Calzada *et al.*, 2001). This region of Cdc6 was identified as a Cdk-binding domain (Elsasser *et al.*, 1996; Perkins *et al.*, 2001; Weinreich *et al.*, 2001). Calzada *et al.* (2001) reported that deletion of this region in the chromosomal copy of *CDC6* (*cdc6Δ2–47*) caused a delay in mitotic exit, and remarkably, combination of the *cdc6Δ2–47* mutation with deletion of *SIC1* absolutely blocked mitotic exit. Cyclin proteolysis is known to be essential for mitotic exit (Wäsch and Cross, 2002), but the *cdc6Δ2–47 sic1Δ* mitotic exit block was shown to occur even in the presence of all cyclin proteolytic machinery. Thus, this result suggested coequal control of Cdk inactivation by cyclin proteolytic machinery and inhibition by stoichiometric binding proteins. This finding was surprising, because it was recently proposed that the primary biological role of Sic1 was in regulating replication origin reloading, not in regulating mitotic exit (Lengronne and Schwob, 2002). In addition, the Cdc6 N terminus is capable in some circumstances of inhibiting completion of mitosis (Weinreich *et al.*, 2001), rather than promoting it as reported (Calzada *et al.*, 2001). Thus, the block to mitotic exit in *cdc6Δ2–47 sic1Δ* strains was a striking and surprising observation.

The essential function of Cdc6 is to promote the assembly of prereplicative complexes (pre-RCs) at origins of DNA replication (Bell and Dutta, 2002). Assembled pre-RCs contain at least ORC, Cdc6, and Mcms and constitute a platform for the DNA replication machinery to come into place. Firing of DNA replication at these sites results in pre-RC disassembly, and reassembly of pre-RCs is prevented by redundant mechanisms resulting from Clb-Cdc28 actions that include phosphorylation of Cdc6 (Nguyen *et al.*, 2001).

Transcription of *CDC6* is most active in late mitosis/early G1 (Zhou and Jong, 1990; Zwerschke *et al.*, 1994) via Swi5 (Piatti *et al.*, 1995), a transcription factor that also activates

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\* Present address: Department of Hematology/Oncology, University Medical Center, 79106 Freiburg Germany.

<sup>†</sup> Corresponding author. E-mail address: [fcross@mail.rockefeller.edu](mailto:fcross@mail.rockefeller.edu).

the transcription of *SIC1* (Knapp *et al.*, 1996; Toyn *et al.*, 1997). In cells that experience a prolonged G1, a second wave of *CDC6* transcription occurs, which is dependent on SBF (Swi4/Swi6) or MBF (Mbp1/Swi6) (Piatti *et al.*, 1995). Cdc6 proteolysis is differentially regulated by different cyclin-Cdc28 kinases (Piatti *et al.*, 1995; Elsasser *et al.*, 1999; Calzada *et al.*, 2000; Drury *et al.*, 2000). As a result of these controls, Cdc6 abundance reaches its maximum in late mitotic cells (Drury *et al.*, 1997). This has the effect of positioning Cdc6 to promote pre-RC assembly after mitosis and also, hypothetically, to regulate mitotic exit.

Here, we evaluate Cdc6 as a Clb2-Cdc28 inhibitor in mitotic exit. We find that although biochemical and genetic experiments demonstrate that Cdc6 has the potential to act in this role, it is not required for this cell cycle step even in the absence of the other known Clb-Cdk inhibitor, Sic1.

## MATERIALS AND METHODS

### Yeast Strain Construction

Standard methods were used for mating, tetrad analysis, and transformations. A list of strains is provided in the supplementary information (Table S1). *CLB2Δdb* and *CLB2Δdb,ken* alleles were constructed as described previously (Wäsch and Cross, 2002). A two-copy integrant of *GAL-SIC1* was required for construction of the *CLB2Δdb,ken* strain (our unpublished observations). *swi5:kanMX* was obtained from D. Stillman. *CDC6* wild-type and mutant plasmids were obtained from M. Weinreich. The *TUB1-GFP* allele marked with *HIS3* in the W303 strain background was obtained from A. Murray. Other plasmids and alleles were laboratory stocks. Protein A tagging of *CDC6*, *cdc6Δ2-49*, *SIC1*, and *CLB2* at the C-terminal end was performed by genomic integration of a DNA sequence that encodes the IgG binding domains of protein A (PRA) from *aureus* and the *HIS3MX* selection marker from *Schizosaccharomyces pombe* (Wach *et al.*, 1997; Rout *et al.*, 2000). Tagging of *CLN2*, *CLB2*, *CLB3*, and *CLB5* at the C-terminal end with nine repeats of the Myc epitope (MYC) was also done by genomic integration of a polymerase chain reaction (PCR) product that also contained the *klTRP1* gene, as described previously (Knop *et al.*, 1999).

### Cell Cycle Blocks for Affinity Purifications

The *CDC6-PRA* and *CDC6-PRA bar1* strains were grown in YPD to a density of  $\sim 2.0 \times 10^7$  cells/ml at 30°C. The *CDC6-PRA cdc20 GALL-CDC20* strain was grown at 30°C in YPG (YPGall) to a density of  $\sim 2.0 \times 10^7$  cells/ml. *CDC6-PRA bar1* strain were arrested by incubating for 2 h at 30°C in YPD (YPDex) containing 100 nM  $\alpha$ -factor (synthesized by The Rockefeller University Protein Resource Center, New York, NY). *CDC6-PRA cdc20 GALL-CDC20* cells were arrested at metaphase by adding 2% glucose to the YPG medium and incubating them for 3.5 h at 30°C. Cells were harvested by centrifugation and resuspended in 20 mM Na-HEPES (pH 7.4), 1.2% polyvinyl-pyrrolidone (Sigma-Aldrich, St. Louis, MO), 0.2 mg/ml phenylmethylsulfonyl fluoride (PMSF), and 4  $\mu$ g/ml pepstatin A (1 ml of buffer/10-g cell pellet). The cell suspension was frozen in liquid nitrogen and lysed by grinding in a mortar grinder (RM100; Retsch, Newtown, PA) that was continuously cooled with liquid nitrogen (Schultz, 1999).

### Affinity Purification and Separation of Protein Complexes

Protein A affinity purification were performed based on a documented method (Aitchison *et al.*, 1996; Rout *et al.*, 1997). Ground cells (4 g) were resuspended in 20 ml of extraction buffer (20 mM Na-HEPES, pH 7.4, 300 mM NaCl, 0.1% Tween 20, 2 mM MgCl<sub>2</sub>, 0.002% DNase I, 0.2 mg/ml PMSF, 4  $\mu$ g/ml pepstatin A) and incubated with gentle agitation at room temperature for 10 min to allow DNase I activity. The lysate was homogenized with a Polytron (PT 10/35; Brinkmann Instruments, Westbury, NY) for  $\sim 20$  s and then rotated for 1 h at 4°C. The soluble fraction was isolated by centrifugation at 3000 rpm (H-1000B; Sorvall, Newton, CT) for 10 min. IgG-DynaBeads were prepared by cross-linking rabbit IgG (ICN, Aurora, OH) to epoxy-activated DynaBeads (DynaL, Lake Success, NY). IgG-DynaBeads (15  $\mu$ g) were added to each lysate, and the suspension was rotated for  $\sim 18$  h at 4°C. The IgG-DynaBeads were collected with a magnet and washed five times with 1 ml of ice-cold extraction buffer. Isolated protein complexes were eluted from the IgG-DynaBeads in 0.5 M NH<sub>4</sub>OH 0.5 mM EDTA for 5 min at room temperature. The eluted proteins were frozen in liquid nitrogen and lyophilized in a SpeedVac (Thermo Savant, Holbrook, NY). Dried protein samples were dissolved in SDS-PAGE loading buffer [78 mM Tris-Cl, 0.003% bromophenol blue, 25 mM Tris base, 6.25 mM Tris (2-carboxyethyl) phosphine, 12.5% glycerol, 2.5% SDS], heated to 95°C for 5 min, and allowed to cool at room temperature for 10 min. Iodoacetamide (25 mM) was then added and samples were

incubated for 30 min at room temperature to modify reduced cysteines. Samples were resolved by SDS-PAGE with a Novex 4-20% Tris-glycine polyacrylamide gel (Invitrogen, Carlsbad, CA). Proteins in the gel were visualized by Coomassie staining with GelCode Blue (Pierce Chemical, Rockford, IL).

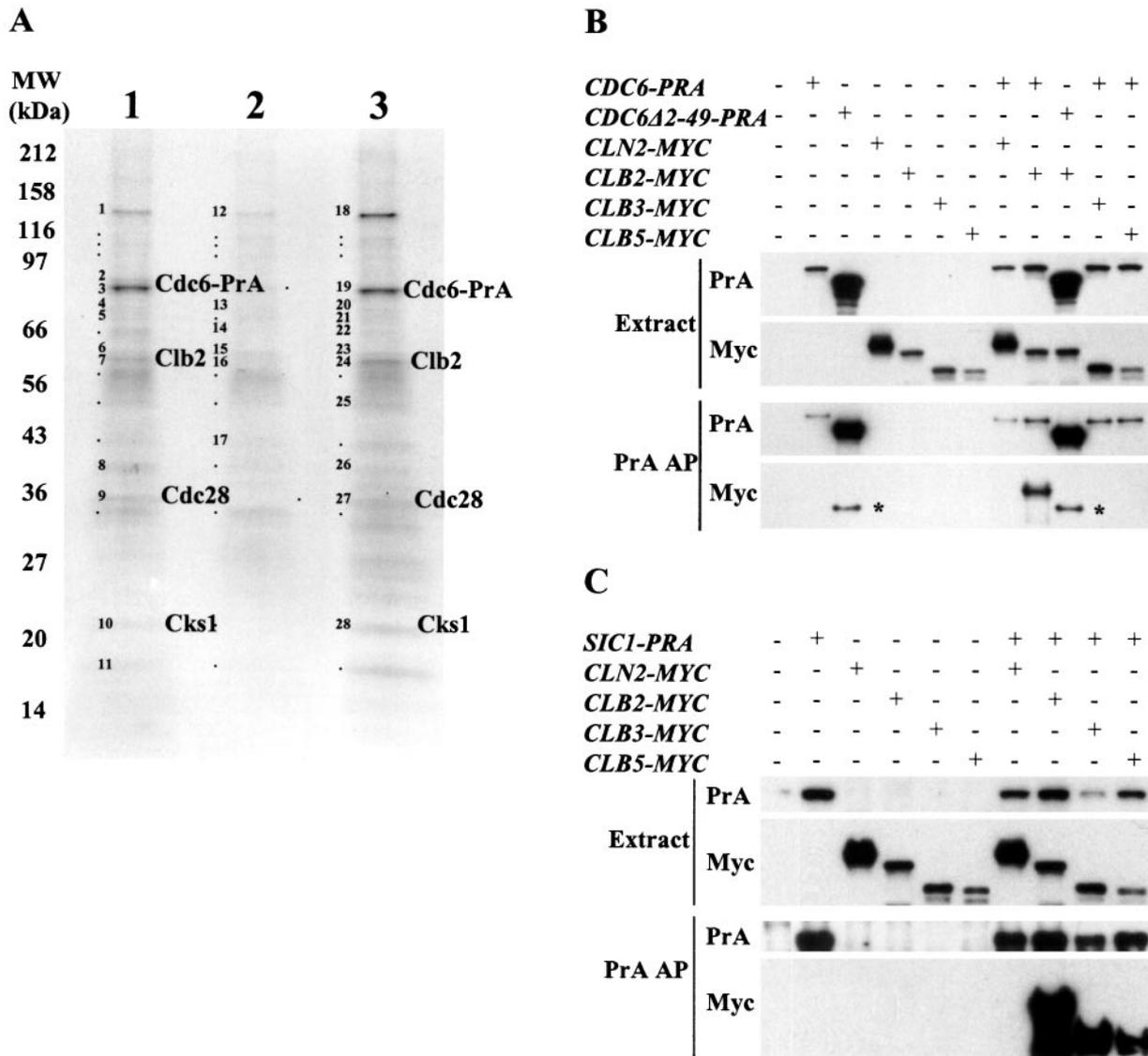
### Mass Spectrometric Identification of Protein Complex Components

Visible protein bands were excised from the Coomassie-stained gel and destained in 55% ammonium bicarbonate (100 mM)/45% acetonitrile with serial washes (4°C). Gel pieces were then dehydrated in acetonitrile, hydrated in 50 mM ammonium bicarbonate, and again dehydrated in acetonitrile. Proteins were digested in-gel with 75 ng of trypsin (Roche Diagnostics, Basel, Switzerland) per gel band in 50 mM ammonium bicarbonate for 6 h at 37°C. Tryptic peptides were extracted from the gel pieces with an 8- $\mu$ l slurry of 1 volume of POROS R2 20 reverse-phase resin (Applied Biosystems, Foster City, CA) to 10 volumes of 5% formic acid/0.2% trifluoroacetic acid (TFA) at 4°C for  $\sim 16$  h. C<sub>18</sub> Ziptips (Millipore, Billerica, MA) were conditioned with sequential washes of 0.1% TFA, 50% methanol/20% acetonitrile/0.1% TFA, and 0.1% TFA. POROS R2 20 resin was transferred to the conditioned Ziptips. The POROS R2 20 resin was washed in the Ziptip twice with 0.1% TFA. Tryptic peptides were eluted from the matrix-assisted laser desorption/ionization (MALDI) target compact disk (Krutchinsky *et al.*, 2001) with one-third saturated 2,5-dihydroxybenzoic acid (Lancaster Synthesis, Windham, NH) in 50% methanol/20% acetonitrile/0.1% TFA.

Tryptic peptides were identified by MALDI mass spectrometric analysis for protein identification (Beavis and Chait, 1996). Mass analysis of the tryptic peptides was performed with a MALDI-quadrupole-quadrupole-time-of-flight mass spectrometer (Centaur Technology, Austin, TX; MDS Sciex, Concord, ON, Canada) modified with a compact disk sample stage as described previously (Krutchinsky *et al.*, 2001). The highly accurate MALDI-mass spectrometry (MS) data was used to search the National Center for Biotechnology Center nonredundant protein database with the program ProFound to identify proteins from the tryptic peptide masses (Zhang and Chait, 2000). In addition to matching peptide masses to proteins, ProFound provides lists of peptide masses that can be used to confirm protein identifications by MS/MS analysis and lists of peptide masses that were not assigned to a protein in the MS analysis. To search for more proteins not identified in the MALDI-MS analysis and to confirm proteins identified in the single-stage MS analysis, amino acid sequence information for the tryptic peptides was obtained by MALDI-MS/MS fragmentation. Using the program M-IT sequencer, confirmation and unassigned masses obtained in the MALDI-quadrupole-quadrupole-time-of-flight analysis were used to prepare instrument files for MALDI-MS/MS analysis (Krutchinsky *et al.*, 2001). MALDI-MS/MS analysis of the same samples on the same MALDI target compact disk was performed using a modified LCQ Deca XP ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) as described previously (Krutchinsky *et al.*, 2001). MALDI-MS/MS data was used to search the National Center for Biotechnology Information nonredundant protein database with the program Sonar (Genomic Solutions, Ann Arbor, MI) to identify proteins from the tryptic peptide fragmentation masses (Field *et al.*, 2002).

### Affinity Purifications and Western Blotting

For the experiments presented in Figure 1, B and C, a 50-ml culture of each strain was grown to mid-log phase. After measuring the OD<sub>600</sub>, cells were harvested from volumes around 50 ml that yielded similar cell masses. Cell pellets of  $\sim 100$   $\mu$ l were washed in TE buffer and resuspended in 250  $\mu$ l of extraction buffer (20 mM K-HEPES, pH 7.4, 110 mM K-acetate, 0.1% Tween 20, 1 mM dithiothreitol, 2  $\mu$ g/ml DNaseI, 4  $\mu$ g/ml pepstatin A, 0.2 mg/ml PMSF, 0.5% protease inhibitor cocktail (Sigma-Aldrich). Acid-washed glass beads (0.15 g) were added, and cell suspensions were vortexed for 5 min. Extraction buffer (500  $\mu$ l) without DNaseI was added, and samples were vortexed for an additional 2 min. Samples were centrifuged for 30 s (tabletop Microfuge E; Beckman Coulter, Fullerton, CA), and the supernatants were removed. An additional 500- $\mu$ l extraction buffer was added, and samples were vortexed for another 2 min. Samples were centrifuged for 30 s and the supernatants were combined. Pooled supernatants were centrifuged for 2 min. Of this final supernatant, 20  $\mu$ l was kept for Western blotting analysis as "extract," and the remainder was submitted to coaffinity purification. For this purpose, rabbit IgG-conjugated magnetic DynaBeads (DynaL) were added, and samples were incubated on a rotator at 4°C for 1 h. Beads were washed six times with 1 ml of extraction buffer without DNaseI. Proteins were eluted twice with 200  $\mu$ l of 0.5 M NH<sub>4</sub>OH 0.5 mM EDTA for 5 min at room temperature. Eluates were pooled and lyophilized using a SpeedVac (Thermo Savant). Desiccated proteins were resuspended in 30  $\mu$ l of SDS-PAGE sample buffer, and extract samples were dissolved in an equivalent volume of 2  $\times$  SDS-PAGE sample buffer. Samples were resolved by SDS-PAGE with a Novex 4-20% Tris-glycine polyacrylamide gel (Invitrogen, Carlsbad, CA) and transferred on nitrocellulose. Tenfold-diluted samples of the purification products were used for the gel stained to the anti-Protein A probing. Western blot analysis was performed, probing for the protein A moiety using a 1:1000 dilution of rabbit IgG (ICN) and probing for the Myc epitope using a 1:1000 dilution of a mouse



**Figure 1.** Cdc6 associates with Clb2. (A) Cdc6 forms a complex with Clb2, Cdc28, and Cks1. Asynchronous *CDC6-PRA* cells (lane 1),  $\alpha$ -factor-arrested *CDC6-PRA bar1* cells (lane2) or glucose-arrested *CDC6-PRA cdc20 GALL-CDC20* cells (lane 3) were submitted to a protein A affinity purification. Bands marked with either a dot or number were excised and analyzed by MALDI-mass spectrometry to identify proteins. Bands containing proteins identified with high confidence are numbered and the identifications are listed in the supplementary Table S1. Bands identified with a dot did not give a high-confidence identification. Proteins that seem to be specific to the Cdc6-PrA pullout are labeled. (B) Cdc6 associates with Clb2 and not with other cyclins and its N terminus is required for this association. *CDC6-PRA* or *cdc6Δ2-49-PRA* or *CDC6-wt* (untagged) cells also expressing *CLN2-MYC*, *CLB2-MYC*, *CLB3-MYC*, or *CLB5-MYC* were submitted to protein A affinity purification. Top, whole cell soluble extracts probed by Western blot for protein A and Myc. Bottom, purified proteins probed by Western blot for protein A and Myc. In the anti-MYC Western blot, the bands indicated by the asterisk (\*) corresponded to the protein A tag from Cdc6Δ2-49-PrA that was recognized by the anti-MYC antibody due to its high abundance. (C) Sic1 associates with Clb2, Clb3, and Clb5 but not Cln2 (methods as in B).

monoclonal anti-Myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in Tris-buffered saline-Tween 20 5% milk (2-h incubation at room temperature). Secondary antibodies were horseradish peroxidase-coupled anti-rabbit and horseradish peroxidase-coupled anti-mouse, respectively, and both used as a 1:1000 dilution in Tris-buffered saline-Tween 20 5% milk (1-h incubation at room temperature). Chemiluminescence was used for the detection (Roche Diagnostics).

**Microscopy and Fluorescence-activated Cell Sorting (FACS)**

Immunofluorescence directed against tubulin and 4,6-diamidino-2-phenylindole (DAPI) DNA staining were carried out as described previously (Jacobson *et al.*, 2000). Fluorescence and differential interference contrast images were

acquired on an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) with a 100 × 1.4 numerical aperture Planapochromat objective (Vermont Optechs, Charlotte, VT), fitted with a Hamamatsu Orca ER cooled charge-coupled device camera (Sciscope Instrument, Iowa City, IA) controlled by MetaVue software (Universal Imaging, Downingtown, PA). DNA content analysis by FACS was performed as described previously (Epstein and Cross, 1992).

**RESULTS**

**Cdc6 Associates Specifically and Stably with Clb2**

Cdc6 has been reported to associate with Cdc28 in vitro and in vivo through its amino terminus (Elsasser *et al.*, 1996,

1999; Calzada *et al.*, 2000). Association between Cdc6 and the Clb5 B-type cyclin has been reported in vitro (Elsasser *et al.*, 1996). To explore potential associations of Cdc6 with cyclins in vivo and at endogenous expression levels, we used strains encoding a protein A fusions of Cdc6 expressed from the endogenous promoter. The Cdc6-PrA fusion protein was functional since *CDC6-PrA* cells grew normally. We affinity-purified Cdc6-PrA and analyzed the composition of the complex by SDS-PAGE and mass spectrometry (Figure 1A). We found that Cdc6 was degraded in an  $\alpha$ -factor arrest, consistent with previous observations (Drury *et al.*, 1997), and we were able to use the band pattern obtained in this condition as an indicator of which bands were contributed from contaminating proteins. All detectable bands were analyzed (supplementary information; Table SII). Strong bands that were observed from the purification done on asynchronous *CDC6-PrA* cells or metaphase-blocked *CDC6-PrA cdc20 GALL-CDC20* cells and not observed from the purification done on  $\alpha$ -factor-arrested *CDC6-PrA bar1* cells were identified as Cdc6-PrA, Clb2, Cdc28, and Cks1. The similar staining intensity of the bands corresponding to the latter three proteins combined with the absence of other strong bands specific to this purification point at the existence of a complex between Cdc6 and a stoichiometric Clb2-Cdc28-Cks1 complex in vivo. All other proteins identified were common contaminants in this procedure, such as abundant ribosomal and metabolic proteins and chaperones (supplementary information; Table SII). Surprisingly, Cdc6-PrA did not copurify with significant amounts of other components of the pre-RC, such as MCM, Cdt1, and ORC complexes, even though these complexes were all extracted and effectively purified using the protein A tag under the same conditions (our unpublished observations).

Cyclins other than Clb2 could have been present in low amounts and could have eluded our mass spectrometric analysis. Therefore, we conducted coaffinity purifications by using strains that expressed Myc-tagged versions of four cyclins or protein A fusions of Cdc6 and Cdc6 $\Delta$ 2-49, or both, all expressed under the control of their endogenous promoters (Figure 1B). We used Cln2-Myc, Clb2-Myc, Clb3-Myc, and Clb5-Myc as a representative set, due to their functional significance (Cross *et al.*, 2002). After protein A affinity purification, purified proteins were resolved on SDS-PAGE and probed for the Myc moiety by Western blotting. Of the four cyclins tested, only Clb2-Myc copurified with Cdc6-PrA, and this association was not observed when Cdc6 $\Delta$ 2-49-PrA was used as a bait, despite the higher abundance of this stabilized mutant protein. Therefore, Cdc6 associates specifically with Clb2 but does not stably associate with Cln2, Clb3, or Clb5 *ex vivo*, and this association is dependent on the amino terminus of Cdc6. For comparison, we performed the same analysis on cells coexpressing Sic1-PrA and the Myc-fusion cyclins (Figure 1C). We verified that Sic1-PrA could interact very strongly with all B-type cyclins tested (Clb2-Myc, Clb3-Myc, and Clb5-Myc) but not with the G1 cyclin Cln2-Myc. Therefore, the specificity of the cyclin-Cdc6 interaction was higher than the Sic1-cyclin interactions. These results contrast with those of Elsasser *et al.* (1996) who proposed that Clb5 could interact with Cdc6 based on *in vitro* binding data.

#### Cdc6 Abundance and Clb2 Binding through the Cell Cycle

Cdc6 should be present at a concentration similar to Clb2 in mitotic cells if it were to contribute significantly to mitotic exit through stoichiometric inhibition of Clb2-Cdk complexes. To test this idea, we used strains encoding protein A

**Table 1.** Quantitation of Cdc6-PrA, Clb2-PrA, and Sic1-PrA

Strain	Protein A-tagged protein	Copies/diploid cell (asynchronous)	
		This study	Cross <i>et al.</i> (2002)
AL4	Cdc6	624 $\pm$ 7 (3)	NA
AL4	Sic1	195 $\pm$ 29 (3)	NA
MK3	Clb2	1,006 $\pm$ 125 (3)	1,128 $\pm$ 231 (4)
MK3	Sic1	265 $\pm$ 29 (3)	214 $\pm$ 42 (5)

AL4, *MAT a/α CDC6-PrA::HIS3MX/CDC6 SIC1-PrA::HIS3MX/SIC1*; MK3, *MAT a/α CLB2-PrA::HIS3MX/CLB2 SIC1-PrA::HIS3MX/SIC1*.

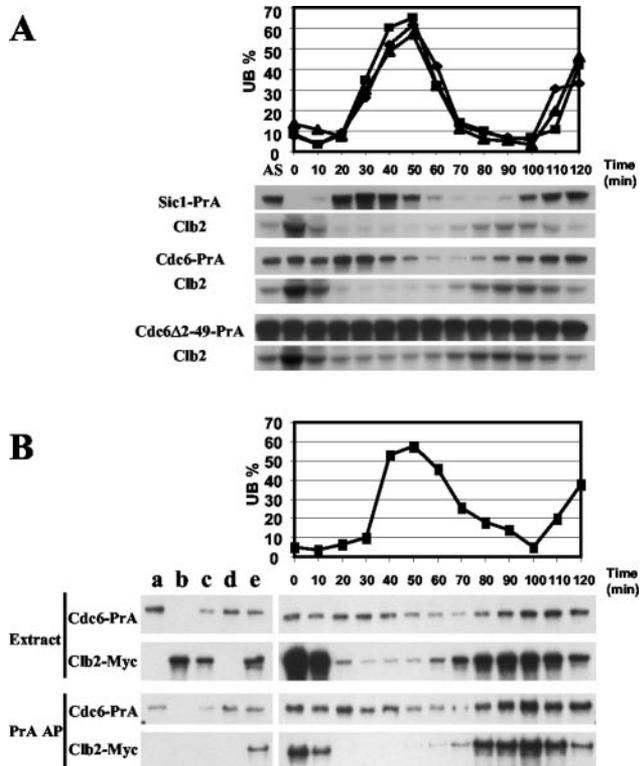
Quantitations were performed on these heterozygous diploid strains as described in text; MK3 was one of the strains tested previously (Cross *et al.*, 2002). Numbers are in estimated copies per cell in asynchronous diploid cells. Published numbers are provided for comparison. The numbers of independent cultures tested are in parentheses.

fusions of both Cdc6 and Clb2 expressed from their endogenous promoters. Using a strategy described elsewhere (Cross *et al.*, 2002), we derived estimates of copy numbers per diploid cell in asynchronous cultures (Table 1). This analysis revealed that Cdc6 has an abundance greater than Sic1 and about two-thirds that of Clb2. Thus, Cdc6 could potentially contribute significant stoichiometric inhibition of Clb/Cdc28 kinase activity.

To act as an inhibitor of Clb2/Cdc28, Cdc6 must also be present at the same time in the cell cycle. Cdc6 has been reported to be present in late mitosis and G1 (Piatti *et al.*, 1995; Drury *et al.*, 1997). Clb2 levels rise in mitotic cells and decrease rapidly before mitotic exit. We examined the abundance patterns of Cdc6 and Clb2 through the cell cycle. We used *CDC6-PrA cdc20 GALL-CDC20* cells that we arrested in metaphase by adding glucose. We released the cells by transfer to galactose medium and assessed the fluctuations of Cdc6-PrA and Clb2 by Western blotting (Figure 2A). Arrested cells displayed high levels of Clb2 and moderate levels of Cdc6-PrA. Cdc6-PrA reached a peak of abundance 20 min after release in galactose, whereas Clb2 disappeared quickly and was almost absent by 20 min. Cdc6-PrA persisted after cell division, reaching its lowest level after 70 min, in freshly budded cells, and then rose. Clb2 stayed at low levels until it reappeared at 70 min as cells budded. These results confirm that periods with high levels of Cdc6 overlap with the period during which Clb2 levels fall, consistent with a role of Cdc6 in Clb2-Cdk1 inhibition.

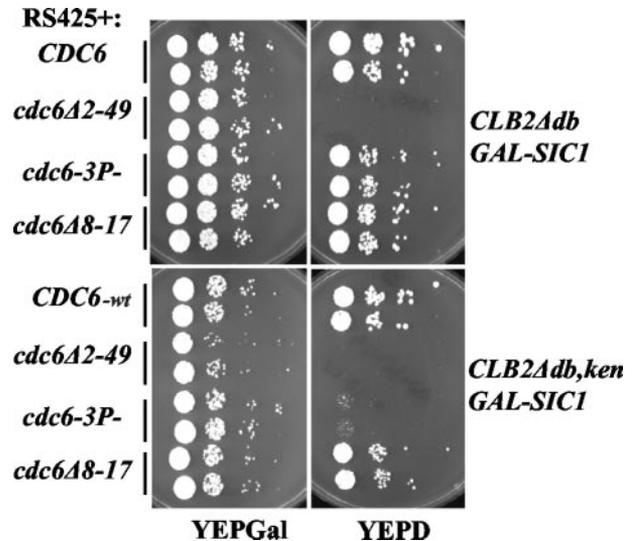
We were interested to observe the accumulation of Cdc6 in the *cdc20*-blocked cultures, in which active Clb-Cdk1 complexes accumulate; in comparison, Sic1-PrA failed to accumulate detectably at this block (Figure 2A,  $t = 0$ ). This observation suggests that "mode 3" proteolysis (Drury *et al.*, 2000), described as controlling Cdc6 levels in cells expressing high Clb levels, is rather inefficient at removing Cdc6 at endogenous expression levels. Mode 3 proteolysis is considered not to require the Cdc6 N terminus, so the persistence of constitutive levels of Cdc6 $\Delta$ 2-49 throughout the cell cycle (Figure 2A) is also consistent with relatively low effectiveness of mode 3 in removing Cdc6 at endogenous expression levels.

To visualize the timing of occurrence of the Cdc6-Clb2 complex, we synchronized *CDC6-PrA CLB2-MYC cdc20*



**Figure 2.** Cdc6 temporally overlaps and associates with Clb2 in mitotic cells. (A) Timing of expression. *CDC6-PRA* (or *cdc6Δ2-49-PRA*, or *SIC1-PRA*) *cdc20 GALL-CDC20* cells were grown in YPG, arrested in YPD for 3 h and released in YPG. Top, budding index (squares, *CDC6-PRA* cells; diamonds, *cdc6Δ2-49-PRA* cells; triangles, *SIC1-PRA* cells). Bottom, Western blot analysis of the cultures probing for protein A and Clb2. In *cdc6Δ2-49-PRA* cells, a breakdown fragment of Cdc6Δ2-49-PrA migrated at a molecular weight identical to Clb2, possibly accounting for the low signal observed for unbudded cells in the Clb2 blot because the protein A could be detected by the Clb2 antibody. (B) Cdc6 associates with Clb2 in mitotic cells. Right, a time course of protein A affinity purification was performed on *CDC6-PRA cdc20 GALL-CDC20* cells grown in YPG, arrested in YPD for 3 h, and released in YPG. Samples were taken every 10 min after release for affinity purification. Left, controls for the affinity purification; all asynchronous cultures. Lane a, *CDC6-PRA* cells; lane b, *CLB2-MYC* cells; lane c, *CDC6-PRA* cells mixed with *CLB2-MYC* cells before cell breakage (control for postlysis binding); lane d, *CDC6-PRA cdc20 GALL-CDC20* cells; and lane e, *CDC6-PRA CLB2-MYC cdc20 GALL-CDC20* cells. Top, budding index; middle, whole cell soluble extracts probed by Western blot for protein A and Myc; bottom, purified proteins probed by Western blot for protein A and Myc.

*GALL-CDC20* cells in metaphase by arresting them in glucose, releasing them in galactose, and then performing protein A affinity purifications on culture samples taken at different time points (Figure 2B). Cdc6-PrA associated with Clb2-Myc at essentially all times when Clb2-Myc was present. We did not attempt to calculate the proportion of total Clb2-Myc that remained stably bound to Cdc6-PrA through this procedure, although it was clearly only a minor fraction based on relative amounts loaded and exposure times used (our unpublished observations). It is hard to interpret this because of possible losses of bound Clb2-Myc during the extensive washes of Cdc6-PrA during the purification.



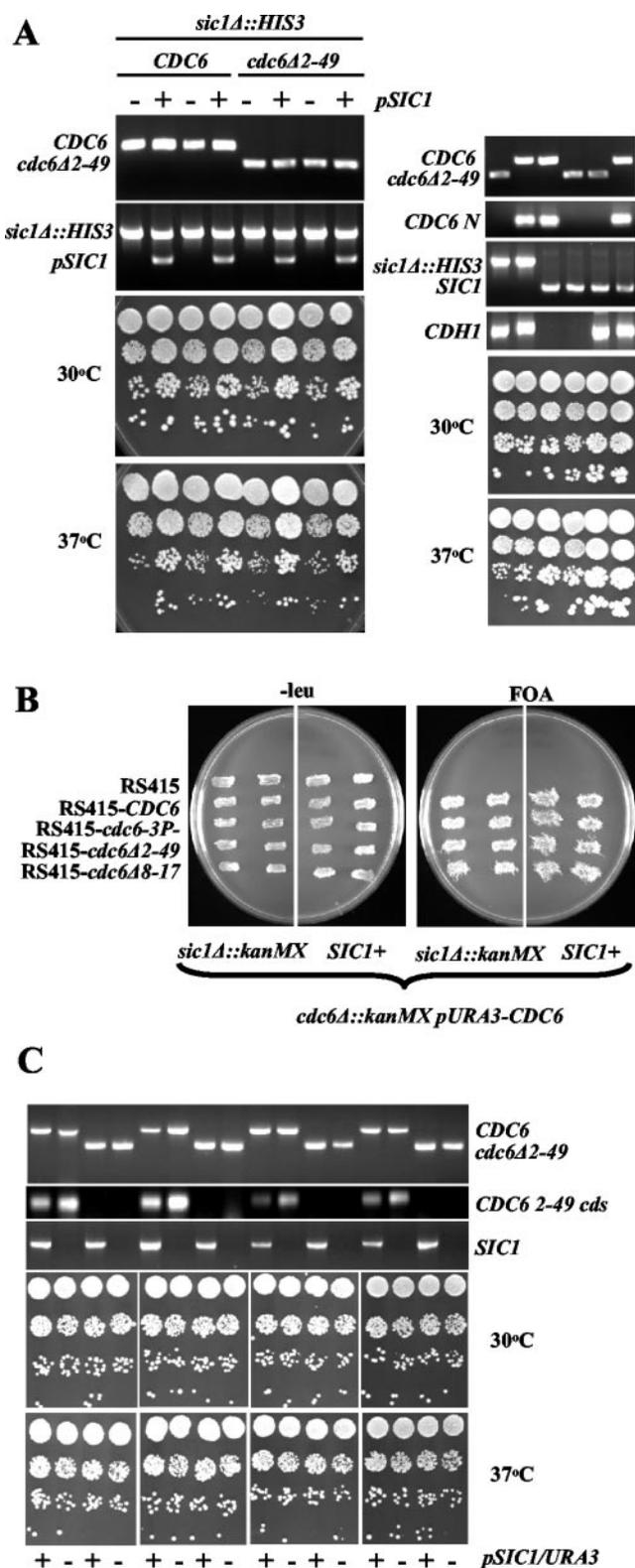
**Figure 3.** Mutants expressing stabilized forms of Clb2 are rescued by *CDC6* in an N terminus-dependent manner. *CLB2Δdb GAL-SIC1* (1 ×) or *CLB2Δdb,ken GAL-SIC1* (2 ×) cells were transformed with plasmids expressing *CDC6*, *cdc6Δ2-49*, *cdc6-3P-*, and *cdc6Δ8-17*. Galactose-grown cultures were serially diluted (10-fold dilutions) on galactose- or glucose-containing rich medium and incubated for 2 d at 30°C.

#### Moderate Overexpression of *CDC6* Helps Cells Attain Mitotic Exit

Strongly overexpressed Cdc6 from the *GAL1* promoter was shown previously to act genetically to inhibit Clb-Cdc28 function and delay entry in mitosis (Bueno and Russell, 1992; Basco *et al.*, 1995; Perkins *et al.*, 2001). Because we observed specific binding of Cdc6 to Clb2, we wanted to see whether a mitotic exit defect specifically due to Clb2 could be overcome by moderate Cdc6 overexpression. To do this, we examined the ability of low- and high-copy plasmids (~1–3 copies and ~10 copies/cell, respectively) containing *CDC6* with its own promoter to rescue the lethal mitotic exit defect associated with removal of the Clb2 destruction box (*CLB2Δdb*) (Wäsch and Cross, 2002). *CDC6* and *SIC1* could both carry out significant rescue of *CLB2Δdb* lethality even at low copy, and rescued very efficiently at high copy (supplementary information; Figure S1). To increase the stringency of the assay, we also tested *CLB2Δdb,ken*, in which the KEN boxes of Clb2, recognized specifically by Cdh1, were also mutated. Rescue of this mutant required higher dosage of both *CDC6* and *SIC1*, consistent with the idea that the low-copy rescue required activation of Cdh1-dependent proteolysis of Clb2Δdb. Notably, *CDC6* was at least as efficient as *SIC1* in these assays.

Rescue by *CDC6* in these assays required the N-terminal 49 amino acids (Figure 3). The N-terminal phosphorylation sites were important for the rescue of *CLB2Δdb,ken*, but not *CLB2Δdb*. Removal of the candidate cyclin interaction sequence in amino acids 8–17 (Weinreich *et al.*, 2001) had only moderate effects on rescue.

Surprisingly, rescue was severely impaired by the K114E mutation, which blocks Cdc6 replicative function (Weinreich *et al.*, 1999) but is outside the Cdk-interacting domain as defined previously (Weinreich *et al.*, 2001) (Figure S2). This suggests that rescue of *CLB2Δdb* lethality may involve the replicative function of Cdc6 to some extent, as well as its



**Figure 4.** *cdc6Δ2-49 sic1* mutants are viable. (A) In the W303 background, a deletion of coding sequence for amino acids 2–49 was introduced in the chromosome with no associated marker, as described in the text and Figure S3. Left, *sic1::HIS3 cdc6Δ2-49 pURA3-SIC1* strains were constructed by tetrad analysis, and loss of the *pSIC1-URA3* plasmid selected on 5-FOA. Strains with and without the plasmid were tested for the *CDC6* N-terminal deletion and

Cdk-inhibitory ability. Perhaps replication origin licensing is somewhat inefficient under these conditions due to residual Clb activity during mitotic exit (Nguyen *et al.*, 2001), yielding an increased requirement for Cdc6 replicative activity. In any case, these results confirm the idea that the Cdc6 N-terminus can contribute to negative regulation of Clb2-Cdk in mitotic exit.

#### Viability of *sic1* Null Strains Lacking the Cdc6 Cdk Inhibitory Domain in the W303, S288C, and BF264-15D Strain Backgrounds

Based on their observation that combining deletion of *SIC1* with deletion of the N terminus of Cdc6 was synthetic lethal, Calzada *et al.* (2001) made the important suggestion that Cdk inhibition by stoichiometric binding inhibitors was required for mitotic exit. However, we found that it was possible to construct this double mutant. In three different genetic backgrounds, viable *sic1Δ cdc6Δ2-49* cells could be generated (Figure 4).

In the W303 background (Figure 4A), we made precise gene replacements of *CDC6* with *cdc6Δ2-49*, *cdc6-3P-* (lacking three Cdk sites in the N terminal 50 amino acids of Cdc6) and *cdc6Δ8-17*, all of which were reported to be defective in Cdk binding and mitotic regulation (Weinreich *et al.*, 2001). The allele construction is described in the supplementary information (Figure S3). These *CDC6* alleles are exact chromosomal gene replacements without associated marker sequences, unlike the *cdc6Δ2-47* allele constructed in Calzada *et al.* (2001), in which a *URA3* gene was inserted 3' to *CDC6*, disrupting the neighboring *YJL193W* open reading frame (Calzada *et al.*, 2001).

We could readily combine all the *cdc6* alleles (*cdc6Δ2-49*, *cdc6-3P-*, *cdc6Δ8-17*, and *cdc6Δ2-47::loxP*) with a *sic1::HIS3* deletion allele, in standard tetrad analysis. In most experiments, we had a *CEN-SIC1/URA3* plasmid segregating, so that we could recover all genotypes (even if *SIC1*-requiring) and test for a *SIC1* requirement by 5-fluoroorotic acid (5-FOA) resistance (selecting plasmid loss events). In 4:0 Ura+ : Ura- tetrads from crosses with *sic1::HIS3* homozygous and *cdc6Δ2-49* segregating, FOA-resistance segregated 4:0 (i.e., *cdc6Δ2-49 sic1Δ* strains did not require the *SIC1* plasmid for viability). In crosses where *cdh1::LEU2* was also segregating, we observed 2:2 FOA-S Leu+ : FOA-R Leu- segregation, independent of *cdc6* genotype (i.e., we could detect a *SIC1* requirement in *cdh1Δ* strains, in the same experiment, pro-

for the *sic1::HIS3* mutation by PCR, and for growth rate by serial dilution at 30 and 37°C. Right, segregants from a cross with *cdh1::LEU2*, *sic1::HIS3*, and *cdc6Δ2-49* segregating were tested by PCR. The second PCR analysis tests specifically for the presence of the coding sequence for amino acids 2–49. The strains were tested for growth rate as in the left panel. (B) In the S288C background (Figure 4B), we made a strain that was *cdc6Δ::kanMX sic1Δ::kanMX pURA3-CDC6*, and a control *cdc6Δ::kanMX SIC1 pURA3-CDC6* strain (complete coding sequence deletions). All *kanMX* disruptions were confirmed by PCR. We transformed these strains with low-copy (*CEN*) plasmids encoding wild-type and the three mutant *CDC6* genes, expressed from the *CDC6* promoter (Weinreich *et al.*, 2001). All transformants, except for the vector controls could readily lose the *URA3/CDC6* plasmid. (C) BF264-15D-congenic background: We backcrossed *cdc6Δ2-49* from W303 (see A) into the BF264-15D congenic strain background (see text). Isogenic pairs of *sic1Δ pURA3-SIC1* segregants (*cdc6Δ2-49* or *CDC6*) from the fifth backcross were selected for *pURA3-SIC1* plasmid loss by FOA and tested for growth rate by serial dilution at 30 and 37°C. PCR characterization of the strains was performed as in A.

**Table 2.** Test of *sic1Δ cdc6Δ2-49* strains for delay in exit from mitosis

Strain	Medium	% Unbudded cells	% Binucleated cells
<i>sic1Δ GAL-SIC1</i>	Gal	31	17
<i>sic1Δ cdc6Δ2-49 GAL-SIC1</i>	Gal	22	16
<i>sic1Δ</i>	Gal	31	17
<i>sic1Δ cdc6Δ2-49</i>	Gal	27	19
<i>sic1Δ GAL-SIC1</i>	Gal → Glu (2.5 h)	26	16
<i>sic1Δ cdc6Δ2-49 GAL-SIC1</i>	Gal → Glu (2.5 h)	28	21
<i>sic1Δ</i>	Gal → Glu (2.5 h)	11	13
<i>sic1Δ cdc6Δ2-49</i>	Gal → Glu (2.5 h)	13	19

Strains of the indicated genotype were grown in the indicated medium and shifted from YEPGal into YEPD (Gal → Glu) for 2.5 h (long enough to quantitatively deplete Sic1 in *GAL-SIC1* strains; Wäsch and Cross, 2002). Note: the reported phenotype of mitotic exit defect in *sic1Δ cdc6Δ2-47* strains was ~100% budded binucleate cells (Calzada *et al.*, 2001).

viding an internal control). Identical results were obtained using the *cdc6-3P-*, *cdc6Δ8-17*, and *cdc6Δ2-47::loxP* alleles (our unpublished observations). We carried out this entire analysis in W303 with independent duplicates of all gene replacements, starting from independent *cdc6Δ2-47::loxP-URA3-loxP* insertions, with identical results.

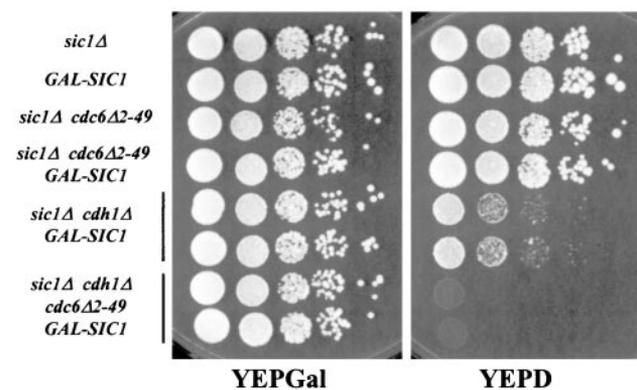
We could also recover *sic1::HIS3 cdc6Δ2-49* segregants without the *SIC1/URA3* plasmid, thus without the use of 5-FOA selection. Among such Ura<sup>-</sup> segregants, 9 of 22 *sic1::HIS3 CDH1*, 6 of 10 *SIC1 cdh1::LEU2*, and 7 of 12 *SIC1 CDH1* segregants contained the *cdc6Δ2-49* mutation. *sic1::HIS3 cdc6Δ2-49* segregants were viable, and displayed only a slight growth defect at 37°C. In cultures grown at 30°C, we observed no excess accumulation of budded binucleate cells with long spindles (the reported lethal phenotype for *sic1Δ cdc6Δ2-47*) (Table 2; our unpublished observations). Accumulation of long spindles monitored using green fluorescent protein (GFP)-labeled tubulin was similar in these two backgrounds (in one experiment, 9% of a cycling log phase *sic1Δ CDC6* strain and 12% of a cycling *sic1Δ cdc6Δ2-49* strain had long spindles). Similar results were obtained with *sic1Δ CDC6 GAL-SIC1* or *sic1Δ cdc6Δ2-49 GAL-SIC1* strains shifted to glucose medium to deplete Sic1 (our unpublished observations). These results contrast with the 100% block as binucleate cells with long spindles reported for *sic1Δ cdc6Δ2-47* (Calzada *et al.*, 2001).

In the S288C background (Figure 4B), we used a plasmid-shuffle strategy to establish that *cdc6Δ::kanMX sic1Δ::kanMX* strains were viable with the three mutant *CDC6* genes, expressed from the *CDC6* promoter (Weinreich *et al.*, 2001) as their sole source of *CDC6*. The mutant transformants grew well, and did not accumulate binucleate cells (our unpublished observations); thus, they did not exhibit a significant delay in mitotic exit. This result extends the lack of requirement for Sic1 or the Cdc6 N terminus to another strain background.

We performed six generations of backcross of *cdc6Δ2-49* from W303 into the BF264-15D strain background (Figure 4C). In each generation, the *cdc6Δ2-49* strain was either *SIC1* or else carried *pURA3-SIC1*, so that selection of modifiers affecting viability or vigor of *sic1Δ cdc6Δ2-49* strains should be avoided. In most backcrosses, the backcrossed *cdc6Δ2-49* strain was also *cdh1Δ*, allowing an internal control for de-

tection of a Sic1 requirement due to *sic1Δ cdh1Δ* spore inviability, as described above. In every generation of backcross, we could readily recover viable *cdc6Δ2-49 sic1Δ* segregants, whereas viable *sic1Δ cdh1Δ* segregants were not recovered. In third, fourth, fifth, and sixth generation backcrosses to BF264-15D, *sic1Δ* segregants lacking the *pURA3-SIC1* plasmid were tested by PCR, and 5 of 7, 6 of 16, 3 of 3, and 4 of 9 were *cdc6Δ2-49*. Note that these segregants were haploids that lacked the *pURA3/SIC1* plasmid from their initial meiotic generation, thus this was an unbiased and unselected sample. These results rule out an unlinked modifier from W303, allowing viability of the double mutant ( $P \ll 0.01$ ), and show that even among strains 98% genetically identical to BF264-15D, *cdc6Δ2-49 sic1Δ* segregants were easily recovered. A W303-specific modifier linked to *SIC1* is ruled out by the backcrossing strategy, because in some crosses the *sic1* allele was from a BF264-15D parent. A W303-specific modifier linked to *CDC6* remains a formal possibility to explain the discrepancy with results in Calzada *et al.* (2001). After the fifth backcross, we compared isogenic pairs of *sic1Δ pURA3-SIC1* segregants (*cdc6Δ2-49* or *CDC6*) before and after selection of loss of the *pURA3-SIC1* plasmid by 5-FOA selection. We observed no significant growth defect for any genotype.

The strains described in Calzada *et al.* (2001) all contained hemagglutinin (HA)-epitope-tagged *CLB2*, to facilitate biochemical analysis. We investigated the possibility that the *CLB2-HA* allele could cause the discrepancy between our results. In the W303 background, we were able to recover viable *CLB2-HA cdc6Δ2-49 sic1Δ* spores, at the expected Mendelian frequency, that generated normal-size colonies. Of 32 *sic1::HIS3* segregants tested by PCR, eight were *cdc6Δ2-49 CLB2-HA*, nine were *CDC6 CLB2-HA*, seven were *cdc6Δ2-49 CLB2*, and eight were *CDC6 CLB2*. A *sic1Δ cdc6Δ2-49 CLB2-HA* strain was backcrossed twice in two independent lines to a *sic1Δ* BF264-15D strain. The results provided no evidence for inviability or any significant partial mitotic exit defect of *sic1Δ cdc6Δ2-49*, with either *CLB2-HA* or *CLB2-wt* (our unpublished observations). The hypothesis that a single-gene suppressor specific to BF264-15D provided inviability of *cdc6Δ2-49 sic1Δ CLB2-HA* was not supported ( $P \ll 0.05$ ; our unpublished observations). Therefore, it is unlikely that the inclusion of HA-tagged Clb2 in the experiments of Calzada *et al.* (2001) are responsible the



**Figure 5.** Removal of the Cdc6 N terminus reduces viability of *sic1Δ cdh1Δ* cells. Galactose-grown cultures were serially diluted (10-fold dilutions) on galactose- or glucose-containing rich medium and incubated for 3 d at 30°C. All strains contained the *TUB1-GFP* allele (used in Figure 6).

discrepancy between their results and ours. This discrepancy remains unexplained at present.

#### **Removal of the Cdc6 N Terminus Confers a Cytokinesis Defect in *sic1Δ cdh1Δ* Cells**

Despite the fact that the *sic1Δ cdc6Δ2-49* mutant was viable, in contradiction to the results of Calzada *et al.* (2001), the ability of Cdc6 to rescue *CLB2Δdb* mitotic exit defects suggest that Cdc6 has the potential to help mitotic exit. We therefore examined more closely the possibility that the Cdc6 N terminus might cooperate with Sic1 under some circumstances. Sic1 is thought to cooperate with Cdh1 in regulating Clb-Cdk activity (see INTRODUCTION), so we asked what would happen in the absence of all three potential regulators.

*sic1Δ cdh1Δ GAL-SIC1* strains proliferate slowly on glucose medium, forming small colonies with >10% plating efficiency (Wäsch and Cross, 2002); this limited proliferation is dependent on the Cdc6 N terminus (Figure 5; Cross, 2003). As shown above, *cdh1Δ cdc6Δ2-49* and *sic1Δ cdc6Δ2-49* double mutants have no significant proliferation defect, so the strong inviability of the triple mutant represents functional overlap among all three genes.

To investigate the basis for the enhanced lethality of this triple mutant, we examined the morphology of *sic1Δ cdh1Δ* with or without the *cdc6Δ2-49* mutation (Figure 6). We used *GAL-SIC1* to allow the culture of these cells in galactose and their subsequent arrest in glucose. Phase contrast microscopy images, Tub1-GFP fluorescence images, and FACS measurements allowed the visualization of cell morphology, spindle morphology, and DNA content of various mutants cultured in galactose or glucose (Figure 6A). In galactose, only minor differences were observed among the *GAL-SIC1*-containing strains (Figure 6A, left).

*sic1Δ* cells showed no clear peak of cells with 1C DNA content, consistent with the role of *SIC1* in preventing DNA replication in early G1 cells by inhibiting Cdk activity (Lengronne and Schwob, 2002). *sic1Δ cdc6Δ2-49 GAL-SIC1* cells (Figure 6A, fourth row) grew well in glucose and seemed identical to *sic1Δ* cells (Figure 6A, first row). *sic1Δ cdh1Δ GAL-SIC1* cells (Figure 6A, fifth row) proliferated in galactose with a somewhat reduced G1 population in comparison to the *sic1Δ GAL-SIC1* cells (Figure 6A, first row). However, in glucose, *sic1Δ cdh1Δ GAL-SIC1* cells (Figure 6A, fifth row) arrested mostly as large-budded cells with replicated DNA and generally short spindles, consistent with previous observations (Wäsch and Cross, 2002). The additional removal of the Cdc6 N terminus had a dramatic effect in this context. In glucose, *sic1Δ cdh1Δ cdc6Δ2-49 GAL-SIC1* cells (Figure 6A, sixth row) reproducibly accumulated as "four-cell body objects" that were resistant to sonication. These objects always had two spindles, one spreading across "cell body 1" and "cell body 2" and one spreading across "cell body 3" and "cell body 4." As was observed with the *sic1Δ cdh1Δ GAL-SIC1* cells in glucose, the spindles were generally short. Consistent with these four-cell body objects, we observed a marked increase in frequency of cells with 4C DNA content.

We used immunofluorescence directed to tubulin and DAPI staining to visualize the nuclei (Figure 6B). For both *sic1Δ cdh1Δ cdc6Δ2-49 GAL-SIC1* cells and *sic1Δ cdh1Δ GAL-SIC1* cells transferred to glucose medium both generally had one short spindle per nucleus. In both cases, this is inconsistent with a mitotic exit defect, because cells did not accumulate as binucleates with long spindles. These results are consistent with previous findings on *sic1Δ cdh1Δ* cells (Wäsch and Cross, 2002). The simplest explanation of these

observations is that the additional removal of the Cdc6 N terminus confers a cytokinesis defect to cells lacking *SIC1* and *CDH1*. This defective cytokinesis could account for the enhancement of lethality of *sic1Δ cdh1Δ* by *cdc6Δ2-49* (Figure 5). The accumulation of 4C DNA content cells suggests that even when cytokinesis is defective, DNA replication in the next cell cycle is not significantly impaired. This is surprising, considering the known role of Clb activity in blocking origin licensing (Nguyen *et al.*, 2001).

#### **CDC6 Overexpression Rescues *sic1Δ cdh1Δ* Cells**

The apparent overlap in function between *SIC1*, *CDH1*, and *CDC6* led us to test whether increased *CDC6* gene dosage could bypass the requirement for either *CDH1* or *SIC1* for viability. We found that extra *CDC6* rescued the reduced viability of *sic1Δ cdh1Δ* cells (Figure 7). Deletion of Cdc6 amino acids 2–49 or mutation of the three Cdk phosphorylation sites within amino acids 2–49 severely reduced the ability of *CDC6* to rescue *sic1Δ cdh1Δ* cells. Deletion of amino acids 8–17 had a lesser but detectable effect (our unpublished observations). These results are consistent with the idea that Sic1, Cdh1, and the Cdk inhibitory domain in the Cdc6 N terminus have overlapping functions.

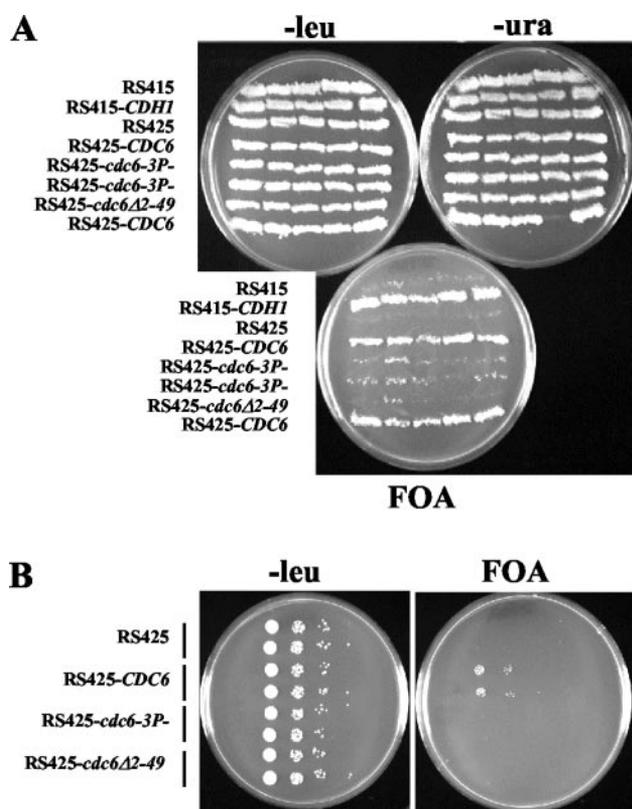
#### ***swi5Δ cdh1Δ* Inviability and Its Rescue by *CDC6***

The results mentioned above demonstrate that Cdc6 has the potential to act as a coinhibitor of Clb-Cdc28 with Sic1. It is interesting that both these proteins are controlled by the Swi5 transcription factor, resulting in a burst of transcription for both at the M/G1 border (Piatti *et al.*, 1995; Knapp *et al.*, 1996; Toyn *et al.*, 1997). However, Swi5 is not essential, suggesting that other mechanisms may provide sufficient Clb-Cdc28 inactivation in its absence. We tested whether Cdh1 could be the relevant activity, and we found that indeed, *swi5Δ cdh1Δ* double mutants were inviable in tetrad analysis (our unpublished observations), whereas both single mutants were fully viable. Lethality could be rescued by *SIC1* overexpression from the *GAL1* promoter (Figure 8A). We used this to determine the lethal phenotype of *swi5Δ cdh1Δ GAL-SIC1* by turning off the *GAL1* promoter with glucose medium. This mutant did not display a rapid specific cell cycle arrest; rather, cells went through a variable number of divisions before ultimately becoming inviable (that is, unable to form colonies even when plated on galactose medium) with mostly replicated DNA (Figure 8B, second row).

This non-Cdc phenotype is difficult to analyze in detail, but the lethality of the double mutant was nevertheless tight enough for us to test the ability of wild-type and mutant *CDC6* and *SIC1* to rescue at varying copy number (Figure 8A). Rescue was quite efficient even with low-copy-number plasmids containing *CDC6* or *SIC1*. Rescue required the Cdc6 N terminus; unlike the results with *CLB2Δdb* (see above), the N-terminal phosphorylation sites and the candidate cyclin-binding sequence (amino acids 8–17) were both strongly required for rescue. In addition, the K114E mutation strongly reduced rescue, suggesting that both the Cdk inhibition and the DNA replication licensing function of Cdc6 were involved. As with rescue of *CLB2Δdb* lethality (see above), this could suggest that inefficient replication origin licensing under these conditions results in an increased requirement for Cdc6 replicative activity.

The *swi5Δ cdh1Δ* cells accumulated as large-budded cells with a short spindle (our unpublished observations), therefore closely resembling *sic1Δ cdh1Δ* cells (Wäsch and Cross, 2002). We tested the effect of removing the Cdc6 N terminus





**Figure 7.** CDC6 rescues *sic1Δ cdh1Δ* cells. A *cdh1Δ sic1Δ* strain carrying a *pSIC1-URA3* plasmid was transformed with *LEU2* plasmids, either vector (RS415, low-copy, or RS425, high-copy), RS415 containing *CDH1* as a positive control, or RS425 carrying wt and mutant *CDC6*. (A) Transformants were patched on *-leu* and then replica-plated to *-ura* (indicating retention of the *pSIC1-URA3* plasmid) or FOA (indicating ability to lose this plasmid). Note that one RS425-*CDC6* transformant spontaneously lost the *pSIC1-URA3* plasmid. (B) Serial dilutions of transformants on *-leu* or FOA.

on the *swi5Δ cdh1Δ* mutant. *cdc6Δ2-49 swi5Δ cdh1Δ GAL-SIC1* cells were viable in galactose but arrested in glucose with a marked increase in 4C DNA (Figure 8B, third row). Like *cdc6Δ2-49 sic1Δ cdh1Δ GAL-SIC1* cells, *cdc6Δ2-49 swi5Δ cdh1Δ GAL-SIC1* cells transferred to glucose medium accumulated as four-cell body objects with generally short spindles (our unpublished observations). Thus, although difficult to interpret in terms of a specific cell cycle requirement, the results with *swi5Δ cdh1Δ* lethality support the idea that Cdc6, Sic1 and Cdh1 can cooperate to regulate Clb-Cdc28 activity and the effect of the loss of all three activities may be a failure of cytokinesis.

#### Functional Overlap between the Cdc6 N Terminus, Sic1, and the Mitotic Exit Network

Cdc14 and Cdc15 are part of the mitotic exit network (Bardin and Amon, 2001). Suggestive of some degree of involvement of the Cdc6 N terminus in mitotic exit, we found that *cdc6Δ2-49* acts as a weak enhancer of the *cdc14-1* (Figure 9A) or *cdc15-2* (Figure 9B) temperature-sensitive mutations, although *sic1Δ* acts as a considerably stronger enhancer. Reciprocally, high-copy *CDC6* but not *cdc6Δ2-49* weakly suppresses *cdc14-1* at semipermissive temperature (Figure 9C). This suppression is much weaker than that by high-copy

*SIC1*. It is also notable that some of the enhancing effects of *cdc6Δ2-49* on *cdc14-1* occur even under conditions of *SIC1* overexpression (Figure 9A, see *cdc6Δ2-49* strains on YEPGal plates, at 30°C), suggesting that the Cdc6 N terminus could have a role in this context that cannot be provided by extra-Sic1. Thus, the Cdc6 N terminus may have the potential to cooperate with the mitotic exit network or with Sic1 or with both, but this is only detectable in highly compromised backgrounds (in the absence of Cdh1, or upon partial inactivation of Cdc14 or Cdc15).

#### DISCUSSION

The possibility of the involvement of Cdc6 in mitotic exit through Cdk inhibition intrigued us for two main reasons. First, it would mean that a protein required for DNA replication would find a second function in another major cellular event, distant both in nature and in time. Second, it would reinforce the idea that Cdk inactivation, through stoichiometric inhibition, is used by the cell as a significant mechanism to achieve mitotic exit. If true, this would contrast with other eukaryotes, for which APC-dependent degradation of B-type cyclins accounts for most or all Cdk inactivation at mitotic exit.

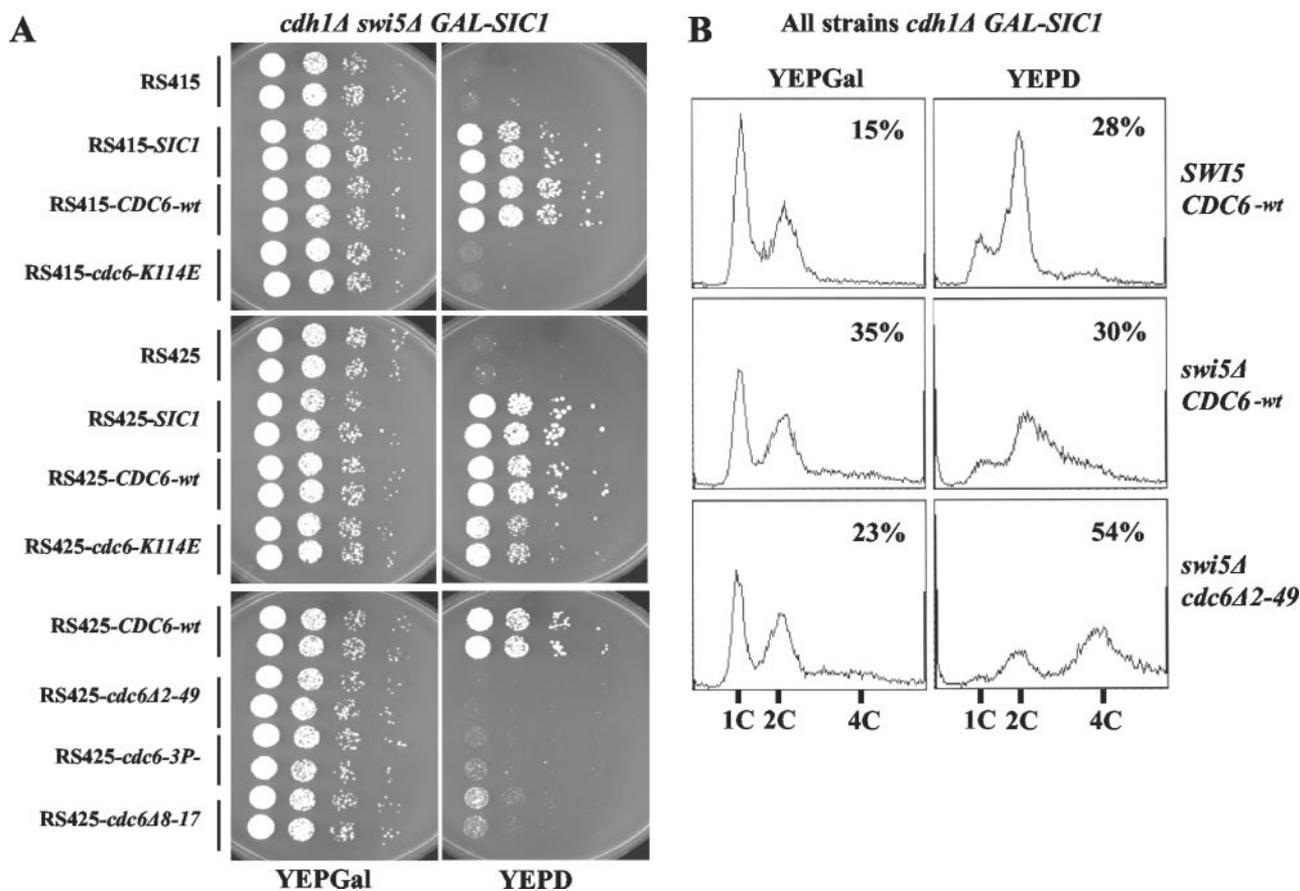
In this work, we show that Cdc6 fills a number of biochemical features consistent with its ability to act as a Clb2-Cdc28 inhibitor contributing to mitotic exit. We also show functional evidence for this role in crippled genetic backgrounds. However, we prove that the function of Cdc6 as Cdk inhibitor is dispensable, even in the absence of Sic1, contradicting a previous report (Calzada *et al.*, 2001).

#### Are the Amounts of Cdc6 Enough to Inhibit Clb2?

Our biochemical observations clearly show that Cdc6 interacts with Clb2 *in vivo*. This is the first set of observations on Cdc6-cyclin interactions made under conditions in which no protein is overexpressed. Quantitative measurements showed that Cdc6 and Clb2 had similar abundances in asynchronous cultures, with Clb2 being in slight excess over Cdc6. We also observed that Clb2 oscillates with higher amplitude than Cdc6 and peaks in budded cells. Therefore, it is reasonable to conclude that Clb2 alone (without counting other mitotic Clbs) exceeds Cdc6 in abundance for most of the time when cells are budded. But if Cdc6 exceeded mitotic Clbs in abundance, sufficient inhibition could potentially be achieved to block mitosis. This is likely to occur when Cdc6 is overexpressed (Bueno and Russell, 1992; Basco *et al.*, 1995; Perkins *et al.*, 2001).

It has been suggested that Cdc6 at normal expression levels is required to prevent mitosis if DNA has not replicated ("reductional mitosis") (Piatti *et al.*, 1995; Weinreich *et al.*, 2001). A novel G1/M checkpoint pathway has been suggested based on the observation that *cdc7* and *dbf4* temperature-sensitive mutants undergo reductional mitosis at the restrictive temperature in some genetic backgrounds, in a way that is independent of the known checkpoint pathways (Toyn *et al.*, 1995). Such a pathway could require Cdc6 (Weinreich *et al.*, 2001). However, it is unclear, based on our quantifications, whether there is enough Cdc6 to account for preventing reductional mitosis if Cdc6 acts a simple stoichiometric inhibitor.

Importantly, at endogenous expression levels, as Clb2 levels fall due to APC activation during mitosis, our quantitation and determination of cell cycle patterns of accumulation (Table 1 and Figure 2) clearly imply that Cdc6 levels will exceed Clb2 levels, allowing the potential for effective



**Figure 8.** *CDH1*, *SWI5*, and *CDC6* interact genetically. (A) *cdh1Δ swi5Δ* mutants are inviable and can be rescued by *SIC1* and by *CDC6*. Rescue by *CDC6* depends on both its N terminus and its DNA-replication activity. *cdh1Δ swi5Δ GAL-SIC1* cells were transformed with plasmids expressing *SIC1*, *CDC6*, *cdc6Δ2-49*, *cdc6-3P-*, *cdc6Δ8-17*, and *cdc6-K114E*. Galactose-grown cultures were serially diluted (10-fold dilutions) on galactose- or glucose-containing rich medium and incubated for 2 d at 30°C. (B) Cells with the indicated genotype were grown in galactose-containing medium and transferred to glucose-containing medium for 3 h. FACS profiles are shown, with the percentage of binucleated cells in the top right corner.

inhibition contributing to mitotic exit. In contrast, Sic1 may not accumulate to significant levels until Clb2 degradation is almost complete (Figure 2), so the primary biological role of Sic1 may be in Clb inhibition in G1, as suggested previously (Wäsch and Cross, 2002; Lengronne and Schwob, 2002).

Although we observe specific Cdc6–Clb2 interaction, the primary function of this interaction may not be inhibition of Clb2-associated kinase activity. It is possible that this interaction is involved in other processes, such as inhibition of pre-replicative complex formation by Clb2 (Nguyen *et al.*, 2001). Further work will be required to evaluate these possibilities.

#### How Much Cdc6 Is Needed for Replication?

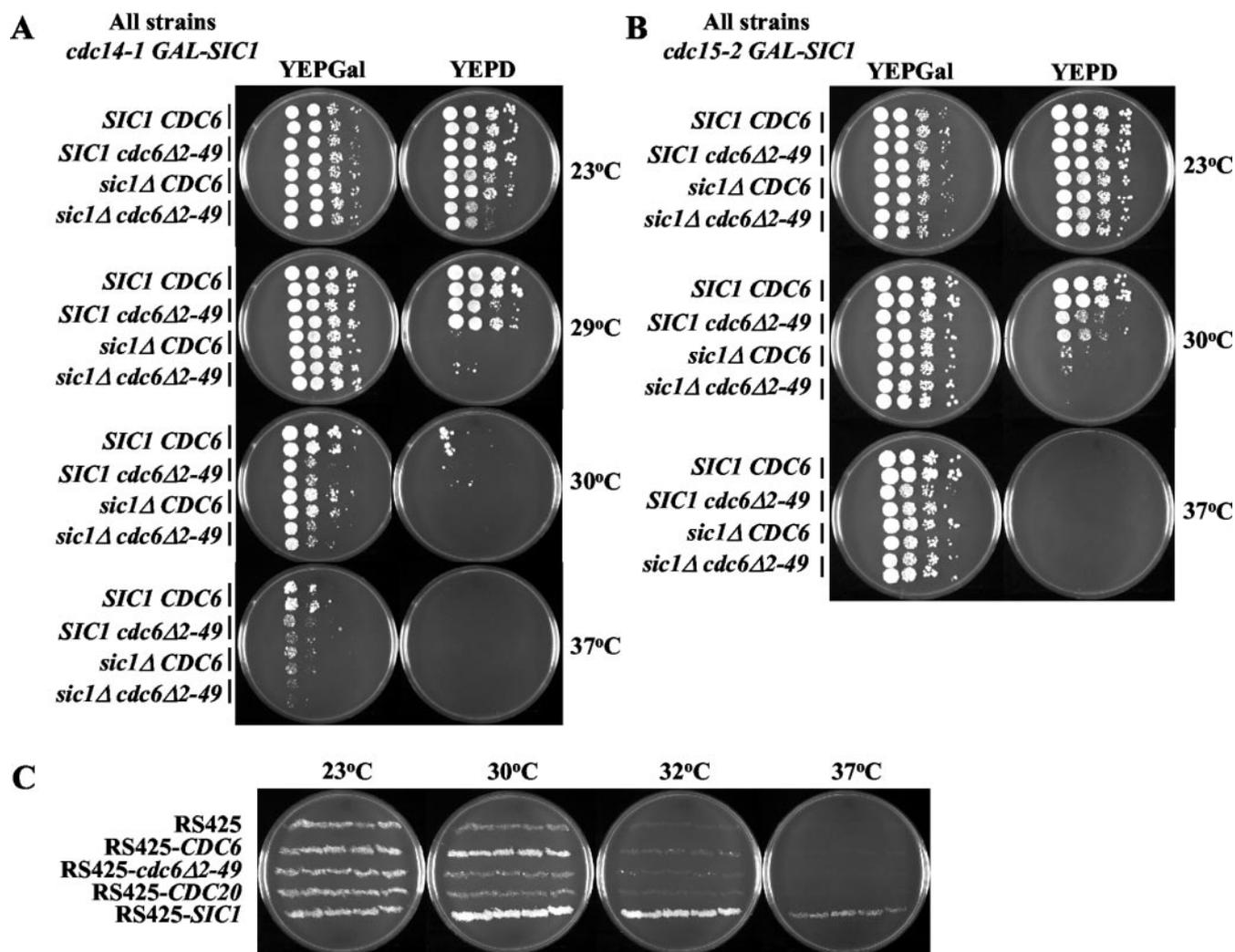
Our quantitation of Cdc6 is relevant to the molecular mechanisms responsible for licensing DNA replication. A recent estimate for the number of functional origins of DNA replication gave a number ~300–400 in haploids (Wyrick *et al.*, 2001). Our estimate of 600–650 for the number of molecules of Cdc6 per diploid cell is in the same narrow range. Therefore, it is possible that only one molecule of Cdc6 is used at each functional origin.

#### Possible Role for Cdk Inhibition by Cdc6 in Mitotic Exit

Overexpression of the well-characterized Clb–Cdk inhibitor *SIC1* can rescue mutants defective in mitotic completion such

as temperature-sensitive mutants of the *DBF2* mitotic kinase (Donovan *et al.*, 1994) and mutants of *CLB2* that fail to degrade Clb2 due to resistance to the APC–proteasome pathway (Wäsch and Cross, 2002). These results suggested that *SIC1* has the potential to promote mitotic exit by lowering mitotic Clb–Cdk activity. In this study, we tested *CDC6* for a similar functional potential using the *CLB2Δdb* and *CLB2Δdb,ken* strains that produce Clb2 proteins that are refractory to APC-mediated degradation. We found that *CDC6*, like *SIC1*, could rescue these mutants, implying that these two Cdk inhibitors have the potential to help mitotic exit through Cdk inhibition.

In addition to testing the ability of *CDC6* to rescue mutants that are defective in mitotic exit, we used a complementary strategy to test the involvement of *CDC6* in mitotic exit. This strategy consisted in testing the effect of deleting the N terminus of Cdc6 in a mutant that barely reaches low enough Cdk activity to exit mitosis, the *cdh1Δ sic1Δ* mutant (Wäsch and Cross, 2002). The inviability of the resulting triple mutant could be explained by a failure in cytokinesis; surprisingly, DNA replication in the succeeding cell cycle was not significantly defective. Consistent with these observations, the reverse experiment showed that extracopies of *CDC6* but not *cdc6Δ2-49* completely rescued the inviability of *cdh1Δ sic1Δ* cells. We also found that the double mutant



**Figure 9.** Genetic interaction of *cdc6Δ2-49* and *sic1Δ* with the mitotic exit network. (A) Interaction with *cdc14-1*. A W303-congenic *cdc14-1* strain was constructed by repeated backcrossing and tetrad analysis, and a *cdc6Δ2-49/+ sic1Δ/+ cdc14-1/+ GAL-SIC1::TRP1/trp1* diploid was constructed. Haploid segregants of the indicated genotype (all *GAL-SIC1 cdc14-1*) were tested for growth by serial dilution and incubation at the indicated temperature, on galactose (*GAL-SIC1* on) or glucose (*GAL-SIC1* off). (B) Interaction with *cdc15-2*. Methods as in A. (C) The indicated plasmids were introduced into a *cdc14-1* strain and replica-plated to plates that were incubated at the indicated temperatures.

*cdh1Δ swi5Δ* showed a striking reduction in viability, which is also rescued by moderate Sic1 or Cdc6 overexpression. This is consistent with the known role of Swi5 as a transcription factor that activates the expression of both *SIC1* and *CDC6* (see INTRODUCTION). In addition, we observed that deletion of the Cdc6 N terminus caused a defect in cytokinesis in the *cdh1Δ swi5Δ* mutant, as with the *cdh1Δ sic1Δ cdc6Δ2-49* mutant.

Another line of evidence pointed at a possible involvement of Cdc6 in mitotic exit, in the context of mutations in the mitotic exit network. We observed an enhanced lethality of *cdc14-1* and *cdc15-2* temperature-sensitive mutants by deletion of the Cdc6 N terminus and a weak suppression of *cdc14-1* by high-copy *CDC6* but not *cdc6Δ2-49* at a semipermissive temperature.

Overall, these experiments support an ancillary role for the Cdc6 N terminus in regulating mitotic exit.

#### Stoichiometric Cdk Inhibitors Are Not Required for Mitotic Exit

Despite the clear potential for the Cdc6 N terminus and Sic1 to function in mitotic exit, we found that the Cdk-inhibitory functions of these genes could be readily removed alone or in combination with no obvious defect in mitotic exit, in three different genetic backgrounds. This clearly contradicts the result of Calzada *et al.* (2001) and demonstrates that Clb-Cdk stoichiometric inhibition by the known inhibitors is not essential for mitotic exit. Because detecting an effect on mitotic exit of removing the Cdc6 N terminus requires highly compromised genetic backgrounds (*cdh1Δ sic1Δ*, *cdh1Δ swi5Δ*, *cdc14ts*, and *cdc15ts*), we conclude that this function of Cdc6 does not contribute significantly to mitotic exit in a wild-type background. The proposal of Calzada *et al.* (2001) represented the first example of essential Cdk control by stoichiometrically binding inhibitors. Because inhibition by Sic1 and Cdc6 is dispensable, as we demonstrated in this study, cyclin

proteolysis dependent on Cdc20 and Cdh1 may be the central and evolutionarily conserved Cdk-inactivating event regulating mitotic exit (Wäsch and Cross, 2002).

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