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Citation for published version:
Rudner, AD, Hardwick, KG & Murray, AW 2000, 'Cdc28 activates exit from mitosis in budding yeast', *Journal of Cell Biology*, vol. 149, no. 7, pp. 1361-76. https://doi.org/10.1083/jcb.149.7.1361

Digital Object Identifier (DOI):
10.1083/jcb.149.7.1361

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Journal of Cell Biology

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Download date: 24. Aug. 2022

Cdc28 Activates Exit from Mitosis in Budding Yeast

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Abstract. The activity of the cyclin-dependent kinase 1 (Cdk1), Cdc28, inhibits the transition from anaphase to G1 in budding yeast. *CDC28-T18V, Y19F* (*CDC28-VF*), a mutant that lacks inhibitory phosphorylation sites, delays the exit from mitosis and is hypersensitive to perturbations that arrest cells in mitosis. Surprisingly, this behavior is not due to a lack of inhibitory phosphorylation or increased kinase activity, but reflects reduced activity of the anaphase-promoting complex (APC), a defect shared with other mutants that lower Cdc28/Clb activity in mitosis. *CDC28-VF* has reduced Cdc20-dependent APC activity in mitosis, but normal Hct1-dependent APC activity in the G1 phase of the cell cycle. The defect in Cdc20-dependent APC activity in *CDC28-VF* correlates with reduced association of Cdc20 with the APC. The defects of *CDC28-VF* suggest that Cdc28 activity is required to induce the metaphase to anaphase transition and initiate the transition from anaphase to G1 in budding yeast.

Key words: anaphase-promoting complex • Hct1 • Cdc20 • Pds1 • sister chromatid separation

Introduction

To exit mitosis, cells must accomplish two tasks: chromosome segregation and inactivation of complexes between mitotic cyclins and cyclin-dependent kinase 1 (Cdk1)[1], known as Cdc28 in budding yeast and Cdc2 in other eukaryotes, which allows the cell cycle to progress into G1. Both steps require the activity of the anaphase-promoting complex (APC) or cyclosome, a multiprotein complex that is required for the ubiquitination of cyclin and other unstable substrates (King et al., 1995; Sudakin et al., 1995; Zachariae and Nasmyth, 1996, 1999; Yanagida et al., 1999). The activity of the APC depends on its interaction with two WD-40 proteins, Cdc20 (Sethi et al., 1991; Sigrist et al., 1995; Visintin et al., 1997; Lorca et al., 1998) and Hct1 (Schwab et al., 1997; Sigrist and Lehner, 1997; Visintin et al., 1997; Yamaguchi et al., 1997; Kitamura et al., 1998). Cdc20 initiates the metaphase to anaphase transition by inducing ubiquitination of the anaphase inhibitor, Pds1 (Cohen-Fix et al., 1996; Funabiki et al., 1997). This reaction causes Pds1 degradation and sister chromatid separation (Funabiki et al., 1996; Ciosk et al., 1998). Cdc20-dependent APC activity is inhibited by the spindle checkpoint, which senses defects in chromosome attachment to the spindle and delays the onset of anaphase until these defects are corrected (Rieder et al., 1995; Fang et al., 1998a; Hardwick, 1998; Hwang et al., 1998; Kim et al., 1998).

The second WD-40 protein, Hct1, also activates the APC (Schwab et al., 1997; Sigrist and Lehner, 1997; Visintin et al., 1997; Kramer et al., 1998). In budding yeast, Hct1-dependent APC activity is necessary for the ubiquitination and degradation of Clb2, the major mitotic cyclin, which causes the sudden drop in the protein kinase activity of Cdc28 at the end of mitosis and keeps the APC active throughout G1 (Zachariae et al., 1998; Jaspersen et al., 1999). The activation of the Hct1-dependent APC depends on the prior activation of the Cdc20/APC, and this dependency helps ensure that the events of mitosis occur in the proper sequence (Lim et al., 1998; Visintin et al., 1998; Shirayama et al., 1999; Yeong et al., 2000).

In budding yeast, active Cdc28 inhibits the transition from anaphase to G1 (Amon, 1997; Li and Cai, 1997). One of the primary targets that Cdc28/Clb2 complexes inhibit is Hct1. Phosphorylation of Hct1 by Cdc28/Clb complexes prevents it from binding to and activating the APC (Zachariae et al., 1998; Jaspersen et al., 1999). This inhibition is opposed by the phosphatase Cdc14, which de-phosphorylates Hct1, initiating a positive feedback loop that drives the cell into G1 (Visintin et al., 1998; Jaspersen et al., 1999). As Hct1 activity rises, the rate of Clb destruction increases, reducing the kinase activity of Cdc28 and further activating Hct1. The CDK inhibitor, Sic1 (Mendenhall, 1993; Donovan et al., 1994), which inhibits Cdc28/Clb

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[1] *Abbreviations used in this paper:* APC, anaphase-promoting complex; Cdk, cyclin-dependent kinase; DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein.

© The Rockefeller University Press, 0021-9525/2000/06/1361/16 $5.00
The Journal of Cell Biology, Volume 149, Number 7, June 26, 2000 1361–1376
http://www.jcb.org

complexes, also participates in this feedback loop, since both its transcription and stability are inhibited by Cdc28/Clb activity (Amon, 1997; Toyn et al., 1997; Visintin et al., 1998).

Does Cdc28 also play a role in inducing anaphase? In frog and clam egg extracts, activation of the APC depends on active Cdc2/Cyclin B complexes (Felix et al., 1990; Hershko et al., 1994; Minshull et al., 1994; Shteinberg and Hershko, 1999). This activating role might be explained by the finding that Cdc2/Cyclin B can phosphorylate subunits of the APC (Patra and Dunphy, 1998; Kotani et al., 1999), and this phosphorylation is correlated with activating the APC and binding of Fizzy, the Cdc20 homologue in frogs (Peters et al., 1996; Fang et al., 1998b; Kotani et al., 1998; Shteinberg et al., 1999). In embryonic cell cycles, no Hct1 homologue is present (Sigrist and Lehner, 1997; Lorca et al., 1998), suggesting that Cdc20-dependent APC activity targets both Pds1 homologues and mitotic cyclins for destruction. In budding yeast, the inability of Cdc20 to catalyze the complete destruction of mitotic cyclins means that Hct1 or Sic1 is required for cells to exit from mitosis (Schwab et al., 1997; Visintin et al., 1997). Since both Hct1 and Sic1 are inhibited by Cdc28, it has been difficult to ask whether a previous step in the exit from mitosis requires Cdc28.

Cdc28/Clb activity is also regulated by inhibitory phosphorylation on residues tyrosine 19, and possibly threonine 18 (Amon et al., 1992; Sorger and Murray, 1992; Booher et al., 1993). Tyrosine 19 is phosphorylated by Swe1 (the homologue of Wee1 in fission yeast) and dephosphorylated by Mih1 (the homologue of Cdc25 in fission yeast; Russell et al., 1989; Booher et al., 1993). Phosphorylation on the homologous sites of fission yeast and vertebrate Cdc2 controls the timing of entry into mitosis and also can be induced by the checkpoints that detect unreplicated or damaged DNA (Gould and Nurse, 1989; Enoch and Nurse, 1990; Norbury et al., 1991; Jin et al., 1996; Rhind et al., 1997). In budding yeast, tyrosine 19 is phosphorylated during S-phase, but *CDC28-T18A, Y19F* (*CDC28-AF*) cells respond normally to the DNA damage checkpoint (Amon et al., 1992; Sorger and Murray, 1992). Inhibitory phosphorylation of Cdc28 is required for the bud emergence or morphogenesis checkpoint, which delays mitosis in cells that have not budded (Lew and Reed, 1993).

Our previous work suggested that inhibitory phosphorylation of Cdc28 might aid in mitotic exit. *cdc55*Δ cells, which lack a B subunit of protein phosphatase 2A (PP2A; Healy et al., 1991), are spindle checkpoint defective and have increased phosphorylation on tyrosine 19 of Cdc28 (Minshull et al., 1996; Wang and Burke, 1997). The premature exit from mitosis in *cdc55*Δ cells with damaged spindles is suppressed by *CDC28-T18V, Y19F* (*CDC28-VF*), suggesting that inhibitory phosphorylation of Cdc28 might work in concert with Hct1 and Sic1 to reduce Cdc28-associated activity at the end of mitosis.

Here, we show that this hypothesis is incorrect. Although *CDC28-VF* has defects in leaving mitosis, these are not due to a lack of inhibitory phosphorylation, but reflect a second defect of *CDC28-VF*. This defect slows the normal activation of the Cdc20-dependent APC and reveals that Cdc28-associated activity is essential for the activation of the APC in mitosis.

Materials and Methods

Strain and Plasmid Construction

Table I lists the strains used in this work. All strains are derivatives of the W303 strain background (W303-1a; Rodney Rothstein, Columbia University, NY). Standard genetic techniques were used to manipulate yeast strains (Sherman et al., 1974) and standard protocols were used for DNA manipulation (Maniatis et al., 1982). All deletions were confirmed by PCR or by mutant phenotype. The sequences of all oligonucleotide primers used in this study are available upon request. The strains TG1 and DH5α were used for all bacterial manipulations.

The strains used for the crosses in Table II were: JM434 (*CDC28-VF*), JM469 (*CDC28-V*), JM467 (*CDC28-F*), ADR1541 (*CDC28-AF*), ADR2035 (*CDC28-F88G*), KH208 (*swe1*Δ), ADR484 (*cdc28-1N*), ADR840 (*cdc28-4*), ADR314 (*clb2*Δ), ADR719 (*cdc23-1*), ADR1147 (*cdc16-1*), LH226 (*doc1-1*), LH125 (*cdc20-1*), ADR1435 (*hct1*Δ), JC126 (*cdc5-1*), ADR1298 (*cdc13-1*), and K1993 (*cdc15-2*). For all crosses, at least 22 tetrads were analyzed.

BAR1 was deleted using pJGsst1 (a gift of Jeremy Thorner, University of California, Berkeley, CA). *PDS1-myc18* strains were made using a *PDS1-myc18* replacement plasmid (Shirayama et al., 1998). *pCUP-GFP12-lacI12, lacO:TRP1* and *lacO:LEU2* were integrated using pSB116 (Biggins et al., 1999), pAFS52, and pAFS59 (Straight et al., 1996), respectively. *GAL-MPS1* strains were made with pAFS120 (Hardwick et al., 1996). *MAD3* was deleted using pKH181 (Hardwick et al., 2000). *swe1*Δ strains were made by crossing JM449 (a gift of Jeremy Minshull, Maxygen, Redwood City, CA) to the appropriate strains. *MIH1* was deleted using pIP33 (a gift of Peter Sorger, Massachusetts Institute of Technology, Cambridge, MA). *CDC55* was deleted using pJM6 (Minshull et al., 1996). *clb2*Δ strains were made by crossing K1890 (Surana et al., 1991) to the appropriate strains. *CDC28-F88G* strains were made by crossing JAU02 (a gift of Jeff Ubersax, University of California, San Francisco, CA) to the appropriate strains. The 2μ-CDC28 plasmid is EF190 (a gift of Eric Foss, Fred Hutchinson Cancer Research Institute, Seattle, WA) and is the *CDC28* gene cloned into pRS425 (Sikorski and Hieter, 1989). *pHIS3-GFP-TUB1* was integrated using pAFS71 (a gift of Aaron Straight, Harvard Medical School, Boston, MA). *cdc20-3* strains were made by crossing K8029 (Shirayama et al., 1998) to the appropriate strains. *pGAL-PDS1-HA* strains were made by crossing RTK43 (Jaspersen et al., 1998) to the appropriate strains.

Mutants in *CDC28-VF-HA*, *CDC28-F-HA*, *CDC28-V-HA*, and *CDC28-HA* were made as described previously (Booher et al., 1993). *CDC28-AF-HA* was made by cloning the HindIII/XhoI fragment from pSF35 (Peter Sorger, MIT, Cambridge, MA) into pRD96 (a gift of Ray Deshaies, California Institute of Technology, Pasadena, CA) cut with HindIII/XhoI to create pAR155, which was used to create a *CDC28-AF-HA* strain as previously described for pRD96 (Booher et al., 1993). The *pGAL-CDC28* plasmids were made as follows: full-length *CDC28-HA* (pAR106) was made by cutting out the 1.1-kb XhoI-HindIII fragment of *CDC28-VF-HA* from pRD96 and replacing it with the same fragment from pRD47. The BstBI-BamHI fragment of pRD96 or pAR106 was then cloned into pDK20 (a gift of Doug Kellogg, University of California, Santa Cruz, CA) cut with SmaI and BamHI, to create pAR109 (yIP-*pGAL-CDC28:URA3*) and pAR108 (yIP-*pGAL-CDC28-VF:URA3*). The plasmids were cut with Stu1 and integrated at the *URA3* locus.

pGAL-CLB2-Δ176 strains were made with pAR39. An EcoRI/BamHI fragment of *CLB2* that lacks the first 176 amino acids was amplified by PCR and cloned into pDK20 cut with EcoRI and BamHI to create pAR39. The plasmid was cut with StuI for integration at the *URA3* locus.

cks1-38 strains are *cks1*Δ::KAN^R covered by *cks1-38* integrated at *TRP1* (pAR183). *CKS1* was deleted by the PCR-targeting method. Diploid cells were transformed with a cassette containing the bacterial *KAN^R* gene, which confers G418 resistance in W303. The cassette was amplified by PCR from pFA6a-kanMX6 (Longtine et al., 1998) with primers that contain the sequences that flank the *CKS1* open reading frame. pAR183 (*cks1-38:TRP*) was constructed by cloning an BamHI/SphI fragment from pSE271 (Tang and Reed, 1993) into YIplac204 (Gietz and Sugino, 1988), the resulting plasmid was cut with EcoRV and integrated into the *CKS1/cks1*Δ::KAN^R heterozygote at the *TRP1* locus. The diploid was sporulated, and a resulting spore, ADR1767, which is temperature sensitive, *TRP$^+$*, and *KAN^R* was used to create the strains used in this study.

HCT1 was deleted using pAR127. An EcoRI/HindIII fragment of the *HCT1* locus was amplified by PCR and cloned into pSK(−) (Stratagene) to create pAR125. An XbaI/SmaI fragment of the *HIS3* gene was then

Table I. Strain List

Name	*MAT*	Relevant genotype*	Source
ADR314	α	*clb2Δ::LEU2*	This study
ADR477	**a**	*CDC28-HA:URA3*	This study
ADR484	α	*cdc28-1N*	This study
ADR509	**a**	*CDC28-VF-HA:URA3*	This study
ADR548	**a**	*CDC28-HA:URA3 leu2-3,112:lacO:LEU2 his3-11,15:pHIS3-GFP-lacI:HIS3*	This study
ADR684	**a**	*swe1Δ::TRP1 CDC28-HA:URA3*	This study
ADR719	**a**	*cdc23-1*	This study
ADR840	α	*cdc28-4*	This study
ADR1100	**a**	*CDC28-V-HA:URA3 swe1Δ::TRP1 ura3-1:pGAL-MPS1:URA3*	This study
ADR1105	**a**	*CDC28-V-HA:URA3 ura3-1:pGAL-MPS1:URA3*	This study
ADR1147	**a**	*cdc16-1*	This study
ADR1248	**a**	*mad3Δ::LEU2 ura3-1:pGAL-MPS1:URA3*	This study
ADR1252	**a**	*CDC28-VF-HA:URA3 bar1Δ*	This study
ADR1298	**a**	*cdc13-1*	This study
ADR1314	**a**	*mih1Δ::LEU2 CDC28-HA:URA3*	This study
ADR1378	**a**	*mih1Δ::LEU2 ura3-1:GAL-MPS1:URA3*	This study
ADR1389	**a**	*CDC28-HA:URA3 bar1Δ*	This study
ADR1435	**a**	*hct1Δ::HIS3*	This study
ADR1506	**a**	*CDC28-AF-HA:URA3 ura3-1:pGAL-MPS1:URA3*	This study
ADR1541	α	*CDC28-AF-HA:URA3*	This study
ADR1606	**a**	*clb2Δ::LEU2 ura3-1:pGAL-MPS1:URA3*	This study
ADR1685	**a**	*cdc23-1 ura3-1:pGAL-CDC28-HA:URA3 trp1-1:lacO:TRP1 his3-11,15:pCUP1-GFP-lacI:HIS3 bar1Δ*	This study
ADR1687	**a**	*cdc23-1 ura3-1:pGAL-CDC28-VF-HA:URA3 trp1-1:lacO:TRP1 his3-11,15:pCUP1-GFP-lacI:HIS3 bar1Δ*	This study
ADR1736	**a**	*cdc15-2 CDC28-VF-HA:URA3 trp1-1:pGAL-PDS1-HA:TRP1 bar1Δ*	This study
ADR1743	**a**	*cdc15-2 CDC28-HA:URA3 trp1-1:pGAL-PDS1-HA:TRP1 bar1Δ*	This study
ADR1774	**a**	*cdc15-2 clb2Δ:LEU2 CDC28-HA:URA3 trp1-1:pGAL-PDS1-HA:TRP1 bar1Δ*	This study
ADR1783	**a**	*cdc20-3 ura3-1:pGAL-CLB2-Δ176:URA3 PDS1-myc18:LEU2 his3-11,15:pHIS3-GFP-TUB1:HIS3 bar1Δ*	This study
ADR1786	**a**	*hct1Δ::HIS3 ura3-1:pGAL-CLB2-Δ176:URA3 PDS1-myc18:LEU2 his3-11,15:pHIS3-GFP-TUB1:HIS3*	This study
ADR1790	**a**	*cdc15-2 CDC20-myc12 CDC28-HA:URA3*	This study
ADR1793	**a**	*cdc15-2 CDC20-myc12 CDC28-VF-HA:URA3*	This study
ADR1857	**a**	*CDC28-VF-HA:URA3 PDS1-myc18:LEU2 trp1-1:lacO:TRP1 his3-11,15:pCUP1-GFP-lacI:HIS3 bar1Δ*	This study
ADR1859	**a**	*CDC28-HA:URA3 PDS1-myc18:LEU2 trp1-1:lacO:TRP1 his3-11,15:pCUP1-GFP-lacI:HIS3 bar1Δ*	This study
ADR1870	**a**	*ura3-1:pGAL-CLB2-Δ176:URA3 PDS1-myc18:LEU2 his3-11,15:pHIS3-GFP-TUB1:HIS3 bar1Δ*	This study
ADR1899	**a**	*cdc28-1N ura3-1:pGAL-MPS1:URA3*	This study
ADR1901	**a**	*cdc28-4 ura3-1:pGAL-MPS1:URA3*	This study
ADR1903	α	*cks1Δ::KAN^R trp1-1:cks1-38:TRP1 ura3-1:pGAL-MPS1:URA3*	This study
ADR1921	**a**	*cdc20-3 trp1-1:pGAL-PDS1-HA:TRP1 bar1Δ*	This study
ADR1925	**a**	*cdc28-13 trp1-1:pGAL-PDS1-HA:TRP1*	This study
ADR1928	**a**	*cdc28-13 hct1Δ::HIS3 trp1-1:pGAL-PDS1-HA:TRP1*	This study
ADR1959	**a**	*CDC28-HA-VF:URA3 trp1-1:pGAL-PDS1-HA:TRP1 bar1Δ*	This study
ADR1968	**a**	*CDC28-HA:URA3 trp1-1:pGAL-PDS1-HA:TRP1 bar1Δ*	This study
ADR2034	**a**	*CDC28-F88G ura3-1:pGAL-MPS1:URA3*	This study
ADR2035	α	*CDC28-F88G ura3-1:pGAL-MPS1:URA3*	This study
JC126	α	*cdc5-1*	Julia Charles
JM434	α	*CDC28-VF-HA:URA3*	Jeremy Minshull
JM445	**a**	*cdc55Δ::HIS3 CDC28-HA:URA3*	Jeremy Minshull
JM467	α	*CDC28-F-HA:URA3*	Jeremy Minshull
JM469	α	*CDC28-V-HA:URA3*	Jeremy Minshull
JM477	**a**	*CDC28-VF-HA:URA3 leu2-3,112:lacO:LEU2 his3-11,15:pHIS3-GFP-lacI:HIS3*	Jeremy Minshull
K1993	**a**	*cdc15-2*	Kim Nasmyth
KH153[‡]	**a**	*ura3-1:pGAL-MPS1:URA3*	this study
KH181	**a**	*CDC28-VF-HA:URA3 ura3-1:pGAL-MPS1:URA3*	This study
KH183	**a**	*CDC28-VF-HA:URA3 mad3Δ::LEU2 ura3-1:pGAL-MPS1:URA3*	This study
KH204	**a**	*CDC28-F-HA:URA3 ura3-1:pGAL-MPS1:URA3*	This study
KH207	**a**	*swe1Δ::TRP1 ura3-1:pGAL-MPS1:URA3*	This study
KH208	α	*swe1Δ::TRP1 ura3-1:pGAL-MPS1:URA3*	This study
LH125	**a**	*cdc20-1*	Lena Hwang
LH226	**a**	*doc1-1*	Lena Hwang

*All strains are isogenic to W303-1a (*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*).
‡All *pGAL-MPS1* strains are derived from crosses with KH153.

cloned into pAR125 cut with SpeI/XmnI, to create pAR127, which replaces the entire *HCT1* open reading frame with *HIS3*. The EcoRI/NotI fragment of pAR127 was used to transform yeast.

CDC20 was tagged at the NH_2 terminus. A cassette containing 12 myc tags (pLH71) was inserted into pCM4 (Hwang et al., 1998) cut with BsteII, to create pLH83. The resultant plasmid was cut with EcoNI and BglII, removing the *CEN-ARS* sequences, blunted, and religated to create pLH92. This plasmid was cut with BspEI for integration at the *CDC20* lo-

Table II. Genetic Interactions of CDC28-VF

	CDC28-VF	*CDC28-V*	*CDC28-F*	*CDC28-AF*	*swe1Δ*	*CDC28-F88G*	*cdc28-1N*	*clb2Δ*	*cdc28-4*
cdc23-1	sl	sl	sl	sl	+	sl	sl	sl	+
cdc16-1	sl	+	+	+	+	+	ND	ND	+
doc1-1	sl								
cdc20-1	sl								
hct1Δ	sl								
cdc5-1	sl								
cdc13-1	+								
cdc15-2	+								

sl, Synthetic lethal; ND, not determined; +, viable.

cus, creating a duplication of *CDC20* and *CDC20-12myc* marked with the *URA3* gene. This strain was grown on 5-fluoroorotic acid to select against the *URA3* gene, and the resulting colonies were screened by Western blot for the presence of *CDC20-12myc* to create LHH371. This strain was crossed to the appropriate strains to create those used in this study.

Physiology

Stock solutions of inhibitors were: 10 mg/ml alpha factor (Biosynthesis); 10 mg/ml nocodazole (Sigma-Aldrich). All stocks were stored at −20°C in DMSO.

For microcolony assays, cells were grown to mid-log phase in yeast extract and peptone (YEP) + 2% raffinose, spotted onto a YEP + 2% galactose plate, and unbudded cells were picked out into a grid with a dissection needle. The number of cells in each microcolony was counted at different times after incubation at 30°C. Each bud is counted as a cell, and the original cells that did not bud are not included in the analysis.

For serial dilution and spotting, cells were prepared in a multiwell dish or a microtiter plate, and using a multiprong applicator, ∼10 μl of each strain and its dilutions were spotted onto various plates and incubated at either 23 or 30°C.

Sister chromatid separation was visualized using a fusion of green fluorescent protein (GFP) and lacI bound to repeats of *lacO*, which had been integrated at specific locations on yeast chromosomes (Straight et al., 1996). Small samples of cells were harvested at the indicated times and either scored live, or fixed for 10 min in 3.7% paraformaldehyde at 4°C, and then washed twice in 0.1 M KPO_4 pH 7.4. The cells were then mounted on slides and viewed by fluorescence microscopy (Nikon). In all experiments, a minimum of 200 cells were counted per time point.

The anaphase arrest of *pGAL-CLB2*-Δ176 was visualized using *pHIS3-GFP-TUB1*. GFP-Tub1 was induced by transferring cells to complete synthetic medium (CSM)-His + 2% galactose + 10 mM 3-aminotriazole for the first 30 min after alpha factor removal. Cells were then transferred into YEP + 2% galactose. Spindle length was scored in living cells by fluorescence microscopy. The anaphase arrest of *cdc15-2* was scored by fixing cells for 5 min in 70% ethanol, washing in 50 mM Tris-Cl, pH 7.5, and then resuspending in 50 mM Tris-Cl, pH 7.4, + 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes). Cells were then mounted on slides and viewed by fluorescence microscopy.

Immunoprecipitation and Western Blots

In experiments where only Western blots are shown, yeast extracts were prepared by bead beating (multitube bead beater; Biospec) frozen cell pellets in 1× SDS sample buffer (2% SDS, 80 mM Tris-Cl, pH 6.8, 10% glycerol, 10 mM EDTA, 0.02% bromophenol blue, 1 mM Na_3VO_4, 1 mM PMSF, and 5 mM NaF) and an equal volume of acid washed glass beads (Biospec) for 1 pulse of 90 s. Samples were then normalized based on OD_{600} readings of the original yeast samples taken during the time course. We have found that this method allows even loading of the samples and works as well as other techniques.

Yeast extracts for immunoprecipitation and Western blots were made by bead beating frozen cell pellets in lysis buffer (100 mM NaCl, 50 mM Tris-Cl, pH 7.5, 50 mM NaF, 50 mM Na-β-glycerophosphate, pH 7.4, 2 mM EGTA, 2 mM EDTA, 0.1% Triton X-100, 1 mM Na_3VO_4, 1 mM PMSF, and leupeptin, pepstatin and chymostatin all at 1 μg/ml) and an excess of acid washed glass beads for 2 pulses of 90 s, incubating on ice for 5 min between each pulse. The resulting lysate was separated from the glass beads and centrifuged in a microfuge at 14,000 rpm for 5 min to remove insoluble material. Protein concentration of each lysate was determined using Bradford reagent (0.04% Coomassie blue G-250 dissolved in 4.75% ethanol, then mixed with 8.5% o-phosphoric acid and H_2O) and samples were normalized based on these measurements. A portion of the lysate was mixed with an equal volume of 2× SDS sample buffer. Standard methods were used for PAGE and protein transfer to nitrocellulose (Schleicher and Schuell; Minshull et al., 1996). Blots were stained with Ponceau S to confirm transfer and equal loading of samples, and then blocked for 30 min in antibody-specific blocking buffer (see below). All antibodies were incubated overnight at 4°C or 2 h at 25°C. After washing in PBST (PBS+ 0.1% Tween-20; Maniatis et al., 1982), the blots were then incubated in HRP-conjugated anti-rabbit or anti-mouse antibodies (Amersham Pharmacia Biotech) at a 1:5,000 dilution in PBST for 30 min at 25°C, washed again, incubated in ECL detection reagents (Amersham Pharmacia Biotech) or Renaissance reagents (NEN Life Science Products), using the manufacturer's instructions, and then exposed to X-OMAT film (Kodak).

The following antibodies were used in Western blots: 9E10 ascites (BabCO) was used at a dilution of 1:1,000 in PBST + 0.02% NaN_3 after blocking in 4% nonfat dried milk in PBST. Affinity-purified rabbit polyclonal anti-Clb2 and anti-Clb3 antibodies (Kellogg and Murray, 1995) were used at a dilution of 1:1,200 in blocking buffer (2% BSA, PBST, 0.5M NaCl, 0.02% NaN_3). Rabbit polyclonal anti-Sic1 serum (a gift of Mike Mendenhall, University of Kentucky, Lexington, KY) was used at 1:1,000 in blocking buffer (4% nonfat dried milk, 2% BSA, PBST, 0.02% NaN_3) + 10 μg/ml cell lysate made from *sic1Δ* cells (JM408, made with MDMp203; a gift of Mike Mendenhall). 12CA5 ascites (BabCO) was used at 1:1,000, rabbit polyclonal anti-Cdc16 and anti-Cdc23 (Lamb et al., 1994) were used at 1:2,000, and anti-Cdc27 (Lamb et al., 1994) was used at 1:2,500. These four antibodies were all diluted in blocking buffer (4% nonfat dried milk, PBST, 0.02% NaN_3).

The remaining lysate was used for immunoprecipitation. 2–20 mg of lysate per sample was used depending on the experiment. 0.33–3 μg of antibody was added to the lysate and incubated on ice for 20 min. Samples were then centrifuged in a microfuge at 14,000 rpm for 5 min at 4°C and transferred to 10–15 μl of protein A CL-4B Sepharose beads (Sigma-Aldrich) that had been equilibrated in lysis buffer. The beads were rotated at 4°C for 1–2 h. The beads were manipulated as described below.

Histone H1 Kinase Assays

For histone H1 kinase reactions, 0.33 μg of anti-Clb2 or anti-Clb3 antibody was used for immunoprecipitation in 1–5 mg of cell lysate. After immunoprecipitation, the beads were washed three times in kinase bead buffer (500 mM NaCl, 50 mM Tris-Cl, pH 7.4, 50 mM NaF, 5 mM EGTA, 5 mM EDTA, 0.1% Triton X-100; transferring the beads to fresh tubes after the second wash) and twice in kinase buffer (80 mM Na-β-glycerophosphate, pH 7.4, 15 mM $MgCl_2$, 20 mM EGTA). All washes were performed on ice. Kinase reactions were performed in 15 μl of kinase buffer containing 1 mM DTT, 25 μM ATP, 2.5 μg histone H1 (Upstate Biotechnology) and 1 μCi of $\gamma[^{32}P]$ATP (Amersham Pharmacia Biotech) and were incubated for 15 min at 25°C. Reactions were stopped by adding 15 μl of 2× SDS sample buffer and heating samples to 99°C for 5 min. Samples were run on a 15% polyacrylamide gel, stained, and dried. Kinase gels were quantified using a Molecular Dynamics PhosphoImager and ImageQuant software.

Phosphotyrosine Detection

For antiphosphotyrosine blots, 2 μg of 12CA5 antibody was used to immunoprecipitate Cdc28-HA or Cdc28-VF-HA from 15–20 mg of cell ly-

sate. The beads were washed three times in kinase bead buffer and twice in PBS. Standard methods were used for PAGE and protein transfer to nitrocellulose. The blot was then blocked in P-Tyr blocking buffer (1% BSA, 10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) and incubated overnight at 4°C in blocking buffer containing an anti-P-Tyr Fab fragment conjugated to HRP, RC20H (Transduction Laboratories), diluted to 1:2,500. The blot was washed five times in TBST (10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 0.1% Tween 20), incubated in SuperSignal chemiluminescent substrate (Pierce Chemical Co.), using the manufacturer's instructions, and then exposed to X-ray film. After exposure, the blot was stripped in an SDS-containing buffer, and reprobed with the 12CA5 antibody to confirm that the same amount of Cdc28 had been immunoprecipitated.

APC Assay

For APC assay, 1 μg of rabbit polyclonal anti-Cdc26 antibody (Hwang and Murray, 1997) was used to immunoprecipitate the APC from 5 mg of cell lysate. The APC assay was conducted as previously described (Charles et al., 1998), except that cells were lysed in the lysis buffer described above, the beads were washed in APC bead buffer (250 mM NaCl, 50 mM Tris-Cl, pH 7.4, 50 mM NaF, 5 mM EGTA, 5 mM EDTA, 0.1% NP-40, 1 mM DTT) three times, and then twice in QA+NaCl buffer (20 mM Tris-Cl, pH 7.6, 100 mM NaCl, 1 mM $MgCl_2$). 25 nM of purified Hct1 (Jaspersen et al., 1999) was added to some samples during the ubiquitination reaction.

Cdc20 Binding to the APC

For Cdc20 binding experiments, 3 μg of anti-Cdc26 antibody was used to immunoprecipitate 10–20 mg of cell lysate. After immunoprecipitation, the beads were washed three times in Cdc20 bead buffer (200 mM NaCl, 50 mM Tris-Cl, pH 7.4, 50 mM NaF, 5 mM EGTA, 5 mM EDTA, 0.1% NP-40, 1 mM DTT), and then twice in low salt kinase buffer (10 mM NaCl, 20 mM Hepes-KOH, pH 7.4, 5 mM $MgCl_2$). The immunoprecipitates were then processed for Western blots as described above.

Results

CDC28-VF Impairs Mitotic Exit

Since *CDC28-VF* suppresses the checkpoint defect of *cdc55Δ* (Minshull et al., 1996), we wondered if the *CDC28-VF* mutant alone might have difficulty leaving mitosis. Fig. 1 A shows that progression through mitosis is delayed in *CDC28-VF*. Wild-type and *CDC28-VF* cells were arrested in G1 by the mating pheromone alpha factor and released into the cell cycle. Once cells had budded, alpha factor was readded to arrest cells that had completed the cycle. This regimen allows us to look clearly at one synchronous cell cycle. *CDC28-VF* cells show a 30-min delay in the degradation of the anaphase inhibitor Pds1 and sister chromatid separation. Clb2 proteolysis and the fall in Clb2-associated kinase activity are delayed by >30 min. These delays can be partially attributed to a 15-min delay in Clb2 accumulation, but the persistence of peak levels of Clb2 for at least 60 min clearly reflects an additional defect in *CDC28-VF*. In addition, mitotic entry is not delayed because short spindles, a marker for mitotic entry, appear at the same time in *CDC28-VF* and wild-type cells (data not shown). *CDC28-VF* does not delay exit from G1, since the CDK inhibitor, Sic1 (Schwob et al., 1994), disappears at the same time (30 min) in wild-type and *CDC28-VF*.

Although *CDC28-VF* delays passage through mitosis, the doubling time of *CDC28-VF* cells is nearly identical to that of wild-type cells (data not shown). This apparent paradox can be explained by the fact that *CDC28-VF* cells exit mitosis at a larger cell size than wild-type cells, and therefore have to grow less in G1 to reach the critical cell size needed to pass Start, the cell cycle transition that commits them to replicating their DNA (Johnston et al., 1977). Thus, the increase in time spent in mitosis is made up by a decrease in time spent in G1.

To see if the mitotic delay in *CDC28-VF* is due to difficulty exiting mitosis, we examined cells that were recovering from activation of the spindle checkpoint. Wild-type and *CDC28-VF* cells were arrested in mitosis by treating them with nocodazole (an inhibitor of microtubule polymerization) for three hours and then released from this arrest into fresh medium containing alpha factor to arrest them in G1 as they left mitosis. Wild-type cells degrade Clb2, inactivate Clb2-associated kinase, and separate their sisters within 90 min of removing nocodazole (Fig. 1 B). *CDC28-VF* cells, however, take 150–180 min to fully escape from the nocodazole arrest.

Since *CDC28-VF* cells are delayed in exiting mitosis, we investigated how they responded to a prolonged mitotic arrest caused by the spindle checkpoint. We examined the response to overexpressing the protein kinase Mps1, which arrests cells in mitosis by activating the checkpoint, but does not damage the spindle (Hardwick et al., 1996). After about eight hours, wild-type cells overcome the arrest, divide, and resume proliferating. In contrast, *CDC28-VF* cells overexpressing Mps1 cannot proliferate (Fig. 2 A); when individual cells are followed microscopically, many never divide, and the remainder go through only one or two divisions (Fig. 2 B and data not shown). The cell cycle arrest and eventual lethality are completely suppressed by the *mad3Δ* mutation, which inactivates the spindle checkpoint (Li and Murray, 1991; Hardwick et al., 2000). *mad3Δ* and *mad3Δ CDC28-VF* cells divide as if there were no activation of the checkpoint. These results show that *CDC28-VF* cells, unlike wild-type, cannot escape from mitosis in the presence of constant stimulation of the spindle checkpoint. *CDC28-VF* cells are also sensitive to other perturbations that activate the spindle checkpoint, including the presence of short linear chromosomes (Wells and Murray, 1996) and mutations that damage the spindle (Hardwick et al., 1999). Like Mps1 overexpression in *CDC28-VF*, these treatments are lethal and cause long delays in mitosis (data not shown).

The Mitotic Defect of CDC28-VF Is Not Caused by a Lack of Inhibitory Phosphorylation

We initially observed that *CDC28-VF* and *cdc55Δ* have opposite effects on the exit from mitosis, consistent with the idea that inhibitory phosphorylation of Cdc28 aids exit from mitosis. More careful examination reveals that the mitotic exit defect in *CDC28-VF* is not due to effects on Cdc28 phosphorylation. Fig. 3 A shows serial dilutions of a panel of mutants, spotted onto galactose-containing plates, which induce Mps1 overexpression, or glucose-containing plates, which do not. *swe1Δ* cells lack the tyrosine kinase that phosphorylates Cdc28 (Booher et al., 1993). Like *CDC28-VF* cells, they have no phosphotyrosine present on Cdc28 (Fig. 3 B), but unlike *CDC28-VF* or *CDC28-F*, they are no more sensitive to Mps1 overexpression than wild-type cells. Since another kinase might phosphorylate threonine 18 in *swe1Δ* cells, we investigated the behavior

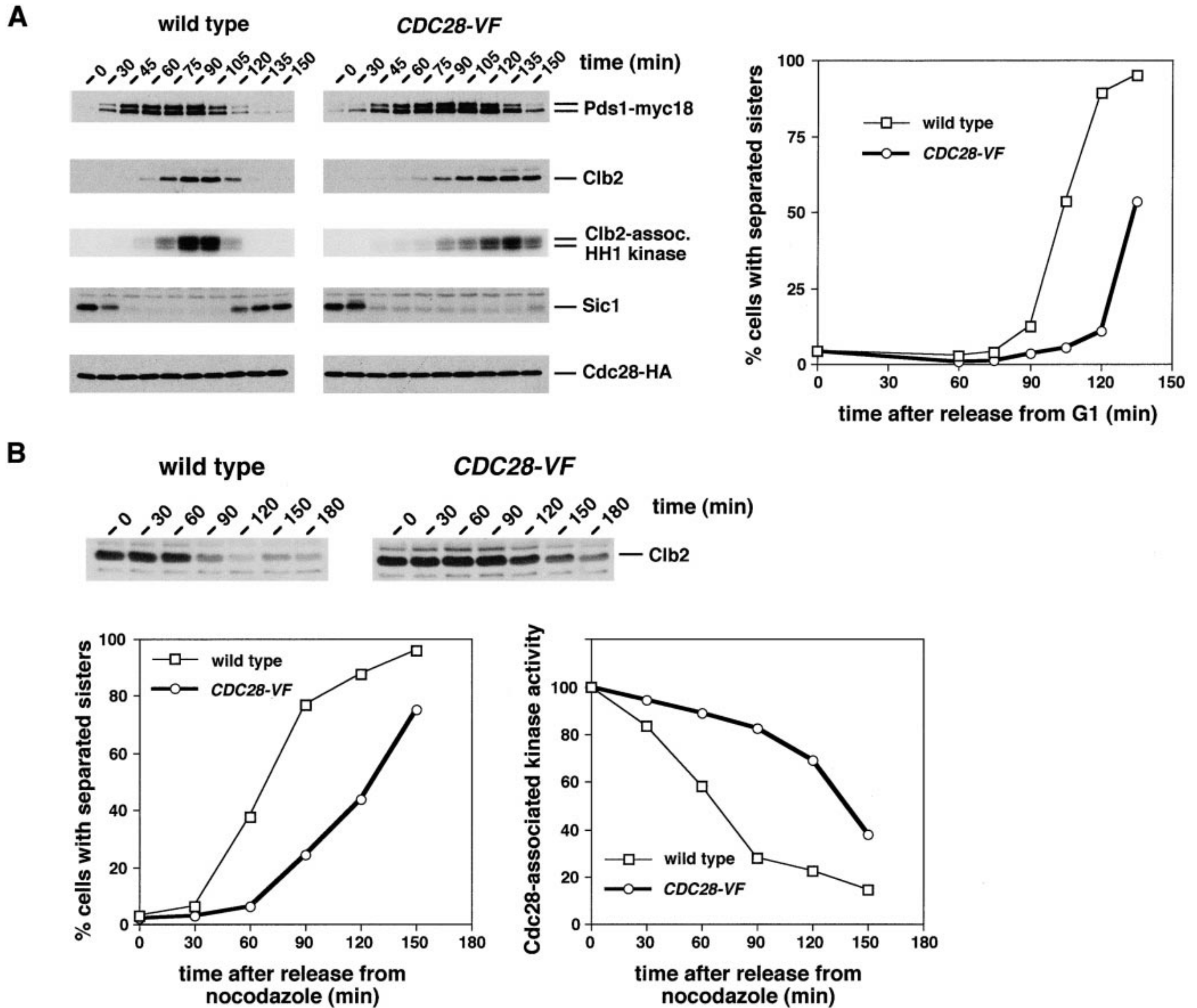
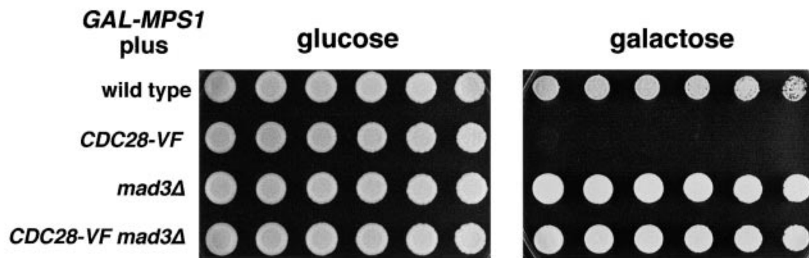


Figure 1. CDC28-VF delays in exit from mitosis. A, Wild-type (ADR1859) and *CDC28-VF* (ADR1857) were grown overnight at 23°C in YPD to mid log phase, were arrested in G1 with alpha factor (1 μg/ml), and at t = 0 the cells were released from the G1 arrest. At t = 90, alpha factor (1.5 μg/ml) was added back to the cultures to rearrest the cells in the next G1. Left, Samples were taken at the indicated times and processed for Western blots and histone H1 kinase assays. Right, Sister chromatid separation was scored by counting the number of fluorescent spots (one or two) of GFP-lacI bound to 256 tandem repeats of *lacO* integrated at the *TRP1* locus. B, Wild-type (ADR548) and *CDC28-VF* (JM477) were grown overnight at 23°C in YEP + 2% glucose to log phase, were arrested in mitosis with nocodazole (10 μg/ml) for 3 h, and at t = 0 the cells were released from the arrest into fresh medium containing alpha factor (10 μg/ml). Samples were taken at the indicated times and processed for Western blots and histone H1 kinase assays. Sister chromatid separation was scored by counting the number of fluorescent spots (one or two) of GFP-lacI bound to 256 tandem repeats of lacO integrated at the *LEU2* locus.

of *swe1*Δ CDC28-V cells, which should lack all inhibitory phosphorylation. *swe1*Δ CDC28-V resemble *CDC28-V* cells, both being only slightly more sensitive to Mps1 overexpression than wild-type cells. *mih1*Δ cells, which like *cdc55*Δ cells have increased inhibitory phosphorylation on Cdc28 (Fig. 3 B), do not have a spindle checkpoint defect as judged by their sensitivity to Mps1 overexpression or to microtubule depolymerizing agents (Fig. 3 A and data not shown).

In principle, the relative insensitivity of *swe1*Δ to Mps1 overexpression could be explained by the existence of other kinases that phosphorylate tyrosine 19 of Cdc28. We do not believe such a kinase exists. We have never detected phosphotyrosine on Cdc28 in *swe1*Δ or *swe1*Δ *mih1*Δ cells (Fig. 3 B and data not shown), and *mih1*Δ cells show a 15-min delay in entering mitosis that is completely suppressed by deleting *SWE1*, suggesting that Swe1 is the only kinase responsible for inhibiting Cdc28 (data not shown). In addition, if such a kinase existed, we would expect *CDC28-AF* (Amon et al., 1992; Sorger and Murray, 1992), which substitutes alanine at position 18 of Cdc28 rather than valine, to behave identically to *CDC28-VF*. However, *CDC28-AF*



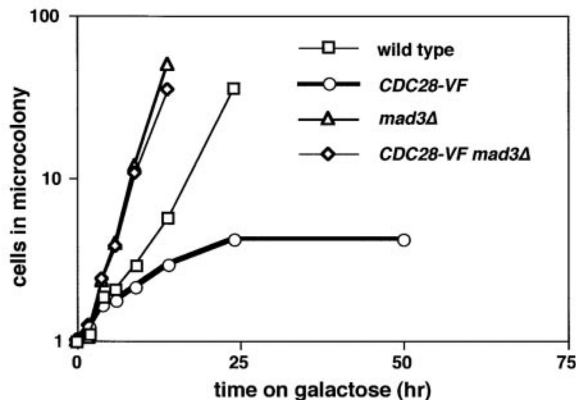


Figure 2. CDC28-VF cannot overcome a spindle checkpoint-dependent arrest. A, Serial dilution assay. All strains contain *pGAL-MPS1.* Wild-type (KH153), *CDC28-VF* (KH181), *mad3*Δ (ADR1248), and *CDC28-VF mad3*Δ (KH183) were grown overnight in YEP + 2% glucose to log phase and adjusted to A_{600} 0.35. Twofold serial dilutions of each strain were prepared in a multiwell dish and then spotted onto YEP + 2% glucose (left) or YEP + 2% galactose (right). The plates were incubated at 30°C for 2 d. B, Microcolony assay. The same strains as in A were grown overnight at 30°C in YEP + 2% raffinose to log phase and at t = 0, unbudded cells were picked out onto YEP + 2% galactose plates. As each cell divided, the number of cells in each microcolony were counted at the indicated times. Large-budded cells were counted as two cells and the original cells that did not bud were not included in the analysis. Each strain has been tested at least three times, following 20–30 cells of each strain in each experiment. In the experiment shown, 30 cells of each strain were followed.

behaves like *CDC28-F*, both of which are less sensitive to Mps1 overexpression than *CDC28-VF* (Fig. 3 A).

We favor the idea that the T18V, Y19F substitution causes a phosphorylation-independent defect in Cdc28. Based on the crystal structure of human Cdk2, tyrosine 19 of Cdc28 is adjacent to the gamma phosphate of bound ATP (De Bondt et al., 1993). Thus, *CDC28-VF*, and to a lesser extent the *CDC28-F* and *CDC28-AF* mutations, might affect ATP binding, substrate binding, catalytic activity, or substrate specificity of Cdc28.

Mitotic Cdc28 Kinase Activity Is Required for Proper Response to the Spindle Checkpoint

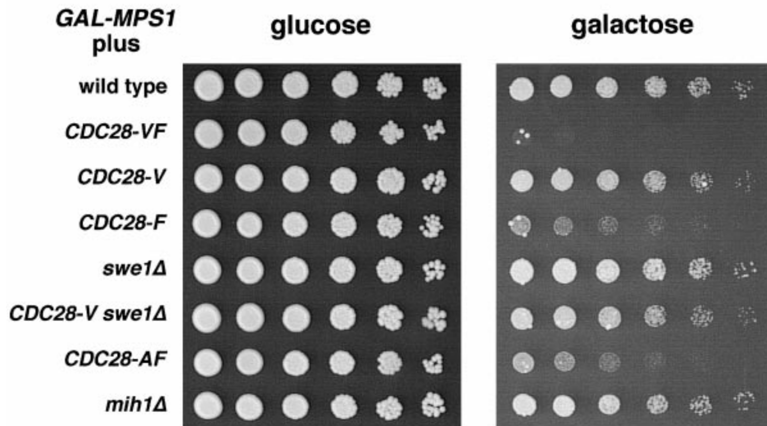
Because the *CDC28-VF* defect is not due to a lack of inhibitory phosphorylation, we asked if other mutations that affect mitotic Cdc28 activity might share phenotypes with *CDC28-VF. cdc28-1N*, *clb2*Δ, and *cks1-38* (a mutant in Cks1, a Cdc28-binding protein required for passage through Start and mitosis) are all more sensitive to Mps1 overexpression than wild-type (Fig. 4 A; Piggott et al., 1982; Hadwiger et al., 1989; Surana et al., 1991; Tang and Reed, 1993). This phenotype is not seen in *cdc28-4*, a temperature-sensitive allele of Cdc28 that prevents passage through Start and is primarily defective in the G1 function of Cdc28 (Reed, 1980).

Does a reduction in mitotic Cdc28 activity cause the *CDC28-VF* phenotype? We have observed that both Clb2- and Clb3-associated kinase activity and total Cdc28-associated kinase activity of *CDC28-VF* cells is lower than wild-type (Fig. 4 B and data not shown). This is seen both in synchronously cycling cells (Fig. 1, Clb2-associated kinase activity) and in cells arrested by the spindle checkpoint (Fig. 4 B). We estimate the specific activity of a Cdc28-VF/Clb complex is roughly half that of a wild-type Cdc28/Clb complex. Because the difference between wild-type and *CDC28-VF* is small, it is difficult to know if it is biologically significant. However, an independent substitution in the ATP binding site of Cdc28, *CDC28-F88G*, has a reduced specific activity in vitro (Bishop et al., 2000) and is as sensitive to overexpression of Mps1 as *CDC28-VF* (Fig. 4 C).

The mitotic defect of *CDC28-VF* is semidominant. The heterozygote *CDC28/CDC28-VF* has an intermediate sensitivity to Mps1 overexpression (data not shown) and overexpression of Cdc28-VF in otherwise wild-type cells creates cells that are fully sensitive to overexpressed Mps1 (data not shown and Fig. 5). These results suggest that Cdc28-VF is a dominant negative mutant, which competes with the wild-type kinase for substrates, mitotic cyclins, and Cks1. In support of this idea, multiple copies of the *CDC28* gene on a 2μ plasmid suppress the lethality of overexpressing Mps1 in a *CDC28-VF* strain (Fig. 4 D). The semidominant phenotype of *CDC28-VF* does not reflect haploinsufficiency of Cdc28, since *CDC28/cdc28*Δ diploids, which contain half as much Cdc28 as *CDC28/CDC28* diploids, do not have a phenotype like *CDC28-VF* (data not shown).

CDC28-VF Is Defective in Activating the APC

Because *CDC28-VF* impairs the exit from mitosis, crippling other pathways involved in this process might kill *CDC28-VF* cells. Our inability to make double mutants between *CDC28-VF* and mutants in the APC supports this idea (Table II). *CDC28-VF* is synthetically lethal in combi-



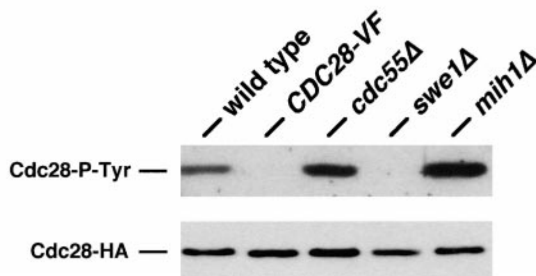


Figure 3. Changes in inhibitory phosphorylation on Cdc28 do not affect sensitivity to the spindle checkpoint. A, Serial dilutions of strains with varying amounts of inhibitory phosphorylation on Cdc28. All strains contain *pGAL-MPS1.* Wild-type (KH153), *CDC28-VF* (KH181), *CDC28-V* (ADR1105), *CDC28-F* (KH204), *swe1*Δ (KH207), *swe1*Δ CDC28-V (ADR1100), *CDC28-AF* (ADR1506), and *mih1*Δ (ADR1378) were grown to saturation for 2 d in YEP + 2% glucose at 30°C, diluted tenfold, and fourfold serial dilutions were prepared in a multiwell dish and spotted onto YEP + 2% glucose (left) or YEP + 2% galactose (right). The plates were incubated at 30°C for 2 d. B, Phosphotyrosine blot. Wild-type (ADR477), *CDC28-VF* (ADR509), *cdc55*Δ (JM445), *swe1*Δ (ADR684), and *mih1*Δ (ADR1314) were grown overnight in YEP + 2% glucose at 23°C to log phase. The cells were harvested, lysed, and Cdc28-HA was immunoprecipitated with the 12CA5 antibody. The immunoprecipitates were run out on a polyacrylamide gel and probed either with a phosphotyrosine antibody (top) or with 12CA5 (bottom).

nation with mutations in components of the APC (*cdc23-1*, *cdc16-1*, *apc10-1,* formerly *doc1-1*; Lamb et al., 1994; Irniger et al., 1995; Hwang and Murray, 1997) and positive regulators of the APC (*cdc5-1*, *hct1*Δ and *cdc20-1*; Schwab et al., 1997; Visintin et al., 1997; Charles et al., 1998; Shirayama et al., 1998). These double mutants are inviable at all temperatures, and although the double mutant spores are able to germinate, they die in microcolonies of large-budded cells (data not shown), indicating a terminal arrest in mitosis. These genetic interactions are specific to the APC and regulators of the APC, because two other mutants that arrest in mitosis, *cdc13-1* (which activates the DNA damage checkpoint) and *cdc15-2* (which arrests cells in anaphase) are both viable in combination with *CDC28-VF.*

The interactions of *CDC28* alleles and *clb2*Δ with Mps1 overexpression and the *cdc23-1* mutant are correlated with each other: *cdc28-1N*, *CDC28-F88G*, and *clb2*Δ are synthetically lethal in combination with *cdc23-1* and cannot proliferate when overexpressing Mps1, whereas *cdc28-4* is viable in combination with *cdc23-1* or Mps1 overexpression (Table II; also, see Irniger et al., 1995). *CDC28-F* and *CDC28-AF*, which have milder phenotypes than *CDC28-VF*, are synthetically lethal with *cdc23-1*, but viable in combination with *cdc16-1*, a weaker APC mutant (Table II). Lastly, *swe1*Δ is viable in combination with *cdc23-1* and *cdc16-1* (Table II), demonstrating that Swe1 is unlikely to have a role in promoting the exit from mitosis.

The genetic interactions between *CDC28-VF* and the APC and its regulators are a mixed blessing. Although they suggest that Cdc28 may help activate the APC, they prevent us from examining the phenotype of a *CDC28-VF* apc^{-} double mutant, since it is impossible to create such a mutant. We overcame this difficulty by exploiting the fact that overexpressing Cdc28-VF creates cells that behave phenotypically like *CDC28-VF.* This overexpression in *cdc23-1* cells at the permissive temperature of 23°C is toxic and no cells survive even a brief 1-h pulse of *CDC28-VF* expression driven by the *GAL1* promoter (data not shown).

We overexpressed *CDC28* and *CDC28-VF* in *cdc23-1* cells that had been arrested by alpha factor and then released them from the arrest into fresh medium at 23°C, adding alpha factor after budding so that cells will rearrest when they reach the next G1. Overexpression of *CDC28-VF* causes a permanent large-budded arrest with high levels of Clb2 and Clb3 (Fig. 5 A). Sic1 levels never rise and Clb2-associated kinase activity never falls. Sister separation and spindle elongation are delayed by 2 h in cells expressing *CDC28-VF* (Fig. 5 B and data not shown). These observations show that the induction of anaphase is delayed and the transition from anaphase to G1 is completely blocked in *cdc23-1 CDC28-VF* cells. These phenotypes are not due to activating the spindle checkpoint because neither *mad2*Δ nor *mad2*Δ *bub2*Δ bypass the arrest (data not shown; Hoyt et al., 1991; Li and Murray, 1991; Alexandru et al., 1999). Therefore, we conclude that *CDC28-VF* is defective in activating both the Cdc20-dependent APC, which triggers anaphase, and the Hct1-dependent APC, which completes cyclin proteolysis.

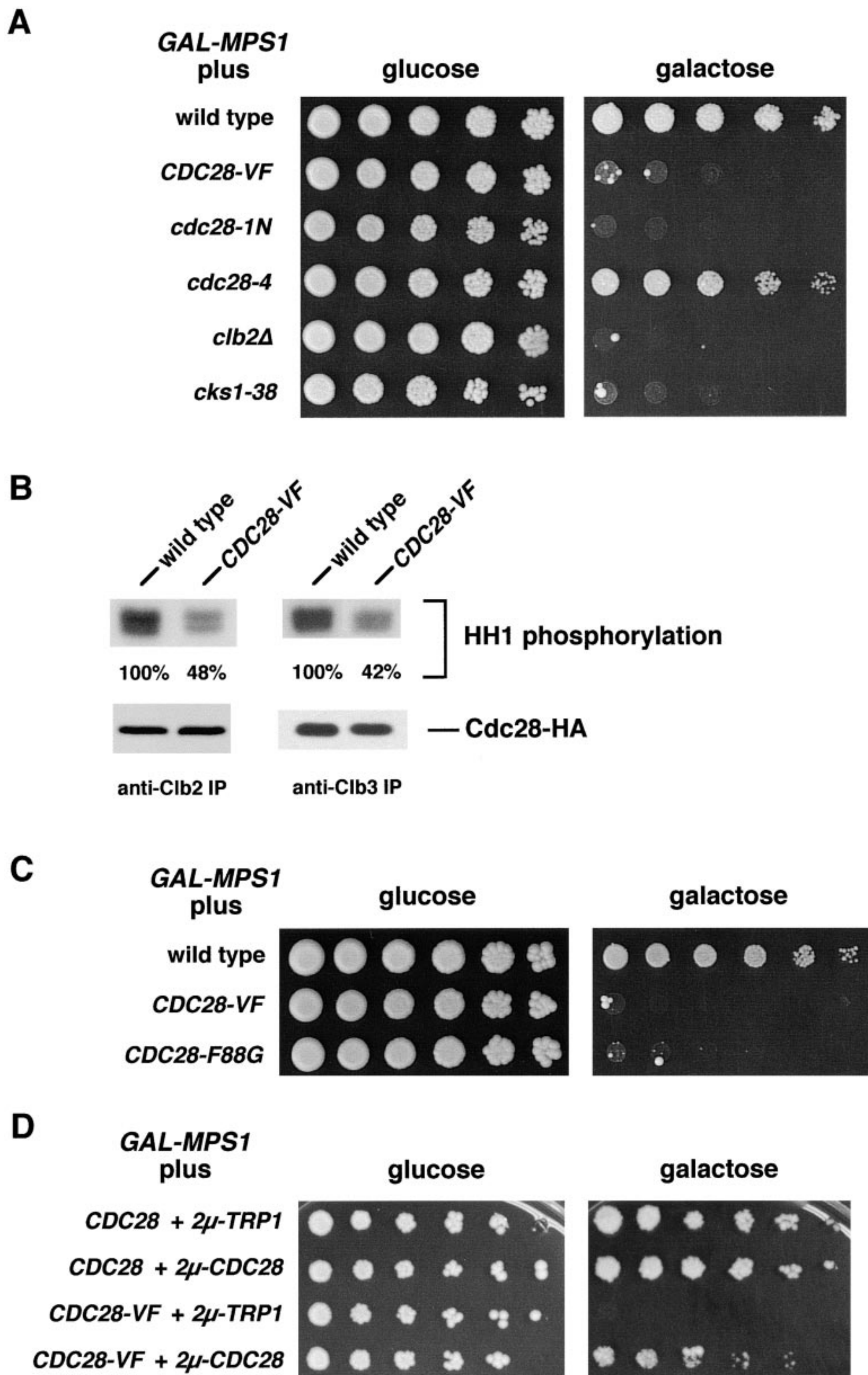
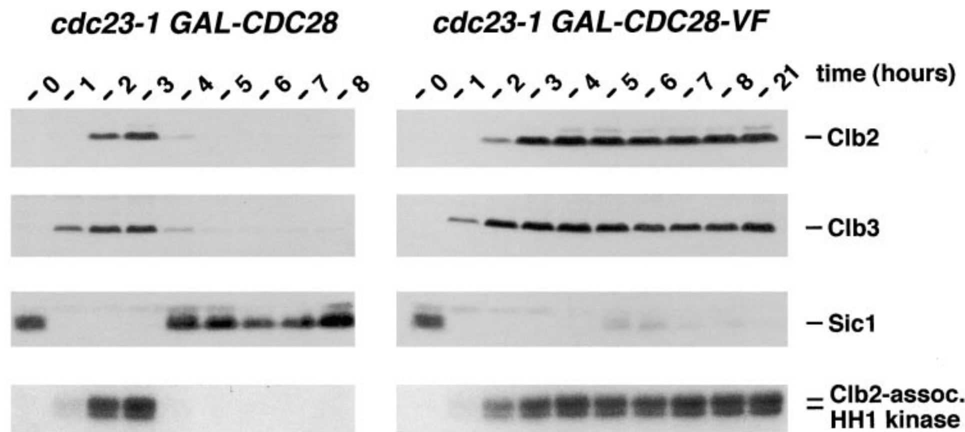


Figure 4. Mutants with defects in mitotic Cdc28 activity resemble *CDC28-VF.* A, Defects in mitotic Cdc28 activity are sensitive to spindle checkpoint-dependent arrest. All strains contain *pGAL-MPS1.* Wild-type (KH153), *CDC28-VF* (KH181), *cdc28-1N* (ADR1899), *cdc28-4* (ADR1901), *clb2*Δ (ADR-1606), and *cks1-38* (ADR-1903) were grown to saturation for 2 d in YEP + 2% glucose at 23°C, diluted tenfold, and fourfold serial dilutions were prepared in a multiwell dish and spotted onto YEP + 2% glucose (left) or YEP + 2% galactose (right). The plates were incubated at 23°C for 2.5 d. B, The specific activity of Cdc28-VF is lower than Cdc28. Wild-type (ADR477) and *CDC28-VF* (ADR509) were grown overnight in YEP + 2% glucose at 23°C to log phase and arrested in mitosis with nocodazole (10 μg/ml) for 3 h. The cells were then harvested, lysed, and Clb2/Cdc28 and Clb3/Cdc28 complexes were immunoprecipitated, and their histone H1 kinase activity was measured. The Western blot (bottom) shows that equal amounts of Cdc28 are precipitated in the two strains, although the kinase activity (top) of Cdc28-VF is reduced relative to wild-type. The activity of wild-type Cdc28 is reported as 100% for both the anti-Clb2 and anti-Clb3 immunoprecipitates. C, *CDC28-F88G* behaves like *CDC28-VF.* All strains contain *pGAL-MPS1.* Wild-type (KH153), *CDC28-VF* (KH181), and *CDC28-F88G* (ADR2034) were grown to saturation for 2 d in YEP + 2% glucose at 30°C, diluted tenfold, and fourfold serial dilutions were prepared in a multiwell dish and spotted onto YEP + 2% glucose (left) or YEP + 2% galactose (right). The plates were incubated at 30°C for 2 d. D, 2μ-CDC28 suppresses *CDC28-VF.* All strains contain *pGAL-MPS1.* Wild-type (KH153) or *CDC28-VF* (KH181) containing either 2μ-CDC28 or an empty 2μ vector were grown to saturation for 2 d in CSM-trp + 2% glucose at 30°C, diluted fivefold, and fourfold serial dilutions were prepared in a multiwell dish and were spotted onto CSM-trp + 2% glucose (left) or CSM-trp + 2% galactose (right). The plates were incubated at 30°C for 2 d.



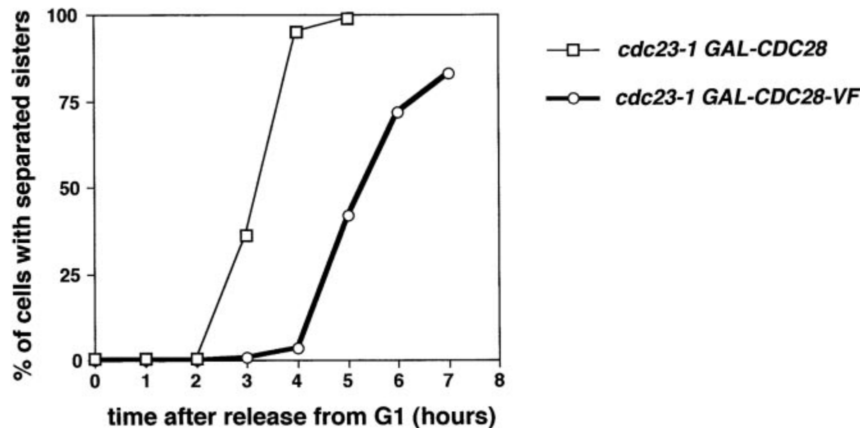


Figure 5. cdc23-1 GAL-CDC28-VF arrest in mitosis at the permissive temperature of 23°C. A, *cdc23-1 GAL-CDC28* (ADR1685) and *cdc23-1 GAL-CDC28-VF* (ADR1687) were grown overnight at 23°C in YEP + 2% raffinose to log phase, arrested in G1 with alpha factor (1 μg/ml) for 3.5 h, and at t = 0 released from the G1 arrest into fresh YEP + 2% galactose. After cells had budded (t = 2), alpha factor (1.5 g/ml) was added back to the cultures to rearrest the cells in the next G1. Samples were taken at the indicated times and processed for Western blots and histone H1 kinase assays. B, Sister chromatid separation was scored by counting the number of fluorescent spots (one or two) of GFP-lacI bound to 256 tandem repeats of *lacO* integrated at the *TRP1* locus.

CDC28-VF Has Normal G1 Hct1-dependent APC Activity

The failure to induce mitotic cyclin proteolysis in *cdc23-1* mutants overexpressing Cdc28-VF could reflect the requirement for Cdc20 activity to activate the Hct1-dependent APC (Visintin et al., 1998; Shirayama et al., 1999; Yeong et al., 2000), or it could reflect a Cdc20-independent defect in the activity of Hct1. To distinguish between these possibilities, we asked if *CDC28-VF* has normal APC activity in G1, a time when all APC activity is Hct1-dependent and Cdc20 is absent (Charles et al., 1998; Prinz et al., 1998; Shirayama et al., 1998; and see below). We immunoprecipitated the APC from alpha factor-arrested cells with antibodies raised against Cdc26, a nonessential component of the APC (Hwang and Murray, 1997), and measured its ability to ubiquitinate an iodinated fragment of sea urchin Cyclin B in a reconstituted ubiquitination assay (Charles et al., 1998). We detected no differences in APC activity in wild-type and *CDC28-VF*, and the activity of immunoprecipitates from both wild-type and *CDC28-VF* could be increased by adding recombinant Hct1 (Fig. 6; Jaspersen et al., 1999). In addition, we have shown that Hct1-dependent proteolysis of Clb2 and Pds1 in G1 is normal in *CDC28-VF* (data not shown and Fig. 7 C). These experiments are consistent with the idea that the *CDC28-VF* mutant has no direct effect on the activity of the Hct1-dependent APC.

Cdc28 Activates the Cdc20-dependent APC

Cdc20 is required for sister chromatid separation (Shirayama et al., 1998). *CDC28-VF* shows delays in sister separation (Figs. 1 and 5), suggesting that Cdc20-dependent APC activity is defective in these cells. We have approached this issue in more detail by examining the half life of Pds1, a substrate of the Cdc20-dependent APC (Visintin et al., 1997; Shirayama et al., 1999; Tinker-Kulberg and Morgan, 1999) during anaphase, a time when the Cdc20-dependent APC is thought to be active (Jaspersen et al., 1998). First, we needed to confirm that degradation of Pds1 in anaphase is due to Cdc20 and not Hct1. We arrested wild-type, *cdc20-3*, and *hct1*Δ strains in anaphase by overexpressing a nondegradable Clb2 (*pGAL-CLB2*-Δ176; Surana et al., 1993). These strains also contained an epitope-tagged form of Pds1 replacing the endogenous gene. When all cells had reached anaphase (Fig. 7 A, t = 0), the cultures were shifted to 37°C to see if Pds1 levels

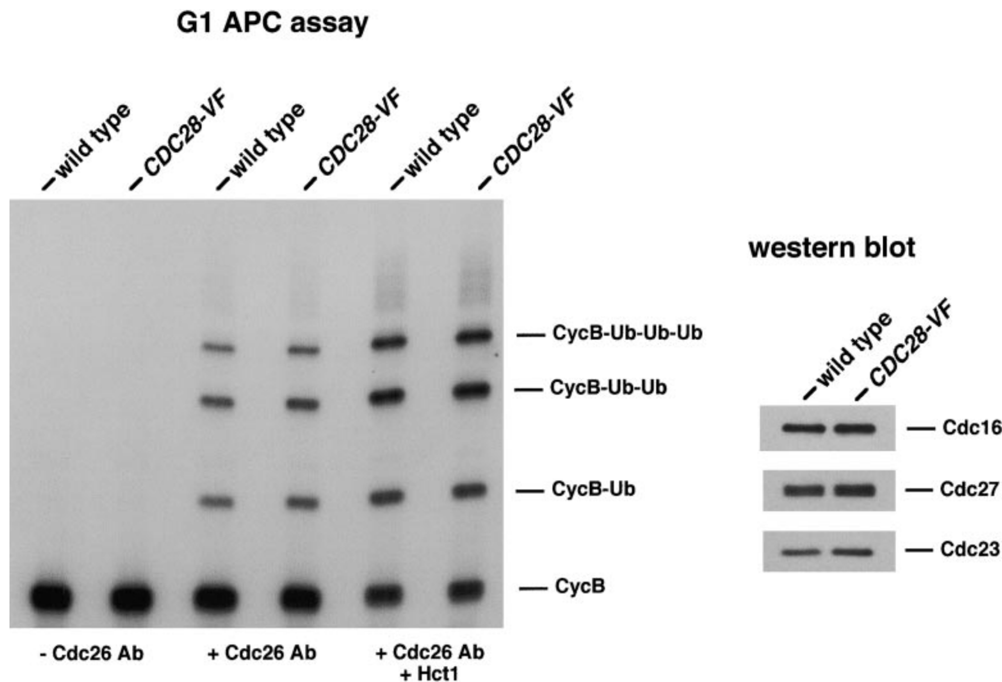


Figure 6. *CDC28-VF* has normal G1 APC activity. Wild-type (ADR1389) and *CDC28-VF* (ADR1252) were grown overnight at 30°C in YEP + 2% glucose to log phase and arrested in G1 with alpha factor (1 μg/ml) for 3 h. The cells were harvested, lysed, and the APC was immunoprecipitated with anti-Cdc26 antibodies. The in vitro ubiquitination activity of the immunoprecipitates was measured in the absence or presence of added Hct1 protein (left). The substrate for the in vitro ubiquitination is an iodinated NH_2-terminal fragment of sea urchin cyclin B (CycB). Western blotting of the immunoprecipitates shows that equal amounts of Cdc16, Cdc23, and Cdc27 are present in the APC isolated from wild-type and *CDC28-VF* cells (right).

would rise in either the *cdc20-3* or *hct1*Δ strain, as an indication that Pds1 had become more stable. Pds1 levels rose in the *cdc20-3* strain, but not in the *hct1*Δ strain, showing that the stability of Pds1 in anaphase is controlled by the Cdc20- rather than the Hct1-dependent APC (Fig. 7 A).

We next examined the half life of Pds1 during an anaphase arrest caused by the *cdc15-2* mutation. Pds1 is unstable during this arrest and Cdc20 is required to exit from the arrest (Jaspersen et al., 1998; Tinker-Kulberg and Morgan, 1999). *cdc15-2*, *cdc15-2 CDC28-VF*, and *cdc15-2 clb2*Δ cells were arrested in anaphase, an epitope-tagged *PDS1* gene driven by the *GAL1* promoter was induced by adding galactose for 1 h, and its expression was terminated by adding glucose. The half life of Pds1 in *cdc15-2* cells in this experiment was <15 min, but was >1.5 h in *CDC28-VF cdc15-2* and *clb2*Δ *cdc15-2* cells (Fig. 7 B), showing that the *CDC28-VF* and *clb2*Δ mutations compromise Cdc20-dependent APC activity.

Earlier studies argued that Hct1 and Cdc20 were specificity factors for the APC, with Cdc20 directing the ubiquitination of Pds1 and Hct1 directing that of mitotic cyclins (Visintin et al., 1997). The instability of Pds1 in G1 cells, which lack detectable Cdc20 (Prinz et al., 1998), prompted us to reexamine this issue. Wild-type, *CDC28-VF*, and *cdc20-3* cells were arrested in G1 by alpha factor, or in the case of *hct1*Δ, which is resistant to alpha factor (Schwab et al., 1997), by the *cdc28-13* mutation (Reed, 1980). Once arrested, wild-type, *CDC28-VF*, and *cdc20-3* cells were shifted to 37°C (for *cdc28-13* and *cdc28-13 hct1*Δ, asynchronous cultures were transferred to 37°C), *pGAL-PDS1* was induced by adding galactose for two hours and then expression was shut off by adding glucose. Pds1 was equally unstable in wild-type, *CDC28-VF*, *cdc20-3*, and *cdc28-13* cells, but was completely stable in the *hct1*Δ *cdc28-13* cells. This control shows that the defect in Pds1 stability in *CDC28-VF* is specific to anaphase and, together with Fig. 7 A, shows that Pds1 is targeted for destruction by the Cdc20-dependent APC in mitosis and by the Hct1-dependent APC in G1. In addition, this experiment reinforces the conclusion derived from in vitro experiments in Fig. 6 that *CDC28-VF* has no defects in Hct1-dependent APC activity in G1. Our results differ from those of Visintin et al. (1997), who found that stability of Pds1 in G1 is regulated by Cdc20. Their results may have been due to incomplete alpha factor arrest of the *cdc20-1* allele used in their study. Our results agree with the recent observation that Clb2 is targeted for destruction by both the Cdc20- and Hct1-dependent forms of the APC (Yeong et al., 2000).

The stabilization of Pds1 in mitotic *CDC28-VF cdc15-2* cells suggests that *CDC28-VF* is defective in the Cdc20-dependent APC. As a first step in investigating the biochemical defect of *CDC28-VF*, we examined the interaction between Cdc20 and the APC in anaphase, a time when the Cdc20-dependent APC is active. We arrested *cdc15-2* and *cdc15-2 CDC28-VF* cells in anaphase, immunoprecipitated the APC with anti-Cdc26 antibodies, and examined the amount of associated Cdc20. Equal amounts of APC was immunoprecipitated from all three strains, but in *CDC28-VF* cells there was less associated Cdc20 (Fig. 8), even though the total level of Cdc20 was similar in wild-type and *CDC28-VF*.

Discussion

Cdc28 Activates the APC

We have shown that the *CDC28-VF* mutant is defective activating the Cdc20-dependent APC. *CDC28-VF* cells show a short delay in exiting mitosis, but this delay be-

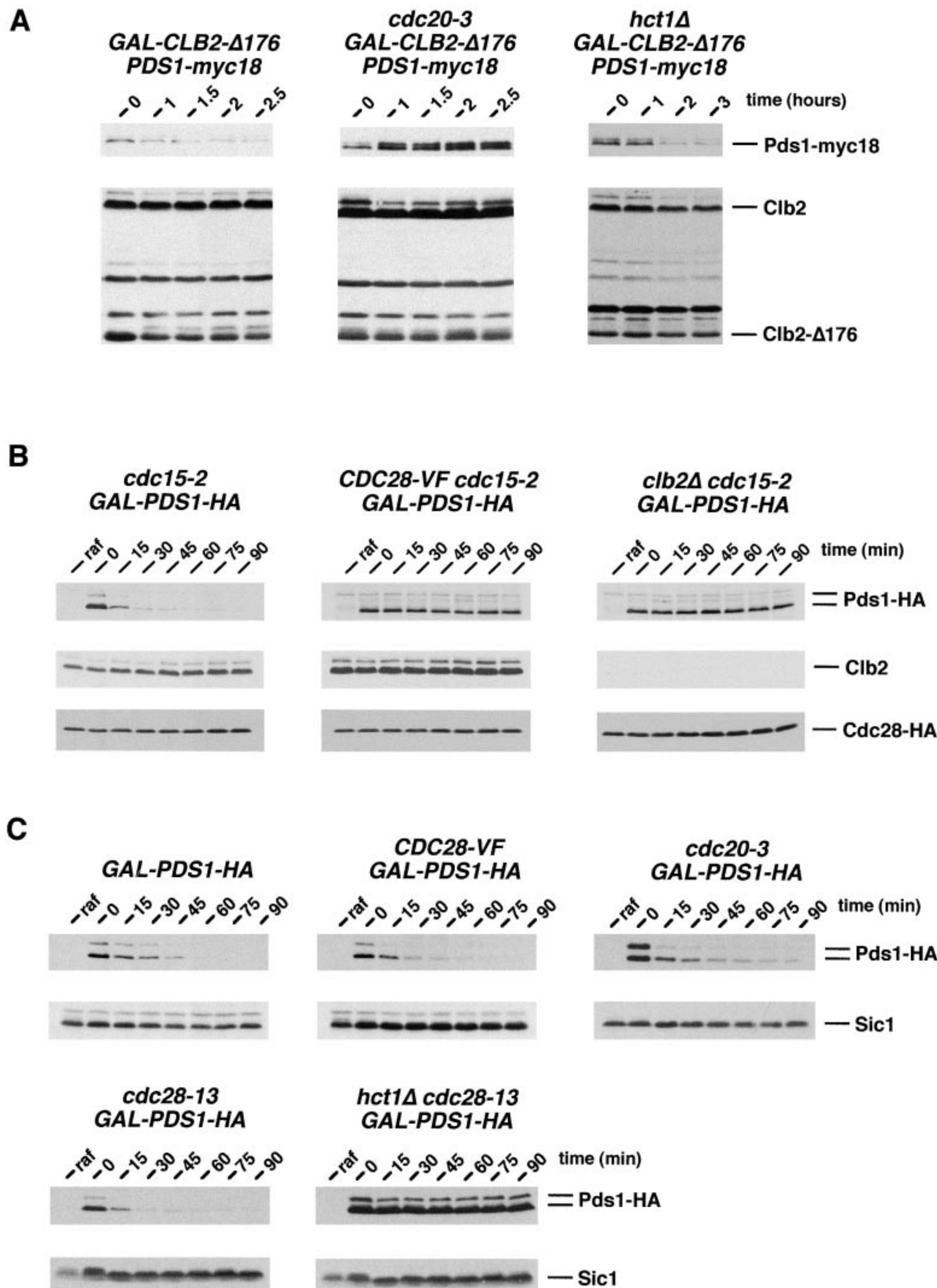


Figure 7. *CDC28-VF* cells have a defective Cdc20-dependent APC. A, Destruction of Pds1 in anaphase depends on *CDC20*. Wild-type (ADR1870), *cdc20-3* (ADR1783), and *hct1*Δ (ADR1786) were grown overnight at 23°C in YEP + 2% raffinose to log phase. The three strains contain an epitope-tagged Pds1 *(PDS1-myc18)*, can overexpress a truncated form of Clb2 (*pGAL-CLB2*-Δ176), which will arrest cells in anaphase, and express a GFP-tagged alpha tubulin (*pHIS3-GFP-TUB1*), which allows the length of the spindle to be easily assessed by microscopy. Wild-type and *cdc20-3* were arrested in G1 by alpha factor (1 μg/ml) for 3.5 h, released from the G1 arrest into YEP + 2% galactose, and at t = 0, when >90% of the cells had reached anaphase (after 5 h, as judged by spindle length), the cultures were shifted to 37°C. *hct1*Δ, which is resistant to alpha factor, was shifted from YEP + 2% raffinose directly to YEP + 2% galactose. Samples were taken at the indicated times and processed for Western blots. B, Pds1 is stable in anaphase in *CDC28-VF* and *clb2*Δ. *cdc15-2 GAL-PDS1-HA* (ADR1743), *cdc15-2 CDC28-VF GAL-PDS1-HA* (ADR1736), and *cdc15-2 clb2*Δ GAL-PDS1-HA (ADR1774) were grown overnight at 23°C in YEP + 2% raffinose to log phase and shifted to 37°C to arrest the cells in anaphase (raf). When >90% of the cells had reached anaphase (after 4 h, as judged by nuclear division, which was scored by DAPI staining), Pds1-HA expression was induced for 1 h by the addition of galactose (to 2%), and at t = 0 its expression was terminated by the addition of glucose (to 2%). Samples were taken at the indicated times and processed for Western blots. Cdc28-HA is shown as a loading control. C, The Hct1-dependent APC regulates Pds1 stability in G1. *CDC28-HA GAL-PDS1-HA* (ADR1968), *CDC28-VF-HA GAL-PDS1-HA* (ADR1959), and *cdc20-3 GAL-PDS1-HA* (ADR1921) were grown overnight at 23°C in YEP + 2% raffinose to log phase and arrested in G1 with alpha factor (1 μg/ml) for 3 h at 23°C, and then shifted to 37°C for an additional 1 h (raf). *cdc28-13 GAL-PDS1-HA* (ADR1925) and *cdc28-13 hct1*Δ GAL-PDS1-HA (ADR1928) were grown overnight at 23°C in YEP + 2% raffinose to log phase and arrested in G1 by shifting the cultures to 37°C for 3.5 h. Pds1-HA expression was induced by addition of galactose (to 2%) for 2 h, and at t = 0 its expression was terminated by the addition of glucose (to 2%). Samples were taken at the indicated times and processed for Western blots. Sic1 is shown as a loading control and as evidence that all cells remain arrested in G1.

comes more severe when the APC is compromised by spindle checkpoint activation or defects in the APC or its regulators. These phenotypes allowed us to show a requirement for Cdc28 in exiting mitosis. The defect in the exit from mitosis in *CDC28-VF* is correlated with reduced binding of Cdc20 to the APC and lower Cdc20-dependent APC activity. *CDC28-VF* cells have normal Hct1-dependent APC activity in G1, suggesting that their failure to

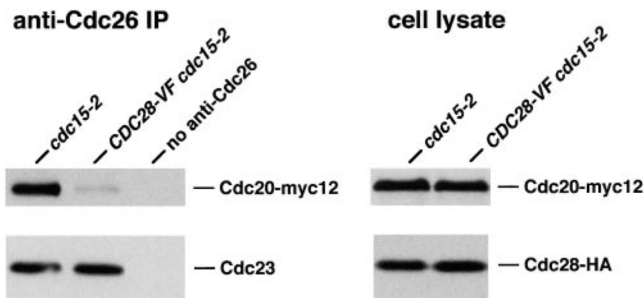


Figure 8. Cdc20 binding to the APC is impaired in *CDC28-VF*. *cdc15-2* (ADR1790) and *cdc15-2 CDC28-VF* (ADR1793) were grown overnight in YEP + 2% glucose at 23°C to log phase. Both strains contain an epitope-tagged Cdc20 *(CDC20-myc12)*. The cultures were shifted to 37°C and after >85% of the cells were arrested in anaphase (4 h, as judged by nuclear division, which was scored by DAPI staining), the cells were harvested, lysed, and the APC immunoprecipitated with anti-Cdc26 antibodies. The amount of Cdc20-myc12 bound to the APC was determined by Western blotting the immunoprecipitates with the 9E10 antibody. Equal amounts of APC were precipitated with the anti-Cdc26 antibodies as judged by Cdc23 levels (left) and equal amounts of cell lysate were used in the immunoprecipitation as judged by Cdc20-myc12 and Cdc28-HA levels (right, cell lysate).

exit mitosis is the result of interrupting the chain of events that normally leads from mitosis to G1: reduced Cdc20-dependent APC activation prevents the activation of Cdc14, which normally leads to activation of Hct1 and increased levels of Sic1, the key steps in inactivating the mitotic activity of Cdc28 (Lim et al., 1998; Visintin et al., 1998; Jaspersen et al., 1999; Shirayama et al., 1999).

Previously, it has been shown that Cdc28 inhibits exit from mitosis. High levels of Cdc28-associated kinase activity cause arrest in anaphase (Holloway et al., 1993; Surana et al., 1993), and inhibiting Cdc28 in mitotically arrested cells activates the APC, driving them through cytokinesis and into G1 (Amon, 1997; Li and Cai, 1997). These observations led to the conclusion that the major role for mitotic Cdc28 in regulating the APC was to inhibit the activity that drove cells out of mitosis. Our results show that Cdc28 has different effects on APC activity at different stages of mitosis. Cdc28/Clb activity activates the Cdc20-dependent APC to induce the metaphase to anaphase transition, but inhibits the Hct1-dependent APC, thus inhibiting cytokinesis and the transition from anaphase to G1. This pattern of regulation helps ensure chromosome segregation precedes cell division and allows budding yeast to regulate these events separately, a useful feature in an organism that specifies the site of cytokinesis long before spindle assembly.

Cdc28 Is Required for Recovery from Checkpoint-dependent Arrest

Proteins that were identified as members of the spindle checkpoint have two roles in keeping cells with depolymerized microtubules from leaving mitosis. Six of them, Mps1, Mad1–Mad3, Bub1, and Bub3, detect kinetochores that are not attached to the spindle (Hoyt et al., 1991; Li and Murray, 1991; Weiss and Winey, 1996) and prevent sister chromatid separation by inhibiting the Cdc20-dependent APC (Hwang et al., 1998; Kim et al., 1998). In contrast, Bub2, also identified as part of the spindle checkpoint (Hoyt et al., 1991), detects an unknown lesion and arrests cells in anaphase, probably by preventing the activation of Cdc14 (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999). The opposition between the inhibition of the Cdc20-dependent APC by the spindle checkpoint and activation by Cdc28 explains why *CDC28-VF* cells, which are defective in activating the Cdc20-dependent APC, have difficulty overcoming a checkpoint-dependent arrest.

We do not know how wild-type cells eventually escape from mitosis despite continued Mps1 overexpression, a process that could be described as adaptation to the spindle checkpoint (Rieder and Palazzo, 1992; Minn et al., 1996; Rudner and Murray, 1996). In particular, we cannot distinguish between constitutive mechanisms, such as stabilization of mitotic cyclins leading to a slow rise in Cdc28/Clb activity that eventually leads to APC activation, and induced mechanisms, such as a slow process initiated by components of the checkpoint that leads to reduced inhibition of Cdc20. Although we initially thought of *CDC28-VF* as an adaptation mutant (Minshull et al., 1996), we now believe that it achieves its effect by reducing the ability of Cdc28 to activate the Cdc20-dependent APC, whether or not the checkpoint is active. It is only when the checkpoint is active, however, that this reduction is sufficient to keep cells from leaving mitosis. This discussion highlights a general difficulty in studying adaptation mutants: the ability to overcome prolonged checkpoint-dependent arrests appears to be sensitive to small, checkpoint-independent defects in basic cell cycle machinery (Toczyski et al., 1997).

Other Mutants Share Phenotypes with CDC28-VF

Genetic analysis of *CDC28* has revealed two types of mutants, those that primarily affect G1 (*cdc28-4, cdc28-13*; Reed, 1980) and those that primarily affect exit from mitosis (*CDC28-VF, cdc28-1N*; Piggott et al., 1982; Surana et al., 1991). The mitotic mutants share phenotypes with *clb2*Δ and *cks1-38*. Do these four mutants, *CDC28-VF*, *cdc28-1N*, *clb2*Δ and *cks1-38*, have a common biochemical defect? *clb2*Δ cells contain no Clb2, and therefore have reduced levels of mitotic Cdc28 activity (Grandin and Reed, 1993). The defects of *cdc28-1N* and *cks1-38*, which arrest cells in mitosis, may have more to do with altering the substrate specificity of Cdc28. When assayed by immunoprecipitation of Clb2-associated kinase, *cdc28-1N* strains have similar kinase activity to wild-type, but they have no kinase activity associated with Cks1-coupled beads (Surana et al., 1991; Kaiser et al., 1999). These results suggest that the primary defect in *cdc28-1N* is its failure to bind Cks1, which has been shown in frog and clam extracts to be essential for exit from mitosis and APC phosphorylation by Cdc2/Cyclin B (Patra and Dunphy, 1996, 1998; Shteinberg and Hershko, 1999).

Our only clue to the biochemical defect of Cdc28-VF is that it appears to have a small reduction in its specific activity (Fig. 4 D). We think this defect may be important because *CDC28-F88G*, a mutant in the ATP binding site of Cdc28, also has reduced specific activity (Bishop et al.,

2000), and shares phenotypes with *CDC28-VF* (Fig. 4 C). The *cdc28-1N* and *cks1-38* mutations may produce their effects by reducing the level of the Cdc28/Clb/Cks1 complexes, whereas in *clb2*Δ cells, the complexes of Cdc28 with the remaining Clb proteins may be less capable of activating the Cdc20-dependent APC.

Although the Cdc28 activity is lower in *CDC28-VF* cells, we do not think that the defect in *CDC28-VF* is simply due to lower total Cdc28 activity per cell. Overexpression of Clb2 and Clb3, which raises Cdc28 activity in cells (Stueland et al., 1993), does not suppress the mitotic defect of *CDC28-VF* (data not shown). This result is consistent with the idea that the specific activity per Cdc28 molecule, not the total Cdc28 activity per cell, is critical for activating the exit from mitosis. An alternative explanation is that the different mutants change the substrate specificity of Cdc28, preventing phosphorylation of important mitotic substrates.

A Cautionary Tale

Protein phosphorylation is a common way of regulating protein activity. Mutating putative phosphorylation sites to nonphosphorylatable residues is a widely used technique for assessing the biological function of phosphorylation of specific proteins (Li et al., 1995; Zachariae et al., 1998; Jaspersen et al., 1999). Our analysis of *CDC28-VF* shows that such mutations can have unanticipated effects that are independent of phosphorylation.

It is difficult to tell how common such effects are. Our observations of *CDC28-VF* suggest that the studies conducted with the *CDC28-AF*, *CDC28-VF*, and *CDC28-F* mutants in budding yeast (as well as experiments with the corresponding mutants in Cdc2) should be reexamined to exclude the possibility that the observed effects of these mutants were due to phosphorylation-independent defects. A simple control is to ask whether the phenotype of the *CDC28* mutants is exactly mimicked by deletion of *SWE1*. If so, the conclusions of the original experiments are secure. If not, phosphorylation-independent effects due to mutating the inhibitory residues may contribute to the observed phenotypes. In the general case, the ideal control is to show that inactivating the kinase that phosphorylates a particular protein produces a similar effect on the substrate's activity as do the phosphorylation site mutants.

Does Cdc28 Phosphorylate the APC?

How does Cdc28 promote anaphase? Experiments in frogs, clams, and mammalian cell culture have all suggested that phosphorylation activates the APC by modifying four of its subunits: Cdc16, Cdc23, Cdc27, and Apc1 (BimE; Hershko et al., 1994; Peters et al., 1996; Kotani et al., 1998, 1999; Patra and Dunphy, 1998). These proteins are phosphorylated during mitosis and the phosphorylated APC has greater Cdc20-dependent activity in vitro, whereas dephosphorylation of purified APC causes a loss of activity (Lahav-Baratz et al., 1995; Shteinberg et al., 1999). Studies in clams have suggested that this phosphorylation is required for proper Cdc20 binding (Shteinberg et al., 1999). In the accompanying paper (Rudner and Murray, 2000, this issue), we show that the APC is phosphorylated by Cdc28 in budding yeast, and that a defect in this phosphorylation causes reduced Cdc20-dependent APC activity and contributes to the *CDC28-VF* phenotype.

We would like to thank Doug Kellogg, Aaron Straight, Lena Hwang, Sue Biggins, Sue Jaspersen, Rachel Tinker-Kulberg, Jeff Ubersax, Dave Morgan, Mike Mendenhall, Ray Deshaies, Bob Booher, Andrew Page, Phil Hieter, Wolfgang Zacharie, Kim Nasmyth, Jeremy Thorner, Peter Sorger, and Steve Reed for yeast strains, plasmids, and antibodies; Jeff Ubersax, Julia Charles, and David Morgan for sharing unpublished results; Jeff Ubersax, Sue Jaspersen, Dave Morgan, and the Murray Lab for critical reading of the manuscript; Jeremy Minshull, Bodo Stern, David Rudner, Alex Szidon, Julia Charles, Rachel Tinker-Kulberg, Hironori Funabiki, Sue Biggins, Marc Lenburg, and Dara Spatz Friedman for invaluable discussions and unwavering support.

This work was supported by grants from the National Institutes of Health and Human Frontiers in Science to A.W. Murray. A.D. Rudner was a pre-doctoral fellow of the Howard Hughes Medical Institute and K.G. Hardwick was a fellow of the Leukemia Society of America during this work.

Submitted: 28 March 2000
Accepted: 17 May 2000

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