P40^{SDB25}, a putative CDK inhibitor, has a role in the M/G₁ transition in *Saccharomyces cerevisiae*

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The *Saccharomyces cerevisiae* protein kinase Dbf2 carries out an essential function in late mitosis, and its kinase activity is cell-cycle regulated around anaphase/telophase. We have isolated *SDB25*, a high copy suppressor of temperature-sensitive *dbf2* mutants, and genetic analysis suggests that the two proteins may function in parallel pathways in late mitosis. *SDB25* encodes p40, a previously characterized substrate and potent inhibitor of Cdc28 kinase activity. Sdb25 is a phosphoprotein, and Sdb25 immunoprecipitates have a histone H1 kinase activity that is *CDC28*-dependent. Remarkably, Sdb25 transcript levels, protein levels, and associated kinase activity are precisely cell-cycle regulated, sharing a common peak in late mitosis. Moreover, Sdb25 protein levels and associated kinase activity are sharply up-regulated at the peak of Dbf2 kinase activity in cells released from a *dbf2* ts block. The Sdb25 protein then disappears around Start in the next cell cycle. This indicates that *SDB25* function is confined to M/G₁, and morphological analysis of *sdb25Δ* cells supports this conclusion. Our data suggest that Sdb25 functions in a pathway in late mitosis leading to the down-regulation of Cdc28 kinase activity as cells traverse the M/G₁ boundary.

[Key Words: Cell cycle; late mitosis; CDK inhibitor; *S. cerevisiae*; pathway]

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In the budding yeast *Saccharomyces cerevisiae*, regulation of the activity of the p34^{CDC28} protein kinase is central to ordered progression through the cell cycle (for review, see Nasmyth 1993). Activation of the kinase is required for traversal of Start in G₁, where commitment to a new round of cell division occurs, and for the initiation of mitosis at the G₂/M boundary. Although Cdc28 is present at a constant level throughout the cell cycle, its histone H1 kinase activity fluctuates periodically (Reed and Wittenberg 1990). The control of Cdc28 function is partly affected by the formation of stage-specific, distinct, heteromeric p34^{CDC28} kinase complexes, with the monomeric form of the protein having little or no activity. At the two major control points, Start and the G₂/M interface, activation of Cdc28 requires its association with different members of a group of unstable proteins, called cyclins (for review, see Lew and Reed 1992). Exit from mitosis may also require deactivation of the kinase, and concomitant destruction of the associated cyclin by proteolysis (Ghiara et al. 1991).

Predating the identification of cyclins as regulatory subunits in p34^{CDC28} heteromeric complexes, two other proteins were shown to be associated with the active form of the kinase. Cks1 is an 18-kD protein that was isolated in a screen for high-copy suppressors of the tem-

perature-sensitive *cdc28-1* mutation (Hadwiger et al. 1989a) and is homologous to the *Schizosaccharomyces pombe* *suc1*⁺ protein that interacts with *cdc2*⁺ (Hayles et al. 1986). Furthermore, it was demonstrated that Cks1 coprecipitated with Cdc28 and associated with a 160-kD complex with Cdc28-dependent kinase activity. Another component of this p34^{CDC28} complex is a 40-kD protein, p40, that is also phosphorylated in vitro, in a Cdc28-specific, cell-cycle-dependent manner (Reed et al. 1985; Wittenberg and Reed 1988; Reed and Wittenberg 1990). Recently, by use of p40 purified from stationary-phase cells, the 40-kD protein has been shown to bind tightly to Cdc28 and to inhibit its kinase activity (Mendenhall 1993). This finding has led to speculation that p40 may function as a cyclin-dependent kinase (CDK) inhibitor (Hunter 1993; Nasmyth and Hunt 1993). A number of CDK inhibitors have recently been discovered that inhibit, by binding stoichiometrically, the kinase activity of a range of CDK complexes (for review, see Pines 1994).

Another protein kinase that has also been implicated in control of the mitotic cell cycle is Dbf2. Initially identified in a screen for mutants defective in S phase, further analysis revealed that in *dbf2* mutant cells, DNA synthesis is delayed, but the cells finally block in mitosis (Johnston et al. 1990). At the restrictive temperature, *dbf2* mutants arrest in late nuclear division with a uniform morphology resembling a dumbbell consisting of large, budded, single cells. Although *dbf2* thermosensi-

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tive (*dbf2-ts*) mutants are lethal, deletion of the gene does not affect cell viability, there being a *DBF2* functional and structural homolog, *DBF20* (Toyn et al. 1991). Either gene can be deleted, but the *dbf2Δdbf20Δ* double mutant is inviable, indicating that both genes have a common, essential function. Unlike *CDC28*, the *DBF2* transcript is expressed under cell-cycle control, with a peak of expression in M phase, whereas *DBF20* is constitutively expressed. Dbf2 kinase activity is cell-cycle regulated in a manner similar to the *DBF2* transcript, although the Dbf2 protein itself is present throughout the cell cycle (Toyn and Johnston 1994). Moreover, the late mitotic genes *CDC5* and *CDC15* are high-copy suppressors of *dbf2* mutations (Kitada et al. 1993), so that Dbf2 may be part of a cell-cycle regulatory network in late mitosis.

In addition to *CDC5* and *CDC15*, several high-copy suppressors of *dbf2* mutants have been isolated from a genomic library, two of which were previously identified genes, *SIT4* and *SPO12* (Parkes and Johnston 1992). *SIT4* has homology to type-2 protein phosphatases (Arndt et al. 1989) and is required for the G₁/S transition (Sutton et al. 1991) and for the normal accumulation of G₁ cyclin RNA (Fernandez-Sarabia et al. 1992). *SPO12* has been implicated in meiosis, mutations in the gene leading to aberrant ascus formation, and is not essential for mitotic growth (Malavasic and Elder 1990). Detailed examination of the *SPO12* transcript shows that it is coordinately regulated with *DBF2*, and recent evidence indicates that *SPO12* may be a regulator of Dbf2 function (Toyn and Johnston 1993).

In this paper we report on another high-copy suppressor of *dbf2*, *SDB25*, which encodes the putative CDK inhibitor protein p40 described above. The cell cycle abundance of Sdb25 transcript and protein levels fluctuate in a cyclin-like manner, and up-regulation of Sdb25 protein levels is confined to late M/early G₁. Coincident with its initial production, Sdb25 associates with a *CDC28*-dependent kinase in late mitosis, although the Sdb25 protein persists until after cells have passed Start in the next cell cycle. Our cell cycle analysis is consistent with Sdb25 being involved in inactivation of Cdc28 kinase as cells traverse the M/G₁ boundary. Moreover, we have found that cells deleted for *SDB25* have a morphology that supports this interpretation of Sdb25 as a negative regulator of Cdc28 kinase activity. The functional relationship between *SDB25* and *DBF2* is not clear, although analysis of the genetic interactions between the two genes indicates that they may function in parallel, partially redundant, pathways in late mitosis. One possibility is that both pathways converge to help down-regulate Cdc28 kinase activity at the end of mitosis.

Results

SDB25 suppression of *dbf2-ts* alleles is copy-number dependent

Yeast genomic libraries in the multicopy vector YRp12 were transformed into strain L182-7A (*dbf2-3 ura3*), and

transformants were screened for plasmids capable of suppressing the *dbf2 ts* phenotype. Restriction mapping of all suppressing plasmids indicated that they contained one of two different regions of chromosomal DNA. These two genomic fragments were named *SDB24* and *SDB25* (suppressor of *dbf2* numbers 4 and 5), respectively. This paper reports on the sequencing and characterization of *SDB25*.

To determine whether the suppression of the *dbf2* phenotype by overexpression of *SDB25* is specific for the *dbf2-3* allele, we transformed strains L119-7D (*dbf2-1 ura3*) and L181-6B (*dbf2-2 ura3*) with YRpSDB25 (YRp12-*SDB25*). None of the resulting *URA*⁺ transformants were observed to grow at 37°C, although expression of *SDB25* from YRp12 allowed growth of *dbf2-3* at 37°C, which is comparable to a wild type. *SDB25* is therefore an allele-specific suppressor, at least when overexpressed on the multicopy plasmid YRp12. Of the other suppressors of *dbf2* (Parkes and Johnston 1992), *SDB21* (*SPO12*) has been studied in most detail, and suppression by *SPO12* requires *DBF20*, the homolog of *DBF2* (Toyn and Johnston 1993). To examine whether *SDB25* has a similar requirement for *DBF20*, YRpSDB25 was introduced into yeast strain JD101 (*dbf2-3 dbf20Δ::TRP1*). As with *SPO12*, we find that YRpSDB25 no longer suppresses the *dbf2-ts* phenotype in cells deleted for *DBF20*. The YRpSDB25 suppression of *dbf2-3* may therefore in some way allow *DBF20* to complete the vital function carried out by these two kinases.

We also tested whether *SDB25* was an allele-specific suppressor when overexpressed on the high-copy plasmid YE24. Surprisingly, we found that YE24SDB25 can suppress all three alleles of *dbf2-ts*, when incubated at 37°C and that the suppression is no longer *DBF20*-dependent. Furthermore, YE24SDB25 can keep alive cells deleted for both *DBF2* and *DBF20*, *dbf2Δdbf20Δ* cells normally being inviable (Fig. 1). Thus, the essential function carried out by the Dbf2/20 kinase can be also be executed by overproducing Sdb25 protein to a sufficient level.

SDB25 encodes a known component of a p34^{CDC28} kinase complex

The *SDB25* gene was located on the library plasmid by transposon mutagenesis, all insertions that destroyed the suppressor activity lying between the *KpnI* and *HpaI* sites on the restriction map (Fig. 2A). The DNA sequence of a 1-kb region around these sites was determined, and one large open reading frame (ORF) of 852 nucleotides was found (Fig. 2B). The predicted molecular mass of Sdb25 is 32.2 kD, and the protein is basic with an estimated pI of 8.9.

Searching the data banks revealed that there was perfect identity between DNA sequence upstream of the *SDB25* gene locus and that of the membrane protein Bos1 gene locus (Shim et al. 1991), indicating that the genetic map position of *SDB25* is adjacent to *BOS1*. Southern blot analysis of *S. cerevisiae* chromosomes separated by pulse-field gel electrophoresis shows that

Figure 1. YEpSDB25 rescues the lethality of *dbf2Δdbf20Δ*. Strain J211-2C (*dbf2Δdbf20Δ*) containing the plasmid pASZ11-DBF2 was transformed with the plasmid YEpSDB25. Two separate red sectors, corresponding to colonies that had lost the pASZ11-DBF2 plasmid, were picked and streaked out onto plates containing FOA (left plate), or one without FOA (right plate). As a control a white colony was also streaked out (upper position). Cells from the red sectors were very clumpy; hence, the heavy inoculum visible on the left plate. The plates were incubated at 25°C for 3 days and then photographed.

SDB25 is physically mapped on chromosome XII (data not shown). Therefore, the *SDB25* locus flanks the divergently expressed gene *BOS1* on chromosome XII.

Searching the existing data banks suggests that *SDB25* is a newly identified gene. However, the identical gene has been independently isolated, named *SIC1*, and the encoded protein is p40 (M. Mendenhall, pers. comm.). The initial identification of p40 was as an endogenous substrate, with an apparent molecular mass of 40 kD, for a kinase activity associated with Cdc28 immune complexes (Reed et al. 1985). Consistent with p40^{*SDB25*} being a substrate of Cdc28 kinase, we find that the *Sdb25* primary amino acid sequence has one perfect match to the proposed consensus motif for cdc2⁺/Cdc28 phosphorylation site S/T-P-X-Z, (where X is a polar amino acid, and Z is generally a basic amino acid) (Fig. 2B) (for review, see Moreno and Nurse 1990).

The finding that a dosage suppressor of the protein kinase *DBF2* is a component of a p34^{*CDC28*} complex suggests that *SDB25* may be functioning in a pathway somehow linking the two enzymes in vivo. Previously it has been shown that overexpressing *DBF2* on a high-copy plasmid did not suppress the *cdc28-4*, *cdc28-13*, or *cdc28-1N* alleles (Kitada et al. 1993). We found that high-copy *SDB25* also failed to suppress a number of *cdc28* alleles including *cdc28-4*, *cdc28-13*, and *cdc28-1N*, and *CDC28* did not suppress any *dbf2-ts* alleles when overexpressed on the high-copy plasmid YEp13 (J. Donovan et al., unpubl.). This would indicate that the two kinases may not be closely associated and/or they do not have significant overlapping substrate specificity.

Cells deleted for SDB25 are viable but have an aberrant size distribution

To examine whether *SDB25* has a function in cell-cycle

A

B

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-130          -110          -90
GGCTTGTGTTTCGCGTCGCAACTTTTGATATTATAAAAGAGAGAAGGCATATCCAAAG
-70          -50          -30
GATAAGGTATTGTTCTTGTAAATCAATCAACCAACCTCTACGGAATTTGACCCCTTGAA
-10          10          30
GCAGGGACTATTACACGAAATGACTCCTCCACCCACCGAGGTCCAGAGGGACTAGGT
          M T P S T P P R S R G T R Y
50          70          90
ACCTTGCGCAGCCTAGTGGCAATAGTCTAGTGGCCCTAATGCAAGGTCAAAGAGCCC
L A Q P S G N T S S A L M Q G Q K T P
110          130          150
CCCCAAAGCCTTCACAGAACCTAGTCCCTGTCCTCCCTCAACAACCTAAGTCTTTTAA
Q K P S Q N L V P V T P S T T K S F K N
170          190          210
ATGCGCCATTATTAGCACCTCCCAATTCGAACATGGGTATGACCTCTCCATTTAATGGC
A P L L A P P N S N M G M T S P F N G L
230          250          270
TTACGCTCCTCAACGCTCGCGTTTCCAAATCTTCAGTGAAGAGGACATATCCAAT
T S P Q R S P F P K S S V K R T L F Q F
290          310          330
TTGAAAGTCATGATAATGGAACAGTAAGGGAAGAGCAGGAACATTGGGTGCTGTAATA
E S H D N G T V R E E Q E P L G R V N R
350          370          390
GGATATTGTTTCCACGCGCAAAATGTGGATATAGATGCAGAGAAGAAGAAGAAG
I L F P T Q Q N V D I D A A E E E E E G
410          430          450
GAGAAGTCTTCTTCCCCCAGCAGACCTACATCTGCCAGGCAGTTACATTTCACTTG
E V L L P P S R P T S A R Q L H L S L E
470          490          510
AAAGAGATGAGTTGATCAGACACATAGAAGAAGATTACTAAGATGTACCTGGTACGC
R D E F D Q T H R K K I T K D V P G T P
530          550          570
CCAGCGACAAGGTGATAACATTGGAATTGGCAAAAATTTGGAATAAATCTCCGAAA
S D K V I T F E L A K N W N N S P K N
590          610          630
ATGACGCCAGGAGTCAAGAAAGTGAAGCAGGAGACATCATCATCAATCCAGTGGGG
D A R S Q E S E D E E D I I N P V R V
650          670          690
TGGGTAAAAATCCCTTTGCATCAGATGAAGTGGTCACTCAGGAAATTAGAAATGAACGTA
G K N P F A S D E L V T Q E I R N E R K
710          730          750
AAAGGGCAATGTTGAGAGAAAACCCAGATATAGAAGACGTAATAACATATGTCAATAAGA
R A M L R E N P D I E D V I T Y V N K K
770          790          810
AGGGAGAGGTGTAGAGAAACGAAGGTTAAGCGATGAAGAAAGAGAAGATTCAAGCCAA
G E V V E K R R L T D E E K R R F K P K
830          850          870
AGGCATTGTTTCAATCTAGGGATCAAGAGCATTAAGAAdATGAAAAAAGTTTCTG
A L F Q S R D Q E H *
890          910          930
GAACGATTATATTTTATTACTACTTATCTACATTTATTGCAAGGGGGC

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Figure 2. Restriction map and nucleotide sequence of the *SDB25* gene. (A) Partial restriction map of the 3.75-kb genomic fragment of YRpSDB25. The solid arrow indicates the ORF of *SDB25* as deduced from the nucleotide sequence. The sequence to the right of the *KpnI* site has been determined previously, and is the upstream of the divergently expressed gene *BOS1*. The ORF of *BOS1* is indicated by the open arrow. (B) Nucleotide and predicted amino acid sequence of the *SDB25* gene. Underlined are the restriction sites *KpnI* (38) and *HpaI* (786), used in constructing the *SDB25* null allele. Enclosed in the box is the putative cdc2⁺/Cdc28 phosphorylation site. The predicted amino acid sequence is shown in single-letter code.

progression, a null allele was created by disrupting the chromosomal copy of a wild-type strain. The *KpnI*–*HpaI* 750-bp fragment, representing ~90% of the *SDB25* ORF, was deleted, and the *TRP1* gene inserted in a plasmid construct that was used to replace one copy of *SDB25* in a diploid. Sporulation of this diploid strain, followed by dissection of 21 tetrads, resulted in four viable spore colonies from each tetrad. Southern hybridization was performed on genomic DNA from a number of *TRP1* spores to confirm that the wild-type *SDB25* gene was replaced with the *sdb25Δ::TRP1* deletion allele (data not shown). Thus, haploids deleted for *SDB25* are viable.

CG379 cells deleted for *SDB25* show no difference in growth rates, compared with an isogenic wild-type strain (doubling time of 140 min at 30°C in rich media). However, microscopic analysis of *sdb25Δ* cells revealed a greater heterogeneity in cell size than with wild type. Unusually, a significant proportion of cells were smaller than average wild-type cells, whereas others, some 6%, were considerably larger (Fig. 3A). In addition, the per-

centage of budded cells was much higher in *sdb25Δ* cells than in the wild-type population being 81% in *sdb25Δ* cells compared with 65% for wild type. FACS analysis is consistent with these observations. A mid-log phase culture of *sdb25Δ* cells consisted almost entirely of cells in S phase, or in G₂/M with a 2C DNA content (Fig. 3B). There were very few cells with a 1C DNA content. The corresponding forward scatter analysis also confirmed our microscopic observations that *sdb25Δ* cells are significantly smaller on average than cells of the control strain (Fig. 3C). One interpretation of these results is that deletion of *SDB25* may have two distinct effects on cell-cycle progression, a delay in G₂/M and/or an acceleration through G₁.

SDB25 has an essential function in cells deleted for DBF2

The genetic data described above suggested that *DBF2* and *SDB25* might interact in vivo. If this were so, it

Figure 3. Morphological and FACS analysis of *sdb25Δ* cells. (A) Mid-log cells in YEPD were harvested, fixed, and photographed by use of phase-contrast microscopy. The field of *sdb25Δ* cells shown was selected to illustrate the heterogeneity in cell size, rather than to represent the statistical distribution as described in the text. (B) Aliquots of fixed cells were also used for FACS analysis, to measure the distribution of DNA content. Cells were prepared for flow cytometry as described in Materials and methods. Horizontal axes, fluorescence (relative DNA content); vertical axis, relative cell numbers. (C) Forward scatter analysis was carried out at the same time as FACS analysis, as an indicator of the relative cell size distribution of the two cultures. Horizontal axis, forward scatter (relative cell size); vertical axis, relative cell number. The reduction in the peak of *sdb25Δ* relative cell number, as compared with wild type reflects the greater variance in size distribution in cells deleted for *SDB25*. Also note the slight elevation of the right-hand side of the *sdb25Δ* tracing above the wild-type tracing, which probably reflects the small percentage of large cells in the *sdb25* culture.

might be anticipated that cells deleted for both *SDB25* and *DBF2* would be severely perturbed, or inviable. A *MATa sdb25Δ::TRP1* was therefore crossed to a *MATαdbf2Δ::URA3* strain, the resulting diploid was sporulated, and 35 asci were dissected and scored for the *URA3* and *TRP1* markers. Altogether 4 nonparental ditypes, 9 parental ditypes, and 22 tetratypes were obtained, from which it could be inferred that a total of 27 spores were deleted for both *SDB25* and *DBF2*. None of the double mutant spores formed colonies, but microscopic examination showed that all had germinated and had subsequently ceased division, though a uniform terminal phenotype was not observed. Thus, *SDB25* has an essential function in a *dbf2Δ* background. To show that this apparent synthetic lethality was not simply a defect in outgrowth of *sdb25Δdbf2Δ* double delete spores, we transformed a *ura3/ura3* diploid heterozygous for *sdb25Δ::TRP1* and *dbf2Δ::LEU2* with *YEpSDB25(URA3)*. The diploid was then sporulated and *URA⁺* spore clones isolated, which were deleted for both *SDB25* and *DBF2*. All such strains failed to grow on agar containing 5-fluoro-otic acid (FOA), which is toxic in the presence of the *URA3* gene. Thus, these strains require the *YEpSDB25* plasmid for viability, confirming that the *sdb25Δdbf2Δ* double delete is lethal. In contrast, there was no essential requirement for *SDB25* in cells deleted for *DBF20* or in cells deleted for *SPO12*.

SDB25 is expressed under cell-cycle control after DBF2 in late mitosis

To investigate the expression of the *SDB25* gene during the cell cycle, Northern blot analysis was carried out on total RNA from synchronized cultures. Cells were synchronized by three distinct methods to reduce the possibility of artifactual results, and more than one cycle was monitored to ensure that the normal pattern of expression was observed. All of the data obtained indicated that *SDB25* is strongly cell-cycle regulated. For instance, in cells synchronized by elutriation the *SDB25* transcript showed more than a 10-fold increase over background levels during the peak of expression (Fig. 4). Comparison with the *DBF2* mRNA shows that *SDB25* peaks later in the cell cycle, very close to the *M/G₁* boundary. This was true in all Northern experiments, with *DBF2* preceding *SDB25* expression, suggesting that Sdb25 acts after Dbf2. Consistent with this, in cells synchronized by temperature arrest of a *dbf2-ts* mutant in late mitosis, the *SDB25* transcript appears almost immediately after the release from the block (data not shown).

Sdb25 is a phosphoprotein

To detect the gene product of *SDB25*, antibodies were generated against the full-length Sdb25 protein, apart from the first 13 amino acids (see Materials and methods). In an immunoblot of *S. cerevisiae* lysates, these antibodies recognized a polypeptide doublet with an apparent molecular mass of 40 kD (Fig. 5A). Both bands were absent when lysates were prepared from

Figure 4. Regulation of the *SDB25* transcript in cells synchronized by elutriation. The culture used in this experiment has been described previously (Kitada et al. 1993). RNA samples were analyzed by Northern hybridization with the indicated probes. RNA levels were quantitated by densitometry from suitably exposed autoradiographs and normalized relative to actin transcript levels. The peak value of *SDB25* and *DBF2* transcript levels were each given a value of 100 for graphical presentational purposes. (□) *DBF2* transcript levels; (■) *SDB25* transcript levels.

sdb25Δ::TRP1 cells and equally enhanced in cells carrying *YEpSDB25* (lanes 2,3). These results are consistent with *SDB25* encoding p40 and demonstrate that the Sdb25 protein migrates slightly anomalously with respect to its predicted molecular mass of 32.2 kD. Treatment of crude cell lysates with phosphatase prior to immunoblotting resulted in the disappearance of the upper band (Fig. 5B, lane 2). Addition of β -glycerophosphate, a competitive inhibitor of phosphatase, to the reaction or treatment with reaction buffer alone did not result in the disappearance of the more slowly migrating band (lanes 3,4). This demonstrates that Sdb25 is a phosphoprotein, the upper band in the doublet being the more phosphorylated form of the protein. However, in many immunoblots performed, no consistent pattern was observed with regard to the intensity of the two bands in the Sdb25 doublet. Thus, we do not draw any physiological conclusions from the fact that overexpressing *SDB25* resulted in both bands being equally enhanced in the immunoblot shown (Fig. 5A).

Sdb25 has an associated kinase activity that is CDC28 dependent

Immune complexes were prepared from *S. cerevisiae* lysates and assayed for histone H1 kinase activity in the

Figure 5. Characterization of Sdb25 antibodies. (A) Sdb25 protein levels in wild-type and *SDB25* overexpressing strains. (Lane 1) CG379 (wild type); (lane 2) JD100 (*sdb25Δ::TRP1*); (lane 3) CG379 carrying YEplacSDB25. Protein extracts were subjected to SDS-PAGE and immunoblotted with rabbit polyclonal anti-Sdb25 serum. Equivalent amounts of protein were loaded for each of the yeast lysates analyzed. The band immediately above Sdb25 appeared in all immunoblots with Sdb25 antibodies and served as an internal loading control. Molecular mass are given in kilodaltons. (B) Phosphorylation state of Sdb25. Extracts from CG379 cells for phosphatase treatment were prepared as described in Materials and methods. (Lane 1) Untreated CG379 extract. In lanes 2 and 4 extracts were treated with calf intestinal phosphatase; in lane 3 phosphatase buffer alone was used, in lane 4, 50 mM β-glycerophosphate was added to the extract before addition of the phosphatase.

presence of [γ -³²P]ATP. Anti-Sdb25 antibodies precipitate a histone H1 kinase activity that cannot be detected in cells deleted for *SDB25* (Fig. 6B, lanes 5,6). Thus, the kinase detected is associated with Sdb25 and does not bind directly to the antibodies. By use of preparations of Sdb25 immunoprecipitates from *cdc28* alleles, we found that the Sdb25-associated kinase activity is almost totally destroyed in *cdc28-4* immunoprecipitates incubated at the nonpermissive temperature (Fig. 6A). A similar, though not so dramatic, effect was observed with *cdc28-13* extracts. We also tested Sdb25 kinase activity in immunoprecipitates prepared from *dbf2Δ* extracts, which have been shown to have a significantly reduced Dbf2 kinase activity (Toyn and Johnston 1994). No significant reduction in Sdb25-associated kinase activity was observed in either *dbf2Δ* or *dbf20Δ* immunoprecipitates, as compared with a wild-type control (Fig. 6B). The Sdb25-associated kinase activity thus exhibits defects only in *cdc28* temperature-sensitive strains, which suggests that Cdc28 is the catalytic subunit in the Sdb25 kinase complex. To confirm this result, we carried out kinase assays in extracts depleted of the Cdc28 protein and found a dramatic reduction in Sdb25-associated kinase activity (data not shown). Taken together with the recent finding that p40^{SDB25} binds tightly to Cdc28 in vitro (Mendenhall 1993), we regard this as convincing evidence that Sdb25 is an in vivo component of a Cdc28 kinase complex.

The Sdb25-associated kinase complex is tightly cell-cycle regulated in late mitosis

In view of the cell-cycle-regulated expression of *SDB25*, both Sdb25 protein levels and the associated kinase activity were examined in cells synchronized by α-factor and compared with fluctuations in transcript levels. A

Figure 6. (A) Sdb25-associated H1 kinase activity is *CDC28*-dependent. Cell lysates were immunoprecipitated with anti-Sdb25 serum and assayed for kinase activity with histone H1. Immunoprecipitates from the indicated strains were split in two and assayed for kinase activity in vitro at either 23°C or 39°C. A gel autoradiograph and corresponding PhosphorImage quantitation are shown. (B) Sdb25-associated H1 kinase activity is not *DBF2* or *DBF20*-dependent. Cell lysates were immunoprecipitated with either anti-Dbf2, or anti-Sdb25, serum and assayed for histone H1 kinase activity. Immunoprecipitates from the indicated strains were assayed for either Dbf2 kinase activity or Sdb25-associated kinase activity. The wild-type control strain used in lanes 1 and 4 is CG379.

strain carrying an epitope-tagged version of Cdc28 was used, enabling total Cdc28 kinase activity to be measured. The Sdb25-associated kinase was found to be active for only a very short period during the cell cycle, at a time when many cells have fully divided chromatin and total Cdc28 kinase activity is decreasing (Fig. 7B). To rule out the possibility that this observed fluctuation might be influenced by the presence of an epitope-tagged version of Cdc28, we carried out a similar analysis in strain CG378 and obtained the same pattern of regulation of Sdb25-associated kinase activity (data not shown). Comparison of the fluctuations in the Sdb25-associated kinase activity and the *SDB25* transcript levels (Fig. 7B,D) shows that both are maximal at approximately the same time, suggesting a causal relationship between both events.

Sdb25 protein levels are up-regulated in late M/early G₁ phase

Following release from the α -factor block, the Sdb25 protein is evident for some 15 min up to about the time of budding (Fig. 7C). This approximates to the beginning of S phase, so that Sdb25 clearly persists while cells are in G₁. Interestingly, the Sdb25-associated kinase activity

has disappeared at this time, so that the kinase is clearly subject to other, independent forms of control. The Sdb25 protein then reappears again at the same time point where the peaks in the kinase activity and transcript levels occur and persists for some 30 min (cf. Fig. 7, B, C, and D). Because the peak in Sdb25-associated kinase activity occurs in late mitosis (see Discussion), Sdb25 must only be present in the mitotic cell cycle for a brief period, from late M phase to shortly after Start in the next cell cycle. To confirm this finding we used temperature-sensitive cell cycle mutants that block in M phase or G₁, when incubated at 37°C. Sdb25 protein is present at very low levels in mutants arrested in late M phase (*dbf2* and *cdc14*) but accumulates in G₁-arrested cells (*cdc28-4* and *cdc28-13*) (Fig. 8). The observed fluctuations in the cell cycle of Sdb25 protein levels are consistent with the earlier finding indicating that p40/Sdb25 is an unstable protein (Mendenhall 1993). Interestingly, analysis of the coding region of *SDB25/SIC1* shows it has three regions with significant PEST scores (amino acids 37–49, 115–141, and 198–212) (Nugroho and Mendenhall 1994). Such regions, rich in proline (P), serine (S), and threonine (T), are present in all G₁ cyclins (Nash et al. 1988; Hadwiger et al. 1989b) and are thought to be signals for proteolysis (Rogers et al. 1986).

Figure 7. Coordinate cell-cycle regulation of Sdb25 transcript levels, protein levels, and associated kinase activity. Cells were synchronized in G₁ phase, by use of α -factor as described in Materials and methods. At the indicated time points following release from α -factor, samples were removed from the culture. (A) Morphological indicators of cell cycle position: (●) Budded cells; (○) divided chromatin. (B) Immunoprecipitates prepared from protein samples were used for histone H1 kinase assays. Kinase assays were quantitated by PhosphorImage analysis and normalized relative to the peak value in each series of assays. For purposes of graphic representation the peak value in each cell cycle assay was given a value of 100. (□) Cdc28 kinase; (■) Sdb25-associated kinase. (C) Protein levels were analyzed by immunoblotting with anti-Sdb25 antibodies. (D) RNA levels were analyzed by Northern blot hybridization with the indicated probes. The first lanes in panels B, C, and D are mid-log kinase activity, protein level, and transcript level, respectively.

Figure 8. Sdb25 protein levels in cell cycle mutants arrested at 37°C. Cultures of the indicated strains were grown to mid-log levels and then split into two. One half was incubated at the nonpermissive temperature 37°C for 3 hr, and the other was grown at 25°C for the same amount of time. Immunoblots were carried out with Sdb25 antibodies, with equivalent amounts of protein being loaded in each lane.

Dbf2 and Sdb25-associated kinases are sequentially activated in late mitosis

Having established the timing of Sdb25 function, we then sought to determine whether activation of Sdb25-associated kinase occurred after activation of the Dbf2 kinase. An experiment similar to that described above suggested that Sdb25 kinase activity appears to peak slightly later than the peak of Dbf2 kinase activity in cells synchronized by α -factor. To examine the relative timing of the two kinase activities more precisely, *dbf2* cells were held at 37°C, the restrictive temperature, for 105 min. By this time >95% of the cells had fully divided chromatin, indicative of an anaphase/telophase arrest point, and both Dbf2 and Sdb25-associated kinase activities had fallen to a minimum. On return to the

permissive temperature, Dbf2 kinase activity rapidly reached a peak within 10 min, coincident with a sharp increase in Sdb25 protein levels. This was followed 5–10 min later by the peak of Sdb25-associated kinase activity, roughly coincident with the peak of Sdb25 protein levels (Fig. 9B). The Sdb25 protein was then degraded, returning to background levels 35 min after cells had been returned to 25°C. Note that cells enlarge during the 37°C holding, and consequently, on release to 25°C, the events of late M phase, G₁, and Start are compressed. On the basis of this experiment it is therefore difficult to determine the precise phase in which Sdb25 is degraded, but these data show that activation of the Sdb25-associated kinase occurs after the Dbf2 kinase. Thus, both at the level of transcription (Fig. 4) and kinase activity, it appears that *SDB25* and *DBF2* are sequentially activated in late mitosis.

Discussion

With the objective of trying to further elucidate the physiological role of the protein kinase Dbf2 in regulation of the yeast cell cycle, we undertook a screen for high-copy suppressors of *dbf2*-ts mutants. This paper reports on the cloning, sequencing, and characterization of *SDB25*, one of five *dbf2* suppressors that were isolated.

Our genetic data indicate that suppression of *dbf2*-ts alleles by overexpression of *SDB25* varies with the copy

Figure 9. Sequential activation of Dbf2 and Sdb25-associated kinase. A culture of strain LJ1 (*dbf2-2*) was synchronized by a temperature shift of *dbf2-2* as described in Materials and Methods. Cell samples were taken every 5 min for just over 1 hr following return to the permissive temperature, and protein extracts were prepared. (A) Morphological indicators of cell cycle position: (●) Budded cells; (○) divide chromatin. (B) Immunoprecipitates prepared from protein samples were used for kinase assays at the time points indicated. Kinase activities were quantitated and normalized as described in Fig. 6. (□) Dbf2 kinase; (■) Sdb25-associated kinase. Protein levels were analyzed by immunoblotting with anti-Sdb25 antibodies, with the nonspecific band appearing above the Sdb25 band serving as a loading control.

number of the plasmid used. That is, YRpSDB25 is an allele-specific suppressor of *dbf2-3*, whereas the higher copy number YEpSDB25 suppresses all three *dbf2-ts* alleles. Furthermore, suppression of *dbf2-3* by YRpSDB25 requires the presence of the Dbf2 structural and functional homolog, Dbf20. But more significantly, introducing YEpSDB25 into cells actually bypasses the normally essential requirement of Dbf2 and Dbf20 for completion of the cell cycle. One straightforward explanation of these data is that Sdb25 and Dbf2/20 are acting in parallel pathways, at the end of mitosis. Thus, when only slightly overexpressed, *SDB25* also requires Dbf20 to act in the alternative pathway, to allow completion of the cell cycle in the absence of a functional Dbf2 protein. However, when highly overexpressed, *SDB25*, alone, allows completion of the essential cell-cycle function common to the two pathways. The fact that *SDB25* has an essential function in a *dbf2Δ* background, *sdb25Δdbf2Δ* cells being unable to grow, is consistent with this interpretation of parallel, partially redundant pathways. From this view, *sdb25Δdbf2Δ* synthetic lethality arises because a component in both pathways is deleted, with the result that a vital cell function cannot be executed. This finding also supports the notion that *SDB25* has a role in the completion of essential mitotic events.

Further evidence for a late mitotic role for *SDB25* comes from examination of the *SDB25* transcript in synchronized cells. This shows that the gene is expressed under cell-cycle regulation, with a peak of expression very close to the M/G₁ boundary. Moreover, the tight correlation between the peaks of *SDB25* expression, Sdb25 protein levels, and Sdb25-associated kinase activity demonstrates that transcriptional activation of *SDB25* is rapidly translated into a functional effect. This also suggests that the primary control over Sdb25 function may be at the transcriptional level, rather than some post-translational effect, such as phosphorylation/dephosphorylation. With two different methods of synchrony, Northern analysis also shows that the peak of *SDB25* expression occurs shortly after the peak of *DBF2* expression. Thus *SDB25* and *DBF2* are not coordinately, but rather sequentially regulated, which may be a transcriptional mechanism for ensuring that Sdb25 functions after Dbf2. The temporally close order of Dbf2 and Sdb25 function is also demonstrated in cells released from a *dbf2*-thermosensitive block, where the Sdb25-associated kinase is activated immediately after the appearance of the Dbf2 kinase, following release from the block. However, neither the genetic interactions described above nor our cell cycle analysis prove anything conclusive about how the function of Sdb25 relates to the function of Dbf2. Using the available antibodies we were unable to demonstrate coimmunoprecipitation of Sdb25 and Dbf2, nor is an Sdb25–protein A fusion protein phosphorylated by the Dbf2 kinase.

Irrespective of the precise physiological relationship between Dbf2 and Sdb25, production of Sdb25 in late M phase appears to result in the protein immediately binding to an active form of a Cdc28 kinase complex. Fur-

thermore, our cell cycle analysis indicates that Sdb25 associates with an active Cdc28 kinase complex for only a short time during the cell cycle. From an earlier, detailed examination of the cell cycle fluctuation of Cdc28 kinase activity with histone H1 as a substrate (Surana et al. 1991), it was concluded that the peak of Cdc28 kinase activity occurs in G₂/M, begins to decline on entry into anaphase, and reaches the lowest level on entry into G₁. This is consistent with a similar analysis (Reed and Wittenberg 1990), which found that the peak of Cdc28 kinase activity occurs in mitosis, and that by the time mitosis has been completed the Cdc28 protein kinase has been inactivated. If these findings can be generalized, then our own analysis (Fig. 7B) would indicate that Sdb25 associates with an active Cdc28 kinase complex at, or more likely after, anaphase has been initiated, but before cells enter G₁.

Mendenhall (1993), has shown recently that purified p40^{SDB25} can inhibit in vitro Cdc28 kinase activity. Although we have no direct evidence to support this conclusion, it is interesting that our cell cycle analysis indicates that Sdb25 is present only when Cdc28 is down-regulated across the M/G₁ interface. Thus, although we have shown that Sdb25 binds to an active kinase at the end of mitosis, its primary role in mitotic cells could well be involved with the maintenance of a deactivated Cdc28 kinase as cells traverse the M/G₁ boundary. Furthermore, the demonstration that Sdb25 has an associated kinase activity that phosphorylates histone H1 in vitro is not incompatible with this suggestion. When assaying Sdb25-associated kinase activity we may be detecting an already inhibited form of Cdc28 kinase activity. From Figure 7 it is clear that the rapid inactivation of the Sdb25-associated kinase at the end of mitosis is not attributable to destruction of Sdb25, which is present in G₁ cells. In respect of this, it would be of interest to determine whether binding of Sdb25 to a Cdc28 kinase complex is coincident with the appearance of Sdb25-associated kinase activity. However, with the available antibodies, we have yet to determine coimmunoprecipitation of p40/Sdb25 and Cdc28, as previous workers have failed to do (Wittenberg and Reed 1988).

Viewing Sdb25 as an inhibitor of Cdc28 kinase activity can help to explain our morphological observations concerning *sdb25Δ* cells, which indicate that deletion of *SDB25* results in two distinct cell-cycle effects. The increase in budded cells and large cells in an *sdb25Δ* culture indicate a G₂/M delay. However, the overall smaller size and decrease in unbudded single cells both imply an acceleration through G₁. Possibly, deletion of *SDB25* results in a phenotype that is a composite of a delay in late M phase, because Cdc28 kinase activity takes longer to decay, and, independently, G₁ speeds up, because Cdc28 kinase is up-regulated more rapidly as cells traverse Start (see Fig. 10B). Note that this interpretation of the *sdb25Δ* phenotype applies to a population of cells, the two partial morphological effects (large budded cells → small cells), being mutually exclusive when applied to successive cycles undergone by an individual cell. Furthermore, the combination of these two effects on the ki-

Figure 10. Speculative model for the role of Sdb25 in a late mitotic pathway. (A) Dbf2 kinase is activated after the metaphase to anaphase transition (Toyn and Johnston 1994), suggesting that its activity may be regulated by a signal transduced via separation of sister chromatids. As Dbf2 kinase activity peaks, Sdb25 is rapidly up-regulated and binds to a CDC28-dependent kinase complex, at a time when total Cdc28 kinase activity is being down-regulated. As discussed in the text, our genetic analysis indicates that Sdb25 and Dbf2 act in parallel pathways, which may converge to help down-regulate Cdc28 at the end of mitosis. However, our data do not exclude the possibility that Sdb25 functions directly downstream in a Dbf2/20-dependent pathway. Once produced Sdb25 persists to approximately Start of the next cell cycle, as depicted by the shaded box. The broken line

linking the two Cdc28 boxes indicates the likely Cdc28 kinase activity as cells traverse M/G₁. As for the role of Sdb25, at present we favor the interpretation that Sdb25 helps inactivate and maintain a down-regulated Cdc28 kinase activity, from late M phase to early G₁. This may facilitate a rapid end to mitosis and a protraction of G₁ prior to Start. Sdb25 may not bind Cdc28 directly; rather it may physically associate with one of the Clb cyclins, the main regulators of mitotic Cdc28 kinase activity. As cells pass Start in G₁, Sdb25 is degraded, suggesting it may be targeted for destruction via the CDC34 ubiquitin conjugating enzyme. (B) Consequences of deleting *SDB25*. On the basis of the model described above, the immediate consequence of deleting *SDB25* is to slow down the passage of cells through the end of mitosis. This would result in the accumulation of budded cells and some enlargement in size as cells continue to grow independently of the ongoing regulatory processes. Separately, deletion of *SDB25* may also result in some cells accelerating through pre-Start G₁, because of the restraining effect of Sdb25 being absent. This would result in such cells having a smaller size on average than wild type.

netics of growth rates could explain why the doubling time is essentially unaltered when *SDB25* is deleted.

In this paper we have presented an initial characterization of a newly identified yeast gene, *SDB25*, which encodes a previously characterized substrate and inhibitor of the Cdc28 kinase. We have shown that Sdb25 has a function in late mitosis, most likely after the metaphase to anaphase transition. This is inferred from the timing of the appearance of the Sdb25-associated kinase complex and from the fact that Sdb25 shows genetic interactions with Dbf2, the function of which is required in late nuclear division (Toyn and Johnston 1994). Our demonstration that the Sdb25-associated kinase is CDC28-dependent suggests Sdb25 may function to regulate the activity of Cdc28 at the end of mitosis. A speculative model, outlining the cell cycle role of Sdb25, is shown in Figure 10A. Formally, formation of the Sdb25-associated, CDC28-dependent, kinase complex could have a positive role in promoting the M/G₁ transition, perhaps by altering the substrate specificity of the kinase. As indicated in the model, we favor an alternative view that Sdb25 may help to down-regulate Cdc28 activity at the end of mitosis. This is consistent with the in vitro properties of p40^{SDB25} being a potent inhibitor of Cdc28 kinase activity (Mendenhall 1993), the morphology of *sdb25Δ* cells, and the timing of Sdb25 expression. Incidentally, if nutrients are limiting, instead of traversing Start, the cell moves into stationary phase in the next cell cycle, when Cdc28 is inactive. Significantly, Sdb25

is present only during these two phases, M/G₁ (our results) and stationary (Mendenhall 1993), strongly suggesting that its physiological role is to maintain an inactive form of Cdc28 prior to commitment to a new cell cycle.

This model also indicates our thinking about the possible mechanism responsible for the cell-cycle regulated degradation of Sdb25. In this respect, a parallel can be drawn with Far1 (McKinney et al. 1993), a negative factor required for cell-cycle arrest (Chang and Herskowitz 1990). Like Sdb25, Far1 is a cell-cycle-regulated phosphoprotein (McKinney et al. 1993) that has been shown to bind and inhibit the Cdc28 protein kinase (Peter et al. 1993). Also, both proteins are degraded in G₁, suggesting that they both may be directed into a proteolytic pathway via the ubiquitin-conjugating enzyme CDC34 (Goebl et al. 1988). CDC34-mediated proteolysis has been proposed recently to account for the disappearance of Far1 after Start (McKinney et al. 1993). Perhaps future work will show that Sdb25 and Far1 are members of a group of cell-cycle-regulated CDK inhibitor proteins that function to modulate the activity of Cdc28 in conjunction with the Clb/Cln cyclin family.

Materials and methods

Culture conditions and general techniques

The relevant genotypes of *S. cerevisiae* strains used in this study

are listed in Table 1. Cells were grown in YEPD (1% Difco yeast extract, 2% Difco Bacto-peptone, and 2% glucose) except when selecting for plasmids; in these cases, they were grown in 0.67% YNB (Difco), 2% glucose, with the appropriate supplemented amino acids. Selection of *ura3* cells that had lost a *URA3* plasmid was accomplished by plating the cells on media containing FOA [Boeke et al. 1984]. Yeast transformations were performed by a modification [Gietz and Sugino 1988] of the lithium acetate method [Ito et al. 1983]. The incubation temperature for yeast cultures was 30°C, except for thermosensitive strains, which were grown at the permissive temperature of 25°C, or a restrictive temperature of 37°C. Cell numbers were determined by use of a particle counter (Coulter Electronics, Dunstable, England).

Construction of the *dbf2Δdbf20Δ* strain containing YEpSDB25 was as follows: Strain J211-2C has the genotype *dbf2Δdbf20Δ ade2 ura3* and is kept alive by the plasmid pASZ11-DBF2 (*CEN ARS ADE2 DBF2*), resulting in white colonies. The plasmid YEpSDB25 was introduced into this strain, and red sectors, corresponding to clones that had lost the pASZ11-DBF2 plasmid, were picked.

All DNA manipulations were as described previously [Maniatis et al. 1982]. The dideoxy chain termination method [Sanger et al. 1977] and double-stranded DNA were used for DNA sequence analysis, using the Sequenase version 2.0 sequencing kit (U.S. Biochemical).

Identification of SDB25

SDB25 was isolated as a single clone with an insert of ~3.75-kb from a *S. cerevisiae* genomic library ligated into the multiple-copy plasmid YRp12 [Barker and Johnston 1983]. To locate the suppressor gene within the genomic fragment, plasmid YRpSDB25 [YRp12 carrying the 3.75-kb insert] was subject to Tn1000 mutagenesis in *Escherichia coli* [Sedgwick and Morgan 1994], and the resulting library of randomly disrupted plasmids was transformed into yeast strain L182-7A (*dbf2-3*). Subsequent replica-plating of the transformants onto YEPD at 37°C and 25°C indicated those plasmids where suppressor function had been disrupted. The transposon sites were located by restriction

analysis of the extracted plasmids, and DNA sequencing analysis was carried out using primers specific to the γ , the δ , and γ sequences of Tn1000.

Synchronization procedures

The elutriator-synchronized cultures used here have been described previously [Kitada et al. 1993]. Synchronous cultures of MATa cells were produced by the α -factor method [Johnston et al. 1990]. Synchronous cultures of *dbf2-2* cells were produced by a temperature-shift method [Creanor and Toyn 1994]. Briefly, a log phase culture of diploid strain LJ1 (homozygous for *dbf2-2*), growing at 25°C in YEPD, was transferred to 37°C for 105 min. The arrested cells were then rapidly cooled to 25°C, the permissive temperature, and cell samples were taken.

Cytological methods

To visualize nuclei, cells were stained with 4,6-diamidino-2-phenylindole (DAPI) [Shero et al. 1991] and observed with a Zeiss Axiophot microscope. The DNA content of individual cells was measured using a FACStar Plus (Becton Dickinson) flow cytometer. Cells were prepared for flow cytometry as described previously [Parkes and Johnston 1992]. Photomicrographs of cells were taken with a phase-contrast microscope with a 40 $\times$ objective.

Gene replacement, epitope tagging, and plasmids

A null allele of *SDB25* was constructed by the one-step gene disruption method [Rothstein 1983]. The 750-bp *KpnI*-*HpaI* fragment of *SDB25* was replaced by an 850-bp fragment containing the *TRP1* gene, this disruption being made in YRpSDB25. The DNA fragment containing *sdb25Δ::TRP1* was generated by *NcoI*-*SspI* digestion and used to transform the *trp1/trp11* diploid CG378/379 (Table 1). Selected diploid prototrophs were determined for *SDB25* deletion by Southern blot analysis. Plasmid YEplacSDB25 was constructed by insertion of the *EcoRI*-*HindIII* 1.8-kb fragment of YRpSDB25, containing *SDB25*, into the

Table 1. Yeast strains used in this study

Strain	Relevant genotype	Source
L119-7D	MATa <i>dbf2-1 ade1 trp1-289 ura3-52</i>	Parkes and Johnston (1992)
L181-6B	MATa <i>dbf2-2 leu2-3,112 trp1-289 ura3-52</i>	Toyn and Johnston (1993)
L182-7A	MATa <i>dbf2-3 ade1,5 leu2-3,112 trp1-289 ura3-52</i>	this study
cdc28-1N	MATa <i>cdc28-1N ade2-1 leu2-3,112 trp1-1 ura3</i>	Kitada et al. (1993)
cdc28-13	MATa <i>cdc28-13 ade1 leu2-3,112 trp1 ura3Δ</i>	Kitada et al. (1993)
L153-6C	MATa <i>cdc28-4 leu2 trp1 ura3 his7 tyr1</i>	this study
L195-6D	MATa <i>cdc14 ade1 ura3 trp1</i>	this study
S7-4A	MATa <i>dbf2Δ::URA3 ade5 his7 leu2-3,112 trp1 ura3</i>	Toyn et al. (1991)
S2-2D	MATa <i>dbf2Δ::LEU2 ade5 leu2 trp1</i>	Toyn et al. (1991)
CG378	MATa <i>ade5 leu2-3,112 trp1-289 ura3-52</i>	C. Giroux (Wayne State University, Detroit, MI)
CG379	MATa <i>leu2-3,112 trp1-289 ura3-52 his7-2</i>	C. Giroux
J99	CG378 <i>dbf20Δ::TRP1</i>	Toyn et al. (1991)
V378	CG378 <i>spo12Δ::TRP1</i>	Parkes and Johnston (1992)
JD100	CG379 <i>sdb25Δ::TRP1</i>	this study
J149	MATa <i>dbf2-2 ade2-101 leu2 trp1 ura3-52</i>	Toyn and Johnston (1993)
LJ1	<i>dbf2-2/dbf2-2</i>	L181-6B $\times$ J149
J103-2A	MATa <i>dbf20Δ::TRP1</i>	J99 $\times$ L181-6B
JD101	MATa <i>dbf2-3 dbf20Δ::TRP1</i>	L182-7A $\times$ J103-2A
J211-2C	MATa <i>ade2-101 ura3 can1 dbf2Δ::LEU2 dbf20Δ::TRP + pASZ11-DBF2</i>	Toyn and Johnston (1993)

multiple cloning site of the high-copy plasmid YEplac181 (Geitz and Sugino 1988). Plasmid YEpSDB25 contains the *SDB25* gene cloned into the high-copy plasmid YEp24. The plasmid pASZ11-DBF2 has been described previously (Toyn and Johnston 1993).

The *CDC28* gene was tagged by insertion of the HA1 epitope (from R. Deshaies and Dr. P. Sorge, University of California, Berkeley), which is recognized by the 12CA5 monoclonal antibody (Wilson et al. 1984). The integrating plasmid pRD86 was used, which contains *URA3* and the carboxy-terminal part of the *CDC28* gene. The plasmid was linearized using the unique *KpnI* site in the *CDC28* gene, before integrative transformation (Rothstein 1983), resulting in one complete epitope-tagged *CDC28* gene, and one amino-terminally truncated *CDC28* gene in the yeast genome.

Northern and Southern blot analysis

Total RNA was extracted from cells as described previously (White et al. 1986). A 5- μ g sample of total RNA, denatured with glyoxal, was separated on a 1.2% agarose gel, transferred to a GeneScreen hybridization membrane (Dupont, NEN Research Products, Boston, MA). Probes for RNA-DNA hybridization were internal fragments from the genes concerned, with the *SDB25* probe being the 750-bp *KpnI*-*HpaI* fragment. DNA was labeled with [α -³²P]TTP by use of an oligo-labeling protocol (White et al. 1986). RNA levels were quantitated with a Personal Densitometer PD-130 (Molecular Dynamics).

Yeast genomic DNA was extracted by the method of Hoffman and Winston (1987). The *SspI*-*NcoI* 3.1-kb fragment of the *SDB25* locus was labeled as for Northern blotting and used as a probe for Southern blot analysis.

Preparation of Sdb25 antiserum

The 2.1-kb *KpnI*-*BglII* fragment of YRpSDB25 was subcloned into pRIT2 (Lowenadler et al. 1986) to give pJ100, which contains a protein A-Sdb25 fusion gene having all but the first 13 amino acid residues of the Sdb25 polypeptide. The fusion protein was induced in *E. coli*, purified using an IgG-Sepharose column (Pharmacia), and injected into rabbits.

Preparation of yeast extracts and immunoblot analysis

Yeast total protein extracts were prepared by resuspending cells in cold lysis buffer (20 mM Tris at pH 7.4, 10 mM EDTA, 100 mM NaCl, 1% Triton X-100, 5% glycerol, 1 mM each of NaF, β -glycerophosphate, Na₃VO₄, EGTA, and sodium pyrophosphate). The following inhibitors were included during cell lysis: 5 μ g/ml each of leupeptin, pepstatin A, chymostatin, aprotinin (Sigma), and AEBSF (Calbiochem). For phosphatase treatment, cells were resuspended in phosphatase buffer (50 mM Tris at pH 8.0, 10% glycerol) plus protease inhibitors as described above. Cells were broken by vortexing with acid-washed glass beads (4 $\times$ 30 sec bursts), after which the lysate was cleared by spinning for 5 min in a benchtop centrifuge. In any given experiment all samples were normalized to contain the same amount of protein (Lowry et al. 1951). Extracts (~100 μ g) used in the phosphatase treatment were incubated at 37°C for 30 min, with 1 unit of calf intestinal alkaline phosphatase (Boehringer Mannheim) in 20 μ l of phosphatase buffer, or mock-treated with 20 μ l of phosphatase buffer. When used, the phosphatase inhibitor β -glycerophosphate (50 mM) was added to 20 μ l of phosphatase buffer.

For immunoblotting, cell extracts containing 100 μ g of total protein were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose (0.45 μ m, Schleicher & Schuell) using a semidry blotting unit (Bio-Rad) according to the manufacturer's instructions. Nitrocellulose blots were blocked by

overnight incubation in PBS/1% nonfat milk and transferred to a 1:25 dilution of Sdb25 antiserum for 2 hr, followed by sequential incubations with antirabbit IgG-conjugated biotin (1:1000), and streptavidin alkaline phosphatase (1:1000) (Sigma). All antibody incubations were done at room temperature with agitation, followed by 30-min washes with several changes of PBS/0.5% BSA buffer. Proteins that interacted with the antibodies were visualized using the chromogenic substrates BCIP/NBT, as described previously (Harlow and Lane 1988).

Protein kinase assays

For Sdb25-associated kinase activity, 1 μ l of Sdb25 antiserum was added to 100 μ g of yeast extract and incubated for 1 hr on ice. Protein A beads (20 μ l of protein A-Sepharose CL-4B; Pharmacia) were then added, and incubation continued with mixing for an additional 30 min. The beads were then washed three times in 1 ml of lysis buffer, followed by one wash in assay buffer (25 mM MOPS at pH 7.2, 60 mM β -glycerophosphate, 15 mM *p*-nitro-phenylphosphate, 15 mM MgCl₂, 5 mM EGTA, 1 mM dithiothreitol, 0.1 mM Na₃VO₄, 10 μ M ATP and 5 μ g/ml each of leupeptin, pepstatin A, chymostatin, and aprontinin). The washed immunoprecipitate was then placed at 30°C and 10 μ l of assay buffer containing 1 μ Ci of [γ -³²P]ATP, and 5 μ g of calf thymus histone H1 (Sigma, type III-S) was added. Incubation was continued for 30 min, and the reaction stopped by the addition of 15 μ l of SDS-polyacrylamide gel sample buffer and heating for 3 min at 90°C prior to electrophoresis.

For Cdc28 kinase assays, 1 μ l (10 μ g of protein) of Mab 12CA5 (ascites fluid, from Babco) was added to 100 μ g of yeast extract from strains containing the epitope-tagged Cdc28, with subsequent steps being essentially the same as for Sdb25-associated kinase assays. Dbf2 kinase assays were as described previously (Toyn and Johnston 1994), being essentially the same as Sdb25-associated and Cdc28 kinase assays. Incorporation of ³²P into histone H1 was quantitated by β -imaging using a PhosphorImager (Molecular Dynamics).

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Note added in proof

The sequence data described in this paper have been submitted to the EMBL data library under accession number X78309.

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