Genetic analysis of Cln/Cdc28 regulation of cell morphogenesis in budding yeast

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The *CLN1*, *CLN2* and *CLN3* gene family of G₁-acting cyclin homologs of *Saccharomyces cerevisiae* is functionally redundant: any one of the three Cln proteins is sufficient for activation of Cdc28p protein kinase activity for cell cycle START. The START event leads to multiple processes (including DNA replication and bud emergence); how Cln/Cdc28 activity activates these processes remains unclear. *CLN3* is substantially different in structure and regulation from *CLN1* and *CLN2*, so its functional redundancy with *CLN1* and *CLN2* is also poorly understood. We have isolated mutations that alter this redundancy, making *CLN3* insufficient for cell viability in the absence of *CLN1* and *CLN2* expression. Mutations causing phenotypes specific for the cell division cycle were analyzed in detail. Mutations in one gene result in complete failure of bud formation, leading to depolarized cell growth. This gene was identified as *BUD2*, previously described as a non-essential gene required for proper bud site selection but not required for budding and viability. Bud2p is probably the GTPase-activating protein for Rsr1p/Bud1p [Park,H., Chant,I. and Herskowitz,I. (1993) *Nature*, 365, 269–274]; we find that Rsr1p is required for the *bud2* lethal phenotype. Mutations in two other genes (*ERC10* and *ERC19*) result in a different morphogenetic defect: failure of cytokinesis resulting in the formation of long multinucleate tubes. These results suggest direct regulation of diverse aspects of bud morphogenesis by Cln/Cdc28p activity.

Key words: *BUD2/erc/GAP/RSR1*/small GTPases

Introduction

In the budding yeast *Saccharomyces cerevisiae*, the Cdc28p protein kinase controls cell cycle progression at the G₁/S and the G₂/M transitions (Nasmyth, 1993). Traversal through the Cdc28p-controlled point in G₁ (called START) commits cells to DNA replication and bud emergence (Pringle and Hartwell, 1981). This transition is controlled by complexes of Cdc28p and three G₁-acting cyclin homologs, Cln1p, Cln2p and Cln3p (Richardson *et al.*, 1989; Wittenberg *et al.*, 1990; Tyers *et al.*, 1993). Transcription and Cdc28p-associated kinase activity of *CLN1* and *CLN2* are cell cycle-regulated, peaking at START, while *CLN3* transcription and

protein kinase activity are constitutive throughout the cell cycle (Wittenberg *et al.*, 1990; Tyers *et al.*, 1993).

When yeast cells traverse START, bud formation is initiated (Pringle and Hartwell, 1981). At least three sets of genes are involved in bud formation. One class of genes is involved in bud site selection. Haploid yeast cells usually bud axially (i.e. subsequent budding occurs next to the previous bud site), while diploid cells bud at opposite poles (Chant and Herskowitz, 1991). Mutations in *RSR1/BUD1*, *BUD2* or *BUD5* cause both haploids and diploids to bud randomly, while mutations in *BUD3* or *BUD4* cause bipolar budding in haploids (Chant and Herskowitz, 1991). Bud site selection is a non-essential process, and in its absence cells bud randomly with no obvious ill effects (Chant and Herskowitz, 1991). Another class of genes is required for budding at any site, independent of bud site selection. These include *CDC24*, *CDC42*, *BEM1* and *BEM2* (Sloat *et al.*, 1981; Adams *et al.*, 1990; Bender and Pringle, 1991). Mutations in these genes lead to an unbudded arrest, but the cells continue to grow in a depolarized fashion, replicate their DNA and undergo nuclear division. The third class are the cytoskeletal components required for budding, which include actin cables and the bud neck filament organizers *CDC3*, *CDC10*, *CDC11* and *CDC12* (Pringle and Hartwell, 1981; Drubin, 1991; Ford and Pringle, 1991; Kim *et al.*, 1991).

Bud site selection and assembly proteins both require small GTPases. Rsr1/Bud1p is a Ras-like GTPase and Cdc42p is a rho-class GTPase (Bender and Pringle, 1989; Johnson and Pringle, 1990). The GTP-bound form of the GTPase is generally considered to be the active form (Bourne *et al.*, 1991). GTPase action generates the GDP-bound form, which is probably inactive. In order to reactivate the protein, GDP must be exchanged for GTP. The guanine nucleotide exchange factor for Bud1p is Bud5p (Chant *et al.*, 1991; Powers *et al.*, 1991). Intrinsic GTPase activity of most of these GTP-binding proteins is quite low, and GTPase-activating proteins (GAPs) are required to stimulate their activity (Bourne *et al.*, 1991). Recently, Bud2p has been identified as the GAP for Bud1p (Park *et al.*, 1993).

Any single *CLN* is sufficient to activate START (Richardson *et al.*, 1989). This functional redundancy is surprising because while Cln1p and Cln2p are structurally homologous and their expression is cell cycle-regulated, Cln3p is only distantly related to Cln1p or Cln2p and its expression is constitutive through the cell cycle (Hadwiger *et al.*, 1989; see above).

We have devised a screen in order to identify potential regulators and downstream targets of specific Cln proteins. We screened for mutants which can no longer survive in the presence of a single *CLN* gene and thus have an elevated requirement for *CLNs* (*erc*). Here we report the isolation of *erc* mutants in a *cln1 cln2 CLN3* background. Of these, two have distinct defects in bud morphogenesis, but do not block DNA replication. The *ERC25* gene was found to be allelic to *BUD2* (Chant and Herskowitz, 1991; Park *et al.*, 1993), and allelic to the *CLA2* gene, which was isolated in

a conceptually similar screen [see accompanying manuscript (Cvrčková and Nasmyth, 1993)].

Results

Isolation and characterization of *erc* mutants

Mutant isolation. *cln1 cln2 CLN3* strains are viable (Richardson *et al.*, 1989), even at 38°C [exhibiting a high plating efficiency despite some morphological abnormalities (Hadwiger *et al.*, 1989; Figures 1, 3 and 4)]. A *cln1 cln2 CLN3* strain with *GAL1::CLN1* on a CEN plasmid was mutagenized with ethylmethanesulfonate (EMS) (see Materials and methods). Mutagenized cells were screened at 38°C for their ability to grow on galactose, which induces over-expression of *CLN1*, but not on glucose, which represses *CLN1* expression. Mutants that were viable on galactose but not on glucose even after loss of the *GAL1::CLN1* plasmid

Table 1. *erc* mutants recovered

	Number of alleles ^a	cdc arrest in glucose ^b
<i>cln3</i>	7 (7)	unbudded, START ^{-c}
<i>swi4</i>	3 (0)	NC ^d
<i>swi6</i>	1 (1)	NC ^d
<i>cdc28</i> ^k	1 (0)	unbudded, START ^{-c}
<i>erc1</i>	6 (5)	— ^e
<i>erc5</i>	3 (1)	—
<i>erc7</i>	1 (0)	—
<i>erc8</i>	4 (0)	—
<i>erc10</i>	4 (3)	tubular, multinucleate
<i>erc11</i>	2 (0)	—
<i>erc12</i>	6 (0)	—
<i>erc14</i>	3 (0)	small unbudded, G ₁ ^f
<i>erc16</i>	2 (0)	—
<i>erc17</i>	6 (0)	—
<i>erc19</i>	2 (0)	tubular, multinucleate ^g
<i>erc20</i>	3 (0)	—
<i>erc21</i>	1 (0)	—
<i>erc22</i>	1 (0)	unbudded, G ₁ ^h
<i>erc23</i>	1 (0)	—
<i>erc24</i>	1 (0)	—
<i>erc25 (bud2)</i>	9 (0)	unbudded, multinucleate ⁱ
<i>erc26</i>	2 (0)	—
<i>erc27</i>	3 (0)	—
<i>erc28</i>	2 (0)	—
Unsorted ^j	29 (NC ^d)	NC ^d
Total number of mutagenized cells screened = 30 000		

^aThe number of alleles is shown; the number that have a tight lethal phenotype at 30°C as well as 38°C is shown in parentheses.

^bcdc arrest is defined as a first cycle arrest with a uniform terminal morphology when the mutants are shifted to glucose medium (YEPD). Cellular morphology and DNA content (determined by FACS analysis; data not shown) are listed.

^cSTART arrest, cells continue growing as elongated shmoos.

^dNC, not characterized.

^eNon-cdc arrest.

^fStrains arrest with 65–86% unbudded cells.

^gCells are tubular in galactose but phenotype is more severe in glucose. Cell morphology makes determination of cdc arrest unclear.

^hStrain arrests with 80–90% unbudded cells. Mutant efficiently rescued by *SIT4* on a CEN plasmid.

ⁱStrains arrest with 80–90% unbudded cells and 45% of cells are binucleate 9 h after shift to glucose.

^jMutants that complement all the complementation groups listed above.

^kSee Cross and Blake (1993).

were discarded. We required that the mutants exhibit a differential plating efficiency under restrictive versus permissive conditions of <1 in 10⁴. We backcrossed at least one member of each complementation group to wild-type, and kept only mutations yielding 2:2 segregation of conditional inviability, indicative of a mutation in a single gene. Seventy-six mutants in 24 complementation groups were identified with this screen (Table I). Unexpectedly,

Fig. 1. Cell morphology of mutants. Strains were all of the genotype *cln1 cln2 CLN3 GAL1::CLN1*. Cultures were grown at 38°C in YEPGal, and switched to pre-warmed YEPD for 8 h before staining of DNA with DAPI and performing fluorescent photomicroscopy. **Left panels:** YEPGal; **right panels:** YEPD. Top: ERC⁺; second row: *bud2::URA3* (*erc25* null allele; see text); third row: *erc10-1*; bottom row: *erc14-1*. The *erc10-1* mutant was incubated at 30°C, since it (but not the other mutants) exhibited a strong mutant phenotype at both 30°C and 38°C.

many of these mutants were viable on glucose at 30°C, despite tight inviability at 38°C. The effect of temperature in this mutant hunt is not understood. It may be relevant in this connection that Tyers *et al.* (1992) showed some intrinsic temperature sensitivity in the Cln3p-associated kinase activity.

Mutations in several genes were expected based on previous work: mutations in *cln3* (Richardson *et al.*, 1989), *cdc28* (Hadwiger *et al.*, 1989), *swi4* and *swi6* (Nasmyth and Dirick, 1991; Ogas *et al.*, 1991) were expected and were recovered based on complementation analysis (Table I). *sit4* mutations increase the CLN requirement (Fernandez-Sarabia *et al.*, 1992); we recovered one mutation (*erc22*) that was efficiently rescued by the *SIT4* gene on a low copy number plasmid.

A subset of erc mutations exhibit a cell division cycle (cdc) mutant phenotype. While START is a CLN-dependent commitment to further cell cycle progression, further processes are required to successfully complete DNA synthesis and cellular division. These downstream events could be genetic targets of CLN action. Thus, it seemed plausible that mutations that affected the ability of CLN3 to carry out some of these specific cell cycle processes could be isolated. Such mutations might have a Cdc⁻ phenotype: arrest with a uniform terminal morphology, implying that the mutated gene is required only at a specific point or for a particular process in the cell division cycle (Pringle and Hartwell, 1981). Excluding mutations in the previously characterized *cln3*, *cdc28*, *swi4*, *swi6* and *sit4* genes (see above), three other *erc* mutations exhibited a Cdc⁻ phenotype (Figure 1). Mutations in *erc10* cause a first-cycle arrest with a multinucleate tubular phenotype upon shift to glucose. Mutation of *CDC3*, *CDC10*, *CDC11* and *CDC12* in a *CLN1 CLN2 CLN3* background results in a similar phenotype (see Introduction). The *erc25* mutation results in first-cycle arrest as large, spherical, unbudded cells, which become multinucleate upon prolonged arrest. This phenotype was similar to that observed with mutants in the bud site assembly genes *CDC24*, *CDC42*, *CDC43* and *BEM1* in a *CLN1 CLN2 CLN3* background (see Introduction). The *erc14* mutation causes a first-cycle arrest as small, mostly unbudded cells (65–85% unbudded).

CLN gene rescue of erc mutants. We examined the ability of different CLN genes on plasmids to rescue these *erc* mutants. While *CLN1*, *CLN2* and *CLN3-2* [a hyperactive allele of *CLN3* (Cross, 1988)] on low copy number CEN plasmids rescued two alleles of *erc25* (*erc25-1* and *erc25-9*), *CLN3* had no rescue activity (data not shown). *erc10* showed a spectrum of CLN gene rescue similar to that of *erc25*. Although both *erc10* and *erc25* mutants were rescued by hyperactive *CLN3-2* at the level of viability, the rescued strains maintained a highly defective cell morphology (data not shown). In contrast, *CLN1* and *CLN2* rescued *erc25* and *erc10* strains to essentially normal cell morphology. Thus full rescue of these *erc* mutations is specific to *CLN1* and *CLN2*. The *erc14* mutant was not rescued by any of the CLN genes on CEN plasmids.

Characterization of ERC25

An *erc25* mutant strain was transformed with a YCp50 genomic library (Cross, 1988) and complementing plasmids were rescued. One plasmid (pBB01) not containing *CLN1*, *CLN2* or *CLN3-2* was subcloned into a *URA3* integrating vector and transformed by *ERC25*-targeted integration into a diploid heterozygous for the *erc25-9* allele. In tetrad analysis from this diploid, the *Ura*⁺ phenotype cosegregated with the *Erc*⁺ phenotype in 12 tetrads. Tetrad analysis of crosses between the marked *URA3::ERC25* and four other *erc25* point mutant alleles showed no recombinant spores in 12 tetrads analyzed per cross. Partial sequence analysis of *ERC25* showed that it had significant homology with GAPs for Ras-like GTP-binding proteins (data not shown). The recently cloned *BUD2* gene was shown to encode a GAP for Rsr1p/Bud1p (Park *et al.*, 1993). Comparison of the sequences (H.O.Park, personal communication) revealed identity between *BUD2* and *ERC25*. This was a surprising result, since in a *CLN1 CLN2 CLN3* background, *bud2* mutants are not lethal, but randomize bud site selection (Chant and Herskowitz, 1991).

F.Cvrčková and K.Nasmyth (see accompanying manuscript) have also isolated *bud2* alleles (under the name 'cla2') in a screen for *cln1 cln2* synthetic lethals, confirming the requirement for *BUD2* in *cln1 cln2* strains. However, we and Cvrčková and Nasmyth both observed efficient com-

Fig. 2. Mapping of *ERC25* to *BUD2*. The upper line shows the map of the subcloned insert used for integration and meiotic mapping of *ERC25*. pBB03 is the minimum subclone able to rescue *erc25-1* and *erc25-9* mutants on glucose at 38°C. Deletion to the right hand *HpaI* site (pBB17.1) inactivated rescue activity, as did substitution of a *URA3/kan^r* cassette for the internal *HpaI* fragment (pBB18). Sequencing revealed the indicated open reading frame, with GAP homology in the center. Comparison of the sequence with the recently isolated *BUD2* sequence (Park *et al.*, 1993; H.O.Park, personal communication) showed identity of the *ERC25*-rescuing activity to *BUD2*. P, *PvuII*; X, *XhoI*; B, *BamHI*; H, *HpaI*; S, *SalI*; Sm, *SmaI* (lost in subcloning).

Fig. 3. Specificity of *CLN* rescue of *bud2::URA3* strains. Strains of the indicated genotype were constructed by tetrad analysis (see Materials and methods). All strains contained the *GAL1::CLN1* cassette (Richardson *et al.*, 1989) integrated at *leu2*. *bud2*: strains contained the *bud2::URA3* allele (see Figure 2 and text). Serial dilutions of YEPGal cultures of strains of the indicated genotypes were spotted onto YEPD (glucose) or YEPGal (galactose) plates and incubated for 3 days at the indicated temperature. All strains were isogenic and typical of a large number of similar strains generated in tetrad analysis.

Fig. 4. Staining of chitin and bud scars. Strains of the indicated genotype were grown in YEPGal at 38°C, then switched to YEPD for 3 h. Chitin and bud scars were stained with Calcofluor as described by Pringle (1991). *BUD2*⁺ strains exhibit clustering of bud sites characteristic of axial budding (Chant and Herskowitz, 1991). *cln1 cln2 CLN3 bud2::URA3 GAL1::CLN1* strains bud randomly in galactose medium ('*GAL1::CLN1* on') and show deposition of chitin over the entire cell surface associated with depolarized growth and failure of budding in glucose medium ('*GAL1::CLN1* off').

plementation between *cla2* and our *erc25* mutations. In addition, upon detailed complementation analysis, some of the *erc25* alleles showed weak complementation in diploids (data not shown). This interallelic complementation may be explainable by examining the structure of Bud2p. The central core of Bud2p contains the GAP catalytic region (Figure 2). This region is flanked by α -helical regions with coiled-coil domains that have weak homology to myosin (data not shown). The carboxyl-terminal flanking region is essential for function (Figure 2). If Bud2p uses these putative myosin-like domains for multimerization, then one could envision mutations that block multimerization in the homozygous state, but which give interallelic complementation.

The *bud2* null phenotype in different *CLN* backgrounds. A deletion allele of *bud2* (Figure 2) was constructed and introduced into a diploid *cln1 cln2 CLN3 ERC⁺ GAL1::CLN1* strain (see Materials and methods). Tetrad analysis revealed that *cln1 cln2 CLN3 bud2::URA3 GAL1::CLN1* cells are

Table II. Suppression of *erc25* by *rsr1::URA3*

Cross No	Genotype	Viability on glucose medium at 38°C in tetrads		
		4:0	3:1 ^a	2:2 ^a
2322	<i>cln1 cln2 CLN3 GAL1::CLN1 RSR1 erc25-9/ERC25 (BUD2)</i>	0	0	23
1486	<i>cln1 cln2 CLN3 GAL1::CLN1 rsr1::URA3/+ erc25-9/ERC25</i>	5	11	2
1485	<i>cln1 cln2 CLN3 GAL1::CLN1 rsr1::URA3/+ erc25-1/ERC25</i>	5	11	3

^aIn 3:1 and 2:2 tetrads, glucose-invisible segregants were always *RSR1⁺* (i.e. *Ura⁻*). 2:2 segregation of *rsr1::URA3* was observed, and in control crosses (like x2322) 2:2 segregation of *erc25* (i.e. glucose-invisible) was observed. The 4:0 tetrads (non-parental ditype) are therefore interpreted as containing two *rsr1::URA3 erc25* segregants, which are suppressed for *erc25* lethality, and two *RSR1⁺ ERC25⁺* (wild-type) segregants. The 2:2 tetrads (parental ditype) are interpreted as containing two *rsr1::URA3 ERC25⁺* segregants, and two *RSR1⁺ erc25* (*Erc⁻* glucose-invisible) segregants. The 3:1 tetrads (tetratype) are interpreted as containing strains of four different genotypes: *ERC25⁺ rsr1::URA3* (glucose-viable), *ERC25⁺ RSR1⁺* (glucose-viable), and *erc25 rsr1::URA3* (glucose-viable) and *erc25 RSR1⁺* (glucose-invisible). Only tetrads with four viable spores and 2:2 segregation of *MAT*, *LEU2* and *rsr1::URA3* were tabulated. These constituted about three-quarters of tetrads dissected in each cross. Most of the excluded tetrads had three viable spores and were not analyzed further.

viable, but sick on glucose at 30°C and inviable on glucose at 38°C (Figure 3). While plating on galactose ('*GAL1::CLN1* on') rescued the lethality at 38°C, it did not rescue the random budding phenotype at 30°C or 38°C (Figure 4, Table II).

The *bud2* null allele was crossed into various *CLN* backgrounds. In these crosses, the conditional *GAL1::CLN1* gene was used to recover potentially lethal combinations; this gene was then inactivated on glucose medium for the rescue test. Strains harboring the *bud2* deletion and containing either *CLN1* or *CLN2* as their sole *CLN* genes grew as well as isogenic *BUD2* strains, while the *bud2* strains containing *CLN3* as their sole *CLN* gene were almost as inviable as strains lacking any *CLN* gene, at 38°C (Figure 3). This demonstrates that one of the *CLN1*, *CLN2* gene pair is necessary and sufficient for *bud2* rescue. Transcriptional control of *CLN1* and *CLN2* (see Introduction) is probably not relevant to *bud2* rescue, because a hybrid gene containing the *CLN3* promoter driving the *CLN2* coding sequence (Valdivieso *et al.*, 1993) efficiently rescued viability of *bud2* null strains at 38°C when present as the sole *CLN* gene (data not shown). Not only were extra copies of *CLN3* on a plasmid unable to rescue the *bud2* lethality at 38°C, but spores expressing *GAL1::CLN3* alone in a *bud2* null background were unable to form colonies even at 30°C (data not shown). Thus, *CLN* rescue appeared to be specific for *CLN1* and *CLN2*.

The relationship of the *Erc⁻* phenotype of *bud2* to random budding and to *RSR1/BUD1*. Since *bud2* mutants in *CLN1 CLN2 CLN3* strains bud randomly (Chant and Herskowitz, 1991), it seemed possible that *cln1 cln2 CLN3* strains might require bud site targeting for efficient budding, unlike *CLN1⁺* or *CLN2⁺* strains. To test this, we deleted the *RSR1/BUD1* gene in a *cln1 cln2 CLN3 GAL1::CLN1* background. *RSR1/BUD1* is a Ras-like protein required for targeted budding (Bender and Pringle, 1989; Chant and Herskowitz, 1991). Deletion of *RSR1/BUD1* did not result in an *Erc⁻* phenotype in this background, although budding was random (data not shown). Thus inactivation of the bud site selection pathway is not sufficient to cause an *Erc⁻* phenotype.

BUD2 has been proposed to encode the GAP for Rsr1p/Bud1p (Park *et al.*, 1993; Bender, 1993). Thus the *Erc⁻* phenotype of *bud2* strains might be due to the accumulation of Rsr1p/Bud1p in the GTP-bound form, due to the lack of GAP activity. The *RSR1^{Val12}* mutant allele of *RSR1* inhibits the GTPase activity of Rsr1p and therefore 'locks' the Rsr1p GTPase in a GTP-bound form (Bourne

Fig. 5. *Erc⁻* phenotype of *RSR1^{Val12}*. A *cln1 cln2 CLN3 GAL1::CLN1 rsr1::URA3* strain was transformed with the pRS314 vector, or the vector containing the *RSR1^{Val12}* gene. Two transformants with each DNA were tested for viability under the indicated conditions as in Figure 3, except that Sc-trp medium requiring retention of the plasmids was used throughout.

Fig. 6. Suppression of *erc25* by *rsr1::URA3*. Segregants from x1485 and x1486 (Table III) were analyzed as in Figure 3. Strains were genotyped as follows: *rsr1::URA3* was indicated by a *Ura*⁺ phenotype. *erc25 rsr1::URA3* strains and *ERC*⁺ *RSR1*⁺ strains were selected from 4:0 tetrads (see Table II). *erc25 RSR1*⁺ and *ERC*⁺ *rsr1::URA3* strains were selected from 2:2 tetrads (see Table II). From both x1485 and x1486, two strains of each genotype were tested. Two dilutions from a 10-fold dilution series are shown for each strain.

et al., 1991; Ruggieri *et al.*, 1992). We introduced *RSR1*^{Val12} on a low copy number CEN plasmid into a *cln1 cln2 CLN3 rsr1::URA3 GAL1::CLN1* strain. *RSR1*^{Val12} generated a significant *Erc*[−] phenotype at 38°C (Figure 5). This phenotype required deletion of the wild-type *RSR1* gene (data not shown). This result is consistent with the idea that accumulation of Rsr1p-GTP might account for the *bud2* *Erc*[−] phenotype.

This hypothesis predicts that deletion of *RSR1* should suppress the *Erc*[−] phenotype of *erc25*. *cln1 cln2 CLN3 GAL1::CLN1* strains that were either *erc25-1* or *erc25-9* were crossed to a *cln1 cln2 CLN3 GAL1::CLN1 rsr1::URA3* strain, and tetrads dissected. In a control cross without *rsr1::URA3* segregating, the *Erc*[−] phenotype (inviability on glucose at 38°C) due to *erc25-9* segregated 2:2 as expected (Table II). In crosses with *rsr1::URA3* and either *erc25-1* or *erc25-9* segregating, the *Erc*[−] phenotype failed to segregate 2:2; instead, a segregation pattern was observed indicating efficient suppression of the *Erc*[−] phenotype of *erc25* by *rsr1::URA3* (Table II; Figure 6).

We therefore conclude that *bud2* mutants exhibit an *Erc*[−] phenotype because of the accumulation of Rsr1p/Bud1p bound to GTP and its inability to hydrolyze the GTP to GDP. The results further support the proposal (Park *et al.*, 1993) that Bud2p is a specific GAP for Rsr1p/Bud1p.

These results suggest crossover of bud-site selection functions with bud-site assembly functions (see Introduction). Consistent with this idea, we have observed partial rescue of *erc25* mutants by a high copy number plasmid containing *CDC42*, or a low copy number CEN plasmid containing *BEM1* (data not shown).

Bud site selection and CLN genes. The identification of *bud2* as an *erc* in this screen might suggest that bud site selection is under *CLN* control. Strains with various combinations of *cln* mutations were tested for budding pattern (Chant and Herskowitz, 1991). *cln1 cln2 CLN3*, *CLN1 CLN2 cln3* and wild-type strains, or *cln1 cln2 cln3* strains overexpressing *CLN1* or *CLN3* all maintained the axial budding phenotype,

Table III. Budding patterns

Genotype	Medium	% Axial ^a
<i>CLN1 CLN2 CLN3 BUD2</i>	YEPD 30°C	78 ± 6 (3)
<i>CLN1 CLN2 CLN3 bud2</i>	YEPD 30°C	18 ± 1 (2)
<i>CLN1 CLN2 cln3 BUD2</i>	YEPD 30°C	71 ± 1 (2)
<i>cln1 cln2 CLN3 BUD2</i>	YEPD 30°C	72 ± 10 (4)
<i>cln1 cln2 CLN3 BUD2 GAL1::CLN1</i>	YEPGal 38°C	73 (1)
<i>cln1 cln2 CLN3 bud2 GAL1::CLN1</i>	YEPGal 38°C	17 (1)
<i>cln1 cln2 cln3 BUD2 GAL1::CLN1</i>	YEPGal 38°C	73 ± 4 (2)
<i>cln1 cln2 cln3 BUD2 GAL1::CLN2</i>	YEPGal 38°C	35 ± 9 (3)
<i>cln1 cln2 cln3 BUD2 GAL1::CLN3</i>	YEPGal 38°C	71 (1)

Cultures were pregrown in the indicated medium (liquid) to log phase, then sonicated briefly and plated on the same medium (solid). 1.5–2.5 h after plating, the plates were examined microscopically. 4-cell/bud microcolonies (200 per strain) were analyzed for budding pattern essentially as described by Chant and Herskowitz (1991).

^aPercentage of microcolonies in which the mother and daughter cells both produced buds in the hemisphere nearest the other cell. The mean, standard deviation and the number of strains tested are presented.

while *bud2* disruption essentially eliminated this pattern (Table III; Figure 4). Axial budding in *cln1 cln2* strains and *cln3* strains was confirmed with Calcofluor staining of bud scars (Chant and Herskowitz, 1991; data not shown). Thus, bud site selection is probably not under the control of a specific *CLN* gene. However, overexpressing *CLN2* from the *GAL1* promoter on galactose induced a significant degree of random budding (Table II), so in some contexts *CLN* function may have a role in the regulation of bud site selection.

Discussion

Specificity of the *erc* screen

Looking for mutants which have elevated *CLN* requirements, we have identified mutations in 24 genes which cause inviability when *CLN3* is the only *CLN* present in the genome, but are rescued by overexpression of *CLN1*. Excluding mutations in genes previously described as

synthetically lethal with *cln1 cln2* (*cln3*, *cdc28*, *swi4*, *swi6*, *sit4*), only three of these mutations have a Cdc^- phenotype. Specificity in a genetic screen is best evaluated at saturation, that is, at the point of having multiple mutant alleles in each gene that can mutate to a given phenotype. We have probably saturated the Erc^- depolarized growth phenotype, with nine mutations falling into only one complementation group (*bud2*) (Table I). This specificity is further confirmed by the fact that a conceptually similar but technically different search for mutants carried out in a different strain background by F.Cvrčková and K.Nasmyth (see accompanying manuscript) identified only the *bud2* mutation as giving the Cdc^- depolarized growth phenotype.

Although the permissive conditions used in this screen allowed gross overexpression of *CLN1* from the *GAL1* promoter (Richardson *et al.*, 1989), this overexpression was not required, at least in the case of *bud2*. The *bud2* mutant is rescued by a single wild-type copy of either *CLN1* or *CLN2*, even in the absence of other *CLNs*, whereas it is not rescued by extra copies of *CLN3* or by *GAL1::CLN3*, and is only very poorly rescued by *CLN3-2* (see above). However, these observations leave the possibility open that *CLN3* is simply a much less active *CLN* gene than *CLN1* or *CLN2*; thus, the difference in rescue activity towards a *bud2* mutant might simply reflect overall 'CLN activity' rather than intrinsic *CLN* specificity. This is a difficult possibility to evaluate because different methods of comparing activity of different *CLN* genes yield different answers.

Tyers *et al.* (1993) showed that using histone H1 as a substrate, Cln1p- or Cln2p-associated kinases were ~200-fold as active as Cln3p-associated kinase. The *GAL1::CLN3* construct strongly increases Cln3p levels (Tyers *et al.*, 1992; Cross and Blake, 1993), but still does not rescue *bud2* null strains (see above). However, the low activity of Cln3p-associated kinase is still consistent with a quantitative model for explaining differential *CLN* gene rescue of *bud2* strains.

Instead of histone H1 kinase activity as an assay for relative Cln function one can use the ability of various *CLNs* to drive the START transition as measured by decreases in cell volume and the length of G_1 . In the absence of any other *CLN* gene, *GAL1::CLN3* or *CLN3-2* are significantly more potent than *CLN1* or *CLN2* (Cross, 1988, 1990; Nash *et al.*, 1988; Hadwiger *et al.*, 1989; Lew and Reed, 1992; F.Cross, unpublished data). This comparison would suggest a high degree of intrinsic *CLN* specificity for the rescue of *bud2* lethality. Possible reasons for the discrepancy between the low *in vitro* kinase activity of *CLN3* and its high biological activity are discussed by Tyers *et al.* (1993).

CLNs and bud site assembly

The bud mutants were originally isolated in a *CLN1 CLN2 CLN3* background as mutants that no longer budded axially (i.e. budding adjacent to the previous bud site) as haploids (Chant and Herskowitz, 1991), but were otherwise viable. However, the work reported here uncovers another potential role for *BUD2*. *cln1 cln2 bud2* mutants arrested as large unbudded cells with delocalized cell growth and multiple nuclei, similar to the phenotype of mutations in genes required for bud site assembly such as *CDC42* and *CDC24* (Pringle and Hartwell, 1981).

Cross-over of function between the bud site selection genes and genes required for bud assembly have been observed

Fig. 7. Two models for the relationship of Cln/Cdc28p activity and bud morphogenesis. (A) In this model, Cln/Cdc28p activity is considered to activate an event called 'START'. The program of bud emergence occurs downstream of, and is dependent on, this event. (B) In this model, Cln/Cdc28p activity independently activates multiple pathways leading to bud morphogenesis. This model appears better able to explain the finding of Cln-specific mutations altering specific aspects of bud morphogenesis.

previously. Certain *cdc24* mutations at permissive temperature induce random budding, as does overexpression of *CDC42* (Sloat *et al.*, 1981; Johnson and Pringle, 1990). *RSR1/BUD1* was originally isolated as a multicopy suppressor of a *cdc24* mutant (Bender and Pringle, 1989). *bem1*, a mutation in a gene required for bud site assembly, has been identified as a synthetic lethal with *bud5* (Chant *et al.*, 1991) suggesting an interaction between these two gene products. Similarly, extra copies of either *CDC42* or *BEM1* partially rescued the lethality of the *bud2* point mutants *erc25-1* and *erc25-9* (data not shown).

The Erc^- phenotype of *bud2* is (i) mimicked in *BUD2* strains by introduction of mutant Rsr1p/Bud1p unable to hydrolyze GTP and (ii) suppressed by deletion of *RSR1/BUD1* (see above). Park *et al.* (1993) proposed a model for Bud2p function in bud site selection in which Rsr1p binds to an essential component for bud site assembly (e.g. Bem1p, Cdc24p or Cdc42p; see Introduction), and brings this essential component to the appropriate bud site; there, Bud2p stimulates GTP hydrolysis of Rsr1p-GTP, causing localized release of the essential component leading to targeted budding. In the absence of Bud2p, essential components must escape binding to Rsr1p-GTP and arrive at a 'random' site resulting in randomized budding. According to this model, our results imply that release of the essential component from Rsr1p-GTP requires *CLN1* or *CLN2* action. Two predictions might follow from this model: first, that overexpression of the essential component(s) might suppress the Erc^- phenotype of *bud2*, and second, that overexpression of *CLN1* or *CLN2* might randomize bud site selection by driving release of the essential component(s) from Rsr1p-GTP at non-targeted sites. Both of these predictions are partially satisfied by results presented above: overexpression of Cdc42p and Bem1p suppress the Erc^- phenotype of *bud2* (data not shown), and overexpression of *CLN2* (but not *CLN1*) does partially randomize bud site selection (Table

III). Further work identifying genetic targets that mediate *CLN1* and *CLN2* rescue of the *bud2* phenotype may identify *CLN*-regulated genes/proteins required for bud site assembly.

The effects of the *CLN* genotype on budding proficiency demonstrated that the *bud2* *Erc*⁻ phenotype are consistent with other data suggesting that *CLN1* and *CLN2* drive hyperpolarized cell growth resulting in elongated buds when overexpressed (Lew and Reed, 1993). *CLN1* and *CLN2*, but not *CLN3*, polarize cortical actin to a pre-bud site. This would be consistent with the model presented above where *CLN1* and *CLN2* activate a function needed for bud site assembly and Bud2p substitutes for that function in *cln1 cln2 CLN3* cells.

Multiple roles for Cln/Cdc28p at START

In Figure 7, two models for the effect of Cln/Cdc28p activity on bud morphogenesis are presented. In model A, Cln/Cdc28p activates START which activates a global program for bud morphogenesis. In this model, bud morphogenesis is 'post-START' without direct input or regulation by Cln/Cdc28p. In model B, Cln/Cdc28p directly activates at least two processes leading to bud morphogenesis. The data presented here support the second model. First, the *bud2* mutants suggest a role for Cln/Cdc28p in polarization of cell growth and bud site assembly. Secondly, the *erc10* mutants suggest that bud site constriction, required for proper cytokinesis, may be controlled by Cln/Cdc28p independently of control of polarized growth. Cln/Cdc28p also activate the processes of spindle pole body duplication and DNA replication (Pringle and Hartwell, 1981). Since these processes continue in the absence of bud morphogenesis (Pringle and Hartwell, 1981; Figure 1), additional targets for Cln/Cdc28p activity are required to account for initiation of the processes at START.

Materials and methods

Cell culture and genetics

Media and genetic methods were as described by Ausubel *et al.* (1987). All yeast strains were isogenic with *BF264-15D*. Mutant *cln1*, *cln2* and *cln3* alleles, and the *GALI::CLN1* overexpression cassette, have been described previously (Richardson *et al.*, 1989; Cross and Tinkelenberg, 1991).

EMS mutagenesis and screening for *erc* mutants

Strain 1242-8B (*cln1 cln2 CLN3 pGALI::CLN3/URA3*) was mutagenized with EMS (Sherman *et al.*, 1986) to an approximate mutagenesis frequency of 1/1000 per locus. Mutagenized cells were plated on YEPGal at 38°C, and colonies were replica-plated to YEPD plates at 38°C. Colonies inviable on YEPD were further characterized. The rapidity of arrest and Cdc specificity of arrest were determined by growing once backcrossed mutant strains to mid-log at 38°C in YEPGal and then simultaneously plating on YEPD plates pre-warmed to 38°C and collecting and resuspending another aliquot in YEPD liquid pre-warmed to 38°C and incubating both cultures at 38°C. At 3 h intervals for 9 h, the budding index of the liquid culture and the number of cell bodies in 100 microcolonies on the YEPD plates were determined.

Cloning of *ERC25*

Mutant strains were transformed with a YCp50 genomic library (Cross, 1988). DNA from the rescued clones was used to transform *Escherichia coli* to rescue the plasmids. *CLN1*, *CLN2* and *CLN3-2 (DAF1)* were screened out by Southern hybridization (Ausubel *et al.*, 1987). The remaining rescued plasmids were then rechecked for complementation of the *Erc*⁻ phenotype and physically mapped to the *S.cerevisiae* genome by Southern hybridization using a Chromoblot (Clontech) and a grid of lambda phage clones (kindly provided by L. Riles). To verify linkage of the cloned gene to *erc25-9*, pBB06 was linearized with *NruI* and transformed into an *erc25-9/+* heterozygous diploid. Specific integration was confirmed by Southern hybridization, and tetrad analysis (Ausubel *et al.*, 1987) was performed.

Plasmid constructions

pBB03 is the 5.5 kb *XhoI* fragment containing *BUD2* from pBB01 subcloned into the *XhoI* site of pRS316 (Sikorski and Hieter, 1989). pBB06 is the 6.0 kb *SalI*–*PvuII* fragment of *BUD2* subcloned into the integrating vector pRS306 (Sikorski and Hieter, 1989). pBB18 was constructed by digesting pBB03 with *HpaI* and substituting the *SmaI*-released *URA3*-kan^r cassette from pJA53 (kindly provided by S. Elledge) for the 2.2 kb internal *HpaI* fragment of *BUD2*. pBB36 is the 1.6 kb *EcoRI*–*SalI* fragment from YCp50-(*RSR1*^{Val12}) (Ruggieri *et al.*, 1992), subcloned into the *EcoRI*–*SalI* sites of pRS314 (Sikorski and Hieter, 1989).

Sequencing

Sequencing was carried out on either plasmid or f1 phage DNA, using Sequenase version 2.0 (US Biochemical) according to the manufacturer's instructions.

Gene disruption

The 5.5 kb *XhoI* fragment from pBB18 containing the *URA3*-kan^r substitution in *BUD2* was isolated from low melting point agarose and transformed into diploid 2330d3.1 (*Erc*⁺ diploid). Gene replacement was verified by Southern hybridization. Diploids with successful replacements were sporulated and tetrads dissected as above.

Calcofluor and DAPI staining

Cells were fixed and stained with Calcofluor (Pringle, 1991) or DAPI (Sherman *et al.*, 1986) as described.

Analysis of budding pattern

Cells were grown to mid-log phase in the appropriate medium and temperature. Cells were then plated on either YEPGal or YEPD plates pre-warmed to the assay temperature. Cells were incubated and allowed to undergo two rounds of budding. The budding pattern was scored essentially according to criteria used in Chant and Herskowitz (1991).

Plating efficiency assays

Stationary phase cells were resuspended in medium without a carbon source and 10-fold serial dilutions were performed. Five microliters from each of these dilutions were plated on the appropriate plates and incubated for 2 days.

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