Bem1p, a scaffold signaling protein, mediates cyclin-dependent control of vacuolar homeostasis in *Saccharomyces cerevisiae*

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How proliferating cells maintain the copy number and overall size of their organelles is not clear. We had previously reported that in the budding yeast *Saccharomyces cerevisiae* the G1 cyclin Cln3p is required for vacuolar (lysosomal) homotypic fusion and loss of Cln3p leads to vacuolar fragmentation. The Cdc42p GTPase is also required for vacuole fusion. Here we show that the scaffold protein Bem1p, a critical regulator of Cdc42p activity, is a downstream effector of Cln3p and the cyclin-dependent kinase (Cdk) Cdc28p. Our results suggest that Bem1p is phosphorylated in a Cdk-dependent manner to promote vacuole fusion. Replacing Ser72 with Asp, to mimic phosphorylation at an optimal Cdk-consensus site located in the first SH3 domain of Bem1p, suppressed vacuolar fragmentation in cells lacking Cln3p. Using in vivo and in vitro assays, we found that Cln3p was unable to promote vacuole fusion in the absence of Bem1p or in the presence of a nonphosphorylatable Bem1p-Ser72Ala mutant. Furthermore, activation of Cdc42p also suppressed vacuolar fragmentation in the absence of Cln3p. Our results provide a mechanism that links cyclin-dependent kinase activity with vacuole fusion through Bem1p and the Cdc42p GTPase cycle.

[**Keywords:** Vacuole; CLN; CDC42; BEM1; cell size; START]

Received August 5, 2005; revised version accepted August 30, 2005.

Overall cell size and macromolecular composition remain unaffected after successive rounds of cell division. This phenomenon also extends to intracellular membrane-bound organelles, because the copy number and size of any given organelle compartment remain constant in dividing cells (Warren and Wickner 1996; Shorter and Warren 2002). Implicit in this central aspect of cellular physiology is a tight coordination between cell division and organelle biogenesis, but in most cases the mechanisms remain mysterious (Warren and Wickner 1996; Shorter and Warren 2002).

Vacuoles in *Saccharomyces cerevisiae* serve as repositories of metabolites and low molecular weight compounds and are analogous to the lysosomes of animal cells, containing numerous hydrolases (Roberts et al. 1991; Jones et al. 1997). The vacuole is a low-copy organelle, and yeast cells typically contain one to three vacuoles. The large size of the vacuolar compartment (~25% of the total cellular volume) (Wiemken and Durr

1974) and the availability of vacuole-specific vital fluorescent dyes facilitate observations of overall vacuolar morphology. Defects in self (homotypic) fusion of vacuolar vesicles lead to vacuolar fragmentation (Seeley et al. 2002). Thus, homotypic fusion is very important for vacuolar homeostasis and it can also be evaluated in vitro (Wickner and Haas 2000). Although daughter cells of certain vacuolar inheritance mutants can be born without a vacuole, they must form a new one before they can pass through a point in late G1 called START and initiate DNA replication and a new round of cell division (Weisman 2003). A recent report also suggested that in *Candida albicans* the vacuolar compartment may impact on cell cycle progression and hyphal development (Barelle et al. 2003). Nonetheless, it is not known how the molecular machinery that regulates cell cycle progression also affects vacuolar biogenesis or vice versa.

In late G1 START completion is mediated by Cdc28p (a cyclin-dependent kinase [Cdk]) in association with one of the G1 cyclins, Cln1,2,3p. Cells lacking all three *CLN* genes are inviable and cannot complete START (Richardson et al. 1989). Cln3p functions upstream of Cln1,2p activating the G1/S transcription program (Dirick et al. 1995), where ~200 genes (*CLN1,2* among them) are tran-

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Article published online ahead of print. Article and publication date are at <http://www.genesdev.org/cgi/doi/10.1101/gad.1361505>.

scribed (Spellman et al. 1998). Cln3p/Cdc28p phosphorylates Whi5p, a repressor of the G1/S transcription factor SBF, thereby releasing Whi5p from SBF and activating START transcription (Costanzo et al. 2004; de Bruin et al. 2004; Schaefer and Breeden 2004). In addition, our earlier findings provided evidence for a novel function of Cln3p in vacuolar homotypic fusion, separate from its role in G1/S transcription and not shared by other G1 cyclins (Han et al. 2003).

A central polarity-establishment factor in a variety of organisms (from yeast to humans) is Cdc42p, a Rho-type small GTPase that orchestrates numerous processes necessary for polarization, such as septin and actin organization and membrane trafficking, in response to cell cycle transitions and environmental cues (Etienne-Manneville 2004; Irazoqui and Lew 2004). Cdc42p membrane localization is not restricted to the plasma membrane but includes internal membranes, notably vacuolar membranes (Richman et al. 2004). Furthermore, Cdc42p is one of several GTPases required for vacuolar homotypic fusion (Eitzen et al. 2001; Muller et al. 2001). Reorganization of vacuole-bound actin is needed for vacuolar homotypic fusion, and proteins of the Cdc42p-dependent processes necessary for actin remodeling are enriched on vacuolar membranes (Eitzen et al. 2002). Among numerous Cdc42p effectors and interacting proteins, the scaffold protein Bem1p is critical for proper Cdc42p activation (Irazoqui et al. 2003). After the Cln3p/Cdc28p-mediated initiation of the G1/S transcription program, the burst of late G1-phase Cdk activity involving Cln1,2p/Cdc28p and Pcl1,2p/Pho85p (another cyclin/Cdk complex) triggers a pathway that leads to phosphorylation of Cdc24p. Cdc24p is a Cdc42p guanine nucleotide exchange factor (GEF). Once at the bud site Cdc24p binds Bem1p, and Cdc42p-dependent actin reorganization necessary for bud emergence takes place (Gulli et al. 2000; Bose et al. 2001; Moffat and Andrews 2004).

In this report we show that Bem1p is required for vacuolar homotypic fusion and that the overall vacuolar compartment in *bem1Δ* cells is enlarged but fragmented, similar to *cln3Δ* cells. Furthermore, Bem1p is phosphorylated in a Cln3p-dependent manner at Ser72. A single S72D substitution in Bem1p (which mimics phosphorylation) or overexpression of *CDC42* or *CDC24* suppresses the vacuolar fragmentation of *cln3Δ* cells. Conversely, substituting Ser72 in Bem1p with Ala blocks the ability of Cln3p to promote vacuole fusion, in vivo and in vitro. The results we report here suggest that Cln3p impacts on vacuolar homeostasis through Bem1p and Cdc42p.

Results

Cln3p is required for an early step of vacuolar homotypic fusion

We had previously shown that Cln3p is required for vacuolar homotypic fusion (Han et al. 2003). Vacuolar homotypic fusion is composed of several distinct se-

quential steps: priming, docking, and fusion (Wickner and Haas 2000). To study the kinetics of the reaction, order of addition experiments can be done, whereby the fusion reaction progressively becomes resistant to the addition of various inhibitors, once the inhibitor-sensitive step has been completed. For example, placement on ice blocks all steps and thus results in a "late-stage" inhibition profile, which is also similar to the GTP-γ-S inhibition profile. There are several GTPases involved in more than one step, including the last fusion step, which leads to a late-stage inhibition profile by GTP-γ-S (Eitzen et al. 2000, 2001). On the other hand, the ionophore FCCP blocks the docking step and it does not significantly inhibit the overall reaction if added late.

Using this approach, we decided to determine the stage of the overall reaction that requires Cln3p for its completion, with cytosol from cells expressing HA-tagged Cln3p. We then added an anti-HA antibody as an inhibitor at various time points during the reaction and measured the overall fusion activity at the end of the 90-min incubation period (Fig. 1). This experiment was

Figure 1. Cln3p is required for an early step during vacuole fusion. (A) A schematic of the experimental strategy is shown. (B) Fusion reactions were incubated at 27°C with cytosol from a *CLN3-HA* strain. At the indicated times of addition (t_1) shown on the X-axis, aliquots were removed and added to tubes containing anti-HA Ab, FCCP, GTP-γ-S, or buffer and incubated at 27°C. The activity from the buffer-containing tube at 27°C was set as 100% fusion activity. Another buffer-containing tube was incubated on ice and its activity was set at 0% fusion activity. As a control, aliquots of reactions with cytosol from the untagged isogenic strain were also added at various time points to tubes containing the anti-HA Ab (●) or buffer, and treated in the same manner as above. Reactions were incubated for a total of 90 min and then assayed for alkaline phosphatase, the relative values of which are shown on the Y-axis. (C) Fusion reactions were performed as described in Materials and Methods, using cytosol from *cln3Δ* cells and recombinant proteins from bacteria as indicated. Fusion was evaluated colorimetrically and the average and standard deviation of the relative fusion activities from at least three independent experiments is shown.

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done in parallel with several other reactions, to compare the profile of Cln3p withdrawal to the profiles of known fusion inhibitors (Fig. 1). From this analysis, it appears that the reaction becomes resistant to removal of Cln3p with kinetics slightly earlier than the kinetics for FCCP addition (Fig. 1). Thus, once docking is complete, Cln3p is not required for vacuolar homotypic fusion.

To further test whether the requirement for Cln3p in vacuole fusion is direct, and not somehow due to indirect effects resulting from Cln3p's role in G1/S transcription, we performed the *in vitro* fusion reaction using cytosol from *cln3Δ* cells, supplemented with Cln3p expressed in bacteria (Fig. 1C). Addition of recombinant Cln3p, but not of the unrelated control protein Dcr2p (Pathak et al. 2004), increased fusion activity (Fig. 1C). These results argue that there is a direct requirement for Cln3p in vacuole fusion.

Bem1p impacts on vacuolar biogenesis, downstream of Cln3p

To understand Cln3p's vacuolar function, we reasoned that one of the numerous known regulators of homotypic fusion might be targeted by Cln3p. Possible downstream effectors of Cln3p/Cdc28p in vacuolar homotypic fusion may display the following properties: Loss-of-function mutations should lead to vacuolar and overall cell size enlargement, loss-of-function mutations should lead to vacuolar fragmentation, and the putative effectors should be Cdk targets. Recent genome-wide studies focused on these properties, namely, altered cell size (Jorgensen et al. 2002; Zhang et al. 2002), fragmented vacuolar morphology (Seeley et al. 2002), and proteins that are phosphorylated by Cdc28p in whole-cell extracts (Ubersax et al. 2003). There are >100 gene products in each data set (Fig. 2A). For example, there were 181 proteins that were significantly phosphorylated by Clb2p/Cdc28p (with a *P*-score ≥ 2 , which is the logarithm of the amount of phosphate incorporated per nanogram of protein). Yet, only Cln3p itself and Bem1p were present in all three data sets (Fig. 2A).

Using the *in vitro* fusion assay, we found that reactions with cytosol from *BEM1-TAP* cells in the presence of inhibitory rabbit IgG (which targets the TAP domain) had significantly reduced fusion activity (Fig. 2B). Loss of Bem1p or Cln3p also leads to vacuolar fragmentation in ~40–60% of the cells (see Fig. 3A,B; Seeley et al. 2002; Han et al. 2003). Furthermore, cells lacking *BEM1* were larger overall (~40%) (Figs. 2, 3) than wild-type cells and, importantly, their vacuolar compartment was also disproportionately enlarged (about twofold) (Figs. 2, 3). Finally, Cdc28p readily phosphorylated Bem1p (*P*-score = 3.4) in the phosphorylation assays by Ubersax et al. (2003). Consequently, we decided to evaluate Bem1p as a putative Cln3p/Cdc28p effector in vacuolar biogenesis.

To test whether Cln3p might mediate its effects in vacuolar biogenesis through Bem1p, we measured the overall cell size and vacuolar size of *CLN3* and *BEM1*

Figure 2. Bem1p is required for vacuole fusion and vacuolar size homeostasis. (A) Venn diagrams from the indicated genome-wide studies; the number of genes corresponding to different phenotypes in each case is shown. (B) Vacuole fusion using cytosol from untagged, *CLN2-TAP*-tagged, or *BEM1-TAP*-tagged strains (see Table 1), carried out in the presence of rabbit IgG antibody. Each measurement was evaluated upon addition of rabbit IgG or buffer. The buffer-containing reactions were then incubated at 27°C or on ice (setting the 100% and 0% values of fusion activity). The IgG-containing reactions were incubated at 27°C. Fusion was evaluated colorimetrically and the average and standard deviation from at least three independent experiments is shown. (C) Cell and vacuole size of live CDCFDA-stained *BEM1+* and *bem1Δ* cells (in the diploid BY4743 background) was evaluated by flow cytometry. The number of cells is shown on the Y-axis, and the X-axis indicates forward angle scattering, which is indicative of cell size (FSC), or vacuolar fluorescence (FL). The average and standard deviation from five independent measurements in each case is shown.

mutant combinations and also microscopically examined their vacuolar morphology. Loss of Cln3p or Bem1p leads to cellular and vacuolar enlargement and vacuolar fragmentation (Fig. 3). Conversely, cells expressing a stabilized form of Cln3p from the dominant gain-of-function *CLN3-2^D* allele are smaller overall (Cross 1988), and their vacuolar compartment is also smaller (see Fig. 3A; Han et al. 2003). However, the *CLN3-2^D* allele was unable to reduce cell and vacuole size in the absence of Bem1p (Fig. 3A). Furthermore, combined loss of Bem1p and Cln3p did not lead to an additive cellular or vacuolar enlargement or vacuolar fragmentation (Fig. 3B). These results are consistent with the idea that Bem1p is required for Cln3p's effects on cell and vacuole size, and that Bem1p and Cln3p do not affect vacuolar biogenesis through separate independent pathways.

Figure 3. Cln3p requires Bem1p to affect cell and vacuole size and vacuolar morphology. (A) The relative vacuole size of the indicated strains (all in the diploid BY4743 background) was measured as in Figure 2, and cell size was measured with a channelyzer. The average and standard deviation from four independent measurements in each case is shown. The indicated strains were also stained with FM4-64 to observe their vacuolar morphology by fluorescence microscopy. The percent of cells with vacuolar fragmentation and the number of cells scored in each case (*n*) is shown. (B) The indicated parameters were measured as in A, using the strains shown (in the haploid BY4741 background).

Bem1p is phosphorylated in a Cdk-dependent manner

It has been previously mentioned in an earlier report that Bem1p might be a phosphoprotein, because phosphatase treatment leads to faster migration of Bem1p during SDS-PAGE (Leeuw et al. 1995). Indeed, immunoprecipitated TAP-tagged Bem1p migrated faster upon phosphatase treatment (Fig. 4A). Bem1p has four potential Cdk phosphorylation sites: two [ST]-P-X-[KR] sites at Thr51 and Ser72 and two [ST]-P sites at Thr26 and Ser60, respectively. As a first step toward establishing possible Cdk-mediated phosphorylation events, we asked whether an antibody directed against phosphorylated [ST]-P recognized Bem1p immunoprecipitated from cell extracts. From these cell extracts we sequentially immunoprecipitated Bem1p-TAP using IgG and calmodulin beads (Fig. 4B). The immunoprecipitated protein was readily recognized by the PAP reagent, via the protein A domain (Fig. 4B). To use the anti-phospho-[ST]-P antibody, after immunoprecipitations with IgG beads, we cleaved the immunoprecipitated product with TEV protease to remove the protein A part of the TAP epitope. Immunoblotting with the anti-phospho-[ST]-P antibody recognized the cleaved Bem1p-CBP (which now lacks the protein A part of the TAP tag) (Fig. 4B). Recognition by the anti-phospho-[ST]-P antibody was phosphorylation dependent, because the signal was significantly reduced after treatment with phosphatase (Fig. 4C). When normalized for Bem1p levels using an antibody that recognizes the remaining part of the TAP tag after TEV cleavage, treatment with phosphatase removed >70% of the signal (Fig. 4C, bottom).

To further evaluate whether Cdc28p activity contributes to Bem1p phosphorylation, we used *cdc28-as1* cells, which express an engineered version of Cdc28p that is inhibited by the ATP analog 1NM-PP1 (Bishop et al. 2000). These cells were then transformed with a low-copy centromeric plasmid carrying cMyc-tagged Bem1p (Irazoqui et al. 2003). Upon treatment with the inhibitory drug, the mobility of Myc-tagged Bem1p increased on SDS-PAGE, consistent with dephosphorylation (Fig. 4D). No such effects were seen upon treatment with DMSO or in the isogenic strain that does not carry the engineered *cdc28-as1* allele (Fig. 4D). We had previously reported that shifting temperature-sensitive *cdc28-1* cells to their nonpermissive temperature led to vacuolar fragmentation (Han et al. 2003). To further confirm that Cdc28p-associated kinase activity is required for vacuole homotypic fusion, we performed the *in vitro* fusion reaction using cytosol from *cdc28-as1* cells, and we found that upon treatment with the inhibitory drug, fusion activity was reduced to ~40% of the activity when DMSO alone was added (Fig. 4E). Together, all of the above results support the hypothesis that Bem1p is phosphorylated in a Cdc28p-dependent manner *in vivo*.

Ser72 is critical for Bem1p's role in vacuolar homeostasis

We then examined whether the Bem1p potential Cdk phosphorylation sites are conserved in other *Saccharomyces* species (Cliften et al. 2003; Kellis et al. 2003) and in a couple of *Candida* species for which genome infor-

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Figure 4. Bem1p might be phosphorylated in a Cdk-dependent manner. (A) Schematic of the TAP-tagged Bem1p. Bem1p-TAP was isolated from cells carrying a chromosomal TAP-tagged *BEM1* copy (*BEM1*-TAP, strain 7499374) (see Table 1) by immunoprecipitation using IgG and treated with calf intestinal phosphatase (CIP) as indicated. The samples were then processed for SDS-PAGE and immunoblotting with the PAP reagent. (B) Bem1p-TAP was isolated by tandem immunoprecipitations using IgG and calmodulin beads. The IgG-immunoprecipitated material was treated with TEV protease as indicated, before immunoprecipitations with calmodulin beads. Immunoblotting was done using the indicated antibodies. (C) The immunoprecipitated samples from Bem1p-TAP cell extracts were treated as in B and also with λ -phosphatase (λ -PPase) and phosphatase inhibitor (PPase-[I]) as indicated. The antibodies used for immunoblotting are shown on the right, and the relative ratios of the signal intensities are shown at the bottom. (D) Extracts from *CDC28*⁺ and *cdc28-as1* cells carrying cMyc-tagged Bem1p on a low-copy plasmid (plasmid pDLB2226) (see Table 2) were prepared using a urea extraction buffer and analyzed by SDS PAGE and immunoblotting with an anti-Myc antibody. The cells were treated with DMSO alone or 1 NM-PP1 (at 5 μ M) for the indicated times. (E) Vacuole fusion activity using cytosol from *cdc28-as1* cells. DMSO alone or 1 NM-PP1 (at 5 μ M for 15 min) was added as indicated. Fusion was evaluated colorimetrically and the average and standard deviation of the relative fusion activities from at least three independent experiments is shown.

mation is available (Fig. 5A). Not surprisingly, all the sites are conserved in the sensu stricto *Saccharomyces* species (*S. mikatae*, *S. kudriavzevii*, *S. paradoxus*, and *S. bayanus*). *S. castellii* is a more distantly related sensu lato *Saccharomyces* species, and alignment of Bem1p to its ortholog in this species is likely to be more useful (Cliften et al. 2003). In *S. castellii* only positions 26 and 72 are conserved, while in the two *Candida* species positions 51 and 72 are conserved (Fig. 5A). The extreme N terminus of the *C. albicans* Bem1p does not show significant conservation with the *S. cerevisiae* Bem1p, and the highlighted position may not correspond to Ser51. Overall, the only conserved site in all of these cases is Ser72 (Fig. 5A), which is a preferred [ST]-P-X-[KR] Cdk consensus site. Ser72 also represents the first amino acid residue of the SH3-1 domain, which spans from position 72 to 132, based on PROSITE software predictions (Gatiker et al. 2002).

To test whether position 72 might be targeted for Cdk-mediated phosphorylation, we replaced Ser72 with Ala (to abolish phosphorylation) or Asp (to mimic phosphorylation) and evaluated the vacuolar morphology of *bem1Δ* or *cln3Δ* cells expressing these Bem1p mutants from low-copy plasmids as C-terminal cMyc fusions (Fig. 5B). The S72A and S72D mutants were expressed at levels similar to the wild-type cMyc-tagged Bem1p, and their subcellular localization was indistinguishable from wild type (data not shown). We noticed that introduction of the otherwise wild-type cMyc-tagged Bem1p on a low-copy centromeric plasmid only partially suppressed the

vacuolar fragmentation of *bem1Δ* cells (Fig. 5B). The plasmid is clearly functional because it does suppress the bud emergence defect of *bem1Δ* cells (see Fig. 7, below; Irazoqui et al. 2003). The lack of strong suppression of the vacuolar fragmentation might be due to the relative underexpression of cMyc-tagged Bem1p from this plasmid compared with endogenous levels (Irazoqui et al. 2003). Consistent with this interpretation, cMyc-tagged Bem1p expressed from a high-copy (2 μ) plasmid at levels no more than twofold higher compared with endogenous Bem1p (Irazoqui et al. 2003) showed strong suppression of the vacuolar fragmentation of *bem1Δ* cells (Fig. 8D, below).

Nonetheless, it is important to note that the fact that wild-type cMyc-tagged Bem1p from a low-copy plasmid only partially suppressed vacuolar fragmentation conveniently allows one to evaluate the effects of the S72D substitution even in *bem1Δ* (but *CLN3*⁺) cells. It was clear that S72D Bem1p suppressed vacuolar fragmentation better than wild-type Bem1p, and, conversely, the S72A Bem1p mutant did not suppress at all the vacuolar fragmentation of *bem1Δ* cells (Fig. 5B,C). Because the S72A mutant is functional in bud emergence (see below, Fig. 7), the lack of suppression in vacuolar fragmentation is unlikely to be due to the production of a nonfunctional unfolded protein. Finally, introduction of the S72D, but not the S72A, Bem1p mutant in *cln3Δ* cells also significantly suppressed their vacuolar fragmentation (Fig. 5B,C).

To further test the role of Ser72, we overexpressed

Figure 5. Ser at position 72 in Bem1p is critical for vacuolar morphology. (A) Bem1p amino acid sequences (positions 24–75) in *Saccharomyces* and *Candida* species. The numbering on top refers to *S. cerevisiae* Bem1p. The amino acids putatively targeted for Cdk-dependent phosphorylation are shown in bold, and the conserved Ser at position 72 is bracketed. (B) Homozygous diploid wild-type (WT), *bem1Δ*, or *cln3Δ* (BY4743 background) were transformed with the low-copy empty vector, wild-type, or mutant cMyc-tagged Bem1p as indicated. The transformants were then stained with FM4-64 to observe their vacuolar morphology by fluorescence microscopy. The percent of cells with vacuolar fragmentation and the number of cells scored in each case (*n*) is shown. Where indicated with an asterisk, the *P* value associated with a χ^2 significance test was found to be significant (2.7×10^{-14}). (C) Representative photographs of the indicated strains from B. The arrow indicates an example of vacuolar fragmentation. (D) Homozygous diploid *bem1Δ* cells (BY4743 background) carrying the *CLN3-2^D* allele on a low-copy plasmid were also transformed with the low-copy wild-type or mutant cMyc-tagged Bem1p as indicated. Vacuolar morphology was evaluated as in B above. (E) Fusion reactions were performed as described in Materials and Methods, using cytosol from cells lacking both *CLN3* and *BEM1* (strain SMY01) (Table 1) and recombinant proteins from bacteria as indicated. The average of the relative activities from two independent experiments is shown in each case. The data from the two experiments had a Pearson correlation value of 0.82, and a paired two-sample *t*-test value of 0.009.

*CLN3*p in cells carrying wild-type or Ser72 Bem1p mutants (Fig. 5D). We found that cells carrying both the *CLN3-2^D* and *BEM1-S72A* alleles had fragmented vacuolar morphology (Fig. 5D). Thus, the S72A substitution

blocks the ability of Cln3p to promote vacuole fusion in vivo. We then performed in vitro fusion reactions using cytosol from cells lacking both Cln3p and Bem1p, supplemented with various combinations of recombinant Cln3p, Bem1p, Bem1p-S72A, and Bem1p-S72D (Fig. 5E). The highest activity was observed upon addition of Bem1p-S72D with or without Cln3p, and the lowest upon addition of Bem1p-S72A. Also, addition of Cln3p alone or with Bem1p-S72A did not increase fusion activity (Fig. 5E). Therefore, these in vitro results completely support our in vivo evidence that Cln3p requires Bem1p to promote fusion. Furthermore, the S72A substitution in Bem1p blocks fusion, while the S72D mutation promotes it. Interestingly, addition of wild-type Bem1p also increased fusion activity in the absence of Cln3p, albeit not to the same level as Bem1p-S72D. Thus, it is possible that in the absence of Cln3p, when large amounts of exogenous Bem1p are added, Bem1p can still be modified in these extracts, perhaps by other cyclin/Cdk complexes. Taken together, our results from the in vitro fusion assays are in very good agreement with our in vivo evidence, and suggest that Cln3p and Bem1p play direct roles in vacuole fusion, with Bem1p acting downstream of Cln3p.

Overall, the evidence supports the notion that the conserved Ser at position 72 in the first SH3 domain of Bem1p might be targeted for Cdk-mediated phosphorylation, and that the phosphorylated form promotes vacuolar homotypic fusion. It is possible that the other sites may also be phosphorylated. However, because the S72D mutant significantly suppressed the vacuolar fragmentation of *cln3Δ* and *bem1Δ* cells in vivo and in vitro, while the S72A mutant did not, it is reasonable to conclude that even if additional sites are phosphorylated, phosphorylation of Ser72 has the most significant biological consequences for vacuolar biogenesis.

We then used 2D gel electrophoresis to better resolve putative Bem1p isoforms (Fig. 6A). Indeed, an acidic isoform present in wild-type Bem1p (Fig. 6A, top panel) was absent in cells lacking Cln3p (Fig. 6A, middle panel) or in cells carrying Bem1p-S72A (Fig. 6A, bottom panel). We also noticed that Bem1p-S72A migrated slightly faster than wild-type Bem1p in standard one-dimensional SDS-PAGE, similar to wild-type Bem1p in *cln3Δ* cells (Fig. 6B). Together, these results strongly suggest that Bem1p is phosphorylated at Ser72 in a Cln3p-dependent manner in vivo.

Finally, we examined whether Bem1p expressed in bacteria could be phosphorylated in vitro. For this experiment we overexpressed *CLN3* from a high-copy plasmid carrying epitope-tagged *CLN3* under the control of a galactose-inducible promoter. It has been previously shown that overexpression of Cln3p allows for the recovery of detectable Cln3p-associated kinase activity against histone H1, and that most of that activity is Cdc28p-dependent (Tyers et al. 1992, 1993). Indeed, the immunoprecipitated Cln3p had associated kinase activity against recombinant Bem1p, albeit to significantly lower levels compared with histone H1 (Fig. 6C). We also performed similar reactions using commercially avail-

Figure 6. (A) Resolution of Bem1p isoforms by 2D gel electrophoresis. Extracts from cells of the indicated *CLN3* genotype were transformed with the low-copy wild-type or mutant cMyc-tagged Bem1p as indicated and subjected to 2D gel electrophoresis and immunoblotting with an anti-Myc antibody as described in the Materials and Methods. A degradation product is indicated by an asterisk (*). A broken line circles the location of the acidic full-length isoform absent from the two lower panels. (B) The samples from A were also analyzed by standard one-dimensional SDS-PAGE and immunoblotting. (C) Phosphorylation of Bem1p in vitro. (Top) Autoradiogram of kinase reactions using recombinant Bem1p as a substrate and commercial preparations of human Cdk/cyclin complexes and Cln3p-associated kinase activity from yeast cells, as indicated. Exposure time was 10 d. (Bottom) Autoradiogram of kinase reactions using histone H1 as a substrate and the same kinase preparations as in the top panel. Exposure time was 4 h.

able preparations of human Cdc2/cyclinB and Cdk2/cyclinA complexes. Surprisingly, Cdc2/cyclinB apparently phosphorylated Bem1p (Fig. 6C). Whether this reflects “background” nonspecific levels of activity of this preparation is unclear at this point. Note that although the Cdc2 signal appears stronger than the signal from Cln3p (Fig. 6C), the commercial Cdc2 preparation we used was much more active against histone H1 than the Cln3p-associated activity we obtained from yeast cells.

Opposing role of Ser72 in bud emergence

Since Bem1p was originally identified for its role in bud emergence (Bender and Pringle 1991), we next examined whether our Bem1p Ser72 mutants have phenotypes associated with bud emergence. To quantify the bud emergence defect we measured the frequency of unbudded cells containing spindles. The S72A mutant was fully functional in bud emergence (Fig. 7), in contrast to its total lack of complementing activity in vacuole fusion (Fig. 5). Interestingly, the S72D substitution did not fully complement the bud emergence defect of *bem1Δ* cells (Fig. 7). Thus, while phosphorylation of Ser72 promotes vacuole fusion, it is not required for and it might even negatively affect bud emergence.

Overexpression of Cdc42p or Cdc24p suppresses the vacuolar fragmentation of *cln3Δ* cells

We next examined whether Cln3p's requirement for vacuolar homotypic fusion might reflect perturbations of Cdc42p activity, since Bem1p is a well-established regulator of Cdc42p, and Cdc42p is also required for the docking step of vacuolar homotypic fusion (Eitzen et al. 2001; Muller et al. 2001). To perhaps bypass the requirement for Cln3p, we activated the Cdc42p GTPase cycle by overexpressing wild-type *CDC42*, or its exchange factor *CDC24*, in *cln3Δ* cells and evaluated their vacuolar morphology. Remarkably, overexpression of *CDC42* or *CDC24* completely suppressed the fragmented vacuolar morphology of *cln3Δ* cells (Fig. 8A,B). On the other hand, overexpression of *CDC42* did not significantly suppress the vacuolar fragmentation of *bem1Δ* cells (Fig. 8B). *CDC24* overexpression weakly suppressed the vacuolar fragmentation in *bem1Δ* cells (Fig. 8B). Overall, overexpression of Cdc42p, in the presence of Bem1p, is sufficient to bypass the requirement for Cln3p in vacuole fusion.

Furthermore, in the in vitro fusion assay, addition of Cdc42p did not rescue the fusion defect of *bem1Δ* cytosolic extracts (Fig. 8C), suggesting again that Bem1p is required for Cdc42p to promote fusion. Interestingly, when the extracts were supplemented with Bem1p-S72A together with Cdc42p there was an increase in fusion activity (~60%) (Fig. 8C), but not to the same extent as when the extracts were supplemented with Bem1p-S72D (see Fig. 5E).

We next examined several other Bem1p amino acid substitutions at positions that are important for Bem1p's biological roles (Irazoqui et al. 2003) for their ability to suppress vacuolar fragmentation of *bem1Δ* cells (Fig. 8D). For example, a P208L substitution affects interactions with various effectors and scaffolds, P355A inter-

Figure 7. Homozygous diploid wild-type (WT) or *bem1Δ* cells (BY4743 background) were transformed with the low-copy empty vector, wild-type, or mutant cMyc-tagged Bem1p as indicated. The cells were grown at 37°C, fixed, and processed for immunofluorescence to visualize tubulin. (A) The percent of unbudded cells with spindles and the number of cells scored in each case (n) is shown. Where indicated with an asterisk, the *P* value associated with a χ^2 significance test was found to be significant (0.017). (B) Representative photographs of the indicated strains from A. Examples of unbudded cells with spindles are outlined.

Figure 8. *CDC42* or *CDC24* overexpression suppresses the vacuolar fragmentation of *cln3Δ* cells. (A) Diploid *cln3Δ* cells (BY4743 background) were transformed with high-copy plasmids as indicated. The transformants were then stained with FM4-64 to observe their vacuolar morphology and photographed through phase optics (top panels) and by fluorescence (bottom panels). (B) The same analysis as in A was also done for *bem1Δ* and wild-type (WT) cells, and the percent of cells with vacuolar fragmentation and the number of cells scored in each case (*n*) is shown. (C) Fusion reactions were performed as described in Materials and Methods, using cytosol from *bem1Δ* cells and recombinant proteins from bacteria as indicated. Fusion was evaluated colorimetrically and the average and standard deviation of the relative fusion activities from at least three independent experiments is shown. (D) Homozygous diploid *bem1Δ* cells (BY4743 background) were transformed with the empty high-copy vector or carrying wild-type or mutant cMyc-tagged Bem1p as indicated. The transformants were then stained with FM4-64 to observe their vacuolar morphology by fluorescence microscopy. The percent of cells with vacuolar fragmentation and the number of cells scored in each case (*n*) is shown. (E) Cells lacking Bem1p are not defective in vacuolar segregation. Exponentially growing cells of the indicated genotype were transferred in medium containing FM4-64 for 1 h, washed, resuspended in fresh medium, and allowed to grow for another 4 h before they were examined microscopically. The number of cells examined is shown in parentheses. The scored cells had a bud diameter ≤ 0.4 of the mother cell's diameter. The percentage of cells with the indicated vacuolar morphology is shown.

feres with a postulated conformational change of Bem1p, R369A inhibits interactions with phosphoinositides, and K482A affects interaction with Cdc24p (Irazoqui et al. 2003). The R369A substitution did not have any effect (Fig. 8D), suggesting perhaps that lipid interactions are not important for Bem1p's function in vacuolar homo-

typic fusion. To varying degrees, the other Bem1p mutants also significantly suppressed vacuolar fragmentation of *bem1Δ* cells (Fig. 8D). Notably, the weakest suppression, albeit still significant, was observed with the P208L substitution (Fig. 8D), which affects protein-protein interactions through the SH3-2 domain (Irazoqui et al. 2003).

Finally, since the actin cytoskeleton plays a major role in vesicle transport in the bud and vacuole inheritance (Weisman 2003; Pruyne et al. 2004), we decided to evaluate vacuolar segregation in *bem1Δ* cells. When we followed the "old" vacuolar compartment synthesized in previous cell divisions, we found that *bem1Δ* and wild-type cells equally distribute their vacuoles between the mother and the bud (Fig. 8E), unlike *cln3Δ* cells, which have a weak but measurable defect (Fig. 8E; Han et al. 2003). Furthermore, overexpression of *cln3Δ* did not alter the vacuolar segregation of *cln3Δ* cells (data not shown). Overall, these results suggest that it is unlikely that the mechanism we describe here linking Cln3p and Bem1p significantly affects vacuole segregation.

Discussion

In this study we present experiments that link the G1 cyclin Cln3p with vacuole fusion, through Bem1p and the Cdc42p GTPase. We discuss our findings in the context of the known roles of these proteins and how they might affect vacuolar homeostasis.

We were initially led to Bem1p because *bem1Δ* cells are large and their vacuolar compartment is also enlarged and fragmented, similar to the situation in *cln3Δ* cells (Fig. 2; Han et al. 2003). The experiments we report here suggest that Bem1p is phosphorylated in a Cdk-dependent manner. The biological significance of Ser72 phosphorylation is underscored by the fact that a substitution to Asp that probably mimics phosphorylation suppresses vacuolar fragmentation, while an Ala substitution does not (Fig. 5). Our data strongly point to the link between Cln3p and modification of Ser72 (Fig. 6). In vitro, however, it appears that multiple Cdk complexes can phosphorylate Bem1p. Addition of Bem1p from bacteria can still promote vacuole fusion in vitro, in the absence of Cln3p (Fig. 5E), suggesting that other cyclin/Cdk complexes might also be able to phosphorylate this exogenous pool of Bem1p. Furthermore, Clb2p/Cdc28p was previously shown to phosphorylate Bem1p (Ubersax et al. 2003) in vitro. This result was further extended in a follow-up study, where Loog and Morgan (2005) found that Clb5p/Cdc28p and Clb2p/Cdc28p phosphorylated Bem1p with equal efficiency. Nonetheless, in living cells Cln3p certainly plays a unique role in vacuolar biogenesis because loss of any other cyclin does not lead to vacuolar fragmentation (Seeley et al. 2002; Han et al. 2003).

While it is thought that overall vacuolar biogenesis is the result of reciprocal control between vacuole fusion and fission (Peters et al. 2004), there is no evidence that either of these processes oscillate in the cell cycle. Vacuolar inheritance is thought to be coordinated with the

cell cycle and we have previously reported that loss of Cln3p leads to mild defects in vacuolar segregation (Fig. 8E; Han et al. 2003), but there are no such defects in *bem1Δ* cells (Fig. 8E). Thus, although the mechanism we describe here linking Cln3p with Bem1p certainly impacts on the “steady-state” overall vacuolar homeostasis, it may not necessarily impart an oscillatory feature to it.

On the other hand, Cln3p's vacuolar roles strongly affect the cell size phenotypes associated with *CLN3* mutations (see Fig. 3; Han et al. 2003). As we have discussed previously (Han et al. 2003), this does not extend to all cell size mutants. Vacuolar enlargement is usually accompanied by cellular enlargement (Efe et al. 2005) but the converse is not necessarily true and not all large cell size mutants have large vacuoles. This is exemplified in double mutant *cln1,2Δ* cells, which are large overall although they have small vacuoles (Han et al. 2003). A general correlation between vacuolar fragmentation and cell size also does not seem to exist (Fig. 2A). All these observations suggest that Cln3p's role in vacuole fusion is separate from and probably does not impact on the other function of Cln3p in accelerating initiation of DNA replication.

How might cyclin-dependent phosphorylation of Bem1p impact on vacuole fusion? Cdc42p is involved in actin dynamics (Etienne-Manneville 2004; Irazoqui and Lew 2004) and vacuole fusion (Eitzen et al. 2001; Muller et al. 2001). Importantly, the temporal requirement of Cln3p or Cdc42p for fusion appears to be similar. Loss of either Cln3p or Cdc42p leads to vacuolar fragmentation, but once the docking step of fusion is completed neither Cln3p (Fig. 1) nor Cdc42p (Eitzen et al. 2001; Muller et al. 2001) are required anymore for fusion. While it is clear that noncytoskeletal actin is found on the surface of vacuoles and remodeling of this actin pool is required for vacuole fusion (Eitzen et al. 2002), it is not known how it contributes to fusion. This requirement is distinct from the actin cytoskeleton-dependent processes of vacuole segregation (Weisman 2003; Pruyne et al. 2004). Since we find no evidence for a defect in vacuolar segregation in *bem1Δ* cells (Fig. 8D), there is no reason to think that the cyclin-dependent phosphorylation of Bem1p we describe here affects this process. Consequently, our data placing Bem1p downstream of Cln3p (Figs. 3, 5) and the ability of Cdc42p to suppress vacuole fragmentation in *cln3Δ* cells (Fig. 8) probably reflect the noncytoskeletal role of actin for vacuole fusion that has been proposed by the Wickner and Mayer laboratories (Eitzen et al. 2001, 2002; Muller et al. 2001).

Organelle-bound actin could also act as a fusion barrier, which must transiently disassemble for vesicles to dock, while later promoting actin assembly is thought to somehow help the docked vesicles to finally fuse (Eitzen 2003). Based on chemical inhibition experiments, it appears that the actin depolymerizing drug latrunculin B inhibits the last fusion step of vacuole homotypic fusion (Eitzen et al. 2001). Interestingly, the F-actin binding and stabilizing drug jasplakinolide inhibited the docking step of fusion only, and not the last latrunculin B-sensitive

step (Eitzen et al. 2001). Given that Cln3p (Fig. 1) and Cdc42p (Eitzen et al. 2001; Muller et al. 2001) are not required after the docking step of fusion, the fact that Ser72 of Bem1p has distinct and even opposing roles in vacuole fusion versus bud emergence (Figs. 5, 7), and the fact that other Bem1p mutants that affect bud emergence do not affect vacuole fusion (Fig. 8), it is possible that the effects we are observing reflect the “actin as a barrier” model of fusion (Eitzen 2003).

Does cyclin-dependent phosphorylation of Bem1p affect other Bem1p-regulated processes? The S72A substitution is incapable of suppressing vacuolar fragmentation in *bem1Δ* cells, but it is still fully active in bud emergence (Figs. 5, 7). On the other hand, the S72D substitution fully suppresses vacuole fragmentation, but it only partially suppresses bud emergence (Figs. 5, 7). These results suggest that Bem1p has distinct roles in bud emergence and vacuole fusion. While presumably locking Ser72 to the phosphorylated state (in the S72D mutant) suffices to suppress vacuolar fragmentation (Fig. 5), it might adversely affect bud emergence (Fig. 7).

The notion that Bem1p's roles in vacuole fusion and bud emergence may be distinct is also suggested by the fact that other Bem1p mutants (for example, the K482A mutant) that were shown to be unable to suppress bud emergence defects (Irazoqui et al. 2003) retain full complementing ability in vacuole fusion (Fig. 8D). Even the P208L mutant, which cannot complement bud emergence (Irazoqui et al. 2003), partially suppressed vacuolar fragmentation (Fig. 8D). The P208L mutant affects interactions between the SH3-2 domain of Bem1p with various effectors, including the Cla4p kinase. Interestingly, loss of Cla4p leads to vacuolar fragmentation (Seeley et al. 2002), and it is possible that interactions mediated by the SH3-2 domain of Bem1p might be important for vacuole fusion, explaining the incomplete suppression of vacuolar fragmentation we got from the P208L mutant (Fig. 8D).

In any case, since the actin remodeling necessary for vacuole fusion is not the same as the rearrangement of the actin cytoskeleton that takes place during bud emergence, probably identical mechanisms/effectors do not operate in both processes. It should also be noted that while Cln1p and Cln2p have established roles in bud emergence (Gulli et al. 2000; Bose et al. 2001; Moffat and Andrews 2004), they do not affect vacuole fusion (Han et al. 2003). Finally, although there are no known protein–protein interactions mediated by the SH3-1 domain of Bem1p, it is possible that Ser72 might impact on vacuole fusion through interactions with effectors that have specific roles in fusion. Given the scaffold role of Bem1p and the fact that addition of Cdc42p in the presence of the Bem1p-S72A mutant did not promote fusion as efficiently as the Bem1p-S72D mutant alone (cf. Figs. 8C and 5E), it is possible that phosphorylation of Ser72 is important for some kind of protein–protein interactions that promote vacuole fusion. Future experiments need to address in full detail the mechanisms that account for the differences between Bem1p's roles in vacuole fusion and bud emergence.

Materials and methods

Strains and DNAs

Cell cultivation, media, and yeast molecular biology techniques were performed as described by Kaiser et al. (1994), unless otherwise indicated. The strains and plasmids used in this study are listed in Tables 1 and 2, respectively. To generate the *bem1Δ cln3Δ* strain (SMY01) we disrupted one *CLN3* copy in diploid *bem1Δ* cells as described previously (Polymenis and Schmidt 1997). The resulting heterozygote was sporulated, and the segregants were obtained by random spore analysis (Kaiser et al. 1994). All the single cyclin deletions and the *bem1Δ* strain in the BY4741 background were obtained by sporulation of the corresponding homozygous diploid deletion strains distributed from Research Genetics. The phenotypes reported for each strain were obtained after examining several independent transformants or segregants for the strain in question.

The putative phosphorylation site amino acid substitutions were introduced in the *BEM1-12MYC* low-copy centromeric plasmid pDLB2226 (Irazoqui et al. 2003). We first PCR amplified *BEM1* sequences from pDLB2226 using forward primers that encoded the desired mutation [BEM1-S72A-FWD, 5'-CCAAAA AACAGACATAATTCTAAAGATATTACTGCTCCAGAGA AAGTTATAAAAAGCCAAATAC-3'; BEM1-S72D-FWD, 5'-CC AAAAAACAGACATAATTCTAAAGATATTACTGATCCAG AGAAAGTTATAAAAAGCCAAATAC-3'], and a reverse primer corresponding to sequences up to position +574 of the *BEM1* ORF [BEM1-(+574)-REV, 5'-CGACCAATTGGCTTAGCAAT GAACC-3']. The PCR products were purified by agarose gel electrophoresis and used as primers in a second PCR reaction with pDLB2226 as template and a forward primer corresponding to *BEM1* sequences up to position -186 [BEM1-(-186)-FWD,

5'-ATTACCCTAAACGGACAAATG-3']. The PCR product of this reaction was also purified by agarose gel electrophoresis and cotransformed into yeast cells together with plasmid pDLB2226, which was previously linearized with *Sma*I and *Hind*III digestion (cutting at positions -166 to +330 of the *BEM1* ORF, respectively). The gap-repaired plasmid derivatives were then recovered from yeast transformants by standard methods (Kaiser et al. 1994).

To construct plasmids for bacterial expression, we digested the BG1805-based plasmids (purchased from Open Biosystems; see Table 2) with *Age*I, which cuts once in the *GAL1* promoter all the BG1805 plasmids used here. The digested plasmids were then transformed into a *Ura*⁻ yeast strain for gap repair together with an oligonucleotide that encodes the arabinose *P_{BAD}* promoter and ribosome-binding site (Guzman et al. 1995) [GAL1-PBAD-FWD, 5'-CGGGAACGGATTAGAAGCCGCCGAGCG GGTGACGCTTTTTATCGCAACTCTCTACTGTTTCTCCA TACCCGTTTTTTTGGATGGAGTGAATATACCTCTATAC TTAAACGTCAAGGAGAA-3']. GAL1-PBAD-FWD at its 5' end carries sequences complementary to *GAL1* sequences upstream of the *Age*I site, and at its 3' end sequences complementary immediately upstream of the ATG start codon of the ORF in the BG1805 plasmids, which correspond to the bacteriophage λ attR1 site.

The mutant plasmid derivatives were sequenced to verify the introduced mutation and the absence of any other mutations at the Genome Technologies Laboratory of Texas A&M University.

Vacuolar morphology and size

For microscopic examination of vacuolar membranes the cells were stained with N-(3-triethylammoniumpropyl)-4-(6-[4-diethylaminophenyl]hexatrienyl)pyridinium dibromide, FM4-64, from Molecular Probes, as described by Han et al. (2003), and then examined microscopically with a Nikon Eclipse TS100 inverted fluorescence microscope.

Vacuolar size was evaluated by flow cytometry after staining with the vital vacuolar stain 5' (and 6')-carboxy-2',7'-dichlorofluorescein diacetate, CDCFDA, from Molecular Probes as described previously (Han et al. 2003).

The mean cell volume of live unfixed samples was measured using a Beckman Coulter Z2 Channelyzer. The data were analyzed using the manufacturer's AccuComp software. The geometric mean is indicated in each case.

In vitro vacuole fusion

Cytosol and vacuoles were prepared and used as described previously (Mayer et al. 1996; Han et al. 2003). Briefly, to measure fusion activity the reaction mixture was equally split, and to each aliquot the same volume of buffer with inhibitor (or antibody) was added. Buffer-added aliquots were incubated at 27°C or on ice, the fusion activities of which were measured after 90 min and set as 100% or 0% fusion activity, respectively. The inhibitor-added aliquot was incubated at 27°C for the same time and its fusion activity was measured to evaluate the relative fusion activity.

Protein analysis

Unless otherwise indicated, the gels for SDS-PAGE (Laemmli 1970) contained 8% of a 29:1 acrylamide/bis-acrylamide solution. Immunoprecipitations and TEV cleavage of TAP-tagged Bem1p were performed according to the protocols and reagents recommended by Rigaut et al. (1999). Briefly, 5×10^9 cells of

Table 1. Strains used in this study

Strain	Relevant genotype	Source
BY4741	<i>MATa hisΔ leu2Δ met15Δ ura3Δ</i>	Res. Genetics
BY4742	<i>MATa his3Δ leu2Δ met15Δ ura3Δ</i>	Res. Genetics
BY4743	BY4741/BY4742	Res. Genetics
W303a	<i>MATa ade2 trp1 leu2 his3 ura3 can1</i>	B. Futcher
GT108	<i>CLN3-3HA::URA3</i> (W303a otherwise)	B. Futcher
7503198	<i>CLN2-TAP::his3MX</i> (BY4741 otherwise)	Open Biosystems
7499374	<i>BEM1-TAP::his3MX</i> (BY4741 otherwise)	Open Biosystems
33340	<i>bem1Δ::kanMX/bem1Δ::kanMX</i> (BY4743 otherwise)	Res. Genetics
DKY6281	<i>MATa lys2 trp1 ura3 his3 leu2 suc2 pho8Δ::TRP1</i>	W. Wickner
BJ3505	<i>MATa lys2 trp1 ura3 his3 gal2 can prb1 pep4Δ::HIS3</i>	W. Wickner
13340	<i>bem1Δ::kanMX</i> (BY4742 otherwise)	Res. Genetics
SMY01	<i>bem1Δ::KanMX cln3Δ::URA3</i> (BY4741 otherwise)	This study
30366	<i>cln3Δ::kanMX/cln3Δ::kanMX</i> (BY4743 otherwise)	Res. Genetics
366	<i>cln3Δ::kanMX</i> (BY4741 otherwise)	Res. Genetics
DOM90	<i>bar1::HisG</i> (W303a otherwise)	D. Morgan
DOM30	<i>cdc28::cdc28-as1</i> (DOM90 otherwise)	D. Morgan

Table 2. *Plasmids used in this study*

Plasmid	Relevant characteristic	Source
p205	CEN [<i>CLN3-2^P</i>] <i>URA3</i>	F. Cross
pDLB2226	CEN [<i>BEM1-12MYC</i>] <i>LEU2</i>	D. Lew
pDLB2226-S72A	CEN [<i>BEM1-S72A-12MYC</i>] <i>LEU2</i>	This study
pDLB2226-S72D	CEN [<i>BEM1-S72D-12MYC</i>] <i>LEU2</i>	This study
pTH113	2 μ [<i>CDC24</i>] <i>LEU2</i>	T. Höfken
pTH114	2 μ [<i>CDC42</i>] <i>LEU2</i>	T. Höfken
pDLB2374	2 μ [<i>BEM1-12MYC</i>] <i>LEU2</i>	D. Lew
pDLB2375	2 μ [<i>BEM1-P208L-12MYC</i>] <i>LEU2</i>	D. Lew
pDLB2377	2 μ [<i>BEM1-P355A-12MYC</i>] <i>LEU2</i>	D. Lew
pDLB2378	2 μ [<i>BEM1-R369A-12MYC</i>] <i>LEU2</i>	D. Lew
pDLB2379	2 μ [<i>BEM1-K482A-12MYC</i>] <i>LEU2</i>	D. Lew
pBAD-DCR2	[<i>P_{BAD}-DCR2-TAG</i>]	This study
pBAD-CLN3	[<i>P_{BAD}-CLN3-TAG</i>]	This study
pBAD-BEM1	[<i>P_{BAD}-BEM1-TAG</i>]	This study
pBAD-BEM1-S72A	[<i>P_{BAD}-BEM1-S72A-TAG</i>]	This study
pBAD-BEM1-S72D	[<i>P_{BAD}-BEM1-S72D-TAG</i>]	This study
pBAD-CDC42	[<i>P_{BAD}-CDC42-TAG</i>]	This study
BG1805-DCR2	2 μ [<i>P_{GALI}-DCR2-TAG</i>] <i>URA3</i>	Open Biosystems
BG1805-CLN3	2 μ [<i>P_{GALI}-CLN3-TAG</i>] <i>URA3</i>	Open Biosystems
BG1805-BEM1	2 μ [<i>P_{GALI}-BEM1-TAG</i>] <i>URA3</i>	Open Biosystems
BG1805-CDC42	2 μ [<i>P_{GALI}-CDC42-TAG</i>] <i>URA3</i>	Open Biosystems

untagged or TAP-tagged *BEM1* cells from the exponential phase of growth were used. Cell extracts were prepared in the presence of protease and phosphatase inhibitor cocktails (Sigma). After the first immunoprecipitation with IgG-agarose beads, the beads were washed twice with RIPA buffer and once with TEV reaction buffer. Eighty units of TEV for 30 min at 30°C were used to cleave the protein A domain. The phosphatase experiment shown in Figure 4A was done as described previously (Liakopoulos et al. 2003). For the phosphatase experiments in Figure 4C, Bem1p-CBP on calmodulin beads was washed three times with RIPA buffer and once with λ -phosphatase reaction buffer. Then 500 U of λ -phosphatase and 10 $\times$ of protease inhibitor cocktail were added and incubated at 30°C for 30 min in the presence or absence of 10 $\times$ phosphatase inhibitor cocktail.

For protein surveillance, cell extracts were prepared using a urea extraction buffer as described by Ubersax et al. (2003). The differences in Bem1p's electrophoretic mobility in 1D SDS-PAGE are better resolved if the samples are desalted prior to electrophoresis (using a kit from Pierce, according to their instructions), and electrophoresis is performed at a constant voltage of 70 V (instead of the typical 200 V applied to minigels).

Two-dimensional gel electrophoresis was performed using carrier ampholytes (pH 4–6; GE Healthcare) in acrylamide tubes according to the method of O'Farrell (1975). Seven-centimeter-long tube gels containing 0.5% ampholytes were focused for ~1000 volt hours without prefocusing. The tubes were extruded and soaked, sequentially, in SDS sample buffer containing dithiothreitol or iodoacetamide. The tube gels containing the reduced and alkylated proteins were subsequently placed on top of 8% polyacrylamide slab minigels and subjected to SDS PAGE (Laemmli 1970). Prior to immunostaining, the proteins were electroblotted onto PVDF membranes (Immobilon P⁸⁰; Millipore) at 100–200 mA overnight using 10 mM CAPS, 10% methanol (pH 11).

For immunostaining, protein A fusion proteins were detected with the peroxidase-anti-peroxidase (PAP) soluble complex reagent from Sigma. The anti-Pgk1p antibody was from Molecular Probes. The anti-phospho-[ST]P, anti-CBP, anti-HA, and anti-Myc antibodies were from Abcam. The horseradish peroxidase-

conjugated secondary antibodies used for immunoblotting were also from Abcam. All antibodies were used at the dilutions recommended by the manufacturers. The blots were processed with reagents from Pierce.

Bacterial expression

The pBAD-based plasmids were transformed into *Escherichia coli* (strain XL1-Blue). For protein expression we followed previously published procedures (Guzman et al. 1995). From 50–100-mL cultures we obtained soluble protein from all the constructs we describe here. The exception was Cln3p, which required larger cultures (0.5–1 L) because the majority of the recombinant protein appeared to be insoluble. We purified the recombinant proteins through their 6xHis epitope, using TALON Co²⁺ affinity beads (BD Biosciences), according to the manufacturer's instructions.

Kinase assays

Human Cdc2/cyclin B and Cdk2/cyclin A were from New England Biolabs, and used at 5 U per reaction. Cln3p-associated activity was obtained from yeast cells (strain W303a) carrying plasmid BG1805-CLN3 (see Table 2), using TALON Co²⁺ affinity beads (BD Biosciences), according to the manufacturer's instructions. The Cln3p-associated activity was from ~10¹⁰ cells initially grown in raffinose, but 4 h prior to harvesting, the culture was induced with 2% galactose. Histone H1 was from Sigma and used as a substrate at 5 μ g per reaction. Bem1p was obtained from bacteria as described above. The reactions also contained 5 μ Ci [γ -³²P]ATP, 100 μ M ATP, 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM EGTA, 2 mM DTT, and 0.01% Brij 35. All the reactions (30 μ L total volume) were performed at room temperature for 20 min.

Immunofluorescence microscopy

Unless otherwise indicated, we followed the protocols of the Botstein laboratory as described at <http://genome-www>.

stanford.edu/group/botlab. DAPI, 4'-diamidino-2-phenylindole, was from Sigma. The anti-tubulin primary antibody and the secondary FITC-conjugated antibody were from Abcam. For the experiment shown in Figure 7, the cells were grown at 37°C to exacerbate the bud emergence defect of *bem1Δ* cells. The samples were examined with a Nikon Eclipse TS100 inverted fluorescence microscope.

Acknowledgments

We thank all the members of the Polymenis laboratory for comments and advice; Elena Lyuksyutova for expert technical assistance with 2D gel electrophoresis; D. Morgan, F. Cross, B. Futcher, T. Höfken, W. Wickner, W. Harper, C. Wittenberg, and D. Lew for generously providing us with reagents; D. Lew, D. Morgan, and C. Wittenberg for useful discussions; and J.C. Hu, M. Kladde, and L. Guarino for help and advice. This work was supported by a grant from the National Institutes of Health (R01-GM062377) to M.P.

References

- Barelle, C.J., Bohula, E.A., Kron, S.J., Wessels, D., Soll, D.R., Schafer, A., Brown, A.J., and Gow, N.A. 2003. Asynchronous cell cycle and asymmetric vacuolar inheritance in true hyphae of *Candida albicans*. *Eukaryot. Cell* **2**: 398–410.
- Bender, A. and Pringle, J.R. 1991. Use of a screen for synthetic lethal and multicopy suppressor mutants to identify two new genes involved in morphogenesis in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **11**: 1295–1305.
- Bishop, A.C., Ubersax, J.A., Petsch, D.T., Matheos, D.P., Gray, N.S., Blethrow, J., Shimizu, E., Tsien, J.Z., Schultz, P.G., Rose, M.D., et al. 2000. A chemical switch for inhibitor-sensitive alleles of any protein kinase. *Nature* **407**: 395–401.
- Bose, I., Irazoqui, J.E., Moskow, J.J., Bardes, E.S., Zyla, T.R., and Lew, D.J. 2001. Assembly of scaffold-mediated complexes containing Cdc42p, the exchange factor Cdc24p, and the effector Cla4p required for cell cycle-regulated phosphorylation of Cdc24p. *J. Biol. Chem.* **276**: 7176–7186.
- Cliften, P., Sudarsanam, P., Desikan, A., Fulton, L., Fulton, B., Majors, J., Waterston, R., Cohen, B.A., and Johnston, M. 2003. Finding functional features in *Saccharomyces* genomes by phylogenetic footprinting. *Science* **301**: 71–76.
- Costanzo, M., Nishikawa, J.L., Tang, X., Millman, J.S., Schub, O., Breikreuz, K., Dewar, D., Rupes, I., Andrews, B., and Tyers, M. 2004. CDK activity antagonizes Whi5, an inhibitor of G1/S transcription in yeast. *Cell* **117**: 899–913.
- Cross, F.R. 1988. *DAF1*, a mutant gene affecting size control, pheromone arrest, and cell cycle kinetics of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **8**: 4675–4684.
- de Bruin, R.A., McDonald, W.H., Kalashnikova, T.I., Yates III, J., and Wittenberg, C. 2004. Cln3 activates G1-specific transcription via phosphorylation of the SBF bound repressor Whi5. *Cell* **117**: 887–898.
- Dirick, L., Bohm, T., and Nasmyth, K. 1995. Roles and regulation of Cln-Cdc28 kinases at the start of the cell cycle of *Saccharomyces cerevisiae*. *EMBO J.* **14**: 4803–4813.
- Efe, J.A., Botelho, R.J., and Emr, S.D. 2005. The Fab1 phosphatidylinositol kinase pathway in the regulation of vacuole morphology. *Curr. Opin. Cell Biol.* **17**: 402–408.
- Eitzen, G. 2003. Actin remodeling to facilitate membrane fusion. *Biochim. Biophys. Acta* **1641**: 175–181.
- Eitzen, G., Will, E., Gallwitz, D., Haas, A., and Wickner, W. 2000. Sequential action of two GTPases to promote vacuole docking and fusion. *EMBO J.* **19**: 6713–6720.
- Eitzen, G., Thorngren, N., and Wickner, W. 2001. Rho1p and Cdc42p act after Ypt7p to regulate vacuole docking. *EMBO J.* **20**: 5650–5656.
- Eitzen, G., Wang, L., Thorngren, N., and Wickner, W. 2002. Remodeling of organelle-bound actin is required for yeast vacuole fusion. *J. Cell Biol.* **158**: 669–679.
- Etienne-Manneville, S. 2004. Cdc42—The centre of polarity. *J. Cell Sci.* **117**: 1291–1300.
- Gattiker, A., Gasteiger, E., and Bairoch, A. 2002. ScanProsite: A reference implementation of a PROSITE scanning tool. *Appl. Bioinformatics* **1**: 107–108.
- Gulli, M.P., Jaquenoud, M., Shimada, Y., Niederhauser, G., Wiget, P., and Peter, M. 2000. Phosphorylation of the Cdc42 exchange factor Cdc24 by the PAK-like kinase Cla4 may regulate polarized growth in yeast. *Mol. Cell* **6**: 1155–1167.
- Guzman, L.M., Belin, D., Carson, M.J., and Beckwith, J. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J. Bacteriol.* **177**: 4121–4130.
- Han, B.-K., Aramayo, R., and Polymenis, M. 2003. The G1 cyclin Cln3p controls vacuolar biogenesis in *Saccharomyces cerevisiae*. *Genetics* **165**: 467–476.
- Irazoqui, J.E. and Lew, D.J. 2004. Polarity establishment in yeast. *J. Cell Sci.* **117**: 2169–2171.
- Irazoqui, J.E., Gladfelter, A.S., and Lew, D.J. 2003. Scaffold-mediated symmetry breaking by Cdc42p. *Nat. Cell Biol.* **5**: 1062–1070.
- Jones, E.W., Webb, G.C., and Hiller, M.A. 1997. Biogenesis and function of the yeast vacuole. In *The molecular biology of the yeast Saccharomyces* (ed. J.R. Pringle et al.), pp. 363–470. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Jorgensen, P., Nishikawa, J.L., Breikreutz, B.J., and Tyers, M. 2002. Systematic identification of pathways that couple cell growth and division in yeast. *Science* **297**: 395–400.
- Kaiser, C., Michaelis, S., and Mitchell, A. 1994. *Methods in yeast genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Kellis, M., Patterson, N., Endrizzi, M., Birren, B., and Lander, E.S. 2003. Sequencing and comparison of yeast species to identify genes and regulatory elements. *Nature* **423**: 241–254.
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.
- Leeuw, T., Fourest-Lieuvin, A., Wu, C., Chenevert, J., Clark, K., Whiteway, M., Thomas, D.Y., and Leberer, E. 1995. Pheromone response in yeast: Association of Bem1p with proteins of the MAP kinase cascade and actin. *Science* **270**: 1210–1213.
- Liakopoulos, D., Kusch, J., Grava, S., Vogel, J., and Barral, Y. 2003. Asymmetric loading of Kar9 onto spindle poles and microtubules ensures proper spindle alignment. *Cell* **112**: 561–574.
- Loog, M. and Morgan, D.O. 2005. Cyclin specificity in the phosphorylation of cyclin-dependent kinase substrates. *Nature* **434**: 104–108.
- Mayer, A., Wickner, W., and Haas, A. 1996. Sec18p (NSF)-driven release of Sec17p (α -SNAP) can precede docking and fusion of yeast vacuoles. *Cell* **85**: 83–94.
- Moffat, J. and Andrews, B. 2004. Late-G1 cyclin-CDK activity is essential for control of cell morphogenesis in budding yeast. *Nat. Cell Biol.* **6**: 59–66.
- Muller, O., Johnson, D.I., and Mayer, A. 2001. Cdc42p functions at the docking stage of yeast vacuole membrane fusion. *EMBO J.* **20**: 5657–5665.

Han et al.

- O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**: 4007–4021.
- Pathak, R., Bogomolnaya, L.M., Guo, J., and Polymenis, M. 2004. Gid8p (Dcr1p) and Dcr2p function in a common pathway to promote START completion in *Saccharomyces cerevisiae*. *Eukaryot. Cell* **3**: 1627–1638.
- Peters, C., Baars, T.L., Buhler, S., and Mayer, A. 2004. Mutual control of membrane fission and fusion proteins. *Cell* **119**: 667–678.
- Polymenis, M. and Schmidt, E.V. 1997. Coupling of cell division to cell growth by translational control of the G1 cyclin CLN3 in yeast. *Genes & Dev.* **11**: 2522–2531.
- Pruyne, D., Legesse-Miller, A., Gao, L., Dong, Y., and Bretscher, A. 2004. Mechanisms of polarized growth and organelle segregation in yeast. *Annu. Rev. Cell Dev. Biol.* **20**: 559–591.
- Richardson, H.E., Wittenberg, C., Cross, F., and Reed, S.I. 1989. An essential G1 function for cyclin-like proteins in yeast. *Cell* **59**: 1127–1133.
- Richman, T.J., Toenjes, K.A., Morales, S.E., Cole, K.C., Wasserman, B.T., Taylor, C.M., Koster, J.A., Whelihan, M.F., and Johnson, D.I. 2004. Analysis of cell-cycle specific localization of the Rdi1p RhoGDI and the structural determinants required for Cdc42p membrane localization and clustering at sites of polarized growth. *Curr. Genet.* **45**: 339–349.
- Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B. 1999. A generic protein purification method for protein complex characterization and proteome exploration. *Nat. Biotechnol.* **17**: 1030–1032.
- Roberts, C.J., Raymond, C.K., Yamashiro, C.T., and Stevens, T.H. 1991. Methods for studying the yeast vacuole. *Methods Enzymol.* **194**: 644–661.
- Schaefer, J.B. and Breeden, L.L. 2004. RB from a bud's eye view. *Cell* **117**: 849–850.
- Seeley, E.S., Kato, M., Margolis, N., Wickner, W., and Eitzen, G. 2002. Genomic analysis of homotypic vacuole fusion. *Mol. Biol. Cell* **13**: 782–794.
- Shorter, J. and Warren, G. 2002. Golgi architecture and inheritance. *Annu. Rev. Cell Dev. Biol.* **18**: 379–420.
- Spellman, P.T., Sherlock, G., Zhang, M.Q., Iyer, V.R., Anders, K., Eisen, M.B., Brown, P.O., Botstein, D., and Futcher, B. 1998. Comprehensive identification of cell cycle-regulated genes of the yeast *Saccharomyces cerevisiae* by microarray hybridization. *Mol. Biol. Cell* **9**: 3273–3297.
- Tyers, M., Tokiwa, G., Nash, R., and Futcher, B. 1992. The Cln3–Cdc28 kinase complex of *S. cerevisiae* is regulated by proteolysis and phosphorylation. *EMBO J.* **11**: 1773–1784.
- Tyers, M., Tokiwa, G., and Futcher, B. 1993. Comparison of the *Saccharomyces cerevisiae* G1 cyclins: Cln3 may be an upstream activator of Cln1, Cln2, and other cyclins. *EMBO J.* **12**: 1955–1968.
- Ubersax, J.A., Woodbury, E.L., Quang, P.N., Paraz, M., Blethrow, J.D., Shah, K., Shokat, K.M., and Morgan, D.O. 2003. Targets of the cyclin-dependent kinase Cdk1. *Nature* **425**: 859–864.
- Warren, G. and Wickner, W. 1996. Organelle inheritance. *Cell* **84**: 395–400.
- Weisman, L.S. 2003. Yeast vacuole inheritance and dynamics. *Annu. Rev. Genet.* **37**: 435–460.
- Wickner, W. and Haas, A. 2000. Yeast homotypic vacuole fusion: A window on organelle trafficking mechanisms. *Annu. Rev. Biochem.* **69**: 247–275.
- Wiemken, A. and Durr, M. 1974. Characterization of amino acid pools in the vacuolar compartment of *Saccharomyces cerevisiae*. *Arch. Microbiol.* **101**: 45–57.
- Zhang, J., Schneider, C., Ottmers, L., Rodriguez, R., Day, A., Markwardt, J., and Schneider, B.L. 2002. Genomic scale mutant hunt identifies cell size homeostasis genes in *S. cerevisiae*. *Curr. Biol.* **12**: 1992–2001.

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Genes Dev. 2005, **19**:

Access the most recent version at doi:[10.1101/gad.1361505](https://doi.org/10.1101/gad.1361505)

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