Synthetic Lethal Analysis Implicates Ste20p, a p21-activated Protein Kinase, in Polarisome Activation

April S. Goehring
David A. Mitchell
Amy Hin Yan Tong
Megan Keniry
The University of Texas Rio Grande Valley
Charles Boone

Follow this and additional works at: https://scholarworks.utrgv.edu/bio_fac

Recommended Citation

This Article is brought to you for free and open access by the College of Sciences at ScholarWorks @ UTRGV. It has been accepted for inclusion in Biology Faculty Publications and Presentations by an authorized administrator of ScholarWorks @ UTRGV. For more information, please contact justin.white@utrgv.edu, william.flores01@utrgv.edu.
Synthetic Lethal Analysis Implicates Ste20p, a p21-activated Protein Kinase, in Polarisome Activation

April S. Goehring,* David A. Mitchell,** Amy Hin Yan Tong,†§ Megan E. Keniry,* Charles Boone,§ and George F. Sprague Jr.*

*Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403-1229; ‡Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario, Canada M5G IL6; and §Department of Medical Genetics and Microbiology, University of Toronto, Toronto, Ontario, Canada M5S 1A8

Submitted June 17, 2002; October 7, 2002; Accepted December 4, 2002

Monitoring Editor: David Drubin

The p21-activated kinases Ste20p and Cla4p carry out undefined functions that are essential for viability during budding in Saccharomyces cerevisiae. To gain insight into the roles of Ste20p, we have used a synthetic lethal mutant screen to identify additional genes that are required in the absence of Cla4p. Altogether, we identified 65 genes, including genes with roles in cell polarity, mitosis, and cell wall maintenance. Herein, we focus on a set that defines a function carried out by Bni1p and several of its interacting proteins. We found that Bni1p and a group of proteins that complex with Bni1p (Bud6p, Spa2p, and Pea2p) are essential in a cla4Δ mutant background. Bni1p, Bud6p, Spa2, and Pea2p are members of a group of polarity determining proteins referred to as the polarisome. Loss of polarisome proteins from a cla4Δ strain causes cells to form elongated buds that have mislocalized septin rings. In contrast, other proteins that interact with or functionally associate with Bni1p and have roles in nuclear migration and cytokinesis, including Num1p and Hof1p, are not essential in the absence of Cla4p. Finally, we have found that Bni1p is phosphorylated in vivo, and a substantial portion of this phosphorylation is dependent on STE20. Together, these results suggest that one function of Ste20p may be to activate the polarisome complex by phosphorylation of Bni1p.

INTRODUCTION

Yeast cells undergo polarized growth during budding and other morphogenetic events in response to intracellular or extracellular cues (Drubin and Nelson, 1996). Polarized cell growth depends on assembly of a polarized actin cytoskeleton, which then directs transport of secretory vesicles containing cell wall and plasma membrane components to the site of growth (Novick and Botstein, 1985; Mulholland et al., 1994; Ayscough et al., 1997; Pruyne et al., 1998). The p21 GTPase Cdc42p plays a critical role in the establishment of subcellular polarity and the execution of subsequent apical growth by regulating the actin cytoskeleton (Adams et al., 1990; Johnson and Pringle, 1990; Ziman et al., 1993; Li et al., 1995; Richman and Johnson, 2000). Cdc42p is also required for septin ring function and for cytokinesis (Richman et al., 1999; Toenjes et al., 1999). How Cdc42p orchestrates these various activities is poorly understood, but some of its target effectors have been identified. For example, Gic1p and Gic2p can bind activated Cdc42p and are important for polarization of the actin cytoskeleton (Brown et al., 1997; Chen et al., 1997). Two other identified effectors for Cdc42p are the related p21-activated protein kinases, Cla4p and Ste20p, both of which interact with activated Cdc42p and localize to sites of polarized growth (Cvrckova et al., 1995; Peter et al., 1996; Leberer et al., 1997; Holly and Blumer, 1999; Mosch et al., 2001). Each kinase has unique roles in the cell. Ste20p functions in pheromone response and haploid invasive growth, whereas Cla4p promotes normal septin function (Ramer and Davis, 1993; Roberts and Fink, 1994; Benton et al., 1997; Tjandra et al., 1998; Gulli et al., 2000; Bose et al., 2001). A cell lacking both kinases is inviable (Cvrckova et al., 1995), demonstrating that Ste20p is essential in the absence of Cla4p (and vice versa). One interpretation of this relationship is that these two kinases share a function that is essential, though other interpretations are possible. Currently, the
only proposed targets of Ste20p and Cla4p are the two myosin I homologs Myo3p and Myo5p. Sites in Myo3p and Myo5p are phosphorylated in vitro by Ste20p and are required for in vivo function (Wu et al., 1997). Myo3p and Myo5p are required for actin patch assembly (Evangelista et al., 2000; Gell et al., 2000; Lechler et al., 2000). However, although an activated allele of Myo3p (MYO3CA) to select stable G418-resistant transformants. The successful creation of the deletion strain was confirmed by PCR, and the protein function was verified in a bni1Δ strain, in which Bni1p is essential (our unpublished data) (Ozaki-Kuroda et al., 1996) that is required to assemble actin cables (Evangelista et al., 2002; Sagot et al., 2002). These cables seem to guide myosin motors that direct secretion, organelle and mRNA inheritance, and mitotic spindle orientation, thereby establishing cell polarity (Evangelista et al., 2002). Bni1p has an intricate network of interactions involving a number of different groups of proteins. One such group of proteins, Bud6p, Spa2p, and Pea2p, complexes with Bni1p to form the "polarisome," which is involved in apical growth (Sheu et al., 1999; Pryue and Bretscher, 2000). We show that Bud6p, Spa2p, and Pea2p are essential in a cla4Δ mutant background with the expectation that the genes identified would suggest the nature of the physiological events that have been perturbed.

Herein, we present the results of two independent synthetic lethal mutant screens. One screen was based on random mutagenesis of the genome by using a red/white colony sectoring assay (Kranz and Holm, 1990; Bender and Fringle, 1991). The second screen used a yeast genome-wide deletion set and evaluated the viability of cla4Δ paired with 4672 different viable deletion strains (Tong et al., 2001). From the collection of genes defined by these screens, we chose a subset for more detailed investigation. BNI1 is at the center of this study and encodes a formin homology protein (Zahm et al., 1997; Fujiwara et al., 2000). Synthetic genetic array analysis (SGA) was also used to identify genes that were essential in a cla4Δ background as described in Tong et al. (2001). Y2928 (MATa cla4Δ-mdt mfa1Δ::MAFLpr-HIS3 can1Δ ura3Δ leu2Δ his3Δ1 lys2Δ) was created in two steps. First, CLA4 was deleted from Y254 by using PCR-based integration with primers (5'-TTTG-GTGTGATTAAATACGCAA GTGAACGTTGAACTATTTGAAGAATG-AGTGCAAAATGGAAACAGCTATG ACCATG-3'). Next, the amplified fragment of the CLA4 locus was cloned into YEPD medium and incubated overnight at 30°C. The lawn of cells was then replica-plated onto YEPD containing 200 μg/ml G418/ geneticin (Invitrogen, Carlsbad, CA) to select stable G418-resistant transformants. The successful creation of the deletion strain was confirmed by PCR, and the protein function was verified in a bni1Δ strain, in which Bni1p is essential (our unpublished data) (Ozaki-Kuroda et al., 2001).

**Synthetic Lethal Mutant Screens**
Two independent methods were used to search for mutations that are lethal in a cla4Δ background. Previously, we described the details of the NCS screen by using the colony sectoring assay (Mitchell and Sprague, 2001). Synthetic genetic array analysis (SGA) was also used to identify genes that were essential in a cla4Δ background as described in Tong et al. (2001). Y2928 (MATa cla4Δ-mdt mfa1Δ::MAFLpr-HIS3 can1Δ ura3Δ leu2Δ his3Δ1 lys2Δ) was created in two steps. First, CLA4 was deleted from Y254 by using PCR-based integration with primers (5'-GACGTTTCATTACACAGCGAAGAAATGACCCCAAGAACCAAATACATGAAATGATAGTATGATTTAGC-3') and the amplified fragment of the CLA4 locus was cloned into YEPD medium and incubated overnight at 30°C. The lawn of cells was then replica-plated onto YEPD containing 200 μg/ml G418/ geneticin (Invitrogen, Carlsbad, CA) to select stable G418-resistant transformants. The successful creation of the deletion strain was confirmed by PCR, and the protein function was verified in a bni1Δ strain, in which Bni1p is essential (our unpublished data) (Ozaki-Kuroda et al., 2001).

**MATERIALS AND METHODS**

**Growth Conditions, Plasmids, and Strains**
Yeast and bacterial strains were propagated using standard methods (Sambrook et al., 1989; Rose et al., 1990). YEPD and SD media were prepared as described previously (Rose et al., 1990). Yeast transformations were performed using modifications of the LiOAc method (Chen et al., 1992; Gietz et al., 1995). Bacterial transformations, DNA preparations, and plasmid constructions were performed by standard methods (Sambrook et al., 1989). The plasmids used in this study, YCPHIScla4-75, pYS9tet1HA-BNI1 (p925), pcle4-75td, and pRS316/AD60CLA4 have been described elsewhere (Cvrckova et al., 1995; Evangelista et al., 1997; Holly and Blumer, 1999; Mitchell and Sprague, 2001). To ensure that the version of cle4-75td we were using was the same as the allele used in Holly and Blumer (1999), we sequenced the pcle4-75td and the cla4-75td alleles rescued from our strains. We found that the cla4-75td alleles rescued from our strains were identical to that of the pcle4-75td from the Blumer laboratory. Strains that were used in this study are listed in Table 1. Gene deletions were constructed by polymerase chain reaction (PCR) (Baudin et al., 1993) by using either the pRS (Sikorski and Hieter, 1989) or pFA6a (Longtine et al., 1998) plasmid series as templates. In all cases, the entire coding region was replaced with the indicated marker, and successful replacement was confirmed by PCR and phenotype when applicable. Single step gene deletion plasmids for swi6Δ-Leu2Δ, spo2Δ::URA3 (p210) and spo2Δ::URA3 (pNV44) were provided by I. Herskowitz and D. Lew (Boorer et al., 1993; Valtz and Herskowitz, 1996). We used bni1Δ::URA3 (p321), a single step gene deletion plasmid, to delete BNI1 (Evangelista et al., 1997). 5-Fluoroorotic acid (5-FOA) (Biovector, Oxford, CT) was used to select for uracil auxotrophs. The COOH-terminal deletion mutant bni1-CTΔ lacks the coding sequence for amino acids 1749–1953 of Bni1p (Lee et al., 1999). bni1Δ::URA3 was created by amplification of the kmnMX6 cassette from pFA6a-kanMX6 together with sequences immediately flanking base pairs 3247–5859 of BNI1 by using the forward primer 5'-ATAATGCACAGGTATACCTGACCGGTCCACCTCTAAA-3' and the reverse primer 5'-GTITTTGATATCAGTTGTGCATATAAATTTTTGTGGTGTTATGAGCCTTGTTAAAC-3' (the sequences flanking base pairs 3247–5859 of BNI1 are underlined) (Longtine et al., 1998). The amplified fragment was transferred into strains SY3357, SY3362, SY3380, and SY3764; the transformants were plated on YEPD medium and incubated overnight at 30°C. The lawn of cells was then replica-plated onto YEPD containing 200 μg/ml G418/ geneticin (Invitrogen, Carlsbad, CA) to select stable G418-resistant transformants. The successful creation of the deletion strain was confirmed by PCR, and the protein function was verified in a bni1Δ strain, in which Bni1p is essential (our unpublished data) (Ozaki-Kuroda et al., 2001).

**Isolation of BNI1, BUD6, and Other NCS Genes**
Wild-type NCS8 and NCS5 were identified as BNI1 and BUD6 by complementation of ncs8Δ (SY3372) and ncs5Δ (SY3369) mutants, respectively. For NCS7 (ATCC no. 77162), an 8.6-kb region shared by all of them was identified and 62 were confirmed by tetrad analysis.

**Isolation of BNI1, BUD6, and Other NCS Genes**
Wild-type NCS8 and NCS5 were identified as BNI1 and BUD6 by complementation of ncs8Δ (SY3372) and ncs5Δ (SY3369) mutants, respectively. For NCS7 (ATCC no. 77162), an 8.6-kb region shared by all of them was identified and 62 were confirmed by tetrad analysis.
Table 1. Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>SY3357</td>
<td>MATa leu2-Δ1 ura3-52 his3-Δ200 trp1-Δ63 ade2-101 mfa2-Δ1 : FUS1-lacZ</td>
<td>Mitchell and Sprague (2001)</td>
</tr>
<tr>
<td>SY3358</td>
<td>MATa leu2-Δ1 ura3-52 his3-Δ200 lys2-801 trp1-Δ63 ade2-101 mfa2-Δ1 : FUS1 lacZ</td>
<td>Mitchell and Sprague (2001)</td>
</tr>
<tr>
<td>SY3362</td>
<td>SY3357 except cla4Δ : TRP1 (pRS316ADE8CLA4)</td>
<td>Mitchell and Sprague (2001)</td>
</tr>
<tr>
<td>SY3364</td>
<td>SY3357 except cla4Δ : TRP1 ncs1Δ : LEU2 (pRS316ADE8CLA4)</td>
<td>Mitchell and Sprague (2001)</td>
</tr>
<tr>
<td>SY3366</td>
<td>SY3357 except cla4Δ : TRP1 ncs2-1 (pRS316ADE8CLA4)</td>
<td>Mitchell and Sprague (2001)</td>
</tr>
<tr>
<td>SY3367</td>
<td>SY3357 except cla4Δ : TRP1 ncs3-1 (pRS316ADE8CLA4)</td>
<td>Mitchell and Sprague (2001)</td>
</tr>
<tr>
<td>SY3368</td>
<td>SY3357 except cla4Δ : TRP1 ncs4-1 (pRS316ADE8CLA4)</td>
<td>Mitchell and Sprague (2001)</td>
</tr>
<tr>
<td>SY3369</td>
<td>SY3357 except cla4Δ : TRP1 ncs5-1 (pRS316ADE8CLA4)</td>
<td>Mitchell and Sprague (2001)</td>
</tr>
<tr>
<td>SY3370</td>
<td>SY3357 except cla4Δ : TRP1 ncs6-1 (pRS316ADE8CLA4)</td>
<td>Mitchell and Sprague (2001)</td>
</tr>
<tr>
<td>SY3371</td>
<td>SY3357 except cla4Δ : TRP1 ncs7-1 (pRS316ADE8CLA4)</td>
<td>Mitchell and Sprague (2001)</td>
</tr>
<tr>
<td>SY3372</td>
<td>SY3357 except cla4Δ : TRP1 ncs8-1 (pRS316ADE8CLA4)</td>
<td>Mitchell and Sprague (2001)</td>
</tr>
<tr>
<td>SY3373</td>
<td>SY3357 except cla4Δ : TRP1 ncs10-1 (pRS316ADE8CLA4)</td>
<td>Mitchell and Sprague (2001)</td>
</tr>
<tr>
<td>SY3380</td>
<td>SY3357 except cla4Δ : TRP1 (YCPHIS3cla4-75)</td>
<td>Mitchell and Sprague (2001)</td>
</tr>
<tr>
<td>SY3403</td>
<td>SY3357 except cla4Δ : TRP1 swe1Δ : LEU2 ncs1Δ : HIS3 (pRS316ADE8CLA4)</td>
<td>Mitchell and Sprague (2001)</td>
</tr>
<tr>
<td>SY3756</td>
<td>SY3357 except cla4Δ : TRP1 bni1Δ : HIS3 (pRS316ADE8CLA4)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3757</td>
<td>SY3357 except cla4Δ : TRP1 bud6Δ : HIS3 (pRS316ADE8CLA4)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3758</td>
<td>SY3357 except cla4Δ : TRP1 spa2Δ : ura3 (pRS316ADE8CLA4)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3759</td>
<td>SY3357 except cla4Δ : TRP1 por2Δ : ura3 (pRS316ADE8CLA4)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3760</td>
<td>SY3357 except cla4Δ : TRP1 ncs1Δ : LEU2 (YCPHIS3cla4-75)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3761</td>
<td>SY3357 except cla4Δ : TRP1 bud6Δ : URA3 (YCPHIS3cla4-75)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3762</td>
<td>SY3357 except cla4Δ : TRP1 spa2Δ : URA3 (YCPHIS3cla4-75)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3763</td>
<td>SY3357 except cla4Δ : TRP1 por2Δ : URA3 (YCPHIS3cla4-75)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3764</td>
<td>SY3357 except cla4Δ : TRP1 ste20Δ : URA3 (YCPHIS3cla4-75)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3766</td>
<td>SY3357 except cla4Δ : TRP1 swe1Δ : LEU2 (pRS316ADE8CLA4)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3767</td>
<td>SY3357 except cla4Δ : TRP1 ste20Δ : TRP1 swe1Δ : LEU2 (pRS316ADE8CLA4)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3768</td>
<td>SY3357 except cla4Δ : TRP1 bni1Δ : ura3 swe1Δ : LEU2 (pRS316ADE8CLA4)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3769</td>
<td>SY3357 except cla4Δ : TRP1 bud6Δ : his3 swe1Δ : LEU2 (pRS316ADE8CLA4)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3770</td>
<td>SY3357 except cla4Δ : TRP1 spa2Δ : ura3 swe1Δ : LEU2 (pRS316ADE8CLA4)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3771</td>
<td>SY3357 except cla4Δ : TRP1 por2Δ : ura3 swe1Δ : LEU2 (pRS316ADE8CLA4)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3772</td>
<td>SY3357 except cla4Δ : TRP1 swe1Δ : LEU2 (YCPHIS3cla4-75)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3773</td>
<td>SY3357 except cla4Δ : TRP1 ste20Δ : URA3 swe1Δ : LEU2 (YCPHIS3cla4-75)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3774</td>
<td>SY3357 except cla4Δ : TRP1 ncs1Δ : LEU2 (YCPHIS3cla4-75)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3775</td>
<td>SY3357 except cla4Δ : TRP1 bud6Δ : ura3 swe1Δ : LEU2 (YCPHIS3cla4-75)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3776</td>
<td>SY3357 except cla4Δ : TRP1 spa2Δ : ura3 swe1Δ : LEU2 (YCPHIS3cla4-75)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3777</td>
<td>SY3357 except cla4Δ : TRP1 por2Δ : ura3 swe1Δ : LEU2 (YCPHIS3cla4-75)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3778</td>
<td>SY3357 except cla4Δ : TRP1 swe1Δ : LEU2 (YCPHIS3cla4-75)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3779</td>
<td>SY3357 except bni1Δ : HIS3 (pY39tet1HA-BNI1)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3780</td>
<td>SY3357 except ste20AΔ : TRP1 bni1Δ : HIS3 (pY39tet1HA-BNI1)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3781</td>
<td>SY3357 except cla4Δ : TRP1 ste20Δ : TRP1 bni1Δ : his3 (YCPHIS3cla4-75)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3782</td>
<td>SY3357 except cla4Δ : TRP1 ste20Δ : kanMX6 bud6Δ : ura3 (YCPHIS3cla4-75)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3783</td>
<td>SY3357 except cla4Δ : TRP1 ste20Δ : kanMX6 spa2Δ : ura3 (YCPHIS3cla4-75)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3784</td>
<td>SY3357 except cla4Δ : TRP1 ste20Δ : kanMX6 por2Δ : ura3 (YCPHIS3cla4-75)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3785</td>
<td>SY3357 except bni1-CT1Δ : kanMX6</td>
<td>This study</td>
</tr>
<tr>
<td>SY3786</td>
<td>SY3357 except cla4Δ : TRP1 bni1-CT1Δ : kanMX6 (pRS316ADE8CLA4)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3787</td>
<td>SY3357 except cla4Δ : TRP1 ste20Δ : TRP1 bni1-CT1Δ : kanMX6 (pRS316ADE8CLA4)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3788</td>
<td>SY3357 except cla4Δ : TRP1 ste20Δ : TRP1 bni1-CT1Δ : kanMX6 (YCPHIS3cla4-75)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3789</td>
<td>SY3357 except cla4Δ : TRP1 ste20Δ : TRP1 bni1-CT1Δ : kanMX6 (YCPHIS3cla4-75)</td>
<td>This study</td>
</tr>
<tr>
<td>SY3790</td>
<td>SY3357 except cla4Δ : TRP1 URA3 : cla4-75-td</td>
<td>This study</td>
</tr>
<tr>
<td>SY3791</td>
<td>SY3357 except cla4Δ : TRP1 bni1Δ : HIS3 URA3 : cla4-75-td</td>
<td>This study</td>
</tr>
<tr>
<td>SY3792</td>
<td>SY3357 except cla4Δ : TRP1 bud6Δ : ura3 URA3 : cla4-75-td</td>
<td>This study</td>
</tr>
<tr>
<td>SY3793</td>
<td>SY3357 except cla4Δ : TRP1 spa2Δ : ura3 URA3 : cla4-75-td</td>
<td>This study</td>
</tr>
<tr>
<td>SY3794</td>
<td>SY3357 except cla4Δ : TRP1 por2Δ : ura3 URA3 : cla4-75-td</td>
<td>This study</td>
</tr>
<tr>
<td>SY3795</td>
<td>SY3357 except cla4Δ : TRP1 swe1Δ : LEU2 : cla4-75-td</td>
<td>This study</td>
</tr>
<tr>
<td>DY759</td>
<td>cry1 ade2-101(αg) his3-11 leu2-3,112 ura3-1</td>
<td>Weiss et al. (2000)</td>
</tr>
<tr>
<td>DY2060</td>
<td>DY759 except cla4Δ : LEU2 ste20Δ : kanMX URA3 : cla4-75-td</td>
<td>Weiss et al. (2000)</td>
</tr>
<tr>
<td>Y2454</td>
<td>MATa mfa1Δ : MFA1pr-HIS3 can1Δ ura3Δ leu2Δ his3Δ1 lys2Δ</td>
<td>This study</td>
</tr>
<tr>
<td>Y2851</td>
<td>Y2454 except cla4Δ : URA3</td>
<td>This study</td>
</tr>
<tr>
<td>Y2928</td>
<td>Y2851 except cla4Δ : natR</td>
<td>This study</td>
</tr>
</tbody>
</table>

* All are derivatives S288C except DY759 and DY2060 (W303 derivatives).
sequenced and found to include BNII. NC58 was shown to be BNII by deletion and linkage analysis (see below). For BUD6, two clones complementing ncs5-1 were found among 8000 library transformants. An 8-kb fragment shared by both complementing plasmids was sequenced. Deletion and subcloning analysis identified BUD6 as the complementing gene. To isolate NC52, strain SY3366, which harbors an ncs2 mutation, was transformed with a high copy YEp13 based library (ATCC no. 37323), yielding six complementing clones in 6000 transformants. A 2-kb fragment containing two overlapping open reading frames (ORFs) shared by all complementing plasmids was sequenced. Deletion analysis identified YNL119w/YNL120c as the complementing ORF(s). For NC53 isolation, 7000 library transformants yielded six complementing clones from a high copy YEp13-based library. A 3.6-kb fragment containing three ORFs shared by all complementing plasmids was sequenced. Deletion and subcloning analysis identified UB4 as the complementing gene. Because ncs4 mutants had a strong mating defect, it seemed reasonable that members of this complementation group could contain mutations in STE20. Indeed, we found that STE20 on a plasmid complemented these mutants. In the case of NC56, transformation of SY3370 with yeast genomic library (ATCC no. 77162) yielded two complementing clones from 9000 transformants. A 6.8-kb fragment containing six ORFs shared by all complementing plasmids was sequenced. Deletion and subcloning analysis identified YGL211w as the ORF containing the complementing gene. In the case of NC510, 16,000 transformants of a yeast genomic library yielded 16 complementing clones. A 4.6-kb fragment containing three ORFs shared by all complementing plasmids was sequenced. Deletion and subcloning analysis identified ELP2 as the complementing gene.

Linkage analysis was performed to verify that the cloned genes represented wild-type versions of the mutant alleles. A HIS3 marker was introduced at the locus of interest in a diploid homozygous for the clα4 mutation and heterozygous for the NCS3 gene of interest. The strain carried plasmid-borne CLA4 so that segregation of the ncs mutation in tetrads could be scored. After sporulation of the marked strains, the Ncs+ phenotype cosegregated with the HIS3 marker in at least 22 tetrads.

**Microscopy**

Standard microscopic techniques were used, and cells were examined using an Axiosplan 2 fluorescence microscope (Carl Zeiss, Thornwood, NY) fitted with an Orca 100 digital camera (Hamamatsu, Bridgewater, NJ). Methods for staining with rhodamine-phalloidin (Molecular Probes, Eugene, OR) to visualize F-actin were described previously (Pringle et al., 1989). All assays were performed in triplicate. Indirect immunofluorescence was performed essentially as described previously (Pringle et al., 1989). All assays were performed in triplicate. Indirect immunofluorescence was performed essentially as described previously (Pringle et al., 1989). All assays were performed in triplicate. Indirect immunofluorescence was performed essentially as described previously (Pringle et al., 1989).

**RESULTS**

Identification of BNII and BUD6 by Synthetic Lethal Interactions with a clα4 Null Mutation

In an effort to identify activators and targets of Ste20p, we screened for mutations that are lethal in combination with a clα4 null mutation by using two independent methods. The first method used a random mutagenesis of the genome and identified synthetic lethal mutations via a red/white colony sectoring assay (Table 2). Such a screen can be expected to identify two classes of genes: 1) genes that encode upstream activators and downstream effectors of STE20, and 2) genes that share a function with CLA4 that is independent of STE20. Previously, we described the isolation of NCS1 (Needs CLA4 to Survive), which falls into the second class (Mitchell and Sprague, 2001). NCS1/RRD1 was unique among NCS genes in that ncs1Δ mutants were not defective for any known STE20 function. NCS1 is a phosphotyrosyl phosphatase activator that may share a function with CLA4 required at the G2/M phase transition (Mitchell and Sprague, 2001).
The screen also yielded complementation groups that exhibited some ste20Δ phenotypes; in many cases, a defect in haploid invasive growth and in some cases a partial defect in mating as well. As described in MATERIALS AND METHODS, molecular cloning identified the genes corresponding to these complementation groups (Table 2). In this study, we chose to concentrate on NCS8/BNI1, both because it is required for efficient mating and because a second complementation group, NCS5/BUD6, encodes a protein known to interact with Bni1p (Evangelista et al., 1997). Bni1p (Bud neck involved protein) is a formin homology protein that interacts with a large number of proteins and has many functions attributed to it. These functions include roles in bipolar bud site selection in diploids, cell polarity, cytokinesis, and spindle alignment during nuclear migration and may all stem from its role in actin cable assembly (Zahner et al., 1996; Lee et al., 1999; Miller et al., 1999; Sheu et al., 2000; Evangelista et al., 1999). Bni1p has been shown to interact by two-hybrid analysis (Evangelista et al., 1997). We also found that loss of Spa2p, another protein that interacts with Bni1p (Fujiwara et al., 1998), is essential in a cla4Δ mutant background. These three proteins, together with a fourth protein Pae2p, form a 12S complex termed the polarisome that has been suggested to promote polarized morphogenesis (Sheu et al., 1998; Pruyne and Bretscher, 2000). We found that Pae2p is likewise essential in a cla4Δ strain.

Table 2. NCS mutants

<table>
<thead>
<tr>
<th>Complementation group</th>
<th>Gene or ORF</th>
<th>Isolates</th>
<th>overcomes synthetic lethality</th>
<th>Invasive growth</th>
<th>Mating competency</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCS1</td>
<td>RRD1</td>
<td>1</td>
<td>Yes</td>
<td>No defect</td>
<td>WT</td>
</tr>
<tr>
<td>NCS2</td>
<td>YNL119w/YNL120c</td>
<td>6</td>
<td>Yes</td>
<td>Defect</td>
<td>WT</td>
</tr>
<tr>
<td>NCS3</td>
<td>UBA4</td>
<td>6</td>
<td>Yes</td>
<td>Defect</td>
<td>WT</td>
</tr>
<tr>
<td>NCS4</td>
<td>STE20</td>
<td>4</td>
<td>No</td>
<td>Defect</td>
<td>1000 fold lower than wild type</td>
</tr>
<tr>
<td>NCS5</td>
<td>BUD6</td>
<td>2</td>
<td>No</td>
<td>Defect</td>
<td>N/D</td>
</tr>
<tr>
<td>NCS6</td>
<td>YGL211w</td>
<td>3</td>
<td>N/D</td>
<td>Defect</td>
<td>N/D</td>
</tr>
<tr>
<td>NCS7</td>
<td>N/D</td>
<td>2</td>
<td>Yes</td>
<td>Defect</td>
<td>WT</td>
</tr>
<tr>
<td>NCS8</td>
<td>BNI1</td>
<td>5</td>
<td>No</td>
<td>Defect</td>
<td>~3-fold lower than wild type</td>
</tr>
<tr>
<td>NCS10</td>
<td>ELP2</td>
<td>2</td>
<td>Yes</td>
<td>Defect</td>
<td>WT</td>
</tr>
</tbody>
</table>

N/D, not determined; WT, wild type.

Polarisome Components Are Essential in a cla4Δ Background

As noted above, loss of either BNI1 or BUD6 is lethal in a cla4Δ strain. Each gene is involved in cell polarity establishment and in bipolar budding (Zahner et al., 1996; Evangelista et al., 1997; Sheu et al., 2000). Moreover, Bni1p and Bud6p have been shown to interact by two-hybrid analysis (Evangelista et al., 1997). We also found that loss of Spa2p, another protein that interacts with Bni1p (Fujiwara et al., 1998), is essential in a cla4Δ mutant background. These three proteins, together with a fourth protein Pae2p, form a 12S complex termed the polarisome that has been suggested to promote polarized morphogenesis (Sheu et al., 1998; Pruyne and Bretscher, 2000). We found that Pae2p is likewise essential in a cla4Δ strain.

To examine the terminal phenotype of cla4Δ mutants lacking polarisome function, we used a plasmid-borne thermosensitive allele of CLA4 (YCPHIS3cla4-75). A striking phenotype of ste20Δ cla4Δ YCPHIS3cla4-75 mutants is the mislocalization of the septin ring (Cvrckova et al., 1995). We therefore examined septin localization in bni1Δ cla4Δ YCPHIS3cla4-75 and in other polarisome cla4Δ double mutants. The septin phenotype of bni1Δ cla4Δ YCPHIS3cla4-75 mutants at the restrictive temperature resembled that of ste20Δ cla4Δ YCPHIS3cla4-75 mutants. The septin ring was formed at the proper time and location. However, as the bud began to grow, the septin ring frequently localized at the tip of the misshapened bud rather than remaining at the bud neck (Figure 2). These results imply that new growth is on the other side of the neck rather than the bud side. Similar phenotypes were observed with bni1Δ cla4Δ YCPHIS3cla4-75, spa2Δ cla4Δ YCPHIS3cla4-75, and pea2Δ cla4Δ YCPHIS3cla4-75 mutants (Figure 2). Other aspects of the polarisome cla4Δ double mutants will be discussed below.
note, however, that wild-type Cla4p and Cla4–75p are also degraded rapidly at 37°C (Figure 3B). The phenotype of bmi1Δ cla4Δ cla4–75-td mutants at the restrictive temperature recapitulated that of cells carrying YCpHIS3cla4–75: the septin ring was mislocalized to the tip of the bud (Figure 3A). Together, these results indicate that the polarisome is essential in the absence of Cla4p and further suggest that Ste20p may activate the polarisome.

Bni1p Is Phosphorylated In Vivo and Phosphorylation Is Partially Dependent on STE20

Because Bni1p and Ste20p colocalize to the bud tip in a Cdc42p-dependent manner (Peter et al., 1996; Leberer et al., 1997; Ozaki-Kuroda et al., 2001) and show similar genetic interactions with CLA4, it seemed plausible that Bni1p and Ste20p might physically interact and that this interaction would be necessary for the essential activity that Bni1p has in the absence Cla4p. To investigate this possibility, we created two-hybrid constructs of full-length, N-terminal, and C-terminal fusions of Ste20p and Bni1p but were unable to detect an interaction. We also failed to detect an interaction using communoprecipitation under a variety of assay conditions. Moreover, the proper localization of Bni1p or Ste20p to the bud tip did not require the presence of the other protein (our unpublished data).

Although we were unable to detect a physical interaction between Ste20p and Bni1p by using the methods described, we considered the possibility that the interaction is transient. In particular, because Ste20p is a protein kinase we asked whether Bni1p is a Ste20p-dependent phosphoprotein. A culture of cells expressing HA-tagged Bni1p was labeled with 32P. Bni1p was immunoprecipitated from the labeled extracts with the HA antibody, and radiolabeled proteins in the immune complexes were visualized by a PhosphorImager and subsequent immunoblot analysis. Bni1p was indeed a phosphoprotein (Figure 4). Moreover, in cells lacking Ste20p, the amount of phosphorylated Bni1p was twofold less than that found in wild-type cells, suggesting that a substantial portion of Bni1p phosphorylation is dependent on Ste20p in vivo (Figure 4).

Septin Ring Mislocalization Is Not the Cause of Lethality in a bmi1Δ cla4Δ Cells

Cells lacking CLA4 exhibit a bud morphology that suggests a defect in the apical-to-isotropic bud transition that occurs
in the G2 phase of the cell cycle. This phenotype is reminiscent of the phenotype conferred by misregulation of Cdc28p kinase activity (Lew and Reed, 1995a,b; McMillan et al., 1998; Richman et al., 1999). Indeed, we showed previously that deletion of SWE1, which encodes a protein kinase thought to be part of a morphogenetic checkpoint that negatively reg-

Figure 2. Morphological phenotypes of bni1Δ cla4Δ, spa2Δ cla4Δ, pea2Δ cla4Δ, and bud6Δ cla4Δ carrying YCpHIS3cla4-75. (A) Exponential cultures of haploid strains SY3380 (cla4Δ), SY3760 (bni1Δ cla4Δ), SY3761 (bud6Δ cla4Δ), SY3762 (spa2Δ cla4Δ), SY3763 (pea2Δ cla4Δ), and SY3764 (ste20Δ cla4Δ) carrying YCpHIS3cla4-75 were grown at 25°C in YEPD, shifted to 37°C for 4 h, fixed, and stained for Cdc3p. (B) Quantitation of Cdc3p mislocalization. For each strain, 250 cells were counted in three independent experiments. (C) Immunoblot analysis by using anti-Cla4p antibodies. Lysates of haploid strains SY3357 (WT; lane 1), SY3380 (cla4Δ; lane 2) SY3764 (ste20Δ cla4Δ; lane 3), SY3760 (bni1Δ cla4Δ; lane 4), and SY3761 (bud6Δ cla4Δ; lane 5) carrying YCpHIS3cla4-75 were analyzed by SDS-PAGE and immunoblot analysis by using affinity purified polyclonal anti-Cla4p antibodies (provided by D. Kellogg, Department of Molecular, Cellular, and Developmental Biology, University of California, Santa Cruz) and monoclonal antibodies to Dpm1p (to confirm equal protein loading). The band corresponding to wild-type Cla4p and Cla4-75p are indicated. All strains expressed similar amounts of Cla4-75p.
ulates Clb1,2p-Cdc28p activity, restores normal bud morphology in cla4Δ mutants (Longtine et al., 2000; Weiss et al., 2000; Mitchell and Sprague, 2001). By bypassing this morphogenetic checkpoint with swe1Δ, we were able to classify the NCS genes into two groups, an NCS1-related group and an STE20-related group. Loss of SWE1 restores normal bud morphology and overcomes the synthetic lethality of ncs1Δ cla4Δ YcPHIS3cla4Δ75 cells. In the case of ste20Δ cla4Δ YcPHIS3cla4Δ75 cells, however, loss of SWE1 restores the localization of the septin ring to the mother bud junction but does not restore viability (Mitchell and Sprague, 2001). To test whether BNI1 is in the NCS1 group or the STE20 group, we deleted SWE1 in bni1Δ cla4Δ YcPHIS3cla4Δ75 cells. The loss of SWE1 from bni1Δ cla4Δ YcPHIS3cla4Δ75 yielded a phenotype similar to that of swe1Δ ste20Δ cla4Δ YcPHIS3cla4Δ75 cells (Figure 5), suggesting that septin ring mislocalization is not the cause of lethality. Furthermore, this result suggests that BNI1 may facilitate or orchestrate some STE20 functions. Similar results were obtained when SWE1 was deleted from pa2Δ cla4Δ, spa2Δ cla4Δ, and bud6Δ cla4Δ cells carrying YcPHIS3cla4Δ75 (Figure 5). Moreover, these polarisome genes constitute a group distinct from other NCS genes based on their genetic interactions with SWE1 (Table 2).
Roles of Bni1p in Spindle Alignment during Nuclear Migration and Cytokinesis Are Not Essential in cla4Δ Cells

In addition to its roles in bipolar budding pattern and apical growth, Bni1p has a role in cytokinesis. Other proteins important for cytokinesis that function with Bni1p are Myo1p, Hof1p, and Bnr1p, a formin homology protein related to Bni1p (Bi et al., 2000). Based on synthetic lethal interactions, BNI1 and MYO1 are believed to be in one functional pathway, whereas HOF1 and BNR1 are in another. Loss of BNI1 is synthetically lethal with bni1Δ and with hof1Δ. However, none of these genes (except BNI1) is essential in a cla4Δ background (our unpublished data). Based on these results, the role of Bni1p in actomyosin contraction during cytokinesis is not essential in the absence of Cla4p.

Bni1p has also been shown to play a role in positioning the mitotic spindle during nuclear migration (Lee et al., 1999; Miller et al., 1999). We investigated whether the genetic interactions between BNI1 and CLA4 are related to the role of BNI1 in nuclear migration by looking for genetic interactions with KIP3. Kip3p is a kinesin-related protein hypothesized to function with Bni1p to organize and position the mitotic spindle. Loss of KIP3 was not synthetically lethal with cla4Δ (our unpublished data). Furthermore, the loss of NUM1, which encodes a protein that controls interaction of bud-neck cytoskeleton with the nucleus in G2 and also interacts with Bni1p, was not synthetically lethal with cla4Δ (our unpublished data).

Bni1p Has Roles Distinct from Spa2p, Pea2p, Bud6p, and Ste20p in cla4Δ Cells

To gain more insight into the role of Bni1p in cla4Δ cells, we examined the terminal phenotype of bni1Δ cla4Δ YCpHIS3cla4-75 mutants in more detail. With respect to the septin ring localization, the terminal phenotype of bni1Δ cla4Δ YCpHIS3cla4-75 cells was similar to ste20Δ cla4Δ YCpHIS3cla4-75 cells, but with respect to other phenotypes, the two phenotypes were distinct. bni1Δ cla4Δ YCpHIS3cla4-75 cells have both wider bud necks and defects in actin localization compared with ste20Δ cla4Δ YCpHIS3cla4-75 at the restrictive temperature (Figure 6). In particular, bni1Δ cla4Δ YCpHIS3cla4-75 mutants had no visible actin cables and only 6% of the cells had organized patches of actin at the tip of the bud. In contrast, cla4Δ single mutants had no observable defects in actin polarization and only 38% of bni1Δ single mutant cells had defects in actin polarization (Figure 6). Thus, it seems that Bni1p is more critical for actin organization in the absence of Cla4p than in wild-type cells, suggesting that Cla4p may also participate in actin organization but that its role in this process is functionally redundant with that of Bni1p.

As previously reported, actin polarization in ste20Δ cla4Δ YCpHIS3cla4-75 cells was indistinguishable from that in cla4Δ mutants, with actin cables traversing from mother to bud and actin patches localized toward the bud tip (Cvrckova et al., 1995). Likewise, spa2Δ cla4Δ and pea2Δ cla4Δ mutants carrying the YCpHIS3cla4-75 construct did not seem to have defects in actin organization compared with wild-type cells or ste20Δ cla4Δ YCpHIS3cla4-75 mutants (Figure 6). bni1Δ cla4Δ YCpHIS3cla4-75 mutants had some noticeable actin defects with fewer actin cables and polarized

**Figure 4.** Bni1p is a phosphoprotein. Cultures of SY3779 (bni1Δ) and SY3780 (bni1Δ ste20Δ) carrying pY39tet1HA-BNI1 and SY3778 (bni1Δ) were incubated with 1 mCi of 32PO4. Rabbit anti-HA antibody was used for immunoprecipitation and an anti-HA monoclonal antibody was used for immunoblot analysis. The relative amount of phospho-Bni1p, normalized to total Bni1p, is indicated below each lane.

**Not All Proteins Involved in Bipolar Budding Pattern Are Essential in cla4Δ Cells**

Recent studies have shown that some mutants defective for bipolar budding pattern selection also show a defect in apical growth and that lengthening the apical growth phase enhances the accuracy of bud site selection (Sheu et al., 2000). Ste20p, Bni1p, Pea2p, Spa2p, and Bud6p have been implicated in apical growth and also have roles in bipolar bud site selection (Snyder, 1989; Valtz and Herskowitz, 1996; Zahner et al., 1996; Evangelista et al., 1997; Sheu et al., 2000). To ascertain whether there is a specific connection between their roles in bipolar budding and the essential function they have in the absence of Cla4p, we looked for genetic interactions between CLA4 and other components of the bipolar bud site machinery. Loss of BUD8 was not synthetically lethal with cla4Δ (Figure 1 and Supplementary Table 1). In addition, the SGA screen showed that the loss of other genes that affect the budding pattern in diploids, such as BUD9, BUD14, BUD16-32 was not synthetically lethal with cla4Δ. These results suggest the roles of Bni1p, Spa2p, Pea2p, and Bud6p in bipolar budding pattern are not essential in the absence of Cla4p.
Figure 5. Loss of SWE1 in bni1Δ cla4Δ restores septin ring localization to the mother-bud junction. (A) Strains SY3766 (cla4Δ swe1Δ), SY3403 (ncs1Δ cla4Δ swe1Δ), SY3767 (ste20Δ cla4Δ swe1Δ), SY3768 (bni1Δ cla4Δ swe1Δ), SY3769 (spa2Δ cla4Δ swe1Δ), SY3770 (pea2Δ cla4Δ swe1Δ), and SY3771 (bud6Δ cla4Δ swe1Δ) carrying pRS316ADE8CLA4 were grown to midlog in YEPD at 30°C. A serial dilution (1/10) was performed starting with 10,000 cells. Cells were spotted onto either YEPD (left) or 5-FOA (right) and grown 3 days at 30°C. (B) Exponential cultures of haploid strains SY3772 (cla4Δ swe1Δ), SY3773 (ste20Δ cla4Δ swe1Δ), SY3774 (bni1Δ cla4Δ swe1Δ), SY3775 (spa2Δ cla4Δ swe1Δ), SY3776 (pea2Δ cla4Δ swe1Δ), and SY3777 (bud6Δ cla4Δ swe1Δ) carrying YCpHIS3cla4-75 were grown at 25°C in YEPD, shifted to 37°C for 4 h, fixed, and stained for Cdc3p. (C) Quantification of Cdc3p mislocalization. For each strain 250 cells were counted in three independent experiments.
actin patches, yet the defects were not as severe as those of
bni1Δ/cla4Δ mutants (Figure 6). Thus, deletion of polarisome genes in
cla4Δ cells leads to two broad phenotypic classes, one that includes
spa2Δ, pea2Δ, and bud6Δ, associated with a more severe terminal phenotype.

To further test for a functional relationship between Ste20p and the polarisome components, we examined the
terminal phenotype of triple mutants containing spa2Δ, pea2Δ, bud6Δ, or bni1Δ in combination with ste20Δ/cla4Δ
YCpHIS3cla4-75. The terminal phenotypes of spa2Δ, pea2Δ, and bud6Δ triple mutants were similar to that of the ste20Δ/cla4Δ YCpHIS3cla4-75 double mutant (Figure 7). Moreover, no synthetic growth defects were observed in strains where the polarisome genes were deleted in combination with ste20Δ. In contrast, the terminal morphology of the bni1Δ triple mutant was more severe than that associated with either the bni1Δ/cla4Δ YCpHIS3cla4-75 or the ste20Δ/cla4Δ YCpHIS3cla4-75 double mutants. The bni1Δ ste20Δ/cla4Δ YCpHIS3cla4-75 cells were large and unbudded with no visible actin cables and mostly unpolarized patches of actin.

Figure 6. bni1Δ/cla4Δ mutants have a severe actin polarization defect. (A) Strains SY3380 (cla4Δ), SY3778 (bni1Δ), SY3764 (ste20Δ/cla4Δ), SY3760 (bni1Δ/cla4Δ), SY3761 (bud6Δ/cla4Δ), SY3762 (spa2Δ/cla4Δ), and SY3763 (pea2Δ/cla4Δ) carrying YCpHIS3cla4-75 were grown at 25°C in YEPD, shifted to 37°C for 4 h, fixed, and stained for F-actin (by using rhodamine-phalloidin). (B) Quantitation of actin polarization. For each strain, 250 cells were counted in three independent experiments.
Figure 7. Terminal phenotype of \textit{bni1\Delta ste20\Delta cla4\Delta}. Exponential cultures of SY3781 (\textit{bni1\Delta cla4\Delta ste20\Delta}), SY3782 (\textit{bud6\Delta cla4\Delta ste20\Delta}), SY3783 (\textit{spa2A cla4\Delta ste20\Delta}), and SY3884 (\textit{pea2A cla4\Delta ste20\Delta}) carrying YCp\textit{HIS3cl4-75} were grown at 25°C in YEPD, shifted to 37°C for 4 h, fixed, and stained for F-actin (by using rhodamine-phalloidin) or for Cdc3p. (B) Quantitation of actin polarization. For each strain, 250 cells were counted in three independent experiments.

**DISCUSSION**

The related protein kinases Ste20p and Cla4p have unique activities, an inference made from the distinct phenotypes of strains lacking an individual kinase. However, a strain lacking both kinases is inviable, implying that there is a physiological connection between their activities. One possibility is that Cla4p and Ste20p share an essential activity. A less constrained interpretation is simply that Ste20p carries out a function that is essential in cells lacking Cla4p cells (and vice versa). In an effort to shed light on Ste20p function, we carried out two independent screens for mutations that are lethal in a \textit{cla4\Delta} background. This effort identified a
surprisingly large number of genes. Herein, we focus on a group of genes whose products are known to interact. Each of the proteins that form this group, Bni1p, Bud6p, Spa2p, and Pea2p, has been implicated in several different facets of cell biology. For example, Bni1p, a formin homology protein, has been implicated in actin polarization, cytokinesis, nuclear migration, and apical growth. Similarly, Bud6p, Spa2p, and Pea2p are involved in budding pattern determination and apical growth. The common denominator among the functions attributed to this set of proteins is involvement in apical growth, and we therefore suggest that proper regulation of this growth is the essential in the absence of Cla4p. We further suggest that the connection between these proteins and the Ste20p/Cla4p essential function is likely to be direct because Bni1p is a Ste20p-dependent phosphoprotein.

**Bni1p Has Roles Distinct from That of Spa2p, Pea2p, Bud6p, or Ste20p in cla4Δ Cells**

The lethality of bni1Δ cla4Δ strains may have more than one cause. As summarized above, we think one aspect of Bni1p function that is required in a cla4Δ mutant background is its participation in apical growth. This interpretation is supported by the finding that deletion of the Bni1p C-terminal 200 amino acids, the region of Bni1p that interacts with Bud6p, is lethal in a cla4Δ mutant background. Indeed, the terminal phenotype of bni1-CTΔ1 cla4Δ YCpHIS3cla4-75 is
similar to the *bud6Δ cla4Δ YCpHIS3cl4a-75* terminal phenotype. The C-terminal region is not only the Bud6p interaction domain on Bni1p, but it is also believed to be an autoinhibitory domain. In the case of other formin homology proteins, this autoinhibitory domain has been shown to interact with the Cdc42p binding domain on the same molecule (Alberts, 2001). Perhaps binding of activated Cdc42p releases the autoinhibitory domain and enables Bni1p to interact with Bud6p and the 12S complex. It will be interesting to determine whether the Ste20p-dependent phosphorylation of Bni1p influences interaction of it with Bud6p or other proteins.

Our results suggest that Bni1p has at least one other function that is important in a *cla4Δ* mutant background. This possibility emerges from the observation that *bni1Δ cla4Δ YCpHIS3cl4a-75* double mutants have additional phenotypes beyond those seen for the *ste20Δ cla4Δ, bni1-CTΔ1 cla4Δ YCpHIS3cl4a-75*, and *bud6Δ cla4Δ YCpHIS3cl4a-75* mutant strains. What is this additional important function? In addition to its role in apical growth, Bni1p also has roles in bud site selection, nuclear migration, cytokinesis, and actin polarization. Genetic tests, coupled with careful examination of the terminal phenotype of the *bni1Δ cla4Δ YCpHIS3cl4a-75* double mutant, point to actin polarization as the likely function. In particular, loss of Hof1p (required for cytokinesis), or Num1p (required for nuclear migration) is synthetically lethal with the absence of *Cla4p*. However, diminution of Cdc42p activity is lethal in a *cla4Δ* mutant background (Cvrckova et al., 1995). Cdc42p interacts with Bni1p and is required for polarization of the actin cytoskeleton. Moreover, *bni1Δ cla4Δ YCpHIS3cl4a-75* mutants contain very few actin cables, whereas *ste20Δ cla4Δ, bud6Δ cla4Δ, pea2Δ cla4Δ*, and *spa2Δ cla4Δ* mutants carrying YCpHIS3cl4a-75 contain abundant cables. Together, these results support the idea that a Bni1p role in actin polarization is critical in the absence of *Cla4p*.

**CLA4 Synthetic Lethal Universe**

The two screens for mutations that are synthetically lethal in a *cla4Δ* mutant background identified a large number of genes. The number of genes is large in absolute terms, but it is surprisingly large compared with the number of genes identified in a complementary synthetically lethal screen. Specifically, in a preliminary effort to identify mutations synthetically lethal with the absence of STE20 by using the colony-sectoring assay, only the *CLA4* gene was identified (Mitchell, Goehring, and Sprague, unpublished data). The functions identified in the *CLA4* synthetic screens reported herein cover a wide spectrum of cell biological processes and include bud emergence (BEM1, BEM2, BEM4), cytokinesis (SHS1), nuclear migration (DYN2, NIPI100, APC9, SLK19), and cell wall maintenance (GIM5, BCK1, CHS3, SKT5/CHS4, CHS5, CHS6, CHS7, FAB1, SLT2, SM11). Not all of the proteins, or even a majority of the proteins, involved in a particular process were identified. This finding implies that lethality does not result because an entire process has become essential in the *cla4Δ* mutant background, but rather implies that a particular activity or role of the protein has become essential.

It is often difficult to identify the targets of signaling proteins. Synthetic lethal screens for genes required in the absence of specific signaling molecules may provide a general means to identify potential downstream targets of the signaling molecule. In this study, we screened for genes that, like *STE20*, were synthetically lethal with *CLA4* and identified the polarisome as a potential target of Ste20p. By extension of this logic, potential downstream targets of Cla4p may be identified in synthetic lethal screens that use query mutations in *STE20* or any one of the other genes identified in the *CLA4* synthetic lethal universe. For example, because *BNII* is synthetic lethal with *CLA4*, the set of ~50 genes that are synthetically lethal with *BNII* (Tong et al., 2001), which includes genes involved in bud emergence, chitin synthase III activity, and the dynemin/dynactin spindle orientation pathway, may be Cla4p targets. Candidate targets are then identified by determining which single mutants exhibit phenotypes that resemble facets of the *cla4Δ* mutant phenotype. Thus, global synthetic lethal networks should be useful for large-scale mapping of functional relationships between signaling molecules and their downstream targets.

**ACKNOWLEDGMENTS**

We thank J. Pringle, I. Herskowitz, I. Pretorius, and D. Lew for providing plasmids. We also thank David Rivers, Hilary Kemp, Greg Smith, and Paul Cullen for helpful comments and suggestions. This work was supported by grant GM-30027 (to G.F.S.) from the National Institutes of Health, National Research Service Award GM-18002-03 (to D.A.M.), training grant 5 T32 GM-07759 (to A.S.G.) from the National Institutes of Health, Canadian Institute of Health Research (to C.B.), and operating grant from the National Cancer Institute of Canada (C.B.).

**REFERENCES**


Chem. 6, 7176–7186.
ity in yeast. Genes Dev. 11, 2972–2982.
associated proteins Gic1 and Gic2 are required for polarized cell
organisms volumes of the BioKnowledge library, an integrated
resource for protein information. Nucleic Acids Res. 29, 75–79.
Cvrcova, F., De Virgilio, C., Manser, E., Pringle, J.R., and Nasmuth,
K. (1995). Ste20-like protein kinases are required for normal local-
ization of cell growth and for cytokinesis in budding yeast. Genes
Dev. 9, 1817–1830.
84, 335–344.
Evangelista, M., Blundell, K., Longtine, M.S., Chow, C.J., Adams,
formin linking Cdc42p and the actin cytoskeleton during cytokine-
Evangelista, M., Klebl, B.M., Tong, A.H., Webb, B.A., Leeuw, T.,
role for myosin-I in actin assembly through interactions with Vrp1p,
Evangelista, M., Pruyne, D., Amberg, D.C., Boone, C., and Bretscher,
A. (2002). Formins direct Arp2/3-independent actin filament assem-
with the dynein intermediate chain Pac1p and cytoplasmic micro-
Fujiwara, T., Tanaka, K., Mino, A., Kikyo, M., Takahashi, K.,
implication in localization of Bni1p at the bud site and regulation of
the actin cytoskeleton in Saccharomyces cerevisiae. Mol. Biol. Cell 9,
1221–1233.
Gagliano, M., Van Dyk, D., Bauer, F.F., Lambrechts, M.G., and
Pretorius, I.S. (1999). Divergent regulation of the evolutionarily
closely related promoters of the Saccharomyces cerevisiae STA2 and
intact SH3 domain is required for myosin-I-induced actin poly-
erization. EMBO J. 19, 4281–4291.
Studies on the transformation of intact yeast cells by the LiAc/SS-
URA3MX cassettes for gene replacement in Saccharomyces cerevisiae.
Yeast 15, 507–511.
yeast vacuolar H+-ATPase occurs in the endoplasmic reticulum and
requires a Vma12p/Vma22p assembly complex. J. Cell Biol. 142,
39–49.
Gulli, M.P., Jaquenoud, M., Shimada, Y., Niederhauser, G., Wiget,
factor Cdc24 by the PAK-like kinase Cla4 may regulate polarized
 cortical protein Num1p is essential for dynein-dependent interac-
Hodges, P.E., McKeel, A.H., Davis, B.P., Payne, W.E., and Garrels,
J.I. (1999). The Yeast Proteome Database (YPD): a model for the or-
ganization and presentation of genome-wide functional data. Nucleic
Acids Res. 27, 69–73.
Holly, S.P., and Blumer, K.J. (1999). PAK-family kinases regulate cell
and actin polarization throughout the cell cycle of Saccharomyces
Cdc42, a Saccharomyces cerevisiae gene involved in the development
Kamei, T., Tanaka, K., Hihara, T., Umikawa, M., Imamura, H.,
a novel Src homology 3 domain-containing Hof1p. Implication in
28345.
approach for identifying yeast homologs of genes from other organ-
Leberer, E., Wu, C., Leeuw, T., Fourest-Lieuvin, A., Segall, J.E., and
yeast type I myosins in Cdc42-dependent actin polymerization.
Lee, L., Klee, S.K., Evangelista, M., Boone, C., and Pellman, D.
(1999). Control of mitotic spindle position by the Saccharomyces
Lew, D.J., and Reed, S.I. (1995b). Cell cycle control of morphogen-
cytoskeleton assembly during polarized cell growth in budding
Longtine, M.S., McKenzie, A., Demarini, D.J., Shah, N.G., Wach,
ules for versatile and economical PCR-based gene deletion and
Longtine, M.S., Theesfeld, C.L., McMillan, J.N., Weaver, E., Pringle,
J.R., and Lew, D.J. (2000). Septin-dependent assembly of a cell-
20, 4049–4061.
checkpoint monitors the actin cytoskeleton in yeast. J. Cell Biol. 142,
1487–1499.
Miller, R.K., Matheos, D., and Rose, M.D. (1999). The cortical local-
alization of the microtubule orientation protein, Kar9p, is dependent
upon actin and proteins required for polarization. J. Cell Biol. 144,
963–975.
phatase activator, Ncs1p (Rdc1p), functions with Cla4p to regulate
the G(2)/M transition in Saccharomyces cerevisiae. Mol. Cell. Biol. 21,
488–500.
Mosch, H.U., Kohler, T., and Braus, G.H. (2001). Different domains of
the essential GTPase Cdc42p required for growth and develop-


