Protein Phosphatase Type 1 Directs Chitin Synthesis at the Bud Neck in *Saccharomyces cerevisiae*

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Submitted February 7, 2008; Revised April 30, 2008; Accepted May 5, 2008
Monitoring Editor: Daniel Lew

Yeast chitin synthase III (CSIII) is targeted to the bud neck, where it is thought to be tethered by the septin-associated protein Bni4. Bni4 also associates with the yeast protein phosphatase (PP1) catalytic subunit, Glc7. To identify regions of Bni4 necessary for its targeting functions, we created a collection of 23 deletion mutants throughout the length of Bni4. Among the deletion variants that retain the ability to associate with the bud neck, only those deficient in Glc7 binding fail to target CSIII to the neck. A chimeric protein composed of the septin Cdc10 and the C-terminal Glc7-binding domain of Bni4 complements the defects of a *bni4Δ* mutant, indicating that the C-terminus of Bni4 is necessary and sufficient to target Glc7 and CSIII to the bud neck. A Cdc10-Glc7 chimera fails to target CSIII to the bud neck but is functional in the presence of the C-terminal Glc7-binding domain of Bni4. The conserved C-terminal PP1-binding domain of mammalian Phactr-1 can functionally substitute for the C-terminus of Bni4. These results suggest that the essential role of Bni4 is to target Glc7 to the neck and activate it toward substrates necessary for CSIII recruitment and synthesis of chitin at the bud neck.

INTRODUCTION

Vesicle targeting and fusion are key regulatory steps in many cellular processes including neurotransmitter release and platelet secretion. Although many of the proteins involved in the secretory pathway have been identified, the final stages of vesicle targeting and fusion are not yet fully understood. Targeting of the chitin synthase machinery in the yeast *Saccharomyces cerevisiae* has served as a model for studying targeted secretion. During vegetative growth, chitin is deposited first as a ring on the mother cell at the base of the incipient bud. This chitin ring will comprise the bud scar in the cell wall, which persists through successive cell divisions. Chitin is again deposited at cytokinesis in the primary septum (Cabib and Duran, 2005). The majority of chitin is synthesized by chitin synthase III (CSIII), one of three chitin synthases in yeast, whose catalytic subunit, Chs3, is targeted to the bud neck for the deposition of the chitin ring (Choi *et al.*, 1994; Chuang and Schekman, 1996; Cos *et al.*, 1998). Chs3 cycles between an endosomal compartment, termed the chitosome, and the cell surface where

it is activated upon association with its regulatory subunit Chs4/Skt5 (Bulawa, 1993; Trilla *et al.*, 1997). Many of the proteins involved in trafficking Chs3 to the plasma membrane have been identified, but it is still not known which proteins are involved in chitosome fusion to the membrane at the bud neck. The cellular distribution of Chs4 differs from Chs3 in that a greater fraction of Chs4 is membrane associated and Chs4 does not appear to be present in cytoplasmic punctae as observed with Chs3 (Reyes *et al.*, 2007). This suggests that Chs3 and Chs4 have different routes of intracellular trafficking to the cell surface. Both Chs3 and Chs4 localize to the presumptive bud site as a ring and remain at the neck of budding cells until buds are medium-sized, when they both dissociate from the neck. At cytokinesis, Chs3 and Chs4 reappear at the neck (Chuang and Schekman, 1996; DeMarini *et al.*, 1997; Santos and Snyder, 1997; Kozubowski *et al.*, 2003). Chs3 and Chs4 are codependent for their recruitment and/or retention at the neck of small-budded cells (DeMarini *et al.*, 1997), but it is not known whether Chs3 and Chs4 are delivered to the neck as a complex or first associate at the neck.

Targeted CSIII activity at the site of bud emergence also requires Bni4, a 100-kDa protein that has been proposed to act as a scaffold to tether Chs4 to septin filaments (DeMarini *et al.*, 1997). Like Chs3 and Chs4, Bni4 localizes as a ring to the presumptive bud site and remains asymmetrically restricted to the mother side of the bud neck. However, Bni4 remains at the bud neck throughout the cell cycle until just before cytokinesis, when its levels drop. *BNI4* null mutants fail to target CSIII to the neck early in the cell cycle, resulting in poorly defined bud scars (DeMarini *et al.*, 1997; Kozubowski *et al.*, 2003). Bni4 also binds to the protein phosphatase type 1 (PP1) catalytic subunit, Glc7. Yeast cells containing a mutation of a conserved PP1/Glc7-binding motif near the C-terminus of Bni4 (Bni4^{V831A/F833A}, hereafter

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E08-02-0130>) on May 14, 2008.

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Abbreviations used: CSIII, chitin synthase III; PP1, protein phosphatase type 1.

referred to as Bni4^{VA/FA}) do not recruit Glc7 or CSIII to the bud neck and exhibit chitin staining similar to *bni4Δ* mutants (Kozubowski *et al.*, 2003). Hyperphosphorylation of Bni4^{VA/FA} and its reduced abundance at the neck led to the proposal that Glc7 is necessary for regulating the association of Bni4 with the septin ring (Kozubowski *et al.*, 2003). However, a more direct role for the phosphatase in CSIII targeting has not been ruled out. Here, we present new data indicating a direct role for Glc7 in recruiting the chitin synthase machinery to the bud neck and propose that the role of Bni4 at the bud neck is not as a tether for CSIII, but rather to target Glc7 to the septin ring and activate it toward one or more substrates necessary to recruit active CSIII.

MATERIALS AND METHODS

Yeast Strains, Media, and General Methods

The yeast strains used in this work are listed in Supplementary Table 1 and are congeneric to KT1112 (*MATa ura3 leu2 his3*; Stuart *et al.*, 1994). Primers are listed in Supplementary Table 2. Yeast strains were grown on YPD medium (2% Bacto peptone, 1% yeast extract, 2% glucose) at 30°C, except where noted. Strains were sporulated at 24°C on medium containing 2% Bacto peptone, 1% yeast extract, and 2% potassium acetate. Synthetic complete medium and media lacking specific amino acids were prepared as described previously (Sherman *et al.*, 1986). Yeast transformation, manipulation of *Escherichia coli*, and the preparation of bacterial growth media were performed as described previously (Maniatis *et al.*, 1989; Kaiser *et al.*, 1994). PCR was used to generate gene deletion, 13-Myc, and fluorescent protein fusion strains. Each amplified cassette was introduced into a diploid strain, drug-resistant or amino acid-prototrophic transformants were sporulated, and haploid meiotic segregants were isolated by tetrad analysis. To generate *bni4Δ::NatMX4*, the deletion cassette was amplified using DNA from the *bni4Δ* strain from the Research Genetics panel (Open Biosystems, Huntsville, AL) with primers SP5-F and SP6-R. Nourseothricin (Nat)-resistant strains were then created by digesting pAG25 (Goldstein and McCusker, 1999) with EcoRI and transforming the Kan-resistant haploids. *CHS3-yEmCitrine* was generated with primers SP94-F and SP95-R, using pKT211 (Sheff and Thorn, 2004) as the template. Ectopically integrated *bni4^{VA/FA}* was described by Kozubowski *et al.* (2003). To create the new ectopically integrated green fluorescent protein (GFP)-tagged and untagged *BNI4* variants, pRS306-based plasmids (described below) were digested with StuI and introduced into JRL360. To create an integrated *GFP-CHS4* fusion, NheI-digested pJL68 (described below) was integrated into yeast. The *chs3Δ::HIS3* allele was derived from backcrosses of DDY181 (DeMarini *et al.*, 1997). Construction of *GLC7-tdimer2*, *GLC7-yEmCitrine*, and *GLC7-13myc* was described previously (Bharucha *et al.*, 2008). The 13Myc integration cassettes were amplified with reverse primer JB6-R and forward primers JB5-F, JB29-F, JB32-F, and JB33-F to generate wild-type *BNI4*-13Myc, *bni4Δ863-892*-13Myc, *bni4Δ873-892*-13Myc, and *bni4Δ882-892*-13Myc, respectively, using pFA6a-13Myc-HIS3MX6 (Longtine *et al.*, 1998) as the template. *bni4Δ863-892-GFP* and *bni4Δ873-892-GFP* were made by amplifying pLK3 (Kozubowski *et al.*, 2003) with reverse primer JB6-R and forward primers JB43-F and JB44-F, respectively. *CDC10-mCFP* was made by amplifying pKT210 (Sheff and Thorn, 2004) with primers JB80-F and CDC10-R.

Plasmid Construction and Site-directed Mutagenesis

Plasmids are listed in Table 1. Standard techniques were used for DNA manipulation (Maniatis *et al.*, 1989). Restriction and modification enzymes were used as recommended by the manufacturers (Promega, Madison, WI; Fermentas, Glen Burnie, MD; and New England Biolabs, Ipswich, MA). To create pJAS14, primers CAT78-F and CAT79-R were used to amplify *BNI4* with NcoI ends from KT1357 (*MATa leu2 his3 ura3 trp1*) (Blocher and Tatchell, 2000) genomic DNA. The *BNI4* fragment was inserted into pGEM-T according to the manufacturer's protocol (Promega, Madison, WI). pLK10 (Kozubowski *et al.*, 2003) was digested with Bpu10I and religated to remove a portion of the *KAN* cassette to create pJL2. The *BNI4* variants were generated using the QuikChange kit (Stratagene, La Jolla, CA) with pJAS14 as the template. These mutations were transferred into yeast transformation vectors (pLK10, pJL2, or p366; DeMarini *et al.*, 1997) by either homologous recombination in yeast or by conventional subcloning. Homologous recombination was performed by cotransforming yeast with mutagenized pJAS14 cut with NcoI and either AatII/NheI-digested pLK10, pJL2 (for GFP-tagging), or AatII/NheI-digested p366. The recombined plasmids were recovered from yeast with the Zymoprep Yeast Plasmid Miniprep kit from Zymo Research (Orange, CA). To transfer the *bni4* mutations to yeast transformation vectors by subcloning, mutagenized pJAS14 was digested with AatII/NheI or AatII/Bsp68I and ligated to AatII/NheI or AatII/Bsp68I cut pJL2 (for GFP-tagging) and p366. To create integrating vectors containing the mutagenized *bni4* sequence, the pJL2-, pLK10-, and p366-based plasmids were digested with

Table 1. Plasmids used in this study

Name	Description	Source
pCZ6	pGEM <i>CDC10</i> -(<i>GDGAGL</i>)- <i>GLC7</i>	This study
pCZ13	<i>GAL1 HA-CDC10</i> -(<i>GDGAGL</i>)- <i>GLC7 TRP1</i>	This study
pJAS14	pGEM <i>BNI4</i>	This study
pJL2	pRS315 <i>CEN LEU2 BNI4-GFP</i>	This study
pJL68	pRS306 <i>GFP-chs4Δ610-696 URA3</i>	This study
pJL149	pGEM <i>CDC10-bni4</i> (823-892)	This study
pJL151	<i>GAL1 HA-CDC10-bni4</i> (823-892) <i>TRP1</i>	This study
pJL168	pGEM <i>CDC10</i>	This study
pJL169	pGEM <i>CDC10-bni4</i> (823-892) <i>V831A/F833A</i>	This study
pJL170	<i>GAL1 HA-CDC10 TRP1</i>	This study
pJL171	<i>GAL1 HA-CDC10-bni4</i> (823-892) <i>V831A/F833A TRP1</i>	This study
pJL179	pGEM <i>CDC10-phactr-1</i> (501-580)	This study
pJL181	<i>GAL1 HA-CDC10-phactr-1</i> (501-580) <i>TRP1</i>	This study
pJL199	pGEM <i>bni4</i> (823-892)	This study
pJL200	<i>GAL1 HA-bni4</i> (823-892) <i>TRP1</i>	This study
pJL201	<i>GAL1 HA-bni4</i> (823-892) <i>LEU2</i>	This study
pJL202	pGEM <i>bni4</i> (823-892) <i>V831A/F833A</i>	This study
pJL203	<i>GAL1 HA-bni4</i> (823-892) <i>V831A/F833A TRP1</i>	This study
pJL204	<i>GAL1 HA-bni4</i> (823-892) <i>V831A/F833A LEU2</i>	This study
YCpIF16	<i>GAL1 HA TRP1</i> expression vector	Foreman and Davis (1994)

XhoI/SpeI and ligated into XhoI/SpeI cut pRS306 (Sikorski and Hieter, 1989). To create a *GFP-CHS4* integrating vector, EcoRI-digested pAR24 (Kozubowski *et al.*, 2003) was ligated into EcoRI cut pRS306 to create pJL68. *CDC10-BNI4*, *CDC10-bni4^{VA/FA}*, *CDC10*, *bni4*(823-892), and *bni4*(823-892) *V831A/F833A* Gal-inducible plasmids were made by nested PCR amplification of genomic DNA from KT1112 or JRL708. For *CDC10-BNI4* and *CDC10-bni4^{VA/FA}*, the first reactions used primer pairs SP121-F/SP116-R and SP117-F/SP122-R. The products were combined for the second PCR reaction with primers SP121-F and SP122-R. For *CDC10*, primers SP121-F and SP131-R were used in a one-step PCR reaction. For *CDC10-GLC7*, primers SP121-F/CZ4-R and CZ5-F/CZ6-R were used for the first PCR reaction with plasmid pKC980 (Karen Clemens and John Cannon, unpublished data) as the template. The two PCR products were combined and SP121-F and CZ6-R were used for the second PCR reaction. For *bni4*(823-892) and *bni4*(823-892) *V831A/F833A*, primers SP138-F and SP122-R were used in a one-step PCR reaction. For *CDC10-PHACTR-1*, p40c1 (kindly provided by Dr. Patrick Allen, Yale University) was used as a template with primers SP121-F/SP133-R and SP132-F/SP134-R for the first reaction, and primers SP121-F and SP134-R were used for the second reaction. The final PCR products were then ligated into pGEM-T according to the manufacturer's protocol (Promega, Madison, WI), creating pJL149 (*CDC10-BNI4*), pJL168 (*CDC10*), pJL169 (*CDC10-bni4^{VA/FA}*), pCZ6 (*CDC10-GLC7*), pJL179 (*CDC10-PHACTR-1*), pJL199 (*bni4*(823-892)), and pJL202 (*bni4*(823-892) *V831A/F833A*). The forward and reverse primers introduced a SacI site before the Start codon and a SpeI restriction site after the Stop codon. pJL151, pJL170, pJL171, pJL181, pJL200, and pJL203 were created by ligating SacI/SpeI fragments from pJL149, pJL168, pJL169, pCZ6, pJL179, pJL199, and pJL202, respectively, into YCpIF16 (Foreman and Davis, 1994). A fragment from pJL200 and pJL203 containing the *GAL1* promoter was ligated as a ApaI/SacI fragment into pUN105 (Elledge and Davis, 1988) to create pJL201 and pJL204, respectively.

Biochemical Procedures

To assess Bni4-GFP and hemagglutinin (HA)-tagged protein levels, total protein was prepared from cultures by lysis in TCA (Davis *et al.*, 1993; Stuart *et al.*, 1994). Proteins were electrophoresed on 8% (Figure 1) or 10% (Figures 3, 5, and 6) polyacrylamide-SDS gels. Immunoblot analysis was performed as described (Stuart *et al.*, 1994) using the 12CA5 anti-HA antibody or BD Living Colors A.v. monoclonal anti-GFP (JL-8) antibody, with subsequent detection

using the Enhanced Chemiluminescence System (Amersham ECL Plus, GE Healthcare, Chalfont St. Giles, United Kingdom). Hsfl expression was used as a loading control (antibody kindly provided by Dr. David Gross, LSUHSC, Shreveport).

To test for coimmunoprecipitation of Glc7-13myc and Bni4-GFP, cells were harvested and lysed as described in Kozubowski *et al.* (2003). Lysates were then subsequently used with the ProFound c-Myc Tag IP/CoIP Application Set according to the manufacturer's instructions (Pierce, Rockford, IL). After adding 2× sample buffer to the beads, 2 μ l β -mercaptoethanol was added, and beads were heated to 100°C for 5 min. Samples were eluted from the columns and electrophoresed on an 8% polyacrylamide-SDS gel. Immunoblot analysis was performed as described above using anti-GFP (JL-8) antibody or anti-Myc 9E10 ascites antibody with subsequent detection using the Enhanced Chemiluminescence System.

Microscopy

Cells were placed onto a pad of 2% agarose in synthetic medium containing 2% glucose or 2% galactose and imaged for GFP, CFP, YFP, and RFP (green, cyan, yellow, and red fluorescent protein, respectively) as previously described (Kozubowski *et al.*, 2003). Fluorescence images in different Z-axis planes (0.5 μ m apart) were acquired using Slidebook software (Olympus Imaging Systems, Melville, NY). Fluorescence levels were quantitated as previously described (Kozubowski *et al.*, 2003) using the average of four adjacent pixels and subtracting background fluorescence from the cytoplasm. Calcofluor staining was done as described previously (Robinson *et al.*, 1999). Images of Calcofluor staining are projections of 20 planes through the Z-axis.

RESULTS

Phenotypic Analysis of BNI4 Deletion Mutants

Bni4 in *S. cerevisiae* consists of 892 amino acids but contains only two recognizable domains or motifs; the C-terminal phosphatase-binding domain (aa residues 830-892) and a

putative coiled-coil domain (aa residues 106-133). To identify functional domains of Bni4, we constructed 23 mutant alleles of *BNI4* containing small, in-frame deletions (10–30 amino acids) in regions that are conserved between members of the *Saccharomyces sensu stricto* group. The underlying rationale for this approach is that mutations in regions of Bni4 that are evolutionarily conserved are more likely to disrupt specific functions of Bni4 than are random mutations. The average level of Bni4 sequence identity within the *S. sensu stricto* species is only 24%, but the regions we targeted are on average 55% identical. In addition, we targeted the coiled-coil domain for deletion (aa 106-135).

All mutant Bni4 alleles, summarized in Table 2 and diagrammed in Figure 1A, were integrated into the genome and expressed from the *BNI4* promoter. The mutant alleles were also integrated as GFP fusions to assay expression and localization. The C-terminal GFP-tag on Bni4 was previously shown not to disturb Bni4 function (Kozubowski *et al.*, 2003). Immunoblot analysis of whole cell extracts revealed that all mutant proteins are stably expressed, although levels varied. For example, Bni4 Δ 499-509 and Bni4 Δ 524-536 accumulate to relatively higher and lower levels, respectively (Figure 1B). We assayed Bni4 protein levels at the necks of small-budded cells by fluorescence microscopy. A summary of the results is shown in Figure 1C and the quantitation of Bni4-GFP levels in all variants is listed in Table 2. Most of the mutations either reduced or eliminated Bni4-GFP accumulation at the neck; only Bni4 Δ 499-509 associated with the

Table 2. Summary of Bni4 variant characterization

Amino acids deleted/mutated	Identity with <i>S. sensu stricto</i> group (%)	Relative levels at the bud neck ^b	Glc7 at the bud neck ^c	Chs4 levels at the bud neck	Phenotype with <i>bni1</i> Δ ^a	
					At 30°C	At 37°C
WT	24	1 $\pm$ 0.21	+	1 $\pm$ 0.23	+	+
<i>bni4</i> Δ	—	—	—	0.04 $\pm$ 0.08	—	—
Δ 10-27	56	0.16 $\pm$ 0.04	NA ^d	NA	+	+/-
Δ 28-43	56	0.23 $\pm$ 0.06	NA	NA	+	+/-
Δ 89-101	85	0.10 $\pm$ 0.04	+	0.62 $\pm$ 0.16	+	+
Δ 106-135	20	0.18 $\pm$ 0.06	+	0.79 $\pm$ 0.16	+	+/-
Δ 158-167	50	0.45 $\pm$ 0.11	NA	0.96 $\pm$ 0.18	+	+/-
Δ 182-191	60	0.42 $\pm$ 0.13	NA	NA	+	+/-
Δ 286-302	35	0.02 $\pm$ 0.01	—	0.10 $\pm$ 0.10	—	—
Δ 339-351	69	0.17 $\pm$ 0.04	NA	NA	+	+/-
Δ 420-432	31	0.35 $\pm$ 0.08	NA	NA	+	+
Δ 480-498	47	0.75 $\pm$ 0.17	NA	NA	+	+
Δ 499-509	73	1.57 $\pm$ 0.29	+	1.16 $\pm$ 0.31	+	+
Δ 510-522	38	0.50 $\pm$ 0.13	NA	NA	+	+
Δ 524-536	54	0.34 $\pm$ 0.12	NA	NA	+	+
Δ 537-549	77	0.64 $\pm$ 0.14	NA	NA	+	+
Δ 550-565	75	0.38 $\pm$ 0.08	NA	NA	+	+
Δ 599-618	20	0.42 $\pm$ 0.11	NA	NA	+	+
Δ 654-667	64	—0.03 $\pm$ 0.03	—	0.44 $\pm$ 0.14	—	—
Δ 749-762	43	0.46 $\pm$ 0.14	NA	NA	+	+
Δ 769-778	30	0.30 $\pm$ 0.09	NA	NA	+	+
Δ 782-795	71	0.53 $\pm$ 0.11	+	0.72 $\pm$ 0.19	+	+/-
V831A/F833A	—	0.06 $\pm$ 0.03	—	0.07 $\pm$ 0.08	—	—
Δ 863-892	70	0.14 $\pm$ 0.05	—	0.06 $\pm$ 0.07	—	—
Δ 873-892	60	0.17 $\pm$ 0.05	—	0.13 $\pm$ 0.06	—	—
Δ 882-892	73	NA ^b	+	0.41 $\pm$ 0.14	+/-	+/-

^a +, no growth defect; +/-, synthetic sick or slow growing; —, synthetic lethal.

^b Values are mean $\pm$ SD.

^c +, detectable at bud neck; —, absent from bud neck.

^d NA, not assayed.

Figure 1. Glc7 levels at the bud neck vary in *bni4* mutants. (A) Diagram of Bni4 mutants used in this study. Regions deleted are shown in blue. (B) Immunoblot of Bni4-GFP variants using α -GFP and α -HSF as a load control. (C) DIC and fluorescence microscopy analysis of representative Bni4-GFP variants. Strains YLK45 (Bni4-GFP), JRL389 (*bni4* Δ 89-101-GFP), JRL837 (*bni4* Δ 286-302-GFP), JRL356 (*bni4* Δ 499-509-GFP), JRL394 (*bni4* Δ 654-667-GFP), YLK84 (*bni4*^{VA/FA}-GFP), KT2652 (*bni4* Δ 863-892-GFP), and KT2650 (*bni4* Δ 873-892-GFP) were grown to midlog phase and imaged by fluorescence microscopy. Fluorescence levels at the bud neck in small-budded cells were quantitated and normalized to wild-type levels. $n > 55$. Error bars, SD. (D) DIC and fluorescence microscopy showing Glc7-mCitrine at the necks of small-budded cells in strains KT2422 (Bni4), JRL729 (*bni4* Δ 89-101), JRL722 (*bni4* Δ 654-667), JRL736 (*bni4*^{VA/FA}), KT2662 (*bni4* Δ 863-892), KT2660 (*bni4* Δ 873-892), KT2747 (*bni4* Δ 882-892), and JRL740 (*bni4* Δ). Bars, 3 μ m.

neck at higher levels. Increased expression of this variant could explain the increased levels at the neck. Two variants, Bni4 Δ 286-302 and Bni4 Δ 654-667, did not accumulate at the bud neck. Bni4^{VA/FA} was previously reported to accumulate at low levels at the bud neck (Kozubowski *et al.*, 2003). We confirmed this result and found that C-terminal truncations of the Glc7-binding domain (Bni4 Δ 863-892 and Bni4 Δ 873-892) also caused accumulation to low levels at the neck, consistent with the hypothesis that Glc7-binding is required for retention of Bni4 at the neck (Kozubowski *et al.*, 2003).

We next examined whether Glc7-mCitrine (a monomeric variant of YFP) is recruited to the bud neck in selected mutants. With the exception of mutants in the Glc7-binding domain, levels of Glc7-mCitrine at the neck roughly parallel Bni4 variant levels. For example, cells bearing Bni4 Δ 89-101 accumulate low levels of Bni4 at the neck and target low levels of Glc7-mCitrine to the neck (Figure 1D). Similarly, Bni4 Δ 654-667 fails to associate with the neck and cells bearing this mutant fail to target Glc7-mCitrine to the neck.

Glc7-mCitrine also was not observed at the neck in most of the Glc7-binding domain mutants (*bni4*^{VA/FA}, *bni4* Δ 863-892, or *bni4* Δ 873-892). The mutant with the smallest C-terminal truncation (*bni4* Δ 882-892) had a low but visible level of Glc7-mCitrine at the neck (Figure 1D).

We used several assays to assess the ability of the Bni4 variants to target CSIII to the bud neck. First, we assayed for rings of GFP-Chs4 and Chs3-mCitrine at the neck in the *bni4* mutants. Strains containing the integrated GFP-CHS4 fusion gene formed normal bud scars, indicating that the gene fusion is functional. Chs4 associates with the neck at cytokinesis independently of Bni4 (Kozubowski *et al.*, 2003; Sanz *et al.*, 2004); therefore we only quantitated GFP-Chs4 levels at the bud neck in small-budded cells. In general, GFP-Chs4 levels in the *bni4* mutants correlate with the ability of the Bni4 variants to target Glc7 to the bud neck (Figure 2A). GFP-Chs4 did not localize to the bud neck in *bni4* Δ , *bni4*^{VA/FA}, or *bni4* Δ 863-892 mutants. Levels of GFP-Chs4 were only slightly above background in *bni4* Δ 873-892 and *bni4* Δ 286-

Figure 2. CSIII recruitment to the bud neck correlates with ability of Bni4 to bind Glc7. (A) DIC and fluorescence microscopy analysis of GFP-Chs4 at the necks of small-budded cells in strains JRL605 (*BNI4*), JRL730 (*bni4Δ89-101*), JRL718 (*bni4Δ654-667*), JRL708 (*bni4VA/FA*), KT2649 (*bni4Δ863-892*), KT2647 (*bni4Δ873-892*), KT2645 (*bni4Δ882-892*), and JRL609 (*bni4Δ*). Graph shows relative values of GFP-Chs4 in the presence of the Bni4 variants compared with wild-type. $n > 24$. Asterisks denote cells late in the cell cycle, when Chs4 is targeted to the actomyosin ring independent of Bni4. Error bars, SD. Significantly different levels; ** $p < 0.001$, according to the unpaired Student's *t* test. (B) Coimmunoprecipitation of Glc7 with Bni4 variants. Strains JRL369 (*BNI4*-GFP), JB412-2D (*GLC7-13myc*), JRL789 (*BNI4*-GFP *GLC7-13myc*), JRL790 (*bni4Δ 89-101*-GFP *GLC7-13myc*), JRL787 (*bni4Δ 654-667*-GFP *GLC7-13myc*), JRL781 (*bni4VA/FA*-GFP *GLC7-13myc*), and JRL784 (*bni4Δ 863-892*-GFP *GLC7-13myc*) were used for immunoprecipitation with α -myc and then subjected to SDS-PAGE. Immunoblots were probed using α -GFP antibody or α -myc antibody. (C) DIC and fluorescence microscopy analysis of Chs3-mCitrine at the necks of small-budded cells in homozygous diploid strains JRL809/JRL810 (*BNI4*), JRL807/JRL808 (*bni4Δ89-101*), JRL813/JRL814 (*bni4Δ499-509*), JRL801/JRL802 (*bni4Δ654-667*), JRL803/JRL804 (*bni4VA/FA*), and JRL811/JRL812 (*bni4Δ*). (D) Calcofluor staining of cells showing chitin in the bud scar. Strains used are same as those in Figure 1D. Bars, 3 μ m.

302 mutants and were ~40% of wild-type levels in *bni4Δ882-892* mutants. Interestingly, the level of GFP-Chs4 at the bud neck in *bni4Δ89-101* mutant cells was 60% of that in wild-type cells, even though Bni4 Δ 89-101-GFP accumulated to the neck at low levels (~10%), similar to the levels of mutants in the Glc7-binding domain. This result suggests that the failure of Bni4^{VA/FA} and other C-terminal variants to target CSIII is not simply due to their failure to associate properly with the bud neck.

Surprisingly, GFP-Chs4 was also present at 40% of wild-type levels in *bni4Δ654-667* mutant cells, even though this variant does not localize to the bud neck (Figure 2A). This variant behaves opposite to *bni4VA/FA*, which accumulates at the bud neck, albeit at low levels, but does not recruit Chs4. One major difference between *bni4Δ654-667* and *bni4VA/FA* is that the former still contains the Glc7-binding region. To test if *bni4Δ654-667* can bind Glc7, we immunoprecipitated Glc7-13Myc and probed for the presence of Bni4. As shown in Figure 2B, Glc7-13Myc associates with Bni4-GFP, Bni4 Δ 89-101-GFP, and Bni4 Δ 654-667-GFP, but not Bni4^{VA/FA}-GFP, or Bni4 Δ 863-892-GFP. Thus, it is possible that Glc7 is targeted to the neck in the presence of Bni4 Δ 654-667-GFP at levels below our limit of detection and this complex may have some activity capable of directing GFP-Chs4 to the bud neck.

We next assayed whether the Bni4 variants can recruit Chs3-mCitrine to the bud neck. Most of the cellular Chs3 is in an endosomal compartment, the chitosome (reviewed in Bartnicki-Garcia, 2006), but a portion of the protein is targeted to the bud neck in a Bni4- and Chs4-dependent manner (Chuang and Schekman, 1996; DeMarini *et al.*, 1997). To better visualize the targeting of Chs3-mCitrine above the background of chitosomal Chs3, we examined Chs3-mCitrine in larger diploid cells. In wild-type, *bni4Δ89-101*, and *bni4Δ499-509* mutant cells, the Chs3-mCitrine ring is clearly observed above the background cytoplasmic fluorescence (Figure 2C). However, these rings are absent in *bni4Δ*, *bni4Δ654-667*, and *bni4VA/FA* mutant cells (Figure 2C). It is interesting that Bni4 Δ 654-667 targets some Chs4 but not Chs3 to the neck, a result not supported by the hypothesis that targeting of Chs4 leads to Chs3 targeting. We have no explanation for this observation, but it suggests to us the possibility that the Bni4-Glc7 complex plays a role in more than one step of the CSIII targeting process.

We also assayed whether the *bni4* mutants properly direct chitin synthesis at the bud neck by examining chitin bud scars by fluorescence microscopy (Figure 2D). As previously shown, *bni4VA/FA* and *bni4Δ* cells have diffuse

and irregular shaped bud scars that are difficult to detect by Calcofluor staining (DeMarini *et al.*, 1997; Kozubowski *et al.*, 2003). *bni4* Δ 654-667, *bni4* Δ 863-892, and *bni4* Δ 873-892 mutant cells show bud scars like those of a *bni4* Δ mutant. In contrast, *bni4* Δ 89-101 mutant cells exhibit wild-type bud scars. *bni4* Δ 882-892 mutants have bud scars intermediate between those of the wild type and those of a *bni4* Δ mutant.

We tested the *BNI4* mutants for synthetic genetic defects in combination with *bni1* Δ , *bni4* Δ , *chs4* Δ , and *chs3* Δ mutants are inviable in the absence of the formin protein Bni1 (Tong *et al.*, 2001), suggesting that *bni1* Δ mutants require targeted CSIII activity for viability. Because *bni4* Δ mutants retain near normal levels of CSIII activity and chitin but are specifically defective in targeting CSIII activity to the bud neck (Sanz *et al.*, 2004), the growth rate of *bni4 bni1* Δ mutants provides a sensitive indicator for Bni4 function. We therefore isolated the *bni4 bni1* Δ double mutants from diploid strains heterozygous for *bni1* Δ and each of our *bni4* mutants. A summary of the growth properties of these mutants is presented in Table 2. *bni1* Δ mutants in combination with *bni4* Δ 286-302, *bni4* Δ 654-667, *bni4* Δ 863-892, or *bni4* Δ 873-892 germinate but fail to grow into macroscopic colonies. All other double mutant pairs grow into colonies but some have noticeable growth defects. For example, *bni1* $\Delta bni4$ Δ 882-892 mutants are slow growing at 30° and 37°C, and other double

mutants grow slowly at 37°C (Table 2). Together, these results demonstrate a relationship between the ability of Bni4 to target Glc7 to the neck and CSIII targeting.

The Glc7-binding Domain of Bni4 Is Sufficient to Recruit CSIII to the Bud Neck

The above data indicate that the C-terminal Glc7-binding domain of Bni4 is essential for CSIII targeting. To test if this domain is sufficient, we constructed a protein fusion, hereafter referred to as Cdc10-Bni4, between the full-length Cdc10 septin and the 70 C-terminal amino acids of Bni4, which contain the Glc7-binding domain. As controls, we also constructed a Cdc10-Bni4 chimera that should not bind Glc7 (Cdc10-Bni4^{VA/FA}) and Cdc10 alone in the same vector. The three alleles were placed under the transcriptional control of a galactose-inducible promoter in a CEN shuttle vector and tagged with the HA epitope at the N-terminus. Immunoblot analysis revealed that all three proteins were expressed at similar levels after 2 h of galactose induction (Figure 3A). The Cdc10-Bni4^{VA/FA} fusion migrates slightly slower than does the Cdc10-Bni4 fusion, suggesting that the Bni4^{VA/FA} portion of the chimera may be hyperphosphorylated, as is the full-length Bni4^{VA/FA} protein (Kozubowski *et al.*, 2003). Cells expressing the *CDC10-BNI4* gene fusion have elongated buds and grow slowly on galactose medium (Figure 3, B and C). Cells expressing Cdc10 or Cdc10-Bni4^{VA/FA} show normal growth rates and normal morphology, indicat-

Figure 3. The C-terminus of Bni4 is necessary and sufficient to recruit Glc7 to the bud neck. (A) Immunoblot of Cdc10-Bni4 fusions after galactose induction. Glu, 2% glucose; Raf, 2% raffinose; 2, 2-h induction 2% galactose; 4, 4-h induction 2% galactose. Asterisks denote nonspecific band. Plasmids pJL170 (Cdc10), pJL151 (Cdc10-Bni4), and pJL171 (Cdc10-Bni4^{VA/FA}) were expressed in strain JRL43 (*bni4* Δ). (B) Colony morphologies of strains JRL873 (*bni4* Δ GFP-*CHS4*) and JRL912 (*chs3* $\Delta bni4$ Δ GFP-*CHS4*) expressing the same Cdc10-fusion plasmids as in Figure 3A or YCpIF16 (empty vector), after 18 h on 2% galactose. Bar, 50 μ m. (C) Growth assays using serial dilutions of strains expressing the Cdc10 fusions described in Figure 3B on selective media containing 2% glucose or 2% galactose. Plates were incubated 72 h at 30°C. (D) Growth assays using serial dilutions on selective media containing 2% glucose or 1.95% sucrose + 0.05% galactose at 24 or 37°C after 72 h. Plasmids described in Figure 3B were expressed in strain KT2328 (*MAT α leu2 his3 trp1 ura3 cdc10-1*). (E) DIC and fluorescence microscopy showing Glc7-timer2 targeting by the Cdc10 fusions. Plasmids described in Figure 3B were introduced into KT2737 (*bni4* Δ *GLC7-timer2* *CDC10-mCFP*). Protein expression was induced by growth in 2% galactose for 1.5 h. Bar, 3 μ m.

sucrose + 0.05% galactose at 24 or 37°C after 72 h. Plasmids described in Figure 3B were expressed in strain KT2328 (*MAT α leu2 his3 trp1 ura3 cdc10-1*). (E) DIC and fluorescence microscopy showing Glc7-timer2 targeting by the Cdc10 fusions. Plasmids described in Figure 3B were introduced into KT2737 (*bni4* Δ *GLC7-timer2* *CDC10-mCFP*). Protein expression was induced by growth in 2% galactose for 1.5 h. Bar, 3 μ m.

ing that the growth and morphological defects induced by *CDC10-BNI4* expression are likely due to Glc7 at the bud neck. The *CDC10* and *CDC10-BNI4^{VA/FA}* chimeras complement the temperature sensitivity of the *cdc10-1* mutant (Figure 3D), indicating these fusion proteins likely associate with the septin ring. The elongated bud phenotype also appears unrelated to aberrant chitin synthesis, as cells lacking *CHS3* and expressing the *CDC10-BNI4* fusion still become elongated (Figure 3B).

We induced the Cdc10 fusions in a *GLC7-tdimer2 CDC10-mCFP bni4Δ* strain to confirm the ability of Cdc10-Bni4 to target Glc7 to the septin ring. Glc7-tdimer2 rather than Glc7-mCitrine was used for these experiments because the former fusion protein accumulates at lower levels in the nucleus (Bharucha *et al.*, 2008), where high fluorescence can obscure the signal at the bud neck. As predicted, Cdc10-Bni4^{VA/FA} is unable to target Glc7 to the neck (Figure 3E). However, Glc7-tdimer2 is targeted to the bud neck by Cdc10-Bni4 (Figure 3E). In cells expressing Cdc10-Bni4, Glc7-tdimer2 was observed on both sides of the bud neck and at all stages of the cell cycle. This is in contrast to the wild-type situation, where Glc7 is restricted to the mother side of the neck. These results confirm that the last 70 amino acids of Bni4 are sufficient to bind Glc7.

We next tested the ability of the Cdc10-Bni4 chimera to recruit a functional CSIII complex to the bud neck. Induction of the Cdc10-Bni4 chimera, but not Cdc10-Bni4^{VA/FA}, results in localization of GFP-Chs4 (Figure 4A) and Chs3-mCitrine (Figure 4B) to the neck of small-budded cells. The recruitment of GFP-Chs4 is abolished in a *chs3Δ* mutant (Figure 4A), indicating that Chs4 targeting in *CDC10-BNI4* cells has the same requirement for Chs3 as in wild-type cells (DeMarini *et al.*, 1997). Interestingly, the temporal dynamics of GFP-Chs4 localization appeared similar in *BNI4* and *CDC10-BNI4* expressing cells. GFP-Chs4 normally appears at the neck at bud emergence and disappears before the disappearance of Bni4 (Kozubowski

et al., 2003). GFP-Chs4 reappears at cytokinesis independent of Bni4 function (Kozubowski *et al.*, 2003; Sanz *et al.*, 2004). In *CDC10-BNI4* cells, GFP-Chs4 is present in small-budded cells and at cytokinesis but was absent from the neck in medium-budded cells (Figure 4C). Thus, Cdc10-Bni4 can target Chs4 to the neck but Chs4 disappearance is regulated independently of Cdc10-Bni4 and Glc7. Calcofluor staining of cells expressing Cdc10-Bni4 revealed normal chitin rings (Figure 4D), indicating that the Cdc10-Bni4 fusion is capable of targeting functional CSIII to the neck.

A Bni4-Glc7 Complex Is Necessary for Proper Chitin Deposition

The above data show that the Glc7-binding domain of Bni4 is necessary and sufficient to target CSIII to the bud neck. To determine if the role of Bni4 is simply to target Glc7 to the neck, we constructed a chimera between Cdc10 and full-length Glc7 (Cdc10-Glc7) and assayed for its ability to target CSIII to the neck. This protein was expressed after 2 h galactose induction at levels similar to that of the Cdc10-Bni4 fusion (Figure 5A). Induction of Cdc10-Glc7 on galactose results in somewhat elongated cells (Figure 5B) and a slightly reduced growth rate (Figure 5C). To test if this chimera is capable of recruiting active CSIII to the bud neck, we induced the Cdc10-Glc7 fusion in a *GFP-CHS4 bni4Δ* strain. Unlike Cdc10-Bni4, the Cdc10-Glc7 fusion does not recruit GFP-Chs4 to the bud neck (Figure 5E, left panel), and Cdc10-Glc7 cells do not synthesize normal bud scars (Figure 5F, left panel). Cdc10-Glc7 recruits another Glc7-binding protein (Sds22-mCitrine) to the neck (data not shown), indicating that the fusion protein localizes to the neck and retains at least some of the normal activity of Glc7.

The above data indicate that simply tethering Glc7 to the neck is not sufficient to recruit CSIII. One explanation for this is that Bni4 may be necessary to alter the specificity of

Figure 4. CSIII targeting by Cdc10-Bni4. (A) DIC and fluorescence microscopy showing GFP-Chs4 targeting to the bud necks of small-budded cells by the Cdc10 fusions. Protein expression was induced by growth in 2% galactose for 1.5 h. Strains and plasmids described in Figure 3B were used. (B) DIC and fluorescence microscopy of Chs3-mCitrine in strains expressing Cdc10-Bni4. Plasmids described in Figure 3B were expressed in strain JRL866 (*bni4Δ CHS3-mCitrine*) by growing cells 2 h in 2% galactose. (C) Time-lapse microscopy of GFP-Chs4 in strain JRL873 (*bni4Δ GFP-CHS4*) containing plasmid

pJL151 (Cdc10-Bni4). Cells were grown in 2% galactose for 2 h before collecting images every 10 min. (D) Calcofluor staining of strains described in Figure 3B. Cells were grown 18 h in 1.95% raffinose + 0.05% galactose and stained with 0.05% Calcofluor in 1× PBS. Bars, 3 μ m.

Figure 5. A Bni4/Glc7 complex is necessary for targeting functional CSIII. (A) Immunoblot analysis of the CDC10-Glc7 chimeric protein after galactose induction. Plasmids pJL170 (Cdc10), pJL151 (Cdc10-Bni4), and pCZ13 (Cdc10-Glc7) were expressed in strain JRL873 (*bni4Δ GFP-CHS4*). Glu, 2% glucose; Raf, 2% raffinose; 2, 2 h induction 2% galactose; 4, 4 h induction 2% galactose. Asterisks denote nonspecific band. (B) Morphology of yeast colonies of strain JRL873 (*bni4Δ GFP-CHS4*) expressing the same Cdc10-fusion plasmids as in Figure 5A after 18 h on 2% galactose. Bar, 50 μ m. (C) Growth assays using serial dilutions of strains described in Figure 5B on selective media containing 2% glucose or 2% galactose. Plates were incubated 72 h at 30°C. (D) DIC and fluorescence microscopy showing Bni4 Δ 286-302-GFP targeting to bud necks of small-budded cells by the Cdc10-Glc7 construct. Plasmids pJL170 (Cdc10) and pCZ13 (Cdc10-Glc7) were expressed in JRL981 (*bni4Δ286-302-GFP*) by growing yeast 2 h in 2% galactose. Bar, 3 μ m. (E) DIC and fluorescence microscopy showing GFP-Chs4 targeting to bud necks of small-budded cells by Cdc10-Glc7 only in the presence of Bni4 Δ 286-302. Cells were grown 2 h in 2% galactose. Strains JRL873 (*bni4Δ GFP-CHS4*) and JRL983 (*bni4Δ286-302 GFP-CHS4*) were transformed with plasmids described in Figure 5A. Asterisk denotes cell late in cell cycle, when Chs4 is targeted to the actomyosin ring independent of Bni4. Bar, 3 μ m. (F) Calcofluor staining of cells described in Figure 5E. Cells were grown 18 h in 1.95% raffinose + 0.05% galactose and stained with 0.05% Calcofluor in 1 $\times$ PBS. Bar, 3 μ m. (G) DIC and fluorescence microscopy showing GFP-Chs4 targeting to bud necks of small-budded cells by Cdc10-Glc7 coexpressed with the C-terminus of WT Bni4. Plasmid pCZ13 (CDC10-GLC7) was expressed alone or in combination with pJL201 (*bni4(823-892)*) or pJL204 (*bni4(823-892 V831A/F833A)*) in strain JRL873 (*bni4Δ GFP-CHS4*). Cells were grown 2 h in 2% galactose. Asterisk denotes cell late in cell cycle when Chs4 is targeted to

Figure 6. The C-terminus of mammalian Phactr-1 can functionally substitute for the C-terminus of Bni4. (A) Clustal W alignment of the C-terminus of Bni4 with the C-termini of the four rat Phactr/Scapinin proteins. The RVXF PP1/Glc7 binding motif is underlined. (B) Immunoblot analysis of the CDC10-Phactr-1 chimeric protein after galactose induction. Plasmids pJL170 (Cdc10), pJL151 (Cdc10-Bni4), and pJL181 (Cdc10-Phactr-1) were expressed in strain JRL43 (*bni4Δ*). Glu, 2% glucose; Raf, 2% raffinose; 2, 2 h induction 2% galactose; 4, 4 h induction 2% galactose. Asterisks denote nonspecific band. HA-Phactr-1 has similar mobility to that of the nonspecific band. (C) Morphology of yeast colonies of strain JRL873 (*bni4Δ GFP-CHS4*) expressing the same Cdc10-fusion plasmids as in Figure 6B or YCpIF16 (empty vector) after 18 h on 2% galactose. Bar, 50 μ m. (D) Growth assays using serial dilutions of strains described in Figure 6C on selective media containing 2% glucose or 2% galactose. Plates were incubated 72 h at 30°C. (E) DIC and fluorescence microscopy showing Glc7-timer2 targeting to bud necks of small-budded cells by the Cdc10-Bni4 and Cdc10-Phactr-1 proteins. Plasmids described in Figure 6B were expressed in KT2737 (*bni4Δ GLC7-timer2 CDC10-mCFP*) by growing yeast 2 h in 2% galactose. Bar, 3 μ m. (F) DIC and fluorescence microscopy showing GFP-Chs4 targeting to bud necks of small-budded cells by the Cdc10-Bni4 and Cdc10-Phactr-1 proteins. Cells were grown 2 h in 2% galactose. Strains and plasmids described in Figure 6C were used. Bar, 3 μ m. (G) Calcofluor staining of cells described in Figure 6C. Cells were grown 18 h in 1.95% raffinose + 0.05% galactose and stained with 0.05% Calcofluor in 1 $\times$ PBS. Bar, 3 μ m.

Glc7 toward its relevant substrates at the bud neck to allow CSIII recruitment. To test this, we first assayed the ability of Cdc10-Glc7 to recruit CSIII in a *bni4Δ286-302* mutant. The Bni4 Δ 286-302 variant does not localize to the bud neck and does not recruit GFP-Chs4 to the bud neck (Figure 1C and Table 2), but it retains the Glc7-binding domain. If the Bni4-Glc7 complex is required for CSIII recruitment, our hypothesis is that the Cdc10-Glc7 will bind Bni4 Δ 286-302, forming a Glc7 holoenzyme able to target CSIII to the neck. As shown in Figure 5D, the induction of Cdc10-Glc7 induces Bni4 Δ 286-302-GFP localization to the bud neck. As expected, Bni4 Δ 286-302-GFP is located on both sides of the bud neck throughout the cell cycle, corresponding to the location of Cdc10-Glc7. Induction of Cdc10-Glc7 in *bni4Δ286-302 GFP-CHS4* cells results in GFP-Chs4 localization to the bud neck (Figure 5E) and visible chitin bud scars (Figure 5F).

To determine if expression of the C-terminus of Bni4 alone could induce Cdc10-Glc7 to recruit CSIII to the neck, we expressed the C-terminus of Bni4 (aa 823–892) with an N-terminal HA epitope tag from a galactose-inducible promoter. Expression of this 12.5-kDa protein alone failed to target CSIII activity to the neck (data not shown). However, coexpression of Bni4(832–892) and Cdc10-Glc7 resulted in the targeting of GFP-Chs4 (Figure 5G) and Chs3-mCitrine (Figure 5H) to small-budded cells and the formation of normal bud scars (Figure 5I). Coexpression of Cdc10-Glc7 with the VA/FA variant of Bni4(832–892) failed to target CSIII components to the neck (Figure 5, G–I). Together, these results suggest that Bni4 not only recruits Glc7 to the neck but also alters its specificity toward those substrate(s) at the bud neck required for proper chitin deposition.

The C-Terminus of Mammalian Phactr-1 Can Functionally Replace Bni4 at the Bud Neck

The recently characterized mammalian Phactr/Scapinin family of proteins (Sagara *et al.*, 2003; Allen *et al.*, 2004) contains a C-terminal PP1-binding domain that is 32% identical to the Glc7-binding domain of Bni4 (Figure 6A) but shares little, if any, similarity with the rest of Bni4. Proteins containing this C-terminal domain are found widely in eu-

Figure 5 (cont). the actomyosin ring independent of Bni4. Bar, 3 μ m. (H) DIC and fluorescence microscopy of Chs3-mCitrine. Plasmids described in Figure 5G were expressed in strain JRL866 (*bni4Δ CHS3-mCitrine*) by growing cells 2 h in 2% galactose. Bar, 3 μ m. (I) Calcofluor staining of strains described in Figure 5G or with plasmid pJL151 (*CDC10-BNI4*). Cells were grown 18 h in 1.95% raffinose + 0.05% galactose and stained with 0.05% Calcofluor in 1 $\times$ PBS. Bar, 3 μ m.

karyotes (Sagara *et al.*, 2003) and in the yeast proteins Afr1 and Yer158c (DeMattei *et al.*, 2000). This domain in Afr1 is necessary to target Glc7 to mating projections and for their normal morphological development (J. Bharucha, J. R. Larson, J. B. Konopka, and K. Tatchell, unpublished data). Phactr (phosphatase and actin regulator) proteins bind actin and PP1 and are expressed most abundantly in the brain. Although not much is known about the physiological roles of the Phactr proteins, a mutation in the PP1-binding domain of Phactr4 is responsible for the serious defects in the early development of the CNS in the *humdy* mouse mutant (Kim *et al.*, 2007). To determine if the PP1-binding domain of Phactr/Scapinin is functionally conserved, we created a fusion protein containing the C-terminal PP1-binding region of rat Phactr-1 (amino acids 501–580) and Cdc10 (Cdc10-Phactr-1). The Cdc10-Phactr-1 fusion protein is expressed at levels similar to those of the other Cdc10 fusions (Figure 6B). Induction of Cdc10-Phactr-1 on galactose results in a greater reduction in growth rate than Cdc10-Bni4 (Figure 6D), but for unknown reasons, it has a relatively minor effect on morphology (Figure 6C). As with Cdc10-Bni4, cells expressing Cdc10-Phactr-1 accumulate Glc7-timer2 and GFP-Chs4 at the bud neck and show normal bud scars on their surfaces (Figure 6, E–G). Thus, the C-terminus of mammalian Phactr-1 can functionally substitute for Bni4 to recruit CSIII and Glc7 to the bud neck.

DISCUSSION

Bni4 was originally described as a scaffold to tether Chs4 to the septins at the neck (DeMarini *et al.*, 1997). Although Glc7 binds directly to Bni4 and localizes to the bud neck in a Bni4-dependent manner (Kozubowski *et al.*, 2003), the role of Glc7 at the neck has not been defined. To examine the function of Bni4, we assayed a collection of Bni4 deletion variants for association with the bud neck and for their ability to target Glc7 and CSIII. Mutant alleles with severe defects in CSIII targeting were limited to two classes: those whose gene products fail to associate with the neck, and presumably septin filaments, and those located at the 3' end of the gene that fail to target Glc7 to the neck. Importantly, no mutants were identified that were specifically defective in targeting Chs4 to the neck, a class of mutant predicted by the current targeting model. Furthermore, most deletion variants near the N-terminus accumulate at the neck at reduced levels similar to that of the Bni4^{VA/FA} variant, yet retain biological activity.

The surprising conclusion of our work is that the C-terminal 70-amino acid Glc7-binding domain of Bni4 is sufficient to target CSIII to the bud neck. What then is the role of the prior 822 amino acids? The most obvious possibility is that the remainder of the protein assures the proper temporal and spatial association of Bni4 with septin neck filaments. Bni4 binds asymmetrically to the septin ring before bud emergence and then only to the mother side of the neck during budding (Kozubowski *et al.*, 2005). Levels of Bni4 at the neck decline as the bud grows and at cytokinesis, little Bni4 is normally associated with the septin rings. Another possibility is that Bni4 has activities in addition to regulating CSIII. Gladfelder *et al.* (2005) have evidence that Bni4 regulates septin functions, and we have noted that *bni4Δ* mutants have a more severe growth defect when combined with *chs3Δ* or *chs4Δ* (unpublished observations). This would not be predicted if Bni4 only regulates CSIII localization. Along similar lines, a *bni4* deletion in *Candida albicans* leads to significantly reduced

formation of hyphae, independent of chitin synthesis (Rowbottom *et al.*, 2004).

The sufficiency of the Glc7/PP1-binding domains of Bni4 and Phactr-1 for CSIII targeting provides important insights into the mechanism of CSIII recruitment to the bud neck. The evidence leads us to argue against a model in which Bni4 simply tethers Chs4 to the neck. Chs4 has been shown to bind Bni4 in two-hybrid studies (DeMarini *et al.*, 1997), but we have been unable to detect Chs4 in coimmunoprecipitation experiments with Bni4 (unpublished observations). Also, there is no simple correlation between Bni4 and Chs4 levels at the bud neck. Several variants, including Bni4Δ89–101, are present at low levels at the neck, presumably because of defects in septin binding, but these variants target Chs4 to the neck at near normal levels, whereas mutants defective in Glc7 binding are completely defective in CSIII targeting. Although the inability of the Cdc10-Bni4^{VA/FA} fusion to target CSIII provides a strong argument that Glc7 plays a direct role in the process, it does not exclude the possibility that another component of CSIII also binds Cdc10-Bni4. However, this possibility is unlikely given that the C-terminus of Phactr-1 is only 32% identical to Bni4 but can target both Glc7 and CSIII to the neck.

The relevant substrates of Bni4-Glc7 are unknown but the ability of this complex to target CSIII to the bud neck suggests several possibilities. Chs3 and Chs4 could be targets of Bni4-Glc7. Both are phosphorylated (Valdivia and Schekman, 2003; Ptacek *et al.*, 2005; Chi *et al.*, 2007), although there is no evidence that their phosphorylation is relevant to their activity or recruitment. Another possibility is that Bni4-Glc7 could directly participate in the fusion of Chs3 vesicles with the membrane at the bud neck. Phosphorylation and dephosphorylation of t-SNAREs play a critical role in membrane fusion (Elbert *et al.*, 2005; Weinberger *et al.*, 2005), and Glc7 is required for a late stage of homotypic vacuole fusion (Peters *et al.*, 1999; Bryant and James, 2003) and transport vesicle fusion (Peters *et al.*, 1999; Bryant and James, 2003). Components of the exocyst complex, Sec3 (Ficarro *et al.*, 2002; Chi *et al.*, 2007; Smolka *et al.*, 2007), Sec5 (Li *et al.*, 2007), Sec8 (Smolka *et al.*, 2007), Sec10 (Gruhler *et al.*, 2005; Li *et al.*, 2007), and Exo84 (Chi *et al.*, 2007; Smolka *et al.*, 2007), are also phosphorylated in vivo.

One prediction of our model is that Glc7 tethered directly to septins should also target CSIII to the bud neck. However, the Cdc10-Glc7 fusion protein does not recruit Chs4 or CSIII to the bud neck, even though it does cause altered bud morphology. Cdc10-Glc7 is able to recruit CSIII in strains containing Bni4Δ286–302, a variant that does not associate with the neck and normally has a null phenotype, or when coexpressed with just the C-terminal 70 a.a. of Bni4. On the basis of these results, we hypothesize that an important role of the Glc7 binding domain of Bni4 is to alter Glc7 substrate specificity. There is ample precedent for such a model. The MYPT1 subunit of myosin phosphatase activates PP1 toward myosin light chain and inhibits the activity toward glycogen synthase (Hartshorne *et al.*, 2004) by changing the conformation of the active site (Terrak *et al.*, 2004). It is unlikely that simply binding to an RVXF-containing protein is sufficient to activate Glc7 toward its appropriate substrates because a protein fusion between Cdc10 and the Glc7-binding domain of Gac1, a glycogen targeting subunit (François *et al.*, 1992; Stuart *et al.*, 1994) is unable to target CSIII to the bud neck (data not shown).

In summary, we propose that the primary role of Bni4 is to target Glc7 to the bud neck in a temporally and spatially

restricted manner and to regulate its activity, allowing it to act on an as yet undefined substrate required to target CSIII to the neck. The observation that a PP1-binding domain from a Phactr/Scapin protein can substitute for the Glc7-binding domain of Bni4 suggests that these vertebrate proteins may have a similar role in regulating PP1 activity.

ACKNOWLEDGMENTS

We thank Patrick Allen (Yale University) for the phactr-1 cDNA clone, John Cannon (University of Missouri) for the GLC7 cDNA clone, David Gross (Louisiana State University Health Sciences Center) for the anti-HSF antibody, and Lucy Robinson for reading the manuscript and thoughtful discussion. This work was supported by the National Institutes of Health Grant GM-47789.

REFERENCES

Allen, P. B., Greenfield, A. T., Svenningsson, P., Haspeslagh, D. C., and Greengard, P. (2004). Phactr1-4, A family of protein phosphatase 1 and actin regulatory proteins. *Proc. Natl. Acad. Sci. USA* 101, 7187–7192.

Bartnicki-Garcia, S. (2006). Chitosomes: past, present and future. *FEMS Yeast Res.* 6, 957–965.

Bharucha, J. P., Larson, J. R., Gao, L., Daves, L. K., and Tatchell, K. (2008). Ypi1, a positive regulator of nuclear protein phosphatase type 1 activity in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 19, 1032–1045.

Bloecher, A., and Tatchell, K. (2000). Dynamic localization of protein phosphatase type 1 in the mitotic cell cycle of *Saccharomyces cerevisiae*. *J. Cell Biol.* 149, 125–140.

Bryant, N. J., and James, D. E. (2003). The Sec1p/Munc18 (SM) protein, Vps45p, cycles on and off membranes during vesicle transport. *J. Cell Biol.* 161, 691–696.

Bulawa, C. E. (1993). Genetics and molecular biology of chitin synthesis in fungi. *Annu. Rev. Microbiol.* 47, 505–534.

Cabib, E., and Duran, A. (2005). Synthase III-dependent chitin is bound to different acceptors depending on location on the cell wall of budding yeast. *J. Biol. Chem.* 280, 9170–9179.

Chi, A., Huttenhower, C., Geer, L. Y., Coon, J. J., Syka, J. E., Bai, D. L., Shabanowitz, J., Burke, D. J., Troyanskaya, O. G., and Hunt, D. F. (2007). Analysis of phosphorylation sites on proteins from *Saccharomyces cerevisiae* by electron transfer dissociation (ETD) mass spectrometry. *Proc. Natl. Acad. Sci. USA* 104, 2193–2198.

Choi, W. J., Sburlati, A., and Cabib, E. (1994). Chitin synthase 3 from yeast has zygotenic properties that depend on both the CAL1 and the CAL3 genes. *Proc. Natl. Acad. Sci. USA* 91, 4727–4730.

Chuang, J. S., and Schekman, R. W. (1996). Differential trafficking and timed localization of two chitin synthase proteins, Chs2p and Chs3p. *J. Cell Biol.* 135, 597–610.

Cos, T., Ford, R. A., Trilla, J. A., Duran, A., Cabib, E., and Roncero, C. (1998). Molecular analysis of Chs3p participation in chitin synthase III activity. *Eur. J. Biochem.* 256, 419–426.

Davis, N. G., Horecka, J. L., and Sprague, G. F., Jr. (1993). Cis- and trans-acting functions required for endocytosis of the yeast pheromone receptors. *J. Cell Biol.* 122, 53–65.

DeMarini, D. J., Adams, A.E.M., Fares, H., De Virgilio, C., Valle, G., Chuang, J. S., and Pringle, J. R. (1997). A septin-based hierarchy of proteins required for localized deposition of chitin in the *Saccharomyces cerevisiae* cell wall. *J. Cell Biol.* 139, 75–93.

DeMattei, C. R., Davis, C. P., and Konopka, J. B. (2000). Point mutations identify a conserved region of the *Saccharomyces cerevisiae* AFR1 gene that is essential for both the pheromone signaling and morphogenesis functions. *Genetics* 155, 43–55.

Elbert, M., Rossi, G., and Brennwald, P. (2005). The yeast par-1 homologs kin1 and kin2 show genetic and physical interactions with components of the exocytic machinery. *Mol. Biol. Cell* 16, 532–549.

Elledge, S. J., and Davis, R. W. (1988). A family of versatile centromeric vectors designed for use in the sectoring-shuffle mutagenesis assay in *Saccharomyces cerevisiae*. *Gene* 70, 303–312.

Ficarro, S. B., McClelland, M. L., Stukenberg, P. T., Burke, D. J., Ross, M. M., Shabanowitz, J., Hunt, D. F., and White, F. M. (2002). Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. *Nat. Biotechnol.* 20, 301–305.

Foreman, P. K., and Davis, R. W. (1994). Cloning vectors for the synthesis of epitope-tagged, truncated and chimeric proteins in *Saccharomyces cerevisiae*. *Gene* 144, 63–68.

François, J. M., Thompson-Jaeger, S., Skroch, J., Zellenka, U., Spevak, W., and Tatchell, K. (1992). GAC1 may encode a regulatory subunit for protein phosphatase type 1 in *Saccharomyces cerevisiae*. *EMBO J.* 11, 87–96.

Gladfelter, A. S., Kozubowski, L., Zyla, T. R., and Lew, D. J. (2005). Interplay between septin organization, cell cycle and cell shape in yeast. *J. Cell Sci.* 118, 1617–1628.

Goldstein, A. L., and McCusker, J. H. (1999). Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* 15, 1541–1553.

Gruhler, A., Olsen, J. V., Mohammed, S., Mortensen, P., Faergeman, N. J., Mann, M., and Jensen, O. N. (2005). Quantitative phosphoproteomics applied to the yeast pheromone signaling pathway. *Mol. Cell Proteomics* 4, 310–327.

Hartshorne, D. J., Ito, M., and Erdodi, F. (2004). Role of protein phosphatase type 1 in contractile functions: myosin phosphatase. *J. Biol. Chem.* 279, 37211–37214.

Kaiser, C., Michaelis, S., and Mitchell, A. (1994). *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Manual*, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Kim, T. H., Goodman, J., Anderson, K. V., and Niswander, L. (2007). Phactr4 regulates neural tube and optic fissure closure by controlling PP1-, Rb-, and E2F1-regulated cell-cycle progression. *Dev. Cell* 13, 87–102.

Kozubowski, L., Larson, J. R., and Tatchell, K. (2005). Role of the septin ring in the asymmetric localization of proteins at the mother-bud neck in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 16, 3455–3466.

Kozubowski, L., Panek, H., Rosenthal, A., Bloecher, A., DeMarini, D. J., and Tatchell, K. (2003). A Bni4-Glc7 phosphatase complex that recruits chitin synthase to the site of bud emergence. *Mol. Biol. Cell* 14, 26–39.

Li, X., Gerber, S. A., Rudner, A. D., Beausoleil, S. A., Haas, W., Villen, J., Elias, J. E., and Gygi, S. P. (2007). Large-scale phosphorylation analysis of alpha-factor-arrested *Saccharomyces cerevisiae*. *J. Proteome. Res.* 6, 1190–1197.

Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* 14, 953–961.

Maniatis, Sambrook, T. J., and Fritsch, E. F. (1989). *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Peters, C., Andrews, P. D., Stark, M. J., Cesaro-Tadic, S., Glatz, A., Podtelejnikov, A., Mann, M., and Mayer, A. (1999). Control of the terminal step of intracellular membrane fusion by protein phosphatase 1. *Science* 285, 1084–1087.

Ptacek, J. *et al.* (2005). Global analysis of protein phosphorylation in yeast. *Nature* 438, 679–684.

Reyes, A., Sanz, M., Duran, A., and Roncero, C. (2007). Chitin synthase III requires Chs4p-dependent translocation of Chs3p into the plasma membrane. *J. Cell Sci.* 120, 1998–2009.

Robinson, L. C., Bradley, C., Bryan, J. D., Jerome, A., Kweon, Y., and Panek, H. R. (1999). The Yck2 yeast casein kinase 1 isoform shows cell cycle-specific localization to sites of polarized growth and is required for proper septin organization. *Mol. Biol. Cell* 10, 1077–1092.

Rowbottom, L., Munro, C. A., and Gow, N. A. (2004). *Candida albicans* mutants in the BNI4 gene have reduced cell-wall chitin and alterations in morphogenesis. *Microbiology* 150, 3243–3252.

Sagara, J., Higuchi, T., Hattori, Y., Moriya, M., Sarvotham, H., Shima, H., Shirato, H., Kikuchi, K., and Taniguchi, S. (2003). Scapin, a putative protein phosphatase-1 regulatory subunit associated with the nuclear nonchromatin structure. *J. Biol. Chem.* 278, 45611–45619.

Santos, B., and Snyder, M. (1997). Targeting of chitin synthase 3 to polarized growth sites in yeast requires Chs5p and Myo2p. *J. Cell Biol.* 136, 95–110.

Sanz, M., Castrejon, F., Duran, A., and Roncero, C. (2004). *Saccharomyces cerevisiae* Bni4p directs the formation of the chitin ring and also participates in the correct assembly of the septum structure. *Microbiology* 150, 3229–3241.

Sheff, M. A., and Thorn, K. S. (2004). Optimized cassettes for fluorescent protein tagging in *Saccharomyces cerevisiae*. *Yeast* 21, 661–670.

Sherman, F., Fink, G. R., and Hicks, J. B. (1986). *Methods in Yeast Genetics*, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

- Sikorski, R. S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122, 19–27.
- Smolka, M. B., Albuquerque, C. P., Chen, S. H., and Zhou, H. (2007). Proteome-wide identification of in vivo targets of DNA damage checkpoint kinases. *Proc. Natl. Acad. Sci. USA* 104, 10364–10369.
- Stuart, J. S., Frederick, D. L., Varner, C. M., and Tatchell, K. (1994). The mutant type 1 protein phosphatase encoded by *glc7-1* from *Saccharomyces cerevisiae* fails to interact productively with the *GAC1*-encoded regulatory subunit. *Mol. Cell Biol.* 14, 896–905.
- Terrak, M., Kerff, F., Langsetmo, K., Tao, T., and Dominguez, R. (2004). Structural basis of protein phosphatase 1 regulation. *Nature* 429, 780–784.
- Tong, A. H. *et al.* (2001). Systematic genetic analysis with ordered arrays of yeast deletion mutants. *Science* 294, 2364–2368.
- Trilla, J. A., Cos, T., Duran, A., and Roncero, C. (1997). Characterization of *CHS4* (*CAL2*), a gene of *Saccharomyces cerevisiae* involved in chitin biosynthesis and allelic to *SKT5* and *CSD4*. *Yeast* 13, 795–807.
- Valdivia, R. H., and Schekman, R. (2003). The yeasts Rho1p and Pkc1p regulate the transport of chitin synthase III (Chs3p) from internal stores to the plasma membrane. *Proc. Natl. Acad. Sci. USA* 100, 10287–10292.
- Weinberger, A., Kamena, F., Kama, R., Spang, A., and Gerst, J. E. (2005). Control of Golgi morphology and function by Sed5 t-SNARE phosphorylation. *Mol. Biol. Cell* 16, 4918–4930.