The Nim1-kinase Gin4 has distinct domains crucial for septin assembly, phospholipid binding, and mitotic exit

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Summary statement: Systematic dissection of the Gin4 kinase in the human pathogenic fungus Candida albicans uncovered three functional domains that interact with distinct cellular components.
Abstract

In fungi, the Nim1 protein kinases, such as Gin4, are important regulators of multiple cell cycle events including the G2-M transition, septin assembly, polarized growth, and cytokinesis. Compelling evidence has linked some key functions of Gin4 with the large C-terminal nonkinase region which, however, was poorly defined. By systematic dissection and functional characterization of the nonkinase region of Gin4 in the human fungal pathogen *Candida albicans*, we report the identification of three novel domains with distinct functions: a lipid-binding domain (LBD), a septin-binding domain (SBD), and a nucleolus-associating domain (NAD). The LBD and SBD are indispensable for Gin4’s function, and they alone could sufficiently restore septin ring assembly in *GIN4* null mutants. The NAD localizes to the periphery of the nucleolus and physically associates with Cdc14, the ultimate effector of the mitotic exit network. Gin4 mutants lacking the NAD are defective in spindle orientation and exit mitosis prematurely. Furthermore, we show that Gin4 is a substrate of Cdc14. These findings provide novel insights into the roles and mechanisms of Nim1-kinases in the regulation of some crucial cell cycle events.
Introduction

Cell division requires temporal precision in the sequential coordination of events which are monitored and enforced by surveillance mechanisms called checkpoints (Hartwell and Weinert, 1989; Lew and Burke, 2003; Murray, 1992; Rudner and Murray, 1996; Zhou and Elledge, 2000). In eukaryotes, although the cyclin-dependent kinase (CDK) is the master cell cycle regulator (Morgan, 1997; Murray, 1994; Murray, 2004; Walworth, 2000), several other protein kinases also play crucial roles in governing various cell cycle events. One example is the Nim1 serine/threonine protein kinases. In *Schizosaccharomyces pombe*, the Nim1-kinases Cdr1 and Cdr2 are essential components of the cell size checkpoint, which promote the G2-M transition by down-regulating the CDK inhibitor Wee1 (Kanoh and Russell, 1998; Moseley et al., 2009). In *Saccharomyces cerevisiae* (*Sc*), a homologous kinase Hsl1 plays a similar role in the morphogenesis checkpoint (Crutchley et al., 2009). Nim1-kinases have also been intimately linked to the regulation of septin organization and dynamics, cytokinesis, and morphogenesis (Altman and Kellogg, 1997; Barral et al., 1999; Li et al., 2012; Mortensen et al., 2002; Wightman et al., 2004). Fungal Nim1-kinases are large proteins of >1000 amino acids with a conserved N-terminal kinase domain followed by an extended poorly characterized C-terminal region (Akada et al., 1997; Asano et al., 2006; Longtine et al., 1998; Mortensen et al., 2002; Okuzaki et al., 1997; Wightman et al., 2004).

The Nim1-kinases are well-established regulators of the septins in both *S. cerevisiae* and the pathogenic fungus *Candida albicans* (*Ca*) (Li et al., 2012; Longtine et al., 1998; Sinha et al., 2007). The filament-forming septins are GTP-binding proteins conserved evolutionarily from yeast to humans (Beise and Trimble, 2011; Fung et al., 2014) and have roles in diverse processes ranging from cytokinesis to cell
morphogenesis (Longtine et al., 1996; Trimble, 1999). In yeasts, septins form scaffolds anchoring several cell cycle regulators (Bridges and Gladfelter, 2015; Crutchley et al., 2009). In humans, septins are associated with several diseases such as cancer (Fung et al., 2014; Hall and Russell, 2004). The septins exist as complexes with multiple levels of organization such as oligomeric rods, filaments, rings, hourglass-shaped collars and gauzes (Barral and Kinoshita, 2008; Weirich et al., 2008). *S. cerevisiae* contains five mitotic septins (Cdc3, Cdc10, Cdc11, Cdc12, and Shs1/Sep7), and the septin cytoskeleton undergoes phase-dependent organizational and dynamic changes during each cell cycle (Dobbelaere et al., 2003; Ong et al., 2014). Septins first form a cap at the nascent bud tip and later transforms into a ring and then an hourglass-shaped collar at the bud neck. The hourglass collar persists throughout mitosis and splits into two rings during cytokinesis before disassembly (Longtine and Bi, 2003). These morphological changes coincide with the dramatic remodelling of septin-filament orientation and crosslinking (Ong et al., 2014). Currently, the mechanisms governing septin assembly, disassembly, and remodelling remain unclear. In *S. cerevisiae*, the CDK Cdc28 directly phosphorylates several septin isoforms including Cdc3 and Cdc11 (Asano et al., 2006; Dobbelaere et al., 2003; Mortensen et al., 2002; Sinha et al., 2007; Tang and Reed, 2002). Cdc28 also acts indirectly via the Cdc42 GTPase which recruits septins to the incipient bud site (Iwase et al., 2006). Other septin-associated protein kinases such as Gin4 and the p21-activated kinase Cla4 are also important septin regulators which target Cdc3, Cdc10, and Shs1 (Altman and Kellogg, 1997; Asano et al., 2006; Bouquin et al., 2000; DeMay et al., 2009; Merlini et al., 2012). The septin ring-to-hourglass transition possibly involves the Elm1 and Gin4 kinases, since cells lacking either kinase failed to assemble the hourglass (Altman and Kellogg, 1997; Asano et
al., 2006; Bouquin et al., 2000; Lee et al., 2002). Splitting of the hourglass was proposed to involve the mitotic exit network (MEN) (Cid et al., 2001; Lippincott et al., 2001).

Recent studies in pathogenic fungi have shed new lights on our understanding of Nim1-kinases and septin regulation (Bridges and Gladfelter, 2014; Gladfelter, 2010; Gonzalez-Novo et al., 2008; Li et al., 2012; Sinha et al., 2007). \( \text{C. albicans} \) can grow in three distinct morphological forms: yeast, pseudohyphae and hyphae (Sudbery, 2011) and has orthologues of all \( \text{S. cerevisiae} \) septins (Warenda and Konopka, 2002). Septin organization and dynamics in \( \text{C. albicans} \) yeast and pseudohyphae resemble that of \( \text{S. cerevisiae} \) (Sudbery, 2001; Sudbery, 2011). However, \( \text{C. albicans} \) hyphae assemble septin structures with localizations and dynamics distinct from the yeast cells (Gonzalez-Novo et al., 2008; Sudbery, 2001). \( \text{C. albicans} \) \( gin4\Delta/\Delta \) mutants exhibit severe defects characterized by extreme bud elongation, and failure in septin ring formation and cytokinesis (Li et al., 2012; Wightman et al., 2004). \( \text{CaGin4} \) phosphorylates the septins Cdc11 and Sep7, regulating the yeast-hyphal transition and Sep7 dynamics respectively (Li et al., 2012; Sinha et al., 2007). However, we observed that \( \text{C. albicans} \) cells expressing a mutant Gin4 lacking the kinase domain seemed able to assemble the septin ring at the bud neck and displayed milder defects than the \( gin4\Delta/\Delta \) mutant, indicating that some important functions of Gin4 are furnished by regions outside the kinase domain (Li et al., 2012). Similar observations were reported in \( \text{S. cerevisiae} \) strains expressing a kinase-dead Gin4 (Longtine et al., 1998). However, the Gin4 nonkinase region remains poorly characterized except for a phospholipid-binding KA1 domain found at the C-terminus of \( \text{S. cerevisiae} \) Nim1-kinases (Moravcevic et al., 2010).
In this study, we have carried out systematic dissection and functional characterization of the nonkinase region of CaGin4 and uncovered new roles for Gin4 in the control of septin organization, plasma membrane (PM) interaction and mitotic exit. We define three novel functional domains: a lipid-binding domain (LBD), a septin-binding domain (SBD), and a nucleolus-associating domain (NAD). The LBD and SBD are indispensable for Gin4’s function, and a fragment containing only the LBD and SBD is sufficient to support septin ring formation at the bud neck. The NAD mediates localization to the periphery of the nucleolus and physically associates with Cdc14. Deleting the NAD causes defects in spindle orientation and premature mitotic exit. We also show that Gin4 is a substrate of Cdc14.

**Results**

**Systematic dissection of the nonkinase domain of CaGin4**

CaGin4 consists of 1349 amino acids (aa) with the kinase domain (aa28-288) located near the N-terminus. To identify functional domains in the nonkinase region (aa289-1349), we first dissected it into three 300-aa segments, CT1 (aa1051-1349), CT2 (aa751-1050) and CT3 (aa451-750) (Fig. 1A). Two sets of truncated alleles were constructed, one with each construct having one of the CT segments deleted ($gin4^{CT1\Delta}$, $gin4^{CT2\Delta}$ or $gin4^{CT3\Delta}$), and in the other, each construct contained only CT1, CT2 or CT3. All the truncated alleles were expressed from the MET3 promoter in a strain that carried a single copy of GIN4 regulated by the MAL2 promoter ($gin4\Delta/P_{MAL2}\cdot GIN4$). The MAL2 promoter allows GIN4 expression (GIN4-ON) in medium with maltose as the sole carbon source and GIN4 repression (GIN4-OFF) by glucose. The GIN4-ON cells were indistinguishable from the wild-type (WT) cells
while the GIN4-OFF cells phenocopied the gin4Δ/Δ mutant. Expressing WT GIN4 from the MET3 promoter fully rescued the defects of the GIN4-OFF cells, while switching off the MET3 promoter led to the gin4Δ/Δ phenotype. Thus, the gin4Δ/P_{MAL2} GIN4 strain allowed us to investigate each gin4 allele in both GIN4-ON and -OFF backgrounds. All the Gin4 proteins carried an N-terminal GFP tag unless indicated otherwise.

We first examined the cellular localization of Gin4^{CT1Δ}, Gin4^{CT2Δ}, and Gin4^{CT3Δ} by fluorescence microscopy. GIN4-ON yeast cells expressing Gin4^{CT1Δ} showed no discernible defects, and GFP-Gin4^{CT1Δ} colocalized with Cdc12-mCherry to the bud neck throughout the cell cycle (Fig.1B, top). However, under GIN4-OFF conditions, the gin4^{CT1Δ} cells exhibited morphological defects similar to the gin4Δ/Δ cells in which no septin ring was formed, and GFP-Gin4^{CT1Δ} colocalized with Cdc12-mCherry to pseudohyphal tips (Fig. 1B, bottom), indicating that gin4^{CT1Δ} cannot support septin ring formation and CT1 is essential for Gin4 function. The ability of Gin4^{CT1Δ} to colocalize with the septins both at the bud neck in GIN4-ON cells and the pseudohyphal tips in GIN4-OFF cells suggests that CT1 is not required for Gin4 to associate with septin complexes. Surprisingly, in GIN4-ON cells, GFP-Gin4^{CT1Δ} often appeared as a single dot in both the mother and daughter compartments or as a pair of closely positioned dots in the mother compartment (Fig. 1B), reminiscent of spindle pole bodies (SPBs).

GIN4-ON yeast cells expressing Gin4^{CT2Δ} showed normal morphology and normal septin rings marked by Cdc12-mCherry (Fig.1C). However, GFP-Gin4^{CT2Δ} did not localize to the bud neck; instead, it was detected in the entire cytoplasm. Also, when GIN4 expression was repressed, gin4^{CT2Δ} cells developed a pseudohyphal morphology which is, however, considerably less severe than gin4Δ/Δ cells. The
pseudohyphae were shorter and had sharper septal constrictions, in which GFP-Gin4$^{CT2\Delta}$ showed the same cytoplasmic localization. Septins, mostly in the form of abnormal rings or aggregates, appeared in the septal region in ~47% of the cells and as a broad crescent at pseudohyphal tips. The data suggest that CT2 may contain motifs required for Gin4 to associate with and facilitate the assembly of septin complexes.

GIN4-ON yeast cells expressing Gin4$^{CT3\Delta}$ showed normal morphology and septin rings (Fig. 1D, top), and GFP-Gin4$^{CT3\Delta}$ colocalized properly with septins to the bud neck. GFP-Gin4$^{CT3\Delta}$ also showed weak localization to the entire plasma membrane (PM). Under GIN4-OFF conditions, the majority of the cells were of the yeast morphology but were obviously larger than GIN4-ON cells, and ~30% of these cells were moderately elongated (Fig. 1D, top). In these cells, septin rings were assembled and localized normally at the bud neck throughout the cell cycle, and cytokinesis occurred successfully. As gin4$^{\Delta/\Delta}$ pseudohyphae do not respond to hyphal induction (Wightman et al., 2004), we tested if gin4$^{CT3\Delta}$ yeast cells can develop hyphae upon serum induction. We found that GIN4-OFF gin4$^{CT3\Delta}$ cells grew true hyphae (Fig. 1D, bottom). However, Gin4$^{CT3\Delta}$ often formed diffuse bands behind the hyphal tip or random cortical aggregates. Also, septin rings failed to form at the septum; instead, they mislocalized with Gin4$^{CT3\Delta}$, suggesting that CT3 might be required for hyphal-specific septin ring assembly.

**CT1 contains a LBD which mediates its PM association**

We next examined the cellular localization of CT1. GIN4-ON cells expressing CT1 were normal in both yeast and hyphal growth; and strikingly, GFP-CT1 localized to the entire PM (Fig. 2A). In comparison, GIN4-OFF CT1 cells grew as pseudohyphae
with cytokinetic defects, and GFP-CT1 also showed the same PM localization. Interestingly, we observed that weak septin ring often existed transiently at the base of newly emerged buds, suggesting CT1 could partially facilitate septin ring assembly.

While our work was in progress, Moravcevic et al. (2010) identified a ~100-aa kinase-associated-1 (KA1) domain at the C-terminus of three *S. cerevisiae* Nim1-kinases Gin4, Kcc4, and Hsl1 and found that the KA1 domain mediates PM association through phospholipid binding. *C. albicans* has orthologues of *Sc* Gin4 and *Sc* Hsl1. Except for the kinase domain, *Ca* Gin4 and *Ca* Hsl1 share poor sequence similarity with their *S. cerevisiae* counterparts. Alignments of *Ca* Gin4 CT1 with the KA1 domains of *Sc* Gin4, *Sc* Kcc4, and *Sc* Hsl1 revealed significant homology in the last 99 aa of *Ca* Gin4 (aa1251-1349), suggesting *Ca* Gin4 also contains a KA1 domain. However, the pairs of basic residues in the *Sc* Kcc4-KA1 domain required for PM association are not conserved in *Ca* Gin4. To determine if the *Ca* Gin4 KA1 domain associates with the PM, we truncated CT1 to CT1.1 (aa1151-1349) and CT1.2 (KA1; aa1251-1349), and GFP-tagged each for expression in *gin4Δ/PMAL2-GIN4* cells. While GFP-CT1.1 was found to localize to the PM, the KA1 fragment localized in the cytoplasm. Therefore, the PM targeting residues lie not in KA1 but in aa1151-1250. Indeed, the aa1151-1250 fragment (CT1.3) localized to the PM. The PM localization was abolished with further truncation of CT1.3 (CT1.3.1 and CT1.3.2) (Fig. 2B). Next, we determined if the basic residues (K1163, K1166, K1167, R1190, K1191, K1197, and K1198) within CT1.3 are required for its PM localization. Unlike *Sc* Kcc4-KA1 and human MARK1-KA1 domains, in which mutating one of several pairs of basic residues was sufficient to abolish the membrane association (Moravcevic et al., 2010), PM localization was completely abolished only when all
seven residues in CT1.3 were replaced with serine (CT1.3_{7S}) (Fig. 2B). Also, unlike *GIN4*-OFF CT1 cells (Fig. 2A), *GIN4*-OFF CT1.3_{7S} cells did not support the formation of transient septin rings (Fig. S1A).

We next investigated if CT1 and CT1.3 can associate with phospholipids. We purified GST-CT1 and GST-CT1.3 from *Escherichia coli* and tested their ability to bind an array of phospholipids using the PIPstrips™ (Fig. 2C). Purified GST was included as the negative control. CT1 exhibited specific affinity to phosphatidylinositol (PtdIns) and phosphoinositides including phosphatidylinositol 3-phosphate [PI(3)P], PI(4)P, PI(5)P, PI(3,4)P₂, PI(3,5)P₂, PI(4,5)P₂ and PI(3,4,5)P₃; and CT1’s affinity for phosphoinositides seemed to increase with the number of negatively-charged phosphates. CT1.3 exhibited affinity for the same set of phospholipids as CT1 except PtdIns which CT1.3 does not bind (Fig. 2C). Our data demonstrate that the CaGin4 CT1.3 has the phospholipid-binding ability and mediates CT1’s PM association.

**CT2 contains a septin-binding domain (SBD)**

Gin4^{CT2Δ}'s inability to colocalize with the septins (Fig. 1C) suggests that CT2 might mediate interaction with the septins. Consistent with this hypothesis, CT2 colocalized with the septins at the bud neck in *GIN4*-ON cells throughout the cell cycle (Fig. 3A), and CT2 disappeared from the bud neck when the septin ring split. Truncating CT2 by 100 aa from the C-terminus (CT2.1) significantly weakened its bud neck localization and caused significant cytoplasmic localization, and further truncation of 100 aa (CT2.2) completely abolished the bud neck localization, suggesting that the entire 300 aa of CT2 is required for its bud-neck localization. To detect CT2’s interaction with the septins, coimmunoprecipitation (Co-IP) was
performed in \textit{GIN4-ON} and -OFF cells coexpressing Cdc12-Myc with GFP-CT2. Cells expressing Cdc12-Myc or GFP-CT2 alone were used as positive and negative controls respectively. Cdc12-Myc was precipitated using anti-Myc (αMyc) beads, and the protein-bound beads were washed with buffers containing either 150 mM or 1 M NaCl. Cdc12-Myc and GFP-CT2 were then probed with αMyc or αGFP respectively in WB analysis. GFP-CT2 was detected in cell lysates expressing Cdc12-Myc and GFP-CT2 but not in those expressing Cdc12-Myc or GFP-CT2 alone (Fig. 3B). CT2-Cdc12 interaction was detected in both \textit{GIN4-ON} and -OFF cells, although more Cdc12 was detected in the former, possibly due to the lack of septin ring assembly in the latter. Gin4-septin interaction remained stable in 1 M NaCl (Li et al., 2012). Similarly, the CT2-Cdc12 interaction was detectable after washes with 1 M NaCl (Fig. 3B). To confirm that the Gin4-septin interaction is directly mediated by CT2, purified GST-CT2 from \textit{E.coli} was used to pull down CT2-binding proteins in cell lysates from either \textit{GIN4-ON} or -OFF cells expressing Cdc12-Myc. Subsequent αMyc WB clearly detected Cdc12-Myc in the pull-down products of GST-CT2 from both \textit{GIN4-ON} and -OFF cells, but not from the products pulled down with the αGST-beads alone (Fig. 3C). Again, the CT2-Cdc12 interaction remained intact after washes with 1 M NaCl. The results show that CT2 contains a septin-binding domain (SBD) which mediates Gin4’s association with Cdc12.

The CT2+1 fragment is sufficient to organize the septin ring at the bud neck

We reported that the Gin4 kinase domain is not required for septin ring assembly at the bud neck in \textit{C. albicans} (Li et al., 2012). Here, we identified a SBD which alone, however, cannot promote septin ring assembly. Also, we observed that expressing CT1 in \textit{GIN4-OFF} cells could support the formation of transient septin rings, suggesting CT1 might play a role in septin ring formation. Consistently, CT1 was
found to coimmunoprecipitate with Cdc12 (Fig. S1B). Next, we asked if a fragment containing only the SBD and LBD would suffice for septin ring assembly. To this end, GFP-CT2+1 (aa751-1349) was coexpressed with Cdc12-mCherry in \textit{GIN4}-ON and -OFF cells. To monitor septin assembly through a cell cycle, we collected \textit{GIN4}-ON G1 cells, released them into \textit{GIN4}-ON or -OFF conditions for further growth and harvested cells at intervals for microscopic examination. \textit{gin4Δ/P:\textit{MAL2}}-\textit{GIN4} cells expressing Cdc12-mCherry were used as a control. Upon release into the \textit{GIN4}-ON condition, the control cells exhibited normal septin ring assembly, splitting and disassembly at expected stages of the cell cycle (Fig. 4A, \textit{GIN4}-ON). However, under \textit{GIN4}-OFF conditions, Cdc12 formed a cap at the incipient bud tip which persisted as the bud elongated, and the septin ring was never formed (Fig. 4A, \textit{GIN4}-OFF). In contrast, in \textit{GIN4}-OFF cells expressing CT2+1, Cdc12 formed a ring at the bud neck and colocalized with CT2+1 throughout bud growth (Fig. 4B); however, the septin ring failed to split, both CT2+1 and Cdc12 did not disassemble after cytokinesis. The \textit{GIN4}-OFF CT2+1 cells grew into chains of moderately elongated yeast cells with septin rings persisting at the septum (Fig. 4B).

An important function of the septin ring is to recruit proteins such as Myo1, a component of the actomyosin ring, to the bud neck. Some septin mutations completely abolished Myo1’s localization and actomyosin ring assembly at the bud neck (Finnigan et al., 2015; Lippincott and Li, 1998). To determine the functionality of the septin ring assembled in CT2+1 cells, we examined Myo1-mCherry localization in \textit{GIN4}-ON and -OFF cells expressing CT2+1. Like in \textit{GIN4}-ON cells, Myo1 and CT2+1 colocalized to the bud neck in \textit{GIN4}-OFF cells, indicating that the assembled septin ring can recruit Myo1. However, Myo1 constriction and disassembly failed to occur in \textit{GIN4}-OFF CT2+1 cells (Fig. 4C). As \textit{gin4Δ/Δ}
pseudohyphae do not respond to hyphal induction (Wightman et al., 2004), we asked if CT2+1 can restore hyphal growth in giz4Δ/Δ cells. In spite of the cytokinesis defects, expressing CT2+1 in GIN4-OFF cells produced separate yeast cells (Fig. S2). These cells responded to hyphal induction rather normally, producing long hyphae (Fig. 4D and E). Hyphal growth in the CT2+1 cells was not due to leaky expression of GIN4 from the MAL2 promoter because GIN4-OFF CT2+1-OFF cells were unresponsive to hyphal induction (Fig. S2). The results demonstrate that the CT2+1 region provides functions required for hyphal development. Interestingly, while CT2+1 and Cdc12 were often seen to colocalize at hyphal tips, septin rings were rarely found even in long hyphae (Fig. 4E). In contrast to the ability of CT2+1 yeast cells to form septin rings, the lack of septin rings in CT2+1 hyphae indicates differential regulation of septin formation between yeast and hyphal growth. In yeast cells, septins within the ring undergo cell-cycle-dependent dynamic changes, which involves regulation by Gin4 (Dobbelaere et al., 2003). To examine if the septin ring promoted by CT2+1 has altered dynamics, we performed fluorescence recovery after photo-bleaching (FRAP) analyses on Cdc12-mCherry in the septin ring. We examined cells with a medium-sized bud in which the septin ring is immobile in WT cells (Dobbelaere et al., 2003). The entire septin ring was photobleached, and the fluorescence recovery monitored over time (Fig. 4F, left). In GIN4-ON WT cells, the fluorescence intensity recovered to ~20% of the pre-bleached level with a slow recovery rate of $t_{1/2}>400s$ ($n=13$). In comparison, fluorescence recovery occurred twice as fast in GIN4-OFF CT2+1 cells ($t_{1/2}<200s$, $n=12$) and reached a higher level of ~40%. Thus, septins in the ring assembled with the help of CT2+1 is more mobile than those in a normal ring. Together, the data demonstrate that the SBD-LBD region alone is sufficient to support the assembly of a partially functional septin ring.
Identification of a nucleolus-associating domain

The larger sizes and mild elongation of $gin4^{CT3\Delta}$ cells suggested possible mitotic defects. Indeed, many $gin4^{CT3\Delta}$ mother cells contained two or more well-separated nuclear masses (Fig. 5A), indicating nuclear division within the mother cell instead of across the bud neck as in WT cells. This phenotype is reminiscent of mutants defective in the spindle position checkpoint (SPC) in S. cerevisiae (Lew and Burke, 2003), suggesting CT3 might contain signals necessary for cell cycle control. To further investigate the mitotic defects in $gin4^{CT3\Delta}$ cells, we tagged Tem1, a SPB-associated GTPase, with mCherry as a cell cycle marker. Using time-lapse microscopy, we monitored Tem1-mCherry through one cell cycle in both $GIN4$-ON and $GIN4$-OFF $gin4^{CT3\Delta}$ cells. We recorded the time elapsed between the time when Tem1 first appeared as a pair of closely positioned dots (completion of SPB duplication) and that when one dot had just crossed the neck. Figure 5B presents a typical $GIN4$-ON cell in which one Tem1 dot had already moved into the daughter cell 30 min after SPB duplication. In comparison, the two Tem1 dots in the $GIN4$-OFF $gin4^{CT3\Delta}$ cell failed to align along the mother-bud axis and started to separate in the mother cell at 80 min (Fig. 5B). The spindle was fully elongated at 1 h 40 min within the mother cell, and the daughter-bound Tem1 dot finally crossed the neck at 2 h. In $GIN4$-ON cells, it took $\sim 24 \pm 10$ min ($n=10$) for the daughter-bound Tem1 to enter the bud after SPB duplication in contrast to $\sim 103 \pm 35$ min ($n=18$) required in $gin4^{CT3\Delta}$ cells. Despite normal septin ring localization and behaviour, the $gin4^{CT3\Delta}$ cells are defective in orientating the spindle poles along the mother-bud axis, leading to spindle elongation within the mother compartment. Our data suggest that CT3 might play a critical role in positioning the spindle and activating mitotic exit.
We examined CT3’s subcellular localization and found that GFP-CT3 appeared as one or two distinct dots in the nucleus in GIN4-ON cells (Fig. 5C) like GFP-Gin4<sup>CT1Δ</sup> (Fig. 1B). Time-lapse monitoring through one cell cycle revealed that CT3 first appeared as a single dot which later divided into two, with one migrating across the bud neck into the daughter cell (Fig. 5D). Moreover, GFP-CT3 never localized to the bud neck. To define the smallest domain responsible for this localization, we truncated CT3 to CT3.1 (aa451-650), CT3.2 (aa451-550), and CT3.3 (aa551-650), and GFP-tagged each in GIN4-ON cells. GFP-CT3.3 behaved like CT3 while CT3.2 localized diffusely in the nucleus (Fig. 5E and Fig. S3A), indicating that CT3.3 contains the signal for the nuclear focal localization. Although the localization pattern is reminiscent of SPBs, GFP-CT3 and Tem1-mCherry signals did not overlap (Fig. S3B). Labelling the nucleolar protein Nop1 with mCherry revealed an association of CT3.3, CT3, and Gin4<sup>CT1Δ</sup> dots with the periphery of the Nop1-labelled area (Fig. 5F). Therefore, we termed CT3.3 the nucleolus-associating domain (NAD).

**Cdc14 associates with the NAD and dephosphorylates Gin4**

Cdc14 is the effector of the MEN and remains sequestered in the nucleolus throughout most of the cell cycle until activation of the MEN triggers its release; and Cdc14 also localizes to the bud neck in late anaphase (Clemente-Blanco et al., 2006; de Bettignies and Johnston, 2003). To determine if the NAD interacts with Cdc14, we first examined if they colocalize in GIN4-ON cells coexpressing Cdc14-GFP with mCherry-CT3 or mCherry-Gin4<sup>CT1Δ</sup>. We found that Cdc14-GFP colocalized with mCherry-CT3 in the nucleus and with mCherry-Gin4<sup>CT1Δ</sup> both in the nucleus and at the bud neck in a population of cells (Fig. 6A & Fig. S3C). Next, we determined if the NAD physically associates with Cdc14. Cdc14-Myc was coexpressed with the various NAD-containing Gin4 constructs, GFP-Gin4, GFP-CT3, GFP-CT3.3, and
GFP-Gin4<sup>CT1Δ</sup>. We performed co-IP in log-phase yeast cells by precipitating the GFP-tagged Gin4 proteins and probing Cdc14 with αMyc WB. Cdc14 was detected in the pull-down products with all the Gin4 variants (Fig. 6B). CT3.3 interaction with Cdc14 was also detected in GIN4-OFF cells (Fig. S4B) although the amount precipitated was less than that from GIN4-ON cells possibly due to the phenotypic consequences of Gin4 depletion. We also conducted co-IP to investigate the interaction of Gin4 or Gin4<sup>CT3Δ</sup> with Cdc14 and found that Cdc14 co-precipitated with both Gin4 and Gin4<sup>CT3Δ</sup> in similar amounts in GIN4-ON and -OFF cells (Fig. S4C).

The data suggest the presence of redundant mechanisms independent of the NAD that mediate Cdc14’s association with Gin4, possibly via components in the septin ring. Consistently, Cdc14 can still localize to the bud neck in GIN4-OFF gin4<sup>CT3Δ</sup> cells (Fig. S4A).

CaGin4 undergoes cell-cycle-dependent phosphorylation by Cdc28 upon entry into mitosis (Li et al., 2012). To show that Gin4 is a substrate of Cdc14, we purified GST-Cdc14 from E. coli and mixed it with immunoprecipitated Gin4-GFP in an in vitro phosphatase assay. Catalytically inactive Cdc14<sup>C275S</sup> was included as a negative control (Bloom et al., 2011). Phosphorylated Gin4 (phos-Gin4) was probed by WB using αPScdk, an antibody that specifically recognizes the phosphoserine within CDK sites (S*PXK/R) (Greig et al., 2015; Li et al., 2012; Zheng et al., 2007). GST-Cdc14 treatment of Gin4-GFP markedly reduced the amount of phos-Gin4, while the mock and GST-Cdc14<sup>C275S</sup> treatments had no effect (Fig. 6C). Lambda phosphatase (λpp), as a positive control, significantly reduced the amount of phos-Gin4. We examined Gin4’s phosphorylation status in WT and cdc14Δ/Δ cells coexpressing GFP-Gin4 and Nop1-mCherry (as a nuclear marker) to show if Cdc14 dephosphorylates Gin4 in vivo. To obtain synchronous cultures, we grew cells to the
stationary phase when >95% of the cells were in G1 and then released them into fresh media for growth at 30°C. We harvested aliquots at intervals and generated budding and nuclear division indices to compare cell cycle progression in the two strains (n>100). We observed only a slight delay in cdc14Δ/Δ cells at the end of the first cell cycle (180 min) (Fig. 6D), consistent with a previous report (Clemente-Blanco et al., 2006). We then immunoprecipitated Gin4-GFP and probed Gin4 with αPScdk WB. Both the WT and cdc14Δ/Δ strains contained untagged and GFP-tagged GIN4. Since Gin4 exists as dimers during mitosis (Mortensen et al., 2002), pulling down Gin4-GFP would also pull down untagged Gin4. Thus, αGFP detected only Gin4-GFP while αPScdk detected phosphoserine residues in both Gin4 and Gin4-GFP, yielding two phos-Gin4 bands (Fig. 6D). As previously reported (Li et al., 2012), levels of both Gin4 and its phosphorylation exhibited cell-cycle dependence in WT cells, peaking at 120-150 min; also, phos-Gin4 was undetectable at 60 min (Fig. 6D). In comparison, phos-Gin4 was readily detected at 60 min in cdc14Δ/Δ cells (Fig. 6D). Moreover, phos-Gin4 levels in cdc14Δ/Δ cells were on average ~1.7 times higher than in WT cells. Hence, we concluded that Cdc14 interacts with and dephosphorylates Gin4 in vivo and the NAD likely mediates this interaction.

Discussion

The role of Nim1 protein kinases in cell cycle control is well-documented in the yeast models S. pombe and S. cerevisiae. However, to date, attention has been focused primarily on their kinase activity. Previously, we discovered association of critical functions with the large C-terminal nonkinase region of CaGin4 and proposed that Gin4 has both kinase-independent and -dependent activities, the former being essential for septin ring assembly and the latter having a role in mitosis (Li et al.,
In this study, we dissected and functionally characterized sub-fragments of the CaGin4 nonkinase region and revealed three distinct functional domains. Our findings shed new lights on the molecular mechanisms by which Gin4 regulates septin assembly and mitosis.

**LBD—a domain that binds phospholipids and mediates association with the PM**

Previous studies in both *S. cerevisiae* and *C. albicans* reported Gin4 localization to specific cellular sites, such as the bud tip and the bud neck, in a cell-cycle-dependent manner. Here, we observed that the C-terminal 300 aa (CT1) of Gin4, when expressed alone, exhibited strong PM association. Although this is consistent with the PM localization of the KA1 domain found in the C-terminus of *ScGin4*, *ScKcc1*, and *ScHsl1* (Moravcevic et al., 2010), we found that the region of *CaGin4* homologous to the KA1 domain was not required for CT1’s PM localization; instead, we mapped the PM targeting motif to the 100-aa region (CT1.3) immediately N-terminal to the KA1 domain. The KA1 domain of *ScKcc4*, *ScGin4* and *ScHsl1* binds phospholipids, especially PS (Moravcevic et al., 2010). We discovered that *CaCT1.3* binds phosphoinositides. We are unable to test the interaction of *CaGin4*’s KA1 domain with phospholipids because repeated efforts failed to obtain soluble GST-KA1 fusion protein. Although both CT1 and CT1.3 showed binding to phosphatidic acid (PA) and phosphatidylserine (PS) in the PIPstrip assays, the control GST exhibited strong binding to PA and PS as well. Nonetheless, we conclude that CT1.3 interacts with phosphoinositides and is crucial for *CaGin4*’s PM association.
SBD—a domain that directly associates with the septins

Septins and Gin4 colocalize throughout most of the cell cycle, and Gin4 was always abundantly immunopurified with the septin complex (Li et al., 2012; Mortensen et al., 2002), suggesting direct Gin4-septin interaction. However, the Gin4 domain responsible for septin association remained undefined. In yeasts, dozens of proteins associate with septins. Although Okuzaki et al. (1997) identified a growth-inhibitory domain in ScGin4 showing the bud-neck localization, no general septin binding motifs have been reported. In this work, we identified that aa751-1050 of CaGin4 contains a potential SBD. This domain, when expressed in GIN4-ON cells, colocalizes with septins throughout the cell cycle and also coimmunoprecipitates with Cdc12. Mutant Gin4 lacking the SBD localizes diffusely to the cytoplasm and fails to support normal septin ring assembly in GIN4-OFF cells. Also, the SBD can directly bind Cdc12 \textit{in vitro}, indicative of a \textit{bona fide} SBD. Despite its ability to bind septins, septin ring assembly is not supported in GIN4-OFF cells expressing SBD alone, suggesting collaboration with other Gin4 domains is required. Indeed, cells expressing the CT2+1 fragment, encompassing only the SBD and LBD, assembled the septin ring and formed neck constrictions, albeit with cytokinesis defects. The formation of transient septin rings in the GIN4-OFF cells expressing CT1 (Fig. 2A) suggested that CT1 might also be able to associate with the septins. Indeed, co-IP experiments confirmed this interaction which, interestingly, occurs to a similar degree in GIN4-ON and GIN4-OFF cells (Fig. S1B). As CT1 does not colocalize with the septin ring, the observation seems to suggest that CT1 might interact with septin molecules in the cytosol but not those in the ring. It is possible that CT1 may have a chaperone-like function that mediates Gin4’s interaction with the septins before
septin ring assembly. Our data indicate that proper septin ring assembly requires \( CaGin4 \)'s interaction with both septins and phosphoinositides. Although septins have a lipid-binding motif near the N-terminus and can self-assemble into filaments and higher-order structures upon contact with lipid membranes, the formation of the septin ring requires more than septin polymerization (Bridges and Gladfelter, 2015; Bridges et al., 2014). Here, we show that, at least in \( C. albicans \), septin ring formation depends on Gin4’s ability to interact with septins and phospholipids. We propose that the SBD-LBD region acts as a chaperon, creating the conditions required for septin ring assembly.

**NAD—a domain that localizes to the periphery of the nucleolus, associates with Cdc14, and has a possible role in mitotic exit**

\( Gin4^{CT1\Delta} \) localizes as a single or a pair of dots in the nucleus, and we identified the smallest motif showing the same localization at aa 551-650 (CT3.3). Although the dots exhibit a SPB-like behaviour, we found that they did not colocalize with the SPB-marker Tem1 but instead localized to a site at the periphery of the nucleolus - the apparatus that sequesters the MEN effector Cdc14 (Segal, 2011; Visintin et al., 1998). Cells expressing Gin4 lacking the NAD (\( gin4^{CT3\Delta} \)) showed frequent premature spindle elongation and nuclear division within the mother cell. Even though a previous study did not detect sequestration of \( CaCdc14 \) in the nucleolus (Clemente-Blanco et al., 2006), we observed colocalization of the NAD and Cdc14 in the periphery of the nucleolus in many cells. Also, co-IP experiments suggest that the NAD and Cdc14 physically associate. We hypothesize that the NAD-mediated interaction of Gin4 with Cdc14 could play a role in mitotic exit although further investigations are necessary to unravel the exact mechanism. We have not been able to detect full-length Gin4 in the nucleus. The nuclear localization of \( Gin4^{CT1\Delta} \)
suggests that full-length Gin4 might enter the nucleus only when its interaction with phospholipids is disrupted at certain stages of the cell cycle, which could be a transient event and affects only a small fraction of Gin4 molecules. Like the LBD of CaGin4, the ScKA1 domain’s PM association was only revealed when truncated versions of the ScNim1-kinases were expressed (Moravcevic et al., 2010). Thus, further investigation is warranted to confirm if full-length Gin4 localizes to the nucleus. However, we cannot rule out the possibility that the observed nucleolar association of the NAD could be an artifact that only occurs in some truncated versions of Gin4.

Cdc14 localizes to the bud neck at cytokinesis in C. albicans (Clemente-Blanco et al., 2006) and also preferentially dephosphorylates phosphorylated CDK sites (Queralt and Uhlmann, 2008; Stegmeier and Amon, 2004). CaGin4 has nine perfect CDK sites with several phosphorylated by Cdc28, making it a potential substrate of Cdc14. Consistently, Cdc14 coimmunoprecipitates with Gin4, and recombinant Cdc14 can dephosphorylate Gin4 in vitro. In S. cerevisiae, Gin4 also associates with Cdc14 (Bloom et al., 2011). Furthermore, in cdc14Δ/Δ mutants, CaGin4 phosphorylation is significantly higher than in WT cells (Fig. 6E). Sanchez-Diaz et al. (2008) proposed that Cdc14 dephosphorylation of CDK substrates in anaphase is central to the initiation of cytokinesis. Our data suggest that Gin4 could be an important Cdc14 target for this mitotic event.

In summary, our studies have uncovered multiple functional domains in the nonkinase region of Gin4. The findings provide new insights into how Gin4 regulates septin assembly and the associated cell cycle events. Figure 7 presents a model describing the role of each Gin4 domain during the cell cycle. At START, the LBD and SBD interact cooperatively with the septins and phospholipids at the presumptive bud site to initiate septin ring assembly at the bud neck. As the cell
cycle progresses, Gin4 kinase activity is activated during mitosis, which stabilizes the septin collar (Li et al., 2012). In late anaphase upon Cdc14’s recruitment to the bud neck, Cdc14 dephosphorylates Gin4 possibly through interaction with the NAD, resulting in Gin4 disassembly from the septin ring. Then cytokinesis ensues, and Gin4 is degraded.

Materials and methods

Strains and culture conditions

All strains used in this study are listed in Table S1.

*C. albicans* strains were routinely grown at 30°C in yeast extract-peptone-dextrose medium (YPD) (2% yeast extract, 1% peptone, and 2% glucose), or in glucose minimal medium (GMM) (2% glucose and 0.67% yeast nitrogen base without amino acids or with required amino acids). For *GIN4*-ON conditions, cells were cultured in maltose minimal medium (MalMM) (2% maltose and 0.67% yeast nitrogen base without amino acids or with required amino acids). For *GIN4*-OFF conditions, cells were grown overnight in GMM at 30°C and reinoculated at 1:10 dilution into fresh GMM for further growth. Hyphal induction was performed in liquid GMM or MalMM supplemented with 20% fetal bovine serum (FBS) and incubating at 37°C.
**Centrifugal elutriation**

50 ml of an overnight yeast culture was reinoculated into 450 ml of fresh media and grown to stationary phase. Cell synchronization is carried out as described in (Bensen et al., 2005).

**Plasmids construction for gene truncation, gene tagging and mutagenesis**

The pClpGFP plasmid (Zheng et al., 2003) was modified to create the truncated \( \text{GIN4} \) mutant constructs. The 3' UTR of \( \text{GAL4} \) was PCR-amplified with the addition of \( \text{PstI} \) and \( \text{MluI} \) sites to the 5' and 3' ends respectively, and cloned into the \( \text{PstI-MluI} \) sites on \( \text{pP}_{\text{MET3}}\text{GFP} \) to generate \( \text{pP}_{\text{MET3}}\text{GFPutr} \). \( \text{GIN4} \) ORF was PCR-amplified from BWP17 genomic DNA using primers that added a \( \text{NarI} \) site to the 5' and a \( \text{PstI} \) site to the 3' ends, and cloned into the \( \text{Clal-PstI} \) site on \( \text{pP}_{\text{MET3}}\text{GFPutr} \) downstream of GFP to yield \( \text{pP}_{\text{MET3}}\text{GFP-GIN4} \). \( \text{pP}_{\text{MET3}}\text{GFP-GIN4} \) was linearized at a unique \( \text{SalI} \) site within the \( \text{MET3} \) promoter for integration into the genome. \( \text{pP}_{\text{MET3}}\text{GFP-gin4}\text{CT2}\Delta \) and \( \text{pP}_{\text{MET3}}\text{GFP-gin4}\text{CT3}\Delta \) plasmids were generated by deleting the CT2 and CT3 region respectively from \( \text{pP}_{\text{MET3}}\text{GFP-GIN4} \) using Quikchange™ site-directed mutagenesis kit (Agilent Technologies, Inc.). The coding region for \( \text{gin4}\text{CT1}\Delta \), CT1, CT2 and CT3 of Gin4 were PCR-amplified using appropriate primers with \( \text{NarI} \) and \( \text{PstI} \) sites added at the 5' and 3' ends respectively, and cloned into the \( \text{Clal-PstI} \) sites on \( \text{pP}_{\text{MET3}}\text{GFPutr} \).

The targeted gene or gene fragment was PCR-amplified and cloned into \( \text{BamHI-Xhol} \) sites of pGEX-4T-1 (GE Life sciences) to generate GST-fusions. Mutation of the basic residues on CT1.3 and generation of the Cdc14\text{C274S} mutant were
performed using the Quikchange™ multi-site directed mutagenesis kit (Agilent Technologies Inc.) according to the manufacturer’s instructions.

**Protein extractions, Western blot (WB) and Coimmunoprecipitation (co-IP)**

Protein work was performed as described previously (Li et al., 2012). IP for GFP-tagged proteins were performed by using either the µMACS GFP Isolation Kit (Miltenyi Biotec) or the GFP-Trap (Chromotek). Protein extraction was performed using lysis buffer containing 150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl pH8.0, complete EDTA-free protease inhibitor cocktail tablets (Roche) and phosSTOP phosphatase inhibitor cocktail tablets (Roche). Protein concentration was estimated using the BCA Protein Assay kit (Thermo Scientific). Approximately 2 mg of proteins were incubated with 50 µl of αGFP microbeads at 4°C for 2 hr. The beads were then washed four times with low-salt wash buffer (150 mM NaCl, 0.1% Triton-X, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH7.2) and once with 20 mM Tris-HCl, pH 7.5 before elution with pre-warmed elution buffer (50 mM Tris-HCl pH6.8, 50 mM DTT, 1% SDS, 1 mM EDTA, 10% glycerol and bromophenol blue ). Wash with high-salt buffer (1 M NaCl) was performed three times, followed once by low-salt wash and once with 20 mM Tris-HCl, pH 7.5 before elution. The eluted proteins were separated on 10% SDS-PAGE gels, and WB performed using appropriate antibodies. For IP of Gin4-GFP in WT (BWP17) and cdc14Δ/Δ cells, stationary phase cells were reinoculated at 1:20 dilution into pre-warmed fresh YPD media. Aliquots of cells were then collected at timed intervals for IP as described above. Protein bands on WB were quantified using ImageJ.
Purification of GST fusion protein

GST-fusion proteins (GST-CT1, GST-CT2, GST-Cdc14 and GST-Cdc14\textsuperscript{C274S}) were expressed in BL21-CodonPlus (DE3)-RIL competent cells (Agilent Technologies). Plasmid-containing cells were cultured in 10 ml LB+Amp media (100 µg/ml ampicillin) at 37°C overnight, reinoculated to 400 ml (1:100) LB+Amp media and grown at 37°C to OD\textsubscript{600} = 0.6-0.8. GST-fusion protein expression was induced at 30°C overnight by adding 1 M IPTG to a final concentration of 0.5 mM. Cell pellets were collected and washed twice with 1x PBS. Protein was extracted by resuspending in 5x volume of lysis buffer (50 mM Tris-HCl pH8.0, 150 mM NaCl, 1% Triton-x 100, 1% glycerol, 4 mM β-mercaptoethanol, 1 mM PMSF and Roche complete EDTA-free protease inhibitor cocktail), sonicated at 30% setting for 30 seconds and kept on ice for 2 min. Repeat sonication six times, spun down cell debris at 15,000 rpm for 30 min at 4°C. Collect supernatant and add 500 ml Glutathione Sepharose 4B beads (GE Healthcare; prewashed with 1x PBS). The mixture was incubated at 4°C on a roller overnight. Beads were collected using a column and washed thrice with cold high-salt buffer (50 mM Tris-HCl pH8.0 and 300 mM NaCl) and once with cold low-salt buffer (50 mM Tris-HCl pH8.0 and 150 mM NaCl). GST-bound beads were incubated with 500 ml of elution buffer (50 mM Tris-HCl pH8.0, 150 mM NaCl and 20 mM reduced glutathione) for 45 min, and supernatant collected. Protein concentrations were determined using NanoDrop™ 1000.
**Protein lipid overlay assay**

GST-CT1’s interaction with phospholipids was investigated using the PIPStrip™ (Echelon Biosciences Inc.) according to manufacturer’s protocol with slight modifications. The PIPstrips were blocked for 1 h in 1% skim dry milk in Tris-Buffered Saline-Tween (TBS-T) at room temperature before incubation with purified GST-CT1 in 5% bovine serum albumin (BSA) in TBS-T at 4°C overnight. The strips were washed 3 times for 10 min with TBS-T and then probed with anti-GST monoclonal antibody (Santa Cruz) at a dilution of 1:1000, followed by anti-mouse IgG-peroxidase antibody at a dilution of 1:5000 (Sigma-Aldrich). Both antibodies were prepared with 5% BSA in TBS-T. ECL detection was carried out with SuperSignal West Pico Chemiluminescent Substrate (Pierce-Thermo Scientific).

**In vitro phosphatase assay**

*In vitro* phosphatase assay were performed in conditions as described in (Bloom et al., 2011). GFP-Gin4 was immunopurified from log-phase cells (JY56), and washed four times with lysis buffer and then twice with 1x phosphatase buffer. λ-phosphatase (NEB) treatment was performed under the same conditions.

**Time-lapse and Fluorescence Microscopy**

Leica DMRXA2 microscope with 100X objective and a Hamamatsu digital camera interfaced with METAMORPH software (Universal Imaging) was used. Cell morphology and fluorescence protein localizations were visualized in living cells without fixing. Nucleus was stained using mounting medium with DAPI (Vectashield®). Time-lapse microscopy was performed on an inverted confocal
laser LSM700 microscope (Carl Zeiss) with attached temperature chamber and a photometrics coolsnap HQ2 digital camera interfaced with METAMORPH software (Universal Imaging). 1X GMM with 2% agarose or 1X MalMM with 2% agarose was spotted on glass slides, and cooled. Live cells were mounted onto the agar and covered with cover slip and sealed. Image recolouring and sum projection of Z-stacks was performed using Fiji (http://fiji.sc/Fiji).

Confocal microscopy and FRAP analysis

Confocal microscopy and FRAP were performed using Olympus Inverted Confocal microscope interfaced with FluoView Imaging System. Analyses were performed with Fiji.
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Competing Interests

The authors declared no competing interests.

Author Contributions

AYJY and WY, conceived and designed the experiments; AYJY and WYM performed the experiments; AYJY, WYM, and WY analyzed the data; and AYJY and WY wrote the paper.

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Figure 1. CT1 and CT2 are indispensable for Gin4 function.

A. Schematic representation of CaGin4 domain organization and construction of truncation mutants.
B. Phenotype of \( gin4^{CT1\Delta} \) expressing cells (JY8) under \( GIN4\)-ON and -OFF conditions. \( GIN4\)-ON cells were grown in maltose minimal medium and \( GIN4\)-OFF cells in glucose minimal medium respectively at 30°C to log-phase. Cells were examined by differential interference contrast (DIC) and fluorescence microscopy. Arrows point to dots formed by GFP- \( Gin4^{CT1\Delta} \). Scale bar: 10 \( \mu \)m throughout the paper unless indicated otherwise.

C. Phenotype of \( gin4^{CT2\Delta} \) expressing cells (JY31). Cells were grown and examined as described in A.

D. Phenotype of \( gin4^{CT3\Delta} \) expressing cells (JY49). For yeast cells, cells were grown as described in A. For hyphal growth, the yeast cells were induced for hyphal growth with 20% serum (FCS) at 37°C for 2 hr.
Figure 2. CT1 contains domains for phospholipid binding and the PM localization.
A. Phenotype of CT1-expressing cells (JY9). Yeast and hyphal cells were grown and examined as described in Figure 1. Arrows point to septin rings at the junction between a new bud and the mother cell.

B. CT1.3 (aa1151-1250) mediates the PM localization. CT1 was truncated into smaller fragments, each being tagged with GFP at the N-terminus and expressed in LCR43 strain. All seven basic residues were replaced with serine in CT1.3 to yield CT1.37S (JY23).

C. Purified GST-CT1 and GST-CT1.3 binds to phosphoinositides in vitro. GST, GST-CT1 and GST-CT1.3 expressed and purified from E. coli were tested for their ability to bind phospholipids by using the PIPstrips™.
Figure 3. CT2 localizes to the bud neck and interacts with septins.

A. CT2 and two sub-fragments (2.1 and 2.2) tagged with N-terminal GFP were expressed in gin4Δ/P_{MAL2}-GIN4 cells coexpressing Cdc12-mCherry (JY35, JY37, and JY39). Cells were grown in GIN4-ON conditions at 30°C for microscopy.

B. Co-IP of CT2 with Cdc12. C-terminally Myc-tagged Cdc12 was expressed in gin4Δ/P_{MAL2}-GIN4 cells coexpressing GFP-CT2 (JY40), gin4Δ/P_{MAL2}-GIN4 cells expressing Cdc12-Myc (JY69) alone were included as a negative control. Left panel, cells were grown under GIN4-ON or -OFF conditions at 30°C to log-phase; Co-IP was carried out with αMyc beads and washed with buffer containing 150 mM NaCl, followed by WB analysis using αGFP or αMyc. Right panel, IP αMyc beads were washed with buffer containing 1 M NaCl before WB analysis.

C. Purified GST-CT2 pulls down Cdc12 from C. albicans cell lysates. GST-CT2 purified from E. coli were incubated with GST-Trap beads. GST-Trap beads
with or without bound GST-CT2 were mixed and incubated with *C. albicans* cell lysates expressing Cdc12-Myc (JY69). After washes with buffer containing 150 mM or 1 M NaCl, proteins on the beads was probed with αMyc or αGST in WB.
Figure 4. The CT2+1 fragment could restore septin ring formation and localization to the bud neck in *GIN4*-OFF yeast cells.

A. Cells expressing Cdc12-mCherry (JY70) were cultured overnight under *GIN4*-ON conditions at 30°C. Elutriated G1 cells were released into *GIN4*-ON or *GIN4*-OFF conditions. Cells at different stages of budding were harvested for microscopy.
B. G1 cells coexpressing Cdc12-mCherry and GFP-CT2+1 (JY71) were prepared as described above and released into GIN4-OFF conditions for further growth. Cells at different stages of bud growth (upper panel) and of a overnight culture (lower panel) were harvested for microscopy.

C. Cells coexpressing Myo1-mCherry and GFP-CT2+1 (JY73) were grown under GIN4-ON or GIN4-OFF conditions at 30°C to log-phase before microscopy. Arrows point to Myo1 at the bud neck.

D. Cells coexpressing Cdc12-mCherry with GFP-Gin4 (JY30) or GFP-CT2+1 (JY71) were grown overnight under GIN4-OFF conditions before shifting to GMM+20% FCS for hyphal induction at 37°C for 2 hr.

E. The same cells as described in D at 3.5 hr of hyphal induction.

F. FRAP analysis of the septin ring in cells expressing WT Gin4 (JY70; n=13) or CT2+1 as the sole Gin4 source (JY71; n=12). Cells with a medium-sized bud were selected for FRAP and the entire septin ring was bleached. Fluorescence recovery was measured at 5-min intervals after photobleaching. Average values were used to generate the curves.
Figure 5. CT3 contains a domain for localization to the nucleolus.

A. GIN4-OFF gin4<sup>CT3Δ</sup> cells exhibited a multi-nucleated phenotype. Yeast cells of strain JY49 grown under GIN4-OFF conditions were DAPI-stained to visualize the nuclei.

B. Time-lapse microscopic examination of the spindle in GIN4-OFF gin4<sup>CT3Δ</sup> cells. Tem1 was C-terminally tagged with mCherry in gin4Δ/P<sub>MAL2</sub>-GIN4 (JY51) and gin4Δ/P<sub>MAL2</sub>-GIN4 gin4<sup>CT3Δ</sup> (JY50) cells. A representative GIN4-ON (upper panel) cell and a GIN4-OFF gin4<sup>CT3Δ</sup> cell (JY50) were shown.
C. CT3 localizes to the nucleus. *GIN4-ON* cells expressing GFP-CT3 (JY43) were stained with DAPI before microscopy.

D. Time-lapse microscopic examination of GFP-CT3 localization during a cell cycle in *GIN4-ON* GFP-CT3 cells (JY43).

E. Truncation of CT3 to locate the domain responsible for nucleolus foci localization. CT3 was truncated into CT3.1 (aa451-650), CT3.2 (aa451-550), and CT3.3 (aa551-650). Each fragment was tagged with GFP at the N-terminus and expressed in *gin4Δ/P* _MAL2-GIN4* cells (JY53, JY54, JY55). Cells were grown in *GIN4-ON* conditions and examined by fluorescence microscopy.

F. CT3.3 colocalizes with Nop1. Nop1 was tagged with mCherry in cells coexpressing GFP-CT3, GFP-CT3.3 and GFP-*Gin4* CTΔ (JY58, JY59, JY60).
Figure 6. Cdc14 physically associates with the NAD and dephosphorylates Gin4

A. Colocalization of CT3 and Cdc14. Cdc14-GFP was expressed from its endogenous promoter in gin4\(^{\Delta}\)/\(\text{P}_{\text{MAL2}}\)-GIN4 cells coexpressing mCherry-CT3
(JY62) or mCherry-Gin4CT1Δ (JY63). Cells were grown in GIN4-ON conditions and examined by fluorescence microscopy.

B. The NAD coimmunoprecipitates with Cdc14. Cdc14 was Myc-tagged C-terminally in gin4Δ/PMAL2-GIN4 cells coexpressing GFP-Gin4, GFP-CT3, GFP-CT3.3 and GFP-Gin4CT1Δ (JY64, JY65, JY66, JY67). Co-IP was carried out in GIN4-ON cells by pulling down proteins using αGFP beads, followed by WB analysis of the precipitates using αMyc or αGFP.

C. Cdc14 can dephosphorylate Gin4 in vitro. GST-Cdc14 and GST-Cdc14C275S were purified from E. coli and mixed with immunopurified GFP-Gin4 (JY56) for in vitro phosphatase assays. Gin4 and phos-Gin4 in the reaction products were detected by WB using αGFP and αPScdk respectively. λ-phosphatase-treated sample was included as a positive control.

D. Involvement of Cdc14 in Gin4 dephosphorylation in vivo. Gin4-GFP was expressed from its endogenous promoter in WT (BWP17) and cdc14Δ/Δ cells, coexpressing Nop1-mCherry (JY72 and JY68). Stationary phase cultures were released into fresh medium for synchronous growth. Aliquots were collected at timed intervals for microscopic evaluation of cell cycle progression (n>100, upper panel). The rest of the cells were subjected to αGFP-IP and WB analysis using αPScdk or αGFP to reveal phos-Gin4 and total Gin4-GFP (lower panel). Phos-Gin4-GFP signal (upper band) was quantified, by analysing with ImageJ and normalizing against the Gin4-GFP signal. The level of Phos-Gin4-GFP at each time point is shown under the blot.
Figure 7. A schematic description of Gin4’s function during a cell cycle.
Figure S1

A. Phenotype of *GIN4*-ON and *GIN4*-OFF CT1.37S cells. Strain co-expressing GFP-CT1.37S and Cdc12-mCherry were grown under *GIN4*-ON and *GIN4*-OFF conditions. Images were taken using epifluorescence microscope. *GIN4*-OFF CT1.37S cells grew as highly elongated pseudohyphae; also, unlike the cells that express CT1 (Fig. 2), Cdc12-mCherry ring was not detected at the base of new buds in *GIN4*-OFF CT1.37S cells. Scale bar =10 μm.

B. Blot for co-immunoprecipitation of CT1 and Cdc12 in *GIN4*-ON and *GIN4*-OFF cells. Immunoprecipitation was performed with αMyc beads from a strain co-expressing GFP-CT1 and Cdc12-Myc in both *GIN4*-ON and *GIN4*-OFF conditions. Strains expressing only GFP-CT1 or Cdc12-Myc were used as negative and positive controls respectively. The blot was probed with αGFP (upper panel) and αMyc (lower panel) antibodies respectively in WB analysis. The data show that GFP-CT1 co-precipitated with Cdc12-Myc in comparable amount in both *GIN4*-ON and *GIN4*-OFF cells.
Figure S2

A. Bright-field images for GIN4-shutdown strain (gin4Δ::P_{MAL2}^:-GIN4) grown overnight (16 h) in GIN4-OFF medium and in GIN4-OFF hyphal-inducing (+FCS) conditions. Overnight cultures of GIN4-OFF gin4Δ::P_{MAL2}^:-GIN4 cells grew as highly filamentous pseudohyphae cells, no single round cells can be seen (left panel). GIN4-OFF pseudohyphae did not produce true hyphae in response to the hyphal induction (right panel). The pseudohyphal tips continued elongation forming elongated cell compartments with unparalleled cell wall and septal constrictions.
B. Bright-field images for CT2+1 strain (gin4∆::P_{MAL2}GIN4 P_{MET3}GFP-CT2+1) grown overnight (16 h) in GIN4-OFF medium and in GIN4-OFF hyphal-inducing (+FCS) conditions. Overnight cultures of GIN4-OFF CT2+1 contained clusters of round cells, with some being separated (left panel, as indicated by arrows); suggesting that these separated round cells were due to the partial functional rescue by CT2+1. GIN4-OFF CT2+1 cells responded to hyphal induction by forming long true hyphae (right panel). The hyphal cells were formed from single round cells with paralleled cell wall and no septal constrictions.

C. Bright-field images for CT2+1 strain (gin4∆::P_{MAL2}GIN4 P_{MET3}GFP-CT2+1) grown overnight (16 h) in GIN4-OFF medium with the addition of cysteine and methionine, and in GIN4-OFF hyphal-inducing (+FCS) conditions with the addition of cysteine and methionine. GIN4-OFF CT2+1-OFF grew as highly elongated pseudohyphae cells and did not form single round cells (left panel). GIN4-OFF CT2+1-OFF cells did not form true hyphae upon hyphal induction (right panel). They formed pseudohyphae with unparalleled cell wall from the pseudohyphal tips similar to cells in A.
Figure S3

A. DAPI-stained images for CT3.1 and CT3.3. Cells expressing either GFP-CT3.1 or GFP-CT3.3 were grown in GIN4-ON conditions and stained with the Vectashield mounting medium containing DAPI. GFP-CT3.1 and GFP-CT3.3 localized to the nucleus. Scale bar =10 µm.

B. Microscopy images showing cellular localization of CT3 and Tem1. Cells co-expressing GFP-CT3 and Tem1-mCherry were imaged using epifluorescence microscopy. The data show that GFP-CT3 does not colocalize with Tem1-mCherry.

C. Epifluorescence images for colocalization of mCherry-CT3 with Cdc14-GFP and colocalization of mCherry-Gin4<sup>CT1Δ</sup> with Cdc14-GFP. Epifluorescence microscopy with Z-stacks was performed on strains co-expressing mCherry-CT3 and Cdc14-GFP or co-expressing mCherry-Gin4<sup>CT1Δ</sup> and Cdc14-GFP. Images shown are projection of sum Z-stack images (5x0.4µm).
Figure S4

A. Cdc14-GFP localizes to the bud neck in GIN4-OFF gin4CT3A cells. Fluorescence microscopy was performed in a strain co-expressing mCherry-Gin4CT3A and Cdc14-GFP. Cells were first grown to large budded cells and images were taken using wide-field epifluorescence microscopy with Z-stacks. Images shown are projection of sum Z-stack images (5x0.4µm). Scale bar = 10 µm.

B. Blot for co-immunoprecipitation of CT3.3 and Cdc14 in GIN4-ON and GIN4-OFF cells. Coimmunoprecipitation experiment was performed with αGFP beads using a strain co-expressing GFP-CT3.3 and Cdc14-Myc. A strain expressing Cdc14-Myc alone was used as negative control. The blot was probed with αMyc and αGFP respectively. In both GIN4-ON and GIN4-OFF conditions, GFP-CT3.3 was able to coprecipitated Cdc14-Myc.

C. Blot for co-immunoprecipitation of Gin4CT3A and Cdc14 in GIN4-ON and GIN4-OFF cells. Coimmunoprecipitation experiment was performed with αGFP beads using a strain coexpressing GFP-Gin4CT3A and Cdc14-Myc, and a strain co-expressing GFP-Gin4 and Cdc14-Myc. A strain expressing Cdc14-Myc only was used as negative control. The blot was probed with αMyc and αGFP respectively. The results show that precipitating GFP-Gin4CT3A and GFP-Gin4 pulled down comparable amounts of Cdc14-Myc in both GIN4-ON and GIN4-OFF cells.
Table S1. *C. albicans* strains used in this study

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Reference
