CDK regulates septin organization through cell-cycle-dependent phosphorylation of the Nim1-related kinase Gin4

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Key words: Cyclin-dependent kinase, Nim1-related kinase, Septins, Cell cycle, Candida albicans

Introduction

Cyclin-dependent kinases (CDKs) control cell cycle progression in eukaryotes (Morgan, 1997; Murray, 2004). This topic has been most extensively studied in the budding yeast Saccharomyces cerevisiae. In this organism the cell cycle is driven essentially by a single CDK, Cdc28, which directly regulates crucial cell cycle events including bud morphogenesis, DNA replication, chromosome segregation and cytokinesis (Lew and Reed, 1993; Eldledge, 1996; Ayad, 2005; Moseley and Nurse, 2009). Identification of CDK substrates has proved to be extremely helpful in understanding how CDKs control diverse cellular processes (Ubersax et al., 2003; Dephoure et al., 2008). To date, approximately 80 CDK targets have been reported in S. cerevisiae, many of which are key components of cellular machines that drive particular cell cycle events (Enserink and Kolodner, 2010). Many control principles first found in S. cerevisiae have later been proved to be evolutionarily conserved.

In recent years, significant progress has also been made in the study of the pathogenic fungus Candida albicans (Berman and Sudbery, 2002; Whiteway and Bachevich, 2007). Taking advantage of certain unique traits of this organism, such as the yeast-to-hypha transition, has revealed new CDK targets and mechanisms previously unknown in other models (Wang, 2009). In particular, studies of the hyphal-specific Cdc28–Hge1 kinase (Zheng et al., 2004) have identified a range of CDK substrates that regulate key aspects of hyphal development including activation of polarized growth (Zheng et al., 2007), vectorial secretion (Bishop et al., 2010), septin dynamics (González-Novoa et al., 2008) and inhibition of cell separation (Wang et al., 2009).

Importantly, the inhibitory phosphorylation of a GTPase-activating protein (GAP) of Cdc42 by Cdc28 in promoting hyphal growth (Zheng et al., 2007) is also used to drive the bud growth in S. cerevisiae (Knaus et al., 2007; Sopko et al., 2007).

Septins are GTP-binding and filament-forming proteins (Faty et al., 2002; McMurray and Thorner, 2009), best known for their roles in cytokinesis (Finger, 2005; Longtine et al., 1996). S. cerevisiae and C. albicans have the same set of mitotic septins including Cdc3, Cdc10, Cdc11, Cdc12 and Sep7 (Shs1) (Finger, 2005; Warenda and Konopka, 2002). In these organisms, the cell cycle starts with the formation of a septin ring at the presumptive bud site (Gladfelter et al., 2001; Caviston et al., 2003; Iwase et al., 2006). When the bud emerges, the ring transforms into a collar-like structure that persists throughout the cell cycle until cytokinesis. The dynamics of septin monomers within the ring also changes in a cell-cycle-dependent manner (Dobbelaere et al., 2003). One role of the septin ring is to recruit, in an orderly manner, proteins that regulate cell cycle progression and cytokinesis (Longtine et al., 2000; Kozubowski et al., 2005). Despite all this knowledge, a key unanswered question is how septin organization and function are regulated in coordination with cell cycle phases. Several protein kinases are known to phosphorylate certain septins and affect their function. In S. cerevisiae, Cdc28 phosphorylates Cdc3 in G1 to promote disassembly of the old septin ring (Tang and Reed, 2002). Shs1 is another Cdc28 target whose phosphorylation affects bud morphogenesis and association of septins with the Nim1-related kinase Gin4 (Egelhofer et al., 2008). Cdc28 also controls septin ring assembly indirectly through the Rho GTPase Cdc42, by...
activating its guanine nucleotide exchange factor Cdc24 (Gulli et al., 2000) and by inhibiting its GAPs (Knaus et al., 2007; Sopko et al., 2007). The active Cdc42 then promotes septin ring assembly at the presumptive bud site (Gladfelter et al., 2001; Caviston et al., 2003; Iwase et al., 2006). Previous work provided evidence that Gin4 and the p21-activated kinase Cla4 target Cdc3, Cdc10 and Shs1 (Mortensen et al., 2002; Dobbelare et al., 2003; Versele and Thorner, 2004; Asano et al., 2006). In C. albicans, deleting GIN4 causes septin disorganization, cell elongation and cytokinetic failure (Wightman et al., 2004). ScGIN4 mutants exhibit similar defects, which are rather milder because of the existence of redundant kinases (Altman and Kellogg, 1997; Barral et al., 1999). We have previously shown that CaGin4 phosphorylates Cdc11 to prime it for further phosphorylation by Cdc28 during hyphal growth (Sinha et al., 2007). These observations indicate that Gin4 plays a conserved role in septin regulation. However, how CDKs and Gin4 coordinate to regulate septin organization and function in a cell-cycle-dependent fashion remains unclear.

In a search of Cdc28 substrates, we found that CaGin4 contains a cluster of nine full consensus CDK phosphorylation sites (S/TPxR/K; referred to as ‘perfect CDK sites’ hereafter) C-terminal to the kinase domain. This raised the possibility that CDKs might regulate the septins through regulatory phosphorylation of Gin4. Here, we report that Cdc28 phosphorylates and activates Gin4, which in turn phosphorylates Sep7, regulating the property of the septin complex. Thus, we have found a new signaling pathway by which a CDK regulates septin organization and function in coordination with cell cycle progression.

**Results**

C. albicans Gin4 is a Cdc28 substrate

C. albicans Gin4 (CaGin4) contains nine perfect CDK sites in a 105 amino-acid (aa; 374–479) region C-terminal to the kinase domain (Fig. 1A) with six of them occurring in tandem. Such dense clustering of CDK sites is characteristic of bona fide CDK substrates (Ubersax et al., 2003; Holt et al., 2009; Enserink and Kolodner, 2010). To confirm this, we first determined whether Gin4 is a phosphoprotein. We immunoprecipitated Myc–Gin4 using anti-Myc beads and then performed western blotting with an antibody (anti-S-P) that preferentially detects phosphorylated Ser (Ser-P) in perfect CDK sites (Zheng et al., 2007; Li et al., 2008).

**Fig. 1. Gin4 is a Cdc28 substrate.** (A) CDK phosphorylation sites in Gin4. The sequence of aa 374–479 that contains nine perfect (in bold) CDK sites. Positions of Ser/Thr in perfect and minimal (SP and TP) CDK sites are indicated. Kc, catalytic kinase domain. (B) Myc–Gin4 was pulled down from log phase yeast cells of strain LCR47 (6Myc-GIN4; see supplementary material Table S1 for a list of all strains used). Half of the sample was treated with l-phosphatase (l-Ppase) and the other half was mock-treated with the phosphatase buffer for 30 minutes at 30°C. Myc–Gin4 was detected with anti-Myc (aMyc) and anti-S-P (aPS) western blotting (WB). (C) Cell lysates (L) were prepared from LCR6 (CDC28-6Myc) and LCR39 (CDC28-6Myc GFP-GIN4) and subjected to anti-GFP (aGFP) IP followed by anti-Myc WB. (D) Left panel: cell lysates were prepared from LCR40 (CLB2-HA GFP-GIN4), JCB64 (CLB2-HA), LCR41 (CLB4-HA GFP-GIN4) and JCB77 (CLB4-HA), and subjected to anti-GFP immunoprecipitation (IP) followed by anti-HA WB. Right panel: cell lysates were prepared from LCR83 (Myc-Cln3), LCR84 (GFP-GIN4 Myc-Cln3), LCR85 (Ccn1-Myc) and LCR86 (GFP-Gin4 Ccn1-6Myc), and subjected to anti-GFP IP followed by anti-Myc WB. (E) Purified GST–Gin4 was phosphorylated with purified Cdc28-as1–Clb2 complexes (LCR82) in the presence of 25 μM 1-NM-PP1 (+) dissolved in DMSO, or DMSO alone (−) in vitro at 30°C for 30 minutes. The reaction buffer contained [γ-32P]ATP. After SDS-PAGE, protein phosphorylation was visualized by autoradiography of the gel. (F) Left panel: cells lysates were prepared from LCR81 (GFP-GIN4 cdc28-as1) and JCB64 (CLB2-HA) were grown in the presence of 25 μM 1-NM-PP1 or DMSO alone for 30 minutes before anti-GFP IP followed by anti-GFP and anti-S-P WB. Right panel: lysates were prepared from LCR44 (GFP-GIN4) and LCR45 (GFP-gin4-10A) for anti-GFP IP followed with anti-GFP and anti-S-P WB. (G) Elutriated G1 cells of LCR47 (6Myc-GIN4) were released into GMM for growth at 30°C. Samples were harvested at intervals to generate a budding index and for anti-Myc IP of Gin4 followed with anti-Myc and anti-S-P WB.

Journal of Cell Science 125 (10)2534
We found that anti-S-P reacted strongly with Gin4 (Fig. 1B) and λ-phosphatase treatment of the precipitate abolished the reaction and increased the electrophoretic mobility of Gin4. The data are thus consistent with Ser phosphorylation in CDK sites.

To determine whether Cdc28 phosphorylates Gin4 in vivo, we first examined whether Cdc28 physically associates with Gin4. Co-immunoprecipitation was conducted using a strain coexpressing GFP–Gin4 and Cdc28–Myc. A strain expressing Cdc28–Myc alone was used as a negative control. We pulled down GFP–Gin4 with a GFP antibody (anti-GFP) from cell lysates and then probed the precipitate by anti-Myc WB. Fig. 1C shows that Cdc28 was detected in the cells coexpressing GFP–Gin4 and Cdc28–Myc but not in the control cells, thus consistent with physical association of Gin4 with Cdc28 in vivo. However, the reciprocal co-immunoprecipitation did not work perhaps because of the strong association of Gin4 with the membrane-associated septin complexes (see below) and weak interaction with Cdc28. We next determined which cyclin mediates this interaction. In S. cerevisiae, Gin4 (ScGin4) was shown to be activated by Cdc28–Clb2 during mitosis (Altman and Kellogg, 1997). Therefore, we tagged the only two B-type cyclins, CaClb2 and CaClb4, individually with a hemagglutinin (HA) epitope in a strain expressing GFP–Gin4. Subsequent co-immunoprecipitation revealed association of both cyclins with Gin4 (Fig. 1D, left panel). However, the association of Gin4 with Clb2 was markedly stronger than with Clb4. This might be explained by the recent finding that Clb2 is the mitotic cyclin in C. albicans (Ofir and Kornitzer, 2010). By contrast, Gin4 association was barely detectable with either one of the two G1 cyclins Ccn1 and Cln3 (Fig. 1D, right panel). To demonstrate direct Cdc28 phosphorylation of Gin4, we conducted in vitro kinase reactions using immunopurified Cdc28-as1–Clb2 (Cdc28as1 is a Cdc28 mutant which is sensitive to the ATP analogue 1-NM-PP1) complexes to phosphorylate a Gin4 fragment (aa 347–649) that contains the nine perfect and one minimal CDK sites. This fragment was purified as a GST fusion protein (Gin4347-649) from Escherichia coli. Phosphorylation was performed in the presence of [γ-32P]ATP and phosphorylated proteins were visualized by autoradiography after SDS PAGE. Fig. 1E shows that the Cdc28-as1–Clb2 complex strongly phosphorylated Gin4347-649 and the reaction was completely blocked by the Cdc28-as1-specific inhibitor 1-NM-PP1 (Bishop et al., 2000), thus demonstrating direct Cdc28 phosphorylation of Gin4 347–649. To obtain evidence that Cdc28 also phosphorylates Gin4 in vivo, we expressed GFP–Gin4 in a cdc28-as1 mutant. 1-NM-PP1 in DMSO or DMSO alone was added to log-phase cells, which were incubated at 30˚C for 30 minutes. As shown in Fig. 1F, left panel, anti-S-P failed to detect Gin4 in 1-NM-PP1-treated cells. Furthermore, anti-S-P did not recognize the Gin4 10A mutant in which all the 10 CDK sites were replaced with Ala (Fig. 1F, right panel).

Mass spectrometry of immunopurified GFP–Gin4 detected phosphorylation in three perfect (T397, S455 and S467) and four minimal CDK sites (S477, S556, S1217 and T778) among dozens of phosphorylation sites (Table 1). Phosphorylation site mapping by tandem mass spectrometry was carried out as described previously (Sinha et al., 2007). Asterisks indicate phosphoserine or phosphothreonine residues. Consensus CDK phosphorylation sites are underlined.

**Table 1. Mass spectrometry mapping of phosphorylation sites in Gin4**

| Sequence of identified peptide | Position of phospho-residues |
|-----------------------------|-----------------------------|
| KYSLN*G*PRR                  | T397                        |
| RRQT*PVLTRR                  | T778                        |
| R*S*PRYS*YKKS                | S455                        |
| R*T*S*AIDLRLDA               | S973                        |
| RLLNS*Q*LNVRD                | S891                        |
| RYSYNSQ*SPTKS                | S473                        |
| RLSVLS*MYST*KE               | T941                        |
| R*T*S*ATFAALCDK             | S634                        |
| R*S*G*S*KYS*LNTG*PRR         | S388, S390, S393, T397     |
| KYPM*S*NEDLIS*EKS            | S294, S300                  |
| RSN*S*QRQFENELPA             | S485                        |
| K*SS*IDELANGTSTSGHRK         | S1098                       |
| RYSYNSQ*PTKS*FYGRR           | S477                        |
| RASHIS*SRPTF*FOYKKS          | S413                        |
| RYS*YNO*TPKS*PGYRR           | S469, S473, S477           |
| KSMVDS*ES*S*A*DDVFDKI        | S1077, S1078, S1080        |
| RASHIS*VS*RPT*S*FOYKKS       | S407, S409, T412, S413     |
| KSMV*S*ES*S*A*DDVFDKI        | S1073, S1076, S1077, S1079 |
| KNDYDDTF*VFS*NSDEVHRK        | T1055, S1058               |
| RGNNSGHDDS*VPPPAHKKV         | S1153                       |
| KST*KS*S*IDELANGTSTSGHRK     | T1094, S1096, S1097        |
| KNDADPNNS*EQELVEDEGIKQ       | S119                        |
| KNDYDDTFV*VNSDEVHRKQ         | S1058                       |
| ROPSSI*S*S*IMS*QSNNHIPOKI    | S10, S11, S12, S15         |
| KLLET*SCOSPHYAAPEIVSGSKLY    | T191                        |
| RALS*EGHASEELTEDVNLKR        | S745                        |
| KSELOQDEET*EKNQGLPYGIERE     | T69                         |
| KLPDGKS*T*KT*KS*S*IDELANGT*STSGHRK | S1093, T1094, S1096, S1097, T1105 |
| KYPM*S*NEDLIS*EKS*LPHPTQYKKS | S300, S303                 |
| KTIENNTNAATNTTQOQLPS*PAESKE  | S1217                       |
| RDYLPEPVTEDSNL*DDYMTEIRK     | T875                        |
| RERDYLPEPVE*DVLNLDYMTIEIRK   | T868                        |
| KAGLAEPEYET*ET*DGEDK*VSIDDLHADRR | T989, T991, S998          |
| RYMD*EPNPQQLQQPASPQPVPEDES*PDLMQSAKI | S555                  |
of Ser-P and Thr-P residues (Table 1). The results demonstrate that at least a subset of the CDK sites in Gin4 is phosphorylated in vivo. Taking together all the data above, led us to conclude that Gin4 is a Cdc28 substrate in vivo.

**Gin4 cellular levels and phosphorylation status are regulated during the cell cycle**

In *S. cerevisiae* Gin4 is phosphorylated during mitosis but its cellular levels are constant throughout the cell cycle (Altman and Kellogg, 1997). To determine whether Gin4 behaves similarly in *C. albicans*, we prepared early G1 cells by centrifugal elutriation, grew them in GMM at 30˚C and took samples at timed intervals for immunoprecipitation of Myc–Gin4. The budding index (Fig. 1G) of the culture showed that cells started to bud at ~90 minutes and underwent cytokinesis between 180 and 210 minutes. WB analysis of the immunoprecipitation products did not detect Gin4 in early G1 cells, consistent with a previous report (Wightman et al., 2004). At 60 and 90 minutes when the majority of the cells were still in G1, Gin4 was detected as a sharp band by anti-Myc but not by anti-S-P, demonstrating that Gin4 exists in hypophosphorylated forms in late G1. Strikingly, at 120 minutes when the majority of cells had already produced a bud, Gin4 was detected strongly by both anti-Myc and anti-S-P as a broad band with a trailing smear, consistent with reduced electrophoretic mobility. The result indicates Gin4 phosphorylation in later stages of the cell cycle, although the experiment could not show whether it happens in mitosis. Gin4 hyperphosphorylation persisted until ~210 minutes, when both its cellular level and degree of phosphorylation decreased substantially. Together, the results indicate that both the protein and phosphorylation level of Gin4 are regulated in a cell-cycle-dependent manner in *C. albicans*.

**Mutation of the CDK sites on Gin4 causes morphological and cytokinetic defects**

To identify the physiological significance of Gin4 phosphorylation by Cdc28, we constructed two GFP-tagged mutant versions of Gin4 in which the 10 CDK sites between aa 374 and 479 were replaced with Ala or Glu, yielding the nonphosphorylatable gin4-10A and the phosphomimetic gin4-10E mutant, respectively. The mutant genes were transformed into the GIN4/Gin4D strain to replace the wild-type (WT) copy of GIN4, yielding strains expressing either gin4-10A or gin4-10E under its native promoter as the sole source of Gin4. To confirm that the two Gin4 proteins were expressed properly, we first performed western blot analysis to examine their expression at timed intervals in synchronized cultures initiated from elutriated G1 cells. We found that Gin4-10A, Gin4-10E and WT Gin4 were expressed to comparable levels throughout the cell cycle and exhibited...
similar phosphorylation levels (Fig. 2A). However, upon closer examination, Gin4 hyperphosphorylation appeared to be moderately reduced in Gin4-10A at 180 and 210 minutes in comparison with WT Gin4 and Gin4-10E, suggesting that phosphorylation at the 10 CDK sites is required for Gin4 to achieve the maximal level of phosphorylation. Next, we examined the morphology and cellular localization of Gin4 in mutant cells. We found that although both Gin4-10E and Gin4-10A appeared to localize normally to the bud neck, they caused morphological and cytokinetic or cell separation defects. However, the gin4-10A mutant exhibited markedly stronger abnormalities than the gin4-10E mutant with considerable cell elongation and swelling and the formation of chains of cells (Fig. 2B), demonstrating that phosphorylation at the CDK sites have physiological roles. The defects were more prominent at 37°C than at 30°C (data not shown).

**Mutation of the CDK sites affects the kinase activity of Gin4**

The close proximity of CDK sites to the kinase domain in Gin4 suggests that Cdc28 might directly regulate the activity of Gin4. To test this idea, we immunopurified Myc-tagged WT Gin4, Gin4-10A and Gin4-10E and assayed their autophosphorylation ability in vitro. We started synchronous cultures with elutriated G1 cells and harvested cells at 90 and 150 minutes. Gin4 autophosphorylation was visualized by autoradiography after SDS-PAGE and the level of phosphorylation was normalized against the amount of Gin4 used in the reaction. Fig. 2C shows that autophosphorylation of Gin4-10A was markedly weaker than that of WT Gin4. At 90 minutes, the level of autophosphorylation of WT Gin4 was twice that of Gin4-10A, and at 150 minutes the difference increased to more than fivefold. Interestingly, Gin4-10E exhibited stronger autophosphorylation than WT Gin4 at 90 minutes, whereas Gin4-10E autophosphorylation was weaker than WT Gin4 at 150 minutes. Our interpretation of the results is that Cdc28 phosphorylation of Gin4 activates the kinase activity of Gin4 as cells progress through the cell cycle; and the replacement of Ser/Thr in the CDK sites with Glu to some extent mimics the effect of CDK phosphorylation, which explains the higher activity of Gin4-10E at 90 minutes when WT Gin4 has not been activated.

**Mutation of the CDK sites of Gin4 affects its association with Sep7**

In both S. cerevisiae and C. albicans, Gin4 associates with septins and regulates their organization (Longtine et al., 1998; Barral et al., 1999; Wightman et al., 2004). It has been reported that Gin4 can bind Sep7 independently of its interaction with other septins and that this interaction depends on the kinase activity of both Gin4 and Cdc28 (Mortensen et al., 2002). We thus hypothesized that Cdc28 might regulate septin organization or function through regulatory phosphorylation of Gin4. To determine whether Cdc28 phosphorylation of Gin4 affects the association of Gin4 with Sep7, we constructed strains coexpressing Sep7–Myc with GFP-tagged WT Gin4, Gin4-10A or Gin4-10E, immunoprecipitated Sep7–Myc and detected Gin4 by anti-GFP WB. Strikingly, although anti-Myc pulled down similar amounts of Sep7 from all cell lysates, the amount of coprecipitated Gin4-10A was markedly lower than that of Gin4 and Gin4-10E (Fig. 2D), indicating that the 10A mutation reduced the association of Gin4 with Sep7. This effect appeared specific for Sep7, because the association of Gin4 with Cdc12 was unaffected by the same mutations (Fig. 2E). Together, the results support the idea that Cdc28 regulates the interaction between Gin4 and Sep7, at least in part, by controlling Gin4 phosphorylation.

**Mutation of the CDK sites of Gin4 affects Sep7 dynamics at the bud neck**

We next conducted fluorescence recovery after photo-bleaching (FRAP) analysis to determine whether mutation of the CDK sites in Gin4 affects the dynamics of Sep7 in the septin ring. To compare septin rings at similar cell-cycle stages, we selected small budded cells (n=10) with a bud diameter of about one third that of the mother cell. We bleached the full ring and then recorded fluorescence recovery at 1-minute intervals. The result showed that the fluorescence intensity of Sep7–GFP recovered to a maximal level of ~32% of the pre-bleach level between 2–3 minutes in WT cells (Fig. 3A,B). This was in stark contrast to a very slow and poor recovery to a maximal level of ~10% in gin4-10A cells. The recovery rate and level in gin4-10E cells were intermediate between WT and gin4-10A cells. Similar results were obtained when half of the septin ring was photo-bleached (data not shown). The data suggest that phosphorylation at the CDK sites of Gin4 has a role in regulating Sep7 dynamics in the septin ring.

Next, we conducted time-lapse microscopy analysis on Sep7 dynamics at the bud neck in live cells. We tagged Sep7 with mCherry and tagged the tubulin protein Tub2 with GFP to monitor the status of the spindle as a cell-cycle marker. Because Gin4 hyperphosphorylation occurs in late stages of the cell cycle, we examined whether it might affect Sep7 disassociation from the septin ring after mitosis. We used the time point when the spindle elongation was first detected to approximate telophase (Fig. 3C). We observed that Sep7 disappeared from the bud neck ~28 minutes after the onset of spindle elongation in WT cells (Fig. 3D). In contrast, this process took only ~10 minutes in gin4-10A cells, indicating premature Sep7 disassociation from the bud neck. Again, the gin4-10E cells exhibited an intermediate phenotype between WT and gin4-10A cells. This is perhaps due to the failure of the E mutation to fully mimic phosphorylation because of differences in the number of charges and structure between the phosphate group and the glutamic residue. Nevertheless, the results support the idea that Cdc28 phosphorylation of Gin4 regulates the timing of Sep7 association from the bud neck.

**Gin4 phosphorylates Sep7**

Gin4 forms a stable complex with septins (Altman and Kellogg, 1997). To examine whether Gin4 can phosphorylate Sep7 and other septins, we purified the Gin4–septin complex by targeting GFP–Gin4 and then examined which components could be phosphorylated by Gin4 in vitro. To reduce binding of non-specific proteins, lysis and wash buffers contained 1 M KCl. The purified proteins were resolved by SDS-PAGE, visualized by Coomassie Blue staining and identified by mass spectrometry (Fig. 4A). The complex appeared ‘clean’, because all the major bands are either septins or well-known septin- or bud-neck-associating proteins, including nearly all the proteins identified previously in S. cerevisiae septin complexes (Altman and Kellogg, 1997; Carroll et al., 1998), such as Cdc3, Cdc10, Cdc11, Cdc12, Sep7, the Cbl2-associated protein Nap1 and the Nim1 kinase Hsl1. The data indicate evolutionary conservation of
this protein complex in fungi. An unexpected finding is Hof1, which is known to localize to the bud neck and has a role in cytokinesis, but has not been found in purified septin complexes previously (Altman and Kellogg, 1997; Carroll et al., 1998; Kaneko et al., 2004). The interaction was further confirmed by co-immunoprecipitation (data not shown).

For in vitro kinase reactions, purified Gin4–septin complexes were incubated in a buffer containing [\(c^{32}\)P]ATP for 30 minutes before SDS-PAGE and autoradiography. Fig. 4B shows that in addition to the strong band of autophosphorylated Gin4, there were two other prominent bands that matched the size of Sep7 and Cdc11. Two other weaker bands were around the sizes of Cdc3 and Nap1; further characterization of which will be published elsewhere. Gin4 phosphorylation of Cdc11 was expected because it was detected previously (Sinha et al., 2007). To confirm Gin4 phosphorylation of Sep7, we repeated the kinase reaction using Gin4–septin complexes purified from a sep7\(^{D}\) mutant. The Sep7 band was not observed. However, when immunopurified GFP–Sep7 was added to the reaction, a new band corresponding to GFP–Sep7 appeared. By contrast, incubating the same amount of immunopurified GFP–Sep7 alone resulted in only weak phosphorylation of some proteins, probably due to the presence of low levels of co-purified Gin4.

To exclude the possibility that the phosphorylation of Sep7 and Cdc11 is the result of other kinases present in the complex, such as Hsl1, we created a catalytically inactive gin4\(^{K57A}\) allele equivalent to Scgin4\(^{K48A}\) (Altman and Kellogg, 1997) and transformed it into the GIN4\(^{\Delta}\) strain to replace the WT copy of GIN4. We made two interesting observations of this strain. First, it showed much milder morphological and cytokinetic defects than Gin4-depleted cells, suggesting that certain important functions of Gin4 might be associated with the nonkinase domain (see evidence in Fig. 5E). Second, the septin complex was largely intact except that the amount of Sep7, Nap1 and Hof1 was reduced (Fig. 4A), indicating that the kinase activity of Gin4 has a role in maintaining the integrity of the septin complex. Importantly, Mortensen et al. also reported complete abolition of the Gin4–Sep7 association in the Scgin4\(^{K48A}\) mutant, indicating that the mechanism governing this interaction is conserved (Mortensen et al., 2002). Kinase assays showed that the K57A mutation nearly completely eliminated the kinase activity (Fig. 4B). These results strongly suggest that Sep7 is a Gin4 substrate in vivo.

Gin4 has functions in G1 essential for septin ring assembly

Previous data on the role of ScGin4 in G1 have been controversial. Kellogg and colleagues showed that Gin4 is activated and physically associates with septins only during mitosis (Mortensen et al., 2002; Altman and Kellogg, 1997). Asano et al. showed that the neck-associated kinase Elm1 phosphorylates and activates Gin4 in mitosis (Asano et al., 2006). However, other studies suggested a role for Gin4 in G1 on the basis of the synthetic lethality of gin4\(^{\Delta}\) with cln1\(^{\Delta}\) cln2\(^{\Delta}\) (Benton et al., 1997), colocalization of Gin4 and septins before bud emergence and evident abnormal septin organization in gin4\(^{\Delta}\) cells with tiny buds (Longtine et al., 1998). In C. albicans,
switching off \textit{GIN4} was shown to impair septin organization, consistent with a role for Gin4 in G1 (Wightman et al., 2004). To further address this controversy and to carry out a more detailed analysis of the role of Gin4 in \textit{C. albicans}, we constructed a \textit{GIN4}-shutoff strain in which one copy of \textit{GIN4} was deleted and the other was placed under the control of the \textit{MAL2} promoter.

Using this strain, we can turn on \textit{GIN4} in media containing maltose as the sole carbon source and shut it off by adding glucose. Because Gin4 is undetectable in early G1 cells, shifting such cells of the \textit{GIN4}-shutoff strain previously grown in maltose medium into glucose medium allowed us to examine the involvement of Gin4 role in septin ring assembly. A quick check of the strain grown in glucose medium revealed strong pseudohyphal growth (Fig. 5A) reminiscent of \textit{gin4} mutants described previously (Wightman et al., 2004), indicating a clean shutoff of the WT \textit{GIN4}. To assess how Gin4 depletion affects septin ring assembly, Cdc12 was tagged with GFP. In maltose medium, Cdc12–GFP localized normally throughout the cell cycle. By contrast, G1 cells released into the glucose medium did not show bud-neck localization of Cdc12–GFP at any stage of the cell cycle; instead it localized persistently at the bud tip (Fig. 5B). Furthermore, the cells were highly elongated and later formed long pseudohyphae-like filaments similar to those shown in Fig. 5A. Other septins localized similarly under the same condition (data not shown). Gin4 depletion in G1 also caused a complete loss of the neck localization of several other proteins including the polarisome protein Spa2 (Zheng et al., 2003), the actomyosin ring component Iqg1 (Li et al., 2008) and the exocyst landmark Sec3 (Li et al., 2007) (see Fig. 6C). The data indicate that Gin4 has functions in G1 essential for septin ring assembly and the construction of a functional neck. Consistently, we detected co-immunoprecipitation of Myc–Gin4 and GFP–Cdc12 well before bud emergence in synchronized cultures (Fig. 5C). Moreover, Cdc12–GFP was found to colocalize perfectly with mCherry-tagged Gin4 at the presumptive bud site and then bud neck throughout the cell cycle until the end of cytokinesis when the septin ring split and the Gin4 ring disappeared (Fig. 5D).
Our data seem to contradict the mitotic activation of Gin4 observed in S. cerevisiae (Altman and Kellogg, 1997; Mortensen et al., 2002). Although Gin4 might be regulated differently in the two organisms, another possibility is that the nonkinase domain of Gin4 plays an essential role in septin ring assembly. This hypothesis is supported by our observations that the gin4<sup>K57A</sup> cells exhibited markedly milder defects than gin4Δ cells. To exclude the possibility that the weak phenotype of the gin4<sup>K57A</sup> mutant is due to its residual kinase activity, we expressed a GIN4-<sup>6Myc</sup> mutant is due to its residual kinase activity, we expressed a GIN4-<sup>6Myc</sup> allele. Cells of this strain were much less elongated under conditions that switched off GIN4 (Fig. 6A). Expression of cdc42<sup>Q61L</sup> also restored septin ring assembly as revealed by tagging Cdc12 with GFP, and greatly improved the bud neck localization of Spa2, Iqg1 and Sec3 (Fig. 6B). Thus, forced activation of CDC42 can substantially restore septin ring assembly and the construction of a functional neck in the absence of Gin4. The results seem to suggest that either Cdc42 and Gin4 act in parallel pathways or that Cdc42 works downstream of Gin4 in this process. However, because cdc42<sup>Q61L</sup> could only partially rescue the phenotype of Gin4 depletion, we favor the idea that the two proteins act in parallel pathways in promoting septin ring assembly.

**Physical interactions of the Cdc42 module with Gin4 and septins**

The crucial role of both Cdc42 and Gin4 in septin ring assembly, along with their colocalization to the presumptive bud site suggest possible physically association of Gin4 with Cdc42 or with one of the Cdc42-associated proteins such as Cdc24, Bem1 and Cla4 (Howell et al., 2009). To test this idea, we constructed strains co-expressing GFP–Gin4 with Cdc42–Myc, Bem1–Myc or Cdc24–HA, and a strain co-expressing Myc–Gin4 and Cla4–GFP. Co-immunoprecipitation experiments detected Gin4 associated only with Bem1 (Fig. 6C) but not with Cdc42, Cdc24 and Cla4 (data not shown). Because studies in S. cerevisiae suggested that Bem1 is a scaffold that has docking sites for Cdc42, Cdc24 and Cla4 together forming a functional module (Bose et al., 2001; Howell et al., 2009), it is likely that Gin4 is associated with this complex through Bem1 in C. albicans. Because Cdc42 overexpression can promote septin ring assembly in the absence of Gin4, we also examined whether...
Cdc42 is able to associate with septins. Indeed, we found that pulling down Cdc12–GFP could co-precipitate Cdc42–Myc (Fig. 6D). Together, the results revealed physical associations of the Cdc42 module with Gin4 and septins. This interaction network might underpin the regulation of septin organization and function.

Discussion
In eukaryotes, the cell-cycle engine controls the assembly of cytoskeletal structures, and in turn the integrity of these structures is monitored by cell-cycle checkpoints that can temporarily halt the engine when detecting problems (Keaton and Lew, 2006; Musacchio and Salmon, 2007). Studies in *S. cerevisiae* have established a signaling pathway that inhibits Cdc28 in response to septin defects (Keaton and Lew, 2006). However, the pathway(s) by which Cdc28 regulates the septins is poorly understood. In this study of *C. albicans*, we have discovered a signaling pathway linking Cdc28 with the septins.

**Gin4 phosphorylation by Cdc28**
In *S. cerevisiae* several protein kinases are involved in Gin4 phosphorylation, including Cla4, Elm1 and Cdc28. Deleting ELM1 or CLA4 leads to Gin4 hypophosphorylation (Sreenivasan and Kellogg, 1999; Tjandra et al., 1998); and inhibition of mitotic Cdc28 abolishes Gin4 hyperphosphorylation (Mortensen et al., 2002). However, the evidence for direct phosphorylation of Gin4 by Cdc28 is controversial. In vitro studies with purified recombinant proteins from insect cells showed that Elm1 directly phosphorylated and activated Gin4, but Cdc28 failed to do so (Asano et al., 2006). The negative result might be due to the absence in the recombinant proteins of additional factors, or a proper structural context required for Cdc28 phosphorylation of Gin4. For example, association with septins or prior phosphorylation of Gin4 by other kinases might be required (Sinha et al., 2007). In support of this argument, purified Cdc28–Cib2 complexes could phosphorylate Gin4 in whole yeast cell extracts (Ubersax et al., 2003). Kellogg and colleagues also thought that Cdc28–Cib2 might phosphorylate Gin4 because of an exact correlation of Gin4 phosphorylation and activation with the rise of Cib2 and their dependence on the mitotic CDK activity (Altman and Kellogg, 1997; Mortensen et al., 2002). Furthermore, ScGin4 contains 11 consensus CDK phosphorylation sites. Two of them are perfect sites residing with four minimal sites in a short region at a position similar to the CDK sites in CaGin4. In light of our new findings, it is highly likely that ScGin4 is also an in vivo Cdc28 substrate. It is noteworthy that mass spectrometry mapped nearly all 13 Elm1 phosphorylation sites on Gin4 to a 200-aa region C-terminal to the kinase domain and, intriguingly, several sites are CDK sites (Asano et al., 2006). Therefore it is possible that the use of recombinant Elm1 and Gin4 might have resulted in phosphorylation at both the CDK and Elm1 sites densely clustered in the same region, causing the observed Gin4 activation in vitro.

**Sep7 is a regulatory target of the Cdc28–Gin4 pathway**
Using purified Gin4–septin complexes in kinase reactions in vitro, we obtained evidence that Gin4 phosphorylates Sep7. Previous studies in *S. cerevisiae* have also reported Gin4 phosphorylation of Sep7 (Mortensen et al., 2002; Dobbelaere et al., 2003; Asano et al., 2006), indicating a conserved regulatory mechanism. One direct consequence of Sep7 phosphorylation by Gin4 appears to be the stabilization of the Gin4–Sep7 association.

We observed that Gin4-10A, which has low kinase activity, exhibited markedly reduced co-immunoprecipitation with Sep7, and when we pulled down Gin4 from the kinase-dead *gin4K57A* mutant, the amount of Sep7 in the Gin4–septin complex was greatly decreased, whereas that of other septins was largely unaffected. Similarly, the Gin4–Sep7 interaction was undetectable in Scgin4*Δ* mutants (Mortensen et al., 2002). Moreover, our results and those for *S. cerevisiae* suggest that Gin4 associates with Sep7 by a mechanism independent of the one that mediates the interaction of Gin4 with other septins. ScGin4 can associate with Sep7 under conditions where no Gin4–Cdc11 interaction can be detected (Mortensen et al., 2002). This specific Gin4–Sep7 interaction might mediate the control of septin organization by Cdc28. Indeed, the septin ring structure in *gin4-10A* cells was less stable than that in WT cells, as demonstrated by FRAP. Furthermore, after mitosis, septin rings disassembled much earlier in *gin4-10A* cells. These results strongly indicate that Cdc28 regulates septin ring structure by phosphorylation-dependent regulation of Gin4 that similarly regulates Sep7.

**Gin4 has kinase-activity-independent functions**
Our observation that the *gin4K57A* and *gin4Δ* mutants exhibit milder defects than *gin4A* mutants suggests that Gin4 has functions independent of its kinase activity. Longtine et al. made a similar observation of ScGin4 and hypothesized that the nonkinase region plays a structural role (Longtine et al., 1998). Our data show that switching off *CAGIN4* in *G1* leads to septin mislocalization to the bud tip. By contrast, both *gin4K57A* and *gin4Δ* alleles support septin ring assembly, indicating that the Gin4 kinase activity is not essential for septin ring assembly. We detected physical association of Gin4 and Cdc12 well before bud emergence, suggesting that the nonkinase region of Gin4 is involved in septin ring assembly from the very beginning. The fact that loss of kinase activity does not significantly affect the association of Gin4 with the core the septins, Cdc3, Cdc10, Cdc11 and Cdc12, further supports a structural role of the nonkinase region in septin organization. However, the *gin4K57A* and *gin4Δ* cells have cytokinetic defects, indicating that the kinase activity is required for septin functions at later stages. In *S. cerevisiae*, Gin4 is phosphorylated and activated during mitosis (Altman and Kellogg, 1997; Mortensen et al., 2002). Although we did not determine the exact time of Gin4 phosphorylation in this study, its hyperphosphorylation was only detected after most cells have budded in a synchronous culture. Previously, we found that Gin4 phosphorylates Cdc11 only during mitosis (Sinha et al., 2007). In *S. cerevisiae*, although Gin4 is not essential for septin ring assembly, *gin4Δ* mutants start to exhibit defects in septin organization early in bud growth (Longtine et al., 1998). Moreover, the synthetic lethality of *gin4Δ* with *cln1Δ cln2Δ* (Benton et al., 1997) and septin colocalization with Gin4 before bud emergence (Longtine et al., 1998) supports a role for Gin4 in G1.

Combining the known roles of Cdc28 and the Cdc42 module in promoting septin ring assembly in *S. cerevisiae* and our new findings here, we propose that Cdc28 regulates septin organization at two discrete times of the cell cycle: G1 and mitosis, and both intimately involve Gin4. The involvement of Gin4 in G1 does not require its kinase activity; instead it plays a structural role in cooperation with the Cdc42 module to promote septin ring assembly. In mitosis, Cdc28–Cib2 phosphorylates and
activates Gin4, which in turn phosphorylates Sep7, regulating the property of the septin complex (Fig. 7).

Materials and Methods

Strains and growth conditions

C. albicans strains (supplementary material Table S1) were grown and G1 cells were prepared by centrifugal elution as described previously (Li et al., 2008).

Gene deletion

Strain D27817 was used to create all the gene deletion mutants in this study. A deletion cassette contained a marker gene, URA3, HIS1 or ARG4 flanked with AB and CD DNA fragments corresponding to the 5' and 3' flanking regions of the target gene, respectively. To construct the GIN4-shutoff strain, a URA3 flipper cassette was constructed by flanking the URA3 flipper (Morschhäuser et al., 1999) with the AB and CD DNA fragments of GIN4. This cassette was used to disrupt one copy of GIN4. The second copy was placed under the control of the MAL2 promoter as described previously (Walther and Wendland, 2008).

Epitope tagging of proteins

To express GFP–Gin4 from the native promoter, a 1206-bp region of the GIN4 promoter was PCR amplified with KpnI and XhoI sites added to the 5' and 3' ends, respectively, cleaved with KpnI and XhoI, and cloned at the KpnI–XhoI sites of the plasmid pClpGFPutr (Zhang et al., 2003), yielding pClpP~gin4GFPutr. The N-terminal region of nucleotides 1–1798 of the GIN4 open reading frame was then PCR amplified with NarI and PstI sites added to the 5' and 3' ends, respectively, cleaved with NarI and PstI, cloned at the Clb5–Prl sites of the plasmid pClpP~gin4GFPutr downstream of GFP. The resulting plasmid, pT~gin4GF–Gin4, was linearized with BamHI at nucleotide 1043 and transformed into the GIN4/ gin4A strain or the Gin4-shutoff strain. The plasmid pT~gin4GF–Gin4 was used to generate GFP–GIN4–10A and GFP–GIN4–10E using the Quick-Change Multi-Site-Directed Mutagenesis Kit (Stratagene). A unique BglII site was generated at nucleotide 1449 for linearization and transformation into the GIN4/shutoff strain. A similar strategy was used to generate Myc–GIN4, Mhc–GIN4–10A and Myc–GIN4–10E.

Co-immunoprecipitation

Generally, 500 μl of the cell pellet was suspended in 1.5 volumes of lysis buffer containing 50 mM Tris, pH 7.5, 1 mM EDTA, 1% NP-40, 150 mM KCl and EDTA-free protease inhibitor mix before incubation with 400 μl anti-GFP-coupled beads (Santa Cruz Biotechnology) at 4°C for 2 hours. The beads were washed four times with 1 ml of the lysis buffer. Proteins were eluted by adding 5× loading dye and boiling for 5 minutes. The supernatant was concentrated by freeze-drying before SDS-PAGE. The gel was stained with Coomassie Blue and protein bands were excised for mass spectrometry identification and phosphorylation mapping as described previously (Li et al., 2008).

In vitro kinase assay activity

For Gin4 autophosphorylation, cells expressing GFP–Gin4 from 1 liter of log culture were extracted in 4 ml lysis buffer containing 50 mM Tris–HCl, pH 7.4, 1% NP-40, 1 M KCl, EDTA-free protease inhibitor mix and incubated with 200 μl anti-GFP-coupled beads (Santa Cruz Biotechnology) at 4°C for 2 hours. The beads were washed four times with the same lysis buffer followed by one wash in 1× standard kinase reaction buffer (Sinha et al., 2007). The beads were resuspended in 200 μl reaction buffer containing 10 μl of [γ-32P]ATP (6000 Ci/mmol) and incubated at 30°C for 30 minutes. The reaction was terminated by two washes with 400 μl reaction buffer and boiling in 1× loading dye for 5 minutes. Phosphorylated proteins were separated by SDS-PAGE and visualized by autoradiography. For Cdc28-as1 phosphorylation of GST–Gin4 fusion proteins, Cdc28-as1–Clb2–Myc was immunopurified using anti-Myc-coupled beads in the presence of 1 M KCl as described previously (Harvey et al., 2005). Equal amounts of purified Cdc28-as1–Clb2–Myc were used to phosphorylate 4 μg of GST–Gin4 in the presence of 25 μM 1-NNPP1 dissolved in DMSO or DMSO alone. The reaction was terminated by boiling in 1× loading dye. Phosphorylation of the GST–Gin4 proteins was visualized by autoradiography of the gel after SDS-PAGE.

FRAP and time-lapse microscopy

FRAP was performed on an inverted confocal laser LSM700 microscope (Carl Zeiss). The septin ring was bleached with a 488 nm laser for 25 iterations at 50% intensity. After the bleaching, images were collected every 30 seconds. Fluorescence intensity was analyzed using ImageJ (http://rsb.info.nih.gov/ij). For each strain, at least ten individual recovery curves were determined. Average values of the bleached region of the septin ring were calculated. A reference cell in the imaging field was used to correct for general bleaching. Time-lapse microscopy was performed as described previously (Li et al., 2008).

Acknowledgements

We thank members of the Wang laboratory for critical reading of the manuscript, and Judith Berman for providing strains.

Funding

This work was funded by the Agency for Sciences, Technology and Research of Singapore.

Supplementary material available online at http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.104497/-/DC1

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Immunopurification and mass spectrometry analysis of Gin4–septin complex

To purify the Gin4–septin complex, cells from 3 liters of log cultures were extracted in 12 ml lysis buffer containing 50 mM Tris–HCl, pH 7.4, 1% NP-40, 1 M KCl and EDTA-free protease inhibitor mix before incubation with 400 μl anti-GFP-coupled beads (Santa Cruz Biotechnology) at 4°C for 2 hours. The beads were washed four times with 1 ml of the lysis buffer. Proteins were eluted by adding 5× loading dye and boiling for 5 minutes. The supernatant was concentrated by freeze-drying before SDS-PAGE. The gel was stained with Coomassie Blue and protein bands were excised for mass spectrometry identification and phosphorylation mapping as described previously (Li et al., 2008).

Co-immunoprecipitation

Generally, 500 μl of the cell pellet was suspended in 1.5 volumes of lysis buffer containing 50 mM Tris, pH 7.5, 1 mM EDTA, 1% NP-40, 150 mM KCl and EDTA-free protease inhibitor mix (Roche). Cells were lysed using Tomy microsmash by five rounds of bead-beating for 45 seconds at 5000 rpm at 4°C with cooling for 1 minute on ice between rounds. Cell lysates were clarified by centrifugal elutriation as described previously (Li et al., 2008).

Materials and Methods

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Funding

This work was funded by the Agency for Sciences, Technology and Research of Singapore.

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