The IQGAP Iqg1 is a regulatory target of CDK for cytokinesis in *Candida albicans*

Chang-Run Li, Yan-Ming Wang and Yue Wang*

Genes and Development Division, Institute of Molecular and Cell Biology, Agency for Science, Technology and Research of Singapore, Singapore, Singapore

Cyclin-dependent kinases (CDKs) drive and coordinate multiple cell-cycle events, including construction and contraction of the actomyosin ring during cytokinesis. However, it remains unclear whether CDKs regulate cytokinesis by directly targeting components of the ring. In a search for proteins containing consensus CDK phosphorylation sites in *Candida albicans*, we found that the IQGAP Iqg1 contains two dense clusters of 19 such sites flanking the actin-interacting CH domain. Here, we show that Iqg1 is indeed a phosphoprotein that undergoes cell-cycle-dependent phosphorylation and can be phosphorylated by purified Clb-Cdc28 kinases *in vitro*. Mass spectrometry identified several phosphoserine and phosphothreonine residues among these CDK sites. Mutating 15 of the CDK phosphorylation sites with alanine markedly reduced Iqg1 phosphorylation *in vivo*. The 15A mutation greatly stabilized Iqg1, caused both premature assembly and delayed disassembly of the actomyosin ring, blocked Iqg1 interaction with the actin-nucleating proteins Bni1 and Bnr1, and resulted in defects in cytokinesis. Our data therefore strongly support the idea that the Cdc28 CDK regulates cytokinesis partly by directly phosphorylating the actomyosin ring component Iqg1.

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Introduction

The actin cytoskeleton has an important function in many cellular processes such as morphogenesis, motility, trafficking, and cytokinesis (Pruyne and Bretscher, 2000). There are two conserved actin-assembly machineries: formins and the actin-related protein 2/3 complex. The former assembles linear actin filaments (Evangelista *et al*, 1997; Pruyn *et al*, 2002), whereas the latter generates actin meshworks (Goley and Welch, 2006). Actin assembly and dynamics are under rigorous spatiotemporal controls. Among many actin regulators, the Rho GTPases Cdc42 and Rac often have an important

function (Tapon and Hall, 1997; Bishop and Hall, 2000; Ridley, 2006). IQGAPs have recently emerged as important regulators of actin (Noritake *et al*, 2005; Brown and Sacks, 2006; Brandt and Grosse, 2007). These conserved proteins contain multiple domains including an N-terminal calponin homology domain (CHD), IQ motifs, and a C-terminal GTPase-activating-protein (GAP)-related domain (GRD). CHD can bind and bundle actin filaments (Fukata *et al*, 1997; Mateer *et al*, 2004), GRD interacts with Cdc42 and Rac (Bashour *et al*, 1997; Swart-Mataraza *et al*, 2002; Noritake *et al*, 2004), and the IQ motifs interact with calmodulin and myosin light chains (Shannon and Li, 1999; Boyne *et al*, 2000). Human IQGAP1 also contains a C-terminal region that mediates interactions with the formin Dia1. IQGAPs form scaffolds that recruit key components of the actin assembly machinery and can promote actin polymerization (Hart *et al*, 1996; Le Clainche *et al*, 2007). Brandt *et al* (2007) recently reported that IQGAP1 is necessary for Dia1 to localize to the leading edge of migrating cells and to the phycoptic cups. IQGAP functions are regulated at multiple levels. Rho GTPases control cellular localizations and functions of mammalian IQGAPs (Briggs and Sacks, 2003; Izumi *et al*, 2004). IQGAP1 is regulated by autoinhibition through intramolecular interactions, which is relieved by protein kinase C ϵ phosphorylation of Ser1443 (Grohmanova *et al*, 2004; Li *et al*, 2005b).

Studies in *Saccharomyces cerevisiae* have provided many key insights into the regulation and function of IQGAPs (Epp and Chant, 1997; Lippincott and Li, 1998; Shannon and Li, 1999; Lippincott *et al*, 2001; Tolliday *et al*, 2002; Ko *et al*, 2007). Iqg1 is the only IQGAP homologue in this yeast and has an important function in cytokinesis. It localizes to the bud neck in anaphase and controls assembly and contraction of the actomyosin ring. After cytokinesis, Iqg1 undergoes ubiquitin-mediated proteolysis (Ko *et al*, 2007). Two formins Bni1 and Bnr1 also localize to the neck and are required for assembling the actomyosin ring (Tolliday *et al*, 2002). In spite of their colocalization and shared cellular functions, it is not clear how Iqg1 and formin cooperate in the dynamics and functions of the ring in a cell-cycle-dependent manner.

The cyclin-dependent kinase (CDK) Cdc28 controls initiation and termination of key cell-cycle events including cytokinesis (Murray, 2004). Satterwhite *et al* (1992) reported that in *Xenopus* the Cdc2 kinase regulates the timing of cytokinesis by phosphorylating the regulatory light chain of myosin-II. Thus, it is an attractive idea that regulation of cytokinesis in yeast might involve Iqg1 phosphorylation by Cdc28. Incidentally, Li and his colleague first noticed the presence of four perfect consensus CDK phosphorylation sites in the N-terminal part of Iqg1 (Lippincott and Li, 1998). However, it remains unknown whether Iqg1 is a direct regulatory target of Cdc28.

In recent years, the polymorphic fungus *Candida albicans* has become a useful model for elucidating mechanisms that control polarized growth (Zheng *et al*, 2004, 2007; Court and

*Corresponding author. Institute of Molecular and Cell Biology, Agency for Science, Technology and Research of Singapore, 61 Biopolis Drive, Singapore 138673, Singapore. Tel.: +65 65869521; Fax: +65 67791117; E-mail: mcbwangy@imcb.a-star.edu.sg

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Sudbery, 2007; Sinha *et al*, 2007). This organism can switch between yeast and hyphal growth. Although core components of the polarity machinery are highly conserved in eukaryotic cells, unique elements have evolved in *C. albicans* specifically for the control of hyphal development. For example, *C. albicans* uses a hypha-specific cyclin-CDK complex, Hgc1-Cdc28, to regulate phosphorylation of two septins Cdc11 (Sinha *et al*, 2007) and Sep7 (González-Novo *et al*, 2008) and the Cdc42 GAP Rga2 to maintain hyphal growth (Zheng *et al*, 2007).

To identify Hgc1-Cdc28 substrates, we have systematically scanned for consensus CDK sites in *C. albicans* proteins that had been implicated in regulating the actin cytoskeleton. One protein we found is Iqg1 whose N-terminal end contains 8 perfect (S/TPxR/K, x is any amino acid (aa)) and 10 minimal (S/TP) consensus sites for CDK phosphorylation. In this study, we took advantage of this property to study whether Cdc28 directly phosphorylates and regulates Iqg1 in *C. albicans*.

Results

Domain organization of *C. albicans* Iqg1

The *C. albicans* genome (Arnaud *et al*, 2007) contains one open-reading frame (orf19.6536) annotated as the orthologue of *S. cerevisiae* (Sc) IQG1. This Calqg1 contains 1647 aa and is ~20% identical to ScIqg1. Sequence analysis using the SMART program (Letunic *et al*, 2006) identified a CHD and a GRD (Figure 1) at positions similar to the same domains in human IQGAP1 and ScIqg1. This program did not find any IQ motifs. However, visual scanning of the sequence revealed several motifs resembling the consensus IQ sequence IQxxxRGxxxRS (Epp and Chant, 1997) in similar regions of ScIqg1 and Calqg1 (Figure 1A and B). Interestingly, the residue in front of the conserved glutamine (Q) is more often to be leucine than isoleucine in the fungal proteins, which may be one reason why the SMART program failed to

identify these motifs. Strikingly, we also found 21 consensus CDK phosphorylation sites, including 8 perfect and 13 minimal ones (Supplementary Table S1). And 11 sites are clustered between aa 39 and 180, and 7 sites between aa 324 and 455, closely flanking CHD. In comparison, ScIqg1 contains 4 perfect and 16 minimal sites, among which all the perfect and 10 minimal sites are also organized in two clusters flanking CHD. In contrast, IQGAP1 contains only four widely scattered minimal sites. The clustering of CDK sites in both ScIqg1 and Calqg1 implies critical functionality in fungi, which might be under the control of CDKs.

Calqg1 cellular localizations

To confirm that Calqg1 is the counterpart of ScIqg1, we first examined its cellular localization. The protein was N-terminal tagged with the green fluorescent protein (GFP) and expressed from its native promoter. GFP-Iqg1 is functional as judged by the lack of any discernible defects when expressed as the sole source of this protein; functionality of all proteins tagged in this study were verified by the same criterion (data not shown). Microscopic examination of yeast cells revealed that Iqg1 localized to the bud neck immediately after bud emergence. The neck localization was observed in all small- to medium-budded and most large-budded cells (Figure 2A; Table I). Although the GFP signal was often seen as a bar across the bud neck, when properly angled, it appeared as a ring. In some large-budded cells, GFP-Iqg1 appeared as a dot in the centre of the neck, presumably the result of contraction of the actomyosin ring, of which Iqg1 is a component (Lippincott and Li, 1998; Shannon and Li, 1999). The Iqg1 ring contraction was confirmed by time-lapse microscopy (Figure 2B). We noticed that the ring fluorescence was weak in cells with small- to medium-sized buds and became markedly stronger in large-budded cells. As a result, time-lapse microscopy often failed to capture the ring in the early stages of cell cycle, giving an impression of sudden appearance of the ring in large-budded cells

Figure 1 (A) Domain organizations of human IQGAP1, ScIqg1, and Calqg1. The numbers denote aa positions. Solid and open triangles indicate perfect and minimal consensus CDK sites, respectively. (B) IQ motifs in each of the proteins above. Numbers indicate positions of the first residue of each motif, and bold letters denote highly conserved residues.

Figure 2 Iqg1 cellular localization. (A) Cells expressing GFP-Iqg1 (strain LCR3, *iqg1Δ/GFP-IQG1*; Table I describes all strains used) were grown in YPD at 30°C to log phase before differential interference contrast (DIC) and fluorescence microscopy. Representative cells at different stages of bud growth are shown. Bars indicate 5 μm throughout the paper. (B) Time-lapse microscopic images (at 3-min intervals) of GFP-Iqg1 show contraction of the Iqg1 ring.

Table I *Candida albicans* strains used in this study

Strain	Genotype	Source
SC5314	Wild type, clinical isolate	
BWP17	<i>ura3/ura3 his1Δ/his1Δ arg4Δ/arg4Δ</i>	Wilson <i>et al</i> (1999)
LCR2	<i>iqg1Δ::ARG4/iqg1Δ::HIS1</i>	This study
LCR3	<i>iqg1Δ::ARG4/GFP-IQG1-URA3</i>	This study
WYL6	Same as BWP17 except <i>BNI1/P_{MET3}-GFP-BNI1-URA3</i>	Li <i>et al</i> (2005a)
LCR4	<i>iqg1Δ::ARG4 iqg1Δ::HIS1 BNI1/P_{MET3}-GFP-BNI1-URA3</i>	This study
WYL18	<i>BNR1/P_{MET3}-GFP-BNR1-URA3</i>	Li <i>et al</i> (2005a)
LCR5	<i>iqg1Δ::ARG4/iqg1Δ::HIS1 BNR1/P_{MET3}-GFP-BNR1-URA3</i>	This study
LCR6	Same as BWP17 except <i>CDC28/CDC28-6Myc-ARG4</i>	This study
LCR7	<i>CDC28/CDC28-6Myc ARG4 IQG1/GFP-IQG1-URA3</i>	This study
JCB64	Same as BWP17 except <i>CLB2/CLB2-HA-URA3</i>	Bensen <i>et al</i> (2005)
JCB77	Same as BWP17 except <i>CLB4/CLB4-HA-URA3</i>	Bensen <i>et al</i> (2005)
LCR8	<i>CLB2/CLB2-HA-URA3 IQG1/GFP-IQG1-ARG4</i>	This study
LCR9	<i>CLB4/CLB4-HA-URA3 IQG1/GFP-IQG1-ARG4</i>	This study
LCR10	<i>cdc28Δ::HIS1/cdc28^{F85G}-ARG4 CLB2/CLB2-HA-URA3</i>	This study
LCR11	<i>cdc28Δ::HIS1/cdc28^{F85G}-ARG4 IQG1/6Myc-IQG1-URA3</i>	This study
LCR12	<i>iqg1Δ::flp/GFP-IQG1-URA3</i>	This study
LCR13	<i>iqg1Δ::flp/GFP-IQG1-15A-URA3</i>	This study
LCR14	<i>iqg1Δ::flp/GFP-IQG1-15E-URA3</i>	This study
LCR17	<i>iqg1Δ::flp/GFP-IQG1-15A-ARG4 BNI1/P_{MET3}-6Myc-BNI1-URA3</i>	This study
LCR18	<i>iqg1Δ::flp/GFP-IQG1-15A-ARG4 BNR1/P_{MET3}-6Myc-BNR1-URA3</i>	This study
LCR19	<i>iqg1Δ::flp/GFP-IQG1-15E-ARG4 BNI1/P_{MET3}-6Myc-BNI1-URA3</i>	This study
LCR20	<i>iqg1Δ::flp/GFP-IQG1-15E-ARG4 BNR1/P_{MET3}-6Myc-BNR1-URA3</i>	This study
LCR22	<i>iqg1Δ::flp/GFP-IQG1-ARG4 BNI1/P_{MET3}-6Myc-BNI1-URA3</i>	This study
LCR23	<i>iqg1Δ::flp/GFP-IQG1-ARG4 BNR1/P_{MET3}-6Myc-BNR1-URA3</i>	This study
LCR24	Same as BWP17 except <i>BNI1/P_{MET3}-6Myc-BNI1-URA3</i>	This study
LCR25	Same as BWP17 except <i>BNR1/P_{MET3}-6Myc-BNR1-URA3</i>	This study
LCR26	<i>iqg1Δ::flp/6Myc-IQG1-ARG4</i>	This study
LCR29	<i>iqg1Δ::flp/6Myc-IQG1-15A-ARG4</i>	This study
LCR30	<i>iqg1Δ::flp/6Myc-IQG1-15A-ARG4 BNI1/P_{MET3}-GFP-BNI1-URA3</i>	This study
LCR31	<i>iqg1Δ::flp/6Myc-IQG1-15A-ARG4 BNR1/P_{MET3}-GFP-BNR1-URA3</i>	This study
LCR32	<i>iqg1Δ::flp/6Myc-IQG1-15E-ARG4</i>	This study
LCR33	<i>iqg1Δ::flp/6Myc-IQG1-15E-ARG4 BNI1/P_{MET3}-GFP-BNI1-URA3</i>	This study
LCR34	<i>iqg1Δ::flp/6Myc-IQG1-15E-ARG4 BNR1/P_{MET3}-GFP-BNR1-URA3</i>	This study
LCR35	Same as BWP17 except <i>MYO1/GFP-MYO1-URA3</i>	This study
LCR36	<i>iqg1Δ::flp/6Myc-IQG1-15A-ARG4 MYO1/GFP-MYO1-URA3</i>	This study
LCR37	<i>iqg1Δ::flp/6Myc-IQG1-15E-ARG4 MYO1/GFP-MYO1-URA3</i>	This study
LCR38	<i>iqg1Δ::ARG4/iqg1Δ::HIS1 MYO1/GFP-MYO1-URA3</i>	This study

Figure 3 *iqg1Δ/Δ* mutants exhibit severe defects in cytokinesis. (A) *iqg1Δ/Δ* cells (LCR2) were grown in YPD at 30°C (left) or in YPD containing 20% serum at 37°C for 2 h (right). The inset shows wild-type (WT) yeast cells (SC5314). (B) The cells shown in (A) were stained with Calcofluor White. (C) *iqg1Δ/Δ* cells were stained with rhodamine-phalloidine. Inset shows large-budded WT cells with an actin ring at the neck. Insets show WT yeast cells (SC5314). (D) *iqg1Δ/Δ* cells were inoculated onto YPD plates and incubated at different temperatures. The bottom panel shows cells grown at 42°C. The inset shows WT yeast cells (SC5314).

(Figure 2B). The data suggest a two-step recruitment of Iqg1 to the neck in *C. albicans*: at bud emergence and in M phase. This differs apparently from ScIqg1, which localizes to the neck only in anaphase (Epp and Chant, 1997; Lippincott and Li, 1998).

***iqg1Δ* mutants are defective in cytokinesis**

To study the functions of Calqg1, we generated *iqg1Δ/Δ* mutants. Several phenotypes were prominent and consistent with severe cytokinetic defects. First, when grown as yeast, most *iqg1Δ/Δ* cells were elongated and formed chains (Figure 3A). However, these cells appeared normal in response to hyphal induction. Second, the chintin dye Calcofluor White failed to stain the septum particularly between cells in a chain (Figure 3B). Third, rhodamine-phalloidine staining could not detect actin rings at the septum (Figure 3C), indicating failure in actomyosin ring assembly. Moreover, the mutant cells are severely swollen with wide necks, suggesting defective bud and neck morphogenesis. Higher temperatures exacerbated the defects as shown by greater cell elongation and loss of viability at 42°C

(Figure 3D). The data are essentially consistent with the phenotype of *Sciqq1Δ* (Epp and Chant, 1997; Lippincott and Li, 1998). We thus conclude that Calqg1 is the functional counterpart of ScIqg1. Interestingly, although the majority of *iqg1Δ/Δ* cells failed to separate after cytokinesis, a small number of single cells (<5%) were observed, suggesting the presence of an Iqg1-independent mechanism(s) for cytokinesis.

***Iqg1* is a phosphoprotein**

The presence of 21 consensus CDK sites in Iqg1 suggested that it might be a Cdc28 substrate. To test this, we tagged Iqg1 with a 6Myc epitope at the N terminus for anti-Myc immunoprecipitation (IP). Phosphorylation was then probed by western blotting using an antibody (α PS) that preferentially recognizes phospho-Ser in consensus CDK sites (Zheng *et al*, 2007). We found that α PS strongly reacted with Iqg1 (Figure 4A), which was abolished by pretreating the precipitate with λ phosphatase (λ PPase). As controls, the Myc antibody detected both λ PPase-treated and λ PPase-untreated Iqg1, and the treated Iqg1 migrated faster as a sharp band in

Figure 4 Evidence that Iqg1 is a Cdc28 substrate. (A) Lysates were prepared from cells expressing Myc-Iqg1 (LCR26) and immunopurified with Myc antibody (Ab). The precipitate was divided into two parts with one treated with λPPase before western blotting using αPS or Myc Ab. (B) Stationary cells were diluted into fresh YPD for further growth at 30°C. Aliquots were harvested at the indicated times for generating the budding index (top) and for IP with Myc Ab, followed by western blotting using αPS or Myc Ab. (C) Stationary cells of LCR11 were first grown in fresh YPD at 30°C for 1.5 or 2.5 h and then grown for 30 min in the presence or absence of 25 μM 1NM-PP1. Then, cells were harvested for IP of Myc-Iqg1 with Myc Ab, followed by western blotting using αPS or Myc Ab. (D) List of perfect and minimal consensus CDK phosphorylation sites on Iqg1 identified by MS. (E) Immunopurified Clb2-HA/Cdc28 (JCB64) and Clb2-HA/Cdc28as (LCR10) complexes were used to phosphorylate immunopurified Myc-Iqg1 (LCR26) in a kinase assay containing γ-³²P-ATP in the presence or absence of 25 μM 1NM-PP1. Phosphorylated proteins were visualized by autoradiography after SDS-PAGE. (F) Log-phase cells expressing GFP-Iqg1 together with Cdc28-Myc (LCR7), Clb2-HA (LCR8), or Clb4-HA (LCR9) were lysed for IP with GFP or HA Ab. Cells expressing Cdc28-Myc (LCR6), Clb2-HA (JCB64), or Clb4-HA (JCB77) alone were used as negative controls. For all coIP experiments described in this study, 2-ml lysate was obtained from a 200-ml culture; 30 μl of the lysate was loaded in lanes labelled as L, and all the precipitate from the rest of the lysate was loaded in lanes labelled as IP. The blots were probed with the appropriate Ab.

contrast to a diffuse band of the untreated Iqg1. To determine when Iqg1 phosphorylation occurs during the cell cycle, stationary-phase yeast cultures containing >95% G1 cells were used to start synchronous cultures in GMM at 30°C. Under this condition, 50% budding occurred at ~1.2 h and one cell-cycle time was ~100 min. We harvested cells at intervals, immunoprecipitated Myc-Iqg1 using Myc antibody, and then probed the precipitate by western blotting using Myc antibody or αPS (Figure 4B). The Myc antibody started to detect Myc-Iqg1 at 1 h, after which the amount of Iqg1 increased gradually and peaked at 2.5 h around the time of cytokinesis. After cytokinesis, the cellular levels of Iqg1 started to decline. Interestingly, αPS could barely detect Myc-Iqg1 at 1 h and started to clearly detect it at 1.5 h, and then the intensity of the signal increased in parallel with the Myc-Iqg1 cellular levels until 3 h when the signal weakened sharply. The results therefore reveal that both the cellular level and phosphorylation status of Iqg1 are regulated in a cell-cycle-dependent manner. To determine whether Cdc28 is involved, we repeated this experiment using a strain coexpressing Myc-Iqg1 and Cdc28as, a mutated Cdc28 sensitive to the ATP analogue 1NM-PP1 (Bishop *et al*, 2000). Owing to the high cost of 1NM-PP1, we examined Iqg1 phosphorylation at only two time points. Again, stationary-phase yeast cells were inoculated into two fresh cultures and grown at 30°C for 1.5 and 2.5 h, respectively, before further dividing each culture into two; to one culture 1NM-PP1 was added and then both cultures were grown for 30 more minutes before IP and western blotting. Figure 4C shows that 1NM-PP1 abolished Iqg1 phosphorylation, consistent with a function of Cdc28 in regulating Iqg1 phosphorylation. However, the failure in detecting Iqg1 by αPS after 1NM-PP1 treatment also suggests Iqg1 dephosphorylation during the time when

Cdc28as was inhibited. This could be either due to the disturbance of a kinase-phosphatase balance that normally determines levels of protein phosphorylation or due to the activation of a phosphatase during the inhibition of Cdc28as. Although we favour the former explanation, we currently have no evidence to exclude the latter.

To map phosphorylation sites, affinity-purified Myc-Iqg1 was analysed by mass spectrometry (MS). The MS data covered ~60% of the Iqg1 sequence and detected phospho-Ser or phospho-Thr at 21 sites (Supplementary Table S2) including four perfect and five minimal CDK sites (Figure 4D). Together, we conclude that Iqg1 is a phosphoprotein and many of the consensus CDK sites are phosphorylated *in vivo*.

Purified Clb2-Cdc28 complex can phosphorylate Iqg1 in vitro

To determine whether Cdc28 can directly phosphorylate Iqg1, we coexpressed Clb2-HA and Cdc28as, purified the Clb2-HA-Cdc28as complex by anti-HA IP (Supplementary Figure S1), and used it to phosphorylate immunopurified Myc-Iqg1 in a kinase assay. Figure 4E shows that the CDK complex could phosphorylate Myc-Iqg1 and the reaction was completely inhibited by 25 μM 1NM-PP1. We had established previously that 1NM-PP1 at concentrations up to 50 μM has no inhibitory effect on WT Cdc28 (Sinha *et al*, 2007; Zheng *et al*, 2007). As negative controls, incubating the purified Myc-Iqg1 alone in the kinase reaction did not cause detectable phosphorylation of Iqg1, nor did the anti-HA precipitate pulled down from cell lysates lacking the HA tag (data not shown). Together, our results strongly support the idea that Iqg1 is a Cdc28 substrate *in vivo*.

Iqg1 physically interacts with Cdc28 and B-type cyclins

To gain further evidence for Iqg1 phosphorylation by Cdc28, we examined whether they associate with each other. A strain was constructed coexpressing GFP-Iqg1 and Cdc28-Myc. We used GFP antibody to pull down GFP-Iqg1 from cell lysates and then used Myc antibody to detect Cdc28-Myc in the precipitate. Figure 4F shows that Cdc28-Myc could indeed coprecipitate with GFP-Iqg1. We also determined whether cyclins are associated with Iqg1. We coexpressed Clb2-HA or Clb4-HA with GFP-Iqg1 and were able to detect coIP of both cyclins with GFP-Iqg1 (Figure 4F). However, such interaction was not detected with the G1 cyclin Ccn1 (data not shown), suggesting that Iqg1 may be specifically regulated by Clb-Cdc28 kinases.

Mutating the CDK sites on Iqg1 causes morphological and cytokinesis defects

To address the biological significance of Iqg1 phosphorylation by CDK, we generated a mutant Iqg1 in which the Ser or Thr residues in all eight perfect CDK sites and the seven minimal sites closest to the N terminus were replaced with Ala (Iqg1-15A). We also constructed a phosphomimetic version replacing the 15 residues with Asp (Iqg1-15E). Each mutant gene was introduced into the *IQG1/iqg1Δ* background to replace the WT copy, yielding strains expressing from the native

promoter, either Iqg1-15A or Iqg1-15E as the sole source of Iqg1. All versions of Iqg1 were tagged at the N terminus with either GFP or 6Myc. We first assessed phosphorylation levels of Iqg1-15A by western blotting using α PS and found that the intensity of the Iqg1-15A band was drastically reduced compared with the WT Iqg1 band, consistent with Iqg1 phosphorylation at the CDK sites *in vivo* (Figure 5A). Microscopic examinations of *iqg1-15A* and *iqg1-15E* cells revealed similar defects in morphology: cell elongation and failure of cytokinesis/separation (Figure 5B). However, the defects were more severe in the *iqg1-15E* mutant. For example, ~50% of the *iqg1-15E* cells had a length-width ratio >2 at 30°C compared with ~16% of the *iqg1-15A* cells, and ~14% of the latter cells grown at 37°C were as long as 30–40 μ m, which was seen only in ~3% of the former cells. In spite of these defects, these mutants, unlike *iqg1Δ/Δ*, were viable at 42°C. Moreover, both mutants were capable of producing separated cells, indicating that many cells could complete cytokinesis. The partial functionality and similar defects of the two Iqg1 mutants suggest that reversible phosphorylation at the CDK sites are important for the functions of Iqg1 in the cell.

Next, we asked whether the CDK-site mutations affect the localization of Iqg1. Examination of asynchronous cultures detected GFP-Iqg1 at the neck of buds of all sizes in both

Figure 5 Mutation of 15 N-terminal CDK phosphorylation sites of Iqg1 causes cytokinetic and morphological defects. (A) The 15A mutation greatly reduced Iqg1 phosphorylation. Cell lysates expressing GFP-Iqg1 (LCR3) or GFP-Iqg1-15A (LCR13) were immunoprecipitated with GFP Ab, and then the precipitate was probed with α PS or GFP Ab in western blotting. (B) DIC and fluorescence images of cells expressing GFP-tagged Iqg1 (LCR3), Iqg1-15A (LCR13), or Iqg1-15E (LCR14). Cells were grown either at 30 or 37°C. Insets show cells with a GFP dot at the centre of the neck, indicating ring contraction. (C) Time-lapse microscopy of cells expressing GFP-Iqg1 (LCR12), GFP-Iqg1-15A (LCR13), or GFP-Iqg1-15E (LCR14). Only cells with normal morphology were selected.

iqg1-15A and *iqg1-15E* mutants, suggesting that the mutations do not significantly affect the general localization pattern of Iqg1 (data not shown). However, time-lapse microscopy revealed prolonged presence of Iqg1-15A at the bud neck. As the microscope setting for this experiment could clearly capture only the image of GFP-Iqg1 in cells with large buds where the fluorescence was strong, we used the first time point when the fluorescence was unambiguously seen to approximate the start of the neck localization of Iqg1. Figure 5C presents representative series of cell images. WT Iqg1 persisted at the neck for 17.5 ± 2.4 min ($n=20$). Strikingly, Iqg1-15A in $\sim 30\%$ of the cells persisted for 30–35 min ($n=20$), which was rarely seen in WT cells, whereas Iqg1-15E disappeared slightly earlier than WT Iqg1 (16.4 ± 1.9 min, $n=20$). The significantly longer persistence of Iqg1-15A suggests that Iqg1 phosphorylation might have an important function in the temporal control of actomyosin-ring assembly or disassembly or both. To address this issue further, we arrested yeast cells with nocodazole in M phase and then released them into drug-free medium to score the cells with a neck actin ring at intervals (Figure 6A). After 2 h of nocodazole treatment, $>95\%$ of the cells of all the three

strains were large-budded with an undivided nucleus, characteristic of M arrest. Under this condition, rhodamine-phalloidine staining detected a neck actin ring in only 4% of WT cells (50 cells per time point). After release, the number of cells with a neck actin ring increased gradually, reached 22% at 30 min and then declined, indicating that actin ring assembly occurs after the mitotic entry, consistent with previous reports for Sclqg1 (Tolliday *et al*, 2002). In comparison, although the *iqg1-15E* cells exhibited a trend similar to WT cells, we clearly detected the actin ring in 22% of the Iqg1-15A cells at the end of the 2-h nocodazole treatment; the percentage of these cells increased gradually after the release and reached 27% at 30 min before declining to 12% at 60 min. The results thus suggest premature assembly of the actomyosin ring in the *iqg1-15A* mutant. When cells were stained with the DNA dye DAPI to visualize nuclei, the percentage of cells with divided nuclei in all strains were similar at all time points after release from the arrest, indicating that the aberrant actin ring behaviour in *iqg1-15A* cells is not a result of disturbed nuclear division.

When we assessed cellular levels of Iqg1-15A and Iqg1-15E in asynchronous cells, we consistently found more Iqg1-15A

Figure 6 The 15A mutation stabilizes Iqg1 and affects the dynamics of the actomyosin ring. (A) Log-phase cells expressing WT Iqg1 (LCR26), Iqg1-15A (LCR29), or Iqg1-15E (LCR32) as the sole source of Iqg1 were arrested with 30 μ M nocodazole for 2 h and then stained with rhodamine-phalloidine to reveal the actin ring at the neck and with 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei. (B) The nocodazole-arrested cells described in (A) were released into drug-free medium for growth at 30°C. Cells were harvested at intervals for actin and nuclear staining. Percentage of cells with a neck actin ring and with divided nuclei was counted. (C) Stationary cells expressing WT Iqg1 (LCR26), Iqg1-15A (LCR29), or Iqg1-15E (LCR32) were released into fresh YPD for growth at 30°C. Aliquots were harvested at 0, 1, 2, and 3 h and lysates were prepared for western blotting using Myc Ab (all Iqg1 proteins have a 6Myc tag). Duplicate blots were probed with PSTAIRE Ab to detect Cdc28 as loading controls. (D) Cell lysates prepared as described in (B) and analysed by western blotting using Myc Ab and PSTAIRE Ab.

and less Iqg1-15E than WT Iqg1 (data not shown). Prompted by the finding that ScIqg1 undergoes APC-mediated proteolysis at the end of cytokinesis (Ko *et al*, 2007), we suspected that the CDK-site mutations might have affected Iqg1 stability. To test this idea, we first determined cellular levels of the Iqg1 mutants during the cell cycle as described in Figure 4B. Stationary-phase cells were released into fresh medium and aliquots were harvested at 1, 2, and 3 h for western blot analysis. We found that whereas both WT Iqg1 and Iqg1-15E were undetectable at 1 h, Iqg1-15A was strongly detected, and its level remained virtually constant throughout the cell cycle (Figure 6C). Furthermore, Iqg1-15E levels were much lower than WT and Iqg1-15A. Next, we repeated the experiment by using cultures released from nocodazole arrest as described in Figure 6B. We found that whereas the levels of all three Iqg1 mutants were similar in the arrested cells, they declined at different rates after release (Figure 6D). The level of WT Iqg1 dropped significantly at 30 min and became barely detectable at 45 min. In comparison, degradation of Iqg1-15E occurred earlier, becoming undetectable at 30 min, whereas the level of Iqg1-15A remained unchanged even at

60 min. Thus, the data suggest that Iqg1 phosphorylation at the CDK sites might have a function in determining Iqg1 degradation after cytokinesis.

Mutations of Iqg1 CDK sites influence the disassembly of the actomyosin ring

To understand the physiological significance of Iqg1 phosphorylation by CDK, we next examined the dynamics of GFP-Myo1. Similar to Iqg1, GFP-Myo1 was found to localize to the bud neck as a ring when the bud had just emerged (data not shown). Time-lapse microscopy revealed that the neck ring contracted to a dot and then disappeared at the end of cytokinesis (Figure 7). The time taken from the start of ring contraction to the disappearance of the dot was 6–9 min ($n = 12$). Interestingly, the GFP-Myo1 ring also exhibited a sudden marked increase in fluorescence intensity during the large-budded stage, reminiscent of the GFP-Iqg1 ring. The neck localization of GFP-Myo1 was largely unaffected in *iqg1Δ/Δ* cells except that the ring did not exhibit the increase in fluorescence intensity and did not contract before disappearance. When we examined GFP-Myo1 in the CDK-site

Figure 7 The CDK-site mutations affect the disassembly of the actomyosin ring. GFP-Myo1 in wild-type (LCR35), *iqg1Δ/Δ* (LCR38), *iqg1-15A* (LCR36), and *iqg1-15E* (LCR37) mutants was examined by time-lapse microscopy.

mutants, we observed the following. First, in *iqg1-15E* cells, GFP-Myo1 disappeared 2–3 min earlier than in WT cells ($n = 10$). Second, in all *iqg1-15A* cells, the GFP-Myo1 dot at the end of the contraction persisted over extended periods of time: the dot did not disappear; instead it was divided into two dots that were inherited one each by the mother and daughter cells. The dots, particularly the ones on the mother side, often persisted into the next cell cycle ($n = 7$ of 12). We did not detect any significant difference in the rate of ring contraction in the mutants, suggesting that the 15A mutations mainly impair the disassembly of the Myo1 ring after contraction. Taken together, our data suggest that CDK phosphorylation of Iqg1 may have an important function in regulating the dynamics of the actomyosin ring. As GFP-Iqg1-15A did not exhibit the same behaviour (see Figure 5C) as GFP-Myo1 in *iqg1-15A* cells after ring contraction, the two proteins might have disassociated after cytokinesis.

The CDK-site mutations markedly affect the association of Iqg1 with formins

The formins are crucial for actin ring assembly in *S. cerevisiae* (Tolliday *et al*, 2002). Inspired by the recent report (Brandt *et al*, 2007) of physical and functional interactions between the mammalian IQGAP1 and Dia1, we examined whether Iqg1 interacts with Bni1 and Bnr1 in *C. albicans*. We coexpressed GFP-Iqg1 with Myc-Bni1 or Myc-Bnr1 and used GFP antibody to pull down GFP-Iqg1 and then Myc antibody to probe Myc-Bni1 or Myc-Bnr1 in the precipitate. This experiment detected Iqg1 coIP with both Bni1 and Bnr1 (Figure 8A and B). The Iqg1-formin interaction was detected also in reciprocal coIP in which the Myc-tagged formins were pulled down first with Myc antibody and followed by probing GFP-Iqg1 with GFP antibody (Supplementary Figure S2). The results thus revealed Iqg1 association with formins. Next, we asked whether this interaction might be regulated by Iqg1 phosphorylation at the CDK sites. To this end, GFP-tagged Iqg1-15A or Iqg1-15E was coexpressed with Myc-Bni1 or Myc-Bnr1. Strikingly, the 15A mutation almost completely abolished the interaction of Iqg1 with both formins (Figure 8C and D). Furthermore, although the cellular levels of Iqg1-15E are lower than the WT Iqg1 (see Figure 6C), the relative amount of formins pulled down with Iqg1-15E was significantly higher than the amount of formins pulled down with the WT Iqg1, suggesting that the 15E mutations enhanced the association of Iqg1 with formin. Thus, the data not only demonstrate the association of Iqg1 with forming, but also suggest that the interaction may be regulated by CDK phosphorylation of Iqg1.

Overexpression of formins partially suppresses the defects of the *iqg1-15A* and *iqg1-15E* mutants

Next, we explored the possible function of Iqg1 in determining the cellular localizations of formin. In *S. cerevisiae*, Bni1 and Bnr1 exhibit distinct localization patterns (Pruyne *et al*, 2004). We reported that CaBni1, similar to ScBni1, localizes to the presumptive bud site, the tips of small- to medium-sized buds and the neck of large-budded cells (Li *et al*, 2005a). However, the localization of CaBnr1 had not been studied in detail. Thus, we first examined GFP-Bnr1 localization in comparison with GFP-Bni1. As the native promoter was too weak for detecting the GFP-tagged formins, we used the stronger *MET3* promoter. We observed that Bnr1 formed a

ring at the bud neck immediately after the bud became visible, and the neck localization persisted throughout the cell cycle until the end of cytokinesis when the Bnr1 ring split into two rings (Figure 8E). In contrast, CaBni1 was seen only at the tips but never at the neck in small- and medium-sized buds; only late in the cell cycle did Bni1 localize to the bud neck. The results show that the *C. albicans* formins exhibit distinct localization patterns similar to their counterparts in *S. cerevisiae*.

We next examined GFP-tagged Bni1 and Bnr1 in *iqg1Δ/Δ*, *iqg1-15A*, and *iqg1-15E* mutants. To minimize the secondary effects, we focused on the normal-looking yeast cells of the mutants. In *iqg1Δ/Δ* cells, Bnr1 exhibited normal localizations, whereas the signal of Bni1 was hard to detect in small buds and was faint at the neck (Figure 8F), suggesting that Iqg1 may influence the localization of Bni1. In the two CDK-site mutants, both formins were found localized normally (data not shown), suggesting that CDK phosphorylation of Iqg1 may not be critical for correct localization of the formins. Unexpectedly, we observed that overexpression of either formin from the *MET3* promoter significantly reduced cell elongation in both *iqg1-15A* and *iqg1-15E* mutants (Figure 8G), indicating a genetic interaction between Iqg1 and formin. We also found that the effect is not due to changes of Iqg1 levels (Figure 8H). Similar expression of the formins in *iqg1Δ/Δ* cells had no obvious effect. Thus, the molecular basis of this genetic interaction remains to be further investigated.

Discussion

Iqg1 is a regulatory target of Cdc28

CDKs have an important function in ensuring orderly occurrence of major cell-cycle events, of which cytokinesis is the last critical step. Although actomyosin-based contractile rings are known as a key component of the cytokinesis apparatus, data remain scant on whether CDKs regulate cytokinesis through direct phosphorylation of constituent molecules of the ring. In this study, we have obtained compelling evidence that the CaIqg1 is a likely substrate of Cdc28. First, sequence analysis of Iqg1 revealed dense clusters of consensus CDK sites. Second, we found that Iqg1 is a phosphoprotein *in vivo* and can be phosphorylated by purified Clb2-Cdc28 kinases *in vitro*. Third, MS analysis mapped several phospho-Ser and phospho-Thr in consensus CDK sites. Furthermore, we have shown that phosphorylation at these sites is of physiological significance. Mutating 15 CDK sites with Ala caused marked reduction in Iqg1 phosphorylation *in vivo*, stabilization of Iqg1, premature assembly and delayed disassembly of the actomyosin structure, and abnormal cytokinesis. Hence, our data strongly support the idea that CDKs regulate cytokinesis partly by direct phosphorylation of actomyosin-ring components (Figure 9).

We observed opposing effect of Iqg1-15A and Iqg1-15E on certain molecular events such as Iqg1 degradation and its interaction with formin, consistent with the view that the hyper and hypophosphorylated forms of Iqg1 have different functions. However, the two mutants also exhibit similar morphological defects, although of different severity: cell elongation and abnormal cytokinesis. This apparent discrepancy is not totally unexpected for the following reasons. On the basis of the observed temporal control of Iqg1 phospho-

Figure 8 Iqg1 interacts physically and genetically with Bni1 and Bnr1. Lysates of cells coexpressing GFP-Iqg1 with (A) Myc-Bnr1 (LCR23) or (B) Myc-Bnr1 (LCR22) were subjected to IP using GFP Ab; lysates from cells expressing Myc-Bnr1 (LCR25) or Myc-Bni1 (LCR24) alone were used as negative controls. (C) Lysates of cells coexpressing Myc-Bnr1 with GFP-Iqg1-15A (LCR18) or with GFP-Iqg1-15E (LCR20) and (D) lysates of cells coexpressing Myc-Bni1 with GFP-Iqg1-15A (LCR17) or with GFP-Iqg1-15E (LCR19) were subjected to IP with GFP Ab, and the blots were probed with Myc or GFP Ab. (E) GFP-Bni1 (WYL6) and GFP-Bnr1 (WYL18) in WT yeast cells with buds of different sizes. (F) GFP-Bni1 (LCR4) and GFP-Bnr1 (LCR5) in *iqg1Δ/Δ* cells. (G) Expression of Bni1 from the *MET3* promoter in *iqg1-15A* (LCR30) and *iqg1-15E* (LCR33) mutants. Expression of Bnr1 (LCR31 and 34) had similar effects (images not shown). (H) Overexpression of *BNI1* does not affect Iqg1 stability. The experiment was performed as described in Figure 6C except using LCR27, LCR30, and LCR33.

rylation (Figure 4B), it is reasonable to hypothesize that the phosphorylation status of Iqg1 may define its function at a specific time of the cell cycle. Thus, neither Iqg1-15A nor

Iqg1-15E can be expected to be fully functional for all events required of Iqg1 throughout the cell cycle. Moreover, replacing 15 residues with either Ala or Glu is likely to cause

Figure 9 Schematic description of Iqg1 phosphorylation by Cdc28 and the cellular events that involve phosphorylated Iqg1.

unfavourable structural changes that somewhat compromise the the function of the protein. Consequently, the partially functional versions of Iqg1 may cause defects in some cellular events that need Iqg1, such as actin polarization, bud and neck construction, and cytokinesis. Many mutants, for example, *gin4Δ* (Wightman *et al*, 2004), *cdc10Δ*, or *cdc11Δ* (Warenda and Konopka, 2002), that affect budding or neck formation cause cell elongation. Unlike ScIqg1, CaIqg1 localizes to the bud neck at bud emergence, suggesting that it may contribute to budding and neck formation. Consistently, *iqg1Δ* cells are severely swollen with broadened necks (Figure 3A). Taken together, the similar morphological defects of *iqg1-15A* and *iqg1-15E* mutants may be attributable to abnormalities in budding or neck formation or both, which may have masked the phenotypes specifically associated with each of the CDK-site mutants at morphological levels.

CDK phosphorylation of Iqg1 may have an important function in disassembly of the actomyosin ring

Previous studies in *S. cerevisiae* and *S. pombe* as well as this work have established an essential function for IQGAPs in the actomyosin-ring-based mechanism of cytokinesis in fungi (Epp and Chant, 1997; Eng *et al*, 1998; Lippincott and Li, 1998). As the central components of the ring are Myo1 and actin, the timing and detailed molecular architecture for assembling the two proteins are predictably important. Myo1 localizes to the bud neck at an early stage of the cell cycle, whereas actin is incorporated into the ring during mitosis (Lippincott and Li, 1998; and this work). Although Iqg1 is apparently not required for Myo1 localization, it has several important functions in the assembly and contraction of the ring. Shannon and Li (1999) presented evidence that CHD is required for recruiting actin filaments, and GRD is required for ring contraction. Our data largely confirmed the functions of Iqg1 in the assembly and contraction of the actomyosin ring; however, we also discovered that Iqg1 may have a function in determining the timely disassembly of the ring after cytokinesis and that this function is most likely under the control of CDKs. We found that although the WT actomyosin ring, labelled with GFP-Myo1, disappeared within 4 min after contraction to a dot, mutating 15 of the consensus CDK sites to Ala in Iqg1 greatly delayed the disappearance of the dot.

We have found that unlike the cyclic behaviour of the WT Iqg1, the cellular levels of Iqg1-15A remained essentially constant throughout the cell cycle. We suspected that the mutant protein may have become resistant to APC-mediated proteolysis that had been shown to occur to ScIqg1 after cytokinesis (Ko *et al*, 2007). Incidentally, the N-terminal region of ScIqg1 appears to have an important function in its ubiquitylation and degradation (Ko *et al*, 2007). The

results of our nocodazole-arrest-and-release experiments support this view. We found that whereas the cellular level of WT Iqg1 dropped significantly at 30 min and was barely detectable at 45 min after release, the level of Iqg1-15A remained largely unchanged for at least 60 min. Interestingly, the 15E phosphomimetic form of Iqg1 was degraded more rapidly than WT Iqg1. Together, the results suggest that Iqg1 phosphorylation at the CDK sites may have an important function in ensuring a timely degradation of Iqg1, which seems to be important for complete disassembly of the actomyosin complex.

Actomyosin-ring-independent mechanisms for cytokinesis

Unlike ScIqg1, CaIqg1 is not essential. Moreover, despite the lack of the actomyosin ring, a small number of *Caig1Δ/Δ* cells can complete cytokinesis, suggesting the presence of a cytokinetic mechanism that can function, although much less efficiently, without both Iqg1 and the actomyosin ring. Actomyosin-ring-independent cytokinesis has also been reported in *S. cerevisiae*. Several studies showed that the ring is not essential in most strain background (Bi *et al*, 1998; Schmidt *et al*, 2002). Ko *et al* (2007) found that *myo1Δ/Δ* strains that express a non-degradable version of Iqg1 or overexpressing Iqg1 produces high percentages of separated cells, thereby implying that Iqg1 has an important function in mediating the actomyosin-ring-independent mechanism of cytokinesis. However, overexpression of Cyk3, a neck-localizing protein with yet unknown function, can suppress the lethality of *iqg1Δ* without restoring the actomyosin ring (Ko *et al*, 2007), suggesting that cytokinesis can occur without both the ring and Iqg1, thereby consistent with our observations in *C. albicans*. Thus, the mechanisms for the actomyosin-ring-independent cytokinesis remain to be elucidated.

Iqg1 physically and genetically interacts with formins

Assembly of the actomyosin ring requires both Iqg1 and formins in *S. cerevisiae*. Tolliday *et al* (2002) suggested that they work synergistically in parallel pathways with formins responsible for creating and Iqg1 for organizing actin filaments. However, it remains possible that Iqg1 and formins may directly interact with and thus regulate each other. Our studies here support this hypothesis. By means of coIP we detected the association of Iqg1 with both Bni1 and Bnr1. And more importantly, we found that Iqg1-15A abolished and Iqg1-15E enhanced this association, suggesting that CDK may regulate the Iqg1-formin interaction through direct phosphorylation of Iqg1. However, the molecular consequence of this interaction remains unclear. In mammalian cells, IQGAP1 is necessary for the subcellular localization of Dial (Brandt *et al*, 2007). We found that Bni1 localization was hard to detect in *iqg1Δ/Δ* cells, suggesting that Iqg1 may have a function in Bni1 localization. However, we did not observe any significant changes in cellular localizations of either formin in *iqg1-15A* and *iqg1-15E* mutants, although the overexpression of formins may have concealed the subtle effects of the CDK-site mutations. As formins are present as components of protein complexes such as polarisomes (Sheu *et al*, 2000) and both formins and Iqg1 interact with Rho GTPases (Kohno *et al*, 1996; Tolliday *et al*, 2002), it is reasonable to hypothesize that the proper interactions of formin with other proteins might partly depend on Iqg1 at the sites of polarized

growth, thereby affecting the quality of morphogenesis and cytokinesis.

Materials and methods

Media and growth conditions

Candida albicans strains were routinely grown in either YPD (1% yeast extract, 2% Bacto peptone, and 2% glucose) or GMM (2% glucose, 1 × yeast nitrogen base). To grow strains defective in uracil, arginine, or histidine synthesis, the required nutrient was added to the GMM. G1 cells were prepared by centrifugal elutriation as described (Sinha *et al*, 2007).

Gene deletion

Gene deletion was done by sequentially deleting the two copies of a gene from BWP17 (Wilson *et al*, 1999). A deletion cassette was constructed by flanking a marker gene, *CaHIS1* or *CaARG4*, with AB and CD DNA fragments (~500 bp each) corresponding to the 5' and 3'-untranslated regions of the target gene, respectively. Transformants were selected using appropriate GMM media. Deletion was verified by Southern blotting or PCR (data not shown).

GFP tagging of proteins

To construct a GFP-Iqg1 fusion protein driven by its native promoter, a 1503-bp fragment of the *IQG1* promoter (nt -1503 to -1, the first nt of the start codon is 1) was amplified by PCR 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 pCIPGFPut in front of GFP (Zheng *et al*, 2003), yielding pCIP_{Iqg1}GFPut. The N-terminal region of nt 1-1260 of the *IQG1* ORF was PCR-amplified with *NarI* and *PstI* sites added to the 5' and 3'-ends respectively, cut with *NarI* and *PstI*, cloned at the *Clal*/*PstI* sites of pCIP_{Iqg1}GFPut downstream of GFP. The resulting plasmid was cut with *BamHI* at nt 648 of the *IQG1* ORF and transformed into the *IQG1/iqg1Δ* strain. To mutate the N-terminal 15 CDK sites, the *IQG1* region of nt 1-1665 was PCR-amplified with *NarI* and *PstI* sites added to the 5'- and 3'-ends, respectively, cut with *NarI* and *PstI* and cloned at the *Clal*/*PstI* sites of pCIP_{Iqg1}GFPut downstream of GFP. The resulting plasmid was used to generate the *GFP-iqg1-15A* and *GFP-iqg1-15E* mutants using the Quik-Change Multi-Site-Directed Mutagenesis Kit (Stratagene). A unique *Sall* site was generated at nt 1157 for linearization and transformation into *IQG1/iqg1Δ* cells.

Similar strategies were used to express GFP-Myo1. The *MYO1* promoter (nt -1352 to -1) was PCR-amplified with *KpnI* and *XhoI* sites added to 5'- and 3'-ends respectively, cut with *KpnI* and *XhoI*, and cloned at the *KpnI*/*XhoI* sites in pCIPGFPut, yielding pCIP_{Myo1}GFPut. The *MYO1* region of nt 1-1057 was PCR-amplified with *NarI* and *PstI* sites added to the 5'- and 3'-ends respectively, cut with *NarI* and *PstI*, cloned at the *Clal*/*PstI* sites of pCIP_{Myo1}GFPut before cleavage with *Clal* at nt 678 of *MYO1* for genomic integration.

To tag Iqg1 with an N-terminal 6Myc epitope, the coding sequence for 6Myc (LDEESILKQE) was synthesized with *XhoI* and *Clal* sites added to 5'- and 3'-ends, respectively, cut with *XhoI* and *Clal*, and cloned at the *XhoI*/*Clal* sites of pCIP_{Iqg1}GFPut, yielding pCIP_{Iqg1}Mycut. The plasmid was cut with *BamHI* at nt 648 of *IQG1* for genomic integration. Similar strategies were used to generate 6Myc-Iqg1-15A and 6Myc-Iqg1-15E.

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Coimmunoprecipitation

Cells were grown in 50 ml GMM overnight and then diluted to 200 ml for 2 h of growth. Cells were harvested and lysed in 1.5 volumes of lysis buffer (50 mM HEPES, pH 7.5, 1 mM EDTA, 5% glycerol, 0.05% Tween 20, 105 mM KCl) with 1 × protease inhibitor mix (Roche). Cells were lysed using Tomy Microsmash by five rounds of 45-s spin at 5000 r.p.m. with 1-min cooling on ice between rounds. The lysate was clarified by spinning at 10000 g twice. Protein concentrations were determined using the Bradford assay. Antibody-conjugated beads (15–50 μl) were washed twice with 500 μl of the lysis buffer, added to 0.4–2 ml of the clarified lysate, and incubated at 4 °C for 2 h. After washing twice with the lysis buffer, the beads were boiled in gel loading buffer.

Immunopurification of Myc-Iqg1

To purify Myc-Iqg1 for MS analysis and the *in vitro* kinase assay, cell lysates were prepared as described above in RIPA buffer ((50 mM Tris-HCl, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 × EDTA-free protease inhibitor mix, and phosphatase inhibitor cocktail (2 mM NaF, 4 mM sodium orthovanadate, 0.2 mM Na₄P₂O₇, 2.3 mM sodium molybdate, and 0.2 mM β-glycerol phosphate)) and incubated with Myc-antibody-coupled beads (Sc40, Santa Cruz Biotechnology) at 4 °C for 1 h. To remove non-specific proteins, the buffer contained 500 mM NaCl. After incubation, the beads were washed four times with wash buffer (50 mM Tris-HCl, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 150 mM NaCl).

Protein dephosphorylation

Dephosphorylation of Myc-Iqg1 was performed immediately after IP on the Myc-antibody-conjugated beads in a 100 μl reaction with ~1000 U of λ-PPase (NEB) at 30 °C for 30 min.

MS mapping of phosphorylation sites

To map phosphorylation sites, Myc-Iqg1 was immunopurified as described above from 14 l of log-phase cells and then processed as described by Sinha *et al* (2007).

Actin, nuclear, and cell wall staining and fluorescence microscopy

Actin, nuclear, and cell wall staining were performed as described (Zheng *et al*, 2003). A Leica DMR fluorescence microscope with ×100 objective and a Hamamatsu digital camera interfaced with METAMORPH software (Universal Imaging) were used for imaging.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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