The shared role of the Rsr1 GTPase and Gic1/Gic2 in Cdc42 polarization

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ABSTRACT The Cdc42 GTPase plays a central role in polarity development in many species. In budding yeast, Cdc42 is essential for polarized growth at the proper site and also for spontaneous cell polarization in the absence of spatial cues. Cdc42 polarization is critical for multiple events in the G1 phase prior to bud emergence, including bud-site assembly, polarization of the actin cytoskeleton, and septin filament assembly to form a ring at the new bud site. Yet the mechanism by which Cdc42 polarizes is not fully understood. Here we report that biphasic Cdc42 polarization in the G1 phase is coupled to stepwise assembly of the septin ring for bud emergence. We show that the Rsr1 GTPase shares a partially redundant role with Gic1 and Gic2, two related Cdc42 effectors, in the first phase of Cdc42 polarization in haploid cells. We propose that the first phase of Cdc42 polarization is mediated by positive feedback loops that function in parallel—one involving Rsr1 via local activation of Cdc42 in response to spatial cues and another involving Gic1 or Gic2 via reduction of diffusion of active Cdc42.

INTRODUCTION

Cell polarization often occurs along a single axis that is generally directed by extra- or intracellular cues. Cells of the budding yeast Saccharomyces cerevisiae are genetically programmed to undergo polarized growth by choosing a bud site, which determines the axis of polarized growth. Selection of a bud site depends on cell type: a and α cells (such as normal haploids) bud in the axial pattern, in which both mother and daughter cells select a new bud site adjacent to their immediately preceding division site. In contrast, a/α cells (such as normal diploids) form buds in the bipolar pattern, in which daughter cells bud peripherally at the pole distal to the division site, and mother cells can choose a bud site near either pole. The Rsr1 GTPase module, which consists of Rsr1 (also known as Bud1), its guanine nucleotide exchange factor (GEF) Bud5, and its GTPase activating protein (GAP) Bud2, functions in directing polarity establishment by guiding Cdc42 and its regulators in response to distinct cortical markers in each cell type (see references in Bi and Park, 2012).

In the absence of spatial cues, yeast cells can still polarize, albeit at a random site. This process, referred to as symmetry breaking, is thought to occur via positive feedback loops involving the scaffold protein Bem1 or the actin cytoskeleton, although several aspects of these mechanisms are under debate (see references in Martin [2015] and Goryachev and Leda [2017]). Cla4, a p21-activated protein kinase (PAK), is one of the Cdc42 effectors, which interact with Cdc42-GTP through the p21-binding domain (PBD), also known as Cdc42/Rac-interactive binding (CRIB) domain (Cvrckova et al., 1995; Benton et al., 1997). Cla4 is suggested to function with Bem1 and Cdc24, a Cdc42 GEF, in symmetry breaking (Kozubowski et al., 2008). Gic1 and Gic2 also interact with Cdc42-GTP and are known to be involved in polarity establishment and septin organization (Brown et al., 1997; Chen et al., 1997; Bi et al., 2000; Jaquenoud and Peter, 2000; Iwase et al., 2006; Sadian et al., 2013).

Despite substantial progress in deciphering the mechanisms underlying Cdc42 polarization, many questions remain. Of particular interest is how specific events during polarity establishment are coordinated with cell-cycle progression in G1, leading to bud
emergence. In haploid cells, which bud in the axial pattern, Cdc42 is activated by Bud3 in early G1 prior to its activation by Cdc24 (Kang et al., 2014). This stepwise Cdc42 activation occurs in correlation with the nuclear exit of the transcriptional repressor Whi5 (Kang et al., 2014), which partitions G1 into two phases—T1 and T2 (Di Talia et al., 2007). While biphasic activation of Cdc42 provides important insights into the temporal steps of bud-site assembly, the underlying mechanism has been less clear. Cells budding in a random pattern such as rsr1Δ exhibit occasional sporadic elevation of Cdc42-GTP during T1. Yet robust Cdc42 polarization occurs similarly during T2 in wild-type (WT) and mutant cells (Lee et al., 2015). These observations raised critical questions: Is stepwise activation of Cdc42 necessary to ensure sequential execution of the processes leading to bud emergence in cells budding in any pattern? If so, is relatively less efficient Cdc42 polarization in rsr1Δ cells during the first phase sufficient for these cells to traverse the G1 phase? Is there an additional mechanism underlying Cdc42 polarization during the first phase of G1? Moreover, what are the sequential processes triggered by stepwise Cdc42 polarization? These questions led us to delve further into stepwise Cdc42 polarization. Here we report that biphasic Cdc42 polarization is coupled to stepwise assembly of a new septin ring. We also find that Rsr1 and Gic proteins have a partially redundant role in promoting Cdc42 polarization in the first phase of G1.

RESULTS AND DISCUSSION
cdc42 alleles that are defective at a distinct phase in G1
To investigate stepwise Cdc42 polarization further, we sought to identify cdc42 alleles that exhibit polarization defects at either the first or second phase of G1. To this end, we examined temperature-sensitive (ts) cdc42 mutants that arrest as unbudded cells at 37°C (Kozminski et al., 2000) by time-lapse imaging of Spa2 fused to green fluorescent protein (GFP) as a marker for bud-site assembly, together with Whi5 fused to mCherry, a red fluorescent protein (RFP) to distinguish the first and second phases. Spa2 is one of the first proteins that localize to the incipient bud site prior to START, a cell-cycle commitment point in G1, and its localization is independent of Bem1 and Cdc24 (Snyder, 1989; Snyder et al., 1991; Arkowitz and Lowe, 1997; Rida and Surana, 2005). Spa2 localized to the sites of polarized growth and to the bud neck during cytokinesis in WT cells after a shift to 37°C (Supplemental Figure S1a), consistent with previous reports. Spa2 localization was partially defective in the cdc42-118 cells at 37°C mainly during the second phase (Figure 1A, b and d), and these cells became arrested with abnormal shapes (Supplemental Figure S1b). In contrast, Spa2 poorly polarized in cdc42-118 cells at 37°C, and Whi5 often stayed longer in the nucleus (Supplemental Figure S1c). The cdc42-118 cells that had already budded at temperature upshift maintained Spa2 polarization in the remaining cell cycle but failed to polarize and arrested as round, unbudded cells in the next G1 phase. A close examination of Spa2 polarization in unbudded cells with or without Whi5 in the nucleus revealed that Spa2 polarization is severely defective in cdc42-118 cells from the first phase (Figure 1A, c and d), despite similar levels of Spa2 in unbudded cells after shifting to 37°C for 2–4 h (Figure 1B). These observations suggest that cdc42-118 is unable to polarize in the first phase, which may lead to the sequential polarization defect in the second phase.

FIGURE 1: Localization of Spa2-GFP and Whi5-RFP in WT and cdc42* mutants at 37°C. (Aa-c) Representative images of each strain at 2 h after shifting to 37°C. Cells with polarized Spa1-GFP during the first (T1) or second phase (T2) of G1 are marked with purple or white arrows, respectively, based on Whi5-RFP localization. Images were deconvolved and summed. Bar, 5 μm. See also Supplemental Figure S1. (Ad) Percentage of unbudded cells with polarized Spa2 at 2 h after shifting to 37°C. Mean ± SEM is shown from analyses of cells in T1 and T2. Student’s t tests were used, with the following notation: ns (not significant) for p ≥ 0.05, ***p < 0.001, and ****p < 0.0001. (B) Normalized global intensity of Spa2-GFP in unbudded cells at 2 h after shifting to 37°C. Mean ± SEM is shown (see details in Materials and Methods).
Cdc42D76A may be defective in its interaction with Gic proteins

To gain a deeper understanding of the first phase of Cdc42 polarization, we further characterized cdc42-118, which encodes Cdc42D76A (Kozminski et al., 2000). A previous genomewide study with cdc42-118 identified synthetic-lethal or synthetic-sick interactions with gic2Δ and rsr1Δ (Kozminski et al., 2003). Extra copies of RSR1 suppress temperature-sensitive growth of cdc42-118 and gic1Δ gic2Δ (Kozminski et al., 2003; Gandhi et al., 2003; Kang et al., 2010), and gic1Δ gic2Δ is synthetic-lethal with rsr1Δ (Kawasaki et al., 2003). Interestingly, we found that extra copies of GIC1 from a low- or high-copy plasmid suppressed temperature-sensitive growth of cdc42-118, while extra copies of GIC2 even made WT cells sick (Figure 2Aa). This web of genetic interactions suggested that the polarity establishment defect of cdc42-118 and gic1Δ gic2Δ may result from a similar molecular deficiency.

Since Cdc42 directly interacts with Rsr1 (Kozminski et al., 2003), we tested whether Cdc42D76A is defective in interaction with Rsr1. After preloading Rsr1 and GST fusions of WT and the mutant Cdc42 with GTPαS or GDP or nucleotide-depleted (–), we incubated with yeast extract containing PBD-RFP at 4°C. Following GST pull-down assays, PBD-RFP and Cdc42 (or Cdc42D76A) were detected with polyclonal antibodies against DsRed (top panel) and GST (bottom panel), respectively. Average relative recovery of PBD is shown below each lane. The WT control lanes have also been used for validation of the PBD-RFP biosensor (Okada et al., 2017). (B) Cdc42D76A interacts with Rsr1 equally well as WT Cdc42. Each purified GTPase, preloaded with GTPαS (T) or GDP (D), was incubated at 24°C in various combinations as indicated. (a) Following GST pull-down assays, Rsr1 and GST-Cdc42 (or -Cdc42D76A) were detected with polyclonal antibodies against Rsr1 (top panel) and GST (bottom panel), respectively. (b) Following GST pull-down assays, His6-Cdc42 (or Cdc42D76A) and GST-Rsr1 were detected with polyclonal antibodies against Cdc42 (top panel) and GST (bottom panel), respectively.

FIGURE 2: Cdc42D76A poorly interacts with PBD. (Aa) Extra copies of GIC1 suppress cdc42-118. A 10-fold serial dilution of each strain carrying an indicated plasmid was incubated at 25 or 36°C for 3 d on SC-Ura. (Ab) Cdc42D76A poorly interacts with PBD-RFP. GST-Cdc42 or -Cdc42D76A, preloaded with GTPαS or GDP or nucleotide-depleted (–), was incubated with yeast extract containing PBD-RFP at 4°C. Following GST pull-down assays, PBD-RFP and Cdc42 (or Cdc42D76A) were detected with polyclonal antibodies against DsRed (top panel) and GST (bottom panel), respectively. (Aa) Following GST pull-down assays, His6-Cdc42 or Cdc42D76A was recovered similarly (Figure 2Ba), suggesting that Cdc42D76A is unlikely defective in interaction with Rsr1. We then tested whether Cdc42D76A is defective in interaction with Gics using PBD-RFP, which contains the Gic2 PBD (Tong et al., 2007). PBD-RFP interacted specifically with Cdc42-GTPαS, as expected, but poorly associated with Cdc42D76A-GTPαS even at 4°C (Figure 2Ab). These data suggest that the poor interaction of Cdc42D76A with Gics may account for its polarization defect in the first phase. Since other Cdc42 effectors also have a similar PBD, we next examined their interactions with Cdc42D76A using yeast extracts carrying tagged Gic2, Cla4, or Ste20. Pull-down assays indicated that Gic2 was most defective in association with Cdc42D76A (Supplemental Figure S2), consistent with the in vitro assay (Figure 2Ab), while Cla4 and Ste20 were also defective in association with Cdc42D76A to different extents. Collectively, these data suggest that Cdc42D76A is primarily, albeit not exclusively, defective in interaction with Gics.
beginning of T₂ (see blue arrows in Figure 3; see Figure 3D), while its peak level during the second phase was elevated ~3–4-fold relative to its intensity at the onset of cytokinesis (Figure 3C; Lee et al., 2015).

Collectively, these results suggest that Rsr1 and Gics share a role in Cdc42 polarization in the first phase of G1.

The role of Rsr1 in Cdc42 polarization depends on spatial cues

If the role of Rsr1 in Cdc42 polarization is dependent on spatial cues, as in the case of bud-site selection, we would expect that gic1Δ gic2Δ would be lethal in the absence of spatial cues (even with WT RSR1). To test this idea, we analyzed meiotic progeny of a diploid generated by crossing gic1Δ gic2Δ with axl2Δ rax1Δ. Deletion of AXL2 and RAX1 is expected to remove both functional axial and bipolar landmarks (Roemer et al., 1996; Kang et al., 2004). We found 6 of 88 meiotic progenies that were expected unambiguously to harbor the axl2Δ rax1Δ gic1Δ gic2Δ quadruple mutation did not germinate after 2.5 d at 30°C, although four spores germinated and formed microcolonies after 4 d (Supplemental Figure S3B). Because of the possible occurrence of spontaneous suppressor mutations and because of the presence of some dead segregants (whose genotype could not be determined), the lethality of the axl2Δ rax1Δ gic1Δ gic2Δ mutant may be higher. Since there were some ambiguities in this genetic analysis, we performed an additional test by generating a $P_{\text{GAL}}$-GIC1 $\text{gic2}\Delta$ axl2Δ rax1Δ strain. When this strain was imaged after turning off GIC1 expression, the majority of these cells arrested as large unbudded cells during a 5–6 h period (89%, $n = 200$; Supplemental Figure S3C). The 11% of cells that had undergone bud emergence did so within 1 h after turning off GIC1 expression, and this is likely due to some remaining Gic1 in these cells, as Gic1 is a relatively stable protein (Höfken and Schiebel, 2004). Therefore, we conclude that the role of Rsr1 in Cdc42 polarization in the first phase depends on spatial cues.

Biphasic Cdc42 polarization may be coupled to stepwise assembly of the septin ring

Why does Cdc42 polarization occur in two steps during G1? Since Cdc42 and Gics are necessary for septin organization (Gladfelter et al., 2002; Iwase et al., 2006; Okada et al., 2013), we tested whether
cells lacking Gics and Rs1 are defective in either recruitment of the septins (appearing as disorganized “clouds”) or ring formation by time-lapse imaging of Cdc3-GFP. Even when both Gics were depleted, new septin clouds often appeared prior to disassembly of the old ring in the presence of RSR1 (98%, n = 16; Figure 4Aa). In contrast, in the absence of Rs1 and Gic2, new septin rings appeared soon after cytokinesis only when GIC1 was expressed (100%, n = 12; Figure 4Ab). When GIC1 expression was turned off, the majority of $P_{GAL}$-GIC1 gic2Δ rsl1Δ cells failed to form new septin clouds or ring after cytokinesis (90%, n = 20; Figure 4Ac); instead, the old septin ring remained in these cells long after cytokinesis, and cells arrested in the unbudded state. In a few cells, new Cdc3-GFP signal appeared after cytokinesis, but the signal was weak and transient without developing into a septin ring. Taken together, these results suggest that Rs1 and Gics share a role in recruitment of new septins in the first phase of Cdc42 polarization. These observations are consistent with the idea that biphasic Cdc42 polarization is coupled to stepwise assembly of the septin ring—first, septin recruitment, and then ring assembly (Iwase et al., 2006)—and is thus unlikely limited to cells budding in the axial pattern.
Overexpression of Cdc42 bypasses requirement of Rsr1 and Gics for septin recruitment
As discussed above, Cdc42 polarization in the first phase and septin recruitment require either Rsr1 or Gics. Although Gics could function in septin recruitment as downstream effectors of Cdc42, we considered another possibility that Gics might share a role with Rsr1 in promoting Cdc42 polarization. If this is the case, then an elevation of Cdc42 might be able to bypass the requirement of Rsr1 and both Gics in septin recruitment. To test this idea, we examined the localization of septin in the P\_GAL-GIC1 gic2Δ rsr1Δ cells carrying a multicopy CDC42 plasmid. Remarkably, these cells were able to recruit new septins even when Rsr1 and both Gics were absent (Figure 4Bb). We compared septin localization patterns at selective time points from time-lapse images. The cells carrying the CDC42 plasmid had either new septin “clouds” or ring within 30 min after cytokinesis even when Rsr1 and both Gics were depleted, unlike cells with a vector control (compare d to b, Figure 4C). This timing of new septin assembly in cells overexpressing Cdc42 was comparable to cells expressing GIC1 (Figure 4, Ba, Ca, and Cc). These results are consistent with the idea that extra copies of Cdc42 allow its polarization and thus trigger septin recruitment in the absence of Gics and Rsr1. Together with the data discussed above, these findings support the idea that Gics and Rsr1 share a role in promoting Cdc42 polarization during T1, which leads to septin recruitment.

Gics may promote Cdc42 polarization by stabilizing Cdc42 on the plasma membrane
How do Gic proteins promote Cdc42 polarization? Although Gics bind to Cdc42-GTP, Gic1 has additional membrane association domains and polarizes even when Cdc42 binding is disrupted (Chen et al., 1997; Takahashi and Pryciak, 2007). We hypothesized that Gics might promote Cdc42 polarization by slowing its mobility on the plasma membrane (PM). To test this idea, we compared Cdc42 dynamics in WT and gic1Δ gic2Δ cells by fluorescence recovery after photobleaching (FRAP) analysis. Indeed, we found that Cdc42 cluster at the incipient bud site recovered faster after bleaching in gic1Δ gic2Δ cells than in WT (Figure 5A). This role of Gics may be more critical at a higher temperature, since haploid gic1Δ gic2Δ cells often fail to establish cell polarity at 37°C (or at 32°C in some strain backgrounds) (Brown et al., 1997; Chen et al., 1997; Bi et al., 2000). It is possible that Cdc42 is more dynamic on the PM at a higher temperature, and thus its polarization is more dependent on Gics even when Rsr1 is present. Consistent with the idea, Cdc42 was more mobile at the incipient bud site at 34°C (a semipermissive temperature for the gic1Δ gic2Δ strain used in this study) than at 22°C (Figure 5Ab).

There has been considerable debate about whether Cdc42 polarizes independently of guanine nucleotide dissociation inhibitors (GDIs) and actin-based trafficking (Marco et al., 2007; Slaughter et al., 2009; Layton et al., 2011; Freisinger et al., 2013; Jose et al., 2013; Klunder et al., 2013; Slaughter et al., 2013; Bendezu et al., 2015; Woods et al., 2016). Interestingly, both fission and budding yeast cells expressing a cdc42 mutation (cdc42-ritC, in which the CAAX sequence is replaced by the C-terminal amphiphilic helix of a Rit GTPase, are able to undergo symmetry breaking, suggesting that Cdc42 can polarize independently of both GDI-mediated membrane extraction of Cdc42-GDP and vesicle trafficking (Bendezu et al., 2015). Since the budding yeast cdc42-ritC mutant is temperature sensitive and exhibits frequent loss of singularity of budding, we speculated that slower mobility of Cdc42-ritC might allow its polarization but result in poor competition between Cdc42 clusters. Indeed, we found that Cdc42-ritC-GFP exhibited much slower recovery than WT Cdc42 after bleaching (Figure 5B).

Since Cdc42-ritC exhibits slower dynamics than WT Cdc42, we wondered whether the cdc42-ritC mutant can bypass requirement of Rsr1 or Gics for its polarization during the first phase of G1. Surprisingly, however, when we tested genetic interactions between cdc42-ritC gic1Δ and gic2Δ, we found all meiotic progeny that could be predicted unambiguously to harbor all cdc42-ritC, gic1Δ, and gic2Δ mutations were inviable (Figure 5Ca). In contrast, a similar genetic test with rsr1Δ indicated that all meiotic progeny (except one) from 88 tetrads, including the triple cdc42-ritC gic1Δ rsr1Δ mutant, were viable (Figure 5Cb). Therefore, polarization of Cdc42-ritC is likely to be mediated by Gics during the first phase of G1. This observation seems counterintuitive because Cdc42-ritC is less mobile than WT Cdc42, which is more mobile in the gic1Δ gic2Δ cells (see Figure 5Ab). A possible explanation for the synthetic lethality of cdc42-ritC gic1Δ gic2Δ is that even a slight increase of its mobility (in the absence of Gic1/2) could be more detrimental to polarization of Cdc42-ritC, which presumably occurs via lateral diffusion and/or GDI-independent exchanges between membrane and cytosol. The cdc42-ritC mutant is also defective in bud-site selection (Bendezu et al., 2015), consistent with our conclusion that its polarization is mediated via Gics, rather than Rsr1, and thus cdc42-ritC is likely inviable in the absence of Gics. Collectively, these results suggest that Gics promote Cdc42 polarization by stabilizing Cdc42 on the PM.

Model for biphasic Cdc42 polarization in G1
In this study, we show that Rsr1 and Gic1/Gic2 share a common role in Cdc42 polarization during the first phase of G1 and that Cdc42 polarization in the first phase is necessary for septin recruitment. We thus suggest that biphasic Cdc42 polarization is coupled to stepwise assembly of the new septin ring and is involved in both spatial cue-dependent and spontaneous cell polarization (Figure 5D). But how Cdc42 polarization promotes septin recruitment remains an open question. Gic proteins are known to be involved in septin assembly by directly interacting with septin subunits and stabilizing the septin complex (Iwase et al., 2006; Sadian et al., 2013). Since overexpression of CDC42 can bypass the requirement of Gic1/2 and Rsr1 for septin recruitment (Figure 4), Cdc42 is able to promote septin recruitment without Gics. An interesting possibility is that Cdc42 may recruit septins directly or via other proteins such as Ax12, as previously suggested (Gao et al., 2007).

While Gic proteins interact with Cdc42-GTP, involvement of Gics in Cdc42 polarization suggests a previously unrecognized positive feedback loop (Figure 5D). Initial stochastic activation of Cdc42 may recruit Gics by interacting with their PBD. The Gic-Cdc42-GTP complex may be stabilized through the interaction with PIP2 on the PM (Takahashi and Pryciak, 2007; Orlando et al., 2008). This stabilization may counteract lateral diffusion of Cdc42, the endocytosis-mediated internalization, and/or GDI-mediated recycling (Ozbudak et al., 2005; Marco et al., 2007; Slaughter et al., 2009; Klunder et al., 2013; Woods et al., 2016), consistent with our FRAP data (Figure 5A).

Cdc42 polarization mediated by Rsr1 may involve a positive feedback loop, which includes spatial cue-dependent recruitment and/or activation of the Rsr1 GTPase module and local activation of Cdc42 (Kozminski et al., 2003; Kang et al., 2014; Lee et al., 2015). Rsr1 may also be involved in the second phase of Cdc42 polarization via interaction between Rsr1-GTP and Cdc24.
FIGURE 5: Gic proteins may promote Cdc42 polarization by stabilizing Cdc42 on the PM. (Aa) FRAP analyses of GFP-Cdc42 at the incipient bud site in WT and gic1Δ gic2Δ cells (mean ± SEM shown). (Ab) Halftimes of GFP-Cdc42 FRAP recovery at indicated sites; all at 22°C, except those indicated at 34°C. For each data point, n = 9–11. Student’s t tests were used, with the following notation: ns (not significant) for p ≥ 0.05, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. (Ba) Halftimes of FRAP recovery of GFP-Cdc42-ritC vs. GFP-Cdc42 at each site. n = 9–11 for each data point. (Bb) FRAP curves of GFP-Cdc42-ritC at each indicated site. See legend to Aa and Ab. (C) Representative tetrads from crosses between cdc42-ritC gic1Δ and gic2Δ (a) and between cdc42-ritC gic1Δ and cdc42-ritC rsr1Δ (b). Segregants marked with green squares and one of each pair marked with green triangles harbor the cdc42-ritC gic1Δ gic2Δ mutation. Segregants marked with red squares harbor the cdc42-ritC gic1Δ rsr1Δ mutation. (D) Model for biphasic Cdc42 polarization coupled to stepwise assembly of a new septin ring. Red and purple rings denote old and new septin rings, respectively. Purple dots and dotted red line denote newly recruited septin “clouds” and disassembling old ring, respectively (see the text).
(Zheng et al., 1995; Park et al., 1997). We anticipate that the first phase of Cdc42 polarization and the initial recruitment of septins do not require Cdc24, because Cdc42 is activated by Bud3 in the first phase (Kang et al., 2014), during which Cdc24 is mostly sequestered within the nucleus in haploid cells (Toenjes et al., 1999; Nern and Arkowitz, 2000; Shimada et al., 2000). The second step of Cdc42 polarization and thus septin ring assembly are likely promoted by Cdc24 and Bem1. Consistent with this timing, Cdc24 activity is stimulated by Bem1 (Smith et al., 2013; Rapali et al., 2017), which associates with Cdc24 after START (Witte et al., 2017). While further investigation is necessary to fully understand the underlying mechanisms, biphasic Cdc42 polarization provides an elegant example of a highly redundant system, which integrates distinct signals to achieve a single biological event.

**MATERIALS AND METHODS**

**Strains, plasmids, and growth conditions**

Standard methods of yeast genetics, DNA manipulation, and growth conditions were used (Guthrie and Fink, 1991). All yeast strains used for imaging express tagged proteins under their native promoters from the chromosomes. Yeast strains were grown in rich yeast medium YPD (yeast extract, peptone, dextrose) or synthetic complete (SC) containing 2% dextrose as a carbon source, unless stated otherwise. Where indicated, SC medium containing 2% galactose (SGal) was used instead of dextrose to turn on the GAL promoter. To track the segregation of auxotrophic markers and to maintain plasmids, strains were cultured in SC medium lacking the appropriate nutrient(s) (e.g., SC-Ura). Strains used in this study are listed in Supplemental Table S1. Multicopy plasmids pRS426-GIC1 and pRS426-GIC2 (2μ, URA3) were kind gifts from P. Pryciak (University of Massachusetts Medical School) (Takahashi and Pryciak, 2007). Plasmids used for expression of GST-Cdc42 and His6-Cdc42 and a multicopy plasmid Yep103-CDC42 were previously described (Kozminski et al., 2003). Plasmids for expression of GST-Cdc42 and His6-Cdc42 and a multicopy plasmid Yep103-CDC42 were previously described (Kozminski et al., 2003). The plasmid used for expression of GST-Rsr1 was previously described (Holding et al., 1991). The plasmids used for expression of movable open reading frame (MORF)-tagged Gic2, Ste20, and Cla4 were previously described (Gelperin et al., 2005) and purchased from Thermo Scientific Open Biosystems.

**Microscopy and image analysis**

Cells were grown in an appropriate synthetic medium overnight and then freshly subcultured for 3–4 h in the same medium. For most time-lapse imaging, images were captured (11 or 13 z-stacks; 0.3-μm step) every 10 or 20 min at 24–25°C using a Nikon E800 microscope fitted with a 100×/1.30 NA oil-immersion objective lens, Hamamatsu ORCA-2 CCD (Hamamatsu Photonics), and Slidebook software (Intelligent Imaging Innovations), and single z-stack images were used to make figures.

To image Spa2-GFP and Whi5-RFP in WT and cdc42 mutants, cells grown at 24–25°C overnight were mounted on glass-bottomed dishes and initially imaged at 25°C for 1 h to ensure healthy growth. Two-color time-lapse imaging was then started at 30 min after temperature upshift to 37°C. Image processing and analyses were performed using ImageJ (National Institutes of Health). Where indicated, images were deconvoluted by the Iterative Constrained Richard-Lucy algorithm using Nis element software. Summed intensity projections of z-stacks of representative time-lapse images were used to generate Figure 1 and Supplemental Figure S1. Images at 2 h after shifting to 37°C were analyzed to compare Spa2-GFP polarization in unbudded cells during T1 (from cytokinesis until mid G1 when Whi5-RFP resides in the nucleus) and T2 (from mid-G1 when Whi5-RFP intensity in the nucleus drops less than 50% until bud emergence or G1 arrest for mutants) (Figure 1). A fluorescence threshold was set to select polarized Spa2-GFP and measure the mean intensity of polarized Spa2-GFP using the summed WT images of z-stacks after background subtraction. The same threshold was also applied to all mutant images that were captured and processed using the same conditions. Unbudded cells (in T1 or T2) that had polarized Spa2-GFP were counted from three to five sets of time-lapse images of each strain, and mean ± SEM is shown by analyzing the following total number of cells at each phase: WT, n = 111 (T1) and 71 (T2), cdc42-123, n = 129 (T1) and 70 (T2), and cdc42-118, n = 245 (T1) and 87 (T2). To compare total Spa2-GFP level in individual cells of each strain after temperature upshift, global intensity of Spa2-GFP in unbudded cells was quantified using the same summed intensity projections (at 2–4 h after temperature upshift to 37°C). A region of interest (ROI) was drawn around the outline of unbudded cells, and integrated density of each ROI was measured in three sets of time-lapse images for each strain, counting a total number of cells: n = 80 (WT), 110 (cdc42-123), and 100 (cdc42-118) (Figure 1B). For quantification of global intensity of GFP-Cdc42 (Supplemental Figure S3Ac), average intensity projections were created from all 15 z-sections at 0.3-μm spacing, and an ROI was drawn around the outline of unbudded cells. WT cells without any fluorescently tagged protein were mixed to capture control cell images together with experimental strains, and the mean intensity of the control cells was used to subtract background.

The PBD fluorescence intensity along the cell cortex was analyzed using single focused z-stack images of HPY2618 (pGAL-GIC1 gicΔ Δ rsr1Δ PBD6×PBD-tomato) when GIC1 was expressed (Gal) from 6 min prior to the onset of cytokinesis (estimated based on PBD distribution) until T1–T2 boundary (estimated based on stabilization of the PBD cluster location) (Okada et al., 2013; Lee et al., 2015) (see Figure 3D). We noticed that the PBD-RFP probe was slightly toxic in this strain background, making cells grow slowly, particularly in galactose-based medium. Images of cells when GIC1 was turned off (Glu) were also analyzed from 6 min prior to cytokinesis and subsequent time points (although the exact cell-cycle stage

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of the end point was not clear due to lack of single PBD cluster development when GIC1 was not expressed. To quantify PBD fluorescence intensity around the cell cortex using ImageJ, freehand lines were drawn by selecting a three-pixel (0.389 μm)-wide region around the cell periphery, and PBD-RFP fluorescence was then measured along the lines for each time point of time-lapse images. Kymographs were generated by displaying PBD intensity in the same scale for both cases when GIC1 was on or off (Figure 3B). Maximum intensity projections of z-stacks were used to make figures of fluorescence images in Figures 3 and 4, except in Figure 3Ab, single z-stack images were used to show PBD fluorescence at the cell periphery more clearly.

To quantify Cdc42 polarization, the fluorescence intensity of PBD-RFP clusters was measured by a threshold method using an ImageJ macro (Okada et al., 2013; Okada et al., 2017). Briefly, mean projections were generated from five best z-sections at each time point, and then a threshold method was used after background subtraction. Mother and daughter cells of HPY2618 were analyzed separately from the onset of cytokinesis until bud emergence when cells were grown in galactose-based medium at 25°C and after shifting to glucose-based medium from the onset of cytokinesis over 4 h at the same temperature. The intensity of PBD-RFP clusters at each time point was normalized to its value at the onset of cytokinesis (t = 0). A representative analysis of PBD-RFP cluster in a mother cell of the PGAL-GIC1 gic2ΔΔ-CBD PW23A LctTomato strain is shown when GIC1 was expressed (Gal) and when GIC1 expression was turned off (Glu) (Figure 3C). These cells rarely underwent new budding when GIC1 expression was turned off.

**FRAP analysis**

Cells were grown in an appropriate synthetic medium overnight and then freshly subcultured for 3–4 h in the same medium. FRAP experiments (and images shown in Figure 5) were performed at 22°C, except those indicated at 34°C, using a spinning disk confocal microscope (Ultra-VIEW VoX CSU-X1 system; Perkin Elmer-Ce tus) equipped with a 100×/1.4 NA Plan Apochromat objective lens (Nikon); 440-, 488-, 515-, and 561-nm solid-state lasers (Modular Laser System 2.0; Perkin Elmer-Cetus), and a back-thinned EM CCD (ImageEM C9100-13; Hamamatsu Photonics) on an inverted microscope (Ti-E; Nikon). Images were captured at a single z-section on a gelatin slab using the photokinesis unit on the Ultra-VIEW VoX confocal system, similarly to the assays described previously (Coffman et al., 2009; Miller et al., 2017). After collecting five prebleach images, selected ROIs were bleached to ≤55% of the original fluorescence intensity. Postbleach images were captured for a duration long enough so the fluorescence recovery curve reached a plateau. For each FRAP experiment, all intensity values (after correcting for background and photobleaching) were normalized, so the prebleaching and the first postbleaching intensities equal 100 and 0%, respectively. To reduce noise, the intensities of every three consecutive postbleach time points were averaged. Then the intensity data from individual ROIs and the average intensity value at each time point across all ROIs were plotted and fitted using the exponential decay equation \( y = m_1 + m_2 \exp(-kt) \), where \( m_3 \) is the off-rate, using Prism 6 (GraphPad Software). The halftime of recovery was calculated using the equation \( t_{1/2} = \ln2/m_3 \).

Halftimes of GFP-Cdc42 FRAP recovery in WT and gic1Δ gic2Δ cells were compared at the following sites: the incipient bud site of unbudded cells (n = 10, WT; n = 11, gic1Δ gic2Δ), bud neck (n = 11, WT; n = 11, gic1Δ gic2Δ), and nonenriched region on the PM of large budded cells (n = 10, WT; n = 11, gic1Δ gic2Δ) at 22°C and also at the incipient bud site of unbudded cells at 34°C (n = 10, WT; n = 9, gic1Δ gic2Δ). Halftimes of GFP-Cdc42-ritC FRAP recovery were also determined at the incipient bud site (n = 10), bud neck (n = 9), and nonenriched region on the PM of large budded cells (n = 8) and compared with those of GFP-Cdc42 at 22°C. Either a FRAP curve (with mean ± SEM) or a bar graph (with median, quartiles, maximum, and minimum) are plotted using Prism 6.

**Protein purification, in vitro binding assay, and immunoblotting**

GST-Rsr1, His6-Gic2Δ, and His6-Gic2ΔD76A were expressed and purified in a protease-deficient Escherichia coli strain (BL21 codon plus), and GST-Cdc42 and GST-Cdc42D76A were expressed from insect cell lines, as previously described (Kozminski et al., 2003). Either purified proteins or high-speed supernatants (2.5 μg total protein per reaction) of the insect cell extracts containing GST-Cdc42 or GST-Cdc42D76A were used in binding assays, as previously described (Kozminski et al., 2003). Briefly, purified GTPases (either with or without GST moiety) were dialyzed overnight at 4°C against a buffer (20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol [DTT], 5 mM MgCl2, 10% glycerol) containing 2.5 μM GDP after purification. Approximately 1 μg of a GST-fusion protein was diluted to a final volume of 50 μl with 50% glutathione Sepharose beads slurry and incubated for 1 h at 4°C. The beads were collected by centrifugation and resuspended in Buffer I (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, 10 mM EDTA, 10% glycerol, 0.1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml aprotinin). After incubation for 1 h at room temperature, Buffer I was substituted with Buffer 1 containing 5 mM MgCl2 plus 0.5 mM GTPγS or 0.5 mM GDP (Roche Diagnostics). After 30 min incubation at 24°C, the beads were resuspended in Buffer I containing 10 mM MgCl2 plus 0.5 mM GTPγS or 0.5 mM GDP instead of 10 mM EDTA and then incubated 20 min at 24°C to stabilize the nucleotide bound state of the GTPase. For in vitro binding reaction, Rsr1 (~400 nM), purified after removal of GST, was preloaded with GTPγS or GDP and incubated with GST-Cdc42 or GST-Cdc42D76A (~400 nM each), which was also preloaded with GTPγS or GDP in various combinations at 24°C. Association of Cdc42 or Cdc42D76A with GST-Rsr1 was determined similarly, except GST- Rsr1, His6-Gic2Δ, and His6-Gic2ΔD76A were used in various combinations, as indicated. The interaction between PBD-RFP and GST-Cdc42 (or GST-Cdc42D76A) was tested by similar binding assays, except that the soluble fraction (S10) of extracts from 80 OD600 units of yeast cells (HPY1231), which express Gic2-PBD-RFP from the chromosome, was used, and the incubation was performed at 4°C. GST-Cdc42, GST-Cdc42D76A, and GST-Rsr1 were detected by immunoblotting with polyclonal antibodies against GST (Santa Cruz Bio technology). Rsr1 (after GST was removed), His6-Gic2Δ, and Gic2- PBD-RFP were detected with polyclonal antibodies against Rsr1 (Park et al., 1997), Cdc42 (Kozminski et al., 2003), and DsRED (Clontech Laboratories), respectively. In vitro binding assays were repeated three times from two independent preparations of purified proteins or yeast extracts.

To test the association of Cdc42 effectors with GST-Cdc42 or GST-Cdc42D76A, Gic2, Cia4, or Ste20 was expressed as a triple affinity tagged (composed of His6-HA epitope-immunoglobulin G (IgG) binding ZZ domain) protein in a yeast strain (Y258) using each MORF plasmid (Gelperin et al., 2005). The pull-down assays of the MORF-tagged proteins were performed with IgG-Sepharose, as previously described (Gelperin et al., 2005). GST fusion proteins were detected as described above, and each MORF-tagged protein was detected with monoclonal anti-HA antibodies (Covance, Emeryville, CA).
**Statistical analysis**

Data analysis was performed using Prism 6 (GraphPad Software). Error bars indicate SEM unless indicated otherwise. The bar graphs of FRAP data show median as a line, quartiles, maximum, and minimum. A two-tailed Student’s t test was performed to determine statistical differences between two sets of data: ns (not significant) for \( p \geq 0.05; * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.\)

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