Adjacent positioning of cellular structures enabled by a Cdc42 GTPase-activating protein–mediated zone of inhibition

Zongtian Tong,1 Xiang-Dong Gao,1,2 Audrey S. Howell,3 Indrani Bose,3 Daniel J. Lew,3 and Erfei Bi1

1Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104
2State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan 430072, China
3Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710

Cells of the budding yeast Saccharomyces cerevisiae are born carrying localized transmembrane landmark proteins that guide the subsequent establishment of a polarity axis and hence polarized growth to form a bud in the next cell cycle. In haploid cells, the relevant landmark proteins are concentrated at the site of the preceding cell division, to which they recruit Cdc24, the guanine nucleotide exchange factor for the conserved polarity regulator Cdc42. However, instead of polarizing at the division site, the new polarity axis is directed next to but not overlapping that site. Here, we show that the Cdc42 guanosine triphosphatase–activating protein (GAP) Rga1 establishes an exclusion zone at the division site that blocks subsequent polarization within that site. In the absence of localized Rga1 GAP activity, new buds do in fact form within the old division site. Thus, Cdc42 activators and GAPs establish concentric zones of action such that polarization is directed to occur adjacent to but not within the previous cell division site.

Introduction

During development and differentiation, it is sometimes important for cells to position specific structures adjacent to but not overlapping each other. For example, in epithelia, apical tight junctions are found next to more basal adherens junctions. In haploid Saccharomyces cerevisiae, new buds form next to the previous cell division site but never within it. Because yeast cell division is accompanied by deposition of specialized “bud scars” on the mother cell wall and “birth scars” on the daughter cell wall (Barton, 1950; Chant and Pringle, 1995), it could be that the scars physically preclude subsequent bud emergence at division sites. Indeed, an early hypothesis to explain reproductive aging in yeast was that bud scars would eventually cover the entire cell wall (Mortimer and Johnson, 1959). However, even if cell wall scars impede budding, how would cells “know” this ahead of time and ensure that polarization sites were positioned next to (rather than overlapping) those sites?

In most fungal and animal cells, a polarity axis chosen in a cell type–specific manner is communicated to a conserved polarity mechanism centered on the Rho family GTPase Cdc42 (Johnson, 1999; Pruyne and Bretscher, 2000; Etienne-Manneville, 2004; Park and Bi, 2007). In S. cerevisiae, the polarity axis for budding is selected by a network of mating type–regulated bud-site selection proteins (Park and Bi, 2007). MATα or α cells bud axially (the new bud forms next to the previous division site) and MATα/α cells bud bipolarly (the new bud forms at either pole of the cell). Landmark proteins concentrated at the chosen sites recruit and activate Cdc24, the guanine nucleotide exchange factor (GEF) for Cdc42, which leads to localized production of GTP-Cdc42, which then directs cytoskeletal polarization and bud formation. However, whereas the landmark proteins localize to the site of cell division, Cdc24 and Cdc42 concentrate at an adjacent spot in preparation for budding. The basis for this avoidance of the previous division site was entirely unknown.

There are thought to be three dedicated GTPase-activating proteins (GAPs) for Cdc42 in yeast: Rga1, Rga2, and Bem3 (Zheng et al., 1994; Stevenson et al., 1995; Smith et al., 2002). All three are large proteins with C-terminal Rho GAP domains. Deletion of these GAPs either singly or in combination does not impair actin polarization but does impair Cdc42-directed assembly of the septin cytoskeleton into a ring at the presumptive bud site (Gladfelter et al., 2002; Smith et al., 2002; Caviston et al., 2003). Rga2 and Bem3 display a similar localization pattern to Cdc42 throughout the cell cycle. Although Rga1 also colocalizes
with Cdc42 at the site of bud emergence, it subsequently spreads throughout the bud cortex and concentrates at the septin ring in the mother-bud neck until the end of the cell cycle (Caviston et al., 2003). Based on this unique localization pattern, we suspected that Rga1 might play a specialized role in addition to its shared roles with the other GAPs. Here, we show that Rga1 specifically prevents Cdc42 activation and thus budding within the old division site.

**Results and discussion**

The most striking phenotype of rga1 Δ single mutants is a paucity of bud scars (Fig. 1 A), which confirms previous findings (Stevenson et al., 1995; Chen et al., 1996). In contrast, rga2 Δ bem3 Δ cells displayed normal scar numbers (Fig. 1 A). The bud scar is a ridge of cell wall material formed by a septin-localized chitin synthase complex during bud emergence (DeMarini et al., 1997). The chitin ring stabilizes the bud neck during bud growth (Schmidt et al., 2003) and remains on the mother cell wall as a bud scar after cell division (Chant and Pringle, 1995). In principle, the dearth of bud scars in rga1 Δ mutants could reflect fewer budding cycles, the ability to form buds without leaving scars, or the occurrence of multiple budding events at the same site. The first possibility predicts that rga1 Δ populations would proliferate much more slowly than wild-type controls, but this was not observed (Chen et al., 1996). The second possibility seemed unlikely, given that all budded cells displayed bright Calcofluor-stained chitin rings on the mother side of the neck (Fig. 1 B). Thus, we tested whether new buds might form at sites of old bud scars. Visualizing bud scars with scanning EM (SEM), we confirmed that wild-type haploid cells displayed nonoverlapping bud scars forming a zigzag line on the cell surface (Fig. 1 C). rga2 Δ bem3 Δ cells displayed a similar pattern, but rga1 Δ cells often displayed more than one bud scar at the base of the bud neck (Fig. 1 C), which is suggestive of repeated budding at the same site. The rga1 Δ rga2 Δ bem3 Δ cells also displayed stacked bud scars, though cells and scars were more irregular in shape (Fig. 1 C), as was expected given the perturbed septin rings in these mutants (Gláfelfer et al., 2002; Smith et al., 2002; Caviston et al., 2003).

To determine whether the stacked bud scars arose by repetitive budding from the same site, we performed 3D time-lapse microscopy on haploid cells expressing a functional GFP-tagged septin. Under optimal growth conditions, the new septin ring in a mother cell forms 5–8 min before the old septin ring at the previous division site disappears (Fig. 1 D; Iwase et al., 2006), providing an opportunity to visualize the new budding event with respect to the old division site. We found that wild-type (n = 7 of 8) and rga2 Δ bem3 Δ (n = 10 of 10) cells formed a new septin ring next to the disassembling old ring (Fig. 1 D). Strikingly, however, most rga1 Δ cells (n = 22 of 30) and rga1 Δ rga2 Δ bem3 Δ cells (n = 7 of 7) formed a new septin ring within the old ring (Fig. 1 D), which indicates that the stacked bud scars are indeed caused by repetitive budding from the same site. To determine where bud emergence occurs in rga1 Δ daughter cells, we isolated newborn daughters by centrifugal elutriation, allowed them to form their first buds, and stained them with fluorescent Con A to visualize birth scars (Lew and Reed, 1993). Birth scars mark the sites on newborn cells that used to be the mother-bud neck and 96.5% of wild-type daughter cells (n = 231) form buds next to the birth scar (Fig. 1 E). Strikingly, 95.9% of rga1 Δ daughter cells (n = 243) formed buds within (rather than next to) the birth scar (Fig. 1 E). This phenotype was confirmed by 3D time-lapse microscopy (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200705160/DC1). We also found that rga1 Δ cells had expanded birth scars with a mean width of 3.8 ± 1.4 μm relative to wild-type birth scars of 1.6 ± 0.3 μm (mean ± standard deviation, n = 20), perhaps because of increased cell surface growth within the birth scar after cytokinesis and/or a mild defect in septin ring assembly. Together, these results demonstrate that Rga1 is necessary to prohibit budding at the previous division site in both mother and daughter cells.

Previous studies have suggested that GAPs can potentially act not only as down-regulators but also as effectors for Cdc42. To determine whether Rga1 function requires GAP activity, we mutated the “arginine finger” motif conserved among Ras, Rho, and Cdc42 GAPs (Ahmadian et al., 1997; Rittinger et al., 1997). As expected, the rga1 R829A GAP domain displayed significantly reduced GAP activity toward Cdc42 in vitro (Fig. 2 A), though it retained the ability to bind GTP-Cdc42 (indeed, the mutant protein pulled down more GTP-Cdc42 than did the wild-type; Fig. 2 B). We then replaced the endogenous RGA1 with full-length epitope-tagged wild-type or R829A mutant genes. These proteins were expressed at comparable levels (Fig. 2 C) and localized similarly throughout the cell cycle (Fig. 2 D), but the arginine finger mutant was completely nonfunctional in terms of the budding pattern (Fig. 2 E and F; and Table I). Thus, the role of Rga1 in preventing polarization at the division site depends on its GAP activity.

The budding-within-the-old-division-site phenotype and its dependency on the GAP activity of Rga1 raise the possibility that the level of GTP-Cdc42 may be elevated at the cell division site in rga1 Δ cells. To examine this possibility, the localization of Gic2–p21 binding domain (PBD)–RFP, a reporter for GTP-Cdc42 (Fig. S1 B), was examined in wild-type and mutant strains (Fig. 3 and Videos 1–4, available at http://www.jcb.org/cgi/content/full/jcb.200705160/DC1). Gic2-PBD-RFP was localized to the presumptive bud site and the bud cortex of predivision cells in all strains. At the time when cells were completing cytokinesis and displayed split septin rings (detected with Cdc3-GFP), Gic2-PBD-RFP was localized adjacent to but outside the old division site in wild-type and most rga2 Δ bem3 Δ cells (Fig. 3, A and B; and Videos 1 and 4). In contrast, most rga1 Δ and rga1 Δ rga2 Δ bem3 Δ cells at this stage showed an increased concentration of Gic2-PBD-RFP within the split septin rings at the division site (Fig. 3, A and B; and Videos 2 and 3). This striking feature was confirmed by the localization of GFP-tagged full-length Gic2 and Ste20, effectors localized through their interaction with GTP-Cdc42 (Fig. S1 C). Thus, Rga1 is uniquely important for clearing GTP-Cdc42 from the bud neck at the end of cytokinesis.

In addition to the C-terminal GAP domain, Rga1 has two N-terminal LIM domains. There is some evidence that these domains may restrict Rga1 GAP function, as their removal leads to synthesis of a truncated protein able to suppress morphogenesis defects caused by mutation of the Rho-GAP Bem2 (Chen et al., 1996). However, we found that deletion of the N-terminal half
Figure 1. **Deletion of RGA1 causes polarization and budding within the previous division site.** (A) Quantitation of bud scar distribution in an asynchronous population of cells from haploid strains YEF473A [wild type], YEF2324 (rga1Δ), YEF2392 (rga2Δ bem3Δ), and YEF2380 (rga1Δ rga2Δ bem3Δ). 200 cells were counted for each strain and unbudded daughter cells were excluded. The cells with only chitin rings at the base of the growing buds were counted as having “0 bud scar.” (B) Chitin staining of wild-type and rga1Δ cells indicated in A. Double chitin rings at the neck of an rga1Δ cell (2) were visualized occasionally when the distance between the rings was large enough to be resolved by light microscopy. (C) SEM observation of bud scars. The same strains described in A were used for SEM. (D) Using the positions of the septin rings as a read-out of the budding patterns in live cells. Cells of haploid strains YZT82 (CDC3-GFP, wild type), YZT55 (rga1Δ CDC3-GFP), and YZT111 (rga2Δ bem3Δ CDC3-GFP) were grown to exponential phase in YM-P medium and observed by 3D time-lapse microscopy at 30°C. Times are given in minutes and seconds after an arbitrary starting point. Arrowheads indicate an old septin ring at the mother side of the bud neck; arrows indicate the nascent septin ring at the new bud site. Views of the 3D images from particular angles are shown: an angled side view of the bud neck of the wild-type cell, an en-face view of the mother side of the bud neck of the rga1Δ cell, and a side view of the bud neck of the rga2Δ bem3Δ cell. Please note that a clear rotation of the mother cell versus the daughter cell occurred after cytokinesis and cell separation at a time between 9 min 17 s and 11 min 45 s for the wild-type cell and between 5 min 29 s and 7 min 31 s for the rga2Δ bem3Δ cell. (E) The first bud of rga1Δ daughter cells forms within the birth scar. Birth scars of representative wild-type (YEF473A) and rga1Δ (YEF2324) cells. Cells 1 and 2 represent off-center and central budding within the birth scar, respectively. Bars, 1 μm.
(or more) of Rga1 rendered the protein nonfunctional in terms of preventing polarization at the previous division site. The truncated protein was stably expressed (Fig. S1 E) but failed to concentrate at the cortical sites where full-length Rga1 was found (Fig. S1 F). Thus, the Rga1 non-GAP domains are important for both localization and function of Rga1. To determine whether proper localization of the Rga1 GAP domain to the septin rings at the end of the cell cycle was sufficient to prevent polarization at the previous division site, we fused the Rga1 GAP domain (residues 700–1007) to the bud-site selection protein Bud3, which is concentrated at the septin rings from G2 through cytokinesis and early G1 (Chant et al., 1995). Remarkably, this fusion was able to fully complement the same-site rebudding phenotype of rga1Δ mutants (Table I). Moreover, the GAP activity of the fusion protein was required because a Bud3-Rga1-GAPR829A mutant failed to restore function. Thus, the only essential role of the N-terminal 70% of Rga1 in preventing polarization at the previous division site is to ensure its localization to that site.

Why is it that only Rga1 (and not Rga2 or Bem3) can block polarization at the previous division site? We found that Rga1 and Rga2 displayed subtly different patterns of localization at the time of cytokinesis even though both concentrate at the mother-bud neck. In large-budded cells with split septin rings (i.e., cells undergoing cytokinesis), Rga1 was concentrated in two rings that lay within the two septin rings (visualized with a functional septin-DsRed.M1 fusion or septin-mCherry), whereas Rga2 was concentrated in a single patch that was either closer to the daughter side of the bud neck or sandwiched by two septin rings (Fig. 4 A). Total Cdc42 was also concentrated on the membranes predominantly appearing as a single patch that was sandwiched by two septin rings (Fig. 4 A). Total Cdc42 was also concentrated on the membranes predominantly appearing as a single patch that was either closer to the daughter side of the bud neck or sandwiched by two septin rings (Fig. 4 A). Total Cdc42 was also concentrated on the membranes predominantly appearing as a single patch that was either closer to the daughter side of the bud neck or sandwiched by two septin rings (Fig. 4 A).
after cytokinesis. To test this hypothesis, we first asked whether the Rga2 GAP domain (residues 712–1009) could block same-site rebudding when fused to Bud3. As with the Bud3-Rga1-GAP fusion described in the previous paragraph (Fig. 4 B), the Bud3-Rga2-GAP fusion protein localized to the septin rings before, during, and after cytokinesis (Fig. S1 G). However, this fusion failed to rescue the same-site rebudding defect (Table I). Similarly, a Bud3-Bem3-GAP fusion protein was unable to rescue the rga1Δ phenotype (Table I). These results suggest either that the Rga1 GAP domain has greater activity than the Rga2 and Bem3 GAP domains or that it plays an additional, unique role in excluding same-site rebudding.

In a complementary approach to determine the importance of the precise septin-associated Rga1 localization, we fused the Rga1 GAP domain to the cytokinesis proteins Mlc2 and Cyk3. Mlc2 is the regulatory light chain of Myo1, the sole type II myosin in budding yeast, and it localizes to the bud neck from bud emergence to the end of the actomyosin contraction (Luo et al., 2004). Cyk3 accumulates in anaphase, localizes with the actomyosin contractile ring during cytokinesis, and then largely disappears (though two faint and fuzzy bands can sometimes be detected after actomyosin ring constriction; Korinek et al., 2000). Mlc2-Rga1-GAP and Cyk3-Rga1-GAP localized similarly to Mlc2 and Cyk3, respectively, thereby changing the Rga1 GAP localization to more closely resemble that of Rga2 and Bem3 during cytokinesis (Fig. 4 B, 1, during actomyosin ring contraction, and 2, presumed to be immediately after the actomyosin ring contraction; and not depicted). Mlc2-Rga1-GAP fusion failed to complement the rga1Δ phenotype (Table I). To our surprise, the Cyk3-Rga1-GAP fusion efficiently suppressed the same-site rebudding pattern defect of rga1Δ cells (Table I). SEM showed that only 1.5% of the cells (n = 197) carrying the Cyk3-Rga1-GAP fusion still budded within the old division site compared with 77% of the rga1Δ cells (n = 31). However, when we tested suppression of the daughter cell budding pattern, only 15.4% of the daughter cells (n = 273) budded axially, 52.7% still budded within the birth scar, and the rest were ambiguous (Fig. 4 C).

The suppression of mother cell budding pattern and the failure to rescue the daughter cell budding pattern were clearly visualized in a single cell by SEM (Fig. 4 C, arrow indicates birth scar). The partial rescue of the daughter cell budding pattern was often associated with the rescue of the enlarged birth scar phenotype (the mean width of the birth scars for Cyk3-rga1700–1007aa cells is 2.8 ± 1.1 μm, n = 20). We speculate that the Cyk3-Rga1-GAP protein was not effectively degraded at the end of cytokinesis in a subset of cells, allowing the remaining fusion protein to clear up the leftover GTP-Cdc42 within the birth scar, eliminating birth scar expansion, and blocking rebudding within the division site.

A major difference between mother and daughter cells is that daughter cells spend a longer time in G1 growing to the critical size before starting the next cell cycle (Johnston et al., 1977). Because Cyk3 is degraded at the end of mitosis, the simplest explanation for the ineffective rescue of the daughter cell budding pattern by the Cyk3-Rga1-GAP fusion protein is that, in daughter cells, the exclusion zone established by Cyk3-Rga1-GAP during cytokinesis has dissipated by the time that the cells initiate the next cell cycle and polarize. In contrast, mother cells begin the next cell cycle almost immediately after cytokinesis, when the exclusion zone is still in effect, so their budding pattern defect is effectively rescued by Cyk3-Rga1-GAP.

In aggregate, our findings indicate that the Rga1 GAP domain must be present at the division site to prevent subsequent polarization toward that site (Fig. 4 D). In daughter cells, which have a longer interval between division and subsequent polarization, it is also important for Rga1 GAP activity to persist after cytokinesis (Fig. 4 D).

We also investigated the role of Rga1 in diploid cells that bud in a bipolar pattern. Haploids with a single copy of RGA1 were unable to suppress the bipolar budding pattern shown by deletion of BUD3, which causes bipolar budding in haploid cells (Chant et al., 1995), similarly decreased the percentage of rga1Δ...
haploid cells that budded within the old division sites (Fig. S2 B). This was expected because, unlike in axially budding cells where bud-site selection proteins always concentrate at the old division site, in bipolar budding, many cells concentrate these factors (and hence Cdc24) at the opposite pole. In such cells, we would not expect Rga1 to be needed. We also examined cells deleted for RSR1, which display a random budding pattern (Bender and Pringle, 1989). Here as well, the rga1Δ phenotype (while present) was quantitatively less penetrant (Fig. S2 B). These findings indicate that deletion of RGA1 causes same-site rebudding in all contexts but that the penetrance of the phenotype is most extreme in axially budding cells, where Rsr1 and associated bud-site selection proteins act to concentrate Cdc24 at the old division site.

Why is it important for yeast cells to avoid rebudding at the same site? In most wild yeast strains, daughter cells remain attached to their mothers for prolonged periods after cytokinesis (laboratory strains have been selected to detach rapidly to reduce clumping and make experimental manipulation easier). Indeed, in some circumstances cells need to remain robustly attached to penetrate solid substrates (e.g., during haploid invasive growth or diploid pseudohyphal growth; Pan et al., 2000; Breitkreutz and Tyers, 2002). Clearly, rebudding at the same site would be impossible if the previous daughter cell continued to occupy that space. Even in our laboratory strain, we observed that in some rga1Δ cells (n = 4 out of 30), new septin rings started to form within the old rings but were then aborted, and new rings then appeared at the opposite pole of the cell (Fig. S2 C). This behavior suggests that the previous division site, with its remnant bud scar, sometimes creates difficulties when attempting to rebud at that site, leading to aborted budding attempts. Therefore, the exclusion zone provided by Rga1 may have evolved to make budding more efficient by avoiding attempts to bud at difficult or occupied sites. Previous work indicated that axial bud-site selection proteins are deposited in a ring at the division site and subsequently recruit and activate Cdc24 (Park and Bi, 2007). Thus, Rga1 and Cdc24 establish concentric zones of negative and positive Cdc42 regulation that lead to the adjacent positioning of cellular structures.

For many families of small monomeric GTPases, there appear to be more GEFs and GAPs than there are G proteins (e.g., ~53 GEFs and 68 GAPs for 17 Rho family GTPases in humans; Bernards, 2003; Bernards and Settleman, 2004). Our work demonstrates that one specific GAP is uniquely used to enforce an exclusion zone for cell polarization within a previous division site, which supports the hypothesis that GAPs play specialized roles. Moreover, the GAP must act at a specific location (the division site) and a specific time in the cell cycle (after cytokinesis). These findings are consistent with the hypothesis that the excess of regulators over G proteins evolved to exert exquisite spatiotemporal control over the activation of the G proteins, enabling each G protein to fulfill several cellular roles.

Materials and methods

Strains and growth conditions
Yeast strains used in this study are listed in Table S1 (available at http://www.jcb.org/cgi/content/full/jcb.200705160/DC1). Standard culture media and genetic techniques were used as described previously (Guthrie and Fink, 1991). For some experiments, yeast was grown in YM-P (Lillie and Pringle, 1980), a rich, buffered liquid medium.

Construction of plasmids and yeast strains
Plasmids YEp181-HA-RGA1 and YEp181-HA-RGA1R829A were constructed as follows: an ~5.8-kb HindIII fragment was subcloned from pALTER-1-HA-RGA1 (Caviston et al., 2003) into a HindIII site in YEp351 to generate YEp351-HA-RGA1. Then, an ~3-kb Xhol-BglII fragment containing N-terminal
HA-RGA1 was subcloned from YEp351-HARGA1 to replace the corresponding fragment in YEp181-RGA1 (Caviston et al., 2003) to generate YEp181-HARGA1. To generate YEp181-HARGA1, an ~1.5 kb BglII fragment containing the R829A site from YEp181-RGA1, which was used to replace the corresponding fragment in YEp181-HARGA1. Yeast strains YZT194 and YZT195 were constructed by integrating HindIII-digested YEp181-HARGA1 and YEp181-HARGA1R829A, respectively, into strain YZ724 (rga1Δ:URA3-KanMX6) by homologous recombination. Plasmid YIp128-CDC3-DsRed.M1 (integrative, LEU2) was constructed by PCR amplifying DsRed.M1 as a 0.7-kb NotI fragment using plasmid pDsRed.M1 (provided by B. Glick, University of Chicago, Chicago, IL) as the template and the following pair of primers: DsRed-1F (5′-TAAGACGCGCCCGATGGACACCGAGGAC-3′; the underlined sequence represents the NotI site and the bold sequence represents the 5′ end coding region of HindIII-digested YEp181-HARGA1 and YEp181-HARGA1R829A, respectively), into strain YZ788 (rga1Δ:URA3-KanMX6) by homologous recombination. Plasmid YIp128-CDC3-DsRed.M1 (integrative, LEU2) was constructed by PCR amplifying DsRed.M1 as a 0.7-kb NotI fragment using plasmid pDsRed.M1 (provided by B. Glick, University of Chicago, Chicago, IL) as the template and the following pair of primers: DsRed-1F (5′-TAAGACGCGCCCGATGGACACCGAGGAC-3′; the underlined sequence represents the NotI site and the bold sequence represents the 5′ end coding region of HindIII-digested YEp181-HARGA1 and YEp181-HARGA1R829A, respectively).
**Sequence** represents the 5′-end coding region of DsRed.M1, excluding the stop codon.

The NotI site, the underlined sequence represents the NotI site, the lower case letters represent dam mutation in order for the normally methylated unique ClaI site to be cleaved. The lower case underlined sequence represents the 5′-end coding region of DsRed.M1, excluding the stop codon.

To generate plasmid pRS426-RGA2-GFP, KanMx6 (2 μm URA3) for the localization experiment, a PCR product was amplified from pFAaA-GFP64L/S65T-KanMx6 (Longtime et al., 1998) using RGA2-2 forward and RGA2-R1 reverse primers. The PCR product encoding the GFP-Dam locus was inserted into SacI- and BamHI-digested pRS315-GFP-RGA1 (Caviston et al., 1998) to generate YIp211-RGA1-C1-GFP. This new plasmid was digested with BamHI and linearized with a unique XhoI site for integration at the RGA1 locus on the chromosome in an integrative vector (integrative, URA3) as used to construct YIp211-PRGA1-GFP. This new plasmid was digested with BamHI and ligated into Yiplac211 (integrative, URA3) to generate YIp211-PRGA1-GFP.

To generate plasmid pRS306-BUD3-C-GFP for the Bud3–Terminal fusion experiment, a PCR-amplified BamHI-EcoRI fragment encoding an 1477–1636 of BUD3 without a stop codon was inserted into the plasmid pRS306-T-C7 vector (integrative, URA3) for the localization experiment, a PCR product was amplified from pFAaA-GFP64L/S65T-KanMx6 (Longtime et al., 1998) using RGA2-2 forward and RGA2-R1 reverse primers. The PCR product encoding the GFP-Dam locus was inserted into SacI- and BamHI-digested pRS315-GFP-RGA1 (Caviston et al., 1998) to generate YIp211-RGA1-C1-GFP. This new plasmid was digested with BamHI and linearized with a unique XhoI site for integration at the RGA1 locus on the chromosome in an integrative vector (integrative, URA3) as used to construct YIp211-PRGA1-GFP. This new plasmid was digested with BamHI and ligated into Yiplac211 (integrative, URA3) to generate YIp211-PRGA1-GFP.

**Localization**

**Three-dimensional time-lapse microscopy** was performed using a microscope system (DeltaVision Spectris; Applied Precision) and a deep-cooled ORCA II-ER disk confocal scanner (PerkinElmer), and a high-resolution 100× Plan Apo objective. Imaging was performed at 30°C. For the two-color localization experiments presented in Figs. 3 A and 4 A and B, images of live cells were acquired digitally by the microscope (Nikon) equipped with a Plan Apo 100× 1.45 NA total internal reflection fluorescence oil immersion objective lens (Nikon), a Yokogawa spinning disk confocal scanner (PerkinElmer), and a deep-cooled ORCA II eXtreme charge-coupled device camera (Hamamatsu). The 488- and 568-nm laser lines of an argon/krypton laser (Melles Griot) were used for excitation of GFP and RFP in combination with a triple-band pass dichroic mirror.
For each cell, 11 images of GFP and RFP at 0.3-μm increments for Fig. 3 A and 20 images of GFP and RFP at 0.2-μm increments for Fig. 4 (A and B) were acquired at 23°C.

Indirect immunofluorescence and bud scar staining
For localization of HA-Rga1 and HA-Rga1ΔR829A, yeast cells grown exponentially in YM-P media at 24°C were fixed by formaldehyde and processed for indirect immunofluorescence microscopy as described previously (Pringle et al., 1991). A mouse monoclonal anti-HA primary antibody (HA-11, Covance) and a secondary Cy3-conjugated donkey anti–mouse IgG antibody (Jackson ImmunoResearch Laboratories) were used. Differential interference contrast and fluorescence microscopy were performed using a microscope (E800; Nikon) with a 60× Plan Apo objective. The images were acquired using Image-Pro Plus software (Media Cybernetics, Inc.).

Bud scars were visualized by fluorescence microscopy using the E800 microscope after staining with Calcofluor (Sigma-Aldrich). Cells were fixed by the addition of formaldehyde to 3.7% and incubation for ~2 h with occasional agitation. Cells were then stained with 0.1% Calcofluor as described previously (Pringle, 1991).

Centrifugal elutriation and birth scar staining
Enrichment of small daughter cells from exponentially growing cultures was achieved by centrifugal elutriation as described previously (Lew and Reed, 1993). After elutriation, cells were grown in rich medium YEPD at 30°C for 100–160 min (100 min after elutriation for the wild-type and rga1Δ cells and 160 min for the CYK3-rga1-GAP cells). Samples were fixed with 3.6% formaldehyde for 2 h at room temperature, washed with 0.1 M KPO₄, pH 7.5, and resuspended in immunofluorescence solution B (0.1 M KPO₄, pH 7.5, and 1.2 M sorbitol). Birth scars were stained with 12.5 μg/ml Alexa 594–ConA (Invitrogen) in immunofluorescence solution B for 20 min. Cells were examined using an AxioImager.A1 (Carl Zeiss, Inc.) with a 100× oil immersion objective. Images were captured using an ORCA cooled charge-coupled device camera and interfaced with MetaMorph software. Images were processed for presentation using Photoshop (Adobe). The means of the width of the birth scars in the post-elutriation cells of the rga1Δ strain were determined by MetaMorph.

Protein assays
Production of recombinant proteins and GAP assays. Production of GST-tagged proteins from E. coli and measurement of the GAP activity were all performed as described previously (Glafeler et al., 2002). To determine the amount of recombinant GST-GAP domain to add to the assay, we first got an approximate estimate using the Bradford assay and then ran 1.5, 2, or 3 μl of wild-type GAP domain to compare to a single amount of mutant GAP domain on a Western blot to fintune the amount. Fig. 2 A shows the relevant lanes from that Western blot spliced next to each other using Photoshop. Similar Western-based quantitation was used to ensure that equal amounts of full-length Rga1 and Rga1-GAP domain, and localization of Rga1-GAP proteins from E. coli were separated by 7% SDS-PAGE and transferred to nitrocellulose. The mean of the width of the traced line was determined by MetaMorph.

Online supplemental material
Fig. S1 shows budding within the birth scar, failure of Cdc24-RBD-RFP localization in the Cdc42 GEF mutant CYK3-rga1-GAP cells and localization of Rga1-GAP domain and Bud3-GFP fusion. Fig. S2 shows the rga1Δ phenotype in diploid cells, the effects of deletion of bud-site selection genes on the rga1Δ phenotype in haploid cells, and an aborted attempt to bud within the old bud scar in a hyperactive rga1Δ cell. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200705160/DC1.

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