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Singularity in Polarization: Rewiring Yeast Cells to Make Two Buds

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SUMMARY

For budding yeast to ensure formation of only one bud, cells must polarize toward one, and only one, site. Polarity establishment involves the Rho family GTPase Cdc42, which concentrates at polarization sites via a positive feedback loop. To assess whether singularity is linked to the specific Cdc42 feedback loop, we disabled the yeast cell's endogenous amplification mechanism and synthetically rewired the cells to employ a different positive feedback loop. Rewired cells violated singularity, occasionally making two buds. Even cells that made only one bud sometimes initiated two clusters of Cdc42, but then one cluster became dominant. Mathematical modeling indicated that, given sufficient time, competition between clusters would promote singularity. In rewired cells, competition occurred slowly and sometimes failed to develop a single "winning" cluster before budding. Slowing competition in normal cells also allowed occasional formation of two buds, suggesting that singularity is enforced by rapid competition between Cdc42 clusters.

INTRODUCTION

A polarized cell usually has a single directional axis: a "front" and a "back." One of the central questions in polarity establishment concerns how cells polarize to one and only one "front" (here referred to as singularity). Singularity does not depend on preoriented polarization cues, because cells deprived of such cues often polarize spontaneously toward a randomly oriented, but nevertheless unique, "front" (Wedlich-Soldner and Li, 2003). The basis for the singularity of polarization remains unclear.

Polarity establishment involves the highly conserved Rho family GTPase, Cdc42p (Etienne-Manneville, 2004). Polarization signals act through Cdc42p-directed guanine nucleotide exchange factors (GEFs) and/or GTPase activating proteins (GAPs) to trigger accumulation of membrane-bound GTP-Cdc42p at the site destined to become the "front" of the cell. GTP-Cdc42p then organizes cytoskeletal elements through various effectors to yield the polarized morphology appropriate to the cell type (Etienne-Manneville, 2004).

The budding yeast *Saccharomyces cerevisiae* is a tractable model for studies of polarity establishment (Park and Bi, 2007). A cell-cycle signal triggers polarization directed toward predictable sites dictated by the "bud site selection" machinery, which employs fixed landmarks that communicate with Cdc42p via the Ras-related GTPase Rsr1p. However, inactivation of Rsr1p does not block polarization: it simply randomizes the budding location. Importantly, such "symmetry breaking" polarization occurs with a timing, efficiency, and singularity similar to that in wild-type cells (Bender and Pringle, 1989; Chant and Herskowitz, 1991).

Theoretical models for symmetry breaking polarization posit that stochastic fluctuations generate small clusters of polarity factors at random sites. In the presence of an autocatalytic amplification mechanism, a stochastic cluster can then grow by positive feedback to generate a dominating asymmetry (Turing, 1952). We recently proposed a molecular mechanism for the positive feedback loop that breaks symmetry in yeast (Goryachev and Pokhilko, 2008; Kozubowski et al., 2008). A key player is the scaffold protein, Bem1p, which links the Cdc42p-directed GEF to a Cdc42p effector kinase (p21-activated kinase [PAK]). GTP-Cdc42p at the cortex can bind PAK and thereby recruit cytoplasmic PAK-Bem1p-GEF complexes, which then induce neighboring Cdc42p molecules to exchange their GDP for GTP, thereby "growing" a cluster of GTP-Cdc42p at the cortex (Figure 1A). In what follows, we refer to this as the "diffusion-mediated" amplification mechanism, as it requires Bem1p complexes to diffuse rapidly in the cytoplasm to successfully locate growing GTP-Cdc42p clusters.

Amplification mechanisms can explain how a random site, benefiting from a stochastic initial advantage, can develop a concentrated cluster of polarity factors. But why does only a single site become the "front"? In the fast block to polyspermy during sea urchin fertilization (another process in which singularity is important), ion fluxes induced by the first sperm to fuse

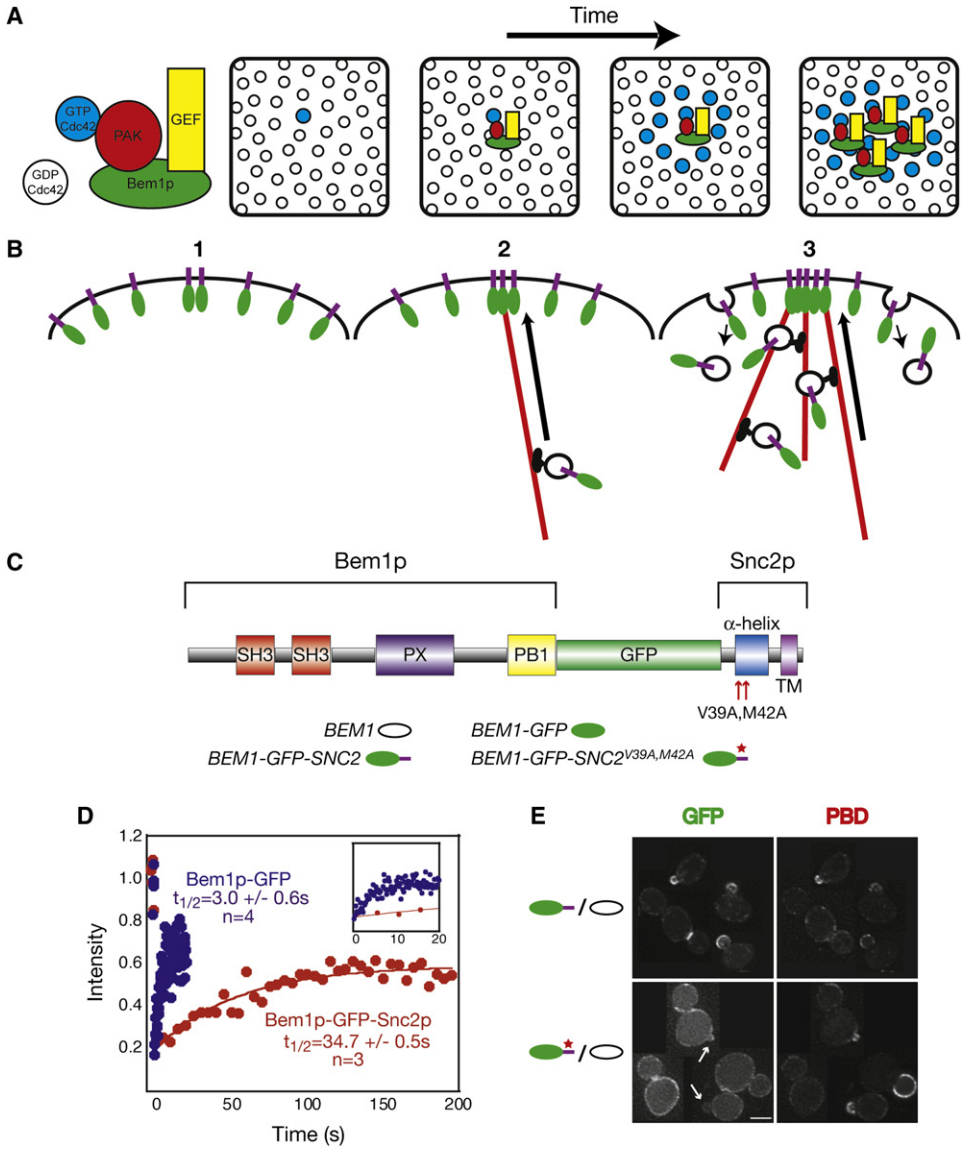

Figure 1. Rewiring the Yeast Polarization Feedback Loop

(A) Diffusion-mediated amplification (Kozubowski et al., 2008). The panels represent sequential snapshots of the plasma membrane as seen from the cell interior. GTP-Cdc42p arising stochastically at random sites may recruit a GEF-PAK complex from the cytoplasm through direct binding of the PAK (a Cdc42p effector). The associated GEF then promotes GTP-loading of Cdc42p in the immediate vicinity, causing growth of a GTP-Cdc42p cluster.

(B) Actin-mediated amplification (Wedlich-Soldner et al., 2003). (1) A membrane protein able to promote actin cable attachment may (2) capture (or nucleate) an actin cable, which then delivers vesicles containing more of the protein toward that site on the plasma membrane, growing a cluster. (3) Slow diffusion and balanced endocytosis maintains the polarized state (Marco et al., 2007).

(C) Synthetic Bem1p-GFP-Snc2p protein used to rewire amplification. Oval, Bem1p; green oval, Bem1p-GFP; green oval with a purple tail, Bem1p-GFP-Snc2p; red star, mutations in the Snc2p endocytosis motif.

(D) FRAP analysis of diploids homozygous for *BEM1-GFP-SNC2* or *BEM1-GFP*. Average intensities plotted relative to prebleach signal. Recovery half-times are indicated (mean ± SD). Inset: same data, expanded timescale.

(E) Bem1p-GFP-Snc2p, but not the endocytosis-deficient Bem1p-GFP-Snc2p^{V39A,M42A}, concentrates at polarization sites (arrows). The scale bar represents 5 μm.

with the egg rapidly make the entire cortex unwelcoming for new sperm (Jaffe, 1976). One could imagine that similarly rapid processes (which could involve ion fluxes, or changes in cell wall tension induced by local cell-wall remodeling (Klis et al., 2006), or other factors) would favor a single cluster of Cdc42p and block others from forming. Another possibility is that singularity could arise via competition between polarity clusters for limiting factors (Goryachev and Pokhilko, 2008). To address the singularity question, yeast geneticists sought to identify mutants that cause cells to form more than one bud, and

discovered a point mutant, *cdc42-22*, in which multiple buds grew simultaneously (Caviston et al., 2002). In that mutant, polarization was no longer dependent on the Cdc42p-directed GEF, suggesting that it must polarize without using the diffusion-mediated mechanism discussed above. Precisely why *cdc42-22* cells make more than one bud is unclear, but one possibility is that the loss of singularity reflects the use of a distinct amplification mechanism to polarize Cdc42p.

To ask whether specific alteration of the Cdc42p amplification mechanism would impact singularity, we created a novel fusion protein designed to "rewire" the endogenous yeast polarization pathway to use an engineered feedback loop to break symmetry. The rewired cells polarize and successfully proliferate, but often polarize to two sites simultaneously and sometimes make two buds. Combined experimental and theoretical analysis of both wild-type and rewired cells suggests that when more than one Cdc42p cluster forms, the amplification mechanisms engender competition between the clusters, eventually producing a single winner. However, if competition is slow (as in rewired cells) and fails to be completed within the time allotted prior to bud emergence, then singularity is violated and two buds are formed. We conclude that singularity is enforced by an intrinsic competitive property of the Cdc42p positive feedback mechanism that underlies polarity establishment.

RESULTS

Rewiring the Yeast Polarization Feedback Loop

Previous work on an artificial system involving overexpression of $Cdc42p^{Q61L}$ (a "constitutively active" GTP-locked mutant that no longer uses a GEF to become GTP loaded) suggested that an alternative amplification pathway, quite distinct from the diffusion-mediated pathway, could be used to grow clusters of GTP-Cdc42p (Wedlich-Soldner et al., 2003). Because overexpression of $Cdc42p^{Q61L}$ is lethal to yeast, we did not use that system, but we did follow the conceptual model emerging from it, which is illustrated in Figure 1B. Here, a membrane-bound polarity factor can influence membrane attachment of actin cables. Actin-mediated delivery of vesicles containing the polarity factor then increases the local concentration of the factor (assuming that it is highly concentrated on vesicles), leading to further actin cable attachment in a positive feedback loop. Eventually, of course, the membrane protein will diffuse away, but a stable focused polarization site can persist if endocytosis removes the polarity factor from the membrane before it diffuses too far (Marco et al., 2007) (Figure 1B).

Actin-mediated amplification (Figure 1B) relies on a protein with the following characteristics: it must (1) traffic at high concentration on secretory vesicles, (2) diffuse slowly in the plasma membrane, (3) enhance the local attachment of actin cables, and (4) undergo endocytosis before it diffuses too far from its site of delivery. The yeast exocytic v-SNAREs (Snc1p and Snc2p) fulfill three (1, 2, and 4) of the four requirements (Valdez-Taubas and Pelham, 2003) but cannot influence actin cables. To create a protein that fulfills all four requirements, we fused the scaffold protein Bem1p (which can influence local actin cable attachment via Cdc42p) to the v-SNARE Snc2p (Figure 1C). Our goal was to drive actin-mediated amplification without the toxic side-effects of $Cdc42p^{Q61L}$ overexpression.

Using fluorescence recovery after photobleaching (FRAP), we found that whereas Bem1p-GFP was highly dynamic (recovery $t_{1/2}$ ~3 s), recovery of Bem1p-GFP-Snc2p was much slower ($t_{1/2}$ ~35 s) (Figure 1D), consistent with previously reported dynamics for Bem1p (Wedlich-Soldner et al., 2004) and v-SNAREs (Valdez-Taubas and Pelham, 2003), respectively. Thus, the dynamics of Bem1p-GFP-Snc2p are dominated by the Snc2p moiety.

When expressed in wild-type cells, Bem1p-GFP-Snc2p was concentrated together with GTP-Cdc42p at prebud sites and bud tips early in the cell cycle, and at the mother-bud neck late in the cell cycle (Figure 1E). GTP-Cdc42p was detected with the PBD-RFP reporter linking the GTP-Cdc42p-binding domain from the effector Gic2p (PBD) to td Tomato (Tong et al., 2007). In principle, polarization could occur either by delivery and endocytosis (Figure 1B) or by lateral diffusion within the membrane and concentration at the polarization site through binding interactions. However, the slow diffusion of integral plasma membrane proteins in yeast ($D = 0.0025\ \mu m^2/s$; Valdez-Taubas and Pelham [2003]) impairs the latter mechanism, and Bem1p-GFP-$Snc2^{V39A,M42A}$p, carrying point mutations that inactivate the Snc2p endocytosis signal (Figure 1C) (Grote et al., 2000; Lewis et al., 2000) was no longer polarized to prebud sites or bud tips (Figure 1E), indicating that its polarization is dependent on recycling.

Because diffusion-mediated amplification (Figure 1A) requires cycling of Bem1p through the cytoplasm, where diffusion is much faster (for GFP, $D = 11\ \mu m^2/s$; Slaughter et al. [2007]), tethering of Bem1p to the membrane would disable this mechanism. At the same time, as a synthetic protein with all four of the requisite properties listed above, Bem1p-GFP-Snc2p should enable actin-mediated amplification (Figure 1B).

Bem1p-GFP-Snc2p Promotes Proliferation of *rsr1*Δ Cells, but Biases Polarization toward the Previous Division Site

To ask whether actin-mediated amplification could replace diffusion-mediated amplification, we replaced one copy of *BEM1* in a diploid with *BEM1-GFP-SNC2*. Upon tetrad dissection, all *BEM1-GFP-SNC2* haploids were viable, even if they lacked spatial cues for bud emergence (Figure 2A). Bem1p-GFP-Snc2p was expressed at similar levels to Bem1p-GFP (Figure 2B), and localized to polarization sites as well as to the mother-bud neck (Figure 2C). GTP-Cdc42p was also polarized at quantitatively similar levels in rewired and wild-type cells (Figure 2D). *BEM1-GFP-SNC2 rsr1*Δ cells proliferated with a normal cell-cycle profile (Figure 2E) and a doubling time only slightly longer than that of controls (102 min versus 90 min). *BEM1-SNC2* lacking the GFP moiety also promoted robust growth (data not shown). As yeast proliferation occurs by budding and absolutely requires polarization, these findings indicate that Bem1p-GFP-Snc2p can establish polarity.

We expected that *BEM1-GFP-SNC2 rsr1*Δ cells would break symmetry and pick random bud sites like *rsr1*Δ cells. However, bud scar and birth scar staining indicated that new buds often formed adjacent to previous division sites (Figure 3A). We

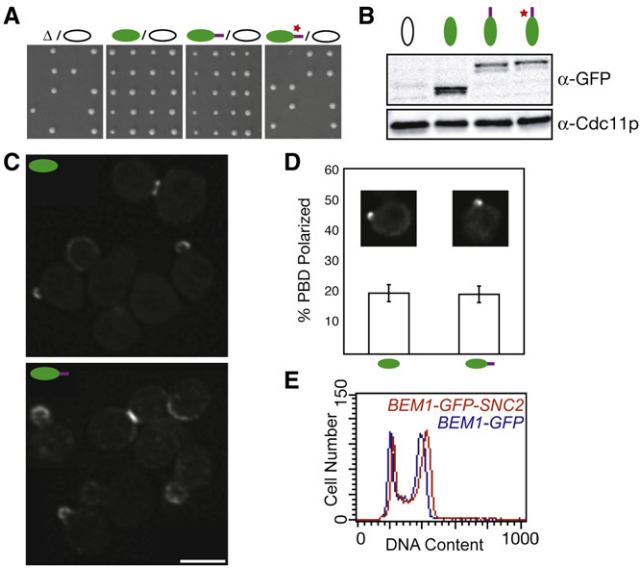

Figure 2. Bem1p-GFP-Snc2p Promotes Polarization and Proliferation of *rsr1*Δ Cells

(A) Tetrads from sporulation of *rsr1*Δ/*rsr1*Δ strains heterozygous for *bem1*Δ, *BEM1-GFP*, *BEM1-GFP-SNC2*, or *BEM1-GFP-SNC2*^{V39A,M42A} as indicated.

(B) Bem1p-GFP, Bem1p-GFP-Snc2p and Bem1p-GFP-Snc2p^{V39A,M42A} are expressed at similar levels. Blot probed with anti-GFP and anti-Cdc11p (loading control).

(C) *BEM1-GFP-SNC2 rsr1*Δ cells display polarized Bem1p-GFP-Snc2p. The scale bar represents 5 μm.

(D) Wild-type and rewired cells polarize comparable amounts of GTP-Cdc42p, assessed with a PBD-RFP probe (mean ± SEM, n = 14). Inset: examples of live cells.

(E) Wild-type and rewired cells displayed a similar cell-cycle profile.

All cells in the figure are *rsr1*Δ.

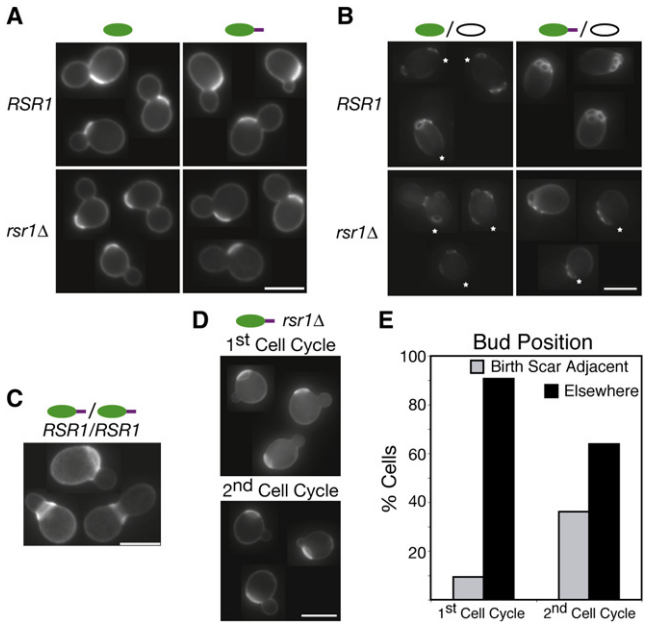

Figure 3. Bem1p-GFP-Snc2p Biases Polarization toward the Previous Division Site, but Can Also Break Symmetry

(A) Budding of *BEM1-GFP-SNC2 rsr1*Δ haploids is biased toward the previous division site. First-time mothers stained to label the cell wall and birth scar (bright patch, which marks previous division site).

(B) Budding of *RSR1/RSR1 BEM1/BEM1-GFP-SNC2* diploids is biased toward the previous division site. Multiple-time mothers stained to label bud scars (location of previous division sites). * indicates birth scars, when not obscured by bud scars.

(C) Budding of *BEM1-GFP-SNC2/BEM1-GFP-SNC2* diploids can occur directly into the birth scar.

(D) After centrifugal elutriation (which lengthens G1 in the first cycle), *BEM1-GFP-SNC2 rsr1*Δ cells break symmetry. In the second cell cycle, budding was again biased. Cells were fixed and stained at 80 min (first cycle) and 180 min (second cycle) after elutriation.

(E) Bud site position for first- and second-time mothers from (D). n > 100.

Scale bars represent 5 μm in all panels.

speculate that because of its concentration at the neck during cytokinesis and its slow diffusion, Bem1p-GFP-Snc2p remains near the division site, seeding polarization toward that site in the next cell cycle. Consistent with this view, when Bem1p-GFP-Snc2p was expressed in wild-type diploid cells (i.e., cells with functional bud site selection and Bem1p, in which daughters almost always bud toward the distal site marked by the landmark Bud8p [Chant and Pringle, 1995; Zahner et al., 1996]), it skewed the budding pattern toward the division site instead (Figure 3B). Some cells even budded directly within the previous division site (Figure 3C), a behavior that is normally prohibited by a Cdc42p GAP (Tong et al., 2007).

Rewired Cells Break Symmetry by Actin-Mediated Positive Feedback

If slow diffusion of Bem1p-GFP-Snc2p from the division site biases polarization toward that site, then lengthening of the early G1 interval (before polarization) should provide time for dissipation of the Bem1p-GFP-Snc2p gradient to a homogeneous distribution, forcing the cells to break symmetry. We used centrifugal elutriation (a size-selection procedure) to isolate small early-G1 daughter cells that have a longer G1 interval, and shifted the cells to 37°C to depolarize actin after elutriation (Lillie and Brown, 1994). *BEM1-GFP-SNC2 rsr1*Δ cells budded at random sites in the first cycle after elutriation, although the preference for the division site returned in the second cycle (Figures 3D and 3E). Thus, Bem1p-GFP-Snc2p can promote symmetry breaking.

If Bem1p-GFP-Snc2p polarizes via actin-mediated amplification instead of diffusion-mediated amplification, then polarity in this strain (unlike in wild-type or *rsr1*Δ cells) should be abolished upon actin depolymerization. Indeed, whereas Latrunculin A-treated control cells polarized Bem1p, GTP-Cdc42p, Spa2p (a polarisome component [Sheu et al., 1998]), and Cdc3p (a septin [Gladfelter et al., 2001]), none of these markers became polarized in Latrunculin A-treated Bem1p-GFP-Snc2p cells (Figures 4A and 4B). At the dose employed (200 μM), Latrunculin A depolymerizes all detectable F-actin (both the cables that mediate vesicle delivery and the cortical patches that mediate endocytosis). Moreover, the endocytosis-deficient Bem1p-GFP-Snc2^{V39A,M42A}p was unable to rescue proliferation of *bem1*Δ *rsr1*Δ cells (Figure 2A), presumably because endocytosis of the construct is key to polarization. These results indicate that

Bem1p-GFP-Snc2p cannot engage an actin-independent polarization mechanism. Thus, Bem1p-GFP-Snc2p can break symmetry, but it does so by actin-mediated positive feedback (Figure 1B).

The demonstration that a synthetic rewiring of the yeast polarization pathway to employ the actin-based mechanism can work to break symmetry provides an important validation of the actin-mediated positive feedback concept (Wedlich-Soldner et al., 2003). Moreover, it indicates that such polarization can occur in a sufficiently rapid timeframe to be useful to yeast, which was unclear from the previous work as polarization of $Cdc42p^{Q61L}$ takes much longer (Gulli et al., 2000; Wedlich-Soldner et al., 2003). We then directly compared the kinetics of polarization in wild-type and rewired cells.

Dynamics of Polarization in Wild-Type and Rewired Cells

Side-by-side comparisons indicated that although superficially similar, polarization in wild-type and rewired cells displayed three notable differences. First, in rewired cells, Bem1p-GFP-Snc2p accumulated at a small focus gradually (Figures 4C and 4D and Movie S1 available online), whereas in wild-type cells, Bem1p-GFP accumulated more abruptly and to a wider zone (~1.9 μm diameter) that subsequently condensed to a small (<1 μm diameter) focus (Figures 4E and 4F and Movie S2). Second, whereas Bem1p-GFP-Snc2p polarization occurred approximately coincident with that of the actin patch marker Abp1p-mCherry, polarization of Bem1p-GFP occurred 3.0 ± 0.41 min (mean ± SEM, n = 12) before that of Abp1p-mCherry (Figures 4C–4G). Third, bud emergence occurred 10.4 ± 0.29 min (mean ± SEM, n = 25) after Bem1p-GFP polarization was first detected, but only 8.2 ± 0.3 min (mean ± SEM, n = 31) after Bem1p-GFP-Snc2p polarization was first detected (Figure 4H). To avoid potential differences stemming from variations in temperature or slide composition, we collected the data in Figure 4H from mixed-cell experiments where *BEM1-GFP rsr1Δ* and *BEM1-GFP-SNC2 rsr1Δ* cells were imaged simultaneously (the cells were distinguished by the presence of an mCherry marker in the *BEM1-GFP-SNC2 rsr1Δ* strain). We also measured the interval between Abp1p polarization and bud emergence, which was similar in wild-type and rewired cells (Figure 4I).

We conclude that in wild-type cells, polarization of Bem1p is rapid and precedes actin polarization by about 3 min, whereas in rewired cells, polarization of Bem1p-GFP-Snc2p is gradual and coincident with actin polarization. In both cases, bud emergence occurs about 8 min after actin polarization. These findings are entirely consistent with (and strongly support) the premise of the rewiring approach: in wild-type cells, diffusion-mediated amplification generates a focus of Bem1p (and GTP-Cdc42p) that subsequently recruits actin, whereas in the rewired cells actin-mediated amplification leads to simultaneous polarization of Bem1p-GFP-Snc2p and actin.

Rewired Cells Sometimes Make Two Buds Simultaneously

In diploid *BEM1-GFP-SNC2 rsr1*Δ cells growing on minimal media at 24°C, 4.9% of budded mothers had two buds (n > 1000). This number was reduced to ~1%–2% under optimal growth conditions (rich media at 30°C), in which the cells budded predominantly toward the division site (see above). Time-lapse analysis revealed that the two buds emerged and grew simultaneously, though at a reduced rate compared to neighboring single-budded cells (Figures 5A and 5B and Movies S3 and S4). The two buds in such cells were both "true buds" in the sense that they displayed polarized actin cables and patches, septin hourglass structures at the neck, and polarized localization of a GTP-Cdc42p reporter as well as Spa2p (Figures 5C and 5D). The location of the two buds relative to each other varied widely: some cells had buds right next to each other while other cells had buds at opposite ends (Figures 5C and 5D). Thus, switching from diffusion-mediated amplification (Figure 1A) to actin-mediated amplification (Figure 1B) caused the occasional breakdown of singularity, suggesting that the normal prohibition restricting cells to form only one bud is conferred by the nature of the amplification mechanism itself.

We tested whether the two-budded phenotype could be suppressed either by reinstating diffusion-mediated positive feedback loop (through addition of *BEM1-GFP*) or by restoring bud site selection (through addition of *RSR1*). *BEM1-GFP* reduced the frequency of two-budded cells (2.5% versus 4.9%, n > 1000), but *RSR1* had little effect (5.6% versus 4.9%, n > 1000).

Competition between Polarization Foci

Time-lapse microscopy of polarization in 144 *BEM1-GFP-SNC2 rsr1Δ* cells revealed that 27 (19%) initiated polarization at two foci, yet only four (2.8%) made two buds. Thus, in a majority of the cells that generated two Bem1p-GFP-Snc2p foci, one of the foci subsequently disappeared, and the cells formed a single bud (Figures 6A and 6B and Movies S5 and S6). Two foci could coexist for up to 10 min before one focus disappeared, leaving a single bud site (see Figure 7F).

In contrast to cells with two foci, we never saw the focus disappear in the 117 cells that only made a single focus. This suggests that the "disappearance" of one focus was due to the presence of the second focus, implicating some form of competition between foci as the basis for the disappearance of one focus.

Cells that established two foci of Bem1p-GFP-Snc2p always did so within <3 min of each other, and in most cases any potential differences in the focus initiation times were not resolved by our 1.5 min image acquisition intervals. Thus, it appears that once a dominant focus becomes established, new foci do not arise. This was not due to progression of the cell cycle (which eventually terminates polarization) because new foci did not arise even if the cell cycle was arrested at a polarizing stage (Figure S1). A competitive mechanism that favors the stronger focus would account for this observation, as newly growing foci would be unable to compete with a well-established focus.

What is the basis for the observed competition between foci? In cells with two foci, secretory vesicles carrying Bem1p-GFP-Snc2p would encounter actin cables oriented toward either focus (Figure 6C). If we assume (as seems likely) that stronger foci (those containing more Bem1p-GFP-Snc2p) sustain more actin cables, then delivery will be biased toward the stronger focus, forming a potential basis for competition (red arrows in Figure 6D). To assess whether this would yield the observed behavior, we turned to mathematical modeling.

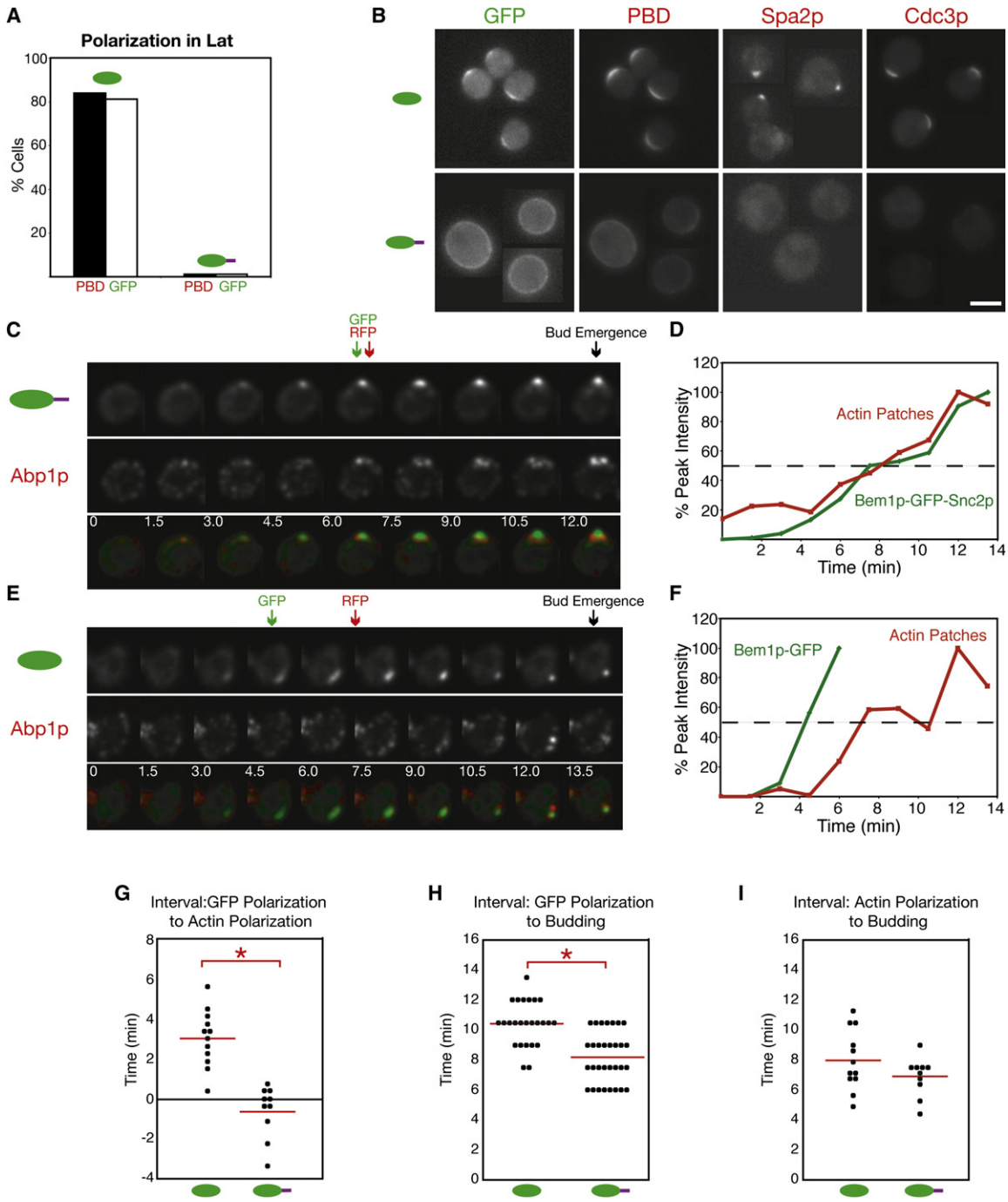

Figure 4. Comparison of Actin-Mediated and Diffusion-Mediated Polarization

(A) Polarization in rewired cells is actin-dependent. Polarization of PBD-RFP and either Bem1p-GFP or Bem1p-GFP-Snc2p was scored after complete actin depolymerization (200 μM Lat-A, 2 hr). n > 90.

(B) Lat-A treated rewired cells do not polarize PBD-RFP, Spa2p-RFP, or Cdc3p-RFP. Montages of live-cell images are shown. The scale bar represents 5 μm.

(C) Dynamics of Bem1p-GFP-Snc2p and actin patch polarization (frames from Movie S1). Actin patches visualized with Abp1p-mCherry. Arrows: times of 50% of peak Bem1p-GFP-Snc2p polarization (green), actin patch polarization (red), and time of bud emergence (black; scored from DIC images). The time is in minutes.

(D) Quantitation of polarization in (C). Integrated GFP or RFP intensity in the focus is plotted as % of peak intensity for that cell.

(E) Dynamics of Bem1p-GFP and actin patch polarization (frames from Movie S2).

(F) Quantitation of polarization in (E).

(G) Interval between polarization of Bem1p-GFP or Bem1p-GFP-Snc2p and actin patches, scored from times when integrated GFP and RFP intensities reached 50% of peak. Line indicates average. * indicates that the difference is statistically significant ($p < 0.001$, Student's t test).

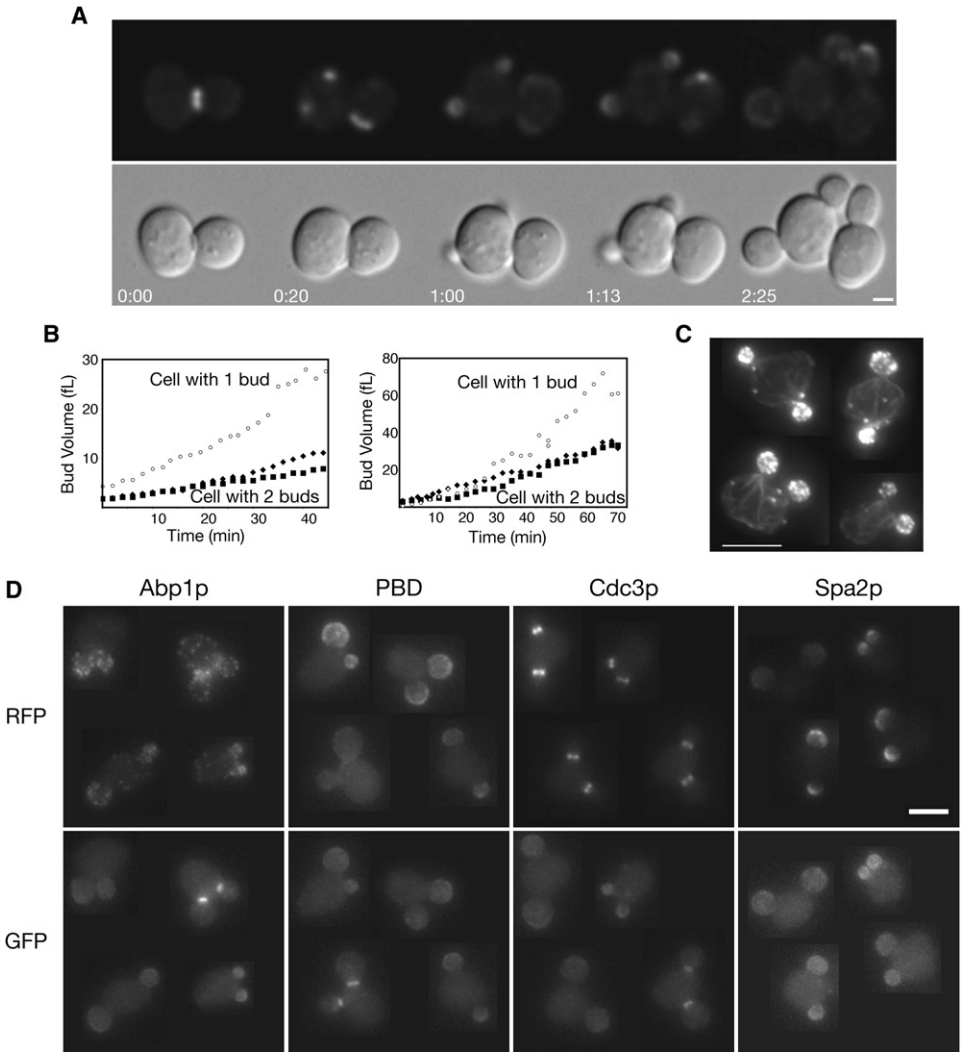

Figure 5. Rewired Cells Violate Singularity

(A) Rewired cells can grow two buds simultaneously. *BEM1-GFP-SNC2* cells (frames from Movie S3). Time is in hours:minutes. The scale bar represents 2 μm.

(B) Comparison of bud growth in side by side one- versus two-bud cells, measured from DIC images (e.g., Movie S4). Open symbols, one-bud cell; closed symbols, two-bud cell. Left, haploid cells. Right, diploid cells.

(C) Actin cables and patches are polarized toward both buds. Cells were fixed and stained with rhodamine-phalloidin. The scale bar represents 5 μm.

(D) Montages of two-bud cells containing *BEM1-GFP-SNC2* (lower panels) and either *ABP1-mCherry*, *CDC3-mCherry*, *PBD-RFP*, or *SPA2-mCherry* (upper panels). The scale bar represents 5 μm.

Mathematical Model for Competition between Bem1p-GFP-Snc2p Foci

A model incorporating delivery of Bem1p-GFP-Snc2p from internal pools to the polarization site by actin cables, diffusion in the plasma membrane, and retrieval by endocytosis (Figure 6D) is presented in the Supplemental Data. We assume that in the relevant timeframe for polarization (a few minutes), the system is at a global steady state in which synthesis and degradation of Bem1p-GFP-Snc2p are balanced, and can be ignored.

The simplest scenario is that delivery of Bem1p-GFP-Snc2p is biased in a manner proportional to the amount of Bem1p-GFP-Snc2p that is already present in each focus. However, because endocytic retrieval of Bem1p-GFP-Snc2p (black arrows in Figure 6D) is also expected to be proportional to the amount already present, this would not necessarily lead to a net change in the

(H) Interval from first detection of polarized GFP signal to bud emergence. Because polarization of Bem1p-GFP is more abrupt than that of Bem1p-GFP-Snc2p, use of the "50% of peak" criterion for GFP polarization would lead to a bigger difference than the "first detection" criterion used here. * indicates that the difference is statistically significant ($p < 0.001$, Student's t test).

(I) Interval from actin patch polarization (50% of peak) to bud emergence. The difference is not statistically significant ($p = 0.14$).

All cells in the figure are *rsr1*Δ.

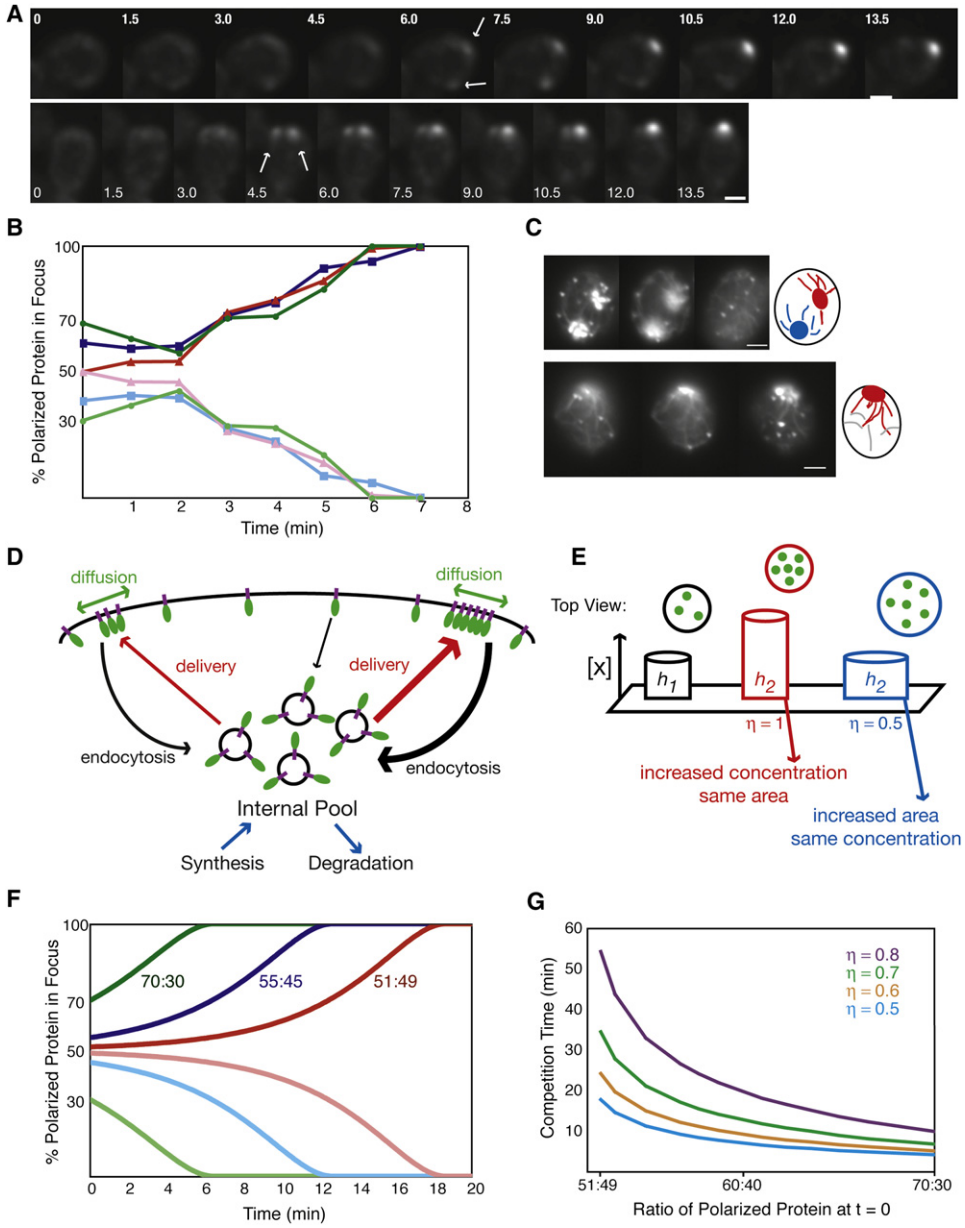

Figure 6. Competition between Foci in Rewired Cells

(A) Rewired *BEM1-GFP-SNC2 rsr1Δ* cells formed two foci but then one focus grew while the other disappeared prior to budding; selected frames from Movies S5 and S6. Time is in minutes. The scale bar represents 2 μm.

(B) Integrated GFP intensity in each focus for three illustrative *BEM1-GFP-SNC2 rsr1Δ* cells. Foci from the same cell are colored in dark versus light red, blue, or green.

(C) Actin cables are directed toward both foci. Selected Z planes of representative one-focus and two-focus *BEM1-GFP-SNC2 rsr1Δ* cells fixed and imaged as in Figure 5C. Tracing shows cables that could be unambiguously assigned to a (color-coded) focus. Grey, no clear attachment.

(D) Model for Bem1p-GFP-Snc2p dynamics in cells with two foci. Red arrows, vesicular trafficking along actin cables; black arrows, endocytosis; green arrows, diffusion in the plane of the membrane.

(E) Effect of focus geometry on diffusion-mediated escape of Bem1p-GFP-Snc2p. Circles represent a top-down view of the cylinders illustrating distribution of Bem1p-GFP-Snc2p (green dots).

(F) Simulation of competition between foci with $\eta = 0.5$. Bem1p-GFP-Snc2p content of foci from the same cell are colored in dark versus light red, blue, or green. Simulations started with the indicated ratios of protein.

(G) Dependence of the competition time on the initial ratio and η.

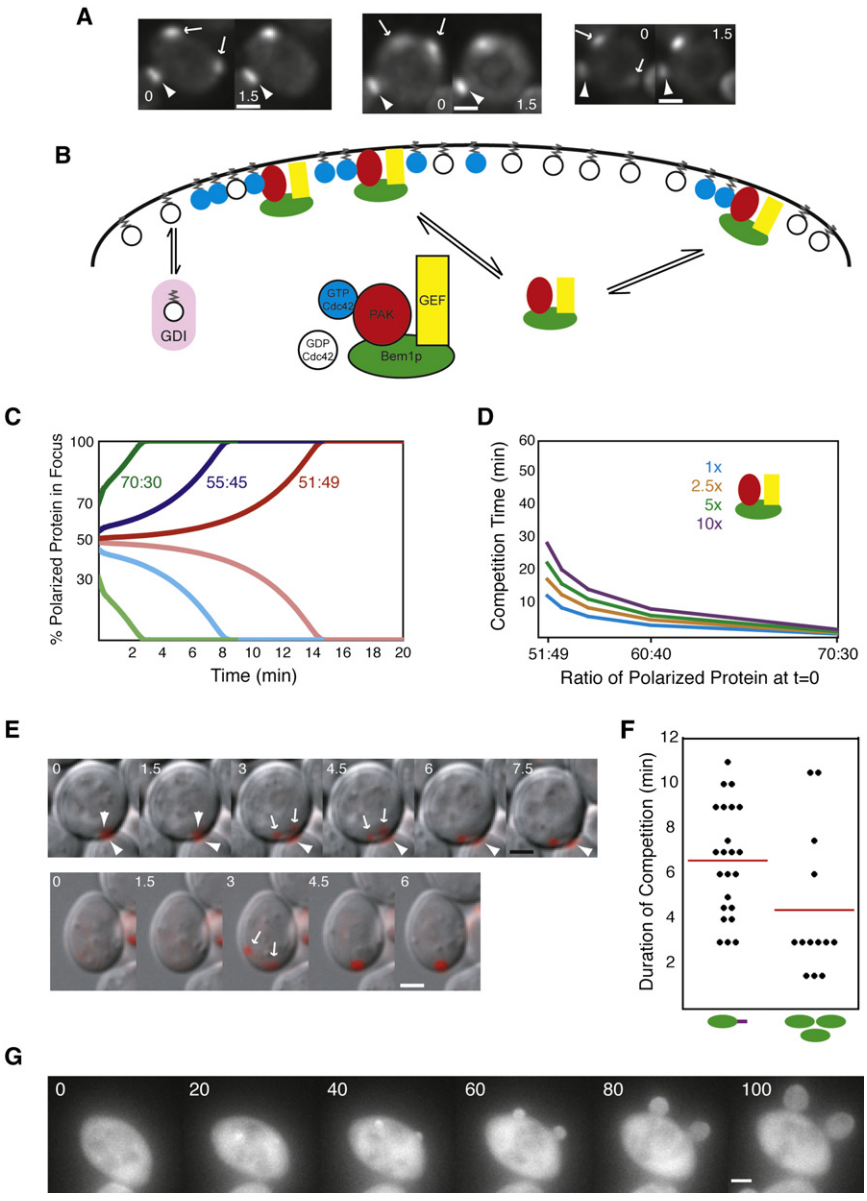

Figure 7. Competition between Foci in Wild-Type Cells and Cells Overexpressing Bem1p

(A) Two-focus (arrows) intermediates in wild-type cells resolve within 1.5 min. Deconvolved images of Bem1p-GFP. Arrowhead, neck. Time is in minutes. The scale bar represents 2 μm.

(B) Model for competition between foci in wild-type cells. A small limiting pool of Bem1p-GEF-PAK complex diffuses rapidly in the cytoplasm and can bind GTP-Cdc42p in either focus. GTP-Cdc42p is concentrated in each focus, and can bind the complex or hydrolyze GTP. GDP-Cdc42p can bind to GDI and be extracted from the membrane or can be re-loaded with GTP by neighboring GEFs.

(C) Simulation of competition between foci. Bem1p content of foci from the same cell are colored in dark versus light red, blue, or green. Simulations started with the indicated ratios of protein.

(D) Dependence of the competition time on the initial ratio and the abundance of the Bem1p-GEF-PAK complex.

(E) Cells overexpressing Bem1p display competition between foci. *SPA2-mCherry* cells containing a high-copy 2 μm-*BEM1-GFP* plasmid were imaged. Spa2p-mCherry (polarity marker)/DIC overlays shown (selected frames from Movie S7). Time is in minutes. The scale bar represents 2 μm.

(F) Quantitation of competition times (interval between first detection of two foci and first detection of a single winning focus). Comparison of rewired (*BEM1-GFP-SNC2 rsr1*Δ) cells and Bem1p overexpressors.

(G) Bem1p overexpressors can violate singularity and make two buds: selected frames from Movie S8. The scale bar represents 2 μm.

relative amounts of protein in the two different foci. The outcome in that scenario would depend on diffusion in the plasma membrane (green arrows in Figure 6D).

The rate of diffusion-mediated "escape" of Bem1p-GFP-Snc2p from a focus will depend on the precise geometry of the focus (i.e., the concentration profile in two dimensions). An unrealistic but instructive geometry is illustrated in Figure 6E, where a reference focus (black) is depicted as a circular region containing evenly-distributed Bem1p-GFP-Snc2p. Here, diffusion-mediated escape of Bem1p-GFP-Snc2p from the focus is proportional both to the length of the circle's perimeter and to the concentration of Bem1p-GFP-Snc2p within the focus.

We now consider two extreme scenarios for the distribution of Bem1p-GFP-Snc2p in a stronger focus (depicted in red or blue in Figure 6E). At one extreme (red), the size (and hence perimeter) of the circle remains unchanged, and the Bem1p-GFP-Snc2p concentration in the circle is higher. In that case, diffusion-mediated escape from each focus will simply be proportional to the total protein content in the focus, just as we assumed for delivery and endocytosis. It can then be demonstrated that in cells with two foci containing amounts of Bem1p-GFP-Snc2p designated as h_1 and h_2, the ratio h_1/h_2 will tend to remain the same regardless of the fraction of Bem1p-GFP-Snc2p in each focus (Supplemental Results, Analysis of the proportional model). Thus, in this scenario there is no net competition between foci.

In the second scenario (Figure 6E, blue), the concentration of Bem1p-GFP-Snc2p in the stronger focus remains the same as in the reference focus, but the area of the circle is increased. Because protein content is proportional to area and diffusion-mediated escape is proportional to the length of the circle's perimeter, in this scenario diffusion-mediated escape would be proportional to [total protein in focus]$^{0.5}$. These extremes (red and blue) allow us to infer the general form of the escape term for a realistic focus geometry in between these extremes: diffusion-mediated escape will be proportional to [total protein

in focus]$^{\eta}$, where $0.5 < \eta < 1$. It can then be shown that the ratio, h_1/h_2, of protein content in one focus to protein content in the other, will change according to

$$\frac{d(h_1/h_2)}{dt} = k\left(\left(\frac{h_1}{h_2}\right) - \left(\frac{h_1}{h_2}\right)^{\eta}\right),$$

where k is a positive quantity dependent on diffusion rate and focus geometry (Supplemental Results, Analysis of the non-proportional model).

This means that if $h_1/h_2 > 1$ (i.e., focus 1 is stronger than focus 2), then $d(h_1/h_2)/dt$ will be positive and focus 1 will grow at the expense of focus 2. Conversely, if $h_1/h_2 < 1$ (i.e., focus 2 is stronger than focus 1), then $d(h_1/h_2)/dt$ will be negative and focus 2 will grow at the expense of focus 1. Unless the foci have precisely equal content ($h_1/h_2 = 1$, in which case $d(h_1/h_2)/dt = 0$), the stronger focus will outcompete the weaker focus. Thus, inclusion of a realistic diffusion scenario ($0.5 < \eta < 1$) in a simple model of Bem1p-GFP-Snc2p behavior is sufficient to promote competition and, given sufficient time, singularity.

To assess the timeframe in which this competitive mechanism would operate, we constrained model parameters on the basis of published observations and experimental data (Figures 1D, 2B, and S2; see the Supplemental Results, Parameter estimation). Examples of model behavior using these parameters and setting $\eta = 0.5$ are shown in Figure 6F. When two foci of Bem1p-GFP-Snc2p were initiated at different ratios, the stronger focus grew while the weaker one disappeared. The bigger the initial asymmetry, the faster the resolution of two foci to one (Figures 6F and 6G). With increasing η, competition took progressively longer (Figure 6G). Thus, with realistic parameter values, competition for Bem1p-GFP-Snc2p can lead to growth of one focus at the expense of the other within a biologically relevant (several minute) timeframe, as long as stronger foci are also larger.

When two foci start out with almost equivalent amounts of Bem1p-GFP-Snc2p, competition takes longer (Figures 6F and 6G), and one would expect two buds to emerge. The fact that we detected two-budded cells shows that competition is not always completed within the allotted interval between polarization and bud emergence. We observed a total of 38 Bem1p-GFP-Snc2p cells in which two buds emerged (including both *RSR1* and *rsr1*Δ strains). In two cases, a tiny bud was then "abandoned," but in the other 36 instances both buds grew for prolonged periods (Figure 5 and Movies S3 and S4), suggesting that competition is terminated soon after bud emergence. Because recycling endosomes and golgi quickly enter small buds (Preuss et al., 1992), the recycling pools of Bem1p-GFP-Snc2p in each bud may become "insulated" from each other soon after bud emergence, terminating competition.

Modeling Competition between Bem1p-GFP Foci in Wild-Type Cells

We then asked whether competition between foci also occurs in wild-type cells as well as in rewired cells. At our time-lapse rates (one Z stack every 1.5 min, which was the fastest rate that did not induce phototoxic damage upon prolonged filming), we observed five apparent instances of two-foci intermediates out of 79 *rsr1*Δ cells containing Bem1p-GFP (6%; Figure 7A). These were fainter than in rewired cells and required deconvolution to detect. By the next time point, these intermediates had resolved to a single focus (Figure 7A), suggesting that competition occurs more rapidly in wild-type than in rewired cells, and resolves within 1.5 min.

A mathematical model of diffusion-mediated amplification (Goryachev and Pokhilko, 2008) suggested that competition between GTP-Cdc42p clusters for a limiting cytoplasmic pool of Bem1p-GEF complexes should lead to the eventual growth of the biggest cluster at the expense of the others (Figure 7B). However, with the parameter values estimated by Goryachev and Pokhilko, the model predicts that competition occurs on a timescale even slower than that of our rewired cell model (Supplemental Results, Modeling competition in the diffusion-mediated amplification system; Figure S3).

Kozubowski et al. (2008) recently showed that an unstable Bem1p-GEF-PAK complex mediates positive feedback, while Goryachev and Pokhilko modeled a stable Bem1p-GEF complex. We altered the model accordingly (Supplemental Results, Adapting the model to account for the Bem1p-GEF-PAK complex). We also re-estimated the GEF and GAP rate constants based on biochemical assays using yeast cell lysates (Supplemental Results, Estimation of GEF and GAP activities; Figure S4). With these modifications, the model predicted faster competition between clusters than the fastest model of the rewired cells ($\eta = 0.5$; compare Figure 7C with Figure 6F), and further tuning of model parameters could make competition even faster (Supplemental Results, Factors affecting competition timescale in the diffusion-mediated model). Thus, competitive features of the diffusion-mediated mechanism might underlie singularity.

Competition between Foci in Cells Overexpressing Bem1p

Mathematical modeling indicated that competition between foci in the diffusion-mediated mechanism would be slower if the abundance of the Bem1p-GEF-PAK complex were increased (Figure 7D). To test this prediction, we increased Bem1p-GFP expression using a high-copy plasmid. Overexpression of Bem1p did not noticeably slow cell proliferation. Most of the overexpressed protein was cytoplasmic, presumably because much of the Bem1p was either monomeric or in distinct complexes that did not polarize. Because of the elevated cytoplasmic background, polarization (though visible) was more difficult to detect, so we added a separate polarity marker, Spa2p-mCherry, to monitor focus formation in these strains. Strikingly, 18 out of 127 (14%) of cells overexpressing Bem1p initially developed two polarization foci, but then (with one exception: see below) one focus grew and the other disappeared prior to budding (Figure 7E and Movie S7). These findings strongly suggest that competition between foci is a feature of the normal polarization process and that competition is slowed by additional Bem1p as predicted by the model.

Competition between foci was somewhat faster in the Bem1p overexpressors than in the rewired cells (Figure 7F). In addition, in a small number of the Bem1p overexpressors we noted three behaviors that we had not seen in the rewired cells. First, in two out of 18 two-foci cells, an initially dimmer focus became

brighter and successfully competed against an initially brighter focus (Figure S5A). Second, in four out of 109 one-focus cells, we observed apparent disappearance of the focus, immediately followed by reappearance of a focus at a distinct site (Figure S5B). Third, in five cells (four of which were initially scored as having only one focus), close examination revealed that at the beginning of focus growth, the cells had two foci close to each other, which appeared to merge forming a single focus at an intermediate position (Figure S5C). Interestingly, this was predicted to occur by the mathematical model (Goryachev and Pokhilko, 2008).

One of the two-focus cells discussed above went on to form two buds, violating the singularity rule. In other experiments (with cells that lacked the Spa2p marker) we also observed rare (<1%) cells budding simultaneously at two sites (Figure 7G and Movie S8). Thus, as in the rewired cells, when competition between foci is too slow, then both foci give rise to buds, violating singularity.

DISCUSSION

Singularity in Polarization Is Guaranteed by Competition between Foci

A key result from this work is that cells synthetically rewired to use a different positive feedback loop to polarize Cdc42p sometimes violated the singularity rule and made two buds simultaneously. This finding suggests that singularity is an intrinsic property of the Cdc42p-amplifying positive feedback system, and that there is no separate singularity-guaranteeing process.

What aspect of the Cdc42p amplification pathway confers singularity? Our findings on both rewired cells polarizing via actin-mediated feedback and Bem1p overexpressors polarizing via diffusion-mediated feedback indicate that at least in some cells, polarization occurs through an intermediate "multiple foci" stage. Thus, the stochastic processes that initiate amplification can occur at more than one site. In most instances where two foci appeared, one focus subsequently grew while the other disappeared, and a single bud emerged from the site of the winning focus. This finding provides strong evidence that foci interact with each other in a competitive manner that leads to the growth of one focus at the expense of the other. Cells that did establish two foci did so almost simultaneously, and new foci no longer appeared once a strong focus had formed, even if the cell cycle was arrested at a polarization-competent stage. This observation is also consistent with a competitive process, as the first focus would effectively prevent the growth of subsequently initiated foci. Thus, singularity can result from a competitive mechanism built into the Cdc42p amplification feedback system.

In *rsr1*Δ cells filmed at 1.5 min time-lapse intervals, the intermediate "multiple foci" stage did not persist beyond a single time point. The transitory nature of this stage suggests that competition occurs very rapidly, making the intermediate difficult to detect. In addition, polarization occurred more abruptly in wild-type than in rewired cells, and this feature would be expected to reduce the incidence of two-spot intermediates. If the first site to begin amplification grows a focus very quickly, then this "first focus" may grow to a dominant size before other sites begin their amplification, precluding growth of subsequent foci.

With current parameter estimates, foci in our mathematical model appear to compete somewhat more slowly than foci in the wild-type cell: according to the model, foci that had starting ratios more equal than 60:40 should have taken longer than 3 min to compete. It is certainly possible to alter parameters so as to speed competition (Supplemental Results, Factors affecting competition timescale in the diffusion-mediated model), and further work may yield more accurate parameter estimates that can explain the rapid competition of wild-type cells. Alternatively, a full accounting of the speed of competition may require that additional aspects of the polarization process become incorporated into the model. One feature absent from the model is noise. In cells, both molecular and vesicular noise would be expected to introduce a random element into the competition. Perhaps noise is responsible for the rare instances (which we cannot currently explain) of cells in which an initially weaker "underdog" focus went on to win the competition.

Given the above considerations, we suggest that the singularity rule boils down to having a rapid competitive mechanism built into the polarization process. Our rewired cells, with a competition mechanism operating in the timeframe of several minutes, disobey singularity in <5% of cells. Bem1p overexpressors, with a faster competition mechanism, disobey singularity in <1% of cells. And wild-type cells, with a competition mechanism that resolves all competitions within 1.5 min, can effectively guarantee singularity.

Basis for Competition between Polarization Foci

In the rewired cells, the simplest scenario is that foci compete via their attached actin cables for the vesicles that deliver additional Bem1p-GFP-Snc2p to the foci. Because foci are constantly losing Bem1p-GFP-Snc2p by diffusion and by endocytosis, delivery of new vesicles is required to maintain a focus. We present a simple mathematical model for such competition and show that if delivery and loss are both proportional to the Bem1p-GFP-Snc2p content of a focus, then the situation is balanced and different foci can coexist indefinitely. However, if stronger foci are also larger in extent (even if only slightly so) than weaker foci, then diffusion-mediated loss will no longer be proportional to protein content, and the stronger focus will grow at the expense of the weaker focus. This simple and realistic assumption about focus geometry suffices to make the two-foci situation competitive rather than balanced. When model parameters were estimated based on experimental findings, the mathematical model was able to promote competition in a relevant timeframe, suggesting that this mechanism is powerful enough to account for competition in the rewired cells. Of course, it remains entirely possible that there are other factors that could enhance competition.

In wild-type cells, mathematical modeling predicted that foci would compete for a limiting pool of cytoplasmic Bem1p-GEF-PAK complex, and that increasing the concentration of the Bem1p-GEF-PAK complex would slow competition. Our finding that cells overexpressing Bem1p displayed readily detectable competition and occasionally violated singularity confirms this

prediction, and supports the validity of the model for diffusion-mediated amplification.

Comparison with *cdc42-22* and Other Mutants

Prior to this work, the major experimental study to address singularity in polarization was focused on *cdc42-22* mutants capable of growing two or even more buds simultaneously (Caviston et al., 2002). In those mutants, polarization was uncoupled from cell-cycle control and new buds continued to emerge and grow throughout the cell cycle. Strikingly, the presence of an established bud did not prevent the initiation of a subsequent bud in the same cell, after which the buds both grew, yielding a remarkable 45% of the population with more than one bud (Caviston et al., 2002). This observation suggests that unlike the cells described in our study, *cdc42-22* mutants do not exhibit significant competition between polarization foci. Because *cdc42-22* mutants no longer need the GEF in order to polarize, they clearly do not use the diffusion-mediated Bem1p-GEF-PAK complex amplification mechanism (Figure 1A). It would be very interesting to determine what amplification mechanism functions in that mutant, and why it is not subject to competition.

Heterozygous diploids containing one copy of *cdc42-22* and one of wild-type *CDC42* obey the singularity rule, leading Caviston et al. (2002) to suggest that wild-type Cdc42p polarizes much more efficiently than Cdc42p-22, so that the polarization site established by the wild-type would (by polarizing associated proteins and downstream factors) deprive the weaker Cdc42p-22 of the wherewithal to establish secondary polarization sites. Interestingly, heterozygous diploids containing one copy of *BEM1* and one of *BEM1-SNC2* were still able to make two-budded cells, albeit at reduced frequency. Thus, the rewired polarization mechanism would appear to operate more efficiently than that of *cdc42-22*, allowing establishment of a second polarization site even when the wild-type mechanism is active.

Occasional multibudded cells have also been reported in other strains that are very sick and slow growing, including *bem1*Δ (Wedlich-Soldner et al., 2004) and *bem2*Δ (Knaus et al., 2007) mutants. In *bem1*Δ mutants (which require Rsr1p to polarize), we also detected very rare two-budded cells in which the buds grew simultaneously, but we noticed that those cells were also multinucleate. Proliferation in the presence of an almost-lethal mutation like *bem1*Δ leads the cells to accumulate a historical legacy of defects (and perhaps also adaptations), including large cell size, abandoned buds, and multiple nuclei. It is therefore difficult to discern why the rare two-budded cells occur in such strains, or what the link to multinuclearity might be. In contrast, the rewired cells and Bem1p-overexpressors discussed above proliferate almost as well as wild-type cells, allowing much cleaner interpretation.

Tuning Competition to Obey or Flout Singularity

Not all polarized cells obey the singularity rule. Filamentous fungi can sustain many growing tips in the same (multinucleate) cell (Harris, 2008), and neurons initially form several neurite extensions from the same cell body (da Silva and Dotti, 2002). Yet, it appears that many of the same polarity regulators that obey singularity in other cell types are similarly employed in these multipolar cells. It may be that evolution has fine-tuned the speed and effectiveness of competition to allow the same molecular elements to promote or disregard singularity in different cell types.

EXPERIMENTAL PROCEDURES

Yeast strains used in this study are listed in the Supplemental Data. Standard media and methods were used for plasmid and yeast genetic manipulations. Immunoblotting was performed as previously described (Keaton et al., 2008; Kozubowski et al., 2008). Further strain construction, image analysis, and experimental details are available in the Supplemental Experimental Procedures.

Analysis of Growth Rate and Cell-Cycle Distribution

Population doubling time was measured by dilution of cultures to 2×10^6 cells/mL in YEPD and growing at 30°C. Aliquots were fixed with 3.7% formaldehyde every 30 min. The absorbance was measured at 600 nm with a Beckman DU 640B Spectrophotometer (Beckman Coulter, Fullerton, CA).

FACS analysis was performed as previously described (Haase and Reed, 2002). The DNA content of 10,000 cells was measured with a Becton Dickinson FACScan and then analyzed with CellQuest software (Becton Dickinson Biosciences, San Jose, CA).

Bud Scar, Birth Scar, and Actin Staining

For visualization of scars, cells were fixed in 3.7% formaldehyde for 1 hr at room temperature, washed, and resuspended in immunofluorescence solution B (IFB: 0.1 M KPO_4, pH 7.5, 1.2 M sorbitol). Birth scars were stained with 12.5 μg/ml Alexa 594-ConA (Invitrogen, Carlsbad, CA) in IFB for 20 min at room temperature. Bud scars were stained with a solution of 0.05% Calcofluor (Sigma-Aldrich, St. Louis, MO) for 30 min at room temperature. For F-actin staining, cells were grown overnight in synthetic complete medium with dextrose at 24°C then fixed and stained with Rhodamine phalloidin (Invitrogen, Carlsbad, CA) as previously described (Amberg, 1998).

Centrifugal Elutriation

Small daughter cells were isolated from exponentially growing cultures by centrifugal elutriation as previously described (Lew and Reed, 1993). After elutriation, cells were grown in YEPD at 37°C for 80 min or 180 min.

Microscopy

For live-cell imaging, exponentially growing cells were mounted on a slide with a slab of synthetic medium solidified with 2% agarose (Denville Scientific, Inc., Metuchen, NJ). Images in Figures 1E, 2C, and 3 were acquired with an AxioImager.A1 (Carl Zeiss, Thornwood, NY) with a 100×/1.4 Plan Apochromat oil immersion objective and an ORCA CCD camera (Hamamatsu, Bridgewater, NJ). Images in Figures 4–7 were acquired with the AxioObserver.Z1 with a similar objective and either a QuantEM backthinned EM-CCD camera (Photometrics, Tucson, AZ) or a Coolsnap ES2 high-resolution CCD camera (Photometrics). All timelapses consisted of DIC and fluorescence (GFP and, where indicated, RFP) images acquired over 11 Z planes with 0.5 μm steps and, excluding Figures 4C and 4E, are displayed as maximum projections. Representative cells were compiled into a single image for presentation with Photoshop (Adobe Systems, San Jose, CA) in Figures 1E, 2C, 3, 4B, 5C, and 5D.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Results, Supplemental Experimental Procedures, five figures, one table, and nine movies and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)01314-2.

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