Regulation of intrinsic polarity establishment by a differentiation-type MAPK pathway in *S. cerevisiae*

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**ABSTRACT**
All cells establish and maintain an axis of polarity that is critical for cell shape and progression through the cell cycle. A well-studied example of polarity establishment is bud emergence in the yeast *Saccharomyces cerevisiae*, which is controlled by the Rho GTPase Cdc42p. The prevailing view of bud emergence does not account for regulation by extrinsic cues. Here, we show that the filamentous growth mitogen activated protein kinase (fMAPK) pathway regulates bud emergence under nutrient-limiting conditions. The fMAPK pathway regulated the expression of polarity targets including the gene encoding a direct effector of Cdc42p, Gic2p. The fMAPK pathway also stimulated GTP-Cdc42p levels, which is a critical determinant of polarity establishment. The fMAPK pathway activity was spatially restricted to bud sites and active during the period of the cell cycle leading up to bud emergence. Time-lapse fluorescence microscopy showed that the fMAPK pathway stimulated the rate of bud emergence during filamentous growth. Unregulated activation of the fMAPK pathway induced multiple rounds of symmetry breaking inside the growing bud. Collectively, our findings identify a new regulatory aspect of bud emergence that sensitizes this essential cellular process to external cues.

**KEY WORDS:** Bud emergence, Cdc42p, Polarity establishment, MAPK, Pseudohyphal growth, Symmetry breaking

**INTRODUCTION**
All cells establish an axis of polarity, which is critical for cell shape, the organization of cellular compartments and progression through the cell cycle (Doerr and Ragkousi, 2019). Cell polarity can be reorganized in response to intrinsic and extrinsic cues and is required during development and for other processes that require dynamic changes in cell shape such as cell migration and differentiation (Henderson et al., 2018; Pei et al., 2019; Piroli et al., 2019). Defects in cell polarity are commonly associated with human disease. For example, the mis-regulation of cell polarity leads to metastasis in many types of cancers (Fomicheva et al., 2019). Defects in cell polarity are also associated with other pathological conditions, such as Alzheimer’s disease (Huang et al., 2019) and autism spectrum disorder (Picone et al., 2019). In the yeast *Saccharomyces cerevisiae*, Cdc42p regulates bud emergence, which is an essential process where daughter cells or buds are produced from mother cells. The pathway that regulates bud emergence has been extensively studied and represents one of the best examples for how cells establish an axis of polarity (Fig. 1A, green pathway). Activation of Cdc42p by the guanine nucleotide exchange factor (GEF) Cdc24p at bud sites – or at random sites in bud-site-selection mutants – results in symmetry breaking, which commits the cell to grow at a particular site. Positive and negative feedback loops promote polarity establishment by amplifying the levels of active or GTP-bound Cdc42p at the incipient bud site (Chiu et al., 2018; Irazoqui et al., 2003; Kozubowski et al., 2008; Woods and Lew, 2019). The GTP-bound conformation of Cdc42p binds effector proteins (Bendezú and Martin, 2013; Guo et al., 2010; McCormack et al., 2013; Wu and Jiang, 2005; Wu et al., 2006; Yang et al., 2007), including Gic1p and Gic2p (Brown et al., 1997; Chen et al., 1997; Iwase et al., 2006; Kawasaki et al., 2003; Liu et al., 2019), p21 activated kinases (PAKs) Ste20p and Cla4p (Cvrckova et al., 1995; Gulli et al., 2000; Hofmann et al., 2004; Simon et al., 1995; Takahashi and Pryciak, 2007), and the formin Bni1p (Evangelista et al., 1997; Jermiss, 1999). The formin Bni1p is activated by the PAKs Ste20p and Cla4p, which, in turn, stimulates the recruitment and activation of Ste20p, the specific Cdc42p effector (Gic2p) (Raman et al., 2007; Yoon and Seger, 2006). In yeast, nutrient limitation induces a Cdc42p-dependent MAPK pathway that regulates filamentous/invasive pseudohyphal growth (Fig. 1A, blue pathway). The MAPK pathway (Adhikari et al., 2015; Borneman et al., 2007; Cullen, 2015; Gancedo, 2001; Gimeno et al., 1992; Leberer et al., 1997; Mösch et al., 1999; Mosch et al., 1996; Pan et al., 2000; Peter et al., 1996; Roberts and Fink, 1994)) Filamentous growth occurs in many fungal species, and in some plant and animal pathogens, filamentous growth is required for virulence (Desai et al., 2014; Lagree and Mitchell, 2017; Lo et al., 1997). At the head of the fMAPK pathway, plasma membrane proteins Msb2p and Sho1p regulate the Cdc42p module, which leads to the recruitment and activation of Ste20p, the specific Cdc42p effector that regulates the fMAPK pathway. Ste20p in turn induces a MAPK cascade composed of Ste11p (MAPKKK), Ste7p (MAPKKK) and Kss1p (MAPK) (Madhani et al., 1997; Roberts and Fink, 1994). MAPK Kss1p regulates the activity of transcription factors Ste12p and Tec1p, as well as transcriptional repressors of factors (Bardwell et al., 1998; Madhani and Fink, 1997;
van der Felden et al., 2014) to regulate expression of target genes that bring about the filamentous cell type (Roberts et al., 2000; Rupp et al., 1999).

Despite the fact that bud emergence is extensively regulated by positive and negative feedback loops and is coordinated with the cell cycle, bud emergence is not known to be regulated by extrinsic factors.
Fig. 1. Role of the fMAPK pathway in rescuing the growth defect of the cdc24-4 mutant. (A) Pathways that regulate bud emergence (green) and filamentous growth (blue). Cdc24p and Cdc42p regulate both pathways. Not all proteins are shown. (B) Growth of the indicated strains relative to wild-type cells. pGFP-MSB2, pSHO1\(^{120L}\), pSTE11-4 and pTEF2-GIC2 were expressed from plasmids (see Table S2) in the indicated strains (see Table S1). Error bars show the standard error of the mean (s.e.m.) for three separate trials. *P<0.01. CTL, plasmid pRS316. (C) Growth of strains relative to the cdc24-4 mutant. This experiment was performed in galactose (GAL) medium, which also compromised the viability of the cdc24-4 mutant, because the pbs2\(\Delta\) mutant has a growth defect at 37°C (Winkler et al., 2002). (D) Inverted maximum intensity projection of GFP-Cdc42p localization in the indicated strains, examined after incubation at 37°C for 4 h. *GFP-Cdc42p clustering. Arrows, sites of bud emergence. Scale bar, 5 μm. (E) Quantification of GFP-Cdc42p clustering. Error bars, s.e.m. for three separate trials. At least 50 cells were counted in each trial. *P<0.05. (F) Cdc12p-GFP localization in the indicated strains, examined after incubation at 37°C for 4 h. Black arrows, Cdc12p-GFP localization in incipient buds; *Cdc12p-GFP localization at mother-bud neck in growing bud; red arrowheads, mislocalized Cdc12p-GFP. Scale bar, 5 μm. (G) Quantification of septin localization; see panel 1E for details.

Results

The fMAPK pathway rescues the bud emergence defect of cdc24-4 through Gic2p

Multicopy Suppressor of Budding Defect 2 (MSB2) was initially characterized as a high-copy suppressor of the budding and growth defects of the cdc24-4 mutant (Bender and Pringle, 1989, 1992). Msb2p was subsequently identified as the mucin-type glycoprotein that regulates the fMAPK pathway (Fig. 1A, blue pathway (Cullen et al., 2004)). To determine whether Msb2p regulates budding through the fMAPK pathway, plasmids carrying alleles that hyperactivate the fMAPK pathway were introduced into the cdc24-4 mutant and examined for growth at 37°C, in synthetic medium lacking uracil to maintain selection for the plasmids, and supplemented with 0.5 M sorbitol, which stabilizes cell integrity (Bender and Pringle, 1989, 1992). Insertion of Green Fluorescent Protein (GFP) into the extracellular domain of Msb2p results in a version of the protein that hyperactivates the fMAPK pathway (Adhikari et al., 2015; Vadaie et al., 2008). pGFP-MSB2 weakly suppressed the growth defect of the cdc24-4 mutant (Fig. 1B). Hyperactive versions of Sho1p (pSHO1\(^{120L}\)) and Ste11p (pSTE11-4) more robustly rescued the growth defect of the cdc24-4 mutant (Fig. 1B). pSTE11-4 also rescued the polarity defect of the cdc24-4 mutant (Fig. S1A) and was used for most of the subsequent experiments. Therefore, activated versions of MAPK pathway components can rescue the growth and polarity defects of a bud emergence mutant.

Msb2p, Sho1p and Ste11p regulate two MAPK pathways [fMAPK and high-osmolarity glycerol response (HOG) (Saito, 2010)]. A pathway-specific regulator of the HOG pathway, Pbs2p (Brewster et al., 1993; Nishimura et al., 2016; Zarrinpar et al., 2004), was not required for growth of the cdc24-4 mutant (Fig. 1C). In fact, the cdc24-4Δ pbs2Δ double mutant grew better than the cdc24-4 single mutant, perhaps because the HOG pathway negatively regulates the fMAPK pathway and its loss results in hyperactive fMAPK pathway activity (Davenport et al., 1999). By comparison, a pathway-specific regulator of the fMAPK pathway, Tec1p (Madhani and Fink, 1997), was required for viability of the cdc24-4 mutant (Fig. 1B). Tec1p functions with another transcription factor, Ste12p (Liu et al., 1993), which was also required for growth of cdc24-4 (Fig. 1C). Ste11p and Ste12p also regulate the mating pathway, but these experiments were carried out in a mating defective strain (ste4Δ). Tec1p was required for suppression of the growth defect of the cdc24-4 mutant by pSTE11-4 (Fig. 1B). pSTE11-4 also induced phosphorylation of the MAP kinase for the fMAPK pathway, Kss1p (P~Kss1p) in the cdc24-4 mutant (Fig. S1B). Therefore, the MAPK pathway that controls filamentous and invasive growth regulates bud emergence in a defined genetic context.

The fMAPK pathway controls filamentous growth by regulating target genes that control cell adhesion (FLO11 (Rupp et al., 1999)), bud-site selection (BUD8 (Cullen and Sprague, 2002; Harkins et al., 2001; Taher et al., 2000)) and cell elongation (CLIN1 (Kron et al., 1994; Loeb et al., 1999; Madhani et al., 1999)). None of these genes was required for viability of the cdc24-4 mutant (Fig. S1C). To identify relevant polarity targets of the fMAPK pathway, gene ontology (GO) term analysis was performed on data generated in Chow et al. (2019). Polarity targets of the fMAPK pathway included genes that regulate bud-site selection (Fig. S1D; AXL2, BUD8, RSR1 and RAX2), polarity establishment (MSB2, GIC2 and RGA1), and septin ring organization (GIC2, AXL2 and RGA1). We explored the roles of many of these genes in this study. We started with GIC2, which encodes a direct effector of Cdc42p that along with GIC1 functions in bud emergence [Fig. 1A, green (Brown et al., 1997; Chen et al., 1997)]. The GIC2 gene was a target of the fMAPK pathway (Fig. S1E; see also Maclsaac et al., 2006).

Gic2p was required for viability of the cdc24-4 mutant (Fig. 1B; Costanzo et al., 2010) and to suppress the growth defect of cdc24-4 by pSTE11-4 (Fig. 1B). Expression of GIC2 from a fMAPK pathway-independent promoter partly rescued the growth defect of the cdc24-4 mutant (Fig. 1B; pTEF2-GIC2) even in cells lacking an intact fMAPK pathway (pTEF2-GIC2 ste12Δ). GIC1 was not a target of the fMAPK pathway (Fig. S1E) and pSTE11-4 suppressed the cdc24-4 gic1Δ mutant better than cdc24-4 gic2Δ (Fig. S1F). Thus, Gic2p is a target of the fMAPK pathway that is required for fMAPK pathway-dependent rescue of the cdc24-4 mutant.

Gic proteins regulate clustering or polarization of Cdc42p at bud sites (Daniels et al., 2018; Kang et al., 2018), which is a critical step in polarity establishment. Time-lapse fluorescence microscopy was performed to examine the viability of the cdc24-4 mutant on SD-URA media. This approach showed that GFP-Cdc42p clustered at a specific site on the cell cortex (Fig. 1D; wild-type, asterisk), from which site new buds emerged. The cdc24-4 mutant was defective for GFP-Cdc42p clustering and bud emergence (Fig. 1D; cdc24-4). Analysis of 30 cells showed this difference to be
statistically significant (Fig. 1E). pSTE11-4 partially restored GFP-Cdc42p clustering and bud emergence to the cdc24-4 mutant (Fig. 1D,E). Rescue of bud emergence by pSTE11-4 was dependent on Gic2p (Fig. 1D,E).

During bud emergence, Gic proteins also regulate formation of the septin ring (Bi and Park, 2012; Iwase et al., 2006; Kang et al., 2018; Okada et al., 2013; Sadian et al., 2013). In wild-type cells, the septin Cdc12p-GFP was localized in a ring prior to bud emergence (Fig. 1F; asterisk) and to the mother-bud neck in budding cells (Fig. 1F; red arrowheads). Based on the analysis of over 150 cells, the localization defect was statistically significant (Fig. 1G). pSTE11-4 partially rescued the septin localization defect of the cdc24-4 mutant (Fig. 1F,G), which was dependent on Gic2p (Fig. 1F,G). The fMAPK pathway did not suppress the growth and morphological defects of the cdc12-6 mutant and septin kinase mutants, elm1Δ and gin4Δ, which indicates that the pathway functions upstream of the septins themselves (Fig. S2). Gic2p also promotes the interaction between Cdc42p and Bni1p (Chen et al., 2012). Bni1p was not required for GFP-Cdc42p clustering or bud emergence (Fig. 1E,F) but was required for septin localization in the cdc24-4 mutant (Fig. 1F,G). Bni1p is required for septin ring assembly (Gladfelter et al., 2005; Kadota et al., 2004). In wild-type cells, Gic2p was required for invasive growth (plate-washing assay; Fig. S3A), colony ruffling (Fig. S3B) and septin organization (Fig. S3C–E) of filamentous cells. Gic2p did not regulate the fMAPK pathway (Fig. S3F). These results establish a new role for the fMAPK pathway in regulating bud emergence by a mechanism that involves the regulated expression of Gic2p. In the next section, we examine the role of the fMAPK pathway in regulating bud emergence in wild-type cells, under conditions of filamentous growth.

The fMAPK pathway regulates GTP-Cdc42p levels during filamentous growth

Like other monomeric GTPases, the exchange of GDP for GTP alters the conformation of Cdc42p and allows the protein to bind effector proteins. During budding, the increase in GTP-Cdc42p levels is critical for symmetry breaking and polarity establishment (Irazoqui et al., 2003; Kozubowski et al., 2008). Msh2p interacts with GTP-Cdc42p to activate the fMAPK pathway (Cullen et al., 2004). During filamentous growth, the level and activity of Msh2p is stimulated by positive feedback (Fig. S1D). To test whether Msh2p and the fMAPK pathway affect the levels of GTP-Cdc42p in the cell, a fluorescent reporter that measures GTP-Cdc42p levels was examined [Gic2p-PBD-tdTomato (Okada et al., 2013)]. Compared with cells undergoing yeast-form growth (YEPD), cells undergoing filamentous growth showed elevated levels of GTP-Cdc42p (Fig. 2A; YEP-GAL). The increase in GTP-Cdc42p levels was dependent on the fMAPK pathway (Fig. 2A) in a manner that was statistically significant (Fig. 2B; YEP-GAL; P<0.00001). As previously shown (Okada et al., 2013), the level of GTP-Cdc42p was also higher in cells responding to the mating pheromone α-factor (Fig. 2A,B). In this case, the increase was independent of the fMAPK pathway.

One caveat in interpreting the above results is that GIC2-PBD-tdTomato is driven by its endogenous promoter, which, as shown above, is regulated by the fMAPK pathway. As a separate test, the activity of a Cdc42p biosensor (Smith et al., 2013) was examined by fluorescence lifetime imaging [FLIM-FRET (Sun et al., 2011)]. In wild-type cells, the Cdc42p biosensor exhibited shorter lifetimes with a version that mimics the GDP-bound conformation (Fig. 2C; Cdc42p<sup>GDP146</sup>) and longer lifetimes with a version that mimics the GTP-bound conformation (Fig. 2C; Cdc42p<sup>GTP461L</sup>) and longer lifetimes with a version that mimics the GDP-bound conformation. In wild-type cells undergoing filamentous growth, the level and activity of Msh2p was statistically significant (Fig. 2B; YEP-GAL; P<0.00001). Activation of the fMAPK pathway reduced the lifetime of the biosensor (Fig. 2C; MSB<sup>2A100-818</sup>). This change corresponded to a 25% increase in the total levels of GTP-Cdc42p, which has previously been shown to...
reflect changes in Cdc42p activity during bud emergence (Smith et al., 2013). Cdc42p activity can also be affected by the Gic proteins (Daniels et al., 2018; Kang et al., 2018; Kawasaki et al., 2003) but the lifetime of the biosensor was not altered in the gic1Δ gic2Δ double mutant (Fig. 2C). Hyperactive versions of Msb2p, Sho1p and Ste11p also partially suppressed the growth defect of the gic1Δ gic2Δ double mutant at 37°C (Fig. S3G; Gandhi et al., 2006), which supports a role for fMAPK in regulating bud emergence that is separate from Gic protein function.

The fMAPK pathway is temporally and spatially regulated to coincide with bud emergence

Bud emergence occurs in the G1 phase of the cell cycle (Gulli et al., 2000; Hartwell et al., 1970; Howell and Lew, 2012; Lew and Reed, 1993; Moran et al., 2019; Pringle et al., 1995). Whether the fMAPK pathway is active in the G1 phase of the cell cycle has not been explored. To address this question, cells were synchronized in the G1 phase of the cell cycle by α-factor (Breeden, 1997), and fMAPK pathway activity was assessed under basal (YEPD) and activating conditions (YEP-GAL) as cells progressed through the cell cycle. An epitope-tagged cyclin, Clb2p-HA, showed the expected pattern of cell-cycle regulation (Cepeda-García, 2017; Cross et al., 2005; Eluere et al., 2007; Imiger et al., 1995; Kuczera et al., 2010; Richardson et al., 1992; Wäsch and Cross, 2002), increasing after α-factor release by 60 min and decreasing by 90 min as cells entered anaphase (Fig. 3A; α-HA). P~Kss1p levels also increased after α-factor release by 80 min and peaked by 100 min (Fig. 3A; P~Kss1p). By comparison, the level of P~Fus3p, which is the MAP kinase that regulates the mating pathway, decreased after release from α-factor (Fig. 3A; P~Fus3p). Conditions that activate the fMAPK pathway (YEP-GAL) led to a delay in Clb2p-HA accumulation (Leitao and Kellogg, 2017) and also showed an increase in P~Kss1p levels after the G1/M phase of the cell cycle, prior to the next round of bud emergence (Fig. 3B). These results demonstrate that the activity of the fMAPK pathway is cell-cycle regulated and increases prior to and during bud emergence.

Bud emergence is spatially regulated in that it results from Cdc42p activation at bud sites (Caviston et al., 2003; Das et al., 2007; Freisinger et al., 2013; Okada et al., 2013). We previously showed that bud-site-selection proteins regulate the fMAPK pathway (Basu et al., 2016), which indicates that the fMAPK pathway is active at sites where bud emergence occurs. In line with this possibility, key regulators of the fMAPK pathway, including Sho1p (see below) and Ste20p are recruited to bud sites prior to bud initiation (Moran et al., 2019). Moreover, polarity targets of the fMAPK pathway included proteins that promote budding at bud sites (Fig. S1D). Polarity targets also included proteins that prevent budding at previous division sites or cytokinesis remnants. These included Rga1p (Fig. S1D), which along with other GTPase activating proteins (GAPs) prevents budding within the existing growth site (Kadota et al., 2004; Miller et al., 2017; Tong et al., 2007), and Rax2p (Fig. S1D), which restricts budding at previous division sites in a complex with Rax1p, Gps1p, Nba1p and Nis1p (Meitinger et al., 2014). Rga1p has been shown to negatively regulate the fMAPK pathway (Smith et al., 2002). Because these were negative regulators of the fMAPK pathway, cells were examined under pathway basal conditions (YEPD). Cells lacking Rax2p, Nba1p or Gps1p also showed elevated fMAPK pathway activity.

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Fig. 3. fMAPK pathway activity in synchronized cells and in mutants that fail to inhibit budding at dormant sites. (A) Immunoblot analysis of wild-type cells synchronized in G1 by α-factor arrest and released in YEPD. Cell extracts were probed at the indicated time points with antibodies to Clb2p-HA (α-HA), P~Kss1p, P~Fus3p, P~Kss1p, and Pgk1p as a control for protein levels. Numbers refer to the ratio of P~Kss1p to Pgk1p relative to 0 min, which was set to 1. BE, bud emergence. (B) Same as panel A, except cells were released into YEP-GAL medium. (C) Immunoblot analysis of wild-type and mutant combinations to assess fMAPK pathway activity. (D) Immunoblot analysis of the role of Msb2p in regulating fMAPK pathway activity in mutants lacking the negative polarity complex. See panel 3A for details.
activity (Fig. 3C; Fig. S4A), which indicates that the negative polarity complex negatively regulates the fMAPK pathway. The elevated activity of the fMAPK pathway in negative polarity complex mutants also required Msh2p (Fig. 3D; Fig. S4A,B). Cells lacking the negative polarity complex did not have an impact on mating (Fig. S4C). Therefore, proteins that spatially promote budding promote fMAPK activity, and proteins that spatially restrict budding also restrict fMAPK pathway activity. Cells lacking other Cdc42p-interacting proteins, Boi1p and Boi2p (Bender et al., 1996; Glomb et al., 2020; Liao et al., 2013; Masgrau et al., 2017) and Msh1p (Bender and Pringle, 1991; Bi et al., 2000; Liao et al., 2013) were examined but did not show altered activity of fMAPK pathway or a difference in invasive growth compared to wild-type cells (Fig. S4D–I). Collectively, the data indicate that fMAPK pathway activity is temporally and spatially regulated to coincide with bud emergence.

The fMAPK pathway stimulates rate of bud emergence during filamentous growth

To further define how the fMAPK pathway regulates bud emergence, the timing of bud emergence was examined using a septin marker (Cdc3p-mCherry) that shows a characteristic localization pattern throughout the cell cycle (Kim et al., 1991; Lippincott et al., 2001). In yeast-form cells grown in SD-URA (GLU), the timing of GFP-Cdc42p clustering, septin recruitment by Cdc3p-mCherry and bud emergence were similar between wild-type cells and an fMAPK pathway mutant (Fig. S5A). In filamentous wild-type cells grown in S-GAL-URA (GAL), GFP-Cdc42p clustering occurred at the incipient site by 10 min [Fig. 4A (green arrowheads) and 4B (Movie 1)] from the preceding round of cytokinesis marked by the septin hourglass split into a double ring (Fig. 4A; set as t=0; Cid et al., 2001; Lippincott et al., 2001). The recruitment of Cdc3p-mCherry also occurred by 10 min [Fig. 4A (red arrowheads) and 4B (Movie 1)]. The average time for bud emergence for 17 wild-type cells was 30 min from the preceding round of cytokinesis (Fig. 4B). Strikingly, in an fMAPK pathway mutant grown under filamentous conditions (GAL), a delay in GFP-Cdc42p clustering, Cdc3p-mCherry recruitment and bud emergence were observed (Fig. 4B). Although initial GFP-Cdc42p clustering and Cdc3p-mCherry recruitment occurred within the same time span (Fig. 4B,C; example 1, green and red arrowheads), some cells showed disappearance and reappearance of the GFP-Cdc42p cluster at the polarity site [Fig. 4C,D (oscillation seen in the pixel intensity graphs); Movies 2–4]. The disappearance and reappearance of the GFP-Cdc42p cluster, referred to here as transient disappearance of the polarity complex, accounted for a significant delay in bud emergence in the fMAPK pathway mutant (Fig. 4B,C). Some cells failed to make buds within the time of the experiment (Fig. 4D; Movies 3,4). In other examples, the initial polarity site disappeared and reappeared at a new site from where the bud emerged [Fig. 4E (in the mother cell, compare the location of green and red arrowheads with the black arrow that marks bud emergence); Movies 5,6]. The transient disappearance of the polarity complex, seen at some level in wild-type cells (although they always made a bud), was increased by 3-fold in the fMAPK mutant (Fig. 4F). Representing GFP-Cdc42p intensity in each cell as the coefficient of variation (CV) of pixel intensity (Lai et al., 2018) over time brought out the polarity defect in the fMAPK mutant relative to the wild-type (Fig. 4G). The CV of pixel intensity also showed a larger change in wild-type (0.16 to 0.23: 7) compared to the ste12A mutant (0.14 to 0.18: 4), which was statistically significant (P<0.01). These results define a function for the fMAPK pathway in stimulating the rate of bud emergence under conditions that promote filamentous growth.

To look at active Cdc42p during bud emergence in filamentous conditions, Gic2p-PBD-tdTomato reporter was co-localized with Sho1p-GFP, an fMAPK pathway regulator which is a direct effector of Msh2p (Cullen et al., 2004; Tatebayashi et al., 2007) that interacts with Cdc24p (Vadaie et al., 2008), Ste20p and Ste11p (Tatebayashi et al., 2006; Zarrinpars et al., 2004). Although Sho1p is known to localize to polarized sites (Pitoniak et al., 2009), whether it localizes to presumptive bud sites is not known. In wild-type cells, Sho1p-GFP was localized to incipient bud sites prior to bud emergence together with GFP-Cdc42p (Fig. 5B,C; wild-type, red arrow). After bud emergence, Sho1p-GFP was found at the bud tips as has been reported previously (Pitoniak et al., 2009). In ste12A mutant cells, Sho1p-GFP initially localized to the incipient bud site but failed to become enriched, and instead migrated back and forth along the distal pole (Fig. 5C; ste12A, black arrows). The low level of active Cdc42p in these cells rapidly disappeared. Bud formation was delayed, and, in the cell shown, bud emergence did not occur within the time of the experiment. Thus, although active Cdc42p clusters at incipient bud sites, an intact fMAPK pathway is required to promote bud emergence under nutrient-limiting conditions. Cells lacking the fMAPK pathway showed a growth defect under filamentous conditions (Fig. 5D; GAL) and a defect in the rate of bud formation (Fig. 5E,F). In particular, at 5, 10 and 18 h, the ste12A mutant formed buds at a slower rate than wild-type cells. Therefore, the fMAPK pathway stimulates the rate of budding during filamentous growth.

Activation of the fMAPK pathway induces growth at multiple sites

Wild-type cells normally grow at a single site due to a regulatory phenomenon known as singularity in budding. Cells containing active versions of Cdc42p bypass this regulation and grow at multiple sites (Caviston et al., 2002; Howell et al., 2012, 2009; Richman and Johnson, 2000; Wedlich-Soldner et al., 2003, 2004). Cells containing hyperactive versions of Msh2p also had this property (Fig. 5A; Basu et al., 2016). Further inspection showed that 16% of cells carrying MSB2Δ100-818 showed multiple growth sites (Fig. 5B; MSB2Δ100-818). Given that the fMAPK pathway regulates bud emergence, the ability of Msh2p to induce multiple growth sites might be mediated by the fMAPK pathway. We found that the formation of multiple growth sites by MSB2Δ100-818 required the fMAPK pathway (Fig. 5B; MSB2Δ100-818 ste12A). Growth at multiple sites was also induced by STE11-4 or over-expression of SHO1 (Fig. 5B). Generally speaking, the formation of multiple growth sites correlated with fMAPK pathway activity (Fig. 5B,C). One exception was GAL-SHO1, which showed high levels of multiple growth site formation (Fig. 5B), yet modestly induced fMAPK pathway activity (Fig. 5C). This may be because over-expression of SHO1 induces a unique cell morphology where cells have hyper-elongated buds (Fig. 5D). The activity of the fMAPK pathway is stimulated by positive feedback, which is evident by immunoblot of the Kss1p protein (Fig. 5C, middle blot), whose levels are controlled by the fMAPK pathway (Roberts et al., 2000). In some fMAPK hyperactive mutants (MSB2Δ100-818 and STE11-4) the levels of total Kss1p were lower than would be expected by positive feedback. Although the reason for this is not known, it could be due to the presence of negative feedback that acts to attenuate the activated pathway. Thus, Msh2p might itself induce multiple growth sites but require the fMAPK pathway for positive feedback. However, this was not the case. MSB2Δ100-818 expressed
Fig. 4. See next page for legend.
from an inducible promoter (pGAL) that did not depend on the fMAPK pathway also required Ste12p to form multiple growth sites (Fig. 5B,C; compare GAL-MSB2Δ100-818 with GAL-MSB2Δ100-818 ste12Δ). Therefore, Msb2p activation itself is not sufficient to induce growth at multiple sites. The fact that MSB2Δ100-818 is more effective at inducing growth at multiple sites than STE11Δ-4 can be explained by fMAPK pathway activity.

Transcriptional targets of the fMAPK pathway may be required for the formation of multiple growth sites. Bni1p and Gic2p were required for multiple growth site formation by the fMAPK pathway (Fig. 5E). High-copy expression of GIC2 itself induced multiple growth sites (Fig. 5E), in line with a previous report (Jaquenoud et al., 1998). The negative polarity complex did not have an impact on growth at multiple sites (Fig. 5B; pSTE11-4 nba1ΔA), although it did stimulate the fMAPK pathway activity (Fig. 5C). Collectively, these results support a role for the fMAPK pathway in the regulation of budding emergence, because activation of the fMAPK pathway can induce growth at multiple sites.

The fMAPK pathway induces symmetry breaking in the growing bud

The multiple growth sites produced by the fMAPK pathway differed in shape from previous reports on multiple bud formation by hyperactivation of Cdc42p (Caviston et al., 2002; Wedlich-Soldner et al., 2003, 2004). Hyperactive Cdc42p induces symmetry breaking in the mother cell to trigger formation of a second bud. By comparison, the hyperactive fMAPK pathway did not affect budding in mother cells. The multiple growth sites were formed inside the growing bud (Fig. 6A). To our knowledge, this new phenotype has not been previously characterized. Growth at a second site within the growing bud was not due to abortive growth at the initial site, because polarity proteins including Cdc24p-GFP (Fig. 5D) localized to multiple sites. Actin dynamics in live cells using Abp140p-YFP, a marker for the actin cables (Asakura et al., 1998; Riedl et al., 2008; Yang and Pon, 2002), showed multiple growth sites in the hyperactive fMAPK mutants compared with wild-type cells (Fig. 6B). Actin staining in filamentous cells using rhodamine phalloidin also showed polarized actin cytoskeleton at more than one site (Fig. S6A–C). Cells expressing MSB2Δ100-818 showed asymmetric clustering of GFP-Cdc42p (Fig. 6C; Movie 8). Over time, GFP-Cdc42p migrated along the bud cortex (Fig. 6C), which was evident by kymograph analysis (Fig. 6D), which allows tracking of changes in protein localization over time (Kaksonen et al., 2003). Sec3p-GFP localization corroborated that these sites were actively growing (Fig. S6C). Some wandering of GFP-Cdc42p occurred in wild-type cells (Movie 7), which might be due to the off-center delivery of vesicles that dilute the polarity complex (Chiou et al., 2017; Dyer et al., 2013).

The hyperactive fMAPK pathway may alter the dynamics of the interaction between Cdc42p and its effectors in a manner that makes the Cdc42p polarity axis ‘forget’ its orientation. This may mimic symmetry breaking occurring but within the bud cortex. In line with this possibility, versions of Cdc42p that contain point mutations in the effector-binding domain (such as Cdc42pΔV160), which impair its interaction with effector proteins (Gladfelter et al., 2002, 2005, 2001), showed a similar phenotype (Fig. 6E). Here multiple protrusions occurred adjacent to the previous polarization site. Similarly, cells expressing MSB2Δ100-818 formed multiple growth projections (Fig. 6A; three, four or five sites). Other genes can induce multiple buds when over-expressed (Sopko et al., 2006), but these did not rescue the growth defect of the cdc24-4 mutant and in most cases made it worse (Fig. S6D). These results demonstrate the importance of a properly regulated fMAPK pathway in bud morphogenesis.

DISCUSSION

Here we show that budding emergence, which is one of the most intensively studied and well understood polarity establishment processes in eukaryotes, is regulated by a MAPK pathway. The fMAPK pathway regulates transcription of polarity target genes and GTP-Cdc42p levels to increase the rate of bud emergence during filamentous growth. In this way, bud emergence is regulated by a pathway whose activity is sensitive to extrinsic cues. Importantly, the ability of the fMAPK pathway to induce the expression of polarity target genes allows the pathway to tailor bud emergence by altering the levels of proteins that act at multiple steps in the polarity pathway.

The fMAPK pathway may also regulate bud emergence during vegetative growth. Ste20p is the first effector recruited by Cdc42p at bud sites (Moran et al., 2019), which is known to activate the fMAPK pathway at bud sites to regulate bud emergence. Ste12p and Tec1p are also required for viability of the cdc24-4 mutant under vegetative growth conditions. A function for the fMAPK pathway in regulating bud emergence might be masked by genetic buffering under normal growth conditions. Indeed, cells lacking TEC1 are synthetically lethal with a diverse class of cytoskeletal and cell-cycle regulatory genes (Costanzo et al., 2010). More recently, Rsr1p in its GDP-locked state has been shown to regulate the timing of Cdc42p polarization in early G1 by interaction with Bem1p (Miller et al., 2019). Rsr1p (Basu et al., 2016) and Bem1p (Basu et al., 2020) both regulate the fMAPK pathway.

A key polarity target of the fMAPK pathway is Gic2p, which is a direct effector of Cdc42p that controls multiple steps in bud emergence. The regulation of GIC2 expression may be a key step in regulating polarity establishment in general. GIC2 expression is regulated by the fMAPK pathway and other proteins that regulate filamentous growth, including Pih1p (Gimeno and Fink, 1994; MacIsaac et al., 2006), Rim101p (Hu et al., 2007; Lamb and Mitchell, 2003), SAGA (Venters et al., 2011) and Rpd3p(L) (Hu et al., 2007; Venters et al., 2011). GIC2 expression is also regulated during mating (Roberts et al., 2000) and the Gic proteins are required for shmoo formation (Brown et al., 1997). Gic2p may function in other contexts as well, and has been implicated as an effector of the protein kinase C pathway (Zanelli and Valentini, 2005). Generally speaking, changes in gene expression may affect the...
regulation of bud emergence during cell differentiation and the response to stress.

Here and in Basu et al. (2016), we identify cross-talk between the polarity pathway and the fMAPK pathway. Cross-talk from the fMAPK pathway to the polarity pathway impacts bud emergence in several ways: (1) the activity of the fMAPK pathway is amplified by positive feedback to stimulate GTP-Cdc42p levels, (2) the fMAPK pathway induces target genes that encode polarity pathway components, and (3) the fMAPK pathway induces target genes that prevent growth at dormant growth sites (Meitinger et al., 2013). We also show that the activity of the fMAPK pathway is critical for proper bud emergence. Too little fMAPK pathway activity causes a delay in bud emergence during filamentous growth, and too much leads to growth at multiple sites. Precise regulation of the fMAPK pathway comes from activation at the right place (incipient bud sites) and time (at M/G1). The fact that the fMAPK pathway is cell-cycle regulated is a novel finding. Cell-cycle regulation of the fMAPK pathway might occur through TEC1, which like other G1 specific genes in the ‘SIC’ cluster (Cho et al., 1998; Spellman et al., 1998; Wittenberg and Reed, 2005) is induced at the M/G1 boundary by the cell-cycle regulated transcription factor Swi5p (Spellman et al., 1998).

During vegetative growth in S. cerevisiae, Cdc42p-dependent budding is dictated by bud-site-selection proteins (Chiou et al., 2017). In other fungal species, diverse mechanisms of polarity control predominate. In Schizosaccharomyces pombe, growth at the two poles is maintained by regulated oscillations in Cdc42p activity (Das et al., 2012). Changes in the orientation of the polarity complex occur during mating (Dyer et al., 2013; Nern and Arkowitz, 2000) and filamentous growth (this study), and may be a common feature of fungal cell differentiation. In the filamentous fungus Ashbya gossypii, Rsr1p regulates symmetric growth cone formation at the hyphal tip (Bauer et al., 2004). In the major human fungal pathogen Candida albicans, Rsr1p and Cdc42p are also required for proper hyphal growth (Si et al., 2016). MAPK pathways may be critical regulators of Cdc42p-dependent morphogenesis during hyphal/pseudohyphal growth. Parallels can also be drawn to higher eukaryotes. In mammals, the ERK pathway is directly involved in breaking radial symmetry of spreading RAT2 fibroblast cells (Klimová et al., 2016). In this case, ERK spatially restricts p190A-RhoGAP activity to limit growth at the cell rear. Functional cross-talk between MAPK pathways and Rho GTPases may constitute an under-explored regulatory circuit in many systems.
Fig. 6. Activation of the fMAPK pathway leads to wandering polarity. (A) Wild-type cells and cells carrying MSB2 Δ100-818 were examined on S-GLU medium by the single cell invasive growth assay. Arrows indicate growth sites. Scale bar, 5 μm. (B) Wild-type and cells containing GAL-SHO1 and GAL-MSB2 harboring Abp140p-YFP, a marker for the actin cytoskeleton, were examined for actin cable dynamics in S-GAL+AA semi-solid agar medium. Scale bar, 5 μm. (C) Time series of growth of wild-type cells and cells harboring MSB2 Δ100-818 expressing GFP-Cdc42p evaluated for multiple growth sites. Scale bar, 5 μm. Time interval: WT, 10 min; MSB2 Δ100-818, 20 min. DIC and inverted maximum intensity projection are shown. (D) Kymograph analysis of the highlighted regions in panel C. Time scale bar, 40 min. (E) Scanning electron micrographs of wild-type and Cdc42p V36T. Scale bar, 5 μm.
**Materials and Methods**

**Strains and plasmids**

Yeast strains are described in Table S1. Plasmids are listed in Table S2. Gene disruptions and *GALI* promoter fusions were made by polymerase chain reaction (PCR)-based methods (Baudin et al., 1993; Longtine et al., 1998). Some gene disruptions were made with antibiotic resistance markers KanMX (Longtine et al., 1998), *HYG* and *NAT* (Goldstein and McCusker, 1999). Internal epitope fusions were made by the pop-in, pop-out strategy (Schneider et al., 1995). Some strains were made ura3- by selection on 5-fluoroorotic acid (5-FOA). Gene disruptions were confirmed by PCR Southern analysis and confirmed by phenotype when applicable.

The pRS series of plasmids (pRS315 and pRS316) have been described (Sikorski and Hieter, 1989). To construct the cassette in PC6077 was replaced with (Sikorski and Hieter, 1989). To construct Southern analysis and confirmed by phenotype when applicable.

**Filamentous growth and mating assays**

Yeast and bacterial strains were manipulated by standard methods (Rose et al., 1990; Sambrook et al., 1989). The single-cell invasive growth assay (Cullen and Sprague, 2000) and the plate-washing assay (Roberts and Fink, 1994) were performed as described. Actin staining by rhodamine phalloidin was performed as described (Yuzuyk and Amberg, 2003). In cells lacking an intact mating pathway (ste4Δ), the FUSI-HIS3 reporter (McCaffrey et al., 1987) was used to evaluate iMAPK pathway activity (Cullen et al., 2004). *FUSI-HIS3* activity was measured by spotting equal concentrations of cells onto SD-HIS medium and SD-HIS medium containing 3-amino-1,2,4-triazole (3-ATA).

** Halo assays were performed as described (Lenes et al., 1987). A saturated culture of cells (A600=0.1) was spread onto YEPD medium and allowed to dry.** 

**Immunoblot analysis**

Immunoblot analysis was used to detect phosphorylated MAP kinases as described (Basu et al., 2016; Lee and Dohlman, 2008; Sabbagh et al., 2001). In cdc2-4 mutant combinations, cells were grown to mid-log in 10 ml SD-URA at 30°C and then incubated at 37°C for 4 h. Cells were harvested by centrifugation. Pellets were washed once with water and flash-frozen in liquid nitrogen. In experiments that did not involve cdc2-4, cells were grown in YEPD or YEP-GAL media for the times indicated. Proteins were precipitated by trichloroacetic acid (TCA) and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide).

**Cell synchronization and cell cycle analysis of MAPK activity**

Cell synchronization experiments were performed as described (Breeden, 1997). The strain harboring Cib2p-HA (PC2744) was transformed with a plasmid containing the STE4 gene (pSTE4; Stevenson et al., 1992). Cells were grown to an optical density (OD) A600 of 0.2 in SD-URA medium. Cells were washed and resuspended in equal volume of YEPD and incubated for 90 min at 30°C. α-Factor was added to a final concentration of 5 μg/ml and the culture was incubated for 90 min to arrest cells in the G1 phase of the cell cycle. Arrested cells were washed twice with water and resuspended in fresh YEPD or YEP-GAL medium to release cells into the cell cycle. Aliquots (10 ml) were harvested every 10 min, flash-frozen in liquid nitrogen and stored at −80°C.

**Immunoblot analysis**

ERK-type MAP kinases (P-Kss1p and P-Fus3p) were detected using p44/42 antibodies (no. 4370; Cell Signaling Technology, Danvers, MA, USA) at a 1:5000 dilution. Kss1p was detected using α-Kss1p antibodies (no. 6775; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:2000 dilution. Cib2p-HA was detected using the α-HA antibody at a 1:5000 dilution (no. 12CA5; Roche Diagnostics). For secondary antibodies, goat anti-rabbit IgG-HRP antibodies were used at a 1:10,000 dilution and 3-amino-1,2,4-triazole (3-ATA). Halo assays were performed as described (Lenes et al., 1987). A saturated culture of cells (A600=0.1) was spread onto YEPD medium and allowed to dry. α-Factor (3 μl and 10 μl, at 1 mg/ml) was spotted on the plates. Plates were incubated at 30°C and photographed at 24 and 48 h. Experiments were performed in triplicate. Halo diameter (in centimeters) was measured by ImageJ analysis and plotted as a function of α-factor concentration. For Lat-A sensitivity, saturated culture of cells (A600=0.1) was spread onto SD-URA-LEU medium containing 0.5 M sorbitol. Lat-A (10 μl; L5163, Sigma) at 0, 0.1, 0.2 and 0.5 mM (in 10 mM DMSO) were spotted on plates. Halo diameter (in centimeters) was measured by ImageJ analysis and plotted as a function of Lat-A concentration.
analysis was performed with Image Lab Software (Bio-Rad). For blots to evaluate phosphorylated MAP kinase proteins, membranes were incubated in 1×TBST (10 mM TRIS–HCl, pH 8, 150 mM NaCl, 0.05% Tween-20) with 5% bovine serum albumin. For other immunoblots, membranes were incubated in 1×TBST with 5% non-fat dried milk. All primary incubations were carried out for 16 h at 4°C. Secondary incubations were carried out at 25°C for 1 h.

Growth assays for temperature-sensitive mutants

Wild-type and temperature-sensitive mutants containing desired plasmids were grown to saturation in SD-URA media. For each strain, 0.1 OD600 of cells were serially diluted four times in distilled water and spotted onto SD-URA-LEU+ 0.5 M sorbitol plates. The plates were incubated at 30 and 37°C. For cdc12-5 suppression, cells were spotted onto SD-URA plates and incubated at 24 and 30°C as the cdc12-6 mutant had a severe growth defect at 30°C and failed to grow completely at 37°C. For all suppression assays, plates were photographed every day for 4 days using an Evolution MP Color Camera (Media Cybernetics) and Q Capture software. Images were imported into ImageJ software. Cells of interest were selected and the measure tool was used to generate values for the integrated density, area and the mean signal intensity. Growth of a colony was quantified by measuring the signal intensity of the background cell. Growth at 37°C was compared with growth at 30°C for each strain and then normalized to wild-type. Quantitation was reported for the day where the temperature-sensitive mutant containing STE11-4 plasmid showed around 60% of growth compared with the wild-type, which was the maximum growth detected across various mutant combinations and independent trials. For most analyses, only the first or the second dilution of cells was used for the quantitation. All growth assays were performed in triplicate. Error bars show standard error of mean (s.e.m.) among the three trials.

Quantitative PCR analysis

Cells for qPCR were concentrated (OD A600=20) and spotted in 10 µl aliquots onto YEP-GAL (2% agar) for 24 h. Cells were spotted in six colonies per plate equidistant to each other and the plate center. All six colonies were harvested for each trial, and two separate trials were compared for each strain. The entire colony surface was scraped into 500 µl of distilled water, harvested by centrifugation, washed and stored at −80°C. RNA was harvested by hot acid phenol chloroform extraction as described previously (Adhikari and Cullen, 2014). Samples were further purified using Qiagen RNaseasy Mini Kit (catalog no. 74104). RNA concentration and purity were measured using NanoDrop (NanoDrop 2000C). RNA stability was determined by 1% agarose Tri-borate-EDTA (TBE, 89 mM Tris base, 89 mM boric acid, 2 mM EDTA) gel electrophoresis. cDNA libraries from RNA samples were generated using iScript Reverse Transcriptase Supermix (no. 1708840; Bio-Rad). qPCR was performed using iTaq Universal SYBR Green Supermix (no. 1725120; Bio-Rad) on a Bio-Rad iCycler. Experiments were performed with biological replicates, and the average of multiple independent experiments was recorded. Primers for pPCR for Gic2 were: forward 5′-GCGGCAACAGACAATCAAAA′-3′ and reverse 5′-GCAAATGTGCATCTGTAAGT′-3′; primers for Gic1 were: forward 5′-GCCGAAACGACAACTCAGAAA′-3′ and reverse 5′-GCTTTCGTGCCAGACCATGTGCTC′-3′; primers for ACT1 were: forward 5′-GGCTTGCCTTGGACTACCTTCCA′-3′ and reverse 5′-GATGGACCACTTTCGATTCC′-3′ as published (Chavel et al., 2010).

DIC and fluorescence microscopy

Differential interference contrast (DIC) and fluorescence microscopy using FITC, Cyan Fluorescent Protein (CFP), Yellow Fluorescent Protein (YFP), rhodamine and DAPI filter sets were performed using an Axioplan 2 fluorescent microscope (Zeiss) with a Plan-Apochromat 100×/1.4 (oil) objective (NA 1.4) (cover slip 0.17) (Zeiss). Digital images were obtained with the Axioham RM camera (Zeiss) and Axiovision 4.4 software. Adjustments to brightness and contrast were made in Adobe Photoshop. Some images were obtained using structural deconvolution with a Zeiss Apotome filter. Multiple polarization events were assigned by examining cells over multiple focal planes by DIC and fluorescence microscopy. Time-lapse microscopy was performed on a Zeiss 710 confocal microscope equipped with a Plan-Apochromat 40×/1.4 oil DIC M27 objective on a heated stage at 37°C. GFP was imaged using 488 nm laser excitation and an emission window from 496 to 572 nm. Time-lapse Z-stacks were captured at 20 and 30 min intervals to monitor bud emergence and Cdc42p clustering.

Budding rate assay

Cells were grown for 16 h on SD+AA medium. Using a toothpick, cells were removed from the plate, washed twice with water and resuspended in 1 ml water. Cells (50 µl) were spread onto SD+AA or S-GAL+AA media and incubated at 30°C. Cells were visualized at 0, 5, 10 and 15 h intervals at 20×. Budding rate, n/(n-1)/n(2), was determined as described (Hall et al., 2014), where n is the number of daughters produced. Budding rate was adjusted for the time interval (5, 5 and 8 h). More than 30 cells were examined for each interval.

Time-lapse microscopy

For time-lapse microscopy, cells were placed onto semi-solid agarose pads that were prepared as described (Skinner et al., 2013) with the following modifications. Approximately 700 µl of SD-URA medium, prepared with agarose (1%), was placed inside 12 mm Nunc glass base dishes (no. 150680; Thermo Fisher Scientific, Waltham, MA, USA) and allowed to set at 25°C for 5 min. For GFP-Cdc42p clustering in cdc24-4 mutant combinations, cells were taken from colonies grown at 30°C for 16 h on SD-URA+G418 semi-solid agar media [2% agarose, 0.67% yeast nitrogen base (YNB) without ammonium sulfate, 0.1% monosodium glutamate (MSG), 2% dextrose, 1× amino acid stock without uracil, 0.36 mg/ml G418], resuspended in 7 µl of synthetic broth (0.67% YNB without ammonium sulfate, 0.1% MSG, 2% dextrose), and placed under the agarose pads by gently lifting the pad with a scalpel. Water (100 µl) was placed in the dish adjacent to the agarose pad to prevent moisture loss, and the Petri dishes were incubated at 37°C for 4 h. For co-localization of Sho1p-GFP and Gic2p-PBD-tdTomato, cells were prepared as above except that they were imaged at 30°C. For GFP-Cdc42p clustering in cells carrying MSB2Δ100-S18, 500 µl of saturated culture was washed twice with water and resuspended in 500 µl water. An aliquot of 5 µl was placed under the agarose pad, and the Petri dish was incubated at 30°C for 10 h before imaging. For strains expressing GFP-Cdc42p and Cdc3p-mCherry, cells were prepared from colonies grown at 30°C for 16 h on SD-URA.

FLIM-FRET analysis

Plasmodia containing the Cdc42p biosensor have been described (Smith et al., 2013) and were a generous gift from Dr Rong Li (Johns Hopkins University, Baltimore, MD, USA). Cells containing pRS316-pACT1-eGFP-CRBCL4A-Cdc42pCAAX-mCherry-CAAX (PC7137) and Q61L (PC7138) or D57Y (PC7139) derivatives were grown on SD-URA medium and placed under the agarose pads. Budding rate assay was performed on the SUNY-Buffalo North Campus Confocal Imaging Facility. FLIM-FRET has been described (Bassard and Halkier, 2018; Gratton et al., 2013; Osterlund et al., 2015; Padilla-Parras et al., 2015; Periasamy and Clegg, 2009; Sun and Periasamy, 2015). FLIM-FRET images were acquired with a Simple Tau TCSPC 150 and HPM-100-40 GaAsP detector (Becker & Hickl, Berlin, Germany) employing the direct coupled port of the LSM 710 and Zeiss Intune Laser excitation at 490 nm. At least 1000 photons per pixel were acquired for the highest count region for each cell.

For confocal microscopy and FLIM, we have used at the SUNY-Buffalo North Campus Confocal Imaging Facility. FLIM-FRET has been described (Bassard and Halkier, 2018; Gratton et al., 2013; Osterlund et al., 2015; Padilla-Parras et al., 2015; Periasamy and Clegg, 2009; Sun and Periasamy, 2015). FLIM-FRET images were acquired with a Simple Tau TCSPC 150 and HPM-100-40 GaAsP detector (Becker & Hickl, Berlin, Germany) employing the direct coupled port of the LSM 710 and Zeiss Intune Laser excitation at 490 nm. At least 1000 photons per pixel were acquired for the highest count region for each cell.

FLIM-FRET data analysis was performed using SPImage 7.3 (Becker & Hickl). Data were fitted by a two-component exponential decay model and automatically generated instrument response function (IRF). Bin factor, shift and offset were adjusted to obtain good fit (low χ²). For measuring the
average lifetime, the decay curve was pooled for the region of interest. The mean fluorescence lifetime for the two-component decay model was calculated according to the equation: $m = \frac{1}{\tau^2} \frac{\mu}{a}$, where $a$ and $\tau$ are the amplitude and lifetime of the ith component and $m$ is the average lifetime of the donor fluorescence.

### Scanning electron microscopy

Scanning electron microscopy was based on established methods (Piccirillo and Honigberg, 2011) and performed as described (Basu et al., 2016). For some experiments, cells were grown for 16 h in liquid medium at 30°C. Cells were washed in 0.1 M sodium phosphate buffer at pH 7.4 and diluted to about 10^6 cells, which were passed over 0.2 µm Whatman nucleopore polycarbonate filter paper (catalog no. 889-78084GE Whatman) with a 10 ml syringe (no. 309604 BD Syringe). Cells were rinsed with one round of buffer by syringe, fixed with 1% glutaraldehyde for 15 min, and rinsed again. Cells were treated by a graded series of ethanol washes (30%, 50, 70, 85 and 100%) by syringe to dehydrate the samples. The filter paper was removed from the holder, placed in a Petri dish and treated with hydrofluorosilane (HMDS). Samples were placed at 4°C for 16 h and imaged the following day. All solutions were filter sterilized before use and stored in clean containers free of corrosion products. Samples were carbon coated and imaged on a field emission scanning electron microscope (Hitachi SU70).

### Image analysis

Gic2p-PBD-tdTomato clustering was quantified as described in Okada et al. (2017) with the following modifications. Raw fluorescence and DIC images were imported in ImageJ. DIC images were used to draw the cell boundary with the polygon tool. The same region of interest (ROI) was applied to the fluorescence image. Signal intensities for all pixels inside the ROI were measured for each sample. Over 60 cells were normalized pixels was used to represent each cell. The background region was subtracted from the selected pixels of the cell. The average lifetime, the decay curve was pooled for the region of interest. The mean fluorescence lifetime for the two-component decay model was calculated according to the equation: $m = \frac{1}{\tau_1^2} \frac{\mu}{a_1} + \frac{1}{\tau_2^2} \frac{\mu}{a_2}$, where $a_1$ and $\tau_1$ are the amplitude and lifetime of the ith component and $m$ is the average lifetime of the donor fluorescence.

### Bioinformatics and statistical analysis

GO term analysis (Ashburner et al., 2000) was performed using the Gene Ontology enrichment analysis and visualizaton algorithm (GOvila) (Eden et al., 2007, 2009) using the two unranked lists mode. Genes encoding proteins associated with cell polarity were identified by GO term analysis (Ashburner et al., 2000; GO:0000282, cellular bud site selection; GO:0030010, establishment of cell polarity; GO:0031106, septin ring organization; GO:0006887, exocytosis; GO:0007120, axial cellular bud site selection; GO:0007121, bipolar cellular bud site selection; GO:0030427, site of polarized growth). GO terms and descriptions come from SGD (http://www.yeastgenome.org). Statistical significance for pairwise comparisons was performed using Student’s t-test in Microsoft Excel. For multiple comparisons, one-way ANOVA with Tukey’s test was performed in Minitab (www.minitab.com).

### Competing interests

The authors declare no competing or financial interests.

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