Arf3p GTPase is a key regulator of Bud2p activation for invasive growth in *Saccharomyces cerevisiae*

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Running title: Role of Arf3p in yeast invasion

Abbreviations: Arf, ADP-ribosylation factor; GEF, guanine nucleotide exchange factor; GAP, GTPase activating protein; GTP, guanosine triphosphate; GDP, guanosine diphosphate.

**ABSTRACT**

The regulation and signaling pathways involved in the invasive growth of yeast have been studied extensively because of their general applicability to fungal pathogenesis. Bud2p, which functions as a GTPase-activating protein (GAP) for Bud1p/Rsr1p, is required for appropriate budding patterns and filamentous growth. However, the regulatory mechanisms leading to Bud2p activation are poorly understood. In this study, we report that Arf3p acts as a regulator of Bud2p activation during invasive growth. Arf3p binds directly to the N-terminal region of Bud2p and promotes its GAP activity both *in vitro* and *in vivo*. Genetic analysis shows that deletion of *BUD1* suppresses the defect of invasive growth in *arf3Δ* or *bud2Δ* cells. Lack of Arf3p, like that of Bud2p, causes the intracellular accumulation of Bud1p-GTP. The Arf3p-Bud2p interaction is important for invasive growth and facilitates the Bud2p-Bud1p association *in vivo*. Finally, we show that under glucose depletion-induced invasion conditions in yeast, more Arf3p is activated to the GTP-bound state and the activation is independent of Arf3p guanine nucleotide-exchange factor (GEF) Yel1p. Thus, we demonstrate that a novel spatial activation of Arf3p plays a role in regulating Bud2p activation during glucose-depletion-induced invasive growth.

Key words: ARF/BUD2/GTPase/cytokinesis/polarity/invasion/GEF/GAP/budding.

**INTRODUCTION**

Cell polarization is crucial for cell proliferation, differentiation, and morphogenesis in both unicellular and multicellular organisms. The determinants of polarity can be either internally or externally derived (Howell and Lew, 2012). In general, polarity establishment can be described as the following steps; the stimulation of spatial cues, the determination of landmark proteins, subsequent recruitment of signaling molecules and asymmetric organization of the cytoskeleton. Of note, small GTPase
modules function as key signaling molecules in polarity establishment (Irazoqui and Lew, 2004; Park and Bi, 2007). The yeast *Saccharomyces cerevisiae* has been used as a model organism for studying the development of polarity, showing three unique life cycle stages of polarized growth: budding, mating, and filamentous growth; among which the mechanisms underlying budding have been the best studied (Park and Bi, 2007). Yeast reproduces via asymmetric division, giving rise to a daughter cell, during which the selection process for determining the budding site is highly organized and exhibits a unique pattern (Park and Bi, 2007; Perez and Rincon, 2010). The machinery required to specify the axial budding pattern of haploid yeast has been identified as a GTPase module composed of Bud1p (Rsr1p), Bud2p, and Bud5p (Bender, 1993). The GTPase Bud1p is activated by its guanine nucleotide exchange factor (GEF), Bud5p, and activated Bud1p subsequently recruits Cdc24p, the GEF for Cdc42p, to the presumptive bud site (Bender, 1993). The GTPase-activating protein (GAP) Bud2p then promotes the hydrolysis of Bud1p-GTP to trigger the activation of Cdc24p, which in turn, activates Cdc42p to regulate the actin reorganization required for polarized growth (Leberer et al., 1996). Therefore, Bud1p GTP-GDP cycling is thought to be necessary for bud site selection and to link spatial landmarks to polarity establishment (Kang et al., 2010; Perez and Rincon, 2010).

ADP-ribosylation factors (Arfs) are highly conserved small GTPases that are major regulators of vesicular trafficking and cytoskeletal reorganization (Chavrier and Menetrey, 2010; Donaldson and Jackson, 2011). Yeast Arf3p is the homologue of mammalian Arf6, which is involved in multiple cellular processes, including cell adhesion, migration, wound healing, membrane ruffling, and metastasis (D'Souza-Schorey and Chavrier, 2006). Although Arf3p and Arf6 are 75% similar in their amino acid sequences, the functions of these two proteins are distinct. Arf3p is involved in actin organization and polarity development during yeast budding (Huang et al., 2003; Zakrzewska et al., 2003; Costa and Ayscough, 2005; Lambert et al., 2007), although the detailed molecular mechanisms underlying the function of Arf3p during polarity development have not been elucidated.

Several fungi, including *S. cerevisiae*, can undergo a developmental switch from vegetative growth to filamentous growth to survive in a stressed environment (Park and Bi, 2007). There are two forms of filamentous growth in *S. cerevisiae*, invasive growth and pseudohyphal growth, depending on ploidy and growth conditions (Pan et al., 2000; Cullen and Sprague, 2012). The regulatory network associated with haploid yeast invasive growth and the cooperative signaling that occurs during this process have been extensively studied, and many key molecules involved in polarity development during yeast vegetative growth have been found to also be required for invasive growth (Pan et al., 2000; Cullen and Sprague, 2002; Palecek et al., 2002).
For example, Bud2p, the critical regulator of bud site selection during yeast budding, has been shown to be required for yeast invasive growth (Jin et al., 2008) and invasive hyphal morphogenesis in *Candida albicans* (Hausauer et al, 2005).

Given these observations, we hypothesized that Arf3p may also participate in the yeast invasive growth and found that the Arf3p-Bud2p interaction is important for invasive growth via regulating Bud2p GAP activity toward Bud1p. Furthermore, we found that the activity of Arf3p is stimulated upon glucose depletion. Together, the results of our study identify Arf3p as an upstream regulator of Bud2p and suggest the existence of a novel small GTPase cascade, Arf3p-Bud2p-Bud1p, which orchestrates polarity development during invasive growth.

**RESULTS**

**Arf3p is required for yeast invasive growth**

Many proteins that are involved in polarized growth in yeast also participate in invasive growth (Cullen and Sprague, 2002). Furthermore, a previous large-scale screening analysis suggested that Bud2p is involved in the maintenance of yeast invasive growth (Park et al., 1993; Cullen and Sprague, 2002; Jin et al., 2008). Since both Arf3p and Bud2p participate in the regulation of polarity process, we speculate that Arf3p may also participate in yeast invasive growth. Therefore, we examined the invasive growth of *ARF3* and *BUD2* deletion mutants using the haploid yeast strain Σ1278b, which is commonly employed to study invasive growth and agar penetration (Gimeno et al., 1992). We found that both the arf3Δ and bud2Δ mutations impaired agar invasion which is a classical phenomenon of yeast invasive growth (Figure 1A). The cell surface flocculin Flo11p is required for yeast invasion (Lo and Dranginis, 1998) and, thus, was used as a control for agar invasion defects (Figure 1A). This phenotype of arf3Δ cells could be significantly restored via the expression of wild-type Arf3p; however, the constitutively active form (Arf3pQ71L), the GTP binding-defective mutant (Arf3pT31N), and the membrane-binding defective mutant (Arf3pG2A) were all unable to restore the phenotype (Figure 1B). These data suggest that dynamic cycling of GTP- and GDP-bound Arf3p at the plasma membrane is required for yeast invasive growth.

**Arf3p directly interacts with Bud2p in a GTP-dependent manner**

A potential physical interaction between Bud2p and Arf3p has been indicated from a large-scale yeast two-hybrid analysis (Hazbun et al., 2003). To explore the underlying mechanism of Arf3p functioning in yeast invasion, we first verified this interaction with yeast two-hybrid analyses. We observed that Arf3p interacts with full-length Bud2p, and the constitutively active mutant Arf3pQ71L, which cannot hydrolyze GTP, strongly interacts with Bud2p (Figure 2A). On the other hand, the constitutively inactive Arf3pT31N mutant, which cannot bind GTP, can not interact
with Bud2p (Figure 2A). The interaction is Arf3p-specific because no interaction was detected between Bud2p and other constitutively active Arf or Arf-like (Ar1) proteins (Figure 2B). To examine the Arf3p-Bud2p interaction \textit{in vivo}, we co-expressed Arf3p (wild-type, Q71L, or T31N) and HA-tagged Bud2p in \textit{arf3Δ} cells. HA-Bud2p was immunoprecipitated and we detected bound Arf3p and Arf3p\textsubscript{Q71L}, but not Arf3p\textsubscript{T31N} (Figure 2C).

Bud2p contains two functional domains: a C2 domain for membrane targeting and a GAP domain to facilitate Bud1p-GTP hydrolysis. To map the Arf3p-interacting region of Bud2p, we expressed and purified various GST-tagged fragments of Bud2p (Bud2N, Bud2C2, Bud2GAP, and Bud2CT), as illustrated in Figure 2D, and examined their interaction with His-tagged Arf3\textsubscript{Q71L} or Arf3\textsubscript{T31N} using \textit{in vitro} pull-down assays. We found that Bud2N (N-terminal 336 amino acids) was able to interact with Arf3\textsubscript{Q71L} (Figure 2E). Further truncation of the N-terminus of Bud2p indicated that the first 120 residues (Bud2N1) are sufficient for the interaction with Arf3\textsubscript{Q71L} (Figure 2F). This interaction was further examined by using purified His-Arf3 pre-bound with GTP\textsubscript{γS} or GDP and GST-Bud2N1. We also found that Bud2N1 specifically interacts with Arf3-GTP\textsubscript{γS} but not Arf3-GDP (Figure S1).

Moreover, a series of N-terminal truncations of Bud2p were examined for potential interaction with Arf3p via yeast two-hybrid analysis, revealing that the N-terminal 40 residues of Bud2p are necessary for interaction with Arf3p (Figure 2G). The requirement for the N-terminal 40 amino acids of Bud2p for interaction with Arf3p was also observed \textit{in vivo} (Figure 2H). These data demonstrate that Arf3p interacts directly with the N-terminal region of Bud2p in a GTP-dependent manner.

To identify the critical amino acids in Arf3p that are responsible for the interaction with Bud2p, we first generated two Arf1p-Arf3p chimeras: ARF3N1C, which contains amino acids 1-77 of Arf3p and amino acids 78-181 of Arf1p, and ARF1N3C, which contains amino acids 1-77 of Arf1p and amino acids 78-183 of Arf3p (Figure S2A). A co-immunoprecipitation experiment, using cells expressing chimeric Arf proteins and HA-Bud2p, demonstrated that the N-terminus of Arf3p are the major region involved in the interaction with Bud2p (Figure S2A). To further identify the specific residues within the N-terminus of Arf3p that is important for this interaction, we aligned the N-terminal sequences of yeast Arf1p and Arf3p with the Arf3p human homolog, Arf6 (Figure S2B). We identified two residues, L23 and I33, in Arf3p, which are conserved in yeast Arf3p and human Arf6, but not yeast Arf1p. Hence, we mutated these two residues in Arf3p to valine, which is the corresponding amino acid in Arf1p, and analyzed the localization and interaction abilities of the mutant proteins. We observed that although Arf3p\textsubscript{L23V}-GFP and Arf3p\textsubscript{I33V}-GFP exhibited similar subcellular distributions compared with wild-type Arf3p based on microscopic
observations and fractionation analysis (Figure S2C and S2D), co-immunoprecipitation analysis revealed that the interaction between Arf3p\textsuperscript{I33V} and HA-Bud2p was weakened (Figure 2I). Interestingly, neither L23 nor I33 of Arf3p was required for interaction with its GEF, Yel1p (Gillingham and Munro, 2007), or its polarization partner, Afi1p (Tsai et al., 2008), in yeast two-hybrid analysis (Figure S2E). These data indicate that I33 of Arf3p specifically contributes to the Arf3p-Bud2p interaction. Collectively, our data demonstrate that I33 of Arf3p and the N-terminal 40 residues of Bud2p are the regions responsible for the Arf3p-Bud2p interaction.

**Arf3p and Bud2p partially colocalize at the emerging bud**
Arf3p localizes to the plasma membrane and is enriched at the emerging bud (Huang et al., 2003); however, Bud2p localizes to the presumptive bud site and subsequently to the mother-bud neck after bud emergence (Park et al., 1999). Therefore, it is not surprising that we observed co-localization of Arf3p-mRFP and GFP-Bud2p at the mother-bud neck (Figure S3A). Nevertheless, upon careful observation, we noticed a subtle difference in the distributions of these two proteins: Arf3p-mRFP was enriched at both the plasma membrane of the daughter cell and the neck, whereas GFP-Bud2p was mainly concentrated at the mother-bud neck (Figure S3). These data indicate that Bud2p and Arf3p only partially co-localize during polarized growth. Indeed, we found that the membrane targeting and polarized distribution of Arf3p and Bud2p are independent of each other, as GFP-Bud2p localized to the bud neck in both wild-type and arf3\textsuperscript{Δ} cells (Figure S3B), and Arf3p-GFP displayed a highly polarized membrane distribution in both wild-type and bud2\textsuperscript{Δ} cells (Figure S3C). These data indicate that the polarized distribution of Arf3p and Bud2p is independent of their physical interaction.

**The Arf3p-Bud2p interaction is important for yeast invasive growth**
Given that Arf3p interacts with Bud2p and both of them share similar phenotypes in yeast invasive growth, we next examined whether the Arf3p-Bud2p interaction is required for yeast invasive growth. We found that agar invasion was restored in bud2\textsuperscript{Δ} cells via the expression of wild-type Bud2p and Bud2p-dN30, but not through the expression of the Arf3p-binding defective mutant Bud2p-dN40 (Figure 3A). Deletion of the N-terminal 40 amino acids of Bud2p may not affect the global conformation of Bud2p because GFP-Bud2p-dN40 was still properly localized to the bud neck (Figure S4A). Additionally, Bud2p-dN40 partially restored (~40%) the budding pattern abnormality of bud2\textsuperscript{Δ} cells (Figure S4B). We further investigated whether expression of the Bud2p-binding defective mutant Arf3p\textsuperscript{I33V} also impairs yeast invasive growth. We observed that the agar invasion defect seen in arf3\textsuperscript{Δ} cells could be restored by ARF3 and ARF3\textsuperscript{L23V}, but not by ARF3\textsuperscript{I33V} (Figure 3B). Notably,
Arf3p<sup>133V</sup> was also unable to rescue the budding pattern abnormality observed in arf3Δ cells (Figure S4C). Together, these data demonstrate that an interaction between Arf3p and Bud2p is required for the proper function of these proteins in yeast invasive growth.

**Accumulation of Bud1p-GTP prevents yeast invasive growth**

Bud2p is a GAP that promotes the hydrolysis of GTP by Bud1p (Park et al., 1993), and Bud5p acts as a GTP-exchange factor that catalyzes the binding of GTP to Bud1p (Chant et al., 1991). Thus, we examined whether the bud1Δ and bud5Δ mutants are also defective in agar invasion. Surprisingly, neither the bud1Δ nor bud5Δ mutant exhibited defects in invasive growth, suggesting that the polarity module involved in bud-site selection functions differently in yeast invasive growth (Figure S5A). Accordingly, overexpression of wild-type or inactive (GDP-bound) Bud1p<sup>K16N</sup> did not affect invasive growth. However, overexpression of the constitutively active (GTP-bound) Bud1p<sup>G12V</sup> significantly interfered with yeast invasive growth (Figure S5B). This result suggests that overproduction of Bud1p-GTP could obstruct yeast invasive growth; therefore, the defect in arf3Δ and bud2Δ cells is due to the accumulation of Bud1p-GTP, and depletion of BUD1 may suppress the invasive growth defect seen in these cells. We generated bud2bud1Δ and arf3bud1Δ double mutant strains to test this hypothesis and observed that deletion of BUD1 in arf3Δ or bud2Δ cells significantly suppresses the defect in agar invasion (Figure 4A). Consistently, we also observed that decreasing Bud1p-GTP levels by deleting BUD5 could suppress the agar invasion defect in bud2Δ cells (Figure S5C). By contrast, flo11bud1Δ mutant cells, similar to flo11Δ cells, exhibit a defect in invasive growth. Notably, we found that deletion of BUD1 in a bud2Δ strain completely restores invasive growth; however, deletion of BUD1 in an arf3Δ strain only partially restores it, suggesting that the accumulation of Bud1p-GTP is just part of Arf3p's role in invasive growth. Furthermore, we observed that overexpression of Bud1p and Bud1p<sup>G12V</sup> caused an invasive growth defect in arf3bud1Δ and bud2bud1Δ cells, while Bud1p<sup>K16N</sup>-overexpressing yeast maintained their agar invasion ability (Figure 4B). These data suggest that the inhibition of yeast invasive growth in bud2Δ and arf3Δ mutant cells occurs via the accumulation of Bud1p-GTP.

**The GAP activity of Bud2p is required for invasive growth**

Given that increased levels of Bud1p-GTP interfere with invasive growth, we hypothesized that the GAP activity of Bud2p is required for this process. To test this hypothesis, we first created a Bud2p GAP-defective mutant. GAP1<sup>IP4BP</sup> IP4BP is a GAP for Rap1, the mammalian homolog of Bud1p, and has functional domains that are similar to those of Bud2p. Two point mutations in GAP1<sup>IP4BP</sup>, GAP1<sup>IP4BP-L484A</sup> and GAP1<sup>IP4BP-R485Q</sup>, significantly impaired its GAP activity (Kupzig et al., 2006). The
L484 and R485 mutations in GAP1IP4BP are equivalent to L681 and R682 in Bud2p (Figure S6A). This leucine residue is highly conserved among members of the GAP family because it stabilizes the arginine finger, which is vital for its GAP activity (Kupzig et al., 2006). To determine whether the L681A and R682Q mutations in Bud2p (Bud2pAQ) cause defects in GAP activity, we immunoprecipitated HA-Bud2p and HA-Bud2pAQ from yeast lysates and incubated them with recombinant [γ-32P]GTP-bound GST-Bud1. The decrease in protein-bound radioactivity was measured and calculated as a measure of GTP hydrolysis. Bud2pAQ showed a significantly reduced ability to promote Bud1p-GTP hydrolysis; thus, Bud2pAQ is indeed a GAP-defective mutant (Figure S6B). We further demonstrated that expression of GAP-defective Bud2pAQ could not rescue the defects in the axial budding pattern or invasive growth in bud2Δ cells (Figure S6C and S6D). Collectively, these data indicate that the GAP activity of Bud2p is required for both types of yeast polarized growth.

**The Arf3p-Bud2p interaction promotes Bud1p-GTP hydrolysis in vivo**

Similar to bud2Δ, the loss of invasive growth in arf3Δ is dependent on the presence of BUD1, suggesting that the equilibrium of GTP- or GDP-bound Bud1p may be altered in arf3Δ cells. To quantify the levels of Bud1p-GTP in vivo, we used recombinant GST-Cdc42-GDP to specifically pull down Bud1p-GTP (Kozminski et al., 2003). We first validated this pull-down assay using yeast cells overexpressing wild-type Bud1p, GTP binding-defective Bud1pK16N, or GTP hydrolysis-deficient Bud1pG12V protein. Bud1pG12V, but not Bud1pK16N, bound specifically to GST-Cdc42-GDP (Figure S7), suggesting that this assay accurately reflects Bud1p-GTP levels in vivo. We therefore used this assay to quantify Bud1p-GTP in HA-Bud1p-expressing wild-type, arf3Δ, bud2Δ, and bud5Δ cells. Compared with wild-type cells, we observed a 4-fold increase in Bud1p-GTP levels in both arf3Δ and bud2Δ cells (Figure 5A). Moreover, Bud1p-GTP was undetectable in bud5Δ cells. These data suggest that Arf3p is required for Bud1p-GTP hydrolysis in vivo. We next reintroduced mutant forms of Arf3p and Bud2p that are unable to interact with each other to determine whether the Arf3p-Bud2p interaction is required for the Bud1p GTP-GDP cycle. The level of Bud1p-GTP was significantly increased in bud2Δ cells expressing Bud2p-dN40 compared with those expressing HA-Bud2p (Figure 5B). Higher levels of Bud1p-GTP were consistently observed in vivo in the Bud2p-interaction-defective mutant Arf3pI33V compared with wild-type Arf3p; however, this phenomenon was not observed for Arf3pL23V, which is capable of binding Bud2p (Figure 5C). Taken together, our results demonstrate that the Arf3p-Bud2p interaction is required to promote Bud1p-GTP hydrolysis in vivo.

**Arf3p directly enhances Bud2p GAP activity in vitro**


Given the direct interaction between Arf3p and Bud2p and the Arf3p requirement for Bud1p-GTP hydrolysis in vivo, we speculated that Arf3p could bind directly to Bud2p to promote Bud1p-GTP hydrolysis. Thus, we measured the GAP activity of Bud2p in the presence of recombinant constitutively active Arf3<sup>Q71L</sup> in vitro. HA-Bud2p was immunoprecipitated from yeast lysates and incubated with different amounts of purified Arf3<sup>Q71L</sup>, and recombinant [γ-<sup>32</sup>P]GTP-bound Bud1p was subsequently added to initiate the reaction. The GAP activity of Bud2p was measured as a function of the decrease in protein-bound radioactivity. Significantly, Bud2p-catalyzed Bud1 GTP hydrolysis was enhanced by Arf3<sup>Q71L</sup> in a dose-dependent manner (Figure 6A). Next, we performed kinetic measurement of GAP activity in the presence of Arf3<sup>Q71L</sup> or Arf3<sup>T31N</sup>. We found that Bud2p GAP activity was promoted by Arf3<sup>Q71L</sup>, but not by Arf3<sup>T31N</sup> (Figure 6B). These data indicate that Arf3<sup>Q71L</sup> directly promotes Bud2p GAP activity in vitro. We further examined the effect of the Arf3p-Bud2p interaction on Bud2p activity in vitro. Purified HA-Bud2p-dN40, which cannot bind Arf3p, exhibited impaired GAP activity compared with HA-Bud2p in vitro (Figure 6C). Moreover, unlike HA-Bud2, the GAP activity of HA-Bud2p-d40N was not enhanced by Arf3<sup>Q71L</sup> (Figure 6D). Taken together, our results demonstrate that active Arf3p directly interacts with Bud2p to promote Bud2p GAP activity.

**Arf3p facilitates the Bud2p-Bud1p association in vivo**

A GAP cascade has been identified in the small GTPase Rab/Ypt family in which Ypt32p recruits and stabilizes Gyp1p, the GAP for Ypt1p, and regulates the progression of membrane trafficking (Rivera-Molina and Novick, 2009). However, the subcellular localization of Bud2p is independent of Arf3p, thus differing from the Ypt GAP cascade. To further examine how activated Arf3p facilitates the GAP activity of Bud2p toward Bud1p, we examined the affinity of Bud2p for Bud1p in the presence and absence of Arf3p. We co-expressed HA-Bud2p and GFP-Bud1p (G12V or K16N) in wild-type or arf3Δ cells and immunoprecipitated HA-Bud2p to detect bound Bud1p. Our data indicated that Bud2p co-precipitated with Bud1p<sup>G12V</sup>, but not Bud1p<sup>K16N</sup>; however, the association between Bud2p and Bud1p<sup>G12V</sup> was diminished in arf3Δ mutant cells (Figure 6E). In addition, we observed that Bud2p-dN40 exhibits decreased Bud1p<sup>G12V</sup> binding in the presence of Arf3p (Figure 6F). Collectively, these data suggest that Arf3p facilitates the interaction between Bud2p and Bud1p, thereby promoting Bud2p GAP activity.

**Arf3p is activated by glucose depletion**

Previous reports have indicated that removal of glucose from the growth medium leads to constitutive invasion in haploid yeast (Cullen and Sprague, 2000). Thus, we examined the effect of glucose on the invasiveness of haploid yeast cells and found that the removal of glucose (only yeast extract and peptone were present in the
medium (YP)) caused constitutive invasion in wild-type yeast cells, in contrast to a glucose-containing medium (YPD), after incubation for 16 hours (Figure 7A). Both arf3Δ and bud2Δ, but not bud1Δ, cells were constitutively defective in YP agar invasion (Figure 7A). The defect in agar invasion exhibited by arf3Δ cells was restored via expression of ARF3 and ARF3L23V, but not of ARF3Q71L, ARF3T31N, and ARF3L23V (Figure S8A). Interestingly, after glucose removal, Arf3p-mRFP and GFP-Bud2p showed dramatic polarization at the plasma membrane of elongated buds, instead of being enriched at the bud neck in large-budded cells (Figure 7B). The polarized distribution of GFP-Bud2p and Arf3p-GFP in YP medium was not observably different in arf3Δ and bud2Δ cells (Figure S8B and S8C), indicating that the intracellular targeting of Arf3p and Bud2p in response to glucose depletion is independent of their interaction.

To examine whether Arf3p activity could be affected by glucose depletion, we measured the amount of active Arf3p using recombinant GST-Afi1N to pull down Arf3p-GTP (Tsai et al., 2008). GST-Afi1N specifically bound to active Arf3pQ71L but not inactive Arf3pT31N (Figure 8A). Using this assay, we observed an increase in Arf3p-GTP when yeast cells were grown in YP (Figure 8A). Importantly, we detected more Arf3p bound to Bud2p in response to glucose depletion (Figure 8B). Consistent with our data, we also observed that the level of Bud1p-GTP in wild-type cells was significantly decreased in response to glucose depletion (Figure 8C). These data suggest that glucose removal activates Arf3p and promotes Arf3p-Bud2p interaction and GTP hydrolysis of Bud1p to initiate yeast invasive signaling.

Glucose depletion induces Arf3p activation via a Yel1p-independent pathway

Yel1p is the only known GEF for Arf3p (Gillingham and Munro, 2007); therefore, it is surprising that the disruption of YEL1 did not affect yeast invasive growth (Figure 9A). Furthermore, even though active Arf3p in yel1Δ cells was significantly reduced (Gillingham and Munro, 2007; Tsai et al., 2008), the interaction between Arf3p and Bud2p remained at a similar level in the absence of Yel1p (Figure 9B). These data suggest that Arf3p activation during invasive growth is independent of Yel1p. Previous studies have shown that Arf3p proteins are recruited to the plasma membranes in their GTP-bound form (Huang et al., 2003) and loss of YEL1 results in the mislocalization of Arf3p to the cytosol (Gillingham and Munro, 2007). We reasoned to further examine the localization of Arf3p in yel1Δ under glucose depletion. Consistent with previous report, the majority of Arf3p is in the cytosol, indicating a decrease of Arf3p-GTP in yel1Δ; however, we still observed an increase in Arf3p polarization on the plasma membrane upon glucose deprivation in the absence of Yel1p (Figure 9C). We obtained additional evidence for this phenomenon by measuring Arf3p activity in yel1Δ cells and found that glucose depletion could still
stimulate Arf3p activation in yellow cells (Figure 9D). Together, these data indicate that the activation of Arf3p during invasive growth does not occur through the GEF activity of Yel1p. We also examined whether other Arf-GEFs, GEA1, GEA2, or SYT1, may be involved in Arf3p activation for invasive growth (Zakrzewska et al., 2003; Chen et al., 2010). Similar to the yellow mutant, yellowgea1Δ, yellowgea2Δ, and yellowsyt1Δ Σ1278b cells remained at a similar level of invasive growth under glucose depletion (Figure S9), suggesting that there is an unknown Arf3p GEF that activates Arf3p under glucose depletion-stimulated invasive growth.

DISCUSSION
In this study, we have identified a novel role for Arf3p in yeast invasive growth. We have shown that Arf3p directly interacts with Bud2p and regulates its GAP activity toward Bud1p by enhancing the association between Bud2p and Bud1p. Finally, we showed that under glucose depletion-stimulated invasive growth, Arf3p activation is stimulated through an unidentified GEF other than Yel1p. Thus, this novel nutrient-stimulated mechanism for the activation of Arf3p and Bud2p provides a functional connection between members of different subfamilies of small GTPases, Arf3p and Bud1p, for the regulation of invasive growth.

The Bud1p GTPase module composed of Bud1p, the GAP Bud2p, and the GEF Bud5p, is a critical regulator of bud site selection linking to polarity establishment during yeast vegetative growth, and the mechanism of its action has been well characterized (Park and Bi, 2007). Bud1p-GTP interacts with Cdc24p and guides it to the presumptive bud site, where Bud2p is localized. The Bud2p-activated hydrolysis of Bud1p-GTP then results in the dissociation of Cdc24p from Bud1p. Cdc24p free of Bud1p subsequently catalyzes the conversion of Cdc42p-GDP to Cdc42p-GTP to mediate actin cytoskeleton reorganization leading to the development of cellular polarity (Butty et al., 2002). Our results indicate that Arf3p modulates Bud1p activity via Bud2p; therefore, it is tempting to assume that Arf3p affects Cdc24p polarization via Bud1p activation. Cdc24p is the Cdc42p GEF and its polarized localization is critical for proper activation of Cdc42p and cytoskeleton reorganization (Park et al., 2002). We hypothesize that Arf3p interacts with Bud2p to promote Bud1p-GTP hydrolysis, which in turn, releases Cdc24p to activate Cdc42p and leads to invasive growth. Similarly, inhibition of the mammalian Arf3p homologue, Arf6, was reported to abolish the polarized recruitment of mammalian Cdc42 to the leading edge of cultured cells (Osmani et al., 2010).

Invasive growth of S. cerevisiae has been used as a model system for studying the filamentous growth of pathogenic yeast and nutrient-stimulated cellular responses, including signaling transduction, cell cycle control, polarized growth, and cell survival. The formation of hyphae in the human pathogen C. albicans is critical
during the infection process (Sudbery, 2011). Notably, *C. albicans bud1Δ/Δ and bud2Δ/Δ* strains are defective in the formation of filamentous hyphae (Hausauer et al., 2005). In addition, AgRsr1p/Bud1p was also reported to be a key regulator of hyphal growth guidance in the model fungus *Ashbya gossypii* (Bauer et al., 2004). We also observed that Bud1p and Bud2p activation is important for the invasive growth of *S. cerevisiae*. Moreover, our preliminary data showed that *C. albicans* failed to generate extended hyphae when two copies of the *CaARF3* gene were deleted. Therefore, we speculate that both Arf3p and Bud2p are required for the invasive growth of *S. cerevisiae* and hyphal guidance in *C. albicans*, suggesting that Arf3p may play an important role during the infection process of microbial pathogens. Interestingly, Arf6, the mammalian homologue of yeast Arf3p, is known to be involved in cell adhesion, migration and metastasis (Sabe, 2003). In addition, previous studies found that the mammalian Bud1p homologue, Rap1, regulates the dynamics of cell adhesion (Bos et al., 2001). Therefore, our studies of Arf3p suggest that Arf6 may regulate Rap1 activity associated with mammalian cell adhesion, migration and invasion.

Alteration of subcellular protein localizations under different physiological conditions is critical for proper protein function. Upon glucose depletion, Arf3p and Bud2p become highly co-localized at the plasma membrane of elongated daughter cells, in contrast to their partial co-localization observed in normal medium. Similarly, our results show that although both *arf3Δ* and *bud2Δ* cells exhibit an abnormal budding pattern during polarized growth, the randomness seen in *bud2Δ* cells is much more severe than that in *arf3Δ* cells. Taken together, these data indicate that Arf3p is not the major regulator of Bud2p activity during polarized growth; however, Arf3p is further activated upon glucose depletion and becomes the main regulator of Bud2p activity during yeast invasion. This speculation is further supported by our observation that Bud2p-dN40, like *arf3Δ*, only exhibits a mild budding pattern abnormality; however, Bud2p-dN40 is completely unable to support invasive growth. Therefore, multiple mechanisms must be employed by Bud2p to establish bud site selection linking to cell polarity under different physiological conditions. While deletion of *BUD1* in a *bud2Δ* strain completely restores invasive growth, deletion of *BUD1* in an *arf3Δ* strain only partially restores it. These results suggest that the regulation of Bud2p GAP activity toward Bud1p is just part of Arf3p’s role in invasive growth. Lsb5p was reported to interact with Arf3p and actin-binding protein Sla1p for regulating membrane trafficking events with the endocytic machinery (Costa et al., 2005). It was shown that deletion of *SLA1* in yeast leads to loss of nitrogen starvation-induced filamentous growth (Wu and Jiang, 2005). A previous study has also shown that altered levels of Arf3p affect plasma membrane PtdIns(4,5)P2 levels and correlate with changes in numbers of endocytic events (Smaczynska-de et al.,
The processes by which activation of Arf3p fosters invasive growth needs to be investigated further. Therefore, there must be multiple effectors employed by Arf3p to regulate invasive growth in yeast.

GTP binding is necessary for the activation of small GTPases and is tightly regulated by GEFs and GAPs. Although Yel1p is the only known Arf3p GEF (Gillingham and Munro, 2007; Smaczynska-de et al., 2008), we found that the deletion of YEL1 in yeast did not reduce invasive growth and Yel1p is not required for Arf3p activation in response to glucose depletion. These data indicate that there is an unknown Arf3p GEF that activates Arf3p during invasive growth. Consistent with this proposal, previous studies also suggested that Yel1p is not the only GEF for Arf3p because some Arf3p can still localize to the plasma membrane in yel1Δ cells (Gillingham and Munro, 2007; Tsai et al., 2008). Notably, our results indicated that the known Arf-GEFs Gea1p, Gea2p, and Syt1p are not responsible for Arf3p activation during invasive growth. Furthermore, we found that lack of Gts1p, the only known Arf3p GAP (Gillingham and Munro, 2007; Smaczynska-de et al., 2008), also did not reduce invasive growth of yeast (our unpublished result). Thus, the activation of small GTPases is regulated by unknown GEFs and GAPs during glucose-depletion-induced invasive growth.

In this study, we identify Arf3p as an upstream regulator of Bud2p and propose the existence of a novel small GTPase cascade, Arf3p-Bud2p-Bud1p, which orchestrates the polarity development during invasive growth. During vegetative growth, Arf3p is only responsible for partial activation of Bud2p leading to yeast axial budding. However, a portion of activated Arf3p, stimulated by glucose depletion, plays a crucial role in attenuating Bud1p activation through promoting the GAP activity of Bud2p involved in invasive growth (Figure 10). After activation via an unidentified GEF under glucose depletion conditions, Arf3p directly binds to Bud2p to promote Bud1p-GTP hydrolysis, which leads to Cdc24p disassociation and Cdc42p activation. Thus, we propose the existence of a hierarchical GTPase cascade involving Arf3p-Bud1p that (1) modulates a unique effector via controlling regulators of Bud1p and (2) coordinates signaling by glucose depletion during yeast invasive growth. Therefore, our results imply that Arf3p GTPases can regulate two different processes by recruiting process-specific effectors. Further studies are required to reveal the full spectrum of Arf3p activation mechanisms under glucose-limiting-induced invasive growth.
MATERIALS AND METHODS

Plasmids, strains, and yeast culture

Standard protocols were used to generate Σ1278b yeast strains via homologous recombination (Longtine et al., 1998). All of the yeast ARF3 plasmids used here encode Arf3p constructs driven by the native ARF3 promoter. The employed plasmids and yeast strains are listed in the Supplementary Tables (Vernet et al., 1987; Gietz and Sugino, 1988; Sikorski and Hieter, 1989). For glucose depletion, Σ1278b yeast cells were cultured in YPD (yeast extract/peptone/dextrose) overnight, washed three times with ddH$_2$O, re-cultured in YP medium (or spotted on YP agar plates), and incubated for 16 h.

In vitro binding assay

E. coli strain BL21 (DE3) (Novagen, La Jolla, CA) was transformed with plasmids including pET32a-ARF3 (Q71L or T31N), pGEX4T-1, and pGEX4T-1 containing various BUD2 fragments, as shown in Fig. 1D. After induction with 500 μM IPTG at 16°C for 8 h, GST fusion proteins or His-tagged proteins were purified from E. coli lysates using Glutathione Sepharose 4B (GE Healthcare Amersham, Piscataway, NJ) or nickel affinity resin (Qiagen, Valencia, CA), respectively, according to the manufacturers’ instructions. In pull-down assays, GST or GST-Bud2 fragments were immobilized on glutathione agarose beads and incubated with His-Arf3 (Q71L or T31N) in binding buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM DTT, and 10 mM MgCl$_2$) for 2 h at 4°C. The beads were washed three times with 1 mL of binding buffer. Bound proteins were then analyzed by Western blotting using anti-His monoclonal antibodies (BD Biosciences, La Jolla, CA).

Yeast two-hybrid assay

The yeast strain YEM1α was co-transformed with different combinations of bait (pEG202) and prey (pACT2) plasmids (Gyuris et al., 1993). The interactions between the bait and prey plasmids were observed using a β-galactosidase assay on His-Leu-plates containing 80 mg/L X-Gal.

Immunoprecipitation

Σ1278b yeast cells were disrupted with glass beads in extraction buffer (PBS containing 1 mM DTT, 5 mM MgCl$_2$, and protease inhibitors). The extracts were cleared via centrifugation at 4,000 rpm for 10 min. Agarose beads conjugated with a monoclonal anti-HA antibody (mouse monoclonal anti-HA agarose antibody) (Sigma, St. Louis, MO) were added to the cleared extracts and incubated at 4°C for 2 h. The beads were washed three times with wash buffer (extraction buffer containing 0.5% NP-40), and the bound complexes were eluted with sample buffer. The bound proteins were subjected to SDS-PAGE and analyzed via Western blotting.

Microscopy
All images of living cells containing GFP-tagged or mRFP-tagged proteins were obtained after growth in synthetic medium to mid-log phase. Fluorescence microscopy was performed with a Zeiss Axioskop microscope equipped with a Cool Snap FX camera. Cells were viewed at a magnification of 100X. For all microscopic examinations, the exposure times and image processing procedures were identical for each sample within an experiment. The light levels were scaled equivalently among all samples within an experiment when the images were exported from the imaging software and when they were subsequently processed with Photoshop. Only the light level min/max settings were adjusted for clarity.

**Calcofluor white staining**

To stain bud scars, Σ1278b yeast cells were grown overnight to the stationary phase. The cells were fixed with 3.7% formaldehyde for 1 h and stained with 1 μg/ml Calcofluor White (Sigma-Aldrich) as described previously (Huang et al., 2003). Stained cells were photographed and quantified via fluorescence microscopy. A minimum of 100 cells was quantified for each experiment.

**Subcellular fractionation**

Σ1278b yeast cells expressing ARF3, ARF3\(^{L23V}\), and ARF3\(^{I33V}\) were harvested and washed once in 10 mM NaN\(_3\)-containing buffer. The cells were incubated with lyticase in 1.2 M sorbitol-containing potassium phosphate buffer to form spheroplasts. The spheroplasts were disrupted by passing them through a 26G needle. The cell lysate was then cleared by centrifugation at 600 g for 5 min to remove debris and unbroken cells. The cleared lysate was next subjected to velocity centrifugation at 13,000 g for 10 min to generate a pellet fraction (P13) and the supernatant fraction (S13). Equal proportions of each fraction were subjected to SDS-PAGE and analyzed via Western blotting using antibodies against Arf3p, Pma1p (plasma membrane marker), and Pgk1p (cytosol marker).

**Invasive growth assays**

The haploid cell invasive assay was performed as described previously (Lo and Dranginis, 1998; Guldal and Broach, 2006). Equal concentrations of Σ1278b yeast cells were spotted onto YPD medium. After incubation for 3 days at 30°C, cells that penetrated the agar were examined after washing cells off the agar surface with water. The samples were photographed before and after washing with a stream of water. To perform a quantitative agar invasion assay, agar blocks containing yeast colonies (before and after washing) were excised and placed into microcentrifuge tubes. Elution buffer containing 0.5 M sodium acetate (pH 7.0) and 1 mM EDTA (pH 8.0) was added and the tubes were incubated at 55°C for 20 min until the agar blocks were completely dissolved. The yeast cell numbers were examined by measuring OD\(_{600}\) values to obtain total cell numbers before and after washing with water. The
percentage of invasive cells observed after washing with water was determined as OD_{600} unit after washing / OD_{600} unit before washing \times 100\%. The data are reported as the mean ± S.D. of three experiments.

**GAP activity assays**

The Bud2p GAP activity assay was performed essentially as described previously (Park *et al.*, 1993). Briefly, GAP activity was assayed by measuring [γ-32P]GTP after a single round of hydrolysis of Bud1-bound GTP by Bud2p. Recombinant GST-Bud1 was incubated in exchange buffer (PBS containing 1 mM EDTA, 0.5 mM MgCl_2, and 1 mM DTT) with [γ-32P]GTP for 25 min at 30°C. HA-Bud2p or HA-Bud2p-dN40 was purified from Σ1278b yeast lysates and co-incubated with GST-Bud1 with [γ-32P]GTP loading at different time points. The reactions were terminated by increasing the MgCl_2 content (5 mM final concentration), and the samples were filtered through a 0.45-μm nitrocellulose membrane. The remaining Bud1-bound radioactivity was detected with a scintillation counter. In the Arf3p-related GAP activity assay, recombinant Arf3, Arf3^{Q71L}, or Arf3^{T31N} was purified and pre-incubated with HA-Bud2p or HA-Bud2p-dN40 for 1 h at 4°C. Data are reported as the mean ± S.D. of three experiments.

**Active Bud1p pull-down assay**

The activation of Bud1p was assayed using a novel pull-down assay. Three Bud1p alleles (wild-type, G12V, and K16N) were tagged with HA and ectopically expressed in bud1Δ cells. Transformed yeast cells of Σ1278b were lysed with glass beads at 4°C in lysis buffer (PBS containing 1 mM DTT, 5 mM MgCl_2, 0.2% NP-40, and protease inhibitors (1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 μM benzamidine, and 1 mM PMSF)). The lysates were centrifuged at 4000 rpm for 5 min, and the clarified lysates were incubated with 5 μg of GST-Cdc42-GDP bound to glutathione-Sepharose beads (GE Healthcare). The beads were washed three times with lysis buffer, and the bound proteins were eluted with sample buffer. The samples were assayed for the presence of Bud1p by Western blotting.

To pull down Bud1p-GTP, yeast cells overexpressing HA-Bud1p under the control of the ADH1 promoter were lysed, and bound proteins were analyzed as described above.

**Active Arf3p pull-down assay**

Three Arf3p alleles (wild-type, Q71L, or T31N) were ectopically expressed in wild-type cells. Σ1278b yeast cells expressing ARF3 were grown in rich medium containing 2% (YPD) or 0% (YP) glucose were lysed with glass beads at 4°C in lysis buffer (PBS containing 1 mM DTT, 2 mM MgCl_2, and protease inhibitors (1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 μM benzamidine, and 1 mM PMSF)). The lysates were centrifuged at 3000 rpm for 5 min, and the clarified lysates
were incubated with 5 μg of GST-Afi1N bound to glutathione-Sepharose beads (GE Healthcare). The beads were washed three times with lysis buffer containing 0.2% Triton X-100, and the bound proteins were eluted with sample buffer. The samples were assayed for the presence of Arf3p by Western blotting.

Acknowledgements
We thank Drs. Hugh R. B. Pelham, Kai Simons, and Roger Y. Tsien for providing the expression plasmids. We also thank Drs. Joel Moss and Randy Haun for their critical review of this manuscript. This work was supported by grants from the National Science Council of Taiwan (NSC-100-2320-B-002-101-MY3) and Yung-Shin Biomedical Research Funds (YSF-86-019) awarded to F.-J. Lee.

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Figure 1: Arf3p is required for yeast invasive growth. (A) Both Arf3p and Bud2p are required for yeast invasive growth. Equal concentrations of wild-type, flo11Δ, bud2Δ, and arf3Δ cells with yeast Σ1278b were spotted onto YPD plates and incubated for 3 days at 30°C. The plates were photographed before (upper panel) and after (lower panel) rinsing with water. (B) Expression of wild-type Arf3p rescues yeast invasive growth in arf3Δ mutant cells. Wild-type or arf3Δ yeast Σ1278b cells transformed with an empty vector and arf3Δ mutant cells transformed with the indicated plasmids were spotted onto YPD plates to examine agar penetration after washing. Protein extracts from the indicated strains were resolved by SDS-PAGE and the indicated proteins were detected by immunoblot with anti-Arf3p and anti-Pgk1p (internal control) antibodies.
Figure 2: Arf3p specifically and directly interacts with Bud2p. (A) Bud2p interacts with Arf3p in a GTP-dependent manner. Bait plasmids (pEG202) containing various ARF3 constructs (wild-type, Q71L, or T31N) were co-transformed with the pJG4-5 plasmid containing BUD2 into yeast YEM1α, and their interactions were analyzed in a β-galactosidase assay. (B) Bud2p specifically interacts with active forms of Arf3p. BUD2/pJG4-5 was co-transformed with different active Arf family members, and their interaction was indicated by the development of a blue color. (C) Arf3p associates with Bud2p in vivo. Each of the indicated Arf3p variants was expressed in
yeast Σ1278b cells with HA-Bud2p. As described in Materials and Methods, HA-Bud2p was immunoprecipitated with an anti-HA antibody and the bound proteins were assayed for the presence of different forms of Arf3p. (D) Schematic representation of the various constructs containing the BUD2 truncation mutants. (E) Arf3p interacts with the N-terminus of Bud2p in vitro. Purified His-Arf3Q71L was incubated with recombinant GST, GST-Bud2-N, GST-Bud2C2, GST-Bud2GAP, or GST-Bud2CT for 1 h. The proteins were pulled down with glutathione-Sepharose 4B beads and visualized via immunoblotting with an anti-His antibody. (F) The N-terminal 120 amino acids of Bud2p interact with Arf3p in vitro. Purified His-Arf3Q71L was incubated with recombinant GST, GST-Bud2-N, GST-Bud2N1, or GSTT-Bud2N2 for 1 h. The proteins were pulled down with glutathione-Sepharose 4B beads and visualized via immunoblotting with an anti-His antibody. (G) Bud2p interacts with Arf3pQ71L via the N-terminal region. Interactions of N-terminal truncations of Bud2p depicted in panel D with the active form of Arf3p were examined using a yeast two-hybrid assay. (H) Bud2p-dN40 dissociates from Arf3p in vivo. Σ1278b yeast cells overexpressing Arf3p were co-transformed with the indicated expression vectors. HA-Bud2p or HA-Bud2p-dN40 was immunoprecipitated with an anti-HA antibody, and the bound proteins were assayed for the presence of Arf3p. (I) Isoleucine 33 of Arf3p is essential for the interaction of Arf3p with Bud2p. The indicated Arf proteins were expressed in Σ1278b yeast cells with HA-Bud2p. HA-Bud2p was immunoprecipitated with an anti-HA antibody, and the bound proteins were assayed by immunoblotting.
**Figure 3:** Arf3p interacts with Bud2p to regulate yeast invasive growth. (A) Expression of Bud2p-dN40 prevents yeast invasive growth in *bud2Δ* cells. Σ1278b yeast cells containing a *BUD2* deletion transformed with different forms of *BUD2* (full length, dN30, or dN40) were spotted onto YPD plates to examine agar penetration and the percentage of invasive cells was quantified as described in the Materials and Methods. (B) Expression of Arf3p*I33V* reduces agar invasion in the *arf3Δ* mutant strain. Σ1278b yeast cells containing an *ARF3* deletion transformed with various *ARF3* alleles (wild-type, L23V, and I33V) driven by the *ARF3* promoter were spotted onto YPD plates to examine agar penetration and the percentage of invasive cells was quantified. Protein extracts from the indicated strains were detected with anti-Arf3p and anti-Pgk1p (internal control). The data are reported as the mean ± S.D. of three experiments. ***, p<0.001**
**Figure 4:** Accumulation of Bud1p-GTP prevents yeast invasive growth. (A) Additional depletion of BUD1 suppresses the defect in agar invasion observed in arf3Δ or bud2Δ mutant cells. The indicated Σ1278b strains were spotted onto YPD plates and incubated for 3 days at 30°C. The plates were photographed before (upper panel) and after (lower panel) rinsing with water. The right panel shows the results of quantification of the percentage of invasive cells. (B) Expression of active Bud1p prevents agar invasion in arf3bud1Δ and bud2bud1Δ double mutant cells. arf3bud1Δ or bud2bud1Δ double mutant yeast Σ1278b cells transformed with a vector control and different alleles of BUD1 (wild-type, G12V, and K16N) were spotted onto YPD plates to examine agar penetration, and the percentage of invasive cells was quantified.
The right panel shows the quantification of the percentage of invasive cells. Data are reported as the mean ± S.D. of three experiments. ***, $p<0.001$
Figure 5: Bud2p interacts with Arf3p to enhance GAP activity leading to Bud1p-GTP hydrolysis in vivo. (A) Elevated Bud1p-GTP levels were detected in arf3Δ and bud2Δ mutant cells. Yeast lysates from the indicated Σ1278b strains expressing HA-Bud1p were prepared and analyzed to detect bound proteins using recombinant GST-Cdc42 purified from E. coli, together with pre-binding with GDP as described in Materials and Methods. The right panel shows the results of quantitative analysis of active Bud1p. (B) Expression of Bud2p-dN40 in bud2Δ cells decreases the content of Bud1p-GTP in vivo. Yeast lysates from the indicated Σ1278b strains expressing HA-Bud1p were prepared and analyzed to detect active Bud1p, as described in Materials and Methods. The right panels show the results of quantitative analysis of
active Bud1p. (C) Expression of Arf3p<sup>133V</sup> in arf3Δ cells increases cellular Bud1p-GTP levels in vivo. Yeast lysates from the indicated Σ1278b strains expressing HA-Bud1p were prepared and analyzed to detect active Bud1p. The right panel shows the results of quantitative analysis of active Bud1p. Data are reported as the mean ± S.D. of three experiments. *, *p<0.05; **, *p<0.01; ***, *p<0.001
Figure 6: Interaction of Arf3p and Bud2p promotes Bud2p GAP activity leading to Bud1p GTP hydrolysis. (A) Arf3p<sup>Q71L</sup> enhances Bud2p GAP activity in a dose-dependent manner. Purified recombinant GST-Bud1 (1 µM) loaded with [γ-<sup>32</sup>P]GTP was incubated with purified HA-Bud2p (5 nM) alone or in the presence of recombinant His-Arf3<sup>Q71L</sup> at 30°C for 10 min. Samples were collected, and protein-bound radioactivity was detected using a nitrocellulose filter-binding assay. (B) Enhancement of Bud2p GAP activity by Arf3p<sup>Q71L</sup>. [γ-<sup>32</sup>P]GTP bound to recombinant GST-Bud1 was incubated with a buffer control or HA-Bud2p (5 nM) with or without 1 µM His-Arf3<sup>Q71L</sup> or His-Arf3<sup>T31N</sup> for the indicated time periods.
After incubation, the hydrolysis of $[^\gamma^{-32}P]GTP$ bound to GST-Bud1 was assayed by measuring radioactivity. (C) Compared to full-length Bud2p, Bud2p-dN40 shows decreased GAP activity. The GAP activity of purified Bud2p or Bud2p-dN40 (50 nM) was assayed using purified GST-Bud1 with bound $[^\gamma^{-32}P]GTP$ as a substrate, as described in the Materials and Methods. The results are representative of three experiments. (D) The GAP activity of Bud2p-dN40 is not enhanced in the presence of Arf3p$^{Q71L}$. Purified recombinant GST-Bud1 (1 μM) loaded with $[^\gamma^{-32}P]GTP$ was incubated with 5 nM HA-Bud2p or HA-Bud2p-dN40 with or without 1 μM Arf3p$^{Q71L}$ or Arf3p$^{T31N}$ for 10 min. Samples were collected to measure protein-bound radioactivity. (E) Arf3p potentiates the association between Bud2p and Bud1p. Different forms of GFP-Bud1p (G12V or K16N) were co-expressed with HA-Bud2p in wild-type or arf3Δ yeast Σ1278b cells. As described in Materials and Methods, HA-Bud2p was immunoprecipitated with an anti-HA antibody, and the bound proteins were assayed for the presence of different forms of Bud1p. (F) Bud2p-dN40 associates with Bud1p more weakly than Bud2p does. Different forms of GFP-Bud1p (G12V or K16N) were co-expressed with full-length (FL) HA-Bud2p or HA-Bud2p-dN40 in yeast Σ1278b cells. HA-Bud2p was immunoprecipitated with an anti-HA antibody and the bound proteins were detected with antibodies against GFP. Data are reported as the mean ± S.D. of the percentage of $[^\gamma^{-32}P]GTP$ hydrolysis (n=3). **, $p<0.01$
Figure 7: Arf3p is activated by glucose depletion. (A) Arf3p and Bud2p are required for yeast invasion during glucose depletion. Equal concentrations of wild-type, arf3Δ, bud2Δ, and bud1Δ yeast Σ1278b cells were spotted onto YPD (2% glucose) or YP (0% glucose) plates and incubated for 16 h at 30°C. The plates were photographed before (upper panel) and after (lower panel) rinsing with water. (B) GFP-Bud2p and Arf3p-mRFP exhibit a polarized distribution in the elongated bud cell under glucose depletion. Wild-type yeast Σ1278b cells were co-transformed with GFP-BUD2/pVT101U and ARF3-mRFP/pHS12. The yeast were grown in rich media with (YPD) or without (YP) glucose for 16 h and observed via fluorescence microscopy. Scale bars: 5 μm.
Figure 8: Glucose depletion induces Arf3p activation to promote Bud1p-GTP hydrolysis. (A) Active forms of Arf3p are elevated under glucose depletion. Σ1278b yeast cells growing in rich medium containing 2% glucose were transferred to rich medium containing no glucose (YD) or rich medium containing 2% glucose (YPD) for 16 h. After the indicated time periods, cellular Arf3p-GTP levels were detected with recombinant GST-Afi1N purified from *E. coli*, as described in the Materials and Methods. The lower panel shows the results of quantitative analysis of active Arf3p. Data are reported as the mean ± S.D. of three experiments. **, p<0.01 (B) Glucose depletion enhances the Arf3p-Bud2p interaction. As described in Materials and Methods, Σ1278b yeast cells cultured in rich medium with (YPD) or without glucose (YP) were harvested; HA-Bud2p was immunoprecipitated using an anti-HA antibody; and bound Arf3p proteins were assayed by immunoblotting. Data are reported as the
mean ± S.D. of three experiments. ***, $p<0.001$. (C) Glucose depletion enhances Bud1p-GTP hydrolysis. As described in the Materials and Methods, wild-type and $arf3\Delta\Sigma1278b$ yeast cells expressing HA-Bud1p cultured in rich medium with (YPD) or without glucose (YP) were harvested and analyzed to detect active Bud1p. Data are reported as the mean ± S.D. of three experiments. **, $p<0.01$. 
Figure 9: Glucose depletion induces Arf3p activation via a Yel1p-independent pathway. (A) Yel1p is not required for agar invasion. Equal concentrations of wild-type, arf3Δ, and yel1Δ Σ1278b yeast cells were spotted onto YPD plates and incubated for 3 days at 30°C. The plates were photographed before (upper panel) and after (lower panel) rinsing with water. (B) Arf3p associates with Bud2p in yel1Δ cells. Arf3p was expressed in arf3Δ and yel1Δ Σ1278b yeast cells with HA-Bud2p. As described in the Experimental Procedures, HA-Bud2p was immunoprecipitated with an anti-HA antibody and the bound proteins were assayed for the presence of different forms of Arf3p. (C) Disruption of YEL1 in yeast cells shows signals that polarize Arf3p-GFP to the bud tip in the absence of glucose. ARF3-GFP/YCplac111 was transformed into wild-type and yel1Δ Σ1278b yeast cells. Yeast cells treated with or without glucose for 16 h were observed by fluorescence microscopy. Scale bars: 5 μm.
(D) Active forms of Arf3p are elevated in yel1Δ cells upon glucose depletion. Wild-type and yel1Δ Σ1278b yeast cells expressing ARF3 and grown in rich medium containing 2% glucose were switched to rich medium containing no carbon source or rich medium containing 2% glucose for 16 h. After the indicated time periods, cellular Arf3p-GTP levels were detected with recombinant GST-Afl1N purified from E. coli as described in the Experimental Procedures. The right panel shows a quantitative analysis of active Arf3p. Data are reported as the mean ± S.D. of three experiments. ***, $p<0.001$
**Figure 10:** A model for the roles of Arf3p in budding polarity and yeast invasion. During polarized growth, Arf3p is activated through the recruitment of Yel1p or another redundant GEF to the bud neck (left panel). When Arfp3-GTP interacts with the N-terminus of Bud2p, Bud2p may strongly associate with Bud1p and efficiently promote GTP hydrolysis activity of Bud1p. Once Bud1p is converted to the inactivated GDP-bound form, it may activate and couple with a downstream Cdc42p GTPase module to maintain budding polarity. Under glucose depletion, an unidentified GEF, other than Yel1p, will be triggered to activate Arf3p (right panel). After its activation, Arf3p directly binds to Bud2p and promotes Bud1p-GTP hydrolysis. Thus, the regeneration of Bud1p-GDP by Bud2p GAP activity via the cooperation of Arf3p may facilitate yeast invasive growth. Arf3p may also alter other unidentified effectors that regulate yeast invasive growth.