Afi1p functions as an Arf3p polarization-specific docking factor for development of polarity

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ADP-ribosylation factors (Arfs) are highly conserved small GTPases and are critical components of vesicle trafficking. Yeast Arf3p, in spite of its similarity to mammalian Arf6, is not required for endocytosis, but is involved in polarity development. In this study, we identified an Arf3p interacting protein 1 (Afi1p), which, through its N-terminal conserved region, specifically interacts with GTP-bound Arf3p. Afi1p is distributed asymmetrically at the plasma membrane and is required for polarized distribution of Arf3p, but not of an Arf3p guanine nucleotide-exchange factor, Yel1p. However, Afi1p is not required for targeting of Arf3p or Yel1p to the plasma membrane. Like arf3 mutant yeast, afi1 mutant yeast exhibited an abnormal budding pattern and partially delayed actin patch polarization. An Afi1p, 38KLGP4A-Afi1p, mutated at the Arf3p-binding region, loses its ability to interact with Arf3p and maintain the polarized distribution of Arf3p. Although 38KLGP4A-Afi1p still possessed a proper polarized distribution, it lost its ability to rescue actin patch polarization in afi1 mutant cells. Our findings demonstrate that Afi1p functions as an Arf3p polarization-specific adapter and participates in development of polarity.

Arfs are guanine nucleotide-binding proteins, which belong to the Ras superfamily. They are critical for vesicular trafficking and organization of the actin cytoskeleton (1). Arfs are activated by guanine nucleotide-exchange factors (GEFs), which facilitate replacement of GDP with GTP, and are inactivated by GTPase-activating proteins (GAPs), which stimulate GDP hydrolysis to GDP (2). Arfs are active in the GTP-bound state and competent to interact with downstream effectors such as coatomer, GAP, phospholipase D (PLD), and phosphatidylinositol-4/5 kinase to initiate vesicle formation or actin cytoskeleton reorganization (3-5). The best known Arf proteins are Arf1 and Arf6; the former is required for intra-Golgi and Golgi-ER trafficking (6), and the latter for membrane trafficking and actin cytoskeleton regulation at the plasma membrane (3,7). Arf6 is involved in many cellular events including constitutive and stimulated endocytosis, membrane ruffling, membrane protrusion, secretory granule exocytosis, and cytokinesis (8).

Although Arf3p is the yeast homologue of mammalian Arf6, the functions of these two proteins are not the same (9). In our previous study, we found that Arf3p was not required for fluid phase Lucifer yellow or FM4-64 endocytosis, or for mating factor receptor internalization. Furthermore, Arf3p was not directly required for reorganization of the yeast actin cytoskeleton (10). In contrast, the cellular location of Arf3p changes during the cell cycle. In G1 phase, Arf3p is present at the plasma membrane and in the cytosol. In S phase, however, it is concentrated at the plasma membrane of the emerging bud. Because of its polarized localization and its critical function in the normal budding pattern of yeast, Arf3p was hypothesized to be a regulator of vesicle trafficking, which is important for polarized growth.

Recently, many studies provide evidence to support the involvement of Arf3p in cell polarity and the organization of the actin cytoskeleton (11). It was found that ARF3 could be a genetic suppressor for ppy1, las17 and vrp1 mutant cells, and Bni1p over-expression could rescue the budding pattern abnormality of an arf3 mutant. In addition, Arf3p shows a genetic interaction with Bud6p and is required for the proper location of GGA-like protein Lsb5p (12). However, the molecular mechanism of how Arf3p participates in polarity establishment and actin organization is still unclear.

To identify regulators and effectors of Arf3p, we used Arf3Q71L-d17N, a constitutively active and N-terminally truncated Arf3p that removes the amphipathic helix, as bait to identify interacting proteins by yeast two-hybrid screening. A hypothetical open reading frame, YOR129c, was identified and named Arf3-interacting protein 1 (AFI1). The predicted amino acid sequence does not contain any recognized structural or functional motifs. We show here that one function of Afi1p is to localize Arf3p at the polarized plasma membrane. We identified a specific conserved sequence at the N-terminus of Afi1p, which is important for its
interaction with Arf3p. An Afi1p mutated at the Arf3p-binding region did not affect its proper polarized distribution, but lost its ability to maintain the polarization of Arf3p and to rescue the defect of actin patch polarization in \textit{af1l} mutant cells. We infer that Afi1p acts a polarization-specific adapter for Arf3p and participates in development of polarity.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Plasmids** - Table 1 lists the yeast strains used in this study. Yeast culture media were prepared as described by Sherman et al. (24). YPD contained 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose. SD contained 0.17% Difco yeast nitrogen base (without amino acids and ammonium sulfate), 0.5% ammonium sulfate, and 2% glucose. Essential nutrients for auxotrophic strains were supplied at the specified concentrations (13). Yeast strains were transformed by the lithium acetate method (13). Plasmids were constructed according to standard protocols (14). Gene disruption was carried out as previously described (15). The primers used for cloning are listed in Table 2, and the plasmids used in this study are listed in Table 3. The mRFP template was provided by Dr. Roger Y. Tsien (16).

**Antibodies** - Monoclonal anti-HA antibody was purchased from Berkeley Antibody Company and polyclonal anti-GFP antibody was purchased from Berkeley Antibody Company. Anti-actin, anti-Pma1p, anti-Emp47p, anti-Pgd1p, and anti-Arf3p antibodies were generated in our laboratory.

**Yeast Two-Hybrid Screening** - Yeast twohybrid screening was performed using the “Interaction Trap” system (17), in which the bait (ARF3Q71LdN17) was fused with the DNA-binding protein LexA on plasmid pEG202. The yeast cDNA library was made using the vector pJG4-5. The library uses the inducible yeast \textit{GAL1} promoter to express proteins as fusions to an acidic domain. Yeast strain YEM1α containing the plasmid pEG202-ARF3Q71LdN17 was transformed with pJG4-5 library plasmids. In the screen, ~500 colonies were recovered from SC-His-Trp-Leu plates and 116 colonies turned blue in the \textit{β}-galactosidase assay. Plasmids were recovered from yeast and transformed into \textit{E. coli} KC8 (pyrF leuB600 trpC hisB463) for selection of library plasmids. Library plasmids containing the insert were re-transformed into DH5α, re-isolated, and sequenced.

**N-terminal GFP Tagging of the Chromosomal \textit{AFI1} Gene** - We attached heterologous DNA, the \textit{GAL1} promoter, and a GFP tag to the Afi1p N-terminus using a PCR-based strategy (18). The PCR products were produced with the primers, and 129CF4 and 129CR5GFP.

**Protein Interaction Analysis** - To analyze the \textit{in vivo} interaction of Arf3p and Afi1p, cells (~50 A600 units) coexpressing Afi1p-3HA and various Arf3p-GFP mutants were harvested and lysed with glass beads in phosphate-buffered saline (PBS) containing 2 mM MgCl2, 5 mM DTT, 0.2% Triton X-100 and a protease inhibitor cocktail, and then centrifuged at 700 x g for 10 min to obtain the cleared lysate (1 ml). Afi1p-3HA was immunoprecipitated with monoclonal anti-HA antibody and washed with binding buffer three times. Denatured samples were analyzed by Western blot to detect the co-precipitation of Arf3p. For detecting the direct interaction between Arf3p and Afi1p, 1 μg purified GST or GST-Afi1N1 was incubated with 1 μg His-Arf3Q71L, His-Arl1Q72L or His-Arl3Q78L in 1 ml of binding buffer (50 mM Tris pH7.5, 150 mM NaCl, 1% TX-100, 1 mM DTT, 10 mM MgCl2). After 1 hr incubation at 4°C, the mixture was washed three times with binding buffer, and the bound proteins were detected by Western blotting.

**Indirect Immunofluorescence Staining** - Cells were grown in YPAD or SD medium and prepared for indirect immunofluorescence staining as described (10,19). Fluorescence microscopy was performed with a Zeiss axioskop (Zeiss, Germany) and images were acquired using a CCD camera (Coolsnap fx). Cells were viewed at a magnification of 1000X.

**Calcofluor White and Phalloidin Staining** - For Calcofluor white staining, cells were fixed with 3.7% formaldehyde and stained with 0.1-1 mg/ml Calcofluor white (Sigma) as described (20). For phalloidin staining, cells were fixed with 3.7% formaldehyde, washed with PBS, and stained with Alexa 594-conjugated phalloidin for 1.5 hour as described (13). Cells were re-suspended in mounting solution and photographed with a Zeiss axioskop and CCD camera at a magnification of 1000X.

**Subcellular Fractionation** - Cells cultures (50 ml) grown in YPAD medium were harvested and subjected subcellular fractionation as described (21). For velocity centrifugation, the lysate was subjected to centrifugation (13,000 x g) for 10 minutes at 4°C to generate the pellet (P13) and supernatant (S13) fractions. The S13 fraction was separated further into membrane (P100) and cytosol (S100) fractions by centrifugation (100,000 x g) for 1 h. Equal proportion of each fraction was subjected to SDS-PAGE.

**RESULT**

Arf3p Interacts with the N-terminus of Afi1p -
To identify proteins that can interact with Arf3p, we used a constitutively-active Arf3p with N-terminal deletion (ARF3Q71LdN17) as bait in a yeast two-hybrid screen and obtained 116 candidates among 1.36 x 10^5 transformants. Of these, 63 were N-terminal fragments of a novel YOR129c gene product with unknown function, which was named Arf3p-interacting protein 1 (AFI1). In yeast two-hybrid experiments, Arf3Q71L, but not Arf3T31N, Arf1Q71L, Arl1Q72L or Arl3Q76L, interacted with the N-terminus of Afi1N (AFI1N), indicating that the interaction of Arf3p and Afi1p is GTP-dependent and specific (Fig. 1A, B). Although Arf1Q71L could not interact with Afi1N, the N-terminally truncated Arf1p; Arf1Q71Ld17N could weakly interact with Afi1N in the yeast two-hybrid assay (Fig. 1B). However, this interaction may be due to the high similarity between Arf1p and Arf3p and does not occur in yeast.

AFI1 encodes a protein of 893 amino acids, with no homology to any known animal or plant sequence or regions of similarity to previously characterized protein domains. However, predicted homologues of AFI1 were found in the fungi Ashbya gossypii (ACR212Cp; 36% identity, score = 838) and Kluyveromyces lactis, (kYOR129C; 35% identity, score = 826) (Fig. S1). In addition, weak homology to a predicted Aspergillus nidulans MesA protein (21% identity, score = 114) was observed. The function of the putative A. gossypii and K. lactis homologues has not been determined. In A. nidulans, MesA promotes the localized assembly of actin cables at polarization sites by facilitating the stable recruitment of a formin protein, SepA (22).

For further mapping the interaction region of Afi1p with Arf3p, different regions of the AFI1 open reading frame (Fig. 1C) were constructed and tested for their interaction with Arf3Q71L in yeast two-hybrid assays. Figure 1D shows that only Afi1N1, the longest N-terminal fragment of Afi1N, could interact with Arf3Q71L, suggesting that the essential interacting sequence may be distributed within this region or that the whole N-terminus is required for proper conformation. This result indicates that no single fragment is sufficient to interact with Arf3p.

To determine the in vivo interaction of Arf3p and Afi1p, we co-expressed C-terminal 3HA-tagged Afi1p and GFP-tagged Arf3p (WT, Q71L, or T31N) in arf3 mutant yeast. Both of these constructs were expressed under the control of their own promoters. Afi1p-3HA was immunoprecipitated with anti-HA antibody and the presence of different bound Arf3p-GFP proteins was determined by immunoblotting (Fig. 2A). Arf3Q71L and to a lesser extend Arf3WT, but not Arf3T31N, bound Afi1p-3HA, suggesting that Arf3p interacts with Afi1p in a GTP-dependent manner. We also expressed and purified recombinant GST-fused Afi1N and His-tagged small G proteins to evaluate their interaction in vitro. As Figure 2B shows, only His-Arf3Q71L, but not His-Arl1Q72L or His-Arl3Q76L could interact with GST-Afi1N. These results support the notion of a specific and direct interaction between Arf3p and Afi1p.

The Expression Level of Afi1p Is Regulated - Although we tried to generate an anti-Afi1p antibody in our laboratory, the titer of the anti-serum was too low to detect endogenous Afi1p. Therefore, for detecting the expression of endogenous Afi1p, three HA-tag sequences were introduced before the stop codon of AFI1 by PCR-based chromosomal integration (YPH250AFI1-3HA). Western blot analysis of cell lysates using monoclonal anti-HA antibody (Fig. S2) showed that cells of early- (lane 1, A600~0.2) and mid- (lane 2, A600~0.6) had similar Afi1p content, whereas late-log phases (lane 3, A600~1.3) and early stationary phase cells (lanes 4, A600~2.0) show a reduced Afi1p level. No Afi1p was detected in cells grown in stationary phase (lanes 5 and 6, A600~4.5). This result indicates that expression of Afi1p, unlike consistent expression of Arf3p, is higher in rapidly proliferating cells than in those in the stationary phase. This expression pattern is similar to the result observed by Wysocka et al. (2003).

Afi1p Is Required for Maintaining a Normal Budding Pattern - Yeast cells select their budding sites according to haploid- or diploid-specific programs. Typically, haploid cells bud in an axial pattern and diploid cells bud in a bipolar pattern (23). Our previous study showed that an ARF3 deletion causes an abnormal budding pattern (10). To examine whether Afi1p also plays a role in yeast polarized growth, we stained haploid cells that had an ARF3 or AFI1 single deletion or double deletion with the chitin dye, Calcofluor white. AFI1 disrupted yeast were generated by homologous recombination and confirmed by PCR (data not shown). AFI1 is a nonessential gene and cells with the double deletion of AFI1 and ARF3 are also viable and exhibited no growth defect at different temperatures, indicating that aif1 is not a synthetic lethal mutation with arf3. Figure 3A and 3B show that ~14% of aif1 cells exhibited a random or bipolar budding pattern, as did arf3 cells, both of which show a significant difference compared to wild-type cells (~3%). Double deletion (aif1arf3) cells also showed a similar level of abnormal budding (~13.5%). Compared to single deletion cells, there was no synergic effect in the aif1arf3 mutant, which suggests that Afi1p and Arf3p might function in the same pathway to maintain the proper budding pattern in yeast.

Afi1p Is Involved in Actin Patch Polarization -
Our previous study showed that an arf3 deletion causes incomplete actin patch polarization in the emerging bud at 34°C (10). To investigate whether Afi1p is also involved in development of actin patch polarization, wild-type, arf3 mutant, afi1 mutant, and afi1arf3 double-mutant cells were grown at 30°C or 34°C, and Alexa-594-conjugated phalloidin was used to visualize F-actin. Both actin cable and actin patch polarization were normal in all strains at 30°C (Fig. 4). However, at 34°C, afi1, arf3 and the double mutants exhibited delay in actin-patch polarization and appeared to have less efficient actin patch enrichment in yeast with small buds. The proportion of abnormal cells in these three mutant strains is not significantly different (Fig. 4B). This temperature-dependent actin polarization defect implies that Afi1p and Arf3p act in the same pathway, which is indirectly involved in early-stage actin patch organization.

**Polarized Distribution of Afi1p on the Plasma Membrane** - To examine the subcellular localization of endogenous Afi1p, we tried to visualize Afi1p-3HA with anti-HA antibody, but no signal was observed. Next, we used a strain carrying a chromosomal C-terminally GFP-tagged Afi1p; however, we still could not observe any GFP signal with microscopy (data not shown).

Because the endogenous Afi1p level is too low to be detected by indirect immunofluorescence staining, a GFP-Afi1p overexpressing strain (YPH250GFPAFI1) was generated by chromosomal integration of the GAL1 promoter and the GFP tag in place of the original AFI1 promoter. Figure S3A shows that overexpressed GFP-Afi1p was localized mainly at the plasma membrane and in the nucleus. In some cells, its enrichment at the nuclear envelope was observed, suggesting that it may be involved in some events in the nucleus or on the nuclear membrane, in addition to the plasma membrane. Plasma membrane and nuclear localizations of GFP-Afi1p are lost upon formaldehyde fixation (Fig. S3B). The yeast (YPH250AFI1-3HA) cell lysate was subjected to differential centrifugation studies. Afi1p-3HA, Arf3p, Pma1p (plasma membrane marker), Emp47p (Golgi marker), and Pgg1p (cytoplasmic marker) in each fraction were identified by Western blot analysis (Fig. S3C). Although little of the endogenous Afi1p was, like Arf3p, detected in the heavy membrane fraction (P13) more than 80% of the Afi1p was found in the cytoplasmic fraction (S100), indicating that the membrane association of Afi1p is different from that of Arf3p. These results indicated that indirect immunofluorescence staining and velocity sedimentation are unsuitable for studying the subcellular localization of Afi1p.

Afi1p-GFP, expressed from the endogenous ARF3 promoter, but not over-expressed from the ADH promoter, showed polarized localization on the plasma membrane (10), suggesting that over-expression would mask the polarization property. To examine whether Afi1p is also asymmetrically distributed on the plasma membrane, many different strategies were utilized, including different fixation methods, different induction time of GFP-Afi1p under the control of the GAL1 promoter, and culturing the yeast containing over-expressed Afi1p-GFP in YPD medium to reduce its plasmid copy number. However, all these strategies failed. Finally, by expressing GFP-Afi1p under the control of its own promoter on a 2μ plasmid YEPplac195, we observed clear polarized distribution of GFP-Afi1p in cells with a lower expression level (Fig. 5A, arrowheads). Although only in the cells having moderate expression of GFP-Afi1p could the enrichment at the plasma membrane of daughter cells and bud necks be observed, this result supports the hypothesis that endogenous Afi1p has a polarized distribution, and this behavior might be required for the polarization of Arf3p. The difference in the expression level of Afi1p under the control of the ADH or its own promoter is dramatic (Fig. 5B).

**Involvement of Afi1p in the Arf3p Polarized Localization** - We previously reported that constitutively active Arf3p (Arf3Q71L) was localized mainly at the plasma membrane (10). Localization of GFP-Afi1p to the plasma membrane supports the idea that Afi1p interacts with active Arf3p at the plasma membrane. To explore the functional significance of the Arf3p-Afi1p interaction, we examined whether Arf3p is involved in the localization of Afi1p or vice versa. The localization of GFP-Afi1p was not affected in the arf3 mutant (Fig. 6A), suggesting that Arf3p is not a determinant of Afi1p localization. We next examined whether Afi1p is required for the plasma membrane targeting of Arf3p. In wild-type cells, Arf3p-GFP, expressed by a low-copy centromeric plasmid under the control of its own promoter was localized at the plasma membrane and concentrated at the emerging buds. In afi1 cells, however, Arf3p-GFP dramatically lost its polarization distribution (Fig. 6B). In addition, constitutively active Arf3p (ARF3Q71L) lost its asymmetric distribution in afi1 mutant cells (Fig. S4). These results suggest that Afi1p may be a determinant for Arf3p targeting to polarized membrane.

**Afi1p Does Not Affect Polarized Localization of Yel1p** - Recently, Yel1p was reported to be a guanine nucleotide exchange factor for Arf3p and was co-localized with Arf3-RFP at the plasma membrane, restricted to the bud neck and the emerging bud tip (24). We wondered whether the presence of Afi1p is also required for the polarization of Yel1p. We expressed
GFP-Yel1p, under the control of the ADH promoter, in wild-type and afi1 deletion strains. GFP-Yel1p was localized to the plasma membrane at the bud and the bud neck in the afi1 mutant (Fig. 7A), indicating that Afi1p does not affect polarized localization of Yel1p. We also determined the localization of GFP-tagged Afi1p in yel1 mutant cells. The polarized distribution of Afi1p was not affected in the absence of Yel1p (Fig. 7B). These results suggest that Afi1p is not required for polarized location of Yel1p and vice versa.

**Yel1p Is Required for Proper Localization of Arf3p and Involved in Actin Patch Polarization**

Yel1p has been shown to be required for the polarized localization of Arf3p to the plasma membrane (24). To confirm this result, we expressed Arf3-GFP in wild-type, yel1 or afi1 mutant strains. Arf3-GFP was localized at the bud neck and bud tip in the wild-type strain and yel1 mutant cells, but lost its polarizing location in afi1 mutant cells. Consistent with previous reports, Arf3-GFP showed cytoplasmic and weak plasma membrane localization in yel1 mutant cells (Fig. 8A). Quantification of the Arf3p fluorescence signals in yel1 and afi1 mutant cells showed that the deletion of YEL1, but not the deletion of AFI1, reduced the plasma membrane association of Arf3p-GFP (Fig. 8B).

We further examined whether the subcellular distribution of endogenous Arf3p was affected in the yel1 and afi1 mutants. Spheroplast homogenates from each strain were fractionated into two fractions, plasma-membrane-rich (P13) and microsome-rich and soluble fraction (S13), by sedimentation centrifugation. The level of Arf3p fluorescence signals in yel1 and afi1 mutant cells showed that the deletion of YEL1, but not the deletion of AFI1, reduced the plasma membrane association of Arf3p-GFP (Fig. 8C). These results suggest that Afi1p and Yel1p may function in different steps in regulating the localization of Arf3p to the plasma membrane.

To examine whether Yel1p, like Arf3p, is also involved in actin patch polarization, the wild-type, arf3 or yel1 cells were grown at 34°C, fixed, and stained with polyclonal anti-actin antibody to detect actin polarization. The arf3 and yel1 cells, but not wild-type cells, showed delayed or reduced actin patch polarization in small buds (Fig. 8D). The proportion of less actin patch in bud sites was not significantly different between arf3 and yel1 mutant cells (Fig. 8E). These results suggest Yel1p and Arf3p may work in the same pathway, which is indirectly involved in actin cytoskeleton organization.

**A Region at the N-Terminus of Afi1p Is Important for Its Localization and the Conserved 38KLGP Amino Acids of Afi1p Are Critical for Its Interaction with Arf3p**

Although Afi1p has no homologue with known function, the homologues from other fungi indicate that the N-terminus is highly conserved among them and might be crucial for its function (Fig. 9A). Therefore, we generated different mutant forms of Afi1p; 1) a truncation of N-terminal 24 amino acids that are not conserved in Afi1p fungal homologues, 2) a deletion of 41 amino acids containing a half of the first conserved box, and 3) an alanine-substitution mutant in the first conserved box, 38KLGP4A (Fig. 9A, box), to investigate the importance of Afi1p N-terminus in determining its localization and interaction with Arf3p. By using yeast two-hybrid analyses, we showed that the non-conserved 1-24 amino acids of Afi1p are not required for the interaction between Afi1p and Arf3p, and proper localization of Afi1p (Fig. 9B, upper panel, 9D). However, the deletion of the N-terminal 41 amino acids of Afi1p could not interact with Arf3p and lost its ability to associate with the plasma membrane. The 38KLGP4A mutant of Afi1p also could not interact with Arf3p, but did not lose its polarized association with the plasma membrane. All these different forms of Afi1p have similar expression levels (Fig. 9B lower panel, 9C). These results indicate that the N-terminal 17 amino acids (25-41) of Afi1p are important for its localization and the conserved 38KLGP amino acids of Afi1p are critical for interaction with Arf3p.

**Interaction with Afi1p Is Important for Polarized Localization and Function of Arf3p**

To determine whether the specific interaction between Afi1p and Arf3p has functional importance, we first integrated plasmid YIplac211, containing wild-type, DN24Af1, or 38KLGP4A mutant forms of Afi1 with its own promoter, into afi1 (YPH250daf1) cells. These strains were then co-expressed with Arf3p-GFP/YCPlac111. Polarized localization of Arf3p-GFP in living cells was observed in afi1 cells expressing wild-type Afi1p and DN24Af1p (Fig. 10A). However, Arf3p-GFP lost its polarized localization in afi1 cells expressing 38KLGP4A-Afi1, indicating that interaction with Afi1p is important for polarized localization of Arf3p.

We next examined whether 38KLGP4A-Afi1 could rescue the defect of actin patch polarization in afi1-deletion cells. The afi1 (YPH250daf1) cells with integrated wild-type Afi1, DN24Af1, and 38KLGP4A-Afi1 were grown at 34°C, fixed, stained with polyclonal anti-actin antibody, and visualized by fluorescence microscopy (Fig. 10B). Wild-type Afi1 and DN24Af1, but not 38KLGP4A-Afi1, could rescue the defect in actin patch polarization in afi1 mutant cells (Fig. 10B). Quantification of actin patches depolarization in the cells was determined and is shown in Fig. 10C. These results indicate that those conserved residues between Afi1p and its fungal homologues are authentic and significant for its function. In addition, the
interaction of Arf3p and Afi1p is critical for the effect of Afi1p on Arf3p location and for actin patch polarization.

**DISCUSSION**

In this study, we show that a novel protein Afi1p (YOR129c) functions as a polarization-specific adapter for Arf3p. Afi1p and Arf3p both are distributed on the polarized plasma membrane and play roles in maintaining normal budding pattern and actin patch polarization. Several lines of evidence support the notion that Afi1p acts as a polarization-specific adapter for Arf3p and participates in development of polarity: (1) Arf3p loses its polarized distribution, but not plasma membrane association, in the absence of Afi1p, (2) a conserved region at Afi1p, critical for its interaction with Arf3p, is required for the polarized distribution of Arf3p, and (3) an Afi1p mutated at the Arf3p-binding region retains its proper polarized distribution, but loses its ability to rescue actin patch polarization in afi1 mutant cells. Furthermore, Afi1p is not required for polarized distribution of Yel1p and vice versa. Taken together, we infer that Arf3p is activated by Yel1p and is recruited via Afi1p to the polarized plasma membrane, where Arf3p modulates development of polarity.

**Arf3p Directly Interacts with Afi1p in a GTP-Dependent Manner** - From yeast two-hybrid assay, we have demonstrated that the N-terminal 25–488 amino acids are necessary and sufficient to interact with active forms of Arf3p (Q71L and Q71LDN17). Other constructs containing either part of the N-terminal 25–488 amino acids could not interact with Arf3p, implying that the N-terminal 25–488 amino acids are essential to form a proper structure/conformation to interact with Arf3p. In addition to Arf3p, the truncated active form of Arf1p also can weakly interact with Afi1p. Concerning the Golgi localization of Arf1p and the plasma membrane/nucleus localization of GFP-Afi1p, it is more likely that Afi1p interacts with Arf3p in vivo rather than with Arf1p. Thus, the interaction of Arf1p and Afi1p in yeast two-hybrid assays may result from the high similarity between Arf1p and Arf3p. In agreement, the interaction of Arf3p with effectors of Arf1p was also observed in our laboratory, such as Gga1p and Gga2p. Combined with the in vivo and in vitro interaction analyses, it is concluded that Afi1p interacts with Arf3p directly and specifically at the plasma membrane.

**Localization of Arf3p and Afi1p at the Polarized Plasma Membrane** - To observe the subcellular localization of Afi1p, GFP-Afi1p was expressed under the control of the GAL1 promoter and was observed to localize at the plasma membrane and nucleus, consistent with the finding of Wysocka et al. (25) in which a C-terminal GFP-fusion protein was used. Although Huh et al. (26) reported that endogenous Afi1p-GFP localized in the cytoplasm, this statement might be an inappropriate conclusion due to the extremely low protein level of endogenous Afi1p, which is difficult to observe. Moreover, we also found that fixation of cells expressing GFP-Afi1p with formaldehyde affects GFP-Afi1p localization, which may be one of the reasons that indirect immunofluorescence staining using an anti-HA antibody could not detect Afi1p-3HA under the control of its own promoter. We also tried to fix cells with glutaraldehyde; however, glutaraldehyde fixation also disturbed GFP-Afi1p localization. Thus, we could not observe the Afi1p location without a GFP tag.

Over-expressed GFP-Afi1p or Arf3p-GFP did not show polarized localization on the plasma membrane (Fig. 5A, (10)). Only in cells having moderate expression of GFP-Afi1p or Arf3p-GFP could these proteins be enriched at the plasma membrane of daughter cells, indicating that over-expression masks the polarization property (Fig. 5 and 6). Monomeric GFP-tagged Afi1p or Arf3p expressed under the control of the ADH promoter lost their property for polarized bud localization, although monomeric GFP-tagged Arf3p could still partially localize to the bud neck. Therefore, we were unable to show their co-localization using different tags (GFP and mRFP) in vivo. From the observation that Arf3p lost its polarized localization in afi1 mutant cells (Fig. 6) and over-expressed Afi1p-mRFP cells (unpublished data), we hypothesize that the polarizing pattern of Arf3p depends on proper polarized distribution of Afi1p.

It has been proposed that there are three general classes of models involved in maintaining proteins in a polarized distribution (27). First, a preexisting stably polarized “anchor” interacts with the protein of interest thereby increasing its local concentration, such as a subset of “bud-site selection” proteins. Second, a “fence” of membrane-associated filaments forms a diffusion barrier, such as the septin-filament system, maintaining the asymmetric distributions of cortical proteins. Third, asymmetric distribution arises from a dynamic process with constitutively polarized secretion and endocytosis, both of which depend on the actin cytoskeleton, such as some SNARE proteins.

The dependence of Afi1p polarized distribution and the independence of actin structure for the asymmetric localization (10) place Arf3p in the first class of polarized protein. However, since Afi1p is quite different from those stably polarized anchor “bud-site selection” proteins and is not an integral membrane protein as well, Afi1p may represent a new class of polarized protein. In addition, the mechanism by which
Afi1p maintains its asymmetric distribution is also intriguing.

**Afi1p and Arf3p Function in the Same Pathway to Regulate Development of Polarity And Actin Patch Polarization** - Both AFII and ARF3 are non-essential genes. Their deletion strains and double deletion strain have no growth defects but exhibit budding pattern abnormality and actin patch depolarization to similar severity; these data suggest that they are both involved in the same polarity establishing pathway. Although it has been suggested that Afi1p might play a role in mating (28), we found, consistent with a previous report (22), that afrl mutants displayed no obvious defects in the formation of mating projections or in mating efficiency (unpublished results). Although it has been reported that Afi1p can interact with a spindle pole body protein Cnm67p (25), this study was unable to demonstrate the cellular function of Afi1p in the spindle pole body. Afi1p was also observed in the nucleus. It is possible that Afi1p shuffles between the nucleus and plasma membrane, similar to other polarity-determining factors such as Cdc24p (29) and mediates initiation and maintenance of polarity establishment.

Over-expression of GFP-Afi1p resulted in an abnormal budding pattern similar to that observed in afrl-deletion cells (unpublished results), implying that the proper protein level of Afi1p is important for its function in polarity development. Two steps are required for polarity formation: choosing and establishing an axis for polarization (30). Polarity selection requires the integration of spatial cues from cortical landmarks, whereas polarity establishment requires many cellular events such as actin cytoskeleton reorganization, vesicle trafficking, and cell wall synthesis. Compared to the severe budding pattern abnormality of mutants lacking cortical landmarks such as Bud3p (31), the abnormal budding pattern resulting from ARF3 or AFII deletion is relatively mild, which implies that both Arf3p and Afi1p are involved in polarity development rather than polarity selection.

In yeast, actin is organized primarily into cortical actin patches and actin cables. Actin patches are discrete F-actin-rich bodies and are sites of plasma membrane invaginations, whereas actin cables are long F-actin bundles that serve as routes for vesicle delivery to actin patches by motor proteins (32). Both structures lie at the cell cortex and polarize to the growing bud which is essential for polarized cell growth. Like the arf3 mutant, the afrl mutant shows incomplete actin patch polarization to the emerging bud at a higher temperature (34°C) and the double deletion of ARF3 and AFII has no synergic effect; these data suggest that Afi1p and Arf3p participate in the same pathway to indirectly regulate actin patch polarization.

Recently, Arf3p was found to be a suppressor of the profilin-deficient phenotype: loss of actin patch polarization to the emerging bud and actin cable formation, and ARF3 deletion exacerbated this phenotype (31). Profilin (PFY1) is an actin monomer-binding protein, implicated in the polymerization of actin filaments. Over-expression of Arf3p in pfy1 can rescue the actin patch polarization phenotype, but not the actin cable formation phenotype (31). This result is consistent with our finding that Arf3p and Afi1p are required for actin patch polarization but not actin cable formation. In addition, it was observed that ARF3 has genetic interaction with the polarity related gene BUD6 (12). Those studies support the cellular function of Arf3p in cell polarity and actin reorganization. Furthermore, we also show an Arf3p guanine nucleotide exchange factor, Yel1p, is involved in actin patch polarization. Thus, although our results cannot provide a direct correlation between Arf3p and actin reorganization, Arf3p and Afi1p may regulate actin patch polarization by regulating other actin-binding proteins or by other indirect methods.

**Interaction with Afi1p Is Important for Polarized Localization and Function of Arf3p** - From the studies utilizing an N-terminal deletion of Afi1p, we show that the N-terminal region (25-41) of Afi1p contains a sequence which is not only important for its localization but also for interacting with Arf3p. We further demonstrated that the 35KLGP sequence of Afi1p is important for interacting with Arf3p, recruiting Arf3p, and maintaining actin patch polarization. Because Afi1p(1-167) or Afi1p(1-334), could not interact with Arf3p, the 35KLGP sequence of Afi1p can not be sufficient to interact with Arf3p. Mutation of this sequence may alter proper conformation within the N-terminus region (25-488), which is sufficient to interact with Arf3p. However, alteration of conformation by this mutant did not appear to affect the polarized localization of Afi1p. This result suggests that conformation or structures of Afi1p required for interacting with Arf3p and docking to its putative receptor or membranes might be different. From our previous work showing that the N-terminal myristate of Arf3p is necessary for its plasma membrane localization (10), this suggests that association of activated Arf3p with the plasma membrane is a prerequisite for its interaction with Afi1p. It is also possible that phospholipids at the polarized plasma membrane binding with Afi1p can participate in this protein/protein interaction.

**Involvement of Afi1p and Yel1p in Polarized Localization of Arf3p** - From the differential centrifugation studies, we show that membrane association of Afi1p (<20%) is much less than that of Arf3p (>70%), suggesting that association of Arf3p with the plasma membrane is...
higher than that of Afi1p. Further, in an afl1 mutant, Arf3p did not dissociate from the plasma membrane and was still uniformly distributed around the plasma membrane in both mother cells and polarized bud cells. These data suggest that activated Arf3p might have different receptor/adaptor, or specific membrane domain for Arf3p docking. In addition, association of Arf3p with the membrane may be different in mother cells and polarized bud cells. It is also possible that association of Afi1p with specific phospholipids on the polarized plasma membrane is a prerequisite for its interaction with activated Arf3p. Because Afi1p is less abundant than Arf3p and expression of Afi1p decreases significantly at stationary phase (Fig. S2), we suspect that Afi1p is a regulated polarization-specific docking factor that recruits activated Arf3p to the polarized membrane and play a specific role in polarized bud cells. It is tempting to speculate that Arf3p plays a different role in non-polarized cells, because of the high abundance of Arf3p in the stationary phase.

Gillingham and Munro work (24) together with our results here showed that in yel1 mutant cells Arf3p was displaced to the cytoplasm and weakly distributed uniformly around the plasma membrane. They speculated that the low levels of residual membrane-bound Arf3p-GFP observed in yel1 mutant cells represent an inactive form of Arf3p, which is only loosely associated with the membrane. However, our data showed that constitutively inactive form of Arf3p, T31N, is diffusely dispersed in the cytoplasm (Fig. S4). Therefore, another explanation is that there might be another redundant GEF for Arf3p existing.

Gillingham and Munro (24) also reported localization of Yel1p was more tightly restricted to the bud neck and bud tip than that of Arf3p, and our results demonstrated that Yel1p was more restricted to the bud neck. They reasoned that following nucleotide exchange, Arf3p can diffuse away from its site of activation before being turned over and released from the membrane. However, our preferable speculation is that Arf3p-GTP may be recruited by Afi1p away from the site of activation, which is performed by Yel1p. This may also argue that the precise subcellular localization of the activated Arf3p, but not the Arf3p activation, is required for the proper initiation of downstream signaling events. However, we cannot rule out that Arf3p might localize to two distinct sub-domains of the plasma membrane.

In this study, Afi1p was identified as an Arf3p-interacting protein that might act as a polarization-specific docking factor of Arf3p. Together, the data presented herein suggest an attractive model in which Afi1p and Yel1p play major roles in the sequential recruitment of Arf3p to the polarized plasma membrane by interacting directly with structures of these molecules (Fig. 11). In step 1, recruitment of Yel1p to the polarized plasma membrane (restricted more at bud neck) causes the switch of Arf3p-GDP to Arf3p-GTP; in step 2, Arf3p-GTP can be recruited away by Afi1p to the polarized bud plasma membrane where Afi1p may serve as a scaffold protein to maintain proper location of Arf3p and other proteins that are crucial for Arf3p’s function on actin patch polarization. Further identification of other interacting molecules of Arf3p and Afi1p may help elucidate the role of Arf3p in polarity development.

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**Footnotes**

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Figure Legends

Figure 1. The N-terminus of Afi1p (Afi1N) interacts with the active form of Arf3p. (A) The Q71L mutant, but not the wild-type or T31N mutant of Arf3p, interacts with Afi1N. Bait plasmids pEG202 containing different forms of Arf3p, wild-type, Arf3Q71L and Arf3T31N, were co-transformed with pJG4-5 plasmid containing the Afi1p N-terminus into yeast YEM1 and their interactions were analyzed by using the β-galactosidase assay. (B) Afi1N specifically interacts with active forms of Arf3p and Arf1p. Afi1N/pJG4-5 was co-transformed with different active Arf family members including the full-length and truncated forms, and an interaction was shown by the development of a blue color. (C) The AFI1 constructs used in the yeast two-hybrid assay. Afi1N is the original interacting region identified in the library screening using ARF3Q71LdN17 as the bait. Constructs used in (D) are shown with their amino acid designation. (D) Afi1p interacts with Arf3p via its N-terminal 1-500 amino-acid region. Different regions of Afi1p illustrated in (C) were constructed and their interaction with the active form of Arf3p was examined.

Figure 2. Afi1p directly interacts with Arf3p in a GTP-dependent manner. (A) Afi1p and Arf3p interact in a GTP-dependent manner in vivo. GFP-tagged proteins of the indicated Arf3p under the control of its own promoter were expressed in YP H250AFI1-3HA. As described in Material and Methods, Afi1p-3HA was immunoprecipitated by anti-HA antibody and the bound proteins were assayed for the existence of different forms of Arf3p-GFP. (B) Afi1p and Arf3p specifically and directly interact with each other in vitro. One g of GST-Afi1N1 or GST was incubated with 1 g of His-Arf3Q71L, His-Arl1Q72L or His-Arl3Q78L at 4 oC for 1 hour. After 3 washes, the bound small G proteins were detected with anti-His antibody.

Figure 3. Both AFI1 and ARF3 disruption result in budding pattern abnormality. (A) arf3, afi1, and arf3afi1 mutant yeast exhibited abnormal budding patterns. Different haploid cells were fixed and stained with Calcofluor white to visualize bud scars. (B) The percentage of yeast exhibiting an abnormal budding pattern in the indicated strains grown in YPAD was assessed and shown graphically. The number of cells counted in each experiment is 100, and data from three independent experiments were calculated. The bar graph presents the mean ± SEM (n = 3).

Figure 4. Both Afi1p and Arf3p are indirectly involved in actin patch polarization. (A) arf3, afi1, and arf3afi1 mutant yeast show depolarized actin patches at 34°C. Different strains grown at 30°C or 34°C were fixed, and stained with Alexa 594-conjugated phalloidin and visualized by fluorescence microscopy. (B) The percentage of actin patch depolarization in indicated strains grown at 34°C was assessed. In each experiment, 100 cells with small buds were examined. The bar graph presents the mean ± SEM (n = 3).

Figure 5. Afi1p exhibits a polarized distribution on the plasma membrane. (A) GFP-Afi1p expressed at a lower level can polarize. Living cells (YPH250dafi1) expressing GFP-Afi1p under the control of the ADH or its own promoter on 2 plasmids were grown to mid-log phase and visualized by fluorescence microscopy. (B) Protein expression of GFP-Afi1p. Cell lysates were extracted from the yeasts in (A) and subjected to immunoblotting. Actin was used as a loading control.

Figure 6. Afi1p is required for proper distribution of Arf3p but not vice versa. (A) AFI1 disruption affects Arf3p-GFP polarized distribution. Wild-type (YPH250) or afi1 (YPH250dafi1) cells expressing Arf3p-GFP under the control of its own promoter were observed by fluorescence microscopy. (B) ARF3 disruption does not affect the localization of GFP-Afi1p. Wild-type (YPH250) or arf3 (YPH250df3) cells expressing GFP-Afi1p under the control of its own promoter were grown to mid-log phase and visualized under the fluorescence microscope. Bars, 5μm.

Figure 7. Afi1p is not required for proper localization of Yel1p and vice versa. (A) AFI1 disruption does not affect the localization of GFP-Yel1p. Wild-type (YPH250) or afi1 (YPH250dafi1) cells expressing Yel1-GFP under the control of the ADH promoter were observed by fluorescence microscopy. (B) YEL1 disruption does not affect the localization of GFP-Afi1p. Wild-type (BY4741) or yel1 (BY4741dyel1) cells expressing GFP-Afi1p under the control of its own promoter were grown to mid-log phase and visualized under the fluorescence microscope. Bars, 5μm.

Figure 8. Yel1p is required for proper location and function of Arf3p (A) Yel1p is required for the
polarized localization of Arf3p. Arf3-GFP under the control of its own promoter was expressed into wild-type (WT), afi1, or yel1 mutant cells. Living yeasts were grown to mid-log phase and examined under the fluorescence microscope. Fluorescence intensities (FI) measured according to pixel brightness were evaluated. (B) Quantification of Arf3-GFP localization in different strains. The ratios of the average of the two fluorescence signals on the plasma membrane (PM) to the average signal of the cytosol (CS) were evaluated. Bars, 10μm. (C) Differential centrifugation analysis. Wild-type yel1-mutant or afi1-mutant cells were grown in YPAD medium, subjected to velocity sedimentation and then separated into P13 and S13 fractions as described in Materials and Methods. Proteins in samples of fractions were analyzed by immunoblotting. The percentage of proteins in P13 and S13 fractions was quantified and indicated. Arf3p, Pma1p (plasma membrane marker), Pgk1p (cytosol marker) were identified with specific antibodies. (D) afi3 and yel1 mutants show depolarized actin patch at 34 ℃, compared with wild-type BY4741. The cells were grown at 34 ℃, fixed, and stained with polyclonal anti-actin antibody and visualized by fluorescence microscopy. (E) The percentage of actin patch depolarization in the indicated strains grown at 34 ℃ was assessed. In each experiment, 100 cells with small buds were examined. The bar graph presents the mean ± SEM (n = 3).

Figure 9. The N-terminal region of Afi1p is conserved and essential for its proper localization. (A) Protein sequence alignment of Afi1p and its homologues from other fungi, ACR212Cp from Ashbya gossypii and kYOR129C from Kluyveromyces lactis. The multiple alignments were assembled by CLUSTAL W (1.83) on EMBL-EBI (http://www.ebi.ac.uk/). The symbol “ * ” indicates identical amino acid, and “ . “ and “ : “ indicate similar residues. The black bars indicate conserved regions and the red box indicates the residues of KLGP4A mutation on Afi1p. Protein expression of different forms of GFP-Afi1p. The full-length (FL), N-terminal 24-amino-acid-deleted (dN24), N-terminal 41-amino-acid-deleted (dN41) and KLGP4A mutant forms of GFP-Afi1p were separately expressed in afi1 (YPH250daf1) cells. Total lysates were prepared and subjected to immunoblotting. Actin was used as a loading control. (B) Interactions between various Afi1p mutants and active Arf3p. The β-galactosidase assay was performed as in Fig. 1. (C) Protein expression of different forms of GFP-Afi1p. The full-length (FL), N-terminal 24-amino-acid-deleted (dN24), N-terminal 41-amino-acid-deleted (dN41) and KLGP4A mutant forms of GFP-Afi1p were separately expressed in afi1 (YPH250daf1) cells. Total lysates were prepared and subjected to immunoblotting. Actin was used as a loading control. (D) Subcellular localization of various forms of GFP-Afi1p. WT, 24-amino-acid-deleted (dN24), 41-amino-acid-deleted (dN41) or KLGP4A mutant forms of GFP-Afi1p on YEPPlac195, under the control of its own promoter on 2μ plasmids, were transformed into afi1 mutant cells. Living cells in mid-log phase were observed by fluorescence microscopy. Bars, 5μm.

Figure 10. KLGP4A mutant form of Afi1p affects the proper localization and function of Arf3p. (A) Effect of KLGP4A mutant of Afi1p on Arf3p localization. Wild-type Afi1p, dN24Afi1p, or KLGP4A mutant form of Afi1p from integrated plasmid YPlac128 under the control of its own promoter were separately co-expressed with Arf3p-GFP/YCPlac111 in afi1 (YPH250daf1) cells. Living cells in mid-log phase were observed by fluorescence microscopy. Bars, 5μm. (B) Effect of KLGP4A mutant of Afi1p on actin patch polarization. Wild-type Afi1p, dN24Afi1p, or KLGP4A mutant form of Afi1p from integrated plasmid YPlac128 under the control of its own promoter was transformed into afi1 (YPH250daf1) cells. The cells grown at 34 ℃ were fixed, stained with polyclonal anti-actin antibody, and visualized by fluorescence microscopy. (C) Quantitation of actin patch depolarization in (KLGP4A mutant forms of GFP-Afi1p). Cells with more actin patches in the mother cell than in the small bud were scored as “depolarization” from three independent experiments. In each experiment, 100 cells with small buds were examined. The bar graph presents the mean ± SEM (n = 3).

Figure 11. A two-step model for the involvement of Afi1p and Yel1p in the polarized localization of Arf3p. Results presented in this study and work from Gillingham and Munro (2007) show roles of Afi1p and Yel1p in regulating the polarized distribution of Arf3p. In step 1, recruitment of Yel1p to the polarized plasma membrane (restricted more at bud neck) causes the switch of Arf3p-GDP to Arf3p-GTP. In step 2, Arf3p-GTP can be recruited away by Afi1p to the polarized bud plasma membrane where Afi1p may serve as a scaffold protein to maintain proper location of Arf3p and other proteins that are crucial for Arf3p’s function on actin patch polarization. Arf3-GFP observed in the yel1 mutant cells represent an inactive form of Arf3p, which is only loosely associated with the membrane. Another explanation is that there might be another redundant GEF for Arf3p existing on the membrane.

Supplemental Figure Legends
Figure S1. **Alignment of Afi1p and its homologues.** ACR212Cp (NP_983614) from *Ashbya gossypii*, kYOR129C (CAG99156) from *Kluyveromyces lactis* and MesA (XP_657872) from *Aspergillus nidulans*, respectively.

Figure S2. **Regulated expression of Afi1p.** Yeast containing YPH250AFI1-3HA was harvested at different growth stages and total proteins were extracted for immunoblotting. Absorbance of yeasts in each lane are: lane 1, ~0.4; lane 2, ~0.6; lane 3, ~1.3; lane 4, ~2.0; lane 5, ~4.5; lane 6, ~6.0. Actin was used as a loading control.

Figure S3. **GFP-Afi1p mainly localizes at the plasma membrane and nucleus.** (A) GFP-Afi1p localization. YPH250GFPAFI1 was grown to mid-log phase, stained with 4 mg/ml DAPI in medium for 2-3 hours, harvested, washed, and visualized by fluorescence microscopy. (B) GFP-Afi1p localization is disrupted by formaldehyde treatment. YPH250GFPAFI1 was grown to mid-log phase, fixed with 3.7% formaldehyde, washed, and visualized under the fluorescence microscope. (C) Differential centrifugation analysis. Afi1-HA from integrated plasmid YIplac128 under the control of its own promoter was transformed into *afi1* (YPH250daf1) cells. The cells were grown in YPAD medium subjected to velocity sedimentation and were separated into P13 and S13 fractions as described in Materials and Methods. Proteins in samples of fractions were analyzed by immunoblotting. HA, Arf3p, Pma1p (plasma membrane marker), Pgk1p (cytosol marker) and Emp47p (Golgi marker) were identified with specific antibodies.

Figure S4. **Afi1p is required for proper localization of constitutively active Arf3p.** Arf3p-GFP, Arf3Q71L-GFP and Arf3T31N-GFP on YCP11, under the control of its own promoter on CEN plasmids were expressed in wild-type (WT) or *afi1* mutant cells. Living cells in mid-log phase were observed under the microscope and photographed. Bar, 5 μm.
### Tables

Table 1. Yeast strains used in this study

| Strain            | Genotype                                                                 |
|-------------------|---------------------------------------------------------------------------|
| YPH250            | MATa ade2, his3, leu2, lys2, trp1, ura3-52, ARF3, AFI1                   |
| YPH250df3         | MATa ade2, his3, leu2, lys2, trp1, ura3-52, arf3, AFI1                  |
| YPH250daf1        | MATa ade2, his3, leu2, lys2, trp1, ura3-52, ARF3, afi1                   |
| YPH250GFPAFI1     | MATa ade2, his3, leu2, lys2, trp1, ura3-52, ARF3, AFI1:: GAL1-GFP-KanMX6 |
| YPH250F3GFPAFI1-6HA | MATa ade2, his3, leu2, lys2, trp1, ura3-52, ARF3::GFP-KanMX6, AFI1::6HA-TRP1 |
| YPH250F3GFPAfi1dC20 | MATa ade2, his3, leu2, lys2, trp1, ura3-52, ARF3::GFP-KanMX6, AFI1::C20::6HA-TRP1 |
| YPH250F3GFPAfi1dC40 | MATa ade2, his3, leu2, lys2, trp1, ura3-52, ARF3::GFP-KanMX6, AFI1::C40::6HA-TRP1 |
| BY4741            | MATa his3, leu2, met15, ura3, ARF3, AFI1                                 |
| BY4741df3         | MATa his3, leu2, met15, ura3, arf3:: KanMX6, AFI1                       |
| BY4741daf1        | MATa his3, leu2, met15, ura3, ARF3, afi1:: KanMX6                      |
| BY4741dyel1       | MATa his3, leu2, met15, ura3, ARF3, AFI1, yel1:: KanMX6                |
| BY4741df3daf1     | MATa his3, leu2, met15, ura3, arf3, afi1:: KanMX6                      |

*ade, adenine-requiring; his, histidine-requiring; leu, leucine-requiring; met, methionine-requiring; lys, lysine-requiring; trp, tryptophan-requiring; ura, uracil-requiring; arf3 represents arf3::hisG; afi1 represents afi1::hphM*
Table 2. Primers used in this study.

| Primer     | Sequence (5’-3’)                                      |
|------------|-------------------------------------------------------|
| Arf3dN17   | GAATTCA GAAATTTAATGCTAGG CTGCTG                   |
| Arf3.C     | TTATTTCTTT TGGACGGTGTG                              |
| Arf3.pro.1 | GAGCTCTGGAGTAAACTCAATGCCACAC                      |
| Arf3.GFP.2 | TGGGCGTCCATGTCGCTG                                 |
| 129C.1     | GAATTCA TGGTCGAGAACAATGAAACTAAAT                |
| 129C.M.1   | GAATTCA TGGAGCAGTAGCGACTGCAAGAATGTC               |
| 129C.M1.1  | GAATTCA TGGGCACATGGAGAAGAACCTTATAT                |
| 129C.M2.1  | GAATTCA TGGAGAGACGCTTATGGGAAATG                  |
| 129C.TH.2  | CTATGTCGCTTTTGGAGAATGTC                            |
| 129CTH.M.2 | CTATGAGAAATGAGGCGCTATGAATATC                      |
| 129CTH.M1.2| CTATTTCTGAATTATCTGAACCTGCC                        |
| 129CTH.M2.2| CTATCTAATCAATTTAAATGCCAAATG                      |
| 129C-S3    | AACGAACAAACAAAACAGATCAGAACATAACCTAAAAAGGAA       |
| 129C-S2    | TGAATTCCTTAATATTGTAATCAGTGAATCAGGACGCAATCG       |
| 129CF4     | TTCTTTCTATAAAAGTGAAGCCTCTATAAAATCAAGAATGCAAGC    |
| 129CR5GFP  | TATCGACCTGGTCGATAGGAGTTATTGTTCTTCGGCAACAT         |
| 129CAG34.3 | TTCTTTCTATAAAAGTGAAGCCTCTATAAAATCAAGAATGCAAGC    |
| 129CAG34.4 | AAGTGTTTGTGCAGCATCTCTCCCCCTTTTGGAAATAGTGTCGTTTT  |
| 129C-Xhol-1| CTTCGAGATGGTTCGAGAGAAGAAGACTAAAAAATAC            |
| 129C-HindII-2 | GAAGCCCTAGCTTCAATGTTGAGGTTTTC                  |
| 129CdN24-1 | GAATTTCTGAGATGCGACCAGTCCAGCTGATATAATTAC         |
| 129CdN41-1 | GAATTCCCTGAGATGTTGAGGTTGAGGCTTATC               |
| 129CKLGP4A-1| TTGTGATAATGCACGGAGCTGACTTTTGGAGCACCACATAC       |
| 129CKLGP4A-2 | GTGCTTCAAAATAGCACTGTCGACTTATGAACTTTC             |
| N-EGFP-BamHI-1 | GGAATCCATGCTCAATGAGAATGAAACTATC       |
| N-EGFP-Xhol-2 | CTCTGAGTTGTCACAGATACCTCAATCCACCAGAATG      |
| AFII- KpnI-2 | CGGCTGTGCGTGGCGCTCAGTGCCTGG                    |
| AFII-N-2   | AAACATTATGTCATATAAAGGGAACAGG                     |
| YELITH-1   | CTCTGAGATGTCGCGCAGGTTGAAACAGG                   |
| YELITH-2   | CTCTGAGAGAACGTTGACGAGAACCACATG                  |
| YEL1-SacI-2 | GAGCTCTTAGAAGATCAGAAGAACCACATG                 |
| Plasmids    | Description                                      | Source or Reference |
|------------|--------------------------------------------------|---------------------|
| pJG4-5     | 2μm TRP1 P_GAL acid bolb B42                     | 33                  |
| pEG202     | 2μm HIS3 P_ADH LEX A DNA binding domain          | 33                  |
| pYEX4T-1   | 2μm URA3 P_CUP1 GST.Tag                         | Clontech            |
| pVT101U    | 2μm URA3 P_ADH                                   | 34                  |
| pVT102U    | 2μm URA3 P_ADH                                   | 34                  |
| pYEura3    | CEN4 URA3 P_GAL                                  | Clontech            |
| YEplac181  | 2μm LEU2                                        | 35                  |
| YEplac195  | 2μm URA3                                        | 35                  |
| Art3pEG    | Art3 on pEG202                                   | This study          |
| Art3Q/LpEG | Art3Q71L on pEG202                               | This study          |
| Art3T/NpEG | Art3T31N on pEG202                               | This study          |
| Art3d17NpEG| Art3d17N on pEG202                               | This study          |
| Art3Q/Ld17NpEG | Art3Q71Ld17N on pEG202                   | This study          |
| Art3T/Nd17NpEG | Art3T31Nd17N on pEG202              | This study          |
| Art1Q/LpEG | Art1Q71L on pEG202                               | This study          |
| GSTArt3    | Art3 on pYEX4T-1                                 | This study          |
| GSTArt3Q/L | Art3Q71L on pYEX4T-1                             | This study          |
| GSTArt3T/N | Art3T31N on pYEX4T-1                             | This study          |
| Afi13HA    | Afi13HA on YIPlac211, AFI1 promoter              | This study          |
| Afi1dN243HA| Afi1dN243HA on YIPlac211, AFI1 promoter          | This study          |
| Afi1KLGP4A 3HA | Afi1 KLGP4A 3HA on YIPlac211, AFI1 promoter  | This study          |
| Art3pYE    | Art3 on pYEura3                                  | 10                  |
| Art3Q/LpYE | Art3Q71L on pYEura3                              | 10                  |
| Art3T/NpYE | Art3T31N on pYEura3                              | 10                  |
| Art3GF     | Art3GF on YEPlac111, ARF3 promoter              | This study          |
| Art3Q/LGF  | Art3Q/LGF on YEPlac111, ARF3 promoter            | This study          |
| Art3T/NGFP | Art3T/NGFP on YEPlac111, ARF3 promoter           | This study          |
| Afi1pJG    | Afi1 on pJG4-5                                   | This study          |
| Afi1N1pJG  | Afi1N1 (1-500 a.a.) on pJG4-5                    | This study          |
| Afi1N2pJG  | Afi1N2 (1-167 a.a.) on pJG4-5                    | This study          |
| Afi1N3pJG  | Afi1N3 (168-334 a.a.) on pJG4-5                  | This study          |
| Afi1N4pJG  | Afi1N4 (335-500 a.a.) on pJG4-5                  | This study          |
| Afi1N5pJG  | Afi1N5 (1-334 a.a.) on pJG4-5                    | This study          |
| Afi1N6pJG  | Afi1N6 (168-500 a.a.) on pJG4-5                  | This study          |
| Afi1CpJG   | Afi1C (501-893 a.a.) on pJG4-5                   | This study          |
| Afi1dNpJG  | Afi1dN (168-893 a.a.) on pJG4-5                  | This study          |
| Afi1dN24pJG| Afi1dN24 on pJG4-5                               | This study          |
| Afi1dN41pJG| Afi1dN41 on pJG4-5                               | This study          |
| Afi1KLGP4ApJG | Afi1KLGP4A on pJG4-5                    | This study          |
| Afi1mRFP   | Afi1mRFP on pVT101U                              | This study          |
| GFP Afi1YEp| GFP Afi1 on YEplac195, AFI1 promoter             | This study          |
| GFP Art1   | GFP Art1 in pVT102U                              | This study          |
| GFP Afi1dN24| GFP Afi1dN24 on YEplac195, AFI1 promoter        | This study          |
| GFP Afi1dN41| GFP Afi1dN41 on YEplac195, AFI1 promoter        | This study          |
| GFP Afi1KLGP4A | GFP Afi1KLGP4A on YEplac195, AFI1 promoter  | This study          |
| GFP Yel1   | GFP Yel1 on pVT101U                              | This study          |
Fig. 2

A

|          | input | bound |
|----------|-------|-------|
|          | W.T.  | Q71L  | T31N |
| Afi1-3HA |       |       |      |
| Arf3-GFP |       |       |      |

B

|          | input | GST | GST-Afi1N1 |
|----------|-------|-----|------------|
|          | Arf3Q71L | Arf1Q72L | Arf3Q78L |
|          | Arf3Q71L | Arf1Q72L | Arf3Q78L |
| Histidine|        |       |            |
| GST-Afi1N1|      |      |            |
| GST       |      |      |            |
Fig. 3

A

WT  arf3  afi1  arf3afi1

B

bud site abnormality (%)

WT  arf3  afi1  arf3afi1
Fig. 5

A

promoter:

ADH

AFII

GFP-Afi1 phase

B

promoter:

ADH AFII

GFP-Afi1 actin
Fig. 7

A

GFP-Yel1

WT       afi1

B

GFP-Afi1

WT       yel1
Fig. S1
Fig. S2

HA-Afl1p

Actin

Arf3p
Af1p functions as an Arf3p polarization-specific docking factor for development of polarity
Pei-Chin Tsai, Szu-Wei Lee, Ya-Wen Liu, Chih-Wen Chu, Kuan-Yu Chen, Jui-Chih Ho and Fang-Jen S. Lee

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