Plasma membrane polarization during mating in yeast cells

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The yeast mating cell provides a simple paradigm for analyzing mechanisms underlying the generation of surface polarity. Endocytic recycling and slow diffusion on the plasma membrane were shown to facilitate polarized surface distribution of Snc1p (Valdez-Taubas, J., and H.R. Pelham. 2003. *Curr. Biol.* 13:1636–1640). Here, we found that polarization of Fus1p, a raft-associated type I transmembrane protein involved in cell fusion, does not depend on endocytosis. Instead, Fus1p localization to the tip of the mating projection was determined by its cytosolic domain, which binds to peripheral proteins involved in mating tip polarization. Furthermore, we provide evidence that the lipid bilayer at the mating projection is more condensed than the plasma membrane enclosing the cell body, and that sphingolipids are required for this lipid organization.

Introduction

Saccharomyces cerevisiae polarizes cell growth to the bud during cell replication and to the mating projection when cells are induced by pheromones to change their shape to form shmoos. This polarization process is characterized by a hierarchy of steps. First, the site on the cell surface is selected by intrinsic and extrinsic cues. This site is marked by the deposition of landmark proteins. Second, cell polarity is established by the activation of small GTPases with CDC42 as the major player. Last, a multiprotein machine is assembled that spools out actin cables to direct post-Golgi traffic to the site of polarized cell growth (Drubin and Nelson, 1996; Madden and Snyder, 1998; Pruyne and Bretscher, 2000; Chang and Peter, 2003).

During budding, membrane traffic is directed by actin cables to the bud, and the septin ring at mother-daughter cell neck region functions as a physical barrier, preventing diffusion of membrane components from the bud to the mother cell (Barral et al., 2000; Takizawa et al., 2000). During mating, the biosynthetic transport is directed to the shmoo tip (Pruyne and Bretscher, 2000). However, there is no diffusion barrier like the septin ring and most proteins diffuse laterally over the entire cell surface. Nevertheless, a specific subset of proteins required for mating is clustered at the tip of the mating projection. We have shown previously that the polarized distribution of membrane components involves raft lipids (sphingolipids and ergosterol) and the actin cytoskeleton (Bagnat and Simons, 2002). Based on these observations, we proposed that lipid rafts are clustered at the tip of the mating projection and that this process is important for the retention of the associated molecules at the mating projection. Recently, it was demonstrated that cycles of endocytosis combined with polarized membrane delivery into the mating projection are able to restrict protein localization to the tips of shmoos (Valdez-Taubas and Pelham, 2003). Here, we report that polarized localization of Fus1p, a type I transmembrane protein involved in cell fusion (Trueheart and Fink, 1989), does not require endocytosis. Instead, the protein is retained at the tip of the mating projection through the interaction of its cytosolic tail with a multiprotein scaffolding machinery. Additionally, we provide evidence that the lipid bilayer at the tip of the mating projection is more ordered than over the cell body and that sphingolipids are required for this specific lipid organization.

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Abbreviations used in this paper: GP, general polarization; SH, Src kinase homology; TMD, transmembrane domain.

The online version of this article contains supplemental material

Results and discussion

To revisit the kinetic recycling model we have analyzed the role of polarized delivery and endocytosis in polarizing Fus1p, a type I transmembrane protein involved in cell fusion (Trueheart and Fink, 1989; Nolan et al., 2006), to the tip of the mating projection. We first analyzed shmoo tip delivery of Fus1p and compared it to another marker protein that is distributed all over the

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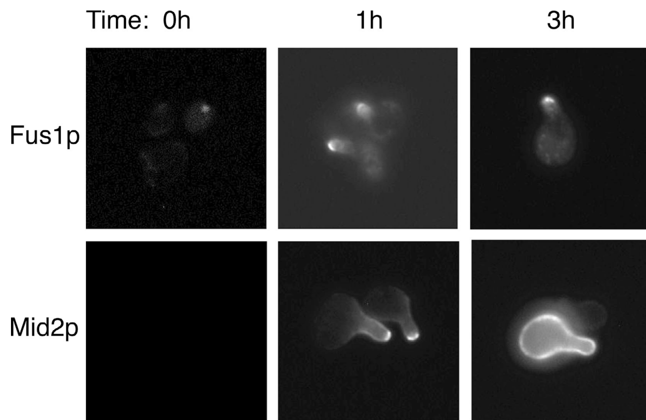


Figure 1. **Polarized exocytosis to the tip of the mating projection.** Localization of GFP-tagged Fus1p and Mid2p at different time points after induction of expression in shmooing cells. Wild-type cells carrying plasmids MBQ30 or MBQ35 were treated with α-factor for 3 h and after that, galactose was added to induce protein expression (for details see Materials and methods).

plasma membrane of mating cells, Mid2p (Fig. 1). Mid2p is a cell wall integrity sensor, and similarly to Fus1p, it is a type I transmembrane protein (Philip and Levin, 2001). 1 h after induction of expression, the marker proteins were delivered to the shmoo tip where both were localized at this point. However, 2 h later Mid2p had diffused over the entire plasma membrane, whereas Fus1p remained at the tip. We then analyzed the effect of endocytosis on the process of Fus1p polarization. We also used Snc1p, a yeast v-SNARE involved in post-Golgi plasma membrane transport, as a second marker protein that is tip localized. It has been shown that Snc1p polarization was abolished after inhibition of endocytosis (Valdez-Taubas and Pelham, 2003). In endocytosis-deficient cells, Snc1p was no longer polarized but was distributed over the plasma membrane of the shmooing cells. In contrast, the polarization of Fus1p to the tip of the mating projection remained normal in *end4Δ* cells (Fig. 2). Thus, there must be an alternative mechanism that maintains biosynthetically delivered Fus1p at the shmoo tip irrespective of ongoing cycles of endocytosis and exocytosis. Important also to note is that most mutants that inhibit endocytosis mate with similar efficiency as wild-type cells (Brizzio et al., 1998). These findings confirm that the kinetic polarization model using endocytosis and polarized exocytosis is involved in local concentration of membrane proteins such as Snc1p. However, this is not the only mechanism used by shmooing cells to polarize their mating machinery.

One reason why Fus1p is retained at the mating tip could be due to interaction with the cell wall, as was demonstrated for glycosylphosphatidylinositol-anchored proteins (De Sampaio et al., 1999). Thus, we constructed fusion proteins between Fus1p and Mid2p where we swapped the extracellular, the transmembrane, and the cytosolic domains of the two proteins (schematically shown in Fig. 3). Analysis of their surface distribution demonstrated that the information for mating tip retention was localized to the cytosolic tail (Fig. 3). The Mid2 protein carrying the cytoplasmic domain from Fus1p was localized to the mating projection. PAGE and Western blot analysis confirmed that this protein displayed a pattern of glycosylation, typical for mature Mid2p (Lommel et al., 2004 and unpublished data). We then analyzed how this chimeric protein behaved in mutants in which endocytosis was inhibited, both in *end4Δ* cells and at the nonpermissive temperature in *end4-1 ts* cells. This chimeric protein behaved like Fus1p and maintained its polarization in endocytosis-deficient cells (Fig. 4 A and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200602007/DC1). Thus, we concluded that the cytosolic tail of Fus1p mediates

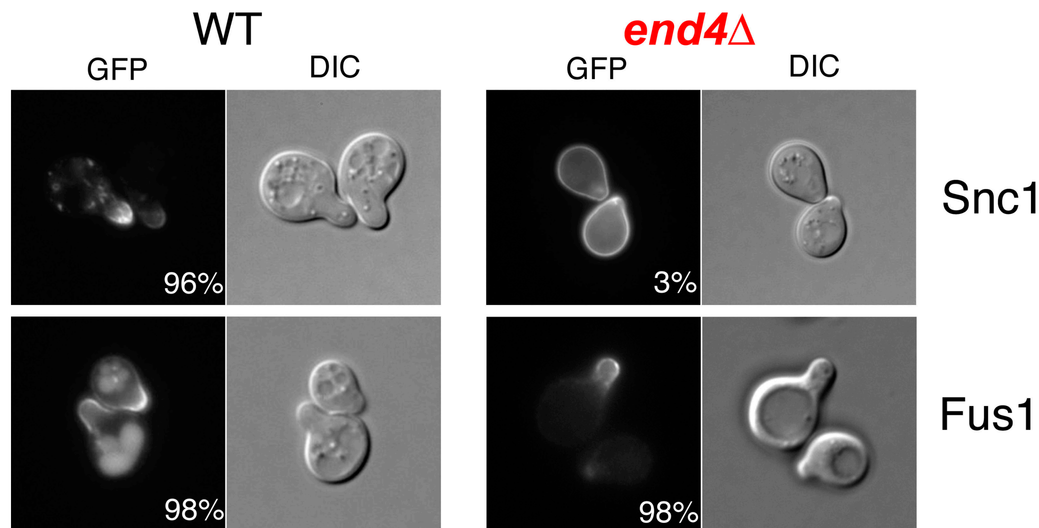


Figure 2. **Polarized distribution of Snc1p, but not of Fus1p, is dependent on endocytosis.** Blocked endocytosis in *end4Δ* disrupted polarized distribution of Snc1p, but did not affect tip localization of Fus1p, compared with wild-type cells. The percentage of cells with fluorescence on the plasma membrane limited to the mating projection is given in the bottom right corner of the GFP images. For Fus1p in wild-type and end4Δ we counted 218 and 327 cells, respectively, and for Snc1p it was 220 and 100 cells, respectively. In wild-type cells, in addition to the plasma membrane, Snc1p was found in intracellular structures due to protein cycling. These structures were not visible in the endocytosis mutant because the protein was trapped on the plasma membrane. Fus1p in wild-type cells was found at the plasma membrane and in the vacuole.

protein retention at the tip and that interactions with the cell wall cannot explain the polarization. The cytosolic tail of Fus1p is 416 amino acids long and contains an Src kinase homology 3 (SH3) domain close to its COOH terminus, followed by a proline-rich domain, both known to be responsible for protein–protein interactions (Tong et al., 2002). We deleted the SH3 domain from the chimeric protein Mid-Fus (used in Fig. 4 A) or Fus1p (not depicted) and saw no effect on polarization. At this time, a report from Nelson et al. (2004) appeared, in which a detailed analysis of the cytoplasmic domain of Fus1p was described. They showed that both domains were important for mating efficiency, but even the double-mutant protein was polarized normally in wild-type cells. Because mutations in these domains prevented protein interaction with the scaffolding machinery (Nelson et al., 2004) we considered the possibility that the double mutant of Fus1p could be polarized via the endocytic recycling mechanism. To test this possibility we expressed the mutated Fus1p in the endocytosis-deficient strain and found that protein polarization was still normal (Fig. 4 B). We concluded that additional sites on the cytoplasmic tail of Fus1p might contribute to Fus1p retention to the mating tip. In a detailed two-hybrid analysis it was demonstrated that the cytosolic tail of Fus1p interacts with several key players in mating polarity, including the GTP-bound form of CDC42, components of the polarisome Pea2p and Bni1p, Fus2p, and Ste5p, the scaffold protein for MAP kinase signaling (Nelson et al., 2004). We analyzed the Mid-Fus protein in *pea2*, *bni1*, *fus2*, *spa2*, *bud6*, *fus1,* and *ste5* deletion mutants. The localization was normal in all mutants accept in *ste5* cells, which did not form shmoos and in *bni1*, which exhibits a subtle defect in protein polarization (Fig. S2). From these data we propose that Fus1p is directly embedded in a dynamic network of protein–protein interactions that is responsible for scaffolding and localization of Fus1 to the shmoo tip as part of the mating machinery.

Based on the findings (1) that polarization of the mating machinery to the shmoo tip is inhibited in *erg6* and in *lcb1-100* cells; (2) that mutations that severely affect the synthesis of the major raft lipids in yeast reduced mating efficiency; and (3) on the polarized distribution of filipin, a molecule that has high affinity for sterols, we postulated previously that raft lipid clustering plays a role in establishing and maintaining mating tip polarization (Bagnat and Simons, 2002). Because partitioning of filipin does not directly correlate with lipid ordering in the bilayer, we took advantage of a dye that does. Laurdan is an environmentally sensitive dye that has a peak of emission shifting from ~500 nm in liquid-disordered membranes to ~440 nm in ordered membrane domains (Gaus et al., 2003). We simultaneously recorded the Laurdan fluorescence intensity in two channels. By expressing a normalized ratio of the two emission regions—the general polarization (GP; see Materials and methods)—Laurdan fluorescence provides a relative measure of lipid order in cell membranes. Importantly, Laurdan does not preferentially partition into a specific lipid phase, nor do GP values depend on the local probe concentration within the membrane (Gaus et al., 2003). The GP images revealed that the membrane at that mating projection is more condensed and ordered than the domain

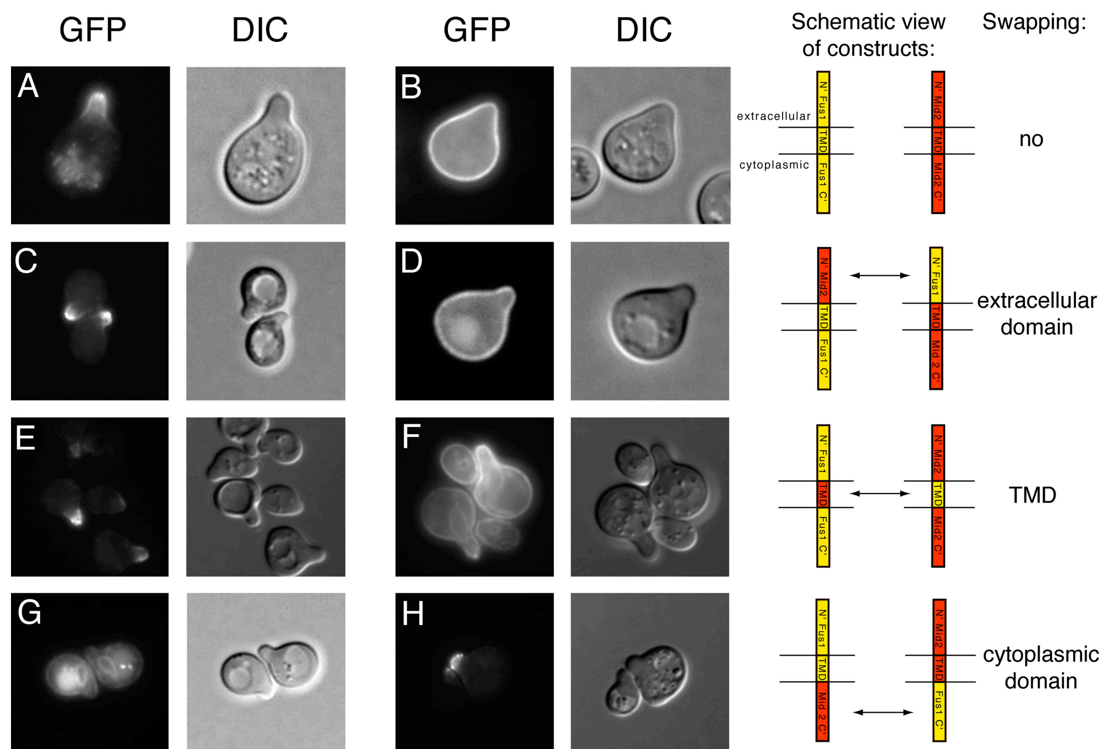
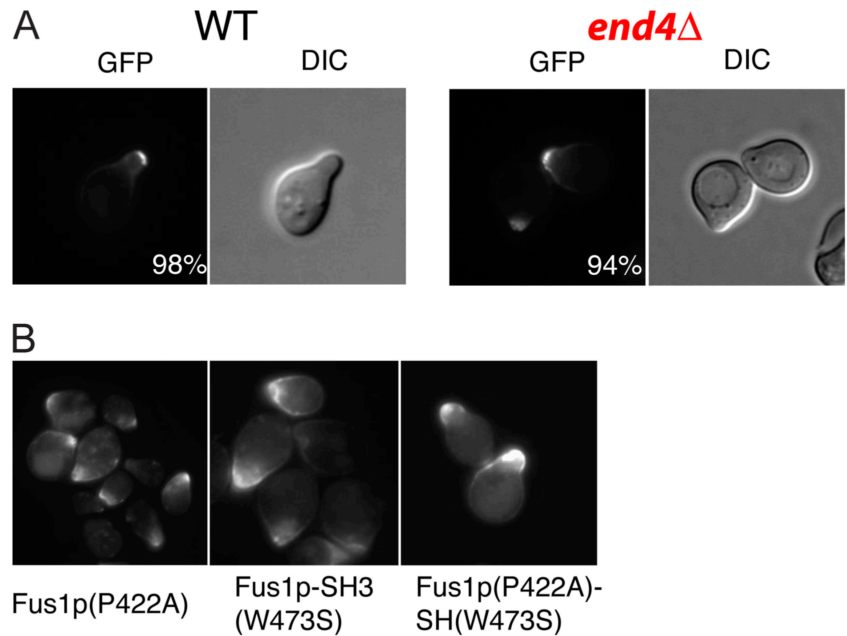


Figure 3. **The cytoplasmic tail of Fus1p is responsible for polarized localization.** Fus1p, Mid2p, and different chimeric proteins were expressed in wild-type cells treated with α-factor. The schematic representation of the expressed fusion proteins is shown on the right and the swapped domains are indicated. The yellow and red colors specify *FUS1* and *MID2* sequences, respectively. Internal membrane staining is seen in F and G, suggesting that protein sorting to the cell surface was compromised. However, the fraction of protein that was delivered to the plasma membrane was not polarized.

Figure 4. **Fus1p does not require its SH3 or the proline-rich domain for polarized localization.** The chimeric Mid(cyt-FusΔSH3) protein, which carries a cytoplasmic tail from Fus1p without SH3 domain, was equally well polarized in the wild-type and *end4Δ* cells (A). The percentage of cells with fluorescence on the plasma membrane limited to the mating projection is indicated (*n* = 361 and 138 for the wild-type and *end4Δ* cells, respectively). Similarly, protein carrying point mutations that affect the function of the proline-rich region or the SH3 domain (Fus1p(P422A) and Fus1p-SH3(W473S), respectively), or the double mutant of Fus1p (Fus1p(P422A)-SH(W473S)), was polarized in *end4Δ* cells (B).



on the opposite site of the cell (Fig. 5). Hence, the membrane at the mating projections displayed the biophysical characteristic that is expected for raft clustering. The coalescence of condensed membrane at the tips of shmoos was also found to occur in the endocytosis-deficient strain but not in the sphingolipid mutant *lcb1-100* (Fig. 5). These data clearly demonstrated the asymmetric organization of the lipids in the plasma membrane of yeast cells during mating. More detailed analysis is needed to understand the molecular mechanisms responsible for the formation and maintenance of the mating projection.

Also, mammalian cells use raft clustering to polarize their cell surfaces during cell migration or cell–cell contacting during immune recognition. In migrating neutrophils it was demonstrated that lipid raft clusters are localized to the rear of the cells in an actin-dependent manner (Seveau et al., 2001). In migrating T-lymphocytes, Gomez-Mouton et al. (2001) showed that two types of raft clusters are assembled at opposite poles, at the leading edge and at the uropod. Recently, it was also demonstrated that when the T cell receptor is activated, a condensed raft cluster is formed at the activation site (Gaus et al., 2005). Each raft-clustering process is specific in that a subset of raft components is included in the assembly, associating and dissociating from the cluster dependent on their raft-partitioning characteristics (Simons and Vaz, 2004; Kusumi et al., 2005) and the kinetics of the protein–protein interactions (Harder, 2004; Douglass and Vale, 2005). This mechanism could also drive the surface polarization during yeast mating (Bagnat and Simons, 2002). The scaffolding of proteins would occur mainly through

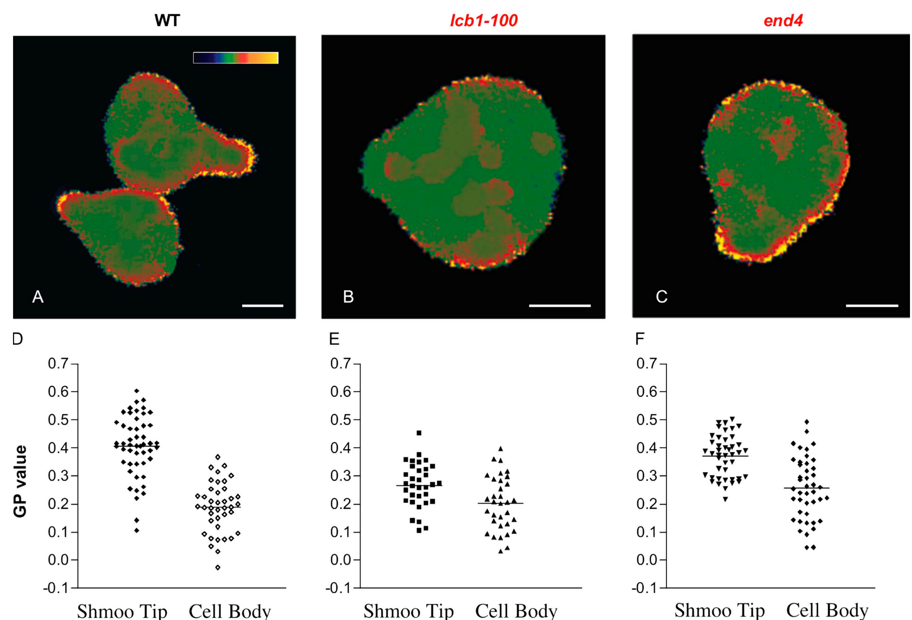


Figure 5. **Membrane condensation at the mating projection.** α-Factor–treated wild-type (A and D), *lcb1-100* (B and E), and *end4Δ* (C and F) cells were killed with 5 mM NaN_3, fixed with 2–4% paraformaldehyde, labeled with 250 μM Laurdan for 5 min, and imaged in water. GP values were calculated from the Laurdan intensity images and pseudocolored as indicated in A (low to high GP values, black to yellow). Bars in A–C = 2 μm. GP values of membranes at the mating tip and at the cell body opposite the mating tip were measured for 40 (D), 28 (E), and 43 (F) individual cells. Horizontal bars in D–F indicate means. Means ± SD are 0.398 ± 0.118 and 0.191 ± 0.090 (D), 0.256 ± 0.143 and 0.202 ± 0.104 (E), and 0.372 ± 0.075 and 0.256 ± 0.111 (F) for mating tip and cell body, respectively. The difference of the GP value at the mating projection between wild-type and sphingolipid mutant cells was statistically significant ($P < 0.001$) but there was no statistically significant difference between wild-type and *end4* cells ($P > 0.05$).

protein–protein interactions. Nevertheless, coming together of proteins with a condensed lipid domain at the mating tip could lead to activation of the mating machinery spatially and temporally by specific lipid–protein interactions (Kalvodova et al., 2005). These interactions could involve integral proteins binding to raft lipids in the bilayer. For instance, the EGF receptor has been shown to be activated by interactions with the ganglioside Gd1a and the glutamate receptor by raft-cholesterol (Eroglu et al., 2003; Liu et al., 2004). Recently, it was demonstrated that yeast Ste5p, a protein that plays a crucial role in pheromone signaling and interacts with the cytoplasmic tail of Fus1p, has a phospholipid binding domain that is necessary for protein localization and signaling (Winters et al., 2005).

The generation of cell surface polarity during yeast mating is thus a complex process involving on one hand endocytosis and recycling and on the other hand establishment of the site where the mating machinery is scaffolded and the membrane reorganized. It is our contention that complex membrane processes such as cell surface polarization are driven by protein–protein and protein–lipid interactions. However, only future work directed specifically toward analysis of these issues will unravel the mechanisms involved.

Materials and methods

Strains and growth conditions

In this study the following yeast strains were used: RH690-15D [wild-type] (*Mata his4, leu2, ura3, lys2, bar1*) was obtained from H. Riezman (University of Basel, Basel, Switzerland), and RH1965 [end4Δ] (*Mata his4, leu2, ura3, lys2, bar1, end4::LEU2*) and RH268-1 [en4-1 ts] (*Mata his4, leu2, ura3, lys2, bar1, end4-1 (ts)*) were obtained from C. Walch-Solimena (MPJ-CBG, Dresden, Germany). 1302-WT (BY4742), *pea2, bni1, fus2, spa2, bud6, fus1 and ste5* deletions are in BY strains derived from S288C (*MATa; his31; leu20; met150; ura30*) and were obtained from EUROSCARF. Cells were grown overnight in yeast extract/peptone (YP) medium containing 2% raffinose (YPRaf) as a carbon source at 24°C. For the induction of expression from the GAL-S promoter, 2% galactose was added. To induce a mating response, 5 μM α-factor (Sigma-Aldrich) was added and cells were incubated for 3 h at 24°C (or as indicated).

Plasmids

Plasmids used in this study are listed in Table I. All constructs created in our lab are based on the centromeric plasmid p416 (Mumberg et al., 1995) and expression was driven from the inducible GAL-S promoter. Plasmids p4269, p4580, and p4667 containing mutants of *FUS1* under control of its own promoter were obtained from the C. Boone lab (University of Toronto, Toronto, Canada; Nelson et al., 2004). The plasmid containing GFP-SNC1 under control of constitutive TPI promoter was obtained from the H. Pelham lab (MRC Laboratory of Molecular Biology, Cambridge, UK; Lewis et al., 2000). Construction of plasmids MBQ30, MBQ35, TPQ53, and TPQ55 was described previously (Bagnat and Simons, 2002; Proszynski et al., 2004, 2005). Plasmids TPQ63, TPQ65, TPQ72, and TPQ57 were created by triple ligation method, where two PCR-amplified fragments of DNA are introduced into a vector (for details see Proszynski et al., 2004). To generate TPQ63, a DNA fragment coding the extracellular domain of Fus1p linked to the transmembrane domain (TMD) from Mid2p (amplified from plasmid TPQ55 with primers containing XbaI and BamHI sites) and a DNA fragment coding the cytoplasmic tail of Fus1p fused to GFP (amplified from TPQ53 with primers containing BglII and HindIII sites) were co-ligated to the MBQ1 vector digested with XbaI–HindIII. To create TPQ65, a fragment of DNA coding the extracellular domain of Mid2p linked to the TMD from Fus1p (amplified from plasmid TPQ53 with XbaI and BamHI sites added on the primers) and a DNA fragment containing the cytoplasmic tail of Mid2p fused to GFP (amplified from MBQ35 with BglII and HindIII sites added on the primers) were coligated into MBQ1 (XbaI–HindIII). To make TPQ72, a DNA fragment coding the extracellular domain and TMD of Fus1p (amplified from plasmid MBQ30 with primers containing XbaI and BamHI sites) and a DNA fragment containing the cytoplasmic tail of Mid2p fused to GFP (prepared as for TPQ65) were co-ligated to the XbaI–HindIII-digested vector MBQ1.

Plasmid TPQ57 was made by co-ligation of a DNA fragment coding GFP (flanked by BamHI–HindIII sites) with a DNA fragment coding the truncated (SH3Δ) version of Fus1p (amplified from MBQ30 with praimers containing XbaI–BglII sites) into the MBQ1 Vector (XbaI–HindIII).

Primers TPQ94 and TPQ97 were constructed by homologus recombination in RH690-15D cells. To generate TPQ94, a DNA fragment (obtained from TPQ63 with BglII–HindIII digestion) coding the TMD from *MID2* and the cytoplasmic tail from *FUS1*, followed by the GFP, was cotransformed with MBQ35 (linearized with BamHI). To create TPQ97, a DNA fragment coding the truncated cytoplasmic tail of Fus1p followed by the GFP coding sequence (PCR amplified from TPQ57) was cotransformed with the NheI-linearized TPQ97. The successful recombination was verified by observation of fluorescence in microscope and sequencing of the plasmids.

Microscopy

Microscopy was performed on live cells, resuspended in water. Images were taken with a microscope (model BX61; Olympus), a camera

Table I. **Plasmids used in this study**

Plasmid name	Expressing	Plasmid type	Source	Original name
MBQ30	Fus1-GFP	centromeric	Our previous studies	no
MBQ35	Mid2-GFP	centromeric	Our previous studies	no
TPQ53	Mid-Fus	centromeric	Our previous studies	no
TPQ55	Fus-Mid	centromeric	Our previous studies	no
TPQ63	Fus(TMD-Mid)	centromeric	This study	no
TPQ65	Mid(TMD-Fus)	centromeric	This study	no
TPQ72	Fus(cyt-Mid)	centromeric	This study	no
TPQ94	Mid(cyt-Fus)	centromeric	This study	no
TPQ97	Mid(cyt-FusΔSH3)	centromeric	This study	no
TPQ57	FusΔSH3	centromeric	This study	no
TPQ115	Fus1p(P422A)-SH3	centromeric	C. Boone lab[a]	p4269
TPQ116	Fus1p-SH3(W473S)	centromeric	C. Boone lab	p4580
TPQ117	Fus1p(P422A)-SH3(W473S)	centromeric	C. Boone lab	p4667
TPQ109	Snc1-GFP	centromeric	H. Pelham lab[b]	TPI-GFP-Snc1
TPQ1	(vector)	centromeric	W. Zachariae lab[c]	p416

[a]University of Toronto, Toronto, Canada.
[b]MRC Laboratory of Molecular Biology, Cambridge, UK.
[c]MPJ-CBG, Dresden, Germany.

(RT Slider SPOT; Diagnostic Instruments, Inc.), and MetaMorph software (Molecular Devices).

For Laurdan microscopy, cells were treated with 5 mM sodium azide and 4% paraformaldehyde for 10 min at 24°C; 250 μM Laurdan (Molecular Probes) was added for a further 5 min at 24°C and cells were washed twice and imaged in water. Laurdan fluorescence was excited at 800 nm with a Verdi/Mira 900 multi-photon laser system and intensity images were recorded simultaneously in the range of 400–445 nm and 445–530 nm for the two channels (Bio-Rad Laboratories), respectively. The generalized polarization GP, defined as

$$GP = \frac{I_{(400\text{-}460)} - I_{(470\text{-}530)}}{I_{(400\text{-}460)} + I_{(470\text{-}530)}}$$

was calculated for each pixel using the two Laurdan intensity images. GP images were pseudocolored in Adobe Photoshop. The GP values were determined at the mating tip or opposite the tip in a region measuring ~1.2 × 0.2 μm, and each data point (or symbol) in the scatter plots represents derivatives from one individual cell. GP values were corrected using the G-factor obtained for Laurdan in DMSO for each experiment. Means and standard deviation of multiple comparisons were compared with one-way ANOVA with Tukey's post-testing assuming Gaussian distributions (PRISM) (Gaus et al., 2003).

Online supplemental material

Fig. S1 shows polarized distribution of chimeric Mid(cyt-FusΔSH3) protein in end4-ts cells. Fig. S2 shows localization of Mid(cyt-FusΔSH3) in bni1Δ and WT cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200602007/DC1.

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