Supporting Information

Garrenton et al. 10.1073/pnas.1005817107

SI Results

The failure of *pik1*^{ts} cells, as well as *mss4*^{ts} and *stt4*^{ts} cells, to express a *FUS1*$_{prom}$-*lacZ* reporter at restrictive temperature was also observed by Winters et al. (1). On the basis of this observation, they concluded that a generally acidic character conferred by any phosphoinositide, and not interaction with PtdIns(4,5)P_2 per se, was necessary for recruitment of Ste5 to the plasma membrane. What they did not appreciate at the time is that, in addition to its function at the Golgi, Pik1 undergoes nucleocytoplasmic shuttling and is the PtdIns 4-kinase responsible for supplying the nuclear pool of PtdIns4P (2). Likewise, Mss4 also undergoes nucleocytoplasmic shuttling (3) and converts the nuclear PtdIns4P to PtdIns(4,5)P_2, and we have demonstrated elsewhere that PtdIns (4,5)P_2-derived inositol-*hexakis*phosphate (IP_6) is required for mRNA export (4). Because expression of β-galactosidase after *FUS1*$_{prom}$-*lacZ* induction requires nuclear export of the *lacZ* transcript and translation of that mRNA in the cytosol, the marked diminution of β-galactosidase production in response to pheromone in both *mss4*^{ts} and *pik1*^{ts} cells at the restrictive temperature is likely due to deficient mRNA export. However, Stt4 is a plasma membrane-associated enzyme that does not undergo nucleocytoplasmic shuttling (5). Hence, the observed lack of β-galactosidase production in response to pheromone in the *stt4*^{ts} mutant at restrictive temperature can be attributed only to defective signaling and not to a lack of mRNA export. Therefore, taken together, the results shown in Fig. 5*C* are fully consistent with the other evidence we have presented in this study showing that Stt4- and Mss4-derived PtdIns(4,5)P_2 at the projection tip is the specific lipid responsible for recruitment and function of Ste5, in keeping with the demonstrated PtdIns(4,5)P_2 binding preference of its PH domain (6).

1. Winters MJ, Lamson RE, Nakanishi H, Neiman AM, Pryciak PM (2005) A membrane binding domain in the ste5 scaffold synergizes with gbetagamma binding to control localization and signaling in pheromone response. *Mol Cell* 20:21–32.
2. Strahl T, Hama H, DeWald DB, Thorner J (2005) Yeast phosphatidylinositol 4-kinase, Pik1, has essential roles at the Golgi and in the nucleus. *J Cell Biol* 171:967–979.
3. Audhya A, Emr SD (2003) Regulation of PI4,5P_2 synthesis by nuclear-cytoplasmic shuttling of the Mss4 lipid kinase. *EMBO J* 22:4223–4236.
4. Weirich CS, et al. (2006) Activation of the DExD/H-box protein Dbp5 by the nuclear-pore protein Gle1 and its coactivator InsP6 is required for mRNA export. *Nat Cell Biol* 8: 668–676.
5. Audhya A, Emr SD (2002) Stt4 PI 4-kinase localizes to the plasma membrane and functions in the Pkc1-mediated MAP kinase cascade. *Dev Cell* 2:593–605.
6. Garrenton LS, Young SL, Thorner J (2006) Function of the MAPK scaffold protein, Ste5, requires a cryptic PH domain. *Genes Dev* 20:1946–1958.

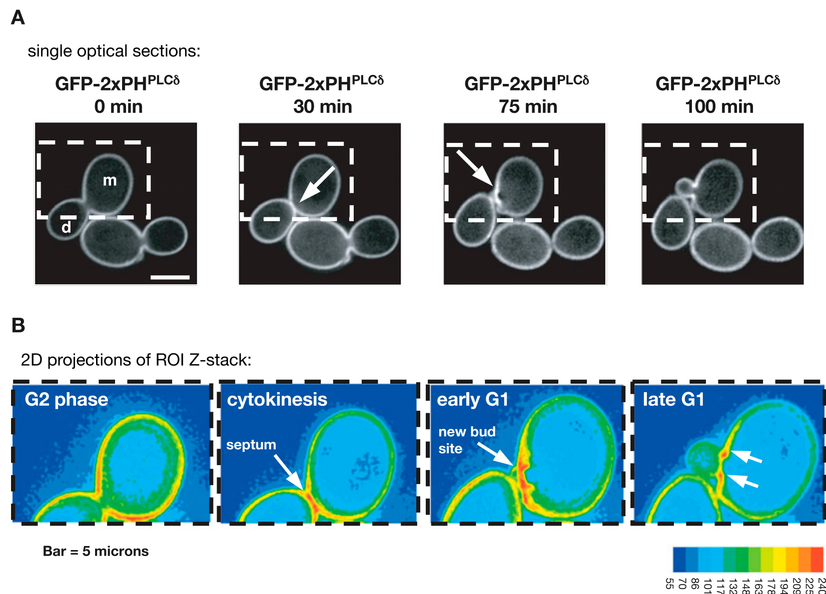

Fig. S1. Distribution of PtdIns(4,5)P_2 at various cell cycle stages assessed by deconvolution fluorescence microscropy. A culture of exponentially growing YCS234 expressing the PtdIns(4,5)P_2-binding fluorescent probe GFP-2XPH$^{PLC\delta1}$ as described in Fig. 1*B* was spread on an agarose pad in SC medium and imaged every 15 min over the course of the experiment. Optical sections (10 × 0.4 μm) were taken through the Z series, with 1-s exposures to visualize the GFP signal. Images were captured using a Deltavision system (Applied Precision) equipped with an Olympus IX-71 microscope, a 1.35 NA 100× Olympus objective, and a CoolSnap HQ camera (Photometrics). 2D projections of the Z series and pixel quantifications were performed using Softworx (Applied Precision). Projections reconstructed from the complete Z stack are shown for representative cells at the indicated phases of the yeast cell division cycle. False color images reflect the indicated scale of measured pixel intensity.

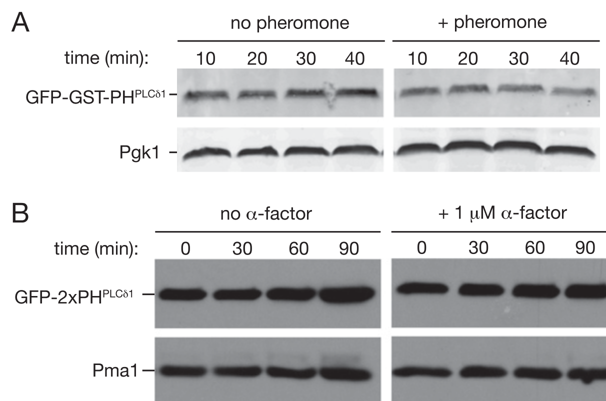

Fig. S2. The PtdIns(4,5)P_2-binding probes are stably expressed in naive and pheromone-treated cells. (*A*) Exponentially growing wild-type *MAT***a** cells (BY4741) carrying a 2-μm DNA vector expressing from the *GAL1* promoter the PtdIns(4,5)P_2-binding GST-GFP-PH$^{PLC\delta1}$ probe were induced by addition of galactose for 30 min and then harvested by brief centrifugation, washed, and resuspended in glucose-containing medium to shut off any further probe expression. After 20 min, the culture was divided into two equal portions, one of which was mixed with YPD and the other mixed with an equivalent volume of YPD containing α-factor-secreting *MAT*α cells (BY4742); samples were withdrawn at the indicated time points, the cells were harvested and lysed, and the resulting extracts were analyzed by SDS/PAGE and immunoblotting with anti-GFP and anti-Pgk1 (as a control for equivalent loading). (*B*) Exponentially growing *MAT***a** *sst1-5* cells (YCS234) carrying a 2-μm DNA vector expressing the PtdIns(4,5)P_2-binding GFP-2xPH$^{PLC\delta1}$ probe were harvested, washed, and resuspended at $A_{600\ nm}$ = 1/mL. The culture was divided into two equal portions, one of which was exposed to 1 μM α-factor. Aliquots were withdrawn at the indicated time points, the cells were harvested by TCA precipitation, acetone washed, and lysed, and the resulting extracts were analyzed by SDS/PAGE and immunoblotting with anti-GFP and anti-Pma1 (gift from G. Sprague, University of Oregon).

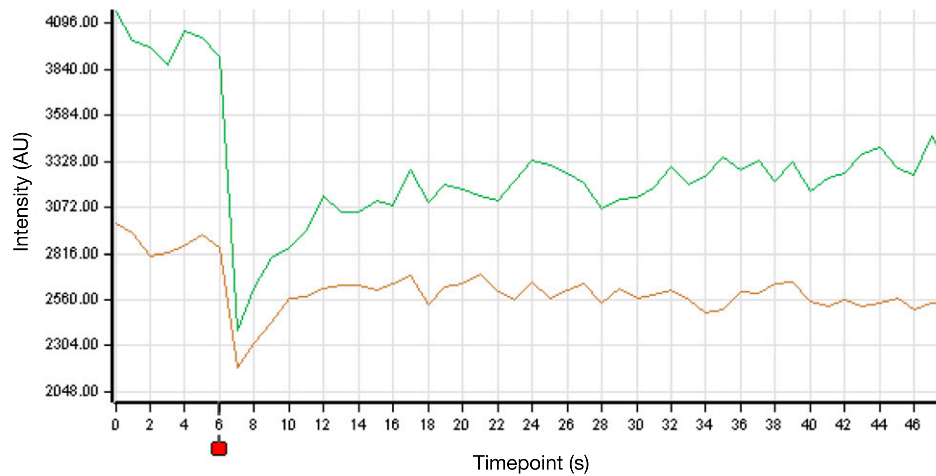

Fig. S3. FRAP analysis of GFP-2XPH$^{PLC\delta1}$ probe binding at different plasma membrane locations. Rates of GFP-2XPH$^{PLC\delta1}$ fluorescence recovery following photobleaching at the mating tip projection (green) and a nonpolarized region (brown) are shown. Data plotted are from the time frames captured in Movie S1. The red square indicates the time point at which the GFP-2XPH$^{PLC\delta1}$ signal was photobleached. FRAP analysis and pixel quantifications were performed using Slidebook 4.1 (Intelligent Imaging Innovations).

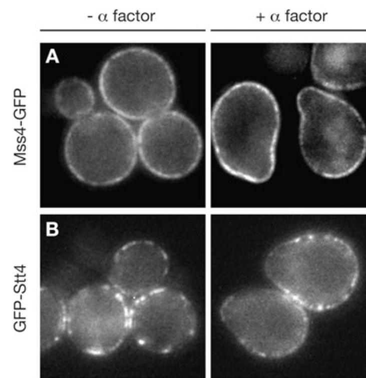

Fig. S4. Pheromone response does not affect the localization of the plasma membrane-associated lipid kinases Stt4 and Mss4. Exponentially growing cultures of *mss4Δ* cells harboring a low-copy (*CEN*) vector expressing Mss4-GFP (*A*) or *stt4Δ* cells harboring a low-copy (*CEN*) vector expressing GFP-Stt4 (*B*), as indicated, were either not treated (*Left*) or treated (*Right*) with 5 μM α-factor for 60 min and examined by standard epifluorescence microscopy.

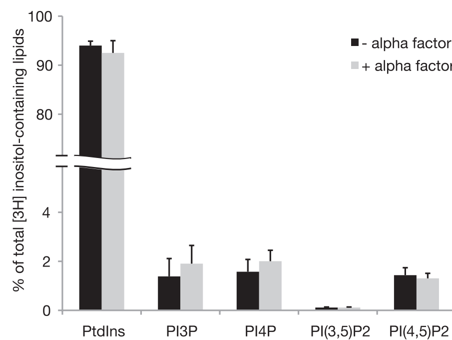

Fig. S5. Rate of phosphoinositide synthesis is not affected by pheromone treatment. Yeast cells (strain YCS234) from an exponentially growing culture (equivalent of $A_{600\ nm}$ = 10) were harvested, washed, and resuspended in inositol-free synthetic medium. These cells were divided into two equal cultures, one of which was exposed to 2 μM α-factor for 15 min, followed by the addition of 50 μCi of *myo*-[2-^{3}H]inositol (Perkin-Elmer), and incubated for an additional 60 min for a total of 75 min either in the absence or in the presence of α-factor. Preparation and analysis of cellular PtdIns-containing species were performed as described previously (1).

1. Stefan CJ, Audhya A, Emr SD (2002) The yeast synaptojanin-like proteins control the cellular distribution of phosphatidylinositol (4,5)-bisphosphate. *Mol Biol Cell* 13:542–557.

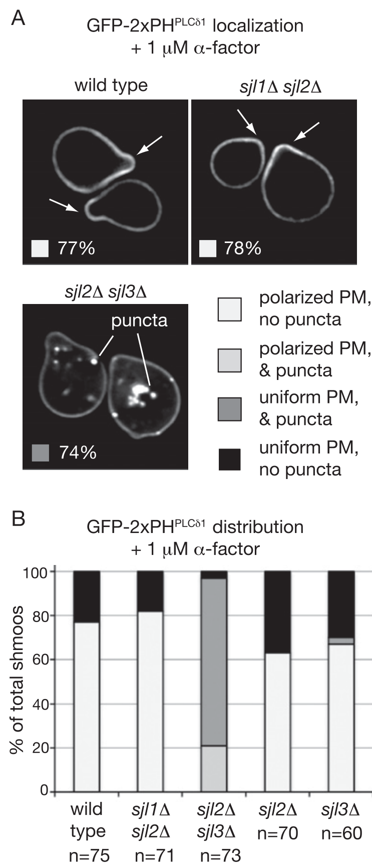

Fig. S6. The PtdIns(4,5)P_2 5-phosphatases Sjl2 and Sjl3 play a role in generating the plasma membrane PtdIns(4,5)P_2 gradient. Exponentially growing YCS234 (wild type), YCS239 (*sjl1*Δ *sjl2*Δ), YCS141 (*sjl2*Δ *sjl3*Δ), YCS236 (*sjl2*Δ), or YCS237 (*sjl3*Δ) cells expressing the PtdIns(4,5)P_2-binding fluorescent probe GFP-2XPH$^{PLC\delta1}$ were exposed to 1 μM α-factor for 90 min before being placed on a coverslip for imaging. Optical sections (8 × 0.4 μm) were taken through the Z series, with 1-s exposures to visualize the GFP signal. Images were captured using a Deltavision system (Applied Precision) equipped with an Olympus IX-71 microscope, a 1.35 NA 100× Olympus objective, and a CoolSnap HQ camera (Photometrics). Analysis of GFP-2XPH$^{PLC\delta1}$ localization was performed using Softworx (Applied Precision). (*A*) Representative single optical sections showing GFP-2XPH$^{PLC\delta1}$ localization in wild-type and mutant cells are provided and (*B*) quantification of the results is shown.

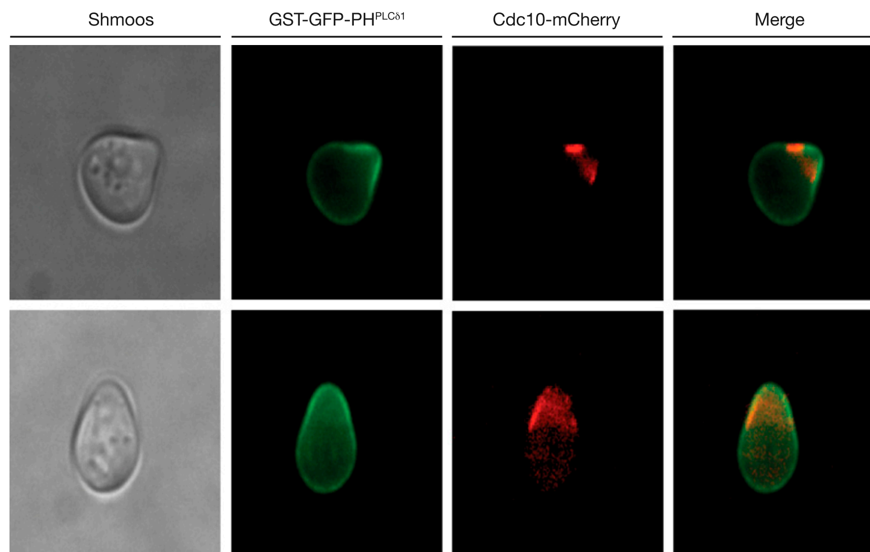

Fig. S7. Septins may serve as a barrier to restrict facile PtdIns(4,5)P_2 diffusion. Strain JTY3992 expressing Cdc10-mCherry from the *CDC10* promoter at the chromosomal *CDC10* locus and carrying plasmid pPP1872 expressing the PtdIns(4,5)P_2-binding GST-GFP-PH^{PLCd1} probe was grown in SCGlc−Ura at 30 °C to saturation. A sample of this stationary phase culture was inoculated onto 5 mL of SCGal−Ura and incubated at 30 °C for 2 h to resume growth and induce probe expression. The cells were then harvested by brief centrifugation, washed, and resuspended in SCRaf-Suc−Ura containing 2% glucose to shut off probe expression. After incubation at room temperature for 10 min, a sample of the culture was spread on an agarose pad on a microscope slide and then exposed to a gradient of pheromone by spotting 1 μL of a 1-mg/mL stock of α-factor at one edge of the pad. After 1.5 h, the cells that had just started to form shmoos (*Upper*) or had clearly formed projections (*Lower*) were visualized under a coverslip using a standard epifluoresence microscope.

Table S1. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype*	Source
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(1)
JTY3602[†]	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 trp1::LEU2*	B. Gullbrand, this laboratory
YLG36[‡]	*MAT***a** *trp1::LEU2 FUS1::FUS1-lacZ::ura3::TRP1*	This study
YPH499	*MAT***a** *ade2-101*^{oc} *his3-Δ200 leu2Δ-1 lys2-801*^{am} *trp1-Δ63 ura3-52*	(2)
BYB88	*MAT***a** *ste4Δ::TRP1 ste5Δ::LYS2*	(3)
JTY3125	*MAT***a** *mss4Δ::HIS3MX6 [CEN LEU2 mss4-102*^{ts}*]*	T. Strahl, this laboratory
YFR201	*MAT***a** *stt4Δ::HIS3 [CEN LEU2 stt4-4*^{ts}*]*	F. Roelants, this laboratory
YES95	*MAT***a** *pik1-63::TRP1*	(4)
YFR203	*MAT***a** *stt4Δ::HIS3 pik1-63::TRP1 [CEN LEU2 stt4-4*^{ts}*]*	F. Roelants, this laboratory
YLG65[§]	*MAT***a** *vps34Δ::HIS3*	This study
YLG70[¶]	*MAT***a** *FUS1::FUS1-lacZ::LYS2*	This study
YLG71[¶]	*MAT***a** *mss4Δ::HIS3MX6 FUS1::FUS1-lacZ::LYS2 [CEN LEU2 mss4-102*^{ts}*]*	This study
YLG72[¶]	*MAT***a** *stt4Δ::HIS3 FUS1::FUS1-lacZ::LYS2 [CEN LEU2 stt4-4*^{ts}*]*	This study
YLG73[¶]	*MAT***a** *pik1-63::TRP1 FUS1::FUS1-lacZ::LYS2*	This study
YLG74[¶]	*MAT***a** *stt4Δ::HIS3 pik1-63::TRP1 FUS1::FUS1-lacZ::LYS2 [CEN LEU2 stt4-4*^{ts}*]*	This study
YLG76[¶,‖]	*MAT***a** *mss4Δ::HIS3MX6 [CEN URA3 MSS4-GFP]*	This study
YLG77[¶,‖]	*MAT***a** *stt4Δ::HIS3 [CEN URA3 GFP-STT4]*	This study
YLG80**	*MAT***a** *ste5Δ*	This study
YLG81**	*MAT***a** *mss4Δ::HIS3MX6 ste5Δ [CEN LEU2 mss4-102*^{ts}*]*	This study
YLG82**	*MAT***a** *stt4Δ::HIS3 ste5Δ [CEN LEU2 stt4-4*^{ts}*]*	This study
YLG83**	*MAT***a** *pik1-63::TRP1 ste5Δ*	This study
YLG84**	*MAT***a** *stt4Δ::HIS3 pik1-63::TRP1 ste5Δ [CEN LEU2 stt4-4*^{ts}*]*	This study
YLG85[§, **]	*MAT***a** *vps34Δ::HIS3 ste5Δ*	This study
JTY3992[††]	BY4741 *cdc10::CDC10-mCherry::KanMX*	This study
SEY6210.1	*MAT***a** *leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	(5)
YCS234	*MAT***a** *sst1-5* derivative of SEY6210.1	This study
YCS236[‡‡]	*MAT***a** *sjl2Δ::HIS3* derivative of YCS234	This study
YCS237[‡‡]	*MAT***a** *sjl3Δ::TRP1* derivative of YCS234	This study
YCS239[‡‡]	*MAT***a** *sjl1Δ::HIS3 sjl2Δ::HIS3* derivative of YCS234	This study
YCS141[‡‡]	*MAT***a** *sjl2Δ::HIS3 sjl3Δ::TRP1* derivative of YCS234	This study

*Unless otherwise noted, all strains are derived from the YPH499 background.

[†]The *TRP1* locus in BY4741 was replaced with *LEU2* by "marker swap" (6), using a XhoI-SmaI fragment of plasmid pTL7, followed by selection for Leu$^+$ transformants, which were subsequently tested to confirm their Trp$^-$ phenotype.

[‡]Integration of the *FUS1::FUS1*$_{prom}$*-lacZ::URA3* reporter gene at the *FUS1* locus in JTY3602 was accomplished by linearizing pSB286 (7) with SphI, followed by selection for Ura$^+$ transformants. When necessary, the *URA3* locus was replaced with TRP1, which was accomplished using the "marker-swap" plasmid pUT11 that had been linearized with SmaI, followed by selection for Ura$^-$ Trp$^+$ transformants as described elsewhere (6).

[§]To disrupt the *VPS34* locus, strains were transformed with an XbaI-SpeI fragment of pKTY37 (8), followed by selection for His$^+$ colonies, and then scored for temperature sensitivity and "nonpink" phenotype. Proper gene replacement was verified by PCR.

[¶]Integration of the *FUS1::FUS1*$_{prom}$*-lacZ::LYS2* reporter gene was accomplished by transformation of pFL-LYS linearized with SphI, followed by selection for Lys$^+$ transformants as described elsewhere (9).

[‖]To generate YLG76 and YLG77, YLG71 and YLG72 were transformed with pCS321 (10) or pRS416-GFP-Stt4 (11), respectively, followed by selection for Ura$^+$ Leu$^-$ colonies.

**To delete the *STE5* locus, strains were transformed with pCJ99 (12) and linearized with BglII, followed by pop-out of the wild-type allele on 5-FOA medium (13). The Ura$^-$ colonies were scored for sterile phenotype and proper gene replacement was verified by complementation.

[††]Generated as described before for constructing a similar derivative of BY4742 (14).

[‡‡]Generated by procedures described previously (15).

1. Brachmann CB, et al. (1998) Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: A useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* 14:115–132.
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6. Cross FR (1997) 'Marker swap' plasmids: Convenient tools for budding yeast molecular genetics. *Yeast* 13:647–653.
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11. Audhya A, Emr SD (2002) Stt4 PI 4-kinase localizes to the plasma membrane and functions in the Pkc1-mediated MAP kinase cascade. *Dev Cell* 2:593–605.
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13. Boeke JD, Trueheart J, Natsoulis G, Fink GR (1987) 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods Enzymol* 154:164–175.
14. McMurray MA, Thorner J (2008) Septin stability and recycling during dynamic structural transitions in cell division and development. *Curr Biol* 18:1203–1208.
15. Stefan CJ, Audhya A, Emr SD (2002) The yeast synaptojanin-like proteins control the cellular distribution of phosphatidylinositol (4,5)-bisphosphate. *Mol Biol Cell* 13:542–557.

Movie S1. Exponentially growing YCS234 cells expressing the PtdIns(4,5)P_2-binding fluorescent probe GFP-2XPH$^{PLC\delta1}$ were exposed to 1 μM α-factor for 90 min before being placed on a coverslip and imaged over the course of a 100-s experiment. Images of single optical sections, using 0.1-s exposures to visualize the GFP signal, were captured every second using a spinning disk confocal system (3i) equipped with a Leica DMI6000B microscope, a 1.4 NA 63× objective, CSU-X1 spinning disk confocal system (Yokogawa), and a QuantEM camera (Photometrics). Regions of interest (ROIs) for FRAP analysis were photobleached by a 0.5-s exposure using a Mosaic system (Photonic Instruments); ROIs for the mating tip projection (green) and a nonpolarized region (brown) are indicated. Five frames per second are shown in this representative movie; data are replotted in Fig. S3. Three other cells were examined similarly and yielded virtually identical results.

Movie SI