Effect of the Pheromone-Responsive G_{α} and Phosphatase Proteins of *Saccharomyces cerevisiae* on the Subcellular Localization of the Fus3 Mitogen-Activated Protein Kinase

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The mating-specific G_{α} protein of *Saccharomyces cerevisiae*, Gpa1, stimulates adaptation to pheromone by a mechanism independent of $G_{\beta\gamma}$ sequestration. Genetic evidence suggests that Gpa1 targets the Fus3 mitogen-activated protein kinase, and it has recently been shown that the two proteins interact in cells responding to pheromone. To test the possibility that Gpa1 downregulates the mating signal by affecting the localization of Fus3, we created a Fus3-green fluorescent protein (GFP) fusion protein. In vegetative cells, Fus3-GFP was found in both the cytoplasm and the nucleus. Pheromone stimulated a measurable increase in the ratio of nuclear to cytoplasmic Fus3-GFP. In contrast, the relative level of nuclear Fus3-GFP decreased as cells recovered from pheromone arrest and did not increase when cells adapted to chronic stimulus were challenged again. Accumulation of Fus3-GFP in the nuclei of stimulated cells was also inhibited by overexpression of either wild-type Gpa1, the E364K hyperadaptive mutant form of Gpa1, or the Msg5 dually specific phosphatase. The effects of Gpa1 and Msg5 on Fus3 are partially interdependent. In a genetic screen for adaptive defective mutants, a nonsense allele of the nucleocytoplasmic transport receptor, Kap104, was identified. Truncation of the Kap104 cargo-binding domain blocked the effect of both Gpa1^{E364K} and Msg5 on Fus3-GFP localization. Based on these results, we propose that Gpa1 and Msg5 work in concert to downregulate the mating signal and that they do so by inhibiting the pheromone-induced increase of Fus3 in the nucleus. Kap104 is required for the G_{α} /phosphatase-mediated effect on Fus3 localization.

When haploid yeast cells of opposite mating type are mixed, they undergo a complex mating reaction, leading to the formation of zygotes. Each mating type constitutively secretes a peptide mating pheromone that triggers cells of the opposite type to arrest in the G_1 phase of the cell cycle, form projections, and induce mating-specific genes in preparation for cell and nuclear fusion. The signal to mate is transmitted across the plasma membrane by a receptor-coupled heterotrimeric G protein. Upon release from G_{α} (Gpa1), the $G_{\beta\gamma}$ dimer (Ste4/Ste18) binds to the Ste5 scaffolding protein and stimulates a Pak kinase (Ste20). These proteins, in turn, are required for activation of the mitogen-activated protein kinase (MAPK) cascade (Ste7, Ste11, and Fus3) that ultimately communicates with regulators of transcription and cell division in the nucleus (reviewed in references 12 and 22).

In addition to the proteins that transmit the mating signal, a number of gene products have been implicated in negative regulation of the pheromone response. These include cell-type-specific proteases that degrade the pheromones (39, 40), elements that upregulate G_1 cyclins (34) and elements that downregulate the pheromone receptors (29, 52), G_{α} (10, 11), $G_{\beta\gamma}$ (27, 48, 59), the Fus3 MAPK (13, 68), and Cdc42 (60). Indirect effects on the mating pathway by global regulators of gene expression (e.g., Cdc36, Cdc39, Srm1, Mot2, and Mot3) have also been detected (5, 8, 21, 23, 31, 38, 47). These various

negative regulators may serve distinct purposes. Some may ensure that the mating signal, which is propagated by constitutively expressed proteins, is not induced inappropriately. Others might provide a link to other signaling pathways. Still others may be involved in turning off the G_1 arrest signal and promoting transition to S phase when the cell is chronically stimulated or immediately after zygote formation.

We are studying adaptive mechanisms that are stimulated by the pheromone-responsive G_{α} protein, Gpa1 (36, 62, 69). Recently, we have shown that Gpa1 binds directly to the mating-specific MAPK, Fus3 (44). Disruption of this interaction by mutation confers increased sensitivity to pheromone and a defect in Gpa1-mediated adaptation. These findings led us to ask whether Gpa1 regulates cell sensitivity to pheromone by affecting the localization of Fus3. Because MAPK signaling modules connect signaling elements located at the plasma membrane to response effectors in the nucleus, it has been presumed that, upon activation, MAPKs must be transported from the cytoplasm to the nucleus. Although some MAPKs clearly undergo signal-induced nuclear translocation (reviewed in references 26 and 50), others constitutively shuttle between the cytoplasm and nucleus and show less obvious nuclear concentration when activated (65). Hog1, the osmoregulatory MAPK of yeast, exemplifies the first class. When cells are subjected to osmotic shock, Hog1 exhibits a dramatic shift from the cytoplasm to the nucleus that is dependent on the Ran GTPase, Gsp1, and the Nmd5 importin. Conversely, Hog1 is actively exported from the nucleus by a mechanism requiring Xpo1/Crm1 as the cells adapt to the environmental change. The translocation of Hog1 is regulated by its phosphorylation

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Relevant genotype	Source or reference
15Dau	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ</i>	49
DSY245	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ</i> YIplac204/ <i>GAL1</i> ^{EG28} - <i>GPA1</i> ^{E364K}	Lab stock
DSY253	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ</i> YIplac128/ <i>GAL1</i> ^{EG28} - <i>GPA1</i> (2X)	Lab stock
DSY278	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ</i> YIplac204/ <i>GAL1</i> ^{EG28} - <i>GPA1</i> ^{E364K} FUS1-LEU2::HIS2	36
A11	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ kap104</i> ^{Δ827-918} YIplac204/ <i>GAL1</i> ^{EG28} - <i>GPA1</i> ^{E364K} FUS1-LEU2::HIS2	This study
ZWY432	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ kap104Δ::URA3</i> YCplac111/ <i>kap104</i> ^{Δ827-918}	This study
IHY169	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ msg5Δ::LEU2</i> YIplac204/ <i>GAL1</i> ^{EG28} - <i>GPA1</i> ^{E364K} FUS1-LEU2::HIS2	This study
HKY137	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ msg5Δ::LEU2</i>	This study
HKY114	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ ptp3Δ::KanMx</i>	This study
HKY161	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ msg5Δ::LEU2 ptp3Δ::KanMx</i>	This study
IHY165	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ fus3Δ::LEU2 kss1Δ::KanMx</i>	This study
IHY205	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ fus3Δ::LEU2 kss1Δ::KanMx</i> YCplac33/ <i>FUS3</i> ^{K42R} -GFP	This study
IHY217	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ fus3Δ::LEU2 kss1Δ::KanMx</i> YIplac204/ <i>GAL1</i> ^{EG28} - <i>GPA1</i> ^{E364K} YCplac33/ <i>FUS3</i> ^{K42R} -GFP	This study
AEY144	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ kap104Δ::HISG</i> YCplac111/ <i>kap104</i> ^{Δ827-918}	This study
AOY135	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ kap104Δ::HISG</i> YIplac204/ <i>GAL1</i> ^{EG28} - <i>GPA1</i> ^{E364K} YCplac111/ <i>kap104</i> ^{Δ827-918}	This study
TBY137	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ msg5Δ::LEU2 ptp3Δ::KanMx</i> YIplac204/ <i>GAL1</i> ^{EG28} - <i>GPA1</i> ^{E364K}	This study
TBY141	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ ptp3Δ::KanMx</i> YIplac204/ <i>GAL1</i> ^{EG28} - <i>GPA1</i> ^{E364K}	This study
TBY147	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ msg5Δ::LEU2</i> YIplac204/ <i>GAL1</i> ^{EG28} - <i>GPA1</i> ^{E364K}	This study
TBY155	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ</i> YIplac204/ <i>GAL1</i> ^{EG28} - <i>GPA1</i> ^{K21E R22E E364K}	This study
TBY163	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ msg5Δ::LEU2 ptp3Δ::KanMx</i> YIplac128/ <i>GAL1</i> ^{EG28} - <i>GPA1</i> (2X)	This study
MMY107	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ gpa1Δ::URA3</i> YIplac128/ <i>GAL1</i> ^{EG43} - <i>GPA1</i> ^{K21E R22E} YCplac22/ <i>GPA1</i> ^{K21E R22E}	44
DSY419	<i>MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ gpa1^{ts}</i> Fus1-lacZ::URA3	Lab stock

state: whereas Hog1 is released from its upstream activator (Pbs2) and moved to the nucleus when phosphorylated (15, 42), the Ptp2 and Ptp3 phosphatases dephosphorylate Hog1 during adaptation to osmotic stress (25, 51). Ptp2 and Ptp3 are also thought to affect the localization of Hog1 by tethering it on opposite sides of the nuclear membrane (42). In contrast to Hog1, Fus3 constitutively shuttles between the cytoplasm and nuclei of vegetative cells and is easily detectable in both compartments. Surprisingly, there is only a slight increase in the nuclear localization of Fus3 in response to pheromone stimulation (6, 65), and there is no apparent change in the rates of Fus3 import or export (65). Here we quantify the signal-induced increase in nuclear Fus3 and show that the relative abundance of Fus3 in the nucleus and cytoplasm is correlated with sensitivity to pheromone and with the duration of the mating response. In addition, we present evidence that Gpa1 and the Msg5 phosphatase negatively regulate the mating signal by antagonizing the accumulation of Fus3 in the nucleus and that this phenomenon is dependent on the yeast homologue of transportin, Kap104.

MATERIALS AND METHODS

Molecular and microbiological techniques. Recombinant DNA techniques were essentially as described by Sambrook et al. (54) and by Ausubel et al. (4). Dideoxy sequence reactions were performed by using the Sequenase version 2.0 kit (U.S. Biochemicals, Cleveland, Ohio).

Yeast transformations were carried out according to the method of Ito et al.

(24). Yeast growth media were prepared as described by Sherman et al. (55). Amino acids were omitted as necessary to select for plasmids. For experiments requiring the induction of the *GAL1* regulated genes, either cells were grown to mid-log phase in sucrose medium and galactose was added to a final concentration of 2% or the cells were cultured overnight in medium containing galactose.

Immunoblots were performed as previously described (35). Strip-purified antiserum raised against Gpa1 (62) and crude antiserum raised against Kap104 (generously provided by John Aitchison) were used as the primary antibodies.

Plasmids and strains. All strains used in the present study were derived from strain 15Dau (*MATa bar1Δ ade1 his2 leu2-3,112 trp1 ura3Δ*), which is isogenic with strain BF264-15D (49), with the exception of the *gpa1* temperature-sensitive strain. The temperature-sensitive allele of *GPA1* was isolated in strain 381G, and the mutant was backcrossed twice to strain 15D. The strains are listed in Table 1. The parent strain for the adaptive-defective (adaptive⁻) mutant hunt, DSY278, was constructed by integrating the *GAL1*^{EG28}-*GPA1*^{E364K} and *FUS1-LEU2* transcriptional fusions at the *TRP1* and *HIS2* loci of 15Dau, respectively (36). Strain DSY278 was shown to contain a single copy of *GAL1*^{EG28}-*GPA1*^{E364K} by southern blot analysis. The A11 mutant strain was backcrossed twice before analysis. Deletion strains were constructed by transformation with disruption plasmids and PCR products containing the yeast markers *URA3* and *LEU2* and the bacterial markers *HISG* and *KanMx* (Table 2). Disruptions were confirmed by genomic PCR analysis and by pheromone response assays. Plasmid DSB174 (YIplac204/*GAL1*^{EG28}-*GPA1*^{E364K}) (62) was integrated into the *TRP1* locus of the *msg5Δ*, *ptp3Δ*, and *msg5Δ ptp3Δ* strains to create strains TBY147, TBY141, and TBY137, respectively. Plasmid TBB105 (YIplac204/*GAL1*^{EG28}-*GPA1*^{K21E R22E E364K}) was integrated into the *TRP1* locus of wild-type cells (strain 15Dau) to create strain TBY155. In all cases, transformants resulting from single-copy integration were identified by Southern blot. To construct strain TBY163, the *msg5Δ ptp3Δ* strain (HKY161) was switched to *MATa* by induction of a *GAL1*-HO plasmid and crossed to strain DSY253 (62), which contains two copies of *GAL1*^{EG28}-*GPA1* integrated at the *LEU2* locus. After sporulation of

TABLE 2. Plasmids used in this study

Plasmid	Marker	Source
pEL4-1	URA3 CEN KAP104	This study
kap104 ^{Δ827-918} /YCplac111	LEU2 CEN kap104 ^{Δ827-918}	This study
KAP104/YIplac211	URA3 integrative KAP104	This study
kap104Δ::URA3/pUC19	URA3 integrative kap104Δ	This study
kap104Δ::hisG-URA3-hisG/pUC19	URA3 integrative kap104Δ	This study
pYES/KAP104	URA3 2μ GAL1-KAP104	This study
piH4.1	URA3 CEN FUS3-GFP	This study
piH4.1AA	URA3 CEN FUS3 ^{T180A Y182A} -GFP	This study
piH4.1KM1-1	URA3 CEN FUS3 ^{K42R} -GFP	This study
YCplac22/FUS3-GFP (AOB117)	TRP1 CEN FUS3-GFP	This study
pRS314Nab2-GFP	URA3 CEN NAB2-GFP	14
pRS316CG/MSG5-GFP (HKB101)	URA3 CEN MSG5-GFP	This study
YCplac111/GAL1-MSG5 (HKB100)	LEU2 CEN GAL1-MSG5	This study
YCplac22/GAL1-MSG5 (AEB101)	TRP1 CEN GAL1-MSG5	This study
YCplac111/Gpa1 ^{E364K} (DSB117)	LEU2 CEN GPA1 ^{E364K}	61
YCplac33/Gpa1 ^{E364K} (HSB105)	URA3 CEN GPA1 ^{E364K}	36
YCplac22/GPA1 ^{K21E R22E}	TRP1 CEN GPA1 ^{K21E R22E}	44
YIplac204/GAL1 ^{EG28} -GPA1 ^{E364K}	TRP1 GPA1 ^{E364K}	62
YIplac204/GAL1 ^{EG28} -GPA1 ^{K21E R22E E364K} (TBB105)	TRP1 GPA1 ^{K21E R22E E364K}	This study
YIplac128/GAL1 ^{EG28} -GPA1	LEU2 GPA1	62
YCplac111/GAL1-PTP3 (TBB104)	LEU2 CEN GAL1-PTP3	This study

the resulting diploid, a *MATa msg5Δ ptp3Δ GAL1^{EG28}-GPA1* segregant was identified by tetrad analysis.

The plasmids used in the present study are listed in Table 2. The key details of newly constructed plasmids are described below.

(i) **pEL4-1.** Plasmid pEL4-1 was recovered in the screen for complementation of the adaptive[−] phenotypes of strain A11. This plasmid contains the 2.75-kb *KAP104* coding sequence on a 4.8-kb genomic insert.

(ii) **KAP104/YIplac211.** For integrative mapping, the *SstI-SphI* fragment containing the coding region of *KAP104* and 2.1 kb of flanking sequence was subcloned from pEL4-1 to YIplac211, an integrative *URA3* vector (18).

(iii) **kap104Δ::URA3/pUC19.** The *SstI-SphI* fragment containing the coding region of *KAP104* and 2.1 kb of flanking sequence was subcloned from pEL4-1 to pUC19 to create KAP104/pUC19. The *KAP104* disruption plasmid, kap104Δ::URA3/pUC19, was then generated by replacing the 1.7-kb *BglII-EcoRV* fragment internal to *KAP104* on pUC19/KAP104 with a 1.1-kb fragment containing the *URA3* gene.

(iv) **kap104Δ::hisG-URA3-hisG/pUC19.** Plasmid pNKY51 (2) was digested with *Bam*HI, and the ends were filled by reaction with Klenow. The 3.8-kb fragment containing *hisG-URA3-hisG* was then released by digestion with *Bgl*II and used to replace the 1.7-kb *EcoRV-Bgl*II fragment internal to the *KAP104* coding region on plasmid KAP104/pUC19.

(v) **pYES/KAP104.** The *KAP104* coding region (from −9 to 3062 where the ATG is at position 1) was amplified by PCR with the following oligonucleotides: 5'-GCG GAT CCT GAA TAA AGA TGG CAT CGA C-3' (which contains a *Bam*HI site) and 5'-GGG CAT GCG CAA AAG TAT TCA AGA ATG-3' (which contains a *Sph*I site). Two independently generated PCR products were subcloned into pYES2.0 (Invitrogen, San Diego, Calif.) at the *Bam*HI and *Sph*I sites, thereby creating transcriptional fusions between *KAP104* and the *GAL1* promoter on the 2μ-based pYES vector.

(vi) **kap104^{Δ827-918}/YCplac111.** The C-terminal truncation allele of *KAP104* was created by PCR mutagenesis by using KAP104/pUC19 as a template and the following oligonucleotides as primers: 5'-TCA GTT GGA ATC ATT GGC AAA TGC-3' and 5'-TAA AAT TTT TTT TAC TGT ATC CTA CG-3'. The C-terminal truncation allele of *KAP104* allele was then moved to YCplac111 (18) as an *SstI-SphI* fragment. Deletion of the 273 target bases was confirmed by sequencing.

(vii) **piH4.1.** The *FUS3* locus was PCR amplified from a genomic template with the following primers: 5'-GGGAATTCGACTACTGGACATTCAGGG-3', which anneals 1,000 bp upstream of the ATG and contains an *Eco*RI site, and 5'-GGCCGCGGACTAAATAATTCGTTCCAAATGAG-3', which anneals to the 3' end of the coding sequence and contains a *Sac*II site. The *FUS3* PCR product was then subcloned to *Eco*RI-*Sac*II-cut pRS316CG (37), thus dropping out the *CUP1* promoter and fusing the green fluorescent protein (GFP) coding sequence, in frame, to the 3' end of *FUS3*.

(viii) **piH4.1AA (FUS3^{T180A Y182A}-GFP) and piH4.1KM1-1 (FUS3^{K42R}-GFP).**

The mutant forms of Fus3-GFP were created by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, Calif.) and plasmid piH4.1 as a template. The primers used to create the T180A Y182A double mutation were 5'-GCAAGCGGCATGCGCCGAGGCTGTGGCCACACGTTG-3' and 5'-CA ACGTGTGGCCACAGCCTCGGCCATGCCGCTTTGC-3'. The primers used to create the K42R mutation were 5'-GGAGAAATCGTGGCAATAAGAAA GATCGAACCATTTCGAT-3' and 5'-ATCGAATGGTTTCGATCTTCTTATT GCCACGATTTCCTCC-3'. The substitutions are underlined.

(ix) **YCplac22/FUS3-GFP (AOB117).** The *Eco*RI-*Sac*I fragment containing the FUS3-GFP translational fusion was moved from piH4.1 to YCplac22 (18).

(x) **YCplac111/GAL1-MSG5 (HKB100) and YCplac22/GAL1-MSG5 (AEB101).** The 0.680kb *Bam*HI-*Eco*RI fragment containing the divergent *GAL1/GAL10* promoter was moved from YCpG2 (43) to the polylinkers of YCplac22 and YCplac111 (18) to create plasmids ZWB159 and ZWB161, respectively. The *MSG5* coding region (−6 to 1470) was amplified from genomic DNA with the following primers: 5'-CGCGGATCCGTGCACATGCAATTTCAC-3', which contains a *Bam*HI site, and 5'-AACTGCAGTTAAGGAAGAAACATCATCTG-3', which contains a *Pst*I site. The *MSG5* PCR product was then digested with *Bam*HI and *Pst*I and ligated to *Bam*HI-*Pst*I-cut ZWB159 and ZWB161 plasmid DNA to create the *GAL1-MSG5* transcriptional fusion vectors.

(xi) **YCplac22/GAL1-PTP3 (TBB104).** The *PTP3* coding region (−238 to 2605) was amplified from genomic DNA by using the following primers: 5'-AAG GATCCTTGCACCGCGCGGATGTAACACTA-3', which contains a *Bam*HI site, and 5'-AACTGCAGTTCTTGACCATGGATATTCG-3', which contains a *Pst*I site. The *PTP3* PCR product was then digested with *Bam*HI and *Pst*I and ligated to the backbone of *Bam*HI-*Pst*I-cut AEB101 plasmid DNA, thereby changing the *GAL1-MSG5* transcriptional fusion to a *GAL1-PTP3* transcriptional fusion.

(xii) **YIplac204/GAL1^{EG28}-GPA1^{K21E R22E E364K} (TBB105).** The *gpa1^{K21E R22E E364K}* allele was isolated from plasmid MMB107 (YCplac22/GPA1^{K21E R22E E364K}) (44) as a *Bam*HI-*Sst*I fragment and used to replace the *GPA1^{E364K}* allele on plasmid DSB174 (YIplac204/GAL1^{EG28}-GPA1^{E364K}) (62). *GAL1^{EG28}* is ca. 18% as active as the native *GAL1* promoter (20).

(xiii) **pRS316CG/MSG5-GFP (HKB101).** The *MSG5* locus (−381 to 1467) was PCR amplified from a genomic template by using the following primers: 5'-CCCTCGAGATGGTCCATCCTGGTAAG-3', which contains an *Ava*I site, and 5'-TCCCGCGGAGGAAGAAACATCATCTGTT-3', which contains a *Sac*II site. The *MSG5* PCR product was then subcloned to *Ava*I-*Sac*II-cut pRS316CG to create an in-frame *MSG5-GFP* translational fusion.

Construction of the A6 genomic library used to clone KAP104. Yeast genomic DNA was extracted from the *STE4* mutant strain A6, a derivative of strain 15Dau, and partially digested with *Sau*3A. The restricted DNA was fractionated by centrifugation through a 10 to 40% sucrose gradient, and 7- to 15-kb fragments were purified and ligated to YCplac33 plasmid DNA (18) digested with *Bam*HI. The ligation mix was then used to transform *Escherichia coli* DH5α. A

FIG. 1. Pheromone induces nuclear accumulation of Fus3. Strains of the indicated genotype were transformed with the indicated GFP reporters and grown to mid-log phase. The cultures were then split and grown with or without the addition of 12 nM α -factor. Images were acquired from the untreated cultures and at various times after the addition of pheromone. The RNCF values were determined as described in Materials and Methods. In panels B to E, distributions of RNCF values are represented in histograms. The number of cells (y axis) are plotted as a function of the RNCF values (x axis). In each panel, the untreated cells are represented in the top histogram, and the cells treated for 3 h with pheromone

total of 54,000 transformants were recovered, of which approximately two-thirds contained an insert. The transformants were washed off the plates and grown in 38 ml of Luria broth plus ampicillin overnight at 37°C, and plasmid DNA was prepared by standard methods. Among 10 randomly picked transformants, the average insert size was 7.2 kb. A library of 33,000 plasmids each containing a genomic insert of roughly 7 kb is expected to saturate the yeast genome (54).

Pheromone response and growth assays. (i) **Halo tests.** Strains were tested for pheromone-induced growth inhibition in standard halo assays as previously described (9).

(ii) **Measurement of *FUS1-lacZ* activity.** *FUS1* promoter activity was assayed in cells transformed with the centromeric reporter plasmid, pSB231 (64); β -galactosidase activity was measured as previously described (57).

(iii) **Single-colony formation assay.** Strain 15Dau was transformed with two independently derived high-copy-number *GAL1-KAP104* vectors, pYES/KAP104-A and pYES/KAP104-B. Transformants were grown to mid-log phase in galactose medium, and ca. 10^3 cells were spread onto plates containing a range of pheromone concentrations. The plates were incubated at 30°C for 6 days, and visible colonies were counted.

Genetic screens and characterization. Backcrosses were performed as described previously (36).

Integrative mapping. To confirm that pEL4-1 contains the gene corresponding to the adaptive⁻ allele in strain A11, plasmid KAP104/YIplac211 was linearized with *Bgl*II, which is in the C terminus of *KAP104*, and used to transform a *MAT α* derivative of strain DSY278. Uracil prototrophs were selected, and the transformants were subsequently crossed to strain A11. Strain A11 is prototrophic for leucine because its adaptive⁻ mutation confers constitutive induction of its integrated *FUS1-LEU2* reporter. The resulting diploid strains were sporulated, dissected, and scored for leucine prototrophy.

Cloning of *KAP104*. The wild-type gene corresponding to the mutant allele in strain A11 was cloned by complementation. The A6 yeast genomic plasmid library was introduced into strain A11, and transformants were selected on synthetic medium lacking uracil and containing α -factor at a concentration of 9 nM. Pheromone resistant transformants were cultured on fresh medium lacking uracil and α -factor and then cured of the transforming plasmids by growth on plates containing 5-fluoroorotic acid. The growth of the cured strains was then compared to the growth of the original transformants on synthetic complete medium containing a 9 nM concentration of α -factor. Only one plasmid of 25,000 screened rendered A11 mutant cells resistant to this dose of pheromone. This plasmid, pEL4-1, was amplified and shown to normalize all of the A11 mutant phenotypes (see below). The 2.75-kb *KAP104* coding sequence was located on the 4.8-kb genomic insert by creating a set of unidirectional nested deletions by using the Exo-Mung deletion kit (Stratagene).

Photomicrographs and quantification of Fus3-GFP localization. Fluorescent and differential interference contrast (DIC) images were acquired by using a Zeiss Axioskop 2 microscope fitted with a Zeiss AxioCam digital camera and then processed by using Zeiss AxioVision software. Localization of Fus3-GFP was quantified from the digital images by using the histogram function of Adobe Photoshop 5.5. A circle of set size was used to sample the brightness of the nucleus and cytoplasm of cells from randomly chosen fields. The ratio of nuclear to cytoplasmic fluorescence was rounded to the nearest 0.1, and values corresponding to at least 25 cells for each strain and condition were plotted in histograms. All such experiments were performed at least three times.

RESULTS

Pheromone induces accumulation of Fus3 in the nucleus.

Upon activation of the mating pathway, Fus3 communicates the mating signal from the cytoplasm to the nucleus by interacting with its downstream effectors, Dig1, Dig2, and Far1 (12). Two studies have reported that the amount of Fus3 in the nucleus increases slightly when cells are treated with pheromone (6, 65). To confirm this observation and to determine whether nuclear accumulation of Fus3 is inhibited by Gpa1, we

constructed an in-frame fusion between the genes encoding Fus3 and GFP. The *FUS3-GFP* hybrid gene proved to encode a functional MAPK, since it was able to fully rescue the response defect conferred by deletion of both *FUS3* and *KSS1* in halo tests (data not shown). Wild-type cells expressing *FUS3-GFP* from a centromeric vector were grown to mid-log phase and examined under a fluorescent microscope before and after pheromone treatment. In the vegetative culture, we observed diffuse fluorescence in both the cytoplasm and nucleoplasm. Upon stimulation with pheromone, which induces *FUS3* transcription, the cells fluoresced more brightly throughout the cytoplasm and nucleoplasm but showed a proportionately greater intensification of the signal in their nuclei. In fact, the mean ratio of nuclear to cytoplasmic fluorescence (RNCF) increased from approximately 1.4 in the vegetative cells to about 1.9 in cells treated with pheromone for 3 h (Fig. 1A). A Student *t* test analysis of the data showed this difference to be highly significant ($P < 0.0001$). The mean RNCF began to rise after 10 min of treatment ($P < 0.0001$ for 1.42 at 10 min versus 1.62 at 20 min) and peaked about 2 h into the time course. Given that the accumulation of activated Fus3 in the nucleus must certainly precede a detectable increase in the mean RNCF, the observed nuclear accumulation of Fus3-GFP occurs early enough to contribute to the induction of mating-specific transcription and cell cycle arrest.

In response to pheromone stimulation, Fus3 is phosphorylated by Ste7 on two residues, T180 and Y182, and thereby activated (17). To determine whether the pheromone-induced increase in the mean RNCF was due specifically to the nuclear accumulation of activated Fus3-GFP, we created a mutant form of the Fus3-GFP reporter, Fus3^{T180A Y182A}-GFP, which cannot be phosphorylated by Ste7. As expected, Fus3^{T180A Y182A}-GFP could not rescue the sterility of the *fus3 Δ kss1 Δ* strain (data not shown). Although the basal nuclear localization of the mutant reporter was normal, the Fus3^{T180A Y182A}-GFP *fus3 Δ kss1 Δ* cells failed to exhibit a significant pheromone-induced increase in the mean RNCF (Fig. 1B) ($P = 0.189$ for 1.8 versus 1.9), a finding consistent with their sterile phenotype. In contrast, Fus3-GFP *fus3 Δ kss1 Δ* cells displayed a significant increase in mean RNCF after pheromone treatment ($P = 0.0001$ for 1.8 versus 2.5) (Fig. 1C). Interestingly, *fus3 Δ KSS1* cells expressing Fus3^{T180A Y182A}-GFP responded almost normally to pheromone due to the functional redundancy of Fus3 and Kss1, and their mean RNCF increased as much as that of *fus3 Δ KSS1* cells expressing Fus3-GFP (compare Fig. 1D and E). This suggests that the activating phosphorylation of Fus3 is not strictly necessary for its pheromone-induced nuclear accumulation. However, it is possible that in the absence of wild-type Fus3, Fus3^{T180A Y182A}-GFP translocates to the nucleus in complex with Kss1. Signal-induced dimerization of mammalian MAPKs is well established (26, 50), and in ERK microinjection studies Khokhlatchev et al. found that the phosphorylation of one

are represented in the bottom histogram. Arrows indicate the mean RNCF for each of the sampled populations. (A) Time course experiment showing Fus3-GFP localization in wild-type cells (○), cells overexpressing Gpa1^{E364K} (□), and cells overexpressing Msg5 (◇). Mean RNCF values were determined before and 10, 20, 40, 60, 90, 120, and 180 min after pheromone treatment; (B) Fus3^{T180A Y182A}-GFP localization in *fus3 Δ kss1 Δ* cells; (C) Fus3-GFP localization in *fus3 Δ kss1 Δ* cells; (D) Fus3^{T180A Y182A}-GFP localization in *fus3 Δ* cells; (E) Fus3-GFP localization in *fus3 Δ* cells.

partner in a MAPK dimer is both necessary and sufficient for nuclear translocation (28). Together, these data demonstrate that Fus3, like other MAPKs, concentrates in the nucleus in response to pathway stimulation but do not allow us to determine whether activating phosphorylation of Fus3 is essential for this phenomenon.

Adaptation to pheromone is correlated with a decrease in nuclear Fus3. Termination of some MAPK-generated signals is correlated with decreased nuclear localization of the MAPK. For example, the Hog1 MAPK of *Saccharomyces cerevisiae* moves to the nucleus when cells are osmotically shocked and leaves the nucleus as the cells adapt to the new culture conditions or when they are returned to medium of normal osmolarity (15, 51). Similarly, growth factor-induced nuclear accumulation of the mammalian p42/p44 MAPK is transient, and adaptation to the stimulus correlates with reappearance of the kinase in the cytoplasm (33). To determine whether the duration of the pheromone response is correlated with the localization of Fus3, we treated mid-log-phase wild-type cells with a dose of pheromone that induces a transient response. Prior to stimulation, the mean RNCf of the culture was 1.6 (Fig. 2A). By 3 h after treatment, about 94% of the cells had arrested in G₁, about 69% of the culture had formed mating projections, and the mean RNCf had risen to 2.4 (Fig. 2B). By 6 h after treatment, the culture had begun to recover. The fraction of arrested cells had decreased to 58%, more than half of the shmooos had budded and, significantly, the mean RNCf had fallen to 1.8 (Fig. 2C). An inference that can be drawn from these results is that yeast cells recover from pheromone stimulation by decreasing the level of nuclear Fus3. To test this idea, we allowed the cells to fully recover and saturate the pheromone-containing medium. At 24 h after the original stimulus, the cells were resuspended in fresh medium and challenged with pheromone again. Cells adapted to chronic pheromone treatment are expected to be unresponsive to further stimulation for up to four generations (46). By 3 h after the second treatment, only 9% of the cells had formed mating projections, and the mean RNCf remained at 1.8 (Fig. 2D). Thus, both recovery from pheromone-induced cell cycle arrest and long-term adaptation to pheromone are correlated with a decreased concentration of Fus3 in the nucleus.

Gpa1 inhibits the pheromone-induced accumulation of Fus3 in the nucleus. Given our recent finding that the activated form of Gpa1 binds directly to the activated form of Fus3 in vivo (44), as well as the results described above, we hypothesized that Gpa1 downregulates the mating signal by inhibiting the pheromone-induced accumulation of Fus3 in the nucleus. To test this idea, we measured the mean RNCf in cells overexpressing wild-type Gpa1, which exhibit a moderate decrease in sensitivity to pheromone (9), and in cells overexpressing the E364K hyperadaptive mutant form of Gpa1, which are completely resistant to pheromone (36). Excess Gpa1 had a moderate inhibitory effect on the pheromone-induced accumulation of Fus3 in the nucleus (Fig. 3A and B; $P = 0.0011$ for the induced values of 2.0 versus 1.7), whereas excess Gpa1^{E364K} almost completely prevented it (Fig. 1A and 3F).

To determine whether the effect of Gpa1 on Fus3 localization depends on Gpa1-Fus3 interaction, we introduced a mutation that disrupts Gpa1-Fus3 binding (K21E R22E) (44) into wild-type Gpa1 and into Gpa1^{E364K}. In *gpa1Δ* cells expressing

FIG. 2. Adaptation to pheromone is correlated with a decrease in nuclear Fus3. Wild-type cells transformed with Fus3-GFP were grown to mid-log phase and treated with 6 nM α -factor. Images were acquired at 0, 3, 6, and 27 h. After 24 h in the original medium, the treated culture was resuspended in fresh medium containing 6 nM α -factor, and images were taken for analysis 3 h later. RNCf values were determined as described in Materials and Methods, and the distribution of values represented in histograms. The number of cells (y axis) are plotted as a function of the RNCf values (x axis). The arrows on the histograms indicate the mean RNCf for each of the sampled populations. The arrows on the DIC images indicate cells that have responded and recovered (budded shmooos). "UB" refers to the percentage of unbudded cells. "S" refers to the percentage of shmooing cells. (A) Vegetative cells; (B) cells 3 h after treatment; (C) cells 6 h after treatment; (D) cells 27 h after first treatment and 3 h after the second treatment.

gpa1^{K21E R22E} from a centromeric vector, the mean RNCf prior to pheromone treatment was not significantly different from that of the control cells ($P = 0.1183$ for the values 1.6 versus 1.7), suggesting that Gpa1 does not affect the basal localization of Fus3 (compare Fig. 3A and C). After pheromone treatment, however, the *gpa1Δ gpa1*^{K21E R22E} cells showed a considerably higher than normal nuclear localization of Fus3-GFP ($P = 0.0043$ for the values 2.0 versus 2.3), a finding consistent with their supersensitivity (44). Moreover, cells overexpressing Gpa1^{K21E R22E E364K}, which binds G $\beta\gamma$ but does not stimulate adaptation to pheromone, showed a clear shift in mean RNCf after pheromone treatment (Fig. 3G), in marked contrast to cells overexpressing Gpa1^{E364K} ($P < 0.0001$ for the induced values of 1.5 versus 1.8). These data

FIG. 3. Gpa1 and Msg5 inhibit the pheromone-induced accumulation of Fus3 in the nucleus. *MATa* strains of the indicated genotype were transformed with the Fus3-GFP reporter and grown to mid-log phase in galactose medium (*GAL1* promoter on). The cultures were then split and grown with or without the addition of 18 nM α -factor. Images were acquired from the untreated cultures and from the treated cultures 3 h after the addition of pheromone. The RNCf values were determined as described in Materials and Methods, and the distribution of values is represented in histograms. The number of cells (y axis) are plotted as a function of the RNCf values (x axis). In each panel, the untreated cells are represented in the histogram on the left, and the treated cells are represented in the histogram on the right. Arrows indicate the mean RNCf for each of the sampled populations. UB refers to the percentage of unbudded cells; S refers to the percentage of shmooing cells. (A) Wild-type cells; (B) *GAL1^{EG28}-GPA1* cells; (C) *GAL1^{EG28}-GPA1^{K21E R22E}* cells; (D) *GAL1-MSG5* cells; (E) *GAL1-PTP3* cells; (F) *GAL1^{EG28}-GPA1^{E364K}* cells; (G) *GAL1^{EG28}-GPA1^{K21E R22E E364K}* cells; (H) *GAL1^{EG28}-GPA1^{E364K} kap104 Δ ::HIS YCplac111/kap104 $\Delta^{827-918}$* cells; (I) *YCplac22/GAL1-MSG5 kap104 Δ ::HIS YCplac111/kap104 $\Delta^{827-918}$* cells.

suggest that Gpa1 affects Fus3 localization by interacting directly with it.

Because Fus3 stimulates its own transcription, its sequestration by Gpa1 is expected to inhibit its expression. Preventing the increase of cytoplasmic Fus3 might, in turn, further limit the pheromone-induced accumulation of Fus3 in the nucleus.

To determine whether Gpa1 can affect the localization of Fus3 without affecting Fus3 expression, we measured basal Fus3 RNCf values in sterile strains. A catalytically inactive form of the reporter, Fus3^{K42R}-GFP, was transformed into a *fus3 Δ kss1 Δ* strain (IHY205) and into an isogenic strain that overexpresses Gpa1^{E364K} (IHY217). Excess Gpa1^{E364K} dramatically

lowered the mean RNCF in sterile cells ($P < 0.0001$ for 1.4 versus 1.6), indicating that Gpa1 can inhibit the nuclear localization of Fus3 independent of its effect on Fus3 expression.

Msg5 and Ptp3 inhibit the accumulation of Fus3 in the nucleus. Fus3 is dephosphorylated and thereby inactivated by the pheromone-inducible, dually specific tyrosine-threonine phosphatase, Msg5, and by two tyrosine phosphatases, Ptp2 and Ptp3 (13, 68). Ptp2 is located in the nucleus (42), Ptp3 is localized to the cytoplasm (42), and Msg5-GFP is found in both compartments (D. E. Stone and H.-J. N. Kim, unpublished results). Because phosphatases are known to regulate the nucleocytoplasmic transport of some MAPKs, we asked whether the localization of Fus3 is controlled by either Msg5 or Ptp3. As shown in Fig. 4A to D, deletion of either *MSG5* or *PTP3* partially increased the basal mean RNCF ($P = 0.0063$ for 1.6 versus 1.8 [*msg5Δ*]; $P = 0.0052$ for 1.6 versus 1.8 [*ptp3Δ*]), and the *msg5Δ ptp3Δ* double deletion conferred full induction (mean RNCF = 2.0) in the absence of pheromone. Conversely, overexpression of Msg5 lowered the mean RNCF of vegetative wild-type cells expressing Fus3-GFP (Fig. 3D) ($P = 0.0046$ for 1.6 versus 1.4) and the mean RNCF of sterile *fus3Δ kss1Δ* cells expressing Fus3^{K42R}-GFP ($P < 0.0001$ for 1.4 versus 1.6). In response to pheromone, wild-type cells overexpressing Msg5 did not fully arrest in G₁ or shmoo, and they did not exhibit the normal increase in the relative level of nuclear Fus3-GFP (Fig. 1A [$P < 0.0004$ for 1.41 versus 1.57] and 3D [$P = 0.022$ for 1.4 versus 1.5]). Interestingly, overexpression of Ptp3 had no effect on the localization of Fus3-GFP (Fig. 3E). Thus, both Ptp3 and Msg5 appear to affect the basal localization of Fus3, whereas Msg5 may be the more important regulator in cells responding to pheromone.

The Gpa1- and Msg5-mediated effects on Fus3 localization are partially interdependent. Both Gpa1 and Msg5 have been implicated in adaptation to pheromone (13, 45, 62). Three observations suggest that they work together to regulate Fus3. First, both the G_α protein and the phosphatase bind to Fus3 (13, 44). Second, both proteins inhibit nuclear localization of the Fus3-GFP reporter. Third, deletion of *MSG5* is partially epistatic to hyperadaptive alleles of *GPA1* in halo tests (13, 69) (Fig. 4I). To further explore the functional relationship between Gpa1 and Msg5, we assessed their interdependence in affecting Fus3 localization. Deletion of *MSG5* had no effect on the ability of Gpa1^{E364K} to inhibit the pheromone-induced increase in nuclear Fus3 (data not shown). Could Ptp3 compensate for the absence of Msg5? Such functional redundancy between phosphatases has been observed in the regulation of both Fus3 and Hog1 (25, 68). Like deletion of *MSG5*, deletion of *PTP3* compromises Gpa1^{E364K}-mediated adaptation in halo tests (Stone and Kim, unpublished) and increases both the basal and the induced RNCF values (Fig. 4C). Although the absence of both phosphatases did not significantly compromise the inhibitory effect of Gpa1^{E364K} on Fus3 localization (compare Fig. 4E and F) ($P < 0.1321$ for the induced values of 1.6 versus 1.5), the *msg5Δ ptp3Δ* double deletion nullified the effect of excess wild-type Gpa1 on the pheromone-induced nuclear accumulation of Fus3-GFP (compare Fig. 4G and 4H) ($P = 0.0001$ for the induced values of 1.7 and 2.0). These results indicate that the effect of Gpa1 on Fus3 localization is partially dependent on Msg5 and/or Ptp3.

In halo tests, Gpa1 adaptive function partially depends on

Msg5; in Fus3 localization assays, Gpa1 function partially depends on Msg5 and/or Ptp3. To determine whether Msg5 adaptive function depends on Gpa1, we transformed the *GALI-MSG5* transcriptional fusion and the *FUS3-GFP* reporter into a *gpa1* temperature-sensitive strain related to strain 15D. The temperature-sensitive form of Gpa1 is partially functional at 23°C and fully inactivated at 37°C (data not shown). Therefore, Fus3-GFP localization was assessed before and after pheromone treatment of log-phase cells cultured at the permissive temperature, and log-phase cells were shifted to the restrictive temperature at time zero. Although the pheromone-induced increase in the mean RNCF of wild-type cells cultured at high and low temperatures was not as great as that observed at 30°C, the effect of the *gpa1* temperature-sensitive mutation (*gpa1^{ts}*) was clear. As shown in Table 3, loss of functional Gpa1 significantly increased the mean RNCF of cells expressing the normal level of Msg5 (when comparing cultures grown at permissive temperature, $P = 0.001$ for 1.6 versus 1.8 and $P = 0.0014$ for 1.8 versus 2.1; when comparing cultures grown at the restrictive temperature, $P = 0.1754$ for 1.7 versus 1.8 and $P = 0.0112$ for 1.9 versus 2.1). This hyperaccumulation of Fus3-GFP to the nuclei of Gpa1-deficient cells was almost completely suppressed by the overexpression of Msg5 at 23°C ($P = 0.0932$ for 1.5 versus 1.6 and $P = 0.0911$ for 1.6 versus 1.7). However, the effect of excess Msg5 on Fus3-GFP localization was significantly diminished in cells cultured at 37°C and therefore completely lacking functional Gpa1 ($P < 0.0001$ for 1.5 versus 1.9 and $P = 0.0048$ for 1.6 versus 1.8). These data indicate that the adaptive function of Msg5 is partially dependent on Gpa1.

Together, the results of the epistasis tests suggest that Msg5 plays a role in Gpa1-mediated adaptation and, in particular, in inhibiting pheromone-induced accumulation of Fus3 in the nucleus. Ptp3 may play a similar role, or it may work in concert with Gpa1 only in the absence of Msg5.

C-terminal truncation of Kap104 confers an adaptive defect and is epistatic to Gpa1^{E364K}. In a genetic screen designed to identify gene products required for Gpa1-mediated adaptation, we isolated a mutant strain, designated A11, that exhibited a recessive supersensitive/adaptive[−] phenotype. When expressed in the A11 background, Gpa1^{E364K} was significantly compromised in its ability to downregulate the pheromone response, as indicated in halo tests (Fig. 5A) and mating-specific transcription assays (data not shown). Segregation analysis indicated that the mutant phenotype was due to mutation of a single locus. The corresponding wild-type gene was cloned by complementation of the adaptive defect, and an integrative mapping strategy was used to confirm the identity of the mutant allele and the cloned gene. The nucleotide sequence of the cloned gene matched that of *KAP104* (for karyopherin protein of 104 kDa), a homologue and functional analogue of the mammalian nuclear import protein, karyopherin-β2 (transportin) (1). Transport receptors of this class bind directly to their cargo molecules and mediate unidirectional movement through the nuclear pore complex (NPC), either into or out of the nucleus. An established function of Kap104 is to import two mRNA binding proteins, Nab2 and Nab4, after they have released their cargo in the cytoplasm (1, 32).

The A11 allele of *KAP104* contains a nonsense mutation and

FIG. 4. Fus3 localization in cells lacking *MSG5* and/or *PTP3*. Strains of the indicated genotype were transformed with the Fus3-GFP reporter and grown to mid-log phase in galactose medium (*GAL1* promoter on). The cultures were then split and grown with or without the addition of 18 nM α -factor. Images were acquired from the untreated cultures and from the treated cultures 3 h after the addition of pheromone. The RNCf values were determined as described in Materials and Methods, and the distribution of values represented in histograms. The number of cells (y axis) are plotted as a function of the RNCf values (x axis). In each panel, the untreated cells are represented in the histogram on the left, and the treated cells are represented in the histogram on the right. Arrows indicate the mean RNCf for each of the sampled populations. (A) Wild-type cells; (B) *msg5Δ* cells; (C) *ptp3Δ* cells; (D) *msg5Δ ptp3Δ* cells; (E) $GAL1^{EG28}$ -GPA1^{E364K} cells; (F) $GAL1^{EG28}$ -GPA1^{E364K} *msg5Δ ptp3Δ* cells; (G) YCplac22/ $GAL1^{EG28}$ -GPA1 cells; (H) YCplac22/ $GAL1^{EG28}$ -GPA1 *msg5Δ ptp3Δ* cells. (I) Halo tests showing the epistatic effect of *msg5Δ* on Gpa1^{E364K}-induced adaptation: wild-type cells (top left), *msg5Δ* cells (top right), YCplac111/Gpa1^{E364K} (61) in wild-type cells (bottom left), and YCplac111/Gpa1^{E364K} in *msg5Δ* cells (bottom right).

TABLE 3. Effect of Msg5 overexpression on Fus3-GFP localization in a *gpa1* temperature-sensitive background

Background	Effect of Msg5 overexpression (mean RNCF)							
	<i>GAL1-MSG5</i> repressed at:				<i>GAL1-MSG5</i> induced at:			
	23°C		37°C		23°C		37°C	
	–	+	–	+	–	+	–	+
<i>gpa1^{ts}</i>	1.8	2.1	1.8	2.1	1.6	1.7	1.9	1.8
<i>GPA1</i>	1.6	1.8	1.7	1.9	1.5	1.6	1.5	1.6

^a +, 3 h after addition of 36 nM pheromone.

is predicted to encode a truncation product lacking the carboxy-terminal 91 residues of the 918-amino-acid Kap104 protein. However, it is possible that a nonsense suppressor intrinsic to this strain could result in the production of some full-length Kap104. The mutant phenotypes of strain A11 might therefore be due to a lower-than-normal level of full-length Kap104 rather than to its complete absence and replacement by the truncation product. To distinguish these possibilities, a deletion allele lacking the last 273 bp of the *KAP104* coding region, *kap104^{Δ827-918}*, was constructed. This allele encodes a shortened form of Kap104, missing the C-terminal 91 amino acids. A centromeric vector containing *kap104^{Δ827-918}* was expressed in a *kap104Δ* background, and these cells were compared to the isogenic wild-type and A11 mutant strains in halo tests. As shown in Fig. 5A, the halo phenotypes of the *kap104^{Δ827-918}* and A11 mutant strains were essentially the same. The two alleles conferred similar degrees of supersensitivity in cells expressing wild-type Gpa1 and had a similar detrimental effect on the hyperadaptive function of Gpa1^{E364K}. Immunoblot analysis demonstrated that strain A11 expresses a shortened form of Kap104 at a level similar to that of the full-length protein in wild-type cells (Fig. 5C) and the normal steady-state level of Gpa1 (Fig. 5B). Therefore, the most likely explanation for the adaptive[–] phenotypes of strain A11 is that the C-terminal one-tenth of Kap104, which comprises about one-quarter of the cargo-binding domain, is critical for proper negative regulation of the pheromone response. Truncation of this domain is epistatic to *GPA1^{E364K}*.

The genetic link between Kap104, a karyopherin, and Gpa1-mediated adaptation led us to wonder whether the adaptive[–] phenotype conferred by *kap104^{Δ827-918}* is due to mislocalization of Fus3. To test this possibility, we transformed the Fus3-GFP reporter into strains AEY144 (relevant genotype *MATa kap104^{Δ827-918}/kap104Δ*) and AOY135 (relevant genotype *MATa GAL1^{EG28}-GPA1^{E364K} kap104^{Δ827-918}/kap104Δ*) and quantified the relative degree of nuclear fluorescence. Although the truncation of Kap104 had no detectable effect on Fus3-GFP localization in *GPA1* cells (data not shown), the *kap104^{Δ827-918}* allele largely suppressed the effect of Gpa1^{E364K} on Fus3 localization (compare Fig. 3F and H) ($P < 0.0001$ for 1.5 versus 1.7, and $P < 0.0001$ for 1.5 versus 2.0). Thus, the loss of Gpa1^{E364K} hyperadaptive function seen in halo tests (Fig. 5) and in culture (Fig. 3F) correlates with restoration of normal Fus3 levels in the nuclei of cells responding to pheromone. Because Gpa1 and Msg5 appear to cooperate to promote an adaptive mechanism involving Fus3 localization, we next sought to determine whether the effect of

Msg5 overexpression on Fus3 localization would also be diminished in *kap104^{Δ827-918}* mutant cells. As can be seen by comparing Fig. 3D and I, this is indeed the case. The C-terminal truncation of Kap104 decreases the ability of excess Msg5 to inhibit both pheromone-induced cell cycle arrest and accumulation of Fus3-GFP in the nucleus ($P = 0.003$ for 1.4 versus 1.6 and $P < 0.0001$ for 1.5 versus 1.8). Together, these results suggest that Gpa1 and Msg5 downregulate the mating signal by antagonizing the signal-induced nuclear translocation of Fus3 and that Kap104 is required for G_α/phosphatase-mediated regulation of Fus3 localization.

Overexpression of Kap104 inhibits growth but potentiates Gpa1-mediated adaptation. Because *kap104^{Δ827-918}* is epistatic to *GPA1^{E364K}*, we wondered whether Kap104 overexpression could enhance Gpa1^{E364K} hyperadaptive function. The *KAP104* coding region was therefore fused to the *GAL1* promoter. Although induction of the *GAL1-KAP104* construct in wild-type cells conferred a severe growth defect (cells overexpressing Kap104 took 7 days to form macroscopic colonies compared to the usual 2 days) and an increased sensitivity to pheromone (data not shown), excess Kap104 was nevertheless able to augment the ability of Gpa1^{E364K} to promote growth at otherwise lethal doses of α -factor. Whereas cells expressing the wild-type level of Gpa1^{E364K} form turbid halos, cells expressing Gpa1^{E364K} in combination with excess Kap104 are completely resistant to pheromone and form no halo at all (Fig. 6A). The combined effect of Gpa1^{E364K} expression and Kap104 overexpression was quantified in a single-colony formation assay (Fig. 6B). Cells expressing the wild-type level of Gpa1^{E364K} and either the wild-type level or the *GAL1*-induced level of Kap104 were spread on plates containing various doses of α -factor, as well as on control plates lacking pheromone. At all three pheromone concentrations, the cells overproducing Kap104 formed significantly more colonies, increasing the plating efficiency by 64% at the lowest dose and almost doubling it at the two higher doses. Immunoblot analysis showed that the steady-state level of Gpa1 was unaffected by the induction of *GAL1-KAP104* (Fig. 6C). Thus, Kap104 overexpression potentiates the adaptive function of Gpa1.

C-terminal truncation of Kap104 results in mislocalization of Nab2. The two known substrates of Kap104 are Nab2 and Nab4. The *kap104^{Δ827-918}* form of Kap104 lacks the C-terminal one-quarter of the substrate-binding domain (HEAT domains 17 and 18) (7) and might therefore be unable to bind its cargo. To test this, we examined the localization of Nab2-GFP in strains A11 and DSY278. As expected, the Nab2 reporter was found concentrated in the nuclei of wild-type cells but spread throughout the cytoplasm of *kap104^{Δ827-918}* cells (Fig. 7).

DISCUSSION

Because MAPK signaling cascades are used by all eukaryotic cells to control growth and differentiation, the timing and duration of MAPK responses must be tightly regulated. For MAPKs that participate in multiple pathways, cross talk must be controlled as well. Although MAPK pathways have been subjected to intense investigation, the ways in which they are regulated are not fully understood. A priori, regulatory mechanisms could involve expression, enzymatic activity, sequestration and/or localization, and stability of MAPKs. Induction of

FIG. 5. Effect of Kap104 truncation on pheromone-induced cell cycle arrest. (A) Strains DSY278 and A11 were transformed with YCplac33 (18) (top row) or YCplac33/Gpa1^{E364K} (36) (bottom row). Strain ZW432 (15Dau *kap104* $\Delta::URA3/kap104^{\Delta 827-918}$) was transformed with YCplac22 (18) (top row) or YCplac22/Gpa1^{E364K} (62) (bottom row). Transformants were cultured and tested in glucose medium. (B) Immunoblot probed with Gpa1 antibody. Protein was extracted from strains DSY278 (left) and A11 (right), both transformed with YCplac33/Gpa1^{E364K}. (C) Immunoblot probed with Kap104 antibody. Protein was extracted from strain DSY278 (left) and strain A11 (right).

MAPK responses is thought to depend on the activation of MAPKs by their MEKs, and their subsequent accumulation in the nucleus, where they phosphorylate transcription factors and other regulatory targets. Such signal-induced localization has been demonstrated in a number of systems (26, 50). Inactivation of MAPKs partially depends on the action of specific protein phosphatases and, in some cases, the regulated export of MAPKs from the nucleus is thought to play a role in down-regulation (15, 33). The yeast mating signal is mediated by a heterotrimeric G protein that communicates with a MAPK cascade composed of Ste11 (MEKK), Ste7 (MEK), and Fus3 (MAPK). The free G $\beta\gamma$ subunit activates the MAPK module, and both the inactive and the active forms of the G α subunit, Gpa1, negatively regulate the response. Prior to this work, genetic evidence suggested that Gpa1 stimulates adaptation by indirectly inactivating Ste4 (G β) (36) and through a mechanism acting at the level of Fus3 (13, 69; A. T. Ellicott and D. E. Stone, unpublished results). In a proteomic screen for gene products that associate with Gpa1 in cells responding to pher-

omone, we identified Fus3 (44). Additional experiments showed that the activated form of Gpa1 binds to the activated form of Fus3 in cells responding to pheromone. This discovery led us to study the relationship between the localization of Fus3 and adaptation to pheromone. We found that the relative level of nuclear Fus3 decreases under conditions in which cells are recovering from pheromone treatment or have adapted to chronic pheromone stimulation and that both Gpa1 and the dual-specific phosphatase Msg5 negatively affect the nuclear localization of Fus3. The adaptive functions of Gpa1 and Msg5 are partially dependent on one another and on the Kap104 importin. To our knowledge, this is the first time that the G α subunit of a heterotrimeric G protein has been shown to affect the subcellular localization of a MAPK.

Gpa1 and Msg5 downregulate the mating signal by affecting the localization of Fus3. Because the expression of Fus3 is induced ~5-fold by pheromone (53), it is difficult to detect signal-induced nuclear localization of Fus3 comparable to that documented for other MAPKs. Using a myc-tagged form of

FIG. 6. Overexpression of Kap104 potentiates Gpa1-mediated adaptation. Strain 15Dau was transformed with the vectors indicated below and the transformants were tested for their ability to grow in a range of pheromone concentrations. The transformants were cultured and assayed in galactose medium. (A) Halo tests: YCplac111/Gpa1^{E364K} and pYES (left); YCplac111/Gpa1^{E364K}, and pYES/KAP104 (right). (B) Single-colony formation assays. The ability to form colonies on medium containing discrete concentrations of α -factor was assayed. The numbers plotted are the percentages of cells of a given strain that formed colonies at the indicated dose. Each datum point is the mean of at least two trials with each of at least six transformants. Plasmids pYES/KAP104-A and pYES/KAP104-B were constructed by using DNA derived from two independent PCR amplifications of the *KAP104* coding region. Columns: 1, pYES; 2, YCplac111/Gpa1^{E364K}; 3, pYES and YCplac111/Gpa1^{E364K}; 4, pYES/KAP104-A and YCplac111/Gpa1^{E364K}; 5, pYES/KAP104-B and YCplac111/Gpa1^{E364K}. (C) Immunoblot probed with Gpa1 antibody. Protein was extracted from strain 15Dau transformed with YCplac111/Gpa1^{E364K} (left) and with pYES/KAP104 and YCplac111/Gpa1^{E364K} (right).

FIG. 7. Effect of *kap104* Δ ⁸²⁷⁻⁹¹⁸ on Nab2-GFP localization. Wild-type and A11 cells transformed with Nab2-GFP were examined in mid-log phase.

Fus3, Elion et al. found that the level of the kinase in the nucleus is slightly enhanced by pheromone treatment (6), and van Drogen et al. came to the same conclusion using a Fus3-GFP reporter (65). To determine whether the nuclear concentration of Fus3 rises and falls as cells respond to pheromone and adapt to it, we developed a quantitative assay that measures the relative proportion of Fus3 in the nucleus and cytoplasm. Although the RNCF values varied considerably within a given culture, four observations indicate that this measurement is sensitive to physiologically relevant changes in Fus3 localization. First, pheromone treatment of wild-type cells causes a 25 to 50% increase in the mean RNCF (Fig. 1 to 4). Statistical analysis showed this difference to be highly significant ($P < 0.0001$). We speculate that the variability of the RNCF values within a given culture is due to the lack of cell cycle synchrony. Second, the increase in nuclear Fus3 is easily detectable after only 20 min of pheromone treatment, suggesting that the buildup of Fus3 in the nucleus is relevant to the induction of mating response. Third, the mean RNCF of wild-type cultures decreases as the cells recover and begin to bud (Fig. 2A to C). Fourth, Fus3 does not concentrate in the nucleus when cells that have adapted to chronic stimulation are rechallenged (Fig. 2D). This observation correlates with the insensitivity of such cells to pheromone-induced cell cycle arrest and may be relevant to the ability of recovered cells to “remember” their exposure to pheromone and remain unresponsive to further stimulation for multiple generations (46).

The availability of a quantitative assay for the relative level of nuclear Fus3 enabled us to ask whether downregulation of the mating signal by Gpa1 and Msg5 is associated with changes in Fus3 localization. The data shown in Fig. 2, 3, and 4 clearly indicate that both of these proteins, when manipulated to promote hyperadaptation, inhibit the pheromone-induced accumulation of Fus3 in the nucleus, and both are dependent on the wild-type function of Kap104 to accomplish this. It is noteworthy that both Gpa1 and Msg5 can inhibit the nuclear localization of Fus3 without affecting Fus3 expression. Is the decreased concentration of nuclear Fus3 in stimulated cells the cause or the result of the mating pathway downregulation?

Although this question cannot be definitively answered on the basis of correlative results, Gpa1 and Msg5 are both known to interact directly with Fus3. Moreover, mutational disruption of the Gpa1-Fus3 interaction increased pheromone sensitivity and localization of Fus3 to the nuclei of stimulated cells (Fig. 3C), implying that Gpa1 regulates the mating signal by directly binding to the MAPK. Therefore, we favor the former possibility: Gpa1 and Msg5 inhibition of Fus3 accumulation in the nucleus is an adaptive mechanism.

It is important to note that the Gpa1 and Msg5 adaptive functions are partially interdependent. Deletion of *MSG5* compromises Gpa1^{E364K}-induced recovery from pheromone arrest (Fig. 4I) and, when combined with the loss of Ptp3, another cytoplasmic phosphatase that targets Fus3, slightly lessens the effect of Gpa1^{E364K} on Fus3 localization (Fig. 4E and F). Furthermore, the *msg5* Δ *ptp3* Δ double deletion is completely epistatic to the effect of excess wild-type Gpa1 on Fus3 localization in stimulated cells (Fig. 4G and H). Conversely, the effect of Msg5 overexpression on Fus3 localization is weakened by inactivation of Gpa1 (Table 3). Together, these results suggest that Msg5 acts with Gpa1 to downregulate the mating signal by inhibiting the nuclear localization of Fus3. Because Gpa1 is found primarily at the plasma membrane (19, 58), its effect on Fus3 localization is likely due to sequestration. Msg5 might also act as a cytoplasmic tether for Fus3, in the same way that Ptp3 serves as a cytoplasmic anchor for the Hog1 MAPK of *S. cerevisiae* (42). Alternatively, or in addition, Msg5 might inhibit nuclear accumulation of Fus3 by dephosphorylating it. Signal-induced nuclear localization of other MAPKs is known to be dependent on their phosphorylation state (for examples, see references 26 and 50), and the same may be true for Fus3. Consistent with this possibility, recent evidence suggests that phosphorylation of Fus3 promotes its release from the Ste5 scaffolding protein (65). Dephosphorylation of Fus3 might therefore promote its binding to Ste5.

Although the data do not yet allow us to formulate a detailed model of the Gpa1-Msg5 functional relationship, we can propose two general possibilities. Gpa1 and Msg5 may act in parallel, separately impinging on Fus3. When the kinase dissociates from one of the regulators, the other may capture it. In this scenario, Msg5 might sequester Fus3 and/or inactivate it. A distinct possibility is that Gpa1 and Msg5 act on Fus3 in a complex. Perhaps Gpa1 facilitates the docking of the phosphatase to the kinase. In this case, the primary role of Msg5 would be dephosphorylation of Fus3 rather than sequestration of it. Coimmunoprecipitation experiments might allow us to detect Gpa1-Fus3-Msg5 complexes if they exist. Whatever the mechanism, it is clear that the role of Msg5 is distinct from that of Ptp3. Whereas deletion of either phosphatase affects the basal RNCF, only overexpression of Msg5 represses the induced RNCF.

Based on photobleaching studies of cells expressing Fus3-GFP, van Drogen et al. concluded that Fus3 shuttles constitutively between the cytoplasm and nucleus and that its rate of transport is not affected by pheromone treatment (65). According to their model, Fus3 is recruited to the plasma membrane in stimulated cells by Ste5, where it is activated by Ste7. Phosphorylation of Fus3 triggers its dissociation from Ste5. Some of the activated kinase molecules then move to the nucleus, whereas some concentrate at the shmoo tip. What

controls this differential localization of activated Fus3? We have shown that the activated form of Gpa1 binds to the activated form of Fus3 in cells responding to pheromone and that the Gpa1-Fus3 interaction is important both in the chemotropic sensing of pheromone and in adaptation to pheromone (44). Moreover, Gpa1 concentrates at the shmoo tips of responding cells (44). Therefore, we propose that Gpa1 captures some of the activated Fus3 molecules as they are released from Ste5, thereby targeting the kinase to its substrates at the plasma membrane and simultaneously modulating the intensity of the mating signal.

Does this mean that Gpa1 inhibits the Fus3-dependent mating responses in the nucleus while at the same time promoting mating-specific events at the plasma membrane? It is interesting to suppose that Msg5, whose expression is strongly induced by pheromone, acts as a temporal switch that alters the consequences of the Gpa1-Fus3 interaction. Early in the mating reaction, when relatively little Msg5 is present, the activated Fus3 that dissociates from Gpa1 is free to move to the nucleus. When the expression of Msg5 is fully induced (near the time of zygote formation), however, any activated Fus3 that dissociates from Gpa1 is sequestered and/or inactivated by the phosphatase. Thus, toward the end of the mating reaction (perhaps as mating partners begin to fuse or in newly formed zygotes), Gpa1 and Msg5 work together to prevent the transport of activated Fus3 to the nucleus. This relieves the cell cycle block and downregulates mating-specific gene expression when these responses are no longer appropriate. Note that in this model, the K_d for the Gpa1-Fus3 interaction determines the relevance of Msg5 to the adaptive function of Gpa1. The model correctly predicts that, in the Fus3-GFP localization assay, Gpa1^{E364K}, which binds Fus3 considerably better than does wild-type Gpa1, is less badly compromised by deletion of *MSG5* and *PTP3* than is wild-type Gpa1 (Fig. 4).

An important point in the model proposed by van Drogen et al. (65) is that pheromone treatment does not alter the rates of nuclear import or export of Fus3. Nevertheless, Fus3 clearly accumulates in the nuclei of cells responding to pheromone. Most likely, this is due to decreased cytoplasmic tethering and/or increased nuclear anchoring of phosphorylated Fus3. There are a number of examples in which transcription factors retain activated MAPKs in the nuclei of yeast and mammalian cells (3, 16, 33). Although van Drogen et al. found that deletion of known Fus3 interactors had no effect on the nucleocytoplasmic distribution of Fus3 (65), their assessment was not quantitative. Differences of 0.2 in the mean RNCF of a cell population are not easily discerned, despite their significance. Alternatively, the retention factors responsible for the pheromone-induced change in Fus3 distribution may not be known.

Involvement of Kap104 in the pheromone response. It is clear from our data that Kap104 is required for proper regulation of the pheromone response. Deletion of the carboxy-terminal 91 amino acids of Kap104, which make up about one-quarter of the presumed cargo-binding domain, confers supersensitivity to pheromone in cells expressing wild-type Gpa1 and compromises the hyperadaptive activity of Gpa1^{E364K} and excess Msg5. These effects of the *kap104*^{Δ827-918} mutation were apparent both in pheromone-induced growth inhibition assays (Fig. 5A) and in pheromone-induced transcriptional assays (data not shown) and could not

be attributed to abnormally low expression of either Gpa1 or *kap104*^{Δ827-918} (Fig. 5B and C). Furthermore, the *kap104*^{Δ827-918} mutation suppressed the effects of excess Gpa1^{E364K} and excess Msg5 on Fus3 localization (Fig. 3H and I). Thus, the epistatic effects of *kap104*^{Δ827-918} on Msg5- and Gpa1-mediated adaptation to pheromone correlate with restoration of normal Fus3 levels in the nuclei of stimulated cells.

A second indication that Gpa1-mediated adaptation depends on Kap104 is that the hyperadaptive activity of Gpa1^{E364K} is potentiated by Kap104 overexpression. Approximately twice as many cells expressing Gpa1^{E364K} and excess Kap104 can overcome what are normally lethal concentrations of pheromone, as can control cells expressing Gpa1^{E364K} and the wild-type level of Kap104 (Fig. 6A and B), despite the decreased growth rate and increased sensitivity to pheromone conferred by Kap104 overexpression. Immunoblot analysis demonstrated that this enhanced resistance to pheromone is not due to an increase in the steady-state level of Gpa1 (Fig. 6C). Thus, changes in both the structure and expression of Kap104 affect the adaptive signaling mechanism(s) stimulated by Gpa1: overexpression of Kap104 enhances recovery, whereas truncation of Kap104 confers an adaptive defect.

How does Kap104 affect Fus3 localization? One possibility is that Kap104 imports a protein that acts as a nuclear tether for Fus3, although this is purely speculative. Another possibility is that Kap104 directly exports Fus3. This is unlikely because there is no precedent for a Ran-dependent karyopherin protein acting as both an importer and an exporter. Based on the structural and biochemical evidence, interaction of a given karyopherin with Ran-GTP in the nucleus is thought to stimulate either cargo release (for an importer) or cargo binding (for an exporter) (see references 7 and 41 and references therein). Kap104 is an import receptor. It is concentrated on the cytosolic side of nuclear pore complexes, where it is thought to interact with the RNA-binding proteins Nab2 and Nab4 (1). Thus far, Nab2 and Nab4 are the only known Kap104 cargo proteins. Moreover, Fus3 does not contain a region similar to the Kap104-binding domain found in Nab2 (56, 63) or a stretch of residues like the M9 sequence of hnRNP A1 (rich in glycine, serine, and asparagine) that mediates the interaction of human transportin with its cargo (67). If Kap104 is not itself the Fus3 export receptor, then perhaps it recycles the unknown transporter. After cargo release, all transport receptors must move back or be moved back through the NPCs to their point of origin. This is true for both importers and exporters. In at least one case, recycling of one transporter depends on the action of another: karyopherin α (importin) is returned to the cytoplasm by the exporter, CAS1/Cse1 (30). A deficiency in recycling the Fus3 exporter could lead to increased levels of Fus3 in the nucleus, just as we observed when *kap104*^{Δ827-918} cells overexpressing Gpa1^{E364K} or Msg5 were challenged with pheromone (Fig. 3). Of course, this explanation assumes that Fus3 is escorted across the nuclear membrane by specific transport receptors, and this has not been established. Fus3 may bind directly to nuclear pore proteins, as does Erk2 (66).

A third way in which Kap104 might affect the pheromone response pathway is by ensuring the proper expression of a key regulator. Because Kap104 is essential for the reimport of Nab2 and Nab4 and thus indirectly responsible for the export

of mature mRNAs, loss of Kap104 function would be expected to adversely affect gene expression. Given that Nab2-GFP is mislocalized in *kap104^{Δ827-918}* cells (Fig. 7), it would be surprising if the mutant cells expressed all genes normally. What genes might be required to limit the amount of nuclear Fus3? We have found that Gpa1 and Msg5 are likely to play a role in controlling the localization of Fus3. However, the steady-state level of Gpa1 is not reduced in mutant strain A11 (Fig. 5B), and the signal intensity of an Msg5-GFP reporter appears to be unaffected by the truncation of Kap104 (data not shown). The phenotypes conferred by *kap104^{Δ827-918}* could also be due to a decrease in the level of an unknown Fus3 exporter, an increase in the level of a Fus3 nuclear tether or, less directly, by loss of transcription factors such as Mot2 or Mot3, which are required for downregulation of the mating signal (5, 8, 21, 23, 31, 38, 47). Although the mechanism is unclear, our data indicate that Kap104 is required for proper regulation of the pheromone response and in particular for the Gpa1/Msg5-mediated effect on Fus3 localization.

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E.B. and I.M.H. contributed equally to this study.

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REFERENCES

- Aitchison, J. D., G. Blobel, and M. P. Rout. 1996. Kap104p: a karyopherin involved in the nuclear transport of messenger RNA binding proteins. *Science* **274**:624–627.
- Alani, E., L. Cao, and N. Kleckner. 1987. A method for gene disruption that allows repeated use of *URA3* selection in the construction of multiply disrupted yeast strains. *Genetics* **116**:541–545.
- Alepuz, P. M., A. Jovanovic, V. Reiser, and G. Ammerer. 2001. Stress-induced MAP kinase Hog1 is part of transcription activation complexes. *Mol. Cell* **7**:767–777.
- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1994. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.
- Cade, R. M., and B. Errede. 1994. *MOT2* encodes a negative regulator of gene expression that affects basal expression of pheromone-responsive genes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **14**:3139–3149.
- Choi, K.-Y., J. E. Kranz, S. K. Mahanty, K.-S. Park, and E. A. Elion. 1999. Characterization of Fus3 localization: active Fus3 localizes in complexes of varying size and specific activity. *Mol. Biol. Cell* **10**:1553–1568.
- Chook, Y. M., and G. Blobel. 1999. Structure of the nuclear transport complex karyopherin-β2Ran-GppNHP. *Nature* **399**:230–237.
- Clark, K. L., and G. F. Sprague. 1989. Yeast pheromone response pathway: characterization of a suppressor that restores mating to receptorless mutants. *Mol. Cell. Biol.* **9**:2682–2694.
- Cole, G. M., D. E. Stone, and S. I. Reed. 1990. Stoichiometry of G protein subunits affects the *Saccharomyces cerevisiae* mating pheromone signal transduction pathway. *Mol. Cell. Biol.* **10**:510–517.
- Dohlman, H. G., D. Apaniesk, Y. Chen, J. Song, and D. Nusskern. 1995. Inhibition of G-protein signaling by mutants with dominant gain-of-function mutations in Sst2p, a pheromone desensitization factor in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **15**:3635–3643.
- Dohlman, H. G., J. Song, D. Ma, W. E. Courchesne, and J. Thorner. 1996. Sst2, a negative regulator of pheromone signaling in the yeast *Saccharomyces cerevisiae*: expression, localization, genetic interaction, and physical association with Gpa1 (G protein α subunit). *Mol. Cell. Biol.* **16**:5194–5209.
- Dohlman, H. G., and J. W. Thorner. 2001. Regulation of G protein-initiated signal transduction in yeast: paradigms and principles. *Annu. Rev. Biochem.* **70**:703–754.
- Doi, K., A. Gartner, G. Ammerer, B. Errede, H. Shinkawa, K. Sugimoto, and K. Matsumoto. 1994. MSG5, a novel protein phosphatase promotes adaptation to pheromone response in *S. cerevisiae*. *EMBO J.* **13**:61–70.
- Duncan, K., J. G. Umen, and C. Guthrie. 2000. A putative ubiquitin ligase required for efficient mRNA export differentially affects hnRNP transport. *Curr. Biol.* **10**:687–696.
- Ferrigno, P., F. Posas, D. Koepp, H. Saito, and P. A. Silver. 1998. Regulated nucleo/cytoplasmic exchange of HOG1 MAPK requires the importin β homologs NMD5 and XPO1. *EMBO J.* **17**:5606–5614.
- Gaits, F., G. Degols, K. Shiozaki, and P. Russell. 1998. Phosphorylation and association with the transcription factor Atf1 regulate localization of Spc1/Sty1 stress-activated kinase in fission yeast. *Genes Dev.* **12**:1464–1473.
- Gartner, A., K. Nasmyth, and G. Ammerer. 1992. Signal transduction in *Saccharomyces cerevisiae* requires tyrosine and threonine phosphorylation of FUS3 and KSS1. *Genes Dev.* **6**:1280–1292.
- Gietz, R. D., and A. Sugino. 1988. New yeast—*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74**:527–534.
- Gillen, K. M., M. Pausch, and H. G. Dohlman. 1998. N-terminal domain of Gpa1 (G protein α subunit) is sufficient for plasma membrane targeting in yeast *Saccharomyces cerevisiae*. *J. Cell Sci.* **111**:3235–3244.
- Giniger, E., and M. Ptashne. 1988. Cooperative DNA binding of the yeast transcriptional activator GAL4. *Proc. Natl. Acad. Sci. USA* **85**:382–386.
- Grishin, A. V., M. Rothenberg, M. A. Downs, and K. J. Blumer. 1998. Mot3, a Zn finger transcription factor that modulates gene expression and attenuates mating pheromone signaling in *Saccharomyces cerevisiae*. *Genetics* **149**:879–892.
- Gustin, M. C., J. Albertyn, M. Alexander, and K. Davenport. 1998. MAP Kinase pathways in the yeast *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **62**:1264–1300.
- Irie, K., K. Yamaguchi, K. Kawase, and K. Matsumoto. 1994. The yeast *MOT2* gene encodes a putative zinc finger protein that serves as a global negative regulator affecting expression of several categories of genes, including mating-pheromone-responsive genes. *Mol. Cell. Biol.* **14**:3150–3157.
- Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153**:163–168.
- Jacoby, T., H. Flanagan, A. Faykin, A. G. Seto, C. Mattison, and I. Ota. 1997. Two protein-tyrosine phosphatases inactivate the osmotic stress response pathway in yeast by targeting the mitogen-activated protein kinase, Hog1. *J. Biol. Chem.* **272**:17749–17755.
- Kaffman, A., and E. K. O'Shea. 1999. Regulation of nuclear localization: a key to a door. *Annu. Rev. Cell Dev. Biol.* **15**:291–339.
- Kao, L.-R., J. Peterson, R. Ji, L. Bender, and A. Bender. 1996. Interactions between the ankyrin repeat-containing protein Akr1p and the pheromone response pathway in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **16**:168–178.
- Khokhlatchev, A. V., B. Canagarajah, J. Wilsbacher, M. Robinson, M. Atkinson, E. Goldsmith, and M. H. Cobb. 1998. Phosphorylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation. *Cell* **93**:605–615.
- Konopka, J. B., D. D. Jenness, and L. H. Hartwell. 1988. The C-terminus of the *Saccharomyces cerevisiae* α-pheromone receptor mediates an adaptive response to pheromone. *Cell* **54**:609–620.
- Kutay, U., F. R. Bischoff, S. Kostka, R. Kraft, and D. Gorlich. 1997. Export of importin α from the nucleus is mediated by a specific nuclear transport factor. *Cell* **90**:1061–1071.
- Leberer, E., D. Dignard, D. Harscu, M. Whiteway, and D. Y. Thomas. 1994. Molecular characterization of *SIG1*, a *Saccharomyces cerevisiae* gene involved in negative regulation of G-protein-mediated signal transduction. *EMBO J.* **13**:3050–3064.
- Lee, D. C. Y., and J. D. Aitchison. 1999. Kap104p-mediated nuclear import. *J. Biol. Chem.* **274**:29031–29037.
- Lenormand, P., J. M. Brondello, A. Brunet, and J. Pouyssegur. 1998. Growth factor-induced p42/p44 MAPK nuclear translocation and retention requires both MAPK activation and neosynthesis of nuclear anchoring proteins. *J. Cell Biol.* **142**:625–633.
- Leza, M. A., and E. A. Elion. 1999. *POG1*, a novel yeast gene, promotes recovery from pheromone arrest via the G1 cyclin *CLN2*. *Genetics* **151**:531–543.
- Li, E., M. J. Cismowski, and D. E. Stone. 1998. Phosphorylation of the pheromone responsive Gβ protein of *Saccharomyces cerevisiae* does not affect its mating-specific signaling function. *Mol. Gen. Genet.* **258**:608–618.
- Li, E., E. Meldrum, H. Stratton, and D. E. Stone. 1998. Substitutions in the pheromone responsive Gβ protein of *Saccharomyces cerevisiae* confer a defect in recovery from pheromone treatment. *Genetics* **148**:947–961.
- Liu, J.-J., and S. Lindquist. 1999. Oligopeptide-repeat expansions modulate “protein only” inheritance in yeast. *Nature* **400**:573–576.
- Lopes, M. D. B., J.-Y. Ho, and S. I. Reed. 1990. Mutations in cell division cycle genes *CDC36* and *CDC39* activate the *Saccharomyces cerevisiae* mating pheromone response pathway. *Mol. Cell. Biol.* **10**:2966–2972.
- MacKay, V. L., S. K. Welch, M. Y. Insley, T. R. Manney, J. Holley, G. C. Saaru, and M. L. Parker. 1988. The *Saccharomyces cerevisiae* *BARI* gene encodes an exported protein with homology to pepsin. *Proc. Natl. Acad. Sci. USA* **85**:55–59.
- Marcus, S., C.-B. Xue, F. Nadier, and J. M. Becker. 1991. Degradation of a-factor by a *Saccharomyces cerevisiae* α-mating-type specific endopeptidase: evidence for a role in recovery of cells from G₁ arrest. *Mol. Cell. Biol.* **11**:1030–1039.

41. Mattaj, J. W., and E. Conti. 1999. Snail mail to the nucleus. *Nature* **399**:208–210.
42. Mattison, C. P., and I. M. Ota. 2000. Two protein tyrosine phosphatases, Ptp2 and Ptp3, modulate the subcellular localization of the Hog1 MAP kinase in yeast. *Genes Dev.* **14**:1229–1235.
43. Mendenhall, M. D., H. E. Richardson, and S. I. Reed. 1988. Dominant negative protein kinase mutations that confer a G1 arrest phenotype. *Proc. Natl. Acad. Sci. USA* **85**:4426–4430.
44. Metodiev, M. V., D. Matheos, M. D. Rose, and D. E. Stone. 2002. Regulation of MAPK function by direct interaction with the mating-specific G α in yeast. *Science* **296**:1483–1486.
45. Miyajima, I., K. Arai, and K. Matsumoto. 1989. *GPA1^{val-50}* mutation in the mating-factor signaling pathway in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **9**:2289–2297.
46. Moore, S. 1984. Yeast cells recover from mating pheromone α factor-induced division arrest by desensitization in the absence of α factor destruction. *J. Biol. Chem.* **259**:1004–1010.
47. Neiman, A. M., F. Chang, K. Komachi, and I. Herskowitz. 1990. *CDC36* and *CDC39* are negative elements in the signal transduction pathway of yeast. *Cell Regul.* **1**:391–401.
48. Pryciak, P. M., and L. H. Hartwell. 1996. *AKR1* encodes a candidate effector of the G $\beta\gamma$ complex in the *Saccharomyces cerevisiae* pheromone response pathway and contributes to control of both cell shape and signal transduction. *Mol. Cell. Biol.* **16**:2614–2626.
49. Reed, S. I., J. A. Hadwiger, and A. T. Lorincz. 1985. Protein kinase activity associated with the product of the yeast cell division cycle gene *CDC28*. *Proc. Natl. Acad. Sci. USA* **82**:4055–4059.
50. Reiser, V., G. Ammerer, and H. Ruis. 1999. Nucleocytoplasmic traffic of MAP kinases. *Gene Expr.* **7**:247–254.
51. Reiser, V., H. Ruis, and G. Ammerer. 1999. Kinase activity-dependent nuclear export opposes stress-induced nuclear accumulation and retention of Hog1 mitogen-activated protein kinase in the budding yeast *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **10**:1147–1161.
52. Reneke, J. E., K. J. Blumer, W. E. Courchesne, and J. Thorner. 1988. The carboxy-terminal segment of the yeast α -factor receptor is a regulatory domain. *Cell* **55**:221–234.
53. Roberts, C. J., B. Nelson, M. J. Marton, R. Stoughton, M. R. Meyer, H. A. Bennet, Y. D. D. He, H. Y. Dai, W. L. Walker, T. R. Hughes, M. Tyers, C. Boone, and S. H. Friend. 2000. Signaling and circuitry of multiple MAPK pathways revealed by a matrix of global gene expression profiles. *Science* **287**:873–880.
54. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
55. Sherman, F., G. R. Fink, and J. B. Hicks. 1986. *Laboratory course manual for methods in yeast genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
56. Siomi, M. C., M. Fromont, J.-C. Rain, L. Wan, F. Weng, P. Legrain, and G. Dreyfuss. 1998. Functional conservation of the transportin nuclear import pathway in divergent organisms. *Mol. Cell. Biol.* **18**:4141–4148.
57. Slater, M. R., and E. A. Craig. 1987. Transcriptional regulation of an hsp70 heat shock gene in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **7**:1906–1916.
58. Song, J., J. Hirschman, K. Gunn, and H. B. Dohlman. 1996. Regulation of membrane and subunit interactions by *N*-myristoylation of a G protein α subunit in yeast. *J. Biol. Chem.* **271**:20273–20283.
59. Spain, B. H., D. Koo, M. Ramakrishnan, B. Dzudzor, and J. Colicelli. 1995. Truncated forms of a novel yeast protein suppress the lethality of a G protein α subunit deficiency by interacting with the β subunit. *J. Biol. Chem.* **270**:25435–25444.
60. Stevenson, B. J., B. Ferguson, C. D. Virgilio, E. Bi, J. R. Pringle, G. Ammerer, and G. F. Sprague. 1995. Mutation of RGA1, which encodes a putative GTPase-activating protein for the polarity-establishment protein Cdc42p, activates the pheromone-response pathway in the yeast *Saccharomyces cerevisiae*. *Genes Dev.* **9**:2949–2963.
61. Stone, D. E., and S. I. Reed. 1990. G protein mutations that alter the pheromone response in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **10**:4439–4446.
62. Stratton, H. F., J. Zhou, S. I. Reed, and D. E. Stone. 1996. The mating-specific G α protein of *Saccharomyces cerevisiae* downregulates the mating signal by a mechanism that is dependent on pheromone and independent of G $\beta\gamma$ sequestration. *Mol. Cell. Biol.* **16**:6325–6337.
63. Truant, R., R. A. Fridell, R. E. Benson, H. Bogerd, and B. R. Cullen. 1998. Identification and functional characterization of a novel nuclear localization signal present in the yeast Nab2 poly(A)⁺ RNA binding protein. *Mol. Cell. Biol.* **18**:1449–1458.
64. Trueheart, J., J. D. Boeke, and G. R. Fink. 1987. Two genes required for cell fusion during yeast conjugation: evidence for a pheromone-induced surface protein. *Mol. Cell. Biol.* **7**:2316–2328.
65. van Drogen, F., V. M. Stucke, G. Jorritsma, and M. Peter. 2001. MAP kinase dynamics in response to pheromones in budding yeast. *Nat. Cell Biol.* **3**:1051–1059.
66. Whitehurst, A. W., J. L. Wilsbacher, Y. You, K. Luby-Phelps, M. S. Moore, and M. H. Cobb. 2002. ERK2 enters the nucleus by a carrier-independent mechanism. *Proc. Natl. Acad. Sci. USA* **99**:7496–7501.
67. Wozniak, R. W., M. P. Rout, and J. D. Aitchison. 1998. Karyopherins and kissing cousins. *Trends Cell Biol.* **8**:184–188.
68. Zhan, X.-L., R. J. Deschenes, and K.-L. Guan. 1997. Differential regulation of FUS3 MAP kinase by tyrosine-specific phosphatases *PTP2/PTP3* and dual-specificity phosphatase *MSG5* in *Saccharomyces cerevisiae*. *Genes Dev.* **11**:1690–1702.
69. Zhou, J., M. Arora, and D. E. Stone. 1999. The yeast pheromone-responsive G α protein stimulates recovery from chronic pheromone treatment by two mechanisms that are activated at distinct levels of stimulus. *Cell Biochem. Biophys.* **30**:193–212.