Nuclear export of Far1p in response to pheromones requires the export receptor Msn5p/Ste21p

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Far1p is a bifunctional protein that is required to arrest the cell cycle and to establish cell polarity during yeast mating. Far1p is localized predominantly in the nucleus but accumulates in the cytoplasm in cells exposed to pheromones. Here we show that Far1p functions in both subcellular compartments: nuclear Far1p is required to arrest the cell cycle, whereas cytoplasmic Far1p is involved in the establishment of cell polarity. The subcellular localization of Far1p is regulated by two mechanisms: (1) Far1p contains a functional bipartite nuclear localization signal (NLS), and (2) Far1p is exported from the nucleus by Msn5p/Ste21p, a member of the exportin family. Cells deleted for Msn5p/Ste21p failed to export Far1p in response to pheromones, whereas overexpression of Msn5p/Ste21p was sufficient to accumulate Far1p in the cytoplasm in the absence of pheromones. Msn5p/Ste21p was localized in the nucleus and interacted with Far1p in a manner dependent on GTP-bound Gsp1p. Two-hybrid analysis identified a small fragment within Far1p that is necessary and sufficient for binding to Msn5p/Ste21p, and is also required to export Far1p in vivo. Finally, similar to Δ*msn5/ste21* strains, cells expressing a mutant Far1p, which can no longer be exported, exhibit a mating defect, but are able to arrest their cell cycle in response to pheromones. Taken together, our results suggest that nuclear export of Far1p by Msn5p/Ste21p coordinates the two separable functions of Far1p during mating.

[*Key Words:* Export; cell cycle; mating; Msn5p/Ste21p; NLS]

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In eukaryotic cells, a double membrane system known as the nuclear envelope separates the nucleus from the cytoplasm, thus forming two distinct subcellular compartments. The localization of proteins to either the nucleus or the cytoplasm can confer regulation of their function (Görlich and Mattaj 1996; Nigg 1997). In many instances this regulation is imposed by the cell cycle stage or by activation of a signal transduction pathway in response to extracellular signals. For example, the transcription factor Swi5p is nuclear only during the G_1 phase of the cell cycle (Moll et al. 1991), whereas multiple mitogen-activated protein kinases (MAPKs) translocate into the nucleus in response to growth factors (Feldherr and Akin 1994).

Yeast-mating pheromones regulate the subcellular localization of Far1p; Far1p is nuclear in the absence of pheromones but is found predominantly in the cytoplasm in cells treated with pheromones (Butty et al. 1998). Pheromones trigger a MAPK signal transduction pathway, which results in transcriptional activation of many genes, cell cycle arrest, and changes in cell polarity and morphology (Sprague and Thorner 1992; Leberer et al. 1997). These responses are initiated by binding of pheromones to a seven-transmembrane receptor, which is coupled to a heterotrimeric G protein. Gβγ then transduces the signal through its effectors Ste5p and Ste20p to a MAPK cascade composed of Ste11p, Ste7p, and Fus3p (Herskowitz 1995; Leeuw et al. 1998). Fus3p is thought to phosphorylate the transcriptional repressors Dig1p and Dig2p resulting in activation of the transcription factor Ste12p (Cook et al. 1996; Pi et al. 1997; Tedford et al. 1997) and regulates the activity of Far1p, which is required to arrest the cell cycle presumably by inhibiting cyclin-dependent kinases (Peter and Herskowitz 1994; Gartner et al. 1998). Far1p also functions as an adaptor that targets cytoplasmic polarity establishment proteins to the heterotrimeric G protein (Butty et al. 1998; Nern and Arkowitz 1999).

We are interested in understanding how the subcellular localization of Far1p is regulated in response to pheromones. Subcellular localization of proteins can be controlled by regulating import into or export from the

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nucleus. Two types of targeting signals mediate nuclear transport of proteins: nuclear localization signal (NLS) sequences promote nuclear import and generally consist of a cluster of basic amino acids (Kalderon et al. 1984; Dingwall and Laskey 1991). Second, nuclear export signal (NES) sequences promote export of proteins from the nucleus to the cytoplasm. A small leucine-rich sequence was the first NES identified, and was shown to be necessary and sufficient to export the human immunodeficient virus (HIV) Rev protein and protein kinase inhibitor (PKI), an inhibitor of cAMP-dependent protein kinase A (Nakielny and Dreyfuss 1997). Targeting signals are recognized by a family of soluble receptors, which are heterodimers consisting of importin α and importin β. The complex translocates with the cargo into or out of the nucleus and disassembles in the new compartment (Nigg 1997). In several cases, binding of import or export receptors to the targeting signal of the cargo is regulated by phosphorylation (Moll et al. 1991; Sidorova et al. 1995; Beals et al. 1997; DeVit et al. 1997; Kaffmann et al. 1998a).

Both genetic and biochemical experiments demonstrate a crucial role of the small GTPase Ran (or Gsp1p in yeast) in both nuclear import and export (Koepp and Silver 1996). Ran is found in the nucleus and cytoplasm, but because the regulators of Ran are localized differentially, the nucleus is thought to contain Ran predominantly in its GTP form, whereas Ran–GDP is predominantly cytoplasmic. This asymmetric distribution of GDP– and GTP–Ran controls assembly and disassembly of transport complexes. Binding of Ran–GTP to importin β family members involved in export promotes interaction with the NES-containing cargo in the nucleoplasm, whereas in the cytoplasm binding of importin β members involved in import allows translocation of NLS-containing proteins into the nucleus. In the nucleus, exchange of Ran–GDP to Ran–GTP by the exchange factor Rcc1 (or Rna1p in yeast) facilitates release of the cargo from importin β. Thus, the nucleotide state of Ran serves as a marker for nuclear and cytoplasmic compartments and imparts directionality to transport processes (Görlich et al. 1996; Izauralde et al. 1997).

Searches of the yeast genome database revealed at least 13 proteins with significant homology to importin β, and several family members have now been shown to function as import or export receptors. The uncharacterized receptors are thought to define additional import and export pathways. A major challenge is to identify targets of the multiple import and export receptors and to understand their role in controlling the subcellular localization of the target proteins in response to extracellular signals.

Here we show that the subcellular localization of Far1p is regulated by two pathways: (1) A bipartite NLS in the amino terminus of Far1p is necessary for efficient import into the nucleus in a cell cycle- and pheromone-independent manner; and (2) we have identified Msn5p/Ste21p as a nuclear export receptor for Far1p. Msn5p/Ste21p was localized in the nucleus and was required to export Far1p in response to pheromones. Msn5p/Ste21p bound Far1p through a novel NES sequence in a manner dependent on the Ran homolog Gsp1p. Accumulation of Far1p in the cytoplasm required activation of the pheromone response pathway but not transcriptional activation of Msn5p/Ste21p, suggesting that post-translational mechanisms regulate relocalization of Far1p in response to pheromones. Finally, our results suggest distinct roles for nuclear and cytoplasmic Far1p during yeast mating and may serve as a paradigm for how cell cycle arrest and polarity establishment are coordinated.

Results

Far1p contains a functional bipartite NLS

To identify sequences within Far1p required for nuclear localization, we determined the subcellular localization of fusions between portions of Far1p and the green fluorescent protein (GFP). We found that the amino-terminal domain of Far1p was required for nuclear localization; deletion of 50 amino-terminal amino acids resulted in a truncated Far1 protein, which was found predominantly in the cytoplasm (Fig. 1A). Importantly, a fusion protein between this amino-terminal domain of Far1p and GFP was found in the nucleus (Fig. 1C), demonstrating that these 50 amino acids of Far1p are not only required but also sufficient for nuclear localization. Closer examination of the sequence revealed two potential bipartite NLSs located between amino acids 11 and 30 (nls1) and 38 and 48 (nls2) of Far1p (Fig. 1B). To address the functional importance of these putative NLS sequences for the localization of Far1p, we mutated the lysine residues 29 and 30 (Far1p–K29A/K30A; nls1) and 41 and 42 (Far1p-R41A/K42A; nls2) to alanine residues. Whereas Far1p–nls2 was still localized predominantly in the nucleus, Far1p–nls1 was found largely in the cytoplasm, even in the absence of pheromones, demonstrating that NLS1 comprises a functional NLS (Fig. 1A). However, some remaining nuclear staining of Far1p–nls1 was still visible, suggesting that NLS2 may contribute to efficient nuclear localization of Far1p. Consistent with this notion, a Far1p mutant protein that has both putative NLS sequences inactivated (Far1p–nls1/nls2) was almost exclusively cytoplasmic (Fig. 1A). NLS1 and NLS2 may function as two separate bipartite NLS or they may be part of the same NLS sequence. Taken together, these results demonstrate that Far1p contains a functional bipartite NLS sequence in the amino-terminal 50 amino acids; NLS1 plays a major role, whereas NLS2 contributes to nuclear localization of Far1p to a minor extent (see also below).

Msn5p/Ste21p functions as an exportin for Far1p

Next, we examined whether Far1p might be exported from the nucleus in response to pheromones. Nuclear export is mediated by exportins, which bind to target proteins and export them in an ATP- and Ran-dependent

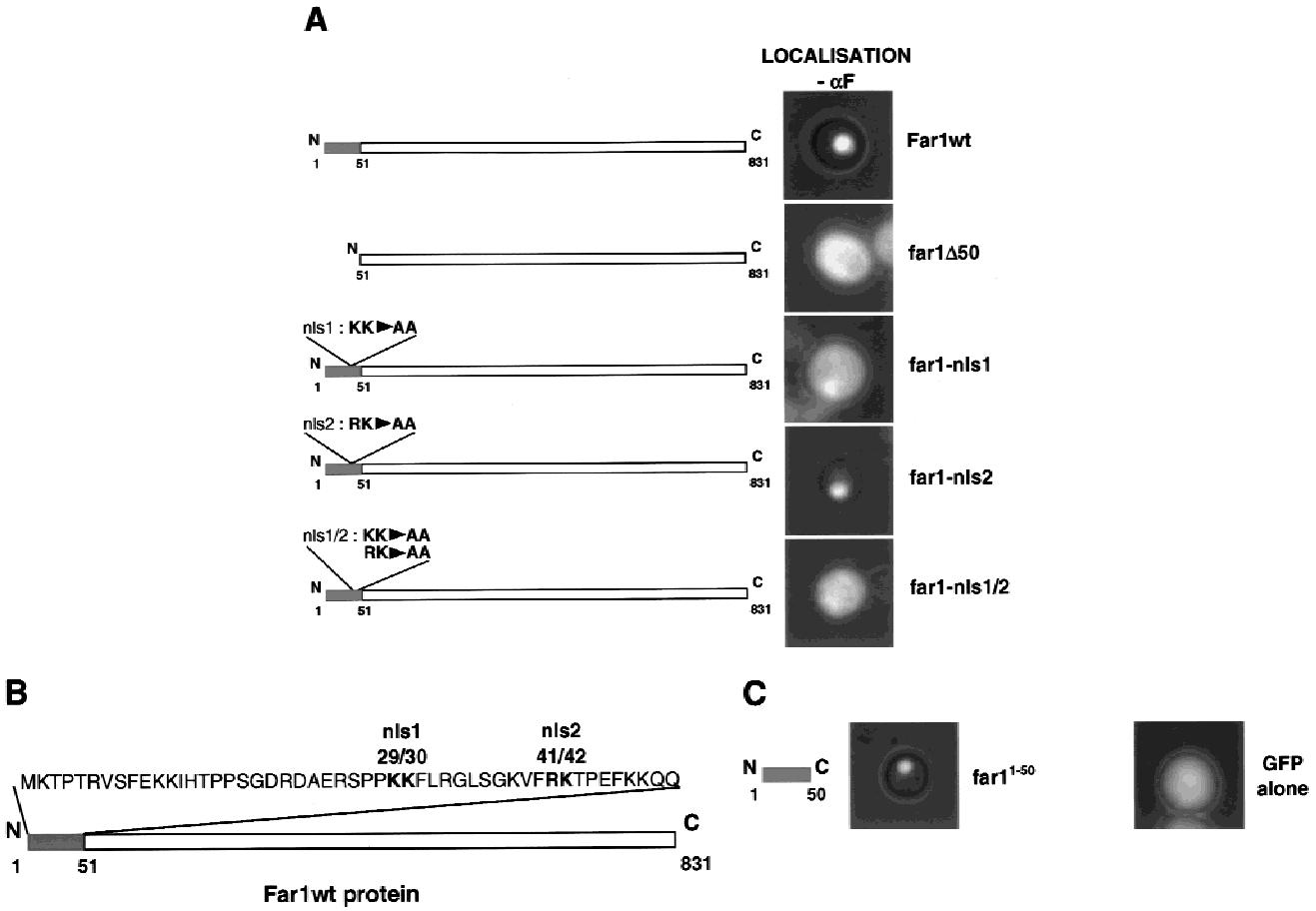

Figure 1. Far1p contains a functional NLS in the amino terminus. (*A*) Wild-type or various Far1p mutant proteins were expressed as fusions to GFP from the inducible *GAL* promoter and visualized by fluorescence microscopy. The introduced mutations are indicated schematically of *left*. Photographs show GFP fluorescence superimposed with phase contrast. (*B*) Schematic representation of Far1p and the 50 amino-terminal amino acids that are required for nuclear localization of Far1p. The mutated basic amino acids that are part of a bipartite nuclear localization signal are highlighted in bold. (*C*) The 50 amino-terminal amino acids of Far1p are sufficient to localize GFP in the nucleus. GFP fused to the 50 amino-terminal amino acids of Far1p (far1$^{1-50}$; *left*) or GFP alone (*right*) were expressed in cells and visualized as described above.

manner (Göhrlich and Mattaj 1996). Because cells lacking *MSN5/STE21* exhibit reduced mating efficiency (Akada et al. 1996) and Msn5p/Ste21p displays significant sequence homology with exportins (Weis 1998) and interacts with Ran–GTP (Göhrlich et al. 1997), we tested whether Msn5p/Ste21p may be involved in localization of Far1p. Interestingly, we observed that Far1p remained exclusively nuclear in Δ*msn5/ste21* cells treated with pheromones (Fig. 2A), suggesting that Msn5p/Ste21p is involved in exporting Far1p. Strikingly, although Far1p–nls1 was predominantly cytoplasmic in wild-type cells, it accumulated in the nucleus of cells deleted for *STE21/MSN5* (Fig. 2B). Both defects were fully corrected by a plasmid expressing endogenous levels of Msn5p/Ste21p (Fig. 2B, right; data not shown), confirming that the defects are caused by lack of Msn5p/Ste21p. We conclude that Msn5p/Ste21p is required for cytoplasmic localization of Far1p in response to pheromones. These results further indicate that Far1p is very dynamic and shuttles between the nucleus and the cytoplasm even in the absence of pheromones.

To test whether expression of Msn5p/Ste21p is sufficient to accumulate Far1p in the cytoplasm, we overexpressed Msn5p/Ste21p from the inducible *GAL* promoter. Strikingly, Far1p relocalized efficiently under these conditions and was found predominantly in the cytoplasm (Fig. 3A). Addition of α-factor further increased the cytoplasmic pool of Far1p, suggesting that pheromones may activate export of Far1p by Msn5p/Ste21p or may inhibit its nuclear import. Moreover, no remaining nuclear staining of Far1p–nls1 was observed in cells overexpressing Msn5p/Ste21p (data not shown). In contrast, overexpression of Msn5p/Ste21p did not alter nuclear localization of Rap1p–Δ303–416–GFP (Fig. 3B), demonstrating that Msn5p/Ste21p is specific and does not perturb indiscriminately nuclear transport. Importantly, overexpression of Msn5p/Ste21p did not activate the pheromone response pathway as measured by the induction of the reporter *FUS1–lacZ* (Fig. 3D). Moreover, expression of Msn5p/Ste21p was able to trigger relocalization of Far1p in strains deleted for *STE7* or *STE20*, demonstrating that activation of the mating

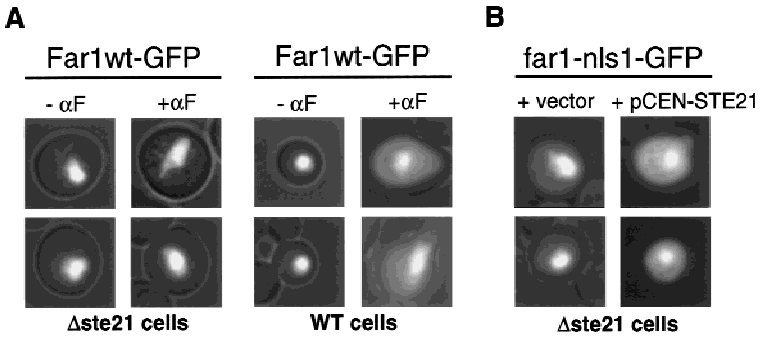

Figure 2. The exportin Msn5p/Ste21p is required to export Far1p. (*A*) Far1p–GFP was expressed in wild-type cells (*right*) or cells deleted for *MSN5/STE21* (*left*) and treated (*right row*) or not treated (*left row*) with α-factor for 6 hr. Photographs show GFP fluorescence superimposed with phase contrast. Note that Far1p–GFP remains nuclear in Δ*msn5/ste21* cells treated with α-factor. (*B*) Δ*msn5/ste21* cells expressing Far1p–nls1–GFP were transformed with either an empty control plasmid (vector; *left row*) or a low copy number plasmid encoding *MSN5/STE21* (pCEN-STE21; *right row*). Cells were analyzed as above. Note that Far1p–nls1–GFP is predominantly nuclear in Δ*msn5/ste21*.

pathway was not required to export Far1p under these conditions (Fig. 3C). Taken together, these results demonstrate that overexpression of Msn5p/Ste21p is sufficient to relocalize Far1p to the cytoplasm in a mating pheromone pathway-independent manner, and strongly suggest that Msn5p/Ste21p functions as an exportin for Far1p in vivo.

Msn5p/Ste21p binds to Far1p in a manner dependent on Gsp1p–GTP

Because exportins have been shown to bind directly to their target proteins in a Ran–GTP-dependent manner, we tested whether Msn5p/Ste21p and Far1p are able to interact with each other by coimmunoprecipitation (Fig. 4) and two-hybrid assays (Table 1; Fig. 5). Myc-tagged Msn5p/Ste21p was immunoprecipitated with 9E10 antibodies from extracts prepared from wild-type cells (Fig. 4A,B, lanes 1–6,8,9) or cells harboring a temperature-sensitive *GSP1* allele (lanes 10–12), which express Far1p from the inducible *GAL* promoter. The immunoprecipitates were then examined for the presence of Far1p by immunoblotting. Far1p readily coimmunoprecipitated with myc-tagged Msn5p/Ste21p (lanes 4,6), whereas no interaction was detected in cells expressing untagged Msn5p/Ste21p (lanes 2,8). Likewise, no interaction between Msn5p/Ste21p and Far1p could be detected when extracts were prepared from *gsp1* cells shifted to 35°C for 3 hr (lanes 10,11), indicating that Gsp1p is required for binding of Far1p to Msn5p/Ste21p. Expression of a GTP-locked mutant form of Gsp1p (Gsp1p–G21V) restored binding of Far1p and Msn5p/Ste21p in *gsp1-1* cells (Fig. 4B, lane 12), although the strain was still unable to grow at the restrictive temperature (data not shown). To further corraborate these results, we performed in vitro binding assays (Fig. 4C): Gsp1p–Myc expressed in *Escherichia coli* was immunoprecipitated, loaded with either GTPγS or GDP, and incubated with yeast extracts containing Msn5p/Ste21p, Far1p, or both proteins as indicated. Interestingly, Far1p bound Gsp1p–Myc only in the presence of Msn5p/Ste21p (cf. Lanes 14 and 18), suggesting that all three proteins together form a complex. Furthermore, Far1p interacted preferentially with Gsp1p–Myc in its GTP-bound form (cf. lanes 17 and 18). Taken together, these results suggest that Far1p and Msn5p/Ste21p interact with each other in a Gsp1p–GTP-dependent manner.

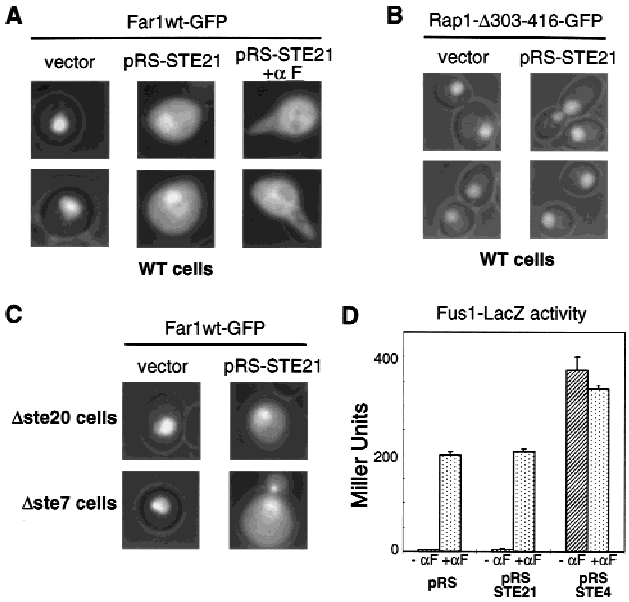

Figure 3. Overexpression of Msn5p/Ste21p is sufficient to export Far1p. (*A,B*) Cells expressing Far1p–GFP (*A*) or for control Rap1p–Δ303–416-GFP (*B*) were transformed with a control plasmid (vector; *left rows*) or a plasmid allowing overexpression of Msn5p/Ste21p from the inducible *GAL* promoter (pRS–STE21; *right rows*). Cells were grown in the presence of galactose and analyzed by fluorescence microscopy. Where indicated α-factor was added for 3 hr. Note that complete cytoplasmic localization of Far1p requires overexpression of Msn5p/Ste21p and addition of α-factor. (*C*) Redistribution of Far1p to the cytoplasm does not require an intact mating pathway. Δ*ste20* (*top*) or Δ*ste7* cells (*bottom*) expressing Far1p–GFP were transformed with a control plasmid (vector; *left row*) or a plasmid allowing overexpression of Msn5p/Ste21p from the inducible *GAL* promoter (pRS–STE21; *right row*) and analyzed as described above. (*D*) The ability of cells carrying a control plasmid (pRS) or plasmids allowing overexpression of Msn5p/Ste21p (pRS–STE21) or Ste4p (pRS–STE4) to induce the reporter *FUS1–lacZ* was determined either in the absence (– αF) or presence of α-factor (+ αF). Bars show mean β-galactosidase activity ±S.D. for four independent transformants. Note that overexpression of Msn5p/Ste21p does not activate the pheromone response pathway.

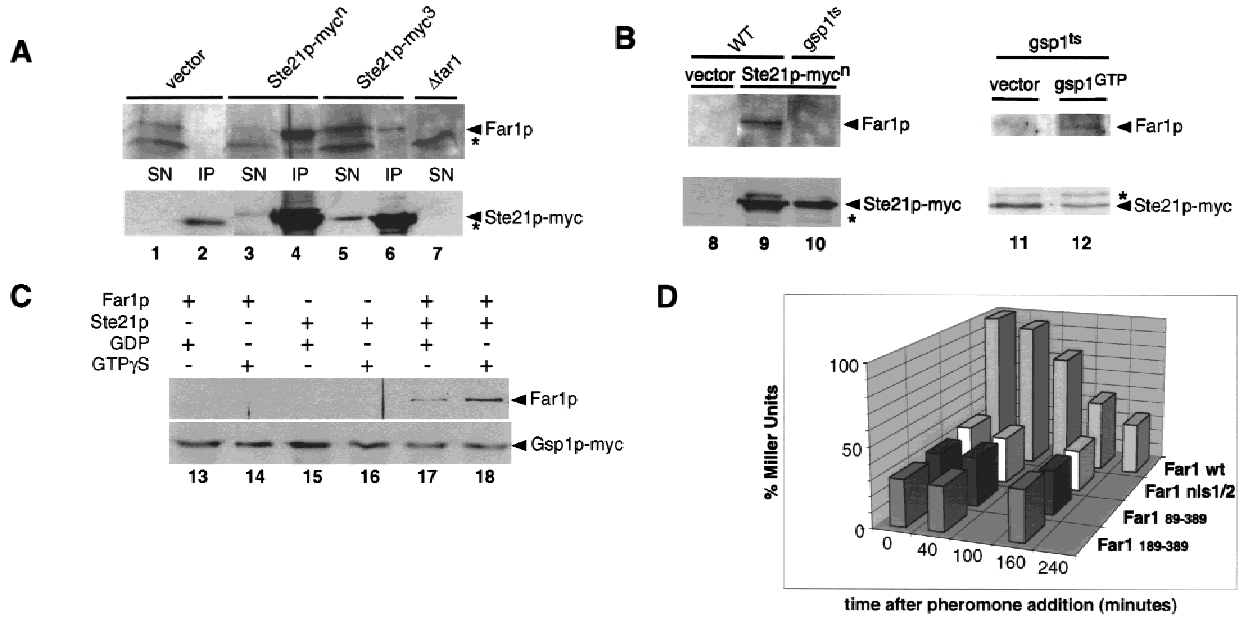

Figure 4. Far1p interacts with Msn5p/Ste21p in a Gsp1p-GTP-dependent manner. (*A*,*B*) Msn5/Ste21p–myc was immunoprecipitated with 9E10 antibodies from extracts prepared from wild-type (K699; lanes *1–6, 8,9*) or temperature-sensitive *gsp1* cells (YSH80; lanes *10–12*) expressing Far1p and either untagged Msn5p/Ste21p (lanes *1,2,8*), Msn5p/Ste21p–myc^n (lanes *3,4,9–12*) or Msn5p/Ste21p–*myc*^{3} (lanes *5,6*). The immunoprecipitates (IP; lanes *2, 4, 6, 8-12*) and an aliquot of the supernatant before immunoprecipitation (SN; lanes *1,3,5,7*) were analyzed for the presence of Far1p (*top*) or Msn5p/Ste21p–myc (*bottom*) by immunoblotting. Expression of Gsp1–GTP (YBM100; gsp1–G21V, lane *12*) restored the ability of Msn5p/Ste21p to interact with Far1p. Cells lacking Far1p (YMP1054, lane *7*) were included to control for the specificity of the antibodies. The arrowhead marks the position of Far1p (*top*) or Msn5p/Ste21p–myc (*bottom*); the asterisk points to the position of proteins that cross-reacts with the antibodies. Note that Far1p and Msn5p/Ste21p interact in a Gsp1p-dependent manner. (*C*) Sepharose beads containing immunoprecipitated Gsp1p–Myc expressed in *E. coli* and loaded with either GTPγS (lanes *14,16,18*) or GDP (lanes *13,15,17*) were incubated with yeast extracts containing as indicated Msn5p/Ste21p and Far1p expressed from the *GAL* promoter. Bound proteins were eluted and analyzed by immunoblotting for the presence of Far1p (*top*) and Gsp1p–Myc (*bottom*). Note that Far1p preferentially bound Gsp1p–GTP, but only in the presence of Msn5p/Ste21p. (*D*) Two-hybrid analysis of Msn5p/Ste21p and either wild-type (wt) or cytoplasmic Far1p mutant proteins in response to pheromones (times in minutes after addition of α-factor). The interaction was quantified as described and shown as percentage of Miller units relative to wild-type controls without pheromones. Note that the interaction between Msn5p/Ste21p and wild-type Far1p but not cytoplasmic mutant forms of Far1p decreases in an α-factor-dependent manner.

Far1p also interacted with Msn5p/Ste21p by two-hybrid assay (Table 1; Fig. 4D and 5). Deletion analysis of Far1p revealed that the domain of Far1p, which binds Msn5p/Ste21p, was located between amino acids 285 and 390 (Table 1). Interestingly, this domain overlaps with the binding site for Cdc28p–Cln2p (Peter et al. 1993; Gartner et al. 1998), suggesting that Msn5p/Ste21p and Cdc28p–Cln2p kinase might compete for binding to Far1p. Although this segment of Far1p does not contain a classic leucine-rich hydrophobic (NES) sequence, we found a motif that is conserved in Far1p from *Candida albicans* and is also present in Ste5p, suggesting that Far1p may use a novel type of NES. Consistent with this notion, we observed that Ste5p–GFP was exported after overexpression of Msn5p/Ste21p, and conversely Ste5p–GFP remained nuclear in Δ*msn5*/Δ*ste21* cells treated with α-factor, suggesting that Ste5p is also a target of Msn5p/Ste21p (data not shown). To test whether this conserved motif is required to export Far1p in vivo we deleted the Msn5p/Ste21p-binding site on Far1p (Far1p–Δ285–390). As shown in Table 1, Far1p–Δ285–390 was unable to interact with Msn5p/Ste21p and importantly, both α-factor treatment and overexpression of Msn5p/Ste21p were unable to export Far1p–Δ285–390 from the nucleus (data not shown, see below). Thus, the ability of Far1p to bind to Msn5p/Ste21p correlates with the ability of Far1p to relocalize to the cytoplasm, suggesting that binding of Far1p to Msn5p/Ste21p is required to export Far1p in response to pheromones. However, this putative NES fused to GFP containing the NLS of Pho4p (Kaffmann et al. 1998a) was only able to induce nuclear export weakly even when Msn5p/Ste21p was overexpressed (data not shown), suggesting that this domain may not be sufficient to function as an export signal in vivo.

Binding of Far1p and Msn5p/Ste21p may not be regulated by pheromones

To test whether the interaction between Far1p and Msn5p/Ste21p is regulated by pheromones we performed coimmunoprecipitation and two-hybrid analysis in cells treated or not treated with α-factor. As shown in Figure 4D, the interaction between wild-type Far1p and Msn5p/

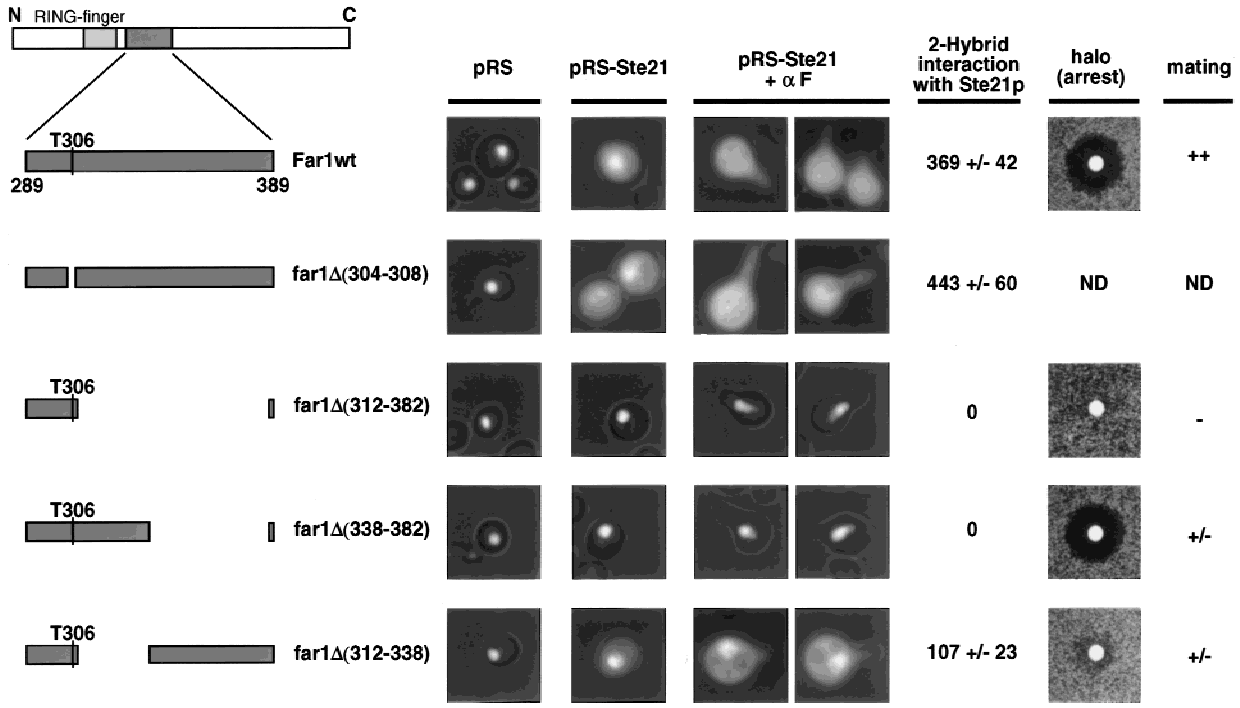

Figure 5. Binding of Far1p and Msn5p/Ste21p is required for efficient mating but not cell cycle arrest. Cells expressing wild-type Far1p–GFP (Far1wt; *top row*) or mutant Far1p–GFP lacking various parts of the Msn5p/Ste21p binding domain (*bottom rows*) were transformed with a control plasmid (pRS; *left row*) or a plasmid expressing Msn5p/Ste21p from the inducible *GAL* promoter (pRS–STE21; *right rows*). Where indicated cells were treated with α-factor for 3 hr. Full-length or the various deletion mutants of Far1p are schematically represented on the left; deleted amino acids are indicated in parentheseis. The Far1p mutants were also tested for their ability to interact with Msn5p/Ste21p by two-hybrid assay; Miller units with standard deviations are shown. Cells deleted for *FAR1* (YMP1054) transformed with low copy number plasmids expressing wild-type Far1p (*top row*) or the indicated Far1p mutant proteins (*bottom rows*) from the endogenous promoter were tested for their ability to arrest the cell cycle by halo assay, or for their ability to mate against the mating tester IH2625. (++) Wild-type mating; (+/–) strongly reduced mating; (–) sterile; (ND) not determined. Note that the Msn5p/Ste21p-binding domain of Far1p is required for nuclear export and efficient mating in vivo.

Ste21p decreased in a time-dependent manner in cells exposed to pheromones with kinetics that mirror the cytoplasmic accumulation of Far1p. We interpret this result to indicate that pheromones do not increase the interaction between Far1p and Msn5p/Ste21p, and that cytoplasmic accumulation of Far1p reduces the transcriptional readout of the two-hybrid assay that occurs in the nucleus. Consistent with this explanation, the interaction between Msn5p/Ste21p and several cytoplasmic Far1p mutants lacking their nuclear localization signal was decreased to levels comparable to wild-type Far1p in pheromone-treated cells, and importantly, no further decrease was observed after pheromone treatment (Fig. 4D). Likewise, Far1p and Msn5p/Ste21p were able to coimmunoprecipitate with similar efficiency in cells treated or not treated with α-factor (data not shown), suggesting that phosphorylation of Far1p does not increase their interaction. In addition, although redistribution of Far1p in response to pheromones was dependent on Fus3p in vivo (M. Blondel and M. Peter, unpubl.), the interaction between Far1p and Msn5p/Ste21p as assayed by two-hybrid analysis was neither dependent on *FUS3* (Table 1) nor on the sites on Far1p, which are phosphorylated by Fus3p in response to pheromones (Table 1; Gartner et al. 1998). Taken together, we conclude that the binding of Far1p and Msn5p/Ste21p may not be regulated by pheromones, although it remains possible that a weak effect could have been masked because the proteins were overexpressed. Therefore, nuclear export of Far1p may be constitutive or regulated by nuclear retention.

The requirements for binding of Far1p to Cdc28p–Clnp and the exportin Msn5p/Ste21p can be mutationally separated

Far1p–Δ285–390 remained in the nucleus in α-factor-treated cells (data not shown), but no longer interacts with Cdc28p–Clnp and therefore is unable to arrest the cell cycle in response to pheromones (Peter et al. 1993). To separate the cell cycle arrest and export functions we constructed several short deletion mutants within this domain (Fig. 5). Any Far1p deletion mutant that removed threonine 306 (T306) was unable to arrest the cell cycle as determined by halo assay (Fig. 5; data not shown), consistent with the result that phosphorylation of T306 by Fus3p regulates its binding to Cdc28p–Clnp (Peter et al. 1993; Gartner et al. 1998). In contrast, Far1p–Δ338–

Table 1. *Two-hybrid analysis of the interaction between Msn5p/Ste21p and Far1p*

Activation domain fusion	Miller units ± S.D.
WT(EGY48)	
Far1p(1–830)	415 ± 117
Far1p(353–830)	0
Far1p(1–389)	877 ± 194
Far1p(174–285)	0
Far1p(89–389)	327 ± 110
Far1p(189–389)	255 ± 71
Far1p(289–389)	253 ± 88
Far1pΔ(285–390)	0
*far1*Δ	
Far1p(1–389)	958 ± 73
Far1p(1–392) A26 A87 A114 A324 V341 A346	1044 ± 78
Far1p(1–392) A26 A87 A114 A306 V341 A346	951 ± 126
*far1*Δ *fus3*Δ	
Far1p(1–830)	492 ± 18
Far1p(1–389)	801 ± 30

Full-length or various mutants of Far1p fused to an activation domain (AD) were tested for their ability to interact with full-length Msn5p/Ste21p fused to a DNA-binding domain by two-hybrid analysis. The numbers in parenthesis indicate the amino acids of Far1p fused to the AD, except in Far1pΔ(285–390), where they indicate the deleted amino acids. The mutations in the Fus3p phosphorylation sites are indicated in small letters (Gartner et al. 1998). Expression of the β-Gal reporter was quantified as described and shown as Miller units with standard deviations. The interaction was tested in either a wild-type strain (EGY48), a strain deleted for *FAR1* (YMP290), or a strain deleted for both *FAR1* and *FUS3* (YMP291). Note that a small domain within Far1p (amino acids 289–389) is necessary and sufficient to bind Msn5p/Ste21p.

382, which lacks the carboxy-terminal half of this domain, was able to arrest efficiently the cell cycle, but failed to interact with Msn5p/Ste21p by two-hybrid assay and as a consequence was unable to exit from the nucleus in vivo (Fig. 5). These experiments demonstrate that the requirements on Far1p for interacting with Cdc28p–Clnp or Msn5p/Ste21p are mutationally separable. Importantly, cells expressing Far1p–Δ338–382 exhibited a bilateral mating defect, demonstrating that nuclear export of Far1p is needed for efficient mating, most likely for oriented cell polarity.

Msn5p/Ste21p is a nuclear protein that is not induced in response to α-factor

Because overexpression of Msn5p/Ste21p was sufficient to export Far1p from the nucleus, we tested whether pheromone may relocalize Far1p by increasing the levels of Msn5p/Ste21p. However, we found that the levels of Msn5p/Ste21p were not altered in response to pheromones (Fig. 6A), suggesting that post-translational modifications of either Far1p or Msn5p/Ste21p regulate export of Far1p in response to pheromones. To examine the subcellular localization of Msn5p/Ste21p, we epitope tagged Msn5p/Ste21p at its amino terminus with GFP and visualized the functional fusion protein by fluorescence microscopy. Consistent with Msn5p/Ste21p functioning as an exportin, the protein was found predominantly in the nucleus (Fig. 6B). We did not observe any differences in localization of Msn5p/Ste21p through the cell cycle or in cells exposed to α-factor (bottom). Similar results were also obtained if Msn5p/Ste21p was localized by indirect immunofluorescence microscopy using myc-tagged Msn5p/Ste21p (data not shown).

Msn5p/Ste21p plays multiple roles during yeast mating

Next, we investigated the mating phenotype of cells deleted for *STE21/MSN5*. As observed previously (Akada et

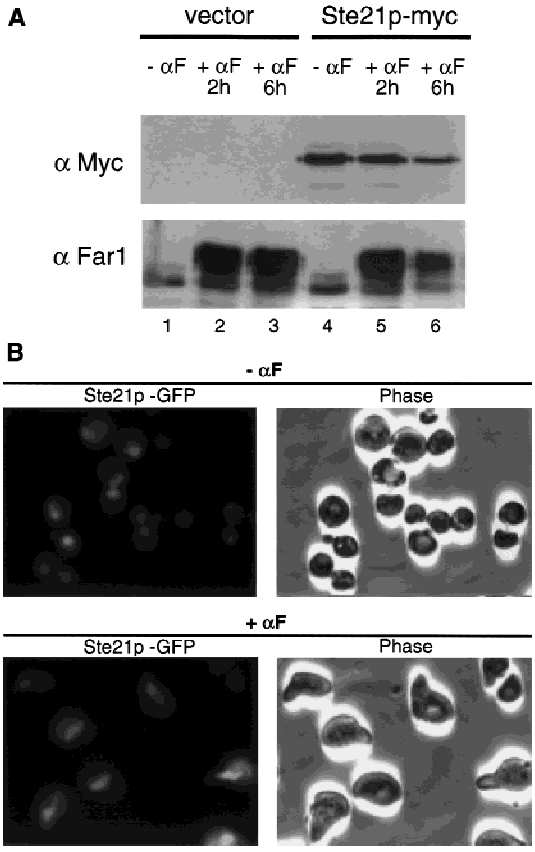

Figure 6. Msn5p/Ste21p is a nuclear protein that is not induced in response to pheromones. (*A*) Cells expressing epitope-tagged Msn5p/Ste21p (Ste21p–myc; lanes *4–6*) or untagged Msn5p/Ste21p (vector; lanes *1–3*) from the endogenous promoter were treated (lanes *2,3,5,6*) or not treated with α-factor for the times indicated (lanes *1,4*). Extracts were immunoblotted with 9E10 antibodies (*top*) or antibodies specific for Far1p (*bottom*). Note that in contrast to Far1p the levels of Msn5p/Ste21p do not increase in α-factor-treated cells. (*B*) Cells expressing Msn5p/Ste21p fused to GFP (Ste21p–GFP) from the endogenous promoter were treated (*top*) or not treated (*bottom*) with α-factor and analyzed by fluorescence microscopy (*left*). The corresponding phase contrast pictures are shown on the right. Note that Msn5p/Ste21p is nuclear in α-factor arrested cells and during all phases of the cell cycle.

al. 1996), Δ*ste21/msn5* cells mate with reduced efficiency (Fig. 7A, top); this mating defect is bilateral as the mating efficiency was decreased dramatically if both mating partners were deleted for *STE21/MSN5* (Fig. 7A, bottom). Interestingly, Δ*msn5/ste21* cells were able to induce the transcripts of *FUS1* and *FAR1* efficiently in response to pheromones (Fig. 7C), demonstrating that Msn5p/Ste21p is not required for signal transduction. Likewise, cells lacking Msn5p/Ste21p were able to arrest the cell cycle in response to pheromones in a *FAR1*-dependent manner (Fig. 7B), suggesting that cytoplasmic Far1p is not required for cell cycle arrest (see also below). However, Δ*msn5/ste21* cells displayed a reduced ability to form mating projections (shmoos), and even after several hours in pheromones >80% of the cells remained unpolarized (Fig. 7D), suggesting that Msn5p/Ste21p is involved in exporting a protein involved in shmoo formation. This protein is unlikely to be Far1p, because Far1p is not needed to form shmoos (Valtz et al. 1995; M. Peter, unpubl.). Consistent with these observations, we found that overexpression of cytoplasmic Far1p was not sufficient to suppress the mating defect of Δ*msn5/ste21* cells (data not shown). In addition, *MSN5/STE21* and *FAR1* were synthetic sterile (data not shown), supporting an additional role of Msn5p/Ste21p during mating. Thus, besides Far1p, Msn5p/Ste21p must export yet unknown targets involved in shmoo formation and perhaps other steps of mating.

Nuclear and cytoplasmic Far1p play distinct roles during mating

Next, we examined the functional importance of Far1p localization for its cell cycle arrest and mating function. Far1p is required for oriented cell polarization during mating (Dorer et al. 1995; Valtz et al. 1995) and is thought to function as a cytoplasmic adaptor that recruits polarity establishment proteins to the site of extracellular signaling marked by the heterotrimeric G protein Gβγ (Bähler and Peter 1999). Several lines of evidence suggest that cytoplasmic Far1p is required for this polarization function. First, like Δ*msn5/ste21* cells, Δ*far1* cells expressing Far1p–Δ338–382 mutant protein, which can no longer be exported in response to pheromones, exhibited a mating defect (see Fig. 5). Second, wild-type cells expressing cytoplasmic Far1p–nls1 or

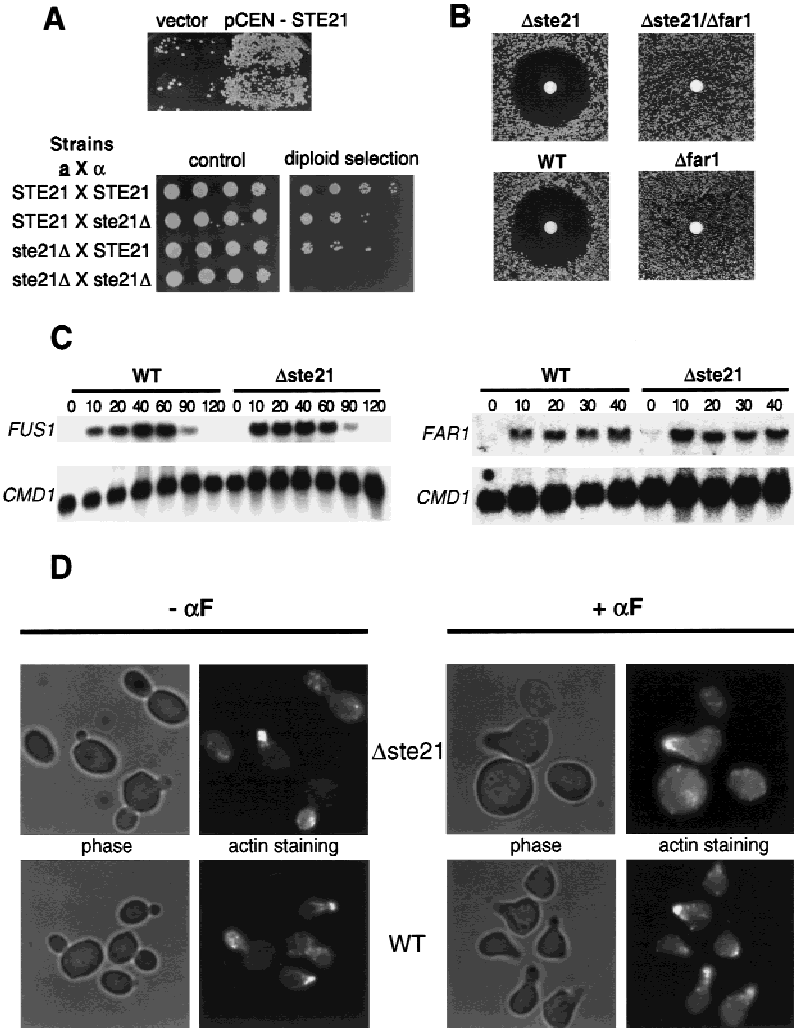

Figure 7. Cells lacking Msn5p/Ste21p exhibit a bilateral mating defect but are able to arrest their cell cycle. (*A*; *top*) Mating assay with the mating tester IH2625 and Δ*msn5/ste21* cells (PAY20) transformed with a control plasmid (vector) or a plasmid carrying *MSN5/STE21* (pCEN–STE21). (*Bottom*) Mating assays with either *MAT***a** or *MAT*α wild-type or Δ*msn5/ste21* cells as indicated. Serial dilutions of the mating reactions were spotted on control plates (*left*) or selective plates where only diploid cells are able to grow (*right*). Note that Δ*msn5/ste21* cells exhibit a bilateral mating defect. (*B*) The indicated strains were analyzed by halo assay for their ability to arrest the cell cycle in response to α-factor. Note that cells lacking Msn5p/Ste21p efficiently arrest their cell cycle in a manner dependent on Far1p. The following strains were analyzed: Δ*ste21* (PAY20); Δ*ste21* Δ*far1* (YMP1067); wt (K699) and Δ*far1* (YMP1054). (*C*) Δ*msn5/ste21* cells (PAY20) were able to efficiently induce mating-specific transcription of *FAR1* (*right*) and *FUS1* (*left*). Northern analysis of total RNA isolated from wild-type (K699; *left* lanes) or Δ*msn5/ste21* cells (PAY20, *right* lanes) treated with α-factor for the times indicated (in minutes). A probe against *CMD1* RNA was used as total RNA loading control (*bottom*). (*D*) The morphology of cells deleted for Msn5p/Ste21p (PAY20; *top row*) or wild-type cells (K699; *bottom row*) in the absence (*left rows*) or presence of α-factor (*right rows*) was analyzed by phase contrast microscopy (*left*). Actin distribution is directed toward the growing bud or shmoo tip as visualized after staining with rhodamine–phalloidin (*right*).

cells overexpressing Msn5p/Ste21p mated with comparable or slightly increased efficiency (Fig. 8E, right column), suggesting that additional Far1p in the cytoplasm may improve the mating function of Far1p. Finally, ~60% of haploid cells overexpressing cytoplasmic Far1p–nls1, but not wild-type Far1p, budded in a bipolar or random instead of axial pattern (Table 2; Chant 1996), suggesting that compartmentalization in the nucleus prevents Far1p from interfering with polarity establishment proteins in the absence of pheromones. Taken together, these results indicate that the polarization function of Far1p during mating requires cytoplasmic Far1p.

In contrast, nuclear Far1p appears to be required for cell cycle arrest in response to pheromones. We have shown above that Δ*ste21/msn5* cells failed to export Far1p but were able to arrest efficiently their cell cycle in

Table 2. *Overexpression of cytoplasmic Far1p interferes with the budding pattern*

Plasmid	Budding pattern (%)			No. of cells counted
	axial	bipolar	random	
pRD53	76	23.5	0.5	200
pRD53–Far1wt	72	26.5	1.5	400
pRD53–*far1–nls1*	34.5	54	11.5	400
pRD53–*far1–nls1,2*	42	44	14	200

Cells deleted for *FAR1* (YACB169) were tranformed with an empty control plasmid (pRD53) or plasmids allowing overexpression of wild-type Far1p, Far1p–nls1 or Far1p–nls1/2 from the inducible *GAL* promoter. Cells were grown in galactose at 30°C, the budding pattern was determined as described and presented as percent of total cells. Only cells with three or more bud scars were included in the analysis.

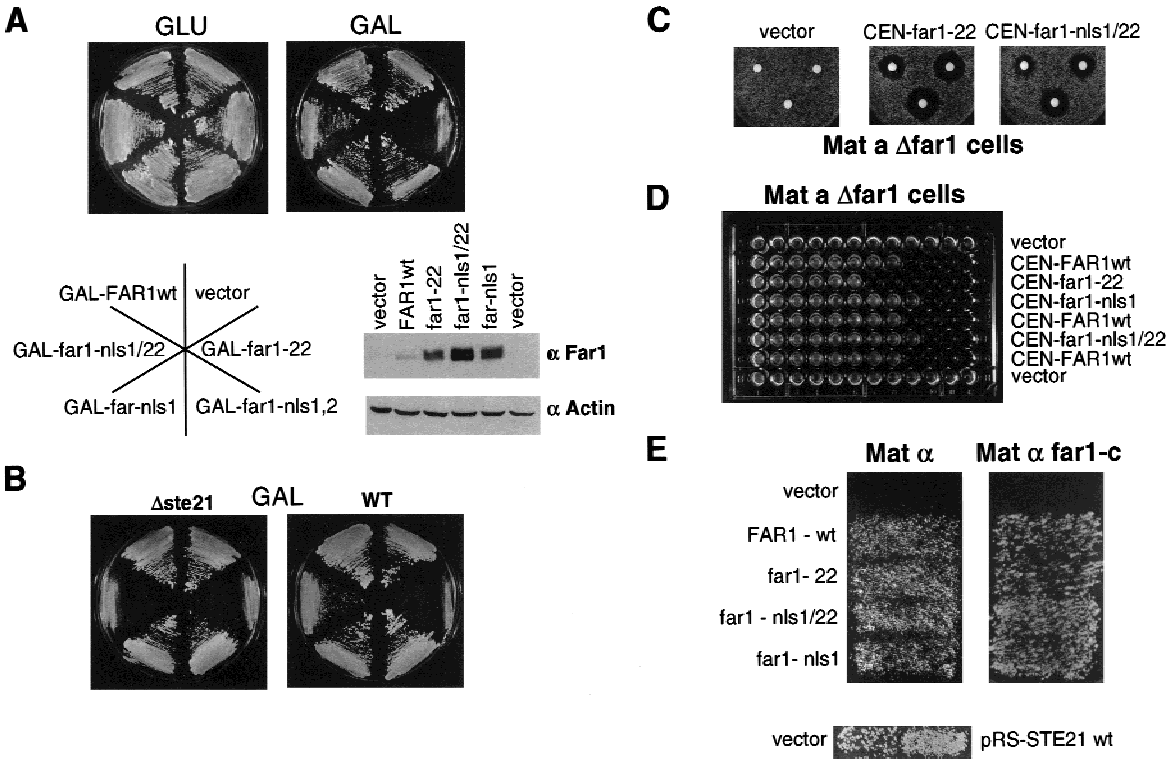

Figure 8. Nuclear Far1p is required to arrest the cell cycle in response to pheromones, whereas cytoplasmic Far1p is necessary for efficient mating. (*A*) Wild-type cells (K699) were transformed with plasmids allowing overexpression of wild type or various mutant Far1p from the inducible *GAL* promoter as indicated, and grown on plates containing glucose (GLU; Far1p not expressed) or galactose (GAL; Far1p expressed). Extracts were prepared from cells and immunoblotted with specific antibodies against Far1p (*top*) or actin (*bottom*). Note that Far1p-22 is only toxic in the presence of a functional NLS. (*B*) Wild-type strains (K699, *right*) or strains deleted for *MSN5/STE21* (Δ*ste21; left*) were transformed with the plasmids as indicated above and grown on media containing galactose. Note that in contrast to wild-type cells Far1–nls1/22 is toxic in Δ*msn5/ste21* cells. (*C*) Cell deleted for *FAR1* but harboring an empty plasmid (vector, *left*) or low copy number plasmids expressing Far1p-22 (*middle*) or Far1p–nls1/22 (*right*) from the endogenous promoter were analyzed by halo assay for their ability to arrest the cell cycle in response to pheromones. The circles contained 2, 10, and 20 μg of α-factor. (*D*) The minimal concentration of α-factor required to arrest cells expressing wild type or the indicated mutant Far1p from the endogenous promoter was determined by serial dilutions as described. Note that efficient cell cycle arrest in response to pheromones requires Far1p with a functional NLS. (*E*) Cells deleted for *FAR1* but harboring an empty plasmid (vector) or low copy number plasmids expressing wild type or the indicated Far1p mutants from the endogenous promoter were analyzed for their ability to mate with wild-type (IH1793; *left*) or mating-reduced *FAR1-C* mating testers (IH2625, *right*). Note that cytoplasmic Far1p does not reduce the mating efficiency. Wild-type cells (K699) harboring an empty plasmid (vector; left patch) or a plasmid allowing overexpression of Msn5p/Ste21p (pRS–STE21; right patch) were mated to wild-type mating testers (IH1793). Note that overexpression of Msn5p/Ste21p slightly increases the mating efficiency.

response to pheromones, suggesting that nuclear Far1p is sufficient for cell cycle arrest. Likewise, Far1p–Δ338–382 can no longer be exported in response to pheromones but is able to fully complement the cell cycle arrest defect of *Δfar1* cells. Previously, we have found that overexpression of a nuclear, stable mutant form of Far1p (Far1p-22) arrests cells in the G_1 phase of the cell cycle by inhibiting the Cdc28p–Clnp kinase (Henchoz et al. 1997). In contrast, cells overexpressing a cytoplasmic double mutant between Far1p-22 and NLS1 (Far1p–nls1/22) were viable and able to divide, although the Far1p–nls1/22 protein was expressed at similar or even higher levels than Far1p-22 (Fig. 8A). Interestingly, cytoplasmic Far1p was stable and no longer subjected to ubiquitin-mediated degradation (M. Blondel and M. Peter, unpubl.), explaining the increased steady-state levels of Far1p–nls1 and Far1p–nls1/22. However, overexpression of Far1p–nls1/22 is toxic in cells deleted for *MSN5/STE21* (Fig. 8B), which accumulated Far1p–nls1/22 in the nucleus (data not shown) because of a defect in the export system, demonstrating that Far1p–nls1/22 is functional and able to arrest the cell cycle when localized in the nucleus. Consistent with these findings, *Δfar1* cells expressing Far1p–nls1/22 from the endogeneous promoter exhibited an eightfold reduced ability to arrest the cell cycle in response to pheromones compared to *Δfar1* cells expressing Far1p-22 (Fig. 8C,D). Taken together, we conclude that nuclear Far1p is required to arrest the cell cycle in response to pheromones.

Discussion

The subcellular localization of Far1p is controlled by activation of the pheromone response pathway during mating; in the absence of pheromones, Far1p is predominantly nuclear, whereas in the presence of pheromones Far1p accumulates in the cytoplasm. We show here that the localization of Far1p is under the control of both nuclear import and export pathways and have identified Msn5p/Ste21p as a specific exportin for Far1p. Export of Far1p in response to pheromones requires Msn5p/Ste21p, which interacts with Far1p in a manner dependent on Gsp1p–GTP. Our results further suggest that nuclear Far1p is required for its cell cycle arrest function, whereas cytoplasmic Far1p interacts with Gβγ and the polarity establishment proteins to orient cell polarity during mating.

The subcellular localization of Far1p is mediated by nuclear import and export

In the absence of pheromones Far1p is localized predominantly in the nucleus (Henchoz et al. 1997). As shown here nuclear localization depends on at least one bipartite NLS, which is located in the amino-terminal domain of Far1p. Far1p lacking this region or harboring point mutations in the basic residues within the bipartite NLS accumulates predominantly in the cytoplasm. NLSs are recognized by import receptors that target specific proteins to the nucleus. For example, Kap123p was shown to be involved in the import of the ribosomal protein L25 (Rout et al. 1997; Schlenstedt et al. 1997), and Pse1p is required to import Pho4p in response to phosphate starvation (Kaffmann et al. 1998a). At present, we do not know which import receptor is required for nuclear localization of Far1p.

In contrast, several lines of evidence strongly suggest that Msn5p/Ste21p functions as an exportin for Far1p, although we cannot rigorously exclude the possibility that Msn5p/Ste21p may indirectly inhibit nuclear import of Far1p. First, cytoplasmic accumulation of Far1p in response to pheromones was abolished in cells deleted for Msn5p/Ste21p. Second, Far1p accumulated in the cytoplasm of cells overexpressing Msn5p/Ste21p even in the absence of pheromones, and third, a cytoplasmic mutant of Far1p that harbors mutations in the major NLS becomes predominantly nuclear when expressed in *Δmsn5/ste21* cells. Fourth, Msn5p/Ste21p is localized in the nucleus, consistent with its proposed function as an exportin for Far1p. Fifth, Far1p coimmunoprecipitated with Msn5p/Ste21p in a manner dependent on GTP-bound Gsp1p and also interacted with Msn5p/Ste21p by two-hybrid analysis. Finally, a small segment of Far1p, which is necessary and sufficient to interact with Msn5p/Ste21p, is required to export Far1p in vivo in response to pheromones. Signals have been defined that target proteins from the nucleus to the cytoplasm (Nakielny and Dreyfuss 1997; Weis 1998). The NES contained in the HIV Rev protein and PKI, an inhibitor of cAMP-dependent protein kinase A, is a small sequence rich in leucine residues, which is bound by the export receptor Crm1p/Xpo1p and Ran–GTP in the nucleus. The interaction domain between Far1p and Msn5p/Ste21p does not contain an obvious leucine-rich NES, suggesting that Far1p uses a novel type of NES. However, the Msn5p/Ste21p-binding domain of Far1p was not able to export efficiently a nuclear GFP fusion protein, suggesting that this motif may not be sufficient to mediate Msn5p/Ste21p-dependent export in vivo. Nevertheless, together with the recently identified Msn5p/Ste21p targets Msn2p, Msn4p, and Pho4p it may be possible to deduce a consensus sequence, which may facilitate the identification of additional proteins exported by Msn5p/Ste21p.

Regulation of the subcellular localization of Far1p by pheromones

The subcellular localization of Far1p is surprisingly dynamic and is regulated by both nuclear import and export. In the absence of α-factor, Far1p is predominantly nuclear and import appears to overcome export. Addition of pheromones shifts the equilibrium and Far1p accumulates in the cytoplasm. It is not understood how pheromones alter this balance, but available results suggest a role for the MAPK Fus3p (M. Blondel and M. Peter, unpubl.). It is possible that Fus3p inhibits nuclear import, increases nuclear export, or both. Fus3p may also regulate the activity of nuclear or cytoplasmic docking sites that may retain Far1p in the nucleus in the absence

of pheromones, or in the cytoplasm in the presence of pheromones (Hood and Silver 1999; Kaffmann and O'Shea 1999).

Nuclear import is regulated in many cases by phosphorylation of sites within or close to the NLS (Moll et al. 1991; Kaffmann et al. 1998a). Two consensus phosphorylation sites for Cdc28p kinase or MAPK are present within the major NLS of Far1p and it has been shown that this region of Far1p is heavily phosphorylated by Cdc28p kinases in vivo and in vitro (McKinney and Cross 1995). Thus, phosphorylation of these sites by Fus3p may prevent nuclear import of Far1p in response to pheromones. Alternatively, because Fus3p is required to inhibit Cdc28p–Clnp activity in response to pheromones (Elion et al. 1990; Peter et al. 1993), it is possible that phosphorylation of Far1p by Cdc28p–Clnp may be required for nuclear import. However, we found that Far1p is localized in the nucleus of G_1 cells arrested by depletion of the G_1 cyclins, suggesting that nuclear import was not dependent on Cdc28p kinase activity (data not shown). In addition, nuclear localization of Far1p is independent of the cell cycle position (Henchoz et al. 1997) and did not require Fus3p or any other component of the mating pathway (data not shown). Finally, a fusion protein between the 50 amino-terminal amino acids of Far1p (containing the NLS and the putative phosphorylation sites) with GFP was localized efficiently in the nucleus of cells treated (data not shown) or not treated with α-factor (Fig. 1C); however, because we do not know whether this amino-terminal fragment of Far1p is phosphorylated efficiently in vivo, we cannot exclude the possibility that phosphorylation of these sites may inhibit nuclear import of full-length Far1p in response to pheromones.

It is clear that nuclear export of Far1p must occur even in the absence of pheromones, because Far1p–nls1 is nuclear in cells lacking Msn5p/Ste21p, whereas it is predominantly cytoplasmic in wild-type cells. Thus, a decrease in the rate of nuclear import can be compensated by decreasing the rate of nuclear export. Overexpression of Msn5p/Ste21p was sufficient to export Far1p in the absence of pheromones or a functional pheromone response pathway, suggesting that increased levels of Msn5p/Ste21p are able to shift the equilibrium. However, endogenous Msn5p/Ste21p levels were not altered in response to pheromones, indicating that post-translational mechanisms may regulate nuclear export of Far1p. Because Msn5p/Ste21p is required to export multiple proteins, not only during mating, but also in response to environmental conditions such as high phosphate levels, we favor a model where regulation of nuclear export occurs at the level of the substrate rather than at the level of the exportin or the export machinery. In support of this notion, recently it has been shown that Msn5p/Ste21p specifically interacts with phosphorylated Pho4p and that phosphorylation of Pho4p is required for its nuclear export in vivo (Kaffmann et al. 1998b). However, although Far1p is a substrate of Fus3p (Peter et al. 1993; Tyers and Futcher 1993; Kranz et al. 1994; Gartner et al. 1998), the interaction between Far1p and Msn5p/Ste21p as assayed by two-hybrid analysis was not increased in response to pheromones and was neither dependent on the presence of Fus3p nor on the pheromone-dependent phosphorylation of Far1p (Table 1). Thus, these results suggest that Fus3p may not regulate directly binding of Far1p with its exportin Msn5p/Ste21p. Further work is required to elucidate the mechanism of regulation of cytoplasmic accumulation of Far1p by pheromones.

Multiple roles of Msn5p/Ste21p during mating and response to various extracellular signals

Several proteins have now been shown to be targets of the exportin Msn5p/Ste21p: the transcription factor Pho4p is exported by Msn5p/Ste21p under high phosphate conditions (Kaffmann et al. 1998b), and Msn5p/Ste21p keeps Msn2p and Msn4p in the cytoplasm in the absence of stress conditions (Gorner et al. 1998; Alepuz et al. 1999). As shown here Msn5p/Ste21p exports Far1p in response to pheromones and cells lacking Msn5p/Ste21p exhibit a bilateral mating defect. The signal transduction and cell cycle arrest functions of *Δmsn5/ste21* cells in response to pheromones are intact, but the cells exhibit a defect in projection formation and in orienting growth toward the mating partner. The latter defect is thought to result from a failure to export Far1p, which is necessary to target the polarity establishment proteins Bem1p, Cdc24p, and Cdc42p to the site of the incoming pheromone signal marked by Gβγ (Butty et al. 1998; Nern and Arkowitz 1999). However, cells lacking Far1p are able to form mating projections and thus Far1p cannot be the only Msn5p/Ste21p target that needs to be exported during mating, an observation that is supported by the synthetic sterility of cells lacking both Msn5p/Ste21p and Far1p. In addition, we found that overexpression of cytoplasmic Far1p was not sufficient to bypass the need for Msn5p/Ste21p during mating, although cytoplasmic Far1p increased the mating efficiency of wild-type cells. Interestingly expression of a membrane-bound version of Ste5p (Ste5p–CTM; Pryciak and Huntress 1998) partially restored shmoo formation in *Δmsn5/ste21* cells (data not shown), suggesting that export of Ste5p may be necessary to form mating projections efficiently.

The function of Far1p is required in two subcellular compartments

Far1p is known to play two separable roles during yeast mating (Valtz et al. 1995): Far1p is required to arrest the cell cycle presumably through the inhibition of the Cdc28p–Clnp kinase (Peter and Herskowitz 1994; Gartner et al. 1998) and Far1p is necessary for oriented cell polarity by linking the polarity establishment proteins to Gβγ (Butty et al. 1998; Nern and Arkowitz 1999). Several lines of evidence suggest that the cell cycle arrest function of Far1p requires nuclear localization of Far1p. First, cells expressing Far1p–nls1 with reduced ability to enter into the nucleus exhibit a modest cell cycle arrest defect

in response to pheromones. Second, a dominant Far1p, which when overexpressed arrests the cell cycle by inhibiting the Cdc28p–Clnp kinase (Henchoz et al. 1997), is no longer toxic if the dominant mutation is combined with a mutation that inactivates the NLS. Finally, cells unable to export Far1p either because they lack the exportin Msn5p/Ste21p or because they express a mutant Far1p deleted for its NES (Far1p–Δ338–382), are able to arrest efficiently the cell cycle in response to pheromones. In contrast, Δ*far1* cells expressing Far1p–Δ338–382 exhibit a mating defect, although these cells are able to arrest and form normal mating projections. Similarly, Δ*msn5/ste21* cells mate with reduced efficiency; we presume that this mating defect is partly due to the requirement of cytoplasmic Far1p to interact with Ste4p to perform its function as an adaptor for polarity establishment (Butty et al. 1998; Nern and Arkowitz 1999). Consistent with that notion, we found that cytoplasmic Far1p was sufficient for the mating function and even slightly increased the mating efficiency. In addition, overexpression of cytoplasmic Far1p interferes with the budding pattern of wild-type cells (Table 2), presumably through its interaction with polarity establishment proteins. It has been found previously that overexpression of a truncated Far1 protein lacking 50 amino acids of the amino terminus interfered with bud formation after release from α-factor arrest (McKinney and Cross 1995). Our results now show that this truncated Far1p protein lacks its major NLS and therefore, may interfere with the polarity establishment machinery during bud formation. Thus, these results suggest that sequestration in the nucleus might prevent Far1p from interacting with polarity establishment proteins in the absence of the physiological stimulus.

On the basis of these results we propose a model for how the cell cycle arrest and polarity establishment functions of Far1p may be coordinated (Fig. 9). In the absence of pheromones, low levels of Far1p are present because the expression of *FAR1* is controlled at the transcriptional level by the pheromone pathway (Chang and Herskowitz 1990; Oehlen et al. 1996). Expressed Far1p is sequestered in an inactive form in the nucleus, where it is unable to interfere with the function of cytoplasmic polarity establishment proteins. Activation of the pheromone response pathway increases expression of Far1p. In addition, Far1p is phosphorylated by Fus3p, which enables Far1p to bind to the Cdc28p–Clnp kinase in the cell nucleus leading to cell cycle arrest (Peter et al. 1993; Gartner et al. 1998). Pheromones also trigger export of Far1p into the cytoplasm by a mechanism that requires the exportin Msn5p/Ste21p. Cytoplasmic Far1p interacts with Bem1p, Cdc24p, and Cdc42p and targets them to the activated heterotrimeric G protein to organize the actin cytoskeleton toward the incoming signal (Arkowitz 1999; Bähler and Peter 1999). Because Cdc28p–Clnp and Msn5p/Ste21p have at least partially overlapping binding sites it is possible that Msn5p/Ste21p and Cdc28p–Clnp compete for binding to Far1p. Such a mechanism may be important to coordinate the cell cycle arrest and polarity functions of Far1p (Fig. 9).

Materials and methods

Yeast strains, genetic manipulations, and database searches

Yeast strains are described in Table 3. The genotypes of the yeast strains are W303, *ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL*$^+$*, psi*$^+$*, ssd1-d2;* A364a, *trp1-289, leu2-3,112, his3-11,15, ura3-52, GAL*$^+$; and EG123: *trp1-Δ99, leu2-Δ1, ura3-52, ade2-101,* unless noted otherwise. Standard yeast growth conditions and genetic manipulations were used as described (Guthrie and Fink 1991). Yeast transformations were performed by lithium acetate procedure (Ito et al. 1983). Strains deleted for *FAR1* marked with *LEU2* or *URA3* were constructed using plasmids pMT870 digested with *Pvu*II, or pFC13 digested with *Not*I. Strains deleted for *MSN5/STE21* were constructed using plasmids pLH64 digested with *Not*I and *Xho*I, and strains deleted for *STE7* were constructed using the plasmid pSL2270 digested with *Pst*I and *Xho*I (Pinten and Sprague 1994). *STE20* was deleted using the kan^R cassette as described in Longtine et al. (1998). Strain YBM100 was constructed by integration of *GAL–gsp1*(*G21V*) at the *URA3* locus of YSH80 by digesting the plasmid pSH125-1 with *Stu*I. Transformants were selected on SD–URA plates at 25°C and tested for their inability to grow on

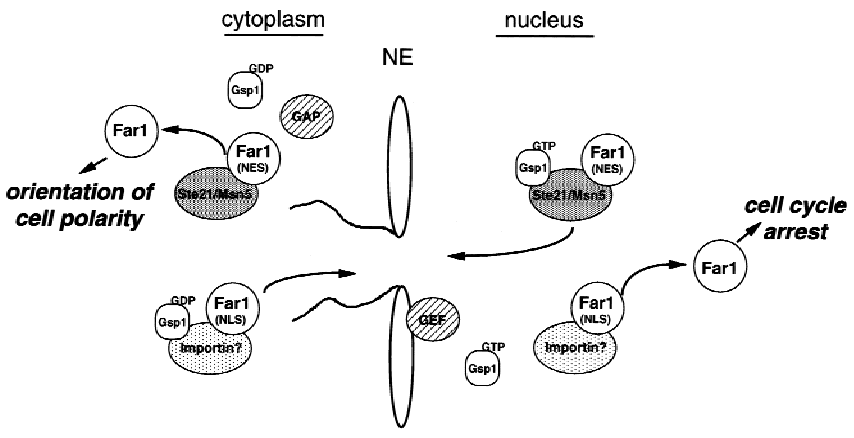

Figure 9. A schematic representation of the Far1p functions. In the absence of pheromones Far1p is exclusively nuclear because of the presence of functional NLS sequences. Nuclear Far1p is inactive as a cyclin-dependent kinase inhibitor (CKI) because it needs to be phosphorylated by the MAPK Fus3p to be able to bind to the Cdc28p–Clnp kinase (Peter et al. 1993; Gartner et al. 1998). Nuclear Far1p interacts with Msn5p/Ste21p in a Gsp1p-dependent manner, which then transports Far1p into the cytoplasm. In the presence of pheromones the balance between import and export is shifted toward export presumably because of activation of Fus3p. Nuclear Far1p is required for cell cycle arrest, whereas cytoplasmic Far1p is needed for the establishment of oriented cell polarity.

Table 3. *Strains list*

Strain name	Relevant genotype	Background	Source
K699	*MAT***a**	W303	Kim Nasmyth (IMP, Vienna, Austria)
YMP1054	*MAT***a** *far1::LEU2*	W303	Henchoz et al. (1997)
PAY20	*MAT***a** *ste21::HIS3*	W303	this study
YMP1067	*MAT***a** *ste21::HIS3 far1::LEU2*	W303	this study
MJ 234	*MAT***a** *fus1::lacZ::URA3*	A364a	this study
GA682	*MAT***a**/α *RAP1Δ303–416-GFP–LEU2::rap1*	SK1	Monika Tsai (ISREC)
YFD162	*MAT***a** *ste7::ADE2 bar1-1 far1Δ*	W303	Frank van Drogen (ISREC)
YFD163	*MAT***a** *ste20::kan*^{R} *bar1-1 far1Δ*	W303	Frank van Drogen
YSH80	*MAT***a** *gsp1::hisG his3-1,15::gsp1ts(HIS3)*	W303	Shai Shaham
IH1793	*MAT*α *lys1*		collection
IH2625	*MAT*α *far1c lys1*		collection
YACB169	*MAT***a** *far1::LEU2*	EG123	this study
LH90	*MAT***a** *ste21::TRP1*	JC-2B	this study
YMP290	*MAT***a** *far1Δ bar1-1*	W303	Gustav Ammerer
YMP291	*MAT***a** *far1Δ fus3Δ bar1-1*	W303	this study
YBM100	*MAT***a** *gsp1::hisG his3-1,15::gsp1ts(HIS3) GAL–gsp1(G21V)–URA3::ura3*	W303	this study

medium containing galactose and thermosensitivity at 37°C. Database searches were performed using the SGD (Stanford University) and the NCBI BLAST programs (National Institutes of Health).

Pheromone response and mating assays

Pheromone response and mating assays were carried out as described (Valtz and Peter 1997). Mating assays were performed with both wild-type (IH1793) and orientation-defective *far1-c* mating testers (IH2625). Quantitative cell cycle arrest assays were performed in microtiter plates as described (Grishin et al. 1998). Each series uses twofold dilutions from well to well starting with 100 μg/ml α-factor; the last well contains no α-factor. To analyze the expression of Msn5p/Ste21p in response to pheromones, cells (LH90) harboring a plasmid-allowing expression of Msn5p/Ste21p from the endogenous promoter (pLH287) were grown in selective media to early log phase, at which time α-factor was added to 25 μg/ml final concentration (time 0). Aliquots were removed after the times indicated and the expression of Msn5p/Ste21p and Far1p was analyzed by immunoblotting as described. Induction of *FAR1* or *FUS1* mRNA in Δ*msn5/ste21* (PAY20) or wild-type cells (K699) was determined by Northern analysis as described previously (Martinez-Pastor et al. 1996).

DNA manipulations

Plasmids are described in Table 4. Standard procedures were used for recombinant DNA manipulations (Sambrook et al. 1989; Ausubel et al. 1991). PCR reactions were performed with the Expand polymerase kit as recommended by the manufacturer (Boehringer Mannheim). Oligonucleotides were synthesized by Genset (France) and the sequences are available upon request. Mutations were introduced by PCR and the correct sequence confirmed by sequencing. Internal deletion mutants of *FAR1* were constructed by PCR by introducing in frame *Bgl*II restriction sites. The *Xho*I–*Sph*I fragment of *FAR1* containing the various mutations was ligated into pTP68 (Henchoz et al. 1997) to express fusions to the GFP (Heim et al. 1995), into pBM18 for expression from the endogenous *FAR1* promoter, and pTP62 (Henchoz et al. 1997) for expression from the inducible *GAL* promoter. For two-hybrid analysis *FAR1* fragments were amplified by PCR, digested with *Nco*I and *Xho*I and subcloned into the two-hybrid vectors pEG203 or pJG4-6 as described previously (Butty et al. 1998). pEG203 and pJG4-6 are derivatives of pEG202 and pJG4-5, respectively (Gyuris et al. 1993), with an altered polylinker. The fragment encoding the Msn5p/Ste21p-binding domain of Far1p (amino acids 289–389) was amplified with specific primers introducing *Hind*III and *Xho*I restriction sites and ligated to a fragment encoding GFP in frame with the nuclear localization signal of Pho4p (Kaffmann et al. 1998a). The resulting fragment was then ligated into pRS416(ADH), resulting into plasmid pNP124. This plasmid allows expression of a PHO4(NLS)–GFP–NES(289–389) fusion protein from the constitutive *ADH* promoter. *STE21* was amplified by PCR, digested with *Xho*I and *Apa*I or *Sal*I and ligated into pEG203 (pBM41) for two-hybrid analysis, or pRS414(G) for expression from the inducible *GAL* promoter (pBM43). To introduce multiple copies of the 9E10 (myc) epitope at the amino terminus of Msn5p/Ste21p, the *Bam*HI site in *MSN5/STE21* was eliminated with a silent mutation, replaced with a new *Bam*HI site at the ATG start codon and ligated with a myc3 cassette flanked by BamHI sites. pLH132 contains one copy, whereas pLH133 contains several copies of the myc cassette. The *Not*I–*Xho*I fragment from pLH132 or pLH133 was also cloned into pRS316 (Sikorski and Hieter 1989) yielding plasmid pLH287 and pRS424 yielding plasmids pBM55 and pBM56. The plasmid allowing expression of Msn5p/Ste21p–GFP was constructed as follows: the *Not*I–*Xho*I fragment from pLH133 was ligated into pRS316 to yield pLH287; pLH287 was then digested with *BamH*I to remove the myc3 cassette and replaced with a GFP fragment isolated from pPP356 (Pryciak and Huntress 1998), to yield plasmid pLH266. Both pLH133 and pLH266 are fully functional and able to complement the mating defect of cells deleted for *MSN5/STE21* (data not shown).

Antibodies and Western blots

Standard procedures were used for yeast cell extract preparation and immuno blotting (Brown et al. 1997; Harlow and Lane 1988). Polyclonal anti-Far1p antibodies have been described previously (Henchoz et al. 1997) and 9E10 antibodies were obtained from the ISREC antibody facility. Antibodies specific for actin

Table 4. *Plasmids list*

Plasmids	Relevant characteristics	Source
pTP68	GAL FAR1wt–GFP URA3 CEN	Henchoz et al. (1997)
pTP487	GAL Far1Δ50–GFP URA3 CEN	this study
pBM9	GAL Far1–NLS1–GFP URA3 CEN	this study
pBM10	GAL Far1–NLS2-GFP URA3 CEN	this study
pBM11	GAL Far1–NLS1/2-GFP URA3 CEN	this study
pTP88	GAL $Far1^{1–50}$–GFP URA3 CEN	this study
pTP419	GAL GFP URA3 CEN	this study
pmsn5-D3	Ste21::HIS3	this study
pBM45	STE21wt LEU2 CEN	this study
pBM43	GAL STE21wt TRP1 CEN	this study
pLH287	STE21-myc^n URA3 CEN	this study
pSL2270	Ste7::ADE2	Printen and Sprague (1994)
pBM41	pLG203 STE21wt	this study
ACB412	pJG4-6 FAR1wt	Butty et al. (1998)
ACB418	pJG4-6 $Far1^{353–830}$	Butty et al. (1998)
ACB413	pJG4-6 $Far1^{1–389}$	Butty et al. (1998)
ACB415	pJG4-6 $Far1^{174–285}$	Butty et al. (1998)
pBC100	pJG4-6 $Far1^{89–389}$	this study
pBC101	pJG4-6 $Far1^{189–389}$	this study
pBC102	pJG4-6 $Far1^{289–389}$	this study
pTP577	pJG4-6 Far1 Δ285–390	this study
GA2162	pJG4-6 Far1 A26 A87 A114 A324 V341 A346	this study
GA2163	pJG4-6 Far1 A26 A87 A114 A306 V341 A346	this study
pLH132	STE21–Myc^3 TRP1 2μ	this study
pLH133	STE21–Myc^n TRP1 2μ	this study
pLH58	STE21wt URA3 CEN	this study
pLH64	STE21::TRP1 LEU2 2μ	this study
pBM56	STE21–Myc^n TRP1 2μ	this study
pBM55	STE21–Myc^3 TRP1 2μ	this study
ACB435	GAL FAR1wt LEU2 CEN	Butty et al. (1998)
pSH125	GAL Gsp1 (G21V) URA3 integrative	this study
pLH266	STE21wt–GFP URA3 CEN	this study
pTP62	GAL FAR1wt URA3 CEN	Henchoz et al. (1997)
pBM5	GAL Far1 NLS1 URA3 CEN	this study
pBM8	GAL Far1 NLS1/2 URA3 CEN	this study
pTP63	GAL Far1-22 URA3 CEN	Henchoz et al. (1997)
pBM14	GAL Far1 NLS1/22 URA3 CEN	this study
pBM18	FAR1wt URA3 CEN	this study
pBM19	Far1–22 URA3 CEN	this study
pBM20	Far1–NLS1 URA3 CEN	this study
pBM29	Far1–NLS1/22 URA3 CEN	this study
pLH59	STE21wt URA3 2μ	this study
pBM58	GAL Far1Δ285–308 GFP URA3 CEN	this study
pBM76	GAL Far1Δ312–382 GFP URA3 CEN	this study
pBM77	GAL Far1Δ338–382 GFP URA3 CEN	this study
pBM78	GAL Far1Δ312–338 GFP URA3 CEN	this study
pBM79	Far1Δ312–382 URA3 CEN	this study
pBM80	Far1Δ338–382 URA3 CEN	this study
pBM81	Far1Δ312–338 URA3 CEN	this study
pTP595	pJG4-6 Far1 Δ312–382	this study
pTP596	pJG4-6 Far1 Δ338–382	this study
pTP597	pJG4-6 Far1 Δ312–338	this study
pTP598	pJG4-6 Far1 Δ304–308	this study
pNP124	ADH Pho4(NLS)–GFP–Far1(NES) URA3 CEN	this study
pTP594	ADH Pho4(NLS)–GFP URA3 CEN	this study
EB0806	pT7-Myc–GSP1	Kaffmann et al. (1998b)

were purchased from Boehringer Mannheim and used as recommended by the manufacturer.

Coimmunoprecipitation and in vitro binding experiments

Wild-type (K699) or *gsp1-1* cells (YSH80) were transformed with a multicopy plasmid expressing myc-tagged Msn5p/Ste21p from the endogenous promoter (pBM56 or pBM55) or for control an empty vector (pRS424) and a plasmid expressing Far1p from the inducible *GAL* promoter (ACB435; Butty et al. 1998). Cells were grown in selective media containing raffinose (2% final

concentration) to early log phase, at which time galactose (2% final concentration) was added for 6 hr at 30°C (25°C for experiments with *gsp1-1* cells followed by a shift at 35°C for 3 hr) to induce expression of Far1p. Cells were pelleted, resuspended in RIPA buffer [50 mM Tris (pH 7.5), 50 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS] containing the protease inhibitors PMSF, aprotinin, leupeptin, and pepstatin (Complete, Boehringer Mannheim), and lysed with a one-shot cell disruptor (Constant Systems Ltd.) set to the maximum pressure (2.8 kBar). The soluble extract was then incubated for 2 hr at 4°C with 9E10 monoclonal antibodies and Sepharose beads coupled to protein G (Pharmacia). The beads were washed four times with RIPA buffer, bound proteins eluted with gel-sample buffer, and subjected to immunoblot analysis with polyclonal antibodies against Far1p and 9E10 antibodies to control for the presence of myc-tagged Msn5p/Ste21p.

In vitro binding assays were carried out as follows: Gsp1p–Myc was immunoprecipitated with 9E10 antibodies from extracts prepared from DH5α cells containing the pT7–MycGsp1 expression plasmid (EB0806). The immunoprecipitate was divided; One-half was incubated for 2 hr at 4°C with 2 mM GTPγS; the other half with 2 mM GDP in 10 mM Tris-HCl (pH 7.5) containing 20 mM EDTA and 2 mM DTT. The reaction was stopped by adding $MgCl_2$ to a final concentration of 50 mM. Yeast extracts were prepared from *ste21*Δ *far1*Δ cells (YMP1067) transformed with control vectors, or plasmids expressing Far1p (CMP62; Henchoz et al. 1997) and Msn5p/Ste21p (pBM43) from the inducible *GAL* promoter. Cells were grown at 30°C to early log phase in selective media containing raffinose (2% final concentration), at which time galactose (2% final concentration) was added for 6 hr. Cells were lysed in phosphate-buffered saline [PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4-$7H_2O$, 1.4 mM KH_2PO_4 (pH 7.3)] containing the protease inhibitors PMSF, aprotinin, leupeptin, and pepstatin (Complete, Boehringer Mannheim) as described above and the extracts incubated for 2 hr at 4°C with Gsp1p–Myc-containing beads. The beads were washed four times with PBS, bound proteins eluted with gel-sample buffer, and analyzed by immunoblotting as described.

Two-hybrid assays

Two-hybrid assays were performed as described (Brown et al. 1997) in EGY48- or W303-based yeast strains using pEG202-based plasmids expressing LexA DNA-binding domain fusions (DBD), and pJG4-5-based plasmids containing fusions to the B42 transcriptional activation domain (AD) (Gyuris et al. 1993). Miller units are averages of at least six independent experiments (with independent colonies) with standard deviations. For experiments with pheromones, cells were grown in selective media containing galactose (2% final concentration) to early log phase, at which time the culture was divided and pheromone (25 μg/ml final concentration) was added to one half. After the times indicated, an aliquot was analyzed as described above. The values were expressed as percent Miller units compared to wild-type controls grown in the absence of pheromones; each time point represents the average of at least four independent experiments (with independent colonies).

Microscopy and budding assays

Yeast actin was visualized with rhodamine–phalloidin (Molecular Probes, Inc., Leiden, The Nctherlands). Briefly, cells were fixed with formaldehyde (3.7% final concentration) for 60 min, washed and stained for 20 min on ice with rhodamine–phalloidin (diluted 1:5 in methanol), washed three times with PBS, and viewed on a Zeiss Axiophot fluorescence microscope. At least 200 cells were counted for the morphological analysis. Proteins tagged with GFP were visualized using a Chroma GFPII filter (excitation 440–470 nm), photographed with a Photometrics CCD camera, and analyzed with Photoshop 4.0 software (Adobe). Photographs shown are overlays of phase contrast and fluorescence images. Cells expressing Far1p–GFP from the inducible *GAL* promoter were grown to early log phase at 25°C in selective media containing raffinose (2% final concentration), at which time galactose was added (2% final concentration) for 6 hr. Where indicated α-factor (25 μg/ml final concentration) was added during the last 3 hr. LH90 cells expressing Msn5p/Ste21p–GFP from the endogenous promoter (pLH58) were grown in selective media at 30°C to early log phase. Where indicated α-factor (25 μg/ml final concentration) was added for 2 hr.

The budding pattern of cells overexpressing wild-type or cytoplasmic mutant forms of Far1p was determined after staining bud scars with calcofluor as described (Guthrie and Fink 1991). Briefly, cells were grown in selective media containing raffinose (2% final concentration) to early log phase, at which time galactose (2% final concentration) was added; cells were kept in log phase for 36 hr by successive dilutions. Cells were stained with calcofluor and counted on a Zeiss Axiophot fluorescence microscope using a UV filter. Only cells with more than three bud scars were included in the analysis.

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