Fus3p and Kss1p Control G1 Arrest in *Saccharomyces cerevisiae* Through a Balance of Distinct Arrest and Proliferative Functions That Operate in Parallel With Far1p

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ABSTRACT

In *Saccharomyces cerevisiae*, mating pheromones activate two MAP kinases (MAPKs), Fus3p and Kss1p, to induce G1 arrest prior to mating. Fus3p is known to promote G1 arrest by activating Far1p, which inhibits three Clnp/Cdc28p kinases. To analyze the contribution of Fus3p and Kss1p to G1 arrest that is independent of Far1p, we constructed *far1 CLN* strains that undergo G1 arrest from increased activation of the mating MAP kinase pathway. We find that Fus3p and Kss1p both control G1 arrest through multiple functions that operate in parallel with Far1p. Fus3p and Kss1p together promote G1 arrest by repressing transcription of G1/S cyclin genes (*CLN1*, *CLN2*, *CLB5*) by a mechanism that blocks their activation by Cln3p/Cdc28p kinase. In addition, Fus3p and Kss1p counteract G1 arrest through overlapping and distinct functions. Fus3p and Kss1p together increase the expression of *CLN3* and *PCL2* genes that promote budding, and Kss1p inhibits the MAP kinase cascade. Strikingly, Fus3p promotes proliferation by a novel function that is not linked to reduced Ste12p activity or increased levels of Cln2p/Cdc28p kinase. Genetic analysis suggests that Fus3p promotes proliferation through activation of Mcm1p transcription factor that upregulates numerous genes in G1 phase. Thus, Fus3p and Kss1p control G1 arrest through a balance of arrest functions that inhibit the Cdc28p machinery and proliferative functions that bypass this inhibition.

IN the presence of mating pheromones, dividing haploid **a** and α cells of *Saccharomyces cerevisiae* stimulate each other to arrest at Start in G1 phase, the commitment point of the cell cycle (Sprague and Thorner 1993). Cell synchronization at Start is required for efficient morphogenesis and fusion between mating cells. However, in the absence of mating, an arrested cell will recover from G1 arrest and resume mitotic growth, even in the presence of mating pheromone. Thus, the decision to arrest in G1 phase could be controlled by a balance between opposing forces that promote arrest and promote proliferation.

The passage through Start is determined by the activity of Cdc28p kinase, which is regulated by three G1 cyclins, Cln1p, Cln2p, and Cln3p, and two G1/S-phase cyclins, Clb5p and Clb6p (Koch and Nasmyth 1994; Cross 1995; Nasmyth 1996). The G1/S-phase cyclins provide both overlapping and unique functions for Start. Cln3p is expressed throughout the cell cycle and appears to play a more important role as a transcriptional regulator of the other G1/S-phase cyclin genes. Cln1p, Cln2p, Clb5p, and Clb6p are expressed periodically in G1 phase, with Cln1p and Cln2p having more important roles in bud emergence, and Clb5p and Clb6p having more important roles in the initiation of DNA replication and spindle pole body duplication. Additional protein kinases may also regulate the G1 to S phase transition in parallel with Cdc28p kinase. Pho85p kinase, in combination with either Pcl1p or Pcl2p cyclin partners, provides functions that overlap with Cln1p/Cdc28p and Cln2p/Cdc28p kinases (Ogas *et al.* 1991; Espinoza *et al.* 1994; Measday *et al.* 1994). Functional overlap also exists for Cla4p kinase (Cvrckova *et al.* 1995) and the Pkc1p pathway (Igual *et al.* 1996; Gray *et al.* 1997).

Many lines of evidence argue that G1 arrest during mating occurs primarily through inhibition of the G1 cyclins. The arrest points of both a *cdc28-4* mutant and a *cln1 cln2 cln3* triple mutant are similar to the mating pheromone arrest point (Reed 1992), and dominant hyperactive alleles of G1 cyclins shorten G1 phase and prevent G1 arrest (Cross 1988; Nash *et al.* 1988). In addition, mutations in individual G1 cyclins restore significant G1 arrest to *fus3* and *far1* mutants that do not properly arrest in the presence of α-factor (Chang and Herskowitz 1990; Elion *et al.* 1990, 1991a). Two levels of G1 cyclin inhibition have been proposed: repression of transcription of the *CLN1* and *CLN2* genes (Wittenberg *et al.* 1990; Elion *et al.* 1991a; Valdivieso *et al.* 1993) and direct inhibition of the three different Clnp/Cdc28p kinases by Far1p (Peter *et al.* 1993; Tyers and Futcher 1993; Peter and Herskowitz 1994). G1 arrest may also involve the regulation of targets other than the G1 cyclins. First, while deletion of *SIC1* restores cell

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division to a *cln1 cln2 cln3* triple mutant, presumably by activation of Clb5p/Cdc28p and Clb6p/Cdc28p kinases, α-factor still inhibits the growth of this strain (Schneider *et al.* 1996; Tyers 1996). Second, a *far3* mutant is resistant to α-factor, although the G1 cyclins appear to be properly regulated (Horecka and Sprague 1996).

G1 arrest in response to mating pheromone is controlled by the mating MAP kinase cascade (Herskowitz 1995). After binding of mating pheromone, the receptor activates a heterotrimeric G-protein which, together with the Ste5p scaffolding protein (Elion 1995), causes sequential activation of a p21-activated kinase homolog (Ste20p), a MAPKKK (Ste11p), a MAPKK (Ste7p), and two MAP kinases (Fus3p and Kss1p). Activation of Fus3p and Kss1p by α-factor is coupled to G1 arrest as well as to other responses required for mating and recovery (*e.g.*, activation of the Ste12p transcription factor, shmoo formation, fusion, and signal attenuation (Elion *et al.* 1990, 1991a, 1993; Gartner *et al.* 1992; Ma *et al.* 1995; Farley *et al.* 1999).

The relative contribution of Fus3p and Kss1p to the control of G1 arrest is not known. Much of the available data support a model in which Fus3p is the major MAP kinase regulator of G1 arrest, with most of the regulation through the control of Far1p. Fus3p phosphorylates Far1p (Elion *et al.* 1993; Peter *et al.* 1993), and this phosphorylation is required to stabilize Far1p (Henchoz *et al.* 1997) and allow its association with the three different Clnp/Cdc28p complexes (Peter *et al.* 1993; Tyers and Futcher 1993; Jeoung *et al.* 1998). Far1p subsequently inhibits the Clnp/Cdc28p kinases through an as-yet-undefined mechanism (Gartner *et al.* 1998). To date, there is no clear evidence that Kss1p plays a direct role in the regulation of Far1p or G1 arrest. While null mutations in *FUS3* cause a G1 arrest defect (Elion *et al.* 1991a) and block pheromone-induced phosphorylation of Far1p and formation of Far1p/Clnp/Cdc28p complexes (Peter *et al.* 1993; Tyers and Futcher 1993), a null mutation in *KSS1* has no obvious effect on G1 arrest (Elion *et al.* 1991a). Kss1p could play an indirect role in regulating G1 arrest, because it is able to activate Ste12p (Elion *et al.* 1991a), which positively regulates the *FAR1* gene (Chang and Herskowitz 1990). This possibility is consistent with the greater α-factor sensitivity of a *fus3* null mutant compared to a *far1* null mutant (Satterberg 1993; Tyers and Futcher 1993) and the ability of overexpressed Ste5p to restore α-factor sensitivity to *fus3* and *far1* null mutants, but not to *fus3 kss1* double mutants (Elion *et al.* 1991b; Leberer *et al.* 1993; Satterberg 1993). However, catalytically inactive Fus3p nearly completely blocks Kss1p from activating the Ste12p-dependent gene *STE2* in the presence of α-factor, arguing that Kss1p normally does not function in the mating pathway in the presence of Fus3p (Madhani *et al.* 1997).

Here we present evidence that Fus3p and Kss1p both control G1 arrest in parallel with Far1p through a combination of functions that both inhibit and promote cell division. Fus3p and Kss1p together promote G1 arrest by repressing transcription of G1/S-phase cyclin genes (*e.g.*, *CLN1*, *CLN2*, *CLB5*) at a step distinct from Cln3p/Cdc28p-mediated activation of Swi4p/Swi6p. This inhibition constitutes a major portion of the cyclin regulation that occurs in the presence of α-factor. Surprisingly, Fus3p and Kss1p also counteract G1 arrest through distinct mechanisms. Kss1p promotes recovery from G1 arrest by inhibiting the MAP kinase cascade at or below Ste11p. By contrast, Fus3p promotes proliferation through a novel function that is not shared by Kss1p and does not involve increasing the level of the G1 cyclins or decreasing Ste12p activity. Genetic suppression tests suggest that this function involves the activation of Mcm1p or genes under its control.

MATERIALS AND METHODS

Media, strains, and yeast strain construction: Yeast strains are described in Table 1. All strains are isogenic derivatives of EY957, which is a *bar1*Δ derivative of W303a. Yeast media were prepared as described (Sherman *et al.* 1986). Plasmids were integrated as described previously for *fus3-6::LEU2*, *fus3-7::HIS3* (pYEE98, pJB225; Elion *et al.* 1990, 1991a), *CLN2-HA LEU2* (MT104; Tyers *et al.* 1993), and *CLN3-1 URA3* (pFC101-1; Cross 1988). All integrations were confirmed by Southern analysis (Sambrook *et al.* 1989).

Halo assays: α-factor sensitivity was measured by a halo assay as described previously (Elion *et al.* 1990), using 50 μl of an overnight culture of yeast cells. α-Factor peptide (synthesized by C. Dahl, Harvard Medical School) was dissolved in 90% methanol and stored at −20°. All halo assays were done at least in duplicate, using 3 μl of 50 μm synthetic α-factor for *bar1* strains and 8 μl of 2 mm α-factor for *BAR1* strains.

Growth conditions: Strains were grown at 30° in selective SC media with 2% dextrose to an A_{600} of 0.5, split in half and incubated in the presence or absence of 100 nm α-factor for 2 hr (unless indicated otherwise), and then harvested. For the *BAR1* strains described in Figure 2, 5 mm α-factor was added for 30 min. The *STE11-4 far1*, *STE11-4 far1 fus3*, and *STE11-4 far1 kss1* strains each contained a *FUS1-HIS3* reporter gene and were grown in media lacking histidine to avoid the propagation of sterile pseudorevertants. The growth rate of these three strains is slower than that of *STE11* strains, with doubling times of 3.2, 3.5, and 2.5 hr for the *STE11-4 far1*, *STE11-4 far1 fus3*, and *STE11-4 far1 kss1*, respectively, compared to 1.5 hr for wild type and *far1* strains. Therefore, a 4-hr α-factor induction point was also done in addition to a 2-hr time point for these slower-growing strains.

Preparation of yeast extracts: Cells were collected at 4°, washed once with cold sterile water, and frozen in dry ice/ethanol. Whole cell extracts were prepared by lysis with glass beads, as described in Surana *et al.* (1991). Protein concentrations were determined using the Bio-Rad (Hercules, CA) protein assay.

β-Galactosidase assays: β-Galactosidase activity was measured as described (Elion *et al.* 1995) using yeast extracts prepared as described above.

Immunoprecipitation, immunoblot analysis, and kinase assays: Immunoprecipitations were performed as described (Elion *et al.* 1993) using 12CA5 mouse monoclonal antibody to detect the HA1 epitope (Field *et al.* 1988). Depending on the abundance of the protein in question, 2 mg (for Cln2-

TABLE 1

Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
Isogenic derivatives of EY699	*MAT***a** *FUS3 KSS1 ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 Gal*$^+$ (W303a)	R. Rothstein
EY725	*kss1*Δ*::URA3*	Elion *et al.* (1991a)
EY1118	*bar1*Δ *his3*Δ*200 lys2::FUS1-HIS3*	Lyons *et al.* (1996)
EY1262	*bar1*Δ *far1*Δ*his3*Δ*200 lys2::FUS1-HIS3*	This study
EY1290	*bar1*Δ *far1*Δ *cln2*Δ *his3*Δ*200 lys2::FUS1-HIS3*	This study
EY1298	*bar1*Δ *STE11-4 far1*Δ *his3*Δ*200 lys2::FUS1-HIS3*	This study
EY1321	*bar1*Δ *STE11-4 far1*Δ *fus3-6::LEU2 his3*Δ*200 lys2::FUS1-HIS3*	This study
EY1335	*bar1*Δ *STE11-4 far1*Δ *kss1::URA3 his3*Δ*200 lys2::FUS1-HIS3*	This study
EY1346	*bar1*Δ *STE11-4 far1*Δ *fus3-6::LEU2 kss1::URA3 his3*Δ*200 lys2::FUS1-HIS3*	This study
EY940	*bar1*Δ *fus3-6::LEU2*	Elion *et al.* (1993)
EY946	*bar1*Δ *fus3-6::LEU2 kss1::URA3*	Elion *et al.* (1993)
EY1155	*bar1*Δ *far1*Δ *fus3-6::LEU2 his3*Δ*200*	This study
EY1163	*bar1*Δ *far1::URA3 kss1::ADE2*	This study
EY1864	*bar1*Δ *far1*Δ *his3*Δ*200 lys2::FUS1-HIS3* + *YEp24*	This study
EY1879	*bar1*Δ *ste2::LEU2*	This study
CY130	*bar1*Δ *his3*Δ*200 lys2::FUS1-HIS3 pho85::LEU2*	This study
CY326	*bar1*Δ *his3*Δ*200 lys2::FUS1-HIS3 CLN2::CLN2-HA LEU2*	This study
CY327	*bar1*Δ *far1*Δ *his3*Δ*200 lys2::FUS1-HIS3 CLN2::CLN2-HA LEU2*	This study
CY328	*bar1*Δ *fus3-7::HIS3 CLN2::CLN2-HA LEU2*	This study
CY358	*bar1*Δ *far1*Δ *fus3-7::HIS3 his3*Δ*200 lys2::FUS1-HIS3 CLN2::CLN2-HA LEU2*	This study
CY329	*bar1*Δ *STE11-4 far1*Δ*his3*Δ*200 lys2::FUS1-HIS3 CLN2::CLN2-HA LEU2*	This study
CY330	*bar1*Δ *STE11-4 far1*Δ *kss1*Δ *his3*Δ*200 lys2::FUS1-HIS3 CLN2::CLN2-HA LEU2*	This study
CY378	*bar1*Δ *STE11-4 far1*Δ *LEU2 fus3-7::HIS3 his3*Δ*200 lys2::FUS1-HIS3 CLN2::CLN2-HA*	This study
CY331	*bar1*Δ *his3*Δ*200 lys2::FUS1-HIS3 URA3::pGAL-CLB5-HA*	This study
CY332	*bar1*Δ *far1*Δ *his3*Δ*200 lys2::FUS1-HIS3 URA3::pGAL-CLB5-HA*	This study
CY385	*bar1*Δ *his3*Δ*200 lys2::FUS1-HIS3 CLN3-1*	This study
CY384	*bar1*Δ *STE11-4 far1*Δ *his3*Δ*200 lys2::FUS1-HIS3 CLN3-1*	This study
Plasmids		
pYBS45	*FUS1-lacZ LYS2 CEN*	Lyons *et al.* (1996)
pJB207	*FUS1-lacZ LEU2 2*μ	Kranz *et al.* (1994)
pYEE98	*fus3-6::LEU2*	Elion *et al.* (1990)
pJB225	*fus3-7::HIS3*	Elion *et al.* (1991a)
pBC65	*kss1::URA3*	Courchesne *et al.* (1989)
MT104	*CLN2-HA LEU2*	Tyers *et al.* (1993)
pYU19	*pho85::LEU2*	A. Toh-e
ES2669	*YIpGAL1-CLB5HA URA3*	E. Schwob
pFC101-1	*CLN3-1 URA3*	Cross (1988)
pAB506	*ste2::LEU2*	J. Konopka
YEp24	*URA3 2*μ	
Ycp50	*URA3 CEN*	
pJB223	*STE5 URA3 2*μ	Kranz *et al.* (1994)
pSL201-5	*GAL1-CLN2HA URA3*	C. Wittenberg
pBA623	*PCL2 URA3 2*μ	B. Andrews
pCC68	*H2A1* + *H2B1 in pBR322*	A. Bortvin and F. Winston
pRT5	*fus3-K42R URA3 CEN*	R. Tung and E. Elion
B3697	*kss1-K42R HIS3 CEN*	Madhani *et al.* (1997)
B1817	*HIS3 CEN*	G. Fink
2μMU	*MCM1 URA3 2*μ	Elble and Tye (1991)

HAp) to 40 mg (for detection of Cln2-HAp in a wild-type strain after α-factor treatment) of protein was used for immunoprecipitation. For quantitation of immunoprecipitated kinase activity, the protein A beads were washed twice more with kinase reaction buffer (20 mm Tris-HCl, pH 7.5, 7.5 mm $MgCl_2$, 0.1 mm EGTA (Mendenhall 1993) and reactions were carried out in 20 μl containing 1.4 μl [γ-^{32}P]ATP (ICN, Costa Mesa, CA; 5000 Ci/mmol),1 μl 10 mm ATP, and 1 μg H1 histone for 15 min at 25° (Surana *et al.* 1991). Reactions were terminated by addition of 2× PAGE sample buffer, boiled for 5 min and loaded onto a 12% SDS-PAGE gel. Phosphorylated H1 was visualized by autoradiography. Quantitations were done with a Molecular Dynamics (Sunnyvale, CA) Phosphorimager. For immunoblot analysis of immunoprecipitated proteins, protein A beads were washed, pelleted, and boiled in sampler buffer immediately before SDS-PAGE (8%, 10%, or 12% depending on the protein) as described in Kranz *et al.* (1994). Blots were developed with an Amersham (Buckinghamshire, UK) ECL kit according to manufacturer's instructions using Fuji RX X-ray film.

Northern analysis: Total RNA was isolated by extraction with hot and acidic phenol as described (Collart and Oliviero 1992), transferred to nitrocellulose, and probed according to Sambrook *et al.* (1989). The following probes were used: 1.3-kb *Eco*RI-*Nco*I *CLN1* from 419, 0.7-kb *Xho*I-*Hin*dIII *CLN2* from 810 (Hadwiger *et al.* 1989), 1.4-kb *Eco*RI-*Xho*I *CLN3* from pFC101-1 (Cross 1988), 1.5-kb *Sal*I-*Bam*HI from Ycp-GAL1-CLB5, 1.3-kb *Bgl*II-*Nsi*I *PCL2* from pBA623, 5.5-kb *Nhe*I-*Sal*I *H2A1* + *H2B1* from pCC68, and 2.0-kb *Xho*I-*Hin*dIII *ACT1* from pYEE15 (Elion *et al.* 1991a). Blots were probed sequentially. Northern blots were reprobed with *ACT1* as a control for loading and retention of RNA on the membrane.

Cell morphology and flow cytometry: Cells were fixed and stained with 4′,6-diamidino-2-phenylindole (DAPI) using a protocol provided by S. Dutcher (Trueheart *et al.* 1987) and then counted after brief sonication to determine the percentage of unbudded cells. For flow cytometric DNA quantitation (FACS) analysis, cells were fixed with 70% ethanol and processed essentially as described (Hutter and Eipel 1979), and then briefly sonicated immediately before DNA quantitation. FACS was done using the Flow Cytometry Facility at the Dana-Farber Cancer Institute.

RESULTS

Hyperactivation of Fus3p and Kss1p by Ste11-4p or excess Ste5p restores G1 arrest to a *far1* mutant: The phenotype of a *far1 cln2* double mutant first suggested that mating pheromone promotes G1 arrest through additional pathways that operate in parallel with Far1p (Chang and Herskowitz 1990). A *far1 cln2* double mutant is completely resistant to α-factor at a concentration that causes a wild-type strain to arrest growth (Figure 1A, left). Unless indicated otherwise, all strains are deleted for the α-factor protease gene, *BAR1*, to avoid complications from recovery as a result of α-factor degradation (Ciejek and Thorner 1979). This α-factor resistance is presumably due to the high levels of Cln3p/Cdc28p and Cln1p/Cdc28p kinase that result from the loss of Far1p inhibition (Peter *et al.* 1993; Tyers and Futcher 1993). However, when the concentration of α-factor is raised to higher levels, a *far1 cln2* double mutant arrests in G1 phase (Figure 1A, panels 2 and 3, and Figure 1B; Chang and Herskowitz 1990). The fact

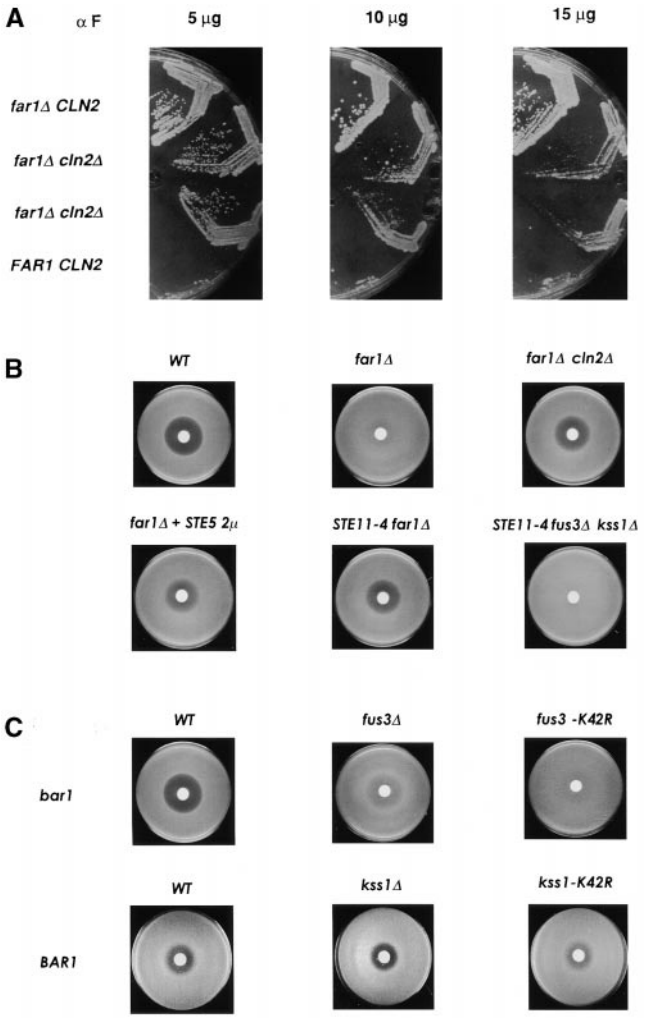

Figure 1.—Suppression of the α-factor-resistant phenotype of a *far1* null. (A) Growth of *far1* strains streaked onto YPD plates with the indicated amount of α-factor. Strains: *FAR1 CLN2* − EY1118; *far1*Δ *CLN2* − EY1262; *far1*Δ *cln2*Δ − EY1290. (B) Hyperactivation of the MAPK cascade by multiple copies of the *STE5* gene or the *STE11-4* mutation restores α-factor sensitivity to a *far1* null. Pheromone sensitivity was tested in halo assays using 3 μl of 50 μm α-factor (materials and methods). Strains: WT − EY1118; *far1*Δ − EY1262; *far1*Δ *cln2*Δ − EY1290; *far1*Δ + *STE5 2*μ − EY1262 + pJB223 (*STE5 URA3 2*μ); *STE11-4 far1*Δ − EY1298; *STE11-4 far1*Δ *fus3*Δ *kss1*Δ − EY1346. Plates were photographed after 24 hr at 30°. (C) Catalytic activity of both Fus3p and Kss1p is required for pheromone-dependent growth arrest. Halo assays were done as in B except that 8 μl of 2 mm α-factor was used for the strains that are *BAR1* (see Table 1). Strains: WT − EY1118 + Ycp50 (*URA3 CEN*); *fus3*Δ − EY940 + Ycp50 (*URA3 CEN*); *fus3*Δ + *fus3-K42R* − EY940 + pRT5 (*fus3K42R URA3 CEN*); WT − EY699 + B1817 (*HIS3 CEN*); *kss1*Δ − EY725 + B1817; *kss1*Δ + *kss1K42R* − EY725 + B3697 (*kss1K42R HIS3 CEN*).

that an increase in α-factor concentration can restore G1 arrest to a *far1 cln2* strain raises the possibility that the MAP kinase cascade activates parallel pathways to regulate G1 arrest.

We directly tested the possibility that Fus3p and Kss1p promote G1 arrest independently of Far1p, by determin-

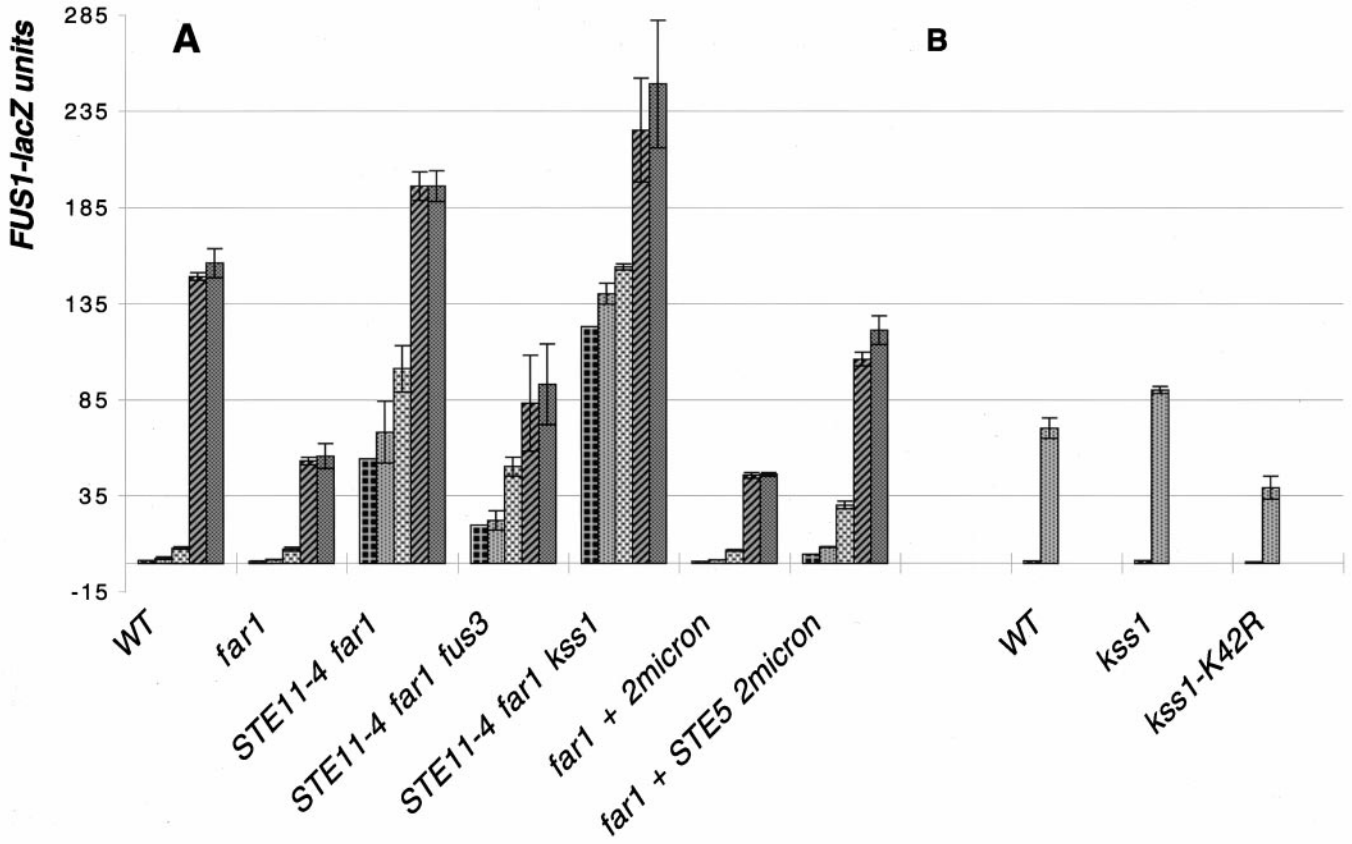

Figure 2.—Level of expression of a *FUS1-lacZ* reporter gene in various strains. β-Galactosidase activity was quantitated as described in Elion *et al.* (1995). The *bar1* strains in A harbor *FUS1-lacZ* on pYBS45 and were induced for 2 hr with either 0, 1, 10, 100, or 500 nm α-factor (shown from left to right). Similar results were found after a 30-min induction. The *BAR1* strains in B harbor *FUS1-lacZ* on pJB207 and were induced with 5 μM α-factor for 30 min. The units shown are the average of at least two independent experiments ± SE. Strain numbers are listed in Figure 1 except for EY1321 (*STE11-4 far1 fus3*) and EY1335 (*STE11-4 far1 kss1*).

ing whether an increase in the level of signaling through the MAPK cascade could restore G1 arrest to a *far1* null mutant. Pathway activity was increased in two independent ways, either by increasing the concentration of Ste5p, a limiting component required for MAP kinase activation (Kranz *et al.* 1994), or by a gain-of-function allele of the MAPKKK Ste11p (*STE11-4*) that causes constitutive hyperactivation of the MAPKK Ste7p that activates Fus3p and Kss1p (Stevenson *et al.* 1992). Overproduction of Ste5p ($STE5^{OP}$) or introduction of *STE11-4* in a *far1* strain increases α-factor-induced *FUS1* expression across a wide range of α-factor concentrations, although *STE11-4* is significantly more potent than $STE5^{OP}$ in the absence of α-factor (Figure 2). Halos assays show that $STE5^{OP}$ and *STE11-4* restore nearly as much α-factor sensitivity to a *far1* null strain as does a deletion in the *CLN2* gene (Figure 1B). *STE11-4* restores more α-factor sensitivity to the *far1* null than does $STE5^{OP}$, consistent with the greater pathway activity in this strain (Figure 2).

The restoration of pheromone sensitivity to a *far1* strain by *STE11-4* or $STE5^{OP}$ requires the components of the mating signal transduction cascade including Fus3p and Kss1p. Deletion of positive regulators of the pathway (*i.e.*, *STE4*, *STE5*, *STE7*, *STE12*) or overexpression of negative regulators (*i.e.*, *GPA1*, *SST2*) blocks the pheromone sensitivity of the *far1 STE11-4* strain. Overexpression of positive regulators that either enhance Ste11p activity (*i.e.*, *STE5*, *STE50*) or the amount of active Ste7p (*i.e.*, *STE7*) further enhances the α-factor sensitivity of the *STE11-4 far1* strain (data not shown). Thus, it is possible to arrest growth in *far1* cells by simply increasing the level of activity of the mating MAP kinase cascade during α-factor induction.

Null mutations in both *FUS3* and *KSS1* completely block the α-factor sensitivity of the *STE11-4 far1* strain, demonstrating that the arrest is completely dependent upon the two mating MAP kinases (Figure 1B). Substitution of catalytically inactive *fus3K42R* for *FUS3* in *STE11 FAR1* and *STE11-4 far1* strains completely blocks α-factor-induced arrest, indicating that Fus3p kinase activity is essential for the arrest (Figure 1C and data not shown). Substitution of catalytically inactive *kss1K42R* for *KSS1* in a *STE11 FAR1* strain causes partial resistance to α-factor and reduced levels of *FUS1* expression (Figure 1C and Figure 2), indicating that Kss1p kinase activity is also required for efficient arrest. *STE11-4* is unlikely to pro-

TABLE 2

Effect of α-factor treatment on budding arrest and inhibition of DNA synthesis

Strain	α-Factor	Unbudded[a] (%)	Wild-type G1 arrest (%)	Δ1C DNA[b]	Wild-type Δ1C DNA (%)
WT	−	39	100	54.5 ± 3.2	100
	+	100			
*far1*Δ	−	45	5	17.1 ± 2.8	31
	+	48			
*fus3*Δ	−	35	43	24.0 ± 2.7	44.5
	+	61			
*far1*Δ *fus3*Δ	−	38	23	6.8 ± 1.4	13
	+	52			
*far1*Δ *kss1*Δ	−	45	2	4.8 ± 0.5	8
	+	46			
*far1*Δ *cln2*Δ	−	43	80	47.5 ± 1.5	84
	+	92			
*far1*Δ + *STE5* 2μ	−	42	56	16.5 ± 1.5	30
	+	76			
*fus3*Δ *kss1*Δ	−	34	0	−1.4 ± 1.1	0
	+	33			
*ste2*Δ	−	26	0	0 ± 4	0
	+	25			
*STE11-4 far1*Δ	−	40	34	22.0 ± 1	42
	+ 2h	61			
	−	45	49		
	+ 4h	75			
*STE11-4 far1*Δ	−	32	43	21	38
*fus3*Δ	+ 2h	58			
	−	38	57		
	+ 4h	73			
*STE11-4 far1*Δ	−	27	39	21.0 ± 1.7	38
*kss1*Δ	+ 2h	51			
	−	28	51		
	+ 4h	59			
*STE11-4 far1*Δ	−	36	5	0 ± 0.5	0
*fus3*Δ *kss1*Δ	+ 2h	39			
	−	38	0		
	+ 4h	35			

[a] Logarithmically growing cells were treated with 100 nm α-factor for the indicated times. Cells were then fixed and counted for the number that were unbudded after brief sonication. Each percentage is an average of at least three separate experiments in which ~200 cells were scored. The standard deviations ranged from <10 to 14% of the mean values.

[b] DNA content was determined by FACS. Δ1C is the percentage change in 1C DNA ± SE. The percentage wild-type Δ1C DNA values are nomalized to the wild-type strain that is assigned as 100. DNA content was also measured for all *STE11-4* strains after the 4-hr treatment with α-factor. The longer exposure to α-factor did not cause a greater inhibition of DNA synthesis. The strains are the same as in Figures 1 and 2 except for EY1155 (*far1*Δ *fus3*Δ), EY1163 (*far1*Δ *kss1*Δ), EY946 (*fus3*Δ *kss1*Δ), and EY1879 (*ste2*Δ).

mote G1 arrest through inappropriate activation of either Mpk1 or Hog1, the other two MAPKs expressed in haploid cells. Mutation of Mpk1, the MAPK in the Pkc1 pathway, does not reduce the ability of *STE11-4* to restore arrest to a *far1* strain (data not shown). Hog1 is an attenuator of the pathway and inhibits Fus3p tyrosine phosphorylation (Hall *et al.* 1996). Thus, Fus3p and Kss1p are specifically required to mediate this arrest.

The increased α-factor sensitivity in the presence of *STE5*^{OP} or *STE11-4* could arise from effects on cell division at any point in the cell cycle. Overexpression of a stable form of Far1p outside of G1 phase leads to G2 arrest, possibly from inappropriate inhibition of Clbp/Cdc28p kinases (McKinney and Cross 1995), and *STE11-4* can cause a G2/M delay during pseudohyphal growth (Kron *et al.* 1994). To determine whether the effects of *STE5*^{OP} and *STE11-4* were G1 phase-specific, we quantitated their effects on budding and DNA synthesis after a short-term exposure to α-factor. For *STE11-4* strains, a 4-hr α-factor induction time point was done in addition to a 2-hr time point, because *STE11-4* causes cells to divide more slowly (3.2-hr doubling time for *STE11-4 far1* compared to 1.5 hr for wild type and *far1* strains; see materials and methods for de-

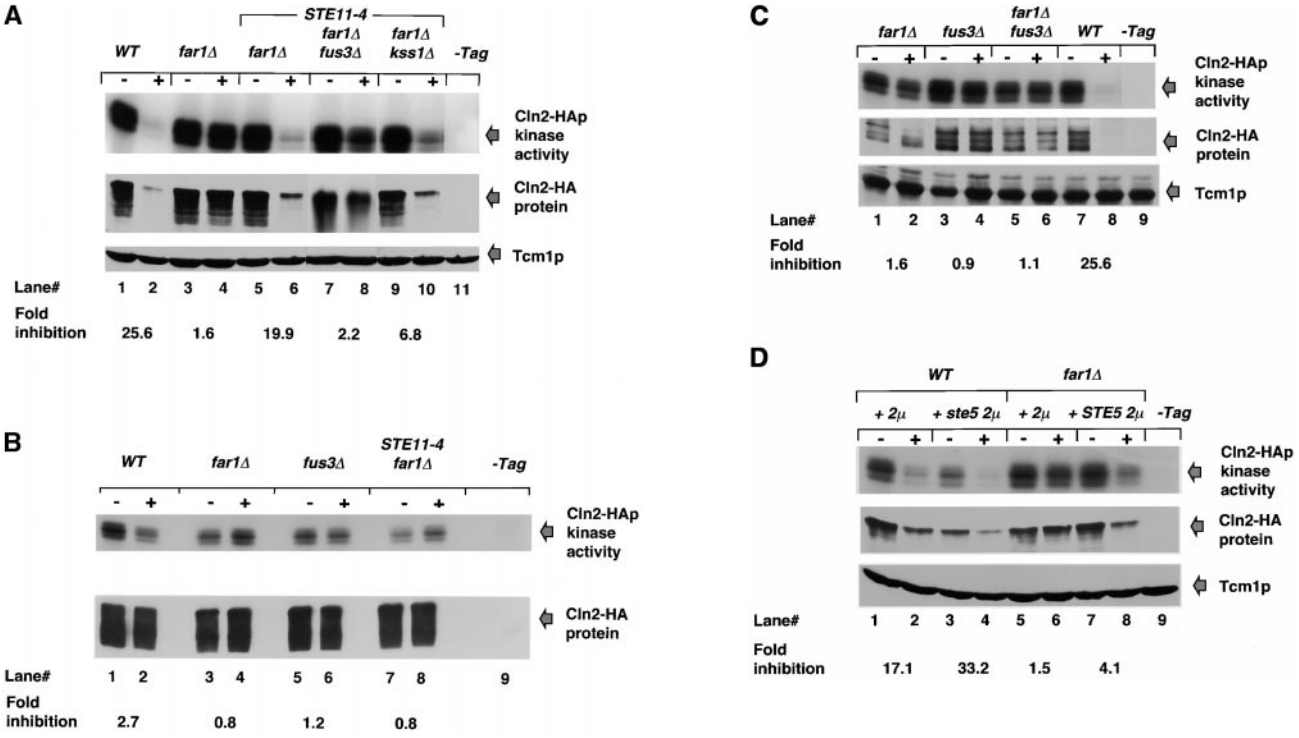

Figure 3.—Cln2p/Cdc28p kinase levels in *far1* and *fus3* strains. (A) STE11-4 restores α-factor-dependent inhibition of Cln2-HAp/Cdc28p kinase in the absence of Far1p. Lanes 1, 2—WT (CY326); lanes 3, 4—*far1*Δ (CY327); lanes 5, 6—*STE11-4 far1*Δ (CY329); lanes 7, 8—*STE11-4 far1*Δ *fus3*Δ (CY378); lanes 9, 10—*STE11-4 far1*Δ *kss1*Δ (CY330); lane 11—no Tag. Exponentially growing strains harboring the *CLN2-HA* gene were treated (+) or not treated (−) with α-factor for 2 hr, and then extracts were prepared and assayed for Cln2-HA kinase activity and Cln2-HA protein. Fold inhibition is the average of two to three experiments. (B) Quantitation of specific activity of Cln2-HAp/Cdc28p kinase in *far1* and *fus3* strains. Strains were treated for 1 hr with α-factor, except for the wild-type strain, which was treated for 15 min. Based on prior normalization, equal amounts of Cln2-HA protein were immunoprecipitated from the different extracts (10–40 mg total protein). After immunoprecipitation and washes, the protein A beads were resuspended in 2 ml of lysis buffer. Of this, 200 μl was used for the Cln2-HA kinase assay, and the remainder was used for the Cln2-HA immunoblot. Strains are in Table 1. Lanes 1, 2—WT; lanes 3, 4—*far1*Δ; lanes 5, 6—*fus3*Δ; lanes 7, 8—*STE11-4 far1*Δ; lane 9—no Tag. (C) Cln2-HAp/Cdc28p kinase activity and Cln2-HA protein in *far1* and *fus3* strains. Lanes 1, 2—*far1*Δ (CY327); lanes 3, 4—*fus3*Δ (CY328); lanes 5, 6—*far1*Δ *fus3*Δ (CY358); lanes 7, 8—WT (CY326); lane 9—no Tag. (D) $STE5^{OP}$ restores of α-factor-dependent inhibition of Cln2-HAp/Cdc28p kinase in the absence of Far1p. Fold inhibition is on the basis of one experiment. Lanes 1, 2—WT + *2*μ (CY326 + Yep24); lanes 3, 4—WT + *STE5-2*μ (CY326 + pJB223); lanes 5, 6—*far1*Δ + *2*μ (CY327 + Yep24); lanes 7, 8—*far1*Δ + *STE5-2*μ (CY327 + pJB223); lane 9—no Tag. All strains were induced with 100 nm of α-factor for 2 hours except for as described in B.

tails). $STE5^{OP}$ and *STE11-4* both cause a *far1* strain to arrest in G1 phase in the presence of α-factor, as shown by a greater accumulation of unbudded cells (Table 2). *STE11-4* somewhat increases the amount of inhibition of DNA synthesis [31% wild-type inhibition for *far1 vs.* 42% wild-type inhibition for *STE11-4 far1*; Table 2, shown as "Δ1C DNA (%)"], while $STE5^{OP}$ has no obvious effect, indicating that most of the arrest is due to a block in budding for both strains. As predicted from the halo assay, a *fus3 kss1* double null mutation completely blocks α-factor-induced inhibition of budding and DNA synthesis by *STE11-4* (*i.e.*, *STE11-4 far1 fus3 kss1* behaves like *ste2* or *fus3 kss1* strains; Table 2) and by $STE5^{OP}$ (data not shown), demonstrating that Fus3p and Kss1p inhibit budding and DNA synthesis in the absence of Far1p.

Elevated signaling is not required for the MAP kinases to inhibit DNA synthesis in a *far1* null: To further confirm that Fus3p and Kss1p promote G1 arrest through mechanisms distinct from Far1p, we compared the arrest behavior of a *far1* single mutant to *fus3 far1*, *kss1 far1*, and *fus3 kss1* double mutants that do not have enhanced levels of signaling. As previously observed (Tyers and Futcher 1993), a *far1* mutant undergoes partial inhibition of DNA synthesis in the presence of α-factor (31% wild-type inhibition; Table 2), although the strain is resistant to α-factor, as measured by a halo assay (Figure 1B) and the accumulation of unbudded cells (Table 2). This partial inhibition is not detected in a *ste2* mutant and therefore requires signal transduction through the α-factor receptor (Table 2). Moreover, deletion of either *FUS3* or *KSS1* reduces the amount of inhibition of DNA synthesis that occurs in a *far1* strain (12% wild type for *fus3 far1* and 8% wild type for *kss1 far1*), and DNA synthesis is not inhibited at all in a *fus3 kss1* double mutant (Table 2). Analysis of the levels of total Cdc28p kinase in these strains shows that these

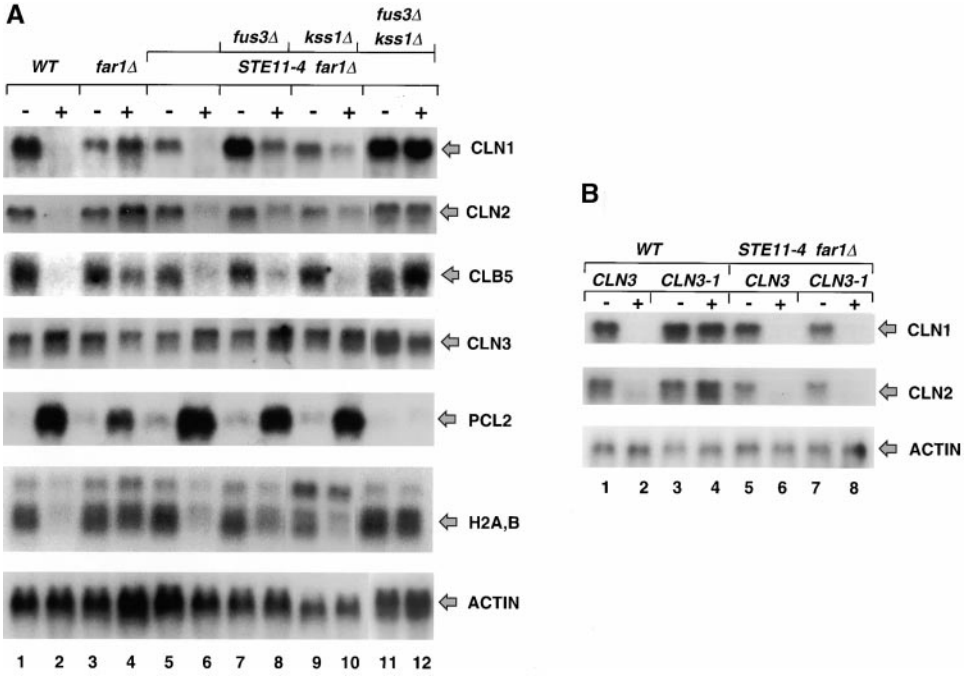

Figure 4.—Fus3p and Kss1p regulate the level of G1/S-phase cyclin mRNAs. (A) Fus3p and Kss1p repress *CLN1*, *CLN2*, *CLB5* and activate *CLN3*, *PCL2*. Lanes 1, 2—WT, lanes 3, 4—*far1*Δ; lanes 5, 6—*STE11-4 far1*Δ, lanes 7, 8—*STE11-4 far1*Δ *fus3*Δ; lanes 9, 10—*STE11-4 far1*Δ *kss1*Δ; lanes 11, 12—*STE11-4 far1*Δ *fus3*Δ *kss1*Δ. (B) *CLN3-1* does not bypass Far1p-independent repression of *CLN1*, *CLN2*. Lanes 1, 2—*CLN3* (EY-1118); lanes 3, 4—*CLN3-1* (CY385); lanes 5, 6—*STE11-4 far1* (EY1298); lanes 7, 8—*STE11-4 far1*Δ *CLN3-1* (CY384). In a parallel experiment, the percentage of unbudded cells in the *STE11-4 far1* and *STE11-4 far1 CLN3-1* strains after a 2-hr exposure to 100 nm of α-factor were found to be: *STE11-4 far1*, 40% − α-factor, 69% + α-factor. *STE11-4 far1 CLN3-1*, 35%; α-factor, 46% + α-factor. For A and B, logarithmically growing strains were induced with 100 nm of α-factor for 2 hr and Northern analysis was performed as described in materials and methods. + indicates α-factor induction. Note that the histone probe detects both *HTA1* and *HTB1* mRNAs. Strain numbers are listed in the legends to Figures 1 and 2.

effects on DNA synthesis are mirrored at the level of Cdc28p kinase activity (data not shown), substantiating the results with the *STE11-4 far1* strain.

Fus3p and Kss1p inhibit Cln2p/Cdc28p kinase independently of Far1p: We next determined whether the level of Cln2p/Cdc28p kinase was reduced in the *STE11-4 far1* strain as an explanation for the increased pheromone sensitivity and budding arrest. An epitope-tagged *CLN2* gene under the control of its own promoter (Tyers *et al.* 1993) was integrated in single copy into the strains to be tested. In a wild-type strain, the steady state levels of Cln2-HAp protein and active Cln2-HAp/Cdc28p kinase are greatly reduced by α-factor, causing an overall 26-fold reduction in the level of Cln2-HAp/Cdc28p kinase (Figure 3A, lanes 1 and 2). As previously reported (Valdivieso *et al.* 1993; Peter and Herskowitz 1994), a *far1* mutant has high levels of Cln2 protein and active Cln2p/Cdc28p kinase (Figure 3A, lanes 3 and 4). *STE11-4* restores a dramatic 20-fold inhibition of Cln2p/Cdc28p kinase to the *far1* strain (Figure 3A, lanes 5 and 6). Similar low levels of Cln2p/Cdc28p kinase are also found in the $STE5^{OP}$ *far1* strain (Figure 3D). Thus, α-factor can inhibit Cln2p/Cdc28p kinase independently of Far1p.

The Far1p-independent inhibition of Cln2p kinase is blocked by a null mutation in *FUS3* (Figure 3A, lanes 7 and 8; *STE11-4 far1 fus3*), demonstrating a clear role for Fus3p in negatively regulating Cln2p/Cdc28p kinase that is distinct from Far1p. Deletion of Kss1p also blocks, to a lesser extent, the inhibition of Cln2p/Cdc28p kinase (Figure 3A, lanes 9 and 10; *STE11-4 far1 kss1*). The level of Cln2p/Cdc28p kinase activity in a *STE11-4 far1 kss1* triple mutant is reproducibly two-fold greater than that of a *STE11-4 far1* double mutant, despite equal levels of Cln2p protein (Figure 3A; compare lanes 5 and 6 with lanes 9 and 10), suggesting that Kss1p modestly inhibits Cln2p/Cdc28p kinase. Thus, both MAP kinases regulate Cln2p/Cdc28p kinase independently of Far1p, possibly at several levels. However, Fus3p plays a much greater role.

Fus3p and Kss1p do not inhibit the specific activity of Cln2p/Cdc28p: We examined the specific activity of Cln2p/Cdc28p kinase in *far1* and *STE11-4 far1* strains to determine whether the MAP kinases inhibit the activity of Cln2p/Cdc28p kinase independently of Far1p (Figure 3B). Large-scale preparations of whole cell extracts were made from wild-type, *far1*, and *STE11-4 far1* strains grown in the absence or presence of α-factor to be able to immunoprecipitate equal amounts of Cln2p under both conditions. A 15-min α-factor induction was done for the wild-type strain, because of the rapid loss of Cln2p, while 1-hr inductions were done for the other strains. Samples were then preequalized so that equal amounts of Cln2p would be immunoprecipitated from each of the extracts (10–40 mg protein; materials and methods). As shown in Figure 3B, a ~3-fold reduction in Cln2p/Cdc28p kinase-specific activity in a wild-type strain is detected after 15 min in α-factor, presumably because of inhibition by Far1p. This level of inhibition may be an underestimate per responding cell, because only a small percentage of cells are at the Start arrest point. Cln2p/Cdc28p kinase has equally high specific

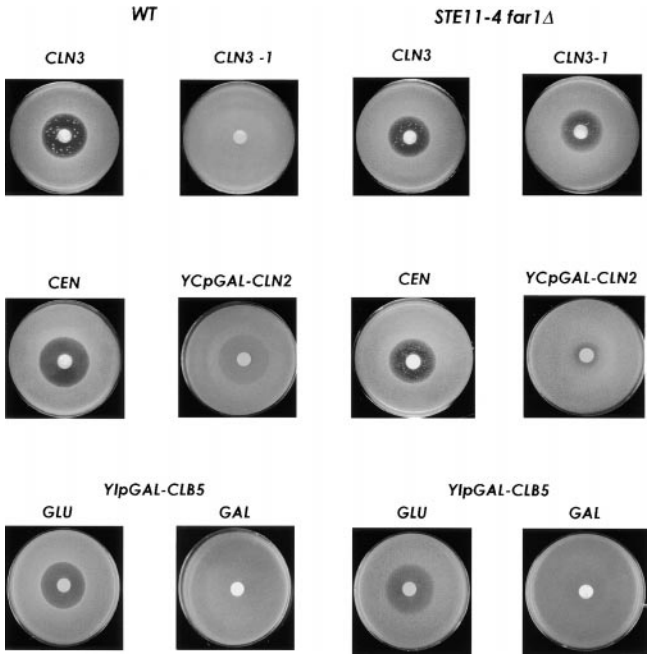

Figure 5.—Dominant *CLN2* and *CLB5* cyclin genes suppress Far1p-independent arrest. Halo assays used 3 μl of 50 μm α-factor. Strains: *CLN3* − EY1118; *CLN3-1* − CY385; *STE11-4 far1*Δ *CLN3* − EY1298; *STE11-4 far1*Δ *CLN3-1* − CY384; WT + *CEN* and *STE11-4 far1* + *CEN* are *EY1118* and *EY1298* bearing *Ycp50*; WT + *pGAL-CLN2* and *STE11-4 far1* + *pGAL-CLN2* are *EY1118* and *EY1298* bearing *pGAL-CLN2 URA3 CEN*. *YIpGAL1-CLB5* strains contain a functional *CLB5-HA* gene (ES2669; Table 1) integrated at the *URA3* locus. Plates were photographed after 24 hr at 30°.

activity in the *far1* and *STE11-4 far1* strains after a 1-hr exposure to α-factor (∼0.8-fold inhibition for both strains). Thus, the enhanced sensitivity and G1 arrest of the *STE11-4 far1* strain is unlikely to be due to an effect on the specific activity of Cln2p/Cdc28p kinase.

Fus3p and Kss1p repress transcription of *CLN1*, *CLN2*, and *CLB5*: The reduction in Cln2p/Cdc28p kinase by hyperactivation of Fus3p and Kss1p by *STE11-4* could be the result of enhanced post-transcriptional inhibition of Cln2p. We therefore determined whether the α-factor-dependent reduction in Cln2p protein detected in the *STE11-4 far1* strain involves more rapid turnover of Cln2 mRNA or protein. On the basis of shut-off experiments using a *GAL1-CLN2-HA* gene, we find no evidence for enhanced post-transcriptional inhibition of Cln2p in the *STE11-4 far1* strain either in the absence or presence of α-factor (data not shown).

We next determined whether transcriptional repression of the G1 cyclin genes is the primary cause of the decreased levels of Cln2p. As previously shown (Nash *et al.* 1988; Wittenberg *et al.* 1990), transcription of the *CLN1* and *CLN2* genes decreases in the presence of α-factor in a wild-type strain (Figure 4A, lanes 1 and 2), while the expression of *CLN3* is slightly increased (Figure 4A). In a *far1* null strain, the addition of α-factor for 2 hr does not reduce expression of either *CLN1* or *CLN2*, nor does it increase the expression of *CLN3* (Figure 4A, lanes 3 and 4). Strikingly, *STE11-4* restores nearly wild-type inhibition of transcription of the *CLN1* and *CLN2* genes to the *far1* null (Figure 4A, lanes 5 and 6), largely accounting for the 26-fold reduction in Cln2p/Cdc28p kinase (Figure 3A). This inhibition contrasts with transcriptional activation of two other cyclin genes that promote budding and are implicated in recovery, *CLN3* and *PCL2* (Figure 4A; Nash *et al.* 1988; Measday *et al.* 1994).

The repression of the *CLN1* and *CLN2* genes is mediated by the combined action of Fus3p and Kss1p. Null mutations in either *FUS3* or *KSS1* partially block the inhibition of transcription in the *STE11-4 far1* strain to similar extents (Figure 4A: lanes 7 and 8, lanes 9 and 10; *STE11-4 far1 fus3* and *STE11-4 far1 kss1*), while null mutations in both *FUS3* and *KSS1* fully block the inhibition (Figure 4A, lanes 11 and 12; *STE11-4 far1 fus3 kss1*). Fus3p and Kss1p also equivalently regulate transcriptional activation of the *CLN3* and *PCL2* genes. This pattern of control contrasts the opposing effects of Fus3p and Kss1p on the transcription of the *FUS1* gene (Elion *et al.* 1991a; Figure 2).

Fus3p and Kss1p also repress transcription of the *CLB5* gene (Figure 4A), with tighter repression than that observed for *CLN2*. In contrast to the pattern of control of *CLN1* and *CLN2*, only a double deletion of *FUS3* and *KSS1* blocks repression of the *CLB5* gene. The transcriptional repression of the *CLB5* gene is unlikely to be an indirect consequence of inhibition of the G1 cyclins, because repression occurs efficiently in the *STE11-4 far1 fus3* strain that has high levels of Cln2p/Cdc28p kinase (Figure 3A). Additional experiments suggest that α-factor does not significantly alter the levels of Clb5p or Clb5p/Cdc28p kinase activity in wild-type or *far1* cells (based on shut-off experiments using *CLB5-HA* expressed from the *GAL1* promoter; data not shown).

Overexpression of either *CLN2* or *CLB5* suppresses Far1p-independent G1 arrest: One could argue that the arrest we detect in the *STE11-4 far1* strain is not tied to the observed transcriptional repression of the G1/S cyclin genes. To test the hypothesis that transcriptional repression of the G1/S cyclin genes is causal to the arrest we observe, we determined whether dominant G1/S cyclin genes that circumvent the transcriptional repression imposed by Fus3p and Kss1p are able to bypass Far1p-independent arrest. As shown in Figure 5, overexpression of either *CLN2* or *CLB5* using the strongly inducible *GAL1* promoter confers α-factor resistance to the *STE11-4 far1* strain in addition to the wild-type strain (*GAL-CLN2*, *YIpGAL-CLB5*; Figure 5). Two additional observations support the view that Far1p-independent arrest involves a G1 arrest block that is a consequence of transcriptional repression of the G1/S genes. First, the pattern of expression of histone H2A and H2B mRNAs (encoded by *HTA1/HTB1*) mirrors that of

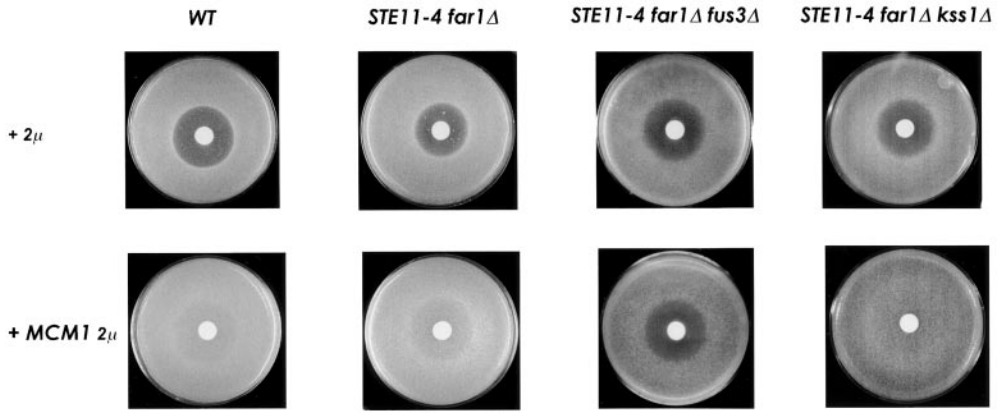

Figure 6.—*MCM1* requires *FUS3* but not *KSS1* to promote proliferation. Strains: WT, *STE11-4 far1*, *STE11-4 far1 fus3*, *STE11-4 far1 kss1* are EY1118, EY1298, EY1321, and EY1335 bearing either Yep24 or *MCM1*-2μ plasmids. Strains were tested for α-factor sensitivity in a halo assay using 3 μl of 50 μm α-factor. Plates were photographed after 36 hr at 30°.

CLN1 and *CLN2* (Figure 4A), consistent with a block at Start in G1 phase. Second, we find that the *STE11-4 far1* strain does not undergo an enhanced loss of viability compared to a *far1* strain after long-term (18-hr) exposure to a high concentration of α-factor (100 nm; data not shown), indicating that the cells are arrested by α-factor rather than dying. Collectively, these findings strongly argue that transcriptional repression of the G1/S cyclin genes is a primary cause of Far1-independent arrest.

Fus3p and Kss1p block Cln3p/Cdc28p from activating the *CLN1* and *CLN2* promoters: Periodic transcription of the *CLN1*, *CLN2*, and *CLB5* genes in G1 phase is controlled by Swi4p/Swi6p and Mbp1p/Swi6p transcription factor complexes (Koch and Nasmyth 1994; Breeden 1996), which are positively regulated by Cln3p/Cdc28p kinase (Cross 1995; Nasmyth 1996). We tested whether Fus3p and Kss1p mediate transcriptional repression of the G1 cyclin genes through inhibition of Cln3p/Cdc28p, by determining whether a dominant *CLN3-1* allele could bypass Far1p-independent inhibition of *CLN1*, *CLN2* transcription. The dominant *CLN3-1* mutation stabilizes Cln3p and allows Cln3p/Cdc28p kinase to hyperactivate transcription of the *CLN1* and *CLN2* genes in the presence of α-factor. This activation requires Swi4p and Swi6p (Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991). Cln3-1p efficiently bypasses transcriptional repression of the *CLN1* and *CLN2* genes, as shown by the high levels of *CLN1* and *CLN2* mRNAs in the *CLN3-1* strain in the presence of α-factor (Figure 4B, lanes 3 and 4) and the resulting α-factor resistance of this strain in a halo assay (Figure 5). Strikingly, however, Cln3-1p does not activate *CLN1* and *CLN2* to an obvious degree in the *STE11-4 far1CLN3-1* strain (Figure 4B, lanes 7 and 8). The absence of transcriptional activation of *CLN1* and *CLN2* correlates with greatly reduced α-factor resistance for this strain compared to the *CLN3-1* strain (Figure 5). Thus, Cln3-1p/Cdc28p is unable to activate the *CLN1*, *CLN2* promoters in the *STE11-4 far1* strain.

One interpretation of this finding is that *STE11-4* inhibits *CLN3-1*, either transcriptionally or post-transcriptionally. Northern analysis demonstrates that the *CLN3* gene is properly upregulated by α-factor in *STE11-4* strains (Figure 4A). Immunoblot analysis shows that the steady state levels of epitope-tagged Cln3p are the same in wild-type as in *STE11-4 far1* strains (data not shown). Two additional observations suggest that the Cln3-1 protein is still functional in the *STE11-4 far1* strain. First, *CLN3-1* does confer some α-factor resistance to the *STE11-4 far1* strain (as shown by the slightly more turbid and smaller diameter halo, Figure 5), consistent with the fact that *CLN3-1* can weakly substitute for *CLN1* and *CLN2* for passage through Start (Cross 1995). Second, *CLN3-1* still promotes budding in the *STE11-4 far1* strain, under the same α-factor conditions that prevent *CLN3-1* from activating the *CLN1*/*CLN2* genes (Figure 4B legend). Collectively, these data argue that Cln3-1p/Cdc28p complexes are selectively blocked for transcriptional activation at the G1/S cyclin promoters.

Fus3p and Kss1p may also promote proliferation in addition to G1 arrest: The results presented thus far show that Fus3p and Kss1p play positive roles in the regulation of Far1-independent G1 arrest. Therefore, we would predict that *STE11-4 far1 fus3* and *STE11-4 far1 kss1* strains should be less sensitive than a *STE11-4 far1* strain in a halo assay because of the elevated levels of the G1/S cyclins. Surprisingly, however, deletion of either *FUS3* or *KSS1* in the *STE11-4 far1* strain causes enhanced α-factor sensitivity in a halo assay (Figure 6), although deletion of both genes causes α-factor resistance (Figure 1B). A trivial explanation of slower growth rate for the more sensitive strains does not account for the increase in α-factor sensitivity, because long-term incubation of the plates does not result in smaller halos. Alternatively, the greater sensitivity might be due to hyperinduction of the Ca^{2+}-dependent pathway (Moser *et al.* 1996) that mediates α-factor-induced cell death (Iida *et al.* 1990). However, viability counts of the *STE11-4 far1 fus3* and *STE11-4 far1 kss1* strains exposed to 100 nm α-factor for 18 hr indicate that the α-factor-induced sensitivity is not due to decreased viability and that the *fus3* null mutation decreases the percentage of α-factor-induced cell death that occurs in the presence of

TABLE 3

Fus3 promotes proliferation through a Cln2/Cdc28-independent mechanism

Strain	α-Factor sensitivity[a]	% Wild-type G1 Arrest[b]	Fold inhibition Cln2/Cdc28[c]	% Wild-type *FUS1-LACZ*[d]
WT	++++	100	26	100
*kss1*Δ	+++++	100	ND	128
*fus3*Δ	++	43	0.93	46[e]
*fus3*Δ *kss1*Δ	−	0	0	<0.1[e]
*far1*Δ	−	5	1.6	36
*fus3*Δ *far1*Δ	+	23	1.1	ND
*kss1*Δ *far1*Δ	+/−	2	ND	ND
*STE11-4 far1*Δ	++	49	20	132
*STE11-4 far1*Δ *fus3*Δ	++++	57	2.2	56
*STE11-4 far1*Δ *kss1*Δ	++++	51	6.8	151

ND, not determined.
[a] Based on halo assays in Figures 1 and 6 and data not shown.
[b] Based on ability to arrest budding; data in Table 2.
[c] Based on data in Figure 3.
[d] Based on data in Figure 2.
[e] From Elion *et al.* (1991a) and Farley *et al.* (1999).

α-factor (data not shown). One interpretation of these observations is that the halo assay represents the net sum of both G1 arrest and proliferative functions, and that Fus3p and Kss1p also promote proliferation in addition to G1 arrest.

Comparative phenotypic analysis of additional *fus3* and *kss1* strains (summarized in Table 3) suggests that Fus3p and Kss1p may counteract G1 arrest through distinct functions. First, a *fus3* null mutant undergoes significantly more budding arrest than does a *far1* mutant, despite slightly higher levels of Cln2p/Cdc28p kinase (Figure 3C) of equivalently high specific activity. Furthermore, a *fus3* null mutation causes enhanced budding arrest in the background of a *far1* null, as shown by the greater partial budding arrest in a *fus3 far1* double mutant compared to a *far1* single mutant. Again, the greater budding arrest occurs despite high levels of active Cln2p/Cdc28p kinase (Figure 3C). Assessment of pathway activity using the *FUS1-lacZ* reporter gene shows that *fus3* and *STE11-4 far1 fus3* strains have reduced Ste12p activity compared to wild-type and *STE11-4 far1* control strains (Figure 2; Elion *et al.* 1991a). These observations suggest that Fus3p regulates proliferation through a function that is not linked to increased G1 cyclin levels or reduced Ste12p activity.

Kss1p appears to counteract G1 arrest by a distinct mechanism. Although *kss1* and *STE11-4 far1 kss1* strains also have enhanced α-factor senstivity (Table 3), the enhanced sensitivity correlates with slower recovery, as shown by a delay in resumption of cell division upon α-factor withdrawal in a *STE11-4 far1 kss1* strain compared to the *STE11-4 far1* strain (Table 4). The delay in recovery correlates with increased pathway activity, as shown by modestly enhanced expression of the *FUS1-lacZ* reporter gene (Table 3; also see Figure 2), increased *PCL2* mRNA levels (Figure 4A), and decreased Cln2p levels (Figure 3A). (The *STE11-4 far1 kss1* strain has less Cln2p compared to the *STE11-4 far1 fus3* strain, despite similar levels of *CLN2* mRNA.) Thus, Kss1p may promote proliferation during recovery through downregulation of the pathway.

Overexpression of *MCM1* suppresses Far1p-independent arrest: We attempted to determine whether Swi4p, Swi6p, and Mcm1p transcription factors that control the G1 to S transition might constitute direct or indirect targets of either Fus3p or Kss1p by testing their ability to confer α-factor resistance to the *STE11-4 far1* strain when overexpressed. Putative targets that might be expected to confer α-factor resistance in this test either

TABLE 4

Part of the pheromone sensitivity of *STE11-4 far1*Δ strains is due to a recovery defect

	Unbudded cells (%)				
	α-Factor		Recovery		
Strain	−	+	1 hr	2 hr	3 hr
WT	41	100	87	17	43
*pho85*Δ	48	98	97	88	48
*STE11-4 far1*Δ	46	83	63	43	41
*STE11-4 far1*Δ *kss1*Δ	28	66	57	52	45
*STE11-4far1*Δ *fus3*Δ	37	73	56	36	17

Logarithmically growing cells were treated with 100 nm α-factor for 4 hr. Cells were washed to remove the α-factor, and then resuspended in fresh media at an A_{600} = 0.5 and allowed to recover. Cells were fixed at the indicated times, stained with DAPI, and then scored to determine the number of unbudded cells. The strains are the same as in Figure 4. *pho85*Δ is CY130.

could be inhibited by Fus3p and Kss1p to promote G1 arrest (such as regulators of *CLN1*, *CLN2*, *CLB5*) or activated to promote recovery (such as regulators of *CLN3*, *PCL2*). Swi4p and Swi6p positively regulate *CLN1*, *CLN2*, *CLB5*, and *PCL2* in addition to other genes (Koch and Nasmyth 1994). Mcm1p is a MADS box regulator of *CLN3*, *SWI4*, and genes involved in DNA synthesis (McInerny *et al.* 1997), cell wall biosynthesis and metabolism (Kuo and Grayhack 1994), the G2/M transition (Althoefer *et al.* 1995), and mating (Elble and Tye 1991; Oehlen *et al.* 1996; Kuo *et al.* 1997). Overexpression of Swi4p and Swi6p, as well as a truncated form of Swi4p (which confers slightly more α-factor resistance than full-length Swi4p in a wild-type strain, L. Breeden personal communication), does not have an effect on the α-factor sensitivity of a *STE11-4 far1* strain (data not shown). In addition, co-overexpression of Swi4p and Swi6p (both under the control of the *GAL1* promoter), even in the presence of *CLN3-1*, does not bypass the arrest of a *STE11-4 far1* strain (data not shown).

By contrast, overexpression of *MCM1* confers significant α-factor resistance to both wild-type and *STE11-4 far1* strains (Figure 6). Mcm1p is unlikely to cause α-factor resistance through inappropriate activation of α-specific genes, because excess Mcm1p does not induce expression of α-factor or inhibit mating in these strains (data not shown). Mcm1p is also unlikely to promote cell division solely through upregulation of *CLN3* and *SWI4*, because overexpression of *SWI4* in the presence of *CLN3-1* has no effect in the *STE11-4 far1* strain and Mcm1p still bypasses G1 arrest in a *cln3*Δ strain (data not shown). In addition, Mcm1p is not bypassing Far1p-independent arrest through overexpression of the G2 cyclins (*i.e.*, *CLB2*; Siegmund and Nasmyth 1996), because a *GAL1-CLB2* gene does not bypass the arrest of either wild-type or *STE11-4 far1* strains (data not shown). These observations suggest that Fus3p and Kss1p may regulate G1 arrest through Mcm1p or genes, in addition to *SWI4* and *CLN3*, that are under Mcm1p control.

Mcm1p requires Fus3p but not Kss1p to counteract G1 arrest: We tested whether Mcm1p can suppress the arrest of *STE11-4 far1 fus3* and *STE11-4 far1 kss1* strains to determine whether Mcm1p strictly requires either Fus3p or Kss1p to promote cell division in the presence of α-factor. Strikingly, excess Mcm1p fails to bypass the α-factor arrest of the *STE11-4 far1 fus3* strain (Figure 6). By contrast, excess Mcm1p efficiently suppresses the α-factor arrest of the *STE11-4 far1 kss1* strain (Figure 6), in addition to that of *kss1*, *hog1*, and *mpk1* deletion strains (data not shown). Thus, Mcm1p specifically requires Fus3p to promote cell division. These findings argue compellingly for a physiologically relevant role for Mcm1p in regulating proliferation in the presence of α-factor. In addition, they argue that Fus3p is required for Mcm1p to promote proliferation, and that this function is not shared by Kss1p.

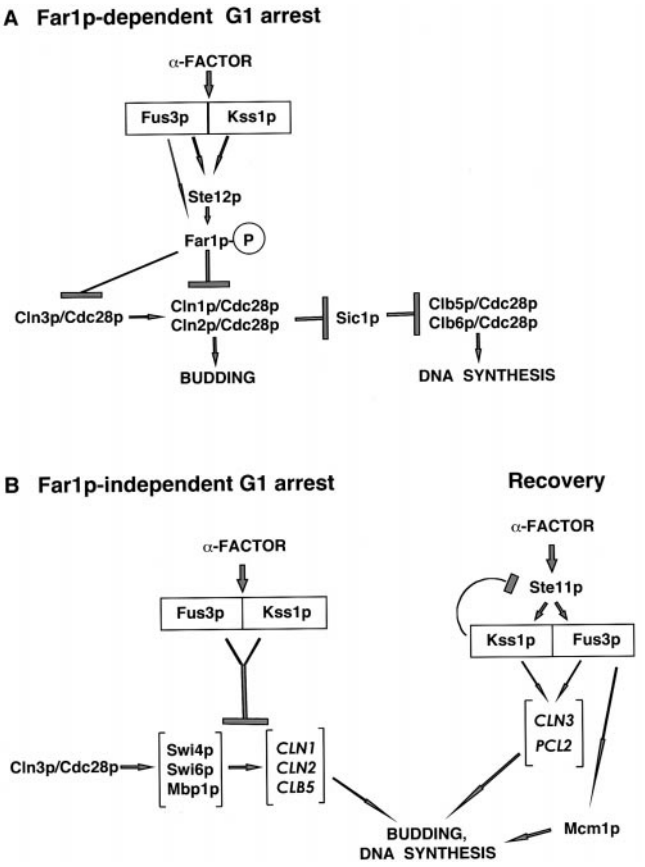

Figure 7.—Summary of levels of control of G1 arrest and proliferation by Fus3p and Kss1p.

DISCUSSION

Fus3p and Kss1p promote G1 arrest independently of Far1p: To analyze the contribution of Fus3p and Kss1p to G1 arrest that is independent of Far1p, we devised *far1* strains with intact G1/S cyclins that undergo G1 arrest as a result of hyperactivation of the mating pathway. Our analysis suggests that Fus3p and Kss1p promote pheromone-induced G1 arrest in at least two ways: through activation of Far1p-dependent inhibition of three G1-cyclin-dependent kinases (Figure 7A), and through Far1p-independent repression of transcription of at least three G1/S cyclin genes (Figure 7B). Whereas Fus3p and Kss1p contribute equally to pheromone-dependent activation of the *FAR1* gene (Farley *et al.* 1999), the vast majority of the control of Far1p is through Fus3p. Fus3p is as essential as Far1p in inhibiting the specific activity of Cln2p/Cdc28p kinase, although Kss1p may weakly inhibit Cln2p/Cdc28p (Figure 3A). Fus3p and Kss1p together repress transcription of the G1/S cyclin genes, although Fus3p has a greater role (Figure 7B). Transcriptional repression is likely to account for the majority of the inhibition of Cln1p/Cdc28p, Cln2p/Cdc28p, and Clb5p/Cdc28p in a wild-type strain, since overexpression of *CLN2* or *CLB5* is sufficient to override Far1p-independent G1 arrest. This

view is consistent with the rapid drop in *CLN1*,*CLN2*, and *CLB5* mRNAs that is induced by α-factor in a wild-type strain (Figure 4; Wittenberg *et al.* 1990) and the greater inhibitory effect of Far1p on Cln3p/Cdc28p than on Cln2p/Cdc28p (Gartner *et al.* 1998; Jeoung *et al.* 1998).

We demonstrate for the first time that Kss1p has a role in regulating pheromone-induced G1 arrest that is distinct from transcriptional control of the *FAR1* gene. The detection of a clear effect of a *kss1* null mutation on G1 arrest necessitated conditions that allowed the evaluation of Far1p-independent arrest in the presence of the G1/S cyclins, presumably because Kss1p functions redundantly with Fus3p and Far1p. The existence of Kss1p-dependent regulatory events that control G1 arrest is supported by the α-factor resistance of a *kss1K42R* mutant (Figure 1C). Our findings argue that Kss1p is required for efficient G1 arrest in a wild-type strain, although Fus3p plays a much greater role, as in mating (Madhani *et al.* 1997; Farley *et al.* 1999).

Fus3p and Kss1p block the ability of Cln3p/Cdc28p to activate Swi4p/Swi6p: How might Fus3p and Kss1p repress the expression of *CLN1*, *CLN2*, and *CLB5*? Cln3p/Cdc28p is a potent activator of transcription of *CLN1* and *CLN2* (Stuart and Wittenberg 1995), making it a potential target of negative control. The fact that hyperactive Cln3-1p/Cdc28p is unable to stimulate expression of *CLN1* and *CLN2* in a *STE11-4 far1* strain in the presence of α-factor, although it does so in a wild-type strain (Figure 4), argues strongly that Fus3p and Kss1p block the function of Cln3p/Cdc28p at the G1 cyclin promoters. However, this inhibition may be indirect, because the majority of α-factor-induced inhibition of Cln3p/Cdc28p is from Far1p (Jeoung *et al.* 1998), *STE11-4* does not lower Cln3p levels (data not shown), and Cln3-1p is still active in the *STE11-4 far1* strain (Figure 4B legend). Thus, Fus3p and Kss1p may inhibit another component of the transcription apparatus or activate a repressor that blocks the expression of the G1/S cyclins. Overexpression of *SWI4* and *SWI6*, either alone or in combination, does not confer α-factor resistance to wild-type or *STE11-4 far1* cells, arguing against the simplest view that Swi4p or Swi6p is a rate-limiting target of the MAP kinases. To date, Dig1p/Rst1p and Dig2p/Rst2p are the only known repressors in the mating pathway (Tedford *et al.* 1997). However, Dig1p/Rst1p and Dig2p/Rst2p may have a function that pertains to the G1 cyclins in addition to Ste12p on the basis of two hybrid interactions with Cln1p and Cln2p (Cook *et al.* 1997; Tedford *et al.* 1997). Alternatively, Fus3p and Kss1p may upregulate a Ste12p-dependent repressor gene. This possibility is consistent with the observation that a *GAL1-STE12* gene induces cells to accumulate in G1 phase in W303a (Dolan 1996), although excess Ste12p does not produce G1 arrest in a W303a *far1* strain (Satterberg 1993).

Fus3p and Kss1p may promote proliferation: Our analysis suggests that the mating MAP kinases also counteract G1 arrest through overlapping and distinct functions. Fus3p and Kss1p together enhance expression of the *CLN3* and *PCL2* genes in the presence of α-factor. This activation is likely to promote recovery, because *cln3* and *pho85* null mutations delay recovery (Table 4; Nash *et al.* 1988). The *PCL2* gene has a pheromone response consensus sequence (TGAAACA) upstream of the ATG, so its activation may occur through Ste12p, as is the case for genes that downregulate the pathway (*i.e.*, *GPA1*, *SST2*, and *MSG5*).

Fus3p may also promote proliferation by a mechanism that does not involve upregulation of Ste12p or the Cdc28p machinery (Figure 7B). A *fus3* null undergoes significant α-factor-dependent inhibition of budding and DNA synthesis, despite reduced Ste12p activity and elevated levels of *CLN1*, *CLN2* mRNAs (Figure 4A), and Cln2p/Cdc28p kinase. These *fus3* null phenotypes are also detected in *far1* and *STE11-4 far1* strains (Table 3), consistent with the loss of a proliferative function. Fus3p may attenuate the pathway activity through its function as a kinase (Gartner *et al.* 1992; Kranz *et al.* 1994; Errede and Ge 1996) or through functions not dependent upon catalytic activity (Farley *et al.* 1999). Fus3p promotes cell division through a function that is not shared by Kss1p, on the basis of the dependency of Mcm1p for Fus3p but not Kss1p. Fus3p is required for vegetative growth (Elion *et al.* 1991a), raising the possibility that these proliferative functions are related.

By contrast, Kss1p appears to promote recovery by inhibiting the activity of the pathway, possibly near the Ste11p step (Figures 4 and 7B). Kss1p also appears to positively regulate the abundance of Cln2p (Figure 3A). Kss1p could inhibit the pathway and increase the level of Cln2p indirectly through its ability to act as a repressor of Ste12p when catalytically inactive (Madhani *et al.* 1997), or because of a distinct function(s) that requires kinase activity.

Fus3p may activate Mcm1p or genes under its control: Fus3p may promote proliferation through the MADS box transcription factor Mcm1p or gene products under its control. Support for this comes from the observation that Mcm1p specifically requires Fus3p, but not Kss1p, Hog1p, or Mpk1p to suppress Far1p-independent G1 arrest. Genetic tests argue that Mcm1p does not promote proliferation solely through upregulation of the *CLN3* and *SWI4* genes, implying that additional Mcm1p-dependent genes are required (such as cell wall and DNA synthesis genes (Kuo and Grayhack 1994; Kuo *et al.* 1997; McInerny *et al.* 1997). Further work is needed to determine which genes must be upregulated by Mcm1p and whether Fus3p regulates Mcm1p or gene products under its control. Mcm1p might be directly phosphorylated by Fus3p in response to pheromone. Mcm1p is known to be phosphorylated in response to another extracellular stimulus, high salt (Kuo *et al.* 1997). Alternatively, Fus3p may activate Mcm1p indi-

rectly through the regulation of a protein required for Mcm1p function.

Are the G1/S cyclins the only targets of negative control? Our experiments reveal, quite surprisingly, that it is possible to inhibit DNA synthesis and budding under conditions of high Cln2p/Cdc28p kinase and not further inhibit DNA synthesis under conditions of reduced Cln2p/Cdc28p kinase. It is possible that conditions that uncouple the control of budding from G1 cyclin levels lead to activation of budding or DNA synthesis checkpoint apparati and cell cycle arrest (Sia *et al.* 1996; Weinert 1998). On the other hand, the absence of a strict correlation among Cln2p/Cdc28p levels, budding arrest, and inhibition of DNA synthesis may hint at the existence of auxiliary mechanisms that inhibit budding and DNA synthesis in a wild-type cell and indicate that Fus3p and Kss1p have additional targets of negative control. This possibility is consistent with the existence of three recessive mutations that confer α-factor resistance to a *far1 cln2* strain without a clear effect on the G1 cyclins or Ste12p activity, *far3* (Horecka and Sprague 1996), *par2*, and *par3* (V. Cherkasova and E. A. Elion, unpublished data). These mutations could either block G1 arrest or promote recovery.

Proper control of Ste11p may be critical for G1 arrest: How does a cell arrest in the presence of α-factor if Fus3p and Kss1p have both cell division arrest and proliferative functions? Our analysis of Far1p-independent arrest suggests that proper regulation of Ste11p may be central to determining whether a cell arrests or divides. First, the ability to restore G1 arrest to a *far1* mutant is specific to Ste5p and Ste11-4p. Overproduction of other rate-limiting components such as Ste4p (Gβ), Ste20p, Bem1p, and Ste12p does not restore G1 arrest to a *far1* mutant (Lyons *et al.* 1996; data not shown). Ste5p and the *STE11-4* mutation both overcome the basal inhibitory state of Ste11p (Stevenson *et al.* 1992; Choi *et al.* 1994), suggesting that an override of negative control of Ste11p is required to induce Far1p-independent arrest. Such an override may circumvent the inhibitory effects of high levels of Cln2p/Cdc28p kinase that accumulate in a *far1* null and that inhibit the activity of Fus3p at the Ste20p/Ste11p step and promote recovery (Wassman and Ammerer 1997; Leza and Elion 1999). It is interesting to speculate that the relative levels of active Fus3p and Kss1p determine whether a cell arrests or divides. For example, a latent increase in the level of activity of proliferative pathways that operate parallel to Cdc28p (Espinoza *et al.* 1994; Measday *et al.* 1994; Cvrckova *et al.* 1995; Igual *et al.* 1996; Zarzov *et al.* 1996; Gray *et al.* 1997) might promote cell division under conditions of low Clnp/Cdc28p and Clbp/Cdc28 kinase. Previous work shows that the strength of signaling through MAP kinases can be a determining factor in differentiation. In PC12 cells, low levels of activation of Erk2p by epithelial growth factor (EGF) result in proliferation, while high levels of activation by nerve growth factor (NGF) result in terminal differentiation into neurons (Marshall 1995).

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LITERATURE CITED

Althoefer, H., A. Schleiffer, K. Wassmann, A. Nordheim and G. Ammerer, 1995 Mcm1 is required to coordinate G2-specific transcription in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **15:** 5917–5928.

Breeden, L., 1996 Start-specific transcription in yeast. Curr. Top. Microbiol. Immunol. **208:** 95–127.

Chang, F., and I. Herskowitz, 1990 Identification of a gene necessary for cell cycle arrest by a negative growth factor of yeast: *FAR1* is an inhibitor of a G1 cyclin, *CLN2*. Cell **63:** 999–1011.

Choi, K. Y., B. Satterberg, D. Lyons and E. A. Elion, 1994 Ste5 tethers multiple protein kinases in the MAP kinase cascade required for mating in *S. cerevisiae*. Cell **78:** 499–512.

Ciejek, E., and J. Thorner, 1979 Recovery of *S. cerevisiae* **a** cells from G1 arrest by α-factor pheromone requires endopeptidase action. Cell **18:** 623–635.

Collart, M. A., and S. Oliviero, 1992 Preparation of yeast RNA, pp. 13.11.1–13.12.4, in *Current Protocols in Molecular Biology*, Vol. 2, edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith and K. Struhl. Massachusetts General Hospital, Harvard Medical School, Boston.

Cook, J. G., L. Bardwell and J. Thorner, 1997 Inhibitory and activating functions for MAPK Kss1 in the *S. cerevisiae* filamentous-growth signalling pathway. Nature **390:** 85–88.

Courchesne, W. E., R. Kunisawa and J. Thorner, 1989 A putative protein kinase overcomes pheromone-induced arrest of cell cycling in *S. cerevisiae*. Cell **58:** 1107–1119.

Cross, F. R., 1988 DAF1, a mutant gene affecting size control, pheromone arrest and cell cycle kinetics of *S. cerevisiae*. Mol. Cell. Biol. **8:** 4675–4684.

Cross, F. R., 1995 Starting the cell cycle: what's the point? Curr. Opin. Cell Biol. **7:** 790–797.

Cross, F. R., and A. H. Tinkelenberg, 1991 A potential positive feedback loop controlling *CLN1* and *CLN2* gene expression at the start of the yeast cell cycle. Cell **65:** 875–883.

Cvrckova, F., C. De Virgilio, E. Manser, J. R. Pringle and K. Nasmyth, 1995 Ste20-like protein kinases are required for normal localization of cell growth and for cytokinesis in budding yeast. Genes Dev. **9:** 1817–1830.

Dirick, L., and K. Nasmyth, 1991 Positive feedback in the activation of G1 cyclins in yeast. Nature **351:** 754–757.

Dolan, J. W., 1996 Novel aspects of pheromone-induced cell-cycle arrest in yeast. Curr. Genet. **30:** 469–475.

Elble, R., and B. K. Tye, 1991 Both activation and repression of **a**-mating-type-specific genes in yeast require transcription factor Mcm1. Proc. Natl. Acad. Sci. USA **88:** 10966–10970.

Elion, E. A., 1995 Ste5p: a meeting place for MAP kinases and their associates. Trends Cell Biol. **5:** 322–327.

Elion, E. A., P. L. Grisafi and G. R. Fink, 1990 FUS3 encodes a cdc2/CDC28-related kinase required for the transition from mitosis into conjugation. Cell **60:** 649–664.

Elion, E. A., J. A. Brill and G. R. Fink, 1991a FUS3 represses CLN1 and CLN2 and in concert with KSS1 promotes signal transduction. Proc. Natl. Acad. Sci. USA **88:** 9392–9396.

Elion, E. A., J. A. Brill and G. R. Fink, 1991b Functional redundancy in the yeast cell cycle: FUS3 and KSS1 have both overlapping and unique functions. Cold Spring Harbor Symp. Quant. Biol. **LVI:** 41–49.

Elion, E. A., B. Satterberg and J. A. Kranz, 1993 FUS3 phosphorylates multiple components of the mating signal transduction cascade: evidence for STE12 and FAR1. Mol. Biol. Cell **4:** 495–510.

Elion, E. A., J. Trueheart and G. R. Fink, 1995 Fus2 localizes near the site of cell fusion and is required for both cell fusion and nuclear alignment during zygote formation. J. Cell Biol. **130:** 1283–1296.

Errede, B., and Q.-Y. Ge, 1996 Feedback regulation of MAP kinase signal pathways. Phil. Trans. R. Soc. Lond. B **131:** 143–149.

Espinoza, F. H., J. Ogas, I. Herskowitz and D. O. Morgan, 1994 Cell cycle control by a complex of the cyclin HCS26 (PCL1) and the kinase PHO85. Science **266:** 1388–1391.

Farley, F. W., B. Satterberg, E. J. Goldsmith and E. A. Elion, 1999 Relative dependence of different outputs of the *S. cerevisiae* pheromone response pathway on the MAP kinase Fus3p. Genetics (in press).

Field, J., J.-I. Nikawa, D. Broek, B. MacDonald, L. Rodgers *et al.*, 1988 Purification of a RAS-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. Mol. Cell. Biol. **8:** 2159–2165.

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.

Gartner, A., A. Jovanovic, D.-I. Jeoung, S. Bourlat, F. R. Cross *et al.*, 1998 Pheromone-dependent G1 cell cycle arrest requires Far1 phosphorylation, but may not involve inhibition of Cdc28-Cln2 kinase, *in vivo*. Mol. Cell. Biol. **18:** 3681–3691.

Gray, J. V., J. P. Ogas, Y. Kamada, M. Stone, D. E. Levin *et al.*, 1997 A role for the Pkc1 MAP kinase pathway of *Saccharomyces cerevisiae* in bud emergence and identification of a putative upstream regulator. EMBO J. **16:** 4924–4937.

Hadwiger, J. A., C. Wittenberg, H. E. Richardson, M. De Barros Lopes and S. I. Reed, 1989 A family of cyclin homologs that control the G1 phase in yeast. Proc. Natl. Acad. Sci. USA **86:** 6255–6259.

Hall, J. P., V. Cherkasova, E. Elion, M. C. Gustin and E. Winter, 1996 The osmoregulatory pathway represses mating pathway activity in *Saccharomyces cerevisiae*: isolation of a *fus3* mutant that is insensitive to the repression mechanism. Mol. Cell. Biol. **16:** 6715–6723.

Henchoz, S., Y. Chi, B. Catarin, I. Herskowitz, R. J. Deshaies *et al.*, 1997 Phosphorylation- and ubiquitin-dependent degradation of the cyclin-dependent kinase inhibitor Far1 in budding yeast. Genes Dev. **11:** 3046–3060.

Herskowitz, I., 1995 MAP kinase pathways in yeast: for mating and more. Cell **80:** 187–197.

Horecka, J., and G. F. Sprague, Jr., 1996 Identification and characterization of *FAR3*, a gene required for pheromone-mediated G1 arrest in *Saccharomyces cerevisiae*. Genetics **144:** 905–921.

Hutter, K. J., and H. E. Eipel, 1979 Microbial determinations by flow cytometry. J. Gen. Microbiol. **113m:** 369–375.

Igual, J. C., A. L. Johnson and L. H. Johnson, 1996 Coordinated regulation of gene expression by the cell cycle transcription factor SWI4 and the protein kinase C MAP kinase pathway for yeast cell integrity. EMBO J. **15:** 5001–5013.

Iida, H., Y. Yagawa and Y. Anraku, 1990 Essential role for induced Ca^{2+} influx followed by $[Ca^{2+}]$ rise in maintaining viability of yeast cells late in the mating pheromone response pathway: a study of $[Ca^{2+}]$ in single *Saccharomyces cerevisiae* cells with imaging of fura-2. J. Biol. Chem. **265:** 13391–13399.

Jeoung, D., L. J. W. M. Oehlen and F. R. Cross, 1998 Cln3-associated kinase activity in *Saccharomyces cerevisiae* is regulated by the mating factor pathway. Mol. Cell. Biol. **18:** 433–441.

Koch, C., and K. Nasmyth, 1994 Cell cycle regulated transcription in yeast. Curr. Opin. Cell Biol. **6:** 451–459.

Kranz, J. A., B. Satterberg and E. A. Elion, 1994 The MAP kinase Fus3 associates with and phosphorylates the upstream signaling component Ste5. Genes Dev. **8:** 313–327.

Kron, S., C. A. Styles and G. R. Fink, 1994 Symmetric cell division in pseudohyphae of the yeast *Saccharomyces cerevisiae*. Mol. Biol. Cell **5:** 1003–1022.

Kuo, M.-H., and E. J. Grayhack, 1994 A library of yeast genomic Mcm1 binding sites contains genes involved in cell cycle control, cell wall and membrane structure, and metabolism. Mol. Cell. Biol. **14:** 348–359.

Kuo, M.-H., E. T. Nadeau and E. J. Grayhack, 1997 Multiple phosphorylated forms of the *Saccharomyces cerevisiae* Mcm1 protein include an isoform induced in response to high salt concentrations. Mol. Cell. Biol. **17:** 819–832.

Leberer, E., C. D. Dignard, D. Harcus, L. Hougan, M. Whiteway *et al.*, 1993 Cloning of *Saccharomyces cerevisiae STE5* as a suppressor of a Ste20 protein kinase mutant: structural and functional similarity of Ste5 and Far1. Mol. Gen. Genet. **241:** 241–254.

Leza, A. M., and E. A. Elion, 1999 *POG1*, a novel yeast gene, promotes recovery from pheromone arrest via the G1 cyclin *CLN2*. Genetics **151:** 531–543.

Lyons, D. M., S. K. Mahanty, K.-Y. Choi, M. Manandhar and E. A. Elion, 1996 The SH3-domain protein Bem1 coordinates mitogen-activated protein kinase cascade activation with cell cycle control. Mol. Cell. Biol. **16:** 4095–4106.

Ma, D., J. G. Cook and J. Thorner, 1995 Phosphorylation and localization of Kss1, a MAP kinase of the *Saccharomyces cerevisiae* pheromone response pathway. Mol. Biol. Cell **6:** 889–909.

Madhani, H. D., C. A. Styles and G. R. Fink, 1997 MAP kinases with distinct inhibitory functions impart signaling specificity during yeast differentiation. Cell **91:** 673–684.

Marshall, C. J., 1995 Specificity of tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell **80:** 179–185.

McInerny, C. J., J. F. Partridge, G. E. Mikesell, D. P. Creemer and L. L. Breeden, 1997 A novel Mcm1-dependent element in the *SWI4*, *CLN3*, *CDC6*, and *CDC47* promoters activates M/G1-specific transcription. Genes Dev. **11:** 1277–1288.

McKinney, J. D., and F. R. Cross, 1995 FAR1 and the G1 phase specificity of cell cycle arrest by mating factor in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **15:** 2509–2516.

Measday, V., L. Moore, J. Ogas, M. Tyers and B. Andrews, 1994 The PCL2 (ORFD)-PHO85 complex: a cell cycle regulator in yeast. Science **266:** 1391–1395.

Mendenhall, M. D., 1993 An inhibitor of $p34^{CDC28}$ protein kinase activity from *Saccharomyces cerevisiae*. Science **259:** 216–219.

Moser, M. J., J. R. Geiser and T. N. Davis, 1996 Ca^{2+}-calmodulin promotes survival of pheromone-induced arrest by activation of calcineurin and Ca^{2+}-calmodulin-dependent protein kinase. Mol. Cell. Biol. **16:** 4824–4831.

Nash, R., G. Tokiwa, S. Anand, K. Erickson and A. B. Futcher, 1988 The *WHI1+* gene of *Saccharomyces cerevisiae* tethers cell division to cell size and is a cyclin homolog. EMBO J. **7:** 4335–4346.

Nasmyth, K., 1996 At the heart of the budding yeast cell cycle. Trends Genet. **12:** 405–412.

Oehlen, L. J., J. D. McKinney and F. R. Cross, 1996 Ste12 and Mcm1 regulate cell cycle-dependent transcription of FAR1. Mol. Cell. Biol. **16:** 2830–2837.

Ogas, J., B. J. Andrews and I. Herskowitz, 1991 Transcriptional activation of CLN1, CLN2, and a putative new G1 cyclin (HCS26) by SWI4, a positive regulator of G1-specific transcription. Cell **66:** 1015–1026.

Peter, M., and I. Herskowitz, 1994 Direct inhibition of the yeast cyclin-dependent kinase Cdc28-Cln by Far1. Science **262:** 566–569.

Peter, M., A. Gartner, J. Horecka, G. Ammerer and I. Herskowitz, 1993 FAR1 links the signal transduction pathway to the cell cycle machinery in yeast. Cell **73:** 747–760.

Reed, S. I., 1992 The role of p34 kinases in the G1 to S-phase transition. Annu. Rev. Cell Biol. **8:** 529–561.

Sambrook, J., E. F. Fritch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*, Ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Satterberg, B., 1993 G1 cell cycle arrest in *S. cerevisiae*. Ph.D. Thesis, Harvard Medical School, Boston.

Schneider, B. L., Q. H. Yang and A. B. Futcher, 1996 Linkage of replication to start by the Cdk inhibitor Sic1. Science **272:** 560–562.

Sherman, F., G. R. Fink and J. B. Hicks, 1986 *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sia, R. A., H. A. Herald and D. J. Lew, 1996 Cdc28 tyrosine phosphorylation and the morphogenesis checkpoint in budding yeast. Mol. Biol. Cell **7:** 1657–1666.

Siegmund, R. F., and K. A. Nasmyth, 1996 The *Saccharomyces cerevisiae* start-specific transcriptional factor Swi4 interacts through the ankyrin repeats with the mitotic Clb2/Cdc28 kinase and through

its conserved carboxy terminus with Swi6. Mol. Cell. Biol. **16:** 2647–2655.

Sprague, G. F., Jr., and J. W. Thorner, 1993 Pheromone response and signal transduction during the mating process of *Saccharomyces cerevisiae*, pp. 657–744, in *The Molecular and Cellular Biology of the Yeast* Saccharomyces, edited by E. Jones, J. Pringle and J. Broach. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Stevenson, B. J., N. Rhodes, B. Errede and G. F. Sprague, Jr., 1992 Constitutive mutants of the protein kinase *STE11* activate the yeast pheromone response pathway in the absence of the G protein. Genes Dev. **6:** 1293–1304.

Stuart, D., and C. Wittenberg, 1995 CLN3, not positive feedback, determines the timing of CLN2 transcription in cycling cells. Genes Dev. **9:** 2780–2794.

Surana, U., H. Robitsch, C. Price, T. Schuster, I. Fitch *et al.*, 1991 The role of Cdc28 and cyclins during mitosis in the budding yeast *S. cerevisiae.* Cell **65:** 145–161.

Tedford, K., S. Kim, D. Sa, K. Stevens and M. Tyers, 1997 Regulation of the mating pheromone and invasive growth responses in yeast by two MAP kinase substrates. Curr. Biol. **7:** 228–238.

Trueheart, J., J. D. Boeke and G.R. Fink, 1987 Two genes required for cell fusion during conjugation: evidence for a pheromone-induced surface protein. Mol. Cell. Biol. **7:** 2316–2328.

Tyers, M., 1996 The cyclin-dependent kinase inhibitor $p40^{SIC1}$ imposes the requirement for Cln G1 cyclin function at Start. Proc. Natl. Acad. Sci. USA **93:** 7772–7776.

Tyers, M., and B. Futcher, 1993 Far1 and Fus3 link the mating pheromone signal transduction pathway to three G1-phase Cdc28 kinase complexes. Mol. Cell. Biol. **13:** 5659–5669.

Tyers, M., G. Tokiwa and B. Futcher, 1993 Comparison of the Saccharomyces cerevisiae G1 cyclins: Cln3 may be an upstream activator of Cln1, Cln2, and other cyclins. EMBO J. **11:** 1773–1784.

Valdivieso, M. H., K. Sugimoto, K.-Y. Jahng, P. M. B. Fernandes and C. Wittenberg, 1993 *FAR1* is required for posttranscriptional regulation of CLN2 gene expression in response to mating pheromone. Mol. Cell. Biol. **13:** 1013–1022.

Wassman, K., and G. Ammerer, 1997 Overexpression of the G1-cyclin CLN2 represses the mating pathway in *Saccharomyces cerevisiae* at the level of the MEKK Ste11. J. Biol. Chem. **272:** 13180–13188.

Weinert, T., 1998 DNA damage and checkpoint pathways: molecular anatomy and interactions with repair. Cell **94:** 555–558.

Wittenberg, C., K. Sugimoto and S. I. Reed, 1990 G1-specific cyclins of *S. cerevisiae:* cell cycle periodicity, regulation by mating pheromone, and association with the p34CDC28 protein kinase. Cell **62:** 225–227.

Zarzov, P., C. Mazzoni and C. Mann, 1996 The SLT2 (MPK1) MAP kinase is activated during periods of polarized cell growth in yeast. EMBO J. **15:** 83–91.

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