Molecular Biology of the Cell
Vol. 10, 3301–3316, October 1999

Regulation of G2/M Progression by the STE Mitogen-activated Protein Kinase Pathway in Budding Yeast Filamentous Growth

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Submitted January 26, 1999; Accepted July 23, 1999
Monitoring Editor: Mark J. Solomon

Inoculation of diploid budding yeast onto nitrogen-poor agar media stimulates a MAPK pathway to promote filamentous growth. Characteristics of filamentous cells include a specific pattern of gene expression, elongated cell shape, polar budding pattern, persistent attachment to the mother cell, and a distinct cell cycle characterized by cell size control at G2/M. Although a requirement for MAPK signaling in filamentous gene expression is well established, the role of this pathway in the regulation of morphogenesis and the cell cycle remains obscure. We find that ectopic activation of the MAPK signal pathway induces a cell cycle shift to G2/M coordinately with other changes characteristic of filamentous growth. These effects are abrogated by overexpression of the yeast mitotic cyclins Clb1 and Clb2. In turn, yeast deficient for Clb2 or carrying *cdc28-1N*, an allele of CDK defective for mitotic functions, display enhanced filamentous differentiation and supersensitivity to the MAPK signal. Importantly, activation of Swe1-mediated inhibitory phosphorylation of Thr-18 and/or Tyr-19 of Cdc28 is not required for the MAPK pathway to affect the G2/M delay. Mutants expressing a nonphosphorylatable mutant Cdc28 or deficient for Swe1 exhibit low-nitrogen-dependent filamentous growth and are further induced by an ectopic MAPK signal. We infer that the MAPK pathway promotes filamentous growth by a novel mechanism that inhibits mitotic cyclin/CDK complexes and thereby modulates cell shape, budding pattern, and cell-cell connections.

INTRODUCTION

When diploid cells of dimorphic strains of the budding yeast *Saccharomyces cerevisiae* are inoculated onto agar media rich in a fermentable carbon source but poor in nitrogen, they can differentiate to enter filamentous growth. Previous studies (reviewed by Kron and Gow, 1995; Madhani and Fink, 1998a) have established that the filamentous cell represents a distinct cell type characterized by an elongated shape, a polar budding pattern, and persistent attachment of each daughter cell to the mother after septation. These features contribute to colony growth as branching filaments that invade the agar. Another characteristic of filamentous growth is that the cell division cycle is regulated by cell size control at mitosis (Kron *et al.*, 1994). Filamentous cells remain budded until the cell doubles in size. Throughout most of this prolonged budded period, the cell exhibits phenotypes suggestive of G2 arrest (Surana *et al.*, 1991), such as a short mitotic spindle, a single rounded nuclear mass, and an elongated, tubular bud. As a result of the long G2/M growth period, daughter cells are born larger than the critical size for progression to S phase at Start. Therefore, newly born daughter cells at the tip of filaments immediately bud and reenter the cell cycle. Thus, the G2/M cell cycle control likely contributes to the capacity of filamentous cells to continue mitotic growth even in the face of nitrogen limitation.

A comparison of the shape and cell division pattern of filamentous cells with the results of Lew and Reed (1993) regarding the effects of different cyclin/CDK complexes on cell morphogenesis suggests that the link between cell cycle and cell shape in filamentous growth may be direct. In the growth pattern of "well-fed" yeast (reviewed by Madden and Snyder, 1998), bud growth is initially polarized to the tip, leading to tube-like growth at S phase and into G2. During mitosis, bud growth becomes isotropic, leading to swelling growth. Finally, septation occurs upon completion of mitotic anaphase. Distinct cyclins combine with the Cdc28 CDK to mediate each of the nuclear transitions (reviewed by Mendenhall and Hodge, 1998). Altered expression of the cyclins or modulation of the activity of the cyclin/Cdc28 complexes has coordinate effects of delaying or accelerating

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both the nuclear transition and the related morphogenetic event (Lew and Reed, 1993). For example, increased expression of G1 cyclins or decreased expression of mitotic cyclins each lead to a delay before the onset of mitosis. This prolongs the period of tubular bud growth, leading to a "filamentous" appearance. It is reasonable to hypothesize that the cell cycle machinery may be a key target of the pathway regulating the morphogenetic events characteristic of filamentous differentiation. Recent data have shown that mutations in G1 cyclins can largely abrogate filamentous growth (Oehlen and Cross, 1998; Loeb *et al.*, 1999), whereas deficiency of the Clb2 mitotic cyclin or induction of inhibitory tyrosine phosphorylation of the principal CDK Cdc28 or a particular point mutation of Cdc28 (Edgington *et al.*, 1999) each significantly induces filamentous differentiation. What remains to be established is any specific link between the low-nitrogen signal and regulation of the cell cycle.

Previous studies of filamentous differentiation have established a branching signal transduction pathway involving elements of the RAS GTPase/cAMP-dependent kinase cascade and the STE MAPK pathway (reviewed by Banuett, 1998). The STE MAPK cascade and gene expression pathway (reviewed by Leberer *et al.*, 1997) is best known for its role in mediating yeast mating response. Pheromones induce a sequence of phosphorylations in the STE MAPK cascade, consisting of a PAK kinase homolog Ste20, a MEK kinase Ste11, a MEK Ste7, and the MAPKs Fus3 and Kss1. The downstream target is a transcriptional activator, Ste12, that binds to pheromone response elements in promoters of effector genes such as Far1 (Hagen *et al.*, 1991). Far1 promotes G1 arrest in pheromone-stimulated cells (Chang and Herskowitz, 1990), acting as a stoichiometric inhibitor that sequesters Cln1,2 G1 cyclin/Cdc28 complexes (Peter and Herskowitz, 1994).

Filamentous differentiation requires only a subset of the STE pathway genes required for mating responses (Madhani and Fink, 1998b). Mutations in the Ste20, Ste11, or Ste7 kinases or the Ste12 transcription factor abrogate filamentous differentiation (Liu *et al.*, 1993; Roberts and Fink, 1994). The MAPK Kss1 serves a dual role in filamentous differentiation as both inhibitor and activator of Ste12-dependent gene expression (Cook *et al.*, 1997; Madhani *et al.*, 1997). Ectopic activation of the STE pathway by dominant active alleles of *STE11* (Liu *et al.*, 1993), *STE7* (Madhani *et al.*, 1997), and *KSS1* (Madhani *et al.*, 1997) or by overexpression of *STE12* (Liu *et al.*, 1993) all promote enhanced filamentous growth and can partially bypass upstream blockades in the pathway.

Ectopic activation of RAS/cAMP signaling can also promote filamentous growth. The dominant active allele *RAS2*-Val19 markedly enhances filamentous growth in nitrogen-starved cells (Gimeno *et al.*, 1992; Mosch *et al.*, 1996) via activation of both cAMP-dependent and MAPK responses (Mosch *et al.*, 1996, 1999). In turn, activation of the STE pathway alone by dominant active alleles such as *STE11-4* (Stevenson *et al.*, 1992; Liu *et al.*, 1993) apparently enhances filamentous growth by cAMP-independent mechanisms. Recent data suggest that RAS signaling induces its cAMP-mediated effects and activates the STE MAPK cascade independently (Mosch *et al.*, 1999; Pan and Heitman, 1999) but that the signals transmitted via these parallel pathways converge on similar targets (Robertson and Fink, 1998; Pan and Heitman, 1999; Rupp *et al.*, 1999).

Thus, elements of the RAS/cAMP and STE MAPK pathways constitute a single "RAS/STE" filamentous response pathway. This compound signaling pathway mediates its effects predominantly via regulation of gene expression. RAS/STE signals induce cell type–specific transcription (Mosch *et al.*, 1996) via a distinct UAS, the filamentous response element (FRE) (Madhani and Fink, 1997), that consists of adjacent Ste12 and Tec1 binding sites. Cells lacking either factor lack FRE-dependent gene expression and are defective in filamentous differentiation (Gavrias *et al.*, 1996; Lo *et al.*, 1997; Madhani and Fink, 1997; Mosch and Fink, 1997). To date, only one effector essential for filamentous differentiation, the *MUC1/FLO11* flocculin (Lambrechts *et al.*, 1996; Lo and Dranginis, 1998), has been shown to be expressed under RAS/STE control via a FRE (Lo and Dranginis, 1998; Rupp *et al.*, 1999). Cells deficient in Flo11 do not remain linked in chains and form only disorganized colonies that fail to invade the substratum. However, these cells remain responsive to RAS/STE signals, retaining the elongated cell shape, polar budding, and G2/M delay characteristic of filamentous cells (Kron and Dranginis, unpublished results). As yet, the genes that mediate these aspects of filamentous development remain to be described.

Potential links between the RAS/STE signal transduction pathway and the cell cycle machinery in the control of filamentous differentiation remain poorly defined. This is surprising, considering the well-established roles for cyclins and CDK in determining yeast cell shape (Lew *et al.*, 1997; Madden and Snyder, 1998; Mendenhall and Hodge, 1998). In pheromone response, ectopic activation of the Cln G1 cyclins can antagonize both gene expression (Oehlen and Cross, 1994) and cell cycle arrest (Edwards *et al.*, 1997; Kron, unpublished results). Recent data have suggested that Cln2 cyclin complexes with Cdc28 CDK mediate this effect by regulation of Ste20 (Oehlen and Cross, 1998; Wu *et al.*, 1998; Leza and Elion, 1999) and/or regulation of Ste11 (Wassmann and Ammerer, 1997). Importantly, Cln1,2/Cdc28 phosphorylation of Ste20 has been proposed to underlie the requirement for Cln G1 cyclins in filamentous differentiation (Oehlen and Cross, 1998). A pathway linking Clb1 and Clb2 mitotic cyclins to morphogenesis via Cdc28 phosphorylation of a Ste20 homolog, Cla4, has been demonstrated (Tjandra *et al.*, 1998), but any relationship to signal transduction remains uncharacterized. A broad interpretation of these studies would place the cell cycle machinery as a regulator acting upstream in the RAS/STE signaling pathway.

An alternative hypothesis based on the role of the CDK inhibitor Far1 in pheromone-induced cell cycle arrest is that the cell cycle effect of RAS/STE signaling may be mediated by a FRE-regulated effector, such as a mitotic inhibitor. Such a factor might inhibit Clb1- and Clb2-dependent forms of the Cdc28 CDK and function to delay both depolarization of the bud and completion of mitosis. To test this hypothesis, we set out to examine the relationship between elements of the cell cycle machinery and the signaling pathways modulating filamentous growth. We observed that 1) ectopic activation of the RAS/STE pathway slows mitotic progression, and 2) modulation of mitotic cyclin expression dramatically affects filamentous differentiation. Cells deficient in mitotic cyclins are activated for filamentous growth, whereas cells overex-

Table 1. Yeast strain list

Strain	Genotype	Source
L5683	*MATα ura3–52*	G.R. Fink
SKY754	*MAT*a *ura3–52*	This work
SKY757	*MAT*a/*MATα ura3–52/ura3–52*	This work
L5791	*MAT*a/*MATα ura3–52/ura3–52 leu2*::hisg/*leu2*::hisg	G.R. Fink
L5624	*MAT*a/*MATα ura3–52/ura3–52 trp1*::hisg/*trp1*::hisg *ste20::TRP1/ste20::TRP1*	G.R. Fink
L5535	*MAT*a/*MATα ura3–52/ura3–52 leu2*::hisg/*leu2*::hisg *ste7::LEU2/ste7::LEU2*	G.R. Fink
L5540	*MAT*a/*MATα ura3–52/ura3–52 leu2*::hisg/*leu2*::hisg *ste11::LEU2/ste11::LEU2*	G.R. Fink
L5537	*MAT*a/*MATα ura3–52/ura3–52 leu2*::hisg/*leu2*::hisg *ste12::LEU2/ste12::LEU2*	G.R. Fink
SKY2183	*MAT*a/*MATα ura3–52/ura3–52 leu2*::hisg/*leu2*::hisg *STE11::STE11–4::LEU2/STE11*	This work
SKY1056	*MAT*a/*MATα ura3–52/ura3–52 CDC28*–T18A, Y19F/*CDC28*–T18A, Y19F	This work
SKY1057	*MAT*a/*MATα ura3–52/ura3–52 leu2*::hisg/*leu2*::hisg *mih1::LEU2/mih1::LEU2*	This work
SKY1058	*MAT*a/*MATα ura3–52/ura3–52 leu2*::hisg/*leu2*::hisg *swe1::LEU2/swe1::LEU2*	This work
SKY776	*MAT*a/*MATα ura3–52/ura3–52 trp1*::hisg/*trp1*::hisg *clb1::TRP1/clb1::TRP1*	This work
SKY778	*MAT*a/*MATα ura3–52/ura3–52 leu2*::hisg/*leu2*::hisg *clb2::LEU2/clb2::LEU2*	This work
SKY2187	*MAT*a/*MATα ura3–52/ura3–52 cdc28–1N/cdc28–1N*	This work
SKY2184	*MAT*a/*MATα ura3–52/ura3–52 leu2*::hisg::*GAL1–CLB1::LEU2/leu2*::hisg	This work
SKY2185	*MAT*a/*MATα ura3–52/ura3–52 leu2*::hisg::*GAL1–CLB2::LEU2/leu2*::hisg	This work
SKY2186	*MAT*a/*MATα ura3–52/ura3–52 leu2*::hisg/*leu2*::hisg *ste7::LEU2/ste7::LEU2 clb2::LEU2/clb2::LEU2*	This work
SKY2188	*MAT*a/*MATα ura3–52/ura3–52 trp1*::hisg/*trp1*::hisg *leu2*::hisg/*leu2*::hisg *ste20::TRP1/ste20::TRP1 clb2::LEU2/clb2::LEU2*	This work
SKY2189	*MAT*a/*MATα ura3–52/ura3–52 leu2*::hisg/*leu2*::hisg *ste12::LEU2/ste12::LEU2 clb2::LEU2/clb2::LEU2*	This work
SKY2225	*MAT*a/*MATα ura3–52/ura3–52 leu2*::hisg/*leu2*::hisg *STE11::STE11–4::LEU2/STE11::STE11–4 CDC28*–T18A, Y19F/*CDC28*–T18A, Y19F	This work
SKY2226	*MAT*a/*MATα ura3–52/ura3–52 leu2*::hisg/*leu2*::hisg *STE11::STE11–4::LEU2/STE11::STE11–4*	This work

All strains are Σ1287b (Grenson *et al.*, 1966) derivatives.

pressing mitotic cyclins cannot form filamentous colonies, even in the presence of an inducing signal. Epistasis analysis places the activity of the cyclins downstream of the RAS/STE signaling pathway. Our results establish a pathway for filamentous differentiation in which the cell cycle engine is a critical target of the RAS/STE pathway involved in promoting the elongated cell shape, polarized cell division, and invasive growth characteristic of the differentiated state.

MATERIALS AND METHODS

Yeast Media

Media were prepared as previously described (Kron *et al.*, 1994) with yeast extract, peptone, and yeast nitrogen base from United States Biochemical (Cleveland, OH). Other reagents were obtained from Fisher Scientific (Pittsburgh, PA) and Sigma (St. Louis, MO). All low-nitrogen media contained 50 μM ammonium sulfate and 6.8 g/l yeast nitrogen base without amino acids or ammonium sulfate and were filter sterilized through a 0.45-μm filter. Synthetic low-ammonia dextrose (SLAD) medium also contained 2% dextrose. Agar (bacteriological, United States Biochemical) was prepared by autoclaving at 4% wt/vol in deionized water and diluting to 2% final concentration with 2× liquid media. Low-ammonia agar media were made using agar washed four times in deionized water before sterilization.

Yeast Strains and Plasmids

All yeast strains were derived in the Σ1278b background (Grenson *et al.*, 1966; Gimeno *et al.*, 1992) using conventional genetic methods. Marker segregation, PCR assay, and/or Southern blotting determined genotypes. Yeast strains are described in Table 1. Transformations were performed by a lithium acetate procedure based on that of Schiestl and Gietz (1989). Plasmids are described in Table 2.

To derive strains that stably express *STE11-4* from the *STE11* chromosomal locus, a 3.6-kilobase *Xba*I fragment from pSL1509 (yCP50-*STE11-4*) (Stevenson *et al.*, 1992) was subcloned into pRS30 (Sikorski and Hieter, 1989) to construct SKB4166, an integrating plasmid carrying the *STE11-4* gene and a *LEU2* marker. A diploid LEU$^-$ strain, L5791, was transformed with SKB4166 guided to one *STE11* locus by digestion at a unique *Nco*I site in the *STE11* ORF. We chose a transformant, SKY2183, that exhibited slow growth in rich media and striking filamentous growth on SLAD plus 1 mM uracil. When transformed with the Ty1-*lacZ* reporter pIL30 (Laloux *et al.*, 1994; Mosch *et al.*, 1996), a high constitutive level of expression was observed. Sporulation of SKY2183 yielded 2:0 LEU$^+$ segregants that when crossed to wild-type haploids dominantly conferred enhanced filamentous growth to the resulting diploids.

A *URA3*-marked centromeric plasmid carrying the dominant *CDC28*-T18A, Y19F allele, SKB4167, was constructed by subcloning a 2.3-kilobase *Xho*I/*Pvu*II fragment from p3303 (Ycp*lac*22 *CDC28*-T18A, Y19F) (Amon *et al.*, 1992) into *Xho*I/*Sma*I-digested pRS316 (Sikorski and Hieter, 1989). To construct a double mutant diploid carrying both *STE11-4* and *CDC28*-T18A, Y19F alleles on both pairs of chromosomes, SKY2183 and SKY1056 were sporulated and dissected. Segregants were then mated and the heterozygous diploid was then sporulated and dissected to yield double mutant *STE11-4*, *CDC28*-T18A, Y19F haploid segregants. These were then mated to each other to create the *STE11-4*/*STE11-4*, *CDC28*-T18A, Y19F/*CDC28*-T18A, Y19F diploid strain SKY2225. SKY2226 (*STE11-4*/*STE11-4*) was formed from LEU$^+$ segregants of SKY2183.

The *cdc28-1N* allele of K406 (Surana *et al.*, 1991) was amplified by high-fidelity PCR and the *Xho*I/*Mun*I fragment was subcloned into pRS413 (Sikorski and Hieter, 1989) to make plasmid SKB4168. The complete ORF of this clone was sequenced and the mutation confirmed. A *URA3*-marked integrating plasmid carrying the *cdc28-1N*

Table 2. Plasmid list

Plasmid	Markers	Source
pIL30	CEN *URA3* Ty1::*TDH3*::*lacZ*	Laloux *et al.* (1994)
pSL1509	YCp50 CEN *URA3 STE11–4*	Stevenson *et al.* (1992)
SKB4166	pRS305 *LEU2 STE11–4*	This work
p3303	plac22 CEN *TRP1 CDC28*–A18F19	Amon *et al.* (1992)
SKB4167	pRS316 CEN *URA3 CDC28*–A18F19	This work
SKB4168	pRS413 CEN *HIS3 cdc28–1N*	This work
SKB4169	pRS306 *URA3 cdc28–1N*	This work
B2255	Ycp50 CEN *URA3 RAS2*–Val19	Fink, personal communication
B2553	2 μm *URA3 STE12*	Fink, personal communication
YIpG2::CLB1	*GAL1::CLB1 LEU2*	Stueland *et al.* (1993)
YIpG2::CLB2	*GAL1::CLB2 LEU2*	Stueland *et al.* (1993)
pSWE1–15	2 μm *LEU2 SWE1*	Booher *et al.* (1993)
pWAS5–1	2 μm *LEU2 MIH1*	Booher, personal communication
pRS305	*LEU2*	Sikorski and Hieter (1989)
pRS413	CEN *HIS3*	Sikorski and Hieter (1989)

allele, SKB4169, was constructed by subcloning a 1.3-kilobase *Bam*HI/*Xho*I fragment from SKB4168 into pRS306 (Sikorski and Hieter, 1989). To create a pair of haploid strains carrying only the *cdc28-1N* allele, SKB4169 was digested with *Hin*dIII and transformed into SKY754 and L5683 to integrate at the *CDC28* locus. Transformants were selected on synthetic media lacking uracil, colony purified, and grown nonselectively for several generations at 22°C. Clones that had evicted the *URA3* plasmid, leaving behind the *cdc28-1N* allele, were selected on media containing 1 mg/ml 5-fluoro-orotic acid and then screened for lack of growth at 36°C. A pair of cells of opposite mating type were then mated to form a *ts* diploid, SKY2187, with the genotype *cdc28-1N/cdc28-1N*.

Strains overexpressing Clb1 and Clb2 were constructed by transforming L5791 with the plasmids YIpG2::CLB1 and YIpG2::CLB2 (Stueland *et al.*, 1993), respectively, each digested with *Bst*EII to direct integration to the *LEU2* locus.

Microscopy Methods

Microcolonies were imaged through the agar and plastic Petri dish with a Zeiss (Thornwood, NY) Axiovert 25 with bright-field illumination and a 32× LD Achroplan objective. Cells from suspension cultures were spread gently onto agar plates and imaged similarly. Pixera VCS image-acquisition software and a Pixera charge-coupled device camera were used to capture images at 1280 ×1024 resolution. Images were converted to gray scale, filtered to remove noise and enhance contrast, cropped, and assembled in Photoshop (Adobe, Mountain View, CA).

Staining of fixed cells with DAPI was carried out essentially as described (Pringle *et al.*, 1991). DAPI-stained experimental and control cells were placed on slides and imaged on a Zeiss Axioskop by phase and epifluorescence microscopy using 365-nm excitation and blue emission filters, a 40× Fluar oil-immersion objective, and 16× eyepieces. The cell shapes and the positions and forms of nuclei in each budded cell were determined. Each cell was classified into groups by apparent bud size/mother cell size ratio and into one of five nuclear morphology classes of the budding cycle: 1) central nucleus, S phase; 2) nucleus at neck in mother cell, S phase/G2; 3) nucleus distributed across neck, G2/metaphase; 4) nucleus stretched along mitotic spindle, anaphase; or 5) separate nuclear masses but no septum formed, telophase. Both as a control for staining and to evaluate nuclear phenotypes, propidium iodide–stained cells used for flow cytometry were examined by epifluorescence microscopy as above but with 546-nm excitation and orange-red emission filters.

Fixed cells grown on rich media were stained with rhodamine or fluorescein phalloidin (Molecular Probes, Eugene, OR) and DAPI as previously described (Pringle *et al.*, 1991) to evaluate nuclear position and reveal altered actin distribution patterns during the cell cycle. Cells from liquid cultures in rich media were prepared for immunofluorescence microscopy and stained with YOL 1/35 anti-tubulin antibody (Kilmartin *et al.*, 1982) (Accurate Chemical and Scientific) and Cy3 goat anti-rat immunoglobulin G polyclonal secondary antibody (Jackson Immunoresearch) and counterstained with DAPI to stain nuclear and mitochondrial DNA essentially as described (Pringle *et al.*, 1991), except that all steps were performed with cells in suspension and gentle centrifugation was used to exchange solutions. Cells were imaged with 546-nm excitation and orange-red emission filters to evaluate tubulin, 365-nm excitation and blue emission filters for DNA, and Nomarski differential interference contrast optics for cell morphology. Calcofluor staining (Pringle, 1991) was performed on exponentially growing cells. Samples were fixed in 3.7% formaldehyde at 25°C for 30 min. Aliquots of $\sim 10^7$ cells were pelleted, resuspended in 1 ml of 0.1 μg/ml Calcofluor staining solution, and incubated for 5 min. The cells were washed three times with 1 ml of water and resuspended to a final volume of 50 μl. Cells were examined to determine bud scar patterns using 365-nm excitation and blue emission filters and Nomarski differential interference contrast optics. Images were recorded using epifluorescence illumination on a Zeiss Axioskop using a 63× PlanApochromat objective, a Sensys 1600 charge-coupled device camera, and IPLab image-acquisition software.

Percentage Elongation on Agar

Yeast strains (SKY757, wild type, and L5535, *ste7/ste7*) were grown overnight to saturation at 30°C in 5 ml of synthetic minimal medium. Cells were washed, diluted to 1000 cells/ml, and spread onto plates of SLAD with uracil. Analysis of cell elongation was performed by microscopic examination using the Zeiss Axiovert 25 and a 32× LD Achroplan objective to image cells through the agar and plastic Petri dish. Scoring was performed by visual estimation of the length-to-width ratios of at least 400 cells. For 6 h, cells were counted in three categories: unbudded, round buds (bud length ≤ twofold bud width), and elongated buds (bud length ≥ twofold bud width).

Flow Cytometry

Cells from log-phase cultures were fixed and stained with propidium iodide to prepare samples for flow cytometry as described

(Hutter and Eipel, 1978). To disrupt cell aggregates, samples were sonicated until judged well dispersed by phase microscopy. Staining was evaluated by epifluorescence imaging using 546-nm excitation and orange emission filters. Samples were analyzed on a FacsScan (Becton-Dickinson, Franklin Lakes, NJ) flow cytometer, and 60,000 events were collected on each sample. The plots presented here represent the gated fluorescence area data. The gated data were analyzed to determine relative DNA content.

Whole Cell Extracts and Protein Analysis

Whole cell extracts were obtained by a method based on that of Amon (1992). Strains were grown overnight to saturation in YPD rich liquid media, diluted 1:50, and regrown to a density of $\sim 10^7$ cells/ml in YPD at 30°C. Cells were chilled, collected by centrifugation, washed in 10 mM Tris-HCl (pH 8.0), and resuspended in 200 μl of buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, 1 mM PMSF, 0.2 mM L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone, 0.025 mM L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone-HCl, 2 mg/ml pepstatin in DMSO). Approximately 0.2 ml of 0.2-mm-diameter acid-washed glass beads was added, cells were vortexed for 5 min, and the extract was cleared by a 5-min centrifugation at 16,000 $\times$ *g* at 4°C. The supernatant was collected and stored at −80°C. Protein concentrations were determined by the Bradford protein assay (Bio-Rad, Richmond, CA) using BSA as a standard.

For Western analysis, protein samples were solubilized with SDS/DTT sample buffer, boiled, and loaded onto 12% bis:acrylamide gels (1:19) with 50 μg of total yeast protein per lane. Samples were electrophoretically separated and transferred to nylon-supported nitrocellulose (Magna). Primary antibodies were, for Cdc28, a mouse anti-PSTAIRE mAb (Calbiochem, La Jolla, CA), and for Clb2, a rabbit polyclonal antibody raised against a TrpE-Clb2 fusion protein (Amon *et al.*, 1994) and affinity purified using the antigen. Immunoreactivity was detected with ^{125}I-protein A (ICN, Costa Mesa, CA), and blots were scanned using a phosphorimager (Molecular Dynamics, Sunnyvale, CA) and analyzed using Imagequant and IPLab (Scanalytics). For analysis of expression from the pIL30 (Laloux *et al.*, 1994) Ty1::*lacZ* reporter, extracts were obtained, assayed for total β-galactosidase activity, and normalized to total protein as described (Alfa *et al.*, 1993).

RESULTS

Activation of the STE MAPK Signal for Filamentous Growth Induces a Delay in G2/M

Wild-type, prototrophic strains constructed in the Σ1278b background exhibit filamentous development when subjected to low-nitrogen stress. When such cells are transferred to nitrogen starvation media (SLAD agar), many cells show a striking change in bud morphology within their first budding cycle, immediately adopting a filamentous growth pattern. In the first cell cycle under nitrogen starvation conditions, 91% of budded wild-type cells (n = 400) formed an elongated bud when examined 4 h after inoculation onto SLAD media. This phenotype is reminiscent of the arrest of cells carrying the *cdc28-1N* mutation (Surana *et al.*, 1991) overexpressing the Swe1 tyrosine kinase (Booher *et al.*, 1993) or overexpressing a G1 cyclin (Lew and Reed, 1993), each of which blocks cell cycle progression at the metaphase-to-anaphase transition by antagonizing Clb1- and Clb2-dependent Cdc28 complexes. When cells lacking elements of the RAS/STE filamentous development pathway are inoculated onto SLAD, the proportion of elongated buds formed is significantly decreased. Only 10% (n = 400) of cells lacking the *STE7* MEK kinase, and thereby defective in RAS/STE signaling, formed elongated buds in their first cell cycles. Rather, most buds emerging from Ste7-deficient mother cells formed small ovoid shapes and soon detached from the mother cell (Figure 1A). This pattern is typical of yeast cell growth on YPD agar media (rich media containing yeast extract, peptone, and dextrose). Nonetheless, the rate of bud emergence in the RAS/STE-signaling mutant was indistinguishable from that in the wild type (wild type, 78% budded at 4 h; *ste7/ste7*, 76% budded at 4 h). A simple inference is that the RAS/STE pathway may antagonize processes that are limiting for completion of mitosis (e.g., accumulation of Clb1,2/Cdc28 complexes) but has little effect on factors limiting Start (e.g., accumulation of Cln1,2/Cdc28 complexes).

To investigate this phenomenon further, we examined the behavior of cells in which the RAS/STE pathway was ectopically induced. We investigated the effect of the dominant activated *STE11-4* mutant allele, T596I (Stevenson *et al.*, 1992), on cell growth in rich media. When introduced as a single copy into diploid cells via integration at the *STE11* locus, *STE11-4* confers enhanced filamentous growth on SLAD media, as previously reported by Liu *et al.* (1993) for the plasmid-borne allele (Figure 1A). We examined several markers for filamentous development in these stable *STE11-4* cells. When inoculated on YPD agar media, this strain (SKY2183) displayed phenotypes characteristic of nitrogen starvation and activation of the RAS/STE pathway, including striking filamentous colony morphology (Figure 1A) and elevated expression of the pIL30 Ty1-*lacZ* FRE reporter. After 2 d of growth on YPD agar, the *STE11-4* cells invaded the agar surface to a greater degree than a wild-type diploid control (Figure 1B). Even in suspension culture in rich media, the *STE11-4* mutant grew as highly elongated cells that remained attached in clusters, forming florets reminiscent of filamentous colonies (Figure 1C). As in bona fide filamentous cells (Kron *et al.*, 1994), *STE11-4* cells exhibited apical polar budding. In the cell clusters and in colonies on agar, buds arose preferentially from the apical end of the mother cell. However, when these cells were examined with the chitin stain Calcofluor to confirm bud-site selection pattern, rather than the usual chitin rings at bud scars, a delocalized distribution of stain was apparent (Figure 2), perhaps consistent with a loss of temporal and/or spatial restriction of chitin deposition. However, staining of *STE11-4* cells with fluorescein phalloidin revealed persistence of an apically polarized distribution of actin cortical patches in large budded cells, as observed in wild-type cells responding to nitrogen starvation (Kron *et al.*, 1994; Cali *et al.*, 1998). Thus, via ectopic activation of the RAS/STE pathway, many features of the filamentous cell phenotype can be dissociated from nitrogen starvation.

Flow cytometry of a *STE11-4/STE11-4* homozygous diploid (SKY2226) growing in rich media demonstrated a predominance of cells with G2/M DNA content (Figure 1D). DAPI staining to determine the distribution of nuclear DNA suggested that most cells with large buds do not have separated nuclear masses or an elongated spindle (Figure 3). These data were confirmed by examination of spindles by anti-tubulin immunofluorescence microscopy. These assays indicated that the *STE11-4* cells do not have any increase in content of anaphase cells, despite their long budded period and G2/M shift. From these data, we infer that the predominant cell type in *STE11-4* cells grown on rich media is a cell

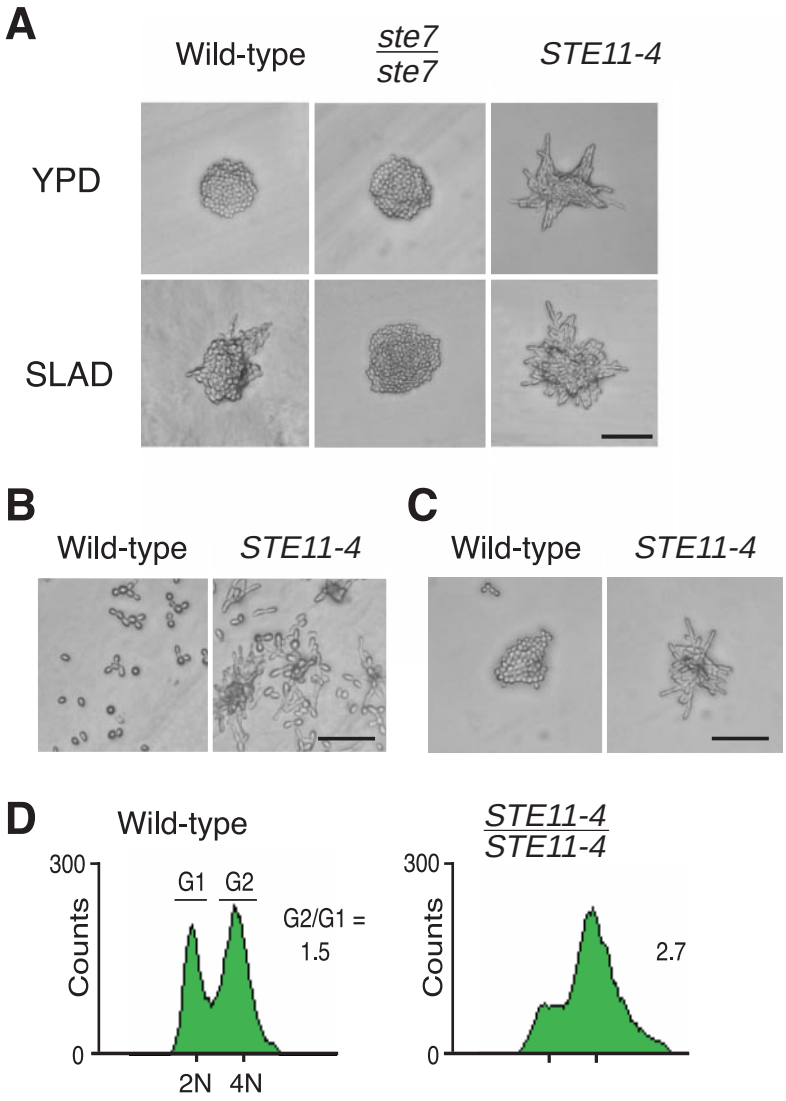

Figure 1. Ectopic activation of the RAS/STE pathway via the *STE11-4* mutation. (A) The dominant active *STE11-4* mutation promotes enhanced filamentous differentiation. Microcolonies formed by the indicated strains (*STE11-4* is the *STE11:: STE11-4:: LEU2/STE11* strain SKY2183) on rich YPD media and low-nitrogen SLAD media reveal that the requirement for nutrient starvation is bypassed by activation of RAS/STE signaling. A cell deficient for *STE7* (*ste7/ste7*, L5535), an element of the RAS/STE pathway, does not exhibit a filamentous phenotype on YPD or SLAD media. Photographs show representative colonies of each strain after 24 h at 22°C. (B) Diploid cell agar invasion assay. Strains were inoculated on YPD agar and grown for 72 h at 30°C. Photographs were taken after the nonadherent cells were washed away with water. The *STE11-4* mutant is hyperinvasive even in the rich media. (C) Cell morphology of mutant cultured in liquid YPD nitrogen-rich media. The *STE11-4* mutant exhibits highly elongated cells that grow as adherent cell aggregates reminiscent of filamentous colonies. This phenotype suggests that the agar signal requirement is also bypassed by ectopic RAS/STE signals. Bars, 50 μm. (D) Cell cycle kinetics of the *STE11-4/STE11-4* homozygous diploid (SKY2226). Flow cytometry was performed on asynchronous cultures growing on YPD rich media to determine the distribution of nuclear DNA content. The *STE11-4* mutant reveals a predominance of cells with G2/M DNA content, which correlates to a large G2/G1 ratio, reflecting the relative percentages of cells falling in the indicated gating intervals of fluorescence intensity.

carrying an elongated bud that has completed DNA synthesis but has yet to leave G2/metaphase, much like filamentous cells cultured on nitrogen starvation media.

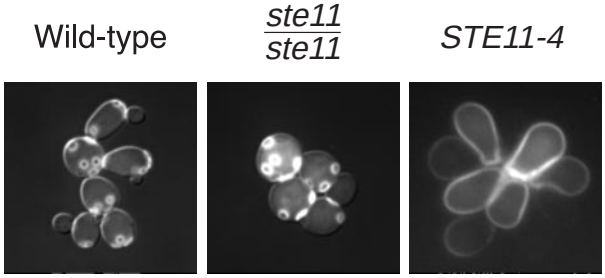

Figure 2. Fluorescence imaging of Calcofluor staining of chitin distribution. The *STE11-4* mutant shows a delocalized distribution of chitin rather than the expected chitin rings that mark bud scars, as is seen in the wild type and the *ste11/ste11* mutant.

These data are consistent with a mechanism by which the RAS/STE pathway negatively regulates the onset of mitotic anaphase, independent of any direct effects of nitrogen starvation. A reasonable target is the abundance or activity of mitosis-promoting factor (MPF), the mitotic form of CDK. We asked whether yeast mitotic cyclins Clb1 and Clb2 and/or the CDK Cdc28 might be regulated by RAS/STE signals.

STE MAPK Signaling Can Promote Filamentous Growth in the Absence of Cdc28 Tyrosine Phosphorylation

Mitotic onset in most eukaryotes is controlled by the reversible inhibitory phosphorylation of a conserved tyrosine and threonine adjacent to the active site in CDKs (Feilotter *et al.*, 1992). Phosphorylation sequesters preformed CDK/cyclin complexes as preMPF and delays anaphase. Our previous studies showed that cells carrying the constitutively active *CDC28*-T18A, Y19F (AF) mutant allele in which the Thr and

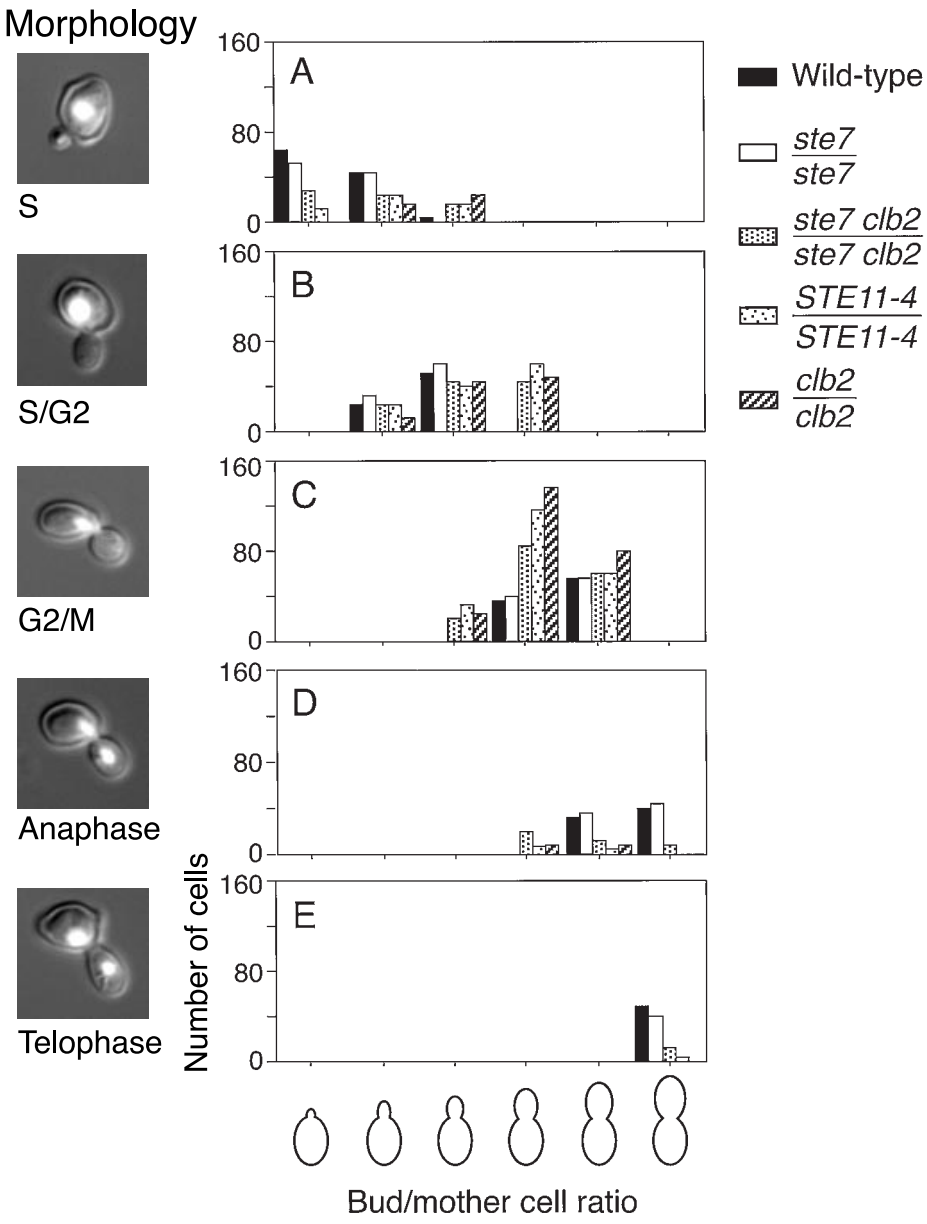

Figure 3. Distribution of nuclear DNA through the budding cycle. Cells were grown on YPD rich media and fixed, and nuclear DNA was stained with DAPI as described in MATERIALS AND METHODS. The cell shapes and the positions and forms of nuclei in 400 budded cells were determined. Each cell was scored individually and classified into groups by apparent bud size/mother cell size ratio and into nuclear morphology classes corresponding to stages in the budding cycle. The histograms plot the number of cells that fall in one morphology class, divided into six groups by their bud size/mother cell size ratio. The five morphology classes plotted are: central nucleus, S phase (A); nucleus at neck in mother cell, S phase/G2 (B); nucleus distributed across neck, G2/metaphase (C); nucleus stretched along mitotic spindle, anaphase (D); and separate nuclear masses but no septum formed, telophase (E). Representative cells are shown to the left of each histogram. These data show that mitotic progression correlates well with bud growth in wild-type cells. By contrast, *STE11-4* and *clb2/clb2* mutants display an overabundance of G2/metaphase nuclei throughout the budded period and a decreased proportion of anaphase and telophase morphologies. This phenotype is suggestive of a postreplication delay analogous to that of filamentous cells cultured on nitrogen starvation media. The *ste7/ste7 clb2/clb2* mutant reveals a slight increase in the proportion of cells in anaphase compared with *clb2/clb2* but still significantly fewer compared with the wild-type control or the *ste7/ste7* mutant.

Tyr residues are substituted with Ala and Phe still form filamentous colonies when inoculated onto low-nitrogen agar, although they are attenuated in their development. This result does not eliminate a pathway whereby the RAS/STE signal for filamentous differentiation promotes phosphorylation of this tyrosine.

We reexamined the behavior of mutants with altered Cdc28 tyrosine phosphorylation and/or altered RAS/STE function exposed to low-nitrogen agar media. We found that cells in which tyrosine phosphorylation is decreased (*CDC28*-T18A, Y19F/*CDC28*-T18A, Y19F, *swe1/swe1*, [2 μm *MIH1*]) are impaired in filamentous development and yield colonies on SLAD media in which cells at the colony periphery lack the degree of cell elongation observed in wild-type strains (data not shown). On the other hand, mutants with constitutively increased tyrosine phosphorylation (*mih1/mih1*, [2 μm *SWE1*]) are moderately enhanced in filamentous growth and produce markedly elongated cells at the colony periphery (data not shown). We confirmed published results that cells lacking certain elements of the RAS/STE pathway (*ste7/ste7*, *ste12/ste12* [see Figure 8A], or *ste11/ste11* [data not shown]) display markedly reduced filamentous development and cells carrying mutations that increase RAS/STE signaling ([*CEN RAS2*-Val19], [*CEN STE11-4*], [2 μm *STE12*] [data not shown]) display dramatically enhanced filamentous growth.

One model consistent with these data, as suggested by Edgington *et al.* (1999), is that inhibition of anaphase by the RAS/STE pathway is mediated by hyperphosphorylation of Thr-18 and/or Tyr-19 of the Cdc28 CDK, affected by activa-

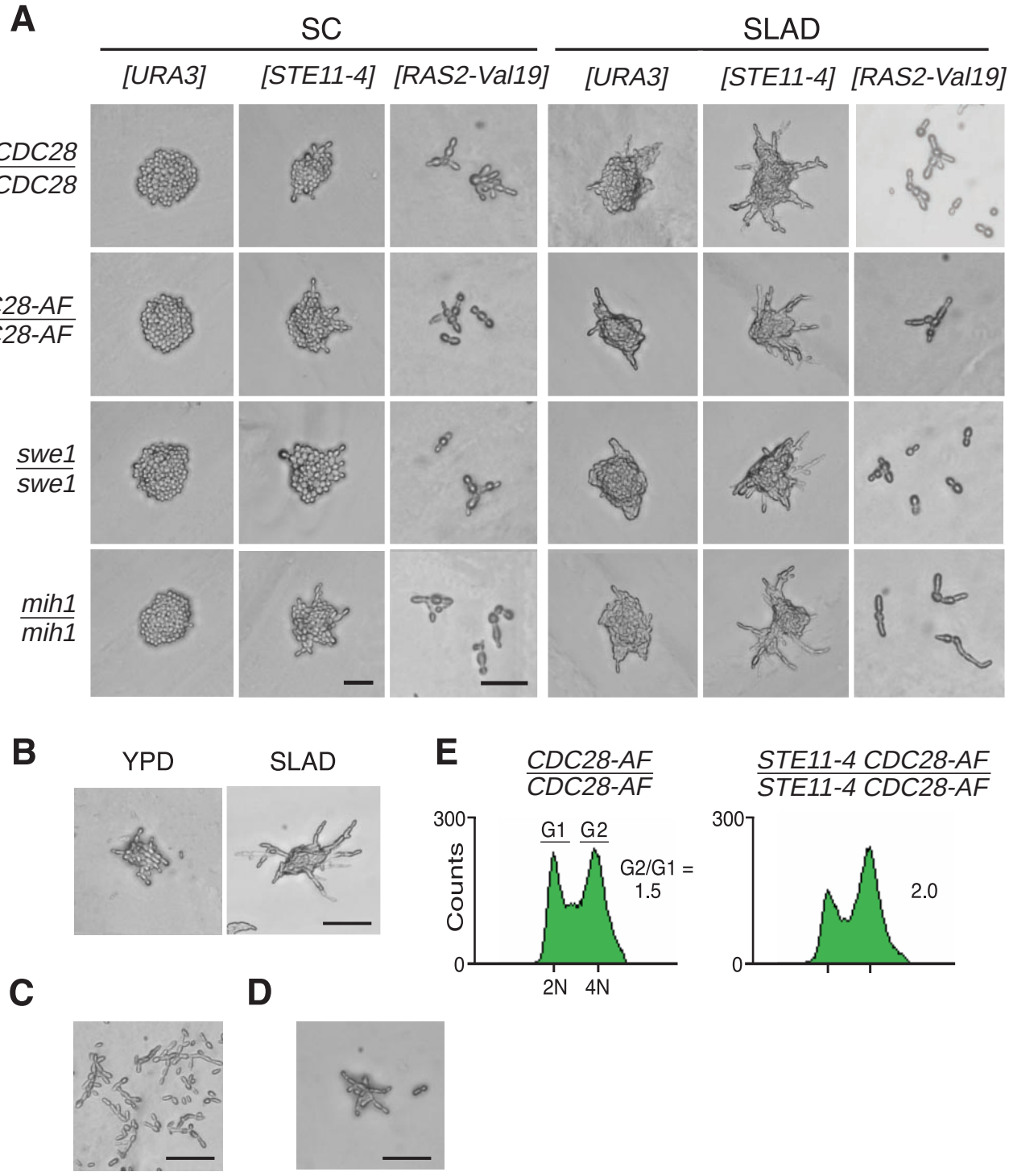

Figure 4.

tion of the Swe1 kinase or inhibition of the Mih1 phosphatase. Alternatively, the RAS/STE pathway and Cdc28 tyrosine phosphorylation may contribute independently to differentiation. In support of the latter possibility, we observed that ectopic activation of the RAS/STE pathway via plasmids containing the *STE11-4* or *RAS2*-Val19 mutations or overexpressing *STE12* suppressed both the impaired filamentation and the decreased cell elongation of *swe1/swe1*

and *CDC28*-T18A, Y19F mutants (Figure 4A). Finally, the double-mutant diploid carrying *CDC28*-T18A, Y19F and *STE11-4* showed enhanced filamentous growth (Figure 4B). Like the *STE11-4* strain, it showed enhanced agar invasion, highly elongated cells that remain attached in clusters, and a G2/M cell cycle shift (Figure 4, C–E). Furthermore, we confirmed our previous observations (Kron *et al.*, 1994) that *CDC28*-T18A, Y19F is dominant with respect to *SWE1*. Overexpression of *SWE1* via an integrated copy of a fusion between the inducible *GAL1,10* promoter and *SWE1* (Booher *et al.*, 1993) did not significantly delay cell division or alter filamentous growth in the *CDC28*-T18A, Y19F mutant. These data suggest that the RAS/STE pathway and Cdc28 tyrosine phosphorylation are independent pathways modulating filamentous growth.

Altered Cyclin Expression Modulates Filamentous Growth

Although control of mitotic anaphase by the RAS/STE pathway may not involve regulation at the level of tyrosine phosphorylation, other modes of regulation of MPF cannot be eliminated. One limiting factor for completion of mitosis is the accumulation of the functionally redundant mitotic cyclins Clb1 and Clb2. We examined whether altering the activity of mitotic kinase by changing the expression of mitotic cyclins might have a significant effect on filamentous development.

Previous studies have shown that deletion or moderate overexpression of either *CLB1* or *CLB2* has only subtle effects on cell growth on rich media (but see Stueland *et al.*, 1993). To test whether altered mitotic cyclin expression might differentially affect filamentous development, we constructed diploid cells deficient for either the *CLB1* or *CLB2* gene. In contrast to other genetic backgrounds, diploid Σ1278b cells carrying a homozygous deletion of *CLB2* exhibited several characteristics of filamentous development even when grown on rich complete media. When inoculated on YPD agar, *clb2/clb2* cells initially formed microcolonies consisting of mostly filamentous cells (Figure 5A). When patches of Clb2-deficient cells were inoculated on YPD agar and allowed to grow for several days, they displayed increased cell adhesion (rough surface and granular consistency) and enhanced agar invasion (Figure 5B) comparable to that displayed by the *STE11-4* strain.

In liquid YPD media, the *clb2/clb2* mutant cells were heterogeneous, with an unusual number of elongated cells that grew as adherent cell aggregates reminiscent of the *STE11-4* florets (Figure 5C). Flow cytometry revealed a marked shift to cells with 4N DNA content (Figure 5D). When inoculated onto SLAD, the *clb2/clb2* mutant exhibited remarkable filamentous growth characterized by highly elongated cells and very few rounded cells in the colonies (Figure 5A), a growth pattern similar to that of the *STE11-4* mutant.

Cells lacking Clb2 are specifically sensitized to the RAS/STE signal. When [*CEN RAS2*-Val19] or [*CEN STE11-4*] was introduced into the *clb2/clb2* mutant cells, a compound effect of dramatic cell elongation and striking filamentation was observed on SLAD media (Figure 5E). Further supporting the hypothesis that diploid cells lacking Clb2 are sensitized to the filamentous growth-inducing signal, we observed that when this mutant is grown in the presence of high-ammonia-containing synthetic media, the cells adopt a rounded shape like that of a wild-type control strain. This suggests that 1) the elongated phenotype on YPD media, a nitrogen source relatively low in ammonia but rich in short peptides and other complex forms of nitrogen, may be due to low but significant basal activity of the signaling pathway, and 2) the presence of ammonia nitrogen might actively repress the signaling pathway, rather than starvation for ammonia nitrogen inducing its function.

Using the same logic that prompted examination of the Clb2 mutant, we tested whether a strain lacking the Clb1 cyclin might also demonstrate induced filamentous growth. A diploid Clb1 mutant constructed by deletion of both *CLB1* alleles showed few or no rich media phenotypes and less cell cycle shift but a moderate induction of filamentous growth on SLAD. This induction was further increased by transformation with the [*CEN RAS2*-Val19] or [*CEN STE11-4*] inducer.

To confirm that the effects of the *CLB1* and *CLB2* deletions were mediated via reduced activation of the Cdc28 CDK, we examined the effect of substituting the temperature-sensitive G2 arrest allele, *cdc28-1N*, for wild-type *CDC28* in an otherwise wild-type strain. Like the Clb2-deficient strain, when grown at a permissive temperature the *cdc28-1N/cdc28-1N* strain exhibited significant cell elongation on YPD media and dramatic filamentous growth on SLAD low-nitrogen media (Figure 5A). The [*CEN STE11-4*]– and [*CEN RAS2*-Val19]–inducing plasmids further enhanced this filamentous phenotype (Figure 5E).

To study the effect of overexpression of mitotic cyclins, we constructed strains carrying integrated copies of fusions between the inducible *GAL1,10* promoter and the *CLB1* and *CLB2* cyclin genes. Although diploid cells carrying an integrated *GAL1::CLB2* construct exhibited normal filamentous growth on repressing SLAD agar media,

Figure 4 (facing page). The RAS/STE pathway and Cdc28 tyrosine phosphorylation independently modulate filamentous growth. (A) Filament formation of Cdc28 tyrosine phosphorylation mutants with altered RAS/STE function on synthetic nitrogen (SC) or nitrogen starvation (SLAD) agar. Mutants with decreased tyrosine phosphorylation lacking the inhibitory Swe1 kinase or its phosphorylation site on Cdc28 (*swe1/swe1* and *CDC28*-T18A, Y19F [AF]) show impaired filamentous development on SC and SLAD media compared with the wild-type strain. On the other hand, lack of the reactivating phosphatase Mih1 (*mih1/mih1*) confers constitutively increased Cdc28 tyrosine phosphorylation and a moderately enhanced filamentous phenotype. Plasmids expressing the *STE11-4* or *RAS2*-Val19 allele suppress the impaired filamentation of both the *swe1/swe1* and *CDC28*-T18A, Y19F strains and further enhance the filamentous phenotype of *mih1/mih1* strains. (B) *STE11-4/STE11-4 CDC28-AF/CDC28-AF* double mutants on YPD or SLAD agar. The nonphosphorylatable CDK mutant *CDC28*-T18A, Y19F does not block an ectopic RAS/STE signal. (C) Agar invasion assay. Cells were treated as described in Figure 1B. Like the *STE11-4* mutant, the double mutant is hyperinvasive in YPD agar. (D) Cell morphology in liquid YPD media. Highly elongated cells that grow as adherent cell aggregates reminiscent of the *STE11-4* florets are apparent in *STE11-4/STE11-4 CDC28-AF/CDC28-AF* double mutant cultures. Bars, 50 μm. (E) Flow cytometry. Cells were prepared as described in MATERIALS AND METHODS. The *STE11--4/STE11-4 CDC28-AF/CDC28-AF* double mutant reveals a marked shift to 4N DNA content relative to the *CDC28-AF/CDC28-AF* control and comparable to that of the *STE11-4/STE11-4* mutant alone.

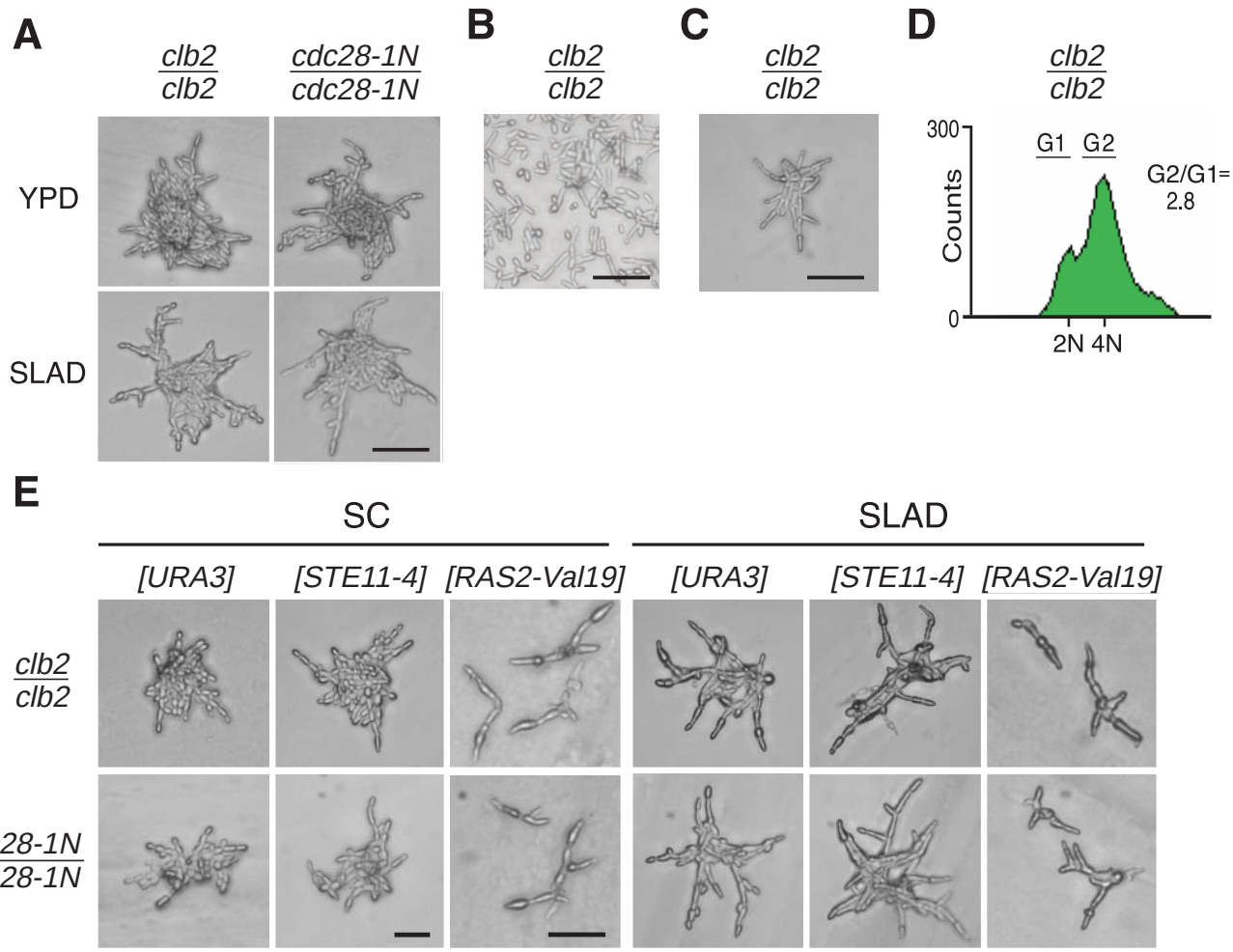

Figure 5. Mutations in mitotic control genes modulate filamentous differentiation. The *clb2/clb2* mutant exhibits exaggerated filamentous development and a growth pattern similar to that of the *STE11-4* mutant. (A) Both the strain carrying the *ts* mitotic arrest allele *cdc28-1N* and the strain deficient in *CLB2* exhibit filamentous growth on YPD media and are further induced by nitrogen starvation. Photographs show representative colonies of each strain after 24 h at 22°C. (B) Agar invasion assay. Cells lacking *CLB2* have enhanced YPD agar invasion comparable to that displayed by the *STE11-4* mutant. (C) Cell morphology in liquid YPD media. The *clb2/clb2* strain grows as highly elongated cells that remain attached in clusters, reminiscent of the *STE11-4* mutant. Bars, 50 μm. (D) Cell cycle kinetics. Like *STE11-4* cells, *CLB2*-deficient cells demonstrate a predominance of cells with G2/M DNA content. (E) Deficiency in mitotic Cdc28 activity conferred by deficiency in the Clb2 mitotic cyclin dramatically enhances filamentous growth on both synthetic nitrogen (SC) and nitrogen starvation (SLAD) media. Activation of the RAS/STE pathway via plasmids containing *STE11-4* or *RAS2*-Val19 augments the striking cell elongation phenotype of these strains. Bars, 50 μm.

induction of Clb2 overexpression by inoculating cells onto synthetic low-ammonia galactose agar media abrogated filamentous growth. Rather than forming the chains of elongated cells characteristic of filamentous growth, these cells formed piles of rounded cells (Figure 6). Diminution of filamentous growth was also observed in cells overexpressing the Clb1 cyclin, whereas overexpression of the Clb3, Clb4, or Clb5 cyclin had only modest effects on PH growth. Finally, overexpression of Clb1 and Clb2 suppressed the filamentous phenotype of *cdc28-1N* cells (data not shown).

These data suggest that the mitotic cyclin Clb2 performs a function in direct antagonism to the RAS/STE pathway. However, although mitotic cyclins may be negatively regulated at some level by the RAS/STE pathway, these results do not indicate that either Clb1 or Clb2 is the sole target of the RAS/STE pathway in the control of anaphase.

One biochemical mechanism consistent with the genetic results described above is that the cell cycle shift observed upon ectopic activation of the RAS/STE pathway, as in the *STE11-4* mutant, might be due to down-regulation of the Clb2 cyclin. A lower cellular content of Clb2 would result in a proportional decrease in Clb2-dependent Cdc28 kinase and thereby slow mitotic entry. Furthermore, low cellular Clb2 content would predict the observed similarity in phenotypes between *STE11-4*–expressing cells and Clb2-deficient cells. Protein extracts were prepared from asynchronous cultures of cells grown on rich YPD media. When we compared levels of Clb2 protein in extracts derived from the wild-type, Ste11-deficient, Clb2-deficient, and *STE11-4*–ex-

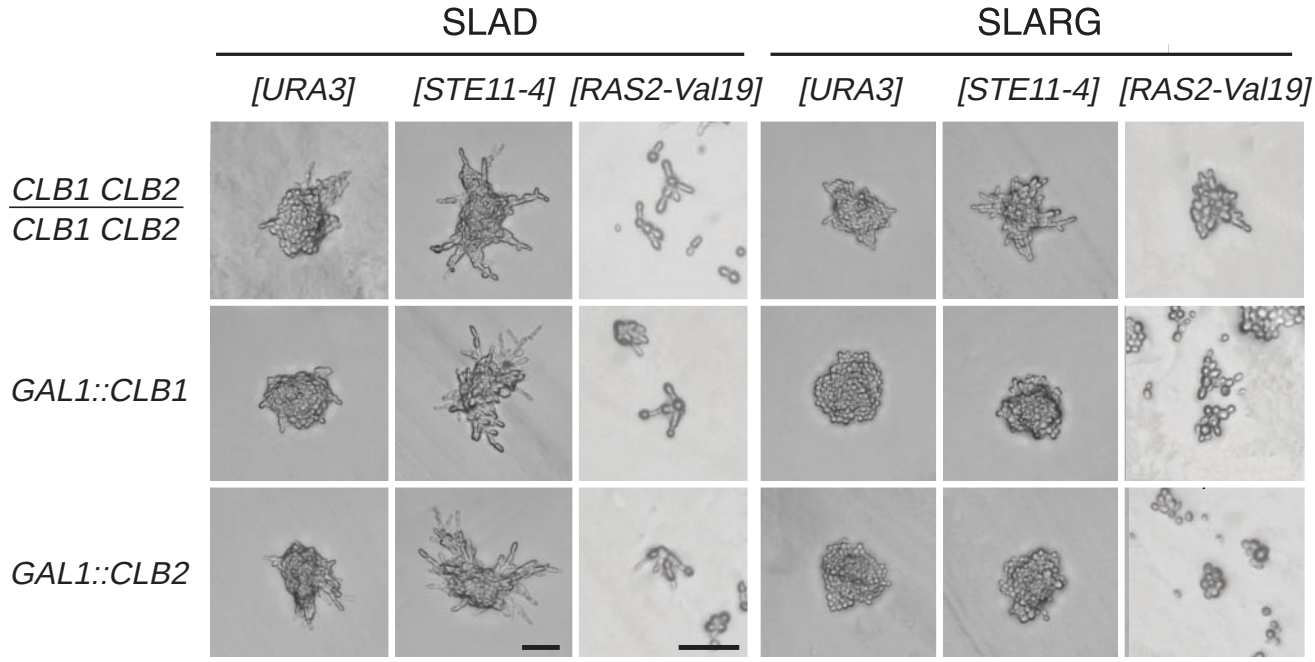

Figure 6. Overexpression of the Clb1 and Clb2 mitotic cyclins abrogates filamentous development and antagonizes RAS/STE signaling. Strains containing integrated copies of the *CLB1* and *CLB2* genes under an inducible *GAL1,10* promoter and a control plasmid or the *STE11-4* or *RAS2*-Val19 allele were grown on nitrogen starvation (SLAD) or synthetic low-ammonia galactose (SLARG) media for 24 h. Both basal and enhanced filamentous growth are abolished by Clb1 or Clb2 overexpression on SLARG media, indicating that the RAS/STE pathway may function upstream of Clb1 and Clb2. Representative colonies of each strain were photographed after 24 h at 22°C. Bars, 50 μm.

pressing strains, we observed the expected absence of Clb2 protein from the Clb2-deficient strain. However, the other strains showed equal levels of expression of Clb2 irrespective of genotype (Figure 7), suggesting that the RAS/STE pathway might have little or no effect on Clb2 cyclin expression or stability.

Epistasis Experiments Place Clb2 Downstream of the RAS/STE Pathway

Were the RAS/STE pathway genetically upstream of Cdc28 and Clb2, one inference would be that loss of function in Clb2, which confers enhanced filamentous growth, might suppress the decreased filamentous growth observed in mutants lacking components of the RAS/STE pathway. We examined mutants lacking the *STE20* MEK kinase kinase, the *STE11* MEK kinase, and the *STE7* MEK. As previously reported (Liu *et al.*, 1993), *ste7/ste7* and *ste12/ste12* mutants each confer decreased filamentous development, and *ste20/ste20* mutants display complete loss of filamentous growth (Figure 8A). Furthermore, all of these mutants were readily washed off the agar with flowing water (Figure 8B) and failed to grow in suspension culture as adherent cell aggregates (Figure 8C). We constructed mutant strains that combined each of the STE kinase mutations with deletion of the *CLB2* cyclin gene and examined their filamentous phenotypes. We found that the *ste20/ste20 clb2/clb2*, *ste7/ste7 clb2/clb2*, and *ste12/ste12 clb2/clb2* double mutants all exhibited a limited form of filamentous differentiation when placed under inducing conditions of low-nitrogen agar (Figure 8A). All of these mutants showed partial agar invasion (Figure 8B) and an elongated cell phenotype (Figure 8C), even in liquid media. By comparison, the *ste20/ste20 clb2/clb2* double mutant showed only a very subtle suppression. The flow cytometry profiles for each double mutant showed a significant shift toward the 4N DNA content (Figure 8D), and we

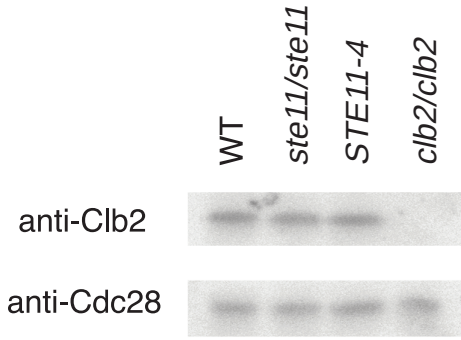

Figure 7. Modulating RAS/STE signaling regulates filamentous growth without altering Clb2 abundance. The anti-PSTAIRE mAb recognizing Cdc28 was used as a loading control. Western analysis of Clb2 and Cdc28 reveals that Clb2 levels are relatively unaffected by RAS/STE signals. WT, wild type.

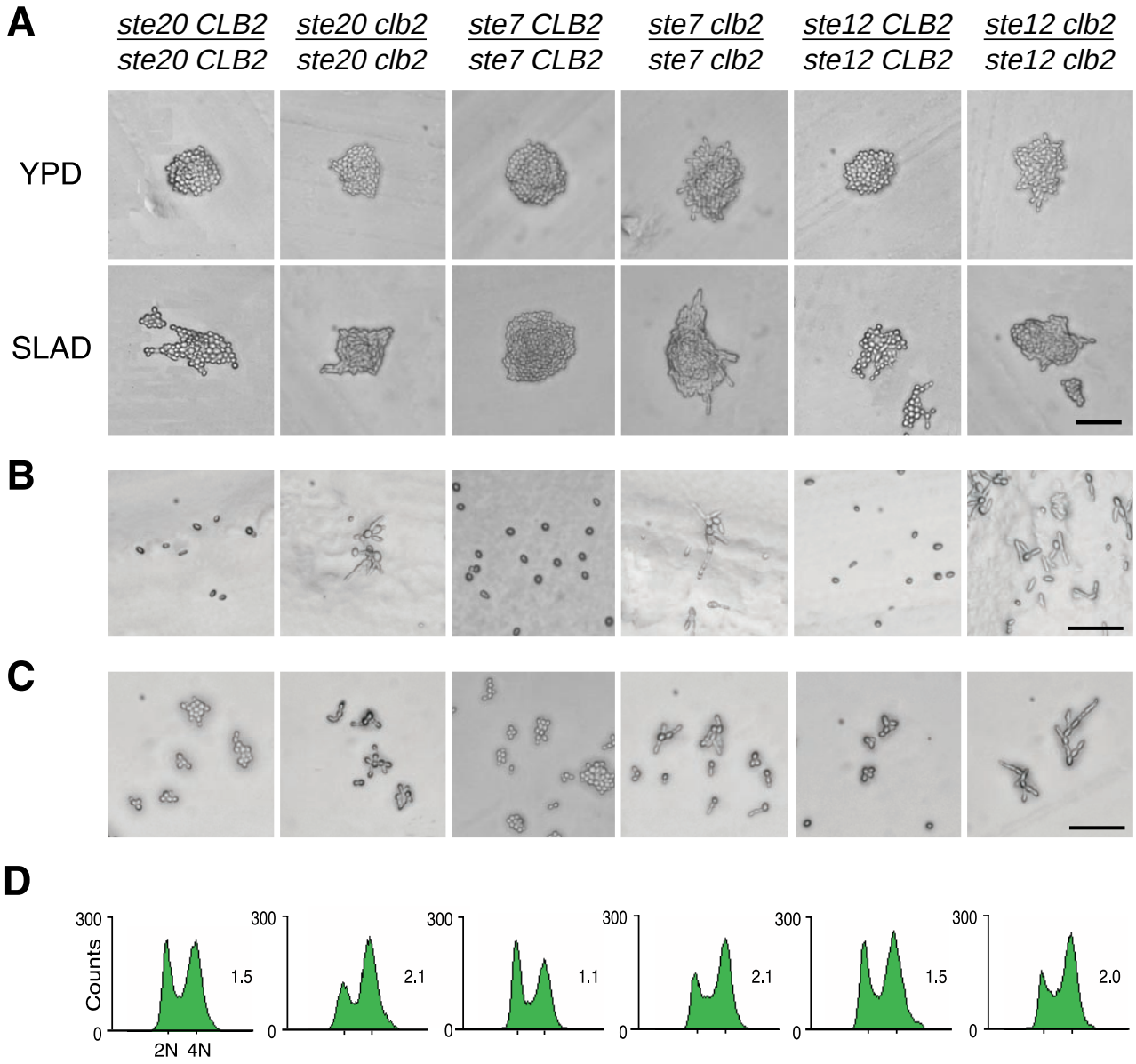

Figure 8. Bypass of mutations in the RAS/STE pathway by Clb2 deficiency. (A) *STE20-*, *STE7-*, and *STE12*-deficient mutants have a decreased filamentous development phenotype. Cells lacking both the Ste20 PAK kinase and the Clb2 mitotic cyclin show only a very subtle suppression, whereas double mutants lacking Ste20 and Clb2, Ste12 and Clb2, or Ste7 and Clb2 exhibit limited filamentous growth on both rich YPD media and low-nitrogen SLAD media. These phenotypes suggest partial bypass of the RAS/STE pathway. Photographs show representative colonies of each strain after 24 h at 22°C. (B) Agar invasion assay. Double mutants lacking both Clb2 and components of the RAS/STE pathway exhibit partial invasion, but *ste7/ste7*, *ste12/ste12*, and *ste20/ste20* mutants confer decreased agar invasion. (C) Cell morphology in liquid YPD media. *ste7/ste7 clb2/clb2*, *ste12/ste12 clb2/clb2*, and *ste20/ste20 clb2/clb2* double mutants display limited cell elongation, but to a greater degree than *ste7/ste7*, *ste12/ste12*, and *ste20/ste20* mutants. Bars, 50 μm. (D) Cell cycle kinetics. A shift toward G2/M DNA content is apparent in cells deficient in both Clb2 mitotic cyclin and elements of the RAS/STE pathway.

observed a decrease in the population of anaphase cells (Figure 3). Thus, the bypass of the STE pathway blockade effected by a deletion of *CLB2* was partial but nonetheless significant.

A complementary test to reveal order of function was to examine whether the induction of filamentous growth by a dominant active allele of a component of the RAS/STE pathway could override the inhibitory effect of Clb1 or Clb2 overexpression. We found that cells carrying the integrated *GAL1::CLB1* and *GAL1::CLB2* constructs failed to form filaments on low-nitrogen galactose-containing media, even if they also carried a [*CEN RAS2*-Val19] or

[*CEN STE11-4*] plasmid (Figure 6). These data suggest that the RAS/STE pathway indeed acts upstream of mitotic cyclins as part of the filamentous differentiation response.

DISCUSSION

Physiological study of filamentous growth has suggested that a change in cell cycle control is critical to the differentiation of rounded yeast cells into elongated filamentous cells. We set out to examine whether modulation of the cell cycle machinery might be both necessary and sufficient for proper morphogenesis in filamentous growth. In particular, we asked whether other cell-autonomous features of filamentous differentiation, such as elongated cell morphology, polar budding, persistent cell connections, and agar invasion, might be downstream of the cell cycle switch.

The STE MAPK Pathway Regulates the G2/M Transition in Diploid Yeast Cells

Previous studies have shown that the low-nitrogen signal for filamentous growth is transduced via a dual signaling pathway involving elements of the STE MAPK– and RAS GTPase/cAMP–dependent kinase cascades. A critical question is whether the cell cycle shift to G2/M in filamentous cells is downstream of the RAS/STE-signaling pathway or is an independent response to nitrogen starvation. The results described here establish a pathway linking the MAPK-signaling cascade directly to the cell cycle machinery.

Our logic is based on the following argument. We have used a dominant activated mutant, *STE11-4*, to produce an ectopic, constitutive signal that stimulates the filamentous development pathway, even in the absence of nitrogen starvation or an agar substrate. Previous studies (Liu *et al.*, 1993) found that the *STE11-4* mutation confers enhanced filamentous growth and promotes expression of the filamentous signaling reporter, Ty1-*lacZ* (Mosch *et al.*, 1996), via its FRE element in a low-nitrogen-independent manner.

We extended these observations by creating diploid yeast strains in which the *STE11-4* mutation is stably expressed from its genomic locus. As expected, these strains exhibit enhanced filamentous growth on low-nitrogen media and display high constitutive expression of the Ty1-*lacZ* reporter. However, we found that such *STE11-4* strains express an ectopic filamentous growth phenotype, even in rich liquid media. Because of persistent attachment to the mother cell after septation, they grow as florets that may contain dozens of cells. *STE11-4* florets formed in rich liquid media are indistinguishable from microcolonies of wild-type cells growing on the surface of low-nitrogen agar. Thus, *STE11-4* is sufficient to replace at least the nitrogen starvation and agar components of the conditions conducive to filamentous growth and to provide a model system that reveals the RAS/STE pathway–specific aspects of filamentous growth.

We examined these *STE11-4* cells for markers of cell cycle progression known to be altered during filamentous differentiation. *STE11-4* filamentous cells grown on rich liquid media are shifted into G2/M. Most cells were budded, many with a single nucleus lodged in the bud neck and a short mitotic spindle spanning the nucleus. Even though many cells had buds nearly equal in size to the mother cell, very few *STE11-4* cells were observed with the elongated mitotic spindle or separated nuclear masses suggestive of anaphase. This finding suggests that a RAS/STE signal alone is sufficient to produce the characteristic cell cycle of filamentous growth.

The RAS/STE-dependent Mitotic Delay Is Not Mediated by Cdc28 Tyr-19 Phosphorylation

A simple model, as suggested by Edgington *et al.* (1999), might explain the link between signal and cell cycle: the physiological pathway from nitrogen starvation to cell cycle may be via a MAPK cascade that promotes increased Tyr-19 phosphorylation of Cdc28. In fact, many observations are consistent with this model. Ectopic activation of the Swe1 kinase by mutation of an inhibitory kinase (Edgington *et al.*, 1999) or overexpression of the Swe1 kinase or lack of the Mih1 phosphatase, as shown here, promote hyperphosphorylation of Cdc28 Tyr-19, delay mitotic onset, and enhance filamentous growth. Mutation of the Cdc28 Tyr-19 residue to Phe or deficiency of the Swe1 kinase each promote mitosis and, as shown here, attenuate filamentous growth. Nonetheless, we observed that the RAS/STE signal can still induce ectopic filamentous development, even in cells lacking the Swe1 kinase or expressing the dominant active *CDC28*-T18A, Y19F mutant lacking the site for inhibitory tyrosine phosphorylation. Although our data and those of Edgington *et al.* (1999) demonstrate that regulation of Cdc28 tyrosine phosphorylation modulates filamentous development, we find that this regulation is neither necessary nor sufficient to confer the mitotic delay or other effects induced by RAS/STE pathway activation. Furthermore, although nitrogen starvation may promote Cdc28 tyrosine phosphorylation and thereby contribute to differentiation, this regulation is not essential for most aspects of filamentous differentiation, including the G2/M cell cycle shift.

Altered Cyclin Expression, Mitotic Cdc28 Activity, and Filamentous Development

Perhaps the most remarkable findings reported here and by Edgington *et al.* (1999) are that simple genetic manipulations to create strains deficient in or overexpressing a single mitotic cyclin are so altered in their developmental responses. Cells lacking the mitotic cyclin Clb2 are highly induced for filamentous growth. When inoculated onto low-nitrogen agar, Clb2-deficient cells form colonies of almost exclusively filamentous cells. In turn, when inoculated onto rich complex agar media (YPD) or even when grown on rich complex liquid media, Clb2 mutants remain highly elongated, bud in a polar pattern, remain attached to their mother cells, and show a dramatic cell cycle shift to G2/M when assayed by flow cytometry. We find that these phenotypes derive in part from supersensitivity to the low-nitrogen signal. Activation of the RAS/STE pathway, as by the *STE11-4* mutation, or expression of *RAS2*-Val19 exacerbates the Clb2 mutant phenotype on low-nitrogen media.

That these effects are due to decreased activation of the mitotic forms of Cdc28 is indicated by the phenotypes conferred by the *cdc28-1N ts* mutation at a permissive temperature. At the nonpermissive temperature (37°C), cells carrying only *cdc28-1N* arrest before mitotic anaphase but with completed DNA synthesis and an assembled mitotic spindle (Surana *et al.*, 1991). Overexpression of mitotic cyclins sup-

presses this *ts* arrest. In turn, cells carrying both *cdc28-1N* and a deficiency for *CLB1* or *CLB2* are nonviable even at 22°C. This synthetic lethality demonstrates a critical defect in mitotic kinase activity in *cdc28-1N* mutants even at the permissive temperature. When yeast cells carrying the *cdc28-1N* mutation were inoculated onto low-nitrogen media and cultured at a permissive temperature, like the Clb2-deficient cells, they were hyperfilamentous. Furthermore, when superstimulated by expression of the *STE11-4*– or *RAS2*-Val19–inducing mutations, like the Clb2-deficient cells, the *cdc28-1N* mutants displayed a dramatically enhanced filamentous phenotype.

As noted above, Myers and colleagues (Edgington *et al.*, 1999) have shown that filamentous growth is modulated by Cdc28 function, but they focus on mitotic functions. They isolated a Cdc28 mutant, *cdc28-127* C127Y, that causes constitutive expression of most filamentous growth characteristics, such as enhanced cell polarity, elongated shape, and G2/M cell cycle delay. Like *cdc28-1N*, *cdc28-127* is defective in mitotic progression. Their data confirm our observations that altered Clb1,2/Cdc28 activity can modulate filamentous growth independent of signal transduction. In contrast to the results reported here, their data indicate a significant role for Cdc28 Tyr-19 phosphorylation by Swe1 kinase in filamentous differentiation. However, as *cdc28-127* remains subject to regulation by Tyr-19 phosphorylation, their data also suggest that there may be multiple means of regulating CDK activity to promote filamentous differentiation.

In reciprocal experiments, we determined that hyperactivity of mitotic cyclins and, by inference, high activity of mitotic kinase can abrogate filamentous development directed by RAS/STE signaling. Cells overexpressing Clb1 or Clb2 maintain a round cell shape and a nonfilamentous growth pattern even in the face of maximal stimulation provided by the nutritional signal of low-nitrogen media combined with dominant active mutations in the RAS/STE signaling pathway. In turn, overexpression of the Clb1 or Clb2 cyclin does not repress the low-nitrogen-induced expression of a FRE reporter (our unpublished results). These data suggest that 1) mitotic functions of the Cdc28 CDK are a critical target of the RAS/STE signal, and 2) repression of mitotic kinase is both necessary and sufficient to induce a coordinated developmental switch characterized by elongated cell shape, polar budding, persistent cell attachment, and G2/M cell cycle delay. This pattern recapitulates key features of filamentous differentiation as stimulated by the physiological signal.

Rather than proving a direct pathway that links RAS/STE to mitotic cyclins, we cannot eliminate the possibility that the effects of altered cyclin activity on filamentous growth, like the effects of modulating Cdc28 Tyr-19 phosphorylation, are simply evidence for a parallel pathway regulating morphogenesis. Other data linking the Cln1 and Cln2 G1 cyclins to filamentous differentiation suggest that their effects may be quite direct, serving as modulators of the RAS/STE response in the signaling pathway (Oehlen and Cross, 1998) and/or as pathway-independent activators of gene expression (Loeb *et al.*, 1999). Nonetheless, neither mechanism adequately addresses the origin of the mitotic delay or its regulation by RAS/STE signals. We propose an alternative model whereby the RAS/STE pathway directly effects repression of mitotic kinase activity in diploid cells. Our hypothesis is based on the paradigm of pheromone response, in which distinct gene products induced by the STE MAPK pathway mediate cell surface (Fus1) and cell cycle (Far1) aspects of differentiation. Far1 serves as an inhibitor of Cln1,Cln2-dependent Cdc28 activity and thereby enforces the G1 arrest of conjugating haploid cells. We imagine a functional equivalent for Far1 acting downstream of the RAS/STE pathway to enforce its cell cycle effects. Coordinately with expression of the cell surface flocculin Flo11 that is required for the increased cell adhesion in filamentous growth, this hypothesized gene product would be under FRE control so that it would be induced in diploid cells upon activation of the RAS/STE pathway to mediate the mitotic delay. As we did not observe any decrease in the steady-state abundance of Clb2 protein in our *STE11-4* cells, we suppose that activation of the RAS/STE pathway neither decreases expression nor increases destruction of mitotic cyclins. An alternative biochemical activity of this factor might be direct, stoichiometric inhibition of Clb1- and Clb2-dependent Cdc28 mitotic kinase activity. As a preliminary experiment to detect regulation at the level of Cdc28 activity, we assayed precipitates of extracts derived from asynchronous cultures of wild-type and RAS/STE pathway mutant yeast strains for histone H1 phosphorylation to quantitate CDK activity. Initial results suggest that although total, $p13^{suc1}$-associated CDK activity may be unaltered, Clb2-associated Cdc28 activity in *STE11-4* cells appears relatively depressed (our unpublished results). Confirmation and characterization of this apparent mitotic inhibition remain to be pursued. In turn, genetic studies may reveal mutations that do not block Flo11 expression but prevent down-regulation of the mitotic activity of Cdc28. Such studies would provide the critical evidence for the hypothesized factor coupling the gene expression program induced by the RAS/STE filamentous signaling pathway to its effects on cell cycle progression.

ACKNOWLEDGMENTS

We thank Ankit Desai for technical assistance. Preliminary studies leading to the results reported here were completed by S.J.K. while a postdoctoral fellow with Gerald R. Fink at the Whitehead Institute (Cambridge, MA). S.J.K. gratefully acknowledges Dr. Fink for his generous support, guidance, and scientific insights and Cora Styles for help with strain constructions and valuable suggestions. We thank A. Amon, R. Booher, B. Errede, D. Lew, H. Liu, H.-U. Mosch, S. Reed, and P. Russel for reagents and helpful comments. This work was supported by grants to S.J.K. from the Arnold and Mabel Beckman Foundation, the Horace W. Goldsmith Foundation, the Cancer Research Foundation, and funds from a Howard Hughes Medical Institute Research Resources for Medical Schools grant to the University of Chicago.

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