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Characterization of Alcohol-induced Filamentous Growth in *Saccharomyces cerevisiae*

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Diploid cells of the budding yeast *Saccharomyces cerevisiae* starved for nitrogen differentiate into a filamentous growth form. Poor carbon sources such as starches can also stimulate filamentation, whereas haploid cells undergo a similar invasive growth response in rich medium. Previous work has demonstrated a role for various alcohols, by-products of amino acid metabolism, in altering cellular morphology. We found that several alcohols, notably isoamyl alcohol and 1-butanol, stimulate filamentous growth in haploid cells in which this differentiation is normally repressed. Butanol also induces cell elongation and changes in budding pattern, leading to a pseudohyphal morphology, even in liquid medium. The filamentous colony morphology and cell elongation require elements of the pheromone-responsive MAPK cascade and TEC1, whereas components of the nutrient-sensing machinery, such as MEP2, GPA2, and GPR1, do not affect this phenomenon. A screen for 1-butanol–insensitive mutants identified additional proteins that regulate polarized growth (BUD8, BEM1, BEM4, and FIG1), mitochondrial function (MSM1, MRP21, and HMI1), and a transcriptional regulator (CHD1). Furthermore, we have also found that ethanol stimulates hyperfilamentation in diploid cells, again in a MAPK-dependent manner. Together, these results suggest that yeast may sense a combination of nutrient limitation and metabolic by-products to regulate differentiation.

INTRODUCTION

Microorganisms either secrete or excrete a wide variety of compounds. Some of these substances result from normal metabolic processes, such as alcohols in fermentative yeast. Other compounds are used to promote survival by coordinating development or differentiation (e.g., cAMP-directed chemotaxis in the slime mold *Dictyostelium discoideum*), by damaging other organisms (e.g., the antifungal, antibiotic, and immunosuppressive drugs FK-506 and rapamycin, produced by species of *Streptomyces*), or by altering the growth environment more directly (e.g., secreted proteases in *Candida albicans*, which promote tissue damage and dispersion of infection). Many of these substances are produced in response to external stimuli: nutrient deprivation stimulates cAMP release in *D. discoideum* and pheromone production in many fungi, whereas host signals stimulate protease secretion in *C. albicans*. The pathways that recognize such signals are of obvious interest but are poorly characterized.

A better understanding of these pathways in fungi is critical, given that morphological differentiation has been correlated with pathogenicity in many species, most conclusively in *C. albicans*, in which it has been shown that mutants unable to filament are avirulent (Lo *et al.*, 1997). Conjugation of compatible cell types in the corn pathogen *Ustilago maydis* results in a filamentous growth form; only this form is virulent, and mutations that block mating differentiation also abrogate pathogenicity (reviewed by Banuett, 1995). Similarly, host invasion in the rice blast fungus *Magnaporthe grisea* is dependent on the formation of a structure known as an appresorium. Several mutations have been isolated that block this morphological differentiation; these mutations all confer an avirulent phenotype (Mitchell and Dean, 1995; Xu and Hamer, 1996). Thus, a further understanding of the signals that stimulate fungal differentiation, and of the pathways that respond to these signals, may aid in the development of strategies to combat fungal diseases in both animals and plants.

The budding yeast *Saccharomyces cerevisiae* has a morphological differentiation pathway similar in both structure and regulation to those mentioned above. This phenomenon—pseudohyphal, or filamentous, growth—is stimulated in diploid cells upon nitrogen starvation. Pseudohyphal cells have an elongated morphology, an altered cell cycle and budding pattern, and enhanced substrate invasion (Gimeno *et al.*, 1992; Kron *et al.*, 1994). The regulation of pseudohyphal

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differentiation is complex, involving at least two partially interconnected pathways, the mating MAPK pathway and a receptor/G protein/cAMP signaling pathway (Liu *et al.*, 1993; Cook *et al.*, 1997; Kübler *et al.*, 1997; Lorenz and Heitman, 1997; Lorenz *et al.*, 2000). The earliest signaling events are not well understood, but the requirement for two cell-surface proteins, the ammonium permease MEP2 and the G protein–coupled receptor GPR1, implicates the existence of extracellular signals (Lorenz and Heitman, 1998a; Lorenz *et al.*, 2000).

Nitrogen starvation is not the only stimulus that promotes filamentous growth. Poorly used carbon sources such as the starch amylopectin have been reported to induce filamentous growth (Lambrechts *et al.*, 1996). A related phenomenon, haploid invasive growth, allows haploid strains to penetrate the agar substrate in rich medium. As in filamentous growth, cells become elongated and alter their budding pattern, processes that require the same elements of the MAPK cascade that regulate pseudohyphal differentiation (Roberts and Fink, 1994).

Several alcohols can also induce morphological abnormalities. Although ethanol is the primary fermentation product in *S. cerevisiae*, yeast produces a wide variety of other alcohols, mostly products of amino acid metabolism known collectively as fusel alcohols. This class of alcohols includes compounds such as isoamyl and isobutyl alcohols that are produced particularly under conditions of nitrogen starvation. Dickinson (1994, 1996) showed that several fusel alcohols can promote an aberrant, elongated morphology in *S. cerevisiae*. In nitrogen-poor conditions, leucine, the precursor of isoamyl alcohol, can also induce elongated cells (Dickinson, 1994).

The morphology of cells growing in the pseudohyphal form is very similar to that of cells exposed to these fusel alcohols. We have further characterized the connection between these alcohol-induced morphological changes and pseudohyphal growth. Several alcohols, notably isoamyl alcohol and butanol, promote a filamentous growth form on solid medium and an elongated and filamentous form in liquid medium. Strikingly, the most dramatic effects are seen in haploid cells in which filamentation is normally repressed. In addition, ethanol, the most prominent alcohol produced by yeast, also enhances filamentous growth in diploid cells. In both of these cases, mutations in the pheromone-responsive MAPK pathway known to block pseudohyphal growth also abrogate alcohol-induced filamentation, indicating a functional link between the nitrogen starvation and alcohol-induced phenomena. These findings suggest that *S. cerevisiae* has co-opted its own metabolic by-products for use as a signaling mechanism to regulate its development under starvation conditions.

MATERIALS AND METHODS

Yeast Strains, Plasmids, and Media

Yeast medium and molecular and genetic methods were as described by Guthrie and Fink (1991). Nitrogen-limiting (SLAD) medium was prepared as described previously (Gimeno *et al.*, 1992; Lorenz and Heitman, 1997). Alcohols were added to agar-containing medium after cooling to a concentration of 1% (vol/vol). It should be noted that because of the volatility of the alcohols, the effective concentration in medium is certainly <1%. Butanol was used in the majority of experiments because it induced abundant filamentation while maintaining a low toxicity to the investigator preparing the medium. It was important to incubate plates containing butanol in air-tight bags or enclosed in parafilm separate from control plates, because butanol induced filamentation even on plates lacking the alcohol when in close proximity, presumably as a result of its volatility.

Yeast strains are described in Table 1. Strains SCY40–SCY46 were constructed through a PCR-mediated disruption method with the use of the G418 resistance cassette (Wach *et al.*, 1994) in haploid strains MLY40 and MLY41. The oligonucleotide primers used to construct these disruptions are listed in Table 2. Briefly, the 5′ and 3′ disruption oligonucleotides, designed to precisely delete the ORF, were used to prime a PCR reaction with the use of plasmid pFA6-KanMX2 (Wach *et al.*, 1994) as a template. PCR products were used to transform haploid strains to G418 resistance. Candidate disruptants were confirmed via PCR with the use of one primer in the 3′ flanking region (outside the ORF) and the 5′ disruption oligonucleotide (see Table 2). In correct disruptants, this PCR produces a product, whereas in inaccurate integrants it does not. Diploid strains homozygous for these deletions were constructed via a cross between independently derived *MAT***a** and *MAT*α haploid deletion strains. YEplac195 (2μ*URA3*; Gietz and Sugino, 1988) was used to complement the Ura$^-$ auxotrophy of these strains in the majority of experiments. The reporter plasmids pJB207 (p*FUS1-lacZ LEU2* CEN) and pIL30-LEU2 (FRE-*lacZ LEU2* CEN) have been described (Trueheart *et al.*, 1987; Laloux *et al.*, 1994; Mösch *et al.*, 1996).

Mutagenesis and Screen for Nonfilamentous Mutants

To find mutants insensitive to butanol, we used a transposon-mediated mutagenesis system (Burns *et al.*, 1994). The transposon-tagged libraries were used to transform strains MLY42 (*MAT*α) and MLY43 (*MAT***a**) carrying a YEplac195 plasmid. Leu$^+$ prototrophs were selected on solid minimal medium plus glucose (YNB medium), pooled, and replated to SLAD + 1% butanol at ~1000 cells per plate. Colonies were screened microscopically after 2–4 d for a nonfilamentous morphology. The insertion points of the transposon were identified as described by Burns *et al.* (1994). Briefly, a bacterial origin of replication and an ampicillin resistance gene were introduced into transposon sequences by integrating plasmid pRSQ2 into each mutant. Genomic DNA was isolated and cleaved with either *Eco*RI or *Hin*dIII, ligated, and used to transform *Escherichia coli* strain DH5α to ampicillin resistance. The only productive ligation products will contain the replication origin and Amp^R inserted into the transposon. These plasmids were sequenced to identify the flanking yeast DNA.

To ensure that the transposon insertion was genetically linked to the nonfilamentous phenotype, we crossed the insertion strains to the parent strains and sporulated and dissected the resulting diploid. For reasons that are not clear, spore viability was quite poor in these diploids, although this lethality was not linked to the transposon insertion (the *LEU2* marker that tags the insertion segregated independently of the spore lethality phenotype). For mutants for which it was difficult to demonstrate linkage via crosses, the candidate gene was disrupted directly with the use of the G418/PCR approach (see above) and analyzed to ensure that the phenotypes of the disruption strains agreed with those of the original insertion mutations.

Photomicroscopy

Whole colony photographs were taken directly on agar plates with a Zeiss (Thornwood, NY) microscope fitted with a 35-mm Nikon (Garden City, NY) camera. Unless indicated otherwise, whole colonies were photographed at ×25 magnification. Single-cell pictures were taken with a Nikon Eclipse E800 microscope. Images were

Table 1. Yeast strains

Strain	Genotype	Reference/Source
Σ1278b series		
MLY40	*ura3-52 MATα*	Lorenz and Heitman, 1997
MLY41	*ura3-52 MAT***a**	Lorenz and Heitman, 1997
MLY42	*ura3-52 Δleu2::hisG MATα*	Lorenz and Heitman, 1997
MLY43	*ura3-52 Δleu2::hisG MAT***a**	Lorenz and Heitman, 1997
MLY61	*ura3-52/ura3-52 MAT***a**/α	Lorenz and Heitman, 1997
MLY97	*ura3-52/ura3-52 Δleu2::hisG/Δleu2::hisG MAT***a**/α	Lorenz and Heitman, 1997
MLY104a	*Δmep1::LEU2 ura3-52 Δleu2::hisG MAT***a**	Lorenz and Heitman, 1998a
MLY108a	*Δmep2::LEU2 ura3-52 Δleu2::hisG MAT***a**	Lorenz and Heitman, 1998a
MLY108a/α	*Δmep2::LEU2/Δmep2::LEU2 ura3-52/ura3-52 Δleu2::hisG/Δleu2::hisG MAT***a**/α	Lorenz and Heitman, 1998a
MLY115a	*Δmep1::LEU2 Δmep2::LEU2 ura3-52 Δleu2::hisG MAT***a**	Lorenz and Heitman, 1998b
MLY115a/α	*Δmep1::LEU2/Δmep1::LEU2 Δmep2::LEU2/Δmep2::LEU2 ura3-52/ura3-52 Δleu2::hisG/Δleu2::hisG MAT***a**/α	Lorenz and Heitman, 1998b
MLY128a	*Δmep3::G418 ura3-52 MAT***a**	Lorenz and Heitman, 1998a
MLY131a	*Δmep1::LEU2 Δmep2::LEU2 Δmep3::G418 Δleu2::hisG ura3-52 MAT***a**	Lorenz and Heitman, 1998a
MLY132a	*Δgpa2::G418 ura3-52 MAT***a**	Lorenz and Heitman, 1997
MLY132a/α	*Δgpa2::G418/Δgpa2::G418 ura3-52/ura3-52 MAT***a**/α	Lorenz and Heitman, 1997
MLY172a	*Δskn7::G418 ura3-52 MAT***a**	Lorenz and Heitman, 1998b
MLY172a/α	*Δskn7::G418/Δskn7::G418 ura3-52/ura3-52 MAT***a**/α	Lorenz and Heitman, 1998b
MLY180a	*Δmsn1::G418 ura3-52 MAT***a**	Lorenz and Heitman, 1998b
MLY180a/α	*Δmsn1::G418/Δmsn1::G418 ura3-52/ura3-52 MAT***a**/α	Lorenz and Heitman, 1998b
MLY182a	*Δphd1::G418 ura3-52 MAT***a**	Lorenz and Heitman, 1998b
MLY182a/α	*Δphd1::G418/Δphd1::G418 ura3-52/ura3-52 MAT***a**/α	Lorenz and Heitman, 1998b
MLY183a	*Δtec1::G418 ura3-52 MAT***a**	Lorenz and Heitman, 1998b
MLY183a/α	*Δtec1::G418/Δtec1::G418 ura3-52/ura3-52 MAT***a**/α	Lorenz and Heitman, 1998b
MLY216a	*Δste12::G418 ura3-52 Δleu2::hisG MAT***a**	Lorenz and Heitman, 1997
MLY216a/α	*Δste12::G418/Δste12::hisG ura3-52/ura3-52 Δleu2::hisG/Δleu2::hisG MAT***a**/α	Lorenz and Heitman, 1997
MLY217a	*Δste11::G418 ura3-52 Δleu2::hisG MAT***a**	Lorenz and Heitman, 1997
MLY218a	*Δste7::G418 ura3-52 Δleu2::hisG MAT***a**	Lorenz and Heitman, 1997
MLY219a	*Δste20::G418 ura3-52 Δleu2::hisG MAT***a**	Lorenz and Heitman, 1997
MLY232a	*Δgpr1::G418 ura3-52 MAT***a**	Lorenz *et al.*, 2000
MLY232a/α	*Δgpr1::G418/Δgpr1::G418 ura3-52/ura3-52 MAT***a**/α	Lorenz *et al.*, 2000
MLY261a	*Δste4::G418 ura3-52 MAT***a**	This study
HLY352	*Δste12::LEU2/Δste12::LEU2 ura3-52/ura3-52 leu2::hisG/Δleu2::hisG MAT***a**/α	Liu *et al.*, 1993
SCY125	*Δash1::G418 ura3-52 MAT***a**	Chandarlapaty and Errede, 1998
XPY95a	*Δflo8::Hyg ura3-52 MAT***a**	Pan and Heitman, 1999
XPY104a	*Δmss11::G418 ura3-52 MAT***a**	Pan and Heitman, unpublished
XPY107a	*Δflo11::Hyg ura3-52 MAT***a**	Pan and Heitman, 1999
SCY40α	*Δmrp21::G418 ura3-52 MATα*	This study
SCY40a/α	*Δmrp21::G418/Δmrp21::G418 ura3-52/ura3-52 MAT***a**/α	This study
SCY41α	*Δhmi1::G418 ura3-52 MATα*	This study
SCY41a/α	*Δhmi1::G418/Δhmi1::G418 ura3-52/ura3-52 MAT***a**/α	This study
SCY42α	*Δmsm1::G418 ura3-52 MATα*	This study
SCY42a/α	*Δmsm1::G418/Δmsm1::G418 ura3-52/ura3-52 MAT***a**/α	This study
SCY44α	*Δfig1::G418 ura3-52 MATα*	This study
SCY44a/α	*Δfig1::G418/Δfig1::G418 ura3-52/ura3-52 MAT***a**/α	This study
SCY45α	*Δbem4::G418 ura3-52 MATα*	This study
SCY45a/α	*Δbem4::G418/Δbem4::G418 ura3-52/ura3-52 MAT***a**/α	This study
SCY46α	*Δbem1::G418 ura3-52 MATα*	This study
SCY46a/α	*Δbem1::G418/Δbem1::G418 ura3-52/ura3-52 MAT***a**/α	This study
27-06	*ste12::Tn-LEU2 ura3-52 Δleu2::hisG MATα*	This study
26-03	*bud8::Tn-LEU2 ura3-52 Δleu2::hisG MATα*	This study
34-16	*chd1::Tn-LEU2 ura3-52 Δleu2::hisG MAT***a**	This study
W303 series		
10556-24D	*ura3-1 can1-100 leu2-3,112 MAT***a**	Fink lab collection
10556-30B	*ura3-1 can1-100 his3-11,15 MATα*	Fink lab collection
S288c series		
FY2	*ura3-52 MATα*	Fink lab collection
FY69	*leu2-1 MAT***a**	Fink lab collection
L5302	*flo8-1::URA3::FLO8*	Liu *et al.*, 1996
L5306	*flo8-1::URA3::FLO8/flo8-1::URA3::FLO8*	Liu *et al.*, 1996

Table 2. Oligonucleotide primers used to create disruption strains

Strain	Gene	Primer	Sequence
SCY40	*MRP21*	5′ disruption	ATGTTGAAGAGCACGCTGAGGCTTTCAAGAATCTCTCTCACAGCTGAAGCTTCGTACGC
		3′ disruption	TCTTGAAGCCCATCATGAATTCCTTCCTATGCCTTTGAGAGCATAGGCCACTAGTGGATCTG
		3′ check	GTATCCTTTCCTCTTGGCATC
SCY41	*YOL095*	5′ disruption	ATGGACAAGCTAACTCCATCTCAATGGAAGGTAATAAATAACAGCTGAAGCTTCGTACGC
		3′ disruption	CTTAGTGAAGAATATGCTCTATAAAATCCAAAATTTTTGCATAGGCCACTAGTGGATCTG
		3′ check	CTATATACGTCTGAAAACGC
SCY42	*MSM1*	5′ disruption	ATGCAATGTCGATCAATTGTGCATCGTCTGTACTCTAAGGTCAGCTGAAGCTTCGTACGC
		3′ disruption	ATGGAATTTTTTTCAAAGGTACTTCTCGGCCTTTCTTGGCATAGGCCACTAGTGGATCTG
		3′ check	TCATATTCGTTTGTTCCTCT
SCY44	*FIG1*	5′ disruption	ATGGTCGCAATCTCAATGATTTGGTTTTTTACCAAGCGTATCAGCTGAAGCTTCGTACGC
		3′ disruption	ACATTATCAATTTCATCTTTCAAGCTTTTCCTATCCCTGCATAGGCCACTAGTGGATCTG
		3′ check	ATGTAGATGAATCCGAAGAG
SCY45	*BEM4*	5′ disruption	TGGATTACGAAGAAATTTTATTTGGTCTGCAACCGATCCTCCAGCTGAAGCTTCGTACGC
		3′ disruption	TAAATTTCCCATTGTTGAAACAACTCTCGCTACCCTTAGCATAGGCCACTAGTGGATCTG
		3′ check	TTGTTCCTCTGGCGTTAAGC
SCY46	*BEM1*	5′ disruption	ACTTCAAACTCTCAAAAAGAGATAGTAATGGGTCGAAGGGCCAGCTGAAGCTTCGTACGC
		3′ disruption	TCCAGAAGTTGATTGGGCTGGAGCCTGCTTGGAGCCCGGCATAGGCCACTAGTGGATCTG
		3′ check	GAAATTTTCAGTTTGGCTTGG
MLY261	*STE4*	5′ disruption	GAAAATACTCAAAAAACTGTACAGCTCAATCAGGTACACATTACGCAGCTGAAGCTTC-GTACGC
		3′ disruption	CACAGTATTTCCAATTCGAAGCTATTGATAACCTGGAGACCATATGCATAGGCCACTAG-TGGATCTG
		5′ check	GAATCCCTTCAATATCGAAAC

captured electronically with the use of a MicroMax digital processor (Princeton Instruments) and OpenLab 2.0.3 software (Improvision).

Phenotypic Assays

Budding Pattern Analysis. To assay bud pattern after exposure to butanol, strains were incubated on YPD with or without 1% butanol for 2 d at 30°C. Strains were then restreaked to the same medium and incubated for two doublings (~3–4 h on YPD, 5–6 h on YPD + 1% butanol). Microcolonies were assayed microscopically for either a compact, axial budding morphology or an elongated, bipolar morphology.

Reporter Gene Assays. To assess signaling stimulated by alcohols, Ura$^-$ Leu$^-$ strains MLY43 (wild-type MAT**a**), MLY61 (wild-type MAT**a**/α), MLY216a (Δ*ste12* MAT**a**), and MLY216a/α (Δ*ste12*/Δ*ste12* MAT**a**/α) were transformed with the *URA3* vector YEplac195 and vectors containing the *FUS1-lacZ* (pJB207) or FRE-*lacZ* (pIL30-LEU2) reporters. Strains were grown in YNB containing 1% (vol/vol) butanol or 5 μg/ml α-factor for either 4 or 24 h at 30°C. β-Galactosidase activity was determined with the use of chlorophenol red-β-galactopyranoside as a substrate, as described (Cardenas *et al.*, 1994).

Haploid Invasion Assays. The ability of haploid strains to invade the agar substrate was determined as described previously (Roberts and Fink, 1994; Cook *et al.*, 1997). Strains were patched to YPD and incubated for 5 d at 30°C. Surface cells were gently washed off, and the plate was incubated for an additional 24 h at 30°C.

Mating Assays. Qualitative mating assays were performed with the use of strains MLY42 + YEplac181 (*ura3-52* Δ*leu2::hisG MAT*α + *LEU2*-2μ) and MLY43 + YEplac195 (*ura3-52* Δ*leu2::hisG MAT***a** + *URA3*-2μ). These strains were incubated together in a patch on YPD with or without 1% (vol/vol) butanol overnight at 30°C and then replica plated to YNB to select for diploids.

RESULTS

Haploid Filamentation Induced by Various Alcohols

Several "fusel" alcohols, such as isoamyl alcohol, *n*-amyl alcohol, and isobutanol, which result from amino acid metabolism, can induce morphological abnormalities in liquid cultures of *S. cerevisiae* (Dickinson, 1994, 1996). Moreover, high levels of leucine, a metabolic precursor of isoamyl alcohol, also induce similar hyphae-like extensions (Dickinson, 1994). Given the morphological similarities between cell shape after alcohol exposure and during filamentous, or pseudohyphal, growth, we further analyzed the effects of various alcohols on the growth form of yeast.

Figure 1 shows the effects of a panel of alcohols on colony morphology on nitrogen-limiting (SLAD) medium. As can be seen, this medium allows diploid cells of the Σ1278b strain background to undergo pseudohyphal differentiation, whereas haploids continue to grow in the yeast form into smooth, round colonies. Several of these alcohols, notably 1-butanol, isobutanol, isoamyl alcohol, and *tert*-amyl alcohol (see the diagrams in Figure 1 for structures of the more complex alcohols), induced haploid cells to form filamentous colonies when present at 1% (vol/vol) on solid SLAD medium. In Figure 1, we present results with *MAT***a** cells; *MAT*α cells filament to a similar degree under these conditions (our unpublished results). Because this differentiation program is normally active only in diploid cells (see the SLAD-only panel in Figure 1) and is repressed in haploid cells, we were surprised to find such vigorous filamentation in haploid cells. These alcohols have little effect on colony morphology of the diploid cell type.

The other notable observation from the experiment shown in Figure 1 is the effect of ethanol on colony morphology. Although haploid strains were unaffected, diploid cells in-

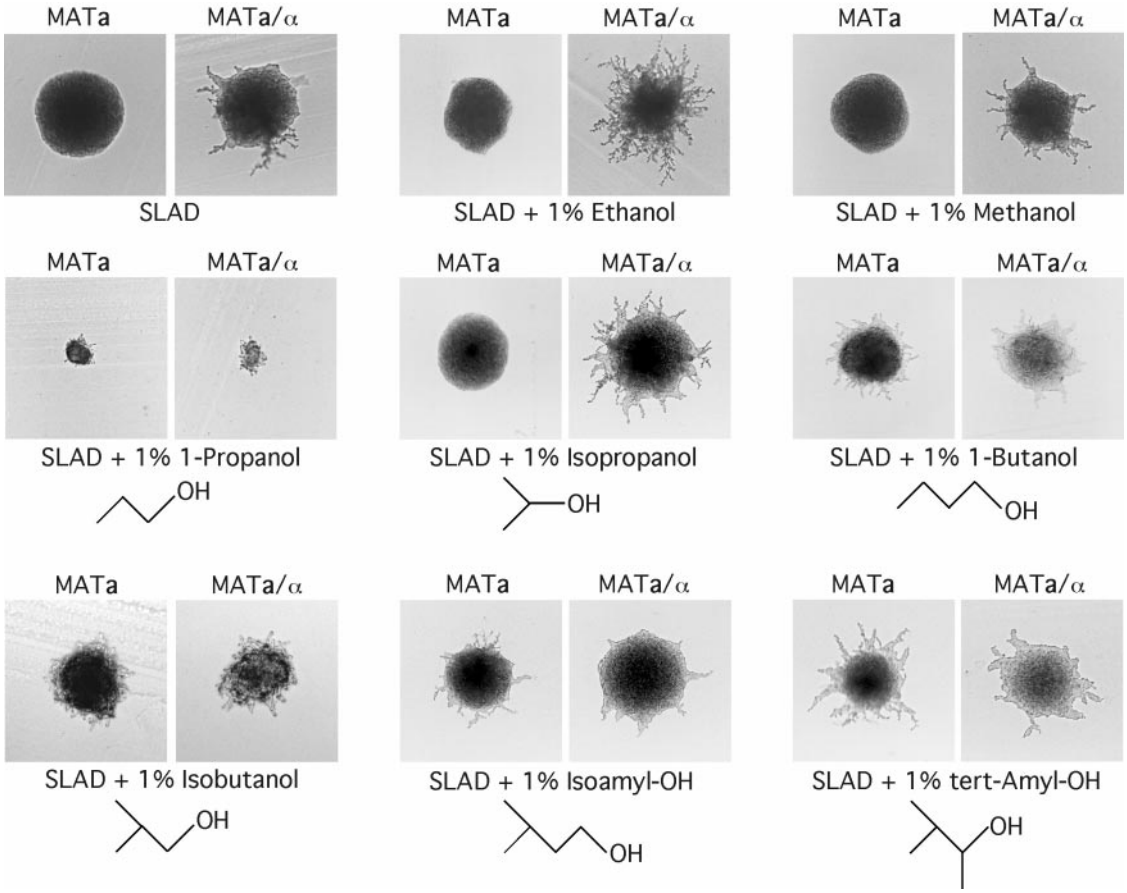

Figure 1. Several alcohols induce haploid filamentation. Wild-type strains MLY41 (*MAT***a**) and MLY61 (*MAT***a**/α) were incubated on solid SLAD medium containing 1% (vol/vol) of the indicated alcohol. The strains were grown at 30°C for 4 d on parafilm-seated plates before being photographed at ×25 magnification. The structures of the less common alcohols are shown below the panels.

cubated on SLAD + 1% (vol/vol) ethanol showed a stimulation of filamentous growth compared with cells grown in alcohol-free conditions. This observation will be examined further at the end of this section.

Filamentous Growth in Liquid Medium

One complication of the analysis of pseudohyphal differentiation is that it occurs only on solid medium. Dickinson (1996) reported that isoamyl alcohol, in particular, would induce morphological abnormalities, described as "hyphal-like extensions," in liquid rich (YPD) medium. We investigated whether these structures were similar in form or regulation to pseudohyphal differentiation.

The time course shown in Figure 2 demonstrates that, at least in this strain background (Σ1278b; in contrast, Dickinson [1996] used the IWP72 background), the phrase "hyphal-like extensions" does not do justice to the morphology of cells grown in liquid YPD + 1% butanol. By 8 h after inoculation into butanol-containing medium, elongated cells can be seen in filamentous clusters. By 30 h, elaborate asters of connected cells have developed. These asters show a remarkable similarity to microcolonies of cells growing in a filamentous form. Although these asters are highly flocculent and sediment readily, they form in liquid medium in rolling cultures. These changes are apparent in both diploids and haploids, but they are far more striking in the haploid cell type. Figure 2 shows results with butanol, although isoamyl alcohol is also effective at inducing these asters (our unpublished results).

The filamentous aster structure shown at the 30-h time point in Figure 2 is by no means rare in these cultures. To assess how quantitative these changes are, we counted the number of distinct cell clusters that contained either 1–19 cells or >20 cells per cluster with the use of haploid strain MLY41 (*MAT***a**) after 30 h of growth in YPD + 1% (vol/vol) butanol. At this time, 38.4% of cell clusters contained >20 cells, and all of these large clusters had an elongated, filamentous form, similar to that shown in Figure 2. This indicates that the majority of cells are incorporated into multicellular asters when exposed to butanol for 30 h. In contrast, only 9.6% of cell clusters from YPD-grown cultures have >20 cells; moreover, these clusters take the form of tight clumps of cells typical of flocculent haploid strains. Thus, butanol exposure both increases the size and changes the structure of cell clumps isolated from liquid rich medium.

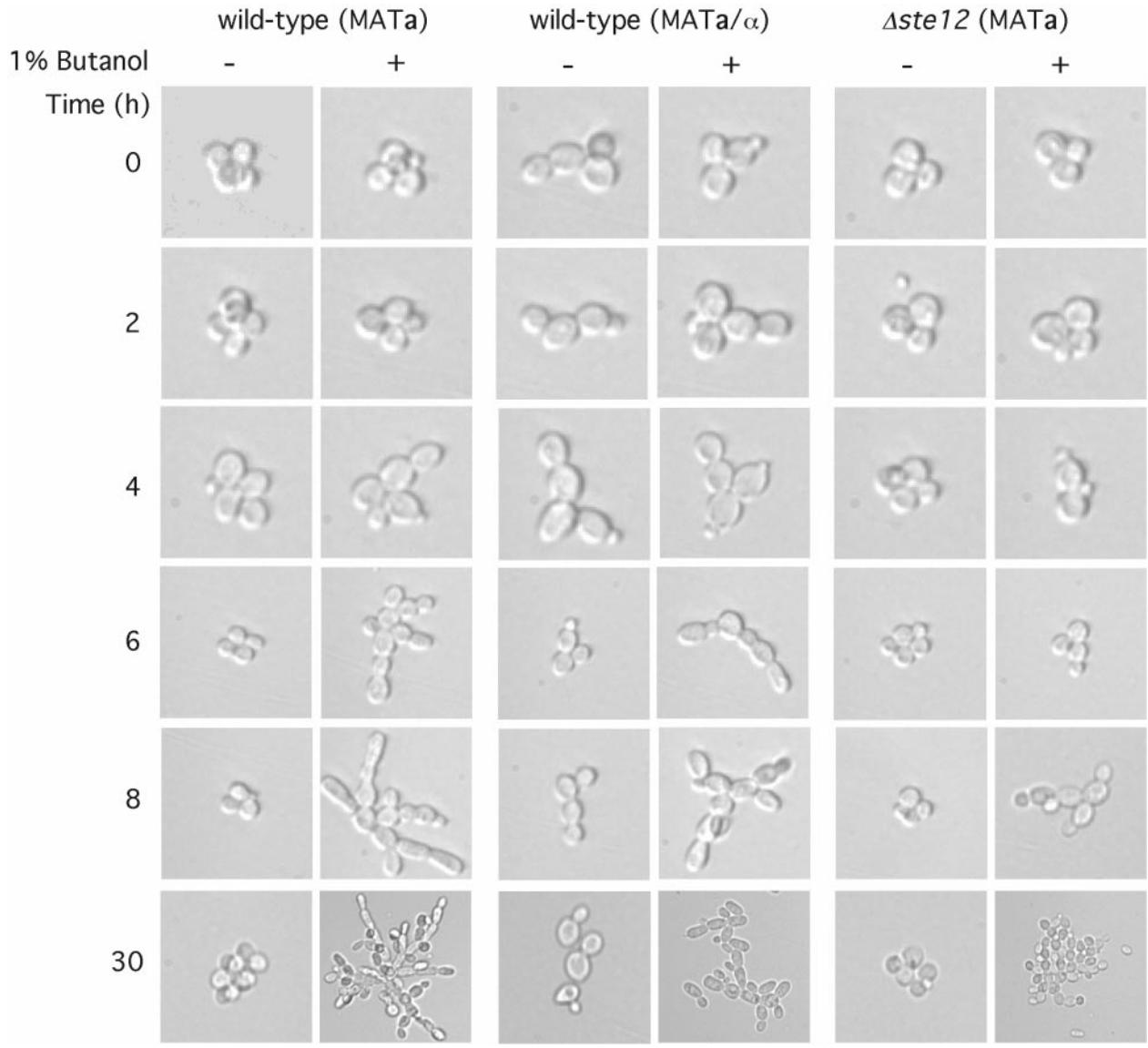

Figure 2. A time course of butanol-induced morphological changes in liquid medium. Strains MLY41 (wild-type *MAT***a**), MLY61 (wild-type *MAT***a**/α), and 27-06 (Δ*ste12 MAT***a**) were grown overnight in liquid YPD medium, diluted into 5 ml of YPD with (+) or without (−) 1% (vol/vol) butanol in 15-ml sealed, screw-cap tubes, and grown at 30°C for the indicated times. Cells were spotted to microscope slides and photographed at ×400 magnification. The images at the 30-h time point are presented at half the magnification of the other panels to show the multicellular asters that develop in the haploid wild-type strain.

Several alterations contribute to the formation of these structures, the most obvious being cellular elongation. However, it is also clear that haploid cells exposed to butanol alter their budding pattern. Haploid cells typically bud in an axial pattern, in which they choose the location for a new bud adjacent to previous bud sites, whereas diploid cells bud in a bipolar manner, in which buds can emerge from either end of the ovoid cell. The axial pattern tends to form tight clusters of cells (see Figure 2; wild-type *MAT***a** without butanol), and the bipolar pattern forms a more elongated cluster of cells. Bipolar buds in cultures of haploid strains can be seen as early as 4 h after exposure to butanol (e.g., compare *MAT***a** with butanol to *MAT***a**/α without butanol at the 4-h time point). The extent and regulation of the budding pattern switch is analyzed further below. Finally, the maintenance of these filamentous structures is undoubtedly aided by the highly flocculent nature of the Σ1278b strain. These asters are difficult to separate by sonication, although they can be micromanipulated apart (our unpublished results). As will be shown below, less flocculent strains also show some of these alterations.

Strains lacking the STE12 transcription factor are deficient in both diploid pseudohyphal differentiation and haploid invasive growth, in addition to being unable to mate (Hart-

Table 3. Butanol-induced budding pattern changes

Strain	Background	Genotype	*MAT*	% Bipolar budding YPD	% Bipolar budding +1% butanol
MLY40	Σ1278b	wt	α	3.9%	97.1%
MLY41	Σ1278b	wt	**a**	4.0	96.1
MLY61	Σ1278b	wt	**a**/α	98.0	97.2
27-06	Σ1278b	Δ*ste12*	**a**	2.4	89.5
10556-24D	W303	wt	**a**	24.0	90.5
10556-30B	W303	wt	α	24.5	93.5
FY2	S288C	wt	α	<0.5	3.3
FY69	S288C	wt	**a**	1.0	4.3

well, 1980; Liu *et al.*, 1993; Roberts and Fink, 1994). Curiously, in the liquid YPD + butanol assay, Δ*ste12* mutants show partial phenotypes (Figure 2). The budding pattern of this haploid strain has clearly switched to the bipolar pattern (see also Table 3), but its cells do not elongate. This pattern is also seen in other *ste* mutants known to affect filamentous growth (we have tested Δ*ste7* and Δ*ste11* mutant strains).

Analysis of Butanol-induced Phenotypes

Strain Background The Σ1278b strain background is commonly used for studies of filamentation because of its vigorous response to nitrogen starvation conditions, and the experiments presented in Figures 1 and 2 used this strain. To address the generality of this phenomenon, we tested strains of the W303 and S288c lineages. Both Σ1278b and W303 show morphological abnormalities after growth for 12 h in liquid YPD + 1% (vol/vol) butanol. W303 is a less flocculent strain than Σ1278b and does not form multicellular asters like Σ1278b, but elongated and misshapen cells can be readily observed (Figure 3A). Dickinson (1996) reported that W303 derivatives formed hyphae-like extensions in response to isoamyl alcohol less readily than the strain background he used regularly (IWP72); although we have not quantitated the frequency of aberrant morphologies in W303, Figures 3 and 4 show some examples of these unconventional morphologies in this strain. In contrast, the cell morphology of S288c is unaffected by butanol (Figure 3A). Even after 30 h of growth in liquid medium, the cellular morphology of S288c-derived strains is indistinguishable between cultures with and without butanol.

When grown on solid nitrogen-limiting SLAD medium, haploid Σ1278b strains form a filamentous colony morphology, whereas W303 strains do not. However, altered cellular morphologies are apparent in both Σ1278b and W303 strains under these conditions. Figure 3B shows cells scraped from solid SLAD medium (with or without butanol) after 4 d of incubation. Elongated cells and morphological abnormalities are readily apparent in Σ1278b and W303 strains grown in the presence of butanol; again, butanol does not affect the morphology of S288c strains, even in the presence of nitrogen starvation.

Strains of the S288c background are unable to undergo pseudohyphal differentiation. The genetic basis for this phenotype was found to be a mutation in a single gene, *FLO8*.

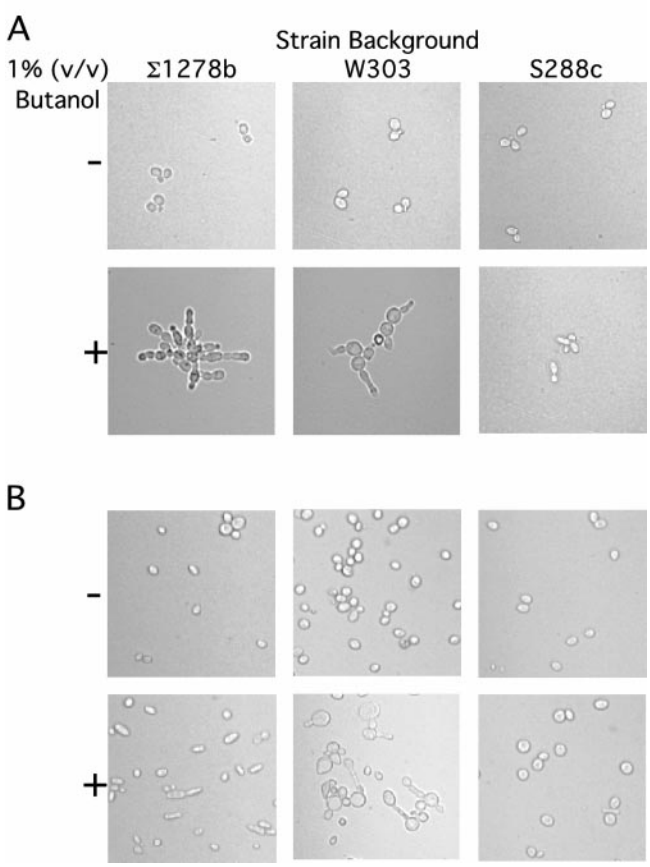

Figure 3. Effect of strain background on butanol-induced morphological changes. (A) Haploid strains MLY41 (Σ1278b), 10556-24D (W303), and FY69 (S288c) were incubated in liquid YPD with (+) or without (−) butanol and analyzed after 12 h for morphological abnormalities, as in Figure 2. (B) The same strains as in A were incubated on solid SLAD medium for 4 d at 30°C, scraped off the agar with a toothpick, and analyzed microscopically, as in Figure 2.

When a functional *FLO8* gene was introduced into S288c, diploid strains were able to form filaments (Liu *et al.*, 1996). We tested *FLO8*$^+$/FLO8$^+$ S288c strains in our assays and found that they did indeed form filaments on low-nitrogen SLAD medium and that a haploid *FLO8*$^+$ S288c filaments on SLAD + 1% butanol. However, *FLO8*$^+$ S288c strains do not form elongated or morphologically abnormal cells in the liquid YPD + 1% butanol assay (our unpublished results). Therefore, there are additional inputs to the alcohol-induced phenotypes in Σ1278b and W303 that are not present in S288c.

Σ1278b cells scraped from SLAD + 1% butanol plates show one of two morphologies: either elongated, cylindrical cells or round, yeast-form cells (see Figure 3B). In contrast, W303 adopts a variety of morphologies vastly different from the typical yeast-form shape (Figure 4). Figure 4A shows cells grown on SLAD for 4 d at 30°C, whereas panels B–I show cells grown on SLAD + 1% butanol. A common growth motif is a large round cell from which a slender projection emerges. These structures are apparent in panels B, C, and D. These projections can appear bent (E) or emerge

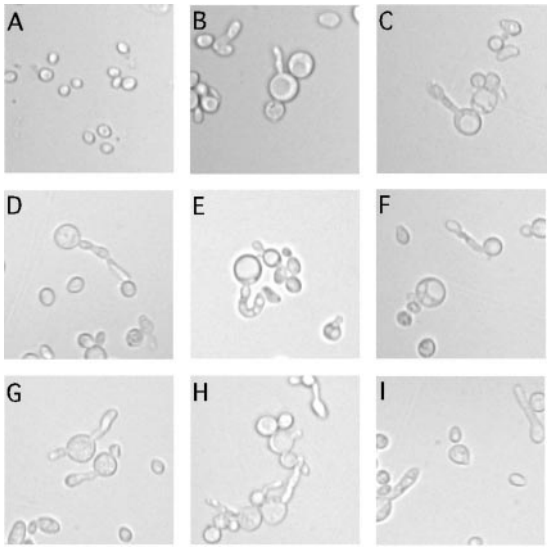

Figure 4. Aberrant morphologies induced in W303 after butanol exposure. Strain 10556-24D, harboring the *URA3* vector YEplac195 and the *LEU2* vector YEplac181, was incubated on solid SLAD medium with or without 1% butanol for 4 d at 30°C. Cells were scraped off the plate and resuspended in 10 μl of water on a microscope slide. (A) Typical cell morphology of this strain on medium lacking butanol. (B–I) Different morphologies of this W303 strain on SLAD + butanol.

from the cell at odd angles (F), and a single cell can have more than one projection (G). These structures are similar in appearance to germ tube formation in *C. albicans* after serum stimulation, but they are unusual for *S. cerevisiae*. In addition, some elongated structures that seem to lack a large cell body can be observed (H and I).

Budding Pattern We assayed the budding pattern of strains grown in the absence or presence of butanol to determine the extent to which haploid strains switch to a bipolar budding pattern and the effect of strain background on this phenomenon. In this assay, strains were incubated on solid YPD with or without 1% butanol for 2 d at 30°C and then restreaked to fresh medium. After 4 h (YPD) or 6 h (YPD + 1% butanol), microcolonies were examined for a tightly clustered (axial) or an elongated (bipolar) pattern. Only microcolonies with four or more cells were counted in this analysis.

Table 3 presents the percentage of microcolonies of each strain showing bipolar budding. Only 4% of Σ1278b haploids (*MAT***a**; 3.9% in *MAT*α) have bipolar budding patterns, whereas 96.1% (*MAT***a**; 97.1% in *MAT*α) bud in a bipolar manner after exposure to butanol. Again, deletion of *STE12* does not affect the butanol-induced switch. The normal diploid bipolar pattern (98.0%) is unaffected by butanol (97.2%). In the W303 lineage, the axial pattern of haploid cells is less uniform (~24% bipolar); nonetheless, butanol increases bipolar budding to >90%. Butanol does not affect budding pattern in strains of the S288c lineage.

Reporter Assays Two reporters have been developed that respond to activation of the STE MAPK pathway. The first uses the promoter of *FUS1*, encoding a cell surface protein that is dramatically up-regulated by pheromone treatment. A *FUS1-lacZ* fusion gene is induced several hundredfold during mating response (Trueheart *et al.*, 1987). The other reporter uses the filamentation response element (FRE) found in the control sequences of the Ty1 retrotransposon (a similar element is found in the *TEC1* promoter; Madhani and Fink, 1997) and has been correlated to MAPK activation in low-nitrogen conditions that stimulate diploid pseudohyphal growth (Laloux *et al.*, 1994; Mösch *et al.*, 1996; Madhani and Fink, 1997). These reporters are specific, in that the *FUS1-lacZ* gene does not respond to nitrogen starvation and the FRE-*lacZ* gene does not respond to pheromone (Mösch *et al.*, 1996; Madhani and Fink, 1997). We grew strains harboring each of these reporter genes in YNB with or without 1% butanol for either 4 or 24 h. At the 24-h time point, the morphology of haploid cells grown in YNB + 1% butanol was similar to the morphology of cells grown in rich (YPD) medium plus butanol, with abundant filamentous asters present. As shown in Figure 5, we found that neither of these reporters is induced by the presence of butanol. Other findings presented here, namely that butanol-treated Δ*ste12* cells do not elongate (Figure 2) and do not form filamentous colonies (see Figure 7), clearly imply a role for STE12 and the MAPK pathway in alcohol-induced filamentation. Yet, the lack of reporter activation after butanol treatment suggests that another avenue of specialization exists to regulate STE12 activity based on an upstream input.

Induction of Filamentous Colony Morphology Filamentation has been difficult to detect in rich medium; one explanation for this finding is that the rapid growth rate of cells in rich conditions allows colonies to overgrow and obscure any filaments that are formed. The central role for nitrogen starvation in regulating diploid pseudohyphal differentiation led us to ask whether nitrogen deprivation was also required for the colony phenotype induced by butanol or whether other starvation signals may allow a filamentous colony morphology after exposure to butanol. To test this question, we incubated strains on solid medium containing altered levels of nitrogen or carbon. The baseline in this experiment is YNB, a synthetic minimal medium with high levels of ammonium sulfate as a nitrogen source and glucose as a carbon source (37 mM ammonium, 2% glucose). We then used medium with 50 μM ammonium and 2% glucose (SLAD) or 37 mM ammonium and either 0.2% or 0.02% glucose. As shown in Figure 6, decreasing the glucose concentration also allows filamentous growth (as assayed by colony morphology) in the presence of butanol, suggesting that this alcohol can enable environmental inputs other than nitrogen starvation to trigger this differentiation pathway. Lambrechts *et al.* (1996) found that poorly used carbon sources such as the starch amylopectin can also induce filamentous growth, but pseudohyphal differentiation has not been observed previously on low-glucose medium. Thus, we conclude that nitrogen starvation, per se, is not required for the haploid, butanol-induced, filamentation phenotype. This phenomenon is also observed after starvation for carbon, and we suggest that it is simply a slow growth rate that is necessary to observe filaments upon butanol treatment.

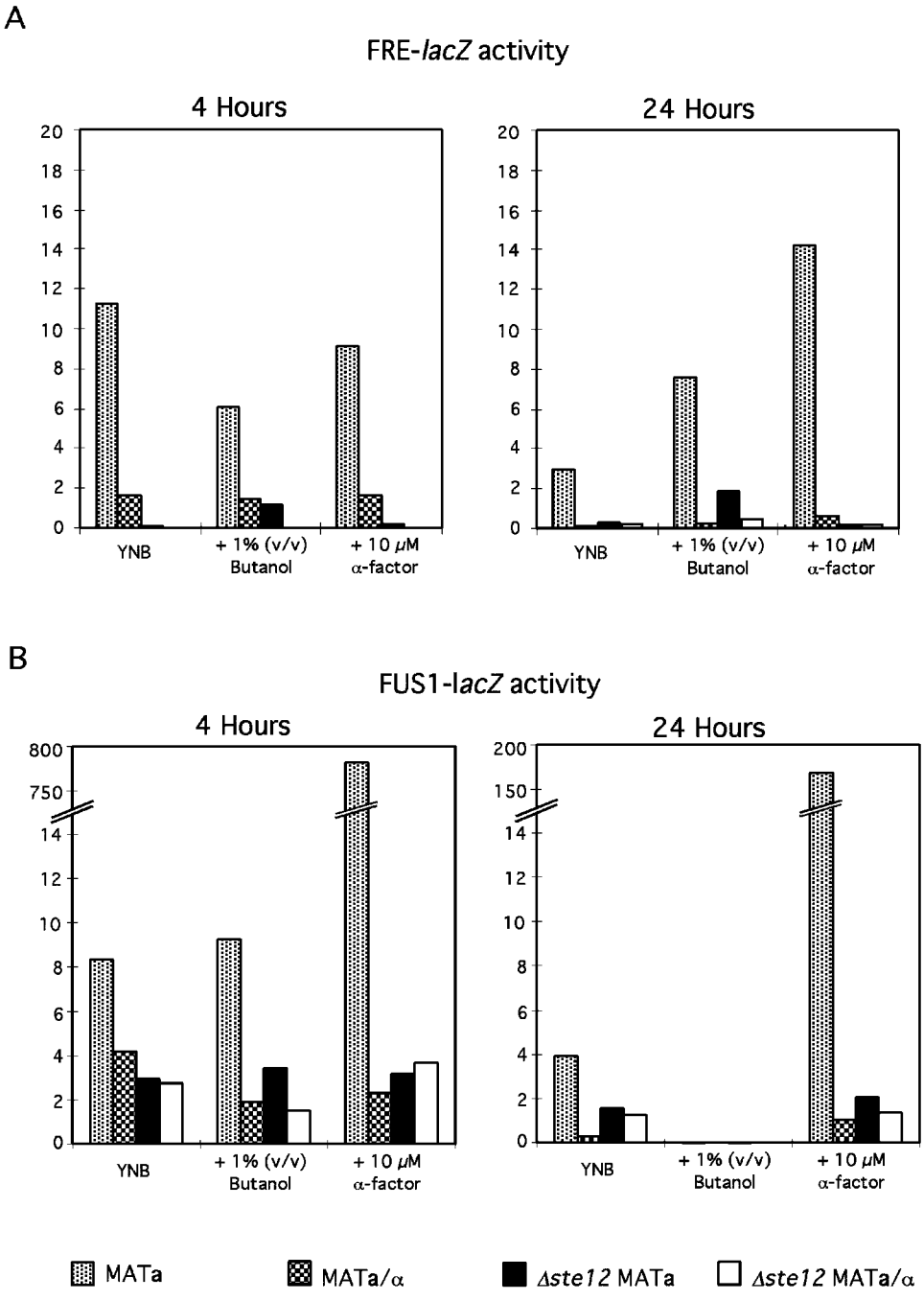

Figure 5. Reporter gene activity after butanol exposure. *MAT***a** and *MAT***a**/α strains, both wild type (MLY41, MLY61) and Δ*ste12* (MLY216a, MLY216a/α), were transformed with an empty *URA3* vector (YEplac195) and a *LEU2* vector containing either the *FUS1-lacZ* reporter (pJB207; top panels) or the FRE-*lacZ* reporter (pIL30-*LEU2*; bottom panels). The strains were then grown in YNB medium overnight (in duplicate) and split three ways; to the three cultures were added nothing, 1% (vol/vol) butanol, or 10 μM α-factor. β-Galactosidase activity was assayed after 4 or 24 h at 30°C in rolling cultures.

Other Phenotypes In addition to the phenotypes described above, we determined whether butanol would affect the processes of haploid invasive growth and mating, both of which require the pheromone-responsive MAPK pathway. The finding that butanol does not affect expression of either the *FUS1-lacZ* or the FRE-*lacZ* reporter gene (Figure 5) suggested that butanol would not alter invasive growth or mating, but each of these processes is complex, and reporter activity may not accurately reflect the effects of butanol. We found that butanol neither inhibits nor enhances haploid invasive growth. Haploid strains continue to invade the agar substrate, although invasion is delayed, presumably as a result of the slower growth rates on media containing butanol. Butanol does not allow diploid strains or haploid Δ*ste12* mutants to invade the agar substrate (our unpublished results). We also investigated mating in the presence of butanol and found that haploid strains are still able to conjugate in the presence of 1% butanol (our unpublished results). Thus, despite the multiple functions of the MAPK

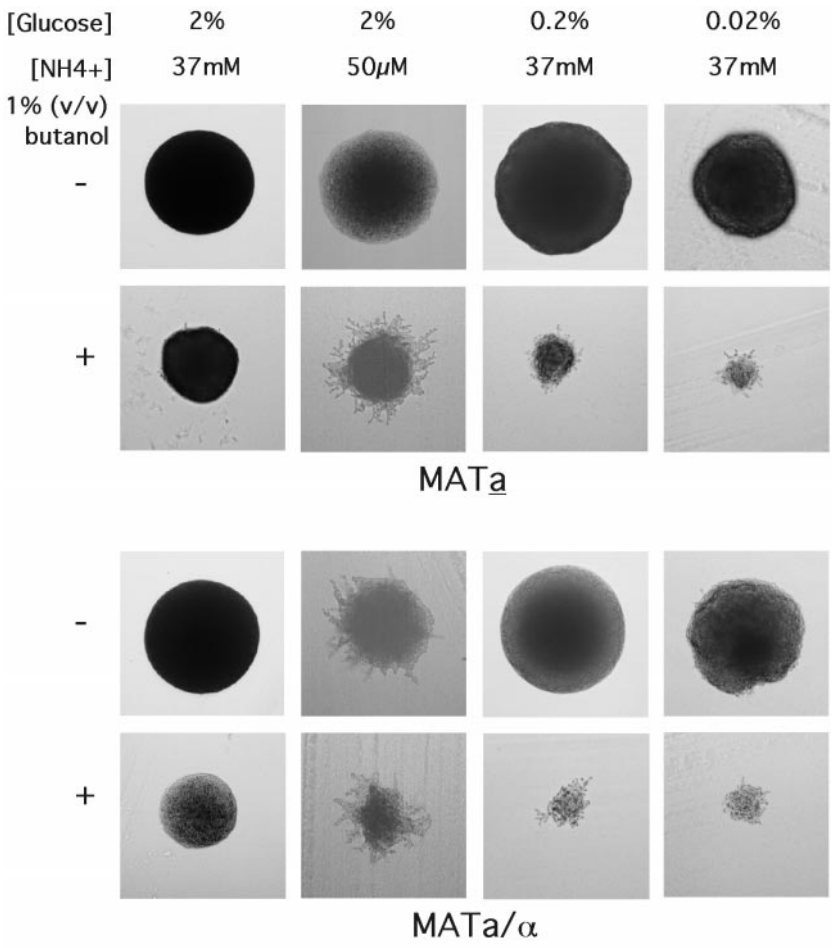

Figure 6. Butanol stimulates filamentation in glucose-poor medium. The *MAT***a** strain MLY41 (top panel) and the *MAT***a**/α strain MLY61 (bottom panel) were incubated on YNB (2% glucose, 37 mM ammonium), SLAD (2% glucose, 50 μM ammonium), or medium with 37 mM ammonium and either 0.2% or 0.02% glucose, either with (+) or without (−) 1% (vol/vol) butanol. Strains were grown for 4 d at 30°C.

pathway, the cell remains able to distinguish between the specific inputs to properly regulate these differentiation events.

Genetic Analysis of Butanol-induced Filamentation

Analysis of Known Mutations A number of mutations have been identified that block filamentation. Among these are elements of the MAPK pathway, including STE12 and its binding partner TEC1, and upstream elements postulated to be part of the nitrogen sensor, such as the G protein/receptor complex GPA2/GPR1 and the ammonium permease MEP2 (Liu *et al.*, 1993; Gavrias *et al.*, 1996; Lorenz and Heitman, 1997, 1998a; Lorenz *et al.*, 2000). When assayed for butanol-induced phenotypes (Figure 7), Δ*ste12* and Δ*tec1* mutations block colony filamentation stimulated by butanol. In some colonies of Δ*ste12* mutant strains grown in the presence of butanol, a weak residual filamentation can be seen, whereas the nonfilamentous phenotype of Δ*tec1* mutant strains is very tight (Figure 7). Differences in the severity of phenotypes conferred by these two mutations have been observed, and we have previously proposed that TEC1 may have STE12-independent functions in the regulation of filamentous growth (Lorenz and Heitman, 1998b). In contrast, butanol still promotes filamentous growth in strains lacking GPA2, GPR1, or MEP2, both on low-nitrogen solid medium (Figure 7) and in liquid rich medium; in fact, in *MAT***a**/α cells, butanol suppresses the pseudohyphal growth defect of Δ*gpa2*/Δ*gpa2*, Δ*gpr1*/Δ*gpr1*, and Δ*mep2*/Δ*mep2* strains on solid SLAD medium (our unpublished results). These findings suggest that this alcohol bypasses the nutrient-sensing apparatus but still requires elements of the MAPK pathway.

Many genes have been found to regulate filamentous growth, in addition to the mutants examined above (Figure 7). We assayed a collection of these strains for butanol-induced filamentous growth on solid SLAD medium. The results of these tests are presented in Table 4. Given the different phenotypes of Δ*mep2*, Δ*gpa2*, and Δ*gpr1* mutants (Figure 7) in nitrogen starvation–induced pseudohyphal growth versus butanol-induced filamentation, it was not surprising to find that several of these additional mutants behave differently in the two assays. Of note is the cell-surface flocculin FLO11, which is required for diploid filamentous growth and haploid invasive growth (Lo and Dranginis, 1998); the *FLO11* gene has a very large and complex promoter that responds to multiple signals, including

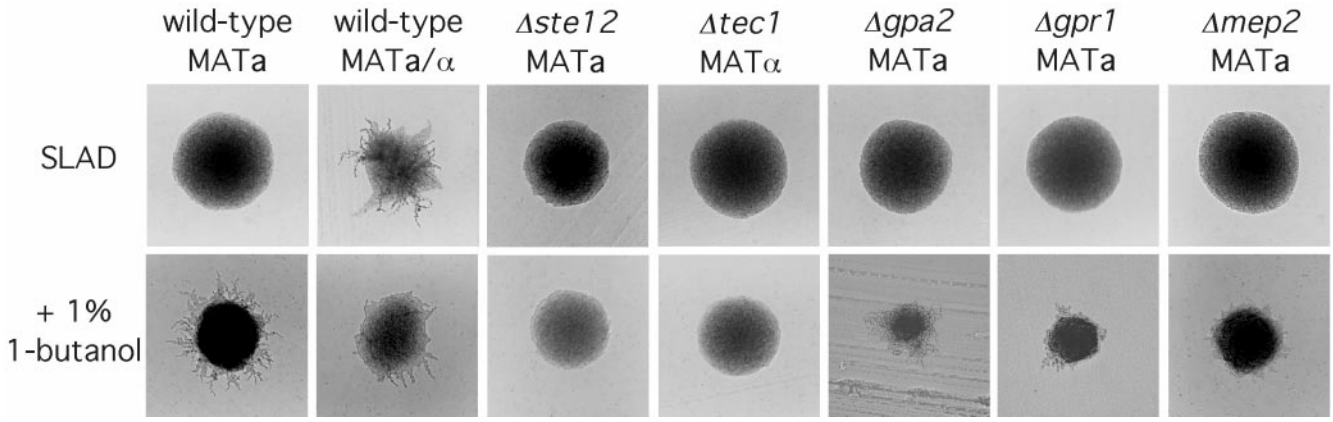

Figure 7. Genetic analysis of the butanol-induced phenomenon. Strains MLY41 (wild-type *MAT***a**), MLY61 (wild-type *MAT***a**/α), 27-06 (Δ*ste12 MAT***a**), MLY183a (Δ*tec1 MAT*α), MLY132a (Δ*gpa2 MAT***a**), MLY232a (Δ*gpr1 MAT***a**), and MLY108a (Δ*mep2 MAT***a**) were incubated on SLAD medium with or without 1% (vol/vol) butanol. After 4 d at 30°C, colonies were photographed at ×25 magnification.

both MAPK and cAMP signaling, and potentially other pathways (Rupp *et al.*, 1999). We found that *flo11* mutant strains are still able to form filaments on SLAD + 1% butanol medium. In addition, the finding that *ste4* mutants have no defects in butanol-induced filamentation indicates that, as for nitrogen-induced pseudohyphal growth, the pheromone-responsive G protein (GPA1, STE4, and STE18) does not regulate filamentation stimulated by butanol. Curiously, although *ste7*, *ste11*, and *ste12* mutants block butanol-induced filamentation to a similar degree, *ste20* mutants have little or no defect in this assay. In this strain background, *ste20* mutants have only a moderate defect in mating (e.g., they are not completely sterile); thus, a STE20 homologue, CLA4 perhaps, may substitute for STE20 to regulate butanol-induced filamentation. This is in marked contrast to diploid pseudohyphal growth, in which Δ*ste20*/Δ*ste20* mutants have a more severe phenotype than other *ste* mutants (Liu *et al.*, 1993), and further suggests that the diploid pseudohyphal and haploid alcohol-induced phenomena are distinct.

Table 4. Effects of various deletions on response to 1-butanol

Genotype	Filamentation in response to 1-butanol (haploid)[a]	Filamentation in response to nitrogen starvation (diploid)[b]	Reference[c]
wt *MAT***a**	+++	−	Gimeno *et al.*, 1992
wt *MAT***a**/α	+++	+++	Gimeno *et al.*, 1992
Δ*mep1*	++	+++	Lorenz and Heitman, 1998a
Δ*mep2*	++	−	Lorenz and Heitman, 1998a
Δ*mep3*	++	+++	Lorenz and Heitman, 1998a
Δ*mep1* Δ*mep2*	+/++	−	Lorenz and Heitman, 1998a
Δ*mep1* Δ*mep2* Δ*mep3*	+/++	−	Lorenz and Heitman, 1998a
Δ*gpa2*	++	−	Lorenz and Heitman, 1997
Δ*gpr1*	++	−	Lorenz *et al.*, 2000
Δ*ste4*	+++	+++	Liu *et al.*, 1993
Δ*ste20*	+++	−	Liu *et al.*, 1993
Δ*ste11*	−/+	−/+	Liu *et al.*, 1993
Δ*ste7*	−/+	−/+	Liu *et al.*, 1993
Δ*ste12*	−/+	−/+	Liu *et al.*, 1993
Δ*tec1*	−	−	Gavrias *et al.*, 1996
Δ*flo11*	++	−	Lo and Dranginis, 1998
Δ*flo8*	++	−	Liu *et al.*, 1996
Δ*msn1*	++	−	Lorenz and Heitman, 1998b
Δ*skn7*	++	−	Lorenz and Heitman, 1998b
Δ*mss11*	++	−	Lorenz and Heitman, 1998b
Δ*ash1*	++	−/+	Chandarlapaty and Errede, 1998

[a] Assays were performed on solid SLAD + 1% (vol/vol) butanol medium.

[b] Assays were performed in homozygous diploid strains on solid SLAD medium.

[c] Reference for the original description of the diploid phenotype.

Screen for Mutations That Block Alcohol-induced Filamentation We next screened for mutations that inhibit butanol-induced filamentation, with the use of a random, transposon-tagged insertional mutagenesis scheme (Burns *et al.*, 1994). We found 12 transposon-linked mutations that, when rescued from the genome, identified nine genes. A few of these represent genes known to regulate filamentous growth, such as *STE12* (Liu *et al.*, 1993), and the polarity establishment gene *BUD8* (Mösch and Fink, 1997), mutation of which disrupts the diploid bipolar budding pattern and causes cell wall defects (Lussier *et al.*, 1997). Although BEM1 and BEM4 had not previously been associated with filamentous growth, this finding was not surprising given their known role in polarity establishment. BEM1, in particular, is required for polarized growth both in budding and in mating response and interacts with STE20, the first kinase necessary for both mating and filamentous/invasive growth (Chenevert *et al.*, 1994; Leberer *et al.*, 1996). *bem1* mutants have delocalized cortical actin and chitin (Chenevert *et al.*, 1992), and it is likely that BEM1 links the MAPK signaling cascade to changes in the actin cytoskeleton. Similarly, BEM4 interacts with Rho-type GTPases that regulate actin cytoskeletal reorganization (Hirano *et al.*, 1996; Mack *et al.*, 1996).

The FIG1 protein has four predicted transmembrane domains and localizes to the cell surface after pheromone exposure. *fig1* mutant strains have weak mating defects and morphological abnormalities upon pheromone exposure, suggesting defects in polarized growth (Erdman *et al.*, 1998), although little is known about the function of the FIG1 protein. Again, given the known roles of these genes in morphogenesis or polarized growth, finding pseudohyphal phenotypes for these mutants was not surprising.

A second group of genes emerged from this screen whose involvement in filamentous growth was less obvious. CHD1 is a member of the chromodomain, helicase, DNA-binding family. Family members are highly conserved from yeast to mammals, although the function of these proteins is not well understood. Several reports propose a role for CHD homologues in regulating chromatin architecture in mouse and *Drosophila* (Stokes and Perry, 1995; Stokes *et al.*, 1996). Yeast *chd1* mutants show mild resistance to 6-azauracil, suggesting alterations in transcriptional efficiency (Woodage *et al.*, 1997). A direct connection between chromosome structure and filamentous growth has not been reported, and a role for CHD1 in filamentous growth was not expected. It is possible that CHD1 directly regulates filamentous growth in its role as a transcription factor by controlling the expression of genes necessary for this response. Alternatively, it may regulate this phenomenon indirectly via reorganization of chromatin structure, thus altering the transcription of effector genes.

The other genes found in this screen, MRP21, MSM1, and HMI1, have mitochondrial functions. MRP21 is a component of the mitochondrial ribosome small subunit, whereas MSM1 is the mitochondrial methionyl-tRNA synthetase. Both of these proteins, then, should affect mitochondrial protein synthesis, and mutations in both *MSM1* and *MRP21* are deficient in respiratory growth (Tzagoloff *et al.*, 1989; GreenWillms *et al.*, 1998), as are the mutations described here. HMI1, an essentially uncharacterized protein, is predicted to be in the mitochondria and has limited homology to helicases (Vandenbol *et al.*, 1995). These mutations suggest a role for respiration in filamentation, although an analysis of mitochondrial function as it relates to pseudohyphal growth has not been reported. Several previous screens failed to find mitochondrial mutations that blocked filamentation. It was initially possible that these genes were specific to alcohol-induced filamentous growth, but, as described below, MSM1 and MRP21 also affect diploid (nitrogen-starved) pseudohyphal differentiation.

We wished to analyze each of the mutations described above for effects on budding pattern, cell elongation, diploid pseudohyphal differentiation, and haploid invasive growth. In the process of determining whether the observed mutant phenotypes were linked to the transposon insertion, we had a great deal of difficulty demonstrating linkage after meiosis because of extremely poor spore viability. (The poor spore viability was not linked to the transposon insertion, because the *LEU2*-marked transposon insertion segregated independently of the spore lethality.) As a result, we created a series of strains with complete deletions in each of these genes. The phenotypes of each of these deletions for diploid pseudohyphal growth, haploid invasive growth, and cell elongation and budding pattern after butanol treatment are listed in Table 5. We assayed cell elongation in liquid culture as shown in Figure 2 and budding pattern as described in Table 3.

We found only two mutations from this screen for which the pseudohyphal and butanol-induced filamentation phenotypes differed, namely, the putative mitochondrial helicase HMI1 and FIG1. The difference between the haploid and diploid phenotypes for *fig1* mutant strains was modest, however. For *hmi1* mutants, in contrast, the difference was significant. It is curious that the three mitochondrial mutants (*hmi1*, *msm1*, and *mrp21*) do not behave similarly, but it should be noted that the connection between HMI1 and mitochondrial function is tenuous. The results presented in Tables 4 and 5 demonstrate that although butanol is able to stimulate filamentous growth of haploid strains containing mutations that block diploid filamentous growth (e.g., Δ*mep2*, Δ*flo11*, and Δ*gpa2*), very few mutations that block butanol-induced filamentation still permit nitrogen-induced filamentation.

Another notable observation from Table 5 is that only a few of these mutations also affect haploid invasive growth. This was surprising, because most of the genes found to regulate diploid pseudohyphal growth also regulate haploid invasion, such as the MAPK pathway and TEC1 (Roberts and Fink, 1994), although this is not universally true, because Δ*mep2* mutations do not alter haploid invasion (Lorenz and Heitman, 1998a). *bem1* mutations inhibit invasion, whereas *bem4* mutations do not; similarly, *msm1* mutations block invasion, but *mrp21* mutations do not. These findings confirm earlier suggestions that only a subset of filamentation genes also regulate invasion. Furthermore, this is the second behavior for which the mitochondrial mutants do not show similar phenotypes, suggesting either that only a specific mitochondrial function is required for invasion or that MSM1 may have a role in nonmitochondrial processes.

Finally, these mutations prevent the filamentous colony morphology in response to butanol, but they have little

Table 5. Summary of mutations not responsive to butanol

Gene	Insertion point[a]	Filamentation (1% butanol)[b]	Pseudohyphal growth[c]	Haploid invasion[c]	Cell elongation[c]	% Bipolar budding[c]	
						YPD	+ 1% butanol
wt (MAT**a**)	NA	+	−	+	+	<1	98
wt (MAT**a**/α)	NA	+	+	−	+	96	97
STE12	−116	−/+	−	−	−	2	90
	+525	−					
	+1562	−					
BUD8[d]	−89	−	−	+	+	2	2
	+723	−					
BEM1	+1611	−	−	−/+	−	<1	81
BEM4	+225	−	−	+	−	1	82
FIG1	−2381	−/+	+/−	+	−	<1	62
CHD1	+89	−	−	−	+	<1	92
MRP21	+125	−/+	−	+/−	+/−	<1	82
MSM1	+1376	−	−	−	+/−	<1	67
HMI1	+1095	−/+	+	+/−	+/−	<1	96

[a] Insertion point indicates the position at which the transposon integrated, relative to the translation start site (+1).

[b] The butanol-induced filamentation response was assayed using the mutants isolated from the transposon-tagged screen. The filamentation phenotypes seen in the screen isolates and the complete knockouts are similar.

[c] These phenotypes were assayed using precise deletions for *BEM1, BEM4, FIG1, MSM1, MRP21,* and *HMI1,* and using screen isolates for *STE12, CHD1,* and *BUD8.*

[d] Several of the phenotypes for *bud8* mutants have been previously reported (Zahner *et al.,* 1996; Mösch and Fink, 1997; Yang *et al.,* 1997).

effect on the switch to a bipolar budding pattern. The notable exception is the *bud8* mutation, but this mutation is known to have defects in both bipolar and unipolar budding (Zahner *et al.*, 1996; Yang *et al.*, 1997). The pseudohyphal, cell elongation, and invasion phenotypes described in Table 5 for *bud8* mutants have been reported previously (Mösch and Fink, 1997). Several other mutations reduce the number of butanol-treated cells that exhibit a bipolar pattern, but none of these reduces the proportion of bipolar cells to <50%, so it is unlikely that this phenomenon is responsible for the nonfilamentous phenotype.

Ethanol-induced Hyperfilamentation

As shown in Figure 1, we identified a role for ethanol in stimulating hyperfilamentation of diploid cells in nitrogen-limiting conditions. As with the butanol-induced phenotypes, we have analyzed the genetic regulation of this phenomenon. Mutations in *STE12* and *TEC1* blocked ethanol-induced hyperfilamentation (Figure 8), as was also the case with butanol, although weak filamentation could be seen in Δ*ste12* mutants (Figure 8). In contrast, ethanol suppressed the pseudohyphal defects of Δ*mep2*/Δ*mep2*, Δ*gpa2*/Δ*gpa2*, and Δ*gpr1*/Δ*gpr1* strains. Again, these data are similar to our findings for the regulation of butanol-induced filamentation

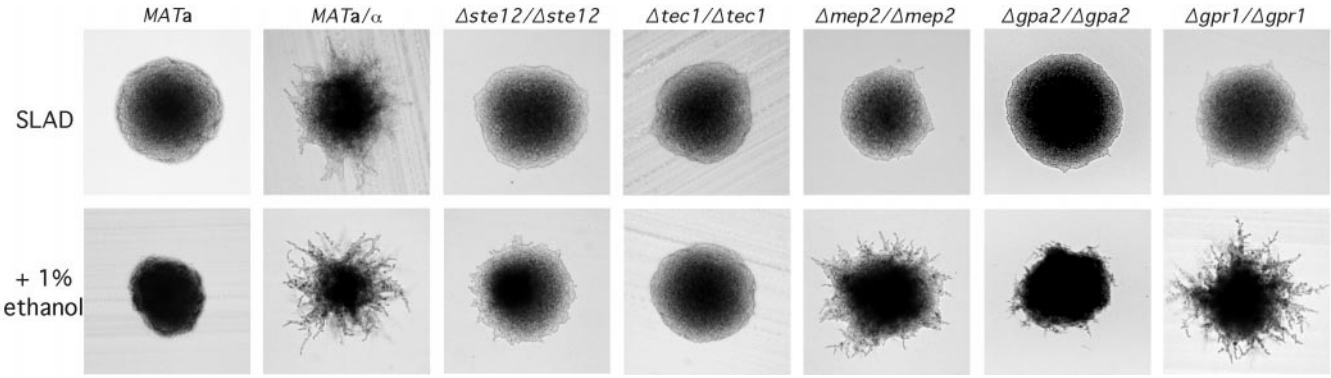

Figure 8. Genetic analysis of the ethanol-induced phenomenon. Strains MLY41 (wild-type *MAT***a**), MLY61 (wild-type *MAT***a**/α), HLY352 (Δ*ste12*/Δ*ste12 MAT***a**/α), MLY183a/α (Δ*tec1*/Δ*tec1 MAT***a**/α), MLY108a/α (Δ*mep2*/Δ*mep2 MAT***a**/α), MLY132a/α (Δ*gpa2*/Δ*gpa2 MAT***a**/α), and MLY232a/α (Δ*gpr1*/Δ*gpr1 MAT***a**/α) were incubated on SLAD medium with or without 1% (vol/vol) ethanol. After 2 d at 30°C, colonies were photographed at ×25 magnification.

in haploid cells, which is a requirement for STE12/TEC1 signaling, but independence from the MEP2 and GPR1/GPA2 nutrient sensors.

DISCUSSION

The data presented here demonstrate a role for various alcohols in both cellular and colony morphology in *S. cerevisiae*. Some of these alcohols are products of amino acid metabolism, such as isoamyl alcohol and butanol, which accumulate specifically in conditions of nitrogen starvation. These alcohols stimulate haploid cells to differentiate into a filamentous form similar to diploid-specific, nitrogen starvation–induced, pseudohyphal development. Both of these phenomena involve an elongated cell morphology, alterations in budding pattern, and a dependence on elements of the pheromone-responsive MAPK pathway.

These phenotypes are strongly dependent on the particular yeast strain under study. The structures formed by strains of the Σ1278b background are strikingly similar to pseudohyphal cells, with elongated, cylindrical cells. W303 derivatives, however, adopt a variety of morphologies, including ellipsoidal yeast-form cells, elongated, cylindrical shapes, and rounded yeast cells projecting a thin, hyphal projection reminiscent of germ tube formation in *C. albicans*. In contrast, strains of the S288c lineage seem completely unaffected by the presence of these alcohols.

This phenotype represents the third process that the pheromone-responsive MAPK pathway regulates in haploid cells. First is mating response, which is activated by pheromone binding to a cell surface receptor/G protein complex and signals through the FUS3 MAPK associated with the STE5 scaffolding protein. Second is haploid invasion, a process stimulated by unknown stimuli, which does not require STE5 and signals primarily through the KSS1 MAPK. Third is butanol-induced filamentation, which also uses the same MAPK pathway. We have not, however, analyzed the roles of STE5, FUS3, and KSS1 in detail. Reporter genes responsive to either pheromone activation (*FUS1-lacZ*) or filamentation/invasion activity (FRE-*lacZ*) do not respond to butanol, indicating again that there is a specialization of this signaling pathway. Furthermore, the phenotypes of Δ*ste20* mutations differ in these assays. In the Σ1278b background, Δ*ste20*/Δ*ste20* mutant strains have a severe defect in pseudohyphal growth—more severe, in fact, that other *ste* mutants. However, Δ*ste20* haploid strains have only a modest defect in mating and essentially no defect in butanol-induced filamentation (our unpublished observations). Thus, there must be pathway-specific specialization at the STE20 step, as there is for STE12 and the MAPKs KSS1 and FUS3.

Although these morphological changes are at least partially dependent on the STE MAPK pathway, they are independent of elements of the nutrient sensors, including GPA2, GPR1, and MEP2. This suggests that the alcohols are bypassing the need for nitrogen starvation, an idea supported by the behavior of strains grown in rich (YPD) liquid medium plus butanol (Figure 2) and by the filamentous colony morphology on nitrogen-rich, glucose-poor medium (Figure 6). This would be similar in concept to haploid invasive growth, in which haploid cells grown on rich solid medium invade the agar substrate. Because this occurs on rich medium, it is unlikely to be a nutrient response, and the mutations in the nutrient-sensing machinery have either no defect in invasive growth (Δ*mep2*; Lorenz and Heitman, 1998a) or a severe defect (Δ*gpa2* and Δ*gpr1*; Pan and Heitman, 1999).

A screen for additional mutants that affect butanol-induced filamentous growth identified several genes not previously appreciated to have filamentation phenotypes. Genes such as *BEM1*, *BEM4*, *BUD8*, and *FIG1* have been implicated in polarized growth; thus, their involvement in this phenomenon is not surprising. The effects of other genes, such as *CHD1*, which encodes a transcription factor homologue, are less obvious. With the exception of only the putative mitochondrial helicase HMI1 (and perhaps FIG1), mutations that block butanol-induced (haploid) filamentation also block nitrogen-induced (diploid) filamentation.

In addition to the haploid phenotypes, we found that ethanol induces hyperfilamentation of diploid strains on low-nitrogen medium. Ethanol has no effect on the colony or cellular morphology of haploid cells. Again, this phenotype requires TEC1 and the pheromone-response elements that regulate filamentous growth, but it does not require GPA2, GPR1, and MEP2. Thus, as with the haploid phenotype, ethanol stimulates filamentation in a manner that bypasses the nutrient-sensing machinery.

Why do these alcohols have effects on colony morphology? It has been suggested that pseudohyphal differentiation is a means by which yeast cells scavenge for nutrients in scarce conditions (Gimeno *et al.*, 1992). Because these alcohols bypass the presumptive nutrient-sensing apparatus, it is possible that they represent an alternative means to sense nutrient availability. As yeast cells metabolize the available nutrients and their own proteins and amino acids as nitrogen sources, the concentration of by-products such as isoamyl alcohol and, in particular, ethanol increases. Yeast may have a mechanism to estimate nutrient availability based on the levels of its own by-products. Alternatively, these alcohols may be toxic to the cell, and high concentrations may stimulate filamentous growth to allow the cell to escape the poisoned environment. Indeed, the presence of butanol, isoamyl alcohol, or ethanol (at high concentrations) slows growth rates, supporting this idea.

A third possibility is that yeast uses the concentration of these alcohols as a mechanism to sense population density and coordinate development appropriately. Quorum sensing of this nature is common in bacteria. Population density is one signal that regulates competence development in *Bacillus subtilis*. A secreted pheromone (ComX) activates a two-component signaling pathway once it passes a threshold concentration (reviewed by Grossman, 1995). Other bacteria, such as *Vibrio fischeri*, control autofluorescence based on population density with the use of secreted autoinducers, mostly related to *N*-acyl homoserine lactones (reviewed by Hellingwerf *et al.*, 1998). Quorum-sensing systems have also been described in both plant and human pathogens (*Agrobacterium tumefaciens* and *Pseudomonas aeruginosa*, respectively) to regulate expression of virulence factor genes.

Whatever the reason for this phenomenon in yeast, there must be a cellular mechanism to sense the presence of these alcohols. Although the STE MAPK pathway is required for the full expression of butanol-induced phenotypes, this pathway is not likely to represent the only butanol-respon-

sive signaling system in yeast. As shown in Figure 2 and Table 3, haploid Δ*ste12* mutants change their budding pattern in response to butanol; thus, this element of the phenotype is necessarily independent of the MAPK pathway. For this reason, we expect there to be a system (or systems) to recognize the presence of these alcohols and transduce a signal to multiple downstream pathways, including the MAPK pathway.

It is possible that, rather than sensing the alcohols themselves, cells sense intermediates in the conversion of amino acids to alcohols. By this model, addition of alcohols to the medium would be expected to increase the concentration of these intermediates in the cell. Several distinct pathways have been identified that convert leucine to isoamyl alcohol or valine to isobutyl alcohol (Dickinson *et al.*, 1997, 1998); the pathways are overlapping and interconnected, supporting the idea that an intermediate could be the relevant signaling molecule. We tend not to support this idea, though, because we also see these affects with 1-butanol and *tert*-amyl alcohol, which, although related to isoamyl and isobutyl alcohols, are not derived from any common amino acids. Moreover, neither α-ketoisocaproic acid (derived from leucine) nor α-ketoisovaleric acid (derived from valine) affected colony morphology when added to solid SLAD medium (Lorenz, Cardenas, and Heitman, unpublished observations).

There are a few precedents for the idea of branched chain amino acids or their derivatives in signaling roles. One study has proposed a role for branched chain amino acids (such as leucine and valine) in the control of translation (Xu *et al.*, 1998). Addition of these amino acids to pancreatic β-cells stimulated phosphorylation of the PHAS-I and $p70^{S6k}$ proteins. Phosphorylated forms of both PHAS-I and $p70^{S6k}$ stimulate translation, PHAS-I via interactions with the mRNA cap-binding protein eIF-4E and $p70^{S6k}$ via phosphorylation of ribosomal protein S6. Although this study correlated the effects with essential versus nonessential amino acids, it also showed that α-ketoisocaproic acid had this effect as well, suggesting that the relevant signaling molecule may be a by-product rather than the amino acids themselves. In microorganisms, branched chain amino acid metabolism has been linked to growth and development in the Gram-negative bacterium *Myxococcus xanthus* (Toal *et al.*, 1995). Mutations at the *esg* locus confer growth defects in minimal medium and defects in aggregation and differentiation during development of multicellular fruiting bodies. The *esg* locus encodes a branched chain keto acid dehydrogenase, an enzyme that converts α-ketoacids (the transamination products of branched chain amino acids) to short, branched chain fatty acids. Indeed, several fatty acids can rescue the developmental defects of *esg* mutants. In this case, the signaling molecule is not an alcohol by-product but a fatty acid; nevertheless, the involvement of branched chain amino acid metabolism is shared between development in *M. xanthus* and the phenotypes described here for *S. cerevisiae*.

Butanol-induced filamentous growth offers an additional advantage that has thus far been impossible in the analysis of filamentation. The recent advent of DNA array or chip experiments presents the possibility of understanding the transcriptional program of filamentous growth. This is undoubtedly complex, because a large number of transcriptional regulators (perhaps 18 to date) have been linked with filamentous growth in many laboratories. Standard pseudohyphal growth is confined to solid medium, and not all cells become elongated or invasive, thus making array experiments extremely difficult. Butanol allows "filamentous" growth in liquid medium, and virtually all cells show some aspects of this behavior, making the butanol-induced phenomenon amenable to array analysis. These experiments are under way and hopefully will shed light on the regulation of filamentous growth.

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REFERENCES

Banuett, F. (1995). Genetics of *Ustilago maydis*, a fungal pathogen that induces tumors in maize. Annu. Rev. Genet. *29*, 179–208.

Burns, N., Grimwade, B., Ross-MacDonald, P.B., Choi, E.Y., and Finberg, K. (1994). Large-scale analysis of gene expression, protein localization, and gene disruption in *Saccharomyces cerevisiae*. Genes Dev. *8*, 1087–1105.

Cardenas, M., Hemenway, C., Muir, R.S., Ye, R., Fiorentino, D., and Heitman, J. (1994). Immunophilins interact with calcineurin in the absence of exogenous immunosuppressive ligands. EMBO J. *13*, 5944–5957.

Chandarlapaty, S., and Errede, B. (1998). Ash1, a daughter cell-specific protein, is required for pseudohyphal growth of *Saccharomyces cerevisiae*. Mol. Cell. Biol. *18*, 2884–2891.

Chenevert, J., Corrado, K., Bender, A., Pringle, J., and Herskowitz, I. (1992). A yeast gene (*BEM1*) necessary for cell polarization whose product contains two SH3 domains. Nature *356*, 77–79.

Chenevert, J., Valtz, N., and Herskowitz, I. (1994). Identification of genes required for normal pheromone-induced cell polarization in *Saccharomyces cerevisiae*. Genetics *136*, 1287–1297.

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

Dickinson, J.R. (1994). Irreversible formation of pseudohyphae by haploid *Saccharomyces cerevisiae*. FEMS Microbiol. Lett. *119*, 99–103.

Dickinson, J.R. (1996). "Fusel" alcohols induce hyphal-like extensions and pseudohyphal formation in yeasts. Microbiology *142*, 1391–1397.

Dickinson, J.R., Harrison, S.J., and Hewlins, M.J.E. (1998). An investigation of the metabolism of valine to isobutyl alcohol in *Saccharomyces cerevisiae*. J. Biol. Chem. *273*, 25751–25756.

Dickinson, J.R., Lanterman, M.M., Danner, D.J., Pearson, B.M., Sanz, P., Harrison, S.J., and Hewlins, M.J.E. (1997). A ^{13}C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae*. J. Biol. Chem. *272*, 26871–26878.

Erdman, S., Lin, L., Malczynski, M., and Snyder, M. (1998). Pheromone-regulated genes required for yeast mating differentiation. J. Cell Biol. *140*, 461–483.

Gavrias, V., Andrianopoulos, A., Gimeno, C.J., and Timberlake, W.W. (1996). *Saccharomyces cerevisiae TEC1* is required for pseudohyphal growth. Mol. Microbiol. *19*, 1255–1263.

Gietz, R.D., and Sugino, A. (1988). New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-bp restriction sites. Gene *74*, 527–534.

Gimeno, C.J., Ljungdahl, P.O., Styles, C.A., and Fink, G.R. (1992). Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and RAS. Cell *68*, 1077–1090.

Green-Willms, N.S., Fox, T.D., and Costanzo, M.C. (1998). Functional interactions between yeast mitochondrial ribosomes and mRNA 5′ untranslated leaders. Mol. Cell. Biol. *18*, 1826–1834.

Grossman, A.D. (1995). Genetic networks controlling the initiation of sporulation and the development of genetic competence in *Bacillus subtilis*. Annu. Rev. Genet. *29*, 477–508.

Guthrie, C., and Fink, G. (1991). Guide to Yeast Genetics and Molecular Biology, San Diego: Academic Press.

Hartwell, L.H. (1980). Mutants of *Saccharomyces cerevisiae* unresponsive to cell division control by polypeptide mating hormone. J. Cell Biol. *85*, 811–822.

Hellingwerf, K.J., Crielaard, W.C., Teixeira de Mattos, M.J., Hoff, W.D., Kort, R., Verhamme, D.T., and Avignone-Rossa, C. (1998). Current topics in signal transduction in bacteria. Antonie Leeuwenhoek *74*, 211–227.

Hirano, H., et al. (1996). ROM7/BEM4 encodes a novel protein that interacts with the Rho1p small GTP-binding protein in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *16*, 4396–4403.

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

Kübler, E., Mösch, H.-U., Rupp, S., and Lisanti, M.P. (1997). Gpa2p, a G-protein alpha-subunit, regulates growth and pseudohyphal development in *Saccharomyces cerevisiae* via a cAMP-dependent mechanism. J. Biol. Chem. *272*, 20321–20323.

Laloux, I., Jacobs, E., and Dubois, E. (1994). Involvement of SRE element of Ty1 transposon in TEC1-dependent transcriptional activation. Nucleic Acids Res. *22*, 999–1005.

Lambrechts, M.G., Bauer, F.F., Marmur, J., and Pretorius, I.S. (1996). Muc1, a mucin-like protein that is regulated by Mss10, is critical for pseudohyphal differentiation in yeast. Proc. Natl. Acad. Sci. USA *93*, 8419–8424.

Leberer, E., Harcus, D., Broadbent, I.D., Clark, K.L., Dignard, D., Ziegelbauer, K., Schmidt, A., Gow, N.A.R., Brown, A.J.P., and Thomas, D.Y. (1996). Signal transduction through homologs of the Ste20p and Ste7p protein kinases can trigger hyphal formation in the pathogenic fungus *Candida albicans*. Proc. Natl. Acad. Sci. USA *93*, 13217–13222.

Liu, H., Styles, C.A., and Fink, G.R. (1993). Elements of the yeast pheromone response pathway required for filamentous growth of diploids. Science *262*, 1741–1744.

Liu, H., Styles, C.A., and Fink, G.R. (1996). *Saccharomyces cerevisiae* S288C has a mutation in *FLO8*, a gene required for filamentous growth. Genetics *144*, 967–978.

Lo, W.S., and Dranginis, A.M. (1998). The cell surface flocculin Flo11 is required for pseudohyphae formation and invasion by *Saccharomyces cerevisiae*. Mol. Biol. Cell *9*, 161–171.

Lorenz, M.C., and Heitman, J. (1997). Yeast pseudohyphal growth is regulated by GPA2, a G protein α homolog. EMBO J. *16*, 7008–7018.

Lorenz, M.C., and Heitman, J. (1998a). The MEP2 ammonium permease regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. EMBO J. *17*, 1236–1247.

Lorenz, M.C., and Heitman, J. (1998b). Regulators of pseudohyphal differentiation in *Saccharomyces cerevisiae* identified through multicopy suppressor analysis in ammonium permease mutant strains. Genetics *150*, 1443–1457.

Lorenz, M.C., Pan, X., Harashima, T., Cardenas, M.E., Xue, Y., Hirsch, J.P., and Heitman, J. (2000). The G protein-coupled receptor Gpr1 is a nutrient sensor that regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. Genetics (*in press*).

Lussier, M., et al. (1997). Large scale identification of genes involved in cell surface biosynthesis and architecture in *Saccharomyces cerevisiae*. Genetics *147*, 435–450.

Mack, D., Nishimura, K., Dennehey, B.K., Arbogast, T., Parkinson, J., Toh-e, A., Pringle, J.R., Bender, A., and Matsui, Y. (1996). Identification of the bud emergence gene *BEM4* and its interactions with rho-type GTPases in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *16*, 4387–4395.

Madhani, H.D., and Fink, G.R. (1997). Combinatorial control required for the specificity of yeast MAPK signaling. Science *275*, 1314–1317.

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

Mitchell, T.K., and Dean, R.A. (1995). The cAMP-dependent protein kinase catalytic subunit is required for appresorium formation and pathogenesis by the rice blast pathogen *Magnaporthe grisea*. Plant Cell *7*, 1869–1878.

Mösch, H.-U., and Fink, G.R. (1997). Dissection of filamentous growth by transposon mutagenesis in *Saccharomyces cerevisiae*. Genetics *145*, 671–684.

Mösch, H.-U., Roberts, R.L., and Fink, G.R. (1996). Ras2 signals via the Cdc42/Ste20/mitogen-activated protein kinase module to induce filamentous growth in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA *93*, 5352–5356.

Pan, X., and Heitman, J. (1999). Cyclic AMP-dependent protein kinase regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *19*, 4874–4887.

Roberts, R.L., and Fink, G.R. (1994). Elements of a single MAP kinase cascade in *Saccharomyces cerevisiae* mediate two developmental programs in the same cell type: mating and invasive growth. Genes Dev. *8*, 2974–2985.

Rupp, S., Summers, E., Lo, H.J., Madhani, H.D., and Fink, G.R. (1999). MAP kinase and cAMP filamentation signaling pathways converge on the unusually large promoter of the yeast *FLO11* gene. EMBO J. *18*, 1257–1269.

Stokes, D.G., and Perry, R.P. (1995). DNA-binding and chromatin localization properties of CHD1. Mol. Cell. Biol. *15*, 2745–2753.

Stokes, D.G., Tartof, K.D., and Perry, R.P. (1996). CHD1 is concentrated in interbands and puffed regions of *Drosophila* polytene chromosomes. Proc. Natl. Acad. Sci. USA *93*, 7137–7142.

Toal, D.R., Clifton, S.W., Rose, B.A., and Downard, J. (1995). The *esg* locus of *Myxococcus xanthus* encodes the E1α and E1β subunits of a branched chain keto acid dehydrogenase. Mol. Microbiol. *16*, 177–189.

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

Tzagoloff, A., Vambutas, A., and Akai, A. (1989). Characterization of *MSM1*, the structural gene for yeast mitochondrial methionyl-tRNA synthetase. Eur. J. Biochem. *179*, 365–371.

Vandenbol, M., Durand, P., Portetelle, D., and Hilger, F. (1995). Sequence analysis of a 44 kb DNA fragment of yeast chromosome XV including the Ty1–H3 retrotransposon, the *suf1*(+) frameshift suppressor gene for tRNA-Gly, the yeast tRNA-Thr-1a and a delta element. Yeast *11*, 1069–1075.

Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994). New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast *10*, 1793–1808.

Woodage, T., Basrai, M.A., Baxevanis, A.D., Hieter, P., and Collins, F.S. (1997). Characterization of the CHD family of proteins. Proc. Natl. Acad. Sci. USA *94*, 11472–11477.

Xu, G., Kwon, G., Marshall, C.A., Lin, T.-A., Lawrence, J.J.C., and McDaniel, M.L. (1998). Branched-chain amino acids are essential in the regulation of PHAS-I and p70 S6 kinase by pancreatic β-cells. J. Biol. Chem. *273*, 28178–28184.

Xu, J.-R., and Hamer, J.E. (1996). MAP kinase and cAMP signaling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. Genes Dev. *10*, 2696–2706.

Yang, Y., Ayscouch, K.R., and Drubin, D.G. (1997). A role for the actin cytoskeleton of *Saccharomyces cerevisiae* in bipolar bud-site selection. J. Cell Biol. *136*, 111–123.

Zahner, J.E., Harkins, H.A., and Pringle, J.R. (1996). Genetic analysis of the bipolar pattern of bud site selection in the yeast *Saccharomyces cerevisiae*. Mol. Cell. Biol. *16*, 1857–1870.