Annual Review of Genetics

The Complex Genetic Basis and Multilayered Regulatory Control of Yeast Pseudohyphal Growth

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Annu. Rev. Genet. 2021. 55:1–21

First published as a Review in Advance on July 19, 2021

The *Annual Review of Genetics* is online at genet.annualreviews.org

https://doi.org/10.1146/annurev-genet-071719-020249

Keywords

filamentous growth, invasive growth, *Saccharomyces cerevisiae*, cell adhesion, *FLO* genes, MAPK

Abstract

Eukaryotic cells are exquisitely responsive to external and internal cues, achieving precise control of seemingly diverse growth processes through a complex interplay of regulatory mechanisms. The budding yeast *Saccharomyces cerevisiae* provides a fascinating model of cell growth in its stress-responsive transition from planktonic single cells to a filamentous pseudohyphal growth form. During pseudohyphal growth, yeast cells undergo changes in morphology, polarity, and adhesion to form extended and invasive multicellular filaments. This pseudohyphal transition has been studied extensively as a model of conserved signaling pathways regulating cell growth and for its relevance in understanding the pathogenicity of the related opportunistic fungus *Candida albicans*, wherein filamentous growth is required for virulence. This review highlights the broad gene set enabling yeast pseudohyphal growth, signaling pathways that regulate this process, the role and regulation of proteins conferring cell adhesion, and interesting regulatory mechanisms enabling the pseudohyphal transition.

INTRODUCTION

Hyphae: highly extended fungal tubes with parallel walls and incomplete septa that can enable intercompartmental cytoplasmic streaming

Pseudohyphae: surface-spreading and invasive filaments of elongated cells that remain connected after cell division

Polarisome: a protein complex that is important in determining cell polarity

As nonmotile microbes, many fungi have evolved dramatic adaptive measures in response to variable nutrient levels and environmental conditions, transitioning from single cells to a scavenging filamentous form capable of invading semisolid substrates. This dimorphism is evident in the baker's yeast *Saccharomyces cerevisiae* (41). *S. cerevisiae* reproduces by budding, with most laboratory strains growing in a unicellular manner. Approximately 100 years ago, however, workers in vineyards began to observe the formation of filaments, or branched chains of elongated yeast cells, upon the inoculation of the yeast into grape juice and upon the completion of primary fermentation without shaking, with the yeast using proline as a primary nitrogen source (47). This dimorphism in *S. cerevisiae* has been intensely studied over the past thirty years, predominantly in the Σ1278b strain of yeast. Gerry Fink's laboratory (41) identified the filamentous response in this genetic background, which had been used previously for analyses of nitrogen assimilation pathways (13, 46). Nitrogen limitation is an induction mechanism for filamentous growth in yeast, and, unlike many commonly used laboratory strains, Σ1278b undergoes a uniform and controlled transition to this filamentous state (41). The pathways now recognized as filamentous growth regulators in yeast are highly conserved, and *S. cerevisiae* has proven to be an informative model of these signaling networks implicated in cancer and other human diseases. The process of filament formation in *S. cerevisiae* is similar to processes of filamentation in the related opportunistic human fungal pathogen *Candida albicans*, in which filamentation is required for virulence (44, 84). Of further significance, the networks regulating filament formation in *S. cerevisiae* are a complex and interconnected tapestry, modeling regulatory mechanisms at play in numerous eukaryotic and metazoan genetic networks.

Filaments in *S. cerevisiae* consist of elongated cells that remain connected after cell division and septum formation. The resulting cellular chains superficially resemble the hyphae of filamentous fungi; however, true hyphae contain highly extended parallel walls with a uniformly narrow morphology and lack of full septal constriction. Pseudohyphal cells in *S. cerevisiae* are uninucleate, while hyphal tubes can be multinucleate (10, 41, 103). The term pseudohyphae acknowledges these distinctions between the filamentous growth states.

Relative to yeast-form cells growing vegetatively under conditions of sufficient nutrients, the pseudohyphal growth mode encompasses changes in cell morphology, cell cycle progression, cell polarity, cell–cell adhesion, and substrate invasion. Pseudohyphal cells are elongated and routinely exhibit a maximum length to maximum width ratio of greater than two. This elongated morphology is due in part to an extended G2 phase, promoting a prolonged period of apical polarized growth at the cell poles. In pseudohyphae, the mother and daughter cells are similar in size upon completion of the cell cycle and septum formation. Consequently, mother and daughter cells initiate the subsequent cell cycle simultaneously, and this synchrony is maintained for the first few cell cycles in pseudohyphae (2, 69, 87). Budding is altered in pseudohyphal cells, with bud emergence occurring predominantly in a distal-unipolar pattern at the opposite end of the cell from the previous bud site (32, 41, 118). In contrast, yeast cells under normal growth conditions bud in either an axial pattern with budding initiated adjacent to the previous bud site or a bipolar pattern of alternated budding at opposite cell poles, depending on whether the cells are haploid or diploid, respectively (22). Yeast haploid cells contain a single **a** or α mating-type allele at the mating-type (MAT) locus, while diploid cells are heterozygous for the MAT**a** and MATα alleles. Cells undergoing pseudohyphal growth exhibit a highly polarized actin cytoskeleton consistent with altered localization of polarisome components and bud-site-selection proteins (32, 113, 130). The altered cell adhesion properties of pseudohyphae are a hallmark of this filamentous growth form. Cells in pseudohyphal filaments remain connected by protein and polysaccharide

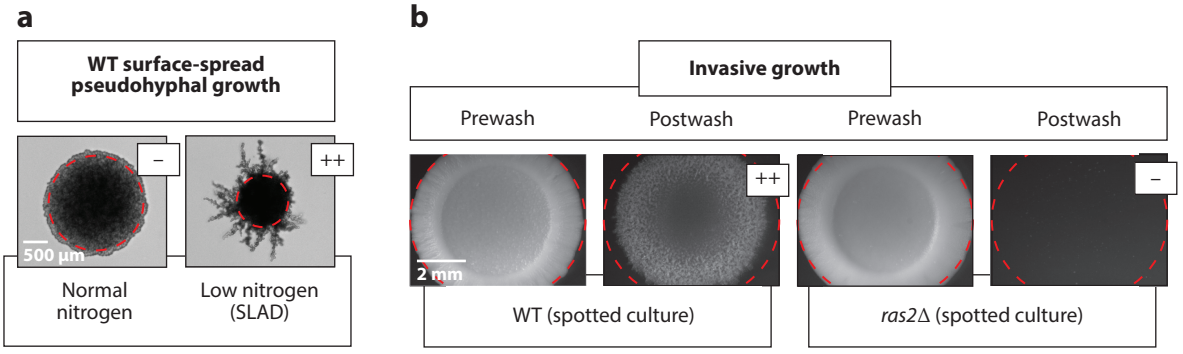

Figure 1

The morphology of filamentous growth in *Saccharomyces cerevisiae*. (*a*) Surface-spread pseudohyphal filaments extend outward from a colony of WT yeast in the Σ1278b genetic background grown on medium with limited ammonium sulfate (SLAD medium). The dashed red line outlines the central colony. The minus symbol denotes a lack of pseudohyphal filamentation; the double plus symbol signifies significant pseudohyphal filamentation. (*b*) Invasive growth of a haploid yeast strain (Σ1278b) grown on standard medium. A spotted culture of a WT strain is shown to the left, and a strain deleted for *RAS2* is shown to the right. Invasive growth is evidenced by yeast present after the surface of the agar plate is washed. The *ras2* deletion strain is defective in invasive growth. The dashed red line outlines the periphery of the spotted culture. Abbreviations: SLAD, synthetic low ammonium dextrose; WT, wild-type.

attachments at the cell surface, which I discuss in depth in the section titled Cell Surface Biology and Adhesins in Pseudohyphal Growth. Strikingly, a strain of *S. cerevisiae* undergoing pseudohyphal growth forms filaments that can extend over a solid agar substrate (**Figure 1*a***) or invade the agar surface (**Figure 1*b***). The extent of pseudohyphal outgrowth from a colony and the degree of its agar invasion are commonly assayed phenotypic readouts of filamentous growth in *S. cerevisiae*.

Researchers have theorized that the collective properties of yeast pseudohyphal growth confer an advantage to *S. cerevisiae* strains outside the laboratory. Pseudohyphal growth has been considered a foraging mechanism or escape method, enabling yeast colonies to extend over a greater area toward nutritive surfaces or away from a deleterious environment. The teleological nature of the argument can be debated, but the filamentous Σ1278b strain of yeast does survive on laboratory media with a limited nitrogen source more effectively than do standard nonfilamentous laboratory derivatives of the S288C background. Presumably, changes in the activity of metabolic pathways in the respective yeast strains are critical in explaining these phenotypes, and further studies of cell metabolism may be informative in this context.

Several conditions can induce filamentous form growth in *S. cerevisiae*, including growth under nutrient stress and elevated levels of quorum-sensing molecules. As indicated above, the decreased availability of fixed nitrogen, typically in the form of ammonium sulfate, has been the condition most commonly used in the laboratory to induce pseudohyphal growth. Diploid strains of the Σ1278b genetic background respond to conditions of nitrogen limitation by extending surface-spread and invasive filaments. Haploid strains of this same genetic background extend invasive filaments into agar on rich growth medium (41) and can grow in filament form in response to nitrogen limitation as well (24, 33). Haploid yeast cultured on media lacking a fermentable carbon source are constitutively invasive, and glucose depletion can induce filamentation in haploid and diploid backgrounds of Σ1278b (31–33). Also, haploid yeast of the filamentous Σ1278b background form surface-spread and invasive filaments in response to several short-chain alcohols, including isoamyl alcohol and 1-butanol. Further, ethanol stimulates hyperfilamentation in diploid cells (90). These alcohols have been proposed to act in yeast as quorum sensors. The aromatic alcohols tryptophol and phenylethyl alcohol activate a quorum-sensing pathway in *S. cerevisiae*

governing filamentation under conditions of low nitrogen (24), although the affected signaling network is distinct from the pathways regulated by the short-chain alcohols above. Consistent with the field, this review distinguishes between the respective filamentous growth forms and strain ploidy if significant with respect to a given study.

Filamentous strains of *S. cerevisiae* can form biofilms. Yeast pseudohyphal growth and biofilm formation are linked, as pseudohyphae are present in biofilms, and partially overlapping gene sets enable and regulate both processes (45, 117, 121). Fungal biofilms have been studied extensively (54, 127). *C. albicans* forms biofilms on a variety of host tissues and implanted biomedical devices, leading to potentially serious infections (114). Biofilms can also act as a protective barrier for the fungus against drug treatment (38). *S. cerevisiae*, however, does not form biofilms as aggressively as *C. albicans*, and the extensive biology of fungal biofilms has been reviewed previously (89, 101); thus, this review does not summarize the literature on yeast biofilms.

This review considers the genetic basis and variability underlying yeast pseudohyphal growth. Signaling pathways controlling pseudohyphal filamentation are summarized briefly, with an emphasis on network interconnections and the broad scope of pseudohyphal growth regulatory control. Genomic and proteomic studies dissecting the mechanisms regulating pseudohyphal growth are highlighted. The yeast cell surface is critical in establishing the altered cell adhesion properties evident in pseudohyphal filaments, and mechanisms through which the adhesins enable pseudohyphal growth are presented.

Biofilm: a mat-like structure with a basement layer of yeast cells and an upper region of filaments embedded in an extracellular matrix

Adhesin: a specialized cell surface protein that enables yeast cell adherence to other cells, tissues, and inert substrates, including plastic and agar

MAPK: mitogen-activated protein kinase

PKA: protein kinase A

AMPK: AMP-activated protein kinase

TOR: target of rapamycin

Scaffold: a protein that binds multiple pathway components to increase local concentrations of the proteins, promoting efficient and specific signaling

SIGNALING PATHWAYS REGULATING YEAST FILAMENTOUS GROWTH

Landmark studies from numerous laboratories have identified a set of signaling pathways central to the regulation of yeast pseudohyphal growth, encompassing the Kss1p mitogen-activated protein kinase (MAPK) pathway, the Ras2p/cAMP-dependent protein kinase A (PKA) pathway, the AMP-activated protein kinase (AMPK) Snf1p pathway, and the target of rapamycin (TOR) pathway (19, 28, 31, 34, 37, 41, 70, 82, 104, 118, 119). A simplified representation of core components within these pathways is provided in **Figure 2**. Collectively, the signaling core is responsive to conditions of nutrient limitation, and functions for these pathways in regulating yeast filamentous growth are reviewed fully by Cullen & Sprague (33).

Nitrogen and glucose levels are physiological regulators of the Kss1p MAPK cascade. Under glucose-limiting conditions, the cell surface glycoprotein Msb2p has been identified as part of a signal transduction mechanism activating the filamentous growth MAPK pathway (30). Proteolytic processing of the Msb2p mucin enables its function, with the plasma membrane proteins Sho1p and Opy2p and the cytoplasmic scaffolding adapter Bem4p, to activate the pathway (30, 93, 108, 137, 146, 148). Msb2p and Bem4p associate with the Rho GTPase Cdc42p, and Cdc42p interaction with Bem4p is required for Kss1p MAPK signaling (7, 30, 74). The cell polarity adapter Bem1p also regulates this pathway through recruitment of the p21-activated kinase Ste20p to the plasma membrane and through interactions with Cdc24p, the guanine nucleotide exchange factor for Cdc42p (77, 144). The bud-site-selection GTPase Rsr1p and polarity landmark proteins function with Cdc24p and Cdc42p to regulate the filamentous growth MAPK pathway, providing part of the molecular basis underlying the altered budding pattern evident during pseudohyphal growth (8). The Kss1p MAPK pathway regulates the expression of cell polarity targets, including the gene encoding Gic2p, a direct effector of Cdc42p (111).

The Kss1p MAPK regulates a large set of effectors, including a heterodimeric transcription factor consisting of Ste12p and Tec1p (4, 9, 40, 82, 94). The Ste12p/Tec1p heterodimer is one of the most widely acknowledged transcriptional effectors of pseudohyphal growth, and Ste12p

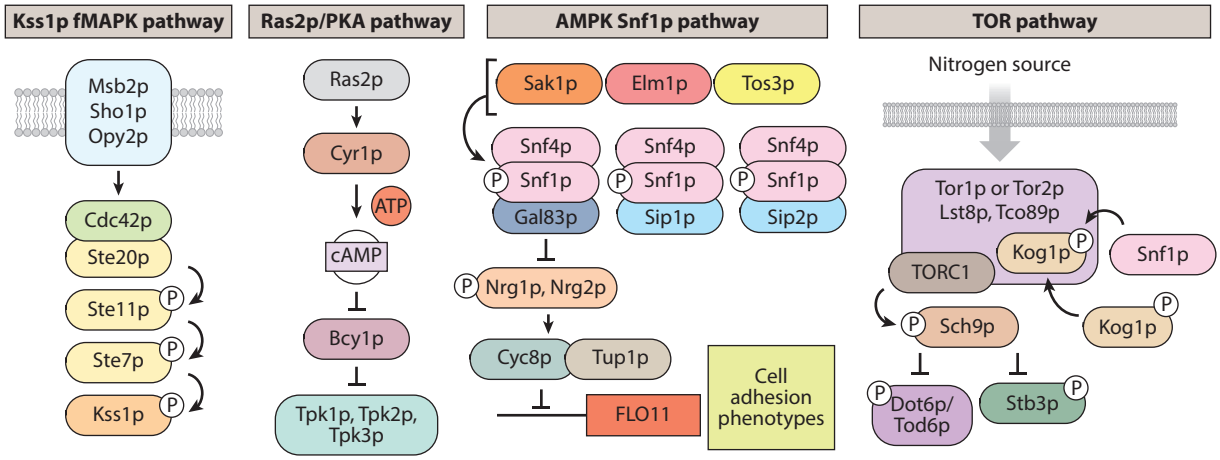

Figure 2

Core signaling pathways required for wild-type pseudohyphal growth. Simplified representations of signaling modules within the indicated pathways are presented. Important phosphorylation events in the respective signaling pathways are indicated. In the interest of simplicity, full upstream inputs and downstream effectors of each signaling module are not depicted. The circled P represents phosphorylation. Abbreviations: AMPK, AMP-activated protein kinase; fMAPK, filamentous growth MAPK pathway; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; TOR, target of rapamycin.

is phosphorylated by activated Kss1p. Ste12p and Tec1p bind cooperatively to filamentation response elements in the promoters of pseudohyphal growth targets (94), with Tec1p specifying the Ste12p heterodimer to filamentation genes. The *FLO11* gene is an important target of the Ste12p/Tec1p heterodimer. *FLO11* encodes a cell surface adhesin required for yeast pseudohyphal growth (86), and its unusually large 2.8-kb promoter has been studied extensively as a downstream target of the Kss1p MAPK, PKA, and Snf1p pathways (120).

The Kss1p MAPK pathway in yeast shares upstream kinases with the mating and high-osmolarity glycerol MAPK pathways; consequently, mechanisms to ensure signaling specificity are critical in ensuring the fidelity of Kss1p-dependent pseudohyphal growth (94). Crosstalk between the mating and filamentous growth pathways is regulated in part through the mating pathway MAPK Fus3p, which, upon activation by mating pheromones, phosphorylates and targets Tec1p for degradation (3). The adapter protein Ste50p activates the MAPKKK Ste11p in the mating, osmoresponsive, and filamentous growth pathways through distinct residues. Genetic studies of *ste50* mutants affecting the Ste50p Ras association domain reveal separable phenotypes with respect to the mating and osmoregulation pathways, identifying clustered residues that form distinct surfaces for potential protein–protein interactions with components of the respective pathways (58, 123). The scaffold proteins Ste5p and Pbs2p assemble pathway-specific complexes for the mating MAPK and osmoregulatory Hog1p pathways, respectively, with Pbs2p acting as the MAPKK and scaffold for the high-osmolarity glycerol pathway (26, 95, 109). The scaffolds are thought to function in part by increasing the local concentration of pathway components, thus facilitating the specific activation of a given signaling module.

Interestingly, Kss1p signaling can also be activated by mild disruptions in the mitotic septins, resulting in a synergistic signaling cycle with activated Kss1p-dependent gene expression leading to exaggerated septin defects and consequent Kss1p hyperactivation. This altered MAPK signaling drives a constitutive and stable pseudohyphal growth state that differs from classically observed pseudohyphal growth in some areas of cell morphology, growth induction, and genetic regulation.

Septin: a eukaryotic GTP-binding protein that can assemble into filaments and ring-like complexes, contributing to cytoskeletal morphology

Filamentous growth driven by septin assembly defects requires several components of the Fus3p mating pathway that are dispensable for classic pseudohyphal growth, and the cells contain septin structures that more closely resemble the highly polarized growth observed in hyphal tubes (67).

Ras2p acts upstream of both the Kss1p MAPK pathway and the cAMP-dependent PKA pathway (97, 98). Activated Ras2p enables increased levels of intracellular cAMP under conditions of glucose limitation through its interaction with adenylate cyclase Cyr1p (133). In *S. cerevisiae*, cAMP-responsive PKA consists of the Bcy1p negative regulatory subunit and three isoforms of the catalytic subunit: Tpk1p, Tpk2p, and Tpk3p (131, 132). Tpk2p phosphorylates the pseudohyphal growth transcription factor Flo8p, regulating a large filamentation gene set that includes *FLO11* (104). *FLO8* is a pseudogene in the nonfilamentous S288C genetic background, from which most common laboratory yeast strains are derived, suggesting its significance as a pseudohyphal growth regulator (83). Flo8p heterodimerizes with the Mss11p transcription factor (66), and both *FLO8* and *MSS11* are required for the wild-type expression of many *FLO* genes mediating adhesion-related phenotypes in yeast pseudohyphal growth (11). Flo8p functions as part of a complex transcriptional network regulating pseudohyphal filamentation. The transcriptional regulator Sok2p is also thought to act downstream of PKA. Sok2p is a negative regulator of filamentation and activates a cascade of transcription factors Phd1p, Ash1p, and Swi5p that is capable of regulating *FLO11* expression independently of the PKA and MAPK pathways (105).

Recently, Ras has been identified as part of the mechanism coordinating fermentation and yeast cell growth. Yeast cells preferentially undergo fermentation rather than aerobic respiration, and the glycolysis intermediate fructose-1,6-bisphosphate binds a mammalian ortholog of Cdc25p, activating Ras and downstream genes important for cell proliferation. Ras targets encompass MAPK/ERK and other proto-oncogenes, suggesting a conserved molecular basis for preferential fermentation in yeast and cancer cells and a mechanism by which enhanced fermentation may stimulate oncogenic potential (106).

The depletion of fermentable carbon sources, such as glucose, also activates the Snf1p kinase pathway. Snf1p is phosphorylated and activated by the upstream kinase Sak1p, Elm1p, or Tos3p (52). Snf1p functions with the activating subunit Snf4p and one of three β-subunit isoforms: Gal83p, Sip1p, or Sip2p (149). *SNF1* is required for pseudohyphal growth and associated changes in cell polarity and adhesion (31). The respective Snf1p-β-subunit forms are at least partially distinct in their functions. Snf1p-Gal83p is required for surface adherence because it antagonizes the transcriptional repression of *FLO11* through the negative regulators Nrg1p and Nrg2p, while Snf1p-Gal83p and Snf1p-Sip2p regulate filamentous morphology independently of *FLO11* (70, 143). Nrg1p and Nrg2p can recruit a complex of the transcriptional regulators Cyc8p and Tup1p at target promoters. Cyc8p and Tup1p act antagonistically with respect to the selection of biofilm versus yeast-form growth modes. Tup1p and Cyc8p are positive and negative regulators, respectively, of cell–cell and cell–surface interactions. Cyc8p represses *FLO11* expression, while Tup1p counters this repression and further protects Flo11p from proteolytic degradation. While this results in an antagonistic effect on biofilm formation, cell flocculation is corepressed by both regulators (100).

TOR is a highly conserved regulator of cell homeostasis from yeasts through metazoans, linking external and intrinsic cellular cues with mechanisms modulating cell growth (reviewed in 43). In yeast, the genes *TOR1* and *TOR2* encode serine/threonine kinases acting in functionally and structurally distinct complexes, TORC1 and TORC2 (50, 72, 88). With respect to pseudohyphal growth, TORC1 is inhibited under conditions of limited availability of fixed nitrogen, and its signaling is required for wild-type pseudohyphal growth (34). The downstream TORC1 network is extensive, but members of the AGC kinase family, including Sch9p, are among the most well-characterized TORC1 substrates (136). TORC1 induces the expression of genes encoding

ribosomal proteins and factors involved in protein synthesis in part through the phosphorylation of Sch9p. The Sch9p kinase, in turn, phosphorylates and inactivates the transcriptional repressors Dot6p/Tod6p and Stb3p (55). TORC1 activity is integrated with the Snf1p and PKA pathways. The Snf1p kinase phosphorylates the TORC1 component Kog1p, driving the formation of Kog1p bodies distinct from TORC1 that act to increase the TORC1 activation threshold in cells that are significantly starved for glucose (56). PKA works in parallel with Sch9p to phosphorylate and inactivate Dot6p/Tod6p (81). Further, the transcriptional profiling of yeast upon the transition from a nonfermentable carbon source to glucose in the presence and absence of PKA and TORC1 inhibitors, individually and in combination, identifies a regulatory circuit integrating PKA and TORC1. PKA drives the expression of important genes in the transition to and from glucose-induced rapid growth, while TORC1 signaling is required for the steady-state expression of these genes. Researchers have proposed that yeast utilizes PKA signaling in this circuit in rapid response to changes in external nutrients and TORC1 signaling to establish stable growth rates responsive to nitrogen source availability (71).

Additional signaling pathways, encompassing at least the retrograde, Rim101p, and Pho85 pathways, contribute to the pseudohyphal growth response (27). Genomic and proteomic studies have been informative in identifying crosstalk between pseudohyphal growth pathways and the broader scope of pseudohyphal growth signaling.

LARGE-SCALE ANALYSES OF YEAST PSEUDOHYPHAL GROWTH

Functional genomic studies have provided unique insight into the genetic basis of pseudohyphal growth. Jin et al. (59) individually introduced into the filamentous Σ1278b background transposon-based disruption alleles affecting 3,627 genes and a set of plasmids enabling the overexpression of 2,043 genes, generating two extensive mutant yeast libraries for phenotypic screening of filamentation defects. In a milestone study for the field, Ryan and colleagues in Charles Boone's laboratory (121) constructed a genome-wide collection of nearly 5,000 gene disruptions in haploid and diploid strains of the Σ1278b background for analysis of surface-spread filamentation, invasive growth, and biofilm phenotypes. This work identified a previously uncharacterized transcription factor, named Mfg1p in the study, as an important activator of pseudohyphal growth. Mfg1p binds Flo8p and Mss11p and regulates the expression of key pseudohyphal growth genes such as *FLO11*. Collectively, the studies highlighted in excess of 700 genes that yield filamentous growth phenotypes upon genetic perturbation. This union gene set, encompassing the core Kss1p MAPK, PKA, Snf1p, and TORC1 pathways discussed previously, indicates an expansive signaling network with the scope to drive altered cell morphology, polarity, adhesion, and metabolism during filamentation. The genetics underlying pseudohyphal growth is complex, but additional genomic and proteomic studies hold the potential to dissect gene relationships within this network.

Systematic phenotypic screening has been employed to further characterize the genetic basis of filamentation-related phenotypes and relevant pathway connections (124). Gene deletion mutants in filamentous backgrounds of *S. cerevisiae* were screened for colony morphology defects, identifying components of the Kss1p MAPK, Snf1p, TORC1, and RIM101 pathways. This study highlighted the importance of the adhesin Flo11p in driving colony morphology, as altered *FLO11* expression driven by modified promoters correlated strongly with colony morphology (142). Functional analysis of transcriptional targets downstream of the Kss1p MAPK pathway identified a role for the transcription factor Sfg1p in regulating *FLO11* expression and in enabling elongated cell morphology. Unexpectedly, the Kss1p MAPK was found to activate genes that are inhibitory to invasive growth, suggesting that the pathway coordinates both positive and negative regulators as a means of achieving fine control of filamentation (138).

Ribonucleoprotein granules: RNA-protein complexes, including stress granules and processing bodies

The broad scope of transcriptional regulation enabling wild-type pseudohyphal growth is evident from transcriptional profiling, which has identified over 800 genes that are differentially expressed during pseudohyphal growth induced by nitrogen limitation relative to growth in media with normal levels of fixed nitrogen (92, 112). Several clever approaches have been used to dissect this regulatory network further. Borneman et al. (12) coupled chromatin immunoprecipitation with DNA microarray analysis to map promoter targets bound by the Ste12p, Tec1p, Sok2p, Phd1p, Mga1p, and Flo8p transcriptional regulators. The transcription factors act as hubs within the pseudohyphal growth regulatory network, with Flo8p directing Mga1p to target promoters. Mayhew & Mitra (96) developed an approach to mark transcription factor binding sites by Ty5 retrotransposon insertion using a chimera of a transcription factor fused to the Ty5 integrase–interacting domain of the heterochromatin protein Sir4p. By this calling card method, Ty5 integration is directed to binding sites of its partner transcription factor, enabling binding site identification. Applied to 14 pseudohyphal growth transcription factors, the analysis identified the binding of the Flo8p-Mss11p-Mfg1p complex at the promoter and termination sequences of *FLO11*. The binding is facilitated by DNA looping and stabilized through an interaction with the nuclear pore complex. The interaction is proposed to constitute a form of transcriptional memory, allowing for the rapid implementation of filamentous growth in cells that have been previously exposed to conditions of nitrogen limitation. The calling card approach was also used to investigate the regulatory mechanisms of the Ste12p transcription factor (150). Point mutants introduced into the *STE12* sequence produced variants with dissimilar binding and expression profiles that converged on small gene subsets to regulate mating and invasion.

Proteomic analyses have informed our understanding of pseudohyphal growth signaling networks. Quantitative phosphoproteomic studies of kinases in the Kss1p MAPK, PKA, Snf1p, and Elm1p pathways have been used to identify the phosphorylation targets of these signaling modules (60, 125). The data sets indicate tens of phosphoproteins regulated by each kinase and suggest previously unstudied regulatory interconnections. Interestingly, MAPK and PKA pathway proteins are required for the wild-type phosphorylation of ribonucleoprotein granule components. Several MAPK pathway proteins and Tpk2p colocalize with protein components of ribonucleoprotein granules induced under conditions of glucose stress, suggesting a potential mechanism coupling pseudohyphal growth with stress-responsive translational regulation (125). MAPK and Snf1p signaling are required for the wild-type phosphorylation of several kinases that act in a pathway for the sequential phosphorylation of soluble inositol (102). The resulting inositol polyphosphate species are important metabolic second messengers, and inositol polyphosphate levels vary in response to conditions of nitrogen limitation. Genetic perturbation of the genes encoding inositol polyphosphate kinases results in pseudohyphal growth phenotypes, and levels of pyrophosphorylated inositol polyphosphates corelate with the degree of observed pseudohyphal growth. Additional studies are underway to define the molecular mechanisms coordinating pseudohyphal growth kinase signaling with the regulation of ribonucleoprotein granule dynamics and inositol polyphosphate signaling (99).

MULTILAYERED REGULATION OF PSEUDOHYPHAL GROWTH

The regulation of pseudohyphal growth extends beyond the traditional control of transcription initiation to encompass posttranscriptional RNA modifications, the activity of noncoding RNAs, and factors that affect translation. Posttranslational histone modifications that affect chromatin architecture also shape yeast cell growth transitions, and these control mechanisms have been reviewed recently (57).

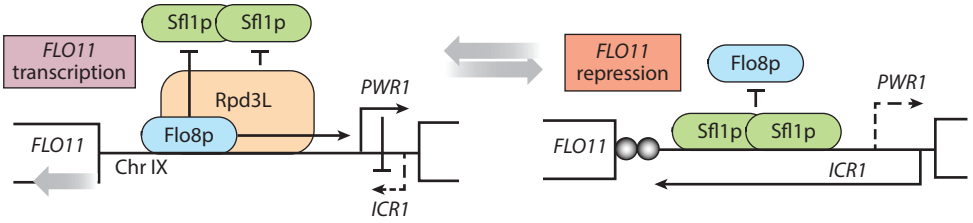

Figure 3

Regulation of *FLO11* transcription through a regulatory circuit involving the noncoding RNAs *ICR1* and *PWR1*. Regulatory interactions allowing *FLO11* transcription are shown to the left, and a repressive state at the *FLO11* locus is shown to the right. Figure is adapted with permission and based on the model presented by Bumgarner and colleagues (16, 17). Figure is not drawn to scale. Abbreviation: Chr, chromosome.

In *S. cerevisiae*, several RNA modifications regulate the pseudohyphal growth transition. RNA methylation contributes to the coordination of pseudohyphal growth and meiotic sporulation in response to nutrient limitation. Yeast cells grown in nutrient-depleted medium establish a commitment point, after which the return to nutrient-rich media initiates a growth form that morphologically and genetically resembles starvation-induced pseudohyphal growth. Lineage restriction during this process is partially dependent on the messenger RNA (mRNA) methyltransferase activity of Ime4p, and the MIS complex of Mum2p, Ime4p, and Slz1p functions in both sporulation and pseudohyphal growth (1). Pseudouridylation is a common RNA modification in yeast transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), and small nuclear RNAs (snRNAs), and notably, the pseudouridylation of residue 28 in the spliceosomal core U6 snRNA is induced during filamentous growth (6). This RNA modification is catalyzed by Pus1p, and *PUS1* mRNA levels are elevated in pseudohyphal growth. U6 snRNA mutants that are strongly pseudouridylated at residue 28 activate pseudohyphal growth in a process dependent upon Pus1p. Further, mutants that block U6-U28 pseudouridylation inhibit pseudohyphal growth. Presumably, the resulting altered splicing of target genes stimulated by U6-U28 pseudouridylation promotes the pseudohyphal growth program.

RNA localization is also an important regulatory mechanism by which pseudohyphal growth is controlled. The RNA-binding protein Khd1p directly interacts with repetitive sequences in *FLO11* mRNA, thereby inhibiting translation and establishing feed-forward repression of *FLO11*. This regulatory mechanism enables altered *FLO11* expression between mother and daughter cells, constructing the asymmetry required for the pseudohyphal growth transition (145).

FLO11 expression is bistable, with variegated transcription established at least in part through the activities of two *cis*-interfering noncoding RNAs transcribed from the *FLO11* promoter (16) (**Figure 3**). *ICR1* and *PWR1* encode long noncoding RNAs transcribed in opposite orientations from the large intergenic region upstream of *FLO11*. *ICR1* transcription is activated by the Sfl1p transcription factor and the absence of Flo8p binding. *ICR1* RNA inhibits *FLO11* transcription by blocking or forcing the dissociation of *trans*-acting factors. *PWR1* transcription is activated by Flo8p. The Flo8p and Sfl1p transcription factors compete for binding upstream of *FLO11*, such that the *ICR1* and *PWR1* transcriptional programs are mutually exclusive. *PWR1* transcription and loss of *ICR1* transcription enable *FLO11* expression. The histone deacetylase Rpd3Lp acts on this locus, with the resulting condensed chromatin potentially inhibiting Sfl1p binding. This conformation enables Flo8p binding and *FLO11* transcription, with *PWR1* transcription and inhibited *ICR1* expression. This genetic circuit toggles between the conversely acting noncoding RNAs, providing a tidy regulatory control mechanism. The contributions of *ICR1* and *PWR1* in enabling the variegated expression pattern of *FLO11* are borne out by single-cell RNA fluorescence in situ

hybridization experiments conducted on thousands of individual yeast cells (17). The results are consistent with the above mechanism establishing Flo11p-dependent phenotypic heterogeneity in clonal cell populations.

Flo11p activity is also modulated by its shedding from cells (63), with Flo11p detectable by western blotting of cultured supernatants. Flo11p shedding attenuates cell adherence, and the shed protein is an essential component of a fluid layer surrounding yeast biofilm-like mats. The furin protease Kex2p is required for the cleavage and maturation of Flo11p.

Translational regulation contributes to the pseudohyphal growth program in *S. cerevisiae*. In evidence, a mutant allele of the yeast *SUP70* gene induces pseudohyphal growth in the absence of nutrient limitation (65). *SUP70* encodes a tRNA that decodes CAG into glutamine. The constitutive pseudohyphal growth phenotype of the *sup70* mutant stems from its decreased efficiency in decoding CAG; thus, the efficiency of translational expression is an important factor enabling the wild-type pseudohyphal response. The *SUP45* gene encodes the yeast translation termination factor eRF1, and nonsense and missense mutations in *SUP45* inhibit surface-spread filamentation and invasive growth (107). The RNA helicase Dhh1p positively regulates the translation of selected mRNAs, including *STE12* transcripts. Recently, mutations affecting the Dhh1p ATPase domains and the Thr16 phosphorylation site were found to produce defects in pseudohyphal colony morphology and agar invasion under conditions of nitrogen limitation, indicating the importance of RNA processing and translation regulation in filamentation (75).

CELL SURFACE BIOLOGY AND ADHESINS IN PSEUDOHYPHAL GROWTH

The yeast cell membrane and cell wall are critical in enabling many of the properties consistent with pseudohyphal growth. To consider changes at the yeast cell periphery that contribute to filamentation, Xu et al. (147) profiled plasma membrane protein preparations from *S. cerevisiae* cells grown under normal conditions and under conditions of nitrogen limitation inducing pseudohyphal growth. The extracted proteins were labeled by isobaric tagging and analyzed by mass spectrometry to identify those that were differentially abundant between the growth conditions. The resulting proteomic profiles encompassed over three hundred proteins and identified differential abundance of a subset of transport proteins required for wild-type pseudohyphal growth, including Mep2p. The yeast Mep2p ammonium transport protein is required for pseudohyphal growth to act as a trigger of filamentation under conditions of ammonium scarcity. As reported by Brito et al. (14), pseudohyphal growth is dependent on the transport activity of Mep2p; conformational changes that accompany substrate translocation through the pore crossing the Mep2p hydrophobic core are required for filamentous signaling. Endocytosis is an important process in polarized growth, and orthologs of proteins in the AP-2 complex for mammalian clathrin-mediated endocytosis are required for wild-type pseudohyphal growth in *S. cerevisiae* and hyphal development in *C. albicans* (23). AP-2 is a heterotetrameric complex that binds endocytic cargo and localizes to sites of endocytosis in vivo. *APM4* encodes the cargo-binding μ subunit of AP-2, and *S. cerevisiae* mutants lacking *APM4* exhibit reduced levels of surface filamentation. The μ homology domain of Apm4p is directly bound by the cell wall stress sensor Mid2p, and Mid2p localization to sites of polarized growth is interrupted in *apm4* mutants. Endocytic recycling of Mid2p as AP-2 cargo, as well as other proteins, may be required for cell wall deposition during polarized cell responses, such as pseudohyphal growth. The composition of the yeast cell membrane is impacted by lipid flippases of the P4 ATPase family, including Dnf3p and the distantly related proteins Dnf1p and Dnf2p, which may be involved in lipid redistribution at sites of polarized growth (122). The Dnf3p flippase catalyzes the translocation of phosphatidylserine and other glycerophospholipids towards

the cytosolic membrane leaflet. Strains deleted for *DNF1*, *DNF2*, and *DNF3* show a pseudohyphal growth defect under conditions of nitrogen limitation, suggesting that these flippases, regulated by Fpk1p and Fpk2p, contribute to the control of pseudohyphal filamentation (39).

Microbial cell surface variability is critical in enabling differential cell properties (reviewed in 139). In the context of yeast pseudohyphal growth, adhesin proteins are of fundamental importance in mediating flocculence and surface contact (141). Adhesins exhibit a common protein structure, containing an amino-terminal domain for lectin-like binding of sugars or peptides, a heavily glycosylated central domain, and a glycosylphosphatidylinositol (GPI)-anchored carboxy-terminal domain. Cleavage of this GPI anchor leaves the adhesin covalently linked to glucans in the cell wall (29, 36, 62, 91). Adhesion is thought to occur through adhesin-mediated binding of cell surface sugars and peptides or by hydrophobic and hydrophilic interactions between the glycosylated central region of the adhesin and abiotic surfaces (61, 129).

In *S. cerevisiae*, the *FLO* gene family encodes a set of adhesins mediating cell adhesion properties (48). *FLO* genes are subtelomeric, and this local chromatin structure with its fluctuating binary expression state contributes to the variegated chromatin silencing of *FLO* genes (49, 116). The yeast *FLO* gene family encompasses *FLO1*, *FLO5*, *FLO9*, *FLO10*, and the aforementioned *FLO11*. Notably, *FLO1* expression in the nonfilamentous S288C strain of yeast is sufficient to restore flocculation, forming biofilm-like cell aggregates with increased resistance to antimicrobials and ethanol. Cells expressing *FLO1* preferentially adhere to other cells expressing *FLO1*, independent of genomic context (126). *FLO11* expression drives cell adhesion to abiotic surfaces and the formation of a yeast velum at liquid-air interfaces (35, 73, 85, 117).

In addition to the variegated expression of *FLO* genes, the internal protein structure of these adhesins generates functional variability in cell properties of adhesion, flocculation, and biofilm formation. The *FLO* genes encode proteins containing 10–20 tandem repeats, each approximately 100 nucleotides in length, oriented in a head-to-tail fashion. This repeat structure can trigger slippage during replication and intragenic variation in repeat number. Since the repeated sequences are very similar within the *FLO* gene family, the repeats may induce crossing over and homologous recombination as well as unequal crossing over and other recombination events. This variability in repeat number has been theorized to be responsible for generating the functional diversity of cell surface antigens that can facilitate yeast environmental adaptation and fungal evasion of the host immune system (140).

As indicated in the section titled Signaling Pathways Regulating Yeast Filamentous Growth, the Flo11p adhesin has been particularly well studied for its significance in integrating pseudohyphal growth signaling pathways and for its importance in enabling pseudohyphal filamentation. Variations in *FLO11* expression levels and sequence underlie many of the flocculation and invasion phenotypes evident in distinct strains of *S. cerevisiae*. Flo11p adhesion domains expressed from different yeast strains preferentially recognized domains of identical sequence from the same strain (5). Recent studies further indicate a role for Flo11p-type cell surface adhesins in kin discrimination. Homotypic interactions between identical Flo11A domains provide a molecular basis for interactions between yeast cells of the same strain or species, resulting in efficient cell aggregation and biofilm formation. Heterotypic interactions between Flo11A domains confer weak adhesive forces, suggesting a mechanism by which yeast strains may be segregated in a heterogeneous population by Flo11A-mediated cell adhesion (15).

Interestingly, many yeast adhesins contain sequences predicted to form β-amyloid-like aggregates (79, 80, 115). These amyloid-forming sequences may allow adhesins to undergo a phase transition to a partially ordered state, with clustered adhesins generating nanodomains on the cell surface. The nanodomains strengthen cell interactions, leading to enhanced biofilm formation. This phase change and fungal adhesin β aggregation can be triggered by the application of shear

Flocculence: calcium-dependent cell–cell adhesion manifested in the appearance of aggregated cells known as flocs

Kin discrimination: the recognition of cells of the same kind in a mixed population

stress resulting from the flow of liquid over a surface. Fungal adhesin aggregation is induced by shear flow, and the resulting cell–cell interactions are sufficiently strong to enable the formation of persistent biofilms under these conditions (20, 21).

Cryptic genetic variation: variation in genome sequences that is typically unexpressed until the environment or genetic background is altered

Adhesin functional diversity is likely driven in part through variation in ligand binding. Hoffmann et al. (51) identified conserved and variable structural motifs contributing to host cell binding and ligand-binding specificity in a family of epithelial adhesins in the pathogenic fungus *Candida glabrata*. Several conserved structural motifs in the ligand-binding pocket of these adhesins confer host cell–binding properties, while the variable loops CBL2 and LI mediate ligand-binding specificity and host cell binding, respectively. The studies suggest that variation in the ligand-binding pockets of adhesins may be an important driver of their functional diversification and evolution.

GENETIC VARIATION UNDERLYING PSEUDOHYPHAL GROWTH PHENOTYPES

The yeast pseudohyphal growth response is a compound phenotype encompassing altered cell morphology, polarity, adhesion, and cell cycle progression through the regulated activity of complex genetic and metabolic signaling networks. Consequently, there is a significant opportunity for variability in the genetic basis of pseudohyphal growth and the observed filamentous phenotypes between yeast strains and species. Analysis of this variability can provide insight into the genetic basis of filamentation and, potentially, the evolution of these signaling networks.

The filamentous Σ1278b strain of *S. cerevisiae* has been sequenced, and allelic differences between filamentous and nonfilamentous yeast strains have been investigated to identify key alleles that enable pseudohyphal growth. By screening deletion libraries in the Σ1278b and nonfilamentous S288C backgrounds, Chin et al. (25) identified the *RPI1* gene for its ability to enable *FLO11* transcription in S288C independent of the Kss1p filamentous growth MAPK pathway. Rpi1p is a transcription factor differentially phosphorylated between the two strains, with its sequence encoding an altered number of internal tandem repeats in the respective backgrounds. Linkage analysis between filamentous and nonfilamentous *S. cerevisiae* strains identifies a premature stop codon in the gene encoding the negative transcriptional regulator Sfl1p in the invasive SK1 genetic background and allelic differences in the polarity gene *PEA2* between the S288C and Σ1278b strains (128). In comparisons of these strains, conditional gene essentiality between the backgrounds typically stems from allelic differences between sets of multiple genomic regions, indicating a complex modifier landscape underlying respective essential gene phenotypes (53). Colony phenotypes in *S. cerevisiae* are established in part through cryptic genetic variation. In a cross of a nonfilamentous laboratory strain with a clinical isolate, a suppressed yeast colony phenotype was dissected, with additional genetic perturbations enabling manifestation of the phenotype. The identified genetic loci indicated *cis*- or *trans*-regulation of *FLO11* transcription, highlighting the importance of *FLO11* in colony-based phenotypes relevant to pseudohyphal growth (76).

Kita et al. (68) used RNA sequencing and full-genome sequencing of 85 diverse *S. cerevisiae* isolates to identify quantitative trait loci that underlie variability in mRNA levels. The analysis identified variants in promoter sequences and in 3′ untranslated regions that affect the RNA binding of PUF family proteins. In isolates from human patients, genes that suppress biofilm formation were typically upregulated, and a single variant in the promoter of the *NIT3* biofilm suppressor showed very strong genome-wide association with *S. cerevisiae* isolates of clinical origin. In a study by Lenhart et al. (78), a diverse collection of *S. cerevisiae* environmental isolates were assayed for natural variation in the phenotype and induction of pseudohyphal growth. The analysis indicated that population origin was significantly linked with the pseudohyphal growth phenotype and that

isolates from West Africa exhibited the strongest association. The environmental isolates, however, typically exhibited little or no pseudohyphal response to exogenous quorum-sensing alcohols. A population of *S. cerevisiae* derived from a highly heterozygous clinical isolate was also used to map loci important for filamentation, identifying sites previously implicated in pseudohyphal growth in Σ1278b.

Comparative analyses of yeast filamentation in distinct fungal species identify commonality and differences in the regulatory networks controlling this response. The fungi *S. cerevisiae*, *C. albicans*, and *Histoplasma capsulatum* are estimated to have diverged from a common ancestor 600 million years ago, but each contains a transcriptional regulator of morphology (Wor1p in *C. albicans*, Ryp1p in *H. capsulatum*, and Mit1p in *S. cerevisiae*) that recognizes a common DNA sequence (18). In *S. cerevisiae*, Mit1p binds to a promoter set encompassing many control regions known to regulate pseudohyphal growth. Interestingly, the gene sets regulated by these orthologous transcription factors exhibit only a partial overlap, despite the strong conservation of binding specificity. The transcription factors may constitute an ancestral regulatory module controlling cell morphology, with gene movement in and out of this regulon contributing to differences in morphological forms between the fungi. *S. cerevisiae* and *Saccharomyces bayanus* are closely related species of budding yeast; however, cAMP-PKA signaling results in opposite filamentous phenotypes in these yeasts (64). Inhibition of PKA signaling in *S. cerevisiae* results in decreased pseudohyphal growth, while the opposite effect is observed in *S. bayanus*. Downstream targets of PKA signaling are divergently regulated in these yeast species, indicating an example of divergent network functions despite conservation of a core signaling module.

MODELING PSEUDOHYPHAL GROWTH

Unique insights into the mechanisms enabling wild-type yeast pseudohyphal growth may be obtained through the quantitative modeling of morphology and relevant cell processes during yeast filamentation. Yeast pseudohyphal growth has been quantified using a two-dimensional agent-based model of yeast cell growth incorporating dimorphic behavior and nutrient diffusion (134). By this model, the nonmotile yeast take up nutrients that diffuse randomly through physical space represented as a lattice. The growth pattern can be specified by adjusting a bias in the direction of cell proliferation. The model accounts for the contributions of uniform growth, diffusion-limited growth encompassing the interaction between microbial cells and a diffusing nutrient, and an intermediate growth mode wherein the aforementioned bias determines the colony morphology. Experimentally observed yeast pseudohyphal colony morphology can be replicated using the model parameters. In a study using a continuous reaction-diffusion model for microbial growth, Tronnolone et al. (135) suggest that diffusion-limited growth on a solid substrate with limited nutrient availability is unlikely to explain the development of pseudohyphal filamentation. Yeast pseudohyphal growth patterns have been characterized using cluster shape primitives, which do not require the use of a predefined feature list or a priori knowledge of the shape (42). This approach is effective in categorizing colony morphology phenotypes and may be of utility in implementing high-throughput morphological assays.

Efforts to model yeast pseudohyphal growth also lay the groundwork for the construction of synthetic gene regulation systems to control filamentation. Controlled expression of the *S. cerevisiae* transcription factors Flo8p and Phd1p can trigger pseudohyphal growth in yeast in diploid and haploid backgrounds in rich media. This system for induced filamentation was used to construct a synthetic genetic timer network to regulate pseudohyphal growth and explore the reversibility of the pseudohyphal transition (110). Collectively, synthetic biology studies hold

potential for the development of genetic circuits that modulate pseudohyphal filamentation, particularly relevant to biotechnology efforts using environmental yeast isolates.

FUTURE DIRECTIONS

The complexity of the yeast pseudohyphal response is important to appreciate. Intricate regulatory mechanisms enable the coordinated control of many cell processes toward this striking morphological transition. This multilayered regulatory control inspires many unresolved questions regarding pseudohyphal growth. Questions persist as to how the respective signaling pathways that are required for pseudohyphal growth are selectively activated and their outputs integrated to regulate the expansive downstream network of effectors carrying out the cell processes that enable filamentation. The yeast pseudohyphal response necessitates substantial changes in cell metabolism, and efforts to understand the metabolomics of pseudohyphal growth promise to highlight previously unknown signaling mechanisms. The complexity of pseudohyphal growth suggests that phenotypic readouts of surface filamentation and invasive growth may need to be supplemented with quantitative analyses of constituent processes of morphology, cell polarity, and cell adhesion to distinguish the granular detail in these signaling systems. Also, while this review has focused on pseudohyphal growth in *S. cerevisiae*, related processes of filamentation in fungal pathogens affecting plants and humans continue to be important focal points for research investigation. In total, our continued understanding of fungal biology is of substantial importance in efforts to improve human health and welfare.

SUMMARY POINTS

1. The pseudohyphal growth response in strains of the budding yeast *Saccharomyces cerevisiae* involves the formation of filamentous chains of connected and elongated cells. Pseudohyphal filaments can spread along the surface of a solid substrate and can invade agar, presumably as a foraging mechanism. Yeast pseudohyphal growth is induced under conditions of nitrogen limitation, by growth in media with a nonfermentable carbon source, or through the presence of alcohols that act as quorum sensors. Cells undergoing pseudohyphal growth exhibit altered cell morphology, cell polarity, cell cycle progression, and cell adhesion properties.
2. More than 700 genes are required for wild-type pseudohyphal growth in *S. cerevisiae*, and extensive mechanisms are in place to enable both regulatory pathway crosstalk and signaling specificity.
3. Yeast pseudohyphal growth is regulated through a complex and interconnected signaling network encompassing the Kss1p mitogen-activated protein kinase (MAPK) pathway, the Ras2p/cAMP-dependent protein kinase A (PKA) pathway, the AMP-activated protein kinase (AMPK) Snf1p pathway, and the target of rapamycin (TOR) pathway. Transcriptional control is mediated through a set of factors, including Ste12p/Tec1p, Flo8p, Mss11p, and Sfl1p. The *FLO11* gene encoding a cell surface adhesin is regulated through these pathways as part of a large set of downstream effectors.
4. The *FLO* gene family contributes to cell adhesion, a hallmark of pseudohyphal growth. The *FLO* genes exhibit variegated expression. *FLO* genes are subtelomeric, and *cis*-interfering long noncoding RNAs encoded in the *FLO11* promoter establish a bistable

switch-like pattern of *FLO11* expression. *FLO* adhesin genes encode proteins with internal repeats that promote variability due to replication slippage and recombination events. The resulting cell surface variability may be important in fungal adaptation to the environment and in host evasion. Adhesins encode sequences with β-amyloid-like properties that may mediate phase transitions important for cell adhesion and biofilm formation.

5. Yeast pseudohyphal growth provides a model regulatory network encompassing well-conserved signaling modules implicated in cancer and other diseases in humans. The transition to a filamentous growth form is present in many fungi, and filamentous development is linked with virulence in fungal pathogens of plants and humans.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

A.K. thanks Paul J. Cullen for helpful discussions and valuable comments regarding the manuscript. Research in the laboratory of A.K. is supported by award 1902359 from the National Science Foundation.

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Annual Review of Genetics

Volume 55, 2021

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Errata

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