A Stress-Responsive Signaling Network Regulating Pseudohyphal Growth and Ribonucleoprotein Granule Abundance in *Saccharomyces cerevisiae*

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ABSTRACT The budding yeast *Saccharomyces cerevisiae* undergoes a stress-responsive transition to a pseudohyphal growth form in which cells elongate and remain connected in multicellular filaments. Pseudohyphal growth is regulated through conserved signaling networks that control cell growth and the response to glucose or nitrogen limitation in metazoans. These networks are incompletely understood, and our studies identify the TORC1- and PKA-regulated kinase Ksp1p as a key stress-responsive signaling effector in the yeast pseudohyphal growth response. The kinase-defective *ksp1*-K47D allele results in decreased pseudohyphal morphology at the cellular and colony level, indicating that Ksp1p kinase signaling is required for pseudohyphal filamentation. To determine the functional consequences of Ksp1p signaling, we implemented transcriptional profiling and quantitative phosphoproteomic analysis of *ksp1*-K47D on a global scale. Ksp1p kinase signaling maintains wild-type transcript levels of many pathways for amino acid synthesis and metabolism, relevant for the regulation of translation under conditions of nutrient stress. Proteins in stress-responsive ribonucleoprotein granules are regulated post-translationally by Ksp1p, and the Ksp1p-dependent phosphorylation sites S176 in eIF4G/Tif4631p and S436 in Pbp1p are required for wild-type levels of pseudohyphal growth and Protein Kinase A pathway activity. Pbp1p and Tif4631p localize in stress granules, and the *ksp1* null mutant shows elevated abundance of Pbp1p puncta relative to wild-type. Collectively, the Ksp1p kinase signaling network integrates polarized pseudohyphal morphogenesis and translational regulation through the stress-responsive transcriptional control of pathways for amino acid metabolism and post-translational modification of translation factors affecting stress granule abundance.

KEYWORDS yeast; functional genomics; proteomics; pseudohyphal growth

The eukaryotic cellular response to nutrient limitation is complex, encompassing programmed changes resulting in reduced protein translation, increased autophagy, an elevated abundance of ribonucleoprotein (RNP) granules, altered anabolism and catabolism of metabolites, and, in some organisms, pronounced morphological changes (Gimeno *et al.* 1992; Gasch *et al.* 2000; Klionsky and Emr 2000; Parker and Sheth 2007). In certain strains of the budding yeast *Saccharomyces cerevisiae*, nitrogen or glucose limitation induces a reversible transition from a yeast-like unicellular form to a filamentous or pseudohyphal morphotype, characterized by the formation of multicellular filaments (Gimeno *et al.* 1992; Cullen and Sprague 2000). Cells undergoing pseudohyphal growth bud distally in a unipolar fashion, exhibiting a polarized actin cytoskeleton, and remain physically connected after cytokinesis (Gimeno and Fink 1994; Kron *et al.* 1994; Lo and Dranginis 1996; Li and Mitchell 1997; Reynolds and Fink 2001; Cullen and Sprague 2002).
Filamentation in *S. cerevisiae* manifests itself principally in two growth forms affected by strain ploidy and nutrient availability. In haploid invasive growth, yeast colonies grown on standard or rich medium form filaments that extend downward, invading agar and other semisoft surfaces (Li and Mitchell 1997; Mösch et al. 1999; Cullen and Sprague 2000, 2002). In diploid pseudohyphal growth, nitrogen or glucose limitation induces the formation of filaments that extend downward from the colony into the substrate and outward over the surface of the agar (Gimeno et al. 1992; Cullen and Sprague 2012; Shively et al. 2013). Some induction stimuli, including the presence of short-chain alcohols that mimic amino acid catabolism observed during nitrogen limitation, can induce both surface filamentation and invasion in haploid yeast (Lorenz et al. 2000). Filament formation is thought to constitute a scavenging mechanism, and a pseudohyphal strain of *S. cerevisiae* grows in culture to a higher optical density than a wild-type, nonfilamentous strain under conditions of extended nitrogen limitation (Gimeno et al. 1992; Ma et al. 2007). Filamentation in *S. cerevisiae* is an informative model of pseudohyphal and hyphal development in the related opportunistic human fungal pathogen *Candida albicans* (Braun and Johnson 1997; Saville et al. 2003; Nobile and Mitchell 2005; Whiteway and Bachewich 2007). Filamentous development in *C. albicans* is required for pathogenicity in a mouse model of disseminated candidiasis (Lo et al. 1997). Yeast pseudohyphal growth has also been intensely studied as a tractable model of highly conserved signaling pathways that regulate metazoan cell growth and stress responses (Minden et al. 1994; Beck and Hall 1999; Westfall et al. 2008; Sengupta et al. 2010; Basu et al. 2016).

Landmark work from numerous groups have identified signaling pathways required for wild-type pseudohyphal growth, including the Kss1p mitogen-activated protein kinase (MAPK) cascade (Liu et al. 1993; Zhou et al. 1993; Roberts and Fink 1994; Cook et al. 1996; Madhani and Fink 1997; Erdman et al. 1998; Erdman and Snyder 2001), the rat sarcoma/cAMP-dependent protein kinase A pathway (Pan and Heitman 1999, 2002; Stanhill et al. 1999), the target of rapamycin (TOR) network (Cutler et al. 2001; Loewith et al. 2002; Braus et al. 2003), and the AMP-activated/sucrose nonfermentable (AMPK/Snf1p) pathway (Kuchin et al. 2002, 2003). Points of crosstalk between these networks can identify important signaling nodes (Borneman et al. 2006), and in this light, the yeast kinase Ksp1p is notable, as recent studies suggest that it is regulated through the PKA and TOR pathways. Ksp1p, a protein of the casein kinase II subfamily, was identified by mass spectrometry in a complex purified by immunoprecipitation of the core TOR Complex 1 protein Kog1p (Laxman and Tu 2011). Ksp1p contains PKA consensus recognition motifs (residues Ser591, Ser624, Ser827, and Ser884) and exhibits decreased phosphorylation at Ser827 and Ser884 upon rapamycin treatment (Soulard et al. 2010). Ksp1p was first identified as a high-copy suppressor of a temperature-sensitive mutation (prp20-10) in the *SRM1/PRP20* gene encoding a nucleotide exchange factor for Ran/GSP1 proteins (Fleischmann et al. 1996). We identified KSP1 in a loss-of-function screen for genes required for wild-type filamentous growth in a haploid derivative of the Σ1278b strain under conditions of butanol induction (Jin et al. 2008). We found that KSP1 was required for the wild-type localization of several pseudohyphal growth signaling proteins, including the Bcy1p regulatory subunit of PKA (Bharucha et al. 2008). Subsequently, Umekawa and Klionsky (2012) reported that KSP1 negatively regulates autophagy, consistent with the observed hypo-phosphorylation of Atg13p in ksp1Δ, and that this suppressive function of Ksp1p is partially activated by PKA.

These studies suggest that KSP1 contributes to eukaryotic cell signaling through stress-responsive pathways that regulate pseudohyphal growth, among other cell processes; however, the functional significance of Ksp1p kinase signaling is unclear, and the scope of the Ksp1p kinase signaling network remains to be determined. Here, we present data indicating the relevance of Ksp1p kinase activity in the yeast pseudohyphal response and globally identify changes in transcript abundance and protein phosphorylation dependent upon Ksp1p kinase signaling under filamentation-inducing conditions. The data identify phosphorylation sites in translation-associated RNP granule proteins that yield pseudohyphal growth phenotypes upon site-specific mutation. We assess the function of Ksp1p signaling in regulating RNP granule abundance and indicate that loss of KSP1 results in elevated Pbp1p-marked granules. Collectively, the work identifies Ksp1p as part of the mechanism linking highly conserved nutrient stress-responsive signaling pathways with the regulation of RNP granules, while identifying the molecular basis of Ksp1p kinase signaling as a TORC1 and PKA downstream effector.

**Materials and Methods**

**Strains, plasmids, and media**

Filamentous yeast strains were derived from the genetic background Σ1278b. Haploid strains were derived from HLY337 and Y825 (Xu et al. 2010). Yeast cells were cultured according to standard techniques as described previously (Guthrie and Fink 1991; Song et al. 2014). Yeast cells were grown in YPD (2% peptone, 1% yeast extract, 2% glucose, and 2% agar as needed) or synthetic complete (SC) medium (0.67% yeast nitrogen base, 2% glucose, 0.2% of the appropriate amino acid dropout mix, and 2% agar, as needed). Low-nitrogen synthetic low-ammonia dextrose (SLAD) media was prepared as follows: 0.17% yeast nitrogen base without amino acids and without ammonium sulfate, 2% glucose, 50 μM ammonium sulfate, and appropriate amino acids to complement auxotrophy. Synthetic low-ammonium low-dextrose (SLALD) media was prepared as described for SLAD media but with 0.05% glucose (Johnson et al. 2014). The plasmid pDS7 (U1A-mCherry) was constructed by amplification of the mCherry protein from plasmid pBS35 (Hailey et al. 2002) using the oligonucleotide primers DSK116 and DSK117, and amplification of the pRP1194.
backbone (Buchan et al. 2010) using oligonucleotide primers DSK114 and DSK115. PCR products were used for Gibson assembly with Gibson Assembly Master Mix (New England Biolabs, Beverly, MA).

**Construction of chromosomal point mutants and gene fusions**

Chromosomal deletions of genes were generated using a one-step PCR-based protocol for gene disruption by amplifying the kanMX6 sequence from plasmid pFA6a-kanMX6 (Longtine et al. 1998). Transformants were selected by plating on YPD medium supplemented with G418. PCR was used to confirm integration of the insertion cassette. Carboxy-terminal GFP fusions were generated by transformation of relevant strains with PCR product after amplification of the GFP-HisMX6 cassette from pFa6a-GFP-HisMX6 (Longtine et al. 1998). Chromosomal point mutants were generated using a URA3-based gene replacement and counter-selection strategy as described previously (Gray et al. 2005; Ma et al. 2008). In brief, the URA3 gene was amplified from pRS406 with primers containing sequence corresponding to 45-bp regions on either side of the mutational target site. Strains were transformed with PCR product for selection on media lacking uracil. Strains with URA3 insertions were transformed with annealed 120-nt oligonucleotides containing the desired mutant sequence and homology to flanking regions. Loss of URA3 was monitored by counterselection on plates supplemented with 5-fluoroarotic acid.

**Surface-spread pseudohyphal growth and invasion assays**

Cultures of diploid wild-type or mutant strains were grown overnight in standard growth media (e.g., YPD media) before being diluted 1:20 in fresh media. Strains were subsequently grown for 4–6 hr prior to harvesting for plating. Cells were washed three times with sterile deionized water and normalized to a final optical density of 1.0 before being diluted and spread onto plates with SLAD medium supplemented with uracil or other amino acids to complement strain auxotrophy. Cells were spread at a density of ~50 cells/plate. Plates were incubated at 30°C until filamentation was observed in wild-type strains, and colonies were then imaged using an upright Nikon Eclipse 80i microscope with CoolSnap ES2 CCD (Photometrics). Surface filamentation was quantified as described (Ryan et al. 2012; Norman et al. 2018). For these analyses, the circumference of a defined area of a colony was measured using ImageJ and compared against the circumference of the same defined area of a wild-type colony. The ratio is calculated from three replicates, and the average and SD are indicated.

Agar invasion was calculated by standard protocols (Ryan et al. 2012; Shively et al. 2013; Norman et al. 2018). A 5 μl aliquot of the culture to be tested was spotted onto a plate and allowed to invade the agar for 2–3 days. Plates were photographed, and surface cells were washed under a gentle stream of water. The washed cultures were imaged again, and the degree of invasive growth was quantified as the mean pixel intensity of the washed spot relative to its previous image. Triplicate replicates were assayed for each strain. To analyze cell morphology, cells were scraped from the edge of a colony and resuspended in a small volume of media before spotting onto a slide for microscopy. Images were captured as described above. Cell dimensions were measured, and height-to-width ratios for individual cells were calculated. Cells with a height-to-width ratio ≥2 were indicative of pseudohyphal growth.

**Transcriptional profiling and analysis**

Single colonies of respective strains (yCK186 and DSY005) were inoculated in YPD media and grown overnight with shaking at 30°C. Subsequently, cells corresponding to an optical density (600 nm) of 1.25 were harvested at 4000 g for 4 min and suspended in 5 ml SLAD media supplemented with uracil, yielding a final OD600 of 0.25/ml. Cultures were grown for an additional 6 hr in SLAD media supplemented with uracil. Cells were then harvested and RNA isolated using the RibobPure Yeast Kit (Invitrogen, Carlsbad, CA) according to manufacturer instructions. RNA concentration was determined using the NanoDrop microvolume spectrophotometer, and the quality of prepared RNA was assessed at the University of Michigan Sequencing Core with the BioAnalyzer platform (Agilent). Libraries for sequencing were prepared according to standard protocols (Song et al. 2014), and sequencing was performed with the Illumina HiSeq 4000 Single-End 51 Cycle platform. Differential transcript abundance at the gene and isoform level was determined using DESeq2 and the Tuxedo pipeline (Trapnell et al. 2013; Love et al. 2014). Lists of differentially abundant transcripts identified by DESeq2 and Tuxedo are available as Supplemental Material. Diagnostic plots for the sequencing generated by each analysis method can also be accessed as Supplemental Material.

Transcripts significantly increased or decreased according to analysis by both DESeq2 and Tuxedo were investigated further for overrepresented biological processes using DAVID 6.8 (Huang da et al. 2009). Enriched gene ontology (GO) terms were summarized using the REVIGO software (Supek et al. 2011).

**Mass spectrometry and analysis**

Wild-type control and ksp1-K47D mutant cells were isotopically labeled with heavy (Lys-8/Arg-10) amino acids in culture. Cell cultures were lysed by bead beating as described previously (Johnson et al. 2014; Shively et al. 2015). Extracted proteins were quantified using the Bradford assay. Equal quantities of proteins from triplicate independent cultures for each strain were treated for disulfide reduction and alkylation; treated protein preparations were digested with N-tosylmocidophenylmethyl ketone (TPMK)-treated trypsin (Worthington Biochemical, Lakewood, NJ). Peptide mixtures were fractionated by strong cation exchange on a PolySulfoethyl A column (150 × 4 mm; PolyLC).
Fractionation protocols are as described (Johnson et al. 2014). Collected fractions were enriched for phosphopeptides using ZrO2 (50 μm inner diameter resin; Glygen). Eluates of enriched phosphopeptides and flow-through from each cation fractionation were analyzed by nano-liquid chromatography–tandem mass spectrometry on a hybrid type mass spectrometer (LTQ-Orbitrap XL; Thermo Fisher Scientific, Waltham, MA) coupled to a nanoLC system (2D nanoLC; Eksigent).

Raw mass spectrometry data were processed and quantified using MaxQuant and the Mascot search engine collectively (Eng et al. 1994; Cox et al. 2009). Mascot searches were performed against a composite database of forward and reverse sequences of verified yeast open reading frames from the Saccharomyces Genome Database. Variable modifications were allowed for oxidation (M) and phosphorylations (STY), as well as a fixed modification of carboxymethylation (C). Peptide, protein, and phosphorylation site identifications were filtered at a false discovery rate of 5%. The MaxQuant normalized heavy:light ratios with significance scores <0.05 were considered significant in this study. The data were further filtered to exclude peptides exhibiting low Mascot scores (<3), high charge states (>5), and long peptide lengths (>40). Proteins were analyzed for enrichment of associated biological functions using tools made available through the Saccharomyces Genome Database, as well as the DAVID software suite (Huang da et al. 2009). Protein-protein interaction networks were generated using Cytoscape software (Killcoyne et al. 2009), and interactions used to construct the network diagram were extracted from BioGRID.

**Reporter assays of FLO11 transcription**

 Diploid strains harboring plasmid pGL669-Z FLO11 6/7 (Aun et al. 2013) were grown overnight in SC media lacking uracil. Cells were washed three times with 2% glucose, diluted 1:5 in SLAD media, and grown at 30°C for 4–6 hr. β-galactosidase assays were performed in triplicate using the Yeast β-Galactosidase Assay Kit (Thermo Scientific) according to manufacturer suggested protocols.

**Fluorescence microscopy**

 Liquid cultures from single colonies were grown in appropriate media overnight. Cultures were subsequently diluted to an optical density (600 nm) of 0.1 and were grown for 24 or 48 hr. Aliquots (1 ml) of the cultures were removed and cells collected by centrifugation at 1000 × g for 1 min. Cells were suspended in 80–100 μl of appropriate SC-based media for imaging using the Deltavision Spectris system (Applied Precision).

**Statistical analysis and error correction**

 The significance of transcriptional profiling data analyzed by DESeq2 was determined using the Wald test P-value for condition vs. control analysis. This test statistic P-value was adjusted using the Benjamini–Hochberg correction as indicated in File S2. Data analysis through the Tuxedo pipeline determines the uncorrected P-value of the test statistic and its false discovery rate–adjusted P-value. Both sets of values are reported in File S4. Quantitative phosphoproteomic data were filtered at a false discovery rate of 5%, and MaxQuant-normalized heavy:light ratios for observed peptides were considered statistically significant at a significance score <0.05 (File S7). GO term enrichment in the set of differentially phosphorylated proteins identified by mass spectrometry was analyzed using the DAVID software suite; adjusted P-values for enriched GO terms were calculated with the Bonferroni correction and the Benjamini–Hochberg procedure, as indicated. For cell morphology and microscopy data, mean values of the control and test samples were measured, and uncorrected P-values were generated using t-tests for the comparison of observed means in two independent samples.

**Data availability**

 Strains and plasmids are available upon request. Supplemental Material, Table S1 lists genotype and source for all strains used in this study. Figure S1 presents phenotypes for ksp1 deletion and kinase-defective mutants under pseudohyphal growth-inducing conditions of nitrogen limitation and limited glucose availability (SLALD medium). Figure S2 presents images indicating that Pbp1p-GFP and U1A-mCherry-bound RNA puncta are elevated in numbers relative to wild type in a homozygous diploid strain deleted for KSP1. Figure S3 presents a diagram of the P_LFO11-6/7-lacZ reporter and indicates reporter activity in a yeast strain deleted for FLO8. Figure S4 presents Western blots indicating that protein levels of ste20p-T203A, tif4631p-S176A, and pbp1-S436A are not significantly different from levels of corresponding wild-type proteins; this further suggests that observed phenotypes for these point mutants do not result from altered expression relative to wild type. File S1 contains detailed descriptions of all supplemental files. File S2 contains statistical analysis of all changes in transcript abundance, both significant and insignificant, between the ksp1-K47D mutant and wild type as determined using DESeq2. File S3 contains boxplots indicating non-normalized counts, depth-normalized counts, and regularized log2-normalized counts for each RNA-sequencing sample analyzed. File S4 contains statistical analysis of all changes in transcript abundance, both significant and insignificant, between the ksp1-K47D mutant and wild type as determined using Tuxedo. File S5 presents summary boxplots of fragments per kilobase of transcript per million mapped reads distribution in log10 scale for each RNA sample. File S6 provides a listing of the union set of genes identified as undergoing statistically significant changes in transcript abundance between ksp1-K47D and wild type by both DESeq2 and Tuxedo analysis. File S7 provides the mass spectrometry data from Mascot and MaxQuant analysis for differentially abundant phosphopeptides between ksp1-K47D and wild type. File S8 provides the protein-protein interactions extracted from BioGRID for the
Results

The kinase activity of Ksp1p is required for pseudohyphal growth

KSP1 encodes a protein of 1029 amino acids, with an estimated molecular mass of 117 kDa. Ksp1p contains a kinase domain, putatively of the casein kinase II family, as part of a strongly conserved region in the amino-terminal half of its protein sequence. The carboxy-terminal half of the protein is enriched in Asn residues and is predicted to bind RNA (Romero et al. 2001; He et al. 2009). Experimental evidence supports this hypothesis; Mitchell et al. (2013) identified Ksp1p as a protein cross-linked to messenger RNA (mRNA). Given the range of functionalities associated with Ksp1p, we sought to determine the contributions of Ksp1p kinase activity toward pseudohyphal growth induced by nutrient limitation.

To address this, we mutated the conserved catalytic lysine residue (Lys47) in the predicted ATP-binding pocket of the Ksp1p kinase domain for phenotypic analysis of pseudohyphal growth. The Lys-to-Asp substitution was achieved by allelic replacement at the native KSP1 locus in a strain of the filamentous Σ1278b genetic background, and the homozygous diploid ksp1-K47D kinase-defective mutant was assayed for surface-spread filament formation under conditions of nitrogen limitation. As indicated in Figure 1, A and B, the kinase-defective ksp1-K47D strain exhibits decreased surface filamentation relative to wild type on medium with low levels of ammonium sulfate as a nitrogen source. This surface filamentation phenotype is also evident in ksp1-K47D under pseudohyphal growth conditions of limited nitrogen and reduced glucose availability (SLALD), although the phenotype is less severe than that observed under conditions of limited nitrogen alone (Figure S1). Cells undergoing pseudohyphal growth are elongated, and the percentage of cells with an elongated morphology is significantly decreased in the ksp1-K47D strain relative to wild type under conditions of nitrogen limitation (Figure 1C). The cell morphology of ksp1 mutants in medium with limited nitrogen and glucose is quantified in Figure S1. Agar filament invasion is also diminished in homozygous diploid ksp1-K47D on low-nitrogen medium (Figure 1D).

A global profile of changes in transcript abundance is dependent upon Ksp1p kinase activity

As a step toward determining the molecular basis of Ksp1p kinase signaling in regulating the pseudohyphal response, we profiled changes in transcript abundance in ksp1-K47D relative to wild type under conditions of reduced nitrogen availability (SLAD media). Cells were grown to log phase in culture for subsequent harvesting, RNA extraction, and high-throughput sequencing (Figure 2A). Transcripts with altered abundance in ksp1-K47D were analyzed for statistically significant enrichment of annotated GO terms and cellular/biochemical properties of the encoded protein products.

In total, we report a union set of 741 transcripts exhibiting altered abundance in the ksp1-K47D mutant as identified through analyses using two independent methods (detailed in Materials and Methods). Approximately 36% of transcripts exhibited decreased abundance in ksp1-K47D relative to wild type, with the remaining 64% of transcripts showing increased abundance. Collectively, transcripts with altered abundance were highly enriched for genes contributing to cellular amino acid biosynthesis and metabolism (Figure 2B); by GO annotation, the latter term encompasses both biosynthetic and catabolic proteins. Affected amino acid biosynthetic and metabolic pathways encompass gene sets responsible for regulating charged/polar amino acids (including lysine, arginine, aspartic acid, glutamine, and sulfur-containing residues such as serine and cysteine), as well as pathways regulating aromatic amino acids and hydrophobic residues (including glycine and branched-chain amino acids). It is also notable that the transcripts annotated as contributing to amino acid biosynthesis or metabolism were predominantly more abundant in the ksp1-K47D mutant relative to wild type. Translation initiation and polyribosome abundance are markedly decreased under many stress conditions in eukaryotes, and the results here indicate a role for Ksp1p in decreasing the transcription of genes contributing to cellular amino acid pools as an output of its kinase signaling activity.

Transcripts exhibiting decreased abundance in ksp1-K47D were enriched for GO terms associated with the morphogenesis checkpoint, DNA replication, and cell wall organization (Figure 2C). The acute onset of starvation activates morphogenesis and DNA replication checkpoints (Giannattasio and Branzei 2017), and the decrease in transcript abundance for related genes in ksp1-K47D may manifest as a defect in the sensing or processing of starvation signals upon impaired Ksp1p kinase signaling. Perhaps expectedly, a significant set of flocculence (FLO) genes show decreased transcript abundance in the ksp1-K47D mutant under conditions that induce pseudohyphal growth. FLO genes are so named because they are required for flocculation, or cell-cell adhesion, which is increased in filamentation-competent strains during pseudohyphal growth (Lo and Dranginis 1998; Halme et al. 2004; Karunanithi et al. 2010). FLO11 is required for wild-type pseudohyphal growth, and its complex and unusually large 3-kb promoter is regulated through transcription factors acting upon by the Kss1p MAPK, PKA, and Snf1p signaling pathways (Madhani et al. 1999; Rupp et al. 1999; Bao et al. 2004; van Dyk et al. 2005). FLO11 transcript abundance is diminished in the ksp1-K47D strain, consistent with the observed decrease in surface-spread and invasive filamentation evident in this mutant (Figure 2C).

In total, these data are consistent with a function for Ksp1p kinase activity in regulating translation and cell morphogenesis
at the transcriptional level. To consider post-translational modifications in phosphorylation achieved directly or indirectly through Ksp1p kinase signaling, we employed an approach using quantitative phosphoproteomics.

**Quantitative phosphoproteomics identifies a set of messenger RNP granule proteins differentially phosphorylated in the ksp1-K47D kinase-defective mutant**

For these studies, we utilized stable isotopic labeling with amino acids in cell culture (SILAC) and mass spectrometry to identify proteins differentially phosphorylated in a strain carrying ksp1-K47D (Ong et al. 2002; Ong and Mann 2006). For SILAC analysis, we generated a haploid strain of the filamentous Σ1278b background deleted for LYS1 and ARG4, making the strain dependent upon exogenously supplied lysine and arginine for protein synthesis. We further deleted KSP1 in this strain and introduced a low-copy, centromeric plasmid carrying the ksp1-K47D allele under transcriptional control of its native promoter. As indicated in Figure 3A, the control strain with wild-type KSP1 was grown in media supplemented with light lysine and arginine, while the strain carrying ksp1-K47D was incubated in media with stable heavy isotopic forms of both amino acids. Filamentous growth was induced in both strains by the addition of the short-chain alcohol butanol. We selected this induction method because the presence of 1% butanol results in a very strong pseudohyphal response for haploid strains in liquid culture, forming extended filaments of elongated cells even without a solid substratum for adherence (Lorenz et al. 2000; Jin et al. 2008). After sufficient growth to ensure protein labeling, cells were harvested and phosphoproteins analyzed by liquid chromatography–tandem mass spectrometry.

By this approach, peptides corresponding to 84 proteins were identified as being hypo-phosphorylated relative to control in the strain with the ksp1-K47D allele, and 65 hyper-phosphorylated proteins were identified. It should be noted that by this approach, direct and indirect targets of Ksp1p kinase activity were identified collectively in the set of proteins hypo-phosphorylated in the ksp1-K47D mutant; presumably, indirect targets of Ksp1p kinase signaling are indicated by the hyper-phosphorylated proteins detected in ksp1-K47D. The full set of differentially phosphorylated proteins identified in this work is indicated in Figure 3B, and sites of differential phosphorylation in each protein are reported in the File S7.

We analyzed the identified set of Ksp1p-dependent phosphoproteins for enrichment of functional annotations, including GO annotations. As shown previously in Figure 2, Ksp1p...
Figure 2 Transcriptional profiling identifies cellular processes affected by Ksp1p kinase signaling. (A) Workflow for the identification and analysis of transcripts differentially abundant in the kinase-defective ksp1-K47D strain relative to wild type. (B) Genes exhibiting differentially abundant transcripts were analyzed for enriched GO terms. Cell processes associated with the biosynthesis and metabolism of amino acid pools were enriched in the data set; relevant GO terms with annotated transcripts are indicated. The P-value associated with each enrichment is shown. Changes in transcript abundance for each gene are represented by the indicated color scale. Changes in transcript abundance were calculated using the base-2 logarithmic scale. (C) Transcripts for known flocculence (GO:0000128) and filamentous growth (GO:0030447) genes were less abundant in ksp1-K47D relative to wild type. GO annotations enriched in the set of transcripts exhibiting decreased abundance in ksp1-K47D and in the transcripts with increased abundance in this mutant are represented in the boxed diagram. The magnitude of enrichment for each term is indicated by the size of each rectangle, with the full box indicating the sum total of enriched GO terms for the respective data set.
signaling is required for wild-type levels of gene transcripts functioning in amino acid biosynthesis and metabolism, relevant in establishing amino acid pools for translation. Consistent with this observation, the Ksp1p-dependent phosphoproteome is also enriched for proteins that regulate translation, but principally as components of cytoplasmic messenger ribonucleoprotein (mRNP) granules that are induced under conditions of cell stress (Figure 4). RNP stress granules are a type of membraneless organelle that forms via phase separation of translationally stalled/inactive RNA and proteins (Buchan and Parker 2009; Buchan et al. 2013). A statistically overrepresented set of mRNP stress granule proteins were identified as being hypo-phosphorylated in ksp1Δ-K47D relative to controls strain. This set of mRNP stress granule proteins includes the helicase Dhh1p, the polyA-binding protein Pbp1p, the p21-activated signaling kinase Ste20p, and the eIF4G translation initiation factor Tif4631p (Buchan et al. 2010, 2011). Using the functional annotation tool DAVID to mine the set of proteins hypo-phosphorylated in ksp1Δ-K47D, we identified additional Ksp1p-dependent phosphoproteins associated with cytoplasmic mRNP granules (Figure 4B). Using the identified core mRNP stress granule proteins to construct a protein-protein interaction network (Figure 4C), the resulting map suggests that Ksp1p signaling affects stress-responsive mRNP granules and that many granule proteins are required for wild-type pseudohyphal growth.

**Sites of Ksp1p-dependent phosphorylation in the stress granule proteins eIF4G/Tif4631p and Pbp1p are required for wild-type pseudohyphal growth**

To determine the pseudohyphal growth-related significance of stress granule proteins dependent upon Ksp1p for wild-type phosphorylation, we generated homozygous diploid strains in the filamentous S1278b background deleted for DHH1, PBP1, STE20, and TIF4631. Deletion of DHH1 did not result in a pseudohyphal growth phenotype; however, loss of the other genes did affect filamentation (Figure 5).

STE20 is required for wild-type pseudohyphal growth and agar invasion (Figure 5A). Ste20p belongs to the p21-activated kinase family and acts immediately upstream of the yeast filamentous growth Kss1p MAPK cascade, phosphorylating the MAPKKK Ste11p (Drogen et al. 2000). Ste20p also phosphorylates the stress granule-localized mRNA decapping protein Dcp2p, and Ste20p has been colocalized with stress granule proteins, including Pub1p-mCherry (Yoon et al. 2010; Mitchell et al. 2013). Notably, we observe that PKA pathway regulation of FLO11 expression is decreased in homozygous diploid ste20Δ/Δ relative to wild type in low-nitrogen media, using a plasmid-based lacZ reporter fusion to a segment of the FLO11 promoter regulated by the PKA pathway (Figure 5B). The reporter contains sequence corresponding to a region 1.0–1.4 kb upstream of the FLO11 initiator ATG, encompassing binding sites for the PKA-regulated transcription factors Flo8p and Sfl1p (Aun et al. 2013).
β-galactosidase levels from the FLO11-lacZ reporter are significantly depressed in a strain deleted for FLO8 (Figure S3). Tif4631p is an ortholog of the eukaryotic translation initiation factor eIF4G isoform 1, acting as a scaffold and interacting with poly(A)-binding protein and components of the mRNA cap-binding complex (Tarun et al. 1997; Gallie and Browning 2001). Tif4631p colocalizes with the stress granule marker Pub1p-mCherry, and a TAP-tagged form of

Figure 4 The Ksp1p-dependent phosphoproteome is enriched for proteins in ribonucleoprotein granules. (A) The GO Biological Process term “stress granule assembly” (GO: 0034063) is enriched in the set of proteins differentially phosphorylated in ksp1-K47D relative to the proteome as a whole. The annotated proteins are Dhh1p, Pbp1p, Ste20p, and Tif4631p. (B) Analysis with the DAVID software suite for data mining validates the above result, identifying enrichment of an annotation cluster with GO 0034063, the Cellular Component term “cytoplasmic stress granule” (GO: 0010494), and the Molecular Function term “mRNA binding” (GO: 0003729). (C) To identify the effect of signaling through these Ksp1p-dependent stress granule phosphoproteins, we constructed an interaction network using Dhh1p, Ksp1p, Pbp1p, Ste20p, and eIF4G/Tif4631p as nodes. Physical interactions extracted from BioGRID are indicated as lines. Ksp1p-dependent phosphoproteins identified in this study are shaded in blue, and proteins required for wild-type pseudohyphal growth are shown in orange. Subsets of functionally related proteins are outlined by dashed lines.
the protein has been used to purify stress granule cores in yeast (Mitchell et al. 2013; Jain et al. 2016). We find that deletion of TIF4631 in a homozygous diploid strain of filamentous S. cerevisiae results in decreased surface filamentation and invasive growth on medium with limited nitrogen (Figure 5A). Homozygous diploid tif4631Δ/Δ exhibits decreased PKA pathway activity relative to wild type under conditions of nitrogen limitation. Tif4631p undergoes Ksp1p-dependent phosphorylation at Ser176, as phosphorylation at this site is decreased in ksp1-K47D. Mutation of Tif4631p Ser176 to nonphosphorylatable alanine (S176A) results in decreased surface filamentation on low-nitrogen medium and decreased PKA pathway activity by the FLO11 promoter-based reporter described above (Figure 5B). Steady-state levels of tif4631p-S176A tagged with the HA epitope at its carboxy terminus did not exhibit a significant change from observed levels of wild-type Tif4631p-HA under identical growth conditions (Figure S4A).

Pbp1p is an ortholog of poly(A)-binding protein, interacting with Pab1p to regulate mRNA polyadenylation (Mangus et al. 1998). Pbp1p is a component of the stress granule core substructure, and Pbp1p carboxy-terminal fluorescent protein fusions are routinely used as stress granule markers in yeast (Shah et al. 2013; Jain et al. 2016). Homozygous deletion of PBP1 results in decreased surface filamentation and agar invasion relative to wild type on medium with low levels of ammonium sulfate as a nitrogen source (Figure 5A). Ser436 in Pbp1p is hyper-phosphorylated in a strain with the kinase-defective ksp1-K47D allele, suggesting an indirect mechanism of Ksp1p regulation at this residue. Mutation of Pbp1p Ser436 to alanine results in increased growth and agar invasion relative to wild type under conditions of nitrogen limitation (Figure 5A). Consistent with this phenotype, the FLO11 promoter-based lacZ reporter is hyperactive relative to wild type under conditions of nitrogen limitation in the homozygous pbp1-S436A mutant (Figure 5B). By Western blotting, levels of pbp1p-S436A tagged at its carboxy terminus with

Figure 5 Ksp1p-dependent phosphorylation sites in the stress granule proteins eIF4G/Tif4631p and Pbp1p are required for wild-type pseudohyphal growth and PKA signaling through FLO11. (A) Surface filamentation phenotypes and agar invasion phenotypes are presented for wild type, ste20Δ/Δ, tif4631Δ/Δ, and pbp1Δ/Δ mutants. Surface filamentation was assayed on medium with low levels of ammonium sulfate (SLAD). + indicates wild-type filamentation; − indicates decreased surface filamentation relative to wild type; +++ indicates increased surface filamentation relative to wild type. The pbp1-S436A mutant grew well beyond the boundaries of the original spotted culture and extended substantial surface filaments that formed a dense surrounding ring, the degree of which is beyond that observed in the wild-type strain. The degree of surface filamentation relative to wild type was quantified as described previously using the circumference (ImageJ) of a defined region from each spotted culture; percentages indicate the mean and SD from four replicate spots. Bar, 1 mm. Agar invasion was quantified for homozygous diploid mutants on low-nitrogen SLAD medium as the average pixel intensity for each spotted culture postwashing relative to the prewashed image as described previously. Wild-type agar invasion is presented in Figure 1. −− indicates severely decreased invasion relative to wild type; −−− indicates severely decreased invasion relative to wild type; +++ indicates increased agar invasion relative to wild type. Bar, 1 mm. (B) PKA pathway activity was estimated for the indicated homozygous diploid mutants in low-nitrogen SLAD media using a lacZ reporter driven by a region of the FLO11 promoter regulated by PKA signaling. β-galactosidase levels are reported as the observed ratio relative to wild type.

714 N. Mutlu et al.
the HA epitope are not significantly different from levels of Pbp1p-HA (Figure S4B), indicating that the observed pbp1-S436A phenotypes are unlikely to result from altered expression of the point mutant.

**KSP1 regulates Pbp1p RNP granule abundance**

RNP stress granule components are enriched above background in the set of proteins constituting the Ksp1p-dependent phosphoproteome, suggesting that Ksp1p may contribute to the regulation of stress-responsive RNP granules. Since the stress granule marker Pbp1p undergoes Ksp1p-dependent phosphorylation, we assessed its localization as a carboxy-terminal fusion to GFP expressed from its native chromosomal locus in diploid wild-type or ksp1Δ/Δ strains. Asterisks indicate Pbp1p foci. The percentage of cells exhibiting Pbp1p foci was calculated for both strains. Cells were grown for an extended period of time in minimal media to induce high optical density/cell number stress. Consequently, cell auto-fluorescence, indicative of dead cells, can be seen in the image. The number of cells counted is indicated, and the percentages are indicated as a ratio relative to wild type, with 20% of cells in the wild-type strain exhibiting Pbp1p-GFP puncta. Bar, 2 μm.

**Figure 6** Loss of KSP1 results in elevated ribonucleoprotein foci marked by Pbp1p. (A) Pbp1p was visualized as a carboxy-terminal fusion to GFP expressed from its native chromosomal locus in diploid wild-type or ksp1Δ/Δ strains. Asterisks indicate Pbp1p foci. The percentage of cells exhibiting Pbp1p foci was calculated for both strains. Cells were grown for an extended period of time in minimal media to induce high optical density/cell number stress. Consequently, cell auto-fluorescence, indicative of dead cells, can be seen in the image. The number of cells counted is indicated, and the percentages are indicated as a ratio relative to wild type, with 20% of cells in the wild-type strain exhibiting Pbp1p-GFP puncta. Bar, 2 μm. (B) The U1A-mCherry binding system was used to visualize mRNA in the homozygous diploid ksp1Δ K47D mutant. Cells were grown in SC media lacking leucine and uracil to maintain selection for the plasmids encoding U1A-mCherry and the modified PGK1 gene. Cells were grown to high optical density as described above. Asterisks indicate U1A-bound mRNA puncta. The fold-increase of cells with puncta in the ksp1Δ K47D mutant relative to wild type is indicated along with the number of cells counted. Bar, 2 μm. P-values (<0.01)
generated a phosphomimetic Ser-to-Asp substitution to determine the effect of phosphorylation at this site on subcellular localization. As indicated in Figure 7A, the Pbp1p-S436D-GFP chimera expressed from its native chromosomal locus in the ksp1-K47D background exhibited 1.8-fold elevated focus abundance relative to a wild-type allele of PBP1. The Ste20p kinase is phosphorylated in a Ksp1p-dependent fashion at Thr203, with the residue hypo-phosphorylated in ksp1-K47D. A carboxy-terminal GFP fusion to a mutated form of this protein with Ala swapped for Thr203 indicates 6.1-fold elevated Ste20p-containing RNP granules (Figure 7B). Consistent with data observed in the fluorescent micrographs, Western blotting indicates that total protein levels of HA-tagged ste20p-T203A-HA are not significantly different from levels of Ste20p-HA (Figure S4A). Collectively, the data are consistent with an observed increase in stress granule RNP foci in a yeast strain defective for Ksp1p signaling. Transcript levels for genes functioning in pathways for amino acid biosynthesis and metabolism were perturbed in a filamentous yeast strain carrying the kinase-defective ksp1-K47D allele. Phosphorylation of a statistically significant set of translation-associated proteins in RNP granules was altered in this mutant. Amino acid metabolism and RNP function contribute to the translational control and repression evident in response to nitrogen limitation. The Ksp1p-dependent phosphorylation sites Ser176 in Tif4631p and Ser436 in the stress granule marker Pbp1p are required for wild-type pseudohyphal growth. Loss of KSP1 results in elevated Pbp1p-marked RNP granules, consistent with a function for Ksp1p in regulating stress granule abundance.

The studies here indicate that Ksp1p kinase signaling contributes to the regulation of many eukaryotic stress responses. Ksp1p is required for pseudohyphal growth, and the Ksp1p-dependent signaling network is enriched in genes contributing to relevant processes, including cell wall organization, DNA replication, and the cell morphogenesis checkpoint. GO terms related to these processes were enriched in previous gene disruption and overexpression screens for pseudohyphal growth regulators (Jin et al. 2008; Ryan et al. 2012; Shively et al. 2013). Genes required for cell-cell adhesion are also enriched in the set of transcripts regulated through Ksp1p signaling. Extending beyond processes intimately associated with pseudohyphal growth, genes

Figure 7 The Ksp1p-dependent Ser436 phosphorylation site in Pbp1p and Thr203 phosphosite in Ste20p are required for the wild-type localization of these respective stress granule-associated proteins. (A) A cassette encoding GFP was integrated at the 3’-end of wild-type PBP1 and in the pbp1-S436D phosphomimetic mutant strains. Plasmids for visualization of modified PGK1 mRNA by U1A-mCherry binding were introduced into each strain. Cells were grown to a high density by culturing the cells for an extended period of time in minimal media as described previously. Asterisks indicate Pbp1p-GFP puncta that also overlap with U1A-mCherry-bound mRNA. The fold-change in cells with GFP puncta relative to wild type is indicated along with the number of cells counted for each strain. Bar, 2 μm. (B) Chromosomal GFP-fusions were generated in a wild-type strain and in a strain with the ste20-T203A mutation by integration of the GFP-encoding cassette at the native STE20 locus as described above. Cells were cultured, imaged, and quantified for GFP and U1A-bound mRNA puncta as indicated above. Wild-type Ste20p puncta are not as easily visualized as Pbp1p puncta (observed in 1% of cells); consequently, the fold-change increase in puncta observed for the ste20-T203A mutant is more dramatic. Bar, 2 μm. P-values (<0.01)

Discussion
The Ksp1p kinase is a putative effector of PKA and TORC1 signaling, but the purview of its regulatory control in eukaryotic stress responses has been largely unclear. Here, we used transcriptional profiling and quantitative phosphoproteomics to determine cellular processes regulated through Ksp1p signaling. Transcript levels for genes functioning in pathways for amino acid biosynthesis and metabolism were perturbed in a filamentous yeast strain carrying the kinase-defective ksp1-K47D allele. Phosphorylation of a statistically significant set of translation-associated proteins in RNP granules was altered in this mutant. Amino acid metabolism and RNP function contribute to the translational control and repression evident in response to nitrogen limitation. The Ksp1p-dependent phosphorylation sites Ser176 in Tif4631p and Ser436 in the stress granule marker Pbp1p are required for wild-type pseudohyphal growth. Loss of KSP1 results in elevated Pbp1p-marked RNP granules, consistent with a function for Ksp1p in regulating stress granule abundance.

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716  N. Mutlu et al.
mediating the cellular response to oxidative stress, autophagy genes, and genes required for sporulation undergo changes in transcript abundance in a strain of yeast with defective Ksp1p kinase activity. This broad function for the Ksp1p regulatory network is consistent with its role in PKA and TORC1 signaling. TORC1 has been proposed to control PKA activity toward selective substrates, and the molecular basis of the Ksp1p signaling network suggests that it may contribute to the integration of PKA and TORC1 signaling, possibly toward the PKA regulatory subunit Bcy1p, which is mislocalized in a ksp1 loss-of-function mutant (Bharucha et al. 2008). Control of FLO11 levels by the PKA-regulated Flo8p and Sfl1p transcription factors is altered in ksp1 mutants and in strains with mutated Ksp1p-dependent phosphosites in Tif4631p and PBP1, although it should be noted that signaling pathways beyond PKA may contribute to the regulation of these transcription factors.

Analysis of the Ksp1p-dependent phosphoproteome was notable in that it identified a significant set of proteins localized to stress granules, including proteins in the translation initiation closed-loop complex, such as elf4F4/Tif4631p, or proteins that bind to this complex, such as Pbp1p and Caf20p. Tif4631p has been immunoprecipitated as part of the proteinaceous stress granule core (Jain et al. 2016). Its Ksp1p-dependent phosphorylation site (Ser176) has not been reported previously and does not lie in a region of Tif4631p corresponding to a known protein domain. Tif4631p Ser176 is required for wild-type pseudohyphal growth but is dispensable for the regulation of stress granule abundance. Tif4631p has been identified previously as an in vitro substrate of Ksp1p (Chang and Huh 2018). The substrate specificity of Ksp1p is unknown, and a substrate recognition motif is not evident in the protein set identified from the phosphoproteomic studies reported here. Consequently, it is unclear if Ser176 is directly phosphorylated by Ksp1p. The observation that Tif4631p Ser176 is required for wild-type pseudohyphal growth but dispensable for its subcellular localization suggests that separate Ksp1p outputs may regulate pseudohyphal growth and RNP abundance. Pbp1p is also a core stress granule protein, and its regulation by Ksp1p is more complex, as Ser 456 is hypo-phosphorylated in ksp1-K47D, while Ser436 is hyper-phosphorylated in this mutant background. Both phosphorylation sites have been identified previously (Holt et al. 2009; Soulard et al. 2010), although we did not observe any pseudohyphal growth phenotypes for Ser456. The Ser436 site is indirectly regulated through Ksp1p signaling. Notably, the phosphomimetic Pbp1p-S436D mutant forms increased RNP granules, similar to the phenotype observed in ksp1-K47D. The Ser436 site in Pbp1p does not conform to the consensus substrate motif for a known signaling kinase, and additional work will be needed to identify its direct regulatory kinase. The data here indicate that Ksp1p kinase signaling results in the phosphorylation of Tif4631p at Ser176 and the dephosphorylation of Pbp1p at Ser436. Both phosphorylation events contribute to the inhibition of RNP granule proliferation.

Recent studies suggest that the regulation of stress granule proliferation and disassembly may be achieved through protein-protein and protein-RNA interactions, with proteins containing prion-like, intrinsically disordered regions contributing significantly to these interactions (Wheeler et al. 2016). Ksp1p was identified in biochemically purified yeast stress granules, and its sequence is predicted to encode a disordered RNA-binding region (Jain et al. 2016). Ksp1p was also identified experimentally as an RNA-binding protein by UV cross-linking and mass spectrometric analysis of proteins from purified RNA-protein complexes (Mitchell et al. 2013). Stress granule core proteins have been hypothesized to nucleate granule assembly; however, stress granule abundance is increased upon loss of KSP1. Consequently, Ksp1p may be a noncanonical stress granule core protein that either inhibits excessive stress granule assembly or facilitates granule disassembly. Studies of RNP dynamics will be necessary to distinguish between these possibilities.

There is precedence for signaling kinases integrating the pseudohyphal response and RNP granule dynamics. We previously colocalized the yeast MAPKs Kss1p and Fus3p and the PKA catalytic subunit Tpk2p with the stress granule and P-body–localized protein Igo1p. Deletion of KSS1 disrupted RNP foci under conditions of glucose limitation, as visualized using the U1A RNA-binding platform (Shively et al. 2015). In yeast, PKA inhibits the formation of large P-body aggregates by phosphorylating the granule scaffolding protein Pat1p (Ramachandran et al. 2011). The degree to which Ksp1p and the signaling kinases above regulate translation through the control of stress granule abundance is an open question. Polyribosome profiling of mutants defective in stress granule assembly does not show obvious phenotypes, which leaves the likely possibility that the translation of specific key transcripts may be regulated through RNP trafficking. Similar analyses may need to be performed for cells exposed to various stresses to assess the mechanistic basis of stress granule and RNP function. The work here underscores the importance of stress-responsive signaling pathways in regulating the abundance of RNPs, while providing a molecular basis for Ksp1p signaling as a node in the eukaryotic stress response network.

Acknowledgments

We thank Roy Parker (University of Colorado) for plasmid reagents and Daniel J. Klionsky, Mara C. Duncan, and Laura J. Olsen (University of Michigan) for helpful comments regarding the manuscript. This work was funded by grant 1R01-A1098450-01A1 (A.K.) from the National Institutes of Health and grant 1902359 from the National Science Foundation (A.K.).

Literature Cited

Aun, A., T. Tamm, and J. Sedman, 2013 Dysfunctional mitochondria modulate cAMP-PKA signaling and filamentous and invasive
growth of Saccharomyces cerevisiae. Genetics 193: 467–481. https://doi.org/10.1534/genetics.112.147389

Bao, M. Z., M. A. Schwartz, G. T. Cantin, J. R. Yates, and H. Madhani, 2004 Pheromone-dependent destruction of the Tec1 translation factor is required for MAP kinase signaling specificity in yeast. Cell 119: 991–1000. https://doi.org/10.1016/j.cell.2004.11.052

Basu, S., N. Vadaie, A. Prabhakar, B. Li, H. Adhikari et al., 2016 Spatial landmarks regulate a Cdc42-dependent MAPK pathway to control differentiation and the response to positional compromise. Proc. Natl. Acad. Sci. USA 113: E2019–E2028. https://doi.org/10.1073/pnas.1522679113

Beck, T., and M. N. Hall, 1999 The TOR signaling pathway controls nuclear localization of nutrient-regulated transcription factors. Nature 402: 689–692. https://doi.org/10.1038/45287

Bharucha, N., J. Ma, C. J. Dobry, S. K. Lawson, Z. Yang et al., 2008 Analysis of the yeast kinome reveals a network of regulated protein localization during filamentous growth. Mol. Biol. Cell 19: 2708–2717. https://doi.org/10.1091/mbc.e07-11-1199

Borneman, A. R., J. A. Leigh-Bell, H. Yu, P. Bertone, M. Gerstein et al., 2006 Target hub proteins serve as master regulators of development in yeast. Genes Dev. 20: 435–448. https://doi.org/10.1101/gad.1389306

Braus, G. H., O. Grundmann, S. Bruckner, and H. U. Mosch, 2003 Amino acid starvation and Gen4p regulate adhesive growth and FLO11 gene expression in Saccharomyces cerevisiae. Mol. Biol. Cell 14: 4272–4284. https://doi.org/10.1091/mbc.E03-01-0042

Brosdy, A. S., and P. A. Silver, 2000 Pre-mRNA processing factors are required for nuclear export. RNA 6: 1737–1749. https://doi.org/10.1017/s1355838200001059

Buchan, J. R., and R. Parker, 2009 Eukaryotic stress granules: the ins and outs of translation. Mol. Cell 36: 932–941. https://doi.org/10.1016/j.molcel.2009.11.020

Buchan, J. R., T. Nissan, and R. Parker, 2010 Analyzing P-bodies and stress granules in Saccharomyces cerevisiae. Methods Enzymol. 470: 619–640. https://doi.org/10.1016/s0076-6879(10)00252-2

Buchan, J. R., J. H. Yoon, and R. Parker, 2011 Stress-specific composition, assembly and kinetics of stress granules in Saccharomyces cerevisiae. J. Cell Sci. 124: 228–239. https://doi.org/10.1242/jcs.078444

Buchan, J. R., R. M. Kolaitis, J. P. Taylor, and R. Parker, 2013 Eukaryotic stress granules are cleared by autophagy and Cdc40/VCP function. Cell 153: 1461–1474. https://doi.org/10.1016/j.cell.2013.05.037

Chang, Y., and W. K. Huh, 2018 Ksp1-dependent phosphorylation of elf4fG modulates post-transcriptional regulation of specific mRNAs under glucose deprivation conditions. Nucleic Acids Res. 46: 3047–3060. https://doi.org/10.1093/nar/gky097

Cook, J. G., L. Bardwell, S. J. Kron, and J. Thorner, 1996 Two novel targets of the MAP kinase Kss1 are negative regulators of invasive growth in the yeast Saccharomyces cerevisiae. Genes Dev. 10: 2831–2848. https://doi.org/10.1101/gad.22831

Cox, J., I. Matic, M. Hilger, N. Nagaraj, M. Selbach et al., 2009 A practical guide to the MaxQuant computational platform for SILAC-based quantitative proteomics. Nat. Protoc. 4: 698–705. https://doi.org/10.1038/nprot.2009.36

Cullen, P. J., and G. F. Sprague, Jr., 2000 Glucose depletion causes haploid invasive growth in yeast. Proc. Natl. Acad. Sci. USA 97: 13619–13624. https://doi.org/10.1073/pnas.240345197

Cullen, P. J., and G. F. Sprague, Jr., 2002 The roles of budsite-selec- tion proteins during haploid invasive growth in yeast. Mol. Biol. Cell 13: 2990–3004. https://doi.org/10.1091/mbc.e02-03-0015

Cullen, P. J., and G. F. Sprague, Jr., 2012 The regulation of filamentous growth in yeast. Genetics 190: 23–49. https://doi.org/10.1534/genetics.111.127456

Cutler, N. S., X. Pan, J. Heitman, and M. E. Cardenas, 2001 The TOR signal transduction cascade controls cellular differentiation in response to nutrients. Mol. Biol. Cell 12: 4103–4113. https://doi.org/10.1091/mbc.12.12.4103

Drogen, F., S. M. O’Rourke, V. M. Stucke, M. Jaquenoud, A. M. Neiman et al., 2000 Phosphorylation of the MEKK Ste11p by the PAK-like kinase Ste20p is required for MAP kinase signaling in vivo. Curr. Biol. 10: 630–639. https://doi.org/10.1016/S0960-9822(00)00511-X

Eng, J. K., A. L. McCormack, and J. R. Yates, 1994 An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. J. Am. Soc. Mass Spectrom. 5: 976–989. https://doi.org/10.1016/1044-0305(94)80016-2

Erdman, S., and M. Snyder, 2001 A filamentous growth response mediated by the yeast mating pathway. Genetics 159: 919–928.

Erdman, S., L. Lin, M. Malczynski, and M. Snyder, 1998 Pheromone-regulated genes required for yeast mating differentiation. J. Cell Biol. 140: 461–483. https://doi.org/10.1083/jcb.140.3.461

Fleischmann, M., I. Stagljar, and M. Aebi, 1996 Allele-specific suppression of a Saccharomyces cerevisiae prp20 mutation by overexpression of a nuclear serine/threonine protein kinase. Mol. Gen. Genet. 250: 614–625.

Gallie, D. R., and K. S. Browning, 2001 elf4fG functionally differs from elfIIs04G in promoting internal initiation, cap-independent translation, and translation of structured mRNAs. J. Biol. Chem. 276: 36951–36960. https://doi.org/10.1074/jbc.m103869200

Gasch, A. P., P. T. Spellman, C. M. Kao, O. Carmel-Harel, M. B. Eisen et al., 2000 Genomic expression programs in the response of yeast cells to environmental changes. Mol. Biol. Cell 11: 4241–4257. https://doi.org/10.1091/mbc.11.12.4241

Giannattasio, M., and D. Branzei, 2017 S-phase checkpoint regulations that preserve replication and chromosome integrity upon dNTP depletion. Cell. Mol. Life Sci. 74: 2361–2380. https://doi.org/10.1007/s00018-017-2474-4

Gimeno, C. J., and G. R. Fink, 1994 Induction of pseudohyphal growth by overexpression of PHD1, a Saccharomyces cerevisiae gene related to transcriptional regulators of fungal development. Mol. Cell. Biol. 14: 2100–2112. https://doi.org/10.1128/MCB.14.12.2100

Gimeno, C. J., P. O. Ljungdahl, C. A. Styles, and G. R. Fink, 1992 Unipolar cell divisions in the yeast S. cerevisiae lead to filamentous growth: regulation by starvation and RAS. Cell 68: 1077–1090. https://doi.org/10.1016/0092-8674(92)90079-R

Gray, M., S. Piccirillo, and S. M. Honigberg, 2005 Two-step method for constructing unmarked insertions, deletions and allele substitutions in the yeast genome. FEMS Microbiol. Lett. 248: 31–36. https://doi.org/10.1016/j.femsle.2005.05.018

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

Hailey, D. W., T. N. Davis, and E. G. D. Muller, 2002 Fluorescence resonance energy transfer using color variants of green fluorescent protein. Methods Enzymol. 351: 34–49. https://doi.org/10.1016/S0076-6879(02)51840-1

Halme, A., S. Bumgarner, C. A. Styles, and G. R. Fink, 2004 Genetic and epigenetic regulation of the FLO gene family generates cell-surface variation in yeast. Cell 116: 415–424. https://doi.org/10.1016/j.cell.2004.05.018
Holt, L. J., B. B. Tuch, J. Villen, A. D. Johnson, S. P. Gygi et al., 2009 Global analysis of Cdk1 substrate phosphorylation sites provides insights into evolution. Science 325: 1682–1686. https://doi.org/10.1126.science.1172867

Huang da, W., B. T. Sherman, and R. A. Lempicki, 2009 Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4: 44–57. https://doi.org/10.1038/nprot.2008.211

Jain, R., J. R. Wheeler, R. W. Walters, A. Agrawal, A. Barsic et al., 2016 ATPase-modulated stress granules contain a diverse proteome and substructure. Cell 164: 487–498. https://doi.org/10.1016/j.cell.2015.12.038

Jin, R., C. J. Dobry, P. J. McCown, and A. Kumar, 2008 Large-scale analysis of yeast filamentous growth by systematic gene disruption and overexpression. Mol. Biol. Cell 19: 284–296. https://doi.org/10.1091/mbc.E07-05-0519

Johnson, C., H. K. Kweon, D. Sheidy, C. A. Shively, D. Mellacheruvu et al., 2014 The yeast sks1p kinase signaling network regulates pseudohyphal growth and glucose response. PLoS Genet. 10: e1004183. https://doi.org/10.1371/journal.pgen.1004183

Karunanithi, S., N. S. Cutler, and J. Heitman, 2000 Characterization of alcohol-induced filamentous growth in Saccharomyces cerevisiae. Mol. Biol. Cell 11: 183–199. https://doi.org/10.1091/mbc.11.1.183

Love, M. I., W. Huber, and S. Anders, 2014 Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15: 550. https://doi.org/10.1186/s13059-014-0550-8

Ma, J., R. Jin, X. Jia, C. J. Dobry, L. Wang et al., 2007 An interrelationship between autophagy and filamentous growth in budding yeast. Genetics 177: 205–214. https://doi.org/10.1534/genetics.107.076596

Ma, J., C. J. Dobry, D. J. Krysan, and A. Kumar, 2008 Unconventional genomic architecture in the budding yeast saccharomyces cerevisiae masks the nested antisense gene NAG1. Eukaryot. Cell 7: 1289–1298. https://doi.org/10.1126/EC.00053-08

Madhani, H. D., and G. R. Fink, 1997 Combinatorial control required for the specificity of yeast MAPK signaling. Science 275: 1314–1317. https://doi.org/10.1126/science.275.5304.1314

Madhani, H. D., T. Galitski, E. S. Lander, and G. R. Fink, 1999 Effectors of a developmental mitogen-activated protein kinase cascade revealed by expression signatures of signaling mutants. Proc. Natl. Acad. Sci. USA 96: 12530–12535. https://doi.org/10.1073/pnas.96.22.12530

Mangus, D. A., N. Amrani, and A. Jacobson, 1998 Pbp1p, a factor interacting with Saccharomyces cerevisiae poly(A)-binding protein, regulates polyadenylation. Mol. Cell. Biol. 18: 7383–7396. https://doi.org/10.1128/MCB.18.12.7383

Minden, A., A. Lin, M. McMahon, C. Lange-Carter, B. Derijard et al., 1994 Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEK. Science 266: 1719–1723. https://doi.org/10.1126/science.7992057

Mitchell, S. F., S. Jain, M. She, and R. Parker, 2013 Global analysis of yeast mRNPs. Nat. Struct. Mol. Biol. 20: 127–133. https://doi.org/10.1038/nsmb.2468

Mösch, H. U., E. Kubler, S. Krappmann, G. R. Fink, and G. H. Braus, 1999 Crosstalk between the Ras2p-controlled mitogen-activated protein kinase and cAMP pathways during invasive growth of Saccharomyces cerevisiae. Mol. Biol. Cell 10: 1325–1335. https://doi.org/10.1091/mbc.10.5.1325

Nobile, C. J., and A. P. Mitchell, 2005 Regulation of cell-surface genes and biofilm formation by the C. albicans transcription factor Bcr1p. Curr. Biol. 15: 1150–1155. https://doi.org/10.1016/j.cub.2005.05.047

Norman, K. L., C. A. Shively, A. J. De La Rocha, N. Mutlu, S. Basu et al., 2018 Inositol polyphosphates regulate and predict yeast pseudohyphal growth phenotypes. PLoS Genet. 14: e1007493. https://doi.org/10.1371/journal.pgen.1007493

Ong, S. E., and M. Mann, 2006 A practical recipe for stable isotope labeling by amino acids in cell culture (SILAC). Nat. Protoc. 1: 2650–2660. https://doi.org/10.1038/nprot.2006.427

Ong, S. E., B. Blagoev, I. Kratchmarova, D. B. Kristensen, H. Steen et al., 2002 Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol. Cell. Proteomics 1: 376–386. https://doi.org/10.1074/mcp.M200025-MCP200

Pan, X., and J. Heitman, 1999 Cyclic AMP-dependent protein kinase regulates pseudohyphal differentiation in Saccharomyces
Rupp, S., E. Summers, H. J. Lo, H. Madhani, and G. Fink, 2011 The cAMP-dependent protein kinase signaling pathway is a key regulator of P body foci formation. Mol. Cell 43: 973–981. https://doi.org/10.1016/j.molcel.2011.06.032

Reynolds, T. B., and G. R. Fink, 2001 ‘Bakers’ yeast, a model for fungal biofilm formation. Science 291: 878–881. https://doi.org/10.1126/science.291.5505.878

Roberts, R. L., and G. R. Fink, 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. https://doi.org/10.1101/gad.8.24.2974

Romero, P., Z. Obradovic, X. Li, E. C. Garner, C. J. Brown et al., 2001 Sequence complexity of disordered protein. Proteins 42: 38–48. https://doi.org/10.1002/1097-0134(20010101)42:1<38::AID-PROT5>3.0.CO;2-3

Rupps, S., E. Summers, H. J. Lo, H. Madhani, and G. Fink, 1999 MAP kinase and cAMP filamentation signaling pathways converge on the unusually large promoter of the yeast FLO11 gene. EMBO J. 18: 1257–1269. https://doi.org/10.1093/emboj/18.5.1257

Ryan, O., R. S. Shapiro, C. F. Kurat, D. Mayhew, A. Baryshnikova et al., 2012 Global gene deletion analysis exploring yeast filamentous growth. Science 337: 1353–1356. https://doi.org/10.1126/science.1224339

Saville, S. P., A. L. Lazzell, C. Monteagudo, and J. L. Lopez-Ribot, 2003 Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of Candida albicans during infection. Eukaryot. Cell 2: 1053–1060. https://doi.org/10.1128/EC.2.5.1053-1060.2003

Sengupta, S., T. R. Peterson, and D. M. Sabatini, 2010 Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. Mol. Cell 40: 310–322. https://doi.org/10.1016/j.molcel.2010.09.026

Shah, K. H., B. Zhang, V. Ramachandran, and P. K. Herman, 2013 Processing body and stress granule assembly occur by independent and differentially regulated pathways in Saccharomyces cerevisiae. Genetics 193: 109–123. https://doi.org/10.1534/genetics.112.146993

Shively, C. A., M. J. Eckwahl, C. J. Dobry, D. Mellacheruvu, A. Nesvizhskii et al., 2013 Genetic networks inducing invasive growth in Saccharomyces cerevisiae identified through systematic genome-wide overexpression. Genetics 193: 1297–1310. https://doi.org/10.1534/genetics.112.147876

Shively, C. A., H. K. Kweon, K. L. Norman, D. Mellacheruvu, T. Xu et al., 2015 Large-scale analysis of kinase signaling in yeast pseudohyphal development identifies regulation of ribonucleoprotein granules. PLoS Genet. 11: e1005564. https://doi.org/10.1371/journal.pgen.1005564

Song, Q., C. Johnson, T. E. Wilson, and A. Kumar, 2014 Pooled segregant sequencing reveals genetic determinants of yeast pseudohyphal growth. PLoS Genet. 10: e1004570. https://doi.org/10.1371/journal.pgen.1004570

Souillard, A., A. Cremonesi, S. Moes, F. Schütz, P. Jenó et al., 2010 The rapamycin-sensitive phosphoproteome reveals that TOR controls protein kinase A toward some but not all substrates. Mol. Biol. Cell 21: 3475–3486. https://doi.org/10.1091/mcb.e10-03-0182

Stanhill, A., N. Schick, and D. Engelberg, 1999 The yeast ras/cyclic AMP pathway induces invasive growth by suppressing the cellular stress response. Mol. Cell. Biol. 19: 7529–7538. https://doi.org/10.1128/MCB.19.11.7529

Supek, F., M. Bosnjak, N. Skunca, and T. Smrč, 2011 REVIGO summarizes and visualizes long lists of gene ontology terms. PLoS One 6: e21800. https://doi.org/10.1371/journal.pone.0021800

Tarun, S. Z., Jr., S. E. Wells, J. A. Deardorff, and A. B. Sachs, 1997 Translation initiation factor eIF4G mediates poly(A)-tail-dependent translation. Proc. Natl. Acad. Sci. USA 94: 9046–9051. https://doi.org/10.1073/pnas.94.17.9046

Trappnell, C., D. G. Hendrickson, M. Sauvageau, L. Goff, J. L. Rinn et al., 2013 Differential analysis of gene regulation at transcript resolution with RNA-seq. Nat. Biotechnol. 31: 46–53. https://doi.org/10.1038/nbt.2450

Umekawa, M., and D. J. Klionsky, 2012 Ksp1 kinase regulates autophagy via the target of rapamycin complex 1 (TORC1) pathway. J. Biol. Chem. 287: 16300–16310. https://doi.org/10.1074/jbc.M111.344952

van Dyk, D., I. S. Pretorius, and F. F. Bauer, 2005 Mss11p is a central element of the regulatory network that controls FLO11 expression and invasive growth in Saccharomyces cerevisiae. Genetics 169: 15476–15488. https://doi.org/10.1534/genetics.112.344952

Whiteway, M., and C. Bachewich, 2007 Morphogenesis in Candida albicans. Annu. Rev. Microbiol. 61: 529–553. https://doi.org/10.1146/annurev.micro.61.080706.093341

Xu, T., C. A. Shively, R. Jin, M. J. Eckwahl, C. J. Dobry et al., 2010 A profile of differentially abundant proteins at the yeast cell periphery during pseudohyphal growth. J. Biol. Chem. 285: 15476–15488. https://doi.org/10.1074/jbc.M110.114926

Yoon, J. H., E. J. Choi, and R. Parker, 2010 Deacetylase activity of Sir2 modulates stress granule assembly and mRNA decay in Saccharomyces cerevisiae. J. Cell Biol. 189: 813–827. https://doi.org/10.1083/jcb.200912019

Zhou, Z., A. Gartner, R. Cade, G. Ammerer, and B. Errede, 1993 Pheromone-induced signal transduction in Saccharomyces cerevisiae requires the sequential function of three protein kinases. Mol. Cell. Biol. 13: 2069–2080. https://doi.org/10.1128/MCB.13.4.2069

Communicating editor: A. Gladfelter