The Yeast Kinase Ksp1 Regulates Cellular Stress Response

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Abstract

Pseudohyphal growth is a morphological transformation in yeast in response to stresses such as glucose or nitrogen deprivation. Instead of exhibiting vegetative growth, cells elongate, and form chains of cells called pseudohyphae under stress. Pseudohyphal growth is regulated by several conserved pathways such as RAS/PKA, Snf1, MAPK, and TOR pathways. Ksp1 kinase activity is necessary for pseudohyphal growth in glucose and nitrogen-deprivation conditions. Under low glucose and nitrogen conditions, when *KSP1* is not present, there is a reduced level of surface pseudohyphal filamentation. Under low nitrogen conditions, the kinase activity of Ksp1 is necessary for cell elongation and wild-type invasive growth. Regulated by PKA, Ksp1 activates TORC1 (Umekawa and Kilonsky 2012). Ksp1 colocalizes with TORC1 and is important for cell survival under rapamycin treatment. Cell growth under rapamycin treatment in a strain with a defective Ksp1 kinase exhibits a differential growth curve compared to wild-type, *ksp1* deletion, or *ksp1*-A827S strains. In this project, several strain and plasmid constructs were made for microscopy and phenotypic analyses, including *CDC33*-mCherry, and Ste20 and Pbp1 were cloned. It was hypothesized that Cdc33 and Ksp1 colocalize to regulate translation under stress, so the Cdc33-mCherry fusion was made to be used to observe Cdc33's localization relative to Ksp1 by fluorescence microscopy. Pbp1, containing Ksp1-dependent phosphorylation sites, and Ste20 defective strains showed a change in surface filamentation and invasion under nitrogen-deprivation conditions compared to wild-type strains. Therefore, Pbp1 and Ste20 were cloned to be introduced into Pbp1 and Ste20 defective strains to observe if wild-type pseudohyphal filamentation is restored. These findings and constructs provide a groundwork for further study of the mechanism by which Ksp1 regulates the yeast pseudohyphal growth response.

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Introduction

Yeast as a model system

Yeast as a model system is useful because it possesses both the genetic complexity of a eukaryote and technical advantages of a prokaryote. Yeast *Saccharomyces cerevisiae* was the first eukaryotic organism sequenced (Goffeau *et al*. 1996). Therefore, it has long been known that many genes in yeast and mammals encode similar proteins (Botstein *et al*. 1997). Many important metabolic pathways are also conserved from yeast to mammals and humans.

In addition, compared to other eukaryotic model organisms, it is easy to manipulate the genetics of yeast due to its rapid growth rate and well-developed DNA transformation system (Sherman 2002). DNA transformation in yeast allows for cloning and mutant isolation. Using DNA transformation, foreign genes can be introduced to yeast cells provided that the DNA to be introduced contains complementary sequences of a targeted area. Due to homologous recombination, a foreign gene sequence with flanking yeast DNA sequence can then be inserted at a specific and corresponding location of the genome. Exploiting this characteristic of yeast, the functional roles and relationships between many genes can be studied.

Another interesting characteristic of yeast is that it has both a haploid and a diploid state. This is especially useful because it is easy to observe recessive loss-of-function alleles in a haploid strain. Diploid strains are useful when trying to determine if a certain mutant allele is dominant or recessive. Complementation assays can also be used with a diploid strain when conducting a genetic screen. Other benefits of yeast include low cost and commercial availability.

Because of all the genetic advantages it possesses, yeast is a suitable model organism for this study.

Nutrient stress responsive pathways

There are many stress responsive pathways in yeast, including the AMP-activated protein kinase (AMPK), mitogen-activated protein kinase (MAPK), protein kinase A (PKA), and target of rapamycin (TOR) pathways. I will provide a brief overview of each here.

AMPK is activated by a low AMP/ATP ratio and regulates cellular energy homeostasis. AMPK is a heterotrimer with one catalytic subunit a and two regulatory subunits β and γ (Mihaylova *et al*. 2011). When the AMP/ATP ratio is low, AMP binds the γ subunit, blocking access to cellular phosphatases and activating AMPK (Hardie 2004). This activation increases catabolic processes such as autophagy and glucose utilization (Hardie 2004). Anabolic activities like glycogen and protein synthesis involving the target of rapamycin (TOR) are downregulated (Hardie 2004).

The MAPK pathway is known for transducing extracellular signals, such as pheromone signals, low nutrient level, and cell wall stress, to cellular responses (Rudolf *et al*. 2007). In the MAPK pathway, MAPK kinase kinase (MAPKKK) activates the MAPK kinase (MAPKK), which activates MAPK. Activation of the MAPK pathway typically leads to the transcription of genes important in cell growth, cell proliferation, and cell differentiation (Santarpia *et al*. 2012). At least four well defined MAPK pathways have been identified in yeast, and one putative activator of the MAPK cascades responsible for mating and pseudohyphal growth is the p21-activated kinase family member Ste20 (STErile protein 20) (Dan *et al* 2011).

The PKA pathway is an intracellular pathway in which signaling molecules bind to a G protein-coupled receptor (GPCR) that leads to the production of cyclic AMP (cAMP) that activates PKA (Sassone-Corsi 2012). Adenylyl cyclase converts ATP into cAMP, which is hydrolyzed by cAMP phosphodiesterase to form 5'-AMP. cAMP activates PKA by binding to its regulatory subunits and releasing the catalytic subunits. The catalytic subunits then enter the nucleus to phosphorylate and activate the cAMP response element-binding protein (CREB). CREB is a transcriptional regulator that can activate some genes with a cAMP response element (CRE).

TOR is a conserved protein kinase that has been identified to be important for cell growth in response to nutrients (Barbet *et al.* 1996; Rohde *et al.* 2001). It was first discovered in *Saccharomyces cerevisiae* in complex with the prolyl isomerase FKBP12 (Heitman *et al.* 1991a). TOR forms two structurally and functionally distinct complexes, TOR complex 1 (TORC1), and TOR complex 2 (TORC2) (Inoue *et al.* 2018). TORC1 has been associated with many cellular activities including, but not limited to, cell growth, ribosome and amino acid biogenesis, stress responses, and autophagy (Loewith *et al.* 2011). On the other hand, TORC2 is involved with actin organization, endocytosis, and sphingolipid biosynthesis (Powers *et al.* 2010). In addition, only TORC1 is sensitive to rapamycin, not TORC2 (Loewith *et al.* 2011).

Pseudohyphal growth and its regulating pathways

Pseudohyphal growth was first discussed by Gimeno *et al.* in 1992. It is a morphological transformation observed in many species of fungi in response to stresses such as glucose or nitrogen limitation. Instead of exhibiting vegetative growth, yeast cells elongate, and form chains of cells called pseudohyphae under stress. Pseudohyphal growth is regulated by conserved

pathways that are important in cancer and neurodegenerative diseases. Therefore, elucidating the detailed mechanisms of pseudohyphal growth contributes to our understanding of these diseases.

In yeast, both diploid and haploid cells form pseudohyphae and have properties of invasive growth. However, haploid strains do not undergo surface filamentation as extensively, particularly on media with limiting amounts of nitrogen (ammonium sulfate) or glucose (Cullen *et al*. 2012). This ability to form pseudohyphae most likely provides an evolutionary advantage, allowing for the acquisition and scavenging of more nutrients when resources are scarce; however, this trait has been selected against in laboratory strains to make it easier to separate cells in culture (Gancedo 2001; Kron 1997).

The transition from vegetative growth to pseudohyphal growth is regulated by several well characterized pathways including the RAS/PKA, sucrose nonfermentable (SNF), MAPK, and TOR pathway.

Pseudohyphal growth and the RAS/PKA pathway

The RAS/PKA pathway regulates pseudohyphal growth in yeast. The GTP-binding protein Ras2 plays a critical role in the transition from vegetative growth to filamentous growth. When decreasing the GTPase activity of Ras2 by mutating its Valine at position 19, enhanced pseudohyphal growth is observed in diploid cells and agar invasion is observed in haploid strains (Gimeno 1992). Without the mutation, diploid cells still form pseudohyphae when starved, but the cells are round, not elongated, and haploid cells do not undergo invasive growth (Kübler *et al*. 1997).

Ras2 activates adenylate cyclase which produces cAMP. The level of cAMP is important for the decision to undergo filamentous growth (Mosch *et al*. 1996). Interestingly, Kayikci and

Magwenw have pointed out in 2018 that cAMP/PKA plays a divergent role in filamentous growth as both inhibiting and hyper-activating the pathway, leading to a more efficient filamentous growth response. Overexpression of the phosphodiesterase Pde2 has been shown to decrease the level of filamentous growth by activating Ras which activates cAMP (Ward *et al.* 1995). In *Saccharomyces cerevisiae*, there are three PKA's: Tpk1, Tpk2, and Tpk3. cAMP binds to the PKA regulatory subunit Bcy1 and activates the catalytic subunits (Pan and Heitman 1999). However, each Tpk contributes to a different phenotype in filamentous growth: deletion of *TPK2* results in no filamentous growth, deletion of *TPK1* results in normal filamentous growth, and deletion of *TPK3* results in hyper-filamentous growth (Robertson and Fink 1998; Pan and Heitman 1999).

Tpk1 and Tpk2 regulate filamentous growth by phosphorylation. The phosphorylation of the transcription factor Flo8 by Tpk2 activates filamentation (Pan and Heitman 1999). The phosphorylation of Yak1 by Tpk1 inhibits Yak1's indirect upregulation of the expression of *FLO11*, which is a GPI-anchored cell surface glycoprotein required for pseudohyphal and invasive growth (Deminoff *et al.* 2006).

Some upstream regulators of the RAS/PKA pathway include a G-protein-coupled receptor (GPCR) Grp1 and its G protein subunits. Grp1 and its Gα subunit Gpa2 regulate filamentous growth positively by enhancing cAMP levels (Lorenz *et al*. 2000b). Gβ subunits Gpb1 and Gpb2 negatively regulate filamentous growth (Harashima and Heitman 2002; Batlle *et al*. 2003).

Pseudohyphal growth and the Snf1 pathway

Snf1 is a kinase required for *Saccharomyces cerevisiae* to adapt to glucose limitation by utilizing other less preferred carbon sources such as sucrose. It is the AMPK homolog in *Saccharomyces cerevisiae*. Snf1 is phosphorylated and activated by Sak1, Tos3, and Elm1. It is inactivated by a phosphatase called Reg1-Glc7 (Hedbacker *et al*. 2008). We now know that glucose limitation can also trigger pseudohyphal growth, and Snf1 regulates two repressors, Nrg1 and Nrg2, at the *FLO11* promoter (Kuchin *et al*. 2002).

Pseudohyphal growth and the MAPK pathway

In the MAPK pathway involved in pseudohyphal growth, Ras2 activates Cdc24, a guanine nucleotide exchange factor, which activates Cdc42 to be its GTP-bound form (Peter *et al*. 1996). Cdc42 displaces Hsl7 and binds Ste20 (Fujita *et al*. 1999). Ste20 then activates Ste11 (MAPKKK), Ste7 (MAPKK), and Kss1 (MAPK) (Bardwell *et al*. 1998). When Kss1 is not phosphorylated, it is bound to Dig1/2 and Ste12, and together they form a complex with Tec1 (Bardwell *et al*. 1998). When Kss1 is phosphorylated by Ste7, Kss1 is able to phosphorylate Ste12 and Dig1/2 (Bardwell *et al*. 1998). Dig1/2 dissociates from the complex and allows Ste12 to bind to the Filamentation and invasive Response Element (FRE) (Bardwell *et al*. 1998). Tec1 binds to the FRE sequence cooperatively with Ste12 (Bardwell *et al*. 1998).

Pseudohyphal growth and the TOR pathway

The TOR pathway regulates cellular differentiation and pseudohyphal growth as nitrogen limitation triggers pseudohyphal growth, and the TOR pathway senses nutrients including amino acid, carbon and nitrogen sources, by regulating both transcription and translation. TOR

regulates the transcription factor Gcn4, a regulator of *FLO11* expression (Braus *et al*. 2003; Boeckstaens *et al*. 2008).

Rapamycin inhibits pseudohyphal growth in *Saccharomyces cerevisiae* (Cutler *et al*. 2001). Rapamycin disrupts the function of eIF4G, or Tif4631 in *Saccharomyces cerevisiae*, and enhances autophagy (Berset *et al*. 1998; Noda and Ohsumi 1998).

Cutler *et al*. in 2001 has shown that Tor1 and Tor2 might regulate pseudohyphal growth with Sit4, a phosphatase, and Tap42, Sit4's regulatory protein. They propose that rapamycin can cause constitutive expression of nitrogen catabolite repression genes to disrupt pseudohyphal growth. One model proposed is that rapamycin induces the nitrogen catabolite repression genes, and the expression of nitrogen catabolite repression genes is necessary for pseudohyphal growth. In the nitrogen catabolite repression pathway, a lack of nitrogen prompts transcription factor Gln3 to be released from binding Ure2 and activates Gln3-dependent genes.

The yeast Ksp1 kinase

KSP1 (Kinase Suppressing *prp20*-10) is a serine/threonine protein kinase in the casein kinase II subfamily. *KSP1* encodes a protein of 1029 amino acids and a molecular mass of 117 kDa. It was first identified as a *prp20*-10 allele-specific high-copy-number suppressor (Fleischmann *et al*. 1996). Ksp1 was then identified, among other kinases such as Bcy1p, Fus3p, Kss1p, Sks1p, and Tpk2p, to be localized in the nucleus during filamentous growth (Bharucha 2008). In 2018, Chang and Huh have shown that eIF4G is a target of Ksp1 and the phosphorylation of eIF4G recruits Dhh1, a DEAD-box helicase, to facilitate mRNA decay and downregulate translation. Their data show that deletion of *KSP1* decreases the interaction between Dhh1 and glycolytic mRNAs under glucose starvation condition.

Umekawa and Kilonsky in 2012 identified *KSP1* to be an important node connecting TORC1 and PKA. Regulated by PKA, *KSP1* activates TORC1 which promotes pseudohyphal growth, and this activation then inhibits autophagy. Experimental data show that PKA phosphorylates Ksp1 at Ser624 and Ser827. Deletion of *KSP1* contributes to the dephosphorylation of the TORC1 substrate Atg13, which associates with autophagy enhancement. Autophagy is a process that allows cells to remove unwanted materials through degradation or recycle cellular components under starvation. A double membrane encloses the damaged proteins, organelles, or pathogens and forms an autophagosome vesicle that is delivered to lysosomes for degradation. Autophagy helps cells maintain their homeostasis and adapt to different nutritional states.

Messenger ribonucleoproteins (mRNPs) are complexes of mRNA and mRNA-binding proteins. Translationally inactive mRNPs can be assembled to form either stress granules or processing bodies (p-bodies). Stress granules can form when translation initiation is halted, or translation initiation rate is decreased as a result of a cell stress response (Kedersha *et al.* 1999). However, stress granules only occur in a specific period of translation initiation. Preventing 60S joining does not induce stress granules (Buchan and Parker 2009). Stress granules consist of translation initiation factors, polyA-binding protein Pabp, and 40S ribosomal subunits (Buchan and Parker 2009). On the contrary, p-bodies are formed under normal conditions, although stresses such as glucose limitation do increase p-body numbers. Compared to the diffuse stress granules, p-bodies have been observed to be more discrete and rounded. P-bodies consist, among other proteins, of decapping proteins, the 5'-3' exonuclease Xrn1, and the Lsm1-7 complex (Decker and Parker 2012).

Pseudohyphal growth is linked to mRNP dynamics because both processes occur in response to similar cell stresses. Further, kinases that regulate filamentous growth have been linked with the regulation of mRNPs. In a filamentous strain of *S. cerevisiae*, Kss1 Fus3, Tpk2, and Ste20 have been found to colocalize with the mRNP protein Igo1 by the Kumar lab. Deletion of *KSS1* results in significantly decreased numbers of mRNPs, visualized by decreased puncta of RNA-binding proteins in cells limited for glucose. mRNP granules have been found to contain the mRNA of translation initiation factors, and these granules target the protein synthesis machinery to sites of polarized growth in filamentous cells (Pizzinga *et al*. 2019). Mutlu *et al.* in 2019 showed that Pbp1p and Tif4631p localize in stress granules, and the *ksp1* null mutant shows elevated abundance of Pbp1p puncta relative to wild-type.

The work presented here is intended to further characterize *KSP1* function as a step towards understanding its mechanism of action in regulating yeast pseudohyphal growth.

Materials and Methods

Yeast growth conditions and media

Yeast cells were cultured according to standard methods (Guthrie and Fink 1991). Yeast cells were grown in YPD (2% peptone, 1% yeast extract, 2% glucose, and 2% agar as appropriate), or SC (0.67% yeast nitrogen base, 2% glucose, 0.2% of the appropriate amino acid dropout mix, and 2% agar as appropriate). Low-nitrogen SLAD media was prepared using 0.17% yeast nitrogen base without amino acids and without ammonium sulfate, 2% glucose, 50μM ammonium sulfate, and appropriate amino acids to complement auxotrophy. Low-nitrogen and low-glucose SLALD media was prepared as described for SLAD media but with 0.05% glucose (Johnson *et al*. 2014).

Yeast transformations

The LiAc-mediated yeast transformation method was used for all strains (Guthrie and Fink 1991). Yeast cells were prepared and suspended in LiAc solutions with plasmid DNA or PCR products. Polyethylene glycol (PEG) and salmon sperm DNA was added to the mixture and incubated at 30°C. Cells were heat shocked at 42°C for 15 minutes to allow DNA to enter the cells. Cells were then plated on the appropriate medium to select for transformants containing the introduced DNA.

Pseudohyphal growth assay

Wild-type or mutant cells were inoculated in YPD media overnight. Strains were diluted 1:20 and grown for 4-6 hours the next day. Cells were washed three times with sterilized and deionized water. The optical density of the cell suspension was normalized to 1.0 before diluting

the strains and spreading the cells onto plates with SLAD media supplemented with uracil or other amino acids to complement strain auxotrophy. Cells were spread at a density of around 50 colonies per plate. Plates were incubated at 30°C until filamentation was observed in wild-type strains. Colonies were 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). The circumference of a colony was measured using ImageJ and compared to the circumference of a wild-type colony. The ratio is calculated from three replicates.

Agar invasion was calculated by standard protocols (Ryan *et al*. 2012; Norman *et al*. 2018). A 5 µl aliquot of a cell suspension was spotted onto a plate and the resulting colony was allowed to invade the agar for 2-3 days. Plates were imaged and surface cells were washed gently with water. Washed cells were imaged. The quantification of invasive growth was done using the mean pixel intensity of the washed sample relative to the unwashed sample. Triplicate experiments were done for each strain. To analyze cell morphology, cells were scraped from the edge of a colony and resuspended in media before spotting on to a glass slide for microscopy imaging. Cell dimensions were measured and used for height-to-width ratio calculations. Cells with a ≥ 2 ratio were defined as exhibiting properties of pseudohyphal growth.

Rapamycin fitness assays

A 96-well plate was prepared with strains DSY160, DSY161, DSY163, and NMY47, which were each suspended in 0, 1, and 5 µg/ml rapamycin in YPD media. Triplicates were done with each strain. A spectrophotometer was used to record the absorbance levels overnight. The mean absorbances were calculated for each strain and were used to generate a growth curve.

Cloning PBP1 and STE20

PBP1 and *STE20* sequences were obtained from the *Saccharomyces* Genome Database (SGD at yeastgenome.org). Using a commercial plasmid editor (Ape), forward and reverse primers for PCR amplification of the genes were designed. *Xho*1 or *Kpn*1 restriction sites and 3-8 bp of additional sequence were added to the 5' end of each primer. PCR products were obtained and purified. PCR products corresponding to *PBP1* and *STE20* sequences and vector pRS416 were digested and ligated according to standard protocols. Recombinant plasmids were transformed into DH5-Alpha competent cells. Transformants were selected on media containing ampicillin (100 micrograms/ml).

Cdc33-mCherry fusion

Yeast strains were generated with sequence encoding the mCherry fluorescent protein integrated at the *CDC33* locus to generate translational fusions to the C-terminus of Cdc33. For this purpose, PCR primers were generated incorporating 40 nucleotides of sequence matching the *CDC33* genomic locus at their 5'-ends and 20 nucleotides matching pBS35 at their 3'-ends. The primers were used to amplify sequence encoding mCherry from the pBS35 plasmid with flanking sequence designed to target the PCR product to the 3'-terminus of *CDC33*. The resulting PCR products were transformed into strains DSY246, DSY247, and DSY248, generating *CDC33*-mCherry fusions.

Table 1: Strains used in this study.

Strain	Genotype	Source
yCK021	*ura3-52/ura3-52 leu2Δ0/LEU2 TRP1/trp1-1 MATa/MATα*	Johnson *et al*., 2014
yCK109	*ura3-52/ura3-52 leu2Δ0/LEU2 TRP1/trp1-1 MATa/MATα ksp1*Δ::*kanMX*/*ksp1*Δ::*kanMX*	Mutlu *et al*. 2019
yCK186	*ura3-52/ura3-52 leu2Δ0/LEU2 TRP1/trp1-1 MATa/MATα ksp1*-K47D/*ksp1*-K47D	Mutlu *et al*. 2019
DSY100	*ura3-52 trp1Δ0 KSP1-GFP-kanMX6*	Daniel Sheidy
DSY130	*ura3Δ0 lys2Δ0 leu2Δ0 his3Δ0 KSP1-GFP CDC33-mCherry MATα*	Daniel Sheidy
Y825	*ura3-52 leu2Δ0 MATa*	Michael Snyder, Stanford University
*ksp1*Δ	*ura3-52 leu2Δ0 ksp1*Δ::*kanMX MATa*	Bharucha *et al.* 2008
ksp1-K47D	*ura3-52 leu2Δ0 ksp1*-K47D *MATa*	Bhaucha *et al.* 2008
ksp1-S827A	*ura3-52 leu2Δ0 ksp1*-S827A *MATa*	Nebibe Mutlu

Table 2: Primers used in this study.

Primer	Sequence
Ste20 forward	CGGGGTACCGAGTATTCGTTTCATTACAG
Ste20 reverse	CCGCTCGAGTTACTTTTGTTTATCATCTT
Pbp1 forward	CGGGGTACCATATCGGAAAGAGATCTAGA
Pbp1 reverse	CCGCTCGAGCTATTTATGGCCACTAGTAC
Cdc33 forward	AGTGCCAATGGTAGACACCCTCAACCATCAATCACCTTGCGGATCCCCGGGTTAATTAA
Cdc33 reverse	GATTAAAATAACAATTATCTTAAGAAAAATTCAGACTATCGAATTCGAGCTCGTTTAAAC

Results

Ksp1 kinase activity is required for wild-type pseudohyphal growth in low nitrogen and low glucose conditions.

In the Kumar lab, *KSP1* was disrupted to observe its role in pseudohyphal growth. A conserved catalytic lysine residue at position 47 was mutated to an aspartic acid because it is in the predicted ATP-binding pocket of Ksp1's kinase domain. A homozygous diploid *ksp1*-K47D and *ksp1* deletion strains were used to study *KSP1* function in pseudohyphal growth under low nitrogen and low glucose conditions.

In Figure 1, I assayed surface filamentation in wild-type and *ksp1* mutants under low nitrogen, and low nitrogen and low glucose conditions, as shown on the colony level. SLAD and SLALD media were used to create an environment with scarce resources. SLAD media contains a lower level of ammonium sulfate to create a low nitrogen environment. SLALD media contains a lower level of ammonium sulfate and a lower level of dextrose to create a low nitrogen and low glucose environment. As shown in the first two columns of Figure 1, in the absence of *KSP1*, filamentation is reduced by at least 62% under low nitrogen conditions. Under low nitrogen conditions in the *ksp1*-K47D strain, filamentation is decreased by at least 85%. This suggests that under low nitrogen conditions, Ksp1 kinase activity is necessary for wild-type pseudohyphal filamentation, as when the kinase domain of Ksp1 is disrupted, there is a reduced level of pseudohyphal filamentation. The second column of Figure 1 shows the magnification of the first column. In the third column of Figure 1, pseudohyphal filamentation in wild-type and *ksp1* mutants under both low nitrogen and low glucose conditions is shown. In the absence of *KSP1*, filamentation is reduced by at least 16% under low nitrogen and low glucose conditions. Under low nitrogen and low glucose conditions in the *ksp1*-K47D strain, filamentation is decreased by

at least 27%. In low nitrogen and low glucose conditions, Ksp1 kinase activity is necessary for wild-type pseudohyphal filamentation, as when the kinase domain of Ksp1 is disrupted, there is a reduced level of pseudohyphal filamentation.

In Figure 2, cell elongation in wild-type and *ksp1* mutants under low nitrogen, and low nitrogen and low glucose conditions, is shown on the single-cell level. Significant cell elongation was defined to be a length versus width ratio of greater than two. As shown in Figure 2, 55% of the wild-type cells were elongated under low nitrogen conditions. In the absence of *KSP1*, only 36% of the cells showed elongation in SLAD media. When the kinase domain of Ksp1 is disrupted, 25% of the cells showed elongation in SLAD media, which is around half of the number in wild-type cells. Consistent with previous observation, the kinase domain of Ksp1 is required for cells to elongate under low nitrogen environments, a characteristic of pseudohyphal filamentation. Under low glucose and low nitrogen conditions, there is less cell elongation overall. In SLALD media which contains less glucose and nitrogen, 25% of the wild-type cells counted showed significant elongation. In the absence of *KSP1*, only 13% of the cells showed elongation in SLALD media, which is significantly lower than wild-type cells. When the kinase domain of Ksp1 is disrupted, 19% of the cells showed elongation in SLALD media. This suggests that, under low nitrogen and low glucose conditions, Ksp1 is required for cell elongation, a characteristic of pseudohyphal growth. Overall, when nutrients are limited, Ksp1 is required for cell elongation, and the kinase activity of Ksp1 is necessary for cell elongation when nitrogen is limited.

In Figure 3, invasive growth assays were done to wild-type and *ksp1* mutants under low nitrogen condition. Cells were washed gently with water, and invasion was determined by the number of cells remaining after wash. In wild-type cells, 98% of cells remained after wash. In

the absence of *KSP1*, 83% of the cells remained after wash. In cells with a disrupted Ksp1 kinase domain, 74% of the cells remained after wash. These results support that the Ksp1 kinase is required for wild-type invasive growth in low nitrogen conditions.

Collectively, Figures 1-3 show that Ksp1 kinase activity is required for wild-type pseudohyphal growth in low nitrogen and low glucose conditions.

Ksp1 could be important for cell survival under rapamycin treatment

Ksp1 activates the TORC1 pathway and is required for pseudohyphal growth. Rapamycin inhibits TORC1, which regulates translation during nutrient-starvation conditions. Rapamycin also has been shown to inhibit pseudohyphal growth (Cutler *et al.* 2001). We hypothesized that Ksp1 is important for cell survival under rapamycin treatment. To investigate this, different mutations of *ksp1* are used to study their effect on the cell's response to rapamycin. *ksp1*-K47D was used to disrupt the ATP-binding pocket of Ksp1's kinase domain. *ksp1*-S827A was used because PKA phosphorylates Ksp1 at Ser827. A 96-well plate was used for spectrophotometry. For each strain, 0, 1, or 5 µg/ml of rapamycin was added to the cell suspensions, and a spectrophotometer was used to measure the absorbance of each strain with each concentration of rapamycin. The mean absorbance of each concentration and triplicate experiments were taken to generate the growth curves in Figure 4. Particularly, when Ksp1 kinase is disrupted, cell growth under rapamycin treatment decreased at first but increased later, unlike what is observed in wild-type, the *ksp1* deletion, or *ksp1*-S827A strains. This could suggest that Ksp1 kinase could be important for rapamycin sensitivity in cells.

Growth curves are determined by the optical density (OD) of cell culture at 600 nm. Optical density describes the logarithmic ratio of light entering yeast cell cultures versus light

passing through them. The yeast growth curve has three phases: lag, logarithmic, and stationary phase. In the lag phase, yeast cells are growing in size but not diving as they are adapting to the environment. In the log phase, they are diving and growing rapidly. In the stationary phase, they have exhausted the resources, so cell division slows down. The experiment lasted around 22 hours. Although *ksp1* kinase-dead cells have shown differential growth properties, a positive control strain *TOR1-1* that is resistant to rapamycin should be tested for comparison to verify further this result.

Construction of plasmids and yeast strains for phenotypic and localization analysis

Mutlu *et al*. showed that Ksp1-dependent phosphorylation sites in the eIF4G Tif4631 and Pbp1 are required for wild-type pseudohyphal growth and PKA signaling through *FLO11* in 2019. The *pbp1* deletion strain showed a decrease in surface filamentation and invasion under nitrogen-deprivation conditions. *pbp1*-S436A showed an increase in surface filamentation and invasion under nitrogen-deprivation conditions. Ser436 in Pbp1 is hyper-phosphorylated in a strain with a kinase-defective allele. Also, the *ste20* deletion strain showed a decrease in surface filamentation and invasion under nitrogen-deprivation conditions. Therefore, I cloned *PBP1* and *STE20* to be introduced into *pbp1* and *ste20* defective strains to observe if wild-type pseudohyphal filamentation is restored. The cloning steps were completed, but we were not able to determine phenotypes of the mutant strains complemented with *PBP1* and *STE20* for inclusion in this thesis.

I also constructed strains for the native expression of Cdc33-mCherry fusion protein for purposes of observing Cdc33 localization relative to Ksp1 by fluorescence microscopy. *CDC33* is the yeast ortholog of translation initiation factor eIF4E. eIF4E binds the 5’ cap of mRNA and

recruits ribosomes to start translation initiation. Cdc33 is a known component of mRNPs, and we hypothesized that Cdc33 and Ksp1 colocalize to regulate translation under stress. Primers containing *CDC33*-matching sequences were used to amplify pBS35 that contains the mCherry sequence and a hygromycin resistance gene. This PCR product was then introduced into a strain of yeast with the *KSP1-GFP* fusion through standard methods for DNA transformation. By homologous recombination, the *CDC33*-mCherry fusion was generated. The integration event was confirmed by PCR, but I was not able to generate localization data for this thesis.

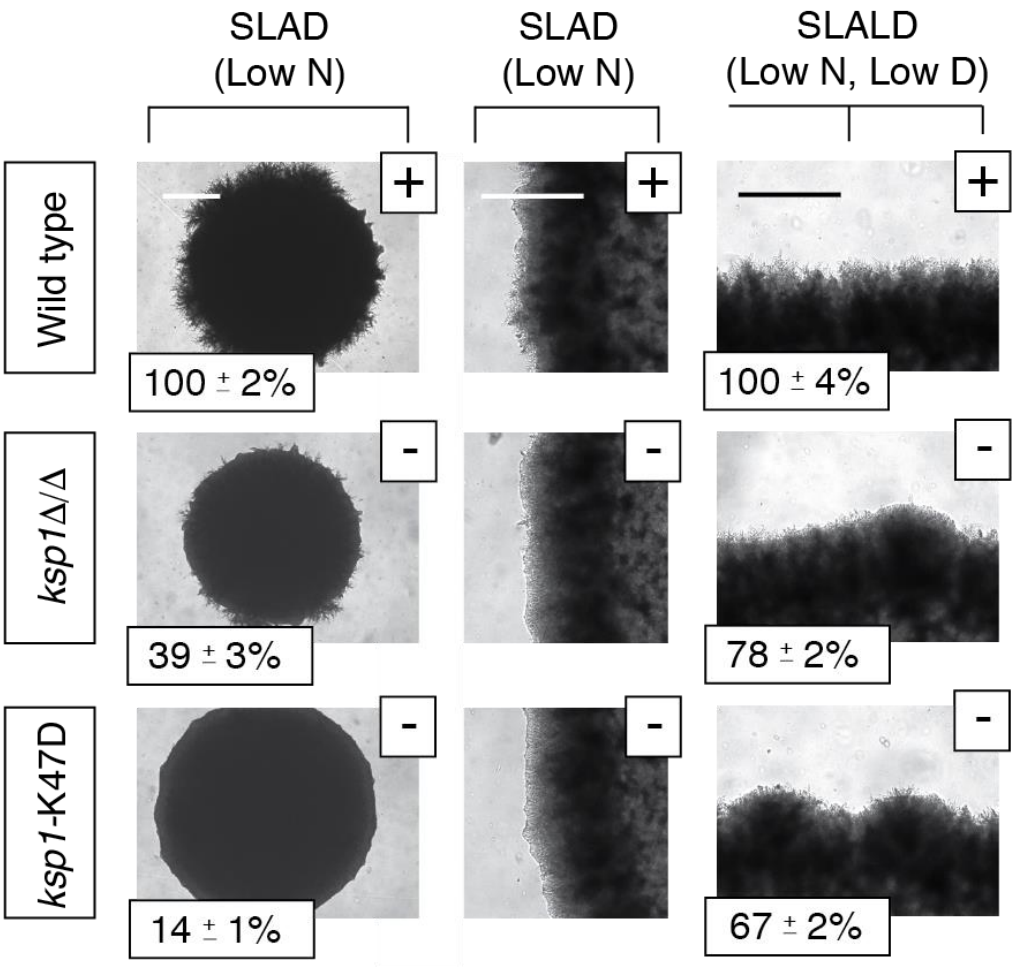

SLAD
(Low N)
SLAD
(Low N)
SLALD
(Low N, Low D)
Wild type
ksp1Δ/Δ
ksp1-K47D
+
+
+
-
-
-
-
-
-
100 ± 2%
100 ± 4%
39 ± 3%
78 ± 2%
14 ± 1%
67 ± 2%

Figure 1

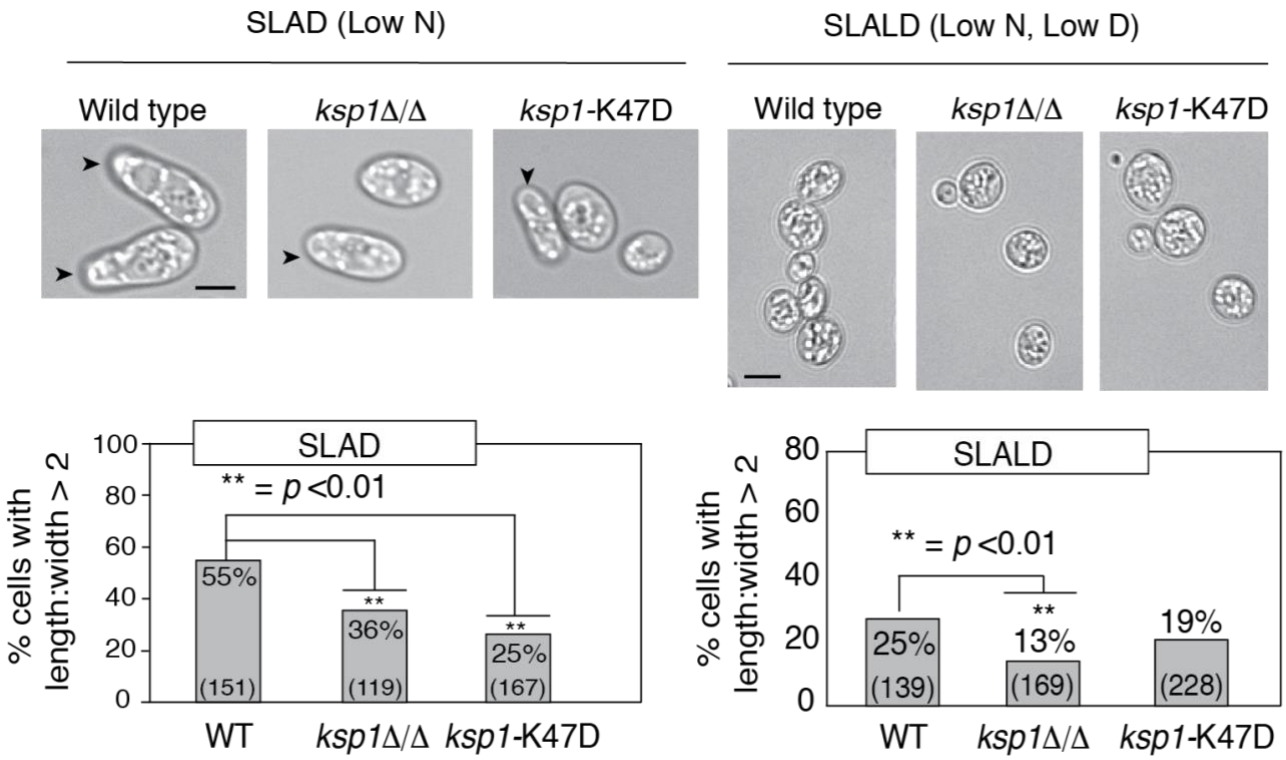

SLAD (Low N)
SLALD (Low N, Low D)
Wild type
ksp1Δ/Δ
ksp1-K47D
Wild type
ksp1Δ/Δ
ksp1-K47D
% cells with length:width > 2
SLAD
** = p <0.01
55%
(151)
36%
(119)
25%
(167)
WT
ksp1Δ/Δ
ksp1-K47D
% cells with length:width > 2
SLALD
** = p <0.01
25%
(139)
13%
(169)
19%
(228)
WT
ksp1Δ/Δ
ksp1-K47D

Figure 2

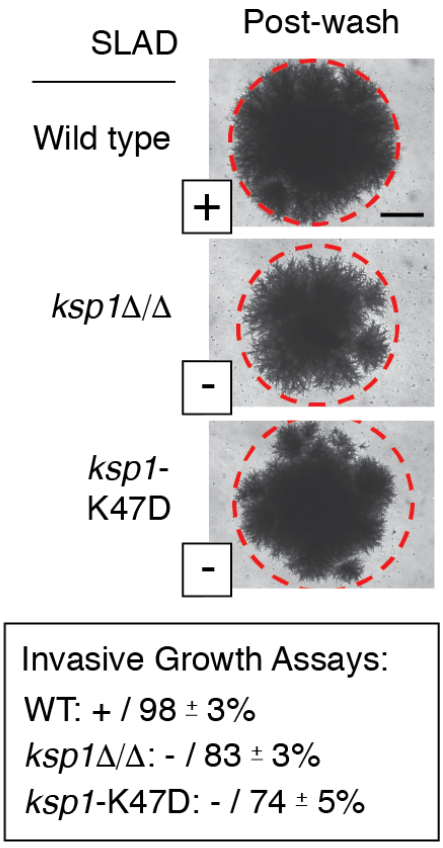
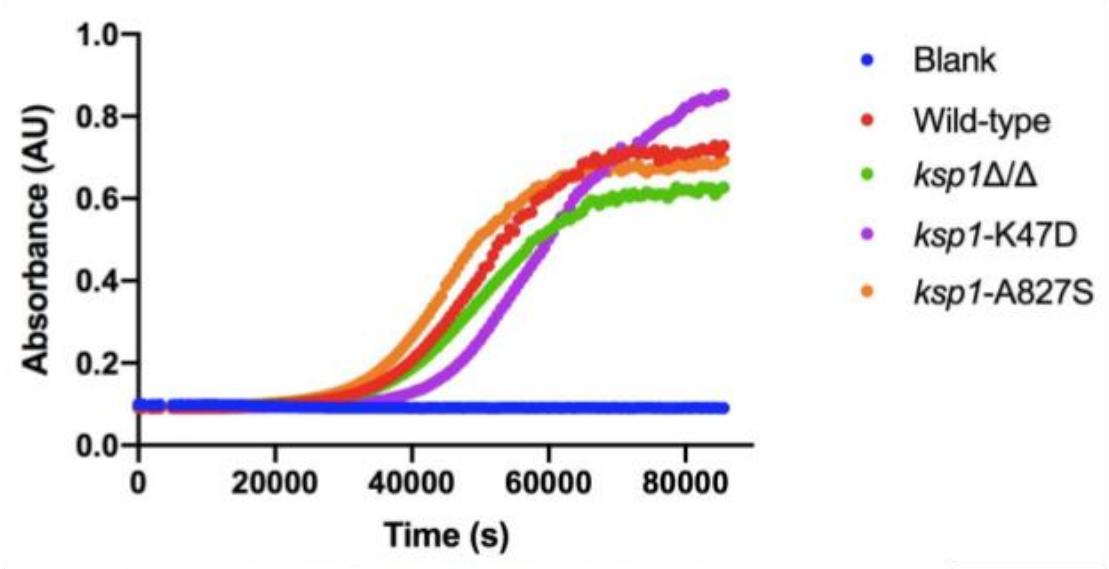

SLAD
Post-wash
Wild type
+
ksp1Δ/Δ
-
ksp1-
K47D
-
Invasive Growth Assays:
WT: + / 98 ± 3%
ksp1Δ/Δ: - / 83 ± 3%
ksp1-K47D: - / 74 ± 5%

Figure 3



Time (s)	0	30601	40593	45589	60577	70569	85558
Blank	0.1	0.091	0.091	0.091	0.091	0.091	0.091
Wild-type	0.093	0.121	0.217	0.313	0.626	0.701	0.728
ksp1 Δ/Δ	0.095	0.119	0.195	0.272	0.528	0.603	0.627
***ksp1*-K47D**	0.091	0.1	0.132	0.179	0.518	0.723	0.853
ksp1-A827S	0.094	0.137	0.277	0.403	0.637	0.667	0.693

Figure 4

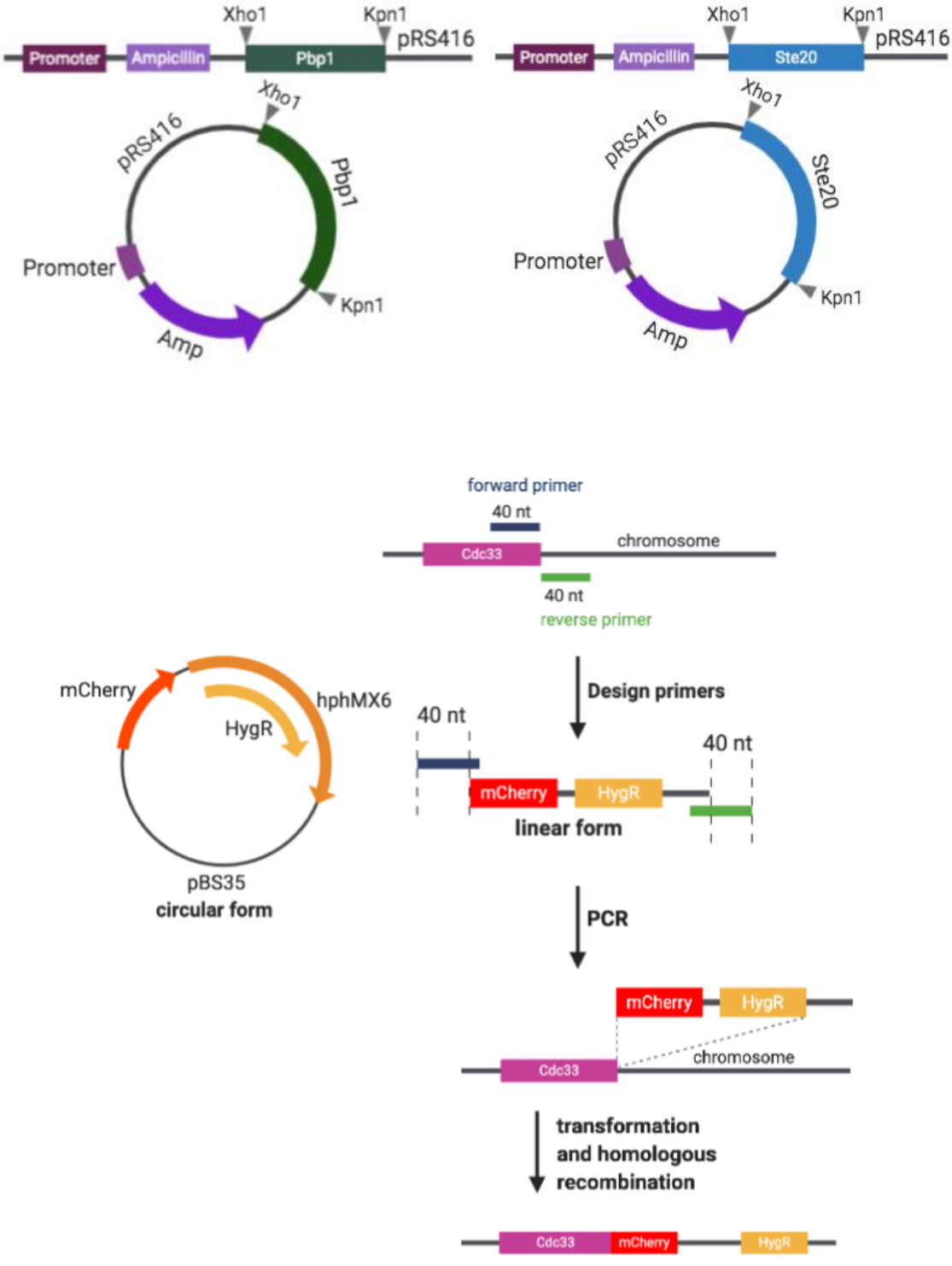

Xho1
Kpn1
pRS416
Promoter
Ampicillin
Pbp1
Xho1
pRS416
Pbp1
Promoter
Kpn1
Amp
Xho1
Kpn1
pRS416
Promoter
Ampicillin
Ste20
Xho1
pRS416
Ste20
Promoter
Kpn1
Amp
forward primer
40 nt
chromosome
Cdc33
40 nt
reverse primer
mCherry
hphMX6
HygR
pBS35
circular form
Design primers
40 nt
40 nt
mCherry
HygR
linear form
PCR
mCherry
HygR
Cdc33
chromosome
transformation
and homologous
recombination
Cdc33
mCherry
HygR

Figure 5

Figure 1: Ksp1 kinase activity is required for wild-type pseudohyphal filamentation under low nitrogen and low glucose conditions. + denotes the wild-type pseudohyphal filamentation. - denotes a reduced level of pseudohyphal filamentation. Filamentation was quantified as the percentage of the circumference for a defined region of the mutant colony relative to the circumference for the corresponding region of a wild-type colony. Percentages indicate the mean of three replicates with standard deviations. All strains are homozygous diploid. Bar = 1 mm. Images acquired and created by Nebibe Mutlu and Angela Hsu.

Figure 2: Ksp1 kinase activity is required for cell elongation under low nitrogen and low glucose conditions. Cells with a length versus width ratio of greater than two are indicated by the arrowheads. A quantification of elongation in cells is shown in the histogram bar for each condition. The number of cells counted is indicated in parentheses. P-values are calculated for pairwise comparisons using two-sample t-tests. Bar = 1μm. Images acquired and created by Nebibe Mutlu and Angela Hsu.

Figure 3: Ksp1 kinase activity is required for wild-type invasion. The quantification was determined by the pixel intensity of washed cells compared to unwashed cells. The dashed red circles indicate the circumference of the spotted culture prior to washing. The means from three replicate experiments with standard deviations are indicated. + denotes wild-type invasion, and - denotes decreased invasion relative to wild-type. Bar = 2 mm. Images acquired and created by Nebibe Mutlu and Angela Hsu.

Figure 4: Growth curves of wild-type and *ksp1* mutants under rapamycin treatment. For each strain, 0, 1, or 5 µg/ml of rapamycin was added to the cell suspensions. A spectrophotometer was used to measure the absorbances of each strain at each concentration of rapamycin. The mean absorbances of each concentration and triplicate experiments were taken to generate the growth curves. The table below the growth curves shows absorbance levels for each strain at specific time points.

Figure 5: Construction of plasmids and strains for phenotypic and localization analysis. In the first row, *PBP1* and *STE20* were cloned using the restriction enzyme method indicated. *Xho*1 and *Kpn*1 restriction sites were incorporated into the primers used to amplify *PBP1* and *STE20* DNA sequences, which are then ligated into the pRS416 plasmid. In the second row, our strategy for generating Cdc33-mCherry fusion protein is indicated. Primers incorporating 40 nucleotides of sequence matching the *CDC33* genomic locus were used to amplify pBS35 which contains the mCherry sequence and a hygromycin-selective gene. This PCR product was then introduced into yeast cells with the *KSP1-GFP* fusion by DNA transformation. Through homologous recombination, the *CDC33-mCherry* fusion construct was generated.

Discussion

Surface filamentation, cell elongation, and invasive growth were studied to understand if Ksp1 kinase activity is necessary for pseudohyphal growth in nutrient-deprivation conditions. Results show that Ksp1 kinase activity is indeed required for wild-type pseudohyphal growth in low nitrogen and low glucose conditions. In nitrogen-deprivation and glucose-deprivation conditions, Ksp1 kinase activity is necessary for wild-type pseudohyphal filamentation as when the kinase domain of Ksp1 is disrupted or when Ksp1 is not present, there is a reduced level of surface pseudohyphal filamentation. Ksp1 is required for cell elongation, and the kinase activity of Ksp1 is necessary for cell elongation when nitrogen is limited. Ksp1 kinase is also required for wild-type invasive growth in low nitrogen conditions. Nitrogen stress signals cells to reduce TORC1 activity, which is also regulated by Ksp1.

Rapamycin fitness assay shows that Ksp1 is important for cell survival under rapamycin treatment. Unlike what is observed in wild-type, *ksp1* deletion, or *ksp1*-A827S strains, in a yeast strain with a defective Ksp1 kinase, cell growth under rapamycin treatment has decreased at first but increased later. This result seems consistent with the idea that Ksp1 colocalizes with TORC1.

Ksp1 is a putative effector of the PKA and TORC1 pathway. Ksp1 integrates PKA and TORC1 signaling as the regulatory subunit of PKA, Bcy1, is mislocalized in cells with a *ksp1* loss-of-function allele (Bharucha *et al*. 2008). Ksp1 is hypophosphorylated under glucose deprivation conditions in a Snf1-dependent and PKA-independent manner, and phosphorylated by TORC1 signaling (Chang and Huh 2018). Ksp1 is the kinase of eIF4G under glucose deprivation conditions and rapamycin treatment (Chang and Huh 2018). Phosphorylation at Ser 624 and Ser 827 of Ksp1 by PKA activates TORC1 signaling (Umekawa and Kilonsky 2012).

Ksp1 was identified among other kinases such as Bcy1, Fus3, Kss1, Sks1, and Tpk2, to be localized in the nucleus during filamentous growth (Bharucha 2008). Fus3 and Kss1 are both MAPKs. Fus3p is involved in the mating MAPK pathway. Kss1 is involved in the filamentation growth MAPK pathway. Nuclear translocation of Ksp1 is necessary for filamentous growth, and Ksp1 is also necessary for the nuclear translocation of Bcy1, Fus3, and Sks1 in filamentous growth (Bharucha 2008).

Under glucose deprivation conditions, Ksp1-dependent phosphorylation of eIF4G promotes degradation of glycolytic mRNAs by recruiting Dhh1 to the glycolytic mRNAs (Chang and Huh 2018). Ksp1 also promotes glucose deprivation-induced degradation of ribosomal protein mRNAs and regulates their translation efficiency (Chang and Huh 2018). Quantitative phosphoproteomic analysis of Ksp1 kinase signaling reveals several important proteins in ribonucleoprotein (RNP) stress granules are hypo-phosphorylated in *ksp1*-K47D (Mutlu *et al*. 2019). Tif4631 and Pbp1 are colocalized to stress granules (Mutlu *et al*. 2019). Ksp1-dependent phosphorylation sites, Ser176 in Tif4631 and Ser436 in Pbp1, are required for wild-type pseudohyphal growth (Mutlu *et al*. 2019). Tif4631 was also reported by Chang and Huh in 2018 to be an *in vitro* substrate of Ksp1. It is necessary for wild-type pseudohyphal growth but not required for the regulation of stress granule abundance. This could suggest these two activities are not regulated by the same pathway related to Ksp1.

Transcriptional profiling and quantitative phosphoproteomics have been done to understand possible Ksp1-regulated cellular processes (Mutlu *et al*. 2019). Different transcript abundances were observed, including an increase of transcript abundance in genes contributing to amino acid biosynthesis. Ksp1 regulates multiple eukaryotic stress responses, including pseudohyphal growth, and possibly cell wall organization, DNA replication, and the cell

morphogenesis checkpoint as demonstrated by the decrease in transcript levels associated with these processes in *ksp1*-K47D mutants. Ksp1 seems to also be associated with cellular responses that are not related to pseudohyphal growth, such as responses to oxidative stress, autophagy, and sporulation, as genes responsible for these activities also have a change of transcript level in yeast cells with a defective *ksp1* allele.

The *ksp1* deletion mutants show an increase in RNP granules marked by Pbp1, which is an observation consistent with the idea that Ksp1 regulates stress granule abundance (Mutlu *et al*. 2019). Pbp1 is another stress granule protein. Its Ksp1-dependent phosphorylation site Ser456 is hypo-phosphorylated in the *ksp1*-K47D mutants, but another site, Ser436, is hyper-phosphorylated in *ksp1*-K47D mutants. The Kumar lab observed that a phosphomimetic Pbp1-S436D mutant, like the *ksp1*-K47D mutant, produces more RNP granules. These results could indicate that the phosphorylation of Ser176 in Tif4631 and dephosphorylation of Ser436 in Pbp1 are both dependent on Ksp1 signaling and inhibit RNP granules. Collectively, the data indicate that Ksp1-dependent phosphorylation regulates stress granule abundance.

Wheeler *et al*. has discussed in 2016 that proteins with disordered domains may contribute to the regulation of stress granule proliferation and disassembly by protein-protein and protein-RNA interactions. Ksp1 might be a suitable candidate because Ksp1 was identified to be an RNA-binding protein by UV cross-linking and mass spectrometry of proteins in RNA-protein complexes (Mitchell *et al*. 2013). Ksp1 was also predicted to have a disordered RNA-binding domain by Jain *et al*. in 2016. Because stress granule abundance is increased when a functional *KSP1* allele is not present, Ksp1 is hypothesized to be responsible for stress granule disassembly or inhibiting stress granule assembly.

In addition, the Kumar lab has found evidence that a signaling kinase can facilitate both pseudohyphal growth and RNA granule abundance. Two MAP kinases in yeast, Kss1 and Fus3, are colocalized to Tpk2, the PKA catalytic subunit, and Igo1p, a stress granule and p-body localized protein. Disrupting *KSS1* perturbs RNP formation as shown by Shively *et al* in 2015. PKA phosphorylates Pat1, a stress granule scaffolding protein, inhibiting p-bodies (Ramachandran *et al*. 2011). Thus, Ksp1 could be another kinase that facilitates both pseudohyphal growth and RNA granule abundance.

Future Studies

In complement to co-localization of fluorescent protein fusions, yeast-two hybrid studies can be used to analyze potential protein-protein interactions between Ksp1 and Cdc33. The Ksp1 protein contains a sequence predicted to bind eIF4Es such as Cdc33. The yeast-two hybrid assay is a method used to detect if there is physical interaction between two proteins. This method utilizes the Gal4 transcription factor, which has a DNA-binding domain and an activation domain. A downstream reporter gene is activated only with the activation of an upstream activating system (UAS) by the binding of Gal4. Two fusion proteins are made. One with a bait fused to the DNA-binding domain (BD). One with a prey fused to the activation domain (AD). If there is interaction between the bait and prey, the transcription factor binds the UAS sequence and activates transcription of the reporter gene.

For this work, *KSP1* can be cloned as a bait, and *CDC33* can be cloned as a prey. The Kumar lab attempted preliminary experiments at assessing the possibility of Ksp1 and Cdc33 interaction by two-hybrid analysis, but no definite conclusions were available at the time of this writing. In particular, control strains with bait and prey alone still need to be analyzed fully, and more stringent conditions for reporter activity may have to be applied to achieve fully interpretable results.

Co-IP can also be used to study interactions between Ksp1 and Cdc33. Co-immunoprecipitation (co-IP) is a common technique for the analysis of protein-protein interactions. Co-IP relies on protein-specific antibodies to indirectly detect proteins that are interacting with the specific target protein. The approach often yields results that are more relevant *in vivo* than two-hybrid data.

Another future direction includes elucidating the detailed mechanisms behind the connection between autophagy and Ksp1. Recent transcriptional profiling data from the Kumar lab indicates that the expression of autophagy genes is upregulated in a strain of yeast with a kinase-defective allele of *KSP1* (Mutlu *et al*. 2019). The mechanism of this upregulation could be studied by deletion of key transcription factors in the *ksp1*-K47D mutant, since deletion of the transcription factor through which Ksp1 is working should interrupt the upregulation of autophagy genes. Deletion mutants can be generated in the *ksp1*-K47D strain using teh gene replacement/integration steps outlined in Figure 5. The resulting data would provide an important advancement in understanding the mechanism of Ksp1 regulation of yeast stress responses.

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