PROTEIN KINASE A-DEPENDENT PHOSPHORYLATION AND DEGRADATION OF CDK8: IMPLICATIONS FOR YEAST FILAMENTOUS GROWTH

by

PEDRO DANIEL MIRA LOURENÇO

B.Sc., The University of British Columbia, 2006

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Biochemistry and Molecular Biology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

June 2008

ABSTRACT

S. cerevisiae have developed the ability to forage for nutrients when presented with conditions of starvation. This dimorphic adaptation is particularly noticeable when yeast are subject to nitrogen depravation and has been termed filamentous growth, as cells form filament-like projections away from the center of the colony. The regulation of this response is under the control of the well-characterized MAPK and cAMP pathways. Previous work showed that Cdk8p phosphorylated a key transcriptional activator of the filamentous response, Ste12p, and subsequently targeted the factor for degradation under conditions of limiting nitrogen.

Data presented in this thesis suggests that Cdk8p is regulated by another kinase, Tpk2p. *In vitro* kinase assays demonstrate that Tpk2p directly phosphorylates Cdk8p on residue Thr37, leading to the destabilization of Cdk8p after growth for 4 hours in SLAD media. Lack of phosphorylation on Thr37 yields a hypo-filamentous phenotype, whereas a phospho-mimic mutant, T37E displays a hyperfilamentous phenotype.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

Wild-type alleles in *Saccharomyces cerevisiae* are represented by italicized capital letters (e.g. *CDK8*), while mutant recessive alleles are denoted in lower case italics (e.g. *cdk8*). Gene products are written with the first letter capitalized, followed by the letter “p” at the end to denote “protein” (e.g. Cdk8p).

ABBREVIATIONS

ATP	adenosone triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphatae
cdc	cell-cycle division
CDK	cyclin dependent kinase
CTD	c-terminal domain
DNA	deoxyribonucleic acid
E. Coli	*Escherichia coli*
FRE	filamentous response element
GDP	guanine diphosphate
GTP	guanine triphosphate
IPTG	isopropyl ß-D-thiogalacto-pyranoside
kb	kilo base pairs
kDa	kilodalton
LB	luria broth
MAPK	mitogen activated protein kinase
mRNA	messenger ribonucleic acid
NAT	nourseothricin
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PIC	pre-initiation complex
PKA	protein kinase A
PRE	pheromone response element
S. cerevisiae	*Saccharomyces cerevisiae*
SD	synthethic dropout
SDS	sodium dodecylsulfate
SDM	site-directed mutagenesis
SLAD	synthetic low ammonium dextrose
RNA	ribonucleic Acid
RNAPII	rNA polymerase II
Thr	threonine
T37A	threonine 37 to alanine 37
T37E	threonine 37 to glutamate 37
TPK	*takashi's protein kinases*
UAS	upstream activating sequence
URA	uracil
YPD	yeast extract peptone dextrose
ZEO	zeocin

ACKNOWLEDGEMENTS

First and foremost, I would like to extent my extreme gratitude to Dr. Ivan Sadowski for the opportunity to study under his supervision, for his daily contagious enthusiasm about science, and for the exceptional guidance only a high-caliber scientist like he could offer. Thank you also to my committee members and excellent scientists Dr. Leann Howe and Dr. Vivien Measday for the fine advice and for bearing with my deadlines.

Thanks also to lab members Dr. Thomas Malcolm, Ting-Cheng Su and Jennifer Parent, for their daily support and encouragement throughout the years I spent in the lab.

CHAPTER 1 – INTRODUCTION

1.1 OBJECTIVE

Cdk8p is an important cyclin-dependent kinase (Cdk) involved in numerous transcriptional processes. While its role in the regulation of the GAL response and in the phosphorylation of the RNA Pol II CTD has been clearly established, little is known about its involvement in the regulation of filamentous growth. Previous work by Nelson et al. (2003) demonstrated that Cdk8p phosphorylates Ste12p, leading to its degradation, and that Cdk8p is destabilized in response to nitrogen depletion. This series of events is thought to regulate the induction of filamentous growth.

My research objectives are to investigate how Cdk8p is destabilized in response to nitrogen depletion, what effects stabilization of Cdk8p has on pseudohyphae formation and to determine what signals upstream of Cdk8p transmit the signal for nitrogen depletion.

1.2 CONTROL OF EUKARYOTIC TRANSCRIPTION

Eukaryotic transcription is a precisely regulated process. Due to limited cellular resources and selective physiological needs, only a subset of genes is expressed at a given time-point. Therefore, mechanisms of transcriptional activation and repression are of utmost importance. Gene-specific activators function to initiate transcription via two main routes: (1) promoting the formation of the pre-initiation complex (PIC) by recruiting the RNA polymerase II holoenzyme complex and its associated factors; and (2) formation of open chromatin structure, which is transcriptionally active (Kornberg 1999). Gene-specific repressors, on the other hand, function by either: (1) inhibiting the formation of the PIC, presumably by preferentially binding to the promoter DNA and preventing the binding of transcriptional activators; and (2) formation of closed chromatin structure, which is transcriptionally inactive due to the inability of the transcriptional machinery to bind to promoters physically interacting with histone proteins (Maldonado et al. 1999).

1.3 GENE-SPECIFIC TRANSCRIPTIONAL ACTIVATORS AND REPRESSORS

Saccharomyces cerevisiae are hypothesized to contain 5,651 genes (Kellis et al. 2003), thus cells are required to regulate gene expression, as simultaneous expression of all genes would result in cellular havoc. Simplistically, each gene is compromised of a coding sequence and an upstream promoter region. An eukaryotic promoter is comprised of a core promoter, which contains the transcription initiation site and the TATA box, and specific sequence elements that can interact with transcriptional activators, enhancers or repressors.

Transcriptional activators and enhancers are factors capable of interacting with upstream activating sequences (UASs) or enhancer sequences, respectively. Activators and enhancer proteins possess DNA-binding domains that specifically recognize and bind to these elements; once bound, these activators form favorable protein-protein interactions with members of the RNA polymerase II

(RNAPII) complex and, thus, facilitate its recruitment to the promoter (Blackwood and Kadonaga 1998). While UASs are typically found within 2kb of the transcription start-site, enhancers have been documented to exist as far away as 85kb.

Transcriptional repressors, on the other hand, interact with upstream repression sequences that inhibit transcription by out-competing activators for promoter binding, preventing recruitment of RNAPII and causing local chromatin structure modifications that prevent binding of either the general transcription machinery or the gene-specific factors (Maldonado et al. 1999), thus exerting their repressive effect.

Transcriptional activators and repressors typically bind to and exert their functions on promoters in a gene-specific manner, thus precisely regulating expression. Therefore, understanding the mechanisms of these proteins and how their activities are regulated is of utmost importance and relevance.

1.4 RNA POLYMERASE II HOLOENZYME

RNA Polymerase II holoenzyme is recruited to specific promoters of protein-coding genes. Due to a complex interplay of factors, the RNA polymerase II complex binds the promoter and initiates transcription.

1.4.1 The Core

The *Saccharomyces cerevisiae* RNA polymerase II (RNAP II) core was initially purified as a 12 subunit complex and is in itself incapable of promoter recognition (Myer and Young 1998). The core RNAP II consists of 12 subunits, Rpb1p – Rpb12p, ranging in size from 6 to 200 kDa. Rpb1p and Rpb2p, the largest and, arguably, the most important subunits, are involved in the binding of DNA and the nucleotide substrates, respectively (Gnatt et al. 2001). The remaining subunits,

Rpb3p – Rpb12p, form a variety of complexes implicated in positioning of the DNA, transcription initiation and stress response (Choder and Young 1993).

1.4.2 RNA Polymerase Carboxy-Terminal Repeat Domain (CTD)

The largest subunit of the RNAP II complex, Rpb1p, contains tandem repeats of the heptapeptide YSPTSPS. In yeast, the CTD is compromised of 26 or 27 repeats of the heptapeptide (Hampsey 1998). The CTD is essential and truncation results in a cold sensitive phenotype (Thompson et al. 1993). Modifications of the CTD alter the activity of the holoenzyme. The hypophosphorylated CTD, termed IIA, is correlated to transcriptionally inactive RNAP II, while the hyperphosphorylated form, IIO, is associated with the transcriptionally active enzyme (Riedl and Egly 2000). The phosphorylated form of the CTD also functions as a binding scaffold for numerous nuclear factors, such as elongation factors and mRNA capping enzyme, which emphasizes the global role of the CTD as a regulator of transcription (Phatnani and Greenleaf 2006).

1.4.3 General Transcription Factors

The general transcription factors were initially purified for their capacity to enhance promoter recognition by the core RNAP II complex. By this assay, five factors were identified: TFIIB, TFIID, TFIIE, TFIIF, TFIIH (Orphanides et al. 1996). The broad roles of the general transcription factors are outlined in table 1.

Table 1. The general transcription factors of RNA Pol II and their functions

Factor	*Gene*	*Function*
TFIIB	SUA7	Transcription start site selection
TFIID	SPT15 TAF1 TAF2 TAF3 TAF4 TAF5 TAF6 TAF7 TAF8 TAF9 TAF10 TAF11 TAF12 TAF13 TAF14	TATA box recognition and binding
TFIIE	TFA1 TFA2	Recruitment of TFIIH
TFIIF	SSU1 TFG2 ANC1	Suppression of non-specific binding of RNAP II; stablization of pre-initiation complex
TFIIH	TFB1 TFB2 TFB3 TFB4 RAD3 SSL1 SSL2 KIN28 CCL1	DNA-dependent ATPase; ATP-dependent helicase; CTD kinase

1.4.4 Mediator Complex

In conjunction to the roles of transcriptional activators and repressors, there exists an additional module involved in regulating transcription. The mediator complex was initially identified in an experiment where it was hypothesized that over-expression of one activator would interfere with the efficiency of another (Gill and Ptashne 1988). All attempts to titrate this effect by addition of known general transcription factors and RNAPII components failed to relieve this phenomena and it was later determined that there was an additional factor contributing to this effect, the mediator.

The mediator complex is comprised of 20 to 30 subunits, depending on the scheme and conditions of purification (Bjorklund and Gustafsson 2005). The mediator complex can function as an intermediary, relaying regulatory information from enhancers or repressors to the basal RNAPII transcription machinery. Given the mediator's ability to interact with both transcriptional activators and repressors, the complex is capable of both gene repression and activation.

Cdk8p, and by association Cyclin C, are components of the eukaryotic mediator complex. Cdk8p has been implicated as a general relay point of environmental stress, as is evidenced by the fact that introduction of kinase-dead alleles results in the de-repression of numerous genes involved in different stress responses (Holstege et al. 1998). It has been noted that Cdk8p levels decrease as yeast cells approximate the diauxic shift (Holstege et al. 1998), that Cyclin C is responsive to a magnitude of cellular stresses (Cooper et al. 1997) and that *cdk8* or *cycc* null strains mimic nutrient starvation phenotypes (Holstege et al. 1998). The mediator, and in particular Cdk8p/CycCp, have been shown to be involved in nutrient sensing and stress-coping responses (Chang et al. 2001; Nelson et al. 2003).

The mediator has the ability to regulate transcription in response to a wide range of environmental signals and stresses, adding to eukaryotic cells' repertoire of transcriptional regulatory mechanisms; thus, highlighting the complex interplay of factors and conditions that are involved in the regulation of gene expression in yeast.

1.5 FILAMENTOUS AND INVASIVE GROWTH

It is well established that in response to the stress of nutrient depletion, *Saccharomyces cerevisiae* adopt a growth pattern and cell morphology that direct daughter cells to grow away from the colony as a means of foraging for nutrients. The pseudohyphae dimorphic change was first identified in the late 19th century (Hansen 1886), but only became notorious when re-discovered and implicated as an adaptation to nitrogen limitation (Gimeno et al. 1992); later evidence also implicated limited carbon source or presence of poor carbon source (Lambrechts et al. 1996) or fusel alcohols (Dickinson 1996) as environmental signals that lead to similar phenotypical responses.

1.5.1 Diploid Pseudohyphal Growth

As a response to nitrogen depletion, diploid yeast strains undergo a series of morphological changes in which progeny cells are continuously placed in locations away from the center of the colony (Gimeno et al. 1992). During diploid pseudohyphal growth, cells become elongated, budding patterns switch from bipolar to unipolar and buds do not separate, thus producing chains of elongated and thin cells called pseudohyphae (Gancedo 2001). Nitrogen-depleted cells also exhibit a modified cell cycle, with progeny cells beginning to bud immediately following cytokinesis (Kron et al. 1994). As demonstrated in Figure 1, the shift in budding pattern results in buds emerging from the same pole of the elongated cell, allowing daughter cells to grow far beyond the perimeter of the colony and to

dig into the agar (Gimeno et al. 1992). This response typically occurs during conditions of nutrient starvation and, therefore, likely allows yeast cells to forage for nutrients.

1.5.2 Haploid Invasive Growth

Unlike their diploid counterparts, haploid yeast cells are unable to form long, protruding filaments under conditions of limiting nutrients (Gimeno et al. 1992). However, their ability to forge for nutrients is still existent, although limited. Phenotypically, haploid cells, when in conditions of depleted nutrients, undergo a series of transformations similar to that of diploids: cells switch from a round to elongated shape; the budding pattern switches from axial to unipolar; and, buds emerge from the same side of the elongated cell (Roberts and Fink 1994). However, colonies fail to exhibit long, protruding filaments, Figure 1. Haploid filaments form and dig into the area confined below the colony and, thus, this response has been appropriately termed invasive growth (Gimeno et al. 1992; Dickinson 1996).

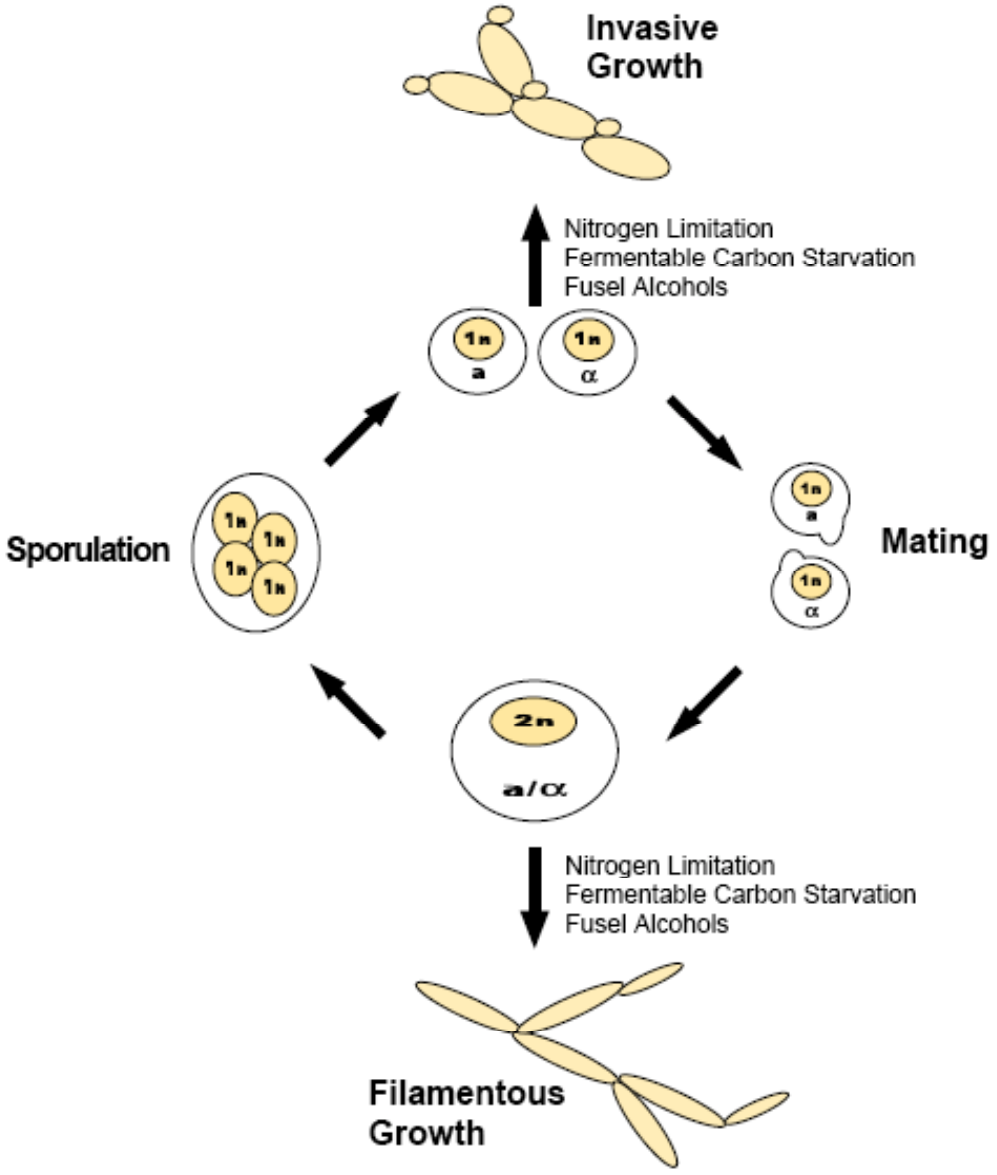

Figure 1.1 Life cycle of yeast *Saccharomyces cerevisiae*

Saccharomyces cerevisiae adopt a growth pattern and cell morphology that direct daughter cells to grow away from the colony as a means of foraging for nutrients when in conditions of limiting nitrogen or fermentable carbon or due to presence of fusel alcohol. Haploid yeast undergo invasive growth, while diploid yeast undergo pseudohyphal growth.

1.5.3 Mitogen-Activated Protein (MAP) Kinase Pathway

Historically, the MAP kinase pathway has been implicated in regulation of the morphogenetic switches of pheromone and filamentous response in yeast. The MAP kinase signaling module is dependent on multiple protein kinases, which are capable of transmitting a signal to their downstream targets via a series of consecutive phosphorylation events, which can directly regulate the various transcriptional activators involved both the pheromone and filamentous response (Levin and Errede 1995).

1.5.3.1 MAPK Pathway in Regulation of Pheromone Response

The MAPK signaling cascade is also implicated in the regulation of the pheromone response, albeit with a few minor difference in relation to its role in the filamentous response. The detection of pheromone and, subsequently, signaling to initiate the pheromone response is triggered when pheromone binds to the to G-coupled receptor of cells of the opposite mating type, Figure 1.2. These peptides are recognized by the opposite mating-type cells and, upon binding, cause a conformational change in the receptors, Ste2p in *MATa* cells and Ste3p in *MAT*α cells (Bender and Sprague 1986).

Peptide mating pheromones, a- and α-factor, are known to cause haploid cells to transiently arrest in G1 phase and express genes required for mating with cells of the opposite mating type. MATa cells produce and secrete a-factor, a 12 amino-acid peptide that stimulates MATα cells (Caldwell et al. 1995). MATα cells, on the other hand, secrete α-factor, a 13 amino acid long peptide, which stimulates MATa cells. Binding of the pheromones to the seven-transmembrane receptors (Ste2p or Ste3p in MATa and MATα cells, respectively) causes Gpa1p (α subunit) to dissociate from Ste4p/Ste18p (β and γ subunits, respectively) (Herskowitz 1995). Dissociated Ste4p/18p interacts with the Rho-family GTPase Cdc24p and the Ste20p kinase, which in turn activate the first component of the MAPK pathway, the MAPKKK Ste11p. Ste11p ultimately phosphorylates and

activates Ste7p (the MAPKK), which subsequently activates Fus3p or Kss1p. Fus3p is the primary MAP kinase responsible for the activation of the mating response, although Kss1p has been found to have redundant roles, however Kss1p does not associate with Ste5p as does Fus1p (Elion et al. 1991). Activated Fus3p can activate two major substrates: Far1p a cyclin-dependent kinase inhibitor required for pheromone-induced cell-cycle arrest in G1 phase, and Ste12p, the transcriptional activator of both the filamentous and the mating-type response.

1.5.3.1 MAPK Pathway in Regulation of Filamentous Response

The MAP kinase pathway involved in the filamentous response includes many of the players implicated in the pheromone response. A proposed model (Figure 1.3) is that the signal for nitrogen starvation is transmitted to Ras2p, a small GTP-binding protein, that, once activated, activates the guanine nucleotide exchange factor Cdc24p (Roberts et al. 1997). Cdc24p facilitates the exchange of GDP for GTP on Cdc42p, enabling it to interact with protein kinase Ste20p and causing the kinase to dissociate from its negative inhibitor Hsl7p (Peter et al. 1996). Activated Ste20p is also found to interact with Bmh1p and Bmh2p, which appear to not be involved with invasive growth, but rather are required for cellular elongation. It is speculated that these proteins might play a role in cell elongation independently of the MAP kinase pathway (Roberts et al. 1997). Activated Ste20p then activates the MAP kinase cascade, leading the phosphorylation and subsequent activation of Ste11p, Ste7p and Kss1p (Gustin et al. 1998). Prior to phosphorylation by Ste7p, Kss1p interacts with transcriptional activator Ste12p and the negative regulators Dig1p and Dig2p, forming a complex that sequesters Ste12p and Tec1p from interacting with the well-defined Filamentous Response Element (FRE) (Cook et al. 1996; Bardwell et al. 1998). Kss1p phosphorylates Ste12p and Dig1/Dig2p, causing dissociation of Dig1p/Dig2p and de-represses the target genes to which Ste12p and Tec1p may bind (Bardwell et al. 1998).

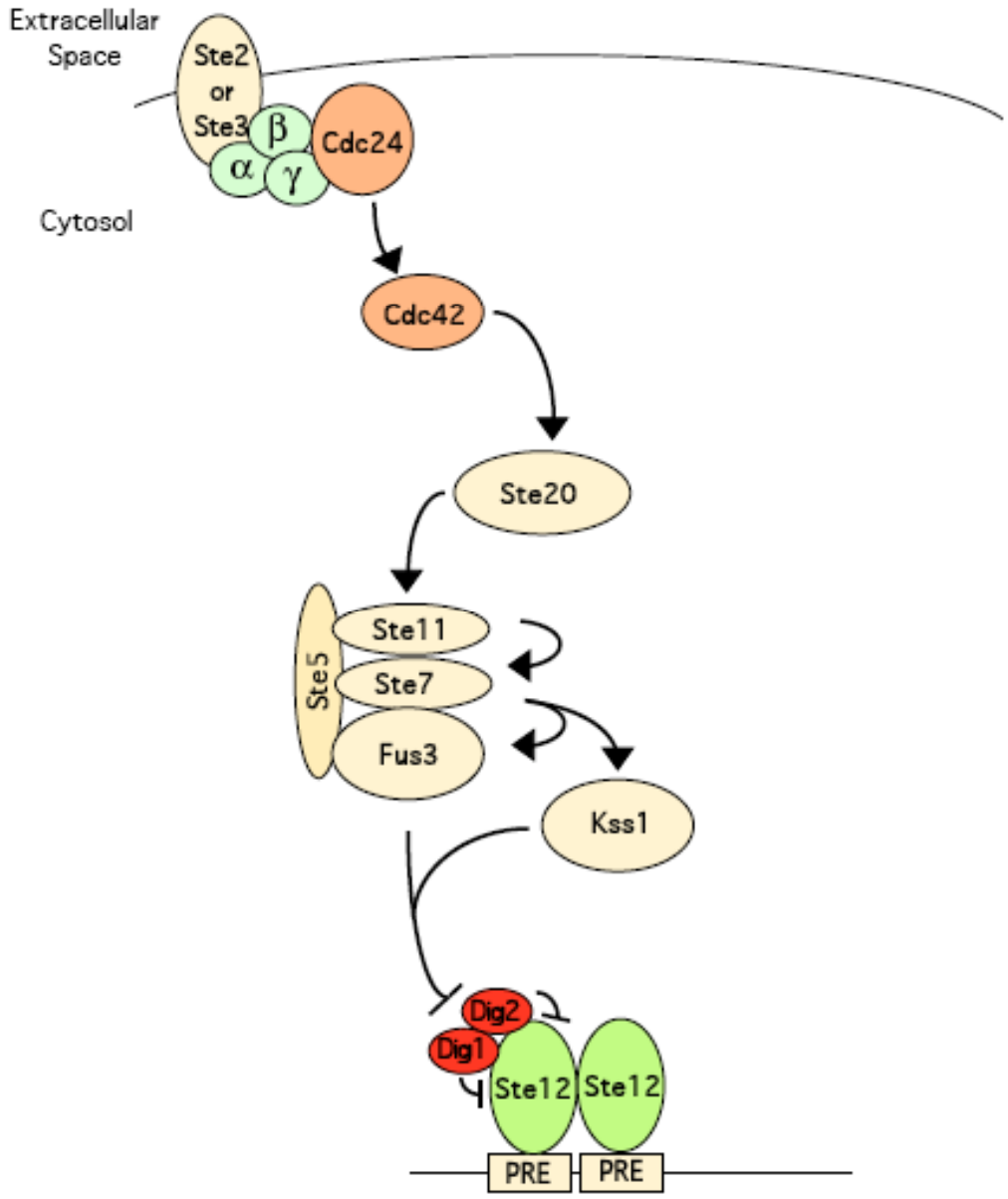

Figure 1.2 Pheromone-dependent activation of Ste12p via MAPK pathway

Presence of pheromone triggers activation of the MAPK signaling cascade to activate either one of the redundant MAP Kinases Kss1p or Fus3p to relieve the inhibitory effects on the transcriptional activator Ste12p, allowing for the transcription of genes involved in mating. See text for details.

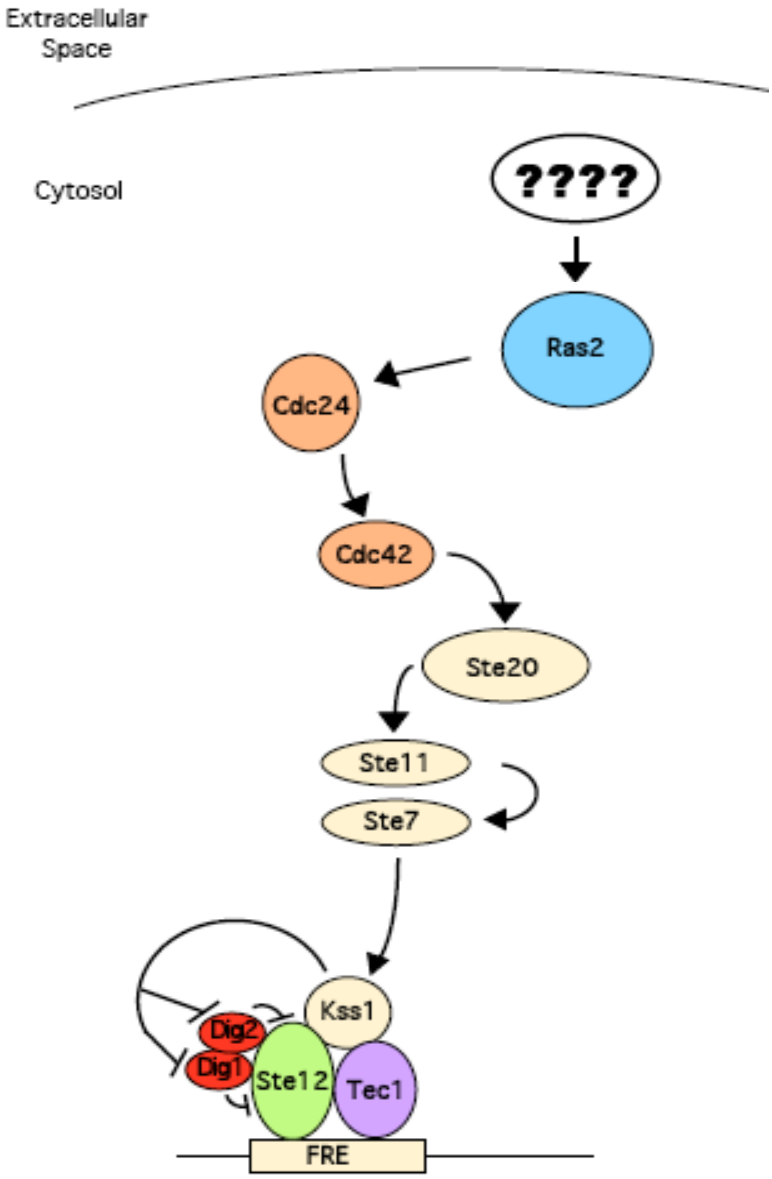

Figure 1.3 MAPK-dependent induction of the filamentous response

It is hypothesized that nitrogen deprivation leads to the activation of the MAPK pathway, resulting in the activation of the MAP kinase Kss1p, which, in turn, relieves the inhibitory effects of Dig1/2p on Ste12p. Note that it is not known how or if nitrogen deprivation stimulates this pathway. See text for details.

1.5.3.2 *Assumed transference of function: MAPK regulation of the filamentous and pheromone response*

As described previously, the MAPK pathway is implicated in activation of both the filamentous and the pheromone response. However, any inference of the MAPK pathway in the filamentous response is far stretched. There are numerous genetic studies implicating that the MAPK pathway is necessary for filament formation, yet, to date, there is no evidence to suggest that nitrogen signaling occurs via the MAPK pathway.

A reasonable explanation is that the relation between filamentous growth and the MAPK pathway is an artifact of the shared requirement for the transcriptional activator Ste12p. The current models propose that both the filamentous and pheromone response are ultimately induced due to activation of Ste12p. However, since the *STE12* promoter contains multiple Ste12p binding sites and *STE12* expression is itself dependent on Ste12p, a basal level of the Ste12p activator is required for *STE12* expression upon stimulation. The same occurs for *TEC1* expression.

Even without pheromone stimulation, there is basal signaling via the MAPK pathway due to spontaneous dissociation of the $\beta\gamma$ subunits from the G-protein coupled receptor (Siekhaus and Drubin 2003). This basal signaling likely allows for a small de-repression of Ste12p by relieving the inhibitory effects of the Dig1/2p and Kss1p proteins. The basal expression of *STE12* is, thus, due to a leaky receptor.

However, in strains that lack components of the MAPK pathway, Ste12p is under constant inhibition by Dig1/2p and Kss1p and, thus, Ste12-dependent expression of *STE12* and *TEC1* is eliminated. Therefore, induction of the filamentous response cannot occur because of the absence of uninhibited Ste12p; thus, the “requirement” of the MAPK pathway for the induction of the filamentous response.

1.5.4 CAMP-Dependent Pathway

Multiple signaling pathways regulate filamentous growth: in addition to the MAP kinase pathway, the cAMP-dependent pathway has also been extensively characterized and implicated with the filamentous response. Cyclic Adenosine Mono-Phosphate (cAMP) is a universal signaling molecule in most, if not all, living organisms. This secondary messenger controls multiple targets by regulating the activity of cAMP-dependent kinases. As depicted in Figure 1.4, Intracellular cAMP levels are dependent on two opposite activities: conversion of ATP into cAMP by adenylate cyclase, which is encoded by the *CYR1* gene in yeast; and reduction in intracellular cAMP levels by action of the phosphodiesterases Pde1p and Pde2p (D'Souza and Heitman 2001).

The activity of the Cyr1p adenylate cyclase is dependent on Ras2p, the small GTP-binding protein also implicated in the MAP kinase pathway (D'Souza and Heitman 2001). Under conditions of nutrient depletion, Gpa2, also regulates cAMP production via the activation of Cyr1p (Colombo et al. 1998). Increased cAMP levels result in the activation of the cAMP-dependent kinase, protein kinase A (PKA). In yeast, the PKA consists of a negative regulatory subunit, Bcy1p, in combination with one of the three isoforms of catalytic subunits: Tpk1p, Tpk2p and Tpk3p (Cannon and Tatchell 1987).

Extracellular cAMP can enhance pseudohyphal differentiation in most wild-type strains and this effect is particularly evident in a *pde2/pde2* mutant diploid. Concomitantly, extracellular cAMP was found to suppress the pseudohyphal differentiation defect in a *gpa2/gpa2* (Lorenz and Heitman 1997). These previous findings serve to highlight the importance of cAMP-dependent kinases in the regulation of the filamentous response.

1.5.4.1 Takashi's Protein Kinases

The major downstream elements directly regulated by cAMP are the cAMP-dependent kinases Tpk1p, Tpk2p, and Tpk3p. These kinases appear to have relatively redundant functions, as deletion of any combination of two isoforms does not result in considerable growth defects, whereas a triple deletion mutant is non-viable (Cannon and Tatchell 1987).

Each of the three isoforms of *TPK1-3* is directly inhibited by the Bcy1p regulatory subunit. Upon binding of cAMP to Bcy1p, a conformational change is induced that causes the regulatory subunit to dissociate from Tpk1p, Tpk2p or Tpk3p, liberating the active kinase (Toda et al. 1987). Of the three isoforms, Tpk2p appears to have the most pronounced effect on filamentation: a *tpk2/tpk2* mutant strain fails to form filaments on nitrogen limiting media, whereas overexpression of *TPK2* shows enhanced pseudohyphal growth (Robertson and Fink 1998). *TPK3*, on the other hand, was found to be an inhibitor of filamentous growth, while a role for *TPK1* is less obvious, as one group has reported no effects of *tpk1* deletions (Robertson and Fink 1998) and another implicated *TPK1* as an inhibitor of the filamentous response, similarly to the effects of *TPK3* (Pan and Heitman 1999).

Tpk1p and Tpk3p are 88% identical over the kinase domain, while Tpk2p is approximately 76% identical to either Tpk1p or Tpk3p (Toda et al. 1987). This extensive similarity may be at the root of the redundancy between the three isoforms. Given the similarity in structure and, most likely, substrate recognition, cellular compartmentalization of the different isoforms is likely to be important with respect to signal specificity. In fact, Tpk2p was the only isoform localized to the nucleus, while Tpk1p and Tpk3p are both cytoplasmic kinases (Huh et al. 2003). Collectively, the three isoforms of TPK have an effect on filamentous growth by regulating multiple targets.

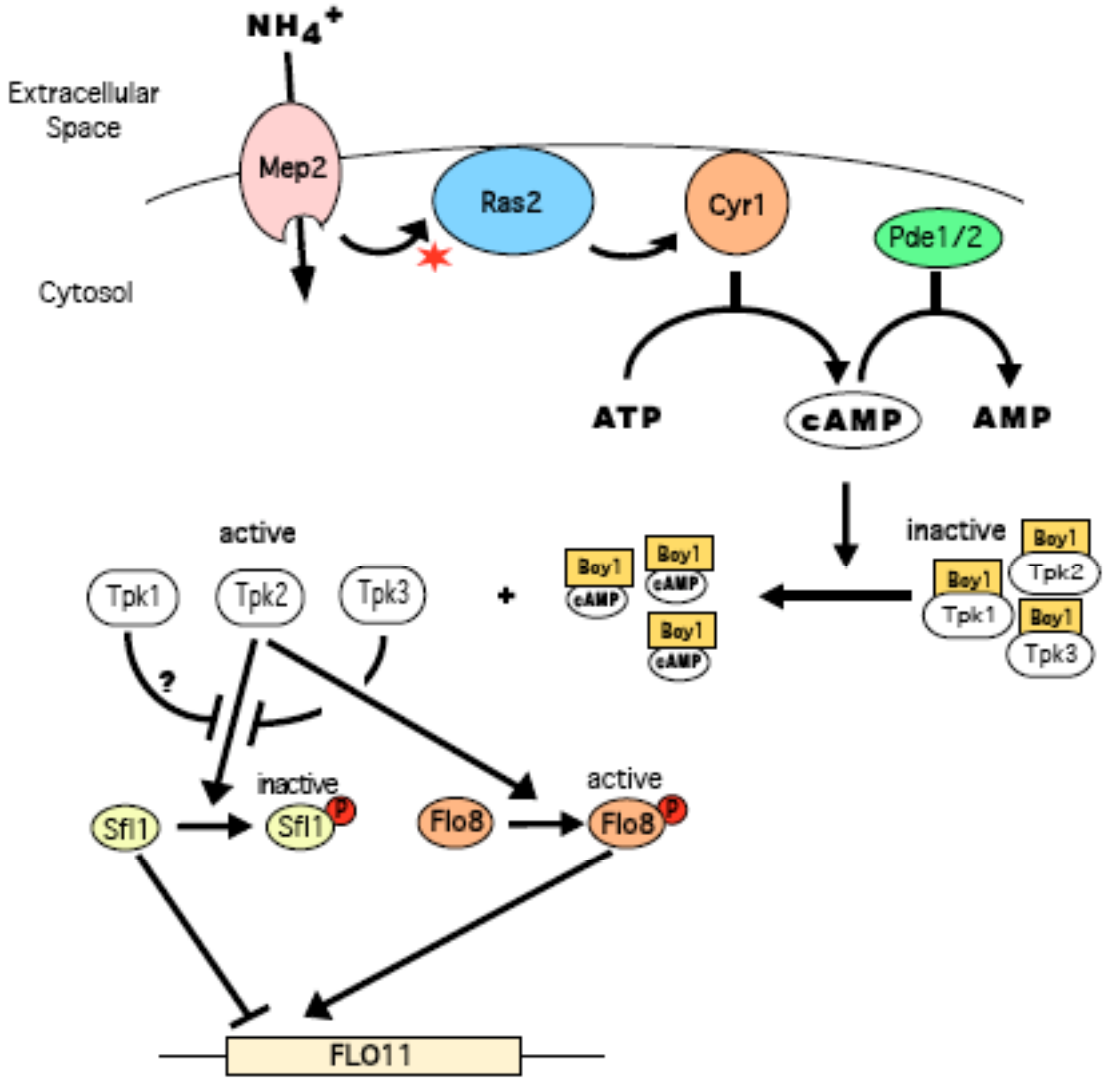

Figure 1.4 cAMP-dependent regulation of filamentous response

The Mep2p ammonia permease senses nitrogen levels. When ammonia is depleted, Mep2p activates Ras2p, which in turn activates the adenylate cyclase Cyr1p, resulting in the accumulation of cAMP. cAMP binds to the PKA inhibitory subunit Bcy1p, resulting in the release of free, active Tpk1p, Tpk2p or Tpk3p. Active Tpk2p phosphorylates the inhibitor Sfl1p, leading to its inactivation; the activator Flo8p is also phosphorylated by Tpk2p, a modification that leads to its activation. Collectively, phosphorylation of these two factors results in the activation of *FLO11*, a hallmark gene of filamenting yeast.

1.5.4.2 Targets of the Tpk isoforms

The *FLO11* gene is required for both haploid invasive and diploid pseudohyphal growth, thus it is an excellent reporter for assaying filamentous responsive transcription. Flo11p is a glycosylphosphatidylinositol-anchored cell-surface protein required for calcium-dependent cell-to-cell adhesion, a process also known as flocculation and highly characteristic of pseudohyphae (Lo and Dranginis 1998). The *FLO11* promoter is one of the largest known yeast promoters, spanning approximately 4kb and containing multiple well characterized positive and negative regulatory elements for many of the factors involved in the filamentous response, such as Ste12p, Tec1p, Flo8p, Phd1p, Sfl1p, and Ash1p (Rupp et al. 1999). The cAMP-dependent pathway has been implicated in regulation of multiple factors involved in the expression of *FLO11*.

Sfl1p is a DNA binding protein involved in the recruitment of the Ssn6-Tup1p repressor to the promoter region of the *FLO11* gene (Figure 1.4) and can, therefore, be regarded as an inhibitor of *FLO11* (Conlan and Tzamarias 2001). In a two-hybrid screen, Tpk2p was found to interact with Sfl1p, thus it is likely that Tpk2p regulate filamentous growth by alleviating repression of the *FLO11* gene via its interaction with Sfl1p (Robertson and Fink 1998). In fact, *FLO11* mRNA levels are increased in a *sfl1* mutant, highlighting the negative effects of this transcriptional regulator. Further studies confirmed this interaction and found that Tpk2p phosphorylates Sfl1p, a modification that prohibits dimerization and DNA binding of this transcriptional repressor (Pan and Heitman 2002).

The activity of Flo8p, a transcriptional activator, is also dependent on the cAMP pathway. Phosphorylation by Tpk2p promotes Flo8p binding and activation of the *FLO11* promoter (Pan and Heitman 2002). In fact, common non-filamenting lab strains from the W303 and S288C backgrounds contain a non-functional allele of *FLO8* which possesses a single point mutation that creates a premature stop codon and significantly reduces the ability of these strains to filament (Liu et al. 1996).

1.5.4.3 Ras2 Dependent Regulation of cAMP

Ras2p is a small GTP-binding protein localized to the plasma membrane. It possesses a C-terminus with membrane anchoring lipids containing palmitoyl and farnesyl groups, which contribute to the cytoplasmic membrane localization of this signaling protein (Bhattacharya et al. 1995). Ras2p has been implicated in numerous signaling pathways, such as sporulation and filamentous growth, via its activating effects on the Cyr1p adenylate cyclase.

The activity of Ras2p is itself dependent on other factors, such as Cdc25p, Ira1p and Ira2p. Cdc25p, a membrane bound guanine nucleotide exchange factor, binds to and activates Ras2p by directly promoting the exchange of GDP for GTP (Broek et al. 1985). Once activated, Ras2p directly stimulates the Cyr1p adenylate cyclase and, thus, cAMP levels are increased (Lai et al. 1993). On the other hand, the GTPases Ira1p and Ira2p can inactivate Ras2p by stimulating the hydrolysis of GTP to GDP (Parrini et al. 1996).

In fact, Ras2p has early been associated with invasive and pseudohyphal growth, as the original results presented by Gimeno et al. (1992) showed that expression of a constitutively active form of Ras2p, the $Ras2^{val19}$ allele, stimulated both types of growth even in nitrogen-rich conditions. Further studies have linked Ras2p activity to Cyr1p and, consequently, cAMP levels: cells harboring the hyperactive $RAS2^{val19}$ allele when over-expressing *PDE2*, a cAMP-degrading phosphodiesterase, were suppressed in terms of pseudohyphal growth, as were cells expressing wild-type *RAS2* (Ward and Garrett 1994; Ward et al. 1995).

1.5.4.4 Nitrogen Sensing

Nutrient sensing in yeast is often associated with nutrient specific transporters or permeases (Kruckeberg et al. 1998). With respect to nitrogen sensing, the ammonium permease Mep2p is responsible for sensing and providing the signal required for pseudohyphal differentiation (Lorenz and Heitman 1998). Three ammonium permeases have been identified to date, Mep1p, Mep2p, and Mep3p, however Mep2p is the only permease involved in sensing ammonium. A *mep2* strain is incapable of filamentous growth in ammonium limiting media, yet cells' ability to filament in response to limitations in other nitrogen sources such as glutamine or aspargaine remains intact. In fact, deletion of *MEP2* does not result in any growth defects, suggesting that Mep2p has redundant functions with Mep1p and Mep3p in terms of ammonium transport, but plays a prominent role in the sensing and signaling of ammonium limitation (Lorenz and Heitman 1998).

The Mep2p permease has been found to interact, via genetic studies, with Ras2p, a small membrane bound GTP-binding protein (Lorenz and Heitman 1998) and this interaction is likely the link between nitrogen sensing and the filamentous signaling cascades. However, the possibility of other signaling intermediates between Mep2p and Ras2p cannot be excluded at this point. Cdc25p, an additional membrane bound guanine nucleotide exchange factor, might also be involved in transmitting the signal from Mep2p to Ras2p.

1.5.5 Snf1 Protein Kinase Pathway

Snf1p, an AMP-activated protein kinase, has been extensively implicated as a key player in the response of yeast to a wide variety of cellular stresses; it has been characterized as a general stress sensor and alters cellular dynamics to cope with the different stress via numerous stress-related processes such as phosphorylation of histone H3; direct regulation of RNA polymerase II holoenzyme; regulation of translation, glycogen and lipid biosynthesis; and regulation of general, salt and heat stress responses (reviewed in (Sanz 2003)).

However, Snf1p is most well characterized due to its role in the signaling of glucose availability. Snf1p kinase is required for regulating the transcriptional changes associated with glucose phosphorylation-dependent derepression via the activation of two transcriptional activators, Cat8p and Sip4p, and inhibition of the transcriptional repressor Mig1p (Vallier and Carlson 1994; Hedges et al. 1995; Conlan and Tzamarias 2001). Further studies linked a single phosphorylation to the increased activity of the kinase in response to glucose deprivation. This phosphorylation has mapped to the conserved activation loop Thr210 residue and it was found to be performed by the redundant upstream kinases Sak1p, Tos3p and Elm1p (Hong et al. 2003; Sutherland et al. 2003)

While historically Snf1p has been implicated in the glucose deprivation response, recent studies have shown that this AMP-activated kinase is also involved in coping with nitrogen limitation and subsequent pseudohyphal differentiation. It has been demonstrated by Kuchin et al. that Snf1p is required for filamentous growth, as is evidenced by the fact that a *snf1* deletion mutant is incapable of forming pseudohyphae (Kuchin et al. 2002). It was later shown that phosphorylation on the Snf1p-activation-loop residue, Thr210, is required for pseudohyphae formation and that nitrogen limitation actually induces phosphorylation of this residue (Orlova et al. 2006). Activation of Snf1p by nitrogen starvation leads to increased activity of this kinase, a requirement for differentiation, as *snf1* deletion mutants expressing the catalytically inactive Snf1-K84R mutant are unable to filament, suggesting that Snf1p is involved in transmission of the signal for nitrogen starvation (Orlova et al. 2006).

1.5.6 Cyclin-Dependent Kinase 8 (Cdk8) Pathway

An additional player involved in the regulation of the filamentous response is Cyclin-Dependent Kinase 8 (Cdk8p) and, by association, Cyclin C. Under normal growth conditions, Cdk8p inhibits filamentous growth by phosphorylating and, consequently, destabilizing Ste12p (Nelson et al. 2003). However, when cells are placed in nitrogen-deprived conditions, Cdk8 is destabilized via what was until now an unknown mechanism and there is loss of Ste12p phosphorylation, resulting in its accumulation.

Ste12p levels are of utmost importance for regulation of the filamentous response. Deletion of *STE12* results in a non-filamenting and non-invasive phenotype, whereas over-expression of the transcription factor yields a significant increase in the rate of filamentation (Liu et al. 1993). Deletion of *CDK8*, on the other hand, results in a hyperfilamentous phenotype, due to the stabilization of Ste12p (Nelson et al. 2003).

However, *cdk8* null diploids are not constitutively filamentous in rich media, suggesting that other players are involved in the regulation of the filamentous response.The role of Cdk8p and Cyclin C in the control of filamentous growth will be discussed in more detail in subsequent sections.

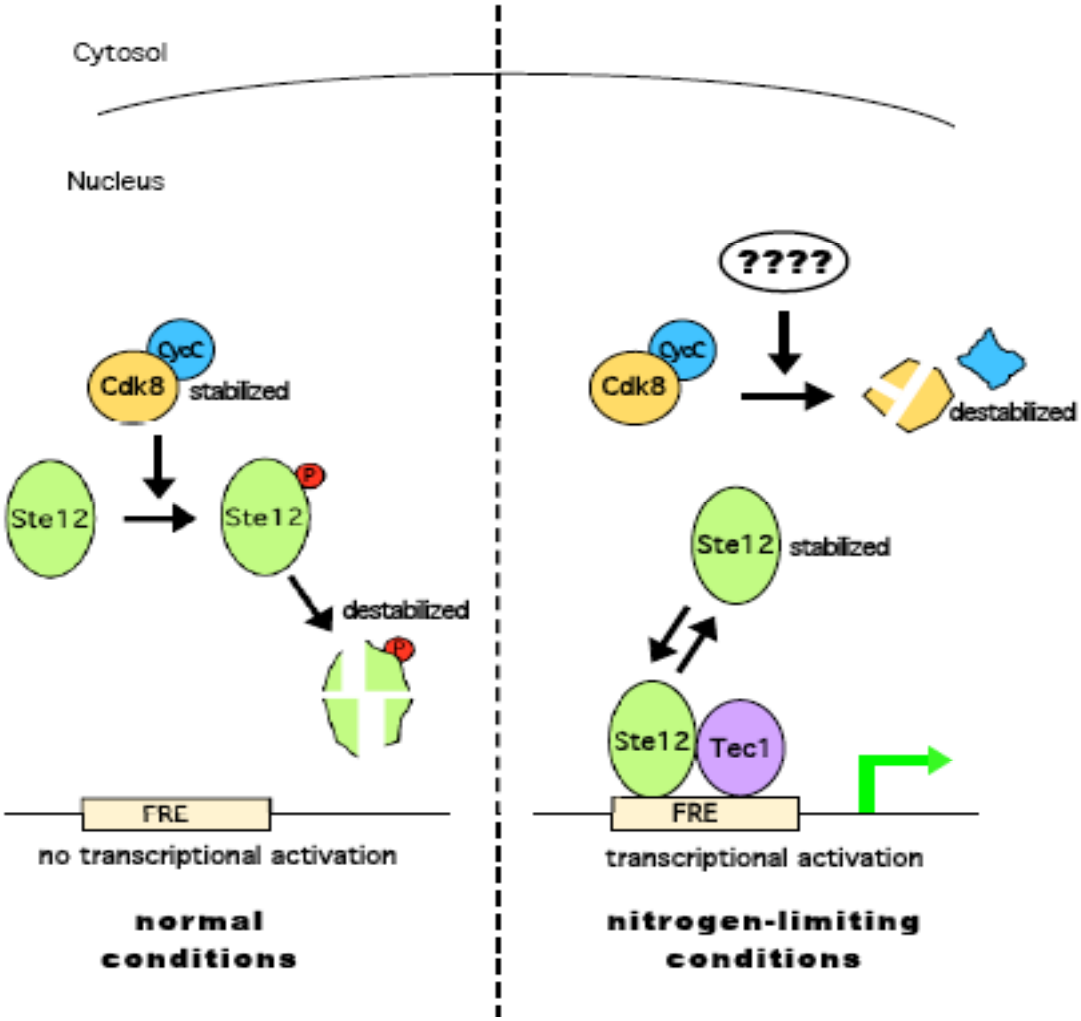

Figure 1.5 Cdk8p represses filamentous growth via an inhibitory phosphorylation on Ste12p

Cdk8p inhibits filamentous growth in cells growing under nitrogen abundant conditions via constitutive phosphorylation of Ste12p and subsequent destabilization. Under conditions of limiting nitrogen, Cdk8p itself becomes destabilized via an unknown mechanism, resulting in loss of Ste12p phosphorylation, ultimately resulting the in the stabilization of the filamentous response transcriptional activator Ste12p.

1.6 CELL-CYCLE DEPENDENCE OF FILAMENTOUS GROWTH

In yeast, there are two major regulatory Cyclin-Dependent Kinases (CDK) that are involved in determining the passage of cells from most stages of the cell-cycle: Cdc28p and Pho85p (Andrews and Measday 1998). There is a growing body of evidence to suggest that CDKs are required for the morphological changes and polarized growth associated with pseudohyphal differentiation (Madden and Snyder 1998). For example, in G1 phase, Cdc28p binding to either Cln1p or Cln2p cyclin is required for polarized growth, and over-expression either cyclin results in hyperpolarization and an elongated morphology. Concomitantly, a similar response is mimicked by delaying entry into G2 by eliminating Clb1p and Clb2p cyclins (Madden and Snyder 1998).

However, the major evidence that the cell cycle is involved in pseudohyphal differentiation can be attributed to the fact that filamenting cells exhibit a modified cell cycle: the daughter cells of filamenting yeast begin to bud immediately following cytokinesis and, thus, bypass the size checkpoint (Kron et al. 1994). Later studies served to further emphasize the role of CDKs in regulating filamentous growth. A strain harbouring the *CDC28*-C127Y mutant, displays a constitutively filamenting morphology, including all previously identified characteristics, such as elongated shape, modified bud-site selection and altered cell cycle (Edgington et al. 1999). Through a series of genetic studies, the authors were also able to implicate Elm1p, Hsl1p and Swe1p as upstream regulators of Cdc28p. According to the model proposed by Edgington et al. (1999), Elm1p, a serine-threonine kinase, activates Hsl1p, another protein kinase. Deletion of either *ELM1 or HSL1* result in constitutive filamentation, whereas dominant mutations of the *HSL1* gene suppress filamentation, even in an *eml1* deletion mutant strain, thus suggesting that Hsl1p is downstream of Elm1p. Hsl1p, once activated, has the ability to phosphorylate another protein kinase, Swe1p, although this modification is thought to inhibit this third kinase. According to this model, Swe1 constitutively phosphorylates Cdc28p, a modification that inhibits induction of the filamentous response. Deletion of either

ELM1 or *HSL1* results in a hyperactive form of Swe1p and the corresponding filamentous phenotype. However, mutations or deletions in *ELM1, HSL1* or *SWE1* all fail to affect the phenotype of the *cdc18*-127 allele, this placing these regulatory kinases upstream of Cdc28p (Edgington et al. 1999).

1.6.1 General Characteristics of Cyclins and Cyclin-Dependent Kinases

Cyclin-Dependent Kinases (CDKs) are a category of serine/threonine protein kinases whose activities are dependent on their association with other regulatory proteins termed cyclins (Pines 1995), which have gained popularity and their name due to their association with the cell cycle. Although the CDKs were originally identified due to their intricate association with the cell cycle, these kinases are constantly being implicated in a wide range of cellular processes, such as in the regulation of filamentous growth.

In addition to conserved catalytic domains, members of the CDK family also share an evolutionarily conserved 16 amino acid sequence near N-terminus, known as the PSTAIRE motif (Li et al. 2004). Another characteristic common to the CDKs is the fact that these kinases phosphorylate S/T-P motifs (Langan et al. 1989); therefore, it is reasonable to expect that additional factors contribute to the specificity of this group of kinases and the interactions with cyclins is an excellent candidate to explain this differential kinase specificity.

Cyclins are proteins whose levels vary throughout the cell cycle, as the name indicates. While only a certain subset actually displays cell cycle-dependent fluctuations, this family of proteins is very diverse, and members of this class range in size from 35 to 90 kDa. However, cyclins do have a conserved 100 residue ‘cyclin box’ domain, which is involved in CDK binding and activation (Lees and Harlow 1993). Cyclin binding results in activation of the kinase domain of the CDK through the induction of conformational changes associated with the protein-protein interaction. The physical contact typically occurs between the aforementioned PSTAIRE motif and the cyclin box domain, which induces a

conformational change that exposes the substrate-binding domain and specific residues that require phosphorylation by CDK-activating kinases, such as Thr169 in Cdc28p (Jeffrey et al. 1995; Russo et al. 1996). The activity of the all CDKs is dependent on their association with a cyclin, as per definition; however the requirement for an activating phosphorylation is not a universal characteristic of all CDKs.

Similarly, the CDKs can be inhibited, via interaction with CDK inhibitors (CKIs). Well-characterized examples of these negative regulators include Far1p, which binds to Cdc28p-G1 cyclin complex to negatively regulate their activity (Peter and Herskowitz 1994) and p40, which inhibits mitotic Cdc28p complexes (Mendenhall and Hodge 1998). Phosphorylations that inhibit the activity of the CDKs have also been documented. For example, phosphorylations on Thr18 and Tyr19 within the ATP binding site of Cdc28p are known to have inhibitory roles (Berry and Gould 1996).

1.6.2 Yeast CDKs

To date, multiple cyclin-dependent kinases have been identified in yeast *S. cerevisiae*. Cdc28p, perhaps the most prominent and important of the yeast CDKs, is involved in regulating cell-cycle progression via its association with 9 different cyclins (Mendenhall and Hodge 1998).

Pho85p is another CDK known to interact with multiple cyclin partners. Pho85p associates with 10 distinct cyclin subunits in response to a wide array of nutritional and environmental conditions and regulates nutritional coping and cell-cycle progression (Huang et al. 2007).

Cdk7p (Kin28p) is known to associate specifically with Ccl1p and is involved with the general transcriptional complex by aiding with the initiation at RNA polymerase II promoters and with recruitment of mRNA processing machinery (Rodriguez et al. 2000).

The CDKS Ctk1p and Bur1p, and their respective cyclins Ctk2p and Bur2p, are also involved with the regulation of transcription. Both Ctk1p and Bur1p are known to phosphorylate the C-terminal repeated domain of the RNA polymerase Rpo21p subunit and this is believed to affect transcription (Lee and Greenleaf 1991; Murray et al. 2001). Cdk8p, which functions in association with Cyclin C, and is discussed in detail in the following section.

1.6.3 Cyclin-Dependent Kinase 8 (CDK8) and Cyclin C

Cdk8p was initially identified as a suppressor of Snf1p (Carlson et al. 1984). Since then, Cdk8p has been isolated in a wide array of screens and its name has morphed over the years to *GIG2, NUT7, SRB10, UME5, RYE5* and *SSN3*. For example, *CDK8* was identified as a suppressor of *SNF1* mutations and, at the time, named *SSN3* (Kuchin et al. 1995).

Cdk8p is a 555 amino-acid threonine/serine protein kinase and shares approximately 40% sequence homology to Cdc28p. This kinase possesses several distinct features common to the general CDK family, such as a non-canonical DFG motif in the VII kinase subdomain and a SACRE sequence in the PSTAIRE domain (Tassan et al. 1995). This information is based on the characterized structure of the highly homologous human Cdk8p counterpart, as, unfortunately, yeast Cdk8p has yet to be crystallized and subject to structural determination.

Cyclin C, on the other hand, is a 323 amino-acid protein required for activation of Cdk8p. Thus, it comes as no surprise that Cyclin C has also been identified, in conjunction with *CDK8*, in the screens mentioned above as *GIG3, NUT9, SRB11, UME3, SSN8* and *RYE2*.

1.6.4 Cdk8-dependent regulation of filamentous growth

Under normal growth conditions, Cdk8p inhibits filamentous growth by phosphorylating and, consequently, destabilizing Ste12p (Nelson et al. 2003). However, when cells are placed in nitrogen-deprived conditions, Cdk8p is destabilized via what was until now an unknown mechanism, and there is loss of Ste12p phosphorylation, resulting in its accumulation.

Ste12p levels are of utmost importance for regulation of the filamentous response. Deletion of *STE12* results in a non-filamenting and non-invasive phenotype, whereas over-expression of the transcription factor yields a significant increase in the rate of filamentation (Liu et al. 1993). Deletion of *CDK8*, on the other hand, results in a hyperfilamentous phenotype, due to the stabilization of Ste12p, when cells are grown in SLAD media (Nelson et al. 2003). In rich media, a *cdk8* null diploid *FLO11* expression is induced 15-fold, even in the absence of nitrogen signaling (Holstege et al. 1998). While *FLO11* expression is induced in the *cdk8* null genotype in rich media, cells are not constitutively filamentous, suggesting that other factors are required for induction of filamentous growth.

1.6.5 Other roles and substrates of Cdk8 in S. cerevisiae

Cdk8p, and by association Cyclin C, are components of the eukaryotic mediator complex. Cdk8p has been implicated as a general relay point of environmental stress, as is evidenced by the fact that introduction of kinase-dead alleles results in the de-repression of numerous genes involved in different stress responses

(Holstege et al. 1998). It has been noted that Cdk8p levels decrease as yeast cells approximate the diauxic shift (Holstege et al. 1998), that Cyclin C is responsive to a magnitude of cellular stresses (Cooper et al. 1997) and that *cdk8* or *cycc* null strains mimic nutrient starvation phenotypes (Holstege et al. 1998). The mediator, and in particular Cdk8p/CycCp, have been shown to be involved in nutrient sensing and stress-coping responses (Chang et al. 2001; Nelson et al. 2003).

More specifically, Cdk8p is involved in phosphorylation of the RNA polymerase II C-terminal domain. Cdk8p has the ability to phosphorylate the CTD prior to formation of the initiation complex on promoter DNA and this phosphorylation leads to the general inhibition of transcription (Hengartner et al. 1998).

In addition to Ste12p, Cdk8p has been found to regulate 3 additional transcriptional activators via phosphorylation. Cdk8-dependent phosphorylation of Gal4p is required for full induction of the GAL response (Rohde et al. 2000); Msn2p and Gcn4p are also phosphorylated by Cdk8p. Phosphorylation of Msn2p promotes nuclear export of the this transcriptional activator, whereas phosphorylation of Gcn4p results in its destabilization (Chi et al. 2001).

Cdk8p has also been found to interact with the transcription factors Sip4p and Sfl1p and regulate their stability; however, current studies have failed to demonstrate a direct phosphorylation by Cdk8 (Song and Carlson 1998; Vincent et al. 2001).

1.7 TRANSCRIPTIONAL REGULATORS OF FILAMENTOUS GROWTH

In the above sections, much emphasis was given to the different signaling pathways that communicate nitrogen limitation to the cells and, therefore, initiate a cascade of events that eventually leads to the up-regulation of the filamentous response. However, the molecular response required for pseudohyphae formation is dependent on the activation or repression of a wide range of specific target genes. This specificity in transcriptional regulation is accomplished via the specific interactions between the filamentous response transcription factors and the filamentous response target gene promoters.

1.7.1 Ste12p and Tec1p

Although Ste12p is heavily implicated with the pheromone response, its role in regulating the transcription of filamentous-specific genes is also preponderant. Ste12p was initially identified as being required for the expression of genes involved in cell mating (Fields and Herskowitz 1985). Further studies came to show that Ste12p was capable of binding specific DNA sequences within pheromone response elements (Dolan et al. 1989), and that pheromone-dependent phosphorylation by the MAP kinase Fus3p was required for positive regulation of this transcription factor (Song et al. 1991). Similarly, Ste12p was also the first transcription factor found to be required for filamentous growth in yeast (Liu et al. 1993). Deletion of *STE12* resulted in a non-filamenting and non-invasive phenotype, whereas over-expression of the transcription factor yielded a significant increase in the rate of filamentation. Later studies by Madhani and Fink (1997) demonstrated that Ste12p has the ability to bind to conserved binding sequences on FREs and that in response to nitrogen deprivation, there is a FRE-dependent induction of the filamentation genes. An FRE consists of a conserved Ste12p binding site (sequence: TGAAACA) closely associated with a Tec1p binding site (sequence: CATTCT) and this element is capable of recruiting, *in vivo* and *in vitro*, Ste12p and Tec1p to their sites on the FRE.

Tec1p functions in a similar manner to Ste12p with respects to activation of the filamentous response. This transcriptional activator was originally identified due to its involvement in the activation of Ty-1 transposons insertions (Laloux et al. 1990). Tec1p possesses a conserved DNA-binding domain, the TEA/ATTS motif, which is known to recognize and bind to CATTCC and CATTCT sequences; expression in yeast of the protein AbaAp, a transcriptional activator from Aspergillus nidulans that contains a TEA/ATTS motif, resulted in induction of pseudohyphal growth, even in the absence of Ste12p (Gavrias et al. 1996). The same group also demonstrated that deletion of *TEC1* results in a significantly reduced ability for yeast cells to filament and penetrate into agar.

Ste12p and Tec1p have been found to co-operatively bind to FREs (Madhani and Fink 1997) and the activation of FRE-mediated genes is dependent on this synergistic relationship. Therefore, it might be the combination of Tec1p on the promoters of filamentation genes with Ste12p that confers specificity in the activation of expression. Ste12p is constitutively bound to 38 promoters, however, upon treatment with the fusel alcohol butanol, which mimics nitrogen-dependent filamentous growth, both Ste12p and Tec1p were found to associate with 57 promoters of genes implicated in filamentous growth (Zeitlinger et al. 2003). This increase in promoter occupancy and recruitment of Tec1p is likely due to the inhibiting effect of Kss1p on inhibitory Dig1p and Dig2p. Without a stimulatory signal, Ste12p complexes to unphosphorylated Kss1p, Dig1p and Dig2p, which serves to inhibit transcription activation and the interaction of Ste12p with Tec1p, as Dig2p and Tec1p both compete for the N-terminal binding sites on Ste12p (Chou et al. 2006). Upon stimulation, Kss1p exerts its effects on Ste12p, causing Dig2p to dissociate and, thus allowing Tec1p to interact with Ste12p and, thus, activate transcription. This response is specific to the filamentous response, as evidenced by the fact that treatment with pheromone results in the accumulation of Ste12p on the promoters of genes involved in mating (Ren et al. 2000).

1.7.2 Msn1

Msn1p and Mss11p are transcriptional activators that when over-expressed strongly activate the expression of *FLO11* and, subsequently, the filamentous response (Lambrechts et al. 1996; Webber et al. 1997). Although these activators have yet to be implicated with the expression of other genes involved in the filamentous response, their role on activation of *FLO11* expression is sufficient to cause the morphological changes associated with pseudohyphae formation. Based on genetic evidence, it seems unlikely that Msn1p and Mss1p are involved regulating the expression of other filamentation genes as is evidenced by the fact their absence can be compensated for by the expression of *FLO11* from an alternative promoter (Gagiano et al. 1999). In addition, Msn1p requires Msn11p in order to induce filamentation; however, over-expression of *MSS11* is able to trigger filamentation. Therefore, is likely that Mss11p is situated downstream of Msn1p. Genetic evidence suggests that Msn1p is downstream of Ras2p and independent of the MAPK pathway (Gagiano et al. 1999). While an exact model for these two transcriptional activators is still to be elucidated, it may be that Mss11p is capable of binding DNA and directly causing activation of *FLO11* expression, while Msn1p may be regulating the activity or stability of Mss11p.

MSS11 was initially characterized as a suppressor of the *STA10* phenotype (Webber et al. 1997). This phenotype is characterized by the repression of the *STA1-3* genes, which encode the extracellular glucoamylase isozymes. In fact, most yeast laboratory strains are repressed in terms of *STA10* expression by an undefined repressor, designated STA10. In fact, the *STA* genes are possible candidates to contribute for the invasive nature of the filamentous response, as the excreted glucoamylases encoded by the *STA1-3* genes might aid in the digestion of the agar and, therefore, facilitate penetration into the agar. Mss11p also possesses a high degree of homology with other well characterized DNA-binding transcriptional activators, such as Flo8p (Kobayashi et al. 1996).

MSN1, on the other hand, was initially identified as a multi-copy suppressor of *snf1* mutants and over-expression of this factor was found to enhance the transcription of various genes involved in nutrient utilization (Estruch and Carlson 1990). Additional experiments demonstrated that Msn1p was capable of activating specific reporter expression when fused to a LexA DNA-binding domain. The latter evidence, and the fact that to date, Msn1p has still not been found to directly bind to DNA suggests that Msn1p is involved in regulating the activity of DNA-binding factors and is not itself a DNA-binding protein. Therefore, based on the genetic information and the functions of Msn1p and Mss11p in response to other cues, the model proposed above is within the logistics of all the evidence gathered to date.

1.7.3 Sf11p

SFL1 was originally identified as a suppressor of flocculation genes (Fujita et al. 1989), and deletion of the *SFL1* gene results in constitutive flocculation in yeast and enhanced pseudohyphae formation (Robertson and Fink 1998). The N-terminal region of Sfl1p is homologous to the DNA-binding domain of other well-characterized transcription factors, such as the heat-shock transcription factor Hsf1p (Fujita et al. 1989).

Under conditions of abundant nitrogen sources, Sfl1p dimerizes and binds to the promoter region of the flocculation genes (Conlan and Tzamarias 2001). Sfl1p inhibits transcription of this class of genes by recruiting the transcriptional co-repressor Cyc8p-Tup1p via a specific domain in the Sfl1p protein. However, under conditions of limiting nitrogen and subsequent activation of the cAMP signaling cascade, activated Tpk2p phosphorylates Sfl1p, a modification that prevents dimerization and the DNA-binding ability of Sfl1p (Pan and Heitman 2002).

It is curious to note that Sfl1p also interacts with the mediator complex, which often exhibits repressive properties (Song and Carlson 1998). While further studies must be performed, this evidence places a filamentous transcription factor in close proximity of Cdk8p and thus further implicates *CDK8* as a general sensor for nitrogen limitation.

1.7.4 Sok2p

SOK2 encodes a transcriptional repressor of filamentation, as evidenced by the fact that diploid *sok2* strains show enhanced pseudohyphae formation (Ward et al. 1995). In addition, while this deletion has an effect on filamentous growth, it also enhances the growth defect of strains with compromised PKA systems. Specifically, deletion of *SOK2* affects two PKA-dependent processes: glycogen accumulation (via regulation of *GAC1*) and increased expression of the heat-shock resistance gene *SSA3*. While the exact mechanism of action of Sok2p has yet to be determined, given the similarities with the DNA-binding domain of Phd1p (Stoldt et al. 1997), it is likely that it directly binds to the promoters of the filamentous genes to cause repression of expression.

1.7.5 Flo8p

FLO8 encodes a transcriptional activator of filamentation. Over-expression of *FLO8* induces flocculation (Kobayashi et al. 1996), while deletion results in the loss of cells to form pseudohyphae (Liu et al. 1996). The activity of Flo8p is dependent on the cAMP pathway, as phosphorylation by Tpk2p upon limiting nitrogen conditions promotes Flo8p binding and activation of the *FLO11* promoter (Pan and Heitman 2002). *FLO8* is of utmost importance for the development of filaments, as can been seen by the fact that most laboratory strains, including the well-characterized strain S288C, are unable to filament in response to different environmental cues due to a premature stop codon in the sequence of FLO8 (Liu et al. 1996).

1.7.6 Ash1p

ASH1 encodes a positive regulator of filamentous growth. Originally identified for its role in the repression of the HO endonuclease involved in the mating-type switching response (Nasmyth 1993), *ASH1* is now being implicated in the regulation of filamentous growth via regulation of *FLO11* expression. Ash1p is required for formation of filaments, as is evidenced by the fact that *ash1* null cells are incapable of forming pseudohyphae and cells over-expressing this transcriptional regulator are hyper-filamentous (Chandarlapaty and Errede 1998). Unfortunately, little is known regarding the regulation and mechanism of action of Ash1p with respect to filamentous growth. However, Ash1p is known to contain a zinc-finger domain (Long et al. 1997), which is a well-characterized DNA-binding motif; thus, it is plausible that Ash1p functions to regulate expression of *FLO11* via its ability to directly bind and activate the *FLO11* promoter. Ash1p is also a known subunit of the Rdp3p histone deacetylase complex (Carrozza et al. 2005), thus its role in chromatin modification might also be a factor involved in the expression of *FLO11*.

1.7.7 Phd1p

PHD1 encodes a possible transcriptional activator of the filamentous response. Originally identified as part of the PHD (pseudohyphal determinant) screen, which aimed to identify regulators of filamentation via gene over-expression studies (Gimeno and Fink 1994). In their initial screen, Gimeno and Fink (1994) identified 7 *S. cerevisiae* genes that when over-expressed cause enhanced filamentation. *ASH1* over-expression, therefore, causes increased filamentation. However, deletion of *PHD1* does not affect the ability of cells to form pseudohyphae.

Based on sequence similarities with StuAp, a well-characterized transcriptional activator from Aspergillus nidulans involved morphogenesis, Phd1p contains two DNA-binding domains, the SWI4p and MBP1p-like motifs (Gimeno and Fink

1994). Thus, it is likely that Phd1p is capable of directly binding DNA. Further studies demonstrated that Phd1p localizes to the nucleus. Thus, it is likely that Phd1p functions to activate transcription by directly binding and activating the promoters of specific effector genes of filamentation.

1.8 EFFECTOR PROTEINS OF FILAMENTOUS GROWTH

1.8.1 FLO11 and the STA1-3 genes

Flo11p is the most extensively characterized effector protein of the filamentous response due to its requirement for pseudohyphae formation and its unusually large promoter that is subject to numerous levels of transcriptional regulation. *FLO11* was initially identified by Lambrechts et al. (1996) as required for the dimorphic switch, as deletion of *FLO11* yielded a non-invasive and non-filamenting phenotype. The *FLO11* gene encodes a membrane-bound mucin protein, which is GPI-anchored to the cell wall and possesses a extra-cellular N-terminal domain (Lambrechts et al. 1996; Lo and Dranginis 1998). Flo11p is likely involved in the regulation of filamentous growth due to its role in the regulation of cell-to-cell attachment, as it allows cells to anchor to each other and provides resistance as cells invade the agar.

The *FLO11* promoter shares a great degree of homology with the promoter for the *STA1-3* genes, which encode starch-degrading glucoamylases. This homology extends over the whole 3kb and expression of the *STA* genes has been found to be dependent on many of the transcriptional activators associated with the filamentous response (Gagiano et al. 1999). Expression of starch-degrading enzymes in response to nutrient or nitrogen deprivation may confer yeast the ability to invade nutrient-rich areas, as production and excretion of these enzymes would facilitate digestion of complex polysaccharide boarders and, thus, penetration of yeast into these compartments.

1.8.2 Other effector proteins involved in pseudohyphae formation

Pseudohyphae formation is a complex, multi-layered event. Since this event is characterized by cellular elongation, unipolar budding patterns, absence of bud separation and altered cell-cycle, multiple players must be involved in the coordination of such morphological changes. It has been suggested that a total of 27 genes are required for the specific morphological changes associated with filamentous growth (reviewed in Gancedo 2001).

For instance, mutations of *BUD1, BUD2* or *BUD5* that cause random cellular distribution of budding site selection prevents the formation of pseudohyphae (Gimeno et al. 1992; Lo et al. 1997). Similar findings have been done that implicate other budding-site selection factors necessary for filament formation. Similarly, multiple genes related to the formation of elongated cells have been found necessary for filament formation. While not completely elucidated, it is likely that elongation is due to a delayed cell cycle and a defective switch from apical to isotropic growth (Gancedo 2001). Thus, the implications of various cell cycle-associated factors in the regulation of pseudohyphae formation. For example, the G1 cyclins Cln1p and Cln2p are required for pseudohyphae formation (Loeb et al. 1999), whereas over-expression of Clb1p or Clb2p, cyclins that activate Cdc28p to promote the transition from G2 to M phase, prevents pseudohyphae formation (Ahn et al. 1999).

Actin, a key cytoskeleton protein, has also been implicated with filament formation. For example, certain alleles of actin have a marked defect in the invasive phenotype of stimulated cells (Cali et al. 1998). The authors suggest that actin is likely participating in the active secretion of hydrolyases to the growth tip, which permit invasive growth, in addition to any role actin may play in altering the cytoskeletal dynamics of filamenting cells.

These factors described in this section constitute only the tip of the iceberg of all players involved in causing the phenotypic changes associated with pseudohyphae formation. However, an in-depth description will not be provided, as this thesis aims to investigate the regulation of filamentous growth at the transcriptional level.

CHAPTER 2 – MATERIALS AND METHODS

2.1 **Media, yeast and *E.coli* manipulation**

2.1.1 Growth media

Standard growth media was used in all experiments involving *S. cerevisiae* and *E. coli.* Depending on the experiment, yeast were grown in either YPD (10 g yeast extract, 20 g peptone, 20 g glucose per liter of solution), synthetic dropout lacking uracil (7 g Difco Yeast Nitrogen Base without amino acids, 20 g glucose, 1 g complete amino-acid mix lacking uracil per liter of solution) or Synthetic low-ammonium dextrose (SLAD) (7 g Difco Yeast Nitrogen Base without amino acids and ammonium sulfate, 10 g glucose, 6 mg of Ultrapure Ammonium Sulfate per liter of solution). For solid media, 20 g of agar was added to each liter of media. For production of SLAD plates, prior to addition to liquid media, the agar was rinsed in dH_20 three times to remove any residual nitrogen. Plates used for yeast mating experiments consisted of YPD plates (pH ~7 by addition of 50 ml of 1M NaPO4, pH 7) with 100 μg/ml of nourseothricin (NAT) and/or 150 μg/ml of Zeocin (ZEO) antibiotics. All *E. coli* work was performed in Luria Broth (LB) media (5 g yeast extract, 10 g tryptone, 10 g NaCl per liter of solution).

2.1.2 Yeast transformation

All yeast transformations were performed using the method conceived by (Gietz and Schiestl 2007). A loop full or an equivalent amount of cells grown over-night in liquid media of the desired yeast were re-suspended in 240 μl of PEG 3500 50% w/v, 36 μl of 1 M Lithium Acetate, 50 μl of boiled salmon-sperm carrier DNA (10 mg/ml) and approximately 2 μg of the intended plasmid. The mixture was vortexed, heat-shocked for 45 minutes at 42°C and plated on the appropriate selective media. Strains with the integrated Cdk8-3xFLAG-1x6HIS wild-type and mutant constructs were generated with digestion of pPL016, pPL017 and pPL018

(*fcy1* disintegrator-based vectors) with restriction enzyme AscI for plasmid linearization prior to yeast transformation, as described (Sadowski et al. 2007).

2.1.3 Yeast mating

For strain construction, prior to mating, all strains were crossed-mated with known tester strains 227a and 227α to ensure compatible mating types and selected on synthetic dropout plates without amino acids. Desired strains were transformed with either pIS419 (NAT resistance) or pIS438 (ZEO resistance). Transformed α-strains were grown overnight in liquid SD –Ura media, cells collected, rinsed in sterile dH20 and plated on appropriate selection plates (YPD containing 100 μg/ml NAT and 150 μg/ml ZEO) to create a lawn of the desired transformed α-type cells. Concomitantly, transformed a-type cells were spotted, allowed to grow on to SD –Ura plates and, subsequently, replica-plated onto the lawn of α-type cells on the antibiotic selective plates.

2.1.4 Filamentous growth assays

Assays for diploid filamentous growth were performed by streaking the appropriate cells on SLAD plates and cells were allowed to grow for 5 days at 30°C. After the indicated incubation period, colonies were photographed directly on the SLAD plates.

2.2 Yeast strains, plasmids, and oligonucleotides

Table 2. Yeast Strains

Yeast Strain	Genotype*	Reference/Source
HLY362	MATa, *ste12::LEU2, leu2::hisG, ura3-52*	G. Fink
L5366	MATa/α, *ura3-52*	G. Fink
L5620	MATa/α, *ste2::Leu2, leu2::hisG, ura3-52*	G. Fink
L5625	MATa/α, *ste11::LEU2, leu2::hisG, ura3-52*	G. Fink
L5628	MATa/α, *fus3::LEU2, leu2::hisG, ura3-52*	G. Fink
L5631	MATa/α, *fus3::LEU2, kss1::URA3, leu2::hisG, ura3-52*	G. Fink
XPY4a	*MATa, ura3-52, tpk1::Kan*	(Pan and Heitman 1999)
XPY4a/α	MATa/α, *tpk1::KAN/tpk1::KAN, ura3-52/ura3-52*	(Pan and Heitman 1999)
XPY5a	MATa, *ura3-52, tpk2::Kan*	(Pan and Heitman 1999)
XPY5a/α	MATa/α, *tpk2::KAN/tpk2::KAN, ura3-52/ura3-52*	(Pan and Heitman 1999)
XPY6a	MATa, *ura3-52, tpk3::Kan*	(Pan and Heitman 1999)
XPY6a/α	MATa/α, *tpk3::KAN/tpk3::KAN, ura3-52/ura3-52*	(Pan and Heitman 1999)
YCN44	MAT a, *ura3-52, cdk8*	(Nelson et al. 2003)
YCN45	MATα, *ura3-52, cdk8*	(Nelson et al. 2003)
YCN60	MATa/α, *ura3-52/ura3-52, cdk8/cdk8*	(Nelson et al. 2003)
YPL01	BY4741, MATa. *mca1, trp1*	This study.
YPL02	BY4741, MATa. *wwm, trp1*	This study.
YPL03	BY4741, MATa. *lap3, trp1*	This study.
YPL04	BY4741, MATa. *nma111, trp1*	This study.
YPL05	BY4741, MATa. *ysp3, trp1*	This study.
YPL016 (WT)	MATa/α, *fcy1::CDK8*	This study.
YPL017 (T37A)	MATa/α, *fcy1::T37A CDK8*	This study.
YPL018 (T37E)	MATa/α, *fcy1::T37E CDK8*	This study.
YPL20 (tpk1; cdk8)	MATa/α, *tpk1::KAN/tpk1::KAN, cdk8/cdk8; ura3-52/ura3-52*	This study.
YPL21 (tpk2; cdk8)	MATa/α, *tpk2::KAN/tpk2::KAN, cdk8/cdk8; ura3-52/ura3-52*	This study.
YPL22 (tpk3; cdk8)	MATa/α, *tpk3::KAN/tpk3::KAN, cdk8/cdk8; ura3-52/ura3-52*	This study.
Z1976	MATa, *his3, leu2, met15, ura3, TPK2::TAP:HIS3*	(Ghaemmaghami et al. 2003

*Unless indicated otherwise, all strains are of the ∑1278 background.

Table 3. Plasmids

Plasmid	Details	Reference
PBHM275	*TEC1 FRE::lacZ* reporter	(Madhani and Fink 1997)
PIS028	2μ *CDK8* C-term 3xHA	I. Sadowski
PJP018	2μ KIN28-6HIS	J. Parent
PMT430	GAL promoter expressing *RAS2*^{val19} mutation	(Deschenes and Broach 1987)
PPL05	2μ h*CDK8* C-term 3xHA	This study.
PPL09	2μ T37A *CDK8* C-term 3xHA	This study.
PPL12	2μ T37E *CDK8* C-term 3xHA	This study.
PPL16	fcy1::*CDK8* C-term 3xFLAG 1x6HIS	This study.
PPL17	fcy1::T37A *CDK8* C-term 3xFLAG 1x6HIS	This study.
PPL18	fcy1::T37E *CDK8* C-term 3xFLAG 1x6HIS	This study.
PSG1	2μ *CDK8* C-term 3xHA	S. Goto
PXP2	2μ TPK1	(Pan and Heitman 1999)
PXP3	2μ TPK2	(Pan and Heitman 1999)
PXP4	2μ TPK3	(Pan and Heitman 1999)

Table 4. Oligonucleotides

Oligo	5’ to 3’ Sequence	Target	Use
OPL09	CGTTCGTTGGAAAGAAAAACGCAATCGGAAG TGTGCATGG	CDK8 mutation T37A	SDM
OPL10	CCATGCACACTTCCGATTGCGTTTTTCTTTCC AACGAACG	CDK8 mutation T37A	SDM
OPL39	CGTTCGTTGGAAAGAAAAACGAAATCGGAAG TGTGCATGG	CDK8 mutation T37E	SDM
OPL40	CCATGCACACTTCCGATTTCGTTTTTCTTTCC AACGAACG	CDK8 mutation T37E	SDM
OPL67	CAAAAATTACGGCTCCTCGCTGCAGACCTGC GAGCAGGGAA	CDK8 mutations T37 to A/E	SDM
OPL68	CCAACGCATTAAATGCATCTATTCTTTTAATT GGATCATAATTTAACAAGTGG	CDK8 mutations T37 to A/E	SDM
OPL69	GGCTCCGGATCCTAATACGACTCACTATAGG GAACAGCCACCATGTATAATGGCAAGGATAG AGCACAAAACTCCTATCAGCC	T7 CDK8	In vitro translation
OPL70	TTTTTTTTTTTTTTTTTTTTTTTTTTACTAACCA TGATGGTGATGGTGGTGTCCCTTATCATCAT CGTCC	T7 CDK8	In vitro translation

2.3 Measurement of transcription

2.3.1 ß-galactosidase assays

Measurement of ß-galactosidase activity in selected yeast strains was performed as described by Olson et al. 2000. Yeast strains used for all ß-galactosidase assays were transformed with pBMH275, plasmid harbouring a TEC1 FRE::lacZ reporter construct (for details see table 1).

2.4 Protein preparation, isolation and manipulation

2.4.1 Site-directed mutagenesis

Site-directed mutagenesis to construct T37A and T37E mutants on pSG1 was performed using the Stratagene QuickChange© protocol with respective mutagenic primers pairs oPL09/oPL10 and oPL39/oPL40 as directed by the manufacturer. Site directed mutagenesis reactions to construct T37A and T37E mutants on pPL16 was performed using the megaprimer method with respective mutagenic primers oPL09/oPL10 and oPL39/oPL40, coupled accordingly to either oPL67 oPL68, as described in detail by (Sarkar and Sommer 1990).

2.4.2 TAP and HIS-tag Purification

Purification of TAP-tagged Tpk1, Tpk2, and Tpk3, was performed as described by (Rigaut et al. 1999). *In vitro* translated 3×Histidine tagged wild-type and T37A Cdk8 proteins were added a 50% slurry of nickel beads (Qiagen), prepared by washing three times in 1× PBS buffer, and equilibrating in low-imidazole storage buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM imidazole, adjusted to pH 8.0). 60µl of the equilibrated beads were added to 30µl of the rabbit reticulocyte mixtures with the appropriate translated protein, and rotated for 3 hours at 4°C. The mix was then spun at 6,000 rpm and the supernatant discarded. The bead pellet was then washed 5× with imidazole wash buffer

(50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole, adjusted to pH 8.0). Wild-type and T37A Cdk8 proteins were then eluted from the beads with 30μl 50mM imidazole elution buffer (50mM NaH_2PO_4, 300mM NaCl, 250mM imidazole, 0.05% Tween-20, adjusted to pH 8.0) by incubating and rotating for 40 minutes at 4°C, and analyzed by SDS-PAGE followed by autoradiography.

2.4.3 Western blotting

For western-blotting of HA or FLAG tagged proteins, approximately 10μg of soluble protein were loaded and resolved on 10% SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes, which were blocked in 5% block solution and incubated with either 1:1000 dilution of monoclonal mouse anti-HA hybridoma supernatant or 1:5000 Sigma monoclonal mouse anti-FLAG antibodies. Following three ten minute washes in TBS-T (0.1 M Tris-HCL pH 8, 0.9% NaCl, 0.1% Tween20), the membranes were incubated with 1:10000 HRP-conjugated goat anti-mouse antibody and protein was detected using standard ECL reagents as per manufacturer's instructions (Pierce).

2.4.4 In Vitro Kinase Assays

In vitro kinase assays with the different forms of purified Cdk8 were performed as described by (Hirst et al. 1999). Approximately 200 ng of purified protein was incubated with 3 μl of purified Tpk2 and 10μCi γ^{32} ATP for 30 minutes at 30°C, prior to analyzing on SDS-PAGE and autoradiography.

2.4.5 Reticulocyte in vitro transcription/translation system

In vitro transcription/translation expression of wild-type, T37A and T37E Cdk8 were carried out using the TNT7 PCR fragment rabbit reticulocyte expression

system from Promega. All PCR products were amplified with primers oPL069 (containing a T7 RNA polymerase promoter) and oPL070 (containing a poly-A tail and T7 terminator sequence) from plasmids pIS427, pPL017 and pPL018. *In vitro* transcription/translation reactions contained 3μl PCR generated template, 40μl of rabbit reticulocyte lysate master mix, 2μl ^{35}S (1mCi stock), 2μl of 1U/μl T7 RNA polymerase, to a final volume of 50μl DNA/RNA with nuclease free dH_2O. The reactions were incubated at 30°C for 90 minutes and analyzed on 12.5% sodium dodecyl-sulphate (SDS) denaturing gels, run at 175V for 1 hour, dried and exposed for up to 2 days on Biomax film (Kodak).

CHAPTER 3 – RESULTS AND DISCUSSION

3.1 *In vivo* degradation of Cdk8p in response to nitrogen depravation

Based on the work performed in the laboratory by a previous student, it was known that in response to nitrogen depravation, there is a transient degradation of Cdk8p, with maximal loss of protein occurring at 4 hours following growth in SLAD media (Figure 3.1), and that degradation of Cdk8p corresponds with the appearance of an additional lower molecular weight protein band in the western blot (S. Goto, unpublished). As proposed at the time, since appearance of this band coincides with disappearance of Cdk8p, this lower molecular weight product likely constitutes a truncated form of Cdk8p. While I was able to reproduce the transient degradation of Cdk8p in response to nitrogen limitation, I was incapable of demonstrating appearance of the truncated fragment. I expect this to be a technical issue related to the antibodies utilized. At the time, Cdk8p was being detected with an α-Cdk8p antibody generously donated to the laboratory, which likely possessed its epitope in the central or N-terminal region of Cdk8p. Unfortunately, the α-Cdk8p antibody previously used was no longer accessible and all subsequent immunoblotting was done with antibodies against C-terminal HA and FLAG tags fused to Cdk8p. A cleavage event must occur in the proximity of the C-terminus, thus cleaving the tag off and rendering it impossible to detect the truncated fragment with α-HA or α-FLAG antibodies.

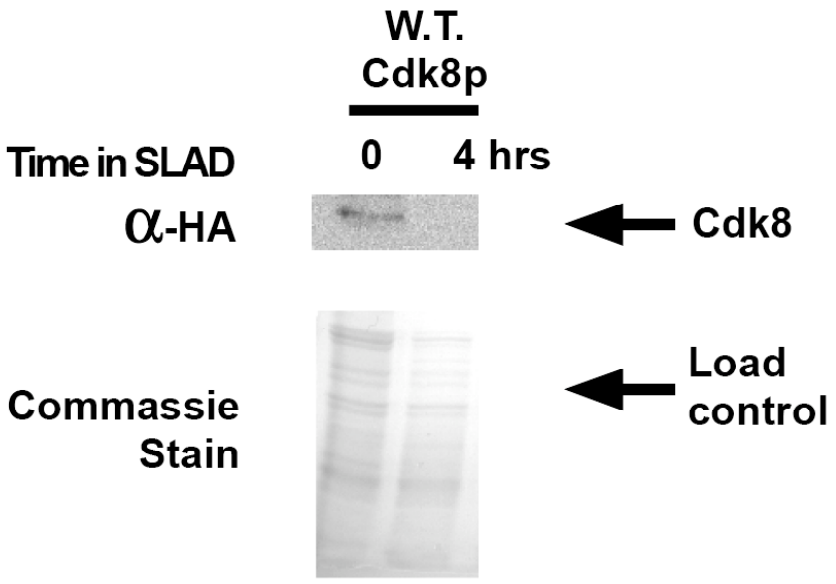

Figure 3.1. **Nitrogen depravation leads to degradation of Cdk8p.** Haploid yeast strain YCN45 (*cdk8*) was transformed with PPL016 (*fcy1::CDK8*) and was grown in SD –URA media, switched to SLAD media (T=0 hrs) and harvested following 4 hours. Cdk8p stability was measured by western blotting whole cell extracts of lysed cells with α-FLAG antibodies.

3.2 *cdk8* null strains hyper-filament and over-expression of *CDK8* inhibits filamentation

The expression of the genes involved in altering the cyto-skeletal dynamics of *S. cerevisiae* in response to nitrogen depletion is under tight transcriptional regulation. The *FLO11* promoter serves as an excellent model to study the ongoing clash between transcriptional activators and transcriptional repressors involved in controlling filamentous growth. Therefore, regulation of the transcriptional activators and repressors is itself of paramount importance. *STE12* is a key player in the activation of filamentous growth, as is evidenced by the fact that deletion of *STE12* yields a non-filamenting phenotype and over-expression results in hyperfilamentation (Liu et al. 1993). Ste12p stability is compromised via phosphorylation by Cdk8p on Ser261 and Ser451 (Nelson et al. 2003).

Ste12p is a transcriptional activator, thus deletion of its direct inhibitor, Cdk8p, results in its stabilization and, consequently permits binding to FRE and activation of the adjacent promoter. As seen in Figure 3.2, YCN60 (*cdk8/cdk8*) transformed with PRS316 (empty vector) yields a hyperfilamenting phenotype, when compared to cells transformed with a plasmid expressing wild-type CDK8, as corroborated by Nelson et al. (2003). This hyperfilamenting phenotype categorizes *CDK8* as a repressor of the filamentous response. However, in *cdk8/cdk8* null strains, filamentation only occurs in SLAD media. Filamentation does not occur in conditions of abundant nitrogen, thus suggesting that additional factors must be involved in inhibiting filamentous growth. The Cdk8p-Ste12p-dependent regulation of filamentous growth is a major component of this tightly regulated response, however, other parallel pathways also exert their effects, namely the Flo8p-Sfl1p-dependent pathway. In cells lacking *cdk8*, under conditions of abundant nitrogen, Tpk2p is inactive and, thus, Sfl1p represses expression of filamentous responsive genes, namely *FLO11*, and Flo8p is unphosphorylated and incapable of binding to DNA. Therefore, despite

stabilization of Ste12p, Sfl1p exerts its inhibitory effects on the *FLO11* promoter and Flo8p is inactive. However, in conditions of limiting nitrogen, Tpk2p becomes activated and phosphorylates Sfl1 and Flo8p. This phosphorylation event stimulates activation of the FLO11 promoter and, coupled with the activation by the largely stabilized pool of Ste12p existent due to lack of phosphorylation-dependent degradation, hyperfilamentation occurs.

On the other hand, over-expression of an inhibitor should, in theory, decrease the activity of any downstream effectors. In yeast L5366 (wild-type) transformed with a plasmid over-expressing *CDK8* (Figure 3.3), there is an evident decrease in the rate of filamentation of cells grown on SLAD media. The hypo-filamenting phenotype observed in cells over-expressing *CDK8* suggests that there is increased kinase activity and subsequent degradation-targeting phosphorylations on Ser261 and Ser 451 of Ste12p (Nelson et al. 2003). The decrease in active Ste12p levels causes a decline FRE occupancy by the transcriptional activator, an event that translates into decreased activation of promoters involved in the filamentous response. Additionally, as seen in Figure 3.2, over-expression of KIN28, which is also a cyclin-dependent kinase, does not affect filamentation, suggesting that Cdk8p has a specific role in the regulation of filamentous growth.

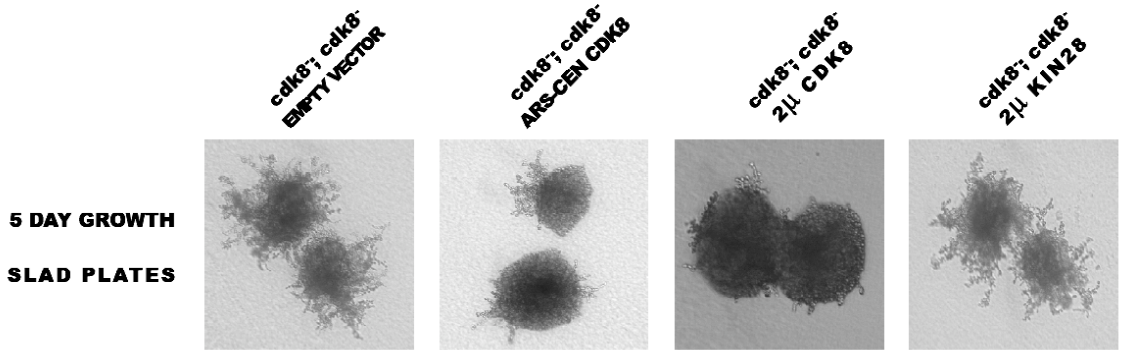

Figure 3.2 CDK8 is an inhibitor of filamentous growth. Yeast strain YCN60 (cdk8/cdk8) was transformed with PRS316 (empty vector control with complementing prototrophic markers); PPL015 (ARS-CEN CDK8); PSG1 (2μ CDK8); and PJP018 (2μ KIN28). Transformed yeast were streaked for single colonies on SLAD plates, incubated days at 30°C and inspected for filamentous growth after 5 days.

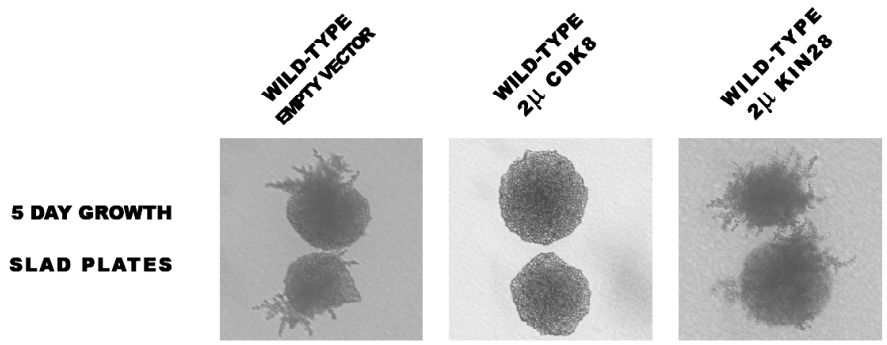

Figure 3.3 CDK8 over-expression inhibits filamentous growth. Yeast strain L5366 (cdk8/cdk8) was transformed with PRS316 (empty vector control with complementing prototrophic markers); PSG1 (2μ CDK8); and PJP018 (2μ KIN28). Transformed yeast were streaked for single colonies on SLAD plates, incubated days at 30°C and inspected for filament growth after 5 days.

3.3 Cdk8 is insensitive to nitrogen depravation in *tpk2* null strains

Given the obvious link between loss of Cdk8p in media depleted for nitrogen, and the hyperfilamenting phenotype of *cdk8* null diploid yeast, it was evident that nitrogen signaling must somehow target Cdk8p for degradation. It was hypothesized that a phosphorylation on Cdk8p would target the enzyme for degradation or truncation. Thus, the rationale was to screen for Cdk8p stability in strains that were lacking kinases that would co-localize with and have access to Cdk8p. The *S. cerevisiae* gene deletion set was utilized to create prototrophic yeast lacking various nuclear kinases. The following strains were screened for lack of Cdk8p destabilization following an incubation a 4 hour incubation in SLAD media: YPL01 (*mca1*); YPL02 (*wwm*); YPL03 (*lap3*); YPL04 (*nma111*); YPL05 (*ysp3*); and XPY5a (*tpk2*). All strains, except XPY5a (*tpk2*) exhibited destabilization of Cdk8 at 4 hours of incubation in SLAD media (data not shown).

In fact, further western-blotting reveled that Cdk8p was, indeed, stabilized in *tpk2* null yeast (Figure 3.4). This result was also in accordance with a previous preliminary screen performed in the lab, which identified *TPK2* as a possible regulator of *CDK8* (Daisy Liu, unpublished). Cdk8p appears insensitive to nitrogen starvation even after a 24-hour incubation in SLAD media (Figure 3.4). Thus, Tpk2p, a nuclear kinase with other known substrates involved in the regulation of the filamentous response, was identified as a possible upstream regulator of Cdk8p stability and the filamentous response. Although pseudohyphae formation and filamentous response, at large, are characteristic of diploid yeast, the same biochemical properties and Cdk8p degradation pattern remain intact in haploid yeast (S. Goto, unpublished); hence, due to technical reasons, haploids were utilized for all western blotting experiments presented here. The question that arouse at this point was whether Tpk2p was exerting its effects on Cdk8p directly or was it merely an upstream kinase involved in a complex cascade of phosphorylation signaling events.

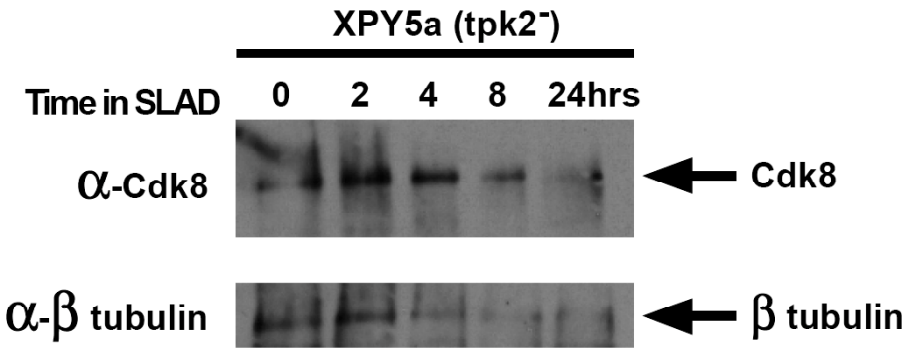

Figure 3.4 Deletion of TPK2 stabilizes CDK8 during nitrogen starvation. Haploid yeast strain XPY5a (*tpk2*) was grown in SD –URA media, switched to SLAD media (T=0 hrs) and harvested at the indicated time points. Cdk8p stability was measured by western blotting whole cell extracts of lysed cells with α-Cdk8 antibodies.

3.4 *tpk2* null strains are nonfilamenting, and overexpression of *TPK2* activates filamentous response in the absence of stimuli

With strains generously donated from the Heitman lab, it became apparent that *TPK2*, in addition to modulating the stability of Cdk8p, it also exerted pronounced effects on the formation of filaments (Figure 3.5). Based on the phenotype of strain XPY5a/α (*tpk2/tpk2*), deletion of *TPK2* results in non-filamenting cells; thus, categorizing Tpk2 as an activator of the filamentous response (Figure 3.5a). XPY4a/α (*tpk1/tpk1*) yeast exhibited wild-type phenotype in relation to filament formation, whereas XPY6a/α (*tpk3/tpk3*) yielded an increase in the rate of filamentation, as seen in Figure 3.5a, rendering it as an inhibitor of filamentous growth, as previously observed (Pan and Heitman 1999).

As expected, a *cdk8/cdk8 tpk2/tpk2* double null was moderately hyperfilamentous (Figure 3.5b). However, the *cdk8/cdk8 tpk2/tpk2* double mutant does not exhibit the same level of hyper-filamentation as does a *cdk8/cdk8* single mutant (Figure 3.5b). One possibility is that this is likely due to the abolishment of the Tpk2-dependent phosphorylations on Sfl1p and Flo8p. Tpk2p likely phosphorylates Sfl1p and this event leads to the inhibition of this transcriptional repressor (Pan and Heitman 2002). Tpk2p also likely phosphorylates and activates Flo8p, a transcriptional activator of *FLO11* (Pan and Heitman 2002). Thus, in a *cdk8/cdk8 tpk2/tpk2* background there are competing forces. The lack of *tpk*2 likely results in the absence of phosphorylation on Sfl1p and Flo8p, while the lack of *cdk8* results in the stabilization of Ste12p. Based on this genetic evidence, it appears that Ste12p exerts a stronger effect on *FLO11* activation than Flo8p and Sfl1p, as Ste12p is itself alone capable of activating filamentous growth, despite Sfl1p repression.

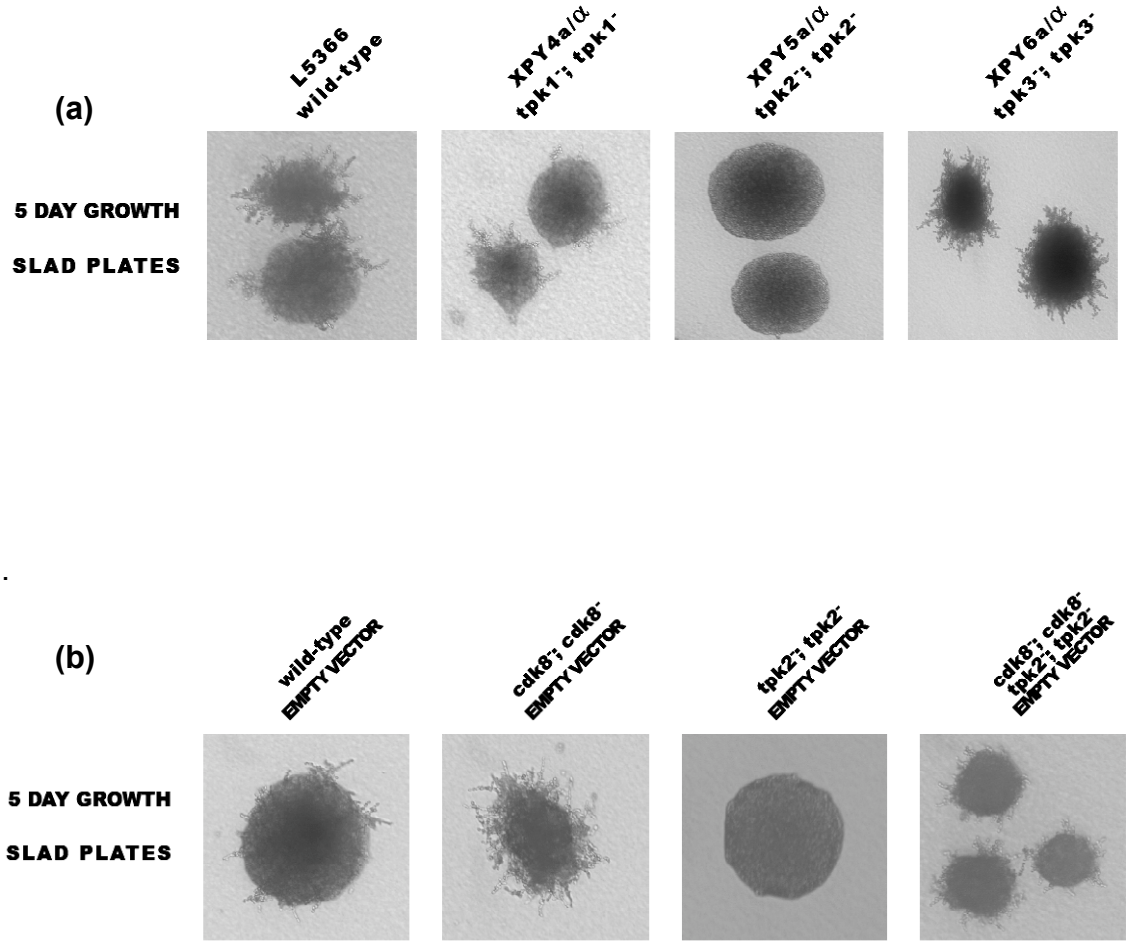

Figure 3.5 *TPK2* is required for filamentous growth and *CDK8* functions downstream of *TPK2*. (a) yeast strains L5366 (wild-type); XPY4a/α (*tpk1/tpk1*); XPY5a/α (*tpk2/tpk2*); and XPY6a/α (*tpk3/tpk3*) were transformed with PRS316 (empty vector control with complementing prototrophic markers). (b) yeast strains L5366 (wild-type); YCN60 (*cdk8/cdk8*); XPY5a/α (*tpk2/tpk2*); and YPL021 (*cdk8/cdk8; tpk2/tpk2*) were also transformed with PRS316. Transformed yeast were streaked for single colonies on SLAD plates, incubated days at 30°C and inspected for filament growth after 5 days

To further investigate the relationship between *TPK2* and *CDK8*, homozygous diploid strains L5366, YCN60, XPY4a/α, XPY5a/α, XPY6a/α, and YPL21 were transformed with PBHM275, a plasmid harbouring *TEC1 FRE::lacZ* reporter. With this construct, activation of filamentous responsive genes could be analyzed in more detail, as β-galactosidase activity ensuing from the TEC1 FRE::lacZ reporter is a direct measure of transcriptional activation from a FRE.

Relative to wild-type, the *tpk2/tpk2* null diploid exhibited a significant decrease in Ste12-dependent expression of filamentous responsive genes in both rich and SLAD media, as is evidenced by the decreased β-galactosidade activity (Figure 3.6a and 3.6b). However, and as expected, in the *cdk8/cdk8 tpk2/tpk2* double null, the FRE-dependent activation was restored to wild-type levels when cells were grown in rich media and slightly increased when cells were grown in SLAD media (Figure 3.6a and 3.6b). This phenomenon likely results from the stabilization and increased FRE binding of Ste12p in the absence of Cdk8p. However, complete restoration of the *cdk8* null phenotype is not restored likely due to the aforementioned lack of Tpk2p-dependent phosphorylation on Sfl1p and Flo8p. Given the pre-supposed upstream regulatory role of Tpk2p on Cdk8p, Flo8p and Sfl1p, it was expected that over-expression of *TPK2* would mimic nitrogen starvation and cause cells to filament even in nitrogen-rich conditions. For this purpose, yeast strains L5366 (wild-type), XPY5a/α (*tpk2/tpk2*) and YCN60 (*cdk8/cdk8*) were transformed with PXP2 (2μ *TPK1*), PXP3 (2μ *TPK2*) and PXP (2μ *TPK3*) in order to induce over-expression of the *TPK* genes. As seen in Figure 3.7, over-expression of *TPK2* in all three strains induced filamentous growth, even on rich media, whereas over-expression of *TPK1* and *TPK3* did not cause filament formation. These transformants all exhibited slower growth rates, likely due to the cellular havoc resulting from altering multiple signaling pathways that involve the TPK genes. Also shown in Figure 3.7, a *tpk3/tpk3* strain exhibits increased FRE-dependent activity, further suggesting that Tpk3p functions as an inhibitor of the filamentous response, while a *tpk1/tpk1* mutant appears to bear no effect on FRE-dependent expression.

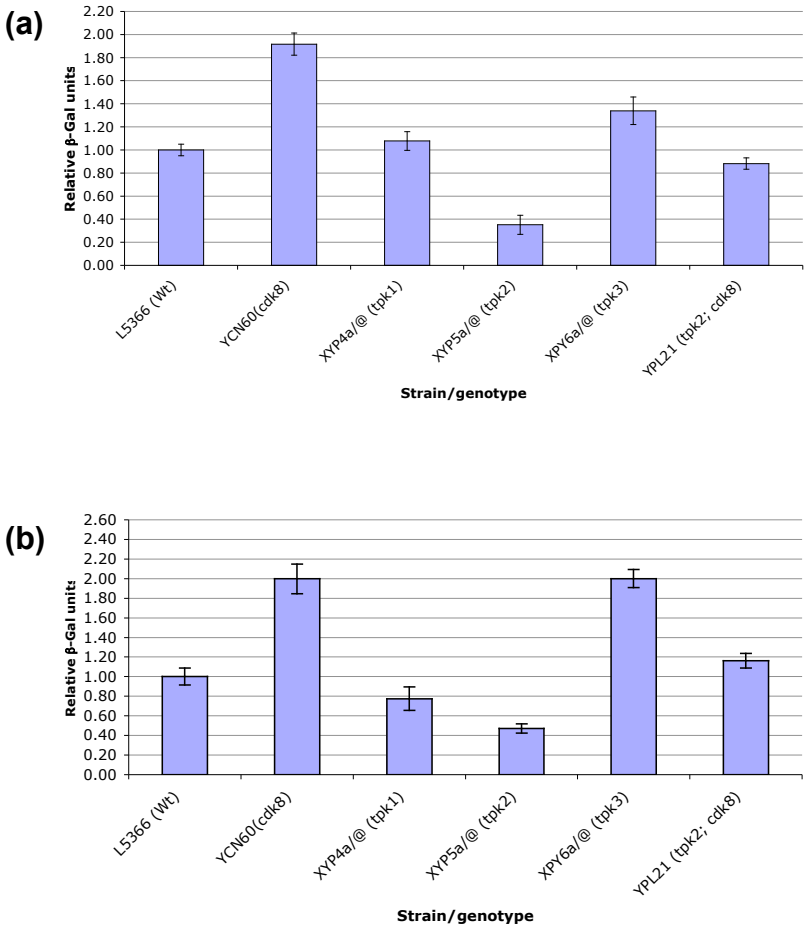

Figure 3.6. Yeast lacking TPK2 show decreased expression of Ste12-dependent filamentous responsive genes. Deletion of *TPK2* decreases FRE-dependent transcription. Homozygous diploid strains L5366, YCN60, XPY4a/α, XPY5a/α, XPY6a/α, and YPL21 were transformed with PBHM275 (*TEC1 FRE::lacZ* reporter). Cells were grown at 30°C in (a) SD media or in (b) SLAD media and assayed for β-galactosidase activity, which was subsequently normalized to the activity of wild-type yeast for each growth media (arbitrarily referenced as 1 unit). Error bars depict the standard error for each category.

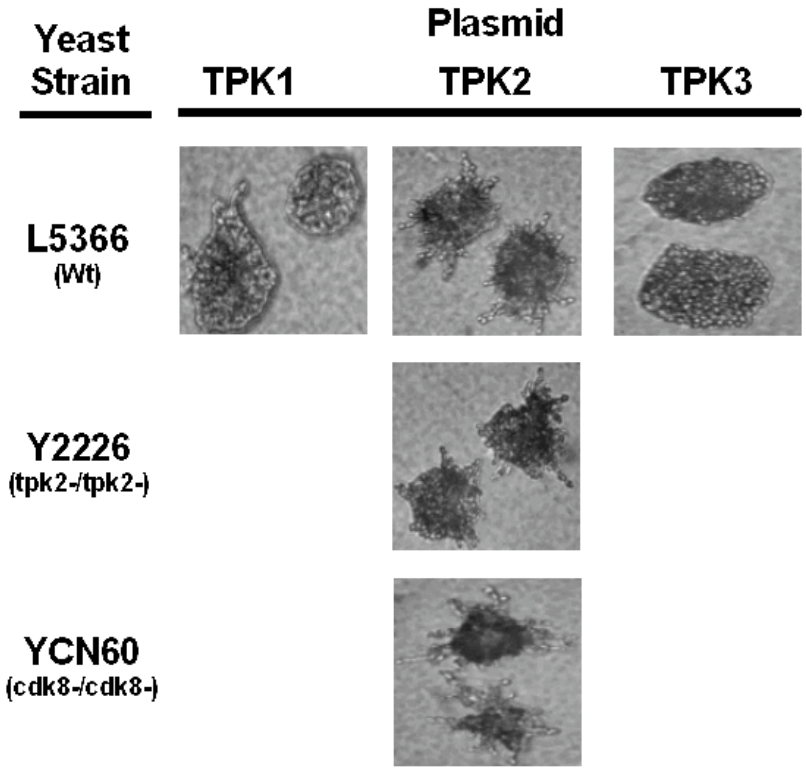

Figure 3.7 TPK2 over-expression induces filament formation in rich media. Yeast strains L5366 (wild-type), XPY5a/α (*tpk2/tpk2*) and YCN60 (*cdk8/cdk8*) were transformed with PXP2 (2μ *TPK1*), PXP3 (2μ *TPK2*) and PXP (2μ *TPK3*). Transformed yeast were streaked for single colonies on SD plates, incubated at 30°C and inspected for filament growth after 3 days.

3.5 Cdk8p contains typical PKA phosphorylation consensus sequence

The genetic evidence for a relation between *CDK8* and *TPK2* was clearly established at this point. However, major questions regarding the actual nature of the interaction between these two kinases still remained unanswered. It was hypothesized that Tpk2p possibly directly phosphorylates Cdk8p, an event that may target Cdk8p for degradation. With this in mind, bioinformatical analysis of the sequence of Cdk8p was performed to identify possible phosphorylation sites. Tpk2p is the yeast orthologue of Protein Kinase A (PKA), which is a serine/threonine kinase with specific consensus phosphorylation sequence of R(R/K/S)X(S/T) (Kim and Johnston 2006). Inspection of the amino acid sequence of Cdk8p revealed one canonical PKA phosphorylation consensus site, in accordance to the defined sequence in human PKA (Figure 3.8) positioned between amino acids 34 and 37. This sequence is Lys_{34}-Lys_{35}-Asn_{36}-Thr_{37}. Given the low occurrence of PKA consensus sequences on Cdk8p, threonine 37 became an extremely good candidate for a phosphorylation site on Cdk8p.

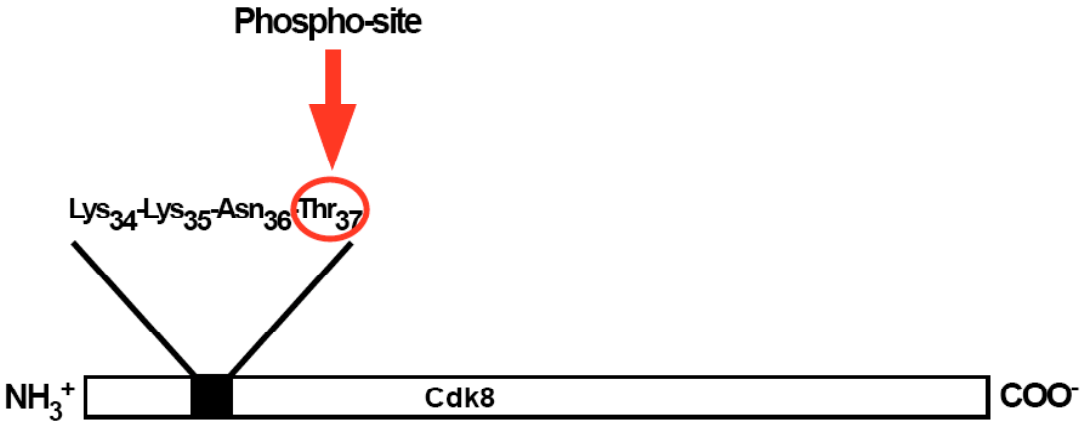

Figure 3.8 Cdk8p contains a typical PKA/TPK consensus phosphorylation site. Schematic representation of Cdk8p and the consensus phosphorylation site [R(R/K/S)X(S/T)] near the N-terminus. Tpk2 is a serine/threonine kinase. The plausible phosphorylation site is threonine 37, the only PKA consensus phosphorylation sequence on Cdk8p.

3.6 T37A and T37E Cdk8 mutants exhibit, respectively, non-filamenting and hyper-filamenting phenotypes

With only one Tpk2p consensus sequence on Cdk8p, it was likely that this site would be at the epitome of the interaction between the two kinases. Thus, in an attempt to further elucidate the exact nature of the interaction between Tpk2p and Cdk8p, the plausible phospho-site was mutated into a non-phosphorylatable residue. There is extensive literature supporting that mutation of a phospho-site, such as serine (S) or threonine (T), into an alanine residue abolishes phosphorylation, as the side chain of alanine (A) is lacks a hydroxyl and prevents formation of a covalent bond with the phosphate group transferred from ATP in the reaction catalyzed by the kinase (Nichols and Matthews 2002) (Morrison et al. 2003). Similarly, numerous studies have shown that phospho-mimic mutations, whereby the phospho-site residues are mutated into glutamate (E) can recapitulate a phenotype caused by the phosphorylation (Maciejewski et al. 1995) (Nichols and Matthews 2002) (Morrison et al. 2003). This mutation is believed to mimic the addition of a phosphate group to the side-chain of a phosphorylatable residue, as it confers a negative charge the amino-acid side chain, similarly to the effects of phosphorylation of serine or threonine.

Threonine 37 was, therefore, mutated into both alanine (T37A) and glutamate (T37E). It was believed that the T37A mutation would mimic the non-phosphorylated state, exhibiting Cdk8p stabilization and reduced rate of filamentation in SLAD media. The T37E mutation, on the other hand, was hypothesized to mimic the phosphorylated state of the kinase, thus resulting in destabilization and increased rate of filamentation in SLAD media.

When the T37A CDK8 mutant was expressed in wild-type yeast expressing endogenous CDK8 or in a *cdk8/cdk8* null strain, filamentation was abolished (Figure 3.9). This implies that phosphorylation on T37 is required in order to induce filament formation. Additionally, when T37E CDK8 was expressed, in the

cdk8/cdk8 null strain, cells became hyper-filamentous, similar to the phenotype exhibited by cells lacking *cdk8* (Figure 3.9). This suggests that the T37E mutation either abolishes kinase activity or targets Cdk8p for degradation. However, T37E CDK8 expressed in a wild-type strain expressing endogenous CDK8 did not display the hyper-filamentous phenotype demonstrated in the *cdk8/cdk8* null background. This is likely due to the fact that endogenous Cdk8p is still active and responsive to nitrogen signaling. Additionally, this implies that the T37A mutant, since it inhibits filamentous growth even wild-type cells, the mutation is dominant to the wild-type. T37A Cdk8p must not, therefore, be subject to the same regulation as wild-type Cdk8p in response to nitrogen limitation.

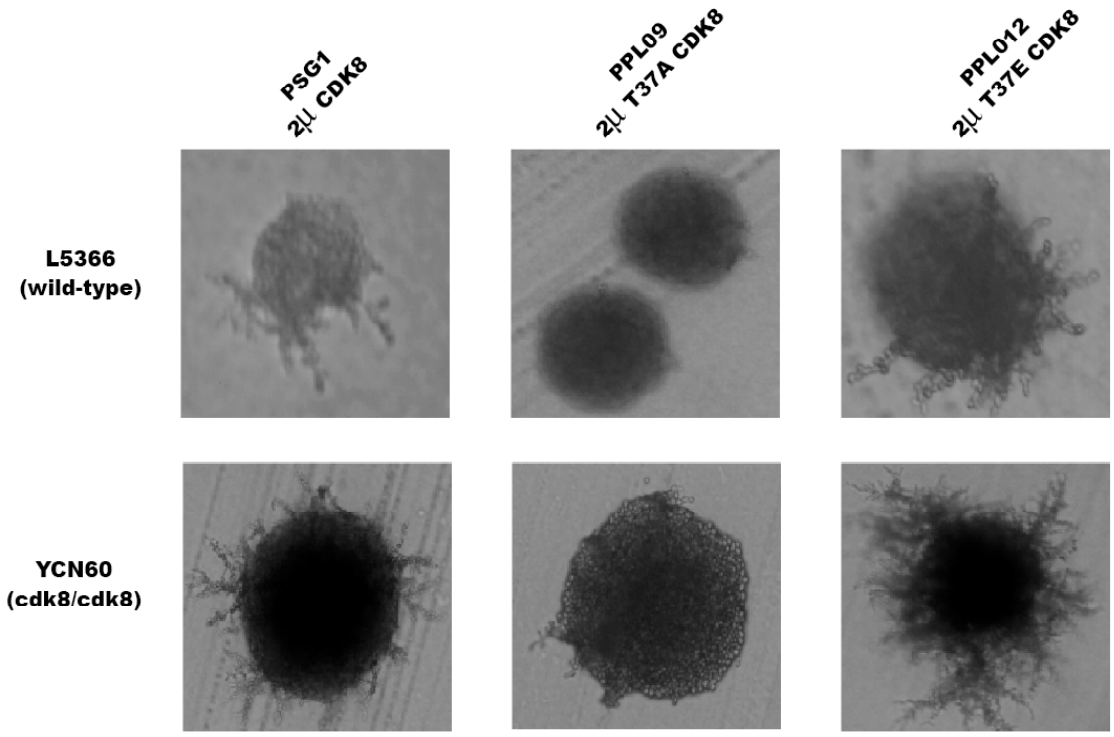

Figure 3.9 T37A Cdk8p mutants are hypofilamentous and T37E Cdk8p mutants are hyperfilamentous. Yeast strains L5366 (wild-type) and YCN60 (*cdk8/cdk8*) were transformed with PSG1 (2μ CDK8), PPL09 (2μ T37A CDK8) and PPL012 (2μ T37E CDK8). Transformed yeast were streaked for single colonies on SLAD plates, incubated at 30°C and inspected for filament growth after 5 days.

3.7 Tpk2p specifically phosphorylates Cdk8p at position Thr37

To determine if Tpk2p was capable of directly phosphorylating T37 of Cdk8p, I produced a wild-type and T37A mutant CDK8 proteins using rabbit reticulocyte lysate-based *in vitro* transcription and translation for use as substrates in *in vitro* kinase reactions with Tpk2p purified from yeast. Wild-type and T37A *CDK8* were expressed using this system and purified via Ni-NTA chromatography. Purified wild-type and T37A Cdk8p were utilized as substrate for an *in vitro* kinase assay (Figure 3.10). Based on this figure, Tpk2p directly phosphorylates wild-type Cdk8p (Figure 3.10, lanes 2 and 3), but not T37A Cdk8p, suggesting that phosphorylation is specific to the T37 phospho-site (Figure 3.10, lane 1). Both wild-type and T37A Cdk8p were present in the kinase reaction, as is evidenced by Figure 3.12c. Further specificity is confirmed by the fact that Tpk2p fails to phosphorylate Ste12p (lane 7).

The possibility of Cdk8p autophosphorylation was eliminated by the inclusion of D290A Cdk8p (Figure 3.10, lane 3). This mutant of CDK8 contains a mutation on the catalytic domain and is, therefore, dead in terms of kinase activity; thus any kinase activity must be due to the presence of Tpk2p in the reaction mixture. Tpk2p functions as an internal control, as this kinase has been found capable of autophosphorylation (Ptacek et al. 2005).

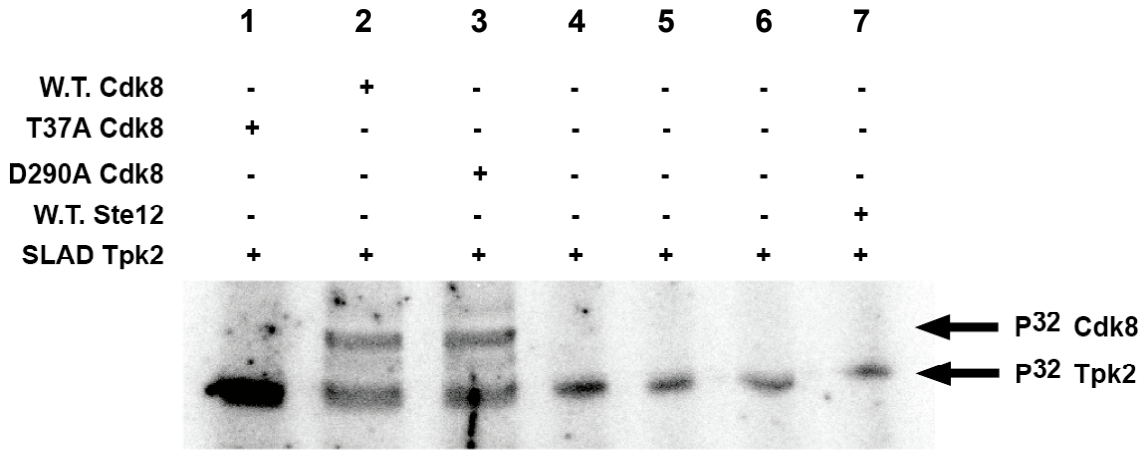

Figure 3.10 Tpk2p phosphorylates Cdk8p *in vitro* specifically on Thr37. Recombinant Cdk8p (wild-type Cdk8p, T37A Cdk8p and D290A Cdk8p) and Ste12p (wild-type) were purified from the rabbit reticulocytes with Ni-NTA agarose. Approximately 200 ng of purified substrate was incubated with 3 μl of purified Tpk2 and 10μCi γ^{32} ATP for 30 minutes at 30°C and loaded on to SDS-PAGE gel. Dried gel was exposed to Biomax (Kodak) film for 48 hours. D290A Cdk8p is kinase-dead. Western-blot of the substrates are shown in Figure 3.12c.

3.8 Phosphorylation of Cdk8p on Thr37 by Tpk2p results in its degradation

Given the strong genetic and *in vitro* biochemical evidence to corroborate the idea that Tpk2p-dependent phosphorylation on Thr37 of Cdk8p causes degradation, it was expected that, *in vivo*, the non-phosphorylated T37A Cdk8p mutant would be stabilized under conditions of limiting nitrogen, whereas the phospho-mimic T37E Cdk8p mutant was expected to have a more rapid turn-over than its wild-type counterpart.

According to Figure 3.11 (lane 4), the non-phosphorylated T37A Cdk8p mutant was, as expected, stabilized despite nitrogen depravation. Even after growth for 4 hours in SLAD media, the T37A mutant appears resistant to nitrogen depletion. This data provides strong support for the model proposed thus far; clearly, lack of phosphorylation on Thr37 abolishes the capacity of Cdk8p to destabilize or to interact with cellular machinery capable of cleaving the kinase.

On the other hand, the phospho-mutant T37E demonstrates a more rapid turnover rate than wild-type Cdk8p (Figure 3.11, lane 2 vs. lane 6). In fact, the stability of the T37E Cdk8p mutant is compromised even in rich media, as detectable levels are reduced, despite the presence of ammonia and other nitrogen-rich compounds (Figure 3.11, lane 5). This data heavily implicates the Thr37 site as a trigger for destabilization and suggests that phosphorylation on Thr37 is necessary and sufficient to target Cdk8p for degradation via an undetermined mechanism. The model for phosphorylation-dependent destabilization of Cdk8p has been gaining consistency with the genetic and biochemical evidence presented in earlier sections of the thesis, especially with the barely detectable levels of T37E Cdk8p protein present in yeast expressing the mutant (Figure 3.11). The reduced levels of the phospho-mimic mutant T37E Cdk8p suggest that phosphorylation leads to destabilization and/or truncation of the kinase via an unknown mechanism. The DNA template utilized to generate the T37E mutant was sequenced and no pre-mature stop codons were detected in this mutant.

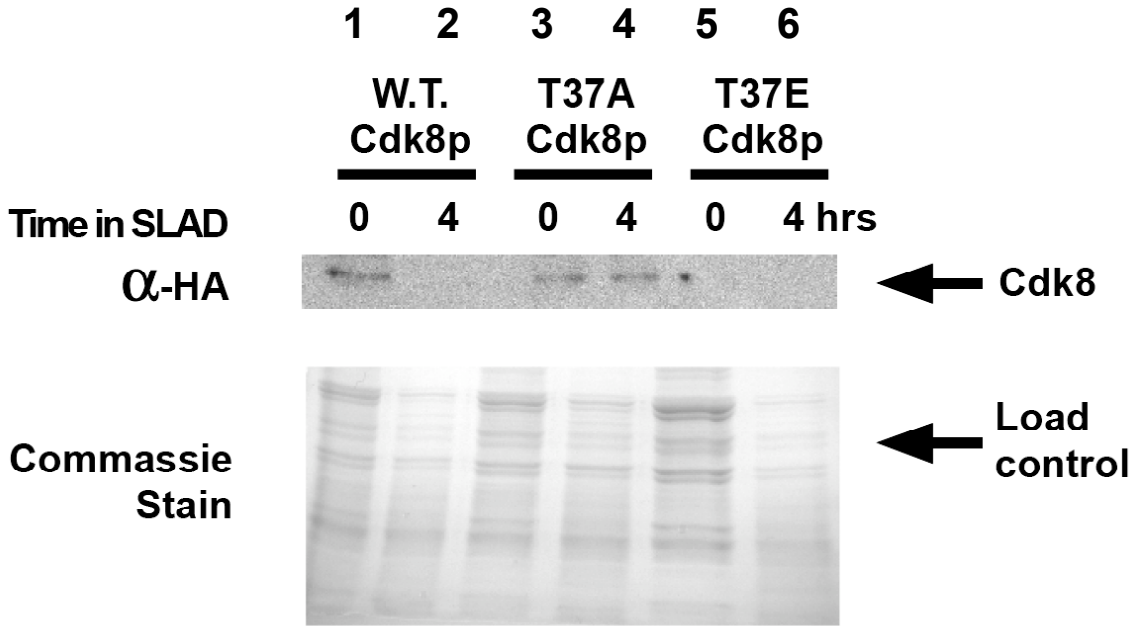

Figure 3.11 T37A Cdk8p is stabilized in SLAD media and T37E Cdk8p is destabilized in rich and SLAD media. Haploid yeast strain YCN45 (*cdk2*) was transformed with PPL016 (*fcy1::CDK8*), PPL017 (*fcy1::T37A CDK8*) or PPL018 (*fcy1::T37E CDK8*) and was grown in SD –URA media, switched to SLAD media (T=0 hrs) and harvested following 4 hours. Cdk8p stability was measured by western blotting whole cell extracts of lysed cells with α-FLAG antibodies

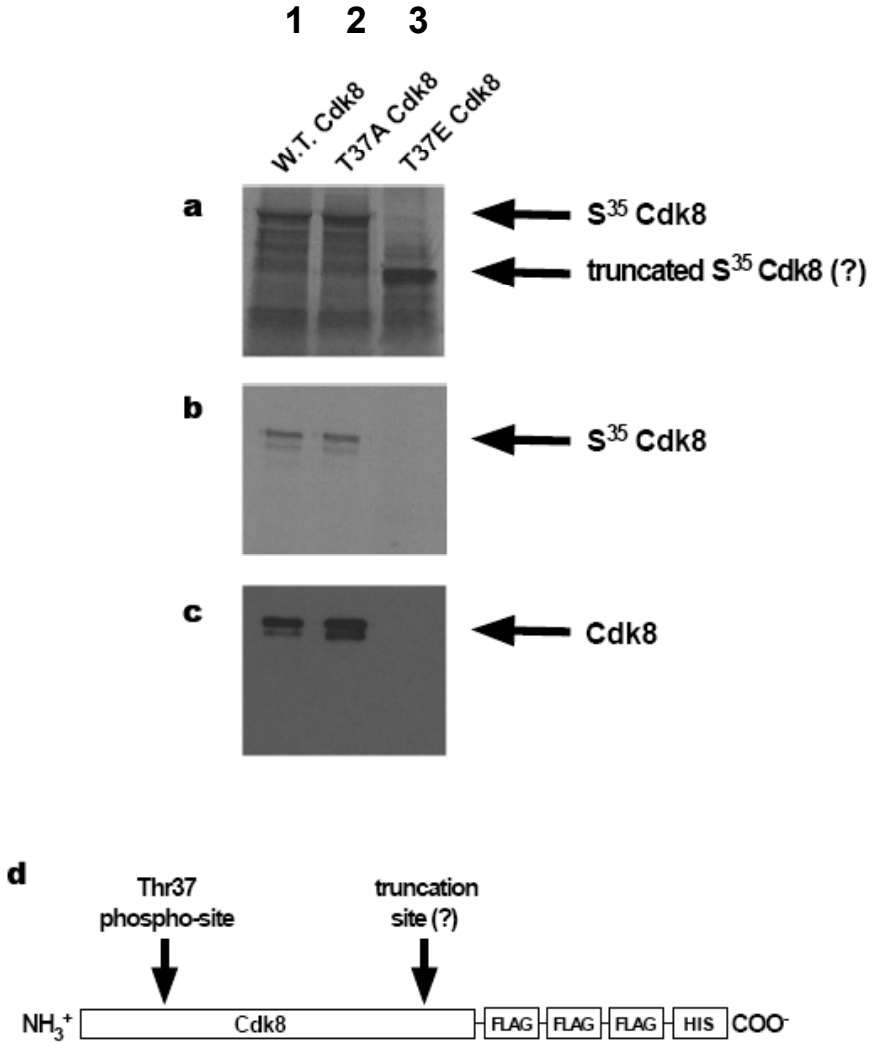

Figure 3.12 Phospho-mimic mutant T37E *CDK8* destabilizes and targets Cdk8p for truncation. (a) *In vitro* translated *CDK8* (wild-type, T37A and T37E) were labeled with S35; (b) crude *in vitro* translated products were purified from the rabbit reticulocyte mix using Ni-NTA agarose; (c) purified proteins were subject to western blotting with α-FLAG; (d) schematic representation of *in vitro* translated *CDK8*; Thr37 is the N-terminal phosphorylation site.

When the different mutants of Cdk8p were expressed in the *in vitro* reticulocyte translation system, wild-type and T37A *CDK8* produced full-length Cdk8p (Figure 3.12a, lanes 1 and 2). However, expression of the T37E *CDK8* mutant yields a fragment of reduced size (Figure 3.12a, lane 3), similarly to what was observed by Susan Goto in her previous work when the truncated Cdk8p product appeared after 4 hours growth in SLAD media. At this point, it was hypothesized that phosphorylation on T37 targeted Cdk8p for degradation via ubiquitination and, subsequently, proteasome truncation. This was considered a very plausible possibility, as rabbit reticulocyte systems are also utilized to study protein degradation events, due to the heavy presence of proteasomes and other degradation machinery in the lysates (Carlson et al. 2005). To test this hypothesis, a known inhibitor of the proteasome, MG132, was added to the rabbit reticulocyte lysate prior to the start of the *in vitro* translation reaction in a gradient fashion at concentrations up to 100 μM, which corresponds to a ten-fold increase relative to the standard concentration utilized. Disappointingly, addition of the proteasome inhibitor MG132 failed to restore full-length Cdk8p synthesis (data not shown). Similarly, to investigate the possibility of a protease-mediated truncation, a protease inhibitor cocktail was added in a gradient fashion and this also failed to eliminate synthesis of the truncated fragment (data not shown).

Given the lack of positive evidence to suggest protease or proteasome mediated truncation of phosphorylated Cdk8p *in vitro*, a model for auto-cleavage gains consistency. Protein auto-cleavage, while not a common process in *S. cerevisae*, has been documented in a wide range of organisms, with the most notorious example in yeast being the auto-cleavage of the enzyme separase (Holland et al. 2007).

3.9 Over-expression of *CDK8* reverses the *tpk3* null phenotype

According to (Pan and Heitman 1999), Tpk3p functions as a general inhibitor of filamentous growth as is evidenced by the hyperfilamentous phenotype of a *tpk3/tpk3* null strain (Figure 3.13). The mechanism of action of Tpk3p has not been elucidated up to this point. The question that arises, therefore, is whether Cdk8p and Tpk3p are part of the same or parallel signaling pathways. To address this issue, yeast of a *tpk3/tpk3* background was transformed with a plasmid over expressing *CDK8*. According to Figure 3.13, over expression of *CDK8* in a *tpk3/tpk3* strain causes a decrease in the rate of filamentation. Although preliminary, this data provides evidence that these two kinases function in parallel to repress filamentation via distinct DNA-binding factors. It is known that Cdk8p exerts its effects via regulation of the transcriptional activator Ste12p, thus it is likely that Tpk3p exerts its effect on a different transcriptional activator. *PHD1* is a transcriptional activator of filamentous response and given the lack of knowledge of the regulatory control of this factor, Tpk3p may be involved in its regulation.

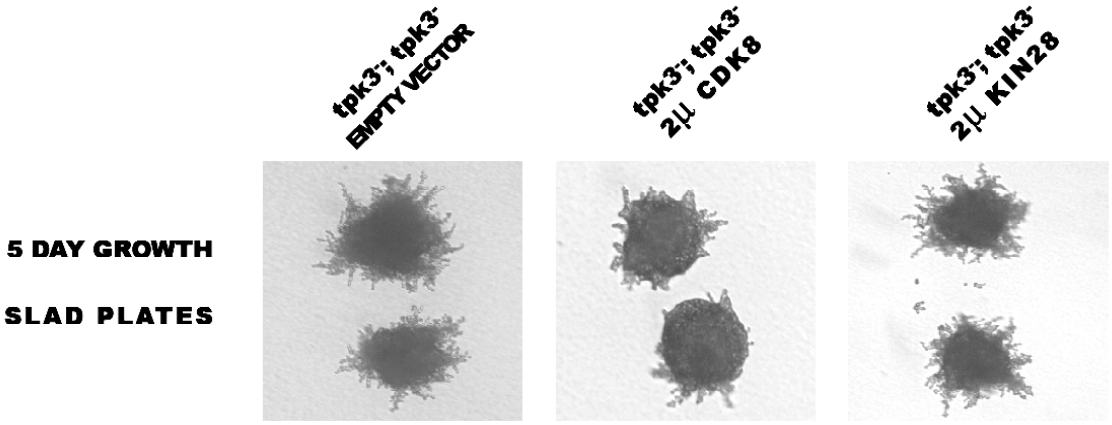

Figure 3.13 TPK3 and CDK8 exert their effects on filamentation via opposing parallel pathways. Yeast strain XPY6a/α (*tpk3/tpk3*) was transformed with PRS316 (empty vector control with complementing prototrophic markers); PSG1 (2μ *CDK8*); and PJP018 (2μ *KIN28*). Transformed yeast were streaked for single colonies on SLAD plates, incubated days at 30°C and inspected for filament growth after 5 days.

3.10 Human *CDK8* complements a *cdk8* null phenotype and is sensitive to nitrogen signaling

It is a well-established notion that there exists a high-degree of homology between *S.cerevisiae* and humans. Statistical studies suggest that approximately 34% of human genes have homologous counterparts in yeast (Botstein et al. 1997). The authors also mention that the value presented is a large underestimate, as genes of uncharacterized function in both humans and S. cerevisiae were not included in this statistical analysis. Given the presence of Cdk8p in the human mediator complex and in NOTCH signaling (Fryer et al. 2004) in humans, a high-degree of homology and retention of function was expected for human CDK8 expressed in yeast.

According to Figure 3.14a, human CDK8 complements a *cdk8/cdk8* null phenotype, thus suggesting homology between the two kinases. The question to address at this moment was whether human Cdk8p was also responsive to nitrogen limitation. Given the fact that there was mild filament formation, it was expected that human Cdk8p was responsive to nitrogen signaling, as the contrary scenario would result in a hypofilamenting phenotype. As demonstrated in Figure 3.14b, there is loss of human Cdk8p in conditions of limiting nitrogen, further corroborating the notion that function is retained between the two organisms.

However, this result opens a new range of questions regarding the complete role of Cdk8p in humans. To date, human Cdk8p has received little attention from the scientific community and if the general characteristics of this kinase determined from S. cerevisiae are retained in humans, CDK8 might have a central role in coordinating stress-related responses in humans.

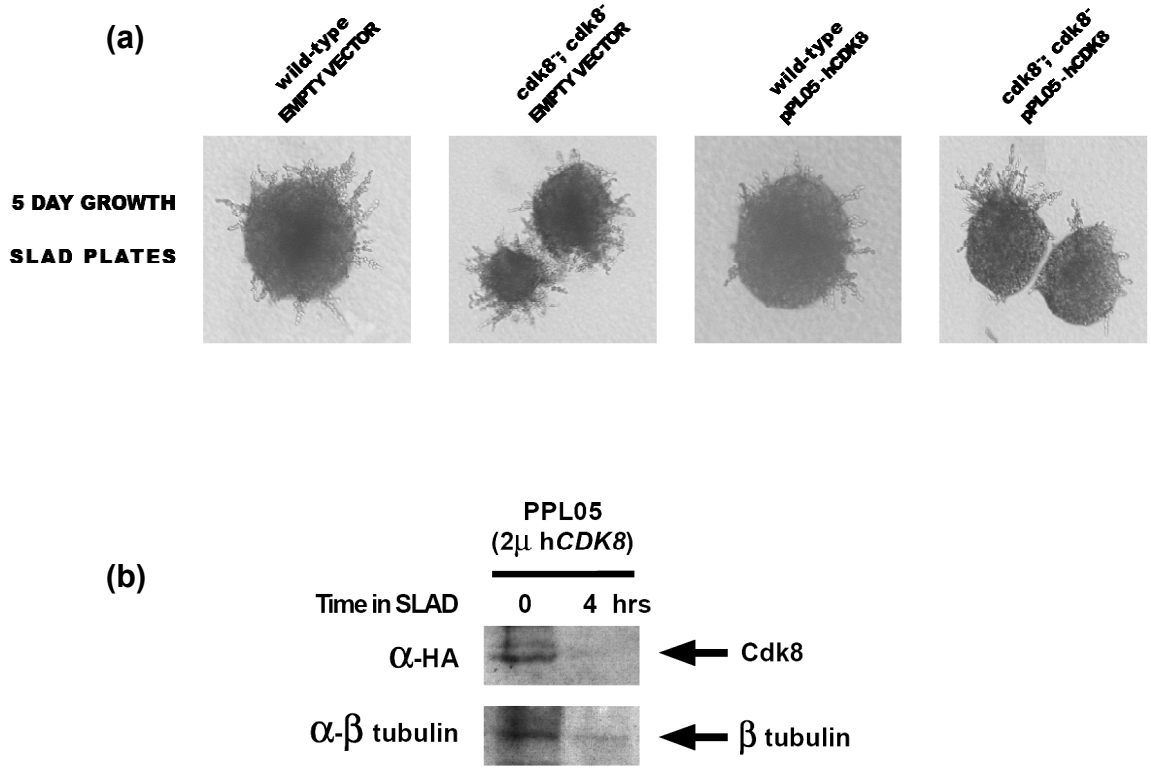

Figure 3.14 Human CDK8 is homologous to *S. cerevisiae* CDK8 and displays sensitivity to nitrogen depletion. (a) yeast strains L5366 (wild-type) and YCN60 (*cdk8/cdk8*) were transformed with PRS316 (empty vector control with complementing prototrophic markers) and PPL05 (2μ hCDK8). Transformed yeast were streaked for single colonies on SLAD plates, incubated days at 30°C and inspected for filament growth after 5 days. (b) Haploid yeast strain YCN45 (*cdk8*) was transformed with PPL05 (2μ hCDK8) and grown in SD –URA media, switched to SLAD media (T=0 hrs) and harvested at the indicated time point. hCdk8p stability was measured by western blotting whole cell extracts of lysed cells with α-HA antibodies.

CHAPTER 4 – CONCLUSIONS AND PERSPECTIVES

Regardless of the exact mechanism that mediates destabilization of Cdk8p in response to limiting nitrogen conditions, my results indicate a novel signaling pathway in *S. cerevisiae* for regulation of filamentous growth. Based on previous studies, a complete signaling cascade can now be described that leads to the activation of Ste12p-dependent filamentous responsive genes. It is known that the membrane-bound ammonium permease, Mep2p, is involved in sensing ammonium levels and relaying this to the small, membrane-bound GTP-binding protein, Ras2p (Lorenz and Heitman 1998). Activated Ras2p, further relays the signal by directly stimulating the adenylate cyclase Cyr1p (Lai et al. 1993), an event that leads to the increase of intracellular cAMP levels cAMP is a major secondary messenger controlling filamentous response, as is evidenced by the fact that exogenous cAMP causes *pde/pde* null yeast to filament. cAMP interacts with Bcy1, the regulatory subunit of Tpk2p, and triggers dissociation of the two proteins, liberating free, active Tpk2p (Toda et al. 1987). Finally, as here indicated, active Tpk2p phosphorylates Cdk8p at position Thr37, a modification that targets Cdk8p for truncation via an undetermined mechanism. Ultimately, loss of Cdk8p results in the stabilization of the transcriptional activator Ste12p, thus activating the expression of filamentous responsive genes (Nelson et al. 2003). A schematic representation of this complete signaling pathway is depicted in Figure 4.1.

While the relation between Tpk2p and Cdk8p has become elucidated, the overall picture of the regulation of filamentous growth still has many questions to address. The complexity in regulation of the filamentous response highlights the importance of maintaining this response under tight transcriptional regulation.

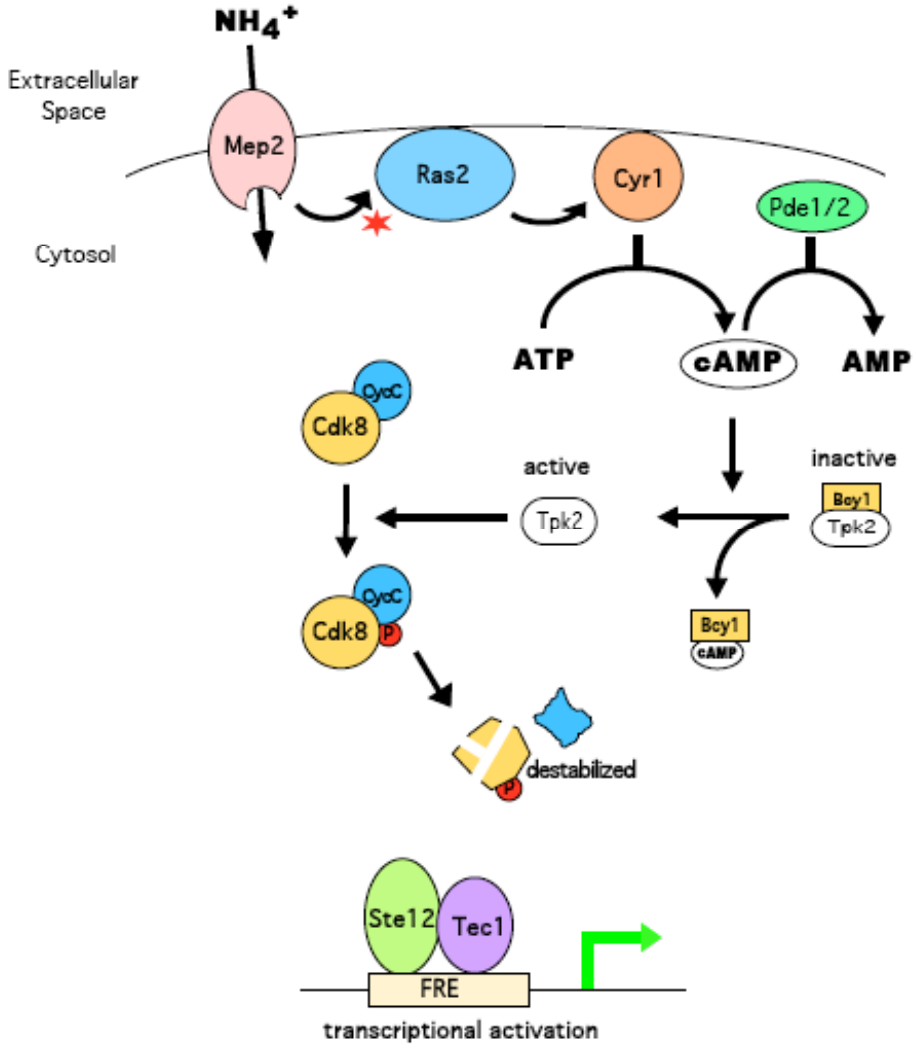

Figure 4.1 Schematic representation of the destabilization of Cdk8p by phosphorylation by Tpk2p. Nitrogen signaling occurs via the cAMP-dependent pathway, resulting in the activation of Tpk2p, a nuclear kinase, which phosphorylates Cdk8p at Thr37. This event is believed to contribute to the destabilization of Cdk8p and, consequently, stabilization of Ste12p. Stabilized Ste12p enhances the expression of FRE-dependent filamentous genes.

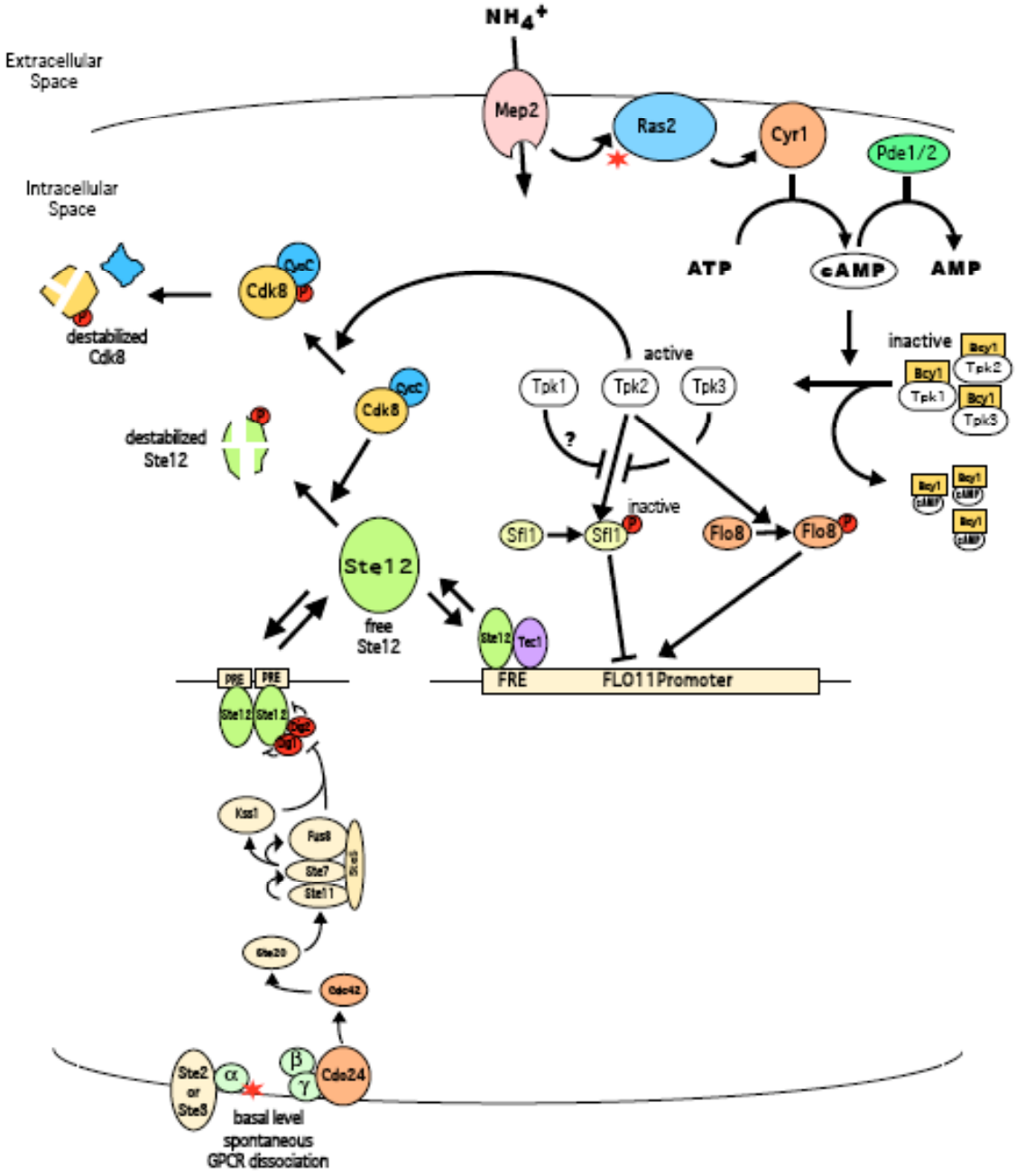

Figure 4.2 Integration of the pathways that regulate filamentous response. Schematic representation of the MAPK, cAMP-dependent Flo8p/Sfl1p, and Cdk8p-dependent pathways exerting their effects on the expression of the hallmark filamentation gene, *FLO11*. Expression of *FLO11* is dependent on the activity of various transcription factors, with Ste12p/Tec1p, Sfl1p and Flo8p being of utmost importance.

Our understanding of the regulation of filamentous growth is further complicated by the fact that while the MAPK pathway is required for filamentous growth, nitrogen signaling is not transmitted through this signaling cascade. Instead, we propose that the MAPK pathway is important because it is required to ensure basal level de-repression of Ste12p, Figure 4.2. Even without pheromone stimulation, there is basal signaling via the MAPK pathway due to spontaneous dissociation of the βγ subunits from the G-protein coupled receptor (Siekhaus and Drubin 2003). This basal signaling likely allows for constitutive basal de-repression of Ste12p by relieving the inhibitory effects of the Dig1/2 and Kss1 proteins. The leaky GCPR, thus, prevents the repression and sequestering of a small pool of Ste12p on the PRE. It is this pool of free, un-repressed Ste12p that is sensitive to the effects of Cdk8p. The proposed model is in agreement with the evidence linking the MAPK to filamentous growth. Genetic evidence demonstrates that strains lacking the major MAPK cascade signal transducers, such as Ste2 or Ste11, do not filament. The reason is likely due to the fact that the basal level of signaling is incapable of reaching its downstream effectors in cells that lack members of the pathway, specifically Ste12p and Tec1p. Therefore, Ste12p (and Tec1p indirectly) is under constant repression by Dig1p/Dig2p and Kss1p, eliminating the formation of a small pool of free, activated Ste12p. Additionally, northern blotting data from our lab has demonstrated that *TEC1* expression is highly dependent upon Ste12p and the basal constitutive MAPK pheromone response signaling. The requirement of the MAPK pathway in activation of the filamentous responses is, therefore, a mere artifact of the shared requirement for Ste12p with the pheromone response. In fact, previous work done in our lab showed that Cdk8p is degraded in nitrogen limiting conditions even in a *ste7/ste7* diploid strain, thus indicating that the MAPK pathway is independent from cAMP-dependent signaling (Daisy Liu, Unpublished).

Another factor that appears to have a pronounced effect on filamentation is Snf1p. Two-hybrid systems and co-immunoprecipitation experiments have

demonstrated a physical interaction between Snf1p and Cdk8p (Kuchin et al. 2000), although the exact nature and extent of this interaction have not yet been elucidated in detail. One possibility is that Snf1p is an upstream regulator of Cdk8p. Yeast that lack *snf1* are hypo-filamenting, whereas over-expression of *SNF1* yields a hyper-filamentous phenotype (Kuchin et al. 2002). Conversely, a strain that lacks *cdk8* exhibits hyper-filamentation (Figure 3.1), while *CDK8* overexpression yields a hypo-filamenting phenotype (Figure 3.2). Based on this genetic evidence, it is plausible that Snf1p is directly phosphorylating Cdk8p and that this event leads to the destabilization of Cdk8p, similarly to the role of Tpk2p presented herein. Thus, Snf1p would function as an inhibitor of Cdk8p and additional pathways involved in glucose and nitrogen sensing would converge on Cdk8p, rendering this kinase as a very versatile and crucial general stress sensor in *S. cerevisiae*.

CHAPTER 5 - FUTURE DIRECTIONS

While the regulation of the filamentous response is a heavily studied and characterized event, many questions still remain unanswered. The previous work performed by Susan Goto (unpublished), demonstrating truncation of Cdk8p in response to nitrogen limitation is an area that requires further investigation. Given the fact that the truncated product forms a product of consistent size, it will prove important to determine the site and mechanism of cleavage. Another issue to address is whether the truncated kinase has any activity that is required for the induction of the filamentous response. Purification and characterization of this lower-molecular weight species is, therefore, of utmost importance.

As discussed in chapter 4, the relation between Snf1p and Cdk8p is very vague. The evidence to suggest interaction between Snf1p and Cdk8p is sufficient to warrant the investigation on the possibility that Snf1p is directly phosphorylating Cdk8p and that this event leads to the destabilization of Cdk8p, similarly to the role of Tpk2p presented herein.

Characterization of the role of Cdk8p in human cells is also of great importance. Cdk8p is a very versatile and crucial general stress sensor in *S. cerevisiae;* thus, to study if its yeast characteristics are retained in humans, it could prove useful in the creation of therapeutics. For instance, Cdk8p in humans may be sensitive hypoxic conditions. Given the fact that the interior of cancerous tumors are often hypoxic, due to the lack of angiogenesis and high O_2 consumption by expedited growth, Cdk8p may be involved in regulating a response that would allows cells starved for O_2 to forage for nutrients in adjacent areas, thus providing a model for metastasis.

REFERENCES

Ahn, S. H., A. Acurio, et al. (1999). "Regulation of G2/M progression by the STE mitogen-activated protein kinase pathway in budding yeast filamentous growth." Mol Biol Cell **10**(10): 3301-16.

Andrews, B. and V. Measday (1998). "The cyclin family of budding yeast: abundant use of a good idea." Trends Genet **14**(2): 66-72.

Bardwell, L., J. G. Cook, et al. (1998). "Repression of yeast Ste12 transcription factor by direct binding of unphosphorylated Kss1 MAPK and its regulation by the Ste7 MEK." Genes Dev **12**(18): 2887-98.

Bender, A. and G. F. Sprague, Jr. (1986). "Yeast peptide pheromones, a-factor and alpha-factor, activate a common response mechanism in their target cells." Cell **47**(6): 929-37.

Berry, L. D. and K. L. Gould (1996). "Regulation of Cdc2 activity by phosphorylation at T14/Y15." Prog Cell Cycle Res **2**: 99-105.

Bhattacharya, S., L. Chen, et al. (1995). "Ras membrane targeting is essential for glucose signaling but not for viability in yeast." Proc Natl Acad Sci U S A **92**(7): 2984-8.

Bjorklund, S. and C. M. Gustafsson (2005). "The yeast Mediator complex and its regulation." Trends Biochem Sci **30**(5): 240-4.

Blackwood, E. M. and J. T. Kadonaga (1998). "Going the distance: a current view of enhancer action." Science **281**(5373): 60-3.

Botstein, D., S. A. Chervitz, et al. (1997). "Yeast as a model organism." Science **277**(5330): 1259-60.

Broek, D., N. Samiy, et al. (1985). "Differential activation of yeast adenylate cyclase by wild-type and mutant RAS proteins." Cell **41**(3): 763-9.

Caldwell, G. A., F. Naider, et al. (1995). "Fungal lipopeptide mating pheromones: a model system for the study of protein prenylation." Microbiol Rev **59**(3): 406-22.

Cali, B. M., T. C. Doyle, et al. (1998). "Multiple functions for actin during filamentous growth of Saccharomyces cerevisiae." Mol Biol Cell **9**(7): 1873-89.

Cannon, J. F. and K. Tatchell (1987). "Characterization of Saccharomyces cerevisiae genes encoding subunits of cyclic AMP-dependent protein kinase." Mol Cell Biol **7**(8): 2653-63.

Carlson, E., N. Bays, et al. (2005). "Reticulocyte lysate as a model system to study endoplasmic reticulum membrane protein degradation." Methods Mol Biol **301**: 185-205.

Carlson, M., B. C. Osmond, et al. (1984). "A suppressor of SNF1 mutations causes constitutive high-level invertase synthesis in yeast." Genetics **107**(1): 19-32.

Carrozza, M. J., L. Florens, et al. (2005). "Stable incorporation of sequence specific repressors Ash1 and Ume6 into the Rpd3L complex." Biochim Biophys Acta **1731**(2): 77-87; discussion 75-6.

Chandarlapaty, S. and B. Errede (1998). "Ash1, a daughter cell-specific protein, is required for pseudohyphal growth of Saccharomyces cerevisiae." Mol Cell Biol **18**(5): 2884-91.

Chang, Y. W., S. C. Howard, et al. (2001). "The rye mutants identify a role for Ssn/Srb proteins of the RNA polymerase II holoenzyme during stationary phase entry in Saccharomyces cerevisiae." Genetics **157**(1): 17-26.

Chi, Y., M. J. Huddleston, et al. (2001). "Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase." Genes Dev **15**(9): 1078-92.

Choder, M. and R. A. Young (1993). "A portion of RNA polymerase II molecules has a component essential for stress responses and stress survival." Mol Cell Biol **13**(11): 6984-91.

Chou, S., S. Lane, et al. (2006). "Regulation of mating and filamentation genes by two distinct Ste12 complexes in Saccharomyces cerevisiae." Mol Cell Biol **26**(13): 4794-805.

Colombo, S., P. Ma, et al. (1998). "Involvement of distinct G-proteins, Gpa2 and Ras, in glucose- and intracellular acidification-induced cAMP signalling in the yeast Saccharomyces cerevisiae." Embo J **17**(12): 3326-41.

Conlan, R. S. and D. Tzamarias (2001). "Sfl1 functions via the co-repressor Ssn6-Tup1 and the cAMP-dependent protein kinase Tpk2." J Mol Biol **309**(5): 1007-15.

Cook, J. G., L. Bardwell, et al. (1996). "Two novel targets of the MAP kinase Kss1 are negative regulators of invasive growth in the yeast Saccharomyces cerevisiae." Genes Dev **10**(22): 2831-48.

Cooper, K. F., M. J. Mallory, et al. (1997). "Stress and developmental regulation of the yeast C-type cyclin Ume3p (Srb11p/Ssn8p)." Embo J **16**(15): 4665-75.

D'Souza, C. A. and J. Heitman (2001). "Conserved cAMP signaling cascades regulate fungal development and virulence." FEMS Microbiol Rev **25**(3): 349-64.

Deschenes, R. J. and J. R. Broach (1987). "Fatty acylation is important but not essential for Saccharomyces cerevisiae RAS function." Mol Cell Biol **7**(7): 2344-51.

Dickinson, J. R. (1996). "'Fusel' alcohols induce hyphal-like extensions and pseudohyphal formation in yeasts." Microbiology **142 (Pt 6)**: 1391-7.

Dolan, J. W., C. Kirkman, et al. (1989). "The yeast STE12 protein binds to the DNA sequence mediating pheromone induction." Proc Natl Acad Sci U S A **86**(15): 5703-7.

Edgington, N. P., M. J. Blacketer, et al. (1999). "Control of Saccharomyces cerevisiae filamentous growth by cyclin-dependent kinase Cdc28." Mol Cell Biol **19**(2): 1369-80.

Elion, E. A., J. A. Brill, et al. (1991). "FUS3 represses CLN1 and CLN2 and in concert with KSS1 promotes signal transduction." Proc Natl Acad Sci U S A **88**(21): 9392-6.

Estruch, F. and M. Carlson (1990). "Increased dosage of the MSN1 gene restores invertase expression in yeast mutants defective in the SNF1 protein kinase." Nucleic Acids Res **18**(23): 6959-64.

Fields, S. and I. Herskowitz (1985). "The yeast STE12 product is required for expression of two sets of cell-type specific genes." Cell **42**(3): 923-30.

Fryer, C. J., J. B. White, et al. (2004). "Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover." Mol Cell **16**(4): 509-20.

Fujita, A., Y. Kikuchi, et al. (1989). "Domains of the SFL1 protein of yeasts are homologous to Myc oncoproteins or yeast heat-shock transcription factor." Gene **85**(2): 321-8.

Gagiano, M., D. van Dyk, et al. (1999). "Msn1p/Mss10p, Mss11p and Muc1p/Flo11p are part of a signal transduction pathway downstream of Mep2p regulating invasive growth and pseudohyphal differentiation in Saccharomyces cerevisiae." Mol Microbiol **31**(1): 103-16.

Gancedo, J. M. (2001). "Control of pseudohyphae formation in Saccharomyces cerevisiae." FEMS Microbiol Rev **25**(1): 107-23.

Gavrias, V., A. Andrianopoulos, et al. (1996). "Saccharomyces cerevisiae TEC1 is required for pseudohyphal growth." Mol Microbiol **19**(6): 1255-63.

Gietz, R. D. and R. H. Schiestl (2007). "High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method." Nat Protoc **2**(1): 31-4.

Gill, G. and M. Ptashne (1988). "Negative effect of the transcriptional activator GAL4." Nature **334**(6184): 721-4.

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**(3): 2100-12.

Gimeno, C. J., P. O. Ljungdahl, et al. (1992). "Unipolar cell divisions in the yeast S. cerevisiae lead to filamentous growth: regulation by starvation and RAS." Cell **68**(6): 1077-90.

Gnatt, A. L., P. Cramer, et al. (2001). "Structural basis of transcription: an RNA polymerase II elongation complex at 3.3 A resolution." Science **292**(5523): 1876-82.

Gustin, M. C., J. Albertyn, et al. (1998). "MAP kinase pathways in the yeast Saccharomyces cerevisiae." Microbiol Mol Biol Rev **62**(4): 1264-300.

Hampsey, M. (1998). "Molecular genetics of the RNA polymerase II general transcriptional machinery." Microbiol Mol Biol Rev **62**(2): 465-503.

Hansen, E. C. (1886). "Recherches sur la physiologie et la morphologie der fermentes alcooliques: VI." C. R. Trav. Lab. Carlsberg **T II**(Les voiles chez genre Saccharomyces): 106-147.

Hedges, D., M. Proft, et al. (1995). "CAT8, a new zinc cluster-encoding gene necessary for derepression of gluconeogenic enzymes in the yeast Saccharomyces cerevisiae." Mol Cell Biol **15**(4): 1915-22.

Hengartner, C. J., V. E. Myer, et al. (1998). "Temporal regulation of RNA polymerase II by Srb10 and Kin28 cyclin-dependent kinases." Mol Cell **2**(1): 43-53.

Herskowitz, I. (1995). "MAP kinase pathways in yeast: for mating and more." Cell **80**(2): 187-97.

Hirst, M., M. S. Kobor, et al. (1999). "GAL4 is regulated by the RNA polymerase II holoenzyme-associated cyclin-dependent protein kinase SRB10/CDK8." Mol Cell **3**(5): 673-8.

Holland, A. J., F. Bottger, et al. (2007). "Protein phosphatase 2A and separase form a complex regulated by separase autocleavage." J Biol Chem **282**(34): 24623-32.

Holstege, F. C., E. G. Jennings, et al. (1998). "Dissecting the regulatory circuitry of a eukaryotic genome." Cell **95**(5): 717-28.

Hong, S. P., F. C. Leiper, et al. (2003). "Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases." Proc Natl Acad Sci U S A **100**(15): 8839-43.

Huang, D., H. Friesen, et al. (2007). "Pho85, a multifunctional cyclin-dependent protein kinase in budding yeast." Mol Microbiol **66**(2): 303-14.

Huh, W. K., J. V. Falvo, et al. (2003). "Global analysis of protein localization in budding yeast." Nature **425**(6959): 686-91.

Jeffrey, P. D., A. A. Russo, et al. (1995). "Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex." Nature **376**(6538): 313-20.

Kellis, M., N. Patterson, et al. (2003). "Sequencing and comparison of yeast species to identify genes and regulatory elements." Nature **423**(6937): 241-54.

Kim, J. H. and M. Johnston (2006). "Two glucose-sensing pathways converge on Rgt1 to regulate expression of glucose transporter genes in Saccharomyces cerevisiae." J Biol Chem **281**(36): 26144-9.

Kobayashi, O., H. Suda, et al. (1996). "Molecular cloning and analysis of the dominant flocculation gene FLO8 from Saccharomyces cerevisiae." Mol Gen Genet **251**(6): 707-15.

Kornberg, R. D. (1999). "Eukaryotic transcriptional control." Trends Cell Biol **9**(12): M46-9.

Kron, S. J., C. A. Styles, et al. (1994). "Symmetric cell division in pseudohyphae of the yeast Saccharomyces cerevisiae." Mol Biol Cell **5**(9): 1003-22.

Kruckeberg, A. L., M. C. Walsh, et al. (1998). "How do yeast cells sense glucose?" Bioessays **20**(12): 972-6.

Kuchin, S., V. K. Vyas, et al. (2002). "Snf1 protein kinase and the repressors Nrg1 and Nrg2 regulate FLO11, haploid invasive growth, and diploid pseudohyphal differentiation." Mol Cell Biol **22**(12): 3994-4000.

Kuchin, S., P. Yeghiayan, et al. (1995). "Cyclin-dependent protein kinase and cyclin homologs SSN3 and SSN8 contribute to transcriptional control in yeast." Proc Natl Acad Sci U S A **92**(9): 4006-10.

Lai, C. C., M. Boguski, et al. (1993). "Influence of guanine nucleotides on complex formation between Ras and CDC25 proteins." Mol Cell Biol **13**(3): 1345-52.

Laloux, I., E. Dubois, et al. (1990). "TEC1, a gene involved in the activation of Ty1 and Ty1-mediated gene expression in Saccharomyces cerevisiae: cloning and molecular analysis." Mol Cell Biol **10**(7): 3541-50.

Lambrechts, M. G., F. F. Bauer, et al. (1996). "Muc1, a mucin-like protein that is regulated by Mss10, is critical for pseudohyphal differentiation in yeast." Proc Natl Acad Sci U S A **93**(16): 8419-24.

Langan, T. A., J. Gautier, et al. (1989). "Mammalian growth-associated H1 histone kinase: a homolog of cdc2+/CDC28 protein kinases controlling mitotic entry in yeast and frog cells." Mol Cell Biol **9**(9): 3860-8.

Lee, J. M. and A. L. Greenleaf (1991). "CTD kinase large subunit is encoded by CTK1, a gene required for normal growth of Saccharomyces cerevisiae." Gene Expr **1**(2): 149-67.

Lees, E. M. and E. Harlow (1993). "Sequences within the conserved cyclin box of human cyclin A are sufficient for binding to and activation of cdc2 kinase." Mol Cell Biol **13**(2): 1194-201.

Levin, D. E. and B. Errede (1995). "The proliferation of MAP kinase signaling pathways in yeast." Curr Opin Cell Biol **7**(2): 197-202.

Li, G. Y., M. Xing, et al. (2004). "A PSTAIRE CDK-like protein localizes in nuclei and cytoplasm of Physarum polycephalum and functions in the mitosis." Cell Res **14**(2): 169-75.

Liu, H., C. A. Styles, et al. (1993). "Elements of the yeast pheromone response pathway required for filamentous growth of diploids." Science **262**(5140): 1741-4.

Liu, H., C. A. Styles, et al. (1996). "Saccharomyces cerevisiae S288C has a mutation in FLO8, a gene required for filamentous growth." Genetics **144**(3): 967-78.

Lo, W. S. and A. M. Dranginis (1998). "The cell surface flocculin Flo11 is required for pseudohyphae formation and invasion by Saccharomyces cerevisiae." Mol Biol Cell **9**(1): 161-71.

Lo, W. S., E. I. Raitses, et al. (1997). "Development of pseudohyphae by embedded haploid and diploid yeast." Curr Genet **32**(3): 197-202.

Loeb, J. D., T. A. Kerentseva, et al. (1999). "Saccharomyces cerevisiae G1 cyclins are differentially involved in invasive and pseudohyphal growth independent of the filamentation mitogen-activated protein kinase pathway." Genetics **153**(4): 1535-46.

Long, R. M., R. H. Singer, et al. (1997). "Mating type switching in yeast controlled by asymmetric localization of ASH1 mRNA." Science **277**(5324): 383-7.

Lorenz, M. C. and J. Heitman (1997). "Yeast pseudohyphal growth is regulated by GPA2, a G protein alpha homolog." Embo J **16**(23): 7008-18.

Lorenz, M. C. and J. Heitman (1998). "The MEP2 ammonium permease regulates pseudohyphal differentiation in Saccharomyces cerevisiae." Embo J **17**(5): 1236-47.

Maciejewski, P. M., F. C. Peterson, et al. (1995). "Mutation of serine 90 to glutamic acid mimics phosphorylation of bovine prolactin." J Biol Chem **270**(46): 27661-5.

Madden, K. and M. Snyder (1998). "Cell polarity and morphogenesis in budding yeast." Annu Rev Microbiol **52**: 687-744.

Madhani, H. D. and G. R. Fink (1997). "Combinatorial control required for the specificity of yeast MAPK signaling." Science **275**(5304): 1314-7.

Maldonado, E., M. Hampsey, et al. (1999). "Repression: targeting the heart of the matter." Cell **99**(5): 455-8.

Mendenhall, M. D. and A. E. Hodge (1998). "Regulation of Cdc28 cyclin-dependent protein kinase activity during the cell cycle of the yeast Saccharomyces cerevisiae." Microbiol Mol Biol Rev **62**(4): 1191-243.

Morrison, L. E., H. E. Hoover, et al. (2003). "Mimicking phosphorylation of alphaB-crystallin on serine-59 is necessary and sufficient to provide

maximal protection of cardiac myocytes from apoptosis." Circ Res **92**(2): 203-11.

Murray, S., R. Udupa, et al. (2001). "Phosphorylation of the RNA polymerase II carboxy-terminal domain by the Bur1 cyclin-dependent kinase." Mol Cell Biol **21**(13): 4089-96.

Myer, V. E. and R. A. Young (1998). "RNA polymerase II holoenzymes and subcomplexes." J Biol Chem **273**(43): 27757-60.

Nasmyth, K. (1993). "Regulating the HO endonuclease in yeast." Curr Opin Genet Dev **3**(2): 286-94.

Nelson, C., S. Goto, et al. (2003). "Srb10/Cdk8 regulates yeast filamentous growth by phosphorylating the transcription factor Ste12." Nature **421**(6919): 187-90.

Nichols, N. M. and K. S. Matthews (2002). "Human p53 phosphorylation mimic, S392E, increases nonspecific DNA affinity and thermal stability." Biochemistry **41**(1): 170-8.

Olson, K. A., C. Nelson, et al. (2000). "Two regulators of Ste12p inhibit pheromone-responsive transcription by separate mechanisms." Mol Cell Biol **20**(12): 4199-209.

Orlova, M., E. Kanter, et al. (2006). "Nitrogen availability and TOR regulate the Snf1 protein kinase in Saccharomyces cerevisiae." Eukaryot Cell **5**(11): 1831-7.

Orphanides, G., T. Lagrange, et al. (1996). "The general transcription factors of RNA polymerase II." Genes Dev **10**(21): 2657-83.

Pan, X. and J. Heitman (1999). "Cyclic AMP-dependent protein kinase regulates pseudohyphal differentiation in Saccharomyces cerevisiae." Mol Cell Biol **19**(7): 4874-87.

Pan, X. and J. Heitman (2002). "Protein kinase A operates a molecular switch that governs yeast pseudohyphal differentiation." Mol Cell Biol **22**(12): 3981-93.

Parrini, M. C., A. Bernardi, et al. (1996). "Determinants of Ras proteins specifying the sensitivity to yeast Ira2p and human p120-GAP." Embo J **15**(5): 1107-11.

Peter, M. and I. Herskowitz (1994). "Joining the complex: cyclin-dependent kinase inhibitory proteins and the cell cycle." Cell **79**(2): 181-4.

Peter, M., A. M. Neiman, et al. (1996). "Functional analysis of the interaction between the small GTP binding protein Cdc42 and the Ste20 protein kinase in yeast." Embo J **15**(24): 7046-59.

Phatnani, H. P. and A. L. Greenleaf (2006). "Phosphorylation and functions of the RNA polymerase II CTD." Genes Dev **20**(21): 2922-36.

Pines, J. (1995). "Cyclins and cyclin-dependent kinases: a biochemical view." Biochem J **308 (Pt 3)**: 697-711.

Ptacek, J., G. Devgan, et al. (2005). "Global analysis of protein phosphorylation in yeast." Nature **438**(7068): 679-84.

Ren, B., F. Robert, et al. (2000). "Genome-wide location and function of DNA binding proteins." Science **290**(5500): 2306-9.

Riedl, T. and J. M. Egly (2000). "Phosphorylation in transcription: the CTD and more." Gene Expr **9**(1-2): 3-13.

Rigaut, G., A. Shevchenko, et al. (1999). "A generic protein purification method for protein complex characterization and proteome exploration." Nat Biotechnol **17**(10): 1030-2.

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**(24): 2974-85.

Roberts, R. L., H. U. Mosch, et al. (1997). "14-3-3 proteins are essential for RAS/MAPK cascade signaling during pseudohyphal development in S. cerevisiae." Cell **89**(7): 1055-65.

Robertson, L. S. and G. R. Fink (1998). "The three yeast A kinases have specific signaling functions in pseudohyphal growth." Proc Natl Acad Sci U S A **95**(23): 13783-7.

Rodriguez, C. R., E. J. Cho, et al. (2000). "Kin28, the TFIIH-associated carboxy-terminal domain kinase, facilitates the recruitment of mRNA processing machinery to RNA polymerase II." Mol Cell Biol **20**(1): 104-12.

Rohde, J. R., J. Trinh, et al. (2000). "Multiple signals regulate GAL transcription in yeast." Mol Cell Biol **20**(11): 3880-6.

Rupp, S., E. Summers, et al. (1999). "MAP kinase and cAMP filamentation signaling pathways converge on the unusually large promoter of the yeast FLO11 gene." Embo J **18**(5): 1257-69.

Russo, A. A., P. D. Jeffrey, et al. (1996). "Structural basis of cyclin-dependent kinase activation by phosphorylation." Nat Struct Biol **3**(8): 696-700.

Sadowski, I., T. C. Su, et al. (2007). "Disintegrator vectors for single-copy yeast chromosomal integration." Yeast **24**(5): 447-55.

Sanz, P. (2003). "Snf1 protein kinase: a key player in the response to cellular stress in yeast." Biochem Soc Trans **31**(Pt 1): 178-81.

Sarkar, G. and S. S. Sommer (1990). "The "megaprimer" method of site-directed mutagenesis." Biotechniques **8**(4): 404-7.

Siekhaus, D. E. and D. G. Drubin (2003). "Spontaneous receptor-independent heterotrimeric G-protein signalling in an RGS mutant." Nat Cell Biol **5**(3): 231-5.

Song, D., J. W. Dolan, et al. (1991). "Pheromone-dependent phosphorylation of the yeast STE12 protein correlates with transcriptional activation." Genes Dev **5**(5): 741-50.

Song, W. and M. Carlson (1998). "Srb/mediator proteins interact functionally and physically with transcriptional repressor Sfl1." Embo J **17**(19): 5757-65.

Stoldt, V. R., A. Sonneborn, et al. (1997). "Efg1p, an essential regulator of morphogenesis of the human pathogen Candida albicans, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi." Embo J **16**(8): 1982-91.

Sutherland, C. M., S. A. Hawley, et al. (2003). "Elm1p is one of three upstream kinases for the Saccharomyces cerevisiae SNF1 complex." Curr Biol **13**(15): 1299-305.

Tassan, J. P., M. Jaquenoud, et al. (1995). "Identification of human cyclin-dependent kinase 8, a putative protein kinase partner for cyclin C." Proc Natl Acad Sci U S A **92**(19): 8871-5.

Thompson, C. M., A. J. Koleske, et al. (1993). "A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast." Cell **73**(7): 1361-75.

Toda, T., S. Cameron, et al. (1987). "Cloning and characterization of BCY1, a locus encoding a regulatory subunit of the cyclic AMP-dependent protein kinase in Saccharomyces cerevisiae." Mol Cell Biol **7**(4): 1371-7.

Toda, T., S. Cameron, et al. (1987). "Three different genes in S. cerevisiae encode the catalytic subunits of the cAMP-dependent protein kinase." Cell **50**(2): 277-87.

Vallier, L. G. and M. Carlson (1994). "Synergistic release from glucose repression by mig1 and ssn mutations in Saccharomyces cerevisiae." Genetics **137**(1): 49-54.

Vincent, O., S. Kuchin, et al. (2001). "Interaction of the Srb10 kinase with Sip4, a transcriptional activator of gluconeogenic genes in Saccharomyces cerevisiae." Mol Cell Biol **21**(17): 5790-6.

Ward, M. P. and S. Garrett (1994). "Suppression of a yeast cyclic AMP-dependent protein kinase defect by overexpression of SOK1, a yeast gene exhibiting sequence similarity to a developmentally regulated mouse gene." Mol Cell Biol **14**(9): 5619-27.

Ward, M. P., C. J. Gimeno, et al. (1995). "SOK2 may regulate cyclic AMP-dependent protein kinase-stimulated growth and pseudohyphal development by repressing transcription." Mol Cell Biol **15**(12): 6854-63.

Webber, A. L., M. G. Lambrechts, et al. (1997). "MSS11, a novel yeast gene involved in the regulation of starch metabolism." Curr Genet **32**(4): 260-6.

Zeitlinger, J., I. Simon, et al. (2003). "Program-specific distribution of a transcription factor dependent on partner transcription factor and MAPK signaling." Cell **113**(3): 395-404.