2013-06-03

Investigation of Multiple Concerted Mechanisms Underlying Stimulus-induced G1 Arrest in Yeast: A Dissertation

Patricia A. Pope
University of Massachusetts Medical School

INVESTIGATION OF MULTIPLE CONCERTED MECHANISMS UNDERLYING STIMULUS-INDUCED G1 ARREST IN YEAST

A Dissertation Presented

By

Patricia A Pope

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

June 3, 2013

Interdisciplinary Graduate Program

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Patricia Anne Pope

The signatures of the Dissertation Defense Committee signifies completion and approval as to style and content of the Dissertation

Peter Pryciak, Ph.D., Thesis Advisor

Jennifer Benanti, Ph.D., Member of Committee

David Levin, Ph.D., Member of Committee

Nicholas Rhind, Ph.D., Member of Committee

William Theurkauf, Ph.D., Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

Dannel McCollum, Ph.D., Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the School

Anthony Carruthers, Ph.D.
Dean of the Graduate School of Biomedical Sciences

Interdisciplinary Graduate Program

June 3, 2013

Acknowledgments

Thank you to Peter for his support and suggestions over the years. Thank you also to my labmates, past and present, for being wonderful colleagues and friends. Thanks to Matt Winters (aka Prince Matthew) and Rachel Lamson for technical (and moral) support, and Samya Bhaduri for discussions both scientific and non-scientific. It has been an absolute privilege to work with all of you.

I need to thank the now former Biotech 4 community for providing a truly spectacular place to work. Everyone's willingness to share expertise, reagents, crazy lunchtime conversations or even the occasional shot of vodka out of a conical tube made it a joy to come to work every day.

Thank you to the members of my thesis committee, whose support has been invaluable. Thanks as well to Dr. David Levin, who has kindly agreed to serve as the outside member of my defense committee.

Finally, I must thank my family for their endless support through all my years of education. Thanks to my brother for always reminding me to not take life quite so seriously, my parents for never doubting me even at those times when I lost faith in myself, and my grandmother for showing me what living life to its fullest really means. And, of course, no mention of my family would be complete without a thanks to my two cats, Dobby and Winky, whose little furry faces were always there to greet me after a long day in lab.

Abstract

Progression through the cell cycle is tightly controlled, and the decision whether or not to enter a new cell cycle can be influenced by both internal and external cues. For budding yeast one such external cue is pheromone treatment, which can induce G1 arrest. Two distinct mechanisms are known to be involved in this arrest, one dependent on the arrest protein Far1 and one independent of Far1, but the exact mechanisms have remained enigmatic. The studies presented here further elucidate both of these mechanisms.

We looked at two distinct aspects of the Far1-independent arrest mechanism. First, we studied the role of the G1/S regulatory system in G1 arrest. We found that deletion of the G1/S transcriptional repressors Whi5 and Stb1 compromises Far1-independent arrest, but only partially, and that this partial arrest failure correlates to partial de-repression of G1/S transcripts. Deletion of the CKI Sic1, however, is more strongly required for arrest in the absence of Far1, though not when Far1 is present. Together, this demonstrates that functionally overlapping regulatory circuits controlling the G1/S transition collectively provide robustness to the G1 arrest response. We also sought to re-examine the phenomenon of pheromone-induced loss of G1/S cyclin proteins, which we suspected could be another Far1-independent arrest mechanism. We confirmed that pheromone treatment has an effect on G1 cyclin protein levels independent of transcriptional control. Our findings suggest that this phenomenon is dependent on SCF^{Grr1} but is at least partly independent of Cdc28

activity, the CDK phosphorylation sites in Cln2, and Far1. We were not, however, able to obtain evidence that pheromone increases the degradation rate of Cln1/2, which raises the possibility that pheromone reduces their synthesis rate instead.

Finally, we also studied the function of Far1 during pheromone-induced G1 arrest. Although it has been assumed that Far1 acts as a G1/S cyclin specific CDK inhibitor, there has been no conclusive evidence that this is the case. Our data, however, suggests that at least part of Far1's function may actually be to interfere with Cln-CDK/substrate interactions since we saw a significant decrease of co-pulldown of Cln2 and substrates after treatment with pheromone. All together, the results presented here demonstrate that there are numerous independent mechanisms in place to help robustly arrest cells in G1.

Table of Contents

List of Tables

List of Figures

Preface

Portions of this dissertation appear in separate publications:

Pope, P. and Pryciak, P.M. Functional overlap among distinct G1/S inhibitors in yeast allows robust G1 arrest and prevents premature cell cycle commitment. (Manuscript in preparation)

CHAPTER I

Introduction

Progression through the cell cycle is tightly controlled, and the transition from G1 to S phase in particular is a critical regulatory step in all organisms. In multicellular eukaryotes, including humans, cells must decide during G1 if they are going to divide, differentiate, or die, and this decision is influenced by a multitude of environmental cues. Improper responses to these external cues can lead to cancer, and many oncogenes and tumor suppressor genes have a link to G1 control. Inappropriate levels of cell cycle control related proteins such as Cyclin D, p27Kip1, and Rb have been linked to cancer (Massague, 2004); demonstrating the importance of appropriate control of the G1/S transition.

The following dissertation describes how one external cue, mating pheromone, can influence the cell cycle of the budding yeast *Saccharomyces cerevisiae*. Specifically, pheromone treatment induces a G1 arrest, and these studies look at two distinct mechanisms involved in this arrest, one dependent on the arrest protein Far1 and one independent of Far1. Although the existence of both of these pathways is well established the exact mechanisms have remained enigmatic. By studying how yeast control the G1/S transition in this setting we hope to shed further light on cell cycle control in all eukaryotic cells

Cell cycle Control in *Saccharomyces cerevisiae*

In all eukaryotes the cell cycle is controlled by cyclin dependent kinases (CDKs) (Morgan, 1997). In budding yeast, the primary CDK responsible for cell cycle control is Cdc28/Cdk1 and its association with a series of nine cyclins determines its specificity at various points in the cell cycle (Cross, 1995; Nasmyth, 1996; Koivomagi et al., 2011). Several waves of CDK activity control when and how the cells progress through the cell cycle, and consequently the expression and activation of cyclins is tightly regulated. There are three G1 cyclins in yeast: Cln1, Cln2, and Cln3. Deletion of all three cyclins causes the cells to permanently arrest in G1 (Hadwiger et al., 1989; Richardson et al., 1989; Cross, 1990). Although the presence of any one of these cyclins is sufficient to promote growth, there are distinct differences between their native functions (Dirick et al., 1995). *CLN3* is expressed at low levels throughout the cell cycle, with a slight peak in M phase that is necessary for initiation of the new cell cycle (Tyers et al., 1993; McInerny et al., 1997; MacKay et al., 2001). The two G1/S cyclins *CLN1* and *CLN2* peak late in G1 and are necessary for propelling the cells through Start, the point at which cells commit to completing a new cell cycle (Hartwell et al., 1974; Wittenberg et al., 1990; Tyers et al., 1993). The remainder of the cell cycle is controlled by a set of six B-type cyclins (Clb1-Clb6) that can be broken down into two subsets, S-phase cyclins (Clb5 and Clb6) and M-phase cyclins (Clb1-Clb4) required for the appropriate timing of mitosis (Nasmyth, 1996;

Bloom and Cross, 2007). Of particular interest to this dissertation is control of the G1/S transition, which will be discussed in detail in the following sections.

Control of the G1/S transition through G1 and G1/S cyclins

The yeast G1 cyclin Cln3 initiates a new cell cycle early in G1 by phosphorylating and deactivating the transcriptional repressor Whi5 (the yeast analog of RB), thereby allowing transcription of a host of G1/S genes including the G1/S cyclins *CLN1* and *CLN2* (Stuart and Wittenberg, 1995; Costanzo et al., 2004; de Bruin et al., 2004; Schaefer and Breeden, 2004). Cln1 and Cln2 can then activate a positive feedback loop by further increasing expression of the G1/S gene set (Skotheim et al., 2008). This process produces a rapid increase in transcription of the G1/S regulon in late G1, causing the cell to progress through Start, the yeast equivalent of the restriction point in higher eukaryotes (Hartwell et al., 1974; Blagosklonny and Pardee, 2002), and begin a new round of DNA replication.

CLN3 transcription is not as strongly cell cycle as the other cyclins (although it is subject to control based on nutrient conditions), so its cyclical function relies more heavily on post-transcriptional control of mRNA and protein abundance and localization (Tyers et al., 1992; Cross and Blake, 1993; Shi and Tu, 2013). In wild type cells Cln3 localizes to cytoplasmic punctae during early G1, and then accumulates in the nucleus in late G1 with a peak at Start (Wang et al., 2004). After Start Cln3 rapidly dissipates from the nucleus. This localization

of Cln3 is controlled at both the mRNA and protein level. Its cytoplasmic sequestration appears to occur by association of Cln3/CDK complexes with the ER, and dissociation of Cln3 from the ER correlates with its rapid entry into the nucleus and recruitment to promoters bound by the transcription factors SBF and MBF (Gari et al., 2001; Wang et al., 2004; Verges et al., 2007).

Once in the nucleus Cln3 is able to phosphorylate Whi5, causing Whi5 to dissociate from the promoters and exit the nucleus, which allows for initiation of the G1/S transcriptional program (de Bruin et al., 2004). It is this Cln3-mediated expulsion of Whi5 that appears to act as the primary cell size control mechanism in yeast cells. Once Whi5 exits the nucleus the time to budding is independent of cell size (Di Talia et al., 2007).

Although Cln3 phosphorylation of Whi5 is a primary regulator of the initial activation of the approximately 200 genes in the G1/S regulon (Spellman et al., 1998), it is not the only factor involved. There are two heterodimeric transcription factors involved in G1/S control known as MBF and SBF (Koch et al., 1993). Each contains a common regulatory subunit Swi6 and specific DNA binding subunits Mbp1 in MBF and Swi4 in SBF that bind specific recognition sites (Iyer et al., 2001). SBF appears to have a predominantly positive role in transcriptional control, whereas MBF seems to function as both a positive and negative regulator (de Bruin et al., 2004; de Bruin et al., 2006). These two transcription factors are aided in their control of G1/S transcription through their interaction with various regulatory proteins. The aforementioned inhibitor Whi5

binds SBF (and possibly MBF) during G1, repressing transcription until it is phosphorylated by Cln3-CDK, which causes it to dissociate from the promoter and leave the nucleus. Another factor, Stb1, binds both SBF and MBF through Swi6 and may have both repressive and activating properties since Stb1 appears to be required both for repression of G1/S transcription in early G1 as well as attainment of maximal levels of MBF-dependent gene expression (Ho et al., 1999; Costanzo et al., 2003; de Bruin et al., 2008). At least part of the repressive action of Whi5 and Stb1 stems from their role in recruiting histone deacetylase complexes to SBF and MBF-bound promoters. The Rpd3 HDAC complex can be found at SBF promoters in early G1, and appears to dissociate in a Cln3-dependent manner (Huang et al., 2009; Takahata et al., 2009; Wang et al., 2009).

After passage through Start the SBF complex dissociates from promoters, thereby stopping expression of those genes. MBF, however, remains associated with its promoters post-Start and transcription is instead constrained by the binding of the repressor Nrm1 (de Bruin et al., 2006).

The two G1/S cyclins Cln1 and Cln2 are at least partially redundant, although there does seem to be some specificity of function. The reason for this specificity is unclear, but there is evidence that localization of the two proteins could be involved (Edgington and Futcher, 2001; Quilis and Igual, 2012) . This observation suggests that the localization of cyclins could play a role in their specificity and function, and may also suggest a means of regulating activity

through regulation of localization, something that could become relevant in Chapter 4 where the role of Far1 in regulating cyclin proteins is discussed.

The important role of all of these factors in controlling the G1/S transition makes them potential targets for regulation during pheromone arrest, a topic that will be addressed further in Chapter 2.

Control of the G1/S transition through the CKI Sic1

Another established aspect of the G1/S regulatory machinery is Sic1. Sic1 is a B-type cyclin specific CDK inhibitor, analogous to mammalian p27(Kip1), that appears to play a role in the timing of B-type cyclin activity (Mendenhall, 1993; Schwob et al., 1994; Barberis et al., 2005). Although low levels of Sic1 are present throughout the cell cycle, peak levels are seen in late M and G1. Transcription of *SIC1* is controlled by the transcription factors Swi5 and Ace2, which activate *SIC1* transcription in late M phase Clb-dependent degradation of Swi5 then shuts off *SIC1* expression in G1 (Knapp et al., 1996; Toyn et al., 1997; Barberis, 2012). Increasing levels of Sic1 in late M appear to have a role in inhibiting Clb2-CDK activity to allow for mitotic exit. It is, however, Sic1's interaction with Clb5 and Clb6 during G1 that is most well characterized, and most relevant to G1/S regulation.

Transcription of the S-phase cyclins Clb5 and Clb6 is controlled by the MBF machinery, and is therefore upregulated at the same time as Cln1 and Cln2 (Schwob and Nasmyth, 1993). Their activity, however, is inhibited during G1 by

the presence of Sic1. During G1 Sic1 binds and inhibits Clb-CDK complexes, and in late G1 CDK-dependent phosphorylation targets Sic1 for ubiquitin-dependent degradation (Feldman et al., 1997; Verma et al., 1997; Nash et al., 2001; Koivomagi et al., 2011). Underlining the importance of Clb5 and Sic1 are numerous associated phenotypes. A strain deleted for all three G1 cyclins is inviable, however, overexpression of *CLB5* or deletion of Sic1 can restore viability (Oehlen et al., 1998). This suggests that control of Sic1/Clb5 may be a major factor of G1/S timing. A strain deleted for *SIC1* has a seventeen-fold increase in incidence of gross chromosomal rearrangements (Lengronne and Schwob, 2002), demonstrating that unscheduled activation of Clb activity in G1 can seriously impact the cell.

Sic1 stability appears to be modulated by carbon source, in cells grown in ethanol Sic1 demonstrates increased stability with a correlated decrease in Clb5 associated kinase activity. Suboptimal carbon sources also affect the cell cycle localization of Sic1, keeping Sic1 cytosolic longer, suggesting that Sic1 is involved in the cellular response to nutrient conditions (Rossi et al., 2005). Growth in a poor carbon source requires an overall slowing of the cell cycle to allow more time for the cell to reach a critical size, and the use of Sic1 as a means to control cell cycle progression in this instance demonstrates the importance of Sic1 in the timing of the G1/S transition. The role Sic plays in pheromone-induced G1 arrest will be discussed further in Chapter 2.

Mating: a yeast differentiation pathway

As with all eukaryotic cells, cell division in yeast is influenced by both external and internal signals, which determine if and when a cell will commit to another cell cycle. In higher eukaryotes these signals include hormones and growth factors, and in yeast they include nutrient levels and other environmental conditions. Another important regulator in yeast is the presence of mating pheromone (Bender and Sprague, 1986). Haploid *Saccharomyces cerevisiae* cells exist in two mating types (*MAT***a** or *MAT*α). Exposure of cells to the mating pheromone of the opposite type (α-factor or a-factor) results in activation of a MAP kinase cascade that leads to significant changes in gene expression patterns (increasing transcription of some genes and repressing others) and cell morphology. As a result, the cells arrest in G1, create mating projections (shmoos), and the two haploid cells fuse to create one diploid cell (Elion, 2000; Dohlman and Thorner, 2001).

The mating response initiates when pheromone is bound by a cell surface G-protein coupled receptor (Ste2 in *MAT***a** or Ste3 in *MAT*α) (Nakayama et al., 1987; Nomoto et al., 1990). Binding causes the release of G$\beta\gamma$ (Ste4 and Ste18) from Gα (Gpa1) on the inner surface of the plasma membrane (Miyajima et al., 1987; Whiteway et al., 1989). This free G$\beta\gamma$ is then able to recruit the cell polarity proteins required for mating projection formation as well as the MAP kinase scaffold protein Ste5 to the membrane along with its associated MAP kinases (Choi et al., 1994; Marcus et al., 1994; Pryciak and Huntress, 1998; Mahanty et

al., 1999; Nern and Arkowitz, 1999; Winters et al., 2005; Lamson et al., 2006). Once the MAP kinase cascade is associated with the membrane, the membrane-bound PAK family kinase Ste20 can then phosphorylate and activate the MAPKKK Ste11, which in turn phosphorylates and activates the MAPKK Ste7 (Neiman and Herskowitz, 1994; Zheng and Guan, 1994; Feng et al., 1998; Leeuw et al., 1998; Drogen et al., 2000). The subsequent activation of the MAPK Fus3 or Kss1 by Ste7 allows them to phosphorylate targets, at consensus S/T-P sites, that play roles in all aspects of the mating response (Errede et al., 1993; Ma et al., 1995; Bardwell et al., 1996). Although the two MAPK proteins are partially redundant Fus3 plays a greater role in the mating response (Elion et al., 1991; Madhani et al., 1997; Cherkasova et al., 1999; Breitkreutz et al., 2001). It is believed that Fus3, once phosphorylated, will dissociate from Ste5 and diffuse through the cytoplasm and nucleus in order to activate downstream effectors of the mating response (Elion et al., 1993; van Drogen et al., 2001).

Downstream effectors of the mating pathway

One known target of the MAPK is the transcriptional activator Ste12. Ste12 binds pheromone response elements (PRE) in the promoters of its target genes (Dolan et al., 1989). Prior to pheromone activation the repressors Dig1 and Dig2 bind Ste12, which keeps transcription shut off (Tedford et al., 1997; Chou et al., 2006). Activated MAPK phosphorylates these repressors, which then dissociate from Ste12, allowing Ste12 to induce transcription of

approximately 200 genes necessary for the mating process including some members of the mating response pathway (Dolan and Fields, 1990; Elion et al., 1993; Roberts et al., 2000).

Another known target of the MAPK is Far1 (Factor arrest), discovered, as its name suggests, in a screen for mutants that no longer arrest is response to pheromone (Chang and Herskowitz, 1990). Activated Far1 is believed to be a CDK inhibitor (CKI) specific for G1-cyclin (Cln)-CDK complexes, and can thus prevent the cyclins from promoting passage through Start (Chang and Herskowitz, 1992; Tyers and Futcher, 1993; Peter and Herskowitz, 1994; Gartner et al., 1998). The mating pathway and cell cycle progression are mutually antagonistic, meaning that components of the mating pathway can inhibit cell cycle progression, and the cell cycle machinery can likewise prevent pheromone pathway activation (Oehlen and Cross, 1994; Oehlen et al., 1998). Cells are only responsive to pheromone in late mitosis and G1, once past Start they will not respond to pheromone until after completion of the division cycle. There are a few ways in which the cell cycle inhibits pheromone signaling. Basal levels of expression of mating genes fluctuate during the cell cycle with a peak in early G1, and they decrease late in G1 when G1/S transcripts including *CLN1* and *CLN2* are at their highest (Oehlen and Cross, 1994; Wassmann and Ammerer, 1997). The expression and stability of Far1 itself is also tied to the cell cycle, the protein accumulates in G1 but is targeted for degradation as cells pass Start in a CDK-dependent manner (McKinney et al., 1993; Henchoz et al., 1997). Another

means of cell cycle regulation of the mating pathway is through regulation of the scaffold protein Ste5 and its recruitment to and association with the plasma membrane (Winters et al., 2005; Bhattacharyya et al., 2006). The G1/S cyclins inhibit the membrane localization of Ste5 by phosphorylating sites flanking a plasma membrane associating domain of Ste5 (Strickfaden et al., 2007). These phosphorylations prevent strong association of Ste5 with the membrane, and thereby prevent signaling through the mating pathway. This mechanism ensures that once a threshold of cyclin activity has been reached, the cell will no longer be able to activate the mating response, allowing it to progress to S phase without interference. If cells are exposed to pheromone in early G1, activated Far1 inhibits G1/S cyclin-CDK activity and thereby prevent this CDK inhibition of Ste5. Although Far1 is important for pheromone-induced G1 arrest, its exact molecular activity has remained somewhat controversial. Early reports suggested that Far1 directly inhibited CDK activity, but later work was unable to confirm this effect (Peter and Herskowitz, 1994; Gartner et al., 1998). Hence, it seems clear that Far1 serves to antagonize Cln-CDK activity in vivo, but the precise molecular mechanism by which this is accomplished is unclear. Chapter 4 of this dissertation returns to this question of Far1's role in CDK inhibition.

Far1 is regulated both positively and negatively following phoshorylation by both MAPKs and CDKs respectively. During pheromone treatment, the MAPK Fus3 phosphorylates Far1 at position T306, which enhances its binding to Cln2 (Gartner et al., 1998); consequently, a *far1-T306A* mutant strain is defective at

pheromone arrest, suggesting that Far1 interaction with Cln2 is vital for G1 arrest. CDK phosphorylation of Far1 at position S87 targets it for degradation; hence, a Far1-S87A mutant protein is significantly stabilized and causes increased sensitivity to pheromone (McKinney et al., 1993; McKinney and Cross, 1995; Henchoz et al., 1997; Doncic et al., 2011).

Although Far1 was originally discovered in a screen for mutants that could not arrest in response to pheromone, it was also observed that deletion of *CLN2* in a *far1*Δ strain could restore some level of G1 arrest (Chang and Herskowitz, 1992). This finding reveals that there must be some additional, Far1-independent route by which the pheromone pathway interferes with the G1/S transition. Indeed, the *far1*Δ arrest defect can also be suppressed by overexpressed or constitutively active forms of pheromone signaling proteins (Cherkasova et al., 1999). Nevertheless, these Far1-independent routes have remained undefined. Potential mechanisms for this Far1-independent arrest will be discussed further in Chapter 2.

Potential targets of this Far1-independent arrest include various aspects of the previously discussed G1/S regulatory machinery. Prior evidence suggested that Cln2 might be destabilized as a result of α-factor arrest (Valdivieso et al., 1993; Lanker et al., 1996). There is also evidence that the ubiquitin ligase SCF^{Grr1}, which is normally responsible for ubiquitination of the G1/S cyclins, is important for pheromone-induced G1 arrest (Schweitzer et al., 2005). Exactly what role Grr1 and/or Cln protein degradation might be playing in the pheromone

response will be further studied in Chapter 3. Additionally, in response to pheromone, the full complement of G1/S genes are not transcribed (Roberts et al., 2000). There is evidence that this process may, at least partially, be Far1-independent since previous work demonstrated that *CLN1* transcription is still repressed in response to pheromone in a *far1Δ cln2Δ* strain (Valdivieso et al., 1993). Whether this repression is the result of an active pheromone-induced mechanism or merely the result of the absence of CDK activation is unclear.

Recently, evidence suggesting that Far1 may have roles outside of the pheromone response has been mounting. Overexpression of *FAR1* produces cells that have increased cell volume, with a corresponding increase in RNA and protein synthesis suggesting a role for Far1 in global control of growth and macromolecule synthesis (Busti et al., 2012). It has also been shown that degradation of Far1, in a Cdc48-dependent manner, is necessary for progression past Start (Fu et al., 2003). Additionally, strains with deletions of various components of the pheromone pathway exhibit larger cell size suggesting that basal signaling through the mating pathway may antagonize growth (Goranov et al., 2009). Taken together, these data provide evidence that Far1 plays a role not only during pheromone response, but also during vegetative growth.

The role of Far1 in polarized growth

Aside from its role in pheromone-induced arrest Far1 is also important for control of polarized growth in both budding and shmooing cells (Valtz et al.,

1995). Polarized growth requires activation of the Rho family GTPase Cdc42 by its GEF Cdc24 to orient the actin cytoskeleton (Etienne-Manneville, 2004), and the proper recruitment of Cdc24 to the site of budding or shmooing is dependent on Far1 (Wiget et al., 2004). During G1, Cdc24 is localized in the nucleus, and becomes cytoplasmic at the time of bud emergence. Nuclear export of Cdc24 coincides with the peak of *CLN* expression, and activation of Cln-CDK activity appears sufficient to promote Cdc24 nuclear export. Far1 is degraded at the time of bud emergence in a CDK-dependent manner, and it appears this degradation of Far1 is critical for Cdc24's export from the nucleus, suggesting that Far1 serves to sequester Cdc24 in the nucleus until the time of bud emergence (Nern and Arkowitz, 2000; Shimada et al., 2000).

Far1 is also involved in the control of Cdc24 during mating, although the mechanism varies from that involved in budding. In response to pheromone a portion of Far1 goes from the nucleus to the cytoplasm, and it is recruited to the plasma membrane by the $G_{\beta\gamma}$ subunit. It appears that in this instance Far1 is able to bring Cdc24 along with it to the site of shmoo formation thereby initiating the pheromone induced polarized growth (Butty et al., 1998; Nern and Arkowitz, 2000; Shimada et al., 2000).

The role of Far1 in polarized growth shows that Far1 influences non-pheromone related cell functions, and also demonstrates how the cell can use localization of Far1 to control cellular functions; something to keep in mind in

Chapter 4 where we'll be discussing how Far1 could be regulated Cln-CDK activity.

A second CDK: Pho85

Budding yeast cells also possess a second CDK, Pho85, originally identified for its role in phosphate metabolism. Pho85 functions with its 10 associated cyclins, known as Pcls, in diverse aspects of nutrient response and cell cycle control (Toh-e et al., 1988; Measday et al., 1997). Deletion of *PHO85* is non-lethal, although the cells do appear to have an extended G1, and deletion of *PHO85* alongside *cln1Δ cln2Δ* is synthetically lethal indicating that the two CDKs, Pho85 and Cdc28, have some overlapping function. Transcription of *PCL1*, *PCL2*, and *PCL9* peaks in G1, similarly to *CLN1* and *CLN2*, making these cyclins the most likely candidates for any role in control of the G1/S transition (Nasmyth and Dirick, 1991; Measday et al., 1994; Measday et al., 1997). Indeed, although a *pcl1Δ pcl2Δ* or a *cln1Δ cln2Δ* mutant is viable a *pcl1Δ pcl2Δ cln1Δ cln2Δ* quadruple mutant is inviable, again suggesting some functional overlap between the two sets of cyclins (Espinoza et al., 1994; Measday et al., 1994). As with *CLN1* and *CLN2*, levels of *PCL1* and *PCL9* decrease after pheromone treatment, PCL2 on the other hand, increases (Measday et al., 1997). There is also mounting evidence that Pho85 can phosphorylate many substrates initially identified as Cdc28 targets, at least in some situations (Huang et al., 2009; Liu et al., 2011; Menoyo et al., 2013). Sic1, for example, can be phosphorylated by

Pcl1-Pho85 along with Cdc28, suggesting that Pho85 may play a role in G1/S control through Sic1 degradation (Nishizawa et al., 1998; Wysocki et al., 2006). There has also been evidence that Pho85 plays a role in regulation of Whi5 (Huang et al., 2009). Pcl9 can be found associated with the promoter of *CLN2* during G1, and this association is dependent on Whi5. Interestingly, although deletion of *CLN3* or *PHO85* causes relatively minor growth defects, the double *pho85Δ cln3Δ* has a much more pronounced growth phenotype, which can be suppressed by further deletion of *WHI5*. Taken together these data suggest that Cln3 and Pho85 work in separate, but converging, pathways to regulate Whi5 (Huang et al., 2009). The existence of an alternate CDK comes into play in later chapters where I'll discuss possible means of regulating Cdc28 targets during pheromone arrest when Cdc28 is normally inhibited.

Other G1 arrest mechanisms in yeast

The mating pathway is not the only pathway known to establish a G1 arrest, and it's possible that the Far1-independent arrest pathways use mechanisms similar to those used by some of these other pathways. The following are a few examples of other G1 arrest mechanisms that have been demonstrated in yeast.

Another MAPK cascade in yeast is the HOG pathway, responsible for mediating the cell's response to high osmolarity. Part of this response is cell cycle arrest in G1, and this arrest is dependent on Sic1 (Escote et al., 2004). In

response to high osmotic stress the MAPK Hog1 directly phosphorylates Sic1 at T173, and this phosphorylation stabilizes Sic1. The absence of Sic1 abolishes the high osmolarity induced G1 arrest, as does replacement of T173 with an alanine. Although this exact mechanism is unlikely to be involved in pheromone arrest, since pheromone-arrested cells in this study did not have the indicated Sic1 phosphorylation, it still serves to demonstrate the critical role Sic1 plays in G1 control and G1 arrest.

Activation of the G1 checkpoint in eukaryotes, often as a result of DNA damage, also results in a G1 delay (Siede et al., 1994; Bartek and Lukas, 2001). In yeast, the most interesting implication of this arrest from a G1/S control standpoint arises not from the mechanism of delay, but from the details of how the cells eventually escape G1 and enter S phase. During the response to DNA damage the cells prevent accumulation of Cln cyclins, thereby preventing inactivation of Sic1 and subsequent activation of Clb-CDK activity. But then how, in the absence of Cln-CDK activity, can the cells ever release from this G1 delay? In a screen for mutants with defects in recovery after DNA damage the CDK Pho85 was recovered. It appears that Pho85 plays a critical role in the cell's exit from the DNA damage checkpoint, and that this function involves interaction with Sic1 (Wysocki et al., 2006). This observation provides further evidence for the importance of Sic1 in controlling G1 arrest, as well as the possibility that Pho85 can play a role in control of the G1/S transition.

CHAPTER II

Functional overlap among distinct G1/S inhibitors in yeast allows robust G1 arrest and prevents premature cell cycle commitment.

The following chapter contains the manuscript:

Pope, P. and Pryciak, P.M. Functional overlap among distinct G1/S inhibitors in yeast allows robust G1 arrest and prevents premature cell cycle commitment. (Manuscript in preparation)

I solely performed all experiments presented. The manuscript was prepared by myself and Dr. Peter Pryciak.

Abstract

In budding yeast, mating pheromones arrest the cell cycle in G1 phase via a pheromone-activated Cdk-inhibitor (CKI) protein, Far1. Yet alternate routes must also exist, because deletion of the cyclin gene *CLN2* restores pheromone arrest to *far1Δ* cells, though by unknown mechanisms. In metazoans, transcriptional repression by Rb proteins and CKI activity of p27/p21 (Kip1/Cip1) proteins together inhibit cell cycle entry. Thus, here we investigated whether yeast pheromone arrest involves the G1/S transcriptional repressors Whi5 and Stb1, or the p27-like CKI protein Sic1. Indeed, *whi5*Δ and *sic1*Δ mutations each caused increased escape from G1 in cells lacking Far1 (i.e., *far1*Δ *cln2*Δ), though not when Far1 was present. Notably, removing the transcriptional repressors caused only partial de-repression of G1/S genes in pheromone-treated cells, and hence only a partial G1 escape phenotype, indicating that pheromone signaling can still inhibit G1/S transcription in their absence. Deletion of *SIC1* had a stronger effect, as it almost entirely abolished pheromone-induced G1 arrest in *far1*Δ *cln2*Δ cells. Therefore, inhibition of B-type cyclin-Cdk activity helps ensure that cell cycle entry can still be blocked when signal-mediated arrest is compromised. Interestingly, although *far1Δ cln2Δ sic1Δ* cells escaped G1 arrest, they failed to proliferate and instead lost viability during pheromone exposure, indicating that exit from G1 is deleterious if pheromone signaling persists. Overall, our findings illustrate how functional overlap among distinct G1/S braking mechanisms can

ensure a robust signal-induced G1 arrest and help prevent premature cell cycle commitment.

Introduction

Cell cycle progression in all organisms is regulated by both internal and external cues. In eukaryotes, the G1 phase of the cell cycle provides a critical period in which cells monitor whether conditions are appropriate for entry into a new division cycle (Morgan, 2007). Signals that control this decision include positive and negative growth factors, differentiation triggers, nutrient levels, and environmental stresses. These regulatory signals either promote or prevent the transition from a stable G1 state to a new round of DNA synthesis and mitosis. Often, cells become insensitive to these regulatory signals once they initiate the G1/S transition, establishing a cell cycle commitment phenomenon known as "Start" in yeast or the "Restriction Point" in animal cells (Hartwell et al., 1974; Pardee, 1974; Cross, 1995; Blagosklonny and Pardee, 2002). Accordingly, signals that influence cell cycle entry generally impact the molecular machinery that controls the G1/S transition. In all eukaryotes, cell cycle transitions are promoted by the activity of cyclin dependent kinases (Cdks) as well as by coordinate changes in gene expression, and these promoting factors are often held in check by inhibitory molecules to ensure that cell cycle progression is carefully controlled (Morgan, 2007). Curiously, the overall architecture of the G1/S regulatory network is strongly conserved throughout eukaryotes, yet some of the individual components may have evolved separately in yeasts versus animals (Cross et al., 2011).

In the budding yeast *Saccharomyces cerevisiae*, a single Cdk, Cdc28, associates with nine different cyclins that help drive distinct cell cycle events (Bloom and Cross, 2007). The transition from G1 to S phase is predominantly controlled by the G1 cyclin Cln3 along with two G1/S cyclins Cln1 and Cln2, whereas the subsequent events of DNA synthesis and mitosis (in S and M phases) are driven by six B-type cyclins (Clb1-Clb6). The decision of yeast cells to enter a new cell cycle can be profoundly influenced by the presence of an external cue known as mating pheromone, which promotes fusion of two haploid mating partner cells (Hartwell, 1973). During this mating reaction, pheromone activates an intracellular signaling pathway that arrests the cell cycle in G1 phase, prior to Start (Dohlman and Thorner, 2001; Bardwell, 2005). An important factor in this G1 arrest pathway is the protein Far1, as *far1*Δ cells do not arrest in response to pheromone (Chang and Herskowitz, 1992). Far1 is thought to be a Cdk inhibitor (CKI) protein that blocks the activity of Cln-Cdc28 complexes and thereby prevents progression through Start (Peter et al., 1993; Tyers and Futcher, 1993; Peter and Herskowitz, 1994; Jeoung et al., 1998), although some findings conflict with this interpretation (Gartner et al., 1998). Notably, however, in some circumstances Far1 is actually dispensable for G1 arrest. For example, removing the G1/S cyclin Cln2 from *far1*Δ cells (i.e., *far1*Δ *cln2*Δ) restores pheromone-induced G1 arrest (Chang and Herskowitz, 1992; Cherkasova et al., 1999). Thus, even in the complete absence of Far1, pheromone signaling still

can interfere with the ability of cells to pass Start and enter a new division cycle. Despite early recognition of this fact, the molecular mechanisms responsible for Far1-independent arrest have remained obscure.

In this study, we wished to probe the Far1-independent arrest mechanisms more closely, in order to better understand how pheromone signaling regulates G1 arrest and to provide more general insights into the multiplicity of factors that control cell cycle commitment decisions. We reasoned that the effects of pheromone signaling might depend on known negative regulators of the G1/S transition that act as “brakes” to antagonize cell cycle entry in many eukaryotes (Morgan, 2007). One such negative regulator is the CKI protein Sic1 (Donovan et al., 1994; Schwob et al., 1994), which is functionally analogous to the mammalian CKI p27(Kip1) (Sherr and Roberts, 1999). During G1, these CKI proteins inhibit Cdks bound to B-type cyclins, and thereby prevent premature entry into S phase. This inhibition is eventually released in late G1, when G1/S cyclin-Cdk activity reaches levels sufficient to target the CKI for degradation (Schwob et al., 1994; Nash et al., 2001; Cross et al., 2007; Koivomagi et al., 2011). Another mode of negative regulation involves transcriptional repression of genes expressed at the G1/S boundary. In animal cells, transcription of G1/S genes is activated by the E2F family of heterodimeric transcription factors and repressed by members of the retinoblastoma protein (Rb) family (Frolov and Dyson, 2004; van den Heuvel and Dyson, 2008). Yeast

cells have an analogous system (Bahler, 2005; Wittenberg and Reed, 2005), in which G1/S transcription is driven by two heterodimeric transcription factors called SBF (Swi4-Swi6) and MBF (Mbp1-Swi6), and is inhibited in early G1 by the repressors Whi5 and Stb1 (Koch et al., 1996; Costanzo et al., 2003; Costanzo et al., 2004; de Bruin et al., 2004; Bean et al., 2005; de Bruin et al., 2008). These repressors block the activity of DNA-bound SBF and MBF in part by recruiting repressive histone deacetylases (Huang et al., 2009; Takahata et al., 2009; Wang et al., 2009); in late G1, they are phosphorylated and inactivated by G1 and G1/S cyclin-Cdk complexes, allowing SBF/MBF-dependent transcription to ensue (Costanzo et al., 2004; de Bruin et al., 2004; Wagner et al., 2009; Doncic et al., 2011). Important targets of SBF and MBF include the G1/S cyclin genes *CLN1* and *CLN2*, which yield increased G1/S Cdk activity, thereby creating a positive feedback loop that helps ensure a decisive and coherent G1/S transition (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991; Skotheim et al., 2008). MBF/SBF-regulated genes are not expressed in pheromone-arrested cells, regardless of whether the arrest is Far1-dependent or Far1-independent (Wittenberg et al., 1990; Cherkasova et al., 1999), but it has been unclear whether this inhibited transcriptional state is a cause or an effect of G1 arrest.

Here, we report that although the transcriptional repressors Whi5 and Stb1 ensure full repression of G1/S transcripts during G1 arrest, pheromone signaling can still inhibit peak G1/S gene expression in their absence. Accordingly,

deletion of these repressors compromises Far1-independent arrest, but only partially. By comparison, the CKI Sic1 is more strongly required for arrest in the absence of Far1, though not when Far1 is present. Our results reveal that multiple, functionally overlapping regulatory circuits controlling the G1/S transition collectively provide robustness to the G1 arrest response, which may help ensure that commitment to cell cycle entry occurs decisively rather than tentatively.

Materials and Methods

Yeast Strains and Plasmids

Standard procedures were used for growth and genetic manipulation of yeast (Rothstein, 1991; Sherman, 2002). Yeast cultures were grown at 30˚C. Yeast strains are listed in Table 1; all were derived from the W303 background (Thomas and Rothstein, 1989) and harbor the *bar1*Δ mutation to block α factor degradation. PCR-mediated gene targeting used methods described previously (Longtine et al., 1998); selectable markers included antibiotic resistance genes (*kanMX6*, *natMX6*) and orthologs of biosynthesis genes from other yeasts (*S. kluyveri HIS3*, *C. glabrata TRP1*, *K. lactis URA3*). To ensure that genetic effects were reproducible, independently derived strains of identical genotype were tested in parallel, and the combined results were averaged. For cell synchronization experiments, the promoter of the essential cell cycle gene *CDC20* was replaced with a regulated promoter (P_{GAL1}) using a PCR-generated cassette marked with the *K. lactis URA3* gene ($URA3^{K.l.}$). For Cdc28 inhibition experiments, the *CDC28* gene was replaced with an ATP analog-sensitive allele *cdc28-as2* via a two-step pop-in/pop-out method (Rothstein, 1991); we used the Cdc28-as2 [F88A] mutant (Colman-Lerner et al., 2005) because in our strains the more severe mutant Cdc28-as1 [F88G] (Bishop et al., 2000) was hypomorphic, as indicated by slow growth and cell shape defects.

Synchronous Culture Assays

As in previous studies (Cosma et al., 2001; Bean et al., 2005; Takahata et al., 2009), cell cultures were synchronized in mitosis and then released for entry into the next cell cycle by using strains in which the *CDC20* gene was under control of the *GAL1* promoter. These P_{GAL1}-*CDC20* strains were grown asynchronously in liquid YPGal medium (containing 2% galactose), and then cultures were arrested in M phase by pelleting and resuspending in YPD medium (2% glucose), followed by incubation for 3 hr. Cultures were released from the M phase block by two rounds of pelleting and washing in YPGal, resuspension in YPGal either with or without α factor (0.2 μM), and incubation (with shaking) for 0 to 240 minutes.

Flow Cytometry and Budding Assays

DNA content was measured by flow cytometry using methods described previously (Haase and Reed, 2002; Strickfaden et al., 2007). Briefly, cell aliquots (0.5 ml) were harvested by centrifugation, resuspended in 0.3 ml sterile water, fixed by addition of 0.7 ml 100% ethanol, mixed by inversion, and incubated overnight at 4˚C. Fixed cells were pelleted, washed once with water, resuspended in 0.5 ml freshly prepared RNase solution (2 mg/ml RNase A in 50 mM Tris-HCl, pH 8.0, 15 mM NaCl), and incubated for 2 hr at 36˚C. They were then pelleted and resupended in 0.2 ml fresh proteinase solution (1 mg/ml proteinase K in 50 mM Tris-HCl pH 8.0), incubated for 1 hr at 36˚C, then pelleted

and resuspended in 0.5 ml 50mM Tris-HCl, pH 7.5, and stored at 4˚C. Before analysis, the suspensions were sonicated (10 pulses with a micro-tip probe), and then 50 µl was mixed with 1 ml fresh Sytox Green solution (1 µM in 50 mM Tris-HCl, pH 7.5), gently vortexed, and analyzed with a Becton-Dickinson FACScan flow cytometer. For experiments that were directly compared with each other, a uniform range of fluorescence values was defined for the 2C peak (generally 100-150 units) and for the total (including 1C, 2C, and intermediate; generally 40-200 units), and then %2C was calculated as 100% x 2C/total.

To analyze cell cycle position by budding, cells aliquots (0.5 ml) were fixed by the addition of formaldehyde to 3.7% final concentration, incubated on nutator (room temperature, 10 min.), washed three times with phosphate-buffered saline (PBS), and resuspended in 500 µl PBS. Fixed cells were spotted onto glass slides and viewed microscopically to score budded and unbudded cells; for each experimental condition, 200 cells were counted.

mRNA Preparation and RT-qPCR Analysis

As described previously (de Bruin et al., 2008), RNA was prepared using a Qiagen RNeasy Plus Mini Kit (#74134). Cells (~5 x 10^7) were harvested by centrifugation and frozen in liquid nitrogen. Cell pellets were resuspended in 400 µl of Qiagen RLT-plus buffer freshly supplemented with β-mercaptoethanol (10 µl per ml of buffer), and transferred to a micro-centrifuge tube. Approximately 400 µl of acid-washed glass beads were added, and cells were lysed by vortexing (4

cycles of 1 min., interspersed with rest periods of 3 min. on ice). The tube was punctured at bottom with a needle, placed in another tube, and centrifuged briefly (10 sec) to transfer the lysate to the fresh tube. Then, cell debris was removed by centrifugation (2 min., full speed). The supernatant was loaded onto Qiagen gDNA Eliminator columns, and then mRNA was prepared according to the manufacturer's instructions, diluted to equal concentrations (0.5 µg/µl), and stored at -70˚C. cDNA was synthesized from the RNA samples using Invitrogen SuperScript VILO cDNA synthesis kit (#11754) as per the manufacturer's instructions, using ~2 µg of RNA per reaction. Products were then diluted to final concentration of 2.5 ng/µl.

Quantitative real-time PCR reactions were performed using Applied Biosystems Power SYBR Green PCR Master Mix (#4367659). Reaction mixtures (15 µl) contained 7.5 µl of SYBR Green reaction mix, 2 µl of primer mix (3 µM each primer), 1 µl of cDNA (2.5 ng), and 4.5 µl of water. Reactions were performed in 96-well plates, in duplicate, using an Applied Biosystems StepOnePlus instrument. Preliminary trials using multiple primers for each gene were performed to identify primer sets with optimal properties (linearity of amplification) for use in all subsequent experiments. Primers are listed in Table 2. The $\Delta\Delta C_T$ method was used to convert real-time amplification kinetics into relative mRNA levels; *ACT1* mRNA served as the internal control.

Cell Viability Assays

Asynchronous cultures were treated with pheromone to a final concentration of 0.2 μM for 1-4 hr. Aliquots were collected before and after treatment, sonicated (7 pulses with a micro-tip probe), diluted in sterile PBS, spread on solid synthetic medium, and incubated for 2 days. Viable colonies were counted and expressed as a percentage of the number formed by equivalent aliquots of the initial, un-treated cultures.

Table 2.1. Yeast strains used in Chapter II

Name	Relevant Genotype*	Source
PPY1716	*MAT***a** *bar1*	1
PPY1748	*MAT***a** *bar1 STE5-8A*	2
PPY1777	*MAT***a** *bar1 far1Δ::ADE2*	2
PPY1789	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6*	2
PPY1867	*MAT***a** *bar1 cln2Δ::kanMX6*	this study
PPY1913	*MAT***a** *bar1 HIS3::P_{GPD1}-CLB5*	2
PPY1918	*MAT***a** *bar1 STE5-8A HIS3::P_{GPD1}-CLB5*	2
PPY2013	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 P_{GAL1}-CDC20::URA3^{KI}*	this study
PPY2014	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 P_{GAL1}-CDC20::URA3^{KI}*	this study
PPY2019	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 mbp1Δ::TRP1^{Cg} P_{GAL1}-CDC20::URA3^{KI}*	this study
PPY2020	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 mbp1Δ::TRP1^{Cg} P_{GAL1}-CDC20::URA3^{KI}*	this study
PPY2043	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 sic1Δ::TRP1^{Cg}*	this study
PPY2063	*MAT***a** *bar1 P_{GAL1}-CDC20::URA3^{KI}*	this study
PPY2064	*MAT***a** *bar1 P_{GAL1}-CDC20::URA3^{KI}*	this study
PPY2068	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 sic1Δ::TRP1^{Cg} P_{GAL1}-CDC20::URA3^{KI}*	this study
PPY2069	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 sic1Δ::TRP1^{Cg} P_{GAL1}-CDC20::URA3^{KI}*	this study
PPY2082	*MAT***a** *bar1 far1Δ::ADE2 P_{GAL1}-CDC20::URA3^{KI}*	this study
PPY2083	*MAT***a** *bar1 far1Δ::ADE2 P_{GAL1}-CDC20::URA3^{KI}*	this study
PPY2085	*MAT***a** *bar1 sic1Δ::TRP1^{Cg}*	this study
PPY2087	*MAT***a** *bar1 far1Δ::ADE2 sic1Δ::TRP1^{Cg}*	this study
PPY2090	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 mbp1Δ::TRP1^{Cg} whi5Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study
PPY2091	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 mbp1Δ::TRP1^{Cg} whi5Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study
PPY2128	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 mbp1Δ::TRP1^{Cg} whi5Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP137	*MAT***a** *bar1 cln2Δ::kanMX6 stb1Δ::natMX6 P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP138	*MAT***a** *bar1 cln2Δ::kanMX6 stb1Δ::natMX6 P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP141	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 stb1Δ::natMX6 P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP142	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 stb1Δ::natMX6 P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP143	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 rpd3Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP144	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 rpd3Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP151	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 whi5Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP152	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 whi5Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP153	*MAT***a** *bar1 cln2Δ::kanMX6 whi5Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP156	*MAT***a** *bar1 cln2Δ::kanMX6 whi5Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP157	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 stb1Δ::natMX6 whi5Δ::HIS3^{Sk}*	this study
YPAP161	*MAT***a** *bar1 cln2Δ::kanMX6 stb1Δ::natMX6 whi5Δ::HIS3^{Sk}*	this study
YPAP165	*MAT***a** *bar1 cln2Δ::kanMX6 P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP166	*MAT***a** *bar1 cln2Δ::kanMX6 P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP167	*MAT***a** *bar1 cln2Δ::kanMX6 stb1Δ::natMX6 whi5Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP168	*MAT***a** *bar1 cln2Δ::kanMX6 stb1Δ::natMX6 whi5Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP171	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 stb1Δ::natMX6 whi5Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP172	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 stb1Δ::natMX6 whi5Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP203	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 rpd3Δ::HIS3^{Sk} whi5Δ::TRP1^{Cg} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP204	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 rpd3Δ::HIS3^{Sk} whi5Δ::TRP1^{Cg} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP208	*MAT***a** *bar1 cln2Δ::natMX6 sic1Δ::TRP1^{Cg}*	this study
YPAP209	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 sic1Δ::TRP1^{Cg} whi5Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP210	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 sic1Δ::TRP1^{Cg} whi5Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP236	*MAT***a** *bar1 cln2Δ::natMX6 sic1Δ::TRP1^{Cg} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP237	*MAT***a** *bar1 cln2Δ::natMX6 sic1Δ::TRP1^{Cg} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP238	*MAT***a** *bar1 cln2Δ::natMX6 sic1Δ::TRP1^{Cg} whi5Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP239	*MAT***a** *bar1 cln2Δ::natMX6 sic1Δ::TRP1^{Cg} whi5Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP240	*MAT***a** *bar1 cln2Δ::kanMX6 cdh1Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP241	*MAT***a** *bar1 cln2Δ::kanMX6 cdh1Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP242	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 cdh1Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP243	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 cdh1Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP244	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 cdh1Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study
YPAP245	*MAT***a** *bar1 far1Δ::ADE2 cln2Δ::kanMX6 cdh1Δ::HIS3^{Sk} P_{GAL1}-CDC20::URA3^{KI}*	this study

* All strains are in the W303 background (*ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1*). In the *P_{GAL1}-CDC20* strains, a cassette containing the *URA3^{KI}* marker and *GAL1* promoter is inserted in place of the *CDC20* promoter at the native *CDC20* locus.

Source: (1)(Zimmerman and Kellogg, 2001) (2)(Strickfaden et al., 2007)

Table 2.2. Oligonucleotide primers used for RT-qPCR analysis

Primer name	Sequence (5' to 3')
CLN1-fw1	CTTTGGTTAGCGGCCAAAAC
CLN1-rev1	AGAAAGGCGTGGAATACGAG
YOX1-up1	AAATAGGCGCTCATCCACAC
YOX1-dn1	ACGTTTTCACGGGAGTCAAC
RNR1-up1	TCGAGGCTGCTTTAGAAACG
RNR1-dn1	GGCAACCAAGAAACAAGAGG
POL1-fw1	TGACATTTGCTCTGGTAGGC
POL1-rev1	CGGCTTATGCTCCTTTTCAC
CDC21-fw1	GGAACCCAGCTGATTTTGAC
CDC21-rev1	CGGATCCTTCTCCTTCTTTG
SIC1-fw1	CCAAAAGCCTTCACAGAACC
SIC1-rev1	GAGAGGTCATACCCATGTTCG
ACT1-fw1	TTCCAGCCTTCTACGTTTCC
ACT1-rev1	CCAGCGTAAATTGGAACGAC

Table 2.3. Plasmids used in Chapter II

Name	Alias	Description	Source
pPP681	pRS316	CEN URA3 vector	1
pPP4042	YCplac33-TEC1	CEN URA3 TEC1	2
pPP4043	YCplac33-tec1-T273M	CEN URA3 TEC1-T273M	2

Source: (1)(Sikorski and Hieter, 1989) (2) (Bao et al., 2004)

Results

Studying Far1-independent G1 arrest using synchronous cultures

In order to test the contribution of various regulatory factors to pheromone arrest, in both the presence and absence of Far1, we used synchronous cultures to measure the duration of G1 phase. The essential cell cycle gene *CDC20* was placed under control of a regulated promoter (P_{GAL1}), allowing cells to be arrested in M phase and then released in the presence or absence of pheromone. Cell cycle progression was monitored by measuring DNA content (using flow cytometry) and/or by scoring budding. This approach offered three useful features: (i) by measuring G1 duration, even relatively subtle and temporary effects on the G1/S transition could be detected; (ii) by using synchronized cells, the uniformity of phenotypes could be readily assessed; and (iii) the specific effects of pheromone on G1 arrest could be distinguished from effects on other cell cycle stages or on cell viability (which will become important later).

In this experimental context (Figure 2.1A), most cells finish mitosis and enter G1 (i.e., 1C DNA) by 30-60 minutes after release from the M-phase block, and then begin a new round of DNA synthesis roughly 30 minutes later (at 90 min). If pheromone was added, wild-type cells completed mitosis normally and

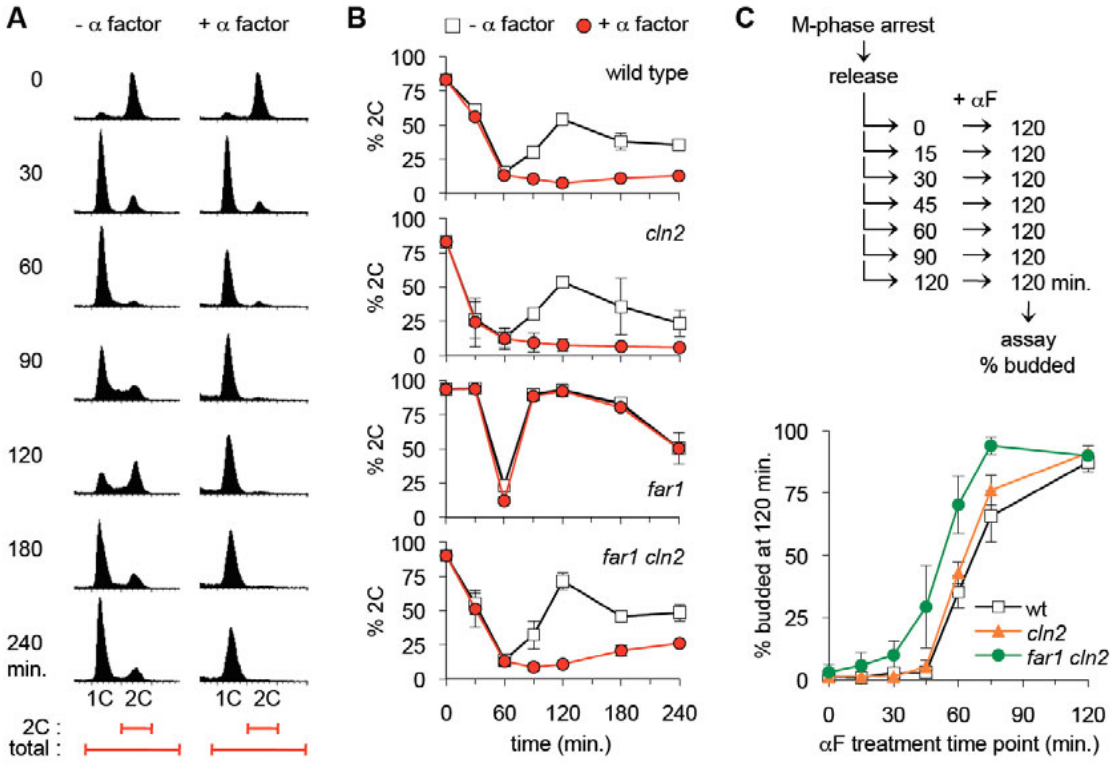
Figure 2.1. Far1-independent arrest and cell cycle commitment in synchronous cultures.

(**A**) Example of synchronous cell cycle progression and G1 arrest. A P_{GAL1}-*CDC20* strain was arrested in M-phase (by transfer to glucose medium) and then released (by return to galactose medium) in the presence or absence of α factor. At the times indicated, DNA content of cells was assayed by flow cytometry. In each histogram, the horizontal axis represents fluorescence, and the vertical dimension shows the number of cells. At bottom is shown the range of fluorescence values used to calculate the proportion of cells with replicated DNA (%2C) in subsequent figures. This example shows a *cln2*Δ strain (YPAP165).

(**B**) The ability of α factor to halt cell cycle progression was analyzed for four strains, using the P_{GAL1}-*CDC20* method described in panel (A). Graphs show mean ± range (n = 2) for wild type and *far1*Δ, or mean ± SD (n = 4) for *cln2*Δ and *far1*Δ *cln2*Δ strains.

(**C**) Cell cycle commitment occurs earlier in the absence of Far1. After releasing P_{GAL1}-*CDC20* cultures from the M-phase block, aliquots were removed at 15-minute intervals and treated with pheromone. At 120 min, cells were scored for whether they had arrested in G1 (unbudded cells) or entered the cell cycle (budded). Graphs show mean ± SD (n = 3).

Figure 2.1. Far1-independent arrest and cell cycle commitment in synchronous cultures.



then arrested in G1 (for > 3 hr). To compare multiple different strains and replicate experiments, we plotted the percentage of cells with 2C DNA content as a function of time (Figure 2.1B); generally, the M-phase arrested cultures were 80-90% 2C, and by 60 minutes after release they had cycled back to G1 and were predominantly 1C (i.e., only 10-15% 2C). As expected, *far1*Δ cells did not arrest in G1 in the presence of pheromone (Figure 2.1B). (In addition, with or without pheromone they showed an accelerated return to the 2C state after completing mitosis, a behavior consistent with previous findings that Far1 can alter the timing of the G1/S transition even without pheromone treatment (Alberghina et al., 2004).) In contrast, when *far1*Δ *cln2*Δ cells were released in the presence of pheromone, they completed mitosis and then remained in G1 for an extended period (Figure 2.1B). The arrest in the *far1*Δ *cln2*Δ strain was not as robust as in wild type or *FAR1 cln2*Δ strains, as evidenced by the gradual increase in cells with 2C DNA content beginning at 120-150 minutes after release. Thus, G1 arrest in the *far1*Δ *cln2*Δ cells is partially leaky, but pheromone clearly imposes a durable G1 delay that affects the majority of cells in the culture.

Separately, we tested how Far1 affects the window of time in which cells commit to a new division cycle (Figure 2.1C). After releasing cultures from the M-phase block, aliquots were removed at short intervals and treated with pheromone to test if the cells could still arrest in G1 or were already committed to

division. In wild type and *cln2*Δ strains, cells transitioned from fully uncommitted (> 95% arrest) to substantially committed (< 65% arrest) between the 45 and 60 minute time points. In the *far1*Δ *cln2*Δ strain, two differences were evident (Figure 2.1C). First, the commitment point occurred roughly 10-15 minutes earlier, as judged by the time at which 50% of cells still arrest. Second, the transition was less sharp, as evidenced by a more gradual increase in the fraction of cells that could not arrest. Together, these data illustrate that Far1 makes the arrest mechanism more potent, as it allows pheromone to arrest cells that have advanced closer to Start, and also more robust, as the arrest is more uniform in *FAR1 cln2*Δ than *far1*Δ *cln2*Δ cells. These findings complement a recent study in which the commitment point could be delayed by a stabilized form of Far1 (Doncic et al., 2011). Nevertheless, despite these clear effects of Far1, our results show that a commitment point still exists in the absence of Far1, albeit one that is advanced so that the time window in which arrest can be imposed is more limited. In experiments to follow, the Far1-independent arrest phenotype in *far1*Δ *cln2*Δ strains serves as sensitized setting in which to test the contribution of other factors that affect the G1/S transition.

Role of G1/S transcriptional repressors in G1 arrest

Previous studies found that expression of G1/S transcripts is inhibited in pheromone-arrested cells (Wittenberg et al., 1990), even when the arrest is Far1-

independent (Cherkasova et al., 1999). Yet it has remained unclear whether this inhibition is a cause or an effect of the G1 arrest. Hence, to determine if Far1-independent arrest relies on control of G1/S transcription, we probed the role of Whi5 and Stb1, the repressors of the G1/S transcription factors SBF and MBF (Figure 2). (Reports vary as to whether Whi5 inhibits only SBF or also MBF, and while Stb1 can bind both complexes it may have a greater negative effect on MBF (Costanzo et al., 2003; Costanzo et al., 2004; de Bruin et al., 2004; Bean et al., 2005; de Bruin et al., 2008; Takahata et al., 2009).) Deletion of *STB1* alone produced no discernable change in either *FAR1 cln2*Δ or *far1*Δ *cln2*Δ strains (Figure 2.2 A-D). Deletion of *WHI5* had no effect in *FAR1 cln2*Δ cells (Figure 2A), but in *far1*Δ *cln2*Δ cells (Figure 2.2C) it allowed a greater fraction of cells to escape the G1 arrest (e.g., 38% vs. 24% 2C at 240 min.). No further enhancement was seen when both Whi5 and Stb1 were removed. Assays using bud emergence as a marker of cell cycle progression yielded similar results (Figure 2.2D), although the escape phenotype caused by removing Whi5 (or both Whi5 and Stb1) was even more evident. Despite these effects, the increased escape phenotypes were only partial, in that pheromone still imposed a significant G1 delay in the majority of cells.

We also tested the role of Mbp1, which is the DNA-binding component of the MBF heterodimer (Bahler, 2005). Although Mbp1 is required for transcriptional activation by MBF, it is also required for full repression of MBF-

Figure 2.2. Partial role for Whi5 in Far1-independent arrest.

(**A**) Removal of Whi5 and/or Stb1 does not affect G1 arrest when Far1 is present. P_{GAL1}-*CDC20* strains in the *FAR1 cln2*Δ background were arrested in M phase and released, in the presence or absence of pheromone. Cell cycle progression and G1 arrest was measured by the flow cytometry assay of DNA content. Graphs show mean ± SEM (n = 4-8).

(**B**) The strains in panel (A) were tested for G1 arrest by the budding assay. Budding was scored 120 minutes after release from M-phase arrest in the presence of α factor. Bars show mean ± SEM (n = 3-4).

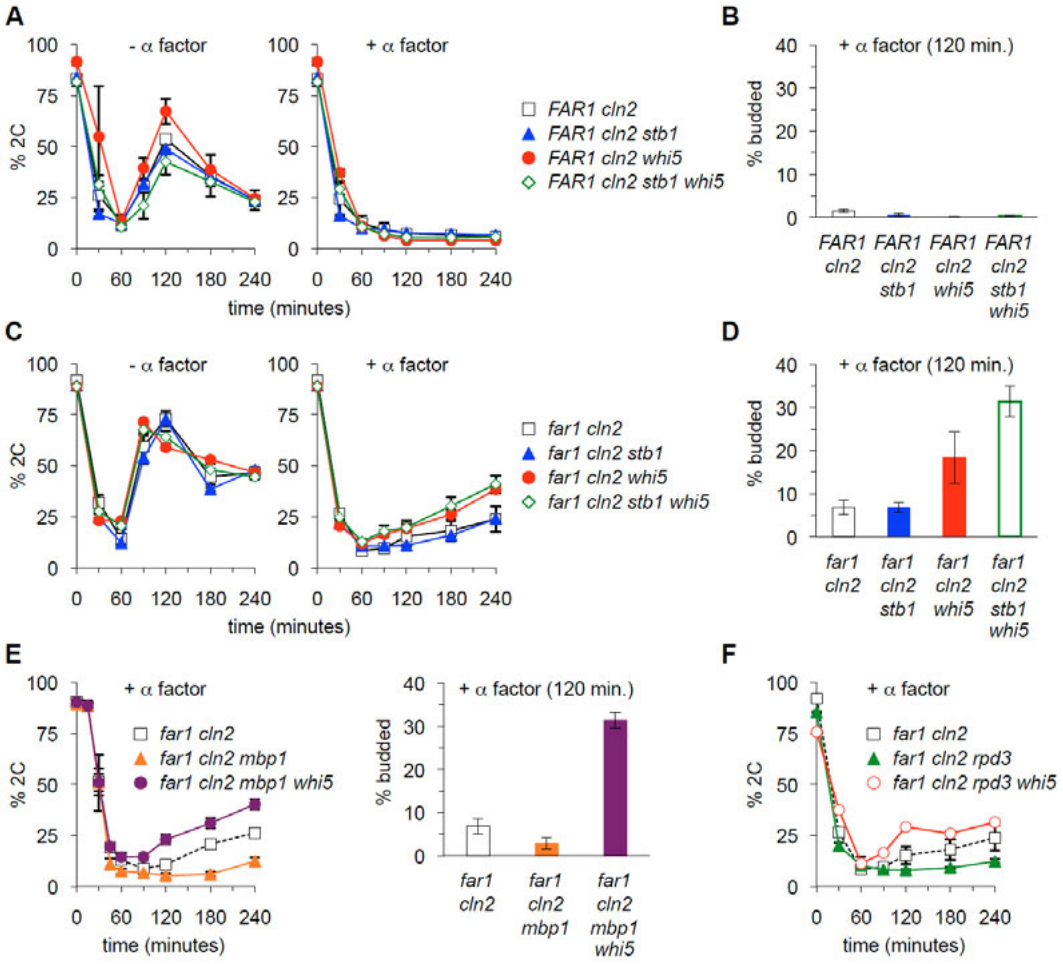
(**C**) Removal of Whi5 partially compromises Far1-independent arrest. Cell cycle progression and G1 arrest was measured in *far1 cln2*Δ strains by the DNA assay. Graphs: mean ± SEM (n = 4-8).

(**D**) Strains from panel (C) were tested for G1 arrest by the budding assay, as in panel (B). Bars show mean ± SEM (n = 6).

(**E**) Mbp1 is not required for Far1-independent arrest or for the role of Whi5. G1 arrest was measured by the DNA (left) and budding (right) assays. Data points: mean ± SEM (n = 3).

(**F**) Rpd3 is not required for the role of Whi5. Graphs show mean ± SEM (n = 4) for *far1*Δ *cln2*Δ and *far1*Δ *cln2*Δ *rpd3*Δ, or mean ± range (n = 2) for *far1*Δ *cln2*Δ *rpd3*Δ *whi5*Δ.

Figure 2.2. Partial role for Whi5 in Far1-independent arrest.



bound genes in early G1 (de Bruin et al., 2006). We found that removing Mbp1 from *far1*Δ *cln2*Δ cells caused a more complete arrest (Figure 2.2E), rather than increased escape, suggesting that its role as an activator outweighs its role as a repressor in this setting. Deleting *WHI5* from these *far1*Δ *cln2*Δ *mbp1*Δ cells allowed increased escape from G1 arrest (Figure 2.2E), but again the effect was partial such that the majority of cells still arrested.

Whi5 and Stb1 repress transcription in part through the recruitment of the histone deacetylase Rpd3 (Huang et al., 2009; Takahata et al., 2009; Wang et al., 2009). To determine if the reduced arrest proficiency of the *far1*Δ *cln2*Δ *whi5*Δ strain was due to the loss of Rpd3 recruitment, we tested *far1*Δ *cln2*Δ *rpd3*Δ strains (Figure 2.2F). In fact, deletion of Rpd3 did not increase escape from G1 arrest; if anything, it seemed to make G1 exit even slower, though this may reflect a slightly decreased growth rate of *rpd3*Δ mutants. Furthermore, Rpd3 is not required for the G1 arrest role of Whi5, because removing Whi5 from the *far1*Δ *cln2*Δ *rpd3*Δ strain still led to increased escape, indicating that the *whi5*Δ phenotype cannot be attributed solely to a defect in Rpd3 recruitment (see Discussion).

Collectively, these results show that Whi5 and Stb1 are not required for the strong G1 arrest seen when Far1 is present, but they contribute to the weaker G1 arrest observed in cells that lack Far1. Even so, their removal from *far1*Δ *cln2*Δ

cells causes only a partial defect in G1 arrest, indicating that pheromone signaling can still inhibit the G1/S transition in the absence of both Far1 and these transcriptional repressors.

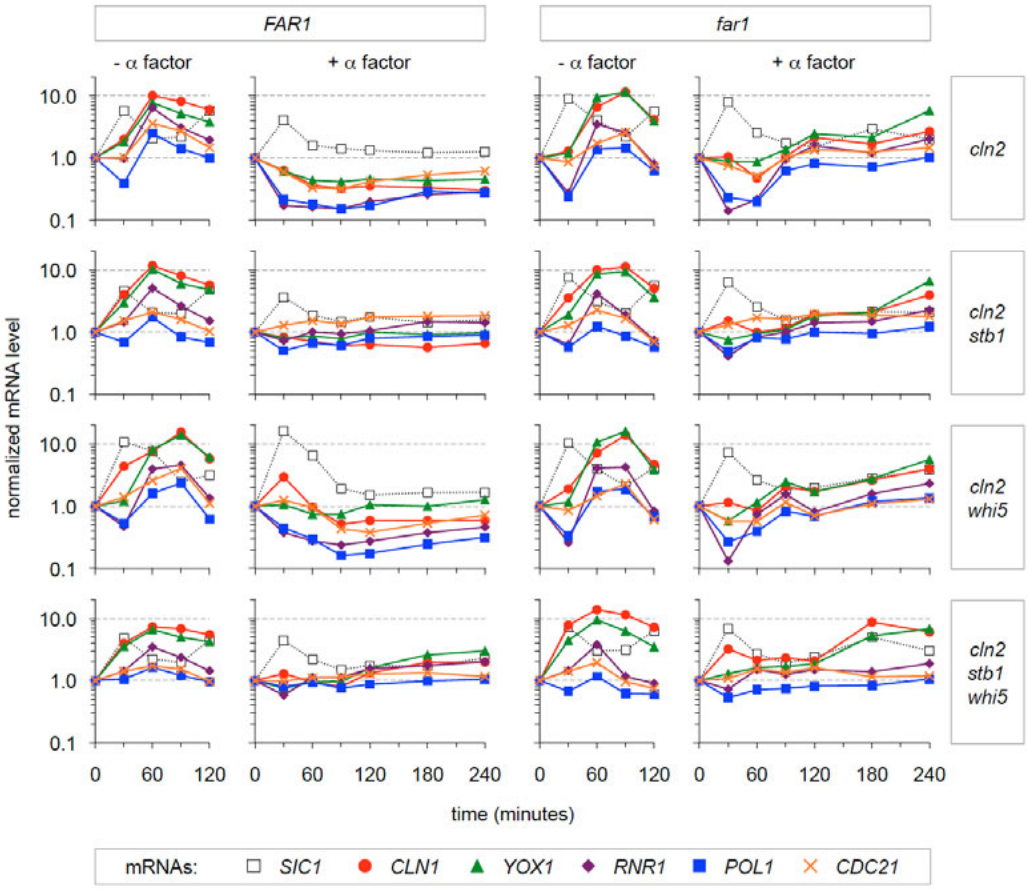
Loss of repressors only partially de-represses transcription

We considered two possible explanations for why removing the transcriptional repressors did not fully eliminate G1 arrest in *far1*Δ *cln2*Δ cells: (a) pheromone signaling might still be able to inhibit G1/S transcription even without the repressors; or (b) the G1/S transcripts could be fully de-repressed but pheromone signaling might exert non-transcriptional effects that inhibit exit from G1. To test these possibilities, we analyzed G1/S transcript levels via RT-qPCR. We chose five representative genes (*CLN1*, *YOX1*, *POL1*, *RNR1*, and *CDC21*) that are induced by SBF and/or MBF at the G1/S transition (Bean et al., 2005; de Bruin et al., 2006; Eser et al., 2011). For comparison, we also monitored a gene expressed at the earlier M/G1 boundary (*SIC1*). We first conducted single time course experiments for eight different strains, in which we analyzed transcript levels at numerous time points in synchronous cultures (Figure 2.3). Then, we analyzed the most informative time points in multiple independent trials (Figure 2.4).

Figure 2.3. Effects of Far1, Whi5, and Stb1 on G1/S mRNA levels.

The effects of Whi5 and Stb1 on G1/S transcript levels were measured in *FAR1 cln2*Δ (left) and *far1*Δ *cln2*Δ (right) backgrounds. P_{GAL1}-*CDC20* strains were arrested in M phase and released, with or without α factor. At 30-minute intervals, mRNA levels were measured by RT-qPCR (see Methods). Five G1/S transcripts (*CLN1*, *YOX1*, *RNR1*, *POL1*, and *CDC21*) and one M/G1 transcript (*SIC1*) were monitored. mRNA levels at each time point were plotted relative to the levels present in the M phase-arrested cultures (t = 0). See Figure 4 for further analyses.

Figure 2.3. Effects of Far1, Whi5, and Stb1 on G1/S mRNA levels.



In cells released from mitosis without pheromone, the M/G1 and G1/S transcripts peaked at 30 and 60 minutes, respectively (Figure 2.3). This agrees with the timing of DNA synthesis and budding (which begin at 60-90 minutes). Adding pheromone prevented the G1/S peak in *FAR1 cln2*Δ cells, and instead these transcripts declined to a minimum at 60 minutes and remained low for up to 4 hours (Figure 2.3). In the absence of Far1 (*far1*Δ *cln2*Δ cells), pheromone still inhibited the G1/S peak, but after 60 minutes these transcripts gradually increased, reaching levels that were higher than the corresponding *FAR1 cln2*Δ cells but below peak levels in untreated cells. This gradual increase is consistent with the leaky arrest phenotype of *far1*Δ *cln2*Δ cells. As expected for transcriptional repressors, removal of Whi5 and/or Stb1 allowed G1/S transcripts to start increasing earlier and/or to reach elevated levels in pheromone-treated cells, and yet pheromone still interfered with their expression. These patterns of partial de-repression were seen in both *FAR1 cln2*Δ and *far1*Δ *cln2*Δ backgrounds, although inhibition by pheromone was generally more potent and durable when Far1 was present. Clearly, pheromone signaling can prevent peak G1/S transcription even without Whi5 and Stb1.

(Note, the drop in G1/S transcript levels from M phase [t=0] to G1 [t=30 min] was unexpected because these genes are not thought to be active during mitosis. This behavior might reflect imperfect initial synchronization in M phase. Alternatively, maximal repression by Whi5 and Stb1 may require DNA binding by

SBF/MBF and nuclear localization of Swi6, which are inhibited in M phase by high Cdk activity (Sidorova et al., 1995; Koch et al., 1996; Queralt and Igual, 2003; Geymonat et al., 2004).)

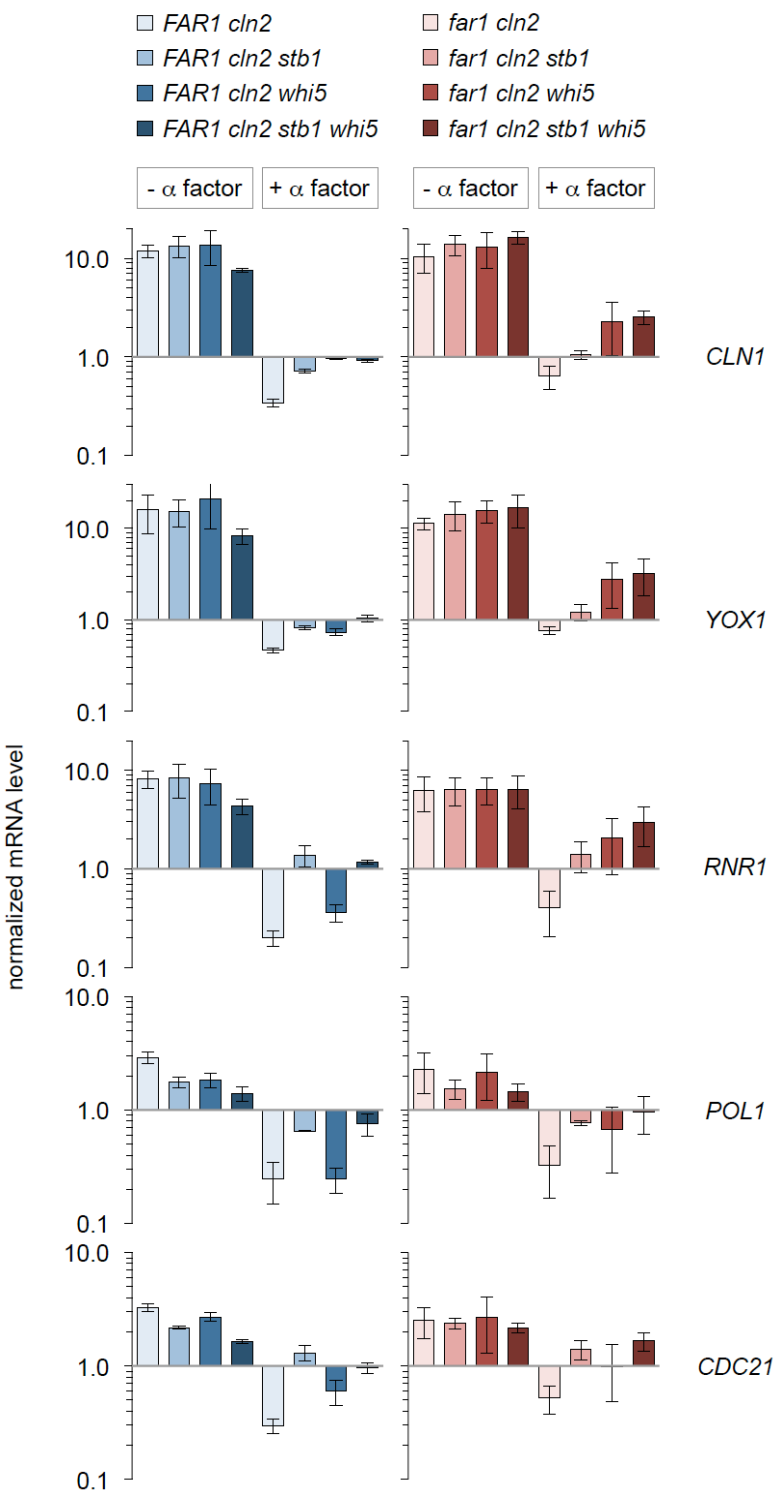
For further analysis we performed multiple repetitions of each time course experiment, and then measured mRNA levels at 60 minutes after release from the M phase block (Figure 2.4), because this was the time of peak expression in the absence of pheromone. Without pheromone, G1/S transcripts reached approximately the same peak level in all strains (Figure 2.4A), consistent with previous findings that Whi5 and Stb1 only slightly affect peak expression (Costanzo et al., 2003; Costanzo et al., 2004; de Bruin et al., 2004; de Bruin et al., 2008; Takahata et al., 2009). But de-repression was clearly evident in the pheromone treated samples, as G1/S transcripts were no longer fully repressed when Whi5 and/or Stb1 were absent. Remarkably, however, even in the absence of both Whi5 and Stb1, G1/S transcript levels in pheromone treated cells did not reach the maximum seen in the untreated samples. Therefore, inactivation of these repressors is not sufficient for full expression of G1/S transcripts, and pheromone signaling can still prevent their full expression. For several genes the highest transcript levels were seen in the *far1*Δ *cln2*Δ *whi5*Δ and *far1*Δ *cln2*Δ *stb1*Δ *whi5*Δ strains, which agrees with the finding that these strains have the strongest G1 escape phenotype, yet the ability of pheromone to

Figure 2.4. Loss of repressors only partially de-represses transcription.

(**A**) G1/S transcripts were assayed at a fixed time corresponding to the transition from G1 to S phase. The P_{GAL1}-*CDC20* arrest/release experiments shown in Figure 3 were repeated three times. mRNA levels were measured before release as well 60 minutes after release in either the presence or absence of α factor. Bars show mRNA levels (mean ± SD; n = 3) at the 60-minute time points, expressed relative to the levels in the M phase-arrested cells (t = 0). The effects of Whi5 and Stb1 were compared in *FAR1 cln2*Δ (left) and *far1*Δ *cln2*Δ (right) backgrounds.

(**B**) Comparison of transcriptional inhibition by pheromone treatment and Cdc28 inhibition.

Figure 2.4. Loss of repressors only partially de-represses transcription.



prevent peak expression in these strains also agrees with the finding that even the strongest G1 escape phenotypes were partial.

Role for CDK activity in repressor-independent transcriptional control

The mRNA analyses revealed a striking synergy between Far1 and Whi5 (Figure 2.4A). That is, for several genes there was an additive effect of removing both proteins (i.e., *far1*Δ *cln2*Δ *whi5*Δ), whereas pheromone treatment could still exert a strong repressive effect if either one was present (i.e., *FAR1 cln2*Δ *whi5*Δ or *far1*Δ *cln2*Δ *WHI5*). Again, this correlates with the arrest behavior, in that removing Whi5 caused a notable escape phenotype only when Far1 was absent. By contrast, the de-repression caused by removing Stb1 was not further enhanced when Far1 was also removed (i.e., compare *FAR1 cln2*Δ *stb1*Δ with *far1*Δ *cln2*Δ *stb1*Δ), and the additive relationship between Far1 and Whi5 did not require Stb1 (i.e., compare *FAR1 cln2*Δ *stb1*Δ *whi5*Δ with *far1*Δ *cln2*Δ *stb1*Δ *whi5*Δ). Together, these results suggest that Far1 and Whi5 contribute additively to transcriptional repression, with a corresponding additive effect on G1 arrest. Because Far1 is thought to inhibit Cln-Cdk activity (Peter et al., 1993; Tyers and Futcher, 1993; Peter and Herskowitz, 1994; Jeoung et al., 1998), the finding that it contributes to transcriptional repression even in the absence of Whi5 and Stb1 suggests that Cdk activity promotes G1/S transcription by additional mechanisms distinct from repressor displacement.

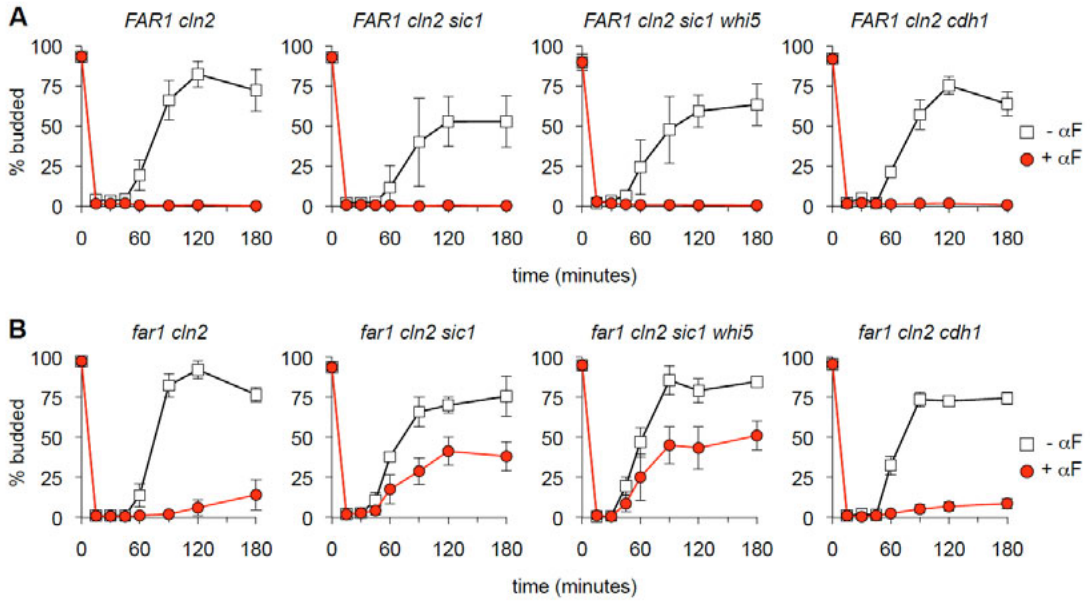
Sic1 plays a strong role in Far1-independent arrest

Another regulator of the G1/S transition is the protein Sic1, a CKI that inhibits S-phase cyclin-Cdks during early G1, and is inactivated in late G1 via phosphorylation by G1/S cyclin-Cdks. Because of its role as an antagonist of the G1/S transition, we tested if Sic1 is involved in the Far1-independent arrest by pheromone (Figure 2.5). (These experiments exclusively used the budding assay because the *sic1*Δ mutation caused extremely broad DNA flow cytometry profiles in P_{GAL1}-*CDC20* strains, which obscured the analysis.) Indeed, although deleting *SIC1* had no impact on G1 arrest when Far1 was present (Figure 2.5A), it had a substantial effect on Far1-independent arrest (Figure 2.5B). That is, in *far1*Δ *cln2*Δ *sic1*Δ cells the ability of pheromone to impose a G1 arrest was strongly disrupted, although it was not eliminated. The residual arrest was not eliminated by further deletion of *WHI5* (Figure 2.5B), and hence we saw no evidence for synergy between Sic1 and Whi5 suggesting that the primary downstream effect of *whi5Δ* is through eventual activation of Sic1 resulting from derepressed *CLN1* expression. Furthermore, removing both Sic1 and Whi5 in a *FAR1* background had no phenotype (Figure 2.5A). Thus, removing any two antagonists of the G1/S transition is not sufficient to cause a G1 escape phenotype; instead, this phenotype is only seen when combining *far1*Δ with either *whi5*Δ or *sic1*Δ mutations. We conclude that the G1/S braking mechanism

Figure 2.5. Strong role for Sic1 in Far1-independent arrest.

P_{GAL1}-CDC20 strains of the indicated genotypes were synchronized and then released into the presence or absence of α factor (αF). Cell cycle progression and G1 arrest was assayed by budding. Panels (**A**) and (**B**) compare results in the *FAR1 cln2*Δ and *far1*Δ *cln2*Δ backgrounds, respectively. All graphs show the mean ± SD (n = 3-4). Note that *far1*Δ *cln2*Δ *cdh1*Δ strains showed phenotypic heterogeneity that was isolate-dependent. Specifically, we tested a total of 12 isolates: six *P_{GAL1}-CDC20* derivatives from each of two independent *far1*Δ *cln2*Δ *cdh1*Δ strains. The results shown are an average of three strains (YPAP242, 244, 245) that represent the majority phenotype seen in 10 of 12 isolates. In 2 of 12 isolates, both derived from the same initial *far1*Δ *cln2*Δ *cdh1*Δ parent strain, we observed a notable escape phenotype (e.g., for YPAP243, ~40% budded cells after 120-180 min. in α factor). The explanation for this heterogeneity is unknown, but the finding that the escape phenotype was observed in only a minority of derivatives (2/6) of one parent strain, and in no derivatives (0/6) of the other, suggests that a rare enhancer mutation may be responsible.

Figure 2.5. Strong role for Sic1 in Far1-independent arrest.



provided by Sic1 allows pheromone to activate a weakened G1 arrest response when Far1 is absent.

For comparison to the findings with Sic1, we tested another antagonist of S- and M-phase cyclins; namely, the APC component Cdh1, which inhibits accumulation of B-type cyclins during G1 (Visintin et al., 1997; Morgan, 2007). We found that removal of Cdh1 in the *far1*Δ *cln2*Δ background caused a negligible change in G1 escape (Figure 2.5B), although for unknown reasons we did observe an increased escape phenotype in rare isolates (see Figure 5 legend). Therefore, these findings suggest that Far1-independent arrest depends more strongly on the inhibitor of Clb-Cdk activity (Sic1) than on the inhibitor of Clb protein accumulation (Cdh1); however, we cannot rule out the possibility that the absence of Cdh1 was to some degree compensated in our experimental system by ectopic expression of its functional relative, Cdc20, which enabled the cell synchronization protocol.

G1 arrest failure can compromise cell viability

Although removing Sic1 allowed *far1*Δ *cln2*Δ cells to escape pheromone-induced G1 arrest more readily, it appeared to cause enhanced sensitivity to pheromone when growth arrest was measured by a long-term “halo assay” (Figure 2.6A). This paradox led us to consider the possibility that the failure of these cells to arrest in G1 causes reduced viability during continuous incubation

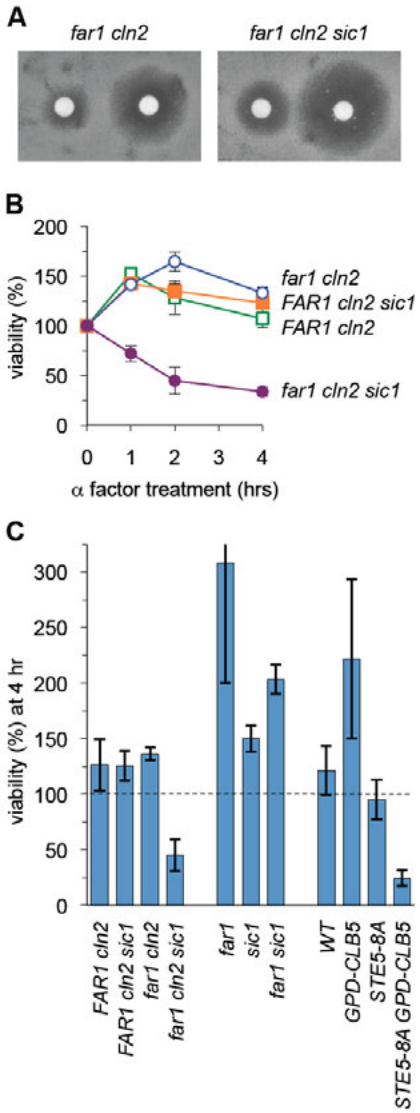
Figure 2.6. Failure to arrest in G1 causes loss of viability during pheromone exposure.

(**A**) Removing Sic1 from *far1*Δ *cln2*Δ cells causes enhanced pheromone sensitivity when measured by a chronic growth arrest ("halo") assay. Cells were spread on solid growth medium, overlaid with filter disks containing 20 µl of 20 µM or 100 µM α factor, and then incubated at 30˚C for 2 days.

(**B**) Pheromone treatment causes loss of viability in *far1*Δ *cln2*Δ *sic1*Δ cells. Asynchronous liquid cultures were incubated with pheromone for 1 to 4 hours, and then cell viability was measured by plating on medium lacking pheromone and counting colony formation (see Methods). Viable cells at each time point were expressed relative to the number present prior to treatment (t = 0). Graphs show mean ± range (n = 2). In parallel cultures incubated without pheromone, no differences in viability were observed among these strains (data not shown).

(**C**) Loss of cell viability is a consequence of escaping G1 arrest without inhibiting pheromone signaling. Asynchronous cultures were incubated with pheromone for 4 hour, and viable cells were measured and expressed relative to the pre-treated cultures (t = 0) as in panel (B). Bars show mean ± SD (n = 4). Strains that continue dividing in the presence of pheromone (e.g., *far1*Δ) show an increased number of viable cells at 4 hours compared to the pre-treated culture. See text for further explanation.

Figure 2.6. Failure to arrest in G1 causes loss of viability during pheromone exposure.



with pheromone. Therefore, we assayed cell viability in asynchronous cultures treated with pheromone for various times (Figure 2.6B). Wild type strains maintained viability for several hours, but the *far1*Δ *cln2*Δ *sic1*Δ strain showed a clear loss of viability after only a short period (2-4 hr). This reduced viability was not seen in the absence of pheromone (see Figure 2.6 legend) or when either Far1 or Sic1 was present (i.e., *FAR1 cln2*Δ *sic1*Δ or *far1*Δ *cln2*Δ *SIC1* strains), suggesting that it is not the presence or absence of either protein *per se* but rather the rapid escape from G1 that ultimately causes reduced viability in the *far1*Δ *cln2*Δ *sic1*Δ strain. Furthermore, simultaneous absence of both proteins was tolerated if strains retained *CLN2* (i.e., *far1*Δ *sic1*Δ strains; Figure 6C). Because Cln2 plays a prominent role in blocking pheromone response as cells pass Start (Oehlen and Cross, 1994; Strickfaden et al., 2007), the combined results suggest that the observed inviability is due to cells exiting G1 without down-regulating pheromone signaling.

These implications were further corroborated by other experiments that did not involve Far1, Sic1, or Cln2. In particular, over-production of the S-phase cyclin Clb5 can override G1 arrest by pheromone (Oehlen et al., 1998), and a Cdk-resistant form of the signaling protein Ste5 (Ste5-8A) prevents pheromone response from being shut down in post-Start cells (Strickfaden et al., 2007). The presence of both features together (i.e., *STE5-8A* P_{GPD1}*-CLB5* double mutants) caused cells to lose viability during pheromone exposure, whereas the single

mutants did not (Figure 2.6C), arguing that inviability is a specific consequence of allowing pheromone signaling to continue in cells that escape the G1 arrest. Altogether, these findings illustrate the physiological importance of maintaining a robust G1 arrest in cells that are undergoing pheromone signaling. They also reveal that, under some circumstances, mutations that compromise G1 arrest would not be identified using only a growth arrest assay (see Discussion).

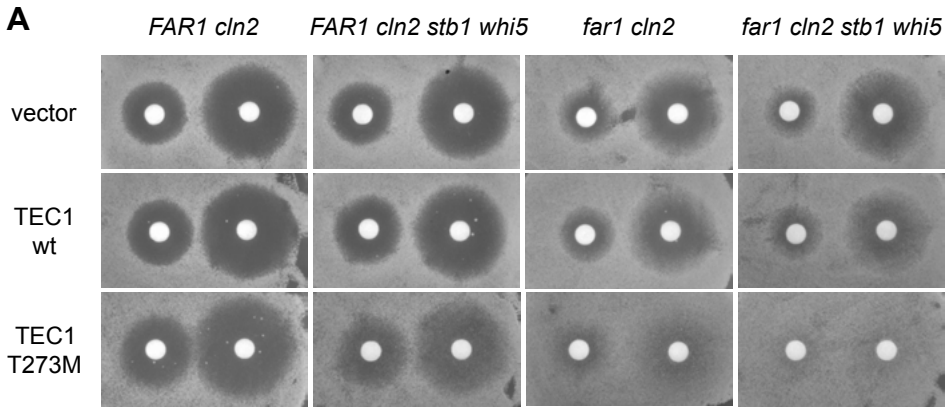
Tec1 antagonizes G1 arrest

One route by which pheromone might affect G1/S transcription independent of the inhibitors Whi5 and Stb1 is the ability of the pheromone-activated MAP kinase Fus3 to trigger degradation of the transcription factor Tec1 (Bao et al., 2004; Bruckner et al., 2004; Chou et al., 2004), which positively regulates *CLN1* expression (Madhani et al., 1999; Bruckner et al., 2004); consequently, this mechanism could also indirectly dampen other G1/S transcripts that are transcriptional targets of Cln1-Cdc28 (Eser et al., 2011). Initially, to test the possibility that Tec1 could play a role in pheromone-induced arrest, we looked at halo assays of *FAR1 cln2Δ, FAR1 cln2Δ whi5Δ stb1Δ, far1Δ cln2Δ,* and *far1Δ cln2Δ whi5Δ stb1Δ* cells expressing either wild type Tec1 or a mutant, stabilized version Tec1-T273M (Figure 2.7). Results of the halos show significantly decreased arrest proficiency in *far1Δ cln2Δ* and *far1Δ cln2Δ whi5Δ stb1Δ* cells expressing Tec1-T273M. It appeared the Tec1-T273M mutant may even have a slight effect in *FAR1 cln2Δ stb1Δ whi5Δ* cells. Based on these

Figure 2.7: Tec1 antagonizes G1 arrest

far1Δ cln2Δ and *far1Δ cln2Δ stb1Δ whi5Δ* cells expressing either a WT or stabilized version of Tec1 show decreased arrest proficiency. *FAR1 cln2Δ, FAR1 cln2Δ whi5Δ stb1Δ, far1Δ cln2Δ,* and *far1Δ cln2Δ whi5Δ stb1Δ* cells harboring either an empty vector or a plasmid expressing WT Tec1 or the stabilized form Tec1-T273M were spread on selective media, and discs containing 20µl or either 20µM or 100µM α factor. (Strains PPY1789, PPY1867, YPAP157, and YPAP161 harbored pPP681, pPP4042, or pPP4043)

Figure 2.7: Tec1 antagonizes G1 arrest



results, we also attempted the block and release assays. Initial results from these experiments appear to support the halo phenotypes, with *far1Δ cln2Δ whi5Δ stb1Δ* cells expressing Tec1-T273M seeming to escape arrest more quickly than their vector expressing counterparts. Quantification and further repeats of this experiment are currently ongoing to confirm these results.

Discussion

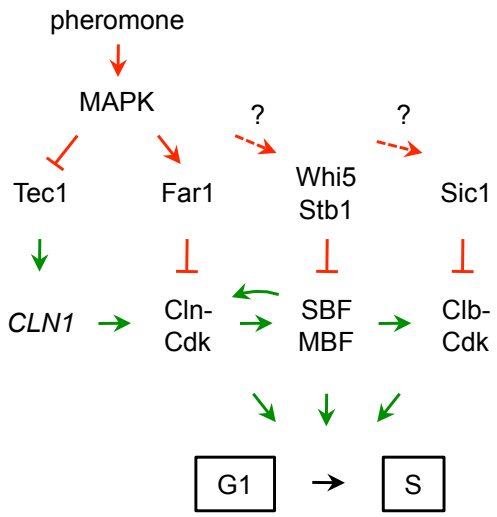
In this study we sought to increase our understanding of the mechanisms that yeast cells use to activate G1 arrest in response to mating pheromones. Previous findings indicate that this arrest does not absolutely require the pheromone-activated CKI Far1, because removal of Cln2 restores pheromone arrest to *far1*Δ cells. Thus, here we compared Far1-dependent and Far1-independent G1 arrest, in terms of their molecular phenotypes and their dependence on other inhibitory factors. We show that Far1 is not absolutely required for establishing a commitment point at Start, but it lengthens the time window in which pheromone can block this commitment step, and it makes G1 arrest more potent. Far1 also makes the G1 arrest process less dependent on other antagonists of the G1/S transition, such as repressors of G1/S transcription and inhibitors of S- and M-phase Cdk activity. Conversely, these repressors and inhibitors are not absolutely required for G1 arrest but they reduce the dependence on Far1. Thus, our findings reveal functional overlap among these distinct G1/S inhibitors, which helps enforce a robust G1 arrest and thereby prevents premature cell cycle commitment in the presence of external anti-proliferative signals (Figure 2.8).

In many eukaryotic systems it is thought that inhibition of G1/S transcription by Rb-like repressors plus inhibition of S- and M-phase Cdk activity

Figure 2.8: A simple illustration of multiple pathways contributing to pheromone arrest.

Regulatory effects that inhibit or promote the G1/S transition are indicated in red or green, respectively. Dashed arrows with question marks emphasize that, although we found roles for Whi5/Stb1 and Sic1 in Far1-independent arrest, it is not known whether pheromone signaling enhances their inhibitory activity or simply depends on their constitutive effects.

Figure 2.8: A simple illustration of multiple pathways contributing to pheromone arrest.



by Cip/Kip-like CKIs are key factors that restrain cell cycle entry and impose a G1 waiting period (Morgan, 2007). In this study we find that pheromone-induced G1 arrest in budding yeast can still be imposed in the absence of the transcriptional repressors Whi5/Stb1 or the CKI Sic1, or even both Whi5 and Sic1 simultaneously. But these negative factors become important for pheromone arrest in *far1*Δ cells. Therefore, although Whi5/Stb1 and Sic1 are not absolutely required for G1 arrest, they help enforce G1 arrest when Far1 is absent. The existence of multiple braking mechanisms may help prevent premature cell cycle entry and expand the commitment decision period, perhaps by imposing a requirement that G1/S Cdk activity exceeds a sufficiently high threshold to counteract multiple antagonists. Such functional overlap, while in principle not required for a basic cell cycle, may increase the opportunities for regulatory control of the G1/S transition and minimize promiscuous division. Indeed, in animals there is evidence that Rb and CKIs can have additive effects in restraining proliferation in undifferentiated cells, or redundant effects in terminally differentiated cells (Brugarolas et al., 1998; Buttitta et al., 2010; Wirt et al., 2010).

It is noteworthy that removal of Whi5 and Stb1 is not sufficient for full expression of G1/S transcripts, despite evident de-repression. Instead, we find that pheromone signaling inhibits expression of G1/S genes even in the absence of both repressors, similar to previous results in *whi5*Δ or *stb1*Δ single mutants (Costanzo et al., 2004; de Bruin et al., 2008). Because this inhibition is maximal

in the presence of Far1 or when Cdc28 is inactivated, it suggests that Cdk activity promotes full G1/S transcription by additional means separate from its role in releasing repression by Whi5/Stb1. This behavior could be explained if SBF and MBF are activated directly via Cdk phosphorylation. Indeed, such a mechanism was proposed prior to the discovery of Whi5 and Stb1, and Cdk phosphorylation sites have been identified in both Swi4 and Swi6, but direct evidence that Cdk phosphorylation of SBF/MBF enhances their activity is still lacking (Sidorova et al., 1995; Wijnen et al., 2002). Previous mutational analyses suggest that Cdk phosphorylation of either Swi6 or Whi5 suffices to dissociate the repressor, and hence that these phosphorylation events act redundantly rather than additively (Costanzo et al., 2004; de Bruin et al., 2004; Wagner et al., 2009). In contrast, our observations clearly argue for a pheromone/Cdk-regulated mechanism that acts in addition to repressor displacement, rather than redundant with it. This is consistent with the results of an experiment done in a strain with inhibitable Cdc28 (cdc28-as2) that suggested that CDK inhibition could still decrease transcription even in the absence of the repressors (results not shown). This additional mechanism could affect SBF/MBF itself, an associated factor such as Msa1 (Ashe et al., 2008), or a separate factor that cooperates with SBF/MBF such as Bck2 or Spt10 (Wijnen and Futcher, 1999; Eriksson et al., 2011). Regardless of the precise mechanism, our mRNA analyses provide a clear molecular readout that correlates with G1 arrest proficiency, and hence provide a quantitative reporter for how potently

pheromone signaling can antagonize the G1/S transition in different genetic backgrounds.

Interestingly, the effect observed upon WHI5 deletion appears to be independent of the HDAC Rpd3, which Whi5 is known to recruit to promoters, suggesting that Whi5 plays a role in repression independent of Rpd3 recruitment. It has been suggested that Whi5 may also recruit an additional HDAC Hos3 (Huang et al., 2009), and that Whi5's presence at promoters may prevent recruitment of the chromatin reorganizing complex FACT (Takahata et al., 2009). Either of these functions, or some yet unidentified role for Whi5, could be contributing to its Rpd3-independent effect on transcription.

Which molecular targets can explain the partial escape phenotypes obtained by removing Sic1 or Whi5 from *far1*Δ *cln2*Δ cells? Ultimately, the key factor dictating whether cells can pass the G1/S transition despite the presence of pheromone may be acquisition of B-type Cdk activity. Indeed, pheromone cannot arrest cells in G1 if the S-phase cyclin Clb5 is expressed from a strong foreign promoter (Oehlen et al., 1998; Strickfaden et al., 2007). This view can explain the effects of removing Sic1, which directly inhibits Clb5-Cdk activity (Schwob et al., 1994). Furthermore, *CLB5* is an SBF-regulated gene, as are *CLN1* and *CLN2*, which engage in a positive feedback loop that enhances their own expression as well as other SBF/MBF-dependent transcripts (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991; Skotheim et al., 2008). Thus, in *far1*Δ *cln2*Δ cells, *CLN1* and *CLB5* expression levels may determine whether

sufficient Clb5-Cdk activity accumulates to pass Start, and hence their increased expression upon removal of Whi5 may cause the escape phenotype. In *far1*Δ single mutants, the presence of Cln2 may tip the balance in favor of Start by a combination of several effects: (i) it might simply increase the total cyclin dosage, equal to an extra copy of *CLN1*; (ii) Cln2 might be a more effective promoter of Start than Cln1 (Tyers and Futcher, 1993), perhaps by providing greater Cdk activity toward key substrates like Whi5 and Sic1; and (iii) Cln2 might counteract pheromone arrest by directly inhibiting pheromone signaling, and it do so more potently than does Cln1 (Oehlen and Cross, 1994; Strickfaden et al., 2007). Thus, when Far1 is absent, the weaker Far1-independent arrest mechanisms can be overridden by Cln2 or by genetic changes that promote Clb5-Cdk activity.

How does pheromone signaling lead to G1 arrest without Far1? Although Whi5 and Sic1 can contribute to pheromone-induced arrest when Far1 is absent, this does not necessarily imply that they are themselves regulated by pheromone. Because Sic1 can be phosphorylated and stabilized by another MAPK, Hog1 (Escote et al., 2004), it is tempting to postulate that a similar effect could be exerted by the pheromone-regulated MAPK, Fus3; yet it is unlikely that the identical mechanism is used because the phosphorylated form of Sic1 was absent in pheromone-arrested cells and can still be induced by Hog1 activation (Escote et al., 2004). Another study, however, reported that the Sic1 N-terminus is partially phosphorylated in pheromone-arrested cells (Koivomagi et al., 2011),

though the sites or responsible kinases are unknown. The effects of pheromone on G1/S transcription – even in the simultaneous absence of Far1, Whi5, and Stb1 – could be due to direct inhibition of transcription per se or an indirect effect of Cdk inhibition. Other pertinent mechanisms include pheromone-induced reduction in Cln protein levels (Valdivieso et al., 1993) or reductions in total protein synthesis rates (Goranov et al., 2009), which may cause a rapid drop in steady state levels of short-lived cell cycle regulators such as cyclins.

The current results from the Tec1 experiments do suggest that pheromone-induced degradation of Tec1 could play a role in G1 arrest. The severely decreased arrest proficiency of cells expressing the stabilized version of Tec1 demonstrates how an abnormally high dosage of Tec1 can interfere with G1 arrest. This suggests that Tec1 could be a critical target of the pheromone response pathway, and that its degradation during pheromone treatment plays a previously unappreciated role in G1 arrest. Since we also observed a synergistic effect of stabilizing Tec1 and absence of the repressors Whi5 and Stb1 it appears likely that control of both of these mechanisms combines to repress G1/S transcription in response to pheromone.

Finally, our findings reveal that robust G1 arrest is physiologically important, because if cell cycle entry occurs when cells are responding to pheromone it can lead to irreversible, lethal effects. In particular, this was observed when *far1*Δ *cln2*Δ *sic1*Δ cells were exposed to pheromone; they were unable to arrest in G1, but they were nevertheless unable to divide and grow.

Similar inviability was observed during pheromone treatment of *STE5-8A* P_{GPD1}-*CLB5* cells, in which G1 arrest is bypassed but signaling cannot be shut down. The exact cause of this inviability is not known, but ongoing studies suggest that pheromone signaling interferes with the proper assembly and/or function of the microtubule-based cytoskeleton during nuclear division (unpublished observations S. Strickfaden & P. Pryciak). Our findings also clarify earlier results that appeared to suggest Sic1 was not required for Far1-independent arrest: namely, pheromone was able to arrest growth of *cln1*Δ *cln2*Δ *cln3*Δ *far1*Δ *sic1*Δ cells (Tyers, 1996). In retrospect, it seems likely that those cells did not arrest in G1, but rather became inviable because of their failure to arrest in the presence of pheromone. The results also illuminate an important consideration when analyzing other mutants for defects in pheromone-mediated arrest: if they allow slippage past the G1/S transition but do not prevent pheromone signaling, then the arrest defect will not be noticeable by growth arrest (e.g., halo) assays. Hence, it is conceivable that a distinct class of pheromone arrest mutants exists that would have gone undetected in prior genetic screens.

CHAPTER III

G1/S cyclin protein levels decrease after pheromone treatment

Abstract

Cln protein levels have been shown to decrease during the response to pheromone, and it has been suggested that this decrease is independent of transcriptional control. It has, however, remained unclear if the effect on Cln protein levels is a result of a directed degradation mechanism or a side effect of another aspect of the pheromone response. Our studies confirmed the previously described results, levels of constitutively expressed Cln proteins decrease after pheromone treatment. Preliminary results suggest an overall decrease in translation rates may be at least partially responsible.

Introduction

As befits an arrest in G1 phase, yeast cells arrested by mating pheromone lack the G1/S cyclin proteins Cln1 and Cln2 (Wittenberg et al., 1990). A transcriptional mechanism clearly contributes to their absence, as the entire set of SBF/MBF-regulated transcripts, which includes *CLN1* and *CLN2*, are not expressed during pheromone arrest (Roberts et al., 2000). However, one previous study found that when *CLN2* was placed under control of a low level promoter not effected by pheromone treatment (from *CLN3*), levels of the Cln2 protein still declined markedly in response to pheromone treatment (Valdivieso et al., 1993), suggesting that pheromone signaling might also exert post-transcriptional control over G1/S cyclin levels. Surprisingly, this post-transcriptional effect was strongly diminished in *far1*Δ cells, leading to the suggestion that it might be mediated by Far1, though by an unspecified mechanism. What was unappreciated at the time of that study, however, was that pheromone signaling is inhibited by G1/S cyclin-CDK activity (Oehlen and Cross, 1994; Wassmann and Ammerer, 1997), and hence, in retrospect, the apparent requirement for Far1 might simply reflect its role as a CDK inhibitor needed to prevent the constitutively-expressed Cln2 from blocking pheromone response. In the course of considering possible mechanisms for Far1-independent arrest by pheromone (see Chapter 2), we wished to revisit this previous observation and probe the responsible mechanism.

Notably, there are well-known routes for post-transcriptional regulation of Cln1 and Cln2. Namely, during a normal cell cycle these cyclins are degraded by a mechanism involving phosphorylation and ubiquitin-mediated proteolysis. The C-terminal tails of Cln1 and Cln2 are phosphorylated in a CDK-dependent manner (Salama et al., 1994; Lanker et al., 1996; Schneider et al., 1998), which targets them for ubiquitination by SCF^{Grr1} (an E3 ubiquitin ligase) and consequent degradation by the proteasome (Barral et al., 1995; Kishi and Yamao, 1998). In G1 these cyclins have a half-life of approximately 8 minutes, and this rapid turnover is dependent on CDK phosphorylation as a form of Cln2 with mutations at its seven CDK consensus sites, $Cln2^{4T3S}$, is significantly more stable ($t_{1/2}$ ~ 60 minutes) (Lanker et al., 1996). Given that MAPKs and CDKs phosphorylate similar sites (i.e., SP or TP), it is conceivable that pheromone could trigger Cln1/2 instability via the same phosphorylation sites and ubiquitination machinery that operate in cycling cells. Moreover, as CDK activity is thought to be inhibited during pheromone-mediated G1 arrest (e.g., via Far1) (Peter and Herskowitz, 1994), any Cln1/2 proteins that are synthesized might be unusually stable in the absence of an alternate mode of degradation, perhaps providing a physiological rationale for a pheromone-stimulated mode.

The experiments in this chapter were aimed at re-examining the phenomenon of pheromone-induced loss of G1/S cyclin proteins, as well as the mechanistic requirements including the role of Far1. We confirmed that pheromone treatment has an effect on G1 cyclin levels independent of

transcriptional control. Our findings suggest that this phenomenon is dependent on SCF^{Grr1} but is at least partly independent of Cdc28 activity, the CDK phosphorylation sites in Cln2, and Far1. We were not able to obtain evidence that pheromone increases the degradation rate of Cln1/2, which raises the possibility that pheromone reduces their synthesis rate instead. Unfortunately, our ability to probe this phenomenon was repeatedly hampered by unusually high day-to-day variability in the severity of effect. Despite considerable effort, we were not able to find a way to control this variability, and consequently we decided to halt further study of this phenomenon. Nevertheless, in order to document the findings for the benefit of future researchers in this area, the primary observations and their potential implications are presented here.

Results

Steady state levels of G1/S cyclin proteins decrease after pheromone treatment

To assess the effects of pheromone on cyclin protein levels in isolation from effects on transcription, we placed the cyclin genes under heterologous control (Figure 1A), such that only the open reading frames (ORFs) of the cyclins were placed between foreign, constitutively-active promoters (P_{CYC1} or P_{ADH1}) and a foreign transcriptional terminator (T_{CYC1}). In addition, each cyclin was tagged at its C-terminus with a myc_{13} epitope. One concern was that constitutive expression of cyclins might interfere with pheromone signaling, specifically through the ability of Cln-CDK's to phosphorylate the scaffold protein Ste5 thereby preventing its pheromone-induced membrane localization. Indeed, ectopic Cln2 expression noticeably reduced pheromone response in wild-type strains, and *far1*Δ strains were even more strongly affected (Figure 3.1B), presumably because Cln-CDK activity is fully uninhibited when Far1 is absent. By contrast, in strains with the *STE5-8A* allele, which encodes a CDK-resistant form of Ste5, cyclin expression had no impact on signaling in either *FAR1* or *far1*Δ backgrounds. Therefore, in all subsequent experiments we studied the effects of pheromone on Cln1/2 protein levels in strains harboring the *STE5-8A* allele.

Figure 3.1: G1/S protein levels decrease after pheromone treatment

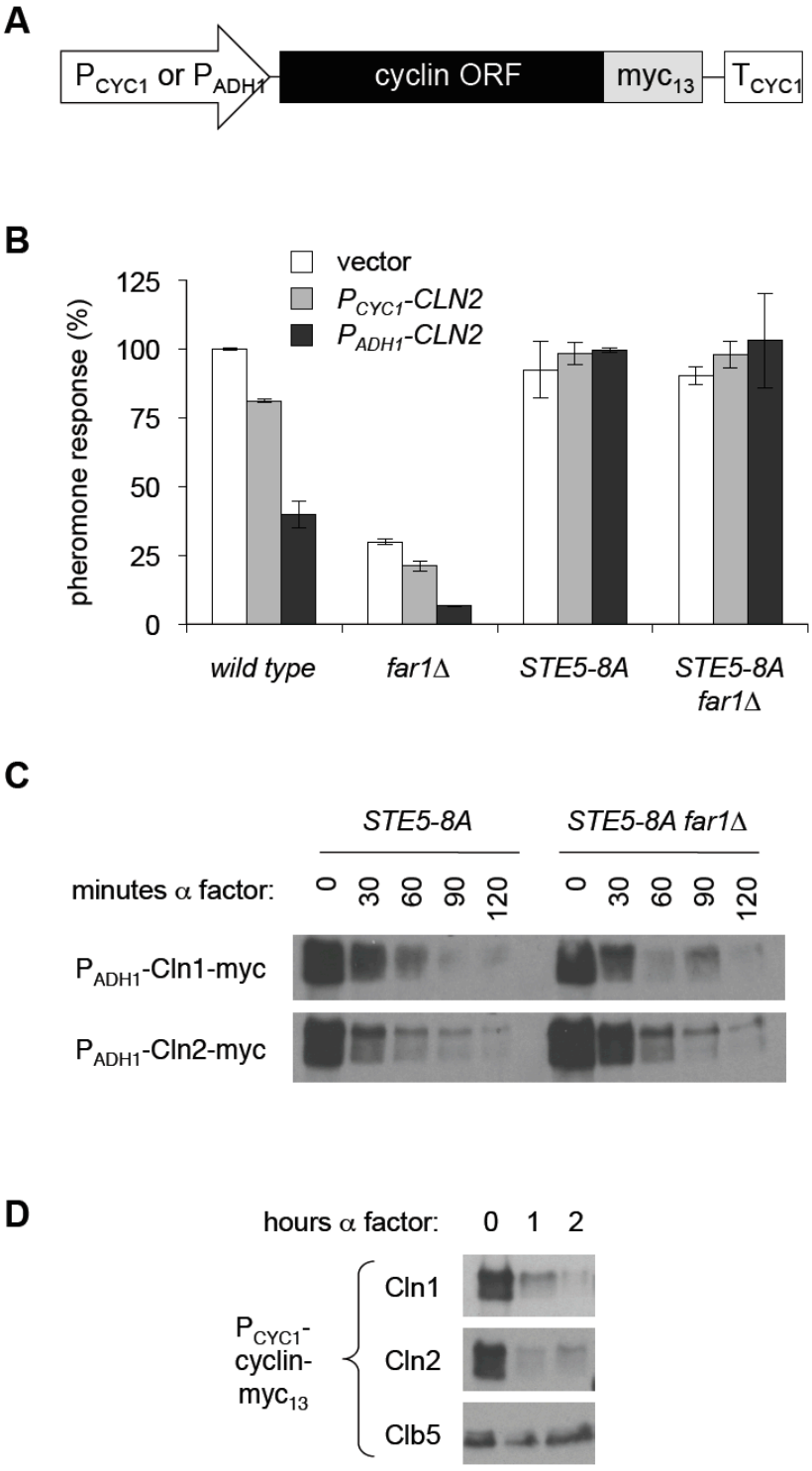
(A) Schematic of constructs used in these experiments containing the Cln1 or Cln2 ORF tagged with a 13myc epitope and under control of a foreign promoter (either ADH1, CYC1, or GAL).

(B) Pheromone signaling is inhibited by Cln2 expression from constitutive promoters in wild type and *far1Δ* cells, but not in *STE5-8A* and *STE5-8A far1Δ*. A plasmid expressing Cln2 under control of either the weaker CYC1 promoter or the stronger ADH1 promoter (or an empty vector) was introduced into the cells, and pheromone response was measured using a transcriptional reporter (*FUS1-lacZ*).

(C) Steady-state levels of Cln1 or Cln2 protein decrease over time after treatment with pheromone. *STE5-8A* and *STE5-8A far1Δ* cells expressing myc tagged Cln1 or Cln2 were treated with pheromone and samples were taken at 30 minute intervals. Samples were analyzed with anti-myc blots.

(D) *STE5-8A* cells expressing a myc-tagged version of Cln1, Cln2, or Clb5 from the constitutive CYC1 promoter were treated with α factor and samples were taken after 1 and 2 hours. Whole cells extracts were analyzed by anti-myc blot.

Figure 3.1: G1/S protein levels decrease after pheromone treatment



In initial experiments, we found that steady state levels of Cln1 and Cln2 were reduced over time after addition of pheromone in both *STE5-8A* and *STE5-8A far1Δ* strains (Figure 3.1C). This result suggested that pheromone affects Cln1 and Cln2 similarly, and that the effect may not require Far1. Early experiments also indicated some cyclin specificity, as pheromone did not appear to affect the S-phase cyclin, Clb5, when expressed from the same foreign context as Cln1/2 (Figure 3.1D). In subsequent experiments in which we quantified protein levels and normalized to an internal control protein (Cdc28 signal), we sometimes observed that the effect of pheromone was greater in *STE5-8A* than *STE5-8A far1*Δ cells (Figure 3.2A,B), suggesting that there may be some partial Far1-dependence. In addition, in some experiments we observed decreased Cln1/2 levels at late times even without pheromone treatment, which we suspected was most likely due to the cultures approaching stationary phase. On balance, these findings provided initial confirmation that pheromone can trigger loss of G1/S cyclin proteins, and so subsequent experiments were aimed at further parsing this phenomenon.

CDK inhibition cannot explain protein loss

It is possible that the pheromone-induced reduction in Cln protein levels could be an indirect result of other responses to pheromone, including inhibition of Cdc28 activity and cell cycle arrest. To assess this possibility we used a strain containing an allele of Cdc28, cdc28-as1, which is specifically inhibited by the

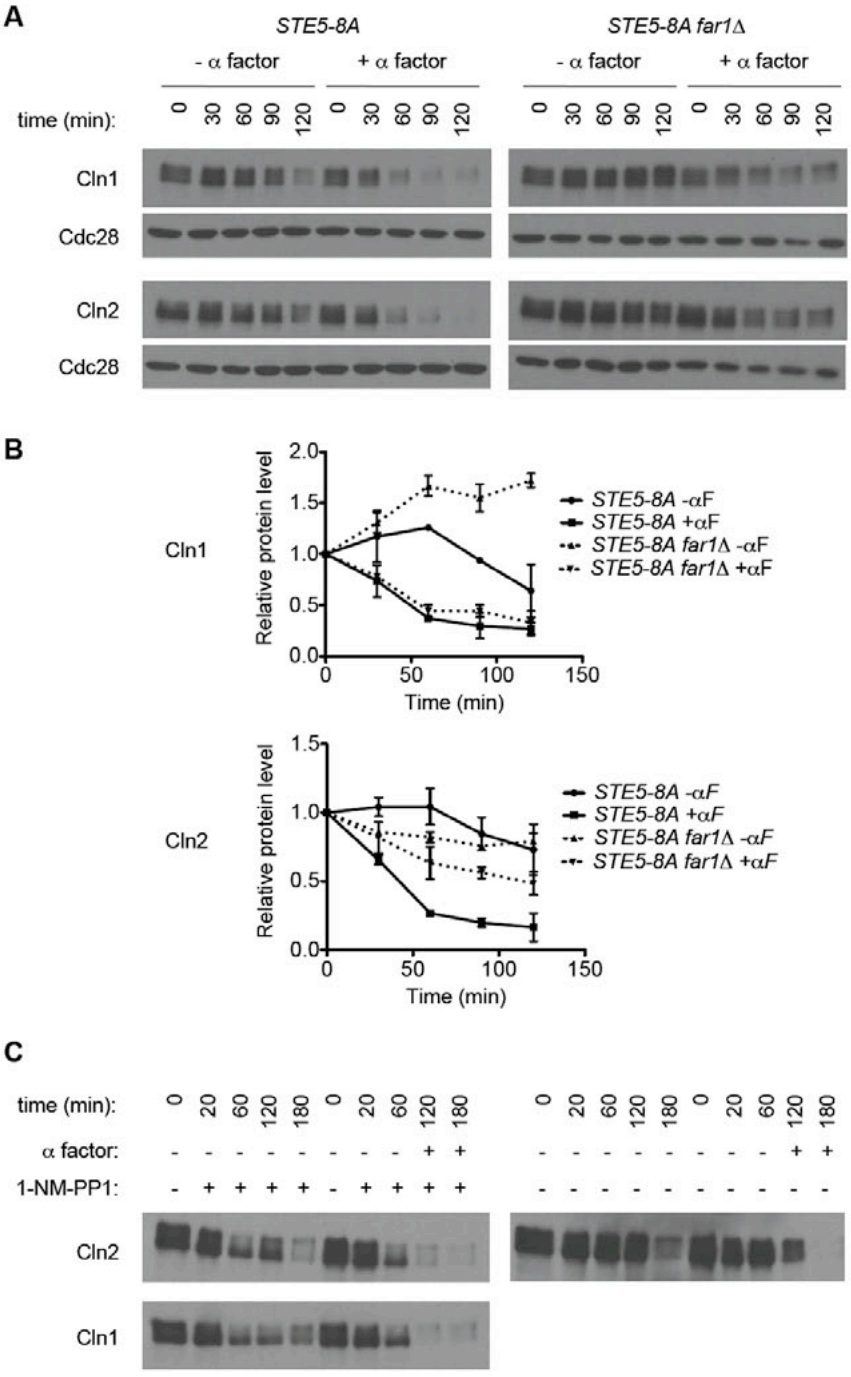
Figure 3.2: Loss of G1/S proteins is Cdc28-independent, but partially dependent on Far1

(A) Steady-state levels of Cln1 and Cln2 followed over time with or without pheromone in *STE5-8A* and *STE5-8A far1Δ* with Cdc28 serving as internal control.

(B) Graphs of results from (A) normalized to the Cdc28 signal

(C) Cdc28 inhibition alone does not cause total cyclin protein loss. Cdc28-as1 cells were treated with 10μM ATP analog 1-NM-PP1 for 60 minutes, then cultures were split and α factor was added to half. Samples were taken at the indicated time points and whole cell extracts were prepared. Samples were analyzed by anti-myc blot. Arrest from Cdc28 inhibition was confirmed via FACS of DNA content after inhibitor treatment.

Figure 3.2: Loss of G1/S proteins is Cdc28-independent, but partially dependent on Far1



ATP analog 1-NM-PP1. We treated cultures with the inhibitor for 60 minutes to inactivate Cdc28 prior to addition of pheromone. Surprisingly, inactivation of Cdc28 alone resulted in somewhat decreased cyclin protein levels, but addition of pheromone caused a stronger decrease (Figure 3.2C). The efficacy of the Cdc28 inhibitor was evident by a shift in the mobility of Cln1 and Cln2 to a faster mobility form, which is diagnostic of reduced CDK phosphorylation. These results demonstrate that CDK inhibition alone is not sufficient to cause the degree of protein loss observed during pheromone treatment, and also suggest that during pheromone treatment cells have mechanisms independent of Cdc28 activity to target Cln proteins for degradation.

Role for the normal cyclin degradation machinery

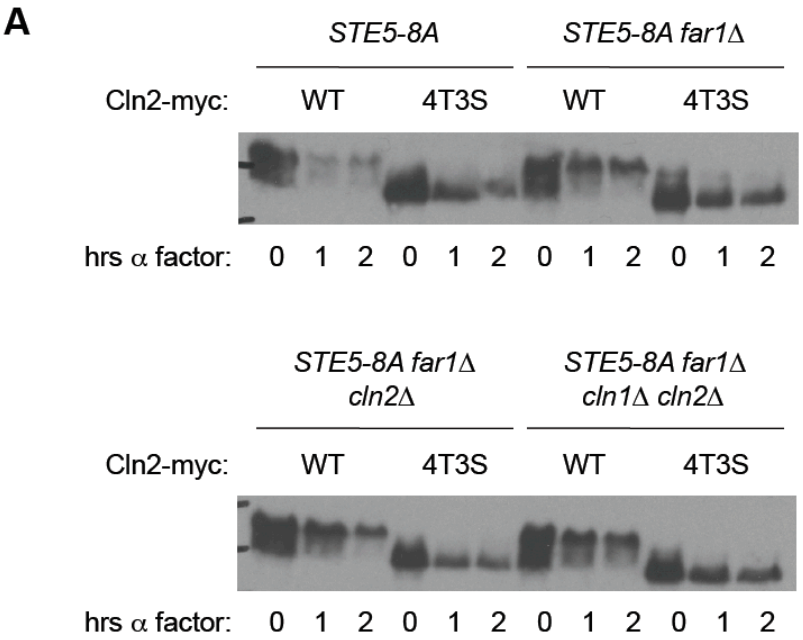
Next, we investigated which aspects of the normal cyclin degradation mechanism are required for the pheromone effects. Because phosphorylation of Cln1/2 at CDK sites normally targets these proteins for ubiquitination by SCF^{Grr1}, we tested the requirement for these CDK sites and Grr1. A mutant form of Cln2 lacking its seven CDK phosphorylation sites, $Cln2^{4T3S}$, is significantly stablized ($t_{1/2}$ > 60 minutes versus < 10min for wild type). Remarkably, steady state levels of $Cln2^{4T3S}$ were still reduced by pheromone treatment, although not as severely as for wild type Cln2 (Figure 3.3A). This result suggests that the CDK phosphorylation sites are not absolutely required for the pheromone effect. In contrast, SCF^{Grr1} appeared to be strongly required, as deletion of *GRR1*

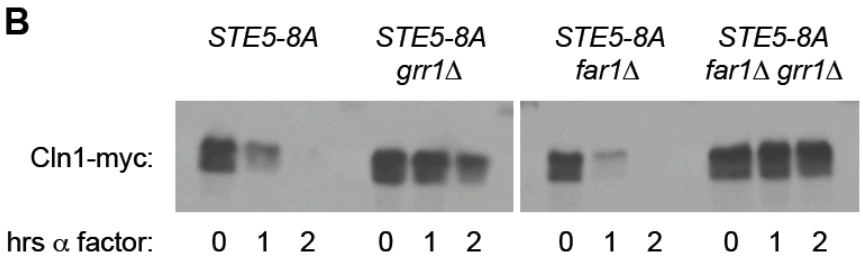
Figure 3.3: Pheromone-induced protein loss requires Grr1 but not CDK consensus sites

(A) $Cln2^{4T3S}$ protein still decreases in response to pheromone even though it lacks the 7 CDK consensus phosphorylation sites. Steady state levels of wild type Cln2 and $Cln2^{4T3S}$ were followed after treatment with pheromone in *STE5-8A*, *STE5-8A grr1Δ*, *STE5-8A far1Δ cln2Δ*, *STE5-8A far1Δ cln1Δ cln2Δ* cells.

(B) Steady state levels of Cln1 no longer drop significantly after pheromone treatment when Grr1 is deleted. *STE5-8A, STE5-8A far1Δ, STE5-8A far1Δ,* and *STE5-8A far1Δ grr1Δ* cells expressing Cln1-myc from a constitutive promoter were treated with pheromone and samples were taken after 1 and 2 hours. Anti-myc blots of whole cell extracts were analyzed to determine protein levels. Cln1 was used in this experiment as opposed to Cln2 so as to limit the detrimental impact of overexpressing Cln protein in *grr1Δ*.

Figure 3.3: Pheromone-induced protein loss requires Grr1 but not CDK consensus sites





significant reduced the effect of pheromone on Cln1 (Figure 3.3B). As Grr1 is generally thought to recognize phospho-peptides in substrate proteins, it is not clear how to explain the seemingly stronger requirement for Grr1 than for the CDK sites. We tested the $Cln2^{4T3S}$ mutant in strains lacking endogenous Cln1/2 proteins to eliminate the possibility of cross-complementation (Figure 3.3A), but we did not test if Grr1 was still required for the loss of the $Cln2^{4T3S}$ mutant.

G1 arrest is not required for cyclin protein loss

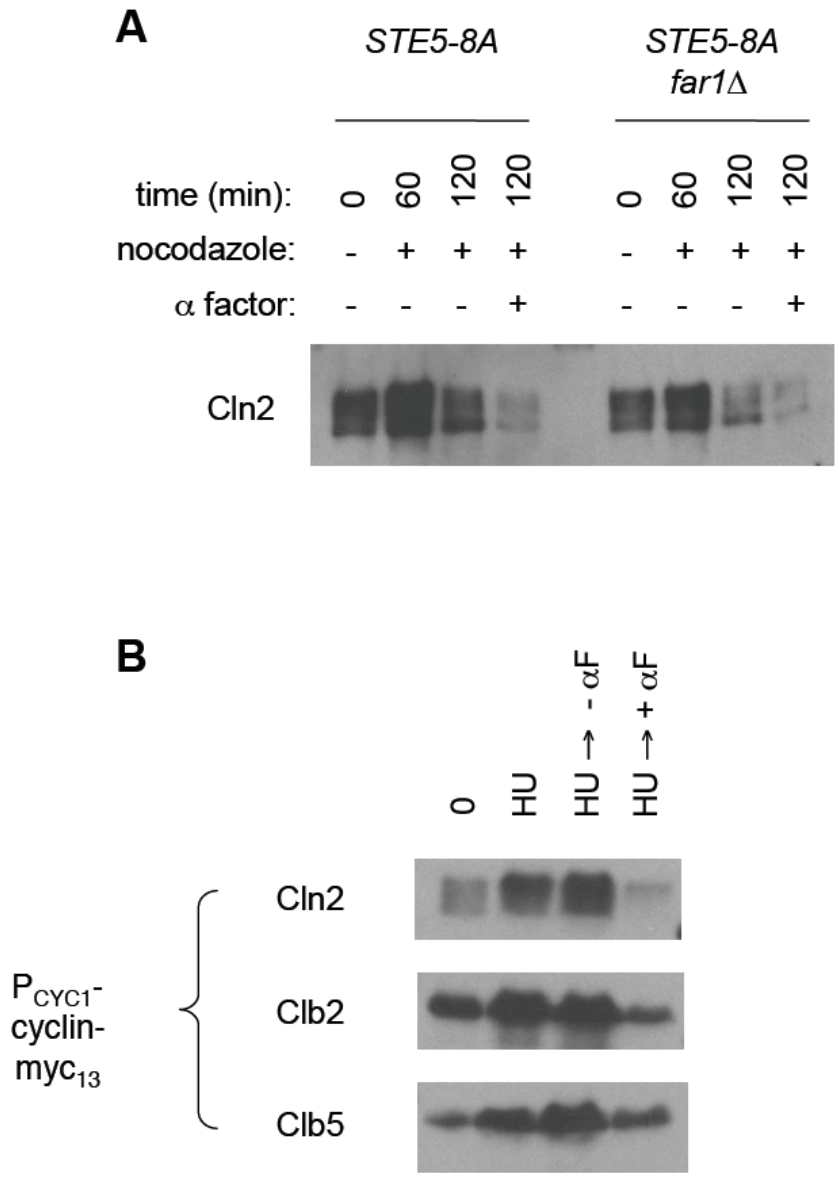
We next wanted to test the G1 specificity of this phenomenon. By first arresting cells in other parts of the cell cycle we hoped to determine if arrest in G1 was required for the pheromone effect. To test G1 phase specificty we looked at levels of constitutively expressed Cln2 in *STE5-8A* and *STE5-8A far1Δ* cells first arrested with nocodozole (Figure 3.4A). Two hours in nocodozole alone decreased steady state protein levels, but addition of pheromone still had an increased effect indicating that 1) the effect on protein levels by pheromone is not dependent on the G1 arrest imposed by pheromone and 2) whatever pheromone is doing to decrease protein levels is not dependent on the cells being in G1. We also looked at the effect of pheromone on cells arrested by hydroxyurea (Figure 3.4B). Here, we compared the effect of pheromone on Cln2, Clb2, and Clb5. If anything it appears that HU arrest actually increases the steady state level of all cyclin proteins, and further treatment with pheromone seems to decrease levels of all of the proteins, not just Cln2. Together these results confirm that the effect

Figure 3.4: Protein levels after arrest with nocodazole or hydroxyurea

(A) Pheromone treatment can still cause decreased Cln2 even when cells are first arrest in mitosis with nocodazole. *STE5-8A* and *STE5-8A far1Δ* cells were treated with nocodazole for 60 minutes prior to addition of α factor. Whole cell extracts were analyzed with anti-myc blots. Cell cycle arrest was confirmed by FACS.

(B) Levels of Cln2, Clb2, and Clb5 proteins decrease in response to pheromone after HU arrest. *STE5-8A* cells harboring the indicated cyclin constructs were treated with hydroxyurea to arrest cells in S-phase prior to addition of pheromone. Whole cell extracts were analyzed by anti-myc blot. Cell cycle arrest was confirmed by FACS.

Figure 3.4: Protein levels after arrest with nocodazole or hydroxyurea



of pheromone on cyclin proteins is independent of G1 arrest, and that this effect may not be specific to the G1/S cyclins.

Unfortunately, in the course of these studies we experienced significant day-to-day variability in the degree of steady-state protein loss observed after pheromone addition. In some experiments the reduction was starkly evident, whereas in other experiments it was almost undetectable. I tested multiple aspects of the experimental procedure to determine if factors such as culture density, nutrient levels, cell lysis procedure, or protein preparation methods could be impacting the overall results. Although there was some dependence on culture density, such that the pheromone effect was strongest in cultures with a higher starting density, this alone was not enough to explain the observed variability. Because we could not identify the source of variability, I was unable to continue any further mechanistic analysis of steady-state protein levels. I did, however, perform some additional experiments aimed at measuring the rate of disappearance of Cln1/2 proteins.

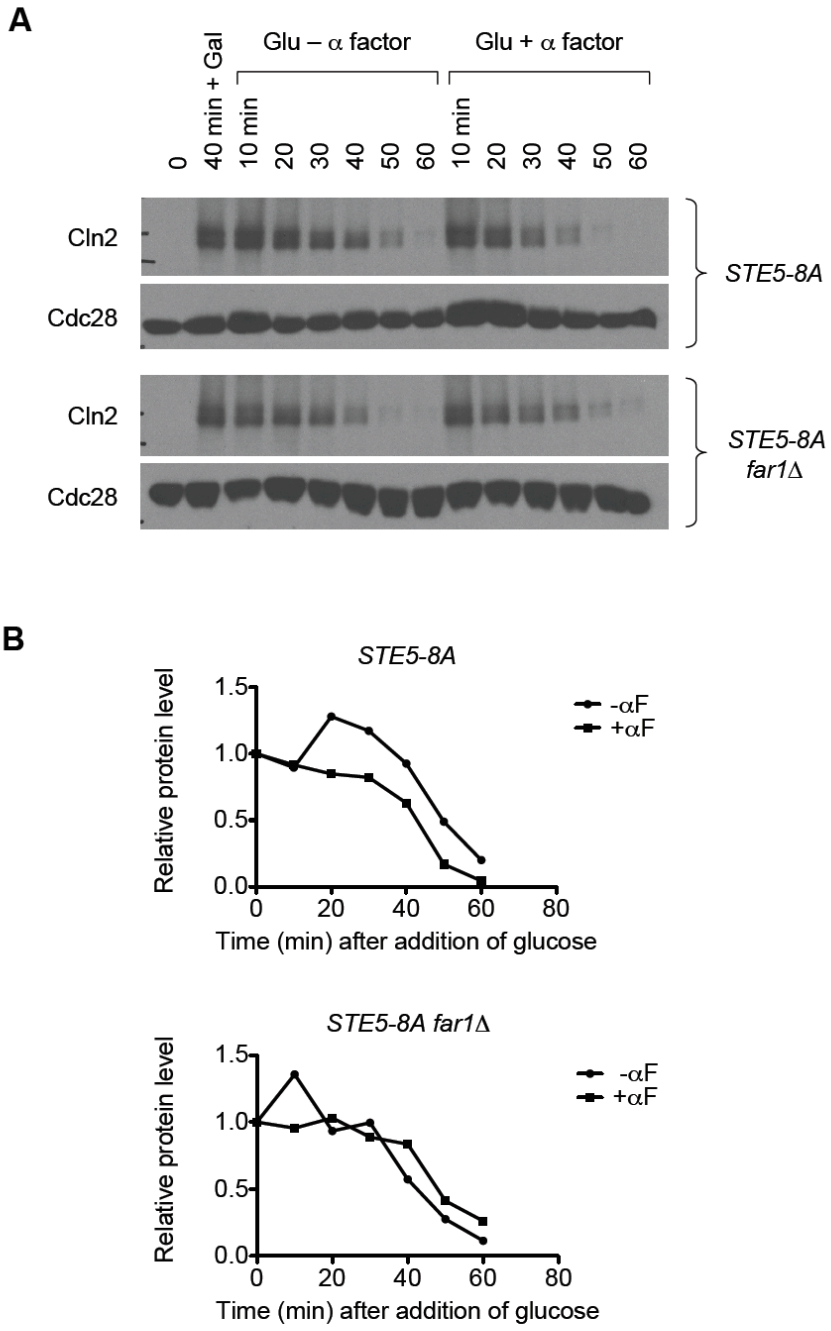
Using transcriptional or translational shut off to study protein decay rates

In one experimental setting, we used a transcriptional pulse-chase system to monitor the decay rate of cyclin proteins. Here, we placed epitope-tagged Cln2 under control of the inducible P_{GAL1} promoter, induced its expression with 2% galactose for 40 minutes, and then shut off expression by adding glucose either

Figure 3.5: Protein levels after transcriptional shut-off

(A) & (B) When Cln2 transcription is shut off, pheromone has only a limited effect on the rate of protein loss. In *STE5-8A* and *STE5-8A far1Δ* cells Cln2 was transiently expressed through induction of a GAL1pr-CLN2-myc construct and then inhibited with glucose in the presence or absence of pheromone. Whole cell extracts were analyzed with anti-myc and anti-Cdc28 blots. Graphs in (B) represent results normalized to Cdc28.

Figure 3.5: Protein levels after transcriptional shut-off



with or without pheromone. Then, the rate of protein loss was monitored. Results in Figure 3.5 show that pheromone treatment did accelerate Cln2 protein loss in *STE5-8A* cells, however, there was no effect of pheromone in *STE5-8A far1Δ* cells. Protein loss does seem to be slightly accelerated in the untreated samples of the *STE5-8A far1Δ* cells as compared to the *STE5-8A* which may be obscuring the results. As this was only a single experiment it is difficult to draw any strong conclusions. However, this experiment did suggest that pheromone can, in fact, impact the rate of cyclin protein disappearance, so we decided to further analyze this phenomenon.

To ask whether pheromone affects Cln2 protein levels by altering the rate of synthesis versus degradation, we used cycloheximide to inhibit synthesis so that we could monitor degradation alone. When cultures were treated with cycloheximide in the presence or absence of pheromone, there seemed to be no difference in rates of Cln2 protein loss (Figure 3.6A). We considered the possibility that the very short half-life of Cln2 (< 10 minutes) in normal cycling cells could obscure detection of any effect of pheromone. Therefore, in an attempt to block CDK-mediated degradation, we inhibited CDK activity by using *cdc28-as2* cells and the ATP analog 1-NM-PP1. We expected that eliminating CDK activity would stabilize the Cln proteins and thereby make any pheromone-dependent changes in protein degradation more evident. Cultures were pre-

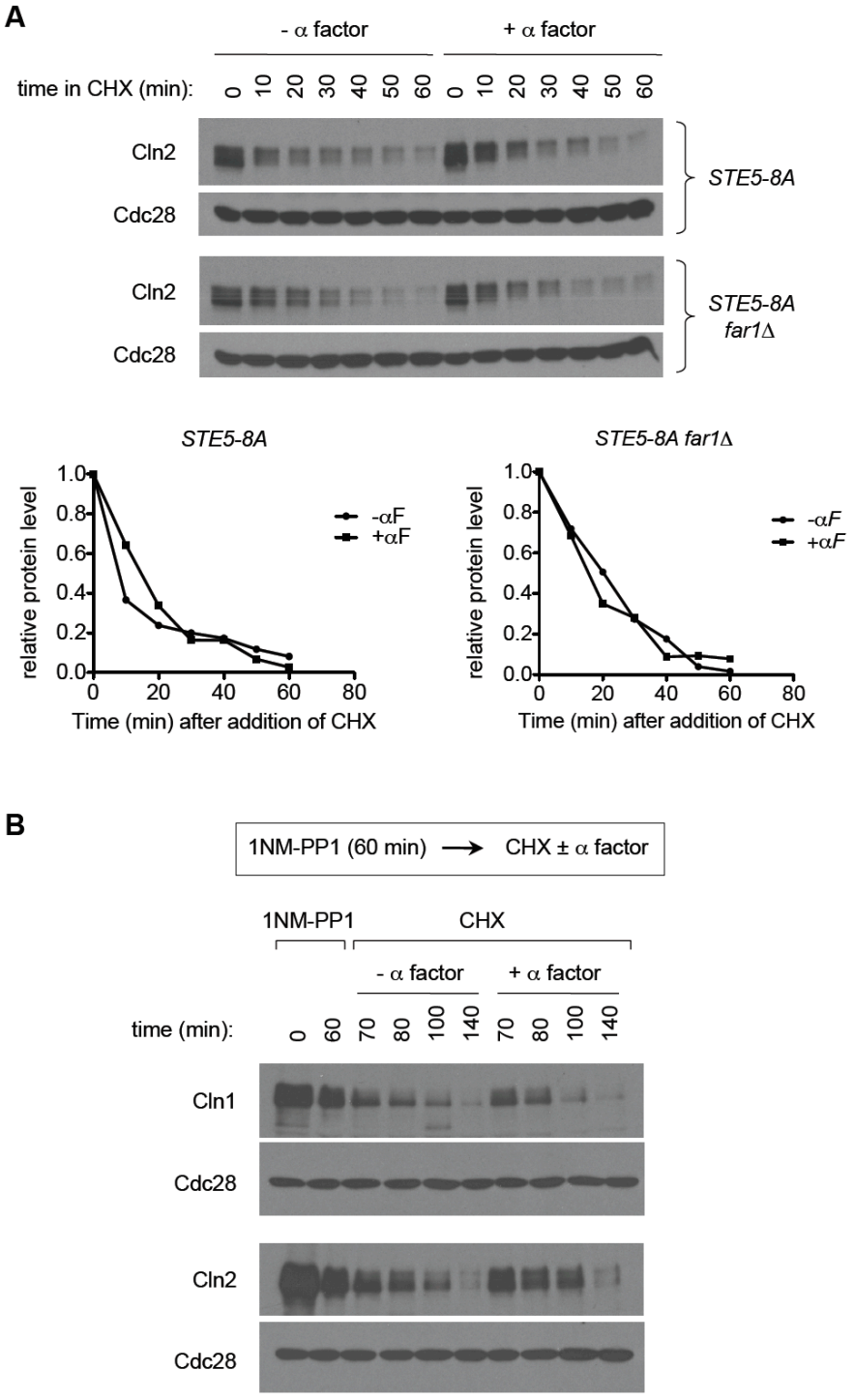
treated with 1-NM-PP1 for 60 minutes prior to addition of cycloheximide and pheromone (Figure 3.6B). Surprisingly, even in the presence of the Cdc28 inhibitor, there was still a very rapid decrease in Cln2 protein levels after addition of cycloheximide. Furthermore, there was no evident effect of pheromone. The rapid decay of cyclin proteins in the absence of CDK activity was unexpected since CDK-dependent phosphorylation of Cln2 is required for its degradation. A simple explanation might be that the inhibition of CDK activity was incomplete and that the residual activity was sufficient to promote Cln2 degradation (see Discussion). Regardless, the continued short half-life of Cln2 makes it difficult to interpret whether pheromone has no effect on degradation or whether the background rate is too fast to allow any increase to be detected. It is worth emphasizing, however, that these results might indicate that pheromone does act by reducing translational synthesis of Cln1/2, rather than increasing their degradation (see Discussion).

Figure 3.6: Protein levels after translational shut-off

(A) When translation is inhibited with cycloheximide pheromone has no measurable effect on rate of Cln protein loss. *STE5-8A* and *STE5-8A far1Δ* cells expressing Cln2-myc were treated with cycloheximide with or without pheromone, and samples were taken at 10 minute intervals. Whole cell extracts were analyzed by anti-myc and anti-Cdc28 blots, and Cln2 protein levels were quantified and normalized to the Cdc28 signal.

(B) Even after inhibition of Cdc28 Cln protein is still rapidly degraded. Cultures were first treated with 1-NM-PP1 to inhibit Cdc28-as2 activity and then treated with cycloheximide with or without pheromone.

Figure 3.6: Protein levels after translational shut-off



Discussion

Overall these results suggest that there is a pheromone-induced effect on protein levels that appears to have some dependence on Far1. However, the phenomenon may not be a result of increased degradation rates as previously hypothesized, but instead may be dependent on pheromone's ability to globally regulate translation.

The results of the steady-state experiments suggested that the destabilization of Cln proteins in response to pheromone is a partially Far1-independent function reliant on Grr1 but at least partially independent of Cdc28 activity and the cyclins' CDK phosphorylation sites. The Cdc28-independence of this phenomenon is consistent with the concept that Cdc28 is inactivated in response to pheromone, and the requirement for Grr1 also seems consistent with our understanding of the Cln degradation pathway. The results obtained from the $Cln2^{4T3S}$ allele, however, were unexpected, and, as discussed below, may indicate that targeted degradation of Cln1/2 is not the primary mechanism at work.

Recent work (published after we discontinued most work on this project) suggests that the pheromone pathway causes a global reduction in protein synthesis, and so this effect may be relevant to the effects of pheromone on

Cln1/2 levels (Goranov et al., 2009) (this effect was specific to pheromone treated samples, and was not simply a result of G1 arrest). In particular, a pheromone-induced reduction in global protein synthesis might cause all short-lived proteins to rapidly shift to a new steady state level (at which synthesis and degradation rates reach equilibrium). This notion could explain why we saw no effect of pheromone on the rate of Cln1/2 degradation (i.e., after cycloheximide addition) but instead only saw effects when Cln1/2 protein synthesis was continued (i.e., the P_{GAL1} transcriptional shut-off experiment, and all steady state experiments including the stabilized $Cln2^{4T3S}$ mutant). Moreover, this view predicts that other short-lived proteins should behave similarly to Cln1/2. We did not test this prediction extensively, although we did observe that pheromone could cause decreased steady-state levels of Clb2 and Clb5 after HU arrest; this result is consistent with the above notion, but is certainly not conclusive. Notably, the recent work suggested that the ability of pheromone to reduce global protein synthesis depends on Far1; though the responsible mechanism is unknown, this finding might explain the partial Far1-dependence we observed in our experiments.

Since Cln1/2 proteins persist in the absence of the ubiquitin ligase SCF^{Grr1} it appears that ubiquitin mediated degradation is required for the effects of pheromone on Cln1/2, although this finding is consistent with multiple models and does not necessarily imply that either Grr1 function or degradation per se are

regulated by pheromone. Because Cln1/2 are ordinarily destabilized by CDK phosphorylation, we were surprised to see relatively rapid continued degradation in experiments where Cdc28 was inhibited. It is conceivable that another kinase was responsible, such as the alternate CDK Pho85. Although there has been no evidence yet presented to suggest Pho85 targets Cln1 and Cln2 proteins, increasing evidence for overlapping function of Pho85 and Cdc28 does suggests it's possible. Alternatively, and probably more likely in this setting, the CDK inhibition may have been incomplete, and any residual activity could potentially be enough to phosphorylate Cln proteins. This possibility may be especially pertinent for substrates like cyclins that are directly associated with the CDK and hence are in high local concentration. In the presence of pheromone, Fus3 or another member of the pheromone signaling cascade could directly phosphorylate cyclin proteins, especially since MAP kinases and CDKs share the same consensus site (i.e, SP or TP). This might relax the requirement for CDK activity, although further testing would be necessary to address this possibility.

As described earlier, we observed wide variability in the Cln1/2 phenotype in pheromone treated cells. In some experiments, the cyclin proteins were rapidly and almost completely lost in response to pheromone, but at other times there was little to no change. One possible contributor to this variation might involve previously observed links between nutrients and Cln protein levels (Baroni et al., 1994; Tokiwa et al., 1994; Barral et al., 1995; Schneider et al., 2004). In

particular, carbon source has been demonstrated to have an impact on Cln protein levels independent of transcription. Steady state protein levels were observed to be six-fold higher in cells grown in glucose versus those grown in ethanol, a difference that correlated with faster growth rates in the glucose cultures (Schneider et al., 2004). This result suggests a link between culture conditions and Cln protein levels that could contribute to day-to-day variability of our results. Although we were unable to conclusively verify that this was the cause, it remains our strongest suspicion that uncontrolled variability in nutrient-based regulation of Cln1/2 protein levels was interfering with our efforts to study pheromone-specific regulation of these same proteins. Future findings that surmount this variability problem may allow these studies to be resumed.

Material and Methods

Yeast Strains

Standard procedures were used for growth and genetic manipulation of yeast (Rothstein, 1991; Sherman, 2002). Yeast cultures were grown at 30˚C. Yeast strains are listed in Table 3.1; all were derived from the W303 background (Thomas and Rothstein, 1989) and harbor the *bar1*Δ mutation to block α factor degradation. PCR-mediated gene targeting used methods described previously (Longtine et al., 1998); selectable markers included an antibiotic resistance gene (*kanMX6*) and biosynthesis genes from S. cerevisiae aa well as orthologs from other yeasts (*ADE2*, *C. glabrata TRP1*). For the cycloheximide and Cdc28 inhibition experiment, the *CDC28* gene was replaced with an ATP analog-sensitive allele *cdc28-as2* via a two-step pop-in/pop-out method (Rothstein, 1991); we used the Cdc28-as2 [F88A] mutant (Colman-Lerner et al., 2005) because in our strains the more severe mutant Cdc28-as1 [F88G] (Bishop et al., 2000) was hypomorphic, as indicated by slow growth and cell shape defects.

Signaling assay

To measure effects of constitutively expressed Cln2 on pheromone response cells were grown in raffinose media, induced with galactose, and then treated with α factor. *FUS1-lacZ* expression was measured by β-galactosidase assay.

Steady state cyclin level

Cultures were grown in synthetic, selective media overnight. In the morning cultures were diluted back to an OD660 of 0.2 and allowed to grow for 3 hours to ensure they were actively growing. Cultures were split, and pheromone was added to half to a final concentration of 0.2 μM. 5mL samples were taken at indicated time points.

For the steady-state hydroxyurea experiments, cultures were treated with 200mM HU for 90 minutes. 5ml samples were taken and then the cultures were split and 0.2 μM pheromone was added to half. 5mL samples were taken after 90 minutes.

For the steady-state nocodazole experiments: after recovery time 5mL samples were taken and the remaining culture was spun down and resuspended in YPD containing 10 μM nocodazole. After 60 minutes 5mL samples were taken and the remaining culture was split in half, with half receiving 0.2 μM pheromone. 5mL samples were taken after 60 minutes.

Steady-state cdc28-as1 experiment

Cultures were grown in synthetic, selective media overnight. In the morning cultures were diluted back to an OD660 of 0.2 and allowed to grow for 3 hours to ensure they were actively growing. Cultures were incubated with 5μM 1-NM-PP1 for 60 min, then cultures were split, and pheromone was added to half to a final concentration of 0.2 μM. Samples were taken at indicated time points.

GAL promoter shutoff

Cultures were grown in synthetic, selective media containing 2% raffinose overnight. In the morning cultures were diluted back to an OD660 of 0.2 and allowed to grow for 3 hours to ensure they were actively growing. A 5mL sample was taken and then galactose (final concentration 2%) was added to induce cyclin expression for 40 minutes. A 5mL sample was removed and the remaining culture was spun down and resuspended in synthetic glucose media to shut off expression. The cultures were then split in half and pheromone was added to half to a final concentration of 0.2 μM. 5mL samples were taken at the indicated time points. Samples were prepared as above.

Cycloheximide treatment, with or without cdc28-as

Cultures were grown in synthetic, selective media overnight. In the morning cultures were diluted back to an OD660 of 0.4. A 5mL sample was removed and the remaining culture was split into two. Both received cycloheximide (5mg/mL final), and one also received 0.2 μM pheromone. 5mL samples were taken at indicated time points, spun, and frozen in liquid nitrogen. Samples were prepared as above.

For experiments in the cdc28-as2 strain, cultures were treated with 10 μM 1-NM-PP1 for 60 minutes prior to addition of cycloheximide and pheromone.

Protein Sample Preparation

For samples in figures 1, 2C, 3, 4 5mL samples were taken and prepared using the NaOH lysis method. Samples were spun down and resuspended in 100µL H_2O. 100µL of 2N NaOH was added, the samples were vortexed, and then allowed to sit at room temperature for 5 min. Samples were then spun for 1 min at room temperature, resuspended in 50µL SDS Sample Buffer and boiled for 3 minutes.

For all other figures the trichloroacetic acid procedure (as described (Lee and Dohlman, 2008)) was used. The 5-10mL samples were spun down and frozen in liquid nitrogen and stored at -80^{o}C. Frozen cell pellets were thawed on ice with 300 µL TCA Buffer (10 mM Tris.HCl, pH 8.0, 10% TCA, 25 mM ammonium acetate, 1 mM Na_2EDTA) and transferred to a pre-chilled microfuge tube. Approximately 200 µL of glass beads were then added, and the samples were vortexed to lyse the cells (five rounds of 1 mintute vortexing with 3 minutes on ice in between). The lysate was transferred to a new microfuge tube and centrifuged at 4˚C for 10 minutes at 16,000 x g. The supernatant was aspirated off and the remaining pellet was resuspended in 150 µL of Resuspension solution (0.1 M Tris.HCl, pH 11.0, 3% SDS). The samples were boiled for 5 min, allowed to cool at room temperature for 5 min, and then centrifuged for 30sec. Then 120µL of the supernatant was transferred to a fresh tube. 20uL was reserved for use in a BCA protein assay, and 100µL 2.5X SDS sample buffer was added to the remaining 100µL of sample. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (#23225).

Western Blots

Either 10μL (for NaOH prepared cells) or 20μg (for TCA prepared cells) of each sample were run out by SDS-PAGE, transferred to PVDF by either semi-dry or submerged transfer method. Myc blots were probed with Rabbit anti-myc (1:200 Santa Cruz Biotechnologies #sc-789) and detected with Goat anti-rabbit AP conjugated secondary antibody (1:3000) and Immun-Star-AP substrate (BioRad #170-5018). Cdc28 blots were probed with goat anti-Cdc28 (1:200 Santa Cruz Biotechnologies #sc-6709) and detected with donkey anti-goat HRP-conjugated secondary (1:3000 sc-2020) and Pierce SuperSignal West Pico (#34080) chemilluminescent reagent.

Blots were quantified using the program ImageJ and this previously described method (Miller, 2007).

Table 3.1. Yeast strains used in this Chapter III

Name	Relevant Genotype*	Source
PPY640	MAT**a** FUS1::FUS1-lacZ::LEU2	1
PPY892	MAT**a** FUS1::FUS1-lacZ::LEU2 far1::ADE2	this study
PPY1748	*MAT**a** bar1 STE5-8A*	2
PPY1778	*MAT**a** bar1 STE5-8A far1Δ::ADE2*	2
PPY1853	*MAT**a** bar1 STE5-8A far1Δ::ADE2 cln2Δ::kanR*	2
PPY2075	MAT**a** FUS1::FUS1-lacZ::LEU2 STE5-8A	this study
PPY2076	MAT**a** FUS1::FUS1-lacZ::LEU2 far1::ADE2 STE5-8A	this study
PPY2179	*MAT**a** bar1 STE5-8A far1Δ::ADE2 cln2Δ::kanR cln1Δ::TRP1^{cg}*	this study
PPY2208	MAT**a** bar1Δ STE5-8A grr1Δ::TRP1^{Cg}	this study
PPY2210	MAT**a** bar1Δ STE5-8A far1::ADE2 grr1Δ::TRP1^{Cg}	this study
PPY2213	*MAT**a*** cdc28-as1	
PPY2270	*MAT**a*** cdc28-as2	this study

* All strains are in the W303 background (*ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1*), except PPY2270 which is BY4741 (MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0)

Source: (1)(Pryciak and Huntress, 1998) (2)(Strickfaden et al., 2007)

Table 3.2. Plasmids used in Chapter III

Name	Alias	Description	Source
pPP3077	pTEFpr-CLN2-myc	CEN URA3 TEF1pr-CLN1-myc13 CYC1term	1
pPP3078	pADHpr-CLN1-myc	CEN URA3 ADH1pr-CLN1-myc13 CYC1term	this study
pPP3079	pADHpr-CLN2-myc	CEN URA3 ADH1pr-CLN2-myc13 CYC1term	this study
pPP3153	pCYC1pr-CLN1-myc	CEN URA3 CYC1pr-CLN1-myc13 CYC1term	1
pPP3154	pCYC1pr-CLB5-myc	CEN URA3 CYC1pr-CLB5-myc13 CYC1term	1
pPP3155	pCYC1pr-CLB2-myc	CEN URA3 CYC1pr-CLB2-myc13 CYC1term	1
pPP3203	pCYC1pr-CLN2-myc	CEN URA3 CYC1pr-CLN2-myc13 CYC1term	1
pPP3204	pCYC1pr-CLN2^{4T3S}-myc	CEN URA3 CYC1pr-CLN2(4T3S)-myc13 CYC1term	this study
pPP3201	pGALpr-CLN2-myc	CEN URA3 GAL1pr-CLN2-myc13 CYC1term	this study

Source: (1)(Bhaduri and Pryciak, 2011)

Strain and Plasmid combinations

Figure 3.1: (B) PPY640, PPY892, PPY2075, PPY2076 harbored pPP681, pPP3079, or pPP3203 (C) PPY1748 and PPY1778 harbored pPP3078 or pPP3079 (D) PPY1748 harbored pPP3153, pPP3154, or pPP3203

Figure 3.2: (A) PPY1748 and PPY1778 harbored pPP3153 or pPP3203 (C) PPY2213 harbored pPP3153 or pPP3203

Figure 3.3: (A) PPY1748, PPY1778, PPY1853, and PPY2179 harbored pPP3203 (B) PPY2208 and PPY2210 harbored pPP3203

Figure 3.4: (A) PPY1748 and PPY1778 harbored pPP3203 (B) PPY1748 harbored pPP3203, pPP3154, or pPP3155

Figure 3.5: PPY1748 and PPY1778 harbored pPP3201

Figure 3.6: (A) PPY1748 and PPY1778 harbored pPP3203 (B) PPY2270 harbored pPP3153 and pPP3203

CHAPTER IV

Far1-dependent G1 arrest in response to pheromone is the result of interference with the ability of Cln proteins to bind with their substrates

The following chapter was prepared by Dr. Peter Pryciak and myself as a partial manuscript to be combined with work of a fellow graduate student, Samyabrata Bhaduri, for future publication. All experiments presented here were completed solely by me.

Abstract

The protein Far1 was originally identified for its role in G1 arrest in response to pheromone. Early studies suggested that Far1 acts as a CDK inhibitor (CKI) protein that directly blocks the activity of Cln-CDK complexes, however, further studies have been unable to conclusively show that Far1 inhibits CDK activity. Although it is clear that Far1's role in G1 arrest is linked to its ability to interact with Cln proteins, exactly how Far1 uses this interaction to eliminate Cln-CDK activity has remained a mystery. Recent revelations regarding the existence of specific docking sites for Cln-CDK binding on certain Cln substrates caused us to revisit this question of Far1's function. Here we report that Far1 is able to interfere with the ability of Cln proteins to dock with substrates, and that this interference results in a correlated decrease in substrate phosphorylation. We also observed that the ability of various Far1 mutants to bind Cln2 correlated with the ability of those cells to arrest in pheromone, suggesting that the ability to interfere with Cln2-substrate binding is critical for Far1's arrest function.

Introduction

In eukaryotic cells, initiation of cell division is regulated by a variety of extracellular signals (Qi and Elion, 2005). During the mating reaction of the budding yeast *Saccharomyces cerevisiae*, extracellular mating pheromones (**a** factor and α factor) activate a signal transduction pathway that blocks entry into a new division cycle and thereby arrests cells in G1 phase (Bardwell, 2005). The protein Far1 (Factor arrest) plays a key role in this process. Far1 was originally identified via a genetic screen for mutants that retained active signaling responses (e.g., pheromone-induced transcription) but were specifically defective at G1 arrest (Chang and Herskowitz, 1990), suggesting that it functioned to connect the signal transduction pathway to the cell cycle (Figure 1A). Far1 function is activated by the pheromone pathway via both increased transcription of the *FAR1* gene and phosphorylation of the Far1 protein by the MAP kinase Fus3 (Chang and Herskowitz, 1990; Peter et al., 1993). Conversely, when cells enter a new division cycle, Far1 is inactivated by CDK phosphorylation, which triggers its degradation (Henchoz et al., 1997).

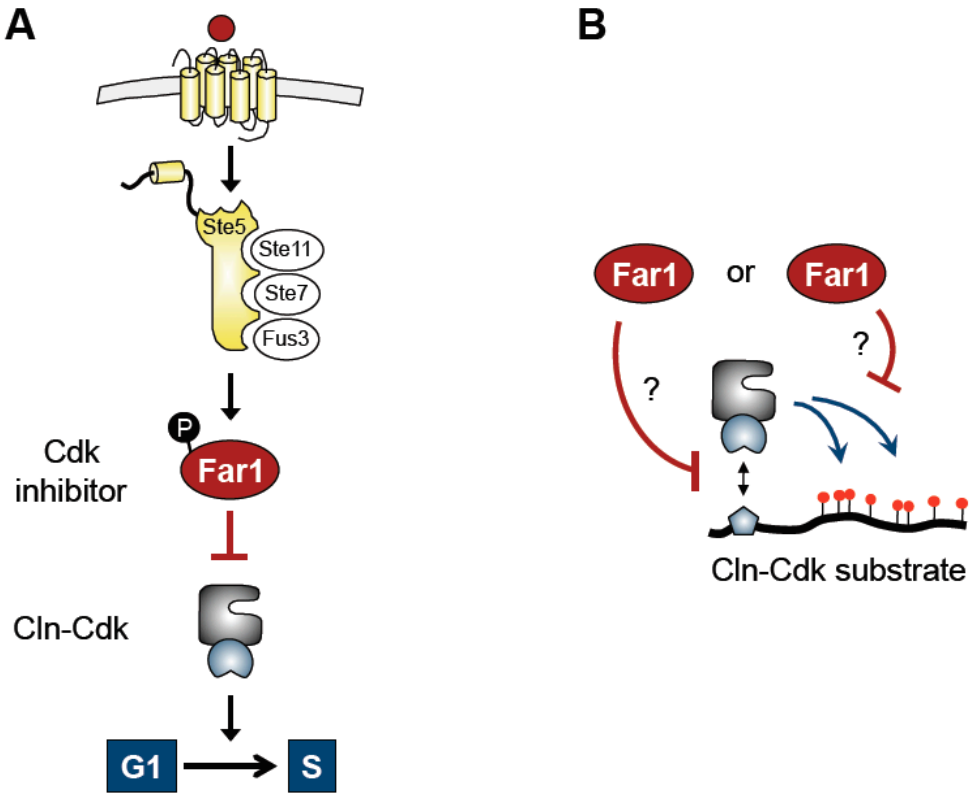
Early studies suggested that Far1 acts as a CDK inhibitor (CKI) protein that directly blocks the activity of cyclin-CDK complexes that contain early-acting cyclins (Cln1, Cln2, Cln3), which function in G1 to promote cell cycle entry. In particular, Far1 was found to bind CDK complexes containing each of these three

Figure 4.1: Schematic of Far1-dependent G1 arrest

(A) Once activated, the mating pathway activates a MAP kinase cascade that then activates Far1 through phosphorylation. Activated Far1 induces G1 arrest through an as yet undefined mechanism.

(B) Two possible mechanisms by which Far1 could induce G1 arrest; by interfering with Cln-CDK/substrate interactions or inhibiting Cln-CDK activity

Figure 4.1: Schematic of Far1-dependent G1 arrest



cyclins in vivo (Tyers and Futcher, 1993), and could inhibit CDK phosphorylation in vitro of generic substrates (e.g., histone H1) when the CDK was associated with the G1/S cyclin Cln2 but not with S- or M-phase cyclins (Clb5 or Clb2, respectively) (Peter and Herskowitz, 1994). Subsequent studies, however, were unable to observe an inhibitory effect of Far1 on Cln2-CDK kinase activity, despite their clear association in a bound complex (Gartner et al., 1998). Still other studies suggested that Far1 might inhibit CDK activity associated with the earliest G1 cyclin, Cln3, or that Far1 regulates post-transcriptional accumulation of the Cln2 protein (Valdivieso et al., 1993; Jeoung et al., 1998). Consequently, although the fact that Far1 plays a role in pheromone-induced G1 arrest has been clear for many years, its precise molecular activity has remained unresolved. Furthermore, to our knowledge there are no published findings indicating that Far1 affects the phosphorylation state of a CDK substrate in vivo.

Recent studies indicate that efficient phosphorylation of some substrates by Cln1/2-CDK requires a specific docking interaction between the cyclin and a short recognition sequence in the substrate that is separate from the phospho-acceptor site(s) (Bhaduri and Pryciak, 2011; Koivomagi et al., 2011). These docking interactions were found to promote phosphorylation of several Cln1/2-specific CDK substrates, both in vivo and in vitro. CDK substrates that contain such docking sites include components of the pheromone signaling pathway (Ste5, Ste20) and regulators of the G1/S transition (Sic1, Whi5) (Bhaduri and

Pryciak, 2011). These findings raise the possibility that the docking interactions might be regulated as an additional layer of control over cell cycle entry. In particular, we considered the possibility that pheromone signaling and/or Far1 might disrupt these docking interactions, either in addition to or as an alternative to direct inhibition of CDK activity per se (Figure 1B). Here, we report that, indeed, Far1 interferes with the ability of Cln2 to bind docking sites in Cln2-CDK substrates, and that this interference can contribute to their reduced phosphorylation *in vivo*.

Results

To assess whether activation of the mating pathway can disrupt docking interactions between Cln2 and substrates, we initially used an assay from our previous studies in which a GST-substrate fusion and an epitope-tagged cyclin were co-expressed and then co-precipitated. One complexity in the current experiments arises from the fact that the mating pathway and Cln2-CDK activity are mutually antagonistic. That is, pheromone-arrested cells do not express the *CLN2* gene and hence lack Cln2 protein, whereas Cln2-expressing cells inhibit pheromone signaling and degrade Far1. Therefore, to allow us to compare a variety of independent conditions without vast changes in protein levels, we circumvented these antagonistic effects as follows: (i) *CLN2* was expressed from a constitutively-active promoter (P_{CYC1} or P_{ADH1}); (ii) the ability of Cln2 to inhibit pheromone signaling was prevented by using strains with the *STE5-8A* allele, which encodes a CDK-resistant form of the pathway scaffold protein, Ste5; and (iii) we initially used strains with the *FAR1-S87A* allele, which is resistant to CDK-triggered degradation.

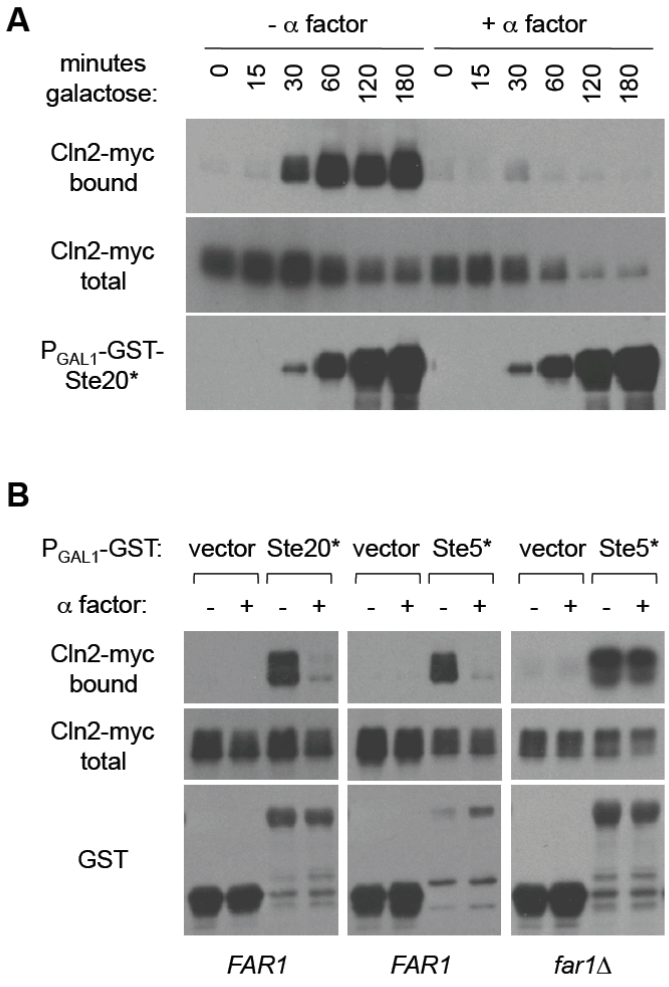
Initial experiments used a GST fusion to a fragment of the Cln2-CDK substrate Ste20 – specifically, the smallest fragment that previously showed strong Cln2 binding (residues 72-333, designated Ste20*) – expressed from a galactose-inducible promoter (P_{GAL1}). We observed Cln2-myc binding after as little as 30 minutes of galactose addition, when GST-Ste20* expression was

Figure 4.2: Far1 prevents Cln-CDK substrate interactions

(A) In the absence of pheromone, Cln2 appears in the bound fraction as soon as detectable levels of GST-Ste20* appear. Cells co-expressing a GAL1pr-GST-Ste20 fusion and Cln2-myc were induced with galactose with or without pheromone and samples were taken at indicated time points. Complexes were recovered using glutathione sepharose beads. Bound and input (5%) were analyzed with anti-myc and anti-GST blots.

(B) In *FAR1* cells pheromone can inhibit Cln-CDK/substrate binding, but inhibition is lost in *far1Δ*. Cells co-expressing chimeric GST-tagged substrates containing the Cln docking site from the indicated (*) protein and myc13-tagged Cln2 were induced with galactose in the presence or absence of pheromone. Glutathione sepharose beads were used to pull down complexes and bound and input (5%) samples were analyzed with anti-myc and anti-GST blots.

Figure 4.2: Far1 prevents Cln-CDK substrate interactions



submaximal (Figure 4.2A). When pheromone (α factor) was added simultaneous with galactose addition, binding of Cln2-myc was inhibited substantially (Figure 4.2A). Pheromone had no impact on expression of the GST fusion, although we frequently observed a reduction in Cln2 levels after prolonged treatments, possibly reflecting a poorly understood post-translational effect of pheromone on Cln2 abundance ((Valdivieso et al., 1993); see Chapter 3); hence, in subsequent experiments we used short treatment times whenever possible to minimize this effect. When we used a GST fusion in which the Ste20 docking site was replaced with one from Ste5 (GST-Ste5*), we found that pheromone inhibited binding of Cln2 to each fusion similarly (Figure 4.2B). Remarkably, however, this inhibition was not observed in *far1*Δ cells (Figure 4.2B), indicating that Far1 is required for the pheromone-induced effect. Together, these findings indicate that the pheromone pathway can disrupt Cln2-substrate binding interactions in a manner that depends on Far1.

Next, we examined the requirement for specific modification sites in Far1. Far1 is regulated in opposite ways by different kinases: phosphorylation at residue T306 by the MAPK Fus3 promotes Far1 arrest activity, whereas phosphorylation at residue S87 by CDK triggers its degradation (Figure 4.3A). We constructed strains with non-phosphorylable Ala mutations at these sites, in the context of full-length Far1 expressed from the native *FAR1* locus. As

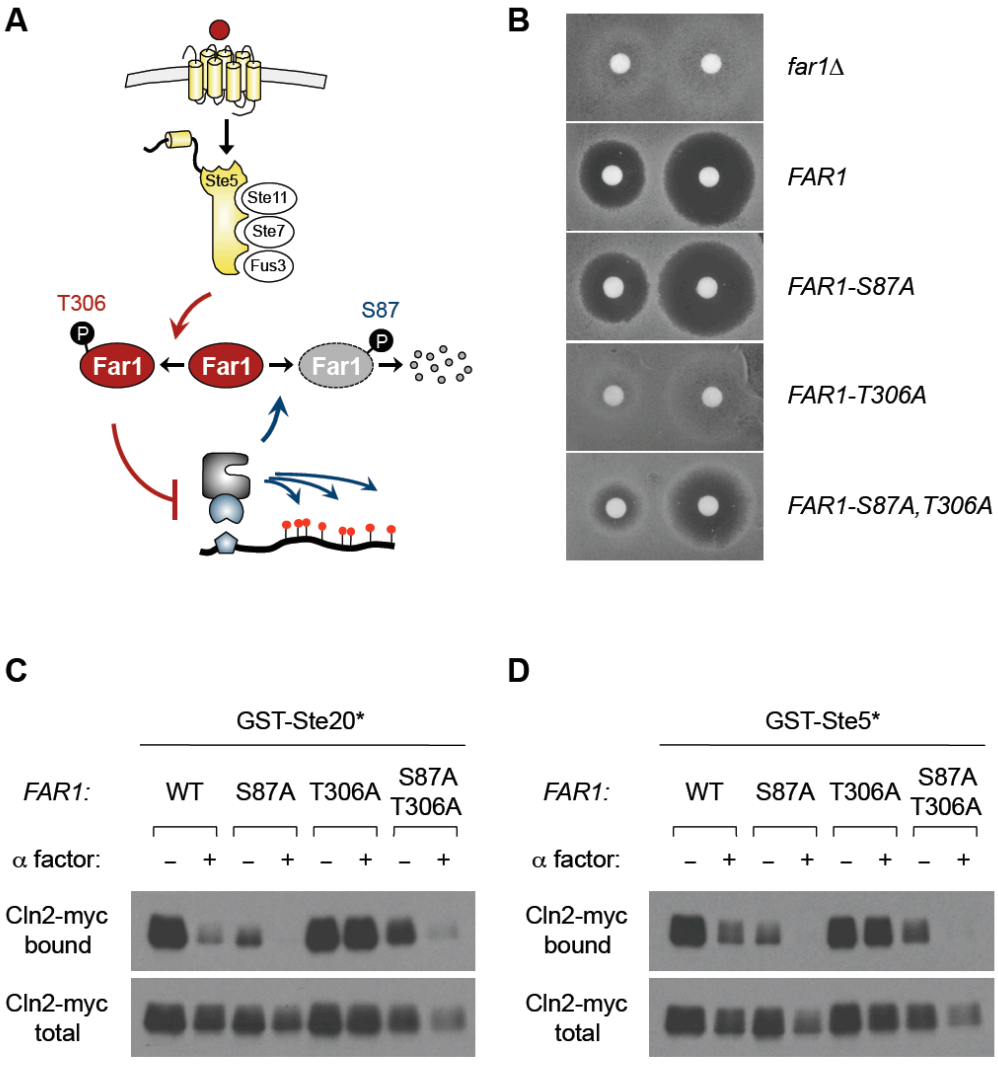
Figure 4.3: Different alleles of Far1 affect G1 arrest as well as Cln-CDK/substrate binding

(A) Pheromone treatment induces phosphorylation of Far1 at T306, and this phosphorylation is critical for the G1 arrest function of Far1. Conversely, Cln-CDK complexes can phosphorylate S87 of Far1 which targets Far1 for degradation.

(B) Different alleles of Far1 can either increase or decrease pheromone sensitivity as judged by zone of inhibition formation. For these assays lawns were spread on agar plates and disks containing 20µl of either 20 or 100µM α factor were added.

(C) (D) Cln2-CDK/substrate binding corresponds to the arrest phenotype. WT, *FAR1-S87A*, *FAR1-T306A*, and *FAR1-S87A,T306A* strains co-expressing a GST-substrate and Cln2-myc constructs were grown in the presence or absence of pheromone to determine the effect of the Far1 genotype on Cln2-CDK/substrate binding. Glutathione sepharose beads were used to pull down complexes and bound and input (2.5%) samples were analyzed with anti-myc and anti-GST blots.

Figure 4.3: Different alleles of Far1 affect G1 arrest as well as Cln-CDK/substrate binding



expected, the T306A mutant was defective at pheromone arrest and the S87A mutant remained functional (Figure 4.3B); the S87A T306A double mutant showed an intermediate phenotype, which indicates that T306 phosphorylation is not absolutely required if Far1 is stabilized by the S87A mutation. These arrest phenotypes match those seen previously when these same mutations were tested in the context of an N-terminal fragment of Far1 (Gartner et al., 1998). When we tested Cln2-substrate interactions in these strains, we observed several notable features common to both the GST-Ste20* and GST-Ste5* partners (Figures 4.3C, 4.3D). First, the T306A mutation in Far1 blocked the ability of pheromone to disrupt the Cln2-substrate interactions. Second, the S87A mutation increased the disruptive effect of pheromone as compared to Far1-WT. Third, this increased potency of the Far1-S87A mutant was evident even in the absence of pheromone. Fourth, the S87A mutation suppressed the defect of the T306A mutation, though not entirely so. Overall, these results parallel the G1 arrest phenotypes, implying that interference with Cln2-substrate docking might contribute to the arrest function of Far1. The ability of the stabilized Far1-S87A mutant to reduce Cln2-substrate binding even in the absence of pheromone was unanticipated, and may indicate that interference with Cln2 is a function of Far1 dosage. Hence, the inhibitory effect of pheromone in these experiments likely reflects both increased expression of Far1 and its phosphorylation by Fus3, which can explain why pheromone still was inhibitory in the S87A T306A double mutant. Finally, although we could not fully eliminate the

effects of pheromone treatment on Cln2 protein levels, the effects on substrate binding generally seemed proportionately stronger. In addition, the effect of the S87A mutant in the absence of pheromone was not accompanied by reductions in Cln2 levels, further suggesting that the effect of Far1 on Cln2-substrate binding is separable from Cln2 abundance.

To confirm these findings, we conducted analogous experiments using an alternative procedure, in which a galactose-inducible GST-Cln2 fusion was used to co-precipitate full-length substrate proteins (Ste20 and Ste5), expressed from their native promoters. To minimize effects of pheromone on Cln2 levels, we used a truncated form of Cln2 (residues 1-372) that lacks destabilizing motifs in its C-terminus. By this method, GST-Cln2 could specifically co-precipitate each substrate protein (Figure 4.4A, 4.4B), and this co-precipitation was disrupted by mutations in their known docking sequences. Then, using this procedure, we again tested the effect of Far1 and its mutant variants (Figure 4.5). Although there was some day-to-day variability in the degree of each effect (bar graphs in Figure 4.5 compile multiple trials shown in Figures 4.6 and 4.7), there were several consistent trends. Binding of Cln2 to each substrate was strongest in *far1*Δ cells, weakest in *FAR1-S87A* cells, and intermediate in *FAR1-WT* cells (Figure 4.5), suggesting that Far1 interferes with docking and that this effect is enhanced by increased Far1 abundance. Interestingly, this trend was apparent

Figure 4.4: Detection of docking-dependent binding between Cln2 and full-length substrate proteins.

GAL promoter driven GST alone or a GST fusion to truncated Cln2 (residues 1-372) were co-expressed with V5-tagged forms of full-length substrates expressed from their native promoters. (A) Ste20 (WT or docking site mutant [mut3]). (B) Ste5 (WT or docking site mutant [LLPP]). Glutathione sepharose beads were used to pull down complexes and bound and input (1.25%) samples were analyzed with anti-V5 and anti-GST blots. The WT and mutant substrates were analyzed in parallel, but were separated by other lanes in the original gels.

Figure 4.4: Detection of docking-dependent binding between Cln2 and full-length substrate proteins.

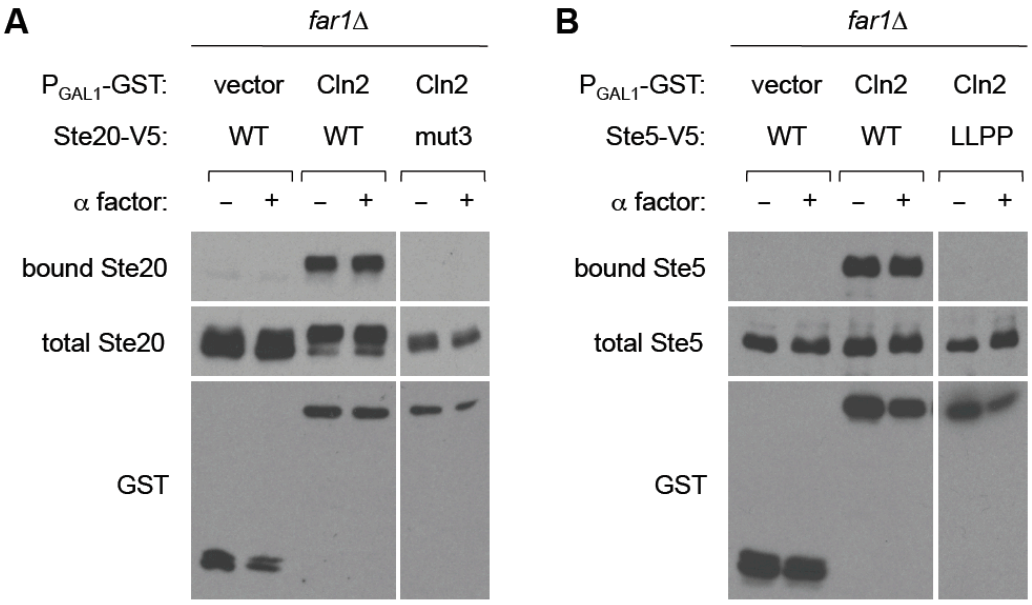


Figure 4.5: The allele of Far1 can affect binding of full-length Ste20 or Ste5 to GST-Cln2

(A) (B)Levels of binding of full-length substrates to Cln-CDK depend on the allele of Far1 present. *far1Δ, WT, FAR1, FAR1-S87A, FAR1-T306A*, and *FAR1-S87A,T306A* cells (all also containing the CDK-resistant *STE5-8A* allele to eliminate potential effects of overexpression of cyclin proteins) co-expressing a V5-tagged substrate (Ste20 in (A) and Ste5 in (B)) and GAL1pr-GST-Cln2 were induced with galactose with or without pheromone and samples were harvested after 1.5 hours. Glutathione sepharose beads were used to pull down complexes and bound and input (1.25%) samples were analyzed by anti-V5, anti-GST, and anti-Cdc28 blots. Cdc28 was used as an internal control for binding. Results in the graphs represent the average of three (A) and four (B) experiments with all blots being shown in Figures 6 and 7 respectively.

Figure 4.5: The allele of Far1 can affect binding of full-length Ste20 or Ste5 to GST-Cln2

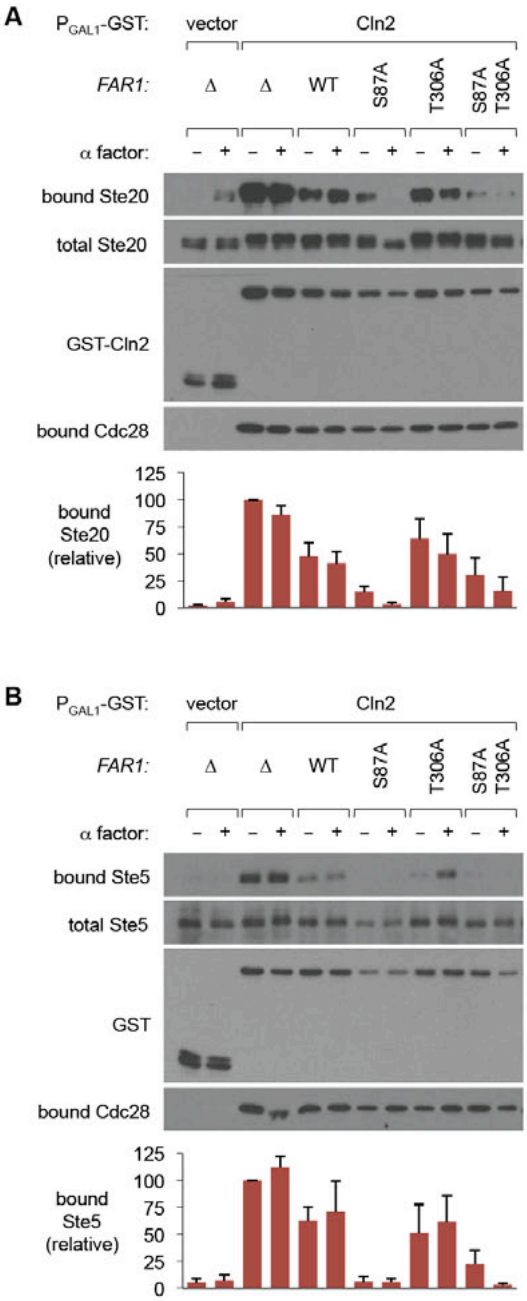


Figure 4.6: Day-to-day variability of the binding phenotype with Ste20 as the substrate

GST-Cln2 was used to pull down full length V5-tagged Ste20. Three individual experiments were completed, and the results from all were average to create the chart in Figure 4.5A.

Figure 4.6: Day-to-day variability of the binding phenotype with Ste20 as the substrate

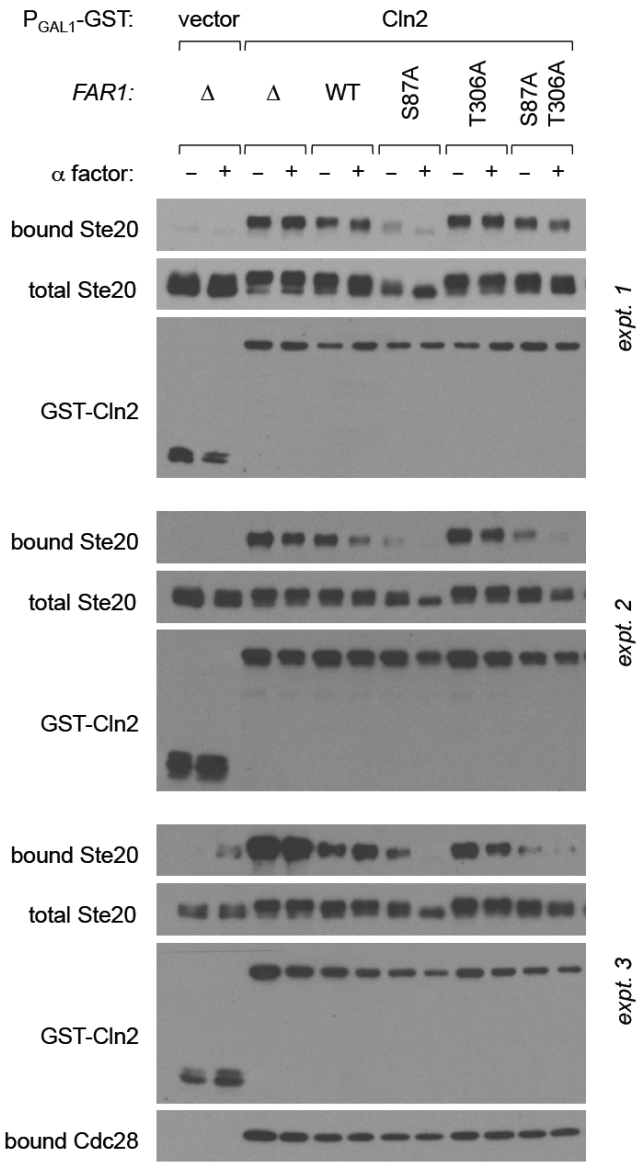
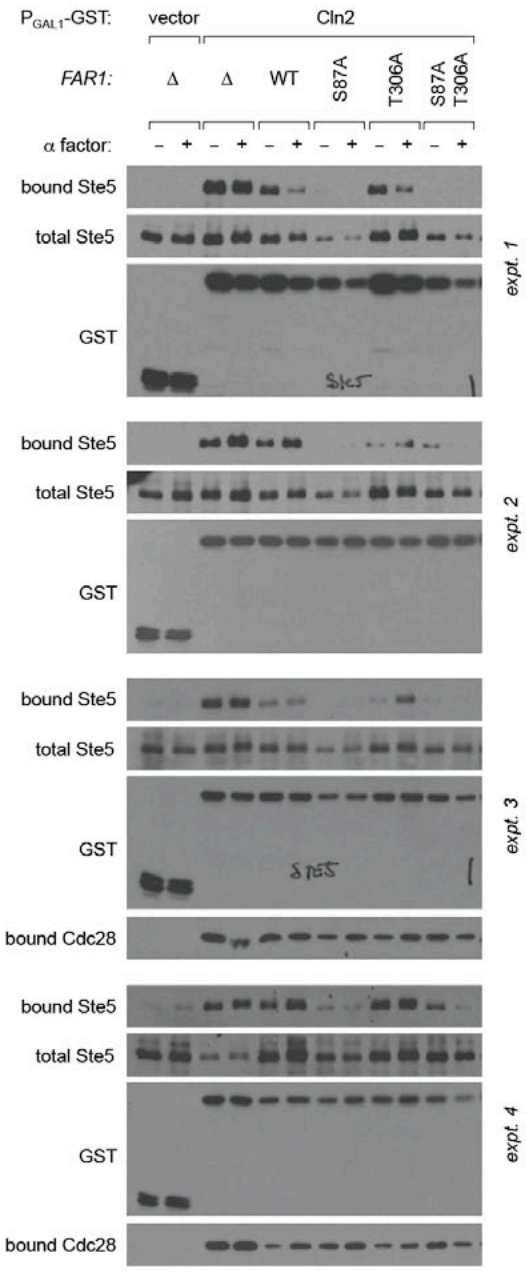


Figure 4.7: Day-to-day variability of the binding phenotype with Ste5 as the substrate

When GST-Cln2 was used to pull down full-length Set5 there was increased day-to-day variability as compared to the Ste20 results. Here blots from four individual experiments are presented to show the degree of this variability.

Figure 4.7: Day-to-day variability of the binding phenotype with Ste5 as the substrate



even in the absence of pheromone treatment. The T306A mutant was usually most similar to WT, but the T306A mutation mildly reduced the interfering activity of the S87A mutant. Importantly, Far1 only affected binding of Cln2 to its substrates and not to its partner CDK molecule, Cdc28 (Figure 4.5). The effect of pheromone was less evident and more variable in these experiments than when using the earlier (reverse) procedure. The reasons for this variability are not clear but may relate to the fact that acute over-expression of Cln2 can drive Far1 phosphorylation and degradation, which may limit the ability of pheromone signaling to increase its levels in the time span of these experiments and/or in the absence of the S87A mutation; hence, the results may primarily reflect the amount of Far1 present at the beginning of the experiment. Overall, however, these results provide further confirmation that Far1 interferes with Cln2-substrate binding interactions.

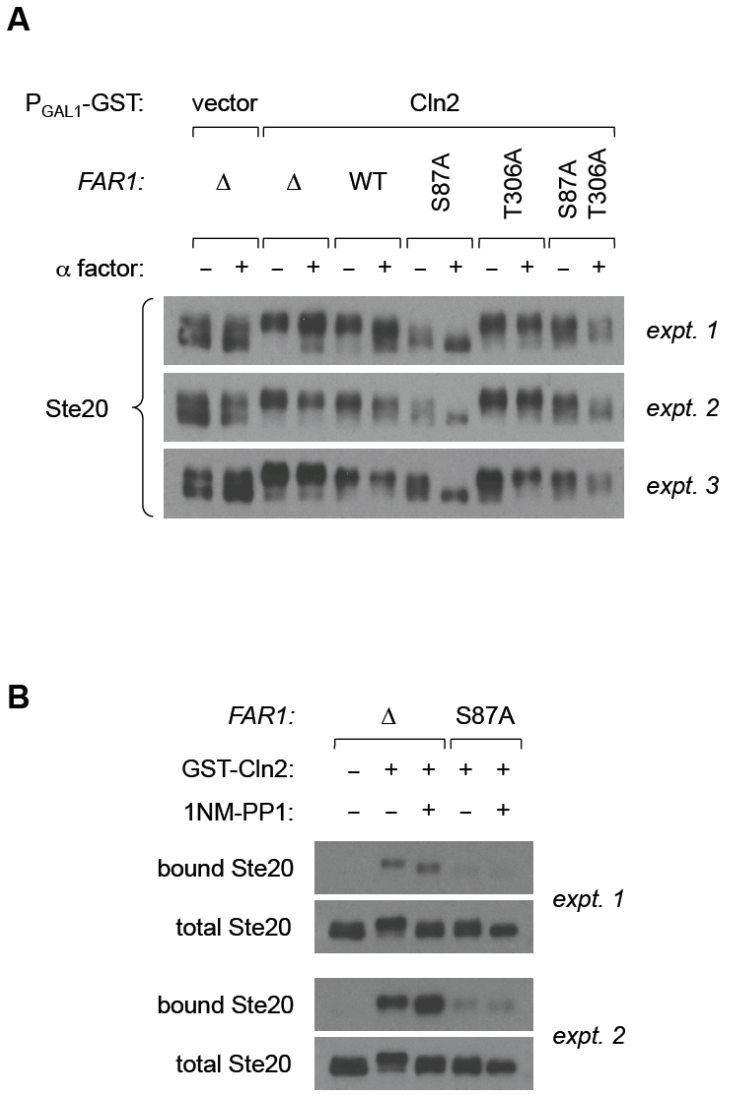
CDK-driven phosphorylation of both Ste20 and Ste5 causes changes in electrophoretic mobility (Bhaduri and Pryciak, 2011), although the effect on Ste20 is easier to detect. In the experiments just described, variations in Ste20 mobility suggested effects on Cln2-CDK activity. To examine this more closely, the same samples were analyzed by electrophoretic conditions that improve the resolution of phosphorylated species in the total Ste20 population (Figure 4.8A). The results showed several reproducible effects of Far1 and pheromone on Cln2-CDK activity. Namely, Cln2 expression in *far1*Δ cells caused Ste20 to

Figure 4.8: *FAR1* genotype and pheromone also affect substrate phosphorylation

(A) To demonstrate changes in phosphorylation, input samples from the binding experiments were run for longer intervals to allow resolution of the phosphoisoforms. The phosphoisoforms of Ste20 present coincide with the binding results, where samples that had the least binding (*FAR1-S87A* +α factor) also showed the strongest reduction in Ste20 phosphorylation, and samples with the strongest binding (*far1Δ* and *FAR1-T306A*) also showed the most phosphorylation.

(B) Cdc28 inhibition does not impact the binding of Cln-CDK and substrates. Cdc28-as2 cells containing GST-Cln2 (or a GST-only vector) and Ste20-V5 constructs were induced with galactose either with or without the ATP analog 1-NM-PP1. Glutathione sepharose beads were used to pull down complexes and bound and input (1.25%) samples were analyzed with anti-GST blots.

Figure 4.8: *FAR1* genotype and pheromone also affect substrate phosphorylation



accumulate in the slowest mobility form, indicative of phosphorylation. Pheromone treatment had no effect in *far1*Δ cells, but could reduce the extent of Ste20 phosphorylation in *FAR1-WT* cells. *FAR1-S87A* cells showed reduced Ste20 phosphorylation even in the absence of pheromone treatment and a further reduction when pheromone was added. The T306A mutant was relatively ineffective at affecting substrate phosphorylation after pheromone treatment, while the S87A T306A double mutant showed a result intermediate between the two single mutants. Collectively, these results suggest parallel effects of Far1 on substrate docking and substrate phosphorylation by Cln2-CDK. To address whether it was more likely that the effects on docking are a cause rather than an effect of phosphorylation, we directly interfered with CDK activity by using *cdc28-as2* cells and the inhibitor compound 1NM-PP1. We found that inactivation of Cdc28-as2 activity (evident in the increased migration of total Ste20) did not itself cause a reduction in Cln2-Ste20 binding, and moreover the ability of the Far1 S87A mutant to disrupt this binding was equally apparent with and without CDK inhibition (Figure 4.8B). Therefore, these results suggest that Far1-mediated interference with Cln2-substrate docking causes reduced substrate phosphorylation, rather than vice-versa. Notably, to our knowledge these results provide the first demonstration that Far1 affects phosphorylation of CDK substrates in vivo, and in a manner regulated by pheromone.

Previous studies showed that Far1 binds Cln-CDK complexes in vivo (Peter et al., 1993; Tyers and Futcher, 1993; Gartner et al., 1998). Our findings that Far1 interferes with Cln2-substrate interactions suggested the possibility that Far1 and substrates might bind Cln2 competitively, and perhaps that Far1 might outcompete substrate binding by virtue of higher concentration or affinity. To address this issue we directly compared the relative levels of these proteins by using the same epitope tag (triple V5) to mark both Far1 and the substrate proteins (all expressed from their native promoters). In whole cell extracts of strains expressing individual tagged proteins, we observed that Ste20 was considerably more abundant than Ste5, and that the levels of Far1 ranged between these two depending on whether it had been induced by pheromone or stabilized by the S87A mutation (Figure 4.9A).

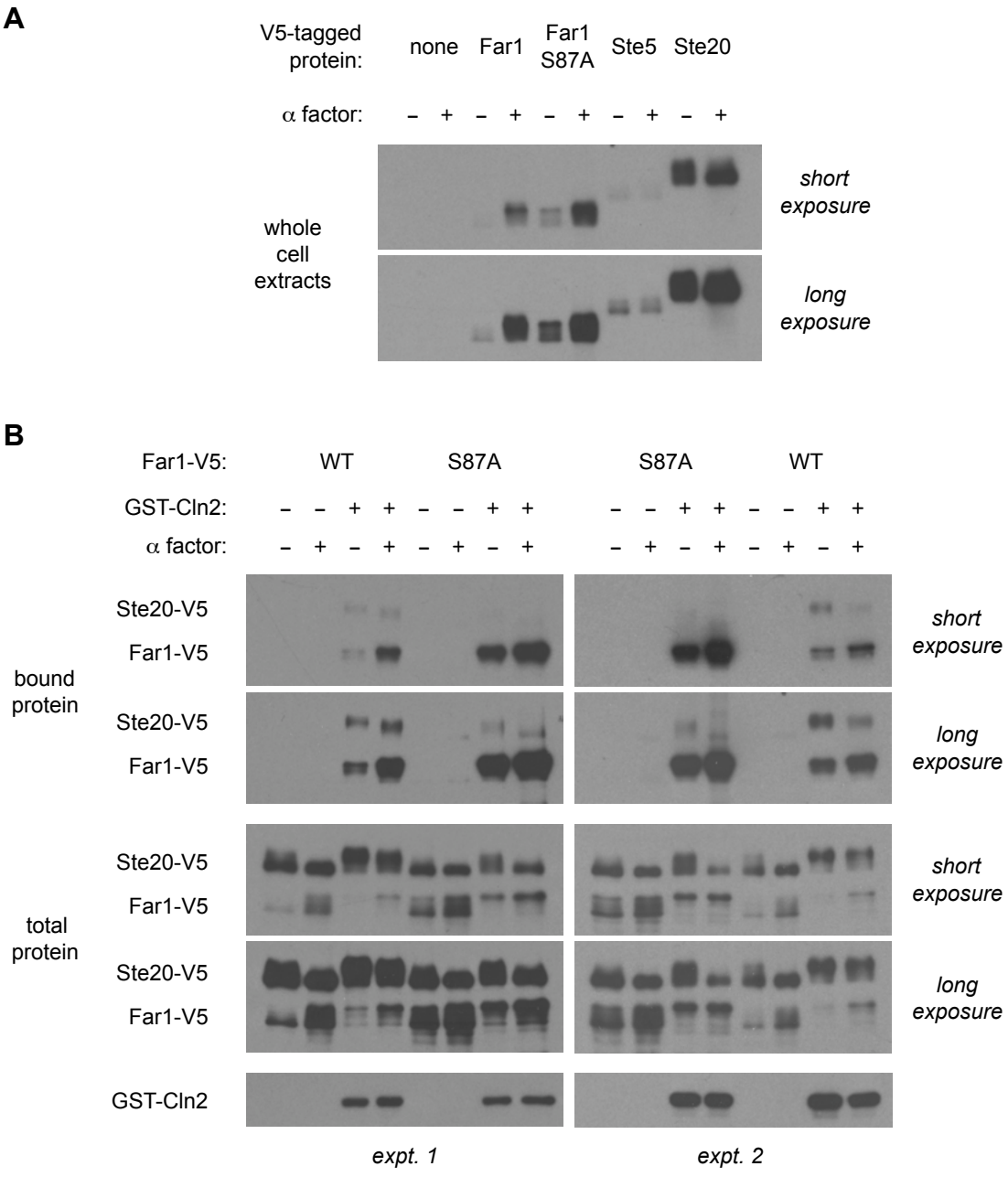
Next, we used cells that simultaneously expressed V5-tagged forms of Far1 and Ste20 to compare their binding to Cln2 (Figure 4.9B). (We chose Ste20 rather than Ste5 because of its easier detection and increased electrophoretic separation from Far1.) Several findings were notable. First, in the total protein samples, the levels and/or migration of both Far1 and Ste20 provided a clear indication of Cln2-CDK activity (Figure 4.9B lower panels). In particular, Cln2 expression triggered a reduction in Far1 levels in a manner that was partially prevented by pheromone and strongly prevented by S87A mutation (although the S87A mutant was still phosphorylated by Cln2-CDK, presumably at other CDK

Figure 4.9: Comparison of Far1 and substrates in whole cell extracts and binding assays

(A) Whole cells extracts of cells with V5 fused to the native Far1 gene (either FAR1 or FAR1-S87A) or expressing V5-tagged Ste20 or Ste5 were analyzed to determine relative amounts of each protein in the cell. 20µg of total protein was run for each sample and the blots were probed with anti-V5 antibody.

(B) More *FAR1-S87A* binds to Cln2 as compared to Far1, and binding further increases with pheromone treatment. Cells with V5 fused to the native Far1 gene (either *FAR1* or *FAR1-S87A*) co-expressing GST-Cln2 and Ste20-V5 constructs were induced with galactose with or without pheromone to determine how the relative levels of bound substrate and Far1 change in the presence or absence of pheromone. Glutathione sepharose beads were used to pull down complexes and bound and input (1.25%) samples were analyzed with anti-V5 and anti-GST blots.

Figure 4.9: Comparison of Far1 and substrates in whole cell extracts and binding assays



sites that are less destabilizing). In addition, as noted earlier, Ste20 mobility served as an indicator of Cln2-CDK activity, in that its phosphorylation was antagonized additively by both pheromone and Far1-S87A. Second, the results clearly indicated that Far1 binds Cln2 more favorably than Ste20, as total Far1-WT was substantially less abundant than Ste20, and yet Far1-WT bound Cln2 at equal or greater levels than Ste20 Figure 4.9B, upper panels). Similarly, Far1-S87A was comparably abundant to Ste20 yet showed disproportionally greater binding to Cln2. Third, when comparing WT and S87A forms of Far1, the increased Cln2 binding of the S87A mutant was accompanied by a reduction in Ste20 binding, consistent with the notion that Far1 outcompetes Ste20 for binding to Cln2. Pheromone treatment induced an increase in Far1-Cln2 binding, although the degree to which this was accompanied by a reduction in Ste20 binding in Far1-WT cells was somewhat variable (Figure 4.9B shows two examples); the source of this variability is not clear, but a possible explanation is that a competitive effect is likely to be most evident when Far1 is in excess of Cln2, and more modest when Cln2 is in excess, and hence the degree of competition may depend on the precise amount of Cln2 that accumulates after acute galactose induction (which may show day-to-day variation). Collectively, therefore, these results suggest that Far1 binds Cln2 in a way that is mutually exclusive with substrate docking, and that the more favorable binding of Far1 allows it to outcompete substrates.

Finally, to explore whether Far1 must be in excess of Cln2 in order to effectively inhibit cell cycle progression, we will perform an additional experiment to compare their relative levels as cells approach the cell cycle commitment point (i.e., “Start”). Specifically, we have prepared strains in which both Far1 (WT or S87A) and Cln2 are tagged with the identical triple-V5 tag. With these, we will prepare synchronous cultures by mitotic block and release (using both *cdc15*^{ts} and P_{GAL1}-*CDC20* methods), and then follow both the ability to arrest in G1 in response to pheromone and the relative levels of Far1 versus Cln2 as a function of time. Our prediction is that pheromone will be able to arrest cells in G1 until Cln2 levels begin to exceed Far1 levels, and that this period will be extended by the Far1 S87A mutation. Due to some delays in strain construction, these experiments will be conducted after submission of this dissertation but should be completed shortly afterward.

Discussion

These studies were undertaken to address long-standing uncertainties about how the yeast pheromone pathway, and specifically the presumed CKI protein Far1, inhibits CDK function in vivo. They were motivated by recent findings that phosphorylation of several Cln1/2-CDK substrates require docking interactions between the cyclin and the substrate (Bhaduri and Pryciak, 2011; Koivomagi et al., 2011), which raised the possibility that these docking interactions might be regulated. Indeed, our findings provide evidence that activation of the pheromone signaling pathway interferes with Cln2-substrate interactions, and that this interference is mediated by Far1. Moreover, from analysis of several Far1 mutants that lack different regulatory phosphorylation sites, the ability of Far1 to disrupt Cln2-substrate docking largely correlates with its ability to mediate pheromone arrest. Finally, by comparing Cln2 binding to Far1 and a substrate (Ste20) in the same cells, we found that Far1 binds Cln2 more favorably (presumably due to stronger protein-protein interaction affinity), and thus competes with substrates for binding to Cln2. Collectively, these observations indicate that Far1 disrupts Cln2-substrate docking interactions. Given the previous evidence that these docking interactions enhance substrate phosphorylation, it seems likely that the ability of Far1 to interfere with docking contributes to a reduction in substrate phosphorylation, either instead of or in addition to a separate ability of Far1 to block CDK activity per se. Disrupting docking may be a particularly effective way to antagonize kinase activity toward

substrates with inherently poor (non-consensus) phosphorylation sites or those that must be phosphorylated at multiple independent sites, as these substrates might be especially dependent on docking. This notion could help explain why inhibition of CDK activity by Far1 has been difficult to detect in some previous studies, as the in vitro assays routinely use histone H1 as a substrate, which has strong (consensus) phosphorylation sites and no docking motif.

The specific mechanism by which Far1 interferes with Cln2 docking interactions is not known. There are no structural data on the Cln2-substrate binding interface, but mutational analyses suggest that the docking sequences are short peptides (e.g., 4-8 residues in length, enriched in Leu and Pro), which presumably bind to a peptide-recognition groove on the cyclin. This would be analogous to the docking interaction between S-phase cyclins (e.g, mammalian CycA or yeast Clb5) and short “RXL” peptides in their substrates. Conceivably, Far1 could outcompete substrate docking by having a similar docking peptide of higher affinity, or by interacting with a larger interface of Cln2 in a way that obscures the peptide recognition site. Previous studies suggested that the ability of Far1 to bind Cln2 requires extensive sequences in two distinct regions (Peter et al., 1993); when considered in light of our finding that Cln2 binds Far1 considerably more strongly than Ste20, this might favor the model that the Far1-Cln2 interaction involves a larger interface on the cyclin than that occupied by the docking motif. Again, this would be analogous to the interaction between

mammalian CKI proteins p21 and p27, which do have RXL-like docking sequences that contribute to cyclin binding and CDK inhibition (Wohlschlegel et al., 2001), but by crystallographic analysis it is clear that this contact with the docking groove is just a small part of an extensive interaction interface that involves both the cyclin and CDK subunits (Russo et al., 1996).

One surprising aspect of our findings was the ability of Far1 (WT or S87A) to at least partially interfere with Cln2-substrate binding even without pheromone treatment. This was unexpected because previous findings implied that Far1 must be activated by pheromone: (a) although *FAR1* transcription is induced by pheromone, over-expression of *FAR1* from a foreign promoter is not sufficient to induce G1 arrest in the absence of pheromone (Chang and Herskowitz, 1992); and (b) Far1 is phosphorylated by Fus3 in response to pheromone, and mutation of this phosphorylation site (T306A) causes a defect in Cln2 binding and pheromone arrest (Gartner et al., 1998). In retrospect, a more accurate interpretation might be that unphosphorylated Far1 is active but is less potent than when it is phosphorylated at T306; this view seems clearly evident in our binding experiments, and is supported by the fact that pheromone can arrest the *FAR1-S87A T306A* double mutant strain, though more weakly than the *FAR1-S87A* single mutant. Over-expression of *FAR1* may not be sufficient to cause G1 arrest because pheromone signaling may still be required to induce Far1-independent effects (see Chapter 2) to efficiently arrest the cell cycle. The

potential for unphosphorylated Far1 to reduce Cln2-CDK activity is also relevant to two other previous studies. In one case, it was observed that *far1*Δ cells show accelerated passage through Start in the absence of pheromone exposure (Alberghina et al., 2004). In another case, when comparing two methods for making the mating pathway resistant to CDK inhibition, namely *FAR1-S87A* versus *STE5-8A*, it was found that only the former was able to extend the Start commitment period (Doncic et al., 2011); it is possible that this was because the critical parameter dictating whether cells are past Start (and hence will not arrest upon pheromone exposure) is the level of Cln1/2-CDK activity present immediately prior to pheromone treatment, and so the key effect of Far1-S87A may have been to reduce Cln1/2-CDK activity before pheromone was added, so that the period in which pheromone can arrest the cell cycle is extended.

Our findings clearly indicate that the ability of Cln2-CDK to phosphorylate Ste20 is inhibited by pheromone and Far1. Remarkably, to our knowledge this is the first demonstration that pheromone and Far1 can block the ability of a cyclin-CDK complex to phosphorylate any substrate in vivo. There are many prior examples in which CDK substrates were unphosphorylated in pheromone-arrested cells, but this is a trivial consequence of the fact that cyclins are not expressed in G1 arrested cells, and hence does not indicate that the kinase activity of cyclin-CDK complexes is reduced. By expressing Cln2 in a manner that was not controlled by pheromone, we were able to observe regulation of

substrate phosphorylation by a set amount of Cln2-CDK in vivo. The findings match previous expectations, but our ability to confirm those expectations is a notable new contribution.

One key issue raised by our findings is the extent to which reduced substrate phosphorylation can be attributed to the disruption of Cln2-substrate binding by Far1, rather than a distinct mechanism whereby Far1 inhibits CDK kinase activity per se. Studies pertinent to this issue are being performed as part of a parallel project being conducted by another graduate student in the lab, Samyabrata Bhaduri, and we plan to combine our findings into a co-authored manuscript for publication. Because his work is distinct from mine, I will not present it in this dissertation, but instead I will briefly summarize his key findings here. (1) He finds that Cln2-CDK phosphorylation of another substrate, based on the Ste5 N-terminus, is antagonized by Far1 in a manner similar to the pattern described above for Ste20. (2) Consistent with the binding results described above, Far1 and Far1-S87A can clearly reduce phosphorylation of this additional substrate even in the absence of pheromone treatment. (3) He has developed a method to link cyclins to their substrates by replacing the native docking interaction with a foreign leucine zipper interaction, and in this context the ability of Far1 to inhibit substrate phosphoryation by Cln2-CDK is greatly reduced but not eliminated, implying that Far1 may interfere with both substrate docking and CDK kinase activity. (4) Unlike the native docking interaction, the leucine zipper

mediated binding interaction between Cln2 and substrate is not inhibited by Far1. (5) Using the leucine zippers to link different cyclins to the same substrate, he can detect substrate phosphorylation driven by multiple classes of cyclins (i.e., Cln3, Cln1, Cln2, Clb5, and Clb2), but Far1 only has a noticeable effect on phosphorylation by the G1/S cyclins (Cln1 and Cln2), indicating that the effects of Far1 on CDK activity are cyclin specific. Thus, these parallel studies support and extend the observations described in this chapter. Collectively, the combined results reveal a previously unsuspected mode of regulation of cyclin-CDK activity, in which an extracellular signal stimulates an intracellular inhibitory factor, Far1, to disrupt the ability of specific cyclin-CDK complexes to both interact with, and hence phosphorylate, their substrates.

Materials and Methods

Yeast Strains and Plasmids

Standard procedures were used for growth and genetic manipulation of yeast (Rothstein, 1991; Sherman, 2002). Yeast cultures were grown at 30˚C. Yeast strains are listed in Table 1; all were derived from the W303 background (Thomas and Rothstein, 1989) and harbor the *bar1*Δ mutation to block α factor degradation. PCR-mediated gene targeting used methods described previously (Longtine et al., 1998); selectable markers included antibiotic resistance genes (*kanMX6*, *hphMX6*) and orthologs of biosynthesis genes from other yeasts (*S. kluyveri HIS3*, *C. glabrata TRP1*, *K. lactis URA3*). For V5 tagging of endogenous Far1 PCR-generated cassette containing the 3xV5 tag marked with the antibiotic resistance gene *kanMX6* was integrated into the genome downstream of the coding sequence. For Cdc28 inhibition experiments, the *CDC28* gene was replaced with an ATP analog-sensitive allele *cdc28-as2* via a two-step pop-in/pop-out method (Rothstein, 1991); we used the Cdc28-as2 [F88A] mutant (Colman-Lerner et al., 2005) because in our strains the more severe mutant Cdc28-as1 [F88G] (Bishop et al., 2000) was hypomorphic, as indicated by slow growth and cell shape defects. The same pop-in/pop-out method was used to create the *FAR1-S87A* and *FAR1-T306A* mutations.

GST Binding Assays

Cultures (25mL) were grown in 2% raffinose, and expression from GAL1pr constructs was induced with 2% galactose with or without 10nM α factor for 1.5-3 hours. For experiments using the *cdc28-as2* allele, cultures were also treated with 10μM or 15 μM 1-NM-PP1. Cells were harvested and stored at -80^{o}C. Cells were lysed by glass bead beating in a non-ionic detergent buffer described previously (Lamson et al., 2002). Glass bead beating was performed using a Fast-Prep apparatus using 2mL tubes and 1 cycle at 4 m/s for 20sec. Prior to GST harvest an aliquot was removed to serve as the input control. GST fusions were collected by binding to glutathione-sepharose beads (GE Healthcare #17-0756-01).

Whole cell extracts

Whole cell extracts were prepared by lysis with trichloroacetic acid, in a method adapted from a previously reported protocol (Lee and Dohlman, 2008). Here, 300μL of TCA Buffer (10 mM Tris.HCl, pH 8.0, 10% TCA, 25 mM ammonium acetate, 1 mM Na_2EDTA) was added directly to frozen cell pellets, and allowed to sit on ice for 10 minutes. Samples were spun for 10 min at 4^{o}C. The pellet was resuspended in 75μL Resuspension Buffer (0.1 M Tris.HCl, pH 11.0, 3% SDS), boiled for 5 min, allowed to cool at room temperature fro 5 minutes, then re-centrifuged for 30sec. 60μL of supernatant was transferred to a new tube, 10μL was reserved for BCA protein concentration assay using Pierce BCA Protein Assay Kit (#23225), and 50μl 2xSDS Sample Buffer was added to the remaining

sample.

Western Blots

Samples were run out by SDS-PAGE, and transferred to PVDF by submerged transfer method. Myc blots were probed with rabbit anti-myc (1:200 Santa Cruz Biotechnologies #sc-789) and detected with goat anti-rabbit HRP conjugated secondary antibody (1:3000, Jackson ImmunoResearch #111-035-144) and Pierce SuperSignal West Pico (#34080) chemilluminescent reagent. V5 blots were probed with mouse anti-V5 (1:5000, Invitrogen #46-0705) and detected using HRP-conjugated goat anti-mouse antibodies (1:3000, BioRad #170-6516) Cdc28 blots were probed with goat anti-Cdc28 (1:200 Santa Cruz Biotechnologies #sc-6709) and detected with donkey anti-goat HRP-conjugated secondary (1:3000 sc-2020). GST tagged proteins were detected with anti-GST (1:1000, Santa Cruz Biotechnologies #sc-138) antibodies, and detected using HRP-conjugated goat anti-mouse antibodies (1:3000, BioRad #170-6516).

Table 4.1. Yeast strains used in Chapter IV

Name	Relevant Genotype*	Source
PPY2268	MAT**a** far1Δ::kanR	J.Benanti
PPY2296	MAT**a** STE5-8A FAR1-S87A	this study
PPY2322	MAT**a** bar1Δ::hphR	this study
PPY2326	MAT**a** bar1Δ::hphR far1Δ::kanR	this study
PPY2327	MAT**a** bar1Δ::hphR STE5-8A far1Δ::kanR	this study
PPY2329	MAT**a** bar1Δ::hphR FAR1-S87A	this study
PPY2330	MAT**a** bar1Δ::hphR STE5-8A FAR1-S87A	this study
PPY2340	MAT**a** bar1Δ::hphR STE5-8A	this study
PPY2354	MAT**a** bar1Δ::hphR FAR1-T306A	this study
PPY2356	MAT**a** bar1Δ::hphR FAR1-S87A,T306A	this study
PPY2358	MAT**a** bar1Δ::hphR STE5-8A FAR1-T306A	this study
PPY2359	MAT**a** bar1Δ::hphR STE5-8A FAR1-S87A,T306A	this study
PPY2369	MAT**a** bar1Δ::hphR STE5-8A far1Δ::kanR cdc28-as2	this study
PPY2371	MAT**a** bar1Δ::hphR STE5-8A FAR1-S87A cdc28-as2	this study
PPY2377	MAT**a** bar1Δ::hphR STE5-8A FAR1-3xV5::kanR	this study
PPY2380	MAT**a** bar1Δ::hphR STE5-8A FAR1-S87A-3xV5::kanR	this study
* All strains are in the BY4741 background (MAT**a** his3Δ1 leu2Δ0 ura3Δ0 met15Δ0)		

Table 4.2. Plasmids used in Chapter IV			
Name	Alias	Description	Source
pPP1843	pUG-GST-GFP	2µm URA3 GAL1pr-GST-GFP vector	1
pPP2154	pHG-GST	CEN HIS3 GAL1pr-GST vector	3
pPP2155	pHG-GST-GFP	CEN HIS3 GAL1pr-GST-GFP vector	this study
pPP2163	pHGT-S20A	CEN HIS3 GAL1pr-GST-ste20(1-333)	2
pPP3152	pH-TEFpr-CLN2-myc	CEN HIS3 TEF1pr-CLN2-myc13 CYC1term	3
pPP3203	pCYC1-CLN2-myc	CEN URA3 CYC1pr-CLN2-myc13 CYC1term	3
pPP3266	pS5kV5	CEN URA3 STE5-3xV5 CYC1term	this study
pPP3267	pRL116V5	CEN URA3 3xV5-STE20	3
pPP3368	pRL116V5-Ala5	CEN URA3 3xV5-STE20(mut3 = SLDDP to AAAAA)	3
pPP3369	pRL116V5-Ala13	CEN URA3 3xV5-STE20(Ala13 = CDK site mutant)	3
pPP3573	pt-HGT-CLN2-t	CEN HIS3 GAL-GST-CLN2 1-372 + TCyc1	this study
pPP3766	pUG-GST-F20L-wt #6-1	2µm URA3 GAL1pr-GST-GFP-ste20(72-118/wt) + ste20(120-333)	this study
pPP3761	pS5kV5-Nhe-P4	CEN URA3 ste5(LLPP-AAAA)-3xV5 CYC1term	3
pPP3771	pUG-GST-F20M-5CSM-wt	2µm URA3 GAL1pr-GST-GFP-ste5(263-335/WT) + ste20(120-333)	3
pPP3825	pHG-GST-S20M-5CSM-WT	CEN HIS3 GAL1pr-GST-ste5(263-335) + ste20(120-333)	this study

Source: (1)(Winters et al., 2005) (2)(Takahashi and Pryciak, 2007) (3)(Bhaduri and Pryciak, 2011)

Strain and Plasmid combinations

Figure 2: (A) PPY2296 harbored pPP3203 and pPP2163 (B) strains PPY2268 and PPY2296 harbored pPP3152 with pPP1843, pPP3766, or pPP3771

Figure 3: (B) strains PPY2322, PPY2326, PPY2329, PPY2354, and PPY2356 (C) PPY2322, PPY2329, PPY2354, and PPY2356 harbored pPP3203 and pPP2163 (D) PPY2322, PPY2329, PPY2354, and PPY2356 harbored pPP3203 and pPP3825

Figure 4: (A) strain PPY2327 harbored pPP2154 or pPP3573 with pPP3267 or pPP3368 (B) strain PPY2327 harbored pPP2154 or pPP3573 with pPP3266 or pPP3761

Figure 5: (A) strains PPY2327, PPY 2330, PPY2340, PPY2358, and PPY2359 harbored pPP2154 or pPP3573 with pPP3267 (A) strains PPY2327, PPY 2330, PPY2340, PPY2358, PPY2359 harbored pPP2154 or pPP3573 with pPP3266

Figure 6: strains PPY2327, PPY 2330, PPY2340, PPY2358, PPY2359 harbored pPP2154 or pPP3573 with pPP3267

Figure 7: (A) strains PPY2327, PPY 2330, PPY2340, PPY2358, PPY2359 harbored pPP2154 or pPP3573 with pPP3266

Figure 8: (A) strains PPY2327, PPY 2330, PPY2340, PPY2358, and PPY2359 harbored pPP2154 or pPP3573 with pPP3267 (B) strains PPY2369 and PPY2371 harbored pPP2154 or pPP3573 with pPP3267

Figure 9: (A) PPY harbored pPP2154 with pPP3266 or pPP3267; PPY2340, PPY2377, and PPY2380 harbored pPP1843 and pPP2155 (B) PPY2377 and PPY2380 harbored pPP3267 with pPP2154 or pPP3573

CHAPTER V

Concluding remarks

Purpose and outcome

Pheromone treatment induces G1 arrest in *Saccharomyces cerevisiae* cells. This fact has been well established, as has the necessity for Far1. How exactly Far1 is able to induce arrest has remained unclear, and Far1-independent mechanisms for arrest, although also clearly present, are even less well understood. Through these studies we were able to uncover details about how both the Far1-dependent and independent arrest mechanisms function, and together our data suggests that these two functions act in concert to robustly arrest cells in G1.

The results presented in Chapter II demonstrate that, in the absence of Far1, G1/S transcriptional regulators and the CKI Sic1 help to enforce G1 arrest. We speculate that the presence of proteins such as Whi5 and Sic1 can help set a higher threshold for Cln/CDK activity that cannot be met once Far1 is activated by pheromone. Additive and/or redundant mechanisms for controlling proliferation have been demonstrated in animal cells, and our results provide clear evidence for similar redundancies in the control of the yeast cell cycle. Cells that rapidly escape G1 arrest (*far1Δ cln2Δ sic1Δ*) actually lose viability demonstrating just how critical it is for the cells to arrest properly, and underlines the necessity of having multiple, redundant G1 arrest mechanisms. Another

important implication of the *far1Δ cln2Δ sic1Δ* results is that using growth arrest assays (such as halo assays) to screen for mutants deficient in pheromone-induced arrest (or potentially any other G1 arrest) may cause us to overlook some mutants, namely those that no longer arrest in G1 but die at a later point in the cell cycle.

In Chapter III we studied the previously established phenomenon of decreased G1/S cyclin protein levels after pheromone treatment. Previous work had suggested that this decrease in protein levels was independent of pheromone-regulation of transcription, and our data confirmed this aspect of the response. We were, however, unable to confirm that the phenomenon is a specific, targeted degradation of the G1/S cyclin proteins. Our data, although not conclusive, does suggest that the observed effect on Cln protein levels may actually be a result of a pheromone-induced global decrease in translation rates. The very short half-lives of Cln proteins would make them more susceptible to changes in translation rates as opposed to more stable proteins such as Cdc28.

Finally, Chapter IV addressed the issue of Far1-dependent arrest. Far1 is known to bind Cln proteins in a pheromone-dependent manner, but the physiological function of this binding has remained unclear. As mentioned, previous data provided conflicting reports on Far1's ability to inhibit CDK activity, leaving the question open as to whether or not Far1 is actually a CKI. Here we present data that suggests that, through Far1, pheromone is able to interfere with Cln-substrate interactions. We saw drastically decreased Cln-substrate binding in

pheromone treated cells, but only when Far1 was present. We also demonstrated that this interference with Cln-substrate binding appears to be a critical function for arrest, as a Cln2-binding compromised allele of Far1 (Far1-T306A) that could no longer strongly inhibit Cln2-substrate interactions also showed reduced pheromone-induced arrest proficiency. We also observed a change in phosphorylation state of substrates that correlates with the binding phenotype; in situations where Far1 caused decreased binding there was also decreased substrate phosphorylation. These results are the first demonstration that pheromone and Far1 can block the ability of a cyclin-CDK complex to phosphorylate any substrate *in vivo*. Our results point towards this binding function of Far1 being a primary means through which Far1 regulates Cln-CDK activity. There is significant evidence that the CIP/KIP family of CKIs in mammalian cells make use of the cyclin docking motif (in this case the RxL motif) and the corresponding binding patch on the cyclin as part of their inhibitory mechanisms. In this case, the inhibitors possess the docking motif, and consequently can bind the cyclins in the same manner as substrates. It has been shown that this RxL motif is critical for p21s inhibition of cyclin E (Adams et al., 1996; Chen et al., 1996), demonstrating the potential importance of cyclin docking for CKI-dependent inhibition. Interestingly, p21 and p27 appear to bind cyclin-CDKs not only through the docking sight but also over larger regions that contact both the cyclin and the CDK (Russo et al., 1996). Part of that binding appears to occlude the catalytic cleft (Russo et al., 1996), suggesting that these

inhibitors have multiple functions that combine to promote CDK inhibition. We do not currently have enough evidence to determine if Far1 acts in a similar manner, using multiple inhibitory mechanisms, but our data do suggest that Far1 has both docking-dependent and docking-independent functions suggesting that a mechanism similar to that of p21/p27 could be in use by Far1.

Taken together all of the data presented here demonstrates how the pheromone response impacts multiple aspects of the G1/S regulatory machinery in order to properly arrest cells in G1.

Future directions

As discussed in Chapter 2, *far1Δ cln2Δ sic1Δ* cells demonstrate an interesting case where although the cells can escape from G1 while treated with pheromone they are unable to successfully complete an additional round of cell division. Based on 2C DNA content and budding pattern the cells appear to pile up post-S-phase. However, viability assays demonstrate that this is not actually a cell cycle arrest, but instead cells are dying at this post-S-phase stage. These results coincide with previous data showing that overexpression of Clb5 in a STE5-8A strain could also cause cells to escape G1 arrest as well as lose viability at a point post-Start. We did not further investigate the mechanism behind this loss of viability, but we suspect mitosis failure due to inappropriate spindle formation may be to blame. Since other cells, including *far1Δ cln2Δ whi5Δ,* can not only escape arrest but also successfully complete another round

of cell division it is clear that the passage through Start in pheromone signaling cells is not sufficient to cause the loss of viability. This instead points to a specific issue arising from inappropriate Clb5 activation without proper activation of other G1/S genes. A comparison of G1/S gene expression in pheromone treated *far1Δ cln2Δ sic1Δ* and *far1Δ cln2Δ whi5Δ* cells could shed some light on the issue, because I suspect differences in gene activation could help explain the differences in viability after G1 escape in these two strains. Some preliminary microscopy work looking at microtubule structures in post-Start arrested cells did suggest that the cells were unable to form proper mitotic spindles, but further work including time-lapse microscopy would be needed to determine exactly what the defect is.

Chapter 3 addressed the role of Far1-dependent G1 arrest, and specifically how Far1 can interfere with Cln/substrate interactions. Work by a fellow lab member looked at another aspect of this phenomenon, that of how Far1 can impact CDK phosphorylation of substrates. What he found was a two-pronged effect: although Far1 does appear to decrease substrate phosphorylation, this is not entirely docking dependent since artificial Cln/substrate interactions induced by leucine zipper fused Cln2 and substrates still show decreased phosphorylation during pheromone treatment. This suggests that Far1 may actually function through at least two mechanisms, interfering with docking and also inhibiting CDK activity. Determination of exactly how Far1 and Cln2 interact could provide further insight into this phenomenon.

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