Regulation of CDK1 Activity during the G1/S Transition in S. cerevisiae through Specific Cyclin-Substrate Docking: A Dissertation

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REGULATION OF CDK1 ACTIVITY DURING THE G1/S TRANSITION IN
S. cerevisiae THROUGH SPECIFIC CYCLIN-SUBSTRATE DOCKING

A Dissertation Presented

By

Samyabrata Bhaduri

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

October 21, 2014

Interdisciplinary Graduate Program
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Abstract

Several cell cycle events require specific forms of the cyclin-CDK complexes. It has been known for some time that cyclins not only contribute by activating the CDK but also by choosing substrates and/or specifying the location of the CDK holoenzyme. There are several examples of B-type cyclins identifying certain peptide motifs in their specific substrates through a conserved region in their structure. Such interactions were not known for the G1 class of cyclins, which are instrumental in helping the cell decide whether or not to commit to a new cell cycle, a function that is non-redundant with B-type cyclins in budding yeast. In this dissertation, I have presented evidence that some G1 cyclins in budding yeast, Cln1/2, specifically identify substrates by interacting with a leucine-proline rich sequence different from the ones used by B-type cyclins. These “LP” type docking motifs determine cyclin specificity, promote phosphorylation of suboptimal CDK sites and multi-site phosphorylation of substrates both \textit{in vivo} and \textit{in vitro}. Subsequently, we have discovered the substrate-binding region in Cln2 and further showed that this region is highly conserved amongst a variety of fungal G1 cyclins from budding yeasts to molds and mushrooms, thus suggesting a conserved function across fungal evolution. Interestingly, this region is close to but not same as the one implicated in B-type cyclins to binding substrates. We discovered that the main effect of obliterating this interaction is to delay cell cycle entry in budding yeast, such that cells begin DNA replication and budding only at
a larger than normal cell size, possibly resulting from incomplete multi-site phosphorylation of several key substrates. The docking-deficient Cln2 was also defective in promoting polarized bud morphogenesis. Quite interestingly, we found that a CDK inhibitor, Far1, could regulate the Cln2-CDK1 activity partly by inhibiting the Cln2-substrate interaction, thus demonstrating that docking interactions can be targets of regulation. Finally, by studying many fungal cyclins exogenously expressed in budding yeast, we discovered that some have the ability to make the CDK hyper-potent, which suggests that these cyclins confer special properties to the CDK. My work provides mechanistic clues for cyclin-specific events during the cell cycle, demonstrates the usefulness of synthetic strategies in problem solving and also possibly resolves long-standing uncertainties regarding functions of some cell cycle proteins.
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Preface

Portions of this dissertation appear in separate publications:


CHAPTER I
Introduction

Eukaryotic cell cycle progression – drivers and regulators

The eukaryotic cell cycle is a highly complex set of sequential events by which living cells duplicate their genomic content and divide it equally between two daughter cells. Most eukaryotes display four distinct cell cycle stages with varying lengths depending on the cell type. The main stages, namely S phase (DNA replication) and M phase (chromosome segregation), are interspersed with two growth phases (G1 before S and G2 before M). The main components that drive the periodic fluctuation of events in all eukaryotic cell cycles are (a) Cyclin-dependent kinases, or CDKs; (b) two sequential waves of transcriptional upregulation and (c) two large-scale protein degradation events (Breeden, 2003; Morgan, 2007; Vodermaier, 2004).

CDKs are the master regulators of the cell cycle with hundreds of potential substrates even in a single cell eukaryote like budding yeast (Holt et al., 2009). As the name suggests, they bind to partner proteins called cyclins for their activity. There are many specialized forms of CDK and activities of different forms rise and fall in a sequential process thereby pushing cells through the cell
cycle (Morgan, 2007). The CDKs help to generate two transcriptional bursts during two distinct stages of the cell cycle. The first and the more pronounced one is at the G1 to S boundary when cells need to massively and robustly upregulate hundreds of transcripts for an irreversible commitment to the cell cycle and successful, error-free DNA replication (Charvin et al., 2010; Morgan, 2007; Novak et al., 2007). This task is carried out by the E2F family of transcription factors in higher eukaryotes and by SBF and MBF transcription complexes in budding yeast (Morgan, 2007). CDKs help this process in the G1 phase by inhibiting the repressors of these transcription factor complexes (retinoblastoma in higher eukaryotes and its analog Whi5 in budding yeast) (Hasan et al., 2013; Morgan, 2007). The second transcriptional wave happens at the G2/M transition and include, in budding yeast the mitotic cyclin Clb2 transcript (Morgan, 2007). There are many other periodically expressed genes in budding yeast cell cycle, but their transcription is not thought to be under CDK control (Orlando et al., 2008).

One key aspect of the cell cycle is irreversibility which is ensured through a series of biochemical switches resulting from factors such as robust positive feedback loops, stoichiometric CDK-inhibitors and proteolytic degradation. In budding yeast, the upstream G1 cyclin-CDK complex turns on the expression of downstream G1/S cyclins. These later G1/S cyclin-CDK complexes drive their own expression (positive-feedback loop) (Skotheim et al., 2008) and promote the
degradation of inhibitors of the S-phase cyclin-CDKs, in addition to turning on the massive SBF/MBF transcriptional program. This sequence of events lets the now-unchallenged S-phase cyclin-CDKs ensure irreversible entry into the cell cycle (Morgan, 2007; Schwob and Nasmyth, 1993; Yang et al., 2013). This irreversible commitment step is aided by proteolytic degradation through the SCF family of ubiquitin ligases, which target, among other things, the S-phase CDK-inhibitor (CKI) and also the G1 cyclins (Morgan, 2007). A Positive feedback loop of a different kind operates at the G2 to M transition. During Xenopus oocyte maturation, the mitotic CDK1-cyclinB complex promotes its own activation by inhibiting Wee1 kinase and activating Cdc25 phosphatase, which control an inhibitory tyrosine phosphorylation site on CDK1. These opposing regulations set up another positive feedback loop and CDK1 remains active even when stimulus is removed (Ferrell; Ferrell and Machleder, 1998; Gould and Nurse, 1989; Morgan, 2007; Potapova et al., 2011; Trunnell et al., 2011). This irreversible process continues until an external factor is introduced. This external factor is the degradation of the mitotic cyclins by another large multi-subunit ubiquitin ligase complex known as the APC (Morgan, 1999; Wasch and Cross, 2002). The APC is involved in promoting chromosome segregation and mitotic exit. From APC activation until the end of G1, mitotic CDK activity will remain low because of the activated status of the APC. The G1 cyclins are refractory to the APC and promote the inhibition of a key APC component (Morgan, 2007), thus allowing the mitotic CDK to get activated again. Interestingly, the mitotic CDK complex
activates the APC, thus promoting its own destruction – a negative feedback loop that generates robust oscillations of mitotic CDK activity (Morgan, 2007). It is worth noting that this Wee1-Cdc25 regulation is not required in budding yeast (Amon et al., 1992), thus bringing out subtle differences between different model systems.

**CDKs and cyclins – mechanisms and controls – general principles**

CDKs are proline-directed serine/threonine kinases, which require binding to cyclin proteins (Morgan, 1997) and the resulting conformational change to become active (De Bondt et al., 1993). In higher eukaryotes, there are multiple CDKs involved in cell cycle control (CDK 1,2,4,6) and multiple cyclins (Cyclin D, E, A, B and their subtypes like Cyclin D1, D2, D3). But in some single-celled eukaryotes like fission yeast and budding yeast, one main CDK, CDK1, controls all of cell cycle in partnership with several periodic cyclins (Figure 1.1) (Morgan, 1997; Morgan, 2007). Once activated, cyclin-CDK complexes phosphorylate proteins at specific serine/threonine residues that are a part of either a full consensus sequence (S/T-P-x-K/R) or a minimal consensus sequence (S/T-P) (Langan et al., 1989; Songyang et al., 1994). Interestingly, a large-scale proteomic screen done in budding yeast showed that about two-thirds of all phosphorylated CDK sites belonged to the minimal consensus category (Holt et al., 2009). Also, one study using human cell lysates show that only 43% of all
Figure 1.1. CDKs and their role in cell-cycle progression

(Reproduced from Bardin and Amon, 2001)

During G1, the levels of G1 cyclins rise, and these cyclins associate with cyclin-dependent kinases (CDKs). Activity of G1 CDKs promotes the passage of cells through START (budding yeast), also known as the restriction point (R) in fission yeast and higher eukaryotes. After passing through this point, a cell is committed to continue through the cell cycle. In budding yeast, the G1 cyclins are Cln1, Cln2 and Cln3. Cln3 is present throughout the cell cycle at low levels, and this cyclin promotes the accumulation of Cln1 and Cln2 (Nasmyth, 1993). In fission yeast, puc1 seems to be important — although not essential — for entry into the cell cycle (Forsburg and Nurse, 1991). In higher eukaryotes, cyclin D-associated kinases are thought to have a function similar to that of Cln3–CDKs in budding yeast, and they are important for promoting the accumulation of the other G1 CDKs, cyclin E–CDK (Morgan, 1997; Nigg, 1995).

S-phase CDKs begin to increase towards the end of G1, and are inactivated during G2 and mitosis. On reaching a critical level, they promote DNA replication. In budding yeast, Clb5 and Clb6-associated kinases are important for promoting DNA synthesis, but in their absence, mitotic CDKs (Clb1, Clb2, Clb3 and Clb4-associated kinases) can support DNA replication. In S. pombe, cig1 and cig2-associated kinases promote DNA replication, but as in S. cerevisiae, the mitotic cyclin cdc13 can function in their absence. In higher eukaryotes, cyclin E–CDKs
— directly or indirectly — and cyclin A–CDKs promote DNA replication (Morgan, 1997).

Mitotic CDKs are activated at the onset of mitosis and, among other processes, promote chromosome condensation, formation of the mitotic spindle and breakdown of the nuclear envelope. These functions are performed by CDKs associated with Clb1, Clb2, Clb3, Clb4 and Clb5 (perhaps also Clb6) in budding yeast, cdc13–cdc2 in fission yeast, and cyclin A and cyclin B-associated kinases in higher eukaryotes. For cells to exit from mitosis, undergo cytokinesis and enter the following G1, mitotic CDKs must be inactivated (Koepp et al., 1999; Morgan, 1997).
Figure 1.1. CDKs and their role in cell-cycle progression

(Reproduced from (Bardin and Amon, 2001)
sites phosphorylated by cyclinA-CDK2 were full consensus sites (Chi et al., 2008). The expression of CDK1 in yeast is not regulated, such that it remains constant during the entire cell cycle and always in excess of its cyclin partners (Mendenhall et al., 1987).

Apart from cyclin binding, the activation state of CDKs is controlled by post-translational modifications (Mendenhall and Hodge, 1998). Full activation of CDKs requires the phosphorylation of a highly conserved threonine residue in the T-loop region near the catalytic cleft by CDK-activating kinase or CAK (Espinoza et al., 1996; Morgan, 1995, 1997). The result of this phosphorylation in the mammalian cyclin A-CDK2 pair, is that the T-loop, which otherwise blocks the catalytic cleft, changes position upon phosphorylation, thereby freeing the active site for substrate binding. Also, the residues responsible for ATP binding change position after this phosphorylation event (Jeffrey et al., 1995; Russo et al., 1996). Other cyclin-CDK pairs are expected to behave in the same manner although differences in activity has been noted between them which probably points to different abilities of different cyclins to activate their CDK partner (Koivomagi et al., 2011b). Budding yeast differs from higher eukaryotes in having the activating phosphorylation event precede the cyclin binding event (Ross et al., 2000). CDK’s activity is negatively regulated by phosphorylation at a conserved Tyr19 residue (and another threonine residue for mammalian CDKs). These phosphorylation sites regulate the orientation of the ATP phosphates and the
affinity for substrate peptides (Welburn et al., 2007). This inhibitory phosphorylation forms the basis of a positive feedback loop that strongly activates the M-phase CDK (mentioned above) at the beginning of mitosis in metazoans but not in budding yeast.

It is interesting to note that not all cyclin-CDK pairs are equally susceptible to this inhibitory phosphorylation. For example, in budding yeast, even though the same CDK1 partners with both G1 phase and M phase cyclins, only the M phase cyclin-CDK complex is inhibited strongly by the inhibitory phosphorylation carried out by Swe1 kinase (Hu and Aparicio, 2005; Keaton et al., 2007). This difference demonstrates that not all CDK complexes are similarly regulated. Also, only the M phase cyclin-CDK complex can phosphorylate and inactivate Swe1, not other CDK complexes (Asano et al., 2005; Bhaduri and Pryciak, 2011; Harvey et al., 2005), thus showing differences in the substrate specificity of different cyclin-CDK complexes (to be discussed in detail later).

Unlike the CDK, cyclin levels are tightly controlled by transcription and proteolysis (Bloom and Cross, 2007; Murray, 2004). Nearly all cyclins are expressed in specific phases during the cell cycle and each cell cycle stage has its associated cyclins (Evans et al., 1983; Murray, 1995; Murray and Kirschner, 1989). Cyclins are rapidly degraded by the proteasome (Glotzer et al., 1991; Murray, 1995) and thus allow the CDK to rapidly exchange their cyclins even
though dissociation rates are low (Kobayashi et al., 1994). All cyclins have a well-conserved domain called the cyclin box fold or CBF (Kobayashi et al., 1992), which binds the CDK and changes the conformation of the active site of the CDK (Jeffrey et al., 1995).

**Budding yeast cyclin-CDK complexes – functions and regulations**

In budding yeast, the major CDK is Cdc28 (also called CDK1), originally discovered by Lee Hartwell in the early seventies (Hartwell, 1974; Hartwell et al., 1974; Hartwell et al., 1973). Cdc28 associates with nine cyclins to carry out different jobs during distinct cell cycle stages (Bloom and Cross, 2007; Cross, 1995a; Enserink and Kolodner, 2010; Koivomagi et al., 2011b; Nasmyth, 1996). Among these cyclins, there are two distinct groups, namely the G1 cyclins (Cln1-Cln3) that control the decision whether or not to begin a new cell cycle, and the B-type cyclins (Clb1-Clb6) that control DNA replication and mitosis. These cyclins are expressed sequentially. Cln3 triggers expression of Cln1/2, which then induce the expression of Clb5 and Clb6, which drive DNA replication but also terminate the expression of Cln1/2 during S phase. The next wave of cyclin expression produces Clb3 and Clb4 at the G2/M stage and finally, Clb1 and Clb2, which take the cell through mitosis (Mendenhall and Hodge, 1998; Morgan, 1997; Pines, 1995). Apart from the G1 cyclin Cln3, all others are expressed in
paralogous pairs (i.e., Cln1/2, Clb1/2, Clb3/4, and Clb5/6) as a result of a whole genome duplication event in the evolution of *Saccharomyces cerevisiae*. Although having any one of the three G1 cyclins is enough to begin a new cell cycle, there are specific differences in their mechanism of action and not all of their functions are redundant (Dirick et al., 1995; Skotheim et al., 2008). Expression of Cln3 is low and more or less constant throughout the cell cycle, peaking slightly in late mitosis and early G1 (MacKay et al., 2001; McInerny et al., 1997; Tyers et al., 1993) where it orchestrates the first step towards cell cycle entry, whereas Cln1 and Cln2 are expressed periodically with a peak in late G1 (Wittenberg et al., 1990). Although originally (in above studies) Cln3 expression was shown to be less periodic than other cyclins, a recent study has shown that Cln3 protein cycles quite a bit with a peak in M phase (Landry et al., 2012). Cln3 and Cln1/2 are thought to promote Start through different mechanisms. Start (restriction point in metazoan cell cycle) is a small window before DNA replication when the cell irreversibly commits to a new cell cycle (Johnson and Skotheim, 2013). Cln3 largely contributes to this event by turning on the expression of some key genes required for cell cycle entry including Cln1/2. Whereas, Cln1/2 can not only drive their own expression (positive feedback loop) (Skotheim et al., 2008) but also control other Start related events such as a) polarized growth for bud emergence; b) spindle pole body duplication and c) promoting degradation of S phase cyclin-CDK inhibitor, Sic1 (Bloom and Cross, 2007; Nasmyth, 1996). Another distinct feature of Cln1/2 is that they can restrict pheromone signaling to
the G1 phase (Oehlen and Cross, 1994; Strickfaden et al., 2007; Wassmann and Ammerer, 1997), a task not fulfilled by Cln3.

Cln3 controls the first step towards cell cycle entry (Stuart and Wittenberg, 1995; Tyers et al., 1993). Many genes (about 200) are transcribed at this stage including key G1/S regulators. These genes are transcriptionally controlled by hetero-dimeric transcription factors SBF (Swi4/Swi6) and MBF (Mbp1/Swi6). SBF and MBF (analogs of E2F in higher eukaryotes) are kept inhibited by a protein called Whi5 (analog of Rb in higher eukaryotes) in the nucleus. The main job of Cln3-CDK1 is to phosphorylate Whi5 and initiate its exit from the nucleus, thus relieving SBF and MBF of repression (Costanzo et al., 2004; de Bruin et al., 2004). Once de-repressed, SBF and MBF start a massive program of transcribing about 200 genes including the G1/S cyclins Cln1 and Cln2 and the S phase cyclins Clb5 and Clb6, in a temporally separated manner. Cln1 and Cln2 are among the earliest transcripts (Eser et al., 2011; Skotheim et al., 2008) and in partnership with the CDK1, they can further phosphorylate Whi5 to completion (Costanzo et al., 2004; Skotheim et al., 2008) and restrict its re-entry to the nucleus (Charvin et al., 2010). Cln1/2-CDK1 can promote their own transcription, thereby setting off a robust positive feedback loop (Figure 1.2A) (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991; Skotheim et al., 2008) resulting in the quick accumulation of strong CDK activity needed to pass through Start,
Figure 1.2. Positive feedback loops operating at G1/S transition.

(A) Model for G1/S transcriptional regulon activation and bud emergence; red lines indicate pathways generating positive feedback in Start transition (reproduced from Skotheim et al., 2008).

(B) Model of Sic1 degradation; red line indicates positive feedback in Sic1 degradation at Start transition (reproduced from (Yang et al., 2013).
Figure 1.2. Positive feedback loops operating at G1/S transition
defined molecularly as the point where roughly 50% of Whi5 has left the nucleus (Doncic et al., 2011). Another very important job that Cln1/2-CDK1 does is to start the multisite phosphorylation of CKI Sic1, which specifically inhibits the S phase Clb5/6-CDK1 complexes. Later, as Clb5/6 gets transcribed in small amounts, Clb5/6-CDK1 completes the phosphorylation of Sic1 and promotes its degradation by the proteasome – another positive feedback loop (Figure 1.2B) (Koivomagi et al., 2011a; Yang et al., 2013). This event contributes to the mechanism of switch-like transition from G1 to S phase (Venta et al., 2012).

Clb5/6 transcription at G1/S is controlled by SBF/MBF (Nasmyth and Dirick, 1991; Schwob and Nasmyth, 1993) but the other four Clbs are controlled through a different group of transcription factors known as the Mcm1-Fkh1/2-Ndd1 complex which turn on the expression of about 35 genes at G2/M including the mitotic cyclins (Futcher, 2002; Spellman et al., 1998; Wittenberg and Reed, 2005). Moreover, SBF/MBF are inactivated by mitotic Clb2-CDK1 (Amon et al., 1993) and Clb2-CDK1 can drive its own transcription by phosphorylating its transcription factors Fkh2 and Ndd1 (Reynolds et al., 2003).

Cyclin abundance is controlled by proteolysis throughout the cell cycle and this negative regulation is very important for generating sharp peaks of specific kinds of CDK activity during distinct cell cycle stages (Bloom and Cross, 2007). Yeast G1/S cyclins have very short half-lives (~ 5-10 minutes) (Barral et al., 1995; Lanker et al., 1996; Salama et al., 1994) and are targeted for degradation by a
ubiquitin ligase complex known as SCF (Skp1/Cdc53-F-box) (Deshaies et al., 1995; Willems et al., 1996). The F-box protein is the substrate-specificity determining factor, which is Grr1 in the case of Cln1/2 (Skowyra et al., 1997; Skowyra et al., 1999). Degradation of Cln2 is preceded by the autophosphorylation of its C-terminus by Cln2-CDK1 at multiple phosphorylation sites encompassing a PEST degradation domain, which is then recognized by Grr1 (Lanker et al., 1996; Salama et al., 1994; Schneider et al., 1998). Cln3 is degraded by not only SCF-Grr1 but also SCF-Cdc4 and its degradation also requires CDK1 dependent phosphorylation of its C-terminus (Landry et al., 2012). SCF also controls the abundance of the B-type cyclin Clb6 through the F-box protein Cdc4 after phosphorylation by CDK1 and another cyclin-dependent kinase Pho85 (Jackson et al., 2006). Other B-type cyclins in budding yeast are degraded by the Anaphase Promoting Complex or APC. At early M phase, APC partners with substrate specific adaptor protein, Cdc20 and targets mitotic cyclins and Clb5 (Shirayama et al., 1999; Visintin et al., 1997; Wasch and Cross, 2002). The mitotic Clb2-CDK1 complex can phosphorylate and activate APC-Cdc20 components, thereby promoting its own degradation (negative feedback loop) (Rudner et al., 2000). During late M phase, APC partners with another adaptor protein Cdh1 with different substrate specificities. APC-Cdh1 can target mitotic cyclins but not Clb5 or Cln1-3. This insensitivity to APC lets G1/S and Clb5 cyclin-CDKs phosphorylate and inactivate Cdh1 at G1/S, thereby allowing the
mitotic Clb2-CDK activity to accumulate at the onset of M phase (Jaspersen et al., 1999; Kramer et al., 2000; Zachariae et al., 1998).

**Specific roles of G1/S CDK at Start transition – substrates of the early CDK**

Over the past two decades, considerable effort has gone into discovering substrates of cyclin-CDK complexes in eukaryotes, and budding yeast has proved to be a valuable model system in this effort. Through these studies, approximately 75 proteins functioning in different critical cell cycle events like DNA replication and morphogenesis in budding yeast have been shown to be under CDK1 control (Enserink and Kolodner, 2010). A similar number of targets have been identified in higher eukaryotes (Blethrow et al., 2008; Errico et al., 2010). However, even conservative estimates predict that CDK1 must have hundreds of targets in any cell. Several large-scale screening and computational studies have generated a larger list of predicted CDK targets and CDK controlled processes (Archambault et al., 2004; Chang et al., 2007; Holt et al., 2009; Moses et al., 2007a; Ubersax et al., 2003). One study in budding yeast (Ubersax et al., 2003) identified 181 targets for the major mitotic cyclin Clb2-CDK. Another large-scale study (Holt et al., 2009) identified 308 substrates containing a total of 547 phosphorylated CDK sites. Similarly, there are some large-scale studies performed in higher eukaryotes to identify CDK substrates. For example, using a gel-shift assay combined with radioactive labeling, many candidates of individual
cyclin-CDK complexes were identified in Xenopus extracts (35 potential substrates for cyclin B-CDK1, 70 for cyclin A-CDK2, and 42 for cyclin E-CDK2) (Errico et al., 2010). There was considerable overlap in substrates between the complexes tested in this study, but one interesting point that emerged is that not everything with a consensus CDK site works as a substrate; instead, some other requirement may need to be met, such as the presence of RxL type cyclin docking motifs (Errico et al., 2010). A study using human cell lysates and an analogue-sensitive allele of CDK2 combined with mass-spectrometry identified 180 potential substrates belonging to various categories including cell cycle progression, mRNA transcription, cellular structure and organization (Chi et al., 2008). One large class of proteins in this study is uncharacterized, thus drawing our attention to the fact that we are yet to fully realize the functions of CDK phosphorylations. More than half of the identified phospho-sites were minimal-consensus (S/TP) sites and about 50% of them contained an RxL type motif distal to the sites themselves (Chi et al., 2008).

The studies described above, as well as others (Blethrow et al., 2008), have identified and/or predicted a large number of targets in both yeast and higher eukaryotes for specific forms of CDK. Of particular relevance to this thesis are substrates of the yeast G1/S cyclin-CDK complexes, and therefore the key substrates and processes are discussed in greater detail in the following paragraphs.
Whi5: Whi5 is an inhibitor of the SBF transcription factor complex, which is key to the G1/S transition. Following mitosis, Whi5 resides in the nucleus and inhibits transcription of the G1/S regulon. Before cells can commit to a new cell cycle at least 50% of Whi5 needs to be exported from the nucleus (Costanzo et al., 2004; Doncic et al., 2011). Phosphorylation of Whi5 promotes its nuclear export. Whi5 has 12 CDK sites and the current understanding suggests that several of these CDK sites need to be phosphorylated to achieve nuclear export and SBF de-repression (Skotheim et al., 2008; Wagner et al., 2009) although there is some ambiguity regarding how many and which sites need to be phosphorylated. Phosphorylation of Whi5 is initiated (but not completed) by Cln3-CDK1 and that phosphorylation is probably sufficient to promote some SBF de-repression and allow some Cln1/2 to be transcribed. Subsequently Cln1/2-CDK1 completes the phosphorylation of Whi5 and fully turns on the transcriptional program. In the absence of Cln1/2, the nuclear exit of Whi5 is not sharp and incomplete as shown by single cell microscopy (Skotheim et al., 2008). In that study, it was also shown that a whi5-6A mutant cannot make a sharp and complete exit from the nucleus at Start and neither can it turn on some of the SBF controlled genes coherently (Skotheim et al., 2008).

Sic1: Sic1 is an inhibitor of S phase Clb5/6-CDK1 complexes and is key to setting the correct timing for the initiation of DNA replication. Sic1 keeps a temporal window in G1 free of Clb5/6-CDK1 activity, which is essential for new
origin licensing (Lengronne and Schwob, 2002). Clb5/6-CDK1 activity can rise only when Sic1 is degraded (Schwob et al., 1994; Verma et al., 1997). Sic1 has nine CDK phosphorylation sites and multiple sites need to be phosphorylated for SCF\(^{Cdc4}\) mediated degradation of Sic1 at the onset of S phase (Feldman et al., 1997; Nash et al., 2001; Verma et al., 1997). Both Cln2-CDK1 and CLB5-CDK1 can phosphorylate Sic1 \textit{in vitro} (Koivomagi et al., 2011a; Skowyra et al., 1997). Although population-average studies (Sedgwick et al., 2006; Verma et al., 1997) in the past had implicated Cln2-CDK1 in Sic1 phosphorylation, recent \textit{in vitro} and single cell \textit{in vivo} time-lapse microscopy studies show that in fact the rate of degradation of Sic1 depends on Clb5-CDK1, not Cln2-CDK1 (Koivomagi et al., 2011a; Yang et al., 2013). However, Cln2-CDK1 mediated initial phosphorylation of Sic1 at certain sites is thought to play a priming role which then allows Clb5-CDK1 to complete the multiple site phosphorylation that is required for efficient Cdc4 recognition (Koivomagi et al., 2011a). An \textit{in vivo} study looking at fluorescently labeled Sic1 in both mother and daughter cells observed that Cln2-CDK1 controls the timing of Sic1 degradation ("defined as the time from Whi5 nuclear exclusion to the point of the fastest Sic1 degradation"), such that when Cln1/2 are absent, this timing is highly variable among cells. Also, removing a Cln2 docking site in Sic1 causes variability in this timing (Yang et al., 2013). The use of two different CDK complexes to separately initiate and then complete substrate phosphorylation is an emerging theme at key cell cycle transition
points (Yang et al., 2013), as with the dual-stage inhibition of Whi5 by Cln3-CDK1 and then Cln1/2-CDK1 (discussed above).

Cdh1: Cdh1 (originally known as Hct1) is an important component of the APC complex and one of its main targets is the mitotic cyclin Clb2 (Baumer et al., 2000). In order to let the mitotic cyclin-CDK activity build up before mitosis, Cdh1 activity needs to be kept in check during G1 and S phase. This regulation is achieved through phosphorylation of Cdh1 by G1/S and S phase cyclin-CDKs (Jaspersen et al., 1999; Zachariae et al., 1998). Cdh1 has eleven CDK sites and the phosphorylation of multiple sites is correlated with its degree of inactivation by CDK (Zachariae et al., 1998). Also, Cln2 has been shown to bind Cdh1 (Archambault et al., 2004) but less is known about the mechanism of Cln2-CDK1 mediated phosphorylation of Cdh1 and it remains to be seen if its phosphorylation depends on docking and processivity mechanisms similar to that in Sic1.

Polarized bud morphogenesis: Several proteins involved in bud emergence are specific targets of Cln1/2-CDK1. Specific cell size and shape are maintained through a coordination of cell growth with the cell-division cycle. But cell growth is not thought to be controlled by the cell cycle (Cross, 1990; Culotti and Hartwell, 1971; Gross et al., 1997; Johnston et al., 1977; Kipreos et al., 1996). G1/S cyclin-CDK1 activity in yeast is thought to promote polarized apical bud growth, which
later shifts to isotropic bud growth during S phase (when Cln1/2 protein levels have gone down). Heightened activity of G1 cyclin-CDK has been shown to drive extremely elongated bud growth (Barral et al., 1995; Lew and Reed, 1993). Recent work (McCusker et al., 2007) has shown disruption of CDK1 activity disrupts the secretory pathway, important for delivering vesicles to the growing bud. Cdc42-GTPase signaling is required for the initiation of polarized bud growth and this process is CDK1 dependent (Butty et al., 2002; Gulli et al., 2000; Lew and Reed, 1993; Moffat and Andrews, 2004). Cdc42 is instrumental in polarized bud emergence in G1 (Adams et al., 1990) and polarized localization of exocyst components Sec4 and Sec15 (Zajac et al., 2005). Cdc42 needs Cdc24-GEF for activation and inhibition of Cdc24 during bud emergence stops polarized growth (Sloat et al., 1981; Sloat and Pringle, 1978). Cdc24 was found to be associated with many other polarity-related proteins such as Bem1, Rga2, Boi1, Boi2 (McCusker et al., 2007). Therefore, it appears that bud emergence requires a large complex containing a GEF, a GAP and a scaffold protein for Cdc42 along with some other proteins needed for actin polarization. Many of these proteins associated with Cdc24, including itself, get hyperphosphorylated at the time of bud emergence (G1/S boundary). The authors went on to further show that these proteins are specific targets of the Cln2-CDK and not Clb2-CDK1 (McCusker et al., 2007). Interestingly, all of these proteins contain multiple CDK sites and it was shown that Cln2-CDK1 mediated multi-phosphorylation of Boi1 is important for maintaining proper polarized bud morphogenesis (McCusker et al., 2007).
Inhibition of pheromone signaling: The pheromone-responsive mating pathway inhibits entry into the cell cycle by activating a CKI protein, Far1 (discussed in detail later and in Chapter 4). In turn, the mating pathway is itself inhibited by the cell cycle, such that pheromone signaling is prevented in cells that have already passed Start (Oehlen and Cross, 1994; Oehlen et al., 1998), which ensures that the cell does not commit to differentiate and divide at the same time. This inhibition is mediated by the G1/S cyclin-CDK complexes, but not by CDK complexes from S or M phases. There are two ways in which G1/S cyclin-CDKs influence the mating pathway. Firstly Cln2-CDK1 promotes the degradation of Far1 as the cells enter G1 (Henchoz et al., 1997; McKinney et al., 1993). Secondly Cln2-CDK1 inhibits the membrane localization of an essential pathway component, the MAPK scaffold protein Ste5 (Bhattacharyya et al., 2006; Winters and Pryciak, 2005). Cln1/2-CDK1 but not other forms of CDK1, phosphorylates the eight CDK sites flanking a membrane localization domain in Ste5. This inhibits the membrane binding of the scaffold protein Ste5 and as a result MAPK signal propagation is stopped (Strickfaden et al., 2007). Interestingly, all of these CDK sites are of the minimal consensus category and Ste5 has a Cln1/2 specific docking motif (Bhaduri and Pryciak, 2011). This mechanism ensures that once proper CDK activity levels are reached, pheromone response is inhibited so that DNA replication can proceed without hinderance. Pheromone signaling can antagonize G1/S cyclin-CDK activity through the CKI
Far1, if cells get exposed to pheromone early on in G1. The molecular mechanism behind Far1’s inhibition of Cln-CDK activity has been a matter of ambiguity because while some early reports showed Far1 as a CKI, later studies failed to confirm that observation (Gartner et al., 1998; Peter and Herskowitz, 1994). Recent work from our group (Pope et al., 2014) has given the first direct in vivo evidence that Far1 can inhibit Cln2-CDK phosphorylation of Ste5. At least part of this mechanism is inhibiting the substrate binding of Cln2, but there is another component of it, which probably affects the activity of the CDK directly (more details later and in Chapter 4)

The two models for regulation of CDK activity during the cell cycle

There are two leading models to explain how different cell cycle events are triggered at different cell cycle stages. One posits different threshold levels of cyclin-CDK activity (the quantitative model), and the other relies on different substrate specificities of different cyclin-CDK complexes (the qualitative model). Studies on the main mitotic cyclin in fission yeast, Cdc13, led early researchers to propose a quantitative model for CDK activity (Fisher and Nurse, 1996). This model stated that low levels of CDK activity is enough to take the cell through the S phase (DNA replication) but is not enough to promote mitosis. Later, CDK activity rises and that, besides promoting mitosis, also prevents re-replication. After mitosis, CDK activity again subsides and the cell gets ready for the next
division cycle. Among other things, this model requires S phase and M phase substrates to respond to different thresholds of CDK activity (namely mitotic substrates will get phosphorylated only by high CDK activity while S phase substrates respond to low CDK activity) (Fisher et al., 2012; Stern and Nurse, 1996; Uhlmann et al., 2011). In support of this model, it has been shown that fission yeast cells lacking three important G1 and S phase cyclins (Puc1, Cig1 & Cig2) are still viable (Martin-Castellanos et al., 2000). More recent work, using an engineered cyclin-CDK fusion protein regulated by different doses of an inhibitor, demonstrated that a fission yeast cell cycle can be driven by a single cyclin-CDK pair (Coudreuse and Nurse, 2010). Several other recent studies show different mitotic events require different levels of CDK1 activity. One study involving the mammalian cyclinB1-CDK1 pair showed that earlier mitotic events required less cyclinB1-CDK1 activity than later ones (Gavet and Pines, 2010). Also, in vitro studies confirmed that late mitotic substrates were phosphorylated by only high levels of cyclinB1-CDK1 activity (Deibler and Kirschner, 2010). Also budding yeast mitotic events like spindle formation and elongation require different levels of mitotic cyclin Clb2 (Oikonomou and Cross, 2011). In this dissertation (Chapter 5) I will present evidence that *S. pombe* Cdc13 may belong to class of special cyclins which can activate the CDK1 kinase to unusually higher levels as compared to other B-type cyclins. Although it’s apparent that different cell-cycle events require different levels of CDK activity, this observation cannot explain all of the specificities in cyclin-CDK
function. For example, most organisms use, not only numerous cyclins, but also several CDKs for their cell cycle progression (Roberts, 1999). In budding yeast, the abundance of different cyclins is quite similar (Cross et al., 2002) thus hinting that there is not much change in net CDK1 activity from G1 to M phase. Therefore, there must be other mechanisms regulating substrate specificities of different cyclin-CDK complexes. In budding yeast, several cell-cycle events require a specific type of cyclin-CDK complex. For example, mitotic events cannot be driven by G1 cyclins and conversely B-type cyclins cannot drive the G1-specific transcriptional program (Nasmyth, 1996; Schwob and Nasmyth, 1993). In fact mitotic cyclins could not even initiate replication properly unless overexpressed (Cross et al., 1999; Nasmyth, 1996). Also, the pheromone response pathway can only be inhibited by G1/S cyclin-CDK complexes (Oehlen et al., 1998). This distinction between G1 and B-type cyclins is likely to be true even for fission yeast, as the absence of the major G1 cyclin, Puc1, causes a cell cycle delay in G1 (Martin-Castellanos et al., 2000). So the quantitative model only pertains to B-type cyclins. A thorough quantitative comparison of activity and substrate specificity between budding yeast S phase Clb5-CDK1 with M phase Clb2-CDK1 complexes showed that even though Clb2 modulates the CDK1 active site towards higher activity than Clb5, some substrates are still more specific towards Clb5-CDK1 (Loog and Morgan, 2005). The intrinsic activity of S phase Clb5-CDK1 is low and hence it phosphorylates several mitotic substrates poorly but for some S phase substrates, this low intrinsic activity is compensated.
by a cyclin-specific docking interaction with the substrates. Conversely, although, Clb2-CDK1 activity is intrinsically high, allowing broader range of substrate selection in mitosis, lack of substrate docking renders it ineffective on several S phase substrates. More recent work has further advanced this model to include that the two methods of substrate targeting changes reciprocally during cell cycle progression (Koivomagi et al., 2011a; Koivomagi et al., 2011b). In this in vitro study, the authors showed, as the cell cycle progresses, the intrinsic activity of each successive cyclin-CDK pair increases but substrate-specific binding decreases. So this new data supports the quantitative model of CDK activity while incorporating the fact that several cell cycle events require cyclin-specific substrate targeting which compensates for low intrinsic CDK activity of early cyclin-CDK complexes (key aspects of the qualitative model). Work to be presented later in this dissertation will further bolster the cyclin-specificity model with respect to a different class of cyclins, namely the G1/S cyclins Cln1 and Cln2 (Bhaduri and Pryciak, 2011). In vivo evidence will be presented to advance the claim that some substrates are specific to the Cln1/2-CDK1 complexes because they have Cln1/2 specific docking motifs (Chapter 2).

**Mechanisms of substrate specificity of CDK complexes**

In spite of the wide functional redundancy within distinct subclasses of cyclins and CDKs, there are many examples of cyclin specific behavior during the cell
cycle. In budding yeast, pheromone response can be inhibited only by Cln1/2-CDK complexes and not by Clb5-CDK or even Cln3-CDK (Oehlen and Cross, 1994). Four G1 cyclin-CDK complexes, namely Cln1/2-CDK1 and Pcl1/2-PHO85, together share an essential function of initiating polarized bud morphogenesis and deletion of all of these four cyclins causes inviability which cannot be rescued by Cln3 or the Clbs (Moffat and Andrews, 2004). The mitotic Clb2-CDK1 complex in yeast cannot trigger the expression of the G1/S regulon, normally turned on by Cln3-CDK1. Instead Clb2-CDK1 can repress the expression of those genes (Amon et al., 1993). Also, Cln1/2-CDK1 promotes polarized bud growth whereas Clbs, especially Clb2-CDK1 promotes isotropic bud growth (Lew and Reed, 1993). From several studies in budding yeast, it was clear that Cln3 and Cln1/2 promote the entry into cell cycle through different mechanisms: Cln3 turns on the massive G1 transcriptional program (Dirick and Nasmyth, 1991; Koch and Nasmyth, 1994; Stuart and Wittenberg, 1995), whereas Cln2 promotes additional G1 events like bud morphogenesis and activation of Clb-CDK complexes (Benton et al., 1993; Cvrckova and Nasmyth, 1993; Lew and Reed, 1993). Then, by putting Cln2 under control of the Cln3 promoter, a subsequent study (Levine et al., 1996) showed that Cln2 and Cln3 still differed in their ability to promote viability in certain genetic backgrounds although this study did not take into account different post-translational regulation of cyclins. This clearly showed that the difference between Cln2 and Cln3 is not simply due to their difference in the timing of expression and of expression levels. Budding yeast
mitotic cyclin, Clb2, expressed from Clb5 promoter, is not as potent as Clb5 in promoting the onset of DNA replication (Cross et al., 1999) and conversely, a proteolysis-resistant Clb5 is unable to inhibit mitotic exit when overexpressed, unlike a similar Clb2 allele (Jacobson et al., 2000). Similarly, in metazoans, cyclin D2 and D3 can prevent differentiation of 32D myeloid cells when stimulated by granulocyte colony-stimulating factor but cyclin D1 is unable to do this job (Kato and Sherr, 1993). In mice, replacing cyclin D1 coding sequence with cyclin E, resulted in normal cell cycle progression, but a detailed investigation found that cyclin E failed to fully recapitulate cyclin D1 specific events like Rb phosphorylation, but instead bypassed the need for cyclin D1 whose main job is to promote cyclin E-CDK2 activity (Geng et al., 1999).

Cyclin-CDKs employ several different mechanisms to specifically select their substrates. The very first selection happens at the level of the CDK active site. In order to get phosphorylated by CDK, a substrate has to have either a full consensus sequence (S/T-P-x-R/K) or at least a minimal consensus sequence (S/T-P) (Beaudette et al., 1993; Nigg, 1993a, b; Songyang et al., 1994). In a crystal structure of metazoan cyclinA with CDK2, a substrate-derived peptide containing the consensus site is shown to present the consensus residues to the active site of the CDK, with no direct contact with cyclinA (Brown et al., 1999). Interestingly, in most cases, CDK phosphorylation sites are located in poorly conserved and intrinsically disordered part of the substrates (Holt et al., 2009;
Moses et al., 2007a; Moses et al., 2007b). One recent study in budding yeast has shown that different cyclins can, however, activate the same CDK1 holoenzyme to different extents, thereby altering the substrate specificity at the active site of the CDK (Koivomagi et al., 2011b). Mechanisms controlling this effect on activity are not well understood but may involve different modulations of the enzymatic efficiency of the CDK active site by different cyclins as observed for mammalian cyclins B1 and A2 with CDK2 (Brown et al., 2007).

Not only can cyclins indirectly influence CDK’s substrate specificity, but they can also perform a more direct role in choosing substrates. Many B-type cyclins contain a hydrophobic patch (hp) composed of some version of a Met-Arg-Ala-Ile-Leu (M-R-A-I-L) sequence that binds to a Arg-x-Leu-Φ or Arg-x-Leu-x Φ (where x is any amino acid and Φ is large hydrophobic amino acid) motif, commonly referred to as the RxL motif (Adams et al., 1996; Cross and Jacobson, 2000; Kelly et al., 1998; Schulman et al., 1998). The RxL docking site has a dramatic impact on the efficiency of phosphorylation of a substrate by some cyclin-CDK complexes while not by others (Schulman et al., 1998; Takeda et al., 2001; Ubersax and Ferrell, 2007). One study found that the RxL motif increased the catalytic efficiency of phosphorylation at a minimal consensus (simply S/T-P) site, but did not affect the full consensus site much (Stevenson-Lindert et al., 2003). This suggests that suboptimal phosphorylation sites coupled with a cyclin specific docking motif can be a method for promoting substrate selectivity of CDK.
complexes. Suboptimal sites will ensure that the phosphorylation site is not favored by any of the CDK forms while a cyclin specific docking motif may increase the local concentration of a particular form of the CDK around the substrate, enough to favor even a slow reaction at the CDK active site. Using linkers of varying length between the RxL motif and the CDK sies, one study suggested that both regions must be simultaneously bound to the cyclin-CDK to maximize the phosphorylation of a substrate (Takeda et al., 2001). The structure of mammalian cyclin A-CDK2 with the CKI p27Kip1 and a peptide for the transcription factor p107, show that the RxL docking site binds to a surface exposed hydrophobic patch (hp) on the cyclin (Brown et al., 1999). This hp site is conserved in all cyclins A, B, D, E, in higher eukaryotes and in S-phase cyclins (Clb5/6) in budding yeast (Brown et al., 1999; Cross and Jacobson, 2000; Cross et al., 1999). Mutating the hp region disrupts function in vivo and reduces enzymatic activity on RxL containing substrates in vitro (Adams et al., 1996; Loog and Morgan, 2005; Schulman et al., 1998). In yeast, the S-phase cyclin Clb5 interacts with a number of substrates in an hp dependent manner (Archambault et al., 2005; Wilmes et al., 2004). On the contrary, budding yeast mitotic cyclins Clb1 and Clb2 do not seem to depend so much on their hydrophobic patch (Loog and Morgan, 2005) in choosing substrates and at the sequence level, the hp region of Clb1/2 looks different than the hp regions of other B-type cyclins (Archambault et al., 2005), although it may be involved in interacting with the Swe1 kinase which is both a substrate and a regulator of Clb2 (Asano et al.,
Recent work on budding yeasts G1/S cyclins, show that they too contain a region which can interact with LLPP type hydrophobic sequences in certain substrates (Bhaduri and Pryciak, 2011; Koivomagi et al., 2011a; Koivomagi et al., 2011b) and we will present evidence in this dissertation that the substrate-binding region in G1/S cyclin Cln2 is close to but not same as the MRAIL region in B-type cyclins (Chapter 3).

The substrate specificity of different cyclin-CDK complexes are also regulated through differential localizations of cyclins (Pines, 1999). Localizations of cyclins to particular compartments may help control their access to both substrates and regulators, and thereby affect their functions. For example, mammalian cyclins B1 and B2 localize to microtubules and the Golgi apparatus, respectively. At the microtubule, cyclin B1 reorganizes nuclear, cytoskeletal and membrane compartments whereas cyclin B2 helps disassemble the Golgi apparatus. By making chimeras between cyclin B1 and B2, it was possible to switch their roles (Draviam et al., 2001). In budding yeast, G1 cyclin Cln3 is primarily nuclear and it correlates well with its role of phosphorylation of the transcriptional repressor Whi5. Forcibly excluding Cln3 from the nucleus makes Cln3-CDK1 largely nonfunctional in this capacity. Likewise, most of Cln2’s functions correlate well with its largely cytoplasmic and bud tip localization pattern. However, restricting Cln2 solely to the cytoplasmic compartment compromises some of its functions,
thus suggesting that Cln2 also has an important nuclear role. Quite interestingly, targeting Cln3 to the cytoplasm gives it a limited ability to carry out some of the cytoplasmic roles of Cln2. For example, cytoplasmically targeted Cln3, but not wild type Cln3, can somewhat rescue a $cln1\,\Delta\,cln2\,\Delta\,pcl1\,\Delta\,pcl2\,\Delta$ cell, which is inviable (Edgington and Futcher, 2001; Miller and Cross, 2000, 2001a, b). These results demonstrate that cyclin localization can control its substrate specificity. There are numerous other examples of how in vivo functions of cyclins are controlled by their subcellular localizations (Baldin et al., 1993; Cardoso et al., 1993; Diehl and Sherr, 1997; Hagting et al., 1998; Hood et al., 2001; Knoblich et al., 1994; Lukas et al., 1994; Maridor et al., 1993; Ohtsubo et al., 1995; Pines and Hunter, 1991).

Of course, apart from the above-mentioned mechanisms, the ability of individual cyclin-CDK complexes to act on particular substrates is also influenced by their patterns of expression; i.e., whether they are co-expressed at the same cell cycle stage, versus at non-overlapping stages, and their different susceptibility to activating or inhibiting factors (Bloom and Cross, 2007).

**Inhibition of CDKs by CKIs**

CDK inhibitors play a very important role in regulating the activity of various cyclin-CDK complexes. In mammalian cells there are two different families, the
INK4 family that inhibits CDK4/6 (Guan et al., 1994; Hannon and Beach, 1994; Hirai et al., 1995; Serrano et al., 1993; Sherr and Roberts, 1999) and the Cip/Kip family that inhibits mainly G1 and S phase cyclin-CDK complexes (el-Deiry et al., 1994; Harper et al., 1993; Polyak et al., 1994a; Polyak et al., 1994b; Toyoshima and Hunter, 1994; Xiong et al., 1993). INK4 proteins make heterodimers with CDK4/6 and force the CDK to assume a conformation where it can no longer bind the cyclin and hence becomes inactive (Brotherton et al., 1998; Russo et al., 1998). Cip/Kip family of CKIs can engage both the cyclin and the CDK subunit (Chen et al., 1995; Luo et al., 1995; Nakanishi et al., 1995) to bring about effective inhibition. In fact the crystal structure of mammalian cyclin A-CDK2 with the CKI p27Kip showed that p27 engaged the cyclin A hydrophobic patch region by presenting a LFG motif and the CDK active site was engaged by a region in p27 that mimicked the interactions of the ATP (Russo et al., 1996). A member of the same family, p21, has been shown to inhibit cyclinB-CDK1 under special circumstances (Charrier-Savournin et al., 2004; Satyanarayana et al., 2008).

In budding yeast there are three known CDK1 inhibitors, which regulate different stages of the cell cycle. One of them, Sic1, inhibits mainly S phase cyclin Clb5/6-CDK1 complexes and regulates the commitment step of the cell cycle (Yang et al., 2013) (discussed above) and another one, Cdc6, likely inhibits mitotic cyclin-CDK1 complexes when overexpressed and helps regulate mitotic exit
Elsasser et al., 1996). A third CKI in budding yeast, Far1, is mainly involved in regulating the mating pathway and is important for pheromone-induced arrest of cell cycle at G1 (Chang and Herskowitz, 1990). Far1 inhibits the Cln1/2-CDK1 complexes (Peter and Herskowitz, 1994) but not others (Chapter 4).

**Focus of this dissertation**

This dissertation focuses on the mechanism of the substrate specificity of budding yeast G1/S cyclin-CDK complexes, the Cln1/2-CDK1 complexes. In Chapter 2, we will present evidence that several CDK1 substrates contain a particular type of docking motif that can only bind to Cln1/2 and not other yeast cyclins. This results in high cyclin specificity during the early cell cycle. One can switch the cyclin specificity simply by switching the docking motif. In Chapter 3, we show that Cln2 contains a highly conserved substrate-docking motif, which is close to, but in a different region than, the hydrophobic patch region found in most B-type cyclins. Mutating this patch in Cln2 has a variety of negative effects on cell cycle progression, the major effect being the significant increase in cell size at the time of commitment to a new cell cycle (Whi5 export, budding and DNA replication). In Chapter 4, we will elucidate the mechanism of action of the CKI Far1. Our results indicate that Far1 inhibits Cln2-CDK1 complex partially by inhibiting Cln2’s substrate binding and partially by inhibiting the activity of the
complex. In Chapter 5, we show that this method of substrate recognition is highly conserved in G1 cyclins of several fungal species as distant as molds and mushrooms and that some of this fungal cyclins have evolved to have unusually high potency, which may give them ability to regulate cell cycle events differently.
CHAPTER II

Cyclin-specific docking motifs promote phosphorylation of yeast signaling proteins by G1/S CDK complexes

The following Chapter contains the manuscript:


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

The eukaryotic cell cycle begins with a burst of CDK phosphorylation. In budding yeast, several CDK substrates are preferentially phosphorylated at the G1/S transition rather than later in the cell cycle when CDK activity levels are high. These early CDK substrates include signaling proteins in the pheromone response pathway. Two such proteins, Ste5 and Ste20, are phosphorylated only when CDK is associated with the G1/S cyclins Cln1 and Cln2, and not G1, S, or M cyclins. The basis of this cyclin specificity is unknown.

Here we show that Ste5 and Ste20 have recognition sequences, or “docking” sites, for the G1/S cyclins. These docking sites, which are distinct from Clb5/cyclin A-binding “RxL” motifs, bind preferentially to Cln2 and Cln1. They strongly enhance Cln2-driven phosphorylation of each substrate in vivo, and function largely independent of position and distance to the CDK sites. We exploited this functional independence to re-wire a CDK regulatory circuit in a way that changes the target of CDK inhibition in the pheromone response pathway. Furthermore, we uncover functionally active Cln2 docking motifs in several other CDK substrates. The docking motifs drive cyclin-specific phosphorylation, and the cyclin preference can be switched by using a distinct motif.

Our findings indicate that some CDK substrates are intrinsically capable of being phosphorylated by several different cyclin-CDK forms, but they are inefficiently
phosphorylated *in vivo* without a cyclin-specific docking site. Docking interactions may play a prevalent but previously unappreciated role in driving phosphorylation of select CDK substrates preferentially at the G1/S transition.
INTRODUCTION

Cyclin-dependent kinases (CDKs) are central regulatory enzymes of the eukaryotic cell cycle (Morgan, 2007). In most eukaryotes, different CDK forms are specialized for driving distinct events in the cell cycle. In metazoans, these forms can differ in both the cyclin subunit and the CDK enzyme. In simpler eukaryotes such as yeasts, a single CDK enzyme associates with multiple different cyclins, which impart different functional properties to the cyclin-CDK complex. The ways in which the cyclin affects these properties are understood partly though still incompletely. For example, cyclins can affect the specific activity of the CDK enzyme, the interaction with particular substrates, or the targeting to distinct subcellular locations (Bloom and Cross, 2007; Ubersax and Ferrell, 2007).

In the budding yeast *Saccharomyces cerevisiae*, a single CDK protein (Cdc28) associates with nine different cyclins (Bloom and Cross, 2007; Enserink and Kolodner, 2010). Six B-type cyclins (Clb1-Clb6) drive DNA synthesis and mitosis (S and M phases), whereas the transition from G1 to S phase is driven by the G1 cyclin Cln3 and the G1/S cyclins Cln1 and Cln2. Although there is functional overlap, these various cyclins are clearly specialized for optimum performance of discrete tasks. Even the three semi-redundant Cln proteins show functional distinctions, as *cln3Δ* and *cln1Δ cln2Δ* cells each display unique
phenotypic defects (Cross, 1988; Moffat and Andrews, 2004; Nash et al., 1988; Skotheim et al., 2008). Interestingly, there are several CDK substrates whose phosphorylation peaks during maximum expression of Cln1/2 (McCusker et al., 2007; Oehlen and Cross, 1998; Strickfaden et al., 2007; Wu et al., 1998), and it is not clear why later cyclins are less effective; e.g., despite the fact that the M-phase cyclin Clb2 confers especially strong CDK activity (Loog and Morgan, 2005), some substrates are phosphorylated more readily by Cln2-Cdc28 than by Clb2-Cdc28. To date, no molecular mechanism explains why any particular substrate is preferentially phosphorylated in a Cln1/2-specific manner.

Cln1/2-specific substrates include proteins in the pheromone response pathway. This signaling pathway triggers a G1 phase arrest that synchronizes cells prior to mating (Bardwell, 2005; Dohlman and Thorner, 2001). In cells that have already begun the cell cycle, this pathway is transiently inactivated so that cell division can conclude (Oehlen and Cross, 1994; Wassmann and Ammerer, 1997). Three proteins in this pathway are phosphorylated at the G1/S transition: Far1 (a CDK inhibitor protein), Ste20 (a PAK-family kinase), and Ste5 (a MAP kinase cascade scaffold protein) (McKinney et al., 1993; Oehlen and Cross, 1998; Strickfaden et al., 2007; Wu et al., 1998). While the role of Ste20 phosphorylation remains unknown (Oda et al., 1999), CDK phosphorylation of Far1 and Ste5 inactivates the cell cycle arrest and signal transduction functions of the pheromone pathway, respectively (Henchoz et al., 1997; McKinney et al.,
1993; Strickfaden et al., 2007). Notably, Ste20 and Ste5 are Cln1/2-specific substrates (Oehlen and Cross, 1998; Strickfaden et al., 2007; Wu et al., 1998), but the mechanistic basis of this specificity is unknown. S-phase cyclins such as mammalian cyclin A and yeast Clb5 have a “hydrophobic patch” that allows them to recognize specific “RxL” motifs in some substrates (Cheng et al., 2006; Schulman et al., 1998; Wilmes et al., 2004). Some related examples exist for other cyclins (Dowdy et al., 1993; Kelly et al., 1998), but not for the yeast G1 or G1/S cyclins. Moreover, as with many CDK substrates, Ste20 and Ste5 are each phosphorylated at a large number of sites, and this can be important for proper regulation (Strickfaden et al., 2007).

In this study we probe the molecular basis of cyclin specificity during G1/S CDK phosphorylation in vivo. We find that both Ste5 and Ste20 have specific recognition sequences, or “docking” sites, that interact preferentially with the cyclin Cln2. In each protein, the docking sites promote efficient phosphorylation of CDK sites, and they do so in a cyclin-specific manner. Furthermore, these docking sites function largely irrespective of distance or orientation relative to the phosphorylation sites, and they are interchangeable between substrates. We identify functionally similar behavior for motifs in several other CDK substrates, suggesting that these docking sites may represent the first of many previously unrecognized recognition sequences for the G1 or G1/S cyclins in yeast.
RESULTS

Cyclin-specific phosphorylation of Ste5 and Ste20.

Pheromone responsiveness rises and falls in opposition to the periodic fluctuations in Cln1/2 levels (Oehlen and Cross, 1994; Wassmann and Ammerer, 1997). Similarly, both Ste20 and Ste5 are phosphorylated in a periodic pattern dependent on Cln1/2 cyclins (Oehlen and Cross, 1998; Strickfaden et al., 2007; Wu et al., 1998). To investigate the basis of this cyclin specificity, we asked if it is an intrinsic feature of the CDK phosphorylation sites themselves or if it involves other structural or functional features of each substrate protein. Ste5 and Ste20 are large, multi-domain proteins (>900 residues) that can be roughly divided into N-terminal regulatory/localization regions and C-terminal catalytic/signaling regions (Figure 2.1A). In each case the CDK sites are concentrated in the N-terminal regions. Using N-terminal fragments of each protein (Ste51-370, Ste51-337, Ste2080-590) as substrates, we found that cyclin specificity was maintained. First, their phosphorylation in synchronous cultures peaked at the G1/S boundary (bud emergence; Figure 2.1B) instead of at times when the M-phase cyclin Clb2 was maximal. Second, when using the inducible GAL1 promoter \( P_{GAL1} \) to drive expression of various cyclins, phosphorylation of the Ste5 and Ste20 fragments was observed only with Cln1 and Cln2, and not with Cln3, Clb5, or Clb2 (Figure 2.1C). (Although unequal levels of cyclins could contribute to these differences, Clb2 was able to drive phosphorylation of the CDK substrate Swe1 [Figure 2.1C] (Harvey et al., 2005), and later results will show activity for both Clb5 and Clb2 in
Figure 2.1. Cyclin specific phosphorylation of Ste5 and Ste20 regulatory domains

(A) Domain structures of Ste5 and Ste20. Red circles indicate CDK sites (Oda et al., 1999; Strickfaden et al., 2007); in Ste20, only the 13 confirmed sites (of 23 possible) are shown.

(B) Phosphorylation of Ste5 and Ste20 fragments in synchronous cdc15-2 cultures, after release from M phase arrest. Full-length Ste20 is shown for comparison; its phosphorylation behavior was described previously (Oehlen and Cross, 1998; Wu et al., 1998). Cell cycle progression was monitored by anti-Clb2 immunoblot and by budding; representative examples are shown.

(C) HA-tagged Ste5 or V5-tagged Ste20 fragments, expressed from native promoters, were monitored after galactose-induced expression of GST-tagged cyclins. Reduced electrophoretic mobility signifies phosphorylation, as confirmed by phosphatase treatment (Appendix Figure 1). For comparison, V5-tagged Swe1 demonstrates activity for Clb2. The relative expression levels for GST-cyclins were highly reproducible; one representative example is shown. A variant of Cln3 lacking ten CDK sites (Cln3^{10A}) was used to increase its stability. Results were similar in sic1Δ cells (Appendix Figure 2), in which the Clb-CDK inhibitor Sic1 is absent.
Figure 2.1. Cyclin specific phosphorylation of Ste5 and Ste20 regulatory domains

A

B

time after cdc15-2\textsuperscript{th} release (min.)

C

\( P_{\text{GAL1-} \text{GST}} \)-cyclin:

- vector
- Clb2
- Clb1
- Clb1\textsuperscript{vA}
- Clb5
- Clb2

- vector
- Clb2
- Clb1
- Clb1\textsuperscript{vA}
- Clb5
- Clb2

GST
similar assays). Thus, stage-specific and cyclin-specific phosphorylation of Ste5 and Ste20 does not require their signaling functions, and the determinants of specificity must lie within their N-terminal fragments.

**A distal recognition sequence promotes CDK phosphorylation of the Ste5 N-terminus.**

We used the Ste5$^{1-337}$ fragment to study the requirements for phosphorylation by Cln2-Cdc28. As expected, phosphorylation required the same 8 CDK sites shown previously to regulate full-length Ste5 (Figure 2.2B) (Strickfaden et al., 2007). Nevertheless, these sites were not sufficient to ensure efficient phosphorylation. By making further truncations, we discovered that phosphorylation required a short stretch of sequence (276-283) far away from the CDK sites (Figure 2.2B). Similarly, alanine replacement of four residues within this region (LLPP) also disrupted phosphorylation (Figure 2.2B). In contrast, internal deletions showed that several other large segments of the Ste5 N-terminus were dispensable (Figure 2.2B). We conclude that efficient Cln2-Cdc28 phosphorylation of Ste5 requires specific sequences that are separate from the phosphorylation sites themselves. For now we tentatively refer to this required region as a Cln2 docking site.

This putative Cln2 docking site lies within a larger inter-domain region of Ste5 with several notable features (Figure 2.3A), including: (i) a MAPK binding site; (ii) four MAPK phosphorylation sites, which possibly could also act as CDK sites due
Figure 2.2. A distal docking motif promotes Cln2-Cdc28 phosphorylation of Ste5

(A) Locations of key Ste5 features and mutations. Residues 275-283 contain the putative Cln2 docking motif required for efficient phosphorylation of the N-terminal CDK sites. Mutations used in later panels are indicated; see Figure 2.3A for details.

(B) Phosphorylation of the Ste5 N-terminus (Ste5\textsuperscript{1-337}) requires both the CDK sites and a distal LLPP motif between residues 275 and 283. Phosphorylation was triggered by galactose-induced expression of a $P_{\text{GAL1}}$-CLN2 construct (+) or a vector control (-).

(C) The role of the LLPP motif is independent of MAPKs ($fus3\Delta kss1\Delta$), the phosphatase Ptc1 ($ptc1\Delta$), and the MAPK binding site (ND mutant). Results show the Ste5\textsuperscript{1-337} fragment except as indicated otherwise. The $fus3\Delta kss1\Delta$ strain (PPY1173) was tested in parallel with a congenic wild-type strain (PPY640). LLPP function also does not require the MAPK phosphorylation sites, but non-phosphorylatable mutations at these sites (4AV) mildly reduce the extent of Cln2-Cdc28 phosphorylation. Also see Figure 2.3B,C.

(D) Cln2-driven phosphorylation of full-length Ste5 (V5-tagged) requires the LLPP motif. For the 8A lanes, a longer exposure (of the same blot) is shown to compensate for imperfect loading.
(E) Mutation of the LLPP motif disrupts the ability of Cln2 to inhibit pheromone signaling. Fus3 phosphorylation was monitored in ste5Δ fus3Δ kss1Δ strains (± P_{GAL1-CLN2}) harboring STE5 variants and wild-type FUS3 on plasmids.

(F) The LLPP motif mediates regulation by Cln2 in the absence of Fus3-Ste5 binding (Ste5 ND mutant) and Fus3 kinase activity (fus3-K42R mutant). Strains (as in panel D) harbored plasmids with the indicated forms of STE5 and FUS3.
Figure 2.2. A distal docking motif promotes Cln2-Cdc28 phosphorylation of Ste5

A  YSPPLLPPP  283
Ste5  PH  VWA
HHH  8A  LLPP
HHH  ND
HHH  4AV
HHH  4E
HHH  Cdk phosphorylation sites
HHH  Cln2 & Ptc1 docking
HHH  MAPK docking
HHH  blocks MAPK phosphorylation
HHH  mimics MAPK phosphorylation

B  P_GAL1 CLN2:
wt  8A  1-337  1-315  1-300  1-283  1-275  1-262
-  -  +  -  -  -  -

P_GAL1 CLN2:
wt  LLPP  8A  LLPP  124  -151  -173  -229  -239
-  -  -  -  -  -  -  -

C  P_GAL1 CLN2:
wild type  fus3-K42R  1-283  1-283  ptc1A
wt  LLPP  wt  LLPP  wt  LLPP  1-315  1-275
-  -  -  -  -  -  -  -

P_GAL1 CLN2:
ND  ND  LLPP  wt  LLPP  4AV  4AV  4E  4E  LLPP
-  -  -  -  -  -  -  -

D  P_GAL1 CLN2:
Ste5 allele:  8A  wt  LLPP  ND  ND  LLPP
full-length Ste5
-  -  -  -  -  -  -  -

E  Ste5 allele:  P_GAL1 CLN2:
0  5  15  30  45  60
-  -  -  -  -  -

α factor (min.)
wt  -
wt  +
8A  +
LLPP  +

phospho-Fus3  total Fus3

F  Ste5 allele:  P_GAL1 CLN2:
FUS3-wt  fus3-K42R
FUS3-wt  fus3-K42R
-  +  +  +  +  +  +  +

+ α factor, 15 min.

phospho-Fus3  total Fus3
to similar target sequences (i.e., SP/TP); and (iii) a binding site for the phosphatase Ptc1 (Bhattacharyya et al., 2006; Malleshaiah et al., 2010). None of these features seemed crucial for docking site function. MAPK binding played no evident role, as the results were unaffected by deleting MAPK genes ($fus3\Delta$ $kss1\Delta$) or by mutating the MAPK binding site (ND mutant) (Figure 2.2C, 2.3B). At the MAPK phosphorylation sites, phospho-mimetic mutations (4E) had no effect (Figure 2.2C), but non-phosphorylatable mutations (4AV) caused a mild reduction; thus, phosphorylation at one or more of these sites (e.g., by a MAPK or CDK) may enhance further CDK phosphorylation elsewhere, or the mutations may mildly disrupt recognition of the docking motif. (All four MAPK sites cannot be required, because the shorter Ste5$^{1-283}$ fragment retains only two sites yet remains a good substrate; Figure 2.2B,C). Finally, although the required LLPP motif overlaps a binding site for Ptc1 (Malleshaiah et al., 2010), it promoted CDK phosphorylation identically in $ptc1\Delta$ cells (Figure 2.2C, 2.3C). Altogether, these data show that recognition of the putative Cln2 docking site can be separated from MAPK and Ptc1 binding, though it may be affected by either the sequence or phosphorylation status of adjacent SP/TP sites. Furthermore, as with the N-terminal fragments, the LLPP motif was also required for CDK phosphorylation of full-length Ste5 (Figure 2.2D).

Next, we tested if the Cln2 docking site is required for CDK inhibition of Ste5 signaling (Strickfaden et al., 2007). Indeed, as with mutation of the CDK sites
Figure 2.3. The Cln2 docking region in Ste5, and requirements for CDK phosphorylation and regulation

(A) Expansion of Ste5 residues 258-337, highlighting the following features: (i) a MAPK binding site (denoted “K” in the schematic above); (ii) four MAPK phosphorylation sites; and (iii) a Ptc1 binding site. Note that the putative Cln2 docking motif identified in this study (LLPP) overlaps the sequence previously implicated in Ptc1 binding (Malleshaiah et al., 2010). Also indicated are the mutations used in Figure 2.2. The ND mutation disrupts MAPK binding (Bhattacharyya et al., 2006), while 4AV and 4E are non-phosphorylatable and phospho-mimetic mutations that also have opposing effects on MAPK binding (Malleshaiah et al., 2010).

(B) The ND and ND/LLPP mutant results from Figure 2.2C are shown here in an expanded form that includes additional controls (wt and LLPP) run alongside on the same gel. Also included on this gel were the wt vs. 8A mutant results that are shown in Figure 2.2B.

(C) The results using the ptc1Δ mutant strain that are shown in Figure 2.2C are shown here in parallel with the same fragments analyzed in a wild type strain, conducted in parallel. Note that both wild type and ptc1Δ strains show the same effects of both truncations (e.g., compare 1-315 vs. 1-275) and point mutations (e.g., compare wt vs. LLPP versions of 1-283). Thus, despite its overlap with the Ptc1 binding site, the LLPP motif controls Cln2-Cdc28 phosphorylation independent of Ptc1.
(D) The ability of \( P_{GAL1}^{\text{CLN2}} \) to inhibit pheromone response is disrupted in a synergistic way by Ste5 mutations in the LLPP motif and the MAPK binding site (ND). These experiments are related to Figure 2.2E-F, but here pheromone response was measured using a transcriptional reporter (\( FUS1^{\text{lacZ}} \)) in either \( FUS3^{\text{KSS1}} \) strains or \( \text{fus3}^{\Delta}\text{kss1}^{\Delta} \) strains with a FUS3-wt plasmid (as in Figure 2.2E-F). Bars, mean ± SD (n = 8 [top] or 3 [others]). As shown in Figure 2.2F, when the kinase activity of Fus3 is eliminated, the ability of Cln2 to inhibit pheromone response is fully dependent on the LLPP motif. But when Fus3 is active the behavior is more complex. This is likely because the LLPP motif also binds Ptc1, which antagonizes Ste5-Fus3 association and limits Fus3 mediated negative feedback (Malleshaiah et al., 2010). Consequently, while the LLPP mutation disrupts inhibition by Cln2, it simultaneously increases Fus3-Ste5 binding and inhibition, which may have a compensatory effect. Hence, the impact of the LLPP mutation on regulation by Cln2 is most clearly revealed in three distinct settings: (i) immediately after pheromone stimulation, before negative feedback kicks in (Figure 2.2E); (ii) when using a kinase-dead form of Fus3 (Figure 2.2F); and (iii) when using the ND mutation to disrupt Fus3-Ste5 binding (Figure 2.2F and this Figure). Finally, because the LLPP mutation seems more effective at restoring Fus3 phosphorylation than transcriptional induction, the transcriptional response may require sustained signaling or it may have a non-linear dependence on Fus3 activation.
Figure 2.3. The Cln2 docking region in Ste5, and requirements for CDK phosphorylation and regulation

A

Ste5

\[\text{Cln2 dock & Ptc1 binding}\]

\[\text{MAPK binding}\]

KIPOSELTSQSRFPY\text{LLPP} GLSYSVP\text{LLPP} wt

KIPOSELTSQSRFPY\text{LLPP} GLSYSVP\text{LLPP} wt

KIPOSELTSQSRFPY\text{LLPP} GLSYSVP\text{LLPP} wt

KIPOSELTSQSRFPY\text{LLPP} GLSYSVP\text{LLPP} wt

KIPOSELTSQSRFPY\text{LLPP} GLSYSVP\text{LLPP} wt

KIPOSELTSQSRFPY\text{LLPP} GLSYSVP\text{LLPP} wt

MAPK phosphorylation sites

B

Ste5 1-337

\[\text{PGAL1-CLN2}\]

\[+\]

\[-\]

\[\text{wt}\]

\[8A\]

\[\text{LLPP}\]

\[\text{ND}\]

\[\text{ND}\]

C

\[\text{P}_{\text{GAL1-CLN2}}\]

\[\text{wt}\]

\[1-283\]

\[1-283\]

\[1-315\]

\[1-275\]

\[\text{LLPP}\]

\[\text{ND}\]

\[\text{LLPP}\]

D

FUS3 KSS1

120 min. α factor

FUS3 kss1Δ

120 min. α factor

FUS3 kss1Δ

30 min. α factor

FUS3 transcriptional response

\(\text{P}_{\text{GAL1-CLN2}}\)

\(\text{wt}\)

\(8A\)

\(\text{LLPP}\)

\(\text{ND}\)

\(\text{ND}\)

\(\text{LLPP}\)
mutation of the LLPP motif restored the ability of pheromone to activate the MAPK Fus3 in $P_{GAL1}$-$CLN2$ cells (Figure 2.2E). However, this restored activation was unusually transient, and the transcriptional response was only partially restored (Figure 2.3D). This complex behavior likely reflects the additional role of the LLPP region in binding to Ptc1, which antagonizes the ability of Fus3 to bind Ste5 and down-regulate signaling (Malleshaiah et al., 2010). That is, although the LLPP mutation disrupts Cln2 docking, it simultaneously leads to excessive binding and inhibition by Fus3, which may have a compensatory effect. To eliminate contributions from Fus3, we used the Ste5 ND mutation to disrupt Fus3 binding (Bhattacharyya et al., 2006), or a kinase-inactive form of Fus3 (K42R). In these contexts, the LLPP mutation clearly blocked the inhibitory effect of Cln2 (Figures 2.2F, 2.3D). Therefore, the LLPP motif can mediate CDK inhibition in the absence of both Fus3 binding and kinase activity, whereas excess Fus3 activity may obscure this role. Although the overlap between Cln2 and Ptc1 sites creates an added layer of complexity, overall these results establish that CDK regulation of Ste5 involves both CDK phosphorylation sites and a separate Cln2 docking motif.

**Cyclin-specific binding sites in Ste20 and Ste5.**

Efforts to test if Ste5 binds Cln2 were hampered by technical issues (e.g., non-specific precipitation), but binding of Ste20 to Cln2 is readily detectable in cell extracts (Archambault et al., 2004; Oda et al., 1999). Therefore, we searched for
the responsible sequences in Ste20 using GST-Ste20 and Cln2-myc\textsubscript{13} fusions. An N-terminal fragment (Ste20\textsuperscript{1-333}) was sufficient to bind Cln2, and this required residues 73-119 (Figure 2.4B). The C-terminal end of this fragment, which harbors a membrane-binding “basic-rich” (BR) domain, was not required but it did enhance binding (Figure 2.5B); hence, further analyses kept the C-terminus fixed at position 333. The required region (73-119) includes a conserved block of \(~\text{20}\) residues (Figure 2.5C), and mutating consecutive sets of residues in this region revealed that an eight-residue stretch (SLDDPIQF) was critical for binding to Cln2 (Figure 2.4Ci). We then asked if the role of this Ste20 motif could be replaced with a small fragment from Ste5 that harbors its putative Cln2 docking site. Indeed, the Ste5 site mediated Cln2 binding, and this required the same LLPP sequence that promotes Ste5 phosphorylation (Figure 2.4Cii).

Furthermore, each sequence preferentially bound to Cln2 over other cyclins (Figure 2.4D), and weakly to Cln1. Collectively, these experiments identify docking sites in both Ste20 and Ste5 that can discriminate among different cyclins to mediate specific binding. The two sites are not highly similar but each contains the motif LxxP\(\Phi\)x\(\Phi\) (where \(\Phi\) is hydrophobic), raising the possibility that a degenerate pattern of hydrophobic side chains forms the Cln2 recognition motif.
Figure 2.4. Cyclin-specific binding by docking motifs in Ste20 and Ste5

(A) The diagram indicates endpoints used for mapping the Cln2-binding region in Ste20, which is outlined in red.

(B) Cells co-expressing GST fusions to Ste20 fragments and Cln2-myc\textsubscript{13} were lysed, and complexes were recovered using glutathione sepharose. Input (5%) and bound proteins were analyzed by anti-myc and anti-GST blots.

(C) Starting with a Ste20\textsuperscript{72-333} fragment, alanine substitutions were made at blocks of residues in the 72-118 region (see Figure 2.5C; numbering starts at 2 because additional flanking mutations were used in other assays). Cln2 binding was tested as in panel B. Separately, the required Ste20 region was replaced by a Ste5\textsuperscript{263-335} fragment (ii), in both wt and LLPP mutant forms, to test the ability of this Ste5 sequence to mediate Cln2 binding.

(D) Ste20 and Ste5 docking sites show cyclin-specific binding. GST alone (-) or GST fusions (+) were used to co-precipitate myc\textsubscript{13}-tagged cyclins (expressed from the CYC1 promoter) in yeast lysates. The GST fusions were to Ste20\textsuperscript{1-333} (Ste20 motif) or to the Ste5\textsuperscript{263-335}-Ste20\textsuperscript{120-333} chimera used in panel Cii (Ste5 motif). Cln3\textsuperscript{10A} showed varying levels of non-specific precipitation but no reproducible binding to either GST fusion.

(E) Cln2-induced phosphorylation was assayed for V5-tagged forms of full-length Ste20 (1-939) or N-terminal fragments (80-590, 80-500), with or without mutations in the docking site (mut3) or the 13 confirmed CDK sites (CDK\textsuperscript{*}).
The Cln2 docking site from Ste20 can drive phosphorylation of a heterologous substrate. Phosphorylation was analyzed using Ste5\(^{1-283}\) (i), Ste5\(^{1-260}\) (ii), and wt or mut3 versions of the Ste20 docking site (residues 80-115) fused to Ste5\(^{1-260}\) (iii).
Figure 2.4. Cyclin-specific binding by docking motifs in Ste20 and Ste5
The Cln2 docking site in Ste20 enhances phosphorylation.

Ste20 contains 23 potential CDK sites, of which at least 13 are used in vivo (Oda et al., 1999). To test if Cln2 docking affects Ste20 phosphorylation, we compared mutations in either the docking site (mut3) or the 13 confirmed CDK sites (CDK*), using full-length Ste20 and several N-terminal fragments (Figure 2.4E). In full-length Ste20 (1-939), the docking site mutation reduced the magnitude of the Cln2-induced mobility shift, but did not eliminate it (i.e., unlike the CDK* mutation). This partial phenotype could signify less efficient use of all sites, or a specific defect at particular sites due to their position or sequence context; in this regard, it is noteworthy that 9 of the 13 confirmed CDK sites are “minimal” sites (S/T-P) whereas only 4 are “consensus” sites (S/T-P-x-K/R). The docking site mutation again caused a partial phenotype for the largest N-terminal fragment (80-590), but it caused a strong disruptive phenotype for smaller fragments such as 80-500 (Figure 2.4E) and 80-333 (Appendix Figure 3). While we did not parse these differences further, we note that the two smaller fragments retain only one consensus CDK site, raising the possibility that docking is especially important for phosphorylating minimal sites. As a separate test of the activity of the Ste20 docking motif, we asked if it could substitute for the analogous site in Ste5. Indeed, the Ste20 motif restored strong phosphorylation to the Ste5^{1-260} fragment, and this activity was blocked by mutations that disrupt Cln2 binding (Figure 2.4Fiii). Thus, the Ste20 docking motif can stimulate Cln2- Cdc28 phosphorylation of native sites in Ste20 or sites
Figure 2.5. Ste20-Cln2 binding and conservation of the Cln2 docking site

(A) Diagram of Ste20 showing the location of the Cln2 docking site outlined in red.

(B) Effect of the Ste20 BR domain on binding between GST-Ste20 fragments and Cln2-myc13. Binding assays were conducted as in Figure 2.4B. The Ste201-254 fragment lacks the BR domain, whereas the BR* derivative has mutations at eight basic residues that provide membrane affinity (Takahashi and Pryciak, 2007). Though the BR domain was not required for Cln2 binding, it did enhance both total binding and capture of the slowest migrating Cln2 species. This could imply some subcellular segregation of different Cln2 phosphoisoforms, or that the polybasic BR domain contributes extra affinity for poly-phosphorylated forms of Cln2. Hence, for simplicity, subsequent analyses kept the C-terminus fixed at position 333.

(C) Conservation of sequences encompassing the Cln2 docking site. The S. cerevisiae Ste20 sequence (at top) was aligned to orthologs from ten other yeasts. Red and blue highlights indicate the most conserved residues. At bottom are residues 80-109 (S. cerevisiae) with brackets indicating blocks of residues replaced with alanines, as used in Figures 2.4C and 2.6C.
Figure 2.5. Ste20-Cln2 binding and conservation of the Cln2 docking site

A

Ste20

1 120 254 333

B

GST-Ste20:

Cln2-myc:

+m + +

myc bound

GST

C

Ste20

kinase
in a heterologous substrate.

**Re-wiring a CDK regulatory circuit via a Ste20^{Ste5PM} chimera.**

CDK phosphorylation of Ste5 serves to inhibit pheromone response at the G1/S transition (Strickfaden et al., 2007). We wished to explore whether the responsible regulatory mechanism could be moved to other proteins, and whether such synthetic approaches (Lim, 2010; Pryciak, 2009) could help further probe the factors controlling CDK regulation. Therefore, we attempted to transfer the regulatory effects of CDK phosphorylation from Ste5 onto Ste20. Our rationale stems from the fact that each protein requires a short membrane-binding motif for its plasma membrane localization and signaling activity (Takahashi and Pryciak, 2007; Winters et al., 2005), and the function of the Ste5 motif, termed the PM domain, is inhibited by phosphorylation at adjacent CDK sites (Strickfaden et al., 2007). Thus, we replaced the membrane-binding (BR) motif in Ste20 with Ste5 fragments that contain both the PM domain and its flanking CDK sites (Figure 2.6A), and then asked if Ste20 now could be inhibited by CDK phosphorylation.

The Ste5 PM domain was a highly effective substitute for the Ste20 BR domain (Figure 2.6B), irrespective of the precise size of the transferred Ste5 fragment. As hypothesized, the function of these Ste20^{Ste5PM} chimeras in the
**Figure 2.6. Re-wiring a CDK regulatory circuit with a Ste20\textsuperscript{Ste5PM} chimera**

(A) The Ste20\textsuperscript{Ste5PM} chimeras. Ste20 residues 124-311, containing the membrane-binding BR domain, were replaced with three fragments from Ste5 that include its membrane-binding PM domain plus 7 or 8 flanking CDK sites.

(B) Pheromone signaling by Ste20\textsuperscript{Ste5PM} chimeras is inhibited by Cln2. Because Cln2-CDK normally inhibits signaling via Ste5, these tests used cells with a non-phosphorylatable Ste5 variant (ste20\textsuperscript{Δ} STE5-8A). Wild-type Ste20 (wt) or Ste20\textsuperscript{Ste5PM} chimeras (from panel A) were introduced on plasmids, and pheromone response was measured using a transcriptional reporter (\textit{FUS1}-\textit{lacZ}). Signaling by all three chimeras (#A, #B, #C) was inhibited by Cln2, but this was blocked by mutations in the CDK phosphorylation sites (7A, 8A). Deletions of the Ste20 N-terminus, made in the #A chimera, show that residues 87-119 are required for regulation by Cln2. Bars, mean ± SD (n = 3).

(C) Sequences required for regulation by Cln2 were analyzed using a chimera similar to #A, containing only Ste20 residues 80-109 upstream of Ste5 1-85 (see Figure 2.7C). Alanine mutations replaced eight blocks of residues (left) or individual residues in the SLDDPIQF motif (right). These were compared to an un-mutated sequence (wt) and a chimera that lacks the sequence entirely (-). Signaling was assayed as in panel B. Bars, mean ± SD (n = 3).

(D) Docking sites from Ste20 (80-115) or Ste5 (257-330) were inserted at different positions (i-iv) into a variant of chimera #A that lacks residues 87-119.
(see panel B). Insertions at position ii also removed residues 1-86. Signaling was assayed as above. Bars, mean ± SD (n = 3).

(E) Bud tip localization of Ste20<sup>Ste5PM</sup> chimera in cycling cells is inhibited via the CDK sites (7A) and the Cln2 docking site (mut3). Strain BY4741 harbored GFP-Ste20 plasmids. Representative images show unfixed cells (left); localization was quantified after formaldehyde fixation (right). Bars, mean ± SD (n = 3 experiments; >150 cells per allele per experiment).

(F) The growth function of Ste20 is inhibited in the Ste20<sup>Ste5PM</sup> chimera, if CDK and docking sites are intact. Serial (1/5x) dilutions of strain KBY211 harboring the indicated Ste20 plasmids were spotted onto plates, and incubated at 25˚ or 36˚ C for 4 days. As a control, the ∆BR allele removes the BR domain in full-length Ste20 (Takahashi and Pryciak, 2007).
Figure 2.6. Re-wiring a CDK regulatory circuit with a Ste20<sup>Ste5PM</sup> chimera
pheromone response pathway was inhibited by expression of Cln2 (Figure 2.6B). (Because Cln2 ordinarily inhibits pheromone response via Ste5, these assays used cells harboring a CDK-resistant derivative, Ste5-8A.) Importantly, this inhibition specifically required intact CDK sites flanking the PM domain (Figure 2.6B), despite the presence of other CDK sites elsewhere in Ste20. Moreover, the inhibition was cyclin-specific and was not accompanied by any reduction in protein levels (Figures 2.7A,B). Thus, these experiments show that the CDK regulatory target in the pheromone pathway can be switched to a different protein, and that the CDK-inhibited domain from Ste5 constitutes a portable regulatory module. Furthermore, inhibition of the Ste20\textsuperscript{Ste5PM} chimera required the region of Ste20 (residues 87-119) that contains its Cln2 docking site (Figure 2.6B, right). This finding establishes two further points: (i) the mere presence of CDK sites in the transferred Ste5 fragment is insufficient to make it an effective target of CDK down-regulation; and (ii) the Cln2 docking site in Ste20 can control functional regulation via CDK phosphorylation.

We used the Ste20\textsuperscript{Ste5PM} chimera to dissect which docking site residues were required for regulatory activity. This function was fully contained in a 30-residue region (80-109) spanning the conserved stretch noted earlier (Figure 2.7C), and it required the same 8-residue motif involved in Cln2 binding (SLDDPIQF) (Figure 2.6C, left). Notably, no single residue in this motif was absolutely required, though partial phenotypes were seen for mutations at the L, P, and F residues
Figure 2.7. Ste20Ste5PM chimeras: cyclin specificity, protein levels, and minimal motif

(A) Inhibition of Ste20Ste5PM signaling is cyclin specific. A ste20Δ STE5-8A strain (PPY2142) was co-transformed with plasmids expressing a Ste20Ste5PM chimera (see Figure 2.6A) and a galactose inducible cyclin construct (or a vector control). Pheromone response was assayed as in Figure 2.6B. Signaling was inhibited strongly by Cln2 and partially by Cln1, each of which was blocked by mutating the CDK sites (7A). Signaling was unaffected by Cln310A or Clb5. Expression of Clb2 inhibited signaling regardless of the CDK sites, and hence this is most likely due to pleiotropic effects on cell physiology resulting from prolonged high levels of Clb2 produced by this construct (which is eventually lethal). Bars, mean ± SD (n = 4).

(B) Protein levels of Ste20Ste5PM chimeras are not affected under conditions in which pheromone signaling is inhibited. Top, the same strains, plasmids, and treatment conditions used to analyze signaling in Figure 2.6B were used to prepare cell extracts for protein analysis by immunoblotting. Equal amounts of protein were loaded in each case (20 µg/lane). Note that in no case does P_{GAL1-CLN2} cause a reduction in protein levels. Also note that in the absence of the PM-proximal CDK sites (7A), the chimera is still phosphorylated at native sites in Ste20 and yet signaling is not inhibited (see Figure 2.6B); this demonstrates that down-regulation is not achieved by phosphorylation elsewhere in the protein but instead specifically requires that phosphates are added adjacent to the PM
domain. *Bottom*, a similar analysis was performed using myc\textsubscript{13}-tagged chimeras, showing results analogous to the GFP-tagged chimeras.

(C) Deletions and truncations were used to show that Ste20 residues 80-109 constitute a minimal motif sufficient to mediate Cln2 inhibition of the chimeras. Signaling was assayed as in Figure 2.6B. Bars, mean ± SD (n = 3-4).
Figure 2.7. Ste20\textsuperscript{Ste5PM} chimeras: cyclin specificity, protein levels, and minimal motif
(Figure 2.6C, right). Thus, Cln2 docking may involve contacts distributed throughout the motif. We also found that docking site function is highly flexible, as it remained active when placed on either side of the PM domain and up to ~280 residues from the nearest CDK site (Figure 2.6D). Moreover, the docking site from Ste5 could also function in the chimera (Figure 2.6D, right). Finally, additional tests showed that the CDK regulation conferred upon the Ste20\textsuperscript{Ste5PM} chimera is not limited to pheromone response or to over-expressed Cln2. Specifically, Ste20 performs additional functions in cycling cells, and its ability to localize to growing bud tips, or to sustain viability in the absence of the related PAK Cla4, requires plasma membrane contact (Leberer et al., 1997; Peter et al., 1996; Takahashi and Pryciak, 2007). These abilities were inhibited in the Ste20\textsuperscript{Ste5PM} chimera in a manner that required both docking and CDK sites (Figure 2.6E, F). Hence, the chimera converts Ste20 from a multi-functional kinase into a form that is restricted to functioning in non-cycling cells. Altogether, the results clearly reveal the feasibility of creating new CDK regulatory circuits via a combination of Cln2 docking and CDK phosphorylation sites, and they establish the Ste20\textsuperscript{Ste5PM} chimera as a functionally flexible platform with which to assay docking site activity.

**Functional Cln2 docking sites in other CDK substrates.**

To assess whether the Cln2 docking behavior seen with Ste5 and Ste20 might be more widespread, we searched for similar sites in other CDK substrates.
Matches to the LxxPΦxΦ motif or the core Ste5 site (LLPP) were too numerous for a proteome-wide analysis. Nevertheless, we scanned the sequences of known Cln1/2-Cdc28 targets and Cln2 binding partners, plus Cdc28 substrates found in large-scale screens (Archambault et al., 2004; Enserink and Kolodner, 2010; Holt et al., 2009; Ubersax et al., 2003), for Leu/Pro-rich sequence motifs that are conserved among fungal orthologs and that lie outside of known or predicted structural domains. Although not comprehensive, as an initial test case we chose seven such sequences (from Sic1, Whi5, Srl3, Bem3, Tus1, Exo84, and Pea2; see Figure 2.9), including one recently implicated in Cln2-Cdc28 phosphorylation of Sic1 (Koivomagi et al., 2011b). We then tested these sequences for their ability to functionally substitute for the Ste5/Ste20 docking sites, using two experimental settings: (i) Cln2 inhibition of signaling by the Ste20<sup>Ste5PM</sup> chimera; and (ii) Cln2-driven phosphorylation of the Ste5 N-terminus.

Remarkably, the majority of these sequences had measurable activity. In the signaling assays (Figure 2.8A), they mediated Cln2 inhibition to varying degrees, from strong (Sic1, Whi5, Exo84) to moderate (Tus1, Pea2) to weak (Bem3); only the Srl3 sequence was ineffective. Importantly, their inhibitory effects were a specific response to Cln2 expression, and no non-specific effects were seen. The three most potent sequences contain exact matches to the Ste5 LLPP motif (Figure 2.9B), though it is notable that each was in fact more potent than the Ste5 sequence in these assays (Figure 2.8A); thus, additional context features
Figure 2.8. Identification of additional candidate Cln2 docking sites

(A) Candidate Cln2 docking sites from seven CDK substrates (see Figure 2.9) were inserted into a Ste20<sup>Ste5PM</sup> chimera lacking the endogenous docking site. These derivatives were compared to chimeras containing the Ste5 or Ste20 docking sites inserted at the same position, or no docking site (none). Cln2 inhibition of pheromone response was assayed as in Figure 2.6. Bars, mean ± SD (n = 5).

(B) The same candidate docking sites used in panel A were inserted at the end of a Ste5 fragment (Ste5<sup>1-260</sup>) that lacks its endogenous docking site, and Cln2-driven phosphorylation was assayed. See Figure 2.9C for additional repetitions.
Figure 2.8. Identification of additional candidate Cln2 docking sites

A

candidate docking sites

\[ \text{myc} \downarrow \text{PM} \downarrow \text{RING} \]

\[ \text{kinase} \]

\[ \begin{array}{c}
\text{pheomone response (\%)} \\
0 \quad 20 \quad 40 \quad 60 \quad 80 \quad 100 \quad 120 \quad 140
\end{array} \]

\[ \begin{array}{c}
\text{insert:} \\
\text{none} \quad \text{Ste5} \quad \text{Ste20} \quad \text{Ste1} \quad \text{Whi5} \quad \text{Sr1} \quad \text{Ben3} \quad \text{Tus1} \quad \text{Exo84} \quad \text{Pep2}
\end{array} \]

B

\[ \begin{array}{c}
\text{Ste5 1-337} \\
\text{Ste5 1-260 + candidate dock}
\end{array} \]

\[ \begin{array}{c}
P_{GAL1}^{\text{CLN2}}: \\
\text{WL} \quad \text{LLPP} \quad \text{Ste20} \quad \text{Ste1} \quad \text{Whi5} \quad \text{Sr1} \quad \text{Ben3} \quad \text{Tus1} \quad \text{Exo84} \quad \text{Pep2}
\end{array} \]

\[ \begin{array}{c}
\text{short exp.} \\
\text{long exp.}
\end{array} \]
likely influence the efficacy of this motif. Similarly, the Pea2 sequence closely resembles the motif in Ste20, and yet was less potent. The phosphorylation assays yielded similar overall results, with a range of activity (Figures 2.8B and 2.9C). The relative activities in the two assays were generally correlated, but there were some discrepancies; for example, the Whi5 and Pea2 sequences were each weaker in the phosphorylation assay than the sequences with which they showed comparable potency in the signaling assay. Together, these results clearly establish the utility of each assay for their ability to rapidly evaluate multiple candidate Cln2 docking sites, and to compare efficacy in parallel. Overall, given the large fraction of sequences that were effective, the findings suggest that Cln2 recognition sites of various strengths may be quite prevalent among Cln-CDK targets.

The phosphorylation role of docking sites is cyclin-specific

Finally, we tested whether the Cln2 docking sites enhance phosphorylation by all forms of cyclin-CDK or only by specific forms. We used the Ste5\(^{1-260}\) fragment as a starting substrate, and monitored its phosphorylation by inducing expression of different cyclins in asynchronous cells (Figure 2.10A). In the absence of any docking site, none of the cyclins drove appreciable phosphorylation. When Cln2 docking sites from Ste5, Ste20, or Exo84 were appended to this substrate, phosphorylation was enhanced in a manner that was clearly cyclin-specific. Cln2 was generally most effective, followed by Cln1. (With the Ste20 site, Clb2 could
Figure 2.9. Sources of candidate Cln2 docking sites from other CDK substrates

(A) For each protein, its structural or functional domains, possible CDK phosphorylation sites, and candidate Cln2 docking motifs are diagrammed. Sequence alignments show the extent of conservation (among fungal orthologs) of the tested regions. Note that Srl3, which is a Cln2 binding protein (Archambault et al., 2004), is a paralog of Whi5; but because the candidate Cln2 docking site in Whi5 is less well conserved in Srl3, we tested a Leu/Pro-rich sequence further downstream. Also note that there are several Cln1/2-Cdc28 targets involved in cell polarization (Rga1, Rga2, Bem2, Bem3, Tus1, Cdc24, Boi1, Boi2) (Archambault et al., 2004; Breitkreutz et al., 2010; Knaus et al., 2007; Kono et al., 2008; McCusker et al., 2007; Sopko et al., 2007; Wai et al., 2009), which we considered good candidates for harboring possible Cln2 docking sites. The large size of some of these proteins made it difficult to identify candidate sequences for our initial survey; hence, we chose two examples (Bem3 and Tus1) in which single regions stood out. Subsequent studies will comprehensively test multiple sequences from such proteins.

(B) Sequences chosen to test for Cln2 docking activity (in Figure 2.8), with red highlighting of the motifs that are most similar to those characterized in Ste5 and Ste20.

(C) Two additional repetitions of the experiments shown in Figure 2.8B are shown here alongside those presented in Figure 2.8B (shown here as repeat #3).
Note the consistency in the extent of phosphorylation, indicating that the relative efficacy of each site is a reproducible feature. Some of the weaker sites (Bem3, Pea2) gave effects that were more detectable in some repeats than others, whereas the Srl3 site was ineffective in all cases.
Figure 2.9. Sources of candidate Cln2 docking sites from other CDK substrates

A

<table>
<thead>
<tr>
<th>Protein</th>
<th>Domain</th>
<th>Cln2 Site</th>
<th>Candidate Docking Motif</th>
</tr>
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<tbody>
<tr>
<td>Sic1</td>
<td>1</td>
<td>121-150</td>
<td>QNVODIAEEEEELEGVLPPP5RPTSARQLH</td>
</tr>
<tr>
<td>Whi5</td>
<td>1</td>
<td>121-150</td>
<td>EEEHEQKXYDHGVMPLPPP7TP55RSE</td>
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<tr>
<td>Sr3</td>
<td>1</td>
<td>135-164</td>
<td>KLSKHEHVAL1PPPPFTEIKRGSB5HFA</td>
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<tr>
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<td>1</td>
<td>99-131</td>
<td>DGRSGY9NDQGWPVLLPPP8A5ERKA5IKSLMLP5PT</td>
</tr>
<tr>
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<tr>
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</tr>
<tr>
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<td>261-290</td>
<td>D56EL55T55P55Q555F55P55Y555PP55F65P55G55L55T55Y55PVE</td>
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</table>

B

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</tr>
<tr>
<td>Ste5</td>
<td>1</td>
<td>261-290</td>
<td>D56EL55T55P55Q555F55P55Y555PP55F65P55G55L55T55Y55PVE</td>
</tr>
</tbody>
</table>

C

Key:
- Globular or functional domain
- Possible Cdk site (S/T-P)
- Candidate Cln2 docking motif

Diagram showing the localization of Cln2 docking sites and their interaction with other proteins.
drive some weak phosphorylation detectable in longer exposures.) As a further test, we fused the same substrate to a different type of docking site: namely, a short sequence containing an "RxL" motif from Fin1, a CDK substrate that is normally preferred by Clb5 (Loog and Morgan, 2005). This motif switched the cyclin preference and caused phosphorylation to be driven strongly by Clb5, plus moderately by Clb2 (Figure 2.10A, bottom). (It is noteworthy that the band patterns were not identical, suggesting different extent or distribution of phosphorylation events.) These results clearly indicate that CDK sites in the Ste5 N-terminus are intrinsically capable of being phosphorylated by several different cyclin-CDK forms, but they are not effectively phosphorylated in vivo in the absence of cyclin-specific docking sites, and hence this provides specificity to the CDK phosphorylation.
Figure 2.10. Docking sites drive cyclin-specific phosphorylation

(A) Phosphorylation of a single substrate (Ste5\textsuperscript{1-260}) was monitored with and without the addition of the indicated docking sites, in cells expressing different \( P_{GAL}$\)-induced cyclins. Docking sites from Ste5, Ste20, and Exo84 preferentially enhance phosphorylation by Cln1/2-Cdc28, whereas an RxL-containing fragment from Fin1 converts the substrate into one that is preferred by Clb5 and Clb2.

(B) Schematic comparison of different cyclin-CDK complexes, with a general model for how cyclin-specific docking interactions can selectively enhance substrate phosphorylation by individual forms of cyclin-CDK.
Figure 2.10. Docking sites drive cyclin-specific phosphorylation
DISCUSSION

In this study we identified docking sites for the yeast G1/S cyclins in several CDK substrates. These sites bind preferentially to Cln2 and enhance phosphorylation of CDK substrates in a cyclin-specific manner \textit{in vivo}. They are also functionally modular, in that they can promote phosphorylation at a variety of distances and positions relative to the CDK sites, and the cyclin specificity of phosphorylation can be switched by exchanging docking sites. We exploited this functional modularity to re-wire a CDK regulatory circuit so as to change the target of Cln2-Cdc28 regulation in the pheromone response pathway, and to identify candidate Cln2 docking sites in several other CDK substrates. The relative ease with which these other sites were found suggests that there may be numerous examples of such sites for the G1 or G1/S cyclins in yeast. Indeed, recent studies from another group show that the Sic1 sequence which we used to drive Cln2-Cdc28 phosphorylation of heterologous substrates does in fact promote phosphorylation of Sic1 itself and can also act as a competitive inhibitor of other Cln2-Cdc28 substrates (Koivomagi et al., 2011b). Collectively, these findings suggest that docking interactions play a prevalent but previously unappreciated role in driving phosphorylation of G1/S CDK substrates.

The use of separate docking and phosphorylation sites offers functional and regulatory flexibility. At one extreme, it can allow different kinases to
phosphorylate the same sites, as we observed by replacing a Cln2 docking site with an RxL motif favored by B-type cyclins. It may also allow the sequence requirements at phosphorylation sites to be relaxed. While most kinases favor certain residues flanking the phosphorylation site (Mok et al., 2010; Ubersax and Ferrell, 2007), this ideal context may not be present or tolerated in all relevant substrates. Indeed, proteome-wide analysis (Holt et al., 2009) suggests that roughly two thirds of CDK sites in yeast are not “consensus” sites (S/T-P-x-K/R). In Ste5, none of the 8 N-terminal CDK sites matches this consensus. The presence of a Cln2 docking site converts the Ste5 N-terminus from a weak substrate into a better substrate, but only for Cln1/2-Cdc28. This enhancement may compensate for poor sequence context, which in turn could minimize use by other cyclin-CDKs, thus providing a regulatory benefit. Other possible benefits of cyclin docking interactions include: (i) they may enhance CDK phosphorylation even for substrates that are not cyclin specific; (ii) they may help drive multi-site phosphorylation of CDK substrates (Salazar et al., 2010); and (iii) they may impart useful regulatory behavior by fostering interplay with competitors or other bound factors. Indeed, the Cln2 docking site in Ste5 overlaps a binding site for the phosphatase Ptc1 (Malleshaiah et al., 2010), and these two factors may compete for access to Ste5 in vivo. Overlapping cyclin and phosphatase binding sites have also been found in the mammalian protein Rb (Hirschi et al., 2010), suggesting a common theme.
Differences in docking strength may impact the extent and/or timing of substrate phosphorylation (Salazar et al., 2010). For example, the docking sites in Ste5 and Ste20 bind stronger to Cln2 than Cln1, and such differences could contribute to disparities in efficacy of these two cyclins (Chang and Herskowitz, 1992; Oehlen and Cross, 1994; Queralt and Igual, 2004). We also observed varying degrees of potency for different Cln2 docking sites, though the responsible sequence features remain uncertain. The Ste5 and Ste20 sites share an LxxPΦxΦ motif and an overall enrichment in Leu, Pro, and/or hydrophobic residues, which was used as a criterion to identify additional sites. An LLPP motif is shared by several of the strong sites, but the docking site in Ste20 does not match this motif and yet is very potent. Thus, deducing the key sequence features of Cln2 docking sites will require subsequent study, as will determination of whether they bind directly to the cyclin versus the cyclin-CDK complex.

Subcellular localization can also contribute to functional specialization of cyclins (Draviam et al., 2001; Edgington and Futcher, 2001; Miller and Cross, 2000). Hence, in addition to driving phosphorylation in cis, some cyclin docking sites may help localize cyclins and/or promote phosphorylation in trans of other proteins in the same complex or subcellular locale (Pascreau et al., 2011). In fact, prior to our discovery that it binds Cln2, we originally found that the Cln2 docking site in Ste20 could trigger hyperpolarized growth when over-expressed
and membrane-localized (Takahashi and Pryciak, 2007); in retrospect, this phenotype could result from the generation of excess cortical binding sites for Cln2, which promotes apical polarized growth (Lew and Reed, 1993). Several Cln1/2-Cdc28 substrates are involved in cell polarity (Enserink and Kolodner, 2010) (see Figure 2.9 legend), so it will be of interest to determine whether they each contain Cln1/2 docking sites or if docking sites in some can serve a scaffolding role that promotes CDK phosphorylation of co-bound or co-localized substrates. Synthetic approaches, such as those described here, can be used to discover these docking sites as well as to characterize their functional properties and activities in a standardized setting, which ultimately can illuminate how the regulatory behavior of native proteins and pathways arises from the combined properties of individual motifs.

ACKNOWLEDGEMENTS

We are grateful to Matthew Winters and Rachel Lamson for technical assistance, and to Mart Loog for discussions and communication of unpublished results. We also thank Jenny Benanti for the Cln310A allele, Patty Pope for cyclin-myc13 constructs, Wendell Lim for the Ste5 ND mutant, and Dan McCollum and Jenny Benanti for helpful discussions.
Materials and Methods

Yeast Strains and Plasmids

Standard procedures were used for growth and genetic manipulation of yeast (Rothstein, 1991; Sherman, 2002). Cells were grown at 30°C in yeast extract/peptone medium with 2% glucose (YPD) or galactose (YPGal), or in synthetic (SC) medium with 2% glucose and/or raffinose. Strains and plasmids are listed in the tables below.

Protein analysis in whole yeast cell lysates

Except for GST co-precipitation assays, protein analysis used whole cell lysates prepared by glass-bead lysis of frozen cell pellets directly in trichloroacetic acid (TCA) solution, as described previously (Lee and Dohlman, 2008), which provides good preservation of protein phosphorylation state. Ten mL of culture (OD$_{660}$ ~ 0.7) was collected by centrifugation, and cell pellets were frozen in liquid nitrogen and stored at -80°C. Frozen pellets were thawed by adding 300 µL of TCA buffer (10 mM Tris.HCl, pH 8.0, 10% TCA, 25 mM ammonium acetate, 1 mM Na$_2$EDTA), transferred to a microcentrifuge tube, lysed by vortexing with glass beads (five 1-min cycles, with chilling on ice for 3 min between cycles). The cell lysate was transferred to a new tube and centrifuged (16,000 x g, 10 min at 4°C). The pellet was resuspended in 150 uL of solution R (0.1 M Tris.HCl, pH 11.0, 3% SDS), boiled for 5 min, cooled to room temperature, and re-centrifuged
for 30 sec. Then, 120 µL of supernatant was transferred to a fresh tube. Aliquots were reserved to determine protein concentration using a detergent-compatible (bicinchoninic acid) assay (Thermo Scientific # 23225), and the remainder was combined with 2x SDS-PAGE sample buffer and boiled for 5 min.

**Phosphorylation Gel Shift Assays**

Cultures harboring HA\textsubscript{3}- or V5\textsubscript{3}-tagged proteins, plus a P\textsubscript{GAL1}\textsuperscript{-}GST-cyclin construct or a vector control, were grown in 2% raffinose media and induced with 2% galactose for 3 hr. Alternatively, to reduce toxic effects of Clb2 expression, the experiments comparing all five P\textsubscript{GAL1}\textsuperscript{-}GST-cyclins in parallel used only 1 hr galactose induction. Wild-type strain BY4741 was used except where noted otherwise. Using samples prepared by the TCA method described above, equivalent amounts of protein (20 µg/lane) were separated by SDS-PAGE, and transferred to PVDF membranes (Immobilon-P; Millipore) using a submerged transfer apparatus. Blots were probed with mouse anti-HA (1:1000, Covance #MMS-101R), anti-V5 (1:5000, Invitrogen #46-0705), or anti-GST (1:1000, Santa Cruz Biotechnologies #sc-138) antibodies, and detected using HRP-conjugated goat anti-mouse antibodies (1:3000, BioRad #170-6516) and Pierce SuperSignal West Pico (#34080) chemilluminescent reagent. To follow phosphorylation in synchronous cultures, cdc15\textsuperscript{-2} strain PPY1762 harboring tagged proteins was grown overnight at 25°C, arrested at 37°C for 3 hr, and then released at 25°C. Aliquots were taken at 20 min intervals, then chilled on ice for 5 min, pelleted,
and frozen in liquid nitrogen. Cell extracts were prepared and analyzed as
described above. In parallel, cells at each time point were fixed with 5%
formaldehyde and scored for small buds by microscopy. Clb2 was detected with
rabbit anti-Clb2 (1:1000, Santa Cruz Biotechnologies #sc-9071) and AP-
conjugated goat anti-rabbit (1:3000, BioRad #170-6518) antibodies, with Immun-
Star-AP substrate (BioRad #170-5018).

Most SDS-PAGE analysis used standard (Laemmli) methods, in which
separating gels included 8% acrylamide [37.5:1 acryl:bis] with 0.1 % SDS.
However, to better separate phosphorlyated forms of large proteins (i.e., full-
length Ste5 and Swe1), a higher acrylamide:bis ratio was used and SDS was
omitted from the gel (i.e., separating gel: 0.375 M Tris-HCl pH 8.8, 7.5 %
acrylamide [43.4:1 acryl:bis]; stacking gel: 0.125 M Tris-HCl pH 6.8, 5 %
acrylamide [37.5:1 acryl:bis]; running buffer: 0.05 M TRIZMA base, 0.38 M
glycine, 0.1 % SDS; electrophoresis: 20 mA for roughly 5 hr).

**Signaling Assays**

To measure effects of $P_{GAL1}$-$CLN2$ on pheromone response, cells were grown in
2% raffinose selective media, induced with 2% galactose, and then treated with $\alpha$
factor. (i) For assays of transcriptional response, 1 hr galactose induction was
followed by 2 hr treatment with 5 $\mu$M $\alpha$ factor; then, $FUS1$-lacZ expression was
measured by $\beta$-galactosidase assay as described previously (Lamson et al.,
2002; Strickfaden et al., 2007). (ii) For assays of MAPK phosphorylation, 75 min.
galactose induction was followed by 0-60 min. treatment with 1 µM α factor. Phosphorylated Fus3 was detected with rabbit anti-phospho-p44/42 (1:1000, Cell Signaling Technology #9101) and HRP-conjugated goat anti-rabbit (1:3000, Jackson ImmunoResearch #111-035-144) antibodies; then, blots were stripped (Thermo Scientific #21059) and probed with goat anti-Fus3 (1:2000, Santa Cruz Biotechnologies #sc-6773) and HRP-conjugated donkey anti-goat (1:3000, Santa Cruz Biotechnologies #sc-2020) antibodies. (Pierce SuperSignal West Pico reagent was used for each.) (iii) For assaying the levels of Ste20Ste5PM chimera proteins, epitope-tagged forms were detected with mouse anti-GFP (1:1000, Clontech #JL-8) and HRP-conjugated goat anti-mouse (1:3000, BioRad #170-6516) antibodies, or rabbit anti-myc (1:200, Santa Cruz Biotechnologies #sc-789) and HRP-conjugated goat anti-rabbit (1:3000, Jackson ImmunoResearch #111-035-144) antibodies (with Pierce SuperSignal West Pico reagent).

**GST Binding Assays**

Cultures (25 ml) of strain BY4741 harboring P_{GAL1}-GST-tagged Ste20 or Ste20^{Ste5} chimeric fragments, with or without cyclin-myct_{13} constructs, were induced with 2% galactose for 3 hr, harvested, and stored at -80°C. Extracts were prepared by glass bead lysis in a non-ionic detergent buffer as described previously (Lamson et al., 2002). Aliquots were reserved to assess input levels. GST fusions were collected by binding to glutathione-sepharose beads (GE Healthcare #17-0756-01) and detected with anti-GST antibodies described above.
and AP-conjugated goat anti-mouse antibodies (1:3000, BioRad #170-6520) and Immun-Star-AP substrate. Cyclin-myc\textsubscript{13} proteins were detected using rabbit anti-myc (1:200, Santa Cruz Biotechnologies #sc-789) and HRP-conjugated goat anti-rabbit (1:3000, Jackson ImmunoResearch #111-035-144) antibodies, and Pierce SuperSignal West Pico (#34080) reagent.
Table 2.1: Yeast strains used in Chapter II

| bkg | strain name | genotype | source $|$ |
|-----|-------------|----------|-----------|
| 1a  | BY4741      | MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 | (1) |
| 1b  | BY4741 ptc1Δ | MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ptc1::kanMX4 | (2) |
| 1c  | BY4741 sic1Δ | MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 sic1Δ::kanMX4 | (2) |
| 2a  | PPY2285     | MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 SWE1-3xV5::kanMX4 | this study |
| 3a  | PPY640      | MATa FUS1::FUS1-lacZ::LEU2 | (3) |
| 3b  | PPY858      | MATa FUS1::FUS1-lacZ::LEU2 ste5::ADE2 | (3) |
| 4a  | PPY1173     | MATa FUS1::FUS1-lacZ::LEU2 fus3::LEU2 kss1::ura3ΔMX4 | (4) |
| 4b  | PPY1665     | MATa FUS1::FUS1-lacZ::LEU2 fus3::LEU2 kss1::ura3ΔMX4 ste5::ADE2 | this study |
| 5a  | PPY1695     | MATa FUS1::FUS1-lacZ::LEU2 ste5::ADE2 his3::Pgal-CLN2 | (5) |
| 5b  | PPY1762     | MATa ade1 cdc15-2 ste5::ADE2 | (5) |
| 6a  | PPY2142     | MATa FUS1::FUS1-lacZ::LEU2 STE5-8A ste20Δ::kanMX4 | this study |
| 6b  | PPY2281     | MATa FUS1::FUS1-lacZ::LEU2 fus3::LEU2 kss1::ura3ΔMX4 ste5::ADE2 his3::Pgal-CLN2 | this study |
| 6c  | KBY211      | MATa ste20::ADE2 clc4::LEU2 + YcpTRP1-clc4-75R | (6) |

* strain background:
(a) S288c (his3Δ1 leu2Δ0 ura3Δ0 met15Δ0)
(b) W303 (ade2-1 his3-115 leu2-3112 trp1-1 ura3-1 can1)

$§$ source: (1) (Brachmann et al., 1998); (2) (Winzeler et al., 1999); (3) (Pryciak and Huntress, 1998); (4) (Winters et al., 2005); (5) (Sticklaken et al., 2007); (6) (Holly and Blumer, 1999).

Table 2.2. Plasmids used in Chapter II

<table>
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§ Source: (1) (Leberer et al., 1997); (2) (Sikorski and Hieter, 1989); (3) (Winters et al., 2005); (4) (Takahashi and Pryciak, 2007); (5) (Mumberg et al., 1995)

** mutations denoted by abbreviated names:

Ste5 LLPP: L278A, L279A, P280A, P281A  
Ste5 ND: L278A, L279A, P280A, P281A  
Ste5 4AV: T267A, S276A, T287V, S329A  
Ste5 4E: T267A, S276A, T287A, S329A  
Ste20 mut1: D80A, D81A, D82A  
Ste20 mut2: N83A, N84A, V85A, V86A  
Ste20 mut3: S87A, L88A, D89A, D90A, P91A  
Ste20 mut4: I92A, Q93A, F94A  
Ste20 mut5: T95A, R96A, V97A  
Ste20 mut6: S98A, S99A, S100A, S101A  
Ste20 mut7: V102A, I103A, S104A, G105A  
Ste20 mut8: M106A, S107A, S108A, S109A  
CHAPTER III

A conserved docking interface in the cyclin Cln2 controls multi-site substrate phosphorylation and ensures timely entry into the cell cycle

The following Chapter contains the manuscript:


The study has been devised and results have been interpreted by myself and Dr. Pryciak. All experiments, except for the ones listed below, have been performed by myself and the manuscript has been prepared by myself and Dr. Pryciak.

Figure 3.5 has been contributed by Brian Gruessner under my guidance.

Figure 3.6 and Figure 3.8D have been contributed by E. Valk and M. Loog.

M.J. Winters has constructed several plasmids used in the study and helped with different experiments.
Abstract

Eukaryotic cell division is driven by cyclin-dependent kinases (CDKs). Distinct cyclin-CDK complexes are specialized to drive different cell cycle events, though the molecular bases for these specializations are only partly understood. In budding yeast, the decision to begin a new cell cycle is regulated by three G1 phase (Cln) cyclins. Recent studies revealed that some CDK substrates contain a novel docking motif that is recognized by Cln1 and Cln2 but not by Cln3 or later S- or M-phase cyclins. Here, to explore the role of this new docking mechanism in the cell cycle, we first show that it is conserved in a distinct cyclin subtype, the fungal Ccn1 group, and then use phylogenetic variation to identify cyclin mutations that disrupt docking. These mutations disrupt binding to multiple substrates as well as the ability to use docking sites to promote efficient, multi-site phosphorylation of substrates in vitro. In cells in which the Cln2 docking function is blocked, we observed reductions in the polarized morphogenesis of daughter buds and reduced ability to fully phosphorylate the G1/S transcriptional repressor Whi5. Furthermore, disruption of Cln2 docking perturbs the coordination between cell size and the G1/S transition such that DNA synthesis and budding are delayed until cells reach a larger size. The findings point to a novel substrate interaction interface on cyclins and indicate that efficient, docking-dependent substrate phosphorylation contributes to punctual cell cycle entry.
Introduction

Cyclin-dependent kinases (CDKs) are central regulators of cell division in eukaryotes (Morgan, 2007). The cyclin subunit has a critical role in triggering CDK kinase activity, but it can also play important regulatory roles by controlling subcellular localization and substrate selection (Bloom and Cross, 2007). Eukaryotic cells invariably have several distinct cyclin-CDK forms that are specialized for particular cell cycle stages. In yeasts, multiple cyclins associate with a single CDK molecule, whereas animal cells have multiple cyclins as well as multiple CDKs. Generally, these different cyclin-CDK forms fall into two broad functional classes: those that control the biochemical events of DNA synthesis and mitosis (in S and M phases), and those that control the decision to begin a new division cycle (in G1 phase). To understand how sequential cell cycle events are properly orchestrated, it is necessary to determine the molecular mechanisms by which distinct cyclin-CDK forms differ functionally from each other. For example, how do early forms of cyclin-CDK, which act in G1 phase to drive cell cycle entry, trigger some events without triggering other events that should occur later in S and M phases? One general class of explanation is that early cell cycle events may rely on cyclin-CDK complexes with low activity but strong substrate selectivity (Koivomagi et al., 2011b; Levine et al., 1996). This study pursues recent discoveries regarding substrate selection by early cyclins.
In the budding yeast *S. cerevisiae*, S and M phases are driven by six B-type cyclins (Clb1-6), whereas the decision to enter a new cell cycle is controlled by three G1 cyclins, Cln1-3 (Bloom and Cross, 2007; Morgan, 2007). The G1 phase constitutes a critical assessment period in which cells determine whether conditions are appropriate to begin a new round of division, and this decision is responsive to both internal and external cues such as nutrient availability, cell size, and inhibitory signals. Ultimately these signals affect the function of Cln1-3, which then drive the CDK phosphorylation events that commit cells to cell cycle entry in a step known as “Start”, followed by the transition from G1 to S phase (Cross, 1995b; Johnson and Skotheim, 2013; Jorgensen and Tyers, 2004). Key CDK substrates in this period are inhibitors of cell cycle entry such as Whi5, a repressor of G1/S transcription (Costanzo et al., 2004; de Bruin et al., 2004), as well as Cdh1 and Sic1, which prevent the expression and activity of Clb cyclins, respectively (Morgan, 2007). Notably, each of these substrates has multiple CDK phosphorylation sites (Nash et al., 2001; Wagner et al., 2009; Zachariae et al., 1998), which may place unique demands on the cyclin-CDK complex to ensure efficient and complete phosphorylation, and also dictate the threshold CDK levels required to trigger the regulatory effect (Koivomagi et al., 2013; Koivomagi et al., 2011a; Nash et al., 2001; Yang et al., 2013).

Despite some functional overlap among Cln1-3 in governing cell cycle entry, these three cyclins have functional distinctions (Bloom and Cross, 2007;
Levine et al., 1996) that contribute to a two-stage commitment process: Cln3 plays an early priming role that initiates expression of Cln1 and Cln2, which further promote their own expression via a positive feedback loop, resulting in a sharp increase in Cln1/2 activity that triggers a decisive entry into the cell cycle (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991; Skotheim et al., 2008). The distinctions between Cln3 and Cln1/2 include different subcellular distributions (Edgington and Futcher, 2001; Miller and Cross, 2000). In addition, recent studies revealed that the Cln1/2 cyclins recognize specific docking motifs in select CDK substrates (Figure 3.1A). These Cln1/2-specific “LP-type” docking motifs (enriched in Leu and Pro residues) are not recognized by either Cln3 or Clb1-6, and hence they promote phosphorylation preferentially by Cln1/2-CDK (Bhaduri and Pryciak, 2011; Koivomagi et al., 2011b). This mechanism is analogous to recognition of RxL motifs by S-phase cyclins such as yeast Clb5 or mammalian CycA (Archambault et al., 2005), but the motifs are unrelated and are not cross-recognized (Bhaduri and Pryciak, 2011; Koivomagi et al., 2011b). Currently, it is unknown what part of the Cln1/2 protein recognizes the LP motifs, or why other cyclins do not recognize them. It is also unknown what cell cycle events depend on docking by Cln1/2. To address these issues, in this study we identify and characterize a docking-defective Cln2 mutant. Our findings uncover a novel substrate-docking interface that is conserved among distinct cyclin subgroups, and demonstrate that this docking function promotes multi-site phosphorylation of substrates and punctual entry into the cell cycle.
Results

Strategic framework for localizing the docking interface in Cln2

Before screening for a Cln2 mutant that is defective at docking, we needed a strategy to distinguish mutants with specific defects in docking from those with more general defects in CDK activation. Our solution was to compare a CDK substrate harboring a native LP docking site with one that instead uses a leucine zipper to promote cyclin-substrate interaction, expecting that the desired class of mutant would only show defects in phosphorylating the former substrate (Figure 3.1B). Indeed, this predicted behavior is exemplified by Cln3, which can phosphorylate a substrate harboring the leucine zipper but does not recognize LP docking sites (Figure 3.1C). Hence, in principle we sought a Cln2 mutant that behaves more like Cln3 in this respect.

Because S-phase cyclins recognize RxL docking motifs via a hydrophobic patch (HP) on their surface, we considered a simple model that the different sequence composition of this region in Cln1/2 cyclins might allow them to recognize LP rather than RxL motifs. But mutations in this part of Cln2 did not confer a specific defect in LP docking, and instead they caused a general mild reduction in all phosphorylation activity, including auto-phosphorylation of the Cln2 C-terminus (Figure 3.1D). Hence, we concluded that LP recognition is encoded elsewhere on Cln2, thus necessitating a broad interrogation of
Figure 3.1. Sequence constraints and evolutionary conservation of docking function.

(A) Interaction of Cln1/2 with “LP” docking sites promotes CDK substrate phosphorylation.

(B) Expected phenotype of a docking-defective Cln1/2 mutant. Defects in docking vs. CDK activity can be discerned by comparing substrates with a docking site vs. a leucine zipper.

(C) Cln3 exemplifies non-docking behavior. Cyclins were expressed as fusions to GST plus a half leucine zipper (GST-[lz]), along with a substrate harboring either the partner half leucine zipper or an LP docking site (see Figure 3.2A). Reduced gel mobility indicates substrate phosphorylation.

(D) Mutations in the hydrophobic patch region of Cln2 (hpm1, 3; see Figure 3.2B) do not confer specific docking defects but rather a mild general reduction in phosphorylation activity.

(E) The C-terminal tail of Cln2 is dispensable for docking. Full-length Cln2 (1-545) and truncated forms were tested for substrate phosphorylation (as in C-D). Truncation endpoints are shown relative to regulatory phosphorylation sites (P-sites) and tandem cyclin box folds (CBF1, 2) predicted for Cln2 (see Figure 3.2C). Lower abundance was observed consistently for the 1-352 fragment.

(F) Conservation of LP docking in Cln1/2 and Ccn1 cyclins. Top, phylogenetic tree of six yeasts, and their G1 cyclins. Some species have two Cln1/2
paralogs, due to whole genome duplication (WGD); where only one is present, it is denoted as Cln12. The Ccn1 group is related but distinct from Cln3 and Cln1/2 groups. Bottom, cyclins from different yeasts were tested in *S. cerevisiae* as GST-(lz) fusions for substrate phosphorylation *in vivo*. All cyclins were truncated to remove their C-terminal tails (see Figure 3.2C).
Figure 3.1. Sequence constraints and evolutionary conservation of docking function.
candidate sequences. To help constrain the search, we tested truncations of the
Cln2 C-terminus, and found that we could dispense with roughly one-third of the
protein (the C-terminal regulatory tail) and retain LP recognition, whereas
truncations that perturb the predicted globular cyclin box fold domains (CBF1, 2)
eliminated all activity (Figure 3.1E). Thus, all positions within the roughly 370-
residue core of Cln2 remained potential candidates.

The contrasting docking ability of Cln1/2 versus Cln3 must arise from
sequence differences, but they were too extensive to explore comprehensively,
and chimeras were non-functional and hence uninformative (unpublished
observations). Therefore, we devised a strategy to exploit natural sequence
variation among fungal G1 cyclins. Namely, by expressing cyclins from other
yeasts in S. cerevisiae, we found that recognition of LP docking sites was
conserved in other Cln1/2 members and lacking among Cln3 members (Figure
3.1F). Moreover, this led us to test a distinct class of G1 cyclin, the Ccn1 group,
which is absent from S. cerevisiae but present in other yeasts such as C.
albicans (Figure 3.1F, top), where it promotes hyphal morphogenesis (Loeb et
al., 1999). Remarkably, we found that Ccn1 members are proficient at using an
LP docking motif to drive substrate phosphorylation (Figure 3.1F). This revealed
that LP docking exists for a class of cyclin other than Cln1/2, and offered a way
to further constrain the possible residues involved in docking.
Figure 3.2. Leucine zippers, hydrophobic patch, and cyclin domains.

(A) Cln2 was fused to a half leucine zipper (lz) at its N-terminus (lz-Cln2) or C-terminus (Cln2-lz), or to no zipper (wt). These were co-expressed with a CDK substrate (Ste5 1-260) harboring no docking site, an LP docking site, or three variants of the partner half leucine zipper (E34[I], E34[V], or E34[N]) that range in binding affinity from 6 to 800 nM (Acharya et al., 2002; Bashor et al., 2008). Reduced gel mobility indicates substrate phosphorylation. The leucine zipper worked best at the Cln2 N-terminus (lz-Cln2) but did not require strong affinity. Hence, all further experiments used the weakest affinity version (E34[N]).

(B) Hydrophobic patch mutations. In S-phase cyclins such as mammalian CycA or yeast Clb5, recognition of RxL docking motifs is mediated by a hydrophobic patch (HP) formed largely by helix α1 (the “MRAIL” motif), and mutation of residues in blue disrupts RxL recognition (Loog and Morgan, 2005; Schulman et al., 1998; Wilmes et al., 2004). To test if the different helix α1 sequence in Cln2 leads to recognition of distinct LP-type docking motifs, we mutated the analogous positions (hpm1) as well as nearby residues (hpm3); see Figure 3.1D. The hpm1 mutations were also tested by other assays in a previous study (Miller et al., 2005).

(C) Yeast cyclin fragments analyzed in Figure 3.1F. Each diagram shows predicted α helices (as boxes) and a plot of protein disorder (highest at top),
obtained using PSIPRED v3.3 and DISOPRED3, respectively (Buchan et al., 2013). Truncation endpoints are shown in text and by red arrows. Helix boxes are colored red and blue (as in panel B) to denote regions predicted to correspond to CBF1 and CBF2 globular domains. These general domain boundaries also agree with separate multiple sequence alignments of over 75 fungal G1 cyclins together and with cyclins of known structure (available on request), as well as with 3D structure prediction results for 8 of the 11 cyclins shown here using I-TASSER (Roy et al., 2010).
Figure 3.2. Leucine zippers, hydrophobic patch, and cyclin domains

A

B

S/M cyclins  N  C
Cln1/2/3  N  C

human CycA2  S  S  C  C
S  Cln2  C
S  Cln2 wt  C

C

Scer Cln2 1-372
Scer Cln1 1-388
Cgl Cln1 1-362
Cgl Cln2 1-371
Agos Cln12 1-364
Sklu Cln12 1-364
Kwal Cln12 1-363
Kwal Ccn1 1-379
Ca b Ccn1 1-469
Scer Cln3 1-395
Agos Cln3 1-324
Identification of a docking defective Cln2 mutant

By performing sequence alignments with multiple members of the Cln1/2, Cln3, and Ccn1 groups, we found that there were many positions where Ccn1 and Cln3 residues were identical (or nearly so), and hence we excluded these from consideration as key residues in LP docking. Then, we scrutinized those positions that correlated with docking ability; i.e., similar in Cln1/2 and Ccn1, but different in Cln3. Based primarily (though not exclusively) on these considerations, twelve Cln2 mutants were designed (Figures 3.3A, 3.4).

These mutants were tested for their ability to phosphorylate substrates with two distinct LP-type docking sites or the control leucine zipper (Figure 3.3B). In addition, because they were initially tested in the context of full-length Cln2, we also monitored their ability to auto-phosphorylate the C-terminus. Several mutants (m1, m2, m5, m6, m9) showed non-specific reduction in phosphorylation of all substrates including the leucine zipper control (note the drop in the upper-most forms), while two mutants (m8, m10) had no detectable activity and no auto-phosphorylation, suggesting severe impairment in CDK activity. By contrast, one mutant, cln2-m4, displayed the desired phenotype, as it showed a specific defect with LP-containing substrates but normal phosphorylation of the control substrate (Figure 3.3B). To explore the generality of this phenotype, we compared the analogous m4 mutation in four distinct cyclins – two Cln1/2
Figure 3.3. Identification of a docking-defective Cln2 mutant.

(A) Twelve regions of Cln2 were mutated, emphasizing residues conserved in
Cln1/2 and Ccn1 groups but not in Cln3 (see Figure 3.4). One example (m4) is illustrated by the sequence alignment, with mutated positions marked by asterisks.

(B) Cln2 mutants were expressed as GST-(lz) fusions to test phosphorylation in vivo of substrates with either of two LP docking sites (from Ste5 or Ste20) or the leucine zipper.

(C) Left, the m4 mutation was tested in four different cyclins (two Cln1/2 and two Ccn1) for effects on docking-dependent phosphorylation. Right, analysis of single mutations at each of the three residues mutated in the m4 mutant.
Figure 3.3. Identification of a docking-defective Cln2 mutant.

A

Cln2

\[\text{CBF1} \quad \text{CBF2}\]

mutant

1 2 3 4 5 6 7 8 9 10 11 12

docking

* **

Scer Cln2
Kwal Cln12
Ag Cln1
Sk Ccn1

Cln1/2 group +
Cln3 group -
Ccn1 group +

B

GST-(lz)-cyclin:

substrate w/ LP\text{Ste5} dock
substrate w/ LP\text{Ste20} dock
substrate w/ leu zipper
GST

C

GST-(lz)-cyclin:

substrate w/ LP dock
leu zipper
GST
Figure 3.4. Site-directed mutations in Cln2.

(A) List of specific residue changes in Cln2 mutants m1-m12.

(B) Comparison of Cln1/2, Cln3, and Ccn1 sequences at positions mutated in cln2 m1-m12. Sequence alignments, created using Clustal Omega (Sievers et al., 2011), compare representatives of each cyclin group in regions encompassing the mutated residues, which are marked with asterisks (or by brackets for large deletions). The top line is S. cerevisiae Cln2. The mutational strategy prioritized residues conserved in Cln1/2 and Ccn1 groups but not in Cln3, but also considered some regions where Cln1/2 and Cln3 differ without requiring clear conservation in Ccn1.
Figure 3.4. Site-directed mutations in Cln2

A

\[ \text{Cln2} \quad \text{CBF1} \quad \text{CBF2} \]

mutant: 1 2 3 4 5 6 7 8 9 10 11 12

m1: Δ1-19
m2: Y20A Y21A S25A N26A A27G
m3: L61A D63A Q64G
m4: R109A L112A R113A
m5: Δ134-152
m6: E163A V165A H166A
m7: V173A D175A M178A
m8: P197A M198A D201A
m9: N209A M212A Y214A
m10: I265K L267G
m11: F300A Q303A D304G
m12: L348A P349G E356A L365A Q366A

B

\[ \text{Cln1/2} \quad \text{Cln3} \quad \text{Ccn1} \]

\[ \text{m1} \quad \text{m2} \quad \text{m3} \]

\[ \text{m4} \quad \text{m5} \]

\[ \text{m6} \quad \text{m7} \]

\[ \text{m8} \quad \text{m9} \]

\[ \text{m10} \quad \text{m11} \quad \text{m12} \]
members and two Ccn1 members (Figure 3.3C, left). Each showed the same behavior, in which the m4 mutant was defective at using a native LP dock but fully competent to use the leucine zipper. We also tested each of the single residue changes in the original m4 triple mutant, and found that one of the changes, L112A, was largely responsible for the defect (Figure 3.3C, right); however, the L112A single mutant was not quite as defective as the m4 triple mutant. We also observed mild defects in each of the other two single mutants (R109A and R113A), and hence we used the original m4 triple mutant for further analyses. Collectively, these findings argue that the m4 mutation disrupts a region of the cyclin with a specific role in utilizing LP docking sites.

**Cln2 docking function is required for interaction with multiple substrates**

To verify that \textit{cln2-m4} was defective at docking interactions, we assayed substrate binding. First, we compared several of the initial Cln2 mutants, expressed as GST fusions, for their ability to co-precipitate Ste5, a Cln2 substrate with an LP docking motif (Bhaduri and Pryciak, 2011). As predicted for a docking mutant, \textit{cln2-m4} showed reduced binding to Ste5 but normal binding to its partner CDK molecule, Cdc28 (Figure 3.5A). The specific nature of this binding phenotype was reinforced by comparison to other mutants: namely, (i) \textit{cln2-m3} showed no binding defects, consistent with it being fully functional in phosphorylation assays; (ii) \textit{cln2-m5} showed reduced binding to both Ste5 and
Cdc28, suggesting a non-specific general defect that agrees with its reduced activity against all substrates; and, (iii) the cln2-m8 and cln2-m10 mutants were defective at binding Cdc28 but bound Ste5 normally, which agrees with their complete inactivity in phosphorylation assays while separately indicating that binding Cdc28 is not required for binding substrates.

We also conducted reciprocal assays in which GST-substrate fusions were used to co-precipitate V5-tagged Cln2 (Figures 3.5B, C), and found that cln2-m4 showed substantially reduced binding to most substrates tested (i.e., Ste20, Ste5, Sic1, Whi5, Rga1, Tus1). The sole exception was Srl3, which binds Cln2 especially strongly; this might indicate a distinct mode of binding or that the reduction in binding is too mild to register in this assay. As a further control, we tested binding to Grr1, an F-box protein that promotes Cln2 ubiquitination; this binding was unaffected by the m4 mutation (Figure 3.5B), consistent with the fact that Grr1 recognizes the phosphorylated C-terminus of Cln2 rather than the globular CBF domains (Berset et al., 2002; Landry et al., 2012). Finally, we also assayed the effect of the m4 mutation on substrate binding by one of the Ccn1 family members, from K. waltii (Figure 3.5D). This cyclin bound only a subset of the substrates that bind Cln2, indicating some divergence in docking motif recognition (to be pursued as part of a separate study), but each of the detectable interactions was disrupted by the ccn1-m4 mutation.
Figure 3.5. Docking-defective Cln2 and Ccn1 mutants shows reduced binding to multiple partners.

(A) Cells (far1Δ) co-expressed a galactose-inducible GST-cyclin (or vector) with V5-tagged Ste5. After galactose induction, GST fusions and co-bound proteins were captured with glutathione-Sepharose. Bound and input proteins were analyzed by immunoblots.

(B) The m4 mutation disrupts Cln2 binding to multiple partners. Cells (far1Δ) expressed V5-tagged Cln2 (wt or m4) and galactose-inducible GST fusions to full-length proteins or N-terminal fragments. Ste5* is a hybrid fragment (Bhaduri and Pryciak, 2011; Pope et al., 2014), in which the Cln2-docking site in the Ste20 N-terminus is replaced with one from Ste5. Grr1ΔF lacks its F-box, to prevent it from driving degradation of Cln2 (Landry et al., 2012).

(C) Binding of V5-tagged Cln2 (wt or m4) to GST fusions in a FAR1 strain, in which binding of Cln2 to some substrates is noticeably weaker than in far1Δ cells (Pope et al., 2014). As in Figure 3.5B, the cln2-m4 mutant shows reduced binding for some substrates (Ste20 N-terminus and full-length Whi5), yet Srl3 binding is not detectably altered.

(D) Binding of V5-tagged K. waltii Ccn1 (wt and m4) to the same set of GST fusion proteins as tested as Figure 3.5B for Cln2 binding. Ccn1 binds only a subset of the proteins bound by Cln2, but in cases where binding is detectable it is disrupted by the m4 mutation. Note that these Ccn1
fragments (1-379) lack the C-terminal tail and the phosphorylation sites therein, explaining why they do not bind Grr1ΔF (Berset et al., 2002; Landry et al., 2012).
Figure 3.5. Docking-defective Cln2 and Ccn1 mutants shows reduced binding to multiple partners.
**In vitro kinase assays confirm a specific defect in docking function**

To probe the biochemical defects of the *cln2-m4* mutant *in vitro*, we purified wt and m4 versions of the Cln2-Cdc28 complex from yeast cells. *cln2-m4* co-purified with normal amounts of Cdc28 and, when using a generic CDK substrate, histone H1, the wt and m4 complexes showed indistinguishable kinase activity. In contrast, however, the *cln2-m4* complex showed defects in activity against other substrates in a manner that indicated a specific failure in LP docking function. Namely, for several substrates (Sic1, Sic1ΔC, Whi5, Stb1), the activity of the *cln2-m4* complex was reduced to a level comparable to that seen when the wt complex was incubated with a competitor LP peptide (Figure 3.6Ai, 3.6B). The competitor peptide had negligible effect on the ability of *cln2-m4* complex to phosphorylate docking dependent substrates, indicating that its reduced activity specifically reflects an inability to recognize the LP docking site to enhance phosphorylation. Similarly, mutating the LP docking site on Sic1 (Sic1-vllpp) reduced phosphorylation by the wt complex, but did not affect phosphorylation by the m4 complex (Figure 3.6Ai, 3.6B).

To further assess the defects of the *cln2-m4* mutant, the products of these kinase reactions were analyzed on Phos-tag gels, which can resolve substrate isoforms that differ in the number of phosphate groups added. The *cln2-m4*
Figure 3.6. Docking promotes multi-site phosphorylation of substrates in *vitro*.

(A) Cln2-Cdc28 complexes containing either wt or m4 Cln2 were purified from yeast cells and assayed for substrate phosphorylation *in vitro*, both with and without a competitor LP peptide. (i) Total \(^{32}\)P incorporation into substrates. (ii) Reaction products were separated on Phos-tag gels to assess the multiplicity of phosphorylation.

(B) Quantification of \(^{32}\)P incorporation from assays as in panel Ai.
Figure 3.6. Docking promotes multi-site phosphorylation of substrates *in vitro*.

A

(i) Cdk substrate: (ii) Cdk substrate:

<table>
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<tr>
<th>substrate</th>
<th>histone H1</th>
<th>Sic1</th>
<th>Sic1ΔC</th>
<th>Sic1ΔC-vllpp</th>
<th>GST-Whi5</th>
<th>6xHis-Stb1</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<tr>
<td>mut4 + LP</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

B

Phosphorylation (relative)
complex was defective at generating multiply-phosphorylated products of several substrates, and instead yielded products modified on few sites. This behavior is similar to that seen when the competitor LP peptide is included, or when the LP docking site is lacking from the substrate (Figure 3.6Aii). Collectively, these \textit{in vitro} findings show that the \textit{cln2-m4} mutant has a specific defect in utilizing LP docking sites to drive substrate phosphorylation extensively at multiple sites.

\textbf{Cln2-substrate docking helps coordinate the G1/S transition with cell size}

To begin assessing how docking contributes to the cellular functions of Cln2, we first asked if \textit{cln2-m4} can support cell growth when expressed from the native \textit{CLN2} promoter. Indeed, the mutant permitted growth when provided as the only G1 cyclin (Figure 3.7A), as well as under conditions where \textit{CLN3} was not sufficient (i.e., \textit{cln1Δ cln2Δ pcl1Δ pcl2Δ}; Figure 3.7B). These results confirm that \textit{cln2-m4} remains generally active, and indicate that docking is not the only function that discriminates Cln1/2 from Cln3.

Because G1 phase is a period of growth before division, defects in the control of cell cycle entry can lead to alterations in cell size (Jorgensen and Tyers, 2004; Turner et al., 2012). When we measured cell volumes in asynchronous cultures (using \textit{cln1Δ} strains to eliminate redundancy between Cln1 and Cln2), we found that \textit{cln2-m4} cultures were shifted toward mildly larger cells (Figure 3.8A, top &
Figure 3.7. Effect of the cln2-m4 mutation on viability and cell size.

(A) Spot growth tests of cyclin plasmids in a cln1Δ cln2Δ cln3Δ background.
Strain PPY2407 (cln1Δ cln2Δ cln3Δ P_{MET3}-CLN2) was transformed with plasmids expressing the indicated CLN2 alleles from the native CLN2 promoter (or a vector control). Cultures were first kept alive by propagation in –Met medium to allow expression of the integrated $P_{MET3}$-CLN2 allele. Then, serial dilutions were spotted onto medium with or without methionine (to repress $P_{MET3}$-CLN2).

(B) Spot growth tests of cyclin plasmids in a cln1Δ cln2Δ pcl1Δ pcl2Δ background.
Strain BY2287A (cln1Δ cln2Δ pcl1Δ pcl2Δ P_{GAL1}-PCL2) was transformed with the indicated CLN2 or CLN3 plasmids, or a vector control. Cultures were first kept alive by propagation in selective medium with raffinose and galactose. Then, serial dilutions were spotted onto solid media either with raffinose + galactose or with glucose (to repress $P_{GAL1}$-PCL2).

(C) The increases in cell volume caused by the cln2-m4 allele were analyzed as in Figure 3.8A, but verified in independent strains. Note that all strains are cln1Δ. Solid and dashed lines show mean ± SEM (n = 3, in YPD).

(D) The increases in diameters of mother and unbudded cells caused by the cln2-m4 allele were analyzed as in Figure 3.8B, but verified using independent strains (all of which are far1Δ cln1Δ). Lines denote the mean ± SD.
(E) The \textit{cln2-m4} allele does not cause an accumulation of 1C or unbudded cells in asynchronous, log-phase cultures (grown in YPD). The graphs plot mean ± SEM (n = 9; left), or mean ± range (n = 2; right).

(F) The \textit{cln2-m4} allele and nutrient source affect cell size independently. Cultures growing in YP or SC media with different carbon source (glucose, galactose, or raffinose) were analyzed. Graphs plot the mean of duplicate measurements for each strain and condition.
Figure 3.7. Effect of the \textit{cln2-m4} mutation on viability and cell size.

**A**

<table>
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<tr>
<th>plasmid:</th>
<th>strain: $\text{cln1}^\Delta \text{cln2}^\Delta \text{cln3}^\Delta P_{\text{MET3}}-\text{CLN2}$</th>
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<td>vector</td>
<td>- methionine</td>
</tr>
<tr>
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<td>+ methionine</td>
</tr>
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**B**

<table>
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</tr>
</thead>
<tbody>
<tr>
<td>vector</td>
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</tr>
<tr>
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<td>glucose</td>
</tr>
<tr>
<td>CLN2 wt</td>
<td>$\text{cln2-m4}$</td>
</tr>
</tbody>
</table>

**C**

- $\text{FAR1 CLN2}$
- $\text{FAR1 cln2-m4}$

**D**

- $\text{far1}^\Delta \text{CLN2}$
- $\text{far1}^\Delta \text{cln2-m4}$

**E**

- $\% \text{ 1C}$
- $\% \text{ unbudded}$

**F**

- $\text{YP YP}$
- Glu Gal
- $\% \text{ of cells}$
Figure 3.8. *cln2-m4* alters the critical cell size for cell cycle entry.

(A) Cell volume is increased in *cln2-m4* strains. Note that all strains are *cln1Δ*. Solid and dashed lines show mean ± SEM (n = 6, in YPD). Results were similar in independent strains; see Figure 3.7C.

(B) Increased diameters of mother and unbudded cells in *cln2-m4* strains compared to *CLN2*-wt, in *far1Δ cln1Δ* background. Cells were grown in SC/raffinose. Lines denote mean ± SD. Results were similar in independent strains; see Figure 3.7D.

(C) *cln2-m4* alters critical size. Small G1 daughter cells (in *far1Δ cln1Δ* background) were isolated by centrifugal elutriation and inoculated in fresh medium (note, *cln2-m4* cells were born larger than wt cells); samples were collected at 15-min. intervals to assay cell volume, budding, and DNA replication. For each strain, we assayed two elutriator fractions (denoted by open and closed symbols) with distinct starting median cell volumes (17 and 23 fL for *CLN2* wt, or 27 and 30 fL for *cln2-m4*), to confirm that the phenotypes relate to size rather than incubation time. Findings were similar in independent strains (not shown).

(D) Time-lapse microscopy was used to monitor the size (diameter) of cells at the times of Whi5 nuclear exit and bud emergence (*left*), as well as the time intervals separating Whi5 exit from Sic1 degradation and budding (*right*).
Only the first G1 of newly born daughter cells was monitored. Lines show mean ± SD.

(E) *cln2-m4* does not alter rate of growth. Median cell volumes from experiment described in Figure 3.8C were plotted against time and slope and $R^2$ values were calculated for each series.

Cln2-wt: Slope = 0.1129 & 0.1374; *cln2-m4*: Slope= 0.1665 & 0.1637,
Cln2-wt: $R^2$ = 0.9718 & 0.9944; *cln2-m4*: $R^2$= 0.9758 & 0.9963.

(F) Summary of phenotypes. In *cln2-m4* cells, Whi5 exit is delayed and Start occurs at a larger cell size. After the delayed Start, subsequent events (DNA replication, bud emergence) occur with relatively normal timing.
Figure 3.8. *cln2-m4* alters the critical cell size for cell cycle entry.
3.7C top: represents two independently derived strains), consistent with a previously documented role for Cln1/2 in setting critical cell size (Dirick et al., 1995; Ferrezuelo et al., 2012; Skotheim et al., 2008). Remarkably, this difference between CLN2 and cln2-m4 cultures was amplified in cells lacking the CDK inhibitor protein Far1 (Figures 3.8A, 3.7C: represents two independently derived strains). We were primed to consider such an effect by our recent finding that Far1 competitively interferes with Cln2-substrate docking (Pope et al., 2014 & Chapter 4). This effect raised the possibility that mutations in the docking interface of Cln2 could simultaneously impair Far1 interaction, so that the resulting defects in positive functions of Cln2 are partly counteracted by reduced inhibition from Far1 (see Discussion). Hence, to avoid differential inhibition, further analysis was conducted using far1Δ strains.

When examined microscopically, mother cells and unbudded cells in cln2-m4 cultures had significantly larger diameters than those in wt cultures (Figures 3.8B, 3.7D: represents two independently derived strains), suggesting that the mutant cells did not begin dividing until they reached a larger size. Indeed, when small daughter G1 cells were isolated by centrifugal elutriation (Figure 3.8C), we observed a clear delay in the G1/S transition such that cln2-m4 cells did not begin budding and DNA synthesis until they reached a roughly one-third greater volume than wt cells (i.e., ~40 vs. 30 fL). The m4 cultures started at larger sizes than wt cultures but rate of growth of both wt and m4 cells were very similar
(Figure 3.8E). Thus, disruption of the docking function of Cln2 skews the calibration between cell size and cell cycle entry. To pinpoint the defect, we conducted time-lapse microscopy with fluorescent forms of two critical G1/S regulators, the transcriptional repressor Whi5 and the CDK inhibitor Sic1. The cln2-m4 cells delayed the nuclear exit of Whi5 until cells were larger, whereas the subsequent degradation of Sic1 and budding occurred with relatively normal timing thereafter (Figure 3.8D). Thus, the predominant effect of cln2-m4 is to delay Start (Figure 3.8F), which coincides with Whi5 exit and the initiation of G1/S transcription (Doncic et al., 2011; Skotheim et al., 2008). It is notable that this delay does not cause an accumulation of excess 1C or unbudded cells in asynchronous cultures (Figure 3.7E), likely because cells that bud at a larger size also produce larger daughters (i.e., with a larger birth size), and hence the time from birth to budding remains comparable. The size threshold for division is also regulated by the availability of nutrients such as carbon source (Jorgensen and Tyers, 2004; Turner et al., 2012), but the cell size effect of the cln2-m4 allele is independent of nutrient regulation, as the mutant cells still shifted to smaller sizes in poor carbon media and were always larger than wt regardless of nutrient conditions (Figure 3.7F). Therefore, nutrient regulation remains intact, but the eventual execution of Start is delayed in cln2-m4 cells.
Docking promotes Whi5 phosphorylation and bud polarization

To compare the potency of cyclins at driving Whi5 phosphorylation in vivo, we used conditions in which any delays in achieving critical cell size were first eliminated. For this, we synchronized cells by a prolonged G1 arrest using mating pheromone. Cells arrested in G1 continue to grow (Goranov et al., 2009; Moore, 1983), and so upon release can begin cell cycle entry without further growth delay (Schwob and Nasmyth, 1993), much as large mother cells do not rely on size and instead use a timer to govern Start (Di Talia et al., 2007). To further equalize initial conditions, and permit comparisons of Cln2 variants both with and without Cln3, we used cells with a separate \( P_{METH}^{-}CLN2 \) construct (Dirick et al., 1995; Skotheim et al., 2008) that was expressed only during initial propagation and then repressed during the experiment.

Using these conditions, we monitored Whi5 gel mobility (Figure 3.9A), which is slowed upon CDK phosphorylation (Costanzo et al., 2004; de Bruin et al., 2004; Wagner et al., 2009). V5-tagged Whi5 was resolved into three species; one in G1 arrested cells, plus two higher forms that appeared after release (Figure 3.9Ai-ii). Notably, in CLN2-wt cells the highest form eventually became the predominant species, whereas this never occurred in cln2-m4 cells and instead the middle form was predominant. This pattern suggests that cln2-m4 cannot drive Whi5 phosphorylation as extensively as wt, in agreement with the in
Figure 3.9. Whi5 phosphorylation and bud polarization depend on Cln2 docking.

(A) CLN3 cln1Δ cln2Δ PMET3-CLN2 and cln3Δ cln1Δ cln2Δ PMET3-CLN2 cells harbored a CLN2 plasmid (wt or m4; native promoter) or empty vector. Cells were arrested in G1 phase with α factor (plus methionine to repress PMET3-CLN2), and then released; aliquots were harvested at times shown. Whi5 phosphorylation was monitored by immunoblotting for Whi5-V5 (i, ii) or Whi5-GFP (iii); Cln2-V5 levels and a loading control (G6PDH) are shown for set (iii).

(B) mRNAs levels were measured, in the cln3Δ background, using conditions as in panel (A). Graphs plot mean ± SEM (n = 5), and asterisks indicate p < 0.05 (t-test) for wt vs. m4.

(C) The cln2-m4 protein shows reduced turnover. Cells harboring a CLN2-V5 plasmid (wt or m4; as in panel Diii), growing in +Met medium, were treated with 50 µg/mL cycloheximide (CHX). Cln2-V5 and G6PDH levels were measured at times indicated. The graph plots the fraction of protein remaining (mean ± SD; n = 4) with exponential trendlines; half-lives (t1/2) were calculated by fitting to an exponential decay (in Prism 7).

(D) cln2-m4 is deficient at driving polarized bud growth. Cells harboring a PGAL1-CLN2 plasmid (wt or m4) were induced with galactose for 1.5 or 3 hr. Images are from 1.5 hr. Plots show individual bud lengths with mean ± SD (n > 150); p = 10^{-29} (1.5 hr) or 10^{-33} (3 hr) by two-tailed t-test.
(E) Cells harboring $P_{\text{GAL1}}$-CLN2 or $P_{\text{GAL1}}$-CCN1 plasmids (as in Figure 3.3C) were induced with galactose for 3.5 hr. Plots show bud lengths with mean ± SD (n > 50); for all pair-wise comparisons of wt versus m4, $p < 10^{-4}$ (two-tailed t-test). Also see Figure 3.10B.
Figure 3.9. Whi5 phosphorylation and bud polarization depend on Cln2 docking.
vitro assays. GFP-tagged Whi5 was resolved into only two species (Figure 3.9Aiii), but again we observed less of the uppermost form in cln2-m4 cells. This reduced phosphorylation of Whi5 was associated with mildly reduced G1/S transcription (Figure 3.9B) and budding (Figure 3.10A), whereas DNA synthesis was unaffected (Figure 3.10A), consistent with prior findings that Cln1/2 have a more unique role in bud emergence whereas expression of Clb5/6 is sufficient to trigger DNA synthesis (Dirick et al., 1995; Moffat and Andrews, 2004; Schwob and Nasmyth, 1993). Furthermore, it is particularly noteworthy that in the absence of Cln2 (i.e., empty vector), phosphorylation of Whi5 by Cln3 was barely detectable (Figure 3.9Aii-iii), despite prior evidence that Cln3 triggers its initial inactivation (de Bruin et al., 2004; Skotheim et al., 2008). This raises the interesting possibility that Cln3-CDK phosphorylates Whi5 on only a fraction of its twelve CDK sites and that Cln1/2-CDK follow with more complete multi-site phosphorylation (see Discussion). Altogether, these findings indicate that cln2-m4 is not entirely defective but is deficient at driving full modification of substrates.

Interestingly, in these synchronous cultures, the cln2-m4 protein was initially expressed on schedule but then persisted long after the wt protein declined (Figure 3.9Aiii), suggesting that it might have a reduced turnover rate. Indeed, using a cycloheximide chase assay, the half-life was roughly doubled for the mutant protein (Figure 3.9C). Hence, Cln2 turnover might be governed by
docking interactions with specific partner proteins (see Discussion). This stabilization is also noteworthy because the increased protein levels or duration in the mutant might partially counteract its defects.

Finally, we analyzed how docking contributes to function of Cln2 in polarized bud growth. Early in the cell cycle, Cln1 and Cln2 have a special role in driving bud emergence as well as highly polarized apical growth of the new buds, which then shifts to an isotropic pattern as Cln1/2 levels decline and Clb1-6 cyclins take their place (Lew and Reed, 1993). If Cln2 is expressed continuously from a foreign promoter (e.g., $P_{GAL1}$), it can drive incessant apical growth and lead to hyperpolarized buds (Lew and Reed, 1993). We found that the ability to drive hyperpolarized growth was greatly diminished for the docking mutant, $cln2-m4$ (Figure 3.9D). Furthermore, the hyperpolarized phenotype was also observed upon expression of other members of the Cln1/2 and Ccn1 subgroups (Figures 3.9E, 3.10B), and in each case this was disrupted by the m4 mutation. Thus, docking helps each of these cyclins drive directionally persistent bud growth, which may be of particular importance in fungi that form hyphal filaments (see Discussion).
Figure 3.10. Analysis of synchronous cultures and cell polarization

(A) Budding and DNA replication, in both CLN3 and cln3Δ backgrounds, were measured following the G1 arrest and release, as in Figure 3.9A. Graphs, mean ± SEM (n = 3-6).

(B) The effect of m4 mutations on the ability of Cln1/2 and Ccn1 cyclins to drive polarized bud growth. The same experiment shown in Figure 3.9E was performed here using only 1.5 hr galactose induction. The graphs plot individual bud lengths with the mean ± SD (n > 50); p < 10^-4, by two-tailed t-test, for all pair-wise comparisons of wt versus m4.
Figure 3.10. Analysis of synchronous cultures and cell polarization (related to Figure 3.9).

A

CLN3 cells

CLN2 wt  cln2-m4  vector

budding (%)  DNA rep. (% 2C)

0  20  40  60  80  0  20  40  60  80
0  30  60  90  120  0  30  60  90  120
minutes  minutes

budding (%)  DNA rep. (% 2C)

0  20  40  60  80  0  20  40  60  80
0  30  60  90  120  0  30  60  90  120
minutes  minutes

B

1.5 hr

bud length (µm)

0  2  4  6  8  10

wt m4  wt m4  wt m4  wt m4  wt m4

P_{GAL1-cyclin:}  vector  Scer  Kwal  Kwal  Calb  Ccn1  Ccn1

wt  m4  wt  m4  wt  m4  wt  m4  wt  m4
Discussion

In this study we have identified a mutant form of the yeast cyclin Cln2 that is deficient in recognition of LP-type docking sites on CDK substrates. The detection of key residues in Cln2 was aided by the discovery that recognition of LP docking motifs is conserved in the distinct Ccn1 group. Mutation of these residues disrupts docking interactions with multiple CDK substrates, and disrupts multi-site phosphorylation of substrates \textit{in vitro}. \textit{In vivo}, these defects hamper the ability of Cln2 to drive polarized bud morphogenesis and to fully phosphorylate a key G1/S regulator. Moreover, disruption of docking by Cln2 alters the calibration of Start to cell size, such that cells delay entry into the cell cycle until they are larger than normal.

Conservation of LP docking in both Cln1/2 and Ccn1 groups attests to a selectively advantageous function. Of note, Cln3 and Cln1/2 groups likely diverged from each other after they split from the Ccn1 group (N. Buchler, personal communication), implying that LP docking existed in the prior common ancestor but was then lost in Cln3. Indeed, most non-yeast fungi (dikarya) harbor only one G1 cyclin, and initial studies suggest that they can recognize LP motifs (S. B. and P.M.P., unpublished observations). Still, LP docking may not be identical in each case, as Ccn1 bound some but not all of the same substrates as Cln1/2 (Figure 3.5D), and we have observed parallel differences in phosphorylation assays (S. B. and P.M.P., unpublished observations). Thus, as
the cyclin groups diverged, they may have developed distinct preferences for LP motif composition. Future elucidation of these specificity determinants can illuminate the evolution of these docking networks.

LP docking motifs are functionally distinct from RxL motifs (Bhaduri and Pryciak, 2011; Koivomagi et al., 2011b), which bind a hydrophobic patch (HP) on S-phase cyclins (Loog and Morgan, 2005; Schulman et al., 1998; Wilmes et al., 2004). A comparison of the HP and m4 regions, using bona-fide structures or models, suggests that they are close but separated (Figure 3.11A, B). The m4 residues begin at a ridge bordering the HP cleft and then proceed along the edge of an adjacent plateau. The non-polar Leu112 residue that is most critical in Cln2 is predicted to be solvent-exposed, and thus is well suited to mediate energetically favorable interactions with Leu/Pro-rich LP motifs. Interestingly, although RxL peptides from CDK substrates do not encroach upon the m4 plateau (Cheng et al., 2006; Lowe et al., 2002), the inhibitor protein p27, which binds cyclin A-CDK2 over a broad interface (Russo et al., 1996), does make contacts in this region as it traverses from the HP cleft toward the CDK (Figure 3.11A). Hence, it is conceivable that LP docking originated from an earlier role in assisting contact with CDK inhibitors, and then evolved an independent function. Indeed, fungal Cln proteins are thought to have evolved from a B-type cyclin precursor (N. Buchler, personal communication). Reconstruction of ancestral
Figure 3.11. Docking interfaces and models.

(A) Structures of a mammalian cyclin A-CDK2 complex, with and without the inhibitor protein p27 (PDB IDs: 1H26, 1JSU), compared to models for S. cerevisiae Cln2 and K. waltii Ccn1 generated by the I-TASSER algorithm (Roy et al., 2010). The hydrophobic patch (HP, yellow) and residues altered in the m4 mutants (red) are highlighted.

(B) Close-up of the boundary between HP and m4 regions in the predicted models, with mutated residues labeled.

(C) General schematic model for accumulation of Cln1/2-CDK activity and substrate phosphorylation in late G1 phase. See text for discussion.

(D) Disruption of Cln2 docking may simultaneously disrupt both positive output and negative regulation. See text for discussion.
Figure 3.11. Docking interfaces and models
cyclins might reveal if any intermediate forms recognized both RxL and LP motifs.

*In vitro*, the docking function of Cln2 was required for the extensive phosphorylation of substrates at multiple sites, which occurs via processive catalysis involving binding of initial phospho-peptides to the Cks1 subunit of the cyclin-CDK-Cks1 complex (Koivomagi et al., 2013; McGrath et al., 2013). Thus, *in vivo*, cell cycle events that require substrates to become highly phosphorylated are likely to be especially dependent on docking, and this may underlie some functional distinctions between different G1 cyclins. In this regard, the Whi5 phosphorylation behavior that we observed *in vivo* is especially noteworthy.

Although Cln3 initiates the inactivation of Whi5 (de Bruin et al., 2004; Skotheim et al., 2008), our analysis of Whi5 gel mobility suggests that Cln3 is not as proficient as Cln2 at triggering extensive modification of Whi5 *in vivo*, and this more potent activity of Cln2 depends at least in part on docking. A superior ability to drive full Whi5 phosphorylation could explain why Cln1/2 accelerate the pace of Whi5 chromatin dissociation and nuclear exit, after being initiated by Cln3 (de Bruin et al., 2004; Skotheim et al., 2008). A two-stage relay, in which Whi5 is phosphorylated partially by Cln3 and then more completely by Cln1/2, would be remarkably analogous to recent findings on Rb phosphorylation by cyclin D and cyclin E in animal cells (Narasimha et al., 2014), and to the sequential phosphorylation of Sic1 by Cln1/2 and Clb5 in yeast (Koivomagi et al., 2011a; Yang et al., 2013).
We found that substrate docking by Cln2 is important for initiating the cell cycle at the proper size. According to current views on cell size control in yeast, Cln1/2 are not expected to be involved in the size sensing mechanism per se but rather in the robust execution of molecular events that drive cell cycle entry once the sensing mechanism is satisfied (Jorgensen and Tyers, 2004; Turner et al., 2012). Thus, the commitment point at Start represents a brief interval in which Cln1/2-CDK activity rapidly accumulates (Figure 3.11C). Accordingly, the docking function of Cln1/2 likely contributes to a sharp and speedy transition by ensuring that full phosphorylation of substrates occurs promptly once the cyclins start to be expressed. Indeed, many key regulators of the G1/S transition are proteins with multiple CDK sites whose complete phosphorylation would benefit from docking, including not only Whi5 but also Sic1 and Cdh1 (Nash et al., 2001; Zachariae et al., 1998), whose inactivation permits the rise in Clb-CDK activity needed for DNA synthesis. Notably, the cln2-m4 mutant showed reduced interactions with some of these key regulators (Figure 3.5). Thus, our results support the view that substrate docking by Cln1/2 helps ensure that the commitment step governing cell cycle entry occurs rapidly and decisively.

It is noteworthy that the cln2-m4 phenotypes were subtler in G1 arrest/release experiments than in the first cycle of new daughter cells. A similar phenomenon was observed previously, in which ectopic Clb5 expression
triggered S phase much more readily in cells that had undergone prolonged G1 arrest than in early G1 daughters (Schwob and Nasmyth, 1993). Because arrested cells continue to grow, they can be past the critical size at the time of release, and this may alter the threshold level of cyclin-CDK activity required to pass Start (Di Talia et al., 2007).

Our observation that Cln2 docking contributes to bud formation and polarized morphogenesis fits with prior findings that Cln2 substrates include several proteins involved in cell polarization such as GEFs and GAPs for Rho GTPases (reviewed in (Enserink and Kolodner, 2010; Wang, 2009)), some examples of which (i.e., Rga1, Tus1) showed reduced binding to cln2-m4. Relatedly, G1 cyclins drive persistent polarized growth in filamentous fungi such as Ashbya gossypii (Hungerbuehler et al., 2007) and Candida albicans (Loeb et al., 1999; Zheng and Wang, 2004), where they phosphorylate secretory proteins and septins (Bishop et al., 2010; Sinha et al., 2007; Zheng et al., 2007). Substrate docking by the Cln1/2 and Ccn1 groups could help maintain high levels of substrate phosphorylation in a localized subcellular domain, and conceivably could co-localize the cyclin with substrates at sites of polarized growth.

Two sources of indirect evidence suggest that the docking interface on Cln2 promotes not only positive functions but also regulation by antagonists (Figure 3.11D). First, the cln2-m4 protein shows reduced turnover, suggesting
that the docking interface might interact with degradation machinery. A likely participant in this process is Cdc48, which binds Cln2 and stimulates its turnover (Archambault et al., 2004); unfortunately, we have not yet obtained sufficiently strong binding with Cdc48 to test if it is disrupted by the m4 mutation. Second, some phenotypic differences between wt and cln2-m4 alleles were greater in \textit{far1Δ} cells. This raises the possibility that, when comparing Cln2-wt and cln2-m4 in \textit{FAR1} cells, the defects in positive functions of cln2-m4 are partly suppressed by reduced inhibition from Far1. Of note, Far1 blocks Cln2-substrate docking (Pope et al., 2014 & Chapter 4), and hence it may engage the docking interface on Cln2. Indeed, in a recently developed \textit{in vitro} assay for Far1 inhibition (E.V. and M.L., in preparation), the cln2-m4 mutation increased the K\textsubscript{i} of Far1 by roughly ten-fold. There are precedents for a single docking region binding both positive and negative factors, including in cyclin-CDK complexes (Lowe et al., 2002; Russo et al., 1996; Schulman et al., 1998) and MAP kinases (Remenyi et al., 2005). Competition among substrates and regulators for a common docking interface may provide a simple mechanism to integrate multiple factors.

In conclusion, our findings uncover a novel substrate-docking interface, conserved among distinct G1 cyclin sub-groups, that contributes to efficient, multi-site phosphorylation of substrates and to the punctual entry into the cell cycle. The patterns of conservation among different G1 cyclins could illuminate further studies into the evolution of docking interfaces, and they also raise the
question of whether the Cln3 group lacks docking entirely or if it has docking interactions that remain to be discovered. In addition, it will be of interest to study the requirement for LP docking by G1 cyclins in filamentous fungi where polarized growth at hyphal tips shows high directional persistence. Finally, these findings provide a useful launch point for future studies into understanding how robust substrate phosphorylation promoted by cyclin-substrate docking interactions influences cell-to-cell variability of Start (Di Talia et al., 2007; Ferrezuelo et al., 2012) and the temporal coherence of distinct events at the G1/S transition (Skotheim et al., 2008).

Acknowledgements

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Materials and Methods

Yeast Strains and Plasmids
Standard procedures were used for growth and genetic manipulation of yeast (Rothstein, 1991; Sherman, 2002). Cells were grown at 30°C in yeast extract/peptone medium with 2% glucose (YPD) or galactose (YPGal), or in synthetic (SC) medium with 2% glucose and/or raffinose. Strains and plasmids are listed in the tables below. The cln2-m4 allele was introduced at the native CLN2 locus by two-step (pop-in/pop-out) allele replacement (Rothstein, 1991), using plasmid pPP4121. PCR-mediated methods (Longtine et al., 1998) were used for gene deletion and tagging endogenous gene loci.

In vivo CDK Phosphorylation Assays
As described previously (Bhaduri and Pryciak, 2011; Pope et al., 2014), cells harboring \( P_{\text{GAL1}} \)-GST-cyclin constructs and HA-tagged CDK substrates were grown in synthetic raffinose media, and then induced with 2% galactose (for 2.5 hr) to drive cyclin expression; substrates were based on the Ste5 N-terminus, with or without docking sites as indicated. Whole cell extracts were prepared, and substrate phosphorylation was assessed by SDS-PAGE and immunoblotting.

Protein Preparation, Binding, and Immunoblotting
Whole cell extracts were prepared by lysis in trichloroacetic acid as described previously (Pope et al., 2014), using 5 or 2 mL cultures (OD\textsubscript{660} ~ 0.6); total
protein concentrations were measured by BCA assay, and equal amounts (usually 20 µg) were loaded per lane.

GST co-precipitation binding assays were performed as described (Pope et al., 2014). Briefly, 10 mL cultures were treated with 2% galactose (1.5 hr) to express GST fusion proteins. Extracts were prepared by glass bead lysis, and GST fusions and co-bound proteins were collected using glutathione-sepharose beads.

For immunoblotting, proteins were resolved by SDS-PAGE and transferred to PVDF in a submerged tank. Primary antibodies were mouse anti-V5 (1:5000, Invitrogen #46-0705), anti-HA (1:1000, Covance #MMS101R), anti-GFP (1:200, Clontech # 632381) or anti-GST (1:1000, Santa Cruz Biotechnologies #sc-138), rabbit anti-G6PDH (1:1000, Sigma #A9521), and goat anti-Cdc28 (1:200 Santa Cruz #sc-6709). HRP-conjugated secondary antibodies were goat anti-mouse (1:3000, BioRad #170-6516), goat anti-rabbit (1:3000, Jackson ImmunoResearch #111-035-144), or donkey anti-goat (1:3000 Santa Cruz #sc-2020). Enhanced chemiluminescent detection used a BioRad Clarity kit (#170-5060).

**Synchronous Cultures**

Small G1 cells were purified by centrifugal elutriation as described previously (Strickfaden et al., 2007). Initial cultures (0.7-1 L) were grown in synthetic
raffinose medium. Elutriated cells were resuspended in YPD and then incubated at 30°C.

For synchronization by G1 arrest/release, CLN3 cln1Δ cln2Δ P_{MET3-CLN2} and cln3Δ cln1Δ cln2Δ P_{MET3-CLN2} cells harbored a CLN2 plasmid (wt or m4; native promoter) or empty vector. Cells were grown in –Met medium, then arrested in G1 phase by transfer to +Met medium (to repress P_{MET3-CLN2}) containing α factor (0.1 µM, 150 min), then released by washing twice and incubating in +Met medium without α factor.

**Flow Cytometry and Budding Assays**

DNA content was measured by flow cytometry using Sytox Green as described previously (Pope and Pryciak, 2013). Budding status was assayed using formaldehyde-fixed cells as described previously (Pope and Pryciak, 2013); 200 cells were counted per condition.

**mRNA Preparation and RT-qPCR Analysis**

RNA was prepared as described previously (Pope and Pryciak, 2013), and cDNA was synthesized from ~1 µg of RNA with a QuantaBio qScript kit (#95048-025); products were diluted to 5 ng/µL. Quantitative real-time PCR was performed using KAPA Biosystems SYBR FAST qPCR Master Mix (#KK4601). Reaction mixtures (10 µL) contained 5 µL of SYBR Green mix, 1.3 µL of primer mix (0.4 µM each primer), 1 µL of cDNA (5 ng), and 2.7 µL of water. Reactions were
performed in 96-well plates, in duplicate, using a BioRad CFX96 instrument; 
ACT1 mRNA served as the internal control. Data were normalized to the average 
maximum wt level.

**Cell Size Measurements**

Cell volume was measured using a Beckman Coulter Multisizer 3 (Beckman 
Coulter). Log-phase cultures were briefly sonicated, and then 10 µL was diluted 
into 10 mL of Isoton II diluent (Beckman Coulter #8546719), and 30,000 cells 
were sized per sample. Particles below 3 µm in diameter were excluded to 
ignore dead cells.

Cell diameters and bud lengths were measured microscopically. Images of 
multiple fields of live cells were captured, and then all relevant cells were 
analyzed in Image J software. Mother cell diameter was defined by a line starting 
at the midpoint of the bud neck, bisecting the mother. Unbudded cells were 
measured using the longest axis evident. Bud lengths were measured from neck 
to tip.

**In Vitro Kinase Assays**

HA₃-tagged Cln2-Cdc28 complexes were purified from yeast (PPY2443, 
PPY2444) using previous immunoaffinity methods (Koivomagi et al., 2013; 
Koivomagi et al., 2011b).
**Time-Lapse Microscopy**

Cells expressing Whi5-mCherry and Sic1-GFP, growing in SC medium, were entrapped in a CellASIC microfluidic device, as in previous studies (Doncic et al., 2011). Images were acquired every 3 min., and multiple fields were followed simultaneously. Using pre-existing software (Doncic et al., 2011; Yang et al., 2013), fluorescence data were analyzed to determine the midpoint times of Whi5 nuclear exit and Sic1 degradation. Phase-contrast images were inspected manually to determine the time of bud emergence, and Image J software was used to measure cell diameters at the midpoint of Whi5 exit and the onset of budding.
Table 3.1. Yeast strains used in Chapter III

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* Strain Background: (a) S288C/BY263 (ade2-107 his3Δ200 leu2-3,112 lys2-801 trp1Δ63 ura3-52 ssd1-d); (b) BY4741 (his3Δ1 leu2Δ0 ura3Δ0 met15Δ0); (c) W303 (ade2-1 his3Δ11,15 leu2-3,112 trp1Δ1 ura3Δ1 can1).  
† Source: (1) Moffat/Andrews; (2) Bachman/Boeke; (3) Pope/Bhaduri; (§) this study, derived from JS146-8C and JS146-16C (Doncic et al.); (†) this study, derived from MK0158 and MK0311 (Koivomagi et al.; Koivomagi et al.); (#) this study.
### Table 3.2.  Plasmids used in Chapter III

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Table 3.3 Oligonucleotide primers used for RT-qPCR analysis in Chapter III

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* Source: (1) (Sikorski and Hieter, 1989); (2) (Bhaduri and Pryciak, 2011); (3) (Takahashi and Pryciak, 2007); (4) (Pope et al., 2014)
CHAPTER IV

Regulation of cyclin-substrate docking by a G1 arrest signaling pathway and the CDK inhibitor Far1

The following Chapter contains the manuscript:


The study has been devised by and all results have been interpreted by myself, Dr. Pope and Dr. Pryciak.

Parts of Figures 4.4, 4.5, 4.6, 4.7 and 4.8 have been contributed by myself.

All other Figures have been contributed by Dr. Pope.

The manuscript had been prepared by myself, Dr. Pope and Dr. Pryciak.
Abstract

Eukaryotic cell division is often regulated by extracellular signals. In budding yeast, signaling from mating pheromones arrests the cell cycle in G1 phase (Bardwell, 2005). This arrest requires the protein Far1 (Chang and Herskowitz, 1990), which is thought to antagonize the G1/S transition by acting as a CDK inhibitor (CKI) (Peter et al., 1993; Peter and Herskowitz, 1994), although the mechanisms remain unresolved (Gartner et al., 1998). Recent studies found that G1/S cyclins (Cln1 and Cln2) recognize CDK substrates via specific docking motifs, which promote substrate phosphorylation in vivo (Bhaduri and Pryciak, 2011; Koivomagi et al., 2011b). Here, we show that these docking interactions are inhibited by pheromone signaling, and that this inhibition requires Far1. Moreover, Far1 mutants that cannot inhibit docking are defective at cell cycle arrest. Consistent with this arrest function, Far1 outcompetes substrates for association with G1/S cyclins in vivo, and it is present in large excess over G1/S cyclins during the pre-commitment period where pheromone can impose G1 arrest. Finally, a comparison of substrates that do and do not require docking suggests that Far1 acts as a multi-mode inhibitor that antagonizes both kinase activity and substrate recognition by Cln1/2-CDK complexes. Our findings uncover a novel mechanism of CDK regulation by external signals, and shed new light on Far1 function to provide a revised view of cell cycle arrest in this model system.
Background and Results

During cell cycle arrest by pheromone, Far1 is thought to act as a CDK inhibitor (CKI) that antagonizes cyclin-CDK complexes containing early cyclins (Cln1, Cln2, Cln3), which function in G1 to drive cell cycle entry (Figure 4.1A). Far1 binds these CDK complexes in vivo (Tyers and Futcher, 1993) and appeared to inhibit Cln2-CDK activity in vitro (Peter and Herskowitz, 1994), but later studies failed to detect this inhibitory effect (Gartner et al., 1998) and others suggested that Far1 might inhibit Cln3-CDK or regulate Cln2 protein levels (Jeoung et al., 1998; Valdivieso et al., 1993). Consequently, the precise effects of pheromone and Far1 on CDK function in vivo have remained unresolved. Recent studies revealed that some Cln-CDK phosphorylation events require docking interactions between Cln1/Cln2 and specific motifs in substrate proteins, including components of the mating pathway (Ste5, Ste20) and regulators of the G1/S transition (Sic1, Whi5) (Bhaduri and Pryciak, 2011; Koivomagi et al., 2011b). Therefore, we asked if 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 4.1B).

To monitor docking, we used an assay in which a GST-substrate fusion and an epitope-tagged cyclin (Cln2) were co-expressed and co-precipitated (Bhaduri and Pryciak, 2011). (Here, we took steps to prevent CLN2 expression and pheromone signaling from interfering with each other; see Experimental
Figure 4.1. Pheromone signaling disrupts Cln2-substrate interactions.

(A) Mating pheromones signal through a MAP kinase cascade, leading to phosphorylation and increased expression of Far1, which is thought to induce G1 arrest by inhibiting Cln-CDK complexes.

(B) Far1 could inhibit cyclin-substrate docking (left) or Cln-CDK kinase activity (right).

(C) Cells harboring Cln2-myc (expressed from the CYC1 promoter; see Figure 4.2) and a galactose-inducible GST fusion to the Ste20 N-terminus (Ste20*) were induced with galactose with or without pheromone (α factor) for varying times. Complexes were captured on glutathione-sepharose. Bound and input samples were analyzed by anti-myc and anti-GST blots.

(D) Binding of Cln2-myc to galactose-inducible GST-Ste20* or GST-Ste5*, induced with or without pheromone. Note that pheromone disruption of Cln2-substrate binding was lost in far1Δ cells.
Figure 4.1. Pheromone signaling disrupts Cln2-substrate interactions.
Figure 4.2. Effects of Cln2 and Far1 on pheromone response.

Pheromone signaling is reduced by deleting FAR1 or expressing CLN2 from foreign, constitutively-active promoters ($P_{CYC1}$ and $P_{ADH1}$). Strains with the indicated genotypes (PPY640, PPY892, PPY2075, PPY2076) harbored plasmids expressing CLN2 from the indicated promoters or a vector control (pPP681, pPP3203, pPP3079). Cells were treated with α factor (5 µM, 2 hr). Pheromone response was assayed using a transcriptional reporter ($FUS1$-$lacZ$); results (mean ± SD; n = 4) were normalized to the wild-type strain with vector. Note that cells lacking Far1 (far1Δ) have reduced signaling, and are sensitive to further reduction by extra CLN2. These features are eliminated by the CDK-resistant STE5-8A allele (Strickfaden et al., 2007), such that the presence or absence of Far1 (or excess CLN2) no longer affects pheromone signaling. Thus, by using STE5-8A strains, it is possible to compare the effect of Far1 on Cln2-substrate interactions without complicating secondary effects on pheromone signaling due to the presence or absence of Far1 and/or Cln2.
Figure 4.2. Effects of Cln2 and Far1 on pheromone response.

A

B

- vector
- $P_{CYC1}$-CLN2
- $P_{ADH1}$-CLN2

pheromone response (%)
Procedures and Figure 4.2.) First, we tested a GST fusion to a Cln2-binding fragment of Ste20 (residues 72-333, designated Ste20*)(Bhaduri and Pryciak, 2011), expressed from an inducible promoter ($P_{\text{GAL1}}$). Without pheromone, we observed Cln2-myc binding as soon as GST-Ste20* expression was detected, but binding was strongly inhibited when pheromone was included (Figure 4.1C). (Note, total Cln2 levels were often reduced by prolonged pheromone treatments, as in earlier studies (Valdivieso et al., 1993), so we used short treatment times where possible to minimize this effect.) Pheromone also inhibited Cln2-myc binding to another, similar GST fusion (GST-Ste5*), in which the Cln2 docking site from Ste20 was replaced with one from Ste5 (Figure 4.1D). Remarkably, this inhibition was not observed in far1Δ cells (Figure 4.1D). Therefore, pheromone signaling can disrupt Cln2-substrate binding interactions in a manner that depends on Far1.

Next, we tested the role of regulatory phosphorylation sites in Far1 (Figure 4.3A): phosphorylation at residue T306 by the MAPK Fus3 promotes Far1 function, whereas phosphorylation at residue S87 by CDK triggers its degradation (Gartner et al., 1998). We introduced non-phosphorylatable Ala mutations at these sites, tested previously in an N-terminal fragment of Far1 (Gartner et al., 1998), into full-length Far1 expressed from the native FAR1 locus. As expected, the T306A mutant was defective at pheromone arrest whereas the
Figure 4.3. Far1 inhibition of docking correlates with G1 arrest ability.

(A) Pheromone triggers phosphorylation of Far1 at T306, which promotes G1 arrest, whereas CDK phosphorylates Far1 at S87, which promotes its degradation (Gartner et al., 1998).

(B) The indicated FAR1 strains were tested for pheromone arrest. Cell lawns were overlaid with disks containing 20 µL of α factor (20 or 100 µM), and incubated at 30˚C for 2 days.

(C, D) Binding of Cln2-myC to GST-Ste20* or GST-Ste5* was analyzed (as in Figure 4.1) using strains with different FAR1 alleles, in the presence or absence of pheromone.

(E) Far1 disrupts Cln2 binding to Sic1 and Whi5. Sic1ΔC (residues 1-214) lacks its CDK-inhibitor domain but includes its Cln1/2 docking site (Bhaduri and Pryciak, 2011; Koivomagi et al., 2011b). Pheromone was omitted from these assays because it affected GST-Whi5 levels. Also see Figure 4.4A.

(F) Far1 disrupts binding of GST-Cln2 to full-length Ste20. Strains harbored a PGAL1-GST-CLN2 plasmid or GST vector, plus V5-tagged Ste20. To reduce effects of pheromone on Cln2 levels, we used a truncated Cln2 (residues 1-372), which lacks its destabilizing C-terminus (Lanker et al., 1996). Cells were induced with galactose ± pheromone; bound complexes were captured and analyzed by anti-V5, anti-GST, and anti-Cdc28 blots. Graphs quantify relative levels of Ste20 binding (mean ± SEM; n = 3). Also see Figure 4.4B-E.
Figure 4.3. Far1 inhibition of docking correlates with G1 arrest ability.

A

B

C

D

E

F
S87A mutant remained functional (Figure 4.3B); the S87A T306A double mutant showed an intermediate phenotype, indicating that T306 phosphorylation is not absolutely required if Far1 is stabilized by the S87A mutation. When we tested Cln2-substrate binding in these strains, we observed several notable features (Figures 4.3C, 4.3D). First, the T306A mutation blocked the ability of pheromone to disrupt Cln2-substrate interactions, whereas the S87A mutation increased this disruptive effect. Second, this increased potency of the Far1-S87A mutant was evident even in the absence of pheromone. Third, the S87A mutation partially suppressed the defect of the T306A mutation, consistent with the arrest phenotypes. (Note that the effect of pheromone in the S87A T306A double mutant cannot be due to Far1 activation by phosphorylation at T306, and instead it may reflect elevated FAR1 transcription (Chang and Herskowitz, 1990).) The ability of Far1-S87A to reduce Cln2-substrate binding even without pheromone was unanticipated, but it may imply that the unmodified wild-type protein is partially active (rather than inactive) and that this activity becomes more evident in the S87A mutant due to higher protein levels or presence in a greater fraction of cells (see below). Overall, the binding results mirror the G1 arrest phenotypes, implying that interference with Cln2-substrate docking relates to the arrest function of Far1. In further support of this view, we found that Far1 (especially Far1-S87A) also disrupted binding of Cln2 to the G1/S regulators Sic1 and Whi5 (Figures 4.3E, 4.4A), which are CDK substrates with Cln1/2 docking sites similar to those in Ste5 and Ste20 (Bhaduri and Pryciak, 2011; Koivomagi et al., 2011b).
Figure 4.4. Effects of Far1 on Cln2-substrate binding interactions.

(A) Far1 inhibits binding of Cln2 to GST-Whi5 fragments. Strains (PPY2322, PPY2326, PPY2329) harbored Cln2-myc (pPP3203) plus GST fusions to Whi5 fragments (pPP4150, pPP4151, pPP4152) or GST alone (pPP2154). Binding was assayed by glutathione-sepharose capture followed by anti-myc and anti-GST blots. Note that Far1-S87A inhibits Cln2 binding to all Whi5 fragments, similar to results using full-length Whi5 (Figure 4.3E), and with all GST-Whi5 fusions a truncated product is also observed (marked by **). Phosphorylation of Whi5 by Cln2-CDK depends on docking (Koivomagi et al., 2011b), and it has at least one docking motif in the 121-150 region (Bhaduri and Pryciak, 2011), but it could also have additional motifs that confer weak binding to the 1-125 fragment.

(B, C) Binding of GST-Cln2 to full-length substrate proteins requires docking sites. Galactose-inducible GST or GST-Cln2 (residues 1-372) was co-expressed in far1Δ cells (PPY2327) with V5-tagged forms of full-length substrates (Ste20 or Ste5) expressed from their native promoters. Binding was assayed by glutathione-sepharose capture followed by anti-V5 and anti-GST blots. In each panel, the WT and mutant substrates were analyzed in parallel but were separated by additional lanes in the original gels. Panel B shows binding to full-length Ste20-WT or a docking site mutant (mut3; (Bhaduri and Pryciak, 2011)). Plasmids were pPP2154, pPP3573, pPP3267, and pPP3368. Panel C shows binding to full-length Ste5-WT or a docking
site mutant (LLPP to AAAA; Bhaduri and Pryciak, 2011). Plasmids were pPP2154, pPP3573, pPP3266, and pPP3761.

(D, E) Far1 inhibits binding of GST-Cln2 to full-length Ste20 and Ste5. Strains with the indicated FAR1 genotype (PPY2327, PPY2330, PPY2340, PPY2358, PPY2359) harbored galactose-inducible GST (pPP2154) or GST-Cln2 (pPP3573), plus V5-tagged Ste20 (pPP3267) or Ste5 (pPP3266). Cells were induced with galactose ± pheromone for 90 minutes, and then bound complexes were captured and analyzed by anti-V5, anti-GST, and anti-Cdc28 blots. Graphs show the relative level of substrate binding (mean ± SEM) from three (D) or four (E) experiments. Panel D is identical to Figure 4.3F and is repeated here for comparison to the other panels.
Figure 4.4. Effects of Far1 on Cln2-substrate binding interactions.
We confirmed these findings via reciprocal assays in which a GST-Cln2 fusion was used to co-precipitate full-length substrates (Ste20 and Ste5). Binding of each substrate to GST-Cln2 required their docking sequences (Figures 4.4B, 4.4C) and was strongest in $far1\Delta$ cells, weakest in $FAR1$-S87A cells, and intermediate in $FAR1$-WT cells (Figure 4.3F, 4.4). This trend was seen even without pheromone treatment, further reinforcing the notion that unmodified Far1 is partially active. Importantly, Far1 did not affect binding of Cln2 to its partner CDK molecule, Cdc28 (Figures 4.3F, 4.4E). The effect of pheromone was less evident in these experiments than when using the previous (reverse) procedure, perhaps because chronic Cln2 expression can induce Far1 degradation, counteracting its activation by pheromone. Overall, however, the results confirm the disruptive effect of Far1 and argue that it blocks interactions between intact Cln2-CDK complexes and their substrates.

Because Far1 binds Cln-CDK complexes (Gartner et al., 1998; Peter et al., 1993; Tyers and Futcher, 1993), we asked if Far1 and substrates bind Cln2 competitively, and if Far1 outcompetes substrates via higher concentration or affinity. First, we compared their concentrations by marking Far1 and substrates with the same epitope tag (3xV5). Far1 levels ranged between those of Ste20 and Ste5, depending on whether it had been induced by pheromone or stabilized by the S87A mutation (Figure 4.5A). Next, we used cells that simultaneously expressed V5-tagged forms of Far1 and Ste20 to compare their binding to Cln2
The results suggest that Far1 binds Cln2 more favorably, as total Far1-WT was much less abundant than Ste20 and yet it bound Cln2 at equal or greater levels. Similarly, Far1-S87A was comparably abundant to Ste20 yet showed disproportionately greater binding to Cln2. When comparing WT and S87A forms of Far1, the increased Cln2 binding to the S87A mutant was accompanied by reduced binding to Ste20, implying that Far1 competes with Ste20. Indeed, pheromone caused increased Cln2-Far1 binding and reduced Cln2-Ste20 binding. Collectively, these results suggest that Far1 binds Cln2 in a way that is mutually exclusive with Cln2-substrate docking, and that the preferential binding of Far1 allows it to outcompete substrates.

We reasoned that Far1 should be in excess of Cln2 in order to effectively outcompete Cln2-substrate interactions. Therefore, we compared their levels as cells approached the critical point of cell cycle commitment, or “Start”. Using synchronous cultures in which both Far1 and Cln2 had the same epitope tag, we monitored protein levels and the ability of cells to arrest in G1 in response to pheromone (Figures 4.5C, 4.6). Far1 was generally in large excess over Cln2 as cells approached Start, and a sharp increase in Cln2 corresponded to the first appearance of committed cells. It did not seem that Cln2 must reach peak levels or exceed Far1 for cells to pass Start, but rather it only must begin to accumulate. This pattern fits previous findings that Start occurs simultaneous with CLN2 promoter firing (Doncic et al., 2011), and is reminiscent of the
Figure 4.5. Far1 outcompetes substrates for binding to Cln2.

(A) Far1, Ste5, and Ste20 were tagged with the identical 3xV5 tag to compare protein levels. Far1 (WT or S87A) was expressed from its native genomic locus; Ste5 and Ste20 were expressed from their native promoters on low copy number plasmids. Whole cell extracts were prepared and equivalent amounts of total protein were analyzed by SDS-PAGE and anti-V5 blots.

(B) Strains with V5-tagged Far1 or Far1-S87A harbored a V5-Ste20 plasmid plus galactose-inducible GST-Cln2 or GST vector. Cells were induced with galactose with or without pheromone, and then binding of Far1 to GST-Cln2 was assayed.

(C) Strains with V5-tagged Far1 and Cln2 were synchronized by arrest in mitosis (using a cdc15-2 mutant). At various times after release, aliquots were taken to assess protein levels and then treated with pheromone to assess whether they could still arrest in G1 or had passed Start (committed). Signal levels in the two blots are directly comparable, as all steps were performed in parallel using equal protein loading. Graphs show mean ± SEM (n = 4-6). See Figure 4.6 for additional tests.
Figure 4.5. Far1 outcompetes substrates for binding to Cln2.

A

V5-tagged protein:

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whole cell extracts

light

dark

B

Far1-V5:

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total protein

Ste20-V5

Far1-V5

bound protein

Ste20-V5

Far1-V5

GST-Cln2

C

M-phase (cdc15) block & release

minutes: 0 10 20 30 40 50 60 70 80 90

Far1-V5

Cln2-V5

Far1-S87A-V5

Cln2-V5

% committed

minutes: 0 30 60 90

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Figure 4.6. Far1 vs. Cln2 levels in synchronous cultures.

Using strains in which Far1 and Cln2 are tagged with the identical 3xV5 tag, cells were synchronized by arrest in mitosis (using either \textit{cdc15} or \textit{P}_{\text{GAL1}}\text{-}\text{CDC20}), and then released. At various times after release, aliquots were taken to assess protein levels and then were treated with pheromone to assess whether they could still arrest in G1 or had passed Start (committed). In each column the blots are directly comparable, as the experiments were performed in parallel, the individual gels had the same amount of protein loaded per lane, and the blots were processed in parallel and exposed simultaneously to a single film. For the same reasons, the four \textit{cdc15}-2 blots are also directly comparable to each other. Results shown are representative of two or more independent experiments. Graphs show mean ± SEM (n = 4-6; left) or mean ± range (n = 2; middle and right). Note that the degree of delay in commitment caused by the Far1-S87A mutant varied with experimental context; it also varied among independent isolates of \textit{BY4741 cdc15}-2 strains, and so we combined results from two \textit{FAR1}-\textit{wt} strains (PPY2393, PPY2394) and four \textit{FAR1}-S87A strains (PPY2395, PPY2396, PPY2425, PPY2426). The data at left are identical to Figure 4.5C and are repeated here to facilitate comparison.
Figure 4.6. Far1 vs. Cln2 levels in synchronous cultures.
mammalian cell restriction point occurring at very low levels of cyclin E (Ekholm et al., 2001; Martinsson et al., 2005). Thus, it may be necessary for Far1 to substantially exceed cyclin levels to prevent Start, whereas cyclin levels may not need to exceed Far1 to pass Start, perhaps because the positive feedback loop governing Cln1/2 expression (Skotheim et al., 2008) makes them destined to overwhelm Far1 once their expression begins. In accord with recent work (Doncic et al., 2011), the Far1-S87A mutant caused mild delays in commitment and Cln2 expression, though to varying degrees (Figures 4.5C, 4.6). Notably, the Far1-S87A protein was not strongly over-expressed compared to peak levels of Far1-WT, but it was present over a broader range of the cell cycle (Figures 4.5C, 4.6). Hence, the increased inhibitory activity of Far1-S87A seen in preceding experiments (using asynchronous cultures) may primarily reflect an increase in the fraction of cells expressing Far1 rather than in its concentration.

CDK phosphorylation of both Ste20 and Ste5 alters their electrophoretic mobility (Bhaduri and Pryciak, 2011; Oehlen and Cross, 1998; Strickfaden et al., 2007; Wu et al., 1998). By using extended electrophoresis to better resolve Ste20 forms, we found that pheromone and Far1 inhibited Cln2-driven phosphorylation (Figures 4.7A, 4.8A). Specifically, Cln2 expression in far1Δ cells converted Ste20 to its slowest mobility, phosphorylated form. Pheromone had no effect in far1Δ cells, but it reduced Ste20 phosphorylation in FAR1-WT cells. In FAR1-S87A cells, Ste20 phosphorylation was reduced even without pheromone
Figure 4.7. Effects of Far1 and pheromone on CDK phosphorylation in vivo using substrates with native and artificial docking interactions.

(A) Extracts of cells harboring V5-Ste20 and galactose-inducible GST-Cln2, as in Figure 4.3F, were analyzed by extended electrophoresis to resolve the extent of Ste20 phosphorylation triggered by Cln2 expression. See Figure 4.8A for replicates.

(B) CDK inhibition alone does not disrupt Cln2-Ste20 binding. Strains with a drug-sensitive CDK (cdc28-as2) harbored V5-tagged Ste20 plus galactose-inducible GST-Cln2 or GST vector. Cells were induced with galactose either with or without the ATP analog 1-NM-PP1 (15 µM), and GST fusions were captured. Total and bound Ste20 were analyzed by anti-V5 blots.

(C) The indicated FAR1 strains harbored a plasmid expressing GST-Cln2 with an attached leucine half-zipper (lz), plus a plasmid expressing an HA-tagged CDK substrate with either the matching half-zipper or an LP-type Cln1/2 docking site (see Figure 4.8Eii). Cultures were pre-incubated for 2 hr ± α factor (0.1 µM), and then induced with galactose for 2 hr. Substrate phosphorylation (Bhaduri and Pryciak, 2011) and GST-Cln2 expression was monitored by anti-HA and anti-GST blots, respectively. Figure 4.8C shows that leucine zipper binding is resistant to pheromone and Far1.

(D) FAR1-S87A and far1Δ strains co-expressing GST-(lz)-cyclins with an HA-tagged substrate (Figure 4.8Eii) were induced with galactose for 2.5 hr. Levels of GST-(lz)-cyclins were monitored in each experiment; one
representative anti-GST blot is shown. Note that, aside from effects of Far1, these results confirm that cyclin docking drives substrate use, because switching the docking site alters which cyclins are effective, as seen previously (Bhaduri and Pryciak, 2011).

(E) GST-(lz)-cyclin plasmids were introduced into a strain with V5-tagged Far1-S87A. Cultures were induced with galactose for 1.5 hr and then association of Far1 with the GST-tagged cyclin was assayed.

(F) Strains co-expressed GST-(lz)-Cln2 with HA-tagged substrates that each show only two mobility forms (see Figure 4.8Eiii), which makes it easier to quantify phosphorylation. Cultures were pre-incubated with α factor (0.1 µM, 30 min.), and then induced with galactose (40 min). Graphs (mean ± SEM, n = 3-4) show the signal in the upper band as a percentage of the total signal (% phos.).
Figure 4.7. Effects of Far1 and pheromone on CDK phosphorylation in vivo using substrates with native and artificial docking interactions.

A. P_{GAL1-GST:} vector Cln2

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B. cdc28-as2

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C. P_{GAL1-GST-(iz)-Cln2:} far1Δ FAR1 FAR1-S87A

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G. P_{GAL1-GST-(iz)-cyclin:} none Cln2 Cln1 Cln3 Clb5 Clb2

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treatment, and pheromone caused a further reduction. The T306A mutant was ineffectual, 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. Chemical inhibition of CDK activity was not sufficient to reduce Cln2-Ste20 binding, and Far1-S87A was equally disruptive with and without CDK inhibition (Figure 4.7B), suggesting that reduced binding causes reduced phosphorylation rather than vice-versa. Notably, to our knowledge these results provide the first demonstration that pheromone and Far1 reduce phosphorylation of CDK substrates in vivo (see Discussion). In contrast, we saw no reduction in phosphorylation of the Cln1/2 C-termini (Figure 4.8B) or Far1 itself (Figure 4.5B, top), indicating that Far1 does not inhibit all Cln-CDK phosphorylation events equally.

Finally, we asked if the ability of Far1 to disrupt substrate phosphorylation is due to inhibition of Cln2-substrate docking, or Cln2-CDK kinase activity, or a combination of both. To address this point, we compared CDK substrates with and without Cln2 docking motifs. Using an approach elaborated in Chapter 3, we replaced a native docking interaction with a foreign leucine zipper (Figure 4.8Eii), thereby allowing phosphorylation of a single substrate to be driven by either a native cyclin docking site or an artificial linkage. Then, we analyzed phosphorylation driven by different cyclins, and the effects of Far1. When the
substrate harbored a native “LP”-type Cln2 docking site, its phosphorylation was inhibited strongly by Far1-S87A, but when the leucine zipper was used, the degree of inhibition was substantially reduced, though not eliminated (Figures 4.7C, 4.7D). These results imply that Far1 inhibits substrate docking strongly, with a residual effect on some non-docking function such as CDK kinase activity. This residual effect might also signify a reduction in kinase processivity mediated by the Cks1 subunit of the CDK complex (Koivomagi et al., 2013; McGrath et al., 2013), though it was still evident when the role of Cks1 was presumably blocked (by changing threonine phosphorylation sites to serine; Figure 4.8D). It is also notable that this residual effect was only seen with the G1/S cyclins Cln1 and Cln2 (Figure 4.7D), even though Far1-S87A could bind all cyclins (Figure 4.7E). Interestingly, however, when the substrate contained an “RxL” docking motif favored by S-phase cyclins such as Clb5 (Bhaduri and Pryciak, 2011; Loog and Morgan, 2005; Wilmes et al., 2004), Far1- S87A could mildly inhibit Clb5-driven phosphorylation (Figure 4.7D), again suggesting that docking-dependent phosphorylation is more susceptible to inhibition by Far1. To help quantify the extent of phosphorylation, we performed related experiments using a variant substrate with only two electrophoretic mobility forms: unphosphorylated and phosphorylated (Figure 4.7F). Here, pheromone treatment of Far1-S87A cells almost completely reversed phosphorylation driven by the native Cln2 docking site (Figure 4.7F, top), but had only a mild effect when the leucine zipper was used (Figure 4.7F, bottom). Collectively, these results suggest that Far1 can
reduce CDK phosphorylation of substrates irrespective of docking, but substrates that require docking are especially sensitive to Far1. Therefore, Far1 may engage cyclin-CDK complexes in a way that simultaneously disrupts both substrate recognition and kinase activity.
Figure 4.8. Pheromone/Far1 effects on CDK phosphorylation in vivo.

(A) Far1 and pheromone inhibit Ste20 phosphorylation by Cln2-CDK. Three repetitions of the experiment in Figure 4.7A are shown to demonstrate reproducibility of the patterns observed.

(B) Far1 and pheromone do not affect phosphorylation of Cln1/2 tails. Cultures of far1Δ or FAR1-S87A strains (PPY2327, PPY2330) harboring PGAL1-GST-cyclin plasmids (pPP3572 or pPP3749) were induced with galactose ± α factor. Extracts were analyzed by anti-GST blot. Bands indicate the extent of phosphorylation at CDK sites in the cyclin C-termini.

(C) Far1 and pheromone do not inhibit leucine zipper-mediated binding. Strains (as in B) expressed GST-(lz)-Cln2 or GST alone, plus a myc-tagged CDK substrate consisting of a Ste20Ste5PM chimera with either an LP-type docking site or a leucine half-zipper (panel Ev). Plasmids: pPP2154, pPP3916; pPP3218, pPP3979. Binding was assayed after induction with galactose ± α factor.

(D) The ability of Far1-S87A to partially reduce phosphorylation driven by a leucine zipper was compared for two substrates (see panel Eiv): one with WT phosphorylation sites (5 SP and 3 TP) and one (“all SP”) in which the 3 TP sites were converted to SP, to block Cks1 from recognizing phospho-Thr as priming sites (Koivomagi et al., 2013; McGrath et al., 2013). Strains: PPY2327, PPY2330. Plasmids: pPP2154, pPP3916; pPP3877, pPP4071.
(E) Substrates used in Figures 4.7 and 4.8. (i) CDK phosphorylation sites and the “LP”-type Cln1/2 docking site in Ste5 (Bhaduri and Pryciak, 2011), from which other substrates are derived. (ii) Top, substrates used in Figures 4.7C-D. The native LP docking site in Ste5 is replaced with motifs shown. The recipient fragment, Ste5 1-260, excludes binding and phosphorylation sites for the MAPK Fus3 (in Ste5 267-330) (Bhattacharyya et al., 2006), preventing pheromone-induced phosphorylation from obscuring changes in CDK-mediated phosphorylation. Bottom, cartoons of cyclin docking. (iii) Substrates used in Figure 4.7F. Deleting the PM/NLS domain (Winters et al., 2005) causes the substrate to show only two mobility forms, which simplifies quantification. The top substrate has an “ND” mutation (Bhattacharyya et al., 2006) to prevent MAPK binding. (iv) Substrates used in Figure 4.8D. In “all SP”, the 3 TP (Thr-Pro) sites in the WT Ste5 sequence are changed to SP (Ser-Pro). (v) Top, Ste20, which is analyzed in Figures 4.7A and 4.8A. Bottom, binding partners used in Figure 4.8C. A Ste20$^{Ste5P}$ chimera (Bhaduri and Pryciak, 2011) acted as recipient for an LP dock or a leucine zipper, to compare effects of Far1 on binding by each motif. (These chimeras were used because the phosphorylation substrates analyzed in Figures 4.7C-D show variable behavior in binding assays.)
Figure 4.8. Pheromone/Far1 effects on CDK phosphorylation in vivo.
Discussion

This study addresses long-standing uncertainties about how yeast pheromone signaling and the presumed CKI protein Far1 promote cell cycle arrest. Our findings reveal an unsuspected mode of CDK regulation, in which an extracellular signal stimulates an inhibitory factor, Far1, to disrupt interactions of specific cyclin-CDK complexes with substrates. Far1 appears to disrupt Cln2-substrate docking by binding Cln2 more favorably so that it outcompetes substrates, and mutant analyses suggest that this effect parallels its ability to mediate G1 arrest. Because Cln1/2 docking enhances substrate phosphorylation (Bhaduri and Pryciak, 2011; Koivomagi et al., 2011b), inhibition of docking by Far1 should contribute to reduced substrate phosphorylation in vivo, in addition to any effects of Far1 on CDK activity per se. Indeed, our findings suggest that Far1 is a multi-mode inhibitor that separately disrupts both CDK activity and substrate docking. This combined effect raises the possibility that Far1 has the strongest inhibitory effect on substrates that are most dependent on docking, which might include proteins with inherently poor (e.g., non-consensus) phosphorylation sites or those that must be phosphorylated at multiple positions. Similar themes could also apply to other kinases.

We find that the ability of Cln1/2-CDK to phosphorylate substrates in vivo can be inhibited by pheromone and Far1. Remarkably, to our knowledge this is
the first such demonstration. Although there are numerous prior examples in which CDK substrates are unphosphorylated in pheromone-arrested cells, this can be explained by the fact that cyclins are not expressed in G1 phase, and hence it does not necessarily indicate that the kinase activity of cyclin-CDK complexes is reduced. Here, by expressing cyclins independent of cell cycle position, we could detect regulation of phosphorylation by a set amount of Cln1/2-CDK in vivo. Also, by linking different cyclins to substrates using a common leucine zipper, we could compare their sensitivity to Far1. Of note, compared to Cln1/2-CDK, Cln3-CDK seemed less susceptible to Far1 inhibition, which could underlie different roles for these cyclins in driving cell cycle re-entry after pheromone arrest (Doncic and Skotheim, 2013).

Surprisingly, Far1 could partially interfere with Cln2-substrate binding and phosphorylation even without pheromone treatment. Prior findings implied that Far1 must be activated, because G1 arrest required pheromone-induced phosphorylation of Far1 at T306 (Gartner et al., 1998) and FAR1 over-expression was not sufficient (Chang and Herskowitz, 1992). We suggest a new interpretation in which unphosphorylated Far1 is partially active but is less potent than when phosphorylated at T306. This view is supported by our binding and phosphorylation data as well as by the partial arrest observed in FAR1-S87A T306A cells, which shows that T306 phosphorylation is not absolutely essential.

It is also relevant to findings that far1Δ cells show accelerated entry into the cell
cycle (Alberghina et al., 2004) and that FAR1-S87A cells show a delay in Start (Doncic et al., 2011). Because Far1 is expressed only during a narrow pre-Start window of the cell cycle (McKinney et al., 1993), the partially active state would normally be restricted to cells poised for G1 arrest, but detection of this state was enhanced when using the stabilized S87A mutant, which is expressed in a larger fraction of cells. This mutant also revealed that inhibitory effects of Far1 are at least partly independent of T306 phosphorylation. Yet, T306 phosphorylation makes Far1 a more potent inhibitor, likely via enhanced binding to CDK complexes (Gartner et al., 1998) and possibly via engaging the phospho-threonine binding pocket in Cks1 (Koivomagi et al., 2013; McGrath et al., 2013).

The specific mechanism by which Far1 disrupts Cln2 docking is not yet known. Currently, there are no structural data on the Cln2-substrate binding interface, but the short docking motifs (Bhaduri and Pryciak, 2011; Koivomagi et al., 2011b) likely bind a peptide-recognition pocket on the cyclin, as with RxL motif recognition by S-phase cyclins (Lowe et al., 2002). Thus, Far1 could displace substrates either by having a higher affinity docking peptide or by interacting with a broader region of Cln2 in a way that obscures peptide recognition. The latter view may be favored by the fact that two separate parts of Far1 are required to bind Cln2 (Peter et al., 1993). This view is also reminiscent of mammalian CKI proteins p21 and p27, whose RxL sequences contribute to cyclin binding and CDK inhibition (Adams et al., 1996; Chen et al., 1996;
Wohlschlegel et al., 2001), but as only a small part of a much larger binding interface involving both the cyclin and CDK subunits (Russo et al., 1996). In fact, such multipartite interactions may have contributed to confusion about inhibitory mechanisms for both Far1 and p21/p27. Namely, complexes of p21 with cyclin-CDK sometimes retained kinase activity (Harper et al., 1995; Zhang et al., 1994), leading to speculation that under such conditions the CKI might contact only the cyclin and not the CDK (Morgan, 1996), a notion later supported by p21 mutants that bind only the cyclin (Chen et al., 1996). Analogous heterogeneity of Far1-Cln-Cdc28 complexes might explain why kinase inhibition was observed in some studies (Peter and Herskowitz, 1994) but not others (Gartner et al., 1998). In addition, the use of generic substrates that do not require docking (e.g., histone H1) would have bypassed the ability of Far1 to regulate this step in either study.

We suggest that Far1 engages the cyclin-CDK complex in a way that disrupts multiple distinct functions, including both substrate docking and kinase activity but perhaps also others such as Cks1-mediated processivity (Koivomagi et al., 2013; McGrath et al., 2013). Multiple concerted effects may help ensure maximal inhibition. Thus, in future studies using *in vitro* assays, it will be important to compare substrates with a range of requirements, in order to dissect the effect of Far1 on total kinase activity, utilization of docking sites, kinase processivity, and multi-site phosphorylation. Investigation of these issues will further illuminate how differential regulation of distinct mechanistic steps in
substrate phosphorylation can provide additional layers of control that fine-tune protein kinase networks.

Acknowledgements

We thank Gustav Ammerer, Alejandro Colman-Lerner and Jan Skotheim for plasmids, Matt Winters for technical assistance, and Dan McCollum for helpful discussions.
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 except as indicated otherwise. Strains and plasmids are listed in tables below. A two-step (pop-in/pop-out) allele replacement method (Rothstein, 1991) was used to introduce the following mutant alleles at their native genomic loci: STE5-8A, FAR1-S87A, FAR1-T306A, FAR1-S87A T306A, cdc15-2, and cdc28-as2. For Cdc28 inhibition 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) caused slow growth and cell shape defects. PCR-mediated gene deletion and tagging used methods described previously (Longtine et al., 1998).

To add epitope tags at endogenous FAR1 and CLN2 loci, a PCR-generated cassette containing the 3xV5 tag and an antibiotic resistance gene (kanMX6 or natMX6) was integrated downstream of the coding sequence. To construct $P_{\text{GAL1}}$-CDC20 strains, the promoter of the essential cell cycle gene CDC20 was replaced with a regulated promoter ($P_{\text{GAL1}}$) using a PCR-generated cassette marked with the K. lactis URA3 gene ($URA3^{Kl}$). To promote cyclin interaction with substrates that lack native docking sites, we used a weak leucine zipper ($K_d \sim 800$ nM) (Acharya et al., 2002; Bashor et al., 2008), wherein two heterodimerizing sequences called R34 and E34(N) (Bashor et al., 2008) were fused to
the cyclin and substrate, respectively; further details will be described elsewhere (S.B. and P.M.P., in preparation).

**GST Co-precipitation Binding Assays**

Cultures (25 mL) were grown in synthetic media with 2% raffinose. Expression of GST fusion proteins from $P_{GAL1}$-GST plasmids was induced with 2% galactose, with or without 10 nM $\alpha$ factor, for 1.5 to 3 hours. For experiments using the $cdc28$-as2 allele, cultures were also treated with 15 $\mu$M 1-NM-PP1. Cells were harvested and stored at -80˚C, then lysed by glass bead beating using a Fast-Prep apparatus (20 sec. at 4 m/s) and a non-ionic detergent buffer described previously (Lamson et al., 2002). Aliquots were removed to provide input controls, and then GST fusions and co-bound proteins were collected using glutathione-sepharose beads (GE Healthcare #17-0756-01).

For experiments in which we wished to examine the effects of pheromone signaling on Cln2 binding interactions, one complexity arises from the fact that the mating pathway and Cln1/2-CDK activity are mutually antagonistic. That is, pheromone-arrested cells do not express the $CLN1/CLN2$ genes and hence lack Cln1/Cln2 proteins (Wittenberg et al., 1990), whereas Cln1/2-expressing cells inhibit pheromone signaling and degrade Far1 (Henchoz et al., 1997; McKinney et al., 1993; Oehlen and Cross, 1994). Therefore, to allow us to vary experimental conditions without affecting protein levels and signaling responses, we circumvented these antagonistic effects as follows: (i) $CLN2$ was expressed
from a constitutively-active promoter ($P_{CYC1}$ or $P_{TEF1}$); (ii) the ability of Cln2 to inhibit pheromone signaling (see Figure 4.2) was prevented by using strains with the $STE5$-8A allele (Strickfaden et al., 2007), which encodes a CDK-resistant form of the pathway scaffold protein, Ste5; and (iii) our initial experiments used strains with the $FAR1$-S87A allele (Gartner et al., 1998), which is resistant to CDK-triggered degradation. Subsequent experiments probed the role of Far1 by using strains with $far1\Delta$ and other $FAR1$ alleles.

**Synchronous Culture Experiments**

For experiments in $P_{GAL1}$-$CDC20$ strains, cells were grown in liquid YPGal medium (containing 2% galactose) and then arrested in M phase by pelleting, resuspending in YPD medium (2% glucose), and incubating for 3 hr. Cultures were released by two rounds of pelleting and washing in YPGal, followed by final resuspension in YPGal. At 10-minute intervals, aliquots were removed to prepare protein samples and test cell cycle commitment. At each time point, 2 mL was pelleted and immediately frozen in liquid nitrogen (and whole cell extracts were prepared later); a separate 3 mL aliquot was treated with $\alpha$ factor (0.2 µM) and incubated at 30°C, and then at 120 minutes after release all treated aliquots were fixed by adding formaldehyde to 3.7%. Cell cycle commitment was scored as the percentage of cells ($n = 200$) that failed to arrest in G1 (i.e., as unbudded cells) after the pheromone treatment.
For experiments in *cdc15-2* strains, cultures were grown at 25°C, arrested at 37°C for 3 hr, then released by transfer to 25°C (using shaking water baths). Aliquots were taken at 10-min intervals to prepare protein samples and test cell cycle commitment as described above, except that 10 µM α factor was used for the *BAR1* strains in the W303 background.

**Whole Cell Extracts**

Whole cell extracts were prepared by a modified version of a previous protocol (Lee and Dohlman, 2008). Here, 300 µL of trichloroacetic acid (TCA) buffer (10 mM Tris-HCl, pH 8.0, 10% TCA, 25 mM ammonium acetate, 1 mM Na₂EDTA) was added directly to frozen cell pellets (usually from 2 mL culture), and incubated on ice for 10 minutes. Samples were pelleted in a microcentrifuge for 10 min at 4°C. The pellet was resuspended in 75 µL Resuspension Buffer (0.1 M Tris.HCl, pH 11.0, 3% SDS), boiled for 5 min, allowed to cool at room temperature for 5 minutes, then re-centrifuged for 30 sec. The supernatant (60 µL) was transferred to a new tube, 10 µL was reserved to assay protein concentration by a Pierce BCA Protein Assay Kit (#23225), and then 50 µL of 2x SDS Sample Buffer was added to the remainder. Equivalent amounts of total protein (generally 20 µg) were loaded in each lane.
Immunoblotting

Protein samples were analyzed by SDS-PAGE, and transferred to PVDF using a submerged tank device. Myc-tagged proteins were detected with rabbit anti-myc (1:200 Santa Cruz Biotechnologies #sc-789) and HRP-conjugated goat anti-rabbit (1:3000, Jackson ImmunoResearch #111-035-144) antibodies. V5-tagged, HA-tagged, or GST-tagged proteins were detected with mouse anti-V5 (1:5000, Invitrogen #46-0705), anti-HA (1:1000, Covance #MMS101R), or anti-GST (1:1000, Santa Cruz Biotechnologies #sc-138) antibodies, followed by HRP-conjugated goat anti-mouse (1:3000, BioRad #170-6516) antibodies. Cdc28 was detected with goat anti-Cdc28 (1:200 Santa Cruz Biotechnologies #sc-6709) and HRP-conjugated donkey anti-goat (1:3000 Santa Cruz Biotechnologies #sc-2020) antibodies. Enhanced chemiluminescent detection used a Pierce SuperSignal West Pico kit (#34080).

Cyclin-CDK Phosphorylation Assays

Following methods established in a previous study (Bhaduri and Pryciak, 2011), cells harboring $P_{GAL1}$-GST-cyclin constructs and HA-tagged CDK substrates were grown in selective media with 2% raffinose and then induced with 2% galactose (for 40 min. to 2.5 hr) to drive cyclin expression. Where indicated, cultures were pre-treated with pheromone prior to galactose addition. Then, whole cell extracts were prepared, and substrate phosphorylation was assessed by SDS-PAGE and immunoblotting. CDK substrates are diagrammed in Figure 4.8E. Note that
most substrate fragments contain an NLS, and similar fragments localize to both nucleus and cytoplasm (Strickfaden et al., 2007; Winters et al., 2005), so in principle they should be accessible to both nuclear and cytoplasmic kinases. Also note that, in order to help express different cyclins at comparable levels, all cyclins in the $P_{GAL1}$-GST-cyclin constructs were truncated to remove destabilizing motifs (see the Plasmids Table for precise boundaries). These truncations remove NLS motifs in some cyclins such as Cln3 (Miller and Cross, 2001b) and Clb2 (Hahn et al., 2008; Hood et al., 2001), but the pattern of substrate specificity seen with these truncated cyclins is the same as that seen earlier with full-length cyclins (Bhaduri and Pryciak, 2011).
### Yeast strains used in Chapter IV

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Table 4.2. Plasmids used in Chapter IV

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<td>pPP3369</td>
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<td>pPP3508</td>
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<td>pPP3571</td>
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<td>pPP3630</td>
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<td>pHG-GST-S20M-5CSM-WT</td>
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<td>CEN HIS3 Pgal1-GST-Whi5(1-172)</td>
<td>this study</td>
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* Source: (1) S korski and Hieter, 1989; (2) Winters et al., 2005; (3) Bhaduri and Pryciak, 2011; (4) Takahashi and Pryciak, 2007; (5) Strickfaden et al., 2007; (6) Colman-Lerner et al., 2005; (7) Doncic et al., 2011; (8) Lu et al., 2012; (9) Alejandro Colman-Lerner lab.
CHAPTER V

Conservation of LP recognition in fungal G1 cyclins
and unusual hyper-potency in some cyclins

The study has been devised and results have been interpreted by myself and Dr. Pryciak. All experiments have been performed by myself.
Introduction:

In Chapters 2 and 3 of this dissertation, we investigated the molecular mechanisms behind the highly specific interactions between yeast G1/S cyclins and their substrates. We found that small hydrophobic motifs in substrates can interact with a distinct region on S. cerevisiae Cln2 and some G1 cyclins from other yeasts. Many fungi that separated from S. cerevisiae before the Whole-Genome-Duplication (WGD) have only one G1/S cyclin often known as Cln12 and one G1 cyclin, Cln3. In many fungi the distinction between G1 and G1/S cyclins are not very clear and they are referred to as G1 cyclins in general. Some yeasts like Candida albicans carry additional G1 cyclins (Figure 5.1 A). Many of these fungal G1 cyclins are important for polarized morphogenesis, which, in some pathogenic species, is linked to virulence. In Ashbya gossypii, deletion of G1 cyclin Cln12 leads to severe morphological defects (Hungerbuehler et al., 2007) with swollen hyphae displaying aberrant branching patterns. The AgCln12 localizes to the hyphal tips (Gladfelter et al., 2006), which is consistent with their potential role in polarity control. In contrast, deletion of AgCln3 does not cause any morphological defect. In the pathogenic yeast Candida albicans, not one but three G1 cyclins have been shown to be important for filamentous hyphal growth, which contribute to its virulence. Apart from the usual G1 cyclin CaCln3 there are two other G1 cyclins, namely Hgc1, which is homologous to S. cerevisiae Cln1 and Cln2, and another cyclin named Ccn1. Deletion of all of these has been
associated with defective hyphal morphogenesis and reduced virulence (Bishop et al., 2010; Chapa y Lazo et al., 2005; Loeb et al., 1999; Zheng and Wang, 2004; Zheng et al., 2007).

Further separated from budding yeast, the fission yeasts *Schizosaccharomyces pombe* and *Schizosaccharomyces japonicus* each have one G1 cyclin known as Puc1 which is a close relative of budding yeast Clns. Deletion of *S. pombe* Puc1 increases the length of G1 and causes cells to undergo S phase at greater cell size than wt cells (quite akin to how *cln2-m4* pushes cells to larger cell size at the time of Start). This delay is completely abolished, however, when the Sic1 equivalent CKI in fission yeast, Rum1, is deleted (Martin-Castellanos et al., 2000). Filamentous growth is also common in bread molds like *Neurospora crassa* although very little is known about the involvement of its only G1 cyclin *NcCln1* in its polarized hyphal morphogenesis.

All of these above fungi belong to the phylum of Ascomycota (Figure 5.2 A), which forms the largest phylum of fungi with over 64000 species. The fungal phyla Basidiomycota, which had separated from the Ascomycota about 400 million years ago, contains sexually and asexually reproducing filamentous fungi including mushrooms like *Coprinopsis cinerea*, economically important smut *Ustilago maydis* (pathogenic to maize) and pathogenic yeasts like *Cryptococcus neoformans*. Several of these organisms simply contain one G1 cyclin. In *U.*
*maydis*, the sole G1 cyclin *UmCln1* has been shown to play an important role in hyphal morphogenesis and mating, which is important for the shift from asexual to sexual reproduction under nutritional stress (the first step in infection) (Castillo-Lluva and Perez-Martin, 2005). In the pathogenic fungus *Cryptococcus neoformans*, which reproduces asexually by budding, deletion of the only G1 cyclin *CnCln1* results in significant increase in cell size at the time of the initiation of DNA replication and budding (quite similar to *cln2-m4* case in Chapter 3), thus suggesting a key role of G1 cyclins in the cell cycle of this organism (Virtudazo et al., 2010).

Based on all of the above observations, it is clear that in many different fungi separated over large evolutionary distances, G1 cyclin-CDK complexes generally play similar roles of controlling polarized morphogenesis of buds or filaments and commitment to cell cycle entry. Our results in the budding yeast model system suggest a role of LP-type docking motifs in Cln2-CDK’s ability to drive polarized growth during budding (Chapter 3). This prompted us to investigate whether the G1 cyclins from different close and distant relatives of *S. cerevisiae* use similar molecular mechanisms to identify their substrates. More specifically, we were interested in knowing whether G1 cyclins from various fungi can use the “LP” type of substrate docking motifs we earlier characterized in Chapter 2. Our initial observations (Chapter 3) indicated that indeed a number of these G1 cyclins from a variety of yeasts are able to use the LP docking motif for substrate
phosphorylation and in fact “m4”-like mutations in those cyclins abrogated this recognition ability but not cyclin function. In this Chapter, we present evidence that not only different ascomycetes yeast, but also different basidiomycetes fungi have G1 cyclins that recognize the LP docking motif. However, there may be additional sequence features necessary for some other G1 cyclins. One remarkable observation that emerged out of these experiments was the hyper-potency of some cyclins belonging to different organisms. They may provide clues to the mechanisms of atypical cell cycle regulation in some yeast like the fission yeast *S. pombe* (Fisher and Nurse, 1996).
Results and Discussions

Conservation of “LP” recognition in foreign G1 cyclins:

In order to investigate whether “LP” recognition is conserved in G1 cyclins across fungal evolution, we expressed several foreign cyclins from yeasts belonging to the Saccharomyces clade and the Candida clade in budding yeast as leucine-zipper fusion proteins (Chapter 3) and tested their ability to phosphorylate substrates containing either “LP” type or leucine-zipper docking sites. Apart from having Cln1/2- and Cln3- like G1 cyclins, some of these yeasts like Candida albicans have other G1 specific cyclins known as Ccn1 and Hgc1 (Figure 5.1 A). We tested Ccn1 cyclins from three different yeasts along with one Hgc1 candidate. All of these cyclins were expressed without the region that controls their stability so as to minimize differences arising from differential control by the proteasomal machinery. The boundaries were decided based on fungal alignments, which showed variable regions rich in CDK phosphorylation sites (for an example of the strategy, see Figure 3.2C). These cyclins were tested for their ability to phosphorylate substrates containing either a native “LP” docking site from Ste5 and Ste20 or a leucine-zipper docking site, which provided for an in vivo control of the activity of these foreign cyclins when expressed in the budding yeast host (Figure 5.1 B). Almost all of these cyclins, with the exception of CaHgc1, were expressed comparably to budding yeast G1 cyclins and they were
Figure 5.1. G1 cyclins from related yeasts have similar docking preferences

(A) Homology tree of fungal (*Sachharomyces*) G1 and B-type cyclins. Some species have additional G1 cyclins called Ccn1. The Ccn1 group is related but distinct from Cln3 and Cln1/2 groups. Adapted from Figure 1 of (Ofir and Kornitzer, 2010)

(B) G1 cyclins from a variety of yeasts were expressed as GST-(lz) fusions to test *in vivo* phosphorylation of substrates with either a defective docking site or two LP docking sites (from Ste5 or Ste20) or the leucine zipper. All cyclins were truncated to remove their C-terminal tails, which control their stability.

Abbreviations: *Sc* = *Saccharomyces cerevisiae*; *Sk* = *Saccharomyces kluyveri*; *Kw* = *Kluyveromyces waltii*; *Ag* = *Ashbya gossypii*; *Ca* = *Candida albicans*. 
Figure 5.1. G1 cyclins from related yeasts have similar docking preferences

A

B

\[ P_{\text{GALP}} \times \text{GST-cyclin:} \]

- Ste5
- Ste20
- Zipper
- Total cyclin

none

ste5

ste20

total cyclin
functionally active as displayed by the phosphorylation of the zippered substrate. In general most cyclins were not able to phosphorylate the substrate when there was no functional docking site present. All Cln1/2 and Ccn1 cyclins were able to use both the Ste5 and the Ste20 docking sites to phosphorylate the CDK sites derived from Ste5 native sequence. It is important to note here that the docking site from Ste5 (LLPP) is slightly different in composition from the Ste20 docking site (SLDDPIQF) but in each case, hydrophobic residues like L and F seemed to be key. However, the two Cln3 cyclins tested here were not able to phosphorylate these substrates in spite of being very potent on the zipper-docking substrate. CaHgc1 was consistently expressed very weakly as compared to the other substrates. As a result, it is not clear whether its inability to phosphorylate native-dock substrates stems from its low expression or true lack of “LP” recognition, although it was able to phosphorylate the zipper-dock substrate somewhat efficiently. One very interesting thing that emerged from this experiment is the hyperpotency of S. kluyveri Ccn1 cyclin. Although all others were unable to phosphorylate a substrate lacking a functional dock, SkCcn1 was able to robustly phosphorylate it and continued to display stronger and more complete phosphorylation on substrates containing both native and zipper-docks, in spite of being expressed at the same levels as other cyclins. This surprising observation suggested that SkCcn1 might have the ability to activate budding yeast CDK1 so strongly that it can phosphorylate minimal consensus CDK sites without the help of any docking motif. This hyperpotency phenotype will be
further explored later in this Chapter. Overall, these observations clearly showed that LP recognition property is well conserved in a wide variety of G1 cyclins which suggests conservation of function in related yeasts.

We wanted to further explore the conservation of “LP” recognition in G1 cyclins of more distantly related fungi. Both fission yeasts and molds are a considerable evolutionary distance away from budding yeasts and their immediate relatives, which are known as Hemiascomycetes (Figure 5.2 A) but still within the larger phylum of Ascomycetes (note, G1 cyclins from different members of Hemiascomycetes were tested in Figure 5.1 B). Outside of this phylum, there is another phylum named Basidiomycetes (Figure 5.2 A), which separated from Ascomycetes about 400 million years ago when plants invaded the earth. Members of Basidiomycetes include mushrooms, rusts, smuts and additional pathogenic budding yeasts. In an experiment very similar to the one described in Figure 5.1 B, we tested several different G1 cyclins belonging to various groups: Puc1s from fission yeasts Schizosaccharomyces pombe and Schizosaccharomyces japonicus; Cln1s from molds Neurospora crassa and Blastomyces dermatitidis; Cln1s from several Basidiomycetes species (smut: Ustilago maydis, mushroom: Coprinopsis cinerea, budding yeast like: Cryptococcus neoformans) (Figure 5.2 A). Each cyclin was expressed as a leucine-zipper fusion protein and tested against substrates containing wt or
**Figure 5.2. G1 cyclins from distant fungi recognize LP-type docking motifs**

(A) Expected Phylogenetic tree of fungi, showing two major phyla Ascomycetes and Basidiomycetes and sub-classes within Ascomycetes. (Adapted from Figure 2 of Hellborg et al., 2008)

(B) G1 cyclins from a variety of distant fungi were expressed as GST-(lz) fusions along with *S. cerevisiae* Cln2 and Cln3 to compare *in vivo* phosphorylation of substrates with either a defective docking site or an LP docking site from Ste5 or the leucine zipper. All cyclins were truncated to remove their C or N-terminal regions that control their stability.

Abbreviations: *Sc* = *Saccharomyces cerevisiae*; *Sp* = *Schizosaccharomyces pombe*; *Sj* = *Schizosaccharomyces japonicus*; *Um* = *Ustilago maydis*; *Cc* = *Coprinopsis cinerea*; *Cn* = *Cryptococcus neoformans*; *Nc* = *Neurospora crassa*; *Bd* = *Blastomyces dermatitidis*. 


Figure 5.2. G1 cyclins from distant fungi recognize LP-type docking motifs

A

B

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<tr>
<th>P_{GAL}^r GST-cyclin:</th>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>zipper</td>
<td></td>
<td></td>
</tr>
<tr>
<td>total cyclin</td>
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Neurospora crassa  
Gibberella zea  
Chalotomium globosum  
Trichoderma reesei  
Magnaporthe grisea  
Phaeosphaeria nodorum  
Aspergillus spp  
Coccidioides immitis  
Histoplasma capsulatum  

Schizosaccharomyces pombe  
Archiascomycetes
mutant native docks from Ste5 or a zipper-dock. Apart from S. pombe Puc1, which was consistently poorly expressed, most cyclins were fully functional in vivo and remarkably were able to phosphorylate a native Ste5 substrate in a docking dependent manner (Figure 5.2 B) whereas ScCLN3 and SjPuc1 were not. This clearly showed that “LP” recognition is highly conserved across large evolutionary distance amongst fungi, raising the possibility that a functional role for this type of docking has also been conserved across fungal evolution. It might be interesting to explore whether this recognition has an in vivo role in these fungi. Since all of these cyclins are involved in polarized morphogenesis in these fungi, “LP” recognition could play a role in the phosphorylation of polarity related proteins by G1 cyclins. A similar role of “LP” recognition was uncovered in budding yeast (Chapter 3). However, none of these new cyclins displayed hyperpotency in substrate phosphorylation. Our results demonstrated that G1 cyclins from fungi which are separated by large evolutionary distance form S. cerevisiae still maintained LP recognition. This argues strongly for the conservation of a functional role of LP recognition in these fungi.

**Differences in sequence requirements between Cln1/2s and Ccn1s**

While we were comparing different classes of G1 cyclins for their ability to use “LP” type docking motifs, we noticed that Ccn1s needed additional sequence features apart from the core “LP” motif which possibly let to somewhat different
Figure 5.3. Clns and Ccn1s have subtly different sequence requirements for docking

Representative cyclins from the Cln1/2 group and the Ccn1 group from different yeasts were expressed as GST-(lz) fusions and compared for their ability to phosphorylate in vivo substrates with either two docking sites from Ste5, different in length or docking sites from Exo84 or Sic1.

Abbreviations: Sc = Saccharomyces cerevisiae; Kw = Kluyveromyces waltii; Ca = Candida albicans.
Figure 5.3. Clns and Ccn1s have subtly different sequence requirements for docking
binding preferences as well (Figure 3.5). We tested “LP” type motifs from three different substrates previously used in Chapter 2, namely from Ste5, Exo84 and Sic1. One of the Ste5 substrates, the Ste5-1-283 contains an intact LLPP motif followed by only two native residues. When we compared two Cln1/2s from two different yeasts with two Ccn1s from two different yeasts, we found that while Cln1/2s recognized all the different docks, Ccn1s only recognized the Ste5 dock but not the docks from Exo84 or Sic1 (Figure 5.3). Ccn1s were able to recognize the truncated Ste5 (1-283) protein, thus suggesting that whatever additional sequence features are required lies within this 1-283 region of Ste5. It cannot be, however, that Ccn1s are using an entirely different sequence for phosphorylating Ste5, because Ccn1s can use a “LP” type docking motif from Ste20 (Figure 5.1B). This brings out subtle differences in substrate recognition between the two classes of G1 cyclins and suggests that in spite of some overlap, these two distinct classes of cyclins probably are able to target different substrates in vivo. This difference further suggests that the Cln1/2 group may have somewhat different functions from the Ccn1 group during cell cycle, with one more suited for a certain role than the other and vice-versa.

**Hyperpotency of some cyclin-CDK complexes in vivo**

Our simple in vivo shift-based phosphorylation assay using a standard substrate -/+ a docking site helped us compare the relative strengths of different cyclin-
Figure 5.4. Hyper-potent cyclins – *S. pombe* Cdc13 & *S. kluyveri* Ccn1

(A) *S. cerevisiae* G1 or B-type cyclins were expressed as GST-(lz) fusions and compared for their ability to phosphorylate *in vivo*, substrates with either an LP docking site from Ste5 or the leucine zipper. All cyclins were truncated to remove their C or N-terminal regions that control their stability.

(B) Full length *S. cerevisiae* G1 cyclin Cln2 and *S. pombe* mitotic cyclin Cdc13 were expressed as GST- fusions and compared for their ability to phosphorylate *in vivo*, substrates with either a wild-type or a mutant LP docking site from Ste5.

(C) *S. cerevisiae* Cln2 and Cln3, *S. Kluyveri* Ccn1 and *S. pombe* Cdc13 were expressed as GST- fusions and compared for their ability to phosphorylate *in vivo*, substrates with either no docking site or an LP docking site from Ste5 or Ste20. Apart from *S. pombe* Cdc13, all other cyclins were truncated to remove their C-terminal regions that control their stability.

Abbreviations: *Sk* = *Saccharomyces kluyveri*; *Sp* = *Schizosaccharomyces pombe*. 
Figure 5.4. Hyper-potent cyclins – *S. pombe* Cdc13 & *S. kluyveri* Ccn1

**A**

- **P$_{GAL1}$-GST-Iz-cyclin:**
  - Ste5$_{1-260}$ + dock:
  - Ste5
  - Leucine zipper
  - Total cyclin

**B**

- Docking site:
  - wt
  - mut.
  - Ste5 1-337
  - P$_{GAL1}$-GST-cyclin:
    - Ste5
    - Cdc5
    - Cdc6
    - Cdc13

**C**

- Docking site:
  - None
  - Ste5
  - Ste20

- P$_{GAL1}$-GST-cyclin:
  - Cdc5
  - Cdc6
  - Cdc13
CDK1 complexes. Most CDK complexes were not able to phosphorylate a substrate without the help of a docking site. However, two complexes were able to, thus exhibiting exceptional potency. In general, although the budding yeast mitotic cyclin Clb2-CDK1 complex showed higher potency than other budding yeast cyclin-CDK complexes on a zipper-dock substrate (evidenced by more complete conversion to the fully phosphorylated form), it was still unable to phosphorylate the substrate in the absence of a functional docking motif (Figure 5.4 A). In contrast, the S. pombe mitotic cyclin, Cdc13, in complex with the budding yeast CDK1, was able to phosphorylate the same substrate even in the absence of a functional docking site (Figure 5.4 B). This hyperpotent behavior was also seen in the case of S. kluyveri G1 cyclin Ccn1, in complex with the budding yeast CDK1 in an earlier experiment (Figure 5.1 B). One question that arises is whether these hyperpotent cyclins still use the native docking motif in spite of being able to phosphorylate without it. So we compared the SkCcn1 and SpCdc13 side by side with ScCln2 and ScCln3 against substrates containing either no functional docking site or docking sites from Ste5 or Ste20 (Figure 5.4 C). Both SkCcn1 and SpCdc13 were able to phosphorylate the substrate that lacked docking, whereas ScCln2 and ScCln3 were not. However, although SpCdc13 was not able to phosphorylate the two +dock substrates any better than ScCln2, SkCcn1 was able to phosphorylate both these substrates more completely with the help of docking sites. This demonstrated that SkCcn1 was still using the “LP” type docking motifs in spite of probably activating the CDK to a
greater extent than other cyclins. However, SpCdc13 did not seem to recognize these LP-type docking motifs. As expected, ScCln3 was unable to use any of these three substrates. Overall, these results clearly point to the fact that different cyclins have the ability to activate the CDK to different extents, which might have interesting consequences for the cell cycle.

The hyperpotency of SpCdc13 is especially interesting because early cell cycle work in S. pombe showed that Cdc13-CDK1 complex was enough to take the cell through all stages of cell cycle (Start-DNA replication-mitosis) (Fisher and Nurse, 1996) in the absence of G1 and S phase cyclins. This helped fuel the idea of the quantitative model for the cell cycle where substrate specificity stemmed from different levels of activity of the CDK complex at the early part versus the later part of the cell cycle and robust oscillations of activity of a single cyclin-CDK module was deemed enough to generate these temporally separated periods of low and high CDK activity. On the contrary, in other eukaryotes including budding yeast, the cell cycle could not be entirely driven by any one type of cyclin-CDK complexes (Table 5.1). To our knowledge, a minimum cyclin-CDK module has not been identified for budding yeast but based on evidences it seems likely that a budding yeast cell cycle can be driven by a combination of one of the three G1 cyclins and an ectopically expressed mitotic cyclin (Clb1 or Clb2)
Table 5.1: Cyclin gene deletion and their phenotypes

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<th>genotype</th>
<th>phenotype</th>
<th>rescue</th>
<th>references</th>
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<tbody>
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<td>budding yeast</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cln1 cln2 cln3Δ</td>
<td>unviable, arrest in G1</td>
<td>sic1Δ, ectopicCLB5</td>
<td>(Epstein and Cross, 1992; Richardson et al., 1989; Schwob and Nasmyth, 1993; Tyers, 1996)</td>
</tr>
<tr>
<td>clb5 clb6Δ</td>
<td>viable, delayed S phase</td>
<td>CLB5promoter-CLB2and swe1Δ</td>
<td>(Hu and Aparicio, 2005; Schwob and Nasmyth, 1993)</td>
</tr>
<tr>
<td>clb3 clb4 clb1 clb2Δ</td>
<td>unviable</td>
<td></td>
<td>(Fitch et al., 1992)</td>
</tr>
<tr>
<td>clb5 clb6 clb3 clb4 clb2ΔGAL-CLB5</td>
<td>unviable, arrest in G2</td>
<td></td>
<td>(Schwob et al., 1994)</td>
</tr>
<tr>
<td>clb5 clb6 clb3 clb4 clb1 clb2ΔGAL-CLB1</td>
<td>viable</td>
<td></td>
<td>(Haase and Reed, 1999)</td>
</tr>
<tr>
<td>fission yeast</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>puc1 cig1 cig2Δ</td>
<td>viable, delay in G1</td>
<td>rum1Δ</td>
<td>(Martin-Castellanos et al., 2000)</td>
</tr>
<tr>
<td>cdc13Δ</td>
<td>unviable</td>
<td></td>
<td>(Hagan et al., 1988)</td>
</tr>
<tr>
<td>mouse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cycD1−/−D2−/− D3−/−</td>
<td>embryos grow but die at mid/late gestation with haematopoietic defects, MEFs are viable</td>
<td></td>
<td>(Kozar et al., 2004)</td>
</tr>
<tr>
<td>cycE1−/−E2−/−</td>
<td>death in late embryogenesis, failure of trophoblast giant cell endoreplication, MEFs are viable</td>
<td>wt placenta rescues embryonic development</td>
<td>(Geng et al., 2003)</td>
</tr>
<tr>
<td>cycA1−/−</td>
<td>viable, male infertile</td>
<td></td>
<td>(Liu et al., 1998)</td>
</tr>
<tr>
<td>cycA2−/−</td>
<td>death after day 5.5 p.c.,cycA1−/− A2−/− MEFs are viable</td>
<td></td>
<td>(Kalaszczynska et al., 2009; Murphy et al., 1997)</td>
</tr>
<tr>
<td>cycB1−/−</td>
<td>death in early embryogenesis</td>
<td></td>
<td>(Brandeis et al., 1998)</td>
</tr>
<tr>
<td>cycB2−/−</td>
<td>viable and fertile</td>
<td></td>
<td>(Brandeis et al., 1998)</td>
</tr>
</tbody>
</table>

Reproduced from (Uhlmann et al., 2011)
(Epstein and Cross, 1992; Fitch et al., 1992; Haase and Reed, 1999; Hu and Aparicio, 2005; Richardson et al., 1989; Schwob et al., 1994; Schwob and Nasmyth, 1993; Tyers, 1996). Although the requirement for G1 cyclins can be bypassed by certain genetic manipulations, these modified cells often lack the fitness of wild type cells (Schwob and Nasmyth, 1993; Skotheim et al., 2008; Tyers, 1996).

The current model for cell cycle regulation says that early events in the cell cycle, like Start and DNA replication, are fueled by a less active but highly specific form of the CDK whereas later events like mitosis are fueled by a highly active form of the CDK which does not have high substrate specificity (Koivomagi et al., 2011b). Our in vivo results with SpCdc13 hints at the possibility that its hyperpotency makes it suitable to drive the cell cycle in a different way than most other cyclin-CDK modules found in other organisms.

In budding yeast, S phase (DNA replication) is normally driven by Clb5/6-CDK1 complexes whereas Mitosis requires mainly Clb1/2-CDK1. Although all of these are B-type cyclins and at least in some cases they can substitute for each other, several differences exist between these two groups such that not all roles of Clb5/6 in the cell cycle are redundant with Clb1/2 and vice versa (reviewed in Chapter 1).
Mechanistically, there are two main differences between these two groups of cyclins. Recent studies have demonstrated the Clb2-CDK1 is a more potent enzyme than Clb5-CDK1, which allows it to have a wider range of substrates (Koivomagi et al., 2011b; Loog and Morgan, 2005). However, some substrates are still more sensitive towards Clb5 because Clb5 can target those substrates through its hydrophobic patch (hp) region whereas Clb2 is not as efficient in this hp-mediated targeting of those substrates (Bhaduri and Pryciak, 2011; Loog and Morgan, 2005). An alignment of different B-type cyclins from a variety of yeasts pointed out that the hp region in the Clb1/2 group from the *Saccharomyces* clade has evolved quite differently from the classical MRAIL like hp of the Clb5/6s, which target RxL type docking sites in substrates (Archambault et al., 2005). It raises the possibility that the hp region in Clb1/2 has evolved to have different functions – it could bind to a different kind of sequence, it could affect the localization of the cyclin or both. Indeed, there is evidence for both roles of the atypical hp region in Clb2 (Bailly et al., 2003; Hu et al., 2008).

One interesting question here is whether a functionally different hydrophobic patch is a specialty of the mitotic cyclins from the *Saccharomyces* clade or all mitotic B-type cyclins in fungi have an altered role for their hp region. Organisms further away from this clade, those belonging to the Basidiomycetes phylum, have only one mitotic cyclin. Future work will aim to investigate whether B-type cyclins from these simpler systems can recognize RxL motifs in substrates.
Another interesting thing that would be looked at in future studies is if other cyclins display hyperpotent behavior. A likely candidate that will be tested along with others is the Cdc13 cyclin from another fission yeast, *S. japonicas*. Also it will be important to test whether this hyperpotent phenotype applies to other CDK phosphorylation sites, for examples, the ones present in Ste20 or Whi5. Currently our observations only pertain to CDK sites in Ste5.

Finally, based on above results, it is clear that “LP” recognition is a highly favored method of substrate selection by fungal G1 cyclins. It will be interesting to know whether this method has an *in vivo* role in these organisms. In *C. albicans*, The Ccn1 cyclin has been shown to be involved in polarized morphogenesis, which is important for its pathogenicity. Future work would aim to address if “LP” recognition is necessary for this role of CaCcn1. Our preliminary results showed that mutating the substrate-binding groove in CaCcn1 significantly reduced its ability to promote hyperpolarized growth when overexpressed in *S. cerevisiae*.

**Acknowledgements**

We thank Rick Baker, Amy Gladfelter, Oliver Rando, and Charlie Specht for fungal DNA samples, Nick Buchler for helpful discussions and Matt Winters for technical assistance.
Materials and Methods

Yeast Strains and Plasmids
Standard procedures were used for growth and genetic manipulation of yeast (Rothstein, 1991; Sherman, 2002). Cells were grown at 30°C in synthetic (SC) medium with 2% glucose and/or raffinose. Strains and plasmids are listed in tables below.

In vivo CDK Phosphorylation Assays
As described previously (Bhaduri and Pryciak, 2011; Pope et al., 2014), cells harboring \( P_{GAL1} \)-GST-cyclin constructs and HA-tagged CDK substrates were grown in synthetic raffinose media, and then induced with 2% galactose (for 2.5 hr) to drive cyclin expression; substrates were based on the Ste5 N-terminus, with or without docking sites as indicated. Whole cell extracts were prepared, and substrate phosphorylation was assessed by SDS-PAGE and immunoblotting.

Whole Cell Extracts
Whole cell extracts (5ml cultures, \( \text{OD}_{660} = 0.6 \)) were prepared by a modified version of a previous protocol (Lee and Dohlman, 2008). Here, 300 µL of trichloroacetic acid (TCA) buffer (10 mM Tris-HCl, pH 8.0, 10% TCA, 25 mM ammonium acetate, 1 mM Na\(_2\)EDTA) was added directly to frozen cell pellets (usually from 2 mL culture), and incubated on ice for 10 minutes. Samples were
pelleted in a microcentrifuge for 10 min at 4°C. The pellet was resuspended in 150 µL Resuspension Buffer (0.1 M Tris.HCl, pH 11.0, 3% SDS), boiled for 5 min, allowed to cool at room temperature for 5 minutes, then re-centrifuged for 30 sec. The supernatant (120 µL) was transferred to a new tube, 20 µL was reserved to assay protein concentration by a Pierce BCA Protein Assay Kit (#23225), and then 100 µL of 2x SDS Sample Buffer was added to the remainder. Equivalent amounts of total protein (generally 20 µg) were loaded in each lane.

**Immunoblotting**

Protein samples were analyzed by SDS-PAGE and transferred to PVDF using a submerged tank device. HA-tagged or GST-tagged proteins were detected with mouse anti-HA (1:1000, Covance #MMS101R) or anti-GST (1:1000, Santa Cruz Biotechnologies #sc-138) antibodies, followed by HRP-conjugated goat anti-mouse (1:3000, BioRad #170-6516) antibodies. Enhanced chemiluminescent detection used a Biorad Clarity kit (#170-5060).
Table 5.2: Yeast strains used in Chapter V

<table>
<thead>
<tr>
<th>Name</th>
<th>Background*</th>
<th>Relevant Genotype</th>
<th>Source†</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4741</td>
<td>(S288c)</td>
<td>MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0</td>
<td>Bachman/Boeke</td>
</tr>
</tbody>
</table>

Table 5.3: Plasmids used in Chapter V

<table>
<thead>
<tr>
<th>Name</th>
<th>Alias</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPP2154</td>
<td>pHG-GST</td>
<td>CEN HIS3 Pgal-GST vector</td>
<td>(1)</td>
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<tr>
<td>pPP3501</td>
<td>pSS5kHA-1-283</td>
<td>CEN URA3 ste5 1-283-3xHA T&lt;sub&gt;CYC1&lt;/sub&gt;</td>
<td>this study</td>
</tr>
<tr>
<td>pPP3509</td>
<td>pH5n-1-337-wt</td>
<td>CEN URA3 ste5 1-337-(w)-3xHA T&lt;sub&gt;CYC1&lt;/sub&gt;</td>
<td>(1)</td>
</tr>
<tr>
<td>pPP3547</td>
<td>pH5n-1-260-S20CSM</td>
<td>CEN URA3 ste5 1-260-(S20C 80-115w)-3xHA T&lt;sub&gt;CYC1&lt;/sub&gt;</td>
<td>(1)</td>
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<tr>
<td>pPP3572</td>
<td>pt-HGT-CLN2-F</td>
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<tr>
<td>pPP3714</td>
<td>pH5n-1-337-P4</td>
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<tr>
<td>pPP3737</td>
<td>pt-HGT-Cdc13</td>
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<td>this study</td>
</tr>
<tr>
<td>pPP3802</td>
<td>pH5n-1-260-dSic1</td>
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<td>(1)</td>
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<tr>
<td>pPP3807</td>
<td>pH5n-1-260-dEco84</td>
<td>CEN URA3 ste5 1-260-(Eco84 281-311)-3xHA T&lt;sub&gt;CYC1&lt;/sub&gt;</td>
<td>(1)</td>
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<tr>
<td>pPP3877</td>
<td>pSS5kHA-1-260-E34(N)</td>
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<td>pPP3916</td>
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<tr>
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<td>CEN HIS3 Pgal-GST-[R34 zipper]-Sklu CLN1(1-264) T&lt;sub&gt;CYC1&lt;/sub&gt;</td>
<td>this study</td>
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<td>pPP3920</td>
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<td>pPP3921</td>
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<td>(2)</td>
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<td>pt-HGT-Rup-CLB2-t</td>
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<td>pPP3957</td>
<td>pt-HGT-Rup-Kwal-CCN1</td>
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<td>pPP3958</td>
<td>pt-HGT-Rup-Sklu-CCN1</td>
<td>CEN HIS3 GAL1pr-GST-[R34 zipper]-SkluCCN1(1-405) T&lt;sub&gt;CYC1&lt;/sub&gt;</td>
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<td>pPP3959</td>
<td>pt-HGT-Rup-Cab1-CCN1-a</td>
<td>CEN HIS3 Pgal-GST-[R34 zipper]-Cab1 CCN1(1-469) T&lt;sub&gt;CYC1&lt;/sub&gt;</td>
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<td>pPP3961</td>
<td>pt-HGT-Rup-Cab1-HGC1</td>
<td>CEN HIS3 GAL1pr-GST-[R34 zipper]-Cab1HGC1(1-406) T&lt;sub&gt;CYC1&lt;/sub&gt;</td>
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<td>pt-HGT-Rup-Agos-CLN3</td>
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<td>(2)</td>
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<td>pPP4130</td>
<td>pt-HGT-Rup-Nc-CLN-t</td>
<td>CEN HIS3 GAL1pr-GST-[R34 zipper]-Nc-aCLN(1-309 w/ exon fusion) T&lt;sub&gt;CYC1&lt;/sub&gt;</td>
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<td>pPP4131</td>
<td>pt-HGT-Rup-Nc-CLN-t</td>
<td>CEN HIS3 GAL1pr-GST-[R34 zipper]-Nc-aCLN(1-309 w/ exon fusion) T&lt;sub&gt;CYC1&lt;/sub&gt;</td>
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<td>pPP4137</td>
<td>pt-HGT-Rup-Sp-Puc1</td>
<td>CEN HIS3 GAL1pr-GST-[R34 zipper]-SpPUC1(FL) T&lt;sub&gt;CYC1&lt;/sub&gt;</td>
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<td>pPP4145</td>
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<td>this study</td>
</tr>
</tbody>
</table>

* Source: (1) Bhaduri and Pryciak, 2011; (2) Pope et al., 2014
CHAPTER VI
Concluding remarks

Purpose and main outcomes:
Several cell cycle events require specific forms of the cyclin-CDK complexes. It has been known for some time that cyclins not only contribute to cell cycle progression by activating the CDK but also by choosing substrates and/or specifying the location of the CDK holoenzyme. There are several examples of B-type cyclins identifying certain peptide motifs in their specific substrates through a conserved region in their structure. However, such interactions were not known for the G1 class of cyclins, which are instrumental in helping the cell decide whether or not to commit to a new cell cycle, a function that is non-redundant with B-type cyclins in budding yeast (reviewed extensively in Chapter 1). In Chapter 2 of this dissertation we have presented evidences that some G1 cyclins in budding yeast, Cln1 and Cln2, identify specific substrates by interacting with a particular type of substrate patch. This interaction is mediated by a conserved region of the cyclins’ structure, and both this region and the substrate patch differ from those used by B-type cyclins. In Chapter 3, we have further shown that the substrate-binding region in Cln1/2 is highly conserved amongst a variety of fungal G1, thus suggesting a conserved function. We discovered that the main effect of disrupting this interaction is to delay cell cycle entry in budding
yeast, possibly resulting from incomplete multi-site phosphorylation of substrates. Also, interestingly, in Chapter 4, we found that a CDK inhibitor can regulate the cyclin-CDK complex by inhibiting cyclin-substrate interaction. In Chapter 5, apart from finding that G1 cyclins from many distant fungi, such as molds and mushrooms, have maintained this substrate interaction function, we discovered that some cyclins have the ability to make the CDK hyperpotent in activity, thus suggesting additional roles for such complexes.

**Salient features of the study and future directions:**

In Chapter 2, we identified short hydrophobic (Leucine and Proline rich) docking sites in several CDK substrates, which specifically bind to budding yeast G1/S cyclins Cln1/2 and enhance phosphorylation of CDK substrates in a cyclin-specific manner *in vivo*. These docking sites are modular in that they can promote phosphorylation from a variety of distances and positions relative to the CDK sites. Furthermore, cyclin specificity of the same phosphorylation sites can be switched simply by exchanging docking sites among different substrates. It was surprising how easily we identified similar “LP” type docking motifs from several CDK substrates suggesting that this type of interaction may be very commonly used by the G1/S cyclin-CDKs in yeast to target their substrates. Subsequent studies from Mart Loog and colleagues have also shown the use of these docking motifs in phosphorylation of CDK substrates by G1/S cyclin-CDKs. Collectively, these findings suggest that docking interactions play a prevalent but
previously unappreciated role in driving phosphorylation of G1/S CDK substrates.

Docking motifs, in principle, are capable of affecting substrate phosphorylation in a number of ways: determining cyclin specificity without any contribution from the CDK sites themselves; promoting phosphorylation of suboptimal CDK sites, which are poorly phosphorylated by most forms of the CDK but form an overwhelming majority of all phosphorylated CDK sites in yeast (Holt et al., 2009); promoting multi-site phosphorylation of substrates in a processive manner (Koivomagi et al., 2013); and introducing additional regulation by interacting with regulators like phosphatases (Malleshaiah et al., 2010).

One key question that arose was how important are these substrate specific docking interactions with G1/S cyclin-CDK for the progression of the cell cycle. The best way to ask this question was to mutate the substrate-binding region in Cln2 and look for cell cycle phenotypes. However, unlike in the substrates where the docking motif occurred in largely unstructured areas, the binding site in the cyclin was envisioned to be a part of highly structured cyclin box folds. Any tampering in this region had the potential to interfere with the function of the cyclin. In the absence of an actual structure of Cln2 with a docking peptide, it was very difficult to predict where in Cln2 one can find the docking region and some initial attempts using targeted or unbiased large-scale mutational approaches failed. So in Chapter 3, we employed a strategy to not only identify different regions to mutate but also to control for any loss of activity upon mutation in vivo.
(detailed in Chapter 3). Through these efforts, we were able to find a Cln2 mutant (m4) that was specifically defective in phosphorylating docking-dependent ("LP" type) substrates but otherwise fully functional. The residues making up this substrate-binding region describe a mild depression near the hp cleft in models of Cln2. A comparison with a bona-fide structure of a B-type cyclin revealed that the m4 region was close to but separate from the well-characterized hp region on B-type cyclins. This m4 mutation caused Cln2 to lose its in vivo interactions with several substrates and also rendered it incapable of promoting multi-site phosphorylation in vitro.

Although the m4 mutations caused disruption of docking dependent binding and phosphorylation, it cannot be ruled out that m4 mutations might not disrupt the actual binding interface in Cln2, instead it might cause a conformation change in Cln2 which masks the actual binding interface. Such possibilities can only be distinguished by solving the crystal structure of Cln2 with a LP peptide.

In vivo, this mutant Cln2 was defective in fully phosphorylating Whi5 after release from pheromone arrest in G1 and also failed to promote robust transcription of several genes. These mechanistic insights were translated into a cell cycle phenotype when we observed that if cells had to rely on this mutant Cln2, they could not start a new cell cycle (budding and DNA replication) until they were substantially bigger in size. One of the roles that Cln2 has always been
associated with is bud morphogenesis (Cvrckova and Nasmyth, 1993). After release from a pheromone arrest in G1, mutant Cln2 was delayed in budding. Also, while overexpressed wild type Cln2 promoted hyperpolarized bud growth, m4 failed to do that as robustly. Interestingly, we mutated the same region in a few other G1 cyclins from neighboring yeasts and in each case the mutant cyclin showed a reduced ability to promote polarized bud morphogenesis. Overall, it appears that docking ability of Cln2 is especially important for the robust execution of several molecular events leading to the commitment point at Start. In this short interval, docking helps promote sharp and full phosphorylation of the early substrates of the CDK, several of which require phosphorylation at multiple CDK sites for their regulation. Therefore our results say that substrate docking of Cln2 is very important for a rapid and decisive commitment to cell cycle entry.

Some phenotypes of m4 were not very pronounced, especially when cells were released from a prolonged pheromone arrest, which helps cells overcome the cell size threshold at cell cycle entry. We hypothesize that inhibitors and negative regulators, which probably make use of the m4 region, cannot inhibit the cln2-m4 allele as strongly they can inhibit the wt allele of Cln2. This difference may lead to attenuation of some of m4 phenotypes. Our hypothesis is based on observations such as the aggravation of m4 phenotypes in the absence of CKI Far1 and the doubling of cln2-m4 half-life when compared to Cln2-wt. The latter observation may indicate that the proteasomal machinery also uses the docking interaction to
identify Cln2 but alternatively the m4 mutations can render Cln2 more stable by some other means, for example, by creasing a more stable folding which is harder to degrade (Nick Pace et al., 2014).

The docking site in Cln2 could also be multipurpose just like the docking sites in its substrates (see above), such that it may also bind not only substrates but also regulators like CDK inhibitors and members of the proteolytic machinery. Indeed two observations indirectly suggest this possibility – one being the increased half-life of \textit{cide2-m4} and the other being the mitigation of some m4 phenotypes (like overall increase in cell size) in the absence of the CDK inhibitor Far1. In fact, Far1 does inhibit substrate binding by Cln2, as shown in Chapter 4. Once stimulated by pheromone, Far1 is able to specifically inhibit Cln2-CDK's interaction with its substrates as well as its ability to phosphorylate docking dependent substrates \textit{in vivo}. Far1 can competitively inhibit this substrate interaction because it can bind Cln2 more strongly than substrates. A mutant Far1, which fails to inhibit Cln2's substrate binding, also fails to promote pheromone arrest. Interestingly, Far1 also separately inhibits another function of the Cln2-CDK complex, most likely CDK activity. Based on our observations, it seems likely that Far1 mediated inhibition is most effective on substrates that will critically depend on docking. Such substrates could contain inherently poor or suboptimal phosphorylation sites and/or might need to be phosphorylated at multiple positions. Another interesting thing that emerged is the specificity of Far1
mediated inhibition of CDK activity – only Cln1/2-CDK complexes were inhibited
while Cln3, Clb5 and Clb2-CDK complexes were not affected even though Far1
could bind all cyclins equally well. Currently it is not known whether Far1 has a
LP docking motif but two independent regions in Far1 have been shown to bind
Cln2 (Peter et al., 1993) thus suggesting that Far1 may employ a larger interface
to interact with Cln2.

Finally, in Chapter 5, we have shown that this substrate binding function is
conserved in G1 cyclins of both yeasts and non-yeast fungi like mushrooms and
molds which shared the last common ancestor with yeasts about 400 million
years ago. This strongly suggests that there is a functional relevance for this
specific docking interaction in these fungi. Quite like budding yeast, in several of
these fungi, G1 cyclins are entrusted with the job of promoting polarized hyphal
morphogenesis, which is a critical requirement for virulence in some of the
pathogenic fungi. It’s likely that similar to budding yeast, this same type of
docking interaction is involved in promoting polarized growth in these other fungi.

Some of these exogenous cyclins demonstrated hyperpotency in activity. Such
hyperpotency likely results from the ability of these cyclins to activate the CDK to
a higher degree, although alternate explanations cannot be ruled out. For
example, these cyclins may change the conformation of the CDK in such a way
that it can bind Cks1 (phosphor-adapter subunit of cyclin-CDK complexes) better
and thus promote more efficient use of phosphorylation sites. Or even, these cyclins could make the cyclin-CDK complex resistant to the CKI Far1.

This hyperpotent behavior also has important implications for cyclin evolution. These hyperpotent cyclins may represent an ancestral form of cyclins, which may have been designed to drive a basic eukaryotic cell cycle completely on their own. But subsequent increase in complications in that basic cell cycle might have fueled the need to generate more specialized cyclin-CDK modules in order to temporally and spatially separate distinct events during the cell cycle. So the modern low-activity but high-specificity cyclins may have evolved from these apparently hyperpotent ancestral cyclins.

Collectively, we have uncovered a novel type of substrate interaction between budding yeast G1/S cyclins and several early substrates of the CDK. This interaction is specific to this type of cyclin and involves a conserved interface on the cyclin, which binds to short leucine-proline rich patches on the substrates. These docking sequences also allow for other regulatory inputs by interacting with inhibitors and regulators, both at the level of the cyclin and the substrates. Our observations lead to several possibilities that warrant future studies. For example, future studies may address how these two different classes of docking interactions evolved, for instance, whether they were both present in some ancient cyclin and diverged into two specific groups at a later time. Also, another
obvious question that arises is whether the Cln3 group has yet another type of docking interaction. Another interesting question is to investigate the role of “LP” docking in polarized growth and virulence in a pathogenic fungus like *Candida albicans*, where the G1 cyclin Ccn1 has been shown to be involved in hyphal growth critical for virulence. Our results in Chapter 3 show that an m4 like mutation in CaCcn1 reduces its ability to promote hyperpolarized bud growth in budding yeast.
CHAPTER VII

Appendix
Figure A1. Phosphorylation of Ste5 N-terminal by Cln2 is reversed by phosphatase treatment

A HA-tagged Ste5 N-terminal fragment, which lacks the NLS domain, resolves into two bands (faster: unphosphorylated; slower: terminally phosphorylated. The HA-tagged Ste5 1-315ΔNLS fragment expressed from its native promoter, was monitored in time course after galactose-induced expression of GST-tagged Cln2. Reduced electrophoretic mobility signifies phosphorylation. The maximally phosphorylated 180m sample was treated with either Calf-intestine Alkaline phosphatase (CIP: NEB # M0290S) (180+CIP) or water (180+mock) for 1 hr. Phosphorylation of the 180m sample is fully reversed by treatment with CIP.
Figure A1. Phosphorylation of Ste5 N-terminal by Cln2 is reversed by phosphatase treatment.
Figure A2. Cyclin specific phosphorylation of Ste20 regulatory domains in the absence of B-type cyclin-CDK inhibitor Sic1

In a strain lacking B-type cyclin-CDK inhibitor Sic1, a V5-tagged Ste20 fragment, expressed from its native promoter, was monitored after galactose-induced expression of GST-tagged cyclins. Reduced electrophoretic mobility signifies phosphorylation. A variant of Cln3 lacking ten CDK sites (Cln3^{10A}) was used to increase its stability. Absence of Sic1 did not alter cyclin specificity of the Ste20 fragment. Results from two independent experiments are shown.
Figure A2. Cyclin specific phosphorylation of Ste20 regulatory domains in the absence of B-type cyclin-CDK inhibitor Sic1
Figure A3. The Cln2 docking site in Ste20 enhances phosphorylation of a Ste20 80-333 N-terminal fragment

\( P_{\text{GAL1}} \)-Cln2-induced phosphorylation was assayed for a V5-tagged form of Ste20 N-terminal fragment (80-333), with or without mutations in the docking site (mut3) or the 13 confirmed CDK sites (CDK\(^+\)). Results from two independent experiments are shown.
Figure A3. The Cln2 docking site in Ste20 enhances phosphorylation of a Ste20 80-333 N-terminal fragment.


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