

Targeted Proteomic Study of the Cyclin-Cdk Module

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Summary

The cell division cycle of the yeast *S. cerevisiae* is driven by one Cdk (cyclin-dependent kinase), which becomes active when bound to one of nine cyclin subunits. Elucidation of Cdk substrates and other Cdk-associated proteins is essential for a full understanding of the cell cycle. Here, we report the results of a targeted proteomics study using affinity purification coupled to mass spectrometry. Our study identified numerous proteins in association with particular cyclin-Cdk complexes. These included phosphorylation substrates, ubiquitination-degradation proteins, adaptors, and inhibitors. Some associations were previously known, and for others, we confirmed their specificity and biological relevance. Using a hypothesis-driven mass spectrometric approach, we also mapped *in vivo* phosphorylation at Cdk consensus motif-containing peptides within several cyclin-associated candidate Cdk substrates. Our results demonstrate that this approach can be used to detect a host of transient and dynamic protein associations within a biological module.

Introduction

All eukaryotic organisms face the same fundamental problems in their somatic cell division cycle. DNA must be faithfully replicated once. Identical sets of genetic material must be physically segregated. Finally, cytokinesis must occur to separate the two progeny cells. These cellular transformations must occur in an orderly, coordinated manner. In all eukaryotic organisms, a cyclin-Cdk module appears to play the most central role in directing this program. An oscillation of cyclin-Cdk activities essentially conducts a symphony of pathways, giving rise to a harmonious cell division cycle. Coordination of a highly complex set of tasks requires multiple avenues of communication involving dynamic and often redundant pathways that involve multiple protein-protein interactions.

Cyclin-dependent kinases are activated upon binding of cyclin subunits. The periodic synthesis and proteolysis of these cyclins ensures an oscillation in Cdk activity necessary to the cell cycle. In the yeast *Saccharomyces cerevisiae*, Cdc28 (Cdk1) is the only Cdk that is clearly involved in cell cycle coordination. It functions with three G1 cyclins (Cln1–3) and six B-type cyclins (Clb1–6). By

contrast, in *Schizosaccharomyces pombe*, one Cdk (Cdc2) functions with one of two G1 cyclins (Cig1 and 2) and one B type cyclin (Cdc13). In vertebrates (including humans), at least eight Cdks function with at least nine cyclins where only some cyclin-Cdk combinations are functional and where tissue specificity adds another level of complexity (reviewed in Morgan, 1997).

The yeast *S. cerevisiae* has provided a simple model for cell cycle studies for more than 30 years. Genes involved in cell cycle functions were initially identified by screens for mutants that failed to proceed through critical cell cycle transitions, followed by complementation of the mutation. This strategy allowed the identification of dozens of genes essential for cell division cycle (CDC genes; Hartwell et al., 1970). Genetic approaches to investigate how specific cyclins participate in the cell cycle have been challenging, most likely because of the cyclins' functional redundancy. Initially, genetic studies suggested that G1 cyclins *CLN1–3* were involved in committing the cell to a division cycle, initiating spindle assembly, budding, and activating the S phase cyclins *CLB5* and *6*. These were found to activate DNA replication initiation and to prevent rereplication. The cyclins *CLB1–4* were found to drive mitosis through the promotion of spindle and bud morphogenesis (reviewed in Miller and Cross, 2001). However, genetic experiments failed to elucidate many of the protein targets of the different cyclin-Cdk complexes. One problem faced by these studies is that communication between proteins tends to be redundant, as crosstalk and backup pathways involving proteins of partially overlapping function can add robustness and adaptability to a living system. Ultimately, the most direct way to study such networks of communicating proteins involves the detection of their interactions.

Understanding the molecular interface between cyclin-Cdk complexes and the cellular events that they control is a central focus of the field today (Nurse, 2000). Phosphorylation targets of cyclin-Cdk complexes have slowly begun to be discovered in a case by case manner. At the same time, a few proteins acting as inhibitors (e.g., Sic1; Donovan et al., 1994; Mendenhall, 1993; Schwob et al., 1994) and adaptors (e.g., Cks1; Morris et al., 2003) have been found. More recently, a study identified around 200 potential Cdk1 phosphorylation substrates in yeast (Ubersax et al., 2003). Only a few studies have begun to address the cyclin specificity of these interactions with targets and the mechanisms contributing to this specificity (reviewed in Miller and Cross, 2001).

In this report, we survey proteins associating with cyclins in *S. cerevisiae* using a single step, Protein A tag affinity purification technique followed by mass spectrometric protein identification. We identified and investigated numerous associations, both known and previously unreported. In addition, we have mapped phosphopeptides containing *in vivo* phosphorylation sites in a subset of cyclin-associated candidate Cdk substrates.

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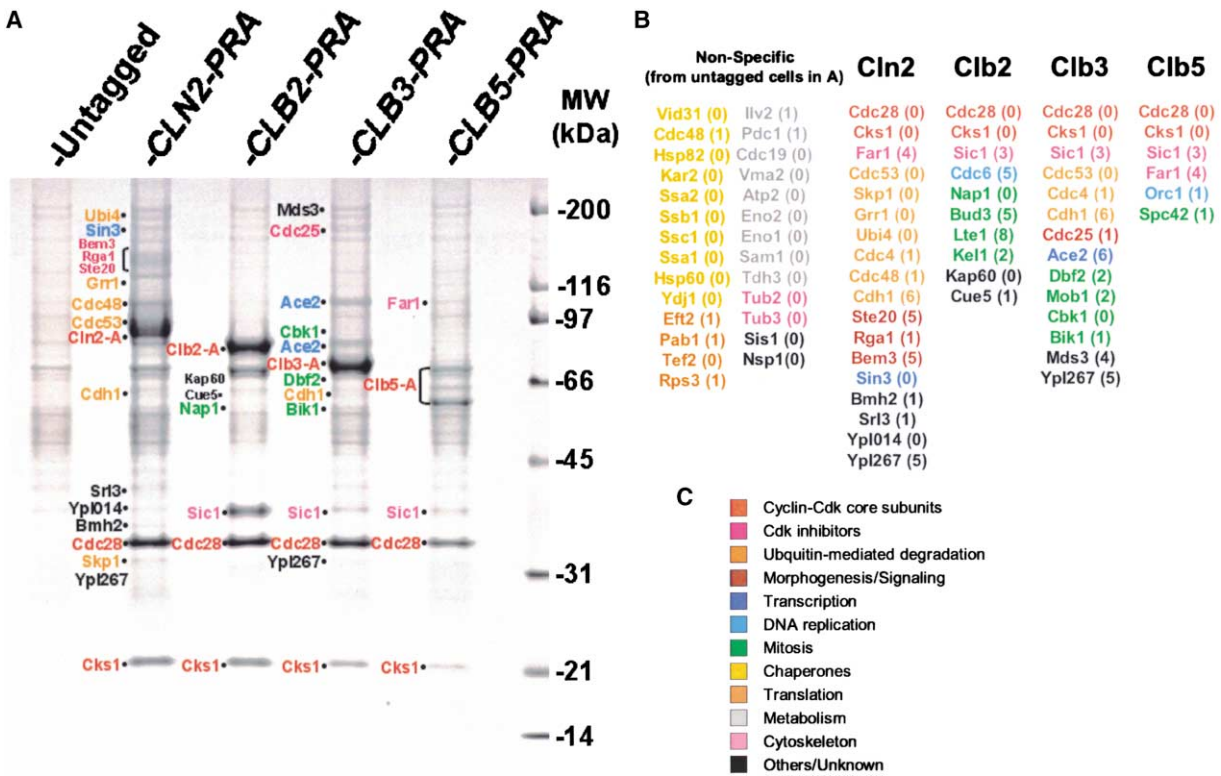


Figure 1. Identification of Cyclin-Associated Proteins by Mass Spectrometry

(A) Example of an analysis. Protein A affinity purifications were performed on untagged, *CLN2-PRA*, *CLB2-PRA*, *CLB3-PRA*, or *CLB5-PRA* strains and eluates were resolved on SDS-PAGE and Coomassie blue stained. Specific cyclin-associated proteins are labeled.

(B) Summary of specific cyclin-associated proteins identified from the gel shown in (A) and from other similar analyses. For a comparison, all proteins identified from the “untagged” lane in (A) are listed. Such proteins were subtracted to generate the lists of cyclin-specific proteins (see text). The numbers in parentheses indicate the number of perfect Cdk phosphorylation consensus motifs present in the protein sequence.

(C) Color code used to classify these proteins by cellular function in (A) and (B).

Results and Discussion

Identification of Cyclin-Cdk-Associated Proteins

In order to identify proteins that associate with cyclin-Cdk complexes, we have constructed strains that express Protein A in fusion with cyclin subunits at their natural genomic loci (Aitchison et al., 1995; Cross et al., 2002). We chose strains expressing Cln2-PrA, Clb2-PrA, Clb3-PrA, and Clb5-PrA as a representative set. Of the four pairs of closely homologous cyclins, Cln2, Clb2, Clb3, and Clb5 are the most abundant and also the most functionally important for cell growth over Cln1, Clb1, Clb4, and Clb6, respectively (Cross et al., 2002). Cln3 was not investigated because of its extremely low abundance (Cross et al., 2002). We performed large-scale affinity purifications on cell lysates from these strains and an untagged strain as a control. Purified complexes were then resolved by SDS-PAGE and proteins were identified by mass spectrometry (Figure 1A; see Experimental Procedures and the Supplemental Data at <http://www.molecule.org/cgi/content/full/14/6/699/DC1>). Numerous proteins from the untagged lane were identified. Due to the high sensitivity of the method (typically in the low femtomole range), multiple proteins were often identified in each gel slice (Supplemental Data and Supplemental Table S1 on *Molecular Cell's* website). The

majority of these proteins were chaperones, metabolic enzymes or translation proteins, occurring at a very high abundance in the cell as suggested by their CAI (codon adaptation index). Identical or similar proteins were found as common contaminants and were filtered out of the data in other studies of protein-protein interactions (Archambault et al., 2003; Ho et al., 2002). Therefore, to increase the stringency of our data set, we assumed that chaperones, metabolic enzymes, translation proteins, and a few other proteins frequently identified with a high CAI were nonspecific contaminants of the purified products (Figure 1B; Supplemental Table S1 on *Molecular Cell's* website). The data set presented in Figure 1A represents the remaining list after removing such contaminants. Figure 1B shows a list of proteins found to associate specifically with cyclins and their number of perfect Cdk phosphorylation consensus motifs: [S/T]-P-X-[K/R] (Nigg, 1993; Holmes and Solomon, 1996). These proteins contained an obvious enrichment for such motifs compared with the contaminants and with the proteome as a whole, suggesting that the cyclin-Cdk complexes were copurifying with their substrates. This list also contains proteins that were identified in other purifications performed under slightly different conditions (Supplemental Data and Supplemental Figure S1 on *Molecular Cell's* website). These results were

highly reproducible when the same conditions were used.

Associating Proteins Have Preferences for Specific Cyclins

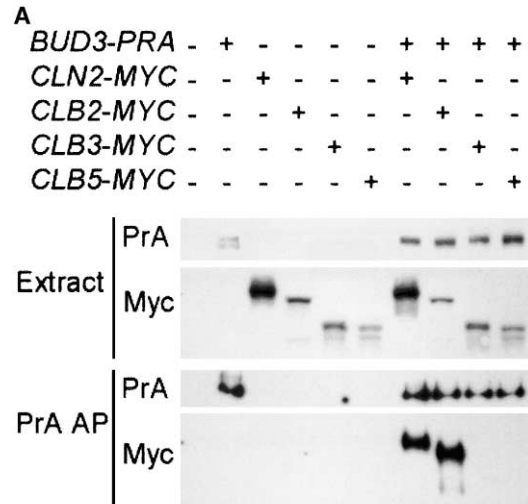
To evaluate the reliability of our mass spectrometric results, we performed a reciprocal copurification and analyzed the products by Western blotting. This test also allowed us to examine the cyclin specificity of the identified associations. For this purpose, we constructed strains that expressed Protein A fusions of cyclin-Cdk-associated proteins together with 9 X Myc fusions of the cyclins Cln2, Clb2, Clb3, and Clb5 (Archambault et al., 2003; Wach et al., 1997). The specificity of this assay is illustrated in Figure 2A, where the copurifications performed with strains encoding Bud3-PrA (Bud3 was found in isolation with Clb2-PrA; Figure 1) and various cyclin-Myc fusions are shown. We tested 12 of the copurifying proteins initially observed by mass spectrometry as well as Swi5 and Sld2, two known substrates of the Cdk that we did not detect by mass spectrometry. The associations observed using this assay (summarized in Figure 2B; raw data in the Supplemental Data and Supplemental Figure S2 on *Molecular Cell's* website) strongly correlated with the associations detected in our mass spectrometric screen (Figure 2B). Importantly, for every protein where an association with at least one cyclin was observed in this assay, the associated cyclin initially detected by mass spectrometry was confirmed, strengthening our confidence in the mass spectrometric data set (even though additional associations were detected). A number of the identified cyclin-associated proteins are known to have upstream functions in cyclin degradation, downstream functions in transcription, DNA replication, morphogenesis or mitotic progression, or Cdk inhibitory functions.

Cdk Inhibitors

Two known Cdk inhibitors were found in association with the cyclins. As expected, the Clb-specific Cdk inhibitor Sic1 (Donovan et al., 1994; Mendenhall, 1993; Schwob et al., 1994) was readily copurified with Clb2, Clb3, and Clb5 but not with Cln2 (Figure 1; Archambault et al., 2003). By contrast, the known Cln-Cdk inhibitor Far1 (Chang and Herskowitz, 1990) was copurified with Cln2 (Figure 1) in the initial mass spectrometric analysis. Far1 also associated with Clb5 (Figures 1 and 2), consistent with a two-hybrid interaction between these two proteins (our unpublished data).

Proteins Involved in the Degradation of Cyclins

All cyclins are targeted for proteasome-dependent degradation through ubiquitination by an E3 ubiquitin ligase complex. While the G1 cyclin Cln2 is ubiquitinated by the SCF^{Grr1} (Skp1-Cullin-F box, functioning with Grr1) enzyme, B type cyclins are ubiquitinated by the APC^{Cdc20} and APC^{Cdh1} enzymes (anaphase-promoting complex, functioning with Cdc20 and Cdh1). The SCF proteins Skp1, Cdc53, and Grr1 were found to copurify with Cln2, likely caught while mediating the ubiquitination of Cln2 (Figure 1); Grr1, an "F box"-containing protein (the F box being an SCF interacting domain), is known to form the bridge between phosphorylated Cln2 and Skp1 (Kishi



B

PrA-tagged Protein assayed	Cln2-Myc	Clb2-Myc	Clb3-Myc	Clb5-Myc
Ace2	+	+	++	-
Bem3	(+)	-	-	-
Bud3	+	++	-	-
Cdc6 *	-	++	-	-
Cdc25	+	-	(+)	-
Cdc48	++	-	-	-
Dbf2	-	+	++	-
Far1	++	-	-	(+)
Grr1	++	-	-	-
Orc6 #	-	-	-	++
Rga1	++	-	-	-
Sic1 *	-	++	++	++
Sld2	-	-	-	++
Swi5	-	-	+	-

Figure 2. Confirmation of Cyclin-Associated Proteins and Examination of Cyclin Specificity

(A) Example of an analysis. Cells expressing *BUD3-PrA*, *CLN2-Myc*, *CLB2-Myc*, *CLB3-Myc*, and *CLB5-Myc*, independently or in combinations, were submitted to Protein A affinity purification and Western blotting for Myc. Bud3-PrA associated with Cln2-Myc and Clb2-Myc in this assay.

(B) Summary of results from this assay. A "+" indicates an association, a "-" indicates no association detected, and a "++" indicates a particularly strong association. Circles indicate the associations originally detected by mass spectrometry (see Figure 1). "##" associations reported in Archambault et al. (2003). "#," Orc1, not Orc6, was detected by mass spectrometry, but Orc6-PrA was used to test the association of the tight ORC complex with cyclins.

and Yamao, 1998; Skowyra et al., 1997). Cdc53 was identified as a Cln2-copurifying protein in a previous study (Willems et al., 1996). We also identified Cdc4 (another F box protein) in association with Cln2 (Supplemental Figure S1 on *Molecular Cell's* website), consistent with the ability of SCF^{Cdc4} to ubiquitinate Cln2 in vitro (Blondel et al., 2000). Cdh1 was also associated with Cln2-Cdc28 (Figure 1), probably as a substrate (Zachariae et al., 1998). Conversely, Cdh1's association with

Cib3 (Supplemental Figure S1 on *Molecular Cell's* website) was observed, most likely because of its role in targeting this cyclin for degradation (Schwab et al., 2001; Visintin et al., 1997). The association of Cdc4 and Cdc53 with Cib3 (Supplemental Figure S1 on *Molecular Cell's* website), could have been bridged by Sic1, which is known to be recognized and ubiquitinated by the SCF^{Cdc4} (Feldman et al., 1997). No other components of APC enzymes other than Cdh1 copurified with the B type cyclins, suggesting that these interactions are more transient.

Cdc48 Interacts Specifically with Cln2-Ubiquitin Conjugates In Vivo

We identified Cdc48 as a major Cln2-associated protein. Originally, the essential gene *CDC48* was isolated from mutants that arrested as budded cells with an undivided nucleus at the restrictive temperatures (Moir et al., 1982). This gene was later found to encode a protein homologous to the mammalian protein VCP (Frohlich et al., 1991). Cdc48 and VCP are members of a superfamily of proteins termed AAA (ATPase associated with various cellular activities), so named for their multiple participations in cell cycle, vesicular transport, mitochondrial functions, peroxisome assembly, and proteolysis (Frohlich, 2001). Cdc48 is a polyubiquitin binding protein that has been proposed to participate in the degradation of substrates of the proteasome (Dai and Li, 2001; Ghislain et al., 1996). Although traces of Cdc48 (a very abundant protein) were present in the control purification from untagged cells, we hypothesized that the clear enrichment observed in the Cln2-PrA purification (Figure 1A) was due to a specific interaction and so we did not discard Cdc48 as a contaminant; perhaps Cdc48 targets ubiquitinated Cln2 for destruction, and it was various ubiquitinated forms of Cln2-PrA that were associating with Cdc48 in the purification (Figure 1A).

Thus, we tested if Cdc48 associated specifically with poly-ubiquitin-Cln2 conjugates and whether Cdc48 associated with other cyclins. The Western blot from Cdc48-PrA purification products revealed the presence of very slow migrating forms of Cln2-Myc, in the 150–200 kDa range (Figure 3A). No signal was observed around 95 kDa, where nonubiquitinated Cln2-Myc normally migrates. In contrast, a similar experiment performed using Grr1-PrA as bait revealed an association with the 95 kDa (nonubiquitinated) form of Cln2-Myc (Supplemental Figure S2E on *Molecular Cell's* website). No association of either Cdc48-PrA or Grr1-PrA with Cib2-Myc, Cib3-Myc, or Cib5-Myc was detected, suggesting that Cdc48 associates preferentially with SCF substrates rather than with APC substrates. Preliminary experiments suggested that Cdc48 does not significantly associate with Sic1 (substrate of SCF^{Cdc4}) in this assay, suggesting some level of specificity for Cdc48 toward specific SCF substrates. Nonetheless, the fact that Cdc48 associates with Far1 (Fu et al., 2003) (substrate of the SCF^{Cdc4}) in addition to Cln2 (substrate of SCF^{Grr1}) suggests that the association of Cdc48 with SCF targets is not restricted by which F box protein subunit is used in the ubiquitin ligation reaction. We demonstrated that the Cdc48-PrA/Ub-Cln2-Myc association occurs in vivo and not postlysis in our coaffinity purification assay by performing an experiment on cell pools containing cells expressing the

tagged proteins separately (Supplemental Figure S3 on *Molecular Cell's* website). It is possible that there is an ATP requirement for this interaction or that the whole pool of Cln2-Ub conjugates is stably bound to Cdc48 before and after the lysis, preventing additional Cdc48 from binding after the lysis.

Cln2-Ub Accumulates in *cdc48-3* Mutant Cells

Based on the above results and on the fact that Cln2 is a constitutively unstable protein (Schneider et al., 1998) that is degraded by a ubiquitination-proteasome pathway, we hypothesized that Cdc48 could function in facilitating the degradation of polyubiquitinated Cln2 by the proteasome. To test this idea, we used a temperature-sensitive allele of Cdc48 (*cdc48-3*) that allows cells to grow at 23°C but causes them to arrest at 37°C as budded cells containing an undivided nucleus (Moir et al., 1982). We grew *cdc48-3 CLN2-MYC* cells at 23°C and then switched half of the culture to 37°C. Fractions of the cultures were collected at different times following the temperature switch. Collected cells were then submitted to a Myc immunoprecipitation (IP). Several features were noted. First, when probed for Myc, the IP of *cdc48-3 CLN2-MYC* revealed a large band that migrated more slowly in the extract from cells incubated at 37°C than in the extract from cells incubated at 23°C (Figure 3B). This indicated that covalently modified forms of Cln2-Myc, possibly phosphoforms, accumulated in the mutant at 37°C. Second, in the IP from *cdc48-3 CLN2-MYC* cells, a strong enrichment for a ubiquitin signal in the ~200 kDa range was observed relative to *CDC48 CLN2-MYC* cells. Thus, the ability to destroy Cln2-ubiquitin conjugates has been impaired by the loss of Cdc48 function. Third, the ubiquitin signals were specific to the *cdc48-3 CLN2-MYC* cells but were observed at both temperatures. This suggests that Cdc48 already presents a loss of activity at 23°C in *cdc48-3* mutants. Fourth, at the nonpermissive temperature the degree of ubiquitination increases (reflected in the shift to higher molecular weight forms of the ubiquitinated Cln2; Figure 3B, bottom panel). Together, these results suggest that Cln2 phosphoforms and polyubiquitinated forms accumulate in the context of a loss of function of Cdc48. This in turn implies that Cdc48 functions downstream of the SCF^{Grr1} ubiquitin ligase in the pathway that allows Cln2 degradation. In a cell cycle block-release experiment (using *CDC48-PRA CLN2-MYC cdc20 GAL-CDC20* cells), we observed that the amounts of Cln2-ubiquitin conjugates that copurify with Cdc48 followed closely the profile of total Cln2 abundance (our unpublished data). Our findings are consistent with Cln2 being constitutively turned over by a fast SCF^{Grr1}-Cdc48-proteasome pathway.

We used another assay to test a role for Cdc48 in Cln2 degradation. It involved the pheromone response pathway, which allows haploid cells to sense the presence of a neighboring cell of the opposite mating type. The pheromone response involves the inactivation of G1 cyclins (such as Cln2), which in turn leads to an arrest in G1 in preparation for the fusion of the two cells (Valdivieso et al., 1993). By contrast, overexpression of *CLN2* represses the mating pathway (Oehlen and Cross, 1994; Wassmann and Ammerer, 1997). Cdc48 has recently been shown to be required for normal degradation

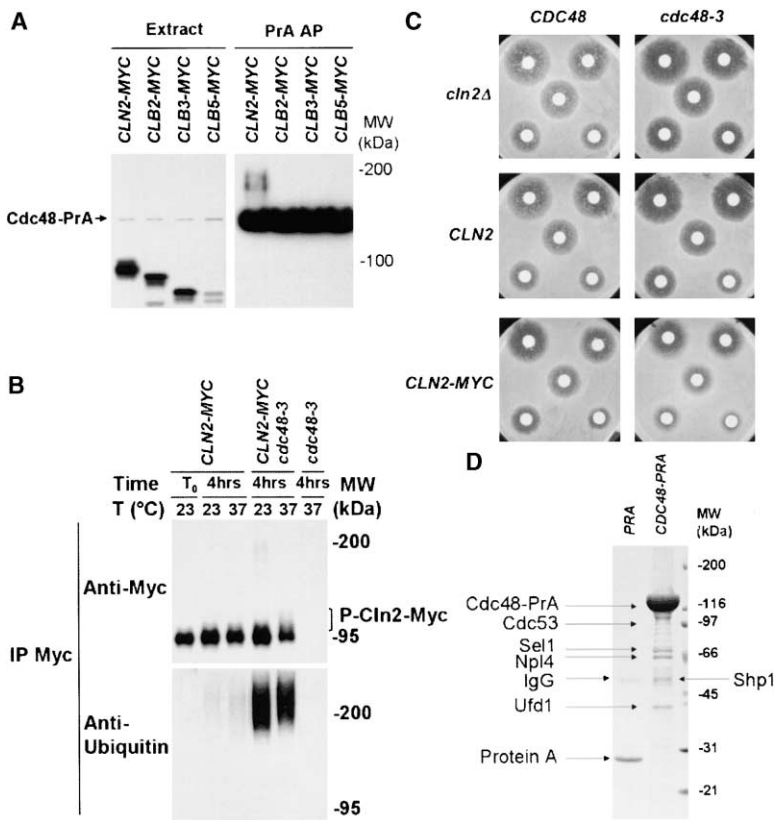


Figure 3. Cdc48 Functions in the Degradation of Cln2-Ub Conjugates In Vivo

(A) Cdc48 associates specifically with slow migrating forms of Cln2. Cells expressing *CDC48-PRA* and *CLN2-MYC*, *CLB2-MYC*, *CLB3-MYC*, or *CLB5-MYC* were submitted to Protein A affinity purification and Western blotting for Myc. Immunoblots for Myc (which also allows weaker detection of the Protein A) are shown for the extract and the purified product.

(B) Ubiquitinated forms of Cln2 accumulate in *cdc48-3* cells. *CLN2-MYC*, *CLN2-MYC cdc48-3*, and *cdc48-3* cells were grown at 23°C and shifted to 37°C (or left at 23°C) for 4 hr. Cell samples from the indicated conditions were used for Myc immunoprecipitations, and the products were probed for Myc and for ubiquitin conjugates.

(C) The sensitivity of *cdc48-3* cells depends on Cln2. *cdc48-3* and *CDC48* (*bar1*, mating type a) cells, containing also *CLN2*, *cln2Δ*, or *CLN2-MYC* alleles, were plated on YPD, and paper circles containing 15 μl of different dilutions of α factor (100, 50, 25, 12.5, or 6.25 mM) were deposited on the plates. Plates were then incubated at 23°C for 2 days.

(D) Identification of Cdc48-associated proteins. Protein A affinity purifications were performed on *CDC48-PRA* or *ZPRp-PRA* cells (control strain expressing Protein A from the *ZPR1* promoter at the *ZPR1* genomic locus). Associated proteins were identified by mass spectrometry. Specifically associated proteins are labeled. Other bands corresponded to proteolytic fragment of Cdc48 or contaminants (see Supplemental Data and Supplemental Table S2 on *Molecular Cell's* website).

of Far1 by the SCF-proteasome degradation pathway (Fu et al., 2003). Therefore, if our hypothesis is right, the result (Figure 3C) should be complicated by the existence of two opposing targets of Cdc48. In fact, the *cdc48-3* and *CDC48* cells (otherwise wild-type) showed little difference in sensitivity to the mating pheromone. However, we found that the sensitivity to α factor of *cdc48-3* cells depended on *CLN2* in an exacerbated manner relative to *CDC48* cells. When *CLN2* was deleted, the *cdc48-3* cells were more sensitive to α factor than *CDC48* cells. In the contrary, when *CLN2-MYC* was present, *cdc48-3* cells were less sensitive to α factor than *CDC48* cells, suggesting that the Myc tag somewhat stabilizes Cln2. These results are consistent with the idea that the sensitivity of cells to the mating pheromone depends on a delicate balance between Cln2 and Far1, both of which depend on Cdc48 for efficient degradation.

Overall, our results point to a previously unidentified function for Cdc48 in the degradation of Cln2-ubiquitin in vivo. We suggest that rather than completely blocking Cln2 degradation, a Cdc48 loss of function shifts the equilibrium of the pathway toward polyubiquitinated forms. Other chaperones could partially substitute for a loss of Cdc48 function in this pathway.

Proteins Complexed with Cdc48

To learn more about the biochemical functions of Cdc48, we affinity purified Cdc48-PrA and identified copurified

proteins by mass spectrometry (Figure 3D; Supplemental Table S2 on *Molecular Cell's* website). Among the major coenriching proteins were Npl4 and Ufd1, consistent with the prior identification of a ternary complex containing Cdc48 (p97), Npl4, and Ufd1 that exists both in mammals (Meyer et al., 2000) and in yeast (Hitchcock et al., 2001). In yeast, this complex was found to mediate the release of ubiquitinated membrane-bound transcription factors (Hitchcock et al., 2001). In addition, we identified Sel1 and Shp1 as major copurifying proteins. *SEL1* was previously identified as a gene whose deletion conferred supersecretion phenotype (Bartkevičiute and Sasnauskas, 2003) and has been proposed to be involved in ER biogenesis (Wright et al., 2003). Sel1 has been localized to the endoplasmic reticulum (ER) (Huh et al., 2003) and could function in protein degradation since the proteasome is more sensitive in a *sel1* mutant (Fleming et al., 2002). Shp1 contains a UBX domain (found in ubiquitin regulatory proteins) and interacts with Cdc48 in a two-hybrid assay (Braun et al., 2002). We also identified Cdc53 (a component of the SCF complex) in association with Cdc48. The Cdc48-Ufd1-Npl4 has been shown to exist in two distinct pools: one being tightly bound to the ER/nuclear membranes and the other being soluble (Hitchcock et al., 2001). We suggest that the ER-bound pool of Cdc48-Ufd1-Npl4 functions with Sel1 in ERAD (endoplasmic reticulum-associated degradation) while the cytosolic pool functions with the SCF (including Cdc53) in the ubiquitin-dependent degra-

dition of proteins, such as Cln2. Future studies should investigate the function of these associations.

Potential Phosphorylation Substrates of Cdc28

The proteins that specifically copurified with cyclins show a strong enrichment for optimal Cdk phosphorylation consensus motifs, corresponding to the sequence [S/T]-P-X-[K/R] (Figure 1B). This sequence is mostly absent or else present only once in the proteins that we labeled as contaminants. This observation reinforces the idea that coenriching proteins are substrates. The average number of [S/T]-P-X-[K/R] Cdk motifs in our specifically associated proteins is 3.0, compared with 0.3 in the whole yeast proteome. There are 24 (69%) proteins with 2 or fewer motifs and 11 (31%) with 3 or greater motifs in our cyclin-associated proteins. There are 6015 (96%) proteins in the proteome with 2 or fewer motifs and 254 (4.0%) with 3 or more. The difference is significant at a level of $p < 1 \times 10^{-6}$. When we consider one or fewer versus two or more motifs, our enrichment for proteins containing such motifs is also very significant. Moreover, 15 of the 24 proteins that contained at least one optimal Cdk phosphorylation consensus motif (namely Far1, Cdh1, Rga1, Bem3, Srl3, Ypl267, Sic1, Bud3, Lte1, Kel1, Ace2, Dbf2, Mob1, Orc1, and Spc42) have been reported to be Cdc28 substrates *in vitro* in a recent study, where a selected fraction of the proteome was assayed (Ubersax et al., 2003). By contrast, none of the proteins that we rejected as contaminants were detected as phosphorylation substrates (Ubersax et al., 2003). The known and potential phosphorylation substrates that we identified as cyclin/Cdc28-associated proteins function in a broad range of cellular events. Below we provide examples of these cyclin-associated proteins that are candidate Cdk substrates (Figure 1) and are involved in transcription, DNA replication, morphogenesis/signaling, and mitotic progression. We also confirmed that some of these candidate Cdk substrates were phosphorylated *in vivo*.

Control of Transcription: Clb3-Cdc28 Contributes to Regulating the Localization of the Transcription Factor Ace2

The G1 transcription factor Ace2 is a strong candidate substrate for Cdc28 phosphorylation because it carries a large number of consensus phosphorylation motifs and has strong homology to the known Cdc28 substrate Swi5. Reports from other groups have suggested the existence of a regulatory connection between Cdc28 and Ace2 (O'Conallain et al., 1999). Much like Swi5 (Moll et al., 1991), Ace2 was thought to be phosphorylated by Cdc28 on or near its nuclear localization signal (NLS), thereby excluding it from the nucleus. In our study, we observed a strong association between Clb3 and Ace2 (Figure 1A) and so hypothesized that Clb3-Cdc28 may function in promoting the cell cycle-regulated nuclear exclusion of Ace2. We therefore visualized the subcellular localization of YFP-Ace2 in wt or in *clb3 clb4* cells. In addition to *CLB3*, we also deleted *CLB4* because of its high similarity to *CLB3*, both in sequence and in function (Miller and Cross, 2001; Morgan, 1997). In a "double blind" experiment, we scored between 1000 and 1200 cells of each type (wt and *clb3 clb4*) for their

enrichment of nuclear YFP-Ace2 fluorescence. In this test, 27% of the *clb3 clb4* cells showed a nuclear signal, compared with 20% for the wt cells, representing a 35% increase in nuclear localization (Figure 4A). Our result is consistent with a role of Clb3,4-Cdc28 in negatively regulating the nuclear localization of Ace2. Because some cytoplasmic Ace2 is still observed in the *clb3 clb4* cells, it seems likely that regulators other than Clb3,4-Cdc28 exist to exclude Ace2 from the nucleus. Some of these regulators may be alternative cyclin-Cdc28 complexes. Consistent with this idea, we found that Ace2-PrA also copurified with Cln2-Myc and Clb2-Myc, although with lesser efficiency when compared with Clb3-Myc (Figure 4B). Moreover, when the same Protein A affinity purification was performed in cells deleted for *CLB3* and *CLB4*, a higher amount of Clb2-Myc reproducibly copurified, suggesting that Clb2 can substitute for the absence of Clb3 and Clb4 (Figure 4B).

To visualize the cell cycle dependence of the Clb3-Ace2 association, we initially used cells tagged on both Ace2 and Clb3 that could be synchronized in mitosis and released into the cell cycle by switching from YEPD to YEPGal medium (strain *ACE2-PRA CLB3-MYC cdc20 GAL-CDC20*). Protein A affinity purification and Myc immunoblotting with samples from the time course showed that the Clb3-Ace2 association occurred in mitosis (when Clb3-Myc is present) and that this association coincided with a decrease in electrophoretic mobility for Ace2 (characteristic of an increase in Ace2 phosphorylation; data not shown).

Interestingly, we found that the Clb3-Ace2 association could occur efficiently post-cell lysis. We used this ability to reconstitute the association in cell lysates to test if the efficiency of the association was dependent on the cell cycle. Thus, we synchronized and then released *ACE2-PRA cdc20 GAL-CDC20* cells, adding asynchronous *CLB3-MYC* cells to samples from the time course immediately before lysis. In this way, the same amount of Clb3-Myc was present in each sample, and we could visualize the changes in efficiency of association independent of protein amounts (Ace2 did not change much in abundance). We observed a marked reduction in association efficiency in G1, at a time when the fast migrating (i.e., likely hypophosphorylated) forms of Ace2 are present (Figure 4C, left). This loss of association was completely dependent on Sic1, as the same experiment performed in parallel with *ACE2-PRA cdc20 GAL-CDC20 sic1* cells resulted in constitutive association between Ace2-PrA and Clb3-Myc (Figure 4C, right). Furthermore, the faster migrating forms of Ace2 were almost absent in these cells throughout the time course. Sic1 is known to function in keeping Clb-Cdc28 activity at its lowest in G1 (Schwob et al., 1994). Therefore, our results suggest that Sic1 allows Ace2 to become dephosphorylated in G1 by inhibiting its interaction with and phosphorylation by the Clb3,4-Cdc28 kinases.

Ace2 Is Phosphorylated at Multiple Sites *In Vivo*

To search for Cdk phosphorylation sites in Ace2, we employed mass spectrometry (Figure 5A). Ace2-PrA expressed in yeast under the endogenous *ACE2* promoter was affinity purified and submitted to a "hypothesis-driven" mass-spectrometric analysis (Kalkum et al.,

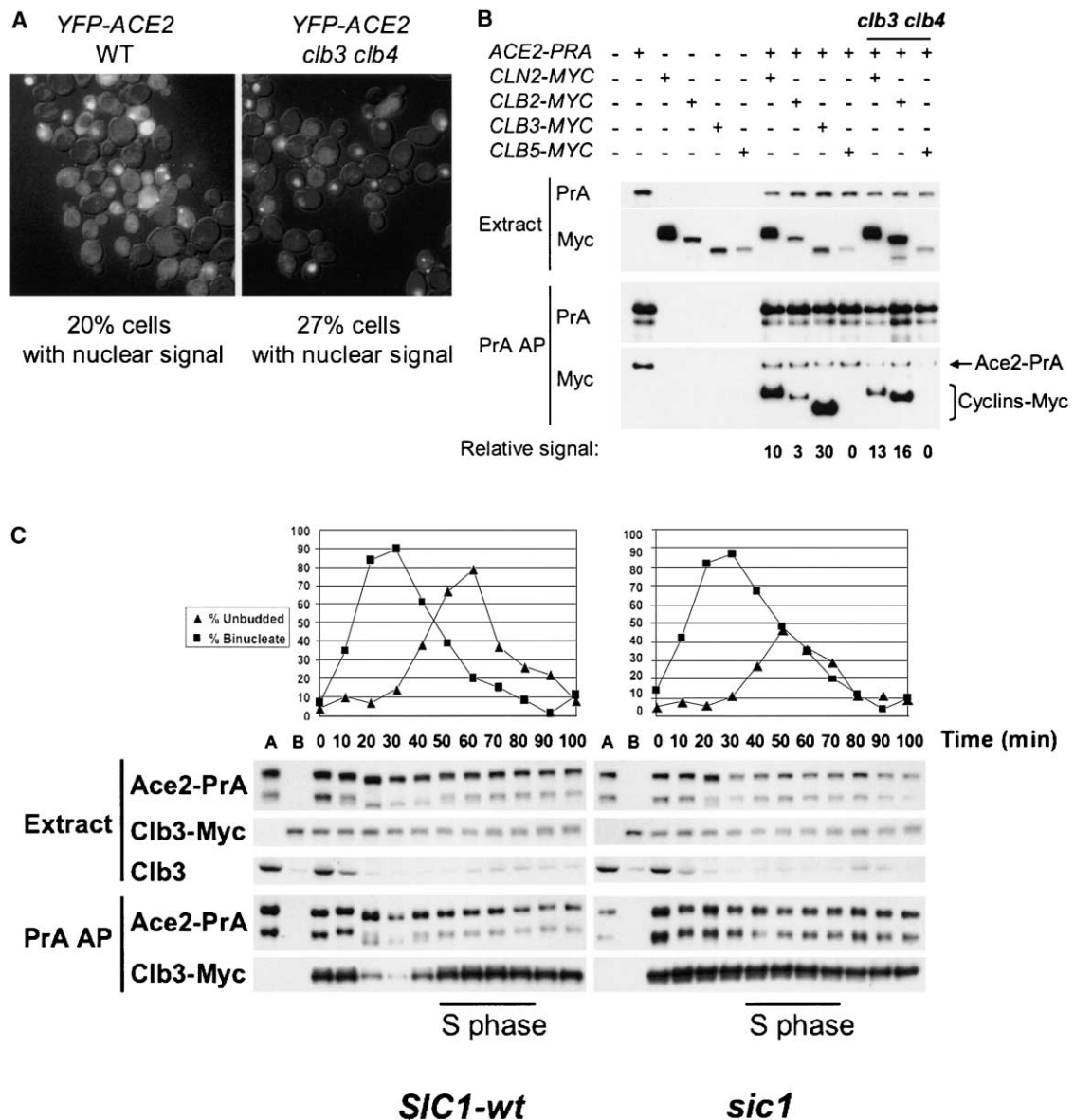


Figure 4. Clb3-Cdc28 Contributes to Regulating the Localization of Ace2

(A) Normal subcellular localization of Ace2 depends on the presence of Clb3 and Clb4. Between 1000 and 1200 asynchronous *YFP-ACE2* cells and *YFP-ACE2 clb3 clb4* cells were scored for the enrichment of nuclear fluorescence signal in double blind experiments. The numbers of 20% and 27% have standard deviations of 1.1% and 1.3%, respectively.

(B) Relative intensities of associations between cyclins and Ace2 in a wt or *clb3 clb4* background. A Protein A affinity purification experiment performed on either wt or *clb3 clb4* cells expressing *ACE2-PRA* and *CLN2-MYC*, *CLB2-MYC*, *CLB3-MYC*, or *CLB5-MYC*. The numbers at the bottom refer to the ratios of cyclin-Myc/Ace2-PrA signals in the above anti-Myc blot from the Protein A purification.

(C) Cell cycle dependence of the competence for association between Ace2 and Clb3. *ACE2-PRA cdc20 GAL-CDC20* cells, *SIC1-wt*, or *sic1* were grown in YEPGal, arrested at mitosis in YEPD for 3 hr, and released in YEPGal. Cells were collected every 10 min and were mixed with asynchronous *CLB3-MYC* cells before being submitted to Protein A affinity purifications. A lanes, arrested *ACE2-PRA cdc20 GAL-CDC20* (*SIC1-wt* or *sic1*) cells were mixed with wt cells. B lanes, wt cells were mixed with *CLB3-MYC* cells. Extracts were probed for Protein A, Myc, and Clb3. Purified products were probed for Protein A and Myc. The percentages of unbudded cells and binucleate cells are shown for the time courses.

2003; Chang et al., 2004). Using this approach, we identified 15 phosphorylated Ace2 peptides, 14 of which contained at least 1 [S/T]-P minimal Cdk phosphorylation consensus motif (Figure 5A). These peptides (containing at least 17 different sites of phosphorylation) covered 26% of the Ace2 sequence and included 16 of its 21 [S/T]-P motifs. For four of these peptides, the phosphor-

ylation could be mapped to specific residues. Serine 80 (in a SP motif), serine 428 (in a SP motif), threonine 501 (in a TPVK motif), and serine 557 (in a SP motif) were identified as four previously unknown *in vivo* phosphorylation sites. Three of the observed phosphopeptides contained serine 714 (in a SPVK motif), one of three proposed Cdk phosphorylation sites present in the con-

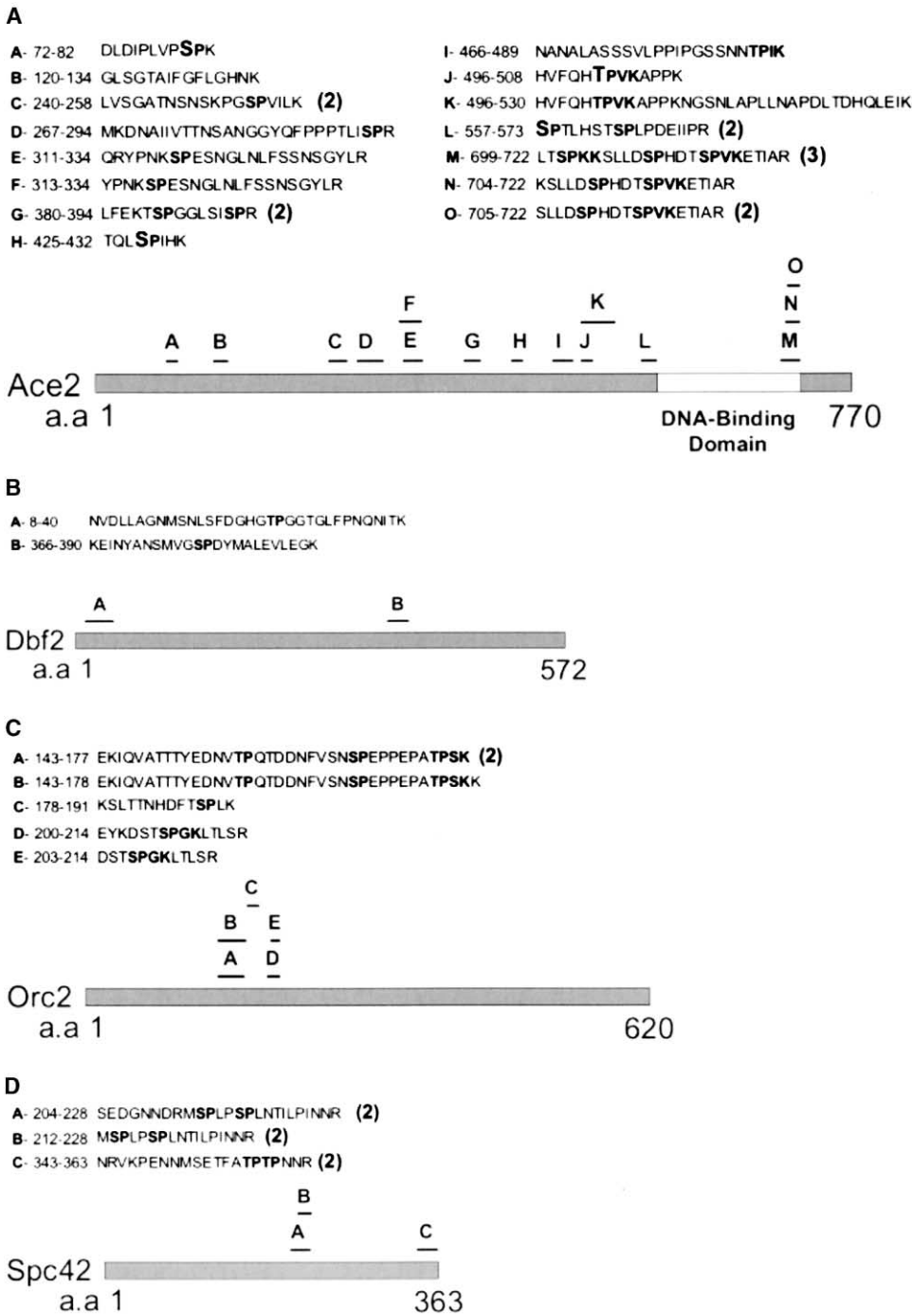


Figure 5. Mapping of Peptides Containing In Vivo Phosphorylation Sites in Cdk Candidate Substrates

(A) Ace2; (B) Dbf2; (C) Orc2; (D) Spc42. Phosphopeptides were mapped from PrA-tagged proteins purified from yeast, using mass spectrometry as described in Chang et al. (2004). The listed peptides were observed to be phosphorylated, some of them bearing two or even three phosphate groups (shown in parentheses). The minimal and optimal Cdk consensus motifs ([S/T]-P and [S/T]-P-X-[K/R]) are shown in bold. Residues that were determined to definitely bear phosphates are shown in bigger font. The numbers on the left of each sequence refer to the amino acid residue numbers and the letters refers to the schematic representation at the bottom, where peptides are mapped on the whole sequence. The DNA binding domain in Ace2 (A) is indicated.

served DNA binding domain also shown to contain an NLS in Swi5 (Moll et al., 1991; O'Conallain et al., 1999). The prevalent idea for the function of Cdc28 on Ace2 is that this NLS is phosphorylated by Cdc28, which causes Ace2 (or Swi5) to be retained in the cytoplasm (Moll et

al., 1991; O'Conallain et al., 1999). Our results show that Ace2 is heavily phosphorylated at multiple sites along its sequence and suggest that Cdc28 is in part responsible for this phosphorylation.

One phosphopeptide (120-134) lacking a [S/T]-P motif

was also identified, indicating that Ace2 can be phosphorylated by at least one other kinase. This kinase could be the Cbk1-Mob2 complex, which functions in targeting Ace2 to daughter cells only at mitotic exit, a mechanism that is required for normal asymmetric growth (Colman-Lerner et al., 2001; Racki et al., 2000; Weiss et al., 2002). In fact, peptide 120–134 contains residue G128, which when mutated to a glutamic acid residue bypasses the requirement for Cbk1 for nuclear localization (Racki et al., 2000; Colman-Lerner et al., 2001). Interestingly, we detected Cbk1 in the Clb3-PrA purification (Figure 1). Instead of being bound directly to Clb3, Cbk1 could have been bound to Ace2, which was present in large amounts in the Clb3-PrA copurification product (Figure 1A).

Other Links between Cyclin-Cdk and Transcription

We observed that Swi5, a close homolog of Ace2, also preferentially associated with Clb3 (Supplemental Figure S2 on *Molecular Cell's* website), suggesting a preferred function for Clb3 in regulating these closely related transcription factors with partially overlapping functions (Doolin et al., 2001). The Cln2-Sin3 association provides another potential connection with transcription. Sin3 is a member of a histone deacetylase complex involved in transcriptional repression (Kadosh and Struhl, 1998) and is phosphorylated at an SP Cdc28 consensus site (Ficarro et al., 2003). These observations lead us to postulate a potential link between Cln2-Cdc28 activity and transcriptional repression by Sin3.

Cyclin-Mediated Control of DNA Replication

We identified two components of the pre-replicative complex (pre-RC; required for DNA replication initiation), Cdc6 and Orc1, in association with Clb2 and Clb5, respectively (Supplemental Figure S1 on *Molecular Cell's* website; Archambault et al., 2003). Cdc6 is a licensing factor required for the loading of the hexameric MCM helicase (Mcm2-7) to form the prereplicative complex, while Orc1 is known to form a stable complex with Orc2–6 (reviewed in Bell and Dutta, 2002). Orc1, Orc2, and Orc6 all contain Cdk consensus phosphorylation motifs and are Cdc28 substrates in vitro (Ubersax et al., 2003). Clb5 interacts specifically with Orc6, and a stable interaction between Clb5-Cdc28 and Orc6 at origins prevents refiring of DNA replication (Wilmes et al., 2004). It is likely that Orc6 as well as other ORC subunits were present in trace amounts in the same purification and that we failed to detect them by mass spectrometry. In such a case, the Clb5-Orc1 association could be bridged by other ORC subunits. Using the same strategy as for Ace2, we identified several in vivo Cdk motif-containing phosphopeptides in Orc2 (Figure 5C) and Orc6 (documented in Chang et al., 2004). We submitted Orc1 to the same analysis and found no phosphopeptides. Phosphopeptides observed in Orc2 and Orc6 contain Cdk consensus sites that were mutated and that were implicated in preventing reinitiation of replication (Nguyen et al., 2001; Wilmes et al., 2004).

We also tested whether a specific cyclin could associate with Sld2, the only known obligatory substrate of Cdc28 in DNA replication (Masumoto et al., 2002). We found that Sld2 preferentially associated with Clb5 (Supplemental Data and Supplemental Figure S2H on *Molec-*

ular Cell's website), consistent with the observation that efficient Sld2 phosphorylation is dependent on *CLB5* and *CLB6* (Masumoto et al., 2002).

Control of Morphogenesis

Cln2-PrA copurified with Ste20, confirming a previous observation (Oda et al., 1999), but Ste20 was not observed in complex with other cyclins tested. Ste20 is a kinase involved in the control of numerous cell growth and pheromone response pathways and is efficiently and specifically phosphorylated by Cln1,2-Cdc28 complexes in vivo (Oehlen and Cross, 1998; Wu et al., 1998). The association of Cln2-Cdc28 with Ste20 may be part of a mechanism allowing Ste20 to switch its function from the mating response pathway to morphogenesis (Oda et al., 1999; Oehlen and Cross, 1998; Wu et al., 1998). Two GAPs (GTPase activating proteins), Rga1 and Bem3, were found to associate with Cln2-PrA (Figure 2 and Supplemental Figure S2 on *Molecular Cell's* website). Rga1 and Bem3 (along with Rga2 and Bem3) stimulate GTP hydrolysis by the Rho-like GTPase Cdc42, which functions in polarity establishment (review in Gulli and Peter, 2001). Since Rga1 acts to repress the pheromone pathway (Stevenson et al., 1995), its phosphorylation by Cln2-Cdc28 could stimulate its function. Consistent with this idea, overexpression of *CLN2* in a *rga1*-deleted strain no longer represses the mating response pathway (Wassmann and Ammerer, 1997). Phosphorylation of Cdc42's GAPs by Cln2-Cdc28 could provide another level of control of the cyclin-Cdk machine over morphogenesis, stimulating polarized growth.

Control of Mitotic Progression

The B type cyclins are essential for mitotic progression and must be inactivated in order to allow termination of mitosis. We found that Nap1 associates specifically with Clb2 (Figure 1), confirming published work (Kellogg et al., 1995). Nap1 was found to be required for the normal mitotic functions of Clb2, including a shift from polarized to isotropic bud growth (Kellogg and Murray, 1995). The axial bud site selection protein Bud3 was also clearly recovered in specific association with Clb2 (Figure 1B and Supplemental Figure S1 on *Molecular Cell's* website). A recent report showed that Bud3 interacts with Clb2 in a two-hybrid assay and that this interaction functions to target Clb2 to the mother bud neck, which seems to stimulate cytokinesis (Bailey et al., 2003). We also identified the karyopherin α (Kap60/Srp1) in association with Clb2, consistent with the existence of both a bud neck-associated pool and a nuclear pool of Clb2—where the nuclearly localized Clb2 is dependent on Kap60-mediated import (Hood et al., 2001).

A series of elegant genetic experiments has suggested that Cln1–3 promote SPB duplication, Clb1–6 promote spindle pole body (SPB) maturation, and mitotic Clb1–4 inhibit SPB licensing and re-reduplication (Haase et al., 2001). Clb5-Cdc28 kinase activity is required for coordinated spindle assembly and orientation (Segal et al., 2000). We found that Clb5 copurifies with Spc42, a phosphoprotein required for SPB duplication. We also observed this interaction in a yeast two-hybrid screen for Clb5 binding proteins (our unpublished data). We identified multiple in vivo phosphorylation at Cdk motif-containing peptides in Spc42 (Figure 5D).

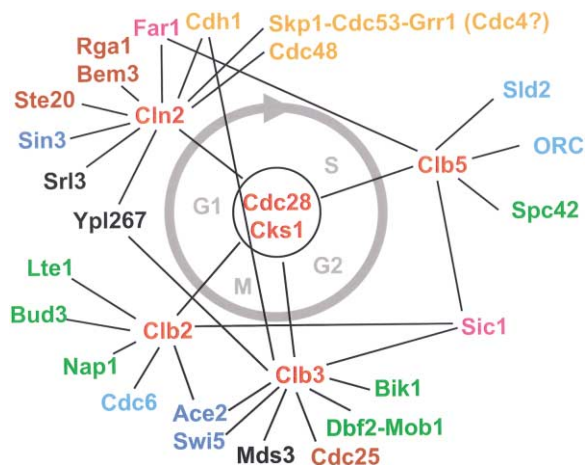


Figure 6. Schematic Organization of Associations with Cyclins Detected in This Study, in the Context of the Cell Cycle Refer to Figure 1C for the color coding.

Mitotic exit and cytokinesis depend on the activation of the mitotic exit network (MEN), composed of the GEF Lte1, the GTPase Tem1, the kinases Cdc5, Cdc15, Dbf2, the Dbf2 binding protein Mob1, and the phosphatase Cdc14 (Bardin and Amon, 2001). Signaling through the MEN leads to Clb-Cdk inactivation, which is required for mitotic exit and cytokinesis. Our finding that Clb2 associates with Lte1 (Figure 1B and Supplemental Figure S1, available on *Molecular Cell's* website), which is enriched for Cdk phosphorylation consensus motifs, suggests that the MEN receives a signal from Clb2-Cdc28. The MEN could also be tuned by Cdk activity at the level of Dbf2 and Mob1 (which form a functional complex; Komarnitsky et al., 1998), as Clb3 copurified with both of these proteins (Figure 1A and Supplemental Figure S1, available on *Molecular Cell's* website). It is formally possible that the kinase Dbf2 (possibly with Mob1) uses the Clb3-Cdc28 complex as a substrate; however, the presence of multiple Cdk consensus motifs in both Dbf2 and Mob1 suggests that they are potential Clb3-Cdc28 substrates. In fact, we observed *in vivo* phosphorylation of Dbf2 in two peptides containing minimal Cdk motifs (Figure 5B). These results suggest the existence of a feedback loop between the MEN and Clb-Cdk activity.

Molecular Mechanisms of Cyclin-Cdk Action

The cyclin-Cdk-associated proteins that we identified (including several known and potential phosphorylation targets) function in several important cell cycle-coordinated cellular events (Figure 6). Some of these targets, such as kinases and proteins of GTPase systems, themselves have a signaling, regulatory function, while other targets, such as subunits of the pre-RC or of the SPB, participate directly in cellular changes. The multiple phosphorylation sites that we have identified in Ace2 suggest a highly complex regulation of Cdk targets, in which it may not always be the degree but the position of the phosphorylation on the target that is important for its regulation. Unraveling this complexity will be a major goal of future work but is beyond the scope of this

study; therefore, we have not evaluated the mechanistic role for the Cdk phosphorylation sites in the regulation of the targets that we have identified. We can speculate that the cyclin-Cdk module could use different molecular mechanisms to act on its targets. On one hand, a cyclin-Cdk complex could act as a kinase on its target, thereby changing the activity of this target effector. On the other hand, it is theoretically possible for a cyclin binding target to function primarily as a “landing pad” or adaptor for a cyclin-Cdk complex. Once bound, the complex could not only phosphorylate the binding target itself but also other proteins in the vicinity; alternatively, the bound cyclin-Cdk module could change (and so regulate) the accessibility of its target for other interactions. By its nature, our study could have enriched for these more stable cyclin-Cdk/target interactions relative to the more transient kinase-substrate interactions.

The Use of Proteomic Tools in Studying Intracellular Signaling

In recent years, a trend has emerged where proteomic strategies are being increasingly used to identify protein interactions taking part in stable complexes. Understandably, there has been a reluctance to apply these methods to identify more dynamic interactions, which are generally expected to be lost during the purification procedures. However, our work and the work of others (Ho et al., 2002) shows that the single-step affinity purification of tagged proteins followed by mass spectrometric identification of associated proteins can indeed be used to identify interactions involved in intracellular signaling. We showed that this approach provides a way to identify proteins functioning directly both upstream of a signaling protein (like the Cln2-Ubiquitin-Cdc48 interaction) or downstream (like the Clb3-Ace2 interaction). Clearly, some kinase-substrate interactions are sufficiently stable throughout the purification procedure (and the techniques employed are sufficiently sensitive) to allow their detection. The use of a single tag makes the purification procedure simpler than the use of a tandem affinity purification (TAP) tag (Honey et al., 2001; Rigaut et al., 1999) of which the serial purification steps involve submitting the complex to different solutions, thus increasing the risk of losing some interactions that might be less stable. The use of a single tag (Flag) was also successfully used to identify several associations with kinases and phosphatases in the proteome-wide study by Ho et al. (2002). The continued exploration of different extracting conditions would almost certainly identify other interactions that were missed in our study.

Our work shows that approaching protein-protein interactions or associations with special care for a specific group, a family, or a module of proteins is proving to be extremely fruitful. Proteome-wide interaction studies have proven to be extremely valuable by their success in identifying hundreds, even thousands, of associations. However, they bear the drawbacks of generating a high proportion of false positives in addition to missing several important biological interactions. The strong interactions between the core subunits of cyclin-Cdk complexes (cyclin, Cdc28, Cks1) and with Sic1 could be detected (although not always) in large-scale studies using affinity purification (Gavin et al., 2002; Ho et al.,

2002) or the two-hybrid system (Ito et al., 2001; Uetz et al., 2000). However, of all the other specific associations with cyclins that we detected in our study, none were detected in those large-scale studies when either cyclins or cyclin-associating proteins were used as baits. Only Srl3 and Ypl014 were found as part of the “Cdc28-associated complex” (Gavin et al., 2002).

Once an associated protein is identified, this protein can in turn be tagged and affinity purified to identify interacting proteins, as we did for Cdc48. This strategy in effect allows a walk through the proteome in action. The genomic tagging at the endogenous locus, unlike overexpression, minimizes the chance of disturbing the physiology of the cell and we assume that the interactions recovered are more likely to be relevant in vivo. As we showed with Ace2 and many other cyclin-associated Cdk candidate substrates, single-step affinity purification using the Protein A tag also allows the identification of in vivo phosphorylation sites on a protein of interest. We are currently exploring ways to perform the phosphosite analysis in a quantitative manner. This will allow us to examine the dynamics of the phosphorylation profile of a protein through the cell cycle.

We emphasize that this strategy has great potential for the study of intracellular signaling. Important pathways often show a high level of redundancy, providing robustness and adaptability to living organisms. Unlike most genetic approaches, the strategy employed here works independently of the existence of “backup pathways” that exist in a signaling network. Restricting a protein-protein association analysis to a specific protein module allows tailoring of the conditions used for the optimal gain of biologically meaningful information. This proteomic approach could be useful to study protein interactions involving a broad range of signaling molecules, such as kinases, phosphatases, and GTPases.

Experimental Procedures

Protein A Affinity Purification for Isolation of Associated Proteins and Protein Identification by Mass Spectrometry

These procedures were performed as described elsewhere (Archambault et al., 2003); modifications are described in the Supplemental Data available on *Molecular Cell's* website.

Coaffinity Purifications, Immunoprecipitation, and Western Blotting

Small-scale Protein A affinity purifications for visualization of copurified Myc-tagged proteins were performed as described (Archambault et al., 2003). Anti-Myc immunoprecipitations were carried out in a similar way. Details are provided in the Supplemental Data available on *Molecular Cell's* website.

Microscopy and FACS

Fluorescence and DIC images were acquired on a Zeiss Axioplan 2 microscope (Carl Zeiss, Inc., Thornwood, NY, USA), with a 100× 1.4 NA Planapochromat objective (Vermont Optechs, Charlotte, VT) fitted with a Hamamatsu digital CCD camera (Sciscope Instrument Co., Iowa City, IA) controlled by Openlab software (Improvision Inc, Lexington, MA). DNA content analysis by FACS was performed as described elsewhere (Epstein and Cross, 1992).

Phosphopeptide Mapping

Phosphopeptide mapping of Ace2-PrA, Dbf2-PrA, Orc2-PrA, and Spc42-PrA was accomplished by hypothesis-driven mass spectrometry as described (Kalkum et al., 2003; Chang et al., 2004) using

an integrated MALDI-QqTOF/MALDI-IT mass spectrometry system (Krutchinsky et al., 2000, 2001; Qin and Chait, 1997).

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