DISSERTATIONES TECHNOLOGIAE UNIVERSITATIS TARTUENSIS
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MARDO KÕIVOMÄGI

Studies on the substrate specificity and multisite phosphorylation mechanisms of cyclin-dependent kinase Cdk1 in *Saccharomyces cerevisiae*

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The current dissertation is based on the following publications referred to in the text by their Roman numbers:

I Avunie-Masala R, Movshovich N, Nissenkorn Y, Gerson-Gurwitz A, Fridman V, **Kõivomägi M**, Loog M, Hoyt MA, Zaritsky A, Gheber L. (2011) Phospho-regulation of kinesin-5 during anaphase spindle elongation. J Cell Sci. 15;124(Pt 6): 873–8.

II **Kõivomägi M**, Valk E, Venta R, Iofik A, Lepiku M, Morgan DO, Loog M. (2011) Dynamics of Cdk1 substrate specificity during the cell cycle. Mol Cell 10;42(5): 610–23.

III **Kõivomägi M**, Valk E, Venta R, Iofik A, Lepiku M, Balog ER, Rubin SM, Morgan DO, Loog M. (2011) Cascades of multisite phosphorylation control Sic1 destruction at the onset of S phase. Nature 12;480(7375): 128–31.

IV **Kõivomägi M**, Loog M (2011) Cdk1: a kinase with changing substrate specificity. Cell Cycle 1;10(21): 3625–6.

V **Kõivomägi M**, Iofik A, Örd M, Valk E, Venta R, Faustova I, Kivi R, Balog ERM, Rubin SM, Loog M. (2013) Multisite phosphorylation networks as signal processors for Cdk1 (Manuscript).

Supporting papers:

VI Venta R, Valk E, **Kõivomägi M**, Loog M. (2012) Double-negative feedback between S-phase cyclin-CDK and CKI generates abruptness in the G1/S switch. Front Physiol 3:459.

VII McGrath D, Balog ERM, **Kõivomägi M**, Lucena R, Mai MV, Hirchi A, Kellogg DR, Loog M, Rubin SM. (2013) Cks Confers Specificity to Cyclin-Dependent Kinase in Multisite Phosphorylation of Cell Cycle Regulatory Proteins (Manuscript).

The articles I–V have been printed with the permission of the copyright owners. My contributions to the papers are as follows:

Ref. I I designed and performed the kinase assay experiments shown in figure 1E.

Ref. II I partially designed and performed the experiments. I analyzed the data and assisted in writing the manuscript.

Ref. III I partially designed the experiments. I performed the experiments, except the isothermal calorimetry and mass-spectrometry experiments. I analyzed the data and assisted in writing the manuscript.

Ref. IV I assisted in writing the manuscript.

Ref. V I partially designed and performed the phosphorylation assays and steady state kinetics experiments. I analyzed the data and assisted in writing the manuscript.

LIST OF ABBREVATIONS

APC	Anaphase promoting complex
CAK	CDK-activating kinase
CDC	Cell division cycle
CDK	Cyclin-dependent kinase
CKI	CDK inhibitor
CKS proteins	Cdc28 kinase subunit proteins
HP	Hydrophobic patch
Hpm	Hydrophobic patch mutant
IDP	Intrinsically disordered protein
LLPP motif	Leu (L, leucine) or Pro (P, proline) rich motif
MAPK	Mitogen activated protein kinase
NLS	Nuclear localization signal
PEST region	Region rich in Pro, Glu, Ser and Thr amino acids
RxL motif	Arg (R, arginine), x amino acid, Leu (L, leucine) containing motif
SCF	Skp1/Cullin/Cdc53/F-box protein
SPB	Spindle pole body

I. INTRODUCTION

The cell cycle is the process by which cells duplicate their contents and then divide to produce a pair of daughter cells. The master regulators of the cell cycle are cyclin dependent kinases (CDKs). CDKs are activated by their periodically accumulating regulatory partners, the cyclins. The enzymatic activity of cyclin-Cdk complexes is tightly controlled by a variety of mechanisms. Substrate targeting by a given cyclin-Cdk complex is mediated by the active site on the CDK and docking sites on the cyclin subunits. Additionally, the presence of a phosphate-binding pocket on the CDK adaptor subunit Cks1 promotes interaction with targets containing multiple phosphorylation sites. In simple eukaryotes, such as budding yeast, a single CDK, Cdk1, enzyme associates with several different cyclins. The combination of rising levels of CDK activity and the distinct substrate specificities of different cyclin-Cdk complexes enables the temporally ordered phosphorylation of the many target proteins that regulate cell cycle events.

Robust inhibition of S-phase CDK activity in the G1 phase of the cell cycle is the major mechanism preventing uncontrolled onset of DNA replication. In budding yeast, S phase is switched on after the rapid proteolytic degradation of the Cdk1 inhibitor Sic1. Sic1 is a stoichometric inhibitor of Clb-Cdk1 complexes. It appears at the end of mitosis, and its destruction at the G1/S boundary is induced by Cdk1-mediated multisite phosphorylation.

The first part of the present dissertation provides an overview of cell cycle control systems, focusing on the different substrate specificities of the various cyclin-Cdk complexes. Next, the CDK inhibitors in yeast and mammalian cells are introduced. Finally, the role of Cks1 as a phosphate binding adaptor molecule for CDK, and the functional implications of this role are reviewed. The original results presented here cover the following areas: a) studies and discussions on the changes in cyclin-Cdk1 substrate specificity during the cell cycle b) *in vivo* and *in vitro* characterization and analysis of multisite phosphorylation of Sic1, and c) characterization of the parameters promoting Cks1-mediated multisite phosphorylation of Cdk1 targets.

2. LITERATURE REVIEW

2.1. The Cell Cycle

The cell cycle is the highly complex process by which all living cells duplicate their contests and distribute them between two daughter cells (Morgan 2007). The cell cycle is typically divided into four distinct phases (Figure 1). The key events of DNA replication and chromosome segregation, which occur (respectively) in the S (DNA synthesis) and M (mitosis) phases of the cell cycle, are separated by gap phases of varying length called G1 and G2. All eukaryotic cell types follow some version of this basic cycle, but the cycle's structure and, regulation, as well as the lenghts of the different phases, may vary. During G1, cells grow and prepare themselves for genome duplication, followed by S phase, when the actual duplication of the genome takes place. In G2, the accuracy of DNA replication is checked as cells prepare for division. Finally, in mitosis, the duplicated genetic material is separated into two daughter cells, and cell division is completed (Forsburg and Nurse 1991; Mendenhall and Hodge 1998).

A classic model system for cell cycle studies is the budding yeast *Saccharomyces cerevisiae*. *S. cerevisiae* is a unicellular fungus, whose cell cycle has a relatively long G1 phase and no clearly defined gap (G2) between S and M phases. Thus, entry into mitosis is not controlled as tightly as it is in other eukaryotic model systems, such as the fission yeast *Schizosaccharomyces pombe* (Hartwell 1974; Morgan 2007). As the name implies, budding yeast cells divide by budding off progeny that are smaller than the mother cells (Hartwell and Unger 1977; Lord and Wheals 1980). To compensate for this difference, and to avoid the problem of getting smaller each time they divide, daughter cells must increase in size and therefore need more time than mother cells to begin next cell cycle (Turner, Ewald et al. 2012). Under certain environmental conditions, budding yeast cells temporarily abandon cell division. In poor nutrient conditions yeast cells arrest as unbudded cells in G1 phase and wait for growth conditions to improve before resuming the cell cycle. Another key environmental influence that interrupts the cell cycle of one cell is proximity to another yeast cell of opposite mating type. These mating partners send out a pheromone signal to arrest each other´s cell cycle in G1 phase and then initiate cell fusion (Herskowitz 1988).

2.2. Cell cycle control system

Cell cycle progression is regulated by a series of biochemical switches that control the order and timing of the major cell cycle events (Hartwell and Weinert 1989; Morgan 2007). These transition points must ensure that cells move unidirectionally through the cell cycle (G1 → S → G2 → M → G1) (Elledge 1996; Morgan 2007). In budding yeast, the first switch point is called Start (Restriction point in mammalian cells), which defines entry into the new cell cycle in late G1 phase. After S phase, the entry into mitosis in most organisms is controlled at the G2/M boundary. Because of budding yeast´s distinc-

tive cellular architecture, the transitions between its S, G2, and M phases are not clearly defined, and cell cycle progression is blocked at the metaphase to anaphase transition, rather than at the G2 to M. Indeed, a unified definition of when *S. cerevisiae* starts mitosis has not been agreed upon (Forsburg and Nurse 1991). Only after successful segregation of sister chromatids can the final event of M phase, cytokinesis, proceed. Defects in the regulation of any of these transitions can result in genomic instability, which, in higher organisms, increases the risk of developing cancer (Sherr 1996; McGowan 2003).

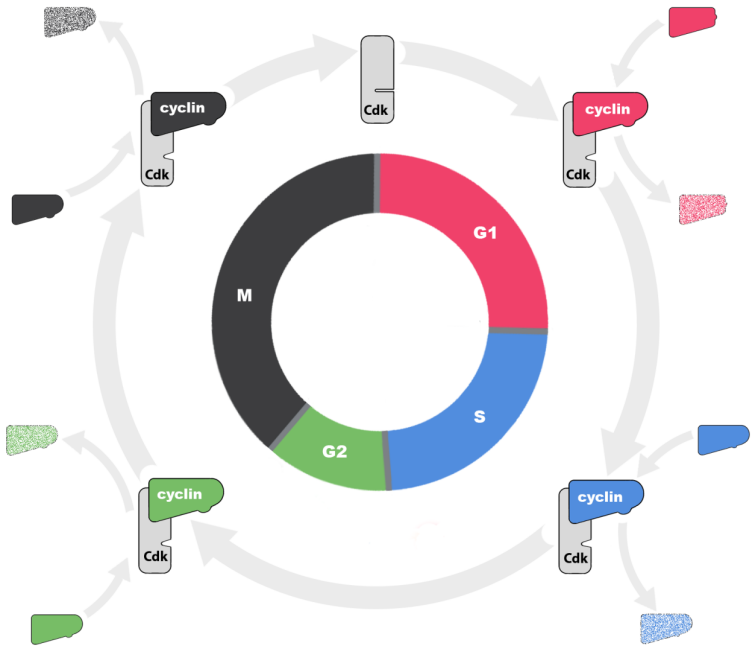

Figure 1. The mitotic cell cycle. The mitotic cell cycle is a sequence of coordinated events that leads to the reproduction of the cell. The cell cycle is divided into 4 phases: G1 →S→G2→M. DNA replication takes place in S phase and the separation of sister chromatids occurs in M phase (mitosis). These two phases are separated by two gap phases, known as G1 and G2. The master regulators of the cell cycle are the cyclin-dependent kinases (CDKs). The catalytic subunit of CDK becomes active when bound to a regulatory cyclin subunit. Each of the cell cycle phases has its specific set of cyclins that are synthesized at the onset of this phase and degraded at the end of the phase. In budding yeast, G1 is driven by the cyclins Cln1,2,3 and S phase by the cyclins Clb5,6. In G2, the cyclins Clb3 and Clb4 are synthesized, and M phase is controlled by cyclins Clb1 and Clb2.

The master regulators of the cell cycle control system are the cyclin-dependent kinases (CDKs), they are activated by periodically synthesized and degraded cyclin partners (Figure 1). During the cell cycle, the rise and fall of CDK activity leads to cyclical changes in the phosphorylation state of diverse targets. This, in

turn, results in the initiation of various cell cycle events (Morgan 2007). Both the production and degradation of the various cyclins are specifically regulated, enabling them to be present at the right time of the cell division cycle. Although cyclin binding is the primary determinant of CDK activity, additional regulatory mechanism exists. CDK activity can be modulated by the binding of adaptor subunits, cyclin-dependent kinase inhibitors (CKIs), or by modifications by other protein kinases (Figure 2). All of these regulators change CDK activity, substrate specificity, or subcellular localization and thereby control progression through cell cycle transition points (Morgan 1997).

2.3. Cyclin-dependent kinases: key regulators of the cell cycle

The cyclin-dependent kinases are a family of proline-directed serine/threonine (Ser/Thr) protein kinases distinguished mainly by their association with cyclins (Morgan 1997). Cyclin binding causes conformational changes in CDK that confer kinase activity to the cyclin-Cdk complex (De Bondt, Rosenblatt et al. 1993). Active kinase complexes are able to phosphorylate Ser (S) or Thr (T) residues in optimal S/T-P-x-K/R (where x is any amino acid) and suboptimal S/T-P consensus motifs (Langan, Gautier et al. 1989; Songyang, Blechner et al. 1994).

Unlike in higher organisms, in budding yeast a single CDK (Cdk1), regulates all phases of the cell division cycle. Cdk1 is activated by different cyclins at different cell cycle phases (Hartwell, Mortimer et al. 1973). In higher eukaryotes, at least six CDKs have been shown to be involved directly in cell cycle control (Nigg 1995; Liu and Kipreos 2000; Malumbres, Harlow et al. 2009; Satyanarayana and Kaldis 2009). Each CDK interacts with a specific subset of cyclins. For example, Cdk1 and Cdk2 both show wide preference in their choice of cyclin partners, binding with cyclins A, B, D and E, whereas Cdk4 and Cdk6 are activated by D-type cyclins (Aleem, Kiyokawa et al. 2005; Hochegger, Takeda et al. 2008).

The first mutant allele of *CDK1* in budding yeast, *CDC28*, was originally found in the early 1970-s by Lee Hartwell in his screen for cell cycle division mutants. The gene encoding *CDK1* is essential and mutant cells arrest early in the cell cycle before Start (Hartwell, Mortimer et al. 1973; Hartwell 1974). It was found that *CDK1* encodes a protein kinase whose activity is regulated through the cell cycle and upon cyclin binding, and that these enzymes are highly conserved in evolution (Beach, Durkacz et al. 1982; Reed, Hadwiger et al. 1985; Wittenberg and Reed 1988; Hadwiger, Wittenberg et al. 1989; Wittenberg and Reed 1989). Although its kinase activity is under complex control, the expression levels of *CDK1* gene are kept constant and its abundance is in excess relative to cyclin partners throughout the cell cycle (Mendenhall, Jones et al. 1987). Therefore, transcriptional and translational regulation of Cdk1 has not been considered important, and apart from cyclin binding, the activity of Cdk1 is controlled mainly at a posttranslational level (Mendenhall and Hodge 1998).

2.3.1. Controlling CDK activity through phosphorylation

For full activation, CDKs require not only the binding of a regulatory cyclin subunit, but also phosphorylation at a conserved Thr residue in the CDK molecule itself (Figure 2) (Morgan 1997). In budding yeast, the activating Thr169 residue is located in a region called T-loop near the entrance of the catalytic cleft: it is phosphorylated by a CDK-activating kinase (CAK) (Morgan 1995; Espinoza, Farrell et al. 1996). The effects of the activating phosphorylation are revealed in the crystallographic structure of the Thr160 (equivalent to budding yeast Thr169) phosphorylated human cyclin A-Cdk2 complex (Russo, Jeffrey et al. 1996). Comparison of this structure with unphosphorylated cyclin A-Cdk2 complex shows that the T-loop region moves due to the phosphorylation and thereby frees the substrate binding site of the kinase. It also changes the positions of amino acid residues responsible for ATP-binding (Jeffrey, Russo et al. 1995; Russo, Jeffrey et al. 1996). In budding yeast, the cyclin-Cdk1 activation pathway differs from that in higher eukaryotes in that, the activating phosphorylation of Cdk1 precedes cyclin binding. This is supported by the fact that a non-phosphorylatable Cdk1 mutant binds cyclin less efficiently compared to wild type control *in vivo* (Ross, Kaldis et al. 2000).

In addition to positive regulation, CDK is also regulated by inhibitory phosphorylation. In yeast cells inhibitory phoshorylation takes place at a single conserved Tyr19 residue. The mammalian version of CDK also has an inhibitory threonine phosphorylation site. These regulatory sites are located near the kinase´s ATP-binding site, and their phosphorylation probably interferes with the orientation of the ATP phosphates and also reduces affinity for substrate peptides/proteins (Welburn, Tucker et al. 2007). Inhibitory phosphorylation is important for DNA damage-induced cell cycle arrest throughout the cell cycle, but its best-characterized function is in controlling the activation of M-phase CDKs at the onset of mitosis.

In budding yeast, Cdk1 is phosphorylated by the Swe1 (the ortholog of Wee1 in budding yeast) tyrosine kinase at Tyr19, and it is dephosphorylated by the Mih1 (the ortholog of Cdc25) phosphatase (Russell, Moreno et al. 1989; Booher, Deshaies et al. 1993). It has been suggested that Swe1 plays a role in cell size control during S/G2/M phases. Loss of Swe1 causes premature mitosis and a reduced cell size (Harvey and Kellogg 2003; Kellogg 2003; Harvey, Charlet et al. 2005). Deletion of Mih1 causes delayed mitosis and shows an increased cell size (Pal, Paraz et al. 2008). Also, it has been proposed that defects in bud morphogenesis engage the morphogenesis checkpoint, which results in activation of Swe1 by an unknown mechanism (Lew and Reed 1995; Lew 2003; McNulty and Lew 2005).

Different cyclin-Cdk1 complexes are differently susceptible to Swe1 promoted inhibition. G1 cyclin-Cdk1 and S phase cyclin-Cdk1 complexes were shown to be weak substrates for inhibitory phosphorylation compared with M phase cyclin Clb2-Cdk1 complexes (Hu and Aparicio 2005; Keaton, Bardes et al. 2007). Consistent with that observation, overexpression of Swe1 results in G2/M phase arrested cells (Booher, Deshaies et al. 1993). Swe1 itself is a

substrate of Cdk1. First, phoshorylation by Clb2-Cdk1 activates Swe1 which holds Clb-Cdk1 complexes in an inactive state. When there is enough Clb2-Cdk1 activity, the phosphorylation of Swe1 rises, this induces a reverse effect and weakens the interaction with Clb2-Cdk1 (Asano, Park et al. 2005; Harvey, Charlet et al. 2005). Furthermore, Swe1 phosphorylation by Clb2-Cdk1 serves as a priming step to promote subsequent polo-like kinase Cdc5-dependent hyperphosphorylation and degradation of Swe1 (Asano, Park et al. 2005). Swe1 degradation is preceded by its relocalization from the nucleus to the mother-bud neck. This relocalization requires Hsl1 (Nim1-related protein kinase) and its association partner Hsl7. Other Hsl1 related kinases Gin4 and Kcc4, in addition to Cla4 (PAK homolog), have been shown to phosphorylate Swe1 (Barral, Parra et al. 1999; Sakchaisri, Asano et al. 2004). The degradation of Swe1 is conducted by two different ubiquitin ligases APC and SCF (Kaiser, Sia et al. 1998; Thornton and Toczyski 2003).

In higher eukaryotes CDK is negatively regulated by the kinases Wee1, Mik1 and Myt1 via phosphorylation of Tyr15 (and adjacent Thr14) (Lundgren, Walworth et al. 1991; Atherton-Fessler, Parker et al. 1993; Mueller, Coleman et al. 1995). This inhibitory phosphorylation is reversed by the protein phosphatase Cdc25 (Honda, Ohba et al. 1993; Sebastian, Kakizuka et al. 1993). Wee1 and related kinases are thought to play a role in mitotic control by holding mitotic cyclin B-Cdk1 complexes in an inactive state. When cells are ready to divide, Cdc25 dephosphorylates CDK to activate cyclin B-Cdk1 complexes. Wee1 and Cdc25 are themselves multisite substrates for cyclin B-Cdk1. When cyclin B-Cdk1 levels reach a certain mitotic threshold, the complex phosphorylates and inhibits Wee1 and activates Cdc25, thereby creating a very powerful activation cascade that abruptly activates more cyclin B-Cdk1 and triggers the start of mitosis (Kellogg 2003; Santos, Wollman et al. 2012).

2.4. Cyclins – activating partners for CDK

Cyclin levels are controlled through regulated transcription, subcellular localization, and timely degradation, which make them present for a limited window of time and in a restricted cell compartment (Murray 2004; Bloom and Cross 2007). Expression of specific cyclins for each cell cycle phase is a common feature of most eukaryotic cell cycles (Evans, Rosenthal et al. 1983; Murray and Kirschner 1989; Hunt and Murray 1993).

Cyclins were first discovered as proteins that appeared and disappeared in synchrony with early embryonic cleavage divisions in sea urchins (Evans, Rosenthal et al. 1983). CDKs can rapidly exchange their cyclin binding partners despite very slow dissociation rates (Kobayashi, Stewart et al. 1994). This is possible due to rapid ubiquitin-mediated degradation of cyclins (Glotzer, Murray et al. 1991; Murray 1995). Cyclin proteins are defined by their ability to bind CDKs and by the presence of a conserved domain called cyclin box, which was revealed by sequence alignment of diverse cyclins (Kobayashi, Stewart et

al. 1992). Cyclin boxes promote binding with CDKs and have a recognizable structural motif called a cyclin fold, which consists of five α-helices (Noble, Endicott et al. 1997). Comparison of crystal structure of cyclin A alone and in complex with Cdk2 reveals that binding with CDK does not affect cyclin conformation (Brown, Noble et al. 1995; Jeffrey, Russo et al. 1995). Rather, cyclin binding has major impact on the conformation of the CDK active site through contacts with its PSTAIRE helix and T-loop (Jeffrey, Russo et al. 1995).

2.4.1. Controlling cyclin abundance through transcription

In budding yeast, cyclins have been classified into two groups: G1 cyclins (Cln1-3) and B-type cyclins (Clb1-6). G1 cyclins participate in the control of the cell cycle from early G1 to DNA replication. The level of G1 cyclins drop dramatically after G1 phase, when their transcription is repressed by mitotic cyclins. B-type cyclins are named after their homology to the cyclin B (mitotic cyclin in higher eukaryotes) and they are expressed in three successive waves from Start to M phase (Mendenhall and Hodge 1998). Eight of these nine cyclins are simultaneously expressed homologous pairs, and these pairs are best distinguished from each other by their expression patterns. The remaining cyclin Cln3 is an upstream regulator of the other G1 cyclins. During G1 and the G1/S transition, Cln1 and Cln2 activate Cdk1. S phase is driven by Clb5 and Clb6, while in G2/M phase Clb3 and Clb4 are expressed. These are finally followed by the mitotic cyclins Clb1 and Clb2 (Figure 1) (Pines 1995; Morgan 1997; Mendenhall and Hodge 1998).

Transcription of *CLN3* gene is detectable throughout most of the cell cycle, peaking in late M/early G1 phase (McInerny, Partridge et al. 1997). Cell cycle entry is initiated by Cln3-Cdk1 (Tyers, Tokiwa et al. 1993; Stuart and Wittenberg 1995). Early cell cycle genes are under the control of the hetero-dimeric transcription factor SBF (composed of Swi4/Swi6) and the related MBF which is formed by Mbp1 and Swi6. The primary role of Cln3-Cdk1 is to phosphorylate the transcriptional inhibitor Whi5, which targets the transcription factors SBF and MBF (Costanzo, Nishikawa et al. 2004; de Bruin, McDonald et al. 2004). Whi5 dissociation from SBF and MBF allows the transcription of about 200 G1/S genes in a temporally organized manner. Amongst earliest transcribed are the two G1 cyclins *CLN1* and *CLN2* (Skotheim, Di Talia et al. 2008; Eser, Falleur-Fettig et al. 2011). After forming active complexes with Cdk1, Cln1,2-Cdk1 are able to promote their own accumulation through a positive feedback loop (Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991; Skotheim, Di Talia et al. 2008). Recently, Start in the budding yeast was quantitatively defined by Skotheim and colleagues as the point where about 50% of Whi5 has translocated out of the nucleus (Doncic, Falleur-Fettig et al. 2011).

Expression of *CLN1* and *CLN2*, which is primarily controlled by SBF, oscillates dramatically through the cell cycle, peaking at Start (Wittenberg, Sugimoto et al. 1990; Tyers, Tokiwa et al. 1992; Stuart and Wittenberg 1995). The first wave of Clb cyclin transcription is controlled by MBF and peaks at

G1/S transition (Nasmyth and Dirick 1991; Schwob and Nasmyth 1993). The other four Clbs appear later, each at times determined by transcriptional control (Andrews and Measday 1998). SBF inactivation is mediated by rising levels of Clb2-Cdk1 (Amon, Tyers et al. 1993). Once activated, Clb2-Cdk1 has the ability to promote its own transcription through the phosphorylation of the transcription factors Fkh2 and Ndd1 (Reynolds, Shi et al. 2003).

2.4.2. Controlling cyclin abundance through proteolysis

Cyclin levels are controlled not only through regulation of their production but through regulation of their destruction, as well. Degradation of the cyclins contributes to the oscillations in CDK activity and sets a requirement for cyclin re-synthesis in each new cell cycle (Figure 2) (Bloom and Cross 2007). Levels of the different cyclin proteins are under tight control of different ubiquitin-dependent proteolysis mechanisms (Deshaies 1997). The G1 cyclins of budding yeast are targets for SCF (Skp1/Cdc53(or cullin)-F-box protein (FBP)) ubiquitin ligase complexes. After the phosphorylation of degradation sites, or degrons, the ubiquitination and degradation of the G1 cyclins Cln1 and Cln2 is mediated by SCF complexes containing the substrate specificity factor Grr1 (Skowyra, Koepp et al. 1999). Degradation of Cln2 depends on its autophosphorylation by active Cln2-Cdk1 (Lanker, Valdivieso et al. 1996). Ubiquitination of Cln3 is mediated by two different SCF ubiquitin ligases, SCF-Cdc4 and SCF-Grr1 (Landry, Doyle et al. 2012) and is triggered by Cdk1-dependent phosphorylation *in cis* (Landry, Doyle et al. 2012). In addition to the G1 cyclins, one B-type cyclin of budding yeast is degraded through the SCF complex: Clb6 is targeted by SCF-Cdc4 complexes. The phosphorylation of Clb6 is mediated by both Cdk1 and another cyclin-dependent kinase Pho85 (Jackson, Reed et al. 2006). The other B-type cyclins are degraded by the Anaphase-Promoting Complex (APC also called the cyclosome). During the early steps of mitosis, the APC, in complex with Cdc20, targets Clb5 and the mitotic cyclins for degradation (Visintin, Prinz et al. 1997; Shirayama, Toth et al. 1999; Wasch and Cross 2002). Later, in M phase, the APC´s substrate specificity is changed as it exchanges the adaptor protein Cdc20 for Cdh1. APC-Cdh1 completes the degradation of the mitotic cyclins and thereby allows cells to complete the cell cycle. In contrast, the Clb5-Cdk1 complexes are not substrates for APC-Cdh1. They can therefore phosphorylate and inactivate Cdh1 at G1/S, allowing accumulation of Clb2 (Zachariae, Schwab et al. 1998; Jaspersen, Charles et al. 1999; Kramer, Scheuringer et al. 2000). Many components of APC-Cdc20 and APC-Cdh1 are differentially phosphorylated and controlled by Cdk1. Clb2-Cdk1 phosphorylates APC-Cdc20 components to activate the APC and facilitate the binding of Cdc20 to the APC *in vivo* (Rudner and Murray 2000).

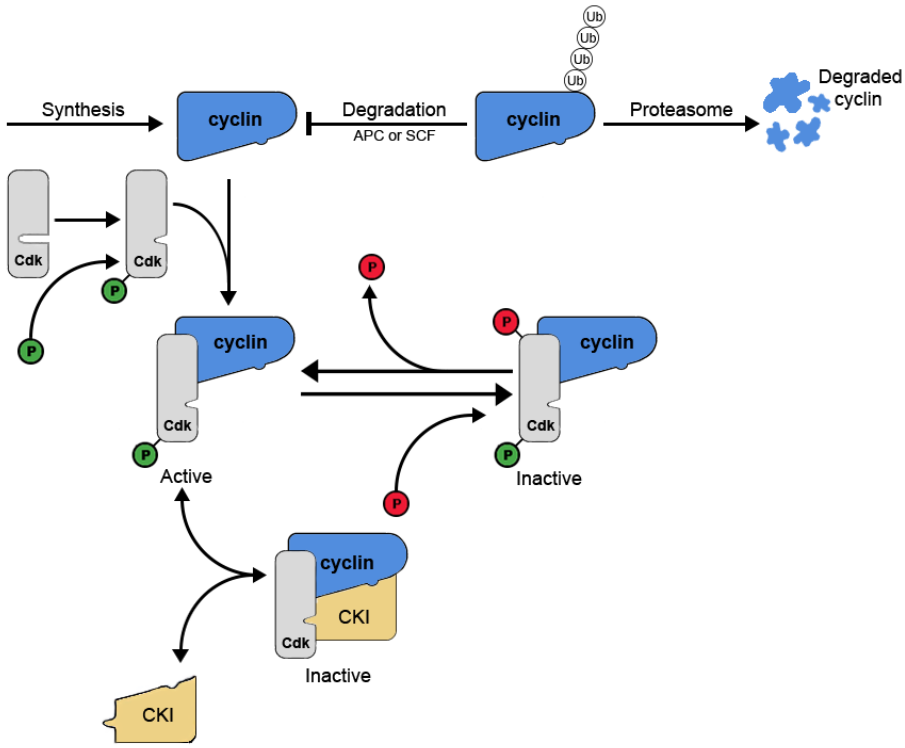

Figure 2. Cyclin-dependent kinase (CDK) activity is regulated at multiple levels. Monomeric CDK lacks activity until it is phosphorylated by CDK-activating kinase (CAK) and associates with a cyclin. The availability of cyclins is controlled by the rates of their synthesis and degradation. Cyclins are targeted for ubiquitin-dependent degradation in the proteasome by two ubiquitin-ligase systems: SCF and APC. The assembled cyclin-Cdk complexes can be inactivated by cyclin-dependent kinase inhibitors (CKIs) or by reversible inhibitory phosphorylation. APC, Anaphase-Promoting Complex; CKI, cyclin-dependent kinase inhibitor; SCF, Skp1-Cullin-Fbox ubiquitin ligase complex; P, phosphorylated residue (green – activating; red – inhibitory); Ub, ubiquitin.

The SCF and APC complexes are E3 ubiquitin ligases that target cell cycle proteins for degradation by the 26S proteasome through the covalent attachment of polyubiquitin chains (Reed 2003). Ubiquitins are attached to lysine residues of target proteins by an enzymatic cascade including three enzyme complexes: i) the ubiquitin-activating enzyme (E1), ii) the ubiquitin-conjugating enzyme (E2), and iii) the ubiquitin-ligase (E3) (Hoyt 1997). The subunits providing substrate specificity to the SCF are called F-box proteins (FBP). Two of them Cdc4 and Grr1 have well characterized roles in budding yeast cell cycle regulation (Skowyra, Craig et al. 1997). Differential localization of FBPs is one way this regulation is accomplished. Cdc4 is localized to the nucleus, whereas Grr1 protein is found in both the nucleus and the cytoplasm (Blondel, Galan

et al. 2000). Most known SCF substrates must be phosphorylated at (phospho)degron sites to be bound by their cognate F-box protein. (Deshaies 1997; Nash, Tang et al. 2001). Binding studies have revealed that Cdc4 binds phosphopeptides containing a single pSer or pThr followed by proline and preceded by hydrophobic residues: I/L-I/L/P-pS/T-P<RKY>$_4$ (where <X> refers to disfavoured residues) (Nash, Tang et al. 2001). In later studies it was found that Cdc4 has a higher affinity for peptides containing two phosphorylated sites (called a diphosphodegron), and this is more important than the actual primary sequence surrounding the degron (Hao, Oehlmann et al. 2007; Bao, Shock et al. 2010). Diphosphodegrons are formed by two phosphates that are separated by two to three amino acids (Hao, Oehlmann et al. 2007). Recently, SCF-Cdc4 substrates such as Sic1, Ash1, Eco1, and Tec1 have been demonstrated to contain diphosphodegrons (Hao, Oehlmann et al. 2007; Bao, Shock et al. 2010; Liu, Larsen et al. 2011; Lyons, Fonslow et al. 2013). In addition, most SCF substrates contain destabilizing PEST regions (regions rich in proline (P), glutamate (E), serine (S) and threonine (T) residues) (Rogers, Wells et al. 1986; Willems, Goh et al. 1999). For example, the G1 cyclins, which have very short half-lives of about 5-10 minutes contain PEST regions in their C-termini (Cross 1988; Nash, Tokiwa et al. 1988; Hadwiger, Wittenberg et al. 1989; Lanker, Valdivieso et al. 1996).

Cdc20 and Cdh1 are the two substrate-specific activators of APC-dependent proteolysis that mediate substrate binding to the APC complex (Visintin, Prinz et al. 1997). Two degradation motifs have been found in APC substrates. A destruction box with the consensus sequence R-x-x-L-x-x-x-x-N (where x is any amino acid) is important for most APC substrates (Glotzer, Murray et al. 1991). In addition, another degradation signal called a KEN box, with the consensus of K-E-N-x-x-x-N (where x is any amino acid) has been identified (Pfleger and Kirschner 2000).

2.4.3. Cyclins can act as localization factors for CDK

Diverse localization of different cyclin-Cdks could regulate their accessibility to specific structures in the cell and to substrates specifically localized to those structures. In budding yeast, the G1 cyclins Cln2 and Cln3 have been shown to localize to different subcellular fractions (Miller and Cross 2000; Edgington and Futcher 2001; Miller and Cross 2001). Cln2 was found to be mainly cytoplasmic but also nuclear (Edgington and Futcher 2001). Its cytoplasmic localization was dependent on phosphorylation: a Cln2 phosphosite mutant exhibited decreased nuclear accumulation of Cln2 (Levine, Huang et al. 1996; Miller and Cross 2000; Miller and Cross 2001). Unlike Cln2, Cln3 has a C-terminal bipartite NLS (nuclear localization signal), and is located only in the nucleus. Deletion of the sequence results in a shift of Cln3 to the cytoplasm (Levine, Huang et al. 1996; Miller and Cross 2000; Miller and Cross 2001).

All mitotic cyclins have a similar localization pattern; mainly nuclear with a small cytoplasmic fraction (Bailly, Cabantous et al. 2003). Additionally, Clb2 is

present at the bud neck during budding (Hood, Hwang et al. 2001). Localization of Clb2-Cdk1 was shown to be independent of its kinase activity but dependent on a hydrophobic patch (HP) in the cyclin, as well as the protein Bud3 (Bailly, Cabantous et al. 2003). Clb5 nuclear localization may be facilitated by the CDK inhibitor Sic1, which binds and inhibits B-type cyclin-Cdk1 complexes (Rossi, Zinzalla et al. 2005). The mitotic Clb4-Cdk1 complex, together with a phospho-adaptor Cks1, has been found to accumulate on budward-directed SPB´s (Spindle pole body). The exact mechanism behind this phenomenon is not well understood, but it might include Kar9 as a transporter (Liakopoulos, Kusch et al. 2003; Maekawa and Schiebel 2004).

2.5. Cyclin-Cdk activity in cell cycle control

Cell cycle events are coordinated by changing cyclin-Cdk activity levels and by different substrate specificities of each cyclin-Cdk. Early results from studies of the fission yeast cell cycle led to the proposal of a quantitative model of CDK regulation (Fisher and Nurse 1996). This model states that in the beginning of the cell cycle the overall level of activity is very low and sufficient only to induce the formation of replication complexes. Thus, S phase (DNA replication) is executed when CDK activity is low, and the subsequent rise in CDK activity, prevents re-replication and promotes mitosis. After completing M phase, the system resets itself, and returns to the low kinase activity state. This model requires either different rates for S- and M-phase targets or different phosphatase specificity towards S- and M-phase targets (Stern and Nurse 1996; Uhlmann, Bouchoux et al. 2011; Fisher, Krasinska et al. 2012). Recent work in fission yeast using an engineered cyclin-Cdk fusion protein and different doses of an inhibitor, which allowed fine-tuning the enzymatic activity of the complex, has provided evidence that, at least in principle, a single cyclin-Cdk can drive the cell division cycle (Coudreuse and Nurse 2010).

Three recent studies have shown that different levels of mitotic Cdk1 activity are required to trigger different events during mitotic entry. It was shown in HeLa cells that increasing levels of cyclin B1-Cdk1 activity coordinate events in prophase. Earlier events required less cyclin B1-Cdk1 activity than later ones (Gavet and Pines 2010). *In vitro* studies showed that higher levels of cyclin B1-Cdk1 activity were needed for phosphorylation of later-acting substrates (Deibler and Kirschner 2010). In budding yeast, the timing of mitotic events like growth polarization, spindle formation, and spindle elongation were shown to depend on different levels of mitotic cyclin Clb2 (Oikonomou and Cross 2011).

In vivo evidence from many organisms hints that numerous cyclins and in some cases several CDKs are required for cell cycle progression (Roberts 1999). Quantitative analysis in budding yeast showed that the abundance of different cyclins is relatively similar (Cross, Archambault et al. 2002). This suggests that the period from G1 to M phase is a state of relatively unchanging

net levels of activated Cdk1. Therefore, in addition to different Cdk1 activity levels, other mechanisms may be required for CDK to coordinate cell cycle events. The biological specificity of cyclins suggests that various cyclin-CDK complexes may have intrinsically distinct substrate preferences, due to differential substrate recognition by different cyclins. For example, in budding yeast, execution of some cell cycle events is dependent on specific cyclin-Cdks. G1 cyclins cannot initiate mitosis, and, conversely, B-type cyclins cannot activate G1-specific transcription (Schwob and Nasmyth 1993; Nasmyth 1996). A large-scale quantitative analysis has shown that different cyclins can simultaneously modulate both CDK active site specificity and cyclin-mediated substrate docking interactions (Loog and Morgan 2005). These two substrate selection mechanisms are mutually compensating: in the case of the S-phase cyclin Clb5-Cdk1, the low intrinsic activity on the active site level was compensated by an efficient cyclin-specific docking interaction for a subset of S-phase targets. Contrarily, the mitotic Clb2-Cdk1 complex has high intrinsic activity on the active site level, enabling broader substrate selection in mitosis. However, this higher intrinsic activity is offset by weaker cyclin specific docking. Further development of the model has indicated that the strength and specificity of the two targeting modes changes reciprocally as the cell cycle progresses. That is, each successive cyclin pair exhibits higher active site specificity and weaker cyclin-mediated binding (Koivomagi and Loog 2011; Koivomagi, Valk et al. 2011). The model includes the principle of gradually increasing active site specificity, which fulfills the core requirement of the rising levels on Cdk1 activity outlined in the quantitative model. Additionally, it also involves different mechanisms of cyclin-specific substrate docking, which compensate for the low intrinsic specificity of Cdk1 in the early stages of the cell cycle for targeting a subset of crucial early targets. The model will be described in detail in the results section of the thesis.

2.6. Substrate recognition specificity of CDKs

Different studies over the years have suggested that cyclin-Cdks recognize their substrates by several mechanisms. The first important aspect of substrate recognition is that the phosphorylation site on the substrate matches the consensus amino acid sequence, which is complementary to the active site of the kinase (Figure 3). The consensus sequence for most cyclin-Cdks is S/T-P-x-R/K (where x is any amino acid) (Beaudette, Lew et al. 1993; Nigg 1993; Songyang, Blechner et al. 1994). A crystal structure of cyclin-Cdk2 complex together with a substrate peptide containing the optimal consensus motif shows that the amino acids forming the consensus sequence bind to the active site of the CDK and do not make direct contact with the cyclin subunit (Brown, Noble et al. 1999). Cyclin-Cdk complexes are also able to phosphorylate target proteins in minimal or suboptimal consensus sequences which consist of S/T-P (Nigg 1993). Some studies indicate that CDKs are able to phosphorylate non-

S/T-P phosphorylation sites, but the mechanisms behind this phenomenon remain unknown (Verma, Annan et al. 1997; Harvey, Charlet et al. 2005; McCusker, Denison et al. 2007; Egelhofer, Villen et al. 2008). Phosphorylation sites are frequently found in poorly conserved, intrinsically disordered regions in substrate proteins (Moses, Heriche et al. 2007; Holt, Tuch et al. 2009).

A systematic study that concentrated on the primary sequence specificities of the protein kinases used a positionally-oriented peptide library approach (Songyang, Blechner et al. 1994). Comparison of cyclin A-Cdk2 and cyclin B-Cdk1 showed that despite being two different kinases that act in different stages of the cell cycle they prefer nearly identical peptide substrates. The consensus motif was found to be K/R-S-P-R/P-R/K/H for cyclin B-Cdk1 substrates (Songyang, Blechner et al. 1994). Also, other approaches, such as GST fusion proteins containing systematic alterations to a consensus phosphorylation site, have been used to determine the specificities of different CDKs bound to various cyclins (Holmes and Solomon 1996). Cyclin A versus cyclin B in complex with Cdk1 showed no differences with respect to the consensus sequence K-S-P-R-K (Holmes and Solomon 1996).

The second important aspect of CDK substrate specificity is that it may involve interaction between the cyclin and docking motifs on the substrate (Figure 3). E, A, and B-type cyclins possess a so-called hydrophobic patch region (hereafter HP) that is located ≈35-40Å away from the active site of CDK and contains an Met-Arg-Ala-Ile-Leu (M-R-A-I-L) sequence conserved among a number of mammalian and yeast cyclins (Adams, Sellers et al. 1996; Kelly, Wolfe et al. 1998; Schulman, Lindstrom et al. 1998; Cross and Jacobson 2000). The HP region recognizes and interacts with target proteins containing the motifs Arg-x-Leu-Φ or Arg-x-Leu-x-Φ (where x is any amino acid and Φ is large hydrophobic amino acid), hereafter RxL. This motif is common to a number of substrates and inhibitors of CDKs. The presence of an RxL binding site increases the efficiency of substrate phosphorylation dramatically, suggesting that this docking site is important for increasing affinity between the substrate and the cyclin-Cdk complex (Schulman, Lindstrom et al. 1998; Takeda, Wohlschlegel et al. 2001; Ubersax and Ferrell 2007). In studies with peptides containing optimal or suboptimal phosphorylation sites, a C-terminally located RxL motif was found to increase catalytic efficiency at the poor phosphorylation site, with a reduced effect at the more consensus-like site (Stevenson-Lindert, Fowler et al. 2003). Based on a study using substrates with linkers of varying length between the RxL motif and CDK phosphorylation site, it was proposed that both sites must be simultaneously bound to the cyclin-Cdk to maximize phosphorylation of the substrate (Takeda, Wohlschlegel et al. 2001). Recent studies in budding yeast have shown that G1 cyclins also possess hydrophobic regions that allow them to recognize an LLPP (Leu-Leu-Pro-Pro) motif in substrate proteins (Bhaduri and Pryciak 2011; Koivomagi, Valk et al. 2011; Koivomagi, Valk et al. 2011).

Structural studies on a complex of cyclin A-Cdk2 with the inhibitor p27Kip1 and a peptide from p107 show that the RxL-containing docking site is located at

an exposed hydrophobic region on the cyclin molecule (Brown, Noble et al. 1999). This hydrophobic site is conserved in cyclins A, B, D, and E in higher eukaryotes and, in the case of budding yeast, in all B-type cyclins including Clb5 (Brown, Noble et al. 1995; Cross, Yuste-Rojas et al. 1999; Cross and Jacobson 2000). Mutations in HP region of the cyclin cause loss of function *in vivo* and reduce enzyme activity against RxL containing substrates *in vitro* (Adams, Sellers et al. 1996; Schulman, Lindstrom et al. 1998; Loog and Morgan 2005). A two-hybrid screen for proteins interacting with Clb5 in an HP-dependent manner identified several potential Clb5-Cdk1 substrates, among them Orc6, Fin1, Yen1 and Far1 (Wilmes, Archambault et al. 2004; Archambault, Buchler et al. 2005). The HP motif in mitotic cyclins Clb1 and Clb2 has evolved differently and might be important for interaction with Swe1, which regulates Cdk1 activity (Hu, Gan et al. 2008).

2.7. Substrates of cyclin-Cdk complexes

To understand how CDKs promote cell cycle progression, it is necessary to identify their physiological targets and to determine how phosphorylation influences the function of these substrates and the cellular events they control (Ubersax and Ferrell 2007). Several studies based on large scale screening methods and computational approaches have provided a list of potential CDK targets in budding yeast (Ubersax, Woodbury et al. 2003; Archambault, Chang et al. 2004; Chang, Begum et al. 2007; Moses, Heriche et al. 2007; Holt, Tuch et al. 2009). So far, detailed reports of about 75 budding yeast CDK substrates phosphorylated *in vivo* have been published (Enserink and Kolodner 2010). A similar number has been described in higher eukaryotes (Blethrow, Glavy et al. 2008; Errico, Deshmukh et al. 2010). However, studies applying global approaches suggest that the number of CDK targets in different model systems could be in the hundreds, if not thousands.

In budding yeast, to search for substrates of Cdk1 in complex with Clb2, the phosphorylation of 522 proteins containing the Cdk1 consensus motif, as well as an additional random set of 173 proteins, were examined. In total, 181 proteins were determined to be Clb2-Cdk1 substrates (Ubersax, Woodbury et al. 2003). 150 of these were also tested in parallel with Clb2- and Clb5-Cdk1 to determine the differences in specificity imposed by the different cyclins. Most of the substrates were better phosphorylated by Clb2-Cdk1, but 36 were more efficiently targeted by Clb5 (Loog and Morgan 2005). Additionally, CDK substrates were identified *in vivo* using a combination of specific CDK inhibition and mass spectrometry. A total of 547 phosphorylation sites on 308 Cdk1 targets were identified (Holt, Tuch et al. 2009).

In *Xenopus* extracts, to identify substrates of various cyclin-Cdk complexes, a shift assay were used. A total of 35 potential substrates for cyclin B-Cdk1, 70 for cyclin A-Cdk2, and 42 for cyclin E-Cdk2 were identified. These substrates were involved in many critical cellular processes, including nuclear assembly,

regulation of CDK activity, cytoskeletal organization, vesicular trafficking, cellular migration, and invasion (Errico, Deshmukh et al. 2010).

In human cell lysates, a screen searching for cyclin A-Cdk2 targets identified 180 potential substrates. These substrates controlled different biological processes, including cell cycle progression, DNA and RNA metabolism, translation, etc. 43% of the sites phosphorylated were optimal consensus sites for CDK. Interestingly, 50% of the non-consensus sites carried at least one optimal RxL motif distal to the phosphorylation site (Chi, Welcker et al. 2008). Another study, using similar methods, identified over 70 substrates for cyclin B-Cdk1 in HeLa cell extracts (Blethrow, Glavy et al. 2008).

CDK targets are found to mediate different processes in all stages of the cell division cycle. In the next paragraphs a selection of key targets are described whose phosphorylation has been characterized in more detail.

2.7.1. CDK targets during G1 phase

In *S. cerevisiae*, entry into the cell cycle is induced by Cln3-Cdk1, which targets Whi5, the repressor of G1/S transcription (Costanzo, Nishikawa et al. 2004; de Bruin, McDonald et al. 2004). The exact mechanism behind Cln3-Cdk1-mediated Whi5 phosphorylation and the subsequent dissociation of Whi5 from SBF complexes remains unknown. It has recently been shown that an activator of the G1-specific transcription factors, Msa1, interacts with SBF and MBF complexes, and this binding promotes proper timing of the G1 transcriptional program (Ashe, de Bruin et al. 2008). It was proposed that Cdk1-dependent phosphorylation of Msa1 in its NLS sequence may induce its nuclear export thereby shutting off the G1 transcriptional program in S phase (Ashe, de Bruin et al. 2008; Kosugi, Hasebe et al. 2009). Another transcriptional activator, Stb1, has been shown to interact with Swi6 to promote the activity of SBF and MBF. Phosphorylation of Stb1 by Cdk1 releases it from promoters (Ho, Costanzo et al. 1999; Costanzo, Schub et al. 2003; de Bruin, Kalashnikova et al. 2008). In addition, other interaction partners of the SBF complex might be regulated by Cdk1. Clb6-Cdk1 complexes have been shown to specifically phosphorylate Swi6 and therefore promote its nuclear export (Geymonat, Spanos et al. 2004).

During pheromone signaling in *S. cerevisiae*, Cln-Cdk1 is thought to negatively control a protein kinase called Ste20, a component of the pheromone response pathway (Wu, Leeuw et al. 1998). Additionally, a scaffold protein, Ste5, that mediates the order of MAPK (Mitogen activated protein kinase) signals in the same pathway was identified as a target of Cln1,2-Cdk1 (Strickfaden, Winters et al. 2007). The phosphorylation of Ste5 blocks its membrane localization, inhibiting pheromone signaling (Winters, Lamson et al. 2005; Strickfaden, Winters et al. 2007). Cln1,2,3-Cdk1 complexes have been proposed to mediate the phosphorylation of a Cdk1 inhibitor and a scaffold protein of the pheromone pathway, Far1, to target it for degradation through the SCF-Cdc4 complex (Gartner, Jovanovic et al. 1998; Jeoung, Oehlen et al. 1998).

During the G1 phase of the cell cycle, cyclin-Cdks trigger critical events that culminate in bud emergence, spindle pole body duplication, and DNA replication. The beginning of bud formation following cell cycle entry represents a dramatic and readily detectable change in cell morphology. Cln1,2,3-Cdk1 activity is crucial for bud formation (Lew and Reed 1993; Moffat and Andrews 2004; McCusker, Denison et al. 2007). In G1, Cln-Cdk1 targets Far1 to allow thereby Cdc24, an exchange factor for the small GTPase Cdc42, to exit the nucleus (Nern and Arkowitz 2000). Membrane clustering and activation of Cdc42 is a key step in cell polarization associated with bud formation. Hydrolysis of GTP to GDP by Cdc42 is stimulated by various GTPase activating proteins (GAPs) that are targets for Cdk1. One of the GAPs, Rga2, was shown to be directly phosphorylated and negatively regulated by Cln1,2-Cdk1. This was shown to restrict the activation of Cdc42 and to prevent bud emergence (McCusker, Denison et al. 2007; Sopko, Huang et al. 2007).

Duplication of the spindle pole body (SPB) is essential for the formation of a bipolar mitotic spindle. SPB duplication begins in G1 and requires Cln-Cdk1 activity. The key candidate target for this process is the SPB component Spc42 (Jaspersen, Huneycutt et al. 2004). Additionally, more than ten potential Cdk1 targets were found in a proteomic screen for phosphorylation sites in SPB components isolated from cells at different stages of the cell cycle (Huisman, Smeets et al. 2007; Keck, Jones et al. 2011).

In mammalian cells, one of the most important substrates in G1 phase for different cyclin-Cdk complexes is the pRb (retinoblastoma tumor suppressor, which functions analogously to Whi5 in budding yeast) protein (Weinberg 1995). pRB contains 16 consensus CDK phosphorylation sites (Lees, Buchkovich et al. 1991). The functional importance of several of these phosphorylation sites was recently demonstrated in a crystallographic study (Burke, Hura et al. 2012; Rubin 2013). During the cell cycle, pRb is hypophosphorylated in early to mid-G1-phase and becomes hyperphosphorylated during mitosis (Arellano and Moreno 1997). pRb is the target of cyclin D1-Cdk4, but it is also a substrate for other cyclin-Cdk complexes, like cyclin E-Cdk2 and cyclin A-Cdk2 (Mittnacht 1998). Several studies have demonstrated that cumulative hyperphosphorylation of pRB at multiple sites is required to liberate bound E2F transcription factor from pRB-E2F complexes (Knudsen and Wang 1996; Knudsen and Wang 1997). The release of E2F allows the transcription of S-phase-specific genes. E2F is itself a substrate for cyclin A-Cdk2: phosphorylation of E2F inhibits its function as transcription factor (Dynlacht, Flores et al. 1994; Xu, Sheppard et al. 1994).

The CDK inhibitor $p27^{Kip1}$ is a key regulator of cell proliferation that binds and inhibits cyclin E-Cdk2 and cyclin A-Cdk2. Tyrosine phosphorylation of $p27^{Kip1}$ in early G1 weakens its inhibitory action towards Cdk2. This allows cyclin E-Cdk2 to phosphorylate $p27^{Kip1}$ at Thr187, which is the recognition signal for SCF-Skp2 ubiquitin ligase (Sheaff, Groudine et al. 1997; Chu, Sun et al. 2007). Additionally, cyclin E-Cdk2 promotes centrosome duplication through the phosphorylation of the centrosomal proteins NPM/B23 (nucleo-

phosmin) and CBP110 (centrosomal protein of 110 kDa) (Okuda, Horn et al. 2000; Chen, Indjeian et al. 2002).

2.7.2. The substrates of CDK in S phase

Cdk1 phosphorylation of key substrates is essential for the initiation of DNA synthesis and for limiting DNA replication to a single round per cycle. DNA replication origins are binding sites for origin recognition complexes (ORC-s, consisting of Orc1-6). ORCs are involved in recruitment of the ATPase Cdc6, Cdt1 (Chromatin licensing and DNA replication factor 1) and the Mcm2-7(Minicromosome maintenance) complex. Together, they form the pre-replication complex (pre-RC) (Diffley 2004). After pre-RCs are formed, the transition to preinitiation complex (pre-IC) takes place (Bell and Dutta 2002). This process is believed to be initiated by Clb5,6-Cdk1 upon destruction of Sic1 (Schwob, Bohm et al. 1994). The initiation of DNA replication is under the control of the essential Clb5-Cdk1 targets Sld2 and Sld3. The phosphorylation of Sld2 at several CDK consensus sites exposes a key residue, T84, - necessary for the formation of the Sld2-Sld3-Dpb11 complex (Masumoto, Muramatsu et al. 2002; Zegerman and Diffley 2007; Tanaka, Umemori et al. 2007). This complex mediates the assembly and activation of the replicative complex (Kang, Galal et al. 2012).

The re-replication of DNA during S phase is prevented by multiple mechanisms. Cdk1 has been shown to phosphorylate the components of pre-RCs: the ORC complex, Cdc6, and the Mcm2-7 complex, which prevents premature reloading of the licensing factors and formation of the pre-replication complex before next G1. Two different subunits of the ORC are phosphorylated by Clb5,6-Cdk1 (Nguyen, Co et al. 2001). Binding between Clb5-Cdk1 and Orc6 is mediated by the interaction of HP-RxL (Wilmes, Archambault et al. 2004). The phosphorylation of Cdc6 by Clb-Cdk1 complexes removes it from replication origins and promotes its ubiquitination and subsequent degradation (Piatti, Lengauer et al. 1995).

A spindle stabilizing protein, Fin1, has been shown to be a target of Clb5-Cdk1. Phosphorylation of Fin1 from S phase through metaphase inhibits its binding to the spindle. After Clb5 degradation in anaphase and activation of Cdc14, Fin1 is dephosphorylated and can associate with the spindle (Woodbury and Morgan 2007).

In higher eukaryotes, cyclin A-Cdk2 activity is needed in the beginning of S phase. In mammalian cells, the ORC subunit Orc1 and Cdt1 are substrates of cyclin A-Cdk1. The phosphorylation of Orc1 prevents its binding to chromatin during mitosis, and Cdt1 is targeted for degradation through the ubiquitin ligase complex of SCF-Skp2 (Li, Vassilev et al. 2004; Liu, Li et al. 2004).

2.7.3. G2/M phase substrates of CDK

Clb3,4-Cdk1 have been shown to phosphorylate the Kar9 protein *in vivo* and this phosphorylation is required for its asymmetrical binding to spindle pole bodies (Liakopoulos, Kusch et al. 2003). The transcription factor Ace2, which is responsible for septum destruction after cytokinesis, coimmunoprecipitates with Clb3. The amount of cells with Ace2 in the nucleus is increased in *clb3*Δ*/clb4*Δ double mutants, suggesting that Clb3-Cdk1 is involved with excluding Ace2 from the nucleus (Archambault, Chang et al. 2004).

2.7.4. Mitotic substrates of CDK

The phosphorylation of the APC components Cdc16, Cdc23, and Cdc27 is required for APC activation and for binding of the activator protein Cdc20 to the APC (Rudner and Murray 2000). Acm1 is an inhibitor of APC-Cdh1. The phosphorylation of Acm1 is thought to play a role in its stabilization, protecting it from proteasome-mediated destruction (Enquist-Newman, Sullivan et al. 2008; Hall, Jeong et al. 2008). The binding of the APC activator Cdh1 to the core complex is also controlled by Cdk1-dependent phosphorylation (Jaspersen, Charles et al. 1999; Crasta, Lim et al. 2008).

The kinesins Kip1 and Cin8 are required for separation of SPBs. Kip1 and Cin8 are both *in vitro* targets for Clb2-Cdk1 (Chee and Haase 2010; Avunie-Masala, Movshovich et al. 2011). The CDK phosphorylation sites in the motor domain of Kip1 were found to be critical for SPB separation (Chee and Haase 2010). Additionally, a Cin8 phosphorylation-deficient mutant changed the normal morphology of spindles (Avunie-Masala, Movshovich et al. 2011).

Several transcriptional regulatory proteins are phosphorylated and controlled by Clb2-Cdk1. For example Clb2-Cdk1 phosphorylates the transcription factor Fkh2 (Pic-Taylor, Darieva et al. 2004) and transcriptional activator Ndd1 (Darieva, Pic-Taylor et al. 2003; Reynolds, Shi et al. 2003). The nuclear localization of the *SIC1* cluster transcription factor Swi5 is controlled by phosphorylation by Clb2-Cdk1 (Moll, Tebb et al. 1991). Recently it was shown that Nrm1, a factor for shutting off the G1 transcriptional program, is stabilized by Clb2-Cdk1-dependent phosphorylation (Ostapenko and Solomon 2011).

In higher eukaryotes, the onset of mitosis requires increased activity of Cdk1 associated with cyclin A and cyclin B, with the cyclin B-Cdk1 complex as the major regulator. Prior to mitosis, cyclin B-Cdk1 is phosphorylated at key residues necessary for nuclear translocation (Toyoshima-Morimoto, Taniguchi et al. 2001; Yang, Song et al. 2001; Santos, Wollman et al. 2012). Once activated the cyclin B-Cdk1 complex promotes several early events of mitosis. For example, phosphorylation of nuclear lamins triggers the dissembly of the lamin filaments (Heald and McKeon 1990). Phosphorylation and activation of condensin is necessary for chromosome condensation (Kimura, Hirano et al. 1998). This is accompanied by hyperphosphorylation of histones and other chromatin-associated proteins (Nigg 1993; Hans and Dimitrov 2001). As

mitosis progresses, cyclin B-Cdk1 phosphorylates many mitosis specific substrates including INCENP (Inner centromer protein) and BubR1 (Mitotic checkpoint serine/threonine-protein kinase BUB1 beta), creating recognition sites for other proteins and causing structural changes that include centrosome separation and spindle assembly (Goto, Kiyono et al. 2006; Wong and Fang 2007). In addition, various components of the regulatory machinery of the cell cycle are controlled by cyclin B-Cdk1 complex activity. These include Cdc25, Wee1, components of APC, separase, and securin (Kumagai and Dunphy 1992; Kramer, Scheuringer et al. 2000; Watanabe, Arai et al. 2004; Gorr, Boos et al. 2005; Watanabe, Arai et al. 2005).

In a screen for mitotic CDK substrates in *Xenopus* embryos, 20 mitotically phosphorylated proteins were found (Stukenberg, Lustig et al. 1997). Closer analyzes revealed that some of them were phosphorylated earlier than others. This lead to the suggestion that there might be different timing of phosphorylation between mitotic targets (Georgi, Stukenberg et al. 2002). For example, targets related to the G2/M transition, like Cdc25 and Wee1, were phosphorylated first. In contrast, Cdc27, a key regulator of mitotic exit, required more time to become fully phosphorylated (Georgi, Stukenberg et al. 2002). In another study, 43 phosphosites were identified in the APC, of which 34 were mitosis-specific. *In vitro*, at least 15 of the mitotic phosphorylation sites were Cdk1-specific. APC components including Apc1, Cdc27, Cdc16, Cdc23, and Apc7 were found to be phosphorylated by Cdk1 (Kraft, Herzog et al. 2003).

2.8. Controlling CDK activity through CKIs

The phosphorylation of CDK targets is temporally regulated by CDK inhibitors (CKI) (Sherr and Roberts 1999). CKIs are proteins that bind and inactivate cyclin-Cdk complexes (Figure 2). They have been found to function in all eukaryotic model systems: keeping, for example, CDK activity low in the G1 phase of the cell cycle, or stopping the cell cycle in response to antimitogenic signals (Morgan 2007). Some CDK inhibitors, like budding yeast Far1 and the INK4 proteins in mammals, respond to extracellular signals. Others, like *S. cerevisiae* Sic1 and its relative in *S. pombe* Rum1 appear to be part of the intrinsic cell cycle machinery (Morgan 2007). The levels of CKIs are tightly controlled by multiple mechanisms including transcription, translation and ubiquitin-mediated proteolysis. In higher eukaryotes, CKIs may not only be involved in cell cycle regulation but also in the regulation of other cellular processes including differentiation, cell migration, senescence, and apoptosis (Denicourt and Dowdy 2004; Besson, Dowdy et al. 2008). Loss of CKIs could be an important factor contributing to uncontrolled cell division and tumorigenesis (Barbacid, Ortega et al. 2005).

2.8.1. CDK inhibitors in mammalian cells

Based on their sequence homology and specificity of action CKI-s can be divided into two distinct families: INK4 (Inhibitors of Cdk4) and Cip/Kip (CDK interacting protein/Kinase inhibitory protein) inhibitors (Sherr and Roberts 1999). The INK4 family members $p16^{INKa}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$ selectively affect the activity of cyclin D-Cdk4,6 complexes (Serrano, Hannon et al. 1993; Guan, Jenkins et al. 1994; Hannon and Beach 1994; Hirai, Roussel et al. 1995). CKIs of the INK4 family are activated after cells sense anti-proliferative signals in the environment. All four INK4 CKI-s share similar structural characteristics and mechanisms of inhibition (Ekholm and Reed 2000). They contain either four ($p15^{INKb}$ and $p16^{INKa}$) or five ($p18^{INKc}$ and $p19^{INKd}$) ankyrin repeats that mediate protein-protein interactions. INK4 proteins have been shown to bind across the back side (non-catalytic) of the target kinase Cdk4 or Cdk6 (Brotherton, Dhanaraj et al. 1998; Russo, Tong et al. 1998). This leads to the formation of Cdk4,6-INK4 heterodimers, in which the CDK subunit is forced into a conformation that cannot bind cyclin and is therefore inactive (Brotherton, Dhanaraj et al. 1998; Russo, Tong et al. 1998).

The Cip/Kip family members $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$ inhibit a broader spectrum of cyclin-Cdk complexes, having higher specificity towards the G1 and S phase kinases compared with the mitotic ones (el-Deiry, Tokino et al. 1993; Harper, Adami et al. 1993; Xiong, Hannon et al. 1993; Polyak, Kato et al. 1994; Toyoshima and Hunter 1994; Lee, Reynisdottir et al. 1995; Matsuoka, Edwards et al. 1995). Cip/Kip inhibitors contain a conserved N-terminal domain that is both necessary and sufficient for inhibition. Their carboxy-terminal regions are variable in length and function (Polyak, Kato et al. 1994; Chen, Jackson et al. 1995; Lee, Reynisdottir et al. 1995; Luo, Hurwitz et al. 1995). The amino-terminal half is composed of two subregions. It contains a short cyclin binding motif and a longer segment that is required for binding to the CDK subunit (Chen, Jackson et al. 1995; Luo, Hurwitz et al. 1995; Nakanishi, Robetorye et al. 1995). The CKIs of the Cip/Kip family can bind cyclin and CDK subunits separately, but they have stronger affinity towards cyclin-Cdk complexes (Harper, Elledge et al. 1995; Lin, Reichner et al. 1996). One of the family members, $p21^{Cip1}$, was shown to effectively inhibit Cdk2, Cdk3, Cdk4 and Cdk6 cyclin-Cdk complexes with a Ki between 0,5-15 nM, but was much less effective toward cyclin B-Cdk1 complexes with a K_i ~400 nM (Harper, Elledge et al. 1995). Although identified primarily as inhibitors, the Cip/Kip CKI-s may also promote cell-cycle entry by activating G1 cyclin-Cdk complexes (Blain, Montalvo et al. 1997; LaBaer, Garrett et al. 1997; Cheng, Olivier et al. 1999). This is possible because unlike most cyclin-Cdk complexes, cyclin D and Cdk4 or Cdk6 have weaker binding affinities for each other, and Cip/Kip proteins can enhance the formation of the active complexes (LaBaer, Garrett et al. 1997; Cheng, Olivier et al. 1999).

2.8.2. CDK inhibitors in yeast

In budding yeast, there are three known inhibitors for cyclin-Cdk1 complexes that are important in cell cycle regulation. Far1 is an important regulator in the mating pathway, arresting cells at Start in response to mating pheromone. Sic1 is necessary in regulating the cell cycle at mitotic exit and between Start and the onset of S phase. The third budding yeast CKI, Cdc6, in addition to functioning as a replication licensing factor, has a role in mitotic exit, helping to inhibit mitotic cyclin-Cdk1 complexes.

FAR1 was originally identified as gene required for cell cycle arrest in response to mating pheromone (Chang and Herskowitz 1990). Later studies revealed that Far1 plays two distinct roles in the pheromone response process (Elion 2000). It physically binds to and inhibits Cln-Cdk1 complexes to mediate pheromone-induced cell cycle arrest (Chang and Herskowitz 1990; Peter and Herskowitz 1994), and it functions as a scaffold protein to establish cell polarity during yeast mating (Valtz, Peter et al. 1995). Deletion of *FAR1* produces no detectable phenotype in cells that have not been exposed to mating pheromone (Peter, Gartner et al. 1993). During the cell cycle Far1 functions only in G1 phase, and its levels are tightly regulated by transcription and post-translational modifications (Elion, Satterberg et al. 1993; McKinney and Cross 1995; Oehlen, McKinney et al. 1996). In normally dividing cells the expression of the *FAR1* gene increases in late mitosis and remains high until the end of G1 (Oehlen, McKinney et al. 1996). This pattern of Far1 accumulation ensures that cells arrest only in G1 in response to mating signal (McKinney and Cross 1995). Far1 cellular localization in unstimulated G1 phase cell is predominantly nuclear, but it constantly shuttles between nucleus and cytoplasm (Blondel, Alepuz et al. 1999; Pines 1999). Nuclear localization of the protein is thought to be required to arrest the cell cycle, whereas cytoplasmic Far1 supports polarized growth towards higher pheromone concentration (Verma, Feldman et al. 1997; Blondel, Alepuz et al. 1999). Upon pheromone sensing there is an approximately fivefold increase in Far1 transcription. This elevated level of the protein is necessary but not sufficient for arrest in G1 (McKinney and Cross 1995; Oehlen, McKinney et al. 1996). To act as an inhibitor of G1 cyclin-Cdk1s, Far1 must be additionally activated post-translationally (Peter, Gartner et al. 1993). The exact molecular mechanism of inhibition remains unclear, but it depends on activated MAPK Fus3, which boosts the transcription of Far1 and also induces phosphorylation of Far1 at Thr306, leading to the inhibition of Cln-Cdk1s (Chang and Herskowitz 1992; Elion, Satterberg et al. 1993; Gartner, Jovanovic et al. 1998). Interestingly, artificial expression of Far1 during the later stages of the cell cycle, in tandem with exposure to mating pheromone, induces cell cycle arrest in post G1 phase cells, showing that activated Far1 may also be capable of inhibiting Clb-Cdk1 complexes (McKinney and Cross 1995). However, it seems that Cln-Cdk1 complexes retain their capacity to phosphorylate and degrade Far1 (McKinney, Chang et al. 1993; Peter, Gartner et al. 1993). This process is controlled via phosphorylation of Ser87 residue on Far1, which results in SCF-Cdc4-dependent ubiquitination and subsequent destruction of the

protein (Henchoz, Chi et al. 1997; Blondel, Galan et al. 2000). This generates a double negative feedback loop between Far1 and Cln-Cdk1 that renders mitosis and mating mutually exclusive: cells commit either to the mitotic cycle or to mating, with no possibility of a mixed state (McKinney, Chang et al. 1993; Doncic, Falleur-Fettig et al. 2011).

In budding yeast, exit from mitosis requires the inactivation of mitotic cyclin-Cdk1 complexes. This is accomplished through cyclin destruction and direct inhibition of Clb-Cdk1s (Donovan, Toyn et al. 1994; Schwab, Lutum et al. 1997; Calzada, Sacristan et al. 2001). It has been shown that, in addition to Sic1, the licensing factor Cdc6 is an important inhibitor of Cdk1 activity (Elsasser, Lou et al. 1996; Calzada, Sacristan et al. 2001). In early studies, it was revealed that the N-terminal region of Cdc6 is important for its association with Cdk1 *in vitro* and *in vivo* (Elsasser, Lou et al. 1996). This was confirmed in later studies, where wt Cdc6 or N-terminal truncations were assayed for interaction with different cyclins *in vivo* (Archambault, Li et al. 2003). The N-terminus of Cdc6 is important for interaction with Clb2-Cdk1 in yeast or cyclin B-Cdk1 in mammals (Elsasser, Lou et al. 1996; Archambault, Li et al. 2003; Mimura, Seki et al. 2004). First, it was reported that Cdc6 can preferentially interact with B-type cyclin-Cdk1 complexes over Cln-Cdk1 complexes. In addition, Cdc6 binding to cyclin-Cdk1 appeared to be weaker than the interaction of Sic1 with the same complexes, because Sic1 was able to displace Cdc6 from the Clb-Cdk1-Cdc6 complexes. The transcription of *CDC6* is controlled by Swi5 and peaks in late mitosis, early G1 phase (Zhou and Jong 1990; Piatti, Lengauer et al. 1995). Overexpression of Cdc6 delays M phase initiation (Bueno and Russell 1992). Like Sic1, Cdc6 is an unstable protein. During the cell cycle, its degradation is regulated by Cdk1-dependent phosphorylation and subsequent ubiquitin-mediated proteolysis, with maximal turnover rate in late G1 and early S phase (Piatti, Lengauer et al. 1995; Elsasser, Lou et al. 1996; Drury, Perkins et al. 1997; Calzada, Sanchez et al. 2000). There are eight Cdk1 consensus sites on Cdc6. These phosphorylation sites, positioned at the N-terminal region and in the middle of the protein, are phosphorylated by Cln-Cdk1 and generate two binding sites for SCF-Cdc4. Through these SCF-Cdc4 phosphodegrons, Cdc6 is targeted for rapid degradation during G1 and S phase (Perkins, Drury et al. 2001). In G2/M phase Cdc6 is phosphorylated by Clb-Cdk1 and destroyed via the SCF-Cdc4 pathway, but the destruction rate is much slower (Perkins, Drury et al. 2001). Although phosphorylation of the N-terminal Cdk1 sites does not form a phosphodegron in G2/M phase, it creates a strong affinity site for Clb2-Cdk1. Binding of Cdc6 with Clb2-Cdk1 removes it from chromatin and keeps it in an inactive state, allowing preRC assembly (Mimura, Seki et al. 2004). The Cdc6 protein is localized to the nucleus, but phosphorylation near its N-terminal NLS may inhibit its nuclear import (Jong, Young et al. 1996; Luo, Elsasser et al. 2003).

So far only one CKI has been identified in fission yeast. The Rum1 protein is a regulator of G1 phase progression and controls DNA replication and mitosis by acting as an inhibitor of Cdk1. It was discovered in a screen for cDNAs that

are lethal when overexpressed in high levels because of the induction of extra rounds of DNA replication (Moreno and Nurse 1994). *RUM1* deleted cells are unable to recognize whether they have duplicated their DNA, and therefore cells that are actually in G1 abberantly enter mitosis (Moreno and Nurse 1994). The overexpression of Rum1 causes cells to continuously replicate their DNA without entering mitosis (hence the name Rum1 – replication uncoupled from mitosis) (Moreno and Nurse 1994). Rum1 is proposed to be structurally and functionally related to the budding yeast Sic1. This is confirmed in experiments where production of *SIC1* rescued the phenotype of *RUM1* deletion and overexpression of *SIC1* induced DNA re-replication, acting similarly to Rum1 in fission yeast (Sanchez-Diaz, Gonzalez et al. 1998). Direct *in vitro* assays have shown that Rum1 is an effective inhibitor for various fission yeast cyclin-Cdk complexes (Correa-Bordes and Nurse 1995; Martin-Castellanos, Labib et al. 1996; Benito, Martin-Castellanos et al. 1998). In fission yeast, Cig1, Cig2, and Cdc13 are B-type cyclins. Cig2 regulates the G1/S transition, while Cdc13 is the mitotic cyclin. Cig1 is thought to have a more minor impact on the onset of S phase. Cig2 and Cdc13 were shown to be inhibited by Rum1, whereas Cig1 was not (Correa-Bordes, Gulli et al. 1997).

The inhibitory domain of Rum1 has been mapped to the middle of the protein and shows 33% identity with the region in Sic1 necessary for the inhibition of B-type cyclin-Cdk1 complexes (Sanchez-Diaz, Gonzalez et al. 1998). Protein levels of Rum1 are sharply periodic. Rum1 begins to accumulate at anaphase, persists in G1, and is sent to degradation during S phase (Benito, Martin-Castellanos et al. 1998). Stabilization of Rum1 in a mutant defective for 26S proteasome function, suggests that its degradation is normally mediated by the ubiquitin-dependent proteasome pathway (Barbacid, Ortega et al. 2005). Phosphorylation of Rum1 by cyclin-Cdk1 complexes at residues Thr58 and Thr62 is also important for targeting the protein for degradation (Benito, Martin-Castellanos et al. 1998). Alanine mutations in one of the two phosphorylated residues cause protein stabilization and induce a cell cycle delay in G1, as well as polyploidization (Barbacid, Ortega et al. 2005). In addition to cyclin-Cdk complexes, MAPK has been demonstrated to phosphorylate N-terminal Thr and Ser residues in Rum1 (Matsuoka, Kiyokawa et al. 2002). This phosphorylation negatively regulates Rum1´s activity as an inhibitor of Cdk1 *in vitro*. Phosphomimetic mutants abolish Rum1 function in yeast cells, showing that phosphorylation by MAPK may affect Rum1 *in vivo* (Matsuoka, Kiyokawa et al. 2002).

2.9. Sic1 as the regulator of the M/G1 and G1/S transitions in the cell cycle

2.9.1. Discovery of Sic1

Sic1 is an inhibitor of Clb-Cdk1 complexes (Mendenhall 1993) that regulates cell cycle progression at the M/G1 and G1/S transitions. It was first discovered as a tight-binding Cdk1 substrate in immunoprecipitated Cdk1 complexes (Reed, Hadwiger et al. 1985). The *SIC1* gene was cloned independently by two research groups. Nugroho and Mendenhall used partial peptide sequence information taken from the purified protein and identified the *SIC1* gene in a λ library of yeast genomic DNA. Donovan and colleagues cloned *SDB25* as a high copy suppressor of temperature-sensitive mutations in the gene encoding the Dbf2 protein kinase. Comparison of the DNA sequence revealed that *SIC1* and *SDB25* were the same gene (Donovan, Toyn et al. 1994; Nugroho and Mendenhall 1994). The *SIC1* open reading frame codes for a hydrophilic protein of 284 residues with a predicted molecular weight of 32,2 kDa (considerably less than the 40 kDa size obtained from SDS-PAGE). Sic1 is intrinsically disordered throughout the polypeptide chain, although the C-terminus is slightly more ordered than the N-terminus (Brocca, Samalikova et al. 2009; Brocca, Testa et al. 2011; Lambrughi, Papaleo et al. 2012). The Sic1 protein has nine CDK consensus phosphorylation sites, seven of which fall within 81 amino acids of the protein´s N-terminus.

One of the important roles of Sic1 is to set the correct timing for the start of DNA replication: it maintains a G1 temporal window free from Clb5,6-Cdk1 activity, which is absolutely necessary for origin licensing (Lengronne and Schwob 2002). In *sic1*Δ cells, DNA synthesis is activated prematurely and is uncontrolled. This results in an extended S phase, a high frequency of broken and lost chromosomes, and inefficient chromosome separation during anaphase (Donovan, Toyn et al. 1994; Lengronne and Schwob 2002; Cross, Schroeder et al. 2007). This might be the reason that *sic1*Δ strains show an altered morphology and frequently arrest permanently in G2 (Nugroho and Mendenhall 1994). The second important role of Sic1 is to suppress the activity of Clb2-Cdk1 in mitotic exit as a parallel mechanism to the APC-Cdh1 dependent destruction of Clb2 (Lopez-Aviles, Kapuy et al. 2009).

2.9.2. Sic1 as an inhibitor of Cdk1

Despite the well-established fact that Sic1 is an inhibitor of Clb-Cdk1 complexes, the molecular mechanism by which Sic1 inhibits its targets´ activity remains largely unknown. Sic1 inhibitory activity is thought to be due to its ability to exclude other substrates from the Cdk1 active site. Kinetic analysis argues that the Ki (inhibition constant) is dependent upon the enzyme concentration and approaches 0,1nM at low Cdk1 concentrations (Mendenhall 1993; Mendenhall, al-Jumaily et al. 1995; Venta, Valk et al. 2012). In another

study, Sic1 was proposed to be a functional homolog of the mammalian cyclin-dependent kinase inhibitor $p21^{Cip1}$. This protein in turn has sequence similarity with the CKI $p27^{Kip1}$ (Barberis, De Gioia et al. 2005). In mammalian cells, progression through S phase is triggered by cyclin A-Cdk2, whose activity is inhibited by $p27^{Kip1}$. The crystal structure of the inhibitory domain of $p27^{Kip1}$ bound to cyclin A-Cdk2 reveals that the N-terminal part of $p27^{Kip1}$ is extended over the surface of the cyclin A-Cdk2 complex, creating hydrophobic contacts with regions on both the cyclin and kinase. According to the inhibitory mechanism proposed for $p27^{Kip1}$, it first occupies a substrate binding site on cyclin A and then binds to the N-terminal lobe of Cdk2, disrupting the active site. Because it also inserts itself into the ATP binding pocket it blocks ATP binding to Cdk2, as well (Russo, Jeffrey et al. 1996). A similar inhibition mechanism has been proposed for Sic1 based on the findings that (i) Sic1 is structurally and functionally related to mammalian $p27^{Kip1}$, sharing a conserved kinase inhibitory domain (KID) and (ii) Sic1 interacts with both the docking site and the catalytic site of the cyclin A-Cdk2 complex (Barberis, De Gioia et al. 2005; Barberis 2012). Preliminary analysis of Sic1 functional domains showed that a C-terminal fragment (residues 160-284) was able to bind Clb5-Cdk1 complexes *in vitro* (Verma, Feldman et al. 1997). A later study further defined the minimal inhibitory domain of Sic1 by showing that a 70 aa fragment of Sic1 from residues 215 to 284 functions *in vivo* as a inhibitor of Clb-Cdk1 complexes (Hodge and Mendenhall 1999).

2.9.3. The rise of Sic1 expression at the M/G1 transition

During the cell cycle, *SIC1* mRNA expression is periodic, peaking shortly after mitosis (Schwob, Bohm et al. 1994). The transcription of *SIC1* is regulated mainly by the activity of Swi5, but also by the Ace2 transcription factor (Knapp, Bhoite et al. 1996; Toyn, Johnson et al. 1997). In a *swi5*Δ mutant, the level of *SIC1* mRNA is decreased to 50% of the control levels, while, in *ace2*Δ cells, *SIC1* transcription is reduced to about 80% of wild-type levels. Deletion of both *SWI5* and *ACE2* genes reduces *SIC1* transcript levels to 20% of that of the wild-type, suggesting that both of these are needed for the activation of *SIC1* (Knapp, Bhoite et al. 1996; Toyn, Johnson et al. 1997). The subcellular localization and activity of Swi5 depends on its phosphorylation state. In the case of high Clb2-Cdk1 activity, Swi5 is phosphorylated (inactive) and retained in the cytoplasm. However, when Clb2-Cdk1 activity is low, Swi5 is dephosphorylated by Cdc14, and the dephosphorylated form is transported to the nucleus. The first burst of nuclear Swi5 generates a positive feedback loop through the produced Sic1 protein that can inhibit residual intact Clb2 in anaphase. This further reduces Swi5 phosphorylation and promotes its nuclear localization. The activation of Sic1 in anaphase is an important event for cell cycle division, because a feedback loop involving Sic1 ensures that mitotic exit is irreversible by preventing resynthesis of mitotic cyclins (Visintin, Craig et al. 1998; Lopez-Aviles, Kapuy et al. 2009). Swi5 has been shown to be a target of

the SCF-Cdc4 ubiquitin ligase, leading to the termination of *SIC1* transcription in the early G1 phase of the cell cycle (Kishi, Ikeda et al. 2008).

2.9.3. Sic1 as a key regulator of the G1/S transition

Sic1 transcription begins in late mitosis, and its protein levels increase until the end of G1 phase, followed by a rapid turnover at the G1/S transition, when Sic1 is phosphorylated by cyclin-Cdk1 complexes and sent to ubiquitin-dependent degradation via the proteasome pathway. The molecular mechanism by which Sic1 controls cell cycle progression has been the subject of many experimental and theoretical studies. These have, so far, focused mainly on the G1 cyclin-Cdk1 threshold that is necessary for timing and coordinating the G1/S transition and destruction of Sic1. In the beginning of the G1/S transition, Clb5,6-Cdk1 complexes, which are required for the initiation of S phase, are held in an inhibited state by Sic1. The G1 cyclin Cln1,2-Cdk1 complexes, which are insensitive to inhibition, phosphorylate Sic1 at multiple sites leading to its degradation (Verma, Annan et al. 1997; Nash, Tang et al. 2001). This model was based on the finding that lethality of the *cln1*Δ*/cln2*Δ*/cln3*Δ triple mutant is supressed by deletion of *SIC1*, although the quadruple mutant is very unhealthy (Schneider, Yang et al. 1996; Tyers 1996). The multisite phosphorylation of Sic1 was thought to set a threshold for Cln1,2-Cdk1 activity and thereby provide ultrasensitive, switch-like activation of Clb5,6-Cdk1 complexes (Nash, Tang et al. 2001). It was found that at least any six of the 9 CDK sites must be targeted for Sic1 degradation, because the phosphorylation of five sites did not restore Sic1 binding to Cdc4. This model predicts that destruction will be slow when up to five sites are phosphorylated in a distributive manner. After this initial lag period, the degradation rate of Sic1 should increase rapidly. The freed Clb5,6-Cdk1 complexes, released from Sic1 inhibition, were shown to be essential for initiating the DNA replication (Schwob and Nasmyth 1993; Schwob, Bohm et al. 1994; Schneider, Yang et al. 1996). A possible positive feedback mechanism of Clb5,6-dependent phosphorylation of Sic1 was proposed, based on the fact that the Clb5-Cdk1 complex is capable of phosphorylating Sic1 *in vitro* (Feldman, Correll et al. 1997; Skowyra, Craig et al. 1997).

2.9.4. SCF-dependent Sic1 degradation

Phosphorylated Sic1 is recognized by the Cdc4 subunit of the SCF ubiquitin ligase, which, in cooperation with E2 enzyme Cdc34, polyubiquitinates Sic1 on its N-terminal lysine residues (Feldman, Correll et al. 1997; Skowyra, Craig et al. 1997). Evidence for this pathway includes the findings that lysine to alanine substitutions in Sic1, as well as inactivation of temperature sensitive SCF components, lead to the stabilization of Sic1 and the failure of cells to enter S phase (Schwob, Bohm et al. 1994). *Cdc4ts*, *cdc34ts,* or *cdc53ts* cells grown at the restrictive temperature show G1 arrest with a multi-budded phenotype. This

phenotype can be suppressed by deletion of the *SIC1* gene (Schwob, Bohm et al. 1994). Together, these experiments indicate that Sic1 is targeted by the Cdc34 degradation pathway as part of G1/S control (Jackson 1996). The *Cdc34ts* phenotype, multi-budded cells with DNA not replicated and spindle pole bodies not separated, is very similar to the phenotype observed in cells deficient for Clb activity or expressing a stable version of Sic1 (Schwob, Bohm et al. 1994). Following polyubiquitination the Sic1 protein is recognized by polyubiquitin-binding factors that target it to the proteasome (Verma, McDonald et al. 2001; Verma, Oania et al. 2004).

Multisite phosphorylation of Sic1 regulates its ubiquitination and degradation. Nash and colleagues proposed that there is only one phosphopeptide binding site on the Cdc4 protein and proposed an allovalent binding model for the interaction between Sic1 and Cdc4 (Nash, Tang et al. 2001; Klein, Pawson et al. 2003; Orlicky, Tang et al. 2003). According to this model, the nine separate, singly-phosphorylated CDK sites with suboptimal specificity towards Cdc4 would have a synergistic effect on the apparent affinity for Cdc4. The multiply-phosphorylated Sic1 is presumed to be kinetically trapped by Cdc4, leading to a high local concentration and high-affinity binding between two proteins (Deshaies and Ferrell 2001; Nash, Tang et al. 2001; Klein, Pawson et al. 2003). Using NMR (Nuclear magnet resonance) studies, it was shown that Sic1 exists in an intrinsically disordered state and it was proposed that its multiply phosphorylated single degrons interact with Cdc4 in dynamic equilibrium (Mittag, Orlicky et al. 2008; Mittag, Marsh et al. 2010; Tang, Orlicky et al. 2012). This model was challenged by Hao and Pavletich, who showed that Cdc4, like its human ortholog Fbw7, is able to bind doubly phosphorylated degrons (Hao, Oehlmann et al. 2007). They found that Sic1 has three possible diphosphodegrons. When these degrons were singly phosphorylated at the primary sites, binding to Cdc4 was weak. However, when both of the sites within the diphosphodegron were phosphorylated, the binding efficiency increased. This strongly suggests that the second phosphate group interacts with Cdc4 (Hao, Oehlmann et al. 2007). Furthermore, Cdc4 dimerization was found to enhance the rate and processivity of Sic1 ubiquitination *in vitro* (Orlicky, Tang et al. 2003; Hao, Oehlmann et al. 2007).

2.9.5. Sic1 as a molecular sensor for different signals

There is evidence that Sic1 is phosphorylated not only by CDK, but by other kinases as well. Following exposure to hyperosmotic stress, cells activate the Hog1 (High osmolarity glycerol response) pathway (Clotet and Posas 2007). Hog1 is a SAPK (Stress-activated protein kinase) that has been reported to act as a central component in the osmotic stress response, delaying cell cycle progression in G1 or at the G2/M transition (Clotet and Posas 2007). In G1 phase, Hog1 induces transient cell cycle arrest through two mechanisms, both of which affect the stability of Sic1 protein. First, Hog1 is able downregulate transcription of G1 cyclins (Cln1 and Cln2) and the S phase cyclin Clb5

(Escote, Zapater et al. 2004; Clotet and Posas 2007; Adrover, Zi et al. 2011). Second, it has been found to directly phosphorylate Sic1 at T173, resulting in its stabilization. Sic1 stabilization then contributes to transient arrest in G1 (Escote, Zapater et al. 2004; Zapater, Clotet et al. 2005). The precise molecular mechanism through which the transient cell cycle arrest is accomplished remains unknown. It has been proposed, based on a yeast two-hybrid binding assay, that T173 phosphorylation might affect Sic1 binding to Cdc4 and thus hamper Sic1 degradation (Escote, Zapater et al. 2004).

Sic1 is also a target for the alternate cyclin-dependent kinase in *S. cerevisiae*, Pho85. *PHO85* is a non-essential gene but it nonetheless has functions multiple pathways as suggested by the pleiotropic phenotype of a *pho85*Δ strain (Huang, Friesen et al. 2007). Pho85 is able to phosphorylate multiple sites on Sic1 *in vitro* and (Nishizawa, Kawasumi et al. 1998). However, the cyclin partner that forms an active complex with Pho85 and targets Sic1 is not known. *In vivo* phosphorylation studies suggest that Pcl1 (Pho85 cyclin) and Pcl2 cyclins, which play a role in cell cycle progression, might be responsible for the activation of Pho85 (Nishizawa, Kawasumi et al. 1998). However, a direct analysis showed no effect of the deletion of Pcl1 and Pcl2 on Sic1 degradation (Moffat and Andrews 2004). Also, a more specific role has been described for Pho85 in the regulation of Sic1 following G1 DNA damage checkpoint activation (Wysocki, Javaheri et al. 2006). The DNA damage checkpoint downregulates G1 cyclin-Cdk1 activity, leading to a delay in the cell cycle. Pho85 is kept active at this time to restart the cell cycle and helps cells to recover from the arrest by compensating for low Cdk1 activity (Wysocki, Javaheri et al. 2006). On the other hand, Pho85 was recently shown to stabilize Cln3. Since Cln3 activates the transcription of *CLN1,2* and *CLB5,6* genes, this suggests that the previously proposed destabilizing effects of Pho85 on Sic1 are likely indirect (Menoyo, Ricco et al. 2013).

Activation of the TOR (Target of rapamycin) pathway by rapamycin also leads to downregulation of the G1 cyclins Cln1-3 and upregulation of Sic1. The rapamycin-sensitive TOR kinase complex is a major regulator of autophagy: it is inhibited when cells are starved, and this allows the induction of autophagy (Wullschleger, Loewith et al. 2006). In rapamycin arrested cells, Sic1 is upregulated: it inhibits Clb5,6-Cdk1 complexes and thereby avoids improper initiation of DNA replication under poor nutrient conditions. Cells deleted for the *SIC1* gene are incapable of rapamycin induced arrest, making them sensitive to a sublethal dose of rapamycin (Zinzalla, Graziola et al. 2007). On the other and, overexpression of Sic1 was shown to induce autophagy. However, the mechanism behind this phenomenon is not known (Yang, Geng et al. 2010).

In addition, Sic1 has been proposed to be a target of CK2 (Casein kinase 2) (Coccetti, Rossi et al. 2004; Barberis, Pagano et al. 2005; Coccetti, Zinzalla et al. 2006; Tripodi, Zinzalla et al. 2007). CK2 is an important regulator of cell cycle progression. It is a constitutively active serine-threonine kinase that has been shown to phosphorylate Sic1 on Ser201 *in vitro*. Sic1 that is phospho-

rylated at this residue has higher affinity for Clb5-Cdk1 complexes; this alters the timing of the G1/S transition (Barberis, Pagano et al. 2005).

The *S. cerevisiae* Ime2 kinase has been well characterized for its role in meiosis. One of its substrates during sporulation is Sic1 (Dirick, Goetsch et al. 1998; Holt, Hutti et al. 2007). Ime2 has been shown to phosphorylate Sic1 at multiple P-x-S/T sites *in vitro* (Sedgwick, Rawluk et al. 2006), even though, Sic1 has been reported to contain only one Ime2 consensus phosphorylation site R-P-x-S/T (where x is any amino acid) (Holt, Hutti et al. 2007). Specificity analysis between Clb2-Cdk1 and Ime2 established Sic1 as an equally good substrate for both kinases (Holt, Hutti et al. 2007). Comparing the Ime2 phosphorylation pattern with that of Cln2-Cdk1, it was shown that they have distinct activities towards Sic1 *in vitro* (Sawarynski, Kaplun et al. 2007). It is thought that Ime2 triggers the destruction of Sic1 and activation of Clb5-dependent kinase in meiotic cells because Cln-Cdk1 complexes are not active during that time (Dirick, Goetsch et al. 1998; Benjamin, Zhang et al. 2003). A recent study suggested that Ime2 does not directly catalyze Sic1 degradation, but may act futher upstream (Brush, Najor et al. 2012).

Several phosphatases like Cdc14 and Dcr2 have been shown to act on Sic1 protein. Cdc14 overexpression has been shown to strongly stabilize Sic1 during mitotic exit (Visintin, Craig et al. 1998). Dcr2 overexpression leads to altered Sic1 stability and therefore causes genomic instability (Pathak, Blank et al. 2007).

2.10. Cks proteins as CDK adaptor molecules

Members of the Cdc28 kinase subunit (Cks) family of small molecular weight proteins (9-18 kDa) are highly conserved in all eukaryotes and are essential for controlled progression through the cell cycle (Pines 1996). Since their discovery over twenty years ago, Cks proteins have been shown to interact with CDKs genetically and physically, but their impact on CDK activity and precise biological function remain unknown. Due to their properties, Cks proteins might be responsible for leading CDKs to phosphorylated substrates and enhancing multisite phosphorylation (Figure 3) (Patra and Dunphy 1998). Additionally, a CDK-independent function of Cks proteins has been described in mammalian cells, where they act as accessory factors linking substrates with ubiquitin ligase complexes (Ganoth, Bornstein et al. 2001). In budding yeast, the Cks1 protein can also act as transcriptional regulator, presumably affecting the expression of many genes. In addition to their role in cell cycle progression, Cks proteins have been extensively studied for their conserved ability to form i) domain-swapped dimers and ii) aggregates in certain conditions (Bader, Seeliger et al. 2006).

2.10.1. Cks proteins in eukaryotic cells

The first Cks protein to be discovered was $p13^{Suc1}$ (Suppressor of $p34^{cdc2}$) (hereafter referred to as Suc1) from *S. pombe*. Suc1 was isolated as a suppressor

of a defective allele of $p34^{cdc2}$ (the Cdk1 homolog in fission yeast, hereafter Cdk1) (Hayles, Beach et al. 1986). It was found that levels of the *SUC1* transcript remain constant during the cell cycle (Hayles, Beach et al. 1986; Hindley, Phear et al. 1987) and that Suc1 is also expressed in stationary-phase cultures (Ducommun, Brambilla et al. 1991). Since the discovery of Suc1 in fission yeast, homologues from other eukaryotic cells have been found, suggesting that the Cks proteins have an essential role in all eukaryotic species. The Cks protein in budding yeast was identified through its strong interaction with Cdk1 (Hadwiger, Wittenberg et al. 1989). Two copies of Cks genes have been identified in mammalian cells and in the nematode *Caenorhabditis elegans* (Richardson, Stueland et al. 1990; Polinko and Strome 2000). The fruit fly *Drosophila melanogaster*, the starfish *Marthasterias glacialis*, the common limpet *Patella vulgate*, and the African clawed frog *Xenopus laevis* all have one Cks protein homolog (Colas, Serras et al. 1993; Finley and Brent 1994; Patra and Dunphy 1996; Vogel, Baratte et al. 2002). Alignment of different Cks protein amino acid sequences reveals a high degree of conservation (Parge, Arvai et al. 1993; Patra and Dunphy 1996; Munoz, Santori et al. 2006). Some of the Cks homologues have insertions at the N-terminus and C-terminus and a longer loop between α-helices, but the core structure a four-stranded β-sheet that generates the typical Cks fold is conserved from yeast to humans.

S. cerevisiae Cks1 is the largest Cks protein found so far, with 150 amino acids (18 kDa). It contains an unusual insertion of 16 glutamine residues (named poly(Q) repeat) at the C-terminus, followed by a sequence rich in glutamines, prolines, and serines. The expression levels of *CKS1* are constant throughout the cell cycle (our unpublished results). *CKS1* was characterized as essential for survival. Overexpression and temperature-sensitive (ts) mutant strains were used to investigate the role of Cks1 (Tang and Reed 1993). A later study showed that *cks1*Δ cells form microcolonies that are slow growing and exhibit a variety of phenotypes consistent with functions previously described for $cks1^{ts}$ mutants (Yu and Reed 2004). The *X. laevis* Cks protein Xe-p9 was first identified through its ability to compensate for the *ts* effect of a fission yeast strain expressing a mutant version of the protein kinase Wee1 and therefore entering mitosis prematurely (Patra and Dunphy 1996).

The human homologues of fission yeast Suc1 were identified in HeLa cells by immunoprecipitation (Draetta, Brizuela et al. 1987). Two human cDNAs were cloned that encode proteins of 9 kDa in size and share 81% sequence identity (Richardson, Stueland et al. 1990). Both human Cks proteins CksHs1 and CksHs2 were shown to functionally complement *CKS1* deletion in *S. cerevisiae*, revealing that their function is highly conserved throughout evolution (Richardson, Stueland et al. 1990). The two human Cks proteins show different expression levels during the cell cycle. *CksHs1* expression is low in G1 and increases about four-fold in G2 and M phase. The expression pattern of CksHs1 has two peaks: a smaller one at the G1/S transition and a larger one near the end of the cell cycle. It has been found that CksHs1 is unstable in G1 phase, and its degradation is mediated by the ubiquitin ligase APC-Cdh1

(Bashir, Dorrello et al. 2004). *CksHs2* transcript levels are barely detectable in G1 and rise about seven-fold to peak in G2 and M phase. CksHs2 expression shows a more linear rise, ending at the end of the cell cycle (Richardson, Stueland et al. 1990). The information about Cks protein functions in mammals is obtained from knock-out (KO) mouse models for both paralogs (Spruck, Strohmaier et al. 2001; Spruck, de Miguel et al. 2003). CksHs1 nullizygous (*CksHs1-/-*) male and female mice are viable and fertile, but they have 10-20% smaller body size than their wild-type kin. The smaller body size is a result of accumulation of $p27^{Kip1}$, which inhibits Cdk2 kinase activity during the mitotic cell cycle (Spruck, Strohmaier et al. 2001). *CksHs2-/-* KO mice showed different phenotypes from *CksHs1-/-* mice. *CksHs2-/-* mice were found to be viable but sterile in both sexes. The sterility was discovered to be due to the failure of the germ cells to progress past the first meiotic metaphase (Spruck, de Miguel et al. 2003). Doubly nullizygous *CksHs1-/- CksHs2-/-* mice have also been generated, but they die before the morula stage, showing a critical role for human Cks paralogs in embryogenesis (Martinsson-Ahlzen, Liberal et al. 2008).

Cks proteins have also been linked to cancer development. All CDK regulators, including Cks proteins, are potential targets in the design of anticancer drugs (Shapiro 2006; Malumbres, Pevarello et al. 2008). Tumor profiling has revealed that both CksHs1 and CksHs2 show altered levels of protein expression in a number of human cancers (Urbanowicz-Kachnowicz, Baghdassarian et al. 1999; Inui, Kitagawa et al. 2003; Kitajima, Kudo et al. 2004; Shapira, Ben-Izhak et al. 2005). For example, overexpression of the human Cks proteins has been observed in prostate cancer (Lan, Zhang et al. 2008). Knockdown of CksHs1 resulted in inhibited proliferation, whereas deletion of CksHs2 led to programmed cell death and inhibited tumorigenicity. These experiments suggest that higher than normal levels of CksHs1 might contribute to uncontrolled cell division; CksHs2 overexpression furthermore protects cells from apoptosis (Lan, Zhang et al. 2008). Overexpression of CksHs2 was associated with aggressive disease progress and poor prognosis in one large breast cancer study (van 't Veer, Dai et al. 2002). CksHs1 has been shown to be overexpressed in many different cancers (Shapira, Ben-Izhak et al. 2005; Slotky, Shapira et al. 2005; Kawakami, Enokida et al. 2007). These examples show that various mechanisms may be invloved in Cks-mediated cancer development (Krishnan, Nair et al. 2010).

2.10.2. Functional roles of Cks proteins

The essential functions of Cks proteins for normal cell cycle progression have been delineated through various genetic and biochemical experiments in different species. Results from various studies indicate that Cks proteins have a role in controlling regulatory pathways which have implications prior to start in G1 and at some points in mitosis. In budding yeast, Cks1 depletion impairs cells´ ability to pass the G1/S and G2/M phase transitions of the cell cycle, ultimately leading to G1 or G2/M arrest, depending on when functional Cks1

protein was lost (Tang and Reed 1993). G2-arrested *cks1*^{ts} mutant cells show high levels of Cdk1 activity towards model substrate H1 protein (Tang and Reed 1993). Overexpression of Cks1 leads to a G2 phase delay (Tang and Reed 1993). In fission yeast, deletion or strong overexpression of Suc1 causes M phase arrest, whereas a mild excess of the protein leads to G2 arrest (cell length is approximately twice normal before division) (Hayles, Aves et al. 1986; Basi and Draetta 1995).

The role of Cks protein in *Xenopus* egg extracts was first described by Patra and Dunphy (Patra and Dunphy 1996). In this study, it was revealed that Xe-p9 has a role in cell cycle transitions. Depletion of *Xenopus* Cks from interphase extracts or overexpression of the same inhibits the progression of mitosis, suggesting that Xe-p9 somehow regulates the activation of cyclin B-Cdk1. Further studies showed that entry into mitosis was impeded due to the accumulation of inhibitory phosphorylation on the Tyr15 of Cdk1. It was suggested that Xe-p9 could control the activity of the Tyr15 kinase Wee1 and the Tyr15 phosphatase Cdc25 through CDK-dependent multiple phosphorylation (Patra, Wang et al. 1999). However, when Tyr15 was mutated to Phe (F) lifting the Wee1-induced inhibition of Cdk1 the depletion of Xe-p9 did not cause any delay in entry into M phase. However, these cells arrested later in mitosis because they failed to initiate the degradation of cyclin B-Cdc2. These results suggest that *Xenopus* Cks is not only required for inactivation of Wee1 and Myt1 and activation of Cdc25, but also for degradation of cyclin B. Xe-p9 seems to activate cyclin B proteolysis by directly promoting the cyclin B-Cdk1-dependent phosphorylation of APC components, including Cdc27 and APC1 (BimE) (Patra and Dunphy 1998).

It has been proposed that Cks proteins may have a role in promoting the multiple phosphorylation of substrates by docking CDKs to partially phosphorylated proteins (Pines 1996). After a cyclin-Cdk-Cks triple complex has phosphorylated one residue in a substrate protein, then the ability of Cks to bind through its anion-binding site phosphates may increase the affinity of the substrate for the kinase complex. This enhanced binding should accelerate the phoshorylation of neighboring sites. Cks-assisted multiphosphorylation of some cell cycle regulatory proteins by CDKs has been observed. So far, Cks proteins have been shown to promote multisite phosphorylation of substrate proteins like Cdc25, Myt1, Wee1 and Cdc27, and APC1 (Patra and Dunphy 1998; Patra, Wang et al. 1999; Ganoth, Bornstein et al. 2001). Further identification of those substrates which bind the phosphate-binding pocket of Cks will contribute to understanding how Cks proteins regulate cell cycle progression.

In budding yeast, the molecular mechanism underlying Cks1´s role in G2/M phase is not entirely clear. It has been shown that Cks1 can promote the degradation of already ubiquitinated Clb2 by the 26S proteasome (Kaiser, Moncollin et al. 1999). It was also proposed that the interaction of Cks1 with the proteasome rather than the APC is required for the proteolysis of mitotic regulators such as Clb2 (Kaiser, Moncollin et al. 1999; Ceccarelli and Mann 2001).

Another role for Cks1 in promoting mitosis has been described. It has been shown that Cks1 may act as a transcriptional modulator by activating expression

of the APC activator Cdc20 (Morris, Kaiser et al. 2003). Cdc20 was found to be a multicopy supressor of *cks1*^{ts} mutants. In *CKS1* defective cells, *CDC20* mRNA expression was at a constitutive, basal leve, unlike in the wild-type situation, where *CDC20* expression was periodic, peaking just before the metaphase-anaphase transition. In chromatin immunoprecipitation (ChIP) experiments it was found that Cks1 immunoprecipitated the *CDC20* promoter region. Also, Cdk1 was found to bind the *CDC20* promoter, but this binding was linked to the presence of Cks1. Therefore it is probable that in addition to cyclin B degradation, activation of *CDC20* transcription is also important for Cks-dependent mitotic progression (Morris, Kaiser et al. 2003).

Later studies have suggested that approximately 25% of the genes in the yeast genome depend on Cks1 for efficient transcription (Yu, Baskerville et al. 2005). One of the genes found when comparing transcriptional activation of wild-type cells with *CKS1* null mutants was *GAL1*. As for *CDC20*, *GAL1* transcriptional activation requires an intact Cdk1-Cks1 complex but does not require its CDK activity (Morris, Kaiser et al. 2003; Yu, Baskerville et al. 2005). Cdk1-Cks1 mediated transcription takes place through the recruitment of the proteasome to actively transcribed promoters. So far the exact function of the proteasome recruitment is unknown. Nonetheless, there is a clear need for proteolytic activity in transcript termination at these sites (Gillette, Gonzalez et al. 2004). There also exists a genetic link between the proteasome and RNA polymerase II-regulated transcription.

An independent role of human CksHs1 protein from CDK has been proposed. CksHs1 can act as an essential factor for SCF-Skp2 (Skp1-Cullin F1 box S phase Kinase associated Protein 2) complex activity. This multisubunit complex is the ubiquitin ligase that targets the CDK inhibitor $p27^{Kip1}$ for proteasome-dependent degradation, thereby freeing CDK activity and letting cells start S phase (Ganoth, Bornstein et al. 2001; Spruck, Strohmaier et al. 2001).

2.10.3. Complex formation between Cks proteins and CDKs

The formation of a complex between cyclin-Cdk and Cks proteins has been studied with a variety of methods. Using the quantitative SILAC method, it was found that all of the cyclins form stable interactions with Cdk1, and at least 50% of cyclin-Cdk1 complexes stoichiometrically bound Cks1 (Kito, Kawaguchi et al. 2008). Considering the time of complex isolation from cells, this implies even higher stoichiometry between cyclin-Cdk1 complexes and Cks1 in the cellular environment. Co-immunoprecipitation studies have also shown that Cks proteins are bound to cyclin-Cdk complexes in yeast (Brizuela, Draetta et al. 1987; Honey, Schneider et al. 2001; Archambault, Chang et al. 2004), in humans (Draetta, Brizuela et al. 1987), and in frog eggs (Patra and Dunphy 1996). Formation of a complex between Cks and cyclin-Cdks is also supported by the use of Cks proteins as an affinity reagent in chromatography to purify different cyclin-Cdk complexes (Vogel and Baratte 1996). Interestingly, it has been

shown that the *Drosophila* Cks homolog interacts with all of the CDKs (Cdk1, Cdk2, Cdk3), except for human Cdk4 (Finley and Brent 1994). In animal cells, this might mean that Cks proteins can associate with only a subset of G1 phase cyclin-Cdk complexes like cyclin A-Cdk2 and cyclin E-Cdk2 (Pines 1996).

In budding yeast, Cks1 has been shown to be an important factor for G1 cyclin-Cdk1 activity. In *Cks1*^{ts} cells the protein kinase activity of the G1 cyclin complexes Cln2-Cdk1 and Cln3-Cdk1 is severely decreased (Reynard, Reynolds et al. 2000). The stabilization of the G1 cyclin-Cdk1 complexes by Cks1 suggests one mechanism that might underlie the requirement for Cks1 proteins in progression through G1 in budding yeast (Reynard, Reynolds et al. 2000). It is not exactly known how Cks1 enhances the interaction of Cln2 with Cdk1, but the stabilization of the complex between Cln2 and Cdk1 could be due to the effect of Cks1 directly interacting with the long C-terminal tail of the Cln2 protein (Reynard, Reynolds et al. 2000). This hypothesis needs further examination, because crystal structures between cyclin A-Cdk2 and Cdk2-CksHs1 shows that cyclin A and CksHs1 bind to opposite sides of Cdk2 (Bourne, Watson et al. 1996).

2.10.4. Crystal structures of Cks proteins

Three dimensional structures of the Cks family of proteins have been solved with the hope of finding answers to the questions raised by genetic and functional. Cks proteins can crystallize into two discrete forms as i) monomers or ii) strand-exchanged dimers (Parge, Arvai et al. 1993; Arvai, Bourne et al. 1995; Bourne, Arvai et al. 1995; Endicott, Noble et al. 1995; Khazanovich, Bateman et al. 1996; Bourne, Watson et al. 2000). The folds of the two conformations are very similar containing usually two, but sometimes three or four, α-helices and four anti-parallel β-sheets. For example, compared with the almost identical CksHs1 and CksHs2, *S. pombe* Suc1 and *S. cerevisiae* Cks1 are found to have two extra insertions of long α-helices at the N-terminus and a large loop between the two conserved α-helices, resulting in an identity of only 53%. Between different Cks proteins a conserved motif with a H-x-P-E-P-H (His-x-Pro-Glu-Pro-His; where x is any amino acid) consensus sequence, named a β-hinge, is located in the C-terminus of the proteins between the third and fourth β-strand (Pines 1996). This region is an important structural determinant in alternate conformations and is differently positioned in monomers and dimers. Dimerization has been observed in yeast Cks1 and Suc1 (Bourne, Arvai et al. 1995; Bourne, Watson et al. 2000), and in human CksHs2 proteins (Parge, Arvai et al. 1993).

The first Cks protein structure obtained was for the human protein CksHs2 (Parge, Arvai et al. 1993). The CksHs2 protein was revealed to have the ability to form not only monomers or dimers but also hexamers, consisting of three dimers. Modelling work suggested that six CDK proteins are able to bind to the hexamer of CksHs2 molecules. This led to the hypothesis that the function of Cks proteins in cell cycle progression may be to act as a hub for CDK multi-

merization. However, later studies have led to the concensus that this hexameric structure is not functionally relevant *in vivo* (Parge, Arvai et al. 1993). The crystal structure of the human CksHs1 protein revealed that this protein takes the conformation of a discrete monomer with the hinge closed in a β-hairpin turn (Arvai, Bourne et al. 1995).

Unlike human CksHs1, which forms a discrete monomer, and CksHs2, which forms strand-exchanged dimers, the structures determined for fission yeast Suc1 revealed that this protein is able to crystallize in both conformations (Bourne, Arvai et al. 1995; Khazanovich, Bateman et al. 1996). Compared to the human CksHs2, Suc1 lacks residues at the N-terminus, six at the C-terminus, and a nine residue loop in the middle of the protein. The domains of the two proteins superimpose well despite the difference in size, but there are differences between the strand-exchanged dimers of the proteins (Khazanovich, Bateman et al. 1996).

Crystal structures of the Cks protein Cks1 from budding yeast have been solved for both a dimeric and a mutant monomeric form (Bourne, Watson et al. 2000; Balog, Saetern et al. 2011). The dimerization of Cks1 is mediated by the C-terminal β-strand (β4), which extends and exchanges with the identical strand from the other subunit of the dimer complex. The subunit folds of Cks1 superimpose well with the Suc1 and CksHs2 structures. However, there are clear differences between the conformations of Cks1 residues Glu89-Cys90 and the equivalent residues in Suc1, Glu86-Val87. Cks1 protein can exist either in a β-hairpin single-domain fold or a β-interchanged dimeric structure (Bourne, Watson et al. 2000). The dimerization constant for budding yeast Cks1 has been proposed to be ~0,4 mM, compared to fission yeast Suc1´s ~2 mM, which is far above the estimated physiological concentration of Cks1, implying that Cks1 is overwhelmingly monomeric *in vivo* (Rousseau, Schymkowitz et al. 2001; Bader, Seeliger et al. 2006).

2.10.4.1. The crystal structure of human CksHs1 in complex with Cdk2 kinase

The crystal structure of the human Cdk2 in complex with the human Cks protein CksHs1 has been determined (Bourne, Watson et al. 1996). The Cdk2 structure consists of an N-terminal and a large C-terminal lobe with the ATP binding site situated in a cleft between the two lobes. CksHs1 interacts with Cdk2 C-terminal lobe in a closed β-hairpin conformation (as a monomer) (Bourne, Watson et al. 1996). Thus, the bound CksHs1 is positioned at the opposite side relative to the structurally similar Cdk2 N-lobe, where the cyclin binding site is located (Jeffrey, Russo et al. 1995). This finding demonstrates that CksHs1 binding has little effect on the formation of cyclin-Cdk complexes. The interface between Cdk2-CksHs1 complex is mainly hydrophobic. The structure of Cdk2 in complex with CksHs1 is superposable with that of free Cdk2, indicating that CksHs1 binding does not give rise to a conformational

change in Cdk2 structure and therefore does not affect binding of other proteins to CDK. However, CksHs1 binding restricts access to CAK, which activates Cdk2 through phosphorylation at Thr160. This leads to the possibility that the activating phosphorylation of the kinase precedes Cks binding to CDK (Bourne, Watson et al. 1996).

It was first hypothesized that there are two regions which act as potential binding sites for Cks proteins on the CDK (Ducommun, Brambilla et al. 1991; Marcote, Knighton et al. 1993). One of these regions corresponds to the observed one in Cdk2-CksHs1 complex (Bourne, Watson et al. 1996), while the other is located at the N-terminal lobe of Cdk2. It has been shown using cross-linking experiments that CDK and Cks form a complex with 1:1 ratio (Ducommun, Brambilla et al. 1991). Given this result it is improbable that exchanged dimers of Suc1 or CksHs2 would bind to CDK. This assumption is now supported by the crystal structure of Cdk2-CksHs1 complex, which shows that Cks protein binds to CDK as a monomer (Bourne, Watson et al. 1996).

2.10.4.2. The crystal structure of Cks reveals an anion-binding site

Crystal structures have also revealed the presence of an anion-binding site capable of interacting with phosphates that might target CDK complexes to other phosphoproteins. A potential binding site for the phosphorylated substrate was first suggested by the presence of the sulfate anion in the crystal structure of the CksHs2 (Parge, Arvai et al. 1993). This and other structures that have been solved, including the human CksHs1, the fission yeast Suc1, and the budding yeast Cks1, confirm the presence of the conserved anion-binding site (Arvai, Bourne et al. 1995; Bourne, Arvai et al. 1995; Endicott, Noble et al. 1995; Khazanovich, Bateman et al. 1996; Bourne, Watson et al. 2000). The crystal structure shows the Cks phosphate-binding site to be on the same side of the CDK catalytic site, thus forming an extended recognition surface for substrates (Bourne, Watson et al. 1996). It has been shown by NMR studies that the Suc1 phosphate-binding region consists of conserved residues which are Arg30, Arg39, Gln78, Trp82 and Arg99 (Landrieu, Odaert et al. 2001). In the budding yeast Cks1 protein the conserved anion-binding pocket is formed by the residues Arg33, Arg42, Ser82, Trp85 and Arg102 (Bourne, Watson et al. 2000; Balog, Saetern et al. 2011). For testing the biological role of the Cks1 anion-binding site, single or triple mutants were generated. In the single mutant, only Arg102 was mutated to alanine. In the triple mutant, residues Arg33 and Ser82 were changed to Glu and Arg102 to Ala (R33E, S82E, R102A). The ability of these two mutants to function *in vivo* was tested in a background where the endogenous *CKS1* gene was disrupted, and cells were kept alive with a plasmid expressing wild-type Cks1. The aim was to see if mutant Cks1 proteins are able to replace wild-type Cks1. These experiments showed that Cks1 protein with a single substitution in the anion-binding pocket was fully functional and able to bind Cdk1 *in vivo*. Cks1 with a triple substitution was not

able to replace the wild-type protein, but preserved Cdk1 binding (Bourne, Watson et al. 2000).

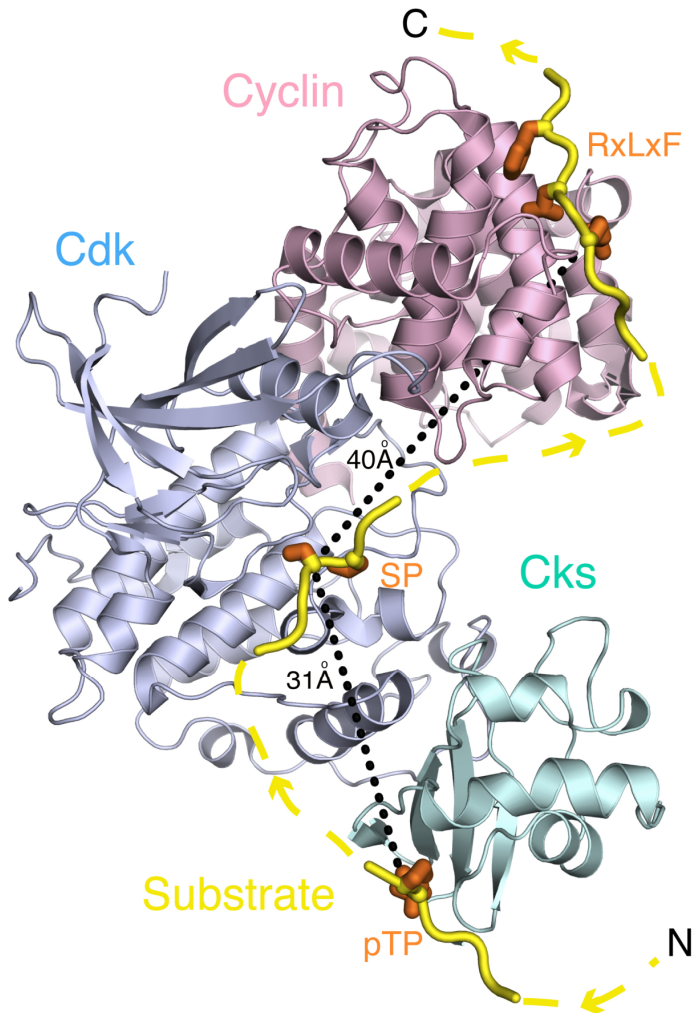

Figure 3. The three substrate interaction sites of the cyclin-CDK-Cks complex. A structural model showing the arrangement of the three key pockets in the cyclin-Cdk-Cks complex that are important for substrate recognition. The substrate specificity of CDK is determined by the active site of the kinase, the docking site on the cyclin, and the phosphate-binding pocket in the CDK adaptor molecule Cks1. The model was created by superimposing domains from crystal structures (PDB codes: 1BUH, 2CCI, in submission) each solved in the presence of the relevant substrate peptide bound to the pocket. The model was made by Dr. Seth M Rubin (UC Santa Cruz).

3. RESULTS AND DISCUSSION

3.1. Objectives of the study

The aim of the studies described in this thesis was to understand different mechanisms underlying the signaling specificity of the master regulator of the cell cycle in S. *cerevisiae*, the cyclin-dependent kinase Cdk1. A second goal was to study CDK targets containing multiple phosphorylation sites and to understand the logic behind multisite phosphorylation networks. The main objectives of the work can be briefly summarized as follows:

1. To analyse the dynamics of the substrate specificity of cyclin-Cdk1 complexes during the cell cycle of budding yeast.
2. To study the mechanism and biological function of multisite phosphorylation of the cyclin-dependent kinase inhibitor Sic1 in the G1/S transition (at the onset of S phase).
3. To identify and study different parameters which determine the dynamics of multisite phosphorylation cascades.

3.2. The cyclins gradually change the activity of Cdk1 (Ref II and IV)

It was shown previously that the substrate targeting specificity of Cdk1 is differentially modulated by different cyclins (Loog and Morgan 2005). The goal of our studies was to provide a full model of the dynamics of Cdk1 specificity during the cell cycle of budding yeast. To that end, we conducted a quantitative analysis of budding yeast Cdk1 specificity in complex with a cyclin from each cell cycle phase. We studied the G1 phase complex Cln2-Cdk1, the S phase complex Clb5-Cdk1, the G2/M complex Clb3-Cdk1, and the mitotic complex Clb2-Cdk1. All four representative cyclin-Cdk1 complexes were purified from yeast cells. For purification of the B-type cyclins Clb5, Clb3, and Clb2, a TAP-tag method was applied (Puig, Caspary et al. 2001). The Cln2-Cdk1 complex was purified by immunoaffinity chromatography using an HA-tag and the corresponding antibody, according to a previously published protocol (McCusker, Denison et al. 2007). To analyse the substrate specificity of the four purified cyclin-Cdk1 complexes at the level of a minimal phosphorylation consensus motif, we performed steady state kinetic analysis using an optimal peptide substrate based on the phosphorylation site of histone H1. H1 peptide is a general, commonly used substrate for CDKs: it is, derived from bovine H1 protein and has the target sequence PKTPKKAKKL (Beaudette, Lew et al. 1993). We measured the steady-state kinetic parameters for each of the four cyclin-Cdk1 complexes and found that each of them exhibited different specificity toward H1 peptide substrate (RefII, Fig. 1C). Remarkably, the specificity (k_{cat}/K_M values) differences followed a gradual rise in the order of appearance of the cyclins during cell cycle progression. The early appearing cyclins showed

lower specificity towards the substrate peptide compared with the later ones. These differences manifested mainly in different K_M values. To show that the observed differences were not caused by different levels of regulatory post-translational modifications (see paragraph 2.3.1 above), we analysed the two known regulatory phosphorylation sites of Cdk1 in budding yeast: the inhibitory site at Tyr19 and the activating site at position Thr169. Western blotting analyses conducted by E. Valk showed that activating phosphorylation was equally present in all enzyme complexes and the observed levels of inhibitory phosphorylation were low and could potentially affect the results in opposite directions. We also analyzed the phosphorylation rates of the inhibitory site at Tyr19 for each of the cyclin-Cdk1 complexes. These experiments showed higher specificity of Swe1 towards the mitotic Clb2-Cdk1 and gradually lower specificity towards earlier complexes. This is in agreement with previously published results showing that Cln2-Cdk1 is a poor substrate for Swe1 (Booher, Deshaies et al. 1993) and Clb5-Cdk1 is less susceptible than Clb2-Cdk1 to inhibition by Swe1 (Hu and Aparicio 2005). These data suggest that both CDK substrates and the Swe1 kinase domain have gradually changing accessibility to the Cdk1 active site during the cell cycle.

These data strongly suggest that cyclins are not simple activators of Cdk1, but that different cyclins can also differentially modulate the intrinsic activity of Cdk1 towards a minimal peptide substrate. The term “intrinsic activity” is used here with respect to the activity measured using the H1-based model substrate. The term “active site specificity” that is used below in this text reflects the possible differences in phosphorylation consensus motifs among the cyclin-Cdk1 complexes relative to the specificity profile defined by H1-peptide as a basal control.

Next we aimed to study the effects of intrinsic activity and docking-site dependence separately.

3.2.1. Cyclin-specific docking motifs of the early cyclin-Cdk1 complexes compensate for poor intrinsic activity on the active site level (Ref II and IV)

The gradual increase of Cdk1 intrinsic activity towards the optimal substrate motif during the progression of the cell cycle could provide an important delay in the accumulation of the high levels of CDK activity required for mitotic processes. This delay mechanism would prevent the premature initiation of mitotic processes in the early cell cycle by CDK. On the other hand, this raises the question of how early cyclin-Cdk complexes with low intrinsic activity can efficiently phosphorylate their substrates, which are required for initiation of Start and S phase. As known from the previous study, Clb5-Cdk1 complexes can compensate for their low intrinsic activity by using an HP docking site on the cyclin surface that binds selectively to substrates containing an RxL motif (Loog and Morgan 2005). On the other hand, Clb2-Cdk1, which is an

intrinsically more potent kinase, seems not to use extra help from the HP docking site.

To study the substrate recognition mechanisms of Cdk1 in more detail we used Sic1, a physiological target and an inhibitor of Cdk1. We designed a series of Sic1-based constructs with mutations in substrate recognition motifs. These constructs were based on a version of Sic1 lacking its C-terminal inhibitory region (Hodge and Mendenhall 1999) (Sic1(1-215), hereafter Sic1ΔC), which was useful as a general tool to analyse individual specificity elements of Cdk1 throughout later studies (Figure 4).

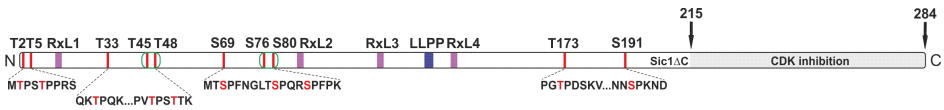

Figure 4. Schematic representation of the CDK phosphorylation sites and the interaction sites of the cyclin-dependent kinase inhibitor Sic1. Sic1 has nine CDK consensus sites: T2, T5, T33, T45, S69, S76, S80, T173, and S191, and one non-CDK consensus site T48, known to be phosphorylated by Cdk1, all shown in red. The Clb5-specific putative RxL docking motifs, of which RxL2 and 3 were found to be functionally important, are shown in purple. A Cln1,2-specific docking motif (LLPP) is shown in blue. The positions of two diphosphodegrons, T45/T48 and S76/S80, are highlighted with green circles. The truncated, non-inhibitory version of Sic1, Sic1ΔC, comprising amino acids 1-215, was used as a basis for substrate constructs throughout the studies.

Using Clb2,3,5-Cdk1 complexes with mutated substrate docking sites and the combinations of Sic1-based substrate constructs with mutated cyclin binding motifs (the RxL motif), we found that HP-RxL docking for efficient substrate phosphorylation was higher when the intrinsic specificity of the complex was lower. Thus, in the case of Clb5- and Clb3-Cdk1, the lower specificity at the active site level was compensated by cyclin-specific docking interactions.

However, there were as yet no specific docking interactions described for the G1 cyclin complexes. As Cln1,2 cyclins do not contain the hydrophobic patch characteristic of the B-type cyclins, it raised the question of the nature of the substrate targeting mechanism of Cln1,2-Cdk1. By searching for potential Cln2 specific docking motifs using truncation mutants based on Sic1 (Sic1 a-g) (II, Fig. 3A) we found a 10 amino acid stretch that enhanced Cln2-Cdk1 specific phosphorylation of Sic1 (II, Fig. 3B). This stretch, with a sequence of VLLPPSRPTS (positions 136-145 of Sic1), contained a group of hydrophobic residues. Alanine substitutions in the first five of them (Sic1ΔC-*vllpp*) abolished Cln2-Cdk1 phosphorylation specificity, but not the specificity of Clb5-, Clb3-, or Clb2-Cdk1 towards Sic1 in *in vitro* kinase assays (II, Fig3 C). A similar effect was observed when a synthetic competitor peptide (hereafter LP peptide), based on the 10 amino acid stretch of Sic1 (II, Fig. 3C), was included in the assay. A similar potential docking region for G1 cyclins was found in the scaffold protein Ste5 and the protein kinase Ste20 by Pryciak and colleagues

(Bhaduri and Pryciak 2011). The data from these simultaneously published studies suggests that motifs containing a series of Leu and Pro residues are likely a universal substrate docking mechanism for G1-specific cyclin-Cdk1 complexes in budding yeast. We also tested a series of potential Cln2-specific targets in the absence and presence of LP peptide and identified several Cln2 specific targets (see paragraph 3.2.3). Collectively, these findings show that docking interactions play an important role in the mechanism by which G1 cyclins drive phosphorylation of a specific set of target proteins.

3.2.2. Different cyclins can modulate the active site specificity of Cyclin-Cdk1 (Ref II and IV)

An important factor in substrate recognition by cyclin-Cdk complexes is the interaction between the substrate consensus phosphorylation sequence, and the CDK active site. Many physiological CDK substrates contain multiple proline and lysine residues in their phosphorylation sites. We asked if these residues, while being an important part of the CDK consensus motif S/T-P-x-K/R (Songyang, Blechner et al. 1994; Holt, Hutti et al. 2007), could have a role in substrate recognition when present in other nearby positions.

To analyse the substrate targeting mechanism relative to a single phosphorylation site, we mutated all the CDK consensus sites in Sic1ΔC to alanines (S/T-P to A-P), except the functionally important site at position T33 (Nash, Tang et al. 2001). By comparing the active site specificity of Cln2- and Clb2-Cdk1 complexes we found that, whereas Clb2-Cdk1 showed a requirement for the lysine at position +3, quite surprisingly the Cln2-Cdk1 exhibited specificity for lysine at positions +2 and +3. The +2 lysine specificity was an exclusive specificity factor of Cln2-Cdk1, compared with B-type cyclins (II, Fig. 4A). By introducing proline into different positions around the T33 site we identified the positive determinant of -2 proline for both Cln2- and Clb2-Cdk1 (II, Fig 4B).

Our results show that, cyclins are not only activating subunits of CDK, but they can also modulate the active site specificity of the CDK towards different phosphorylation motifs. We can conclude that Cln2-Cdk1 has both overlapping and distinct consensus motif requirements compared with S-phase and mitotic cyclin-Cdk1 complexes. This type of Cln2 specificity may be an important determinant in G1/S-phase substrates, which must be phosphorylated to start the G1 specific transcription program and to regulate other G1 processes. Indeed, a number of G1-specific targets contain sites with the exclusively Cln2-specific motifs S/T-P-K/R-x (where x is any amino acid).

3.2.3. Search for cyclin-specific Cdk1 targets (Ref I, II and IV)

Having determined the general rules for cyclin-specific substrate phosphorylation, we intended to test the specificity of a larger number of physiological substrates. The potential candidates were chosen to identify Cln2 or Clb2

specific targets. For this we studied a large set of known targets for Cln2- and Clb-Cdk1s, as well as a number of unknown ORFs (open reading frames) with at least five S/T-P phosphorylation sites. The substrate proteins were expressed and purified from bacterial cells. For specificity analysis, the rates of substrate phosphorylation were followed for the four representative cyclin-Cdk1 complexes. Relative specificity values for different substrates revealed several types of cyclin specificity profiles. Based on these profiles, we proposed a classification for Cdk1 targets based on four distinct groups.

Type I substrates are proteins with high specificity for the G1 complex Cln2-Cdk1 (II, Fig. 6A). Several of these substrates were related to G1-specific transcriptional control, including Whi5, Stb1, Xbp1, Msa1, Tos8 and Yhp1. Remarkably, the substrate specificity of type I targets was largely dependent on the LLPP docking interaction. The presence of LP competitor peptide in kinase assays reduced the phosphorylation of Whi5, Stb1, Pds1, and Yhp1 in the case of Cln2-Cdk1 but not in the case Clb5,3,2-Cdk1 (II, Fig. 6A). The LP peptide-dependent loss of phosphorylation of Whi5 is in agreement with another study, where the potential Whi5 LLPP was shown to replace the functional LLPP region of Ste5 protein (Bhaduri and Pryciak 2011).

The substrates specific for the S-phase complex Clb5-Cdk1 and S/G2 complex Clb3-Cdk1 were termed Type II substrates (II, Fig. 6B). The specificity of these targets depends on the docking interaction between the hydrophobic patch of the cyclin and the substrate. A triple mutation in the hydrophobic patch region (hereafter hpm) abolished interaction with the substrate protein RxL motif. This docking mechanism compensates for the poor specificity of Clb5-Cdk1 towards these targets on the phosphorylation consensus site level. This group contained the spindle-stabilizing protein Fin1, which must be fully phosphorylated in the beginning of the cell cycle as described in paragraph 2.7.2. Additionally, two members of the ORC complex Orc2 and Orc6 showed Clb5 specificity. The phosphorylation of Orc6 was dependent on the HP-RxL interaction, as also shown previously (Wilmes, Archambault et al. 2004). More members of this group have been identified in a proteomic screen (Loog and Morgan 2005).

A small group designated as Type III targets was found to be specific for the S/G2 complex Clb3-Cdk1 while showing weak specificity for Clb5- or Clb2-Cdk1. This finding was surprising, as there was no information about Clb3 specific functions or substrates. The mechanism of Clb3-specific recognition of these targets was dependent on the hydrophobic patch of Clb3. In one of these substrates, a novel type of Clb3-specific recognition motif was mapped that was distinct from the conventional RxL motif (our unpublished results). This group contained a protein of unknown function Ypr174c, the transcription factor Ash1, and the putative transcription factor Tos4 (II, Fig. 6C).

The targets specific for the mitotic complex Clb2-Cdk1 were termed Type IV substrates (II, Fig. 6D). These proteins showed overall cyclin specificity that matched with the pattern observed for the H1 model peptide (paragraph 3.2). The high intrinsic specificity of Clb2-Cdk1 towards the consensus phospho-

rylation motif is sufficient for efficient phosphorylation of these substrates, and the additional support from the cyclin-dependent docking interactions is not used.

Additionally, we have studied cyclin specificity in the phosphorylation of the kinesin motor protein Cin8. A truncated version of Cin8, Cin8-590 (which contains the motor domain), showed higher specificity towards the mitotic complex Clb2-Cdk1 than towards the earlier complexes Clb5-Cdk1 and Clb3-Cdk1 (I, Fig. 1E). Therefore, Cin8 belongs to the Type IV category of substrates, which was found to be in agreement with its *in vivo* phosphorylation profile in late mitosis (Avunie-Masala, Movshovich et al. 2011). When all Cdk1 consensus sites were mutated to alanines in Cin8-590, the phosphorylation signal was lost for all tested cyclin-Cdk1 complexes (I, Fig. 1E). Our results are in agreement with another study, where full length Cin8 protein was shown to be a target of Clb2-Cdk1 *in vitro* (Chee and Haase 2010).

3.3. Multisite phosphorylation mechanism of Sic1 (Ref III)

Cellular biochemical switches exist within intracellular signaling networks to make binary decisions. Multisite phosphorylation has been proposed as a mechanism for generating switch-like responses from graded inputs (Ferrell 1996; Nash, Tang et al. 2001; Thomson and Gunawardena 2009). To investigate switch-like behavior arising from multisite phosphorylation, we studied the phosphorylation dynamics of Sic1, a protein which is both a substrate and an inhibitor of Clb-Cdk1 complexes in budding yeast. Furthermore, Sic1 plays an important role in the regulation of the cell cycle, and it is considered a functional homologue of $p27^{Kip1}$ in higher eukaryotes. Despite the biological significance of Sic1, little is known regarding its mechanism of multisite phosphorylation. Fundamental insights into multisite phosphorylation obtained from the Sic1 system might be applicable to other multisite phosphoproteins.

In budding yeast, DNA replication is initiated by Clb5,6-Cdk1 complexes. The activity of Clb5,6-Cdk1 is inhibited in G1 phase by the stoichometric inhibitor Sic1. At the G1/S transition, Sic1 is rapidly phosphorylated by Cdk1. The phosphorylation of two diphosphodegrons in the N-terminal phosphorylation cluster promotes its ubiquitination by an SCF-Cdc4 complex and subsequent degradation by the proteasome. It has been suggested that phosphorylation of Sic1 is performed by the G1-specific Cln1,2-Cdk1 complex, which is not inhibited by Sic1.

To study the multisite phosphorylation mechanisms of Sic1, we used the non-inhibitory truncated version of Sic1 (Sic1ΔC) (Figure 4). Strikingly, kinetic analysis performed using the purified cyclin-Cdk1 complexes revealed that the hyperphosphorylated species accumulated abruptly at the early stages of unphosphorylated substrate consumption (III, Fig. 1A,B,D). We found that this pattern of highly phosphorylated forms depended on Cks1 (III, Fig. 1A), the

phosphoadaptor subunit of the Cdk1 complex. When alanine mutations were introduced to the phosphate-binding pocket of Cks1 (hereafter Cks1*mut*) considerably reduced accumulation of multiphosphorylated forms of Sic1 was observed (III, Fig. 1A). These results suggest that Cks1 enforces phosphor-dependent cooperativity or processivity in Sic1 multiphosphorylation by docking with intermediately-phosphorylated forms of Sic1 via its phosphate-binding pocket.

Next, we aimed to confirm that the phosphate-binding pocket of Cks1 is indeed responsible for phosphorylation-dependent degradation of Sic1 *in vivo*. Because *CKS1* deletion is lethal to cells (Tang and Reed 2002), we used a strain in which the promoter of *CKS1* was replaced with a galactose inducible pGALL promoter (Mumberg, Muller et al. 1994; Janke, Magiera et al. 2004). This allowed us to repress the expression of endogenous *CKS1* and replace it with the expression of Cks1wt or Cks1*mut* under another promoter. After the shut-off of endogenous *CKS1* expression, Sic1 protein levels were stabilized, and expressing Cks1wt from the plasmid restored the rapid degradation profile for Sic1. However, replacing the expression of the endogeneous Cks1 with Cks1*mut* resulted in stabilization of Sic1 (Figure 5). These results confirm that Cks1 with an intact phospho-binding pocket is required for phosphorylation and degradation of Sic1 *in vivo*.

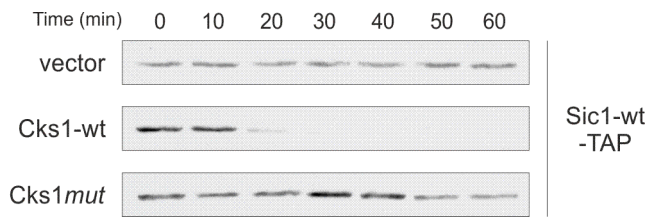

Figure 5. The effect of the phosphate-binding pocket of Cks1 on the degradation of endogenous Sic1. Cks1wt or Cks1mut were expressed from a CEN vector under a constitutive *pADH1* promoter. The endogenous *CKS1* was under a *pGALL* promoter, and its expression was repressed by growing the cells in media containing glucose. Cells were arrested in G1 using α-factor. After release of cells from arrest by removal of α-factor, the endogenous Sic1 levels were followed by western blotting. In cells expressing the wild type Cks1, the degradation rate of Sic1 was identical to that in wild-type cells (III, Fig. 3E). However, Sic1 was stabilized in the absence of Cks1 (vector) or in cells expressing only Cks1*mut*.

Additionally, we performed a viability assay to study the importance of the Cks1 phospho-binding pocket in suppressing the levels of overexpressed Sic1. It was found that the co-overexpression of Cks1*mut* with Sic1 was lethal to cells. This result additionally confirmed that the Cks1 phospho-binding pocket is responsible for efficient phosphorylation and degradation of Sic1 (Figure 6).

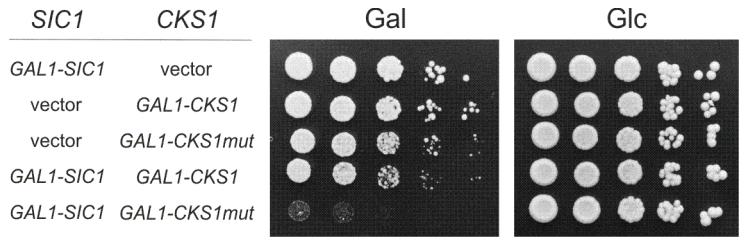

Figure 6. The importance of the Cks1 phosphate-binding pocket for suppression of Sic1-dependent inhibition of cell cycle progression. Viability assay using overexpression of both Sic1 (in CEN vector) and Cks1wt or Cks1*mut* from a pGAL1 promoter (in 2-micron vector). The expression of Cks1*mut* severely suppressed the viability of cells overexpressing Sic1. The cells were spotted as serial dilutions on selective synthetic plates containing glucose or raffinose and galactose as the main carbon source. Cell growth was monitored for two days at 30 °C.

In order to identify additional docking interactions that might influence the multiphosphorylation dynamics of Sic1, we studied the process with respect to cyclin-dependent substrate interactions. Sic1 has four potential RxL docking motifs (Figure 4). We found that rapid Sic1 phosphorylation by Clb5-Cdk1 depends on the HP-RxL interaction. Alanine mutations in the HP motif of the cyclin or in the RxL docking site of Sic1 considerably reduced the phosphorylation rate. Subsequently, we mapped the two RxL motifs responsible for Sic1 phosphorylation and degradation *in vivo* (data not shown). However, mutations of all four RxL motifs produced an even stronger effect in viability assays (data not shown). *In vitro* kinase assays using Clb5-Cdk1 and the version of Sic1 with mutated RxL motifs showed less abrupt production of multiphosphorylated forms, indicating that semi-processive multiphosphorylation of Sic1 requires both Cks1-dependent and HP-RxL-dependent docking. Additionally, mutation of the Cln2-specific LLPP docking motif in Sic1 (see section 3.2.1 above), also reduced the accumulation of highly phosphorylated species (data not shown). Taken together, these data indicate both Cln2-Cdk1 and Clb5-Cdk1 use cyclin specific docking motifs, in addition to Cks1-dependent phospho-priming for semi-processive multiphosphorylation of Sic1.

3.3.1. Phosphorylation of suboptimal degron sites is mediated by phosphorylated priming sites (Ref III and VI)

Sic1 has nine CDK consensus sites, as shown in figure 4. It was found that Clb5-Cdk1 phosphorylated only four of the sites (T5, T33, S76, and S80) efficiently (III, Fig. 2B). The Clb5-specificity of these sites was dependent on RxL2 and RxL3 motifs (III, Fig. 2B). On the other hand, Cln2-Cdk1 showed considerable specificity only towards the N-terminal site T5 (III, Fig. 2B). These results show that cyclin-specific docking motifs direct the phospho-

rylation of certain primary sites. We proposed that these N-terminal sites may act as priming sites for Cks1-dependent phosphorylation of additional sites in Sic1. To test this idea, we constructed Sic1 variants in which all CDK sites except a triple cluster (S69/S76/S80), containing the diphosphodegron pS76/pS80, were mutated to alanines. The phosphorylation of this construct S69/S76/S80-Sic1ΔC showed no apparent Cks1-dependent potentiation (III, Fig. 2C). However, the Cks1-dependent abrupt accumulation of multiphosphorylated forms could be restored by adding back single N-terminal CDK sites including T5, T33, or T45 (III, Fig. 2C). These results confirm that N-terminal sites are able to act as priming sites for Cks1-dependent phosphorylation of C-terminal sites. The Cks1-dependent docking effect was very powerful, as it was able to cause the efficient phosphorylation of a non-CDK site T48, which does not contain the minimal consensus motif S/T-P for CDK.

In vivo studies showed that cells overexpressing a Sic1 variant containing only the triple cluster S69/S76/S80 were inviable (III, Fig. 2D). Adding back one of the N-terminal primer sites, T5, T33, or T45, did not rescue the inviability of cells (III, Fig 2D). However, viability improved when we restored two N-terminal primer sites: T33 and T45 (III, Fig. 2D). We proposed that T33 might act as a primer for both diphosphodegrons, pT45/pT48 and pS76/pS80. The non-CDK site T48 has been shown to be phosphorylated *in vivo* (Verma, Annan et al. 1997). Indeed, alanine mutation of T48 in the background of T33/T45/S69/S76/S80 showed a strong growth-suppressing effect (III, Fig. 2F). To study the different roles of diphosphodegrons pT45/pT48 and pS76/pS80, we used western blotting of Phos-tag SDS-PAGE to determine the contribution of each diphosphodegron to the phosphorylation and degradation of Sic1. We constructed versions of Sic1ΔC fused with a 3HA-tag and compared the versions with different alanine mutation in one of the diphosphodegron sites T48, S80, or both. Western blotting experiments from cells expressing constructs under a constitutive promoter indicated that both diphosphodegrons are required for proper Sic1 destruction (III, Fig. 2G).

The earlier model of Sic1 regulation proposed that at least six sites must be simultaneously and randomly phosphorylated *in vivo* to cause the binding of Sic1 to SCF-Cdc4 ubiquitin ligase and initiate the degradation of Sic1 (Nash, Tang et al. 2001). This was questioned by binding studies that revealed the potential requirement of closely positioned pairs of phosphorylated sites (pT5/pS9; pT45/pT48; pS76/pS80) for SCF-Cdc4 binding (Hao, Oehlmann et al. 2007). We propose a model that combines these two findings. In the proposed model, the N-terminal sites T5, T33, and T45 act as priming sites for a Cks1-dependent processive phosphorylation cascade that results in efficient phosphorylation of the diphosphodegrons to provide the proper degradation of Sic1.

3.3.2. Differential roles of Cln2- and Clb5-Cdk1 in the multiphosphorylation of Sic1 (Ref III and VI)

Next we aimed to study the relative impact and potentially different roles of Cln2-Cdk1 and Clb5-Cdk1 in the phosphorylation of Sic1. To map the order of Cks1-mediated phosphorylation events, we developed a method to determine the apparent rate constants for each step. The obtained results revealed differences between Cln2-Cdk1 and Clb5-Cdk1 (III, Fig. 3B and 3C). Clb5-Cdk1 was more potent compared with Cln2-Cdk1 in phosphorylating the critical diphosphodegron pair pS76/pS80. This is accomplished by simultaneous use of T5 or T33 as priming sites for Cks1-dependent docking (III, Fig. 3B) and the RxL motifs for cyclin-dependent docking (data not shown). Also, the initial phosphorylation of the priming sites T5 and T33 themselves was more efficient for Clb5-Cdk1 due to its use of the two RxL docking motifs.

We propose that in late G1 the Clb5-Cdk1 complex is inactive and the Sic1 phosphorylation cascade starts with the phosphorylation of T5 by Cln2-Cdk1. This step is followed by docking-enhanced phosphorylations, leading to a form with phosphorylated sites pT5/pT33/pT45/pS76. As Cln2-Cdk1 has a weaker ability to phosphorylate the priming sites, as well as the paired diphosphodegrons, it is incapable of initiating Sic1 degradation alone. However, rising levels of Clb5-Cdk1 can use these pre-phosphorylated sites as a platform to mediate fast phosphorylation of diphosphodegrons and set the point of abrupt G1/S transition through a positive feedback mechanism. This model predicts that the Cln2-Cdk1 may be able to drive Sic1 degradation when limiting suboptimal diphosphodegrons are changed to optimal sites for Cln2-Cdk1. To test this, we modified the construct where Clb5-specific RxL sites were removed by introducing the exclusively Cln2-specific determinant motif S/T-P-R/K-A in the positions of the suboptimal diphosphodegron sites T48A and S80 and in a suboptimal site S69 making them optimal phosphorylation sites for Cln2-Cdk1. Strikingly, a strain expressing the resultant construct showed almost complete rescue of the viability defect caused by the initial mutation of Clb5-specific RxL motifs (III, Fig. 3D). Finally, further mutation of the Cln2-specific docking site (LLPP) in this construct caused inviability of the cells, which was not observed when the docking site was mutated in the context of initial wild type sequence of Sic1. This result indicates that we had artificially rewired the cascade to become mostly dependent on Cln2-Cdk1 instead of Clb5-Cdk1. Importantly, these results are unlikely due to improved binding to ubiquitin ligase SCF-Cdc4, because any basic amino acid downstream from the phosphoacceptor pS or pT residue is known to be a negative determinant for Cdc4 binding (Nash, Tang et al. 2001). Our results suggest that Cln2-Cdk1 is not able to drive the degradation of Sic1 alone, because the Cln2-dependent cascade is not efficient enough to provide sufficient rates for the final rate-limiting phosphorylation steps of the phosphodegrons. Clb5-Cdk1 is able to phosphorylate critical phosphodegrons with sufficient rates, which are

accelerated through positive feedback of the emerging free Clb5-Cdk1 that is released from the inhibitory complex.

To further test the proposed model, and to precisely determine the relative impact of Cln2-Cdk1 and Clb5-Cdk1 in the phosphorylation and degradation of Sic1, we analysed the degradation of endogeneous Sic1. We found that degradation was delayed when either Cln-specific or Clb-specific docking sites were mutated in Sic1 (III, Fig. 3E). These findings confirmed that both Cln2-Cdk1 and Clb5-Cdk1 have a role in Sic1 degradation. However, when we inhibited all Clb-Cdk1 activity by overexpressing a non-degradable version of Sic1 (Sic1ΔN (215-284)) under the pGAL1 promoter (Hodge and Mendenhall 1999), we observed the stabilization of endogenous Sic1 (III, Fig. 3F). This result indicates that the key trigger for Sic1 degradation in the G1/S transition is emerging Clb5-Cdk1 activity. Finally, when the rate-limiting degron sites were changed to become Cln2-specific, as described above, the Cln2-Cdk1 was able to degrade Sic1 even in the complete absence of Clb5-Cdk1 (III, Fig. 3G).

3.4. The requirement for phospho-threonine over phospho-serine in Cks1-dependent docking of multisite targets of Cdk1 (Ref V)

To analyze the determinants required for the binding of Cks1 to the phosphorylated priming sites, we tested different amino acid substitutions around the N-terminal priming site T33 in different Sic1ΔC constructs. Strikingly, however, we found that when Thr at position 33 was replaced by Ser, no Cks1-dependent phosphorylation of the secondary site was observed (V, Fig. 2C). This result suggested that the phosphate-binding pocket of Cdk1 binds phospho-threonine but not phospho-serine. We also constructed a set of mutants with positional variations around T33 site. We found that -2 proline residue enhanced the interaction with phospho-epitope with Cks1 (data not shown).

Next, we mutated all CDK consensus sites containing threonines in Sic1 to serines (Ser-Sic1ΔC). The abrupt accumulation of multiply phosphorylated forms was severely suppressed in case of the Ser-Sic1ΔC. The effect was comparable with that of Cks1*mut* as seen in III, Fig. 1A. The quantifications revealed that the serine phosphorylation sites are not less specific direct targets of Cdk1, indicating that only the secondary Cks1-dependent docking steps were affected by the replacement mutation (data not shown). To confirm that Cks1 specificity is also an important factor for Cks1-dependent phosphorylation of Sic1 *in vivo*, we overexpressed the all-Ser form of Sic1 (Sic1-Ser) in yeast cells. Cells expressing Sic1-Ser were inviable (V, Fig. 2D), indicating, that Cdk1 is not able to phosphorylate the phosphodegrons of Sic1 to a sufficient level without the Cks1-dependent cascade. The phosphorylation of a Sic1-Ser construct follows a distributive phosphorylation mode, in which the phosphorylation of each site is independent of previous phosphorylation.

The strong preference of Cks1 for phosphorylated Thr sites reveals previously unrecognized complexity in the phosphorylation of CDK targets and suggests a mechanism that could allow CDK to differentially regulate multisite substrates.

3.4.1. Analysis of different parameters that define the outcome of multisite phosphorylation (Ref V)

The majority of known Cdk1 targets contain multiple phosphorylation sites that are usually clustered in intrinsically disordered regions (Holt, Tuch et al. 2009). The phosphorylation dynamics of these clusters of sites is likely controlled by various parameters. In the case that the sites in a cluster are phosphorylated sequentially in a Cks1-dependent manner, the cluster becomes a network with different connectivities between the sites. There are several structural parameters that could control the phosphorylation rate through the networks.

One of the parameters investigated was the distance between the priming phosphorylation site and the secondary phosphorylation site in Cks1-dependent phosphorylation steps. We created a series of constructs based on Sic1ΔC containing two phosphorylation sites. First, the priming site with an optimal consensus motif was left at a fixed position, and, second, we placed an acceptor site with suboptimal CDK consensus motif at different distances along the polypeptide chain. Due to its intrinsically disordered nature (Brocca, Samalikova et al. 2009; Mittag, Marsh et al. 2010), Sic1ΔC is an excellent tool to study such distance requirements. At certain distances, a strong signal of doubly phosphorylated species was detected. This was shown to be dependent on Cks1 (VI, Fig. 3B), which confirmed that it was the result of a two-step cascade, where a priming site was targeted before the phosphorylation of the secondary site (VI, Fig. 3B). Strikingly, the Cks1-dependent secondary phosphorylation step indicated sharp dependence on the distance between the priming site and the secondary site. For all three cyclin-Cdk1 complexes tested, the peak optimum distance was from 12 to 16 amino acids downstream of the priming site (VI, Fig. 3C-E). Between 10 and 12 amino acids, a sharp rise in the capability of cyclin-Cdk1 complexes to phosphorylate the secondary site was observed. The rate of the secondary phosphorylation started to decline after a distance of 20 to 30 amino acids N-terminal from the priming site. The distance dependence of secondary site phosphorylation was almost identical for all three cyclin-Cdk1 complexes, showing that the Cdk1-Cks1 interface does not depend on cyclin specificity. As seen in the model based on the crystal structures of cyclin A-Cdk2-Cks1 (modelled by Dr. Seth M. Rubin), the bound Cks with its cationic pocket forms a continuous surface with CDK and its active site (Figure 3). The shortest distance between the CDK active site and the Cks phosphate binding pocket is 31 Å. However, as is shown in figure 3, the peptide linker between two sites would need to take a route different from the shortest distance

(assuming that the flexible and, intrinsically disordered linkers would extend on average 4 Å/residue).

We also tested if the distance between the priming site and the secondary site is critical for multisite phosphorylation *in vivo*. As previously shown, Sic1 degradation is dependent on the phosphorylation of its diphosphodegrons, pT45/pT48 and pS76/pS80. Efficient phosphorylation of these degrons requires N-terminal sites that, after becoming phosphorylated, serve as Cks1-mediated docking sites for the cyclin-Cdk1-Cks1 complex. We used viability assays with a version of Sic1 containing the minimal set of 5 phosphorylation sites needed for viability: T33, T45, T48, S76, and S80. Surprisingly, changing the distance between the priming site T33 and the degron by only two amino acids toward either the N- or C-terminus caused lethality to cells (VI, Fig. 4A). In these constructs, the Cks1 docking distance perfectly fits the optimum of 12-16 amino acids, obtained from *in vitro* assays, suggesting that it is an important factor for the phosphorylaton of the diphosphodegron. The T48 site in the diphospho-degron T45/T48 is a non-CDK consensus site, whose phosphorylation could be even more sensitive to the Cks1-dependent docking distance, compared with the T-P site used in kinase assay. The importance of the distance was further proved by the fact that moving the position of the degron T45/T48 by 10 amino acids downstream in a Sic1 version containing all nine Cdk1 sites severely reduced the viability of the cells (V, Fig. 4B). One possible explanation as to why Cks1-dependent phosphorylation has been evolved may be the ability to target diphosphodegrons that contain non-CDK sites. Directing the crucial signals to sites with no proline in position +1 would prevent the other proline-directed kinases (e. g. MAP kinases) from prematurely triggering cell cycle transitions.

The second parameter that may influence the phosphorylation of a CDK site is its distance from the docking site. In Sic1, there are two Clb5-specific RxL docking sites and a single Cln2-specific LLPP motif. We analysed the distance requirement between a docking site and a phosphorylation site in constructs containing only one RxL motif and the LLPP motif. We varied the position of the optimal CDK site in Sic1 along the Sic1ΔC polypeptide, while the position of the docking site (RxL and LLPP) was fixed. In the case of Clb5-Cdk1, we observed an abrupt rise in the phosphorylation rates when the phosphoacceptor site was 16-20 amino acids N-terminal from the RxL docking motif (VI, Fig. 5B). However, Clb2-Cdk1 showed only small increase in rates (V, Fig. 5C) within the same distance variations, which is consistent with our previous results indicating that Clb2-Cdk1 has a much weaker ability to use HP for potentiation of substrate phosphorylation (Loog and Morgan 2005). The observed minimal distance of 16 amino acids is also in agreement with previously observed result for cyclin E- and cyclin A-Cdk2 complexes (Takeda, Wohlschlegel et al. 2001). As described in paragraph 2.6, the shortest distance between the CDK active site and HP on the cyclin is 40 Å. Our observed minimum distance was 16 amino acids (about 64 Å), suggesting that the polypeptide chain takes a longer path, as presented in figure 3. The strict

distance requirements show that the phosphorylation site and RxL docking motif may bind simultaneously with the cyclin-Cdk1 complex.

In case of Cln2-Cdk1, it seems that LLPP motif can potentiate phosphorylation of sites placed either N- or C-terminal to the docking site (V, Fig. 5D). These results suggest that the LLPP site is less directionally deterministic than the HP-RxL docking interaction.

3.4.2. Screen for substrates that show Cks1 dependent processivity (Ref V)

So far, we had established that the multisite phosphorylation of Sic1 is mediated by Cks1. The role of Cks1 in promoting substrate protein phosphorylation has been addressed also in two earlier studies (Patra and Dunphy 1998; Patra, Wang et al. 1999). However, this phenomenon had not been studied for a large set of Cdk1 targets. If Cks1-dependent phosphorylation were observed for a broad range of Cdk1 targets it could provide a mechanistic basis for the threshold model described in paragraph 2.5. The parameters that control the phosphorylation of multisite targets may generate a wide range of different output signals, acting as amplifiers of the small changes in the CDK input activities.

For a larger scale analysis, we chose a set of confirmed or potential Cdk1 targets containing multiple phosphorylation sites. In the phosphorylation assays we used Cks1wt or Cks1*mut*, which lacks a functional phosphate-binding pocket. The cyclin-Cdk1 complexes that were chosen to test different substrates were based on the cyclin specificity profiles described in more detail in paragraph 3.2.3. For all three of the cyclin-Cdk1 complexes used in the assays, some targets were more dependent on Cks1-mediated multisite phosphorylation than others (V, Fig. 1A). In the subset of substrates tested with Cln2-Cdk1, the transcriptional regulator Stb1 and an S-phase specific transcription factor Hcm1 showed the largest differences in the phosphorylation patterns for Cks1wt and Cks1*mut*. Both of these targets contain two optimal CDK sites with threonines, which after being phosphorylated may act as efficient priming sites for the subsequent steps of Cks1-mediated phosphorylation cascades. The phosphorylation pattern with Cks1wt and Cks1*mut* was similar in all four targets, that all lack CDK sites based on threonine (V, Fig. 1A). In case of Clb5-Cdk1, Sic1, and the kinetochore protein, Cnn1 showed high Cks1-dependent phosphorylation. Interestingly, the phosphorylation of two proteins involved in DNA replication, Orc6 and Sld2, was not affected by Cks1 (V, Fig. 1A). In a subset tested with Clb2-Cdk1, almost all substrates were phosphorylated in a Cks1-dependent manner, except the transcription factor Swi5. Interestingly, Whi5 showed a Cks1-dependent effect with Clb2-Cdk1, but a much weaker effect with Cln2-Cdk1. The differences amongst the targets hint that the multisite networks may have different patterns which are affected by the network parameters discussed in earlier paragraphs. These patterns may have functional importance in regulating different cell cycle transitions or responding to the signals of different cyclin-Cdk1 complexes.

4. CONCLUSIONS

To briefly summarize the results of this study:

1. In the course on our studies on cyclin specificity in Cdk1 substrate phosphorylation, we have found that the activity of Cdk1 towards the consensus phosphorylation motif increased gradually, following the order of appearance of the cyclins in the cell cycle (0,1 (Cln2)< 0,34 (Clb5)< 1,28 (Clb3)< 4,1 (Clb2)). We identified a novel docking motif that compensates for the weak intrinsic specificity of Cln2-Cdk1 towards its targets in G1 phase. Additionally, we found that Cln2-Cdk1 has consensus site specificity distinct from that of B-type cyclin-Cdk1 complexes, suggesting that, in addition to their CDK-activating function, cyclins can also differentially modulate the phosphorylation consensus motifs of different cyclin-Cdk1 complexes. In a screen for cyclin-specific physiological targets, we identified several Cln2, Clb3, and Clb2 specific Cdk1 substrates. Additionally, we proposed a classification system for Cdk1 targets based on their cyclin specificity profile. Based on the obtained results, we proposed a model of describing the dynamics of Cdk1 specificity during cell cycle progression. In addition to gradually rising Cdk1 activity levels, the changing pattern of cyclin specificity, supported by cyclin-specific docking sites, exists to facilitate ordered progression through phosphorylation switches.

2. In our studies on the mechanisms behind the multisite phosphorylation of Sic1, we performed a detailed mapping of the events that eventually cause the phospho-dependent degradation of Sic1. We proposed that Sic1 destruction at the onset of S phase depends on a complex process, in which both Cln2-Cdk1 and Clb5-Cdk1 mediate a semi-processive multi-phosphorylation cascade that leads to the phosphorylation of specific diphosphodegrons. We found that the cascade is shaped by a precisely orientated docking interaction mediated by cyclin-specific docking sites in Sic1 and by Cks1, the phosphoadaptor subunit of the Cdk1 complex. We have found that the increase in specificity due to Cks1-dependent docking is great enough that it can promote efficient phosphorylation of the non-CDK consensus sites, leading to the rise of diphosphodegrons. The mechanistic studies on Sic1 phosphorylation suggested that Cln2-Cdk1 acts as a priming kinase, phosphorylating a set of N-terminal priming sites, necessary for Cks1-dependent phosphorylation. More importantly, Clb5-dependent phosphorylation of Sic1 creates a positive feedback loop, which is the main driving force behind the abrupt switch-like destruction of Sic1 at G1/S transition.

3. We have studied different structural parameters which determine the ability of Cdk1 to produce multi-phosphorylated output for its targets. The parameters that control Cdk1-dependent multisite cascades include the distances between the phosphorylation sites, the positions of docking sites relative to

phosphorylation sites, the number of serines versus threonines in the clusters, Cks1 consensus site specificity, and the processivity factors at each phosphorylation step. Our studies show that Cks1 has a strong preference for pThr over pSer as its docking sites. Additionally Cks1 prefers phosphor-sites with proline at the -2 position.

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SUMMARY IN ESTONIAN

Uurimustöö *Saccharomyces cerevisiae* tsükliinist sõltuva kinaasi Cdk1 substraadispetsiifilisusest ja multifosforüleerimise mehhanismist

Raku jagunemistsükkel ehk rakutsükkel on protsess, mille käigus rakk kahekordistab oma sisu ja seejärel jaguneb kaheks. Rakutsükli saab jagada neljaks erinevaks etapiks: G1-, S-, G2- ja M-faasiks. Võtmetähtsusega sündmused – DNA replikatsioon ja kromosoomide segregatsioon ning järgnev tsütoplasma jagunemine – toimuvad vastavalt S- ja M-faasis. S- ja M-faas on teineteisest eraldatud vaheetappide ehk G1- ja G2-faasiga. Rakutsükli faaside vaheldumine on reguleeritud kontrollsüsteemi poolt, mille peamisteks komponentideks on tsükliinist sõltuvad kinaasid (*cyclin-dependent kinase*; CDK). CDK valkude aktiivsuse ostsillatsioon sõltub erinevate regulatoorsete subühikute ehk tsükliinide olemasolust erinevates rakutsükli etappides. Tsükliinid võib jaotada kolme klassi: G1-tsükliinid, mis seonduvad CDK-dega G1-faasis, S-faasi tsükliinid, mis kontrollivad DNA replikatsiooni, ja mitootilised ehk B-tüüpi tsükliinid, mis aktiveerivad CDK-d rakutsükli G2- ja M-faasis. CDK-de ensümaatilist aktiivsus reguleeritakse nelja erineva mehhanismi abil: tsükliini seondumine, aktiveeriv või inhibeeriv fosforüleerimine ja seondumine inhibiitorvalkudega. Aktiivsed tsükliin-CDK kompleksid toimivad lülititena, lisades teistele valkudele fosfaatrühmi ning muutes seeläbi nende omadusi. Enamus substraatvalke sisaldavad mitmeid CDK poolt äratuntavaid fosforüleerimise konsensusjärjestusi S/T-P-x-K/R (kus x võibolla ükskõik milline aminohape), milles aminohapped seriin (S) või treoniin (T) käituvad fosfaadi aktseptorina. Lisaks kuulub tsükliin-CDK kompleksi veel CDK adaptorvalk Cks, moodustades kolmikkompleksi tsükliin-CDK-Cks. Cks võib seonduda juba fosforüleeritud valkudega, aidates kaasa substraatide multi-fosforüleerimisele. Üldiselt määravad tsükliin-CDK-Cks komplekside substraadi spetsiifilisust kolm äratundmismotiivi: 1) Tsükliinil asuv hüdrofoobne tasku, mis interakteerub substraatidel oleva RxL (arginiin, ükskõik milline aminohape, leutsiin) motiiviga, 2) CDK aktiivsait, mis seondub sihtmärkvalgu konsensusjärjestusega ja 3) Cks-e katioonne tasku, mis seondub juba fosforüleeritud seriini või treoniini fosfaatrühma ja ümbritseva konsensusjärjestusega.

Üheks mudelorganismiks, kus rakutsükli toimimismehhanisme uurida, on pagaripärm *Saccharomyces cerevisiae*. Erinevalt imetajatest leidub *S. cerevisieae*-s ainult üks tsükliinist sõltuv kinaas, Cdk1, mis interakteerub erinevatel rakutsükli etappidel üheksa erineva tükliiniga (Cln1–3 ja Clb1–6) ning adaptorvalgu Cks1-ga. Tsükliinid Cln1-3 on aktiivsed G1 faasis ja G1/S faasi ülemineku. Clb5 ja 6 vastutavad korrektse S-faasi sisenemise ja läbimise eest. Clb3 ja Clb4 osalevad G2/M üleminekul. Clb1 ja Clb2 aga kontrollivad mitootiliste rakkude saatust.

Käesoleva eksperimentaalse töö esimene osa keskendub küsimusele, kuidas muutub erinevate tsükliin-Cdk1 komplekside aktiivsus *S. cerevisiae* rakutsükli

käigus. Me leidsime, et tsükliin-CDK komplekside aktiivsus optimaalse fosforüleerimisjärjestuse suhtes kasvab rakutsükli käigus graduaalselt. Me identifitseerisime substraatvalkudes uudse G1 tsükliinide seondumisjärjestuse, mis aitab kompenseerida nende nõrka aktiivsaidi spetsiifikat rakutsükli varastel etappidel. Lisaks leidsime, et G1 tsükliin-Cdk1 komplekside konsensusjärjestuse spetsiifika on erinev B-tüüpi tsükliin-Cdk1-e omast. Substraatvalkude laiapõhjalise analüüsi tulemusel suutsime identifitseerida erinevate tsükliin-Cdk1 komplekside spetsiifilisi füsioloogilisi sihtmärkvalke. Lähtuvalt oma andmetest pakume välja mudeli, mille kohaselt on rakutsükli progressiooniks olulised nii graduaalselt tõusev Cdk1 aktiivsus kui ka rakutsükli käigus muutuv tsükliinispetsiifika.

Enamus CDK sihtmärkvalkudest sisaldavad mitmeid fosforüleerimisjärjestusi ning seetõttu keskendusime eksperimentaalse töö teises osas multifosforüleerimise mehhanismi detailsele uurimisele CDK inhibiitorvalgu Sic1-e näitel. B-tüüpi tsükliin-CDK komplekside inhibiitori Sic1-e tase hakkab tõusma mitoosi lõpus ja valk püsib aktiivsena hilise G1 faasini, kus toimub Sic1-e fosforüleerimisest sõltuv lagundamine. Spetsiifilistest lagundamisjärjestustest ehk degronitest fosforüleeritud Sic1 ära tundmine toimub läbi Cdc4, mis on ubikuitiini ligaasi SCF-i (Skp1/Cdc53/F-box) spetsiifilisusfaktor. Ubikuitineeritud Sic1-e lagundamine toimub üle proteasoomi raja. Oma töös uurisime põhjalikult erinevate tsükliin-CDK komplekside poolt läbiviidavat Sic1 fosforüleerimist. Leidsime, et G1/S üleminekul on oluline roll nii Cln2-Cdk1 (G1 tsükliin-CDK kompleks) kui ka Clb5-Cdk1 (S tüskliin-CDK kompleks) kompleksidel, sest mõlemad osalevad Sic1-e semi-protsessiivsel fosforüleerimisel. Avastasime, et Sic1-e fosforüleerimise kaskaad on sõltuv nii CDK adaptorvalgust Cks1-st kui ka tsükliinispetsiifilistest seondumisjärjestustest Sic1-s. Sic1-e multifosforüleerimise mehhansimi uurimine viis mudelini, mille kohaselt Cln2-Cdk1 toimib fosforüleerimise kaskaadis kui praimerkinaas Clb5-Cdk1-le, fosforüleerides efektiivsemalt neid fosforüleerimissaite, mis ei vii Sic1-e lagundamisele. Clb5-Cdk1 saab seda platformi kasutada kiireks Sic1-e fosforüleerimiseks ning juba Sic1-e inhibitsiooni alt vabanenud Clb5-Cdk1 tagab läbi positiivse tagasiside mehhanismi kiire Sic1-e lagundamise ja pöördumatu G1/S ülemineku.

Eksperimentaalse töö kolmandas osas uurisime erinevaid parameetreid, mis mõjutavad Cdk1 poolt läbiviidavat substraatvalkude multifosforüleerimist. Elemendid, mis määravad tsükliin-Cdk1-Cks1-st sõltuva multifosforüleerimise on järgmised: distantsid erinevate fosforüleerimisjärjestuste vahel, tsükliini seodumisjärjestuste positsioon fosforüleerimissaitide suhtes, Cks1 konsensusjärjestuse erinev spetsiifika, seriini- ja treoniinijääkide esinemise suhe CDK konsensusjärjestustes ja iga fosforüleerimisetapi protsessiivsusfaktor. Oma töös leidsime üllatusena, et Cks1 omab tugevat eelistust fosforüleeritud treoniini jääkide suhtes. Praimeri ja aktseptori fosforüleerimisjärjestuste vahelise distantsi uurimisel leidsime, et Cks1-e poolt vahendatud fosforüleerimine toimub suunas N-terminusest C-terminusse. Lisaks näitasime minimaaldistantsi nõuet ning kitsast optimumi kahe fosforüleerimisjärjestuse vahel. Tsükliini spetsiifilise seondumisjärjestuse mõju uurimine näitas erinevusi eri tsükliin-

CDK komplekside vahel. Leidsime, et G1 tsükliin-CDK kompleksid on võimelised fosforüleerima seondumisjärjestustest nii N- kui ka C-terminuse poole jäävaid fosforüleerimisjärjestusi. B-tüüpi tsükliinidest uuritud Clb5-e puhul toimub fosforüleerimine peamiselt seondumisjärjestusest N-terminuse poole, omades minimaaldistantsi nõuet fosforüleerimisjärjestuse ja tsükliinspetsiifilise seondumismotiivi vahel. Me pakkusime välja mudeli, mille kohaselt uuritud parameetrid kontrollivad kollektiivselt multifosforüleeritavate võrgustike võimet differentsaalselt töödelda Cdk1 signaale. See omadus võimaldab nendel võrgustikel korraldada rakutsükli erinevate protsesside õigeaegset käivitumist.

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PUBLICATIONS

I

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Phospho-regulation of kinesin-5 during anaphase spindle elongation

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Summary

The kinesin-5 *Saccharomyces cerevisiae* homologue Cin8 is shown here to be differentially phosphorylated during late anaphase at Cdk1-specific sites located in its motor domain. Wild-type Cin8 binds to the early-anaphase spindles and detaches from the spindles at late anaphase, whereas the phosphorylation-deficient Cin8-3A mutant protein remains attached to a larger region of the spindle and spindle poles for prolonged periods. This localization of Cin8-3A causes faster spindle elongation and longer anaphase spindles, which have aberrant morphology. By contrast, the phospho-mimic Cin8-3D mutant exhibits reduced binding to the spindles. In the absence of the kinesin-5 homologue Kip1, cells expressing Cin8-3D exhibit spindle assembly defects and are not viable at 37°C as a result of spindle collapse. We propose that dephosphorylation of Cin8 promotes its binding to the spindle microtubules before the onset of anaphase. In mid to late anaphase, phosphorylation of Cin8 causes its detachment from the spindles, which reduces the spindle elongation rate and aids in maintaining spindle morphology.

Key words: Cdk1, Cin8, Kinesin-5, Microtubules, Mitosis

Introduction

Evolutionary conserved homotetrameric kinesin-5 motor proteins have major roles in mitotic spindle morphogenesis (Blangy et al., 1995; Goshima and Vale, 2005; Hagan and Yanagida, 1992; Heck et al., 1993; Hoyt et al., 1992). These motors crosslink and slide antiparallel microtubules (Gheber et al., 1999; Kapitein et al., 2005) originating from opposite spindle poles, thereby performing their mitotic functions (Kashina et al., 1997). In addition to their well-established roles in spindle assembly (Hoyt et al., 1992), kinesin-5 motor proteins also have a role in anaphase B spindle elongation in the budding yeast *Saccharomyces cerevisiae* (Gerson-Gurwitz et al., 2009; Movshovich et al., 2008; Saunders et al., 1995; Straight et al., 1998) and other organisms (Sharp et al., 1999; Touitou et al., 2001). Although kinesin-5 motor proteins perform essential functions at several stages of spindle dynamics, and their levels are regulated in a cell-cycle-dependent manner (Gordon and Roof, 2001; Hildebrandt and Hoyt, 2001; Spellman et al., 1998), regulation of their function is not well understood. Previous reports have indicated that kinesin-5 motors are phosphorylated by mitotic kinases (Blangy et al., 1995; Chee and Haase, 2010; Garcia et al., 2009; Giet et al., 1999; Sawin and Mitchison, 1995; Sharp et al., 1999), but the mechanism by which phosphorylation regulates kinesin-5 functions is not fully understood. Here we examine the role of phosphorylation in controlling the intracellular function of the *S. cerevisiae* kinesin-5 homologue Cin8.

Results and Discussion

Cin8 is phosphorylated during anaphase in its motor domain by Cdk1

To examine the phosphorylation of Cin8 as a function of cell cycle progression, we used cells expressing Myc-tagged Cin8 that were arrested at different stages of the cell cycle. Fractionation of the cell extracts by SDS-PAGE followed by western blot analysis demonstrated that in cells arrested at late anaphase by any one of several temperature-sensitive mutations [*cdc15-2*, *cdc14-1* (Jaspersen et al., 1998) and *cdc5-7* (Park et al., 2003)], Cin8 exhibits a slow-migrating form (Fig. 1A), indicative of protein phosphorylation. This band-shift was observed for Cin8 produced either from a CEN (centromere) or 2 μm plasmid tagged with the Myc epitope either at its N- or C- terminus (Fig. 1) and for Cin8–BCP (Gheber et al., 1999) (data not shown). The slow-migrating band of Cin8 was not present in cells arrested in S-phase or in metaphase (Fig. 1B), and was abolished by treatment of the cell extracts with phosphatase (Fig. 1C). These results indicate that Cin8 is differentially phosphorylated during anaphase.

The amino acid sequence of Cin8 contains five putative phosphorylation sites for the conserved cyclin-dependent kinase 1 Cdk1 (Cdc28 in *S. cerevisiae*) ([S/T]-Px-[R/K] or [S/T]-P-[R/K]) (Langan et al., 1989; Shenoy et al., 1989). Three sites are located in the motor domain and two are in the stalk and tail. Mutation of all five amino acids to alanine (Cin8-5A) or of the three located in the motor domain (Cin8-3A) but not of the two in the stalk and tail

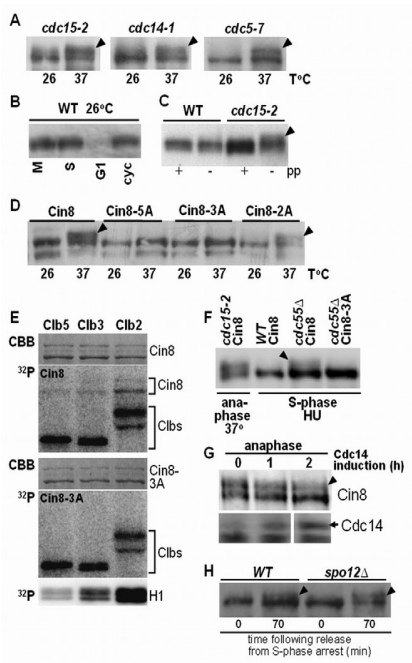

Fig. 1. Cin8 is differentially phosphorylated during anaphase. Band-retardation assay in whole extracts of cells producing 6Myc–Cin8. Extracts were fractionated on SDS-PAGE and examined by Western blot. Arrowheads indicate the slow-migrating band of Cin8. (**A**) Cells expressing *cdc15-2*, *cdc14-1* or *cdc5-7* and containing 6Myc–Cin8 were grown at 26°C (permissive) and 37°C (restrictive). (**B**) Wild-type cells were either cycling or arrested at metaphase (by nocodazole), S-phase (by hydroxyurea) and G1 (by α-factor). (**C**) WT cells or *cdc15-2* cells were grown at 37°C for 4 hours. The extracts were either treated with CIP phosphatase (pp) (+) or not (–). (**D**) *cdc15-2* cells expressing either WT Cin8, Cin8-5A (S277, T285, S493, S736, S1010), Cin8-3A (S277, T285, S493) or Cin8-2A (S736, S1010) (indicated on top) were grown at 26°C or 37°C (indicated at the bottom). (**E**) In vitro phosphorylation of bacterially expressed Cin8-590 by Cdk1 using equal concentrations of the Cdk1 complexes with cyclin Clb2, Clb3 or Clb5 (indicated on top). Coomassie Brilliant Blue (CBB) staining and ^{32}P autoradiograms of SDS-PAGE fractionation of phosphorylation reaction mixtures are shown (indicated on the left). Proteins corresponding to the various bands are indicated on the right, based on their predicted size. The relative specificity pattern obtained using equal concentrations of the kinase complexes with a reference substrate Histone H1 is shown in the bottom panel (H1). (**F**) Effect of CDC55 deletion on band-retardation assay of Cin8. Genotypes of cells and variants of Cin8–13Myc are indicated at the top; the various modes of arrest are indicated at the bottom. (**G**) Effect of Cdc14 overexpression on the slow-migrating band of Cin8–13Myc. 3HA–Cdc14 was produced from a galactose-inducible promoter. Cells were arrested either in S-phase or at late anaphase (indicated on top). In anaphase-arrested cells, Cdc14 was induced by addition of galactose for the times indicated above. The western blots of Cin8–13Myc (top) and 3HA–Cdc14 (bottom) are shown. (**H**) 6Myc–Cin8 was expressed in WT or *spo12Δ* cells (indicated on top). Cells were arrested in S-phase and released to fresh medium for 70 minutes (bottom).

(Cin8-2A), almost completely abolished the slow-migrating band of Cin8 at the end of anaphase (Fig. 1D). To examine whether Cin8 is phosphorylated by Cdk1, we performed an in vitro phosphorylation assay using a bacterially expressed truncated form of Cin8, which contains its catalytic motor domain (Cin8-590) (Hildebrandt et al., 2006). Cin8-590 was indeed phosphorylated by Cdk1, and the mitotic complex Clb2–Cdk1 showed higher specificity relative to the earlier complexes Clb3–Cdk1 and Clb5–Cdk1 (Fig. 1E). Moreover, alanine replacement of the three Cdk1-specific sites that abolish the slow-migrating band of Cin8 during anaphase (Fig. 1D) completely eliminated the phosphorylation of Cin8-3A-590 by the Clb2–Cdk1 in vitro (Fig. 1E). Consistent with these findings, it has been recently reported that Cin8 is a substrate of Clb2–Cdk1 both in vivo and in vitro (Chee and Haase, 2010). However, this recent report (Chee and Haase, 2010) does not demonstrate specifically that sites in the Cin8 motor domain are targets for Cdk1 phosphorylation. Our results indicate that during anaphase, Cin8 is phosphorylated by Clb2–Cdk1 at site(s) located within its motor domain. Moreover, in agreement with previous reports that a low Clb2–Cdk1 activity is maintained throughout anaphase (Queralt et al., 2006), our data suggest that this activity is sufficient for accumulation of the phosphorylated form of Cin8 at the end of anaphase. Alternatively, during anaphase, the same sites might be phosphorylated by an additional kinase.

Two of the three sites that abolish the Cin8 phosphorylation, S277 and T285, are located within the uniquely large loop 8 (aa 255–353) of Cin8 motor domain (Hoyt et al., 1992). The third, S493 is only 13 residues away from loop 12 and α-helix 5 in the kinesin motor domain, which are important for the interaction with microtubules (Kull et al., 1996; Sablin et al., 1996; Woehlke et al., 1997). Interestingly, S493 itself is conserved among the kinesin family members and, as in Cin8, is part of a consensus sequence for phosphorylation by Cdk1 also in the second *S. cerevisiae* kinesin-5 homologue Kip1.

Because Cdk1 is activated before anaphase (Grandin and Reed, 1993), the finding that phosphorylated Cin8 is accumulated only during anaphase (Fig. 1A,B) suggests that prior to anaphase, it is dephosphorylated by one or more phosphatases. Therefore, we examined whether the separase-dependent Ser/Thr-specific PP2A^{Cdc55} phosphatase, which dephosphorylates Cdk1 sites before anaphase onset (Queralt et al., 2006), affects the phosphorylation of Cin8. Indeed, a reproducible slow-migrating band of Cin8 was observed for *cdc55Δ* but not for WT cells arrested in S-phase with short spindles (Fig. 1F). This band was abolished when the phosphorylation-deficient mutant Cin8-3A was expressed in *cdc55Δ* cells (Fig. 1F). These results indicate that the PP2A^{Cdc55} phosphatase is involved, at least in part, in Cin8 dephosphorylation before anaphase onset. A similar regulation mechanism was previously demonstrated for inhibition of Cdk1-dependent Net1 phosphorylation before anaphase by the PP2A^{Cdc55} phosphatase (Queralt et al., 2006). Our data therefore suggest that inactivation of separase-dependent PP2A^{Cdc55} promotes phosphorylation of a number of Cdk1 targets, including Cin8, whose phosphorylation is required for anaphase progression.

To examine the possibility that the conserved Cdc14 phosphatase (Stegmeier et al., 2002) dephosphorylates Cin8 during anaphase,

the band-retardation assay was performed in cells synchronized in late anaphase by *cdc15-2* and then Cdc14 was overexpressed from a galactose-inducible promoter, while holding the late-anaphase arrest. Overexpression of Cdc14 reduced the appearance of the slow-migrating band of Cin8 in a time-dependent manner (Fig. 1G). Quantitative analysis indicates that without Cdc14 overexpression, the ratio of intensities between the slow-migrating (phosphorylation) and the low bands of Cin8 is ~1, whereas after overexpression of Cdc14 for 2 hours, this ratio drops to ~0.5.

To further explore the involvement of Cdc14 in Cin8 dephosphorylation, we examined Cin8 phosphorylation during anaphase progression in WT cells versus cells deleted for *SPO12*, a member of the early-anaphase Cdc14 activation pathway (Stegmeier et al., 2002). Seventy minutes after release from S-phase arrest, when ~60% of cells are in anaphase (not shown), a reproducible slow-migrating band appeared in both *WT* and *spo12Δ* cells (Fig. 1H). Quantitative analysis showed that in the absence of Spo12, the ratio between the slow-migrating and the low bands of Cin8 increased by 40% (Fig. 1H), indicating the deletion of *SPO12* enhances Cin8 phosphorylation. Taken together, these results show that Cdc14 is involved in Cin8 dephosphorylation during anaphase.

Cdk1 phosphorylation of Cin8 affects its localization to the mitotic spindle

Monitoring of the localization of Cin8–3GFP to the anaphase spindles in unsynchronized *WT* cells revealed that in early-mid anaphase, when spindles are shorter than 4.5–5 μm, WT Cin8 binds to a large portion of the spindle, as well as near the spindle poles (Fig. 2Aa,Ba). As spindles elongate, Cin8 was concentrated at a decreasing portion of the middle region. At late anaphase, when spindles are longer than ~8 μm, Cin8 was barely detectable at the middle spindle and was diffusively localized near the spindle poles (Fig. 2Aa). Consistent with the localization pattern in cells arrested in late anaphase by the *cdc15-2*, *cdc14-1* and *cdc5-7* mutations, when significant phosphorylation of Cin8 is detectable (Fig. 1A), Cin8 localized diffusively near the spindle poles and was not focused at the midzone (supplementary material Fig. S1).

The Cin8-2A mutant, which does not affect Cin8 phosphorylation during anaphase (Fig. 1D), exhibited similar spindle localization to that of WT Cin8 (Fig. 2Ab). Compared with WT Cin8 and Cin8-2A, the phosphorylation-deficient Cin8-3A mutant (Fig. 1D) remained localized near the spindle poles, occupied a larger portion of the middle spindle during mid-late anaphase and remained localized to this region for a longer period of time (Fig. 2Ac,Bb). Similarly to the Cin8-3A mutant, in cells that lack the Cdk1 cyclin Clb2 (*clb2Δ*), which is involved in Cin8 phosphorylation (Fig. 1E), *WT* Cin8 occupied a large portion of the anaphase spindles (supplementary material Fig. S2B). By contrast, the phospho-mimic Cin8-3D, where Cdk1 sites in the motor domain are replaced by aspartic acid, failed to bind to the spindle and exhibited diffusive localization at early anaphase (Fig. 2Ad). Also, in *cdc55Δ* cells, where Cin8 phosphorylation is increased (Fig. 1F), partial diffusive localization of Cin8 in early anaphase was observed (supplementary material Fig. S2C). Thus, taken together, our results indicate that Cin8 dephosphorylation at early anaphase is required for its attachment to the spindle, whereas phosphorylation of Cin8 leads to its detachment from the spindle at late anaphase.

Next, viability of *cin8Δ kip1Δ* cells was examined in which *CIN8* shuffle plasmid (Gheber et al., 1999) was replaced by a centromeric plasmid expressing *CIN8*, *cin8-3A* or *cin8-3D*. Cin8 and Cin8-3A cells remained viable at all examined temperatures, but cells

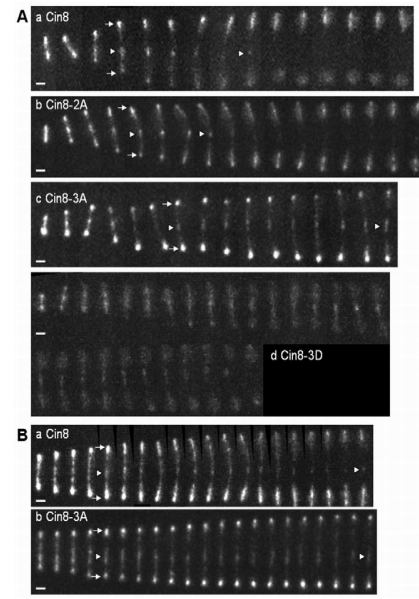

Fig. 2. Anaphase spindle localization of phosphorylation mutants of Cin8–3GFP. (**A**) a, Cin8; b, Cin8-2A, c, Cin8-3A, d, Cin8-3D. Time interval, 1 minute. (**B**) a, Cin8; b, Cin8-3A; time interval, 20 seconds. Scale bar: 1 μm. Arrows indicate spindle poles; arrowheads indicate the midzone.

producing Cin8-3D were not viable at 37°C (supplementary material Fig. S3A). At room temperature, ~65% of the *cin8Δ kip1Δ* cells expressing *cin8-3D* were large-budded with monopolar spindles, compared with only ~10% of the cells expressing *CIN8*. At 37°C, the cells producing Cin8-3D arrested as large-budded cells with collapsed spindles (supplementary material Fig. S3B). These findings indicate that in the absence of Kip1, the reduced binding of Cin8-3D to the spindle interferes with spindle assembly and suggest that dephosphorylation of Cin8 is required to assemble mitotic spindles and maintain their bipolar structures before anaphase.

The phosphorylation-dependent affinity of Cin8 to the spindles might be explained by two mechanisms. The additional negative charge caused by phosphorylation (or mutation to aspartic acid) of residues in the motor domain might directly reduce the affinity of Cin8 for MTs, causing its detachment from the spindle. This is opposed to what has been shown thus far for several kinesin-5 family members, namely that a C-terminal tail domain phosphorylation promotes localization to the mitotic spindle (Blangy et al., 1995; Cahu et al., 2008; Sawin and Mitchison, 1995; Sharp et al., 1999). Here, we show for the first time that phosphorylation of the kinesin-5 Cin8 negatively regulates its localization to the mitotic spindle: its prevention increases Cin8 binding to the spindle. A similar effect of phosphorylation has been shown for the *Caenorhabditis elegans* kinesin-6 homologue ZEN-4: the motor domain phosphorylation by Cdk1 reduced the affinity of ZEN-4 for MTs (Mishima et al., 2004). In addition,

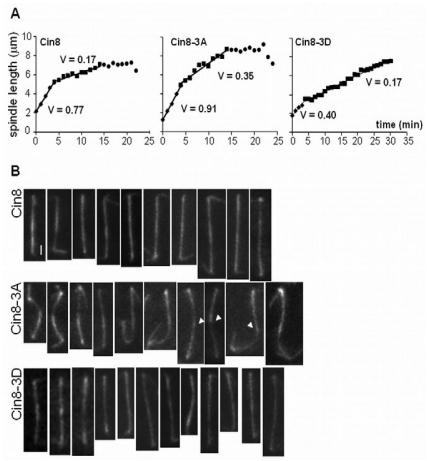

Fig. 3. Anaphase spindle elongation and morphology in cells that express phosphorylation mutants of Cin8. (A) Representative spindle-elongation kinetics in cells producing WT Cin8 (left), Cin8-3A (middle) and Cin8-3D (right). V, rate of spindle elongation (μm/minute). The phases of spindle elongation are indicated as follows: diamonds, fast phase; squares, slow phase; circles, post-anaphase. (B) Long anaphase spindle morphologies in cells expressing tubulin–GFP and Cin8 (top), Cin8-3A (middle) or Cin8-3D (bottom). Scale bar: 1 μm. Arrowheads indicate small overlapping region of the iMTs.

phosphorylation of the conserved serine residue in the motor domain of the *Drosophila melanogaster* kinesin-13, KLP10A, alters the interaction of KLP10A with the MTs and diminishes its MT-depolymerizing activity (Mennella et al., 2009).

An alternative role for Cin8 phosphorylation might be to reduce interactions between Cin8 and accessory spindle binding protein(s). It has recently been shown that Cin8 interacts directly with the conserved MT bundling and midzone-organizing protein Ase1, an interaction that is dependent on Ase1 dephosphorylation (Khmelinskii et al., 2009): delivery of Cin8 to the spindle seems to be controlled by dephosphorylation of Ase1 (Khmelinskii et al., 2009). Our data strongly indicate that spindle detachment of Cin8 is regulated by its own phosphorylation. It is possible that the interaction between Cin8 and Ase1 depends on the phosphorylation state of both proteins, as is the case for the fission yeast kinesin-6 and Ase1 (Fu et al., 2009), and that Cin8 phosphorylation reduces its interaction with Ase1.

Cdk1 phosphorylation of Cin8 regulates its function in mitotic spindle morphogenesis

In *S. cerevisiae* cells, anaphase B consists of two phases, a fast phase followed by a slow one, with Cin8 contributing mainly to the elongation during the fast phase (Straight et al., 1998). To examine the role of phosphorylation in Cin8 function on the spindle, we monitored the anaphase spindle elongation kinetics and the spindle morphology. In Cin8-3D cells (Fig. 2Ad), as well as in *cdc55Δ* cells (supplementary material Fig. S2C), where Cin8 exhibits diminished binding to the spindle compared with WT cells, the spindle elongation rate was considerably slower (Fig. 3A, Table 1 and supplementary material Fig. S2C). However, in Cin8-3A and *clb2Δ* cells, where Cin8 occupies a larger portion of the middle spindle (Fig. 2Ac,Bb and supplementary material Fig. S2B), the spindle elongation rate was considerably faster (Fig. 3A, Table 1 and supplementary material Fig. S2B). Also, in Cin8-3A cells, the overlapping region of antiparallel interpolar MTs (iMTs) is significantly decreased (Fig. 3B, arrowheads and Table 1) and the spindle disassembly is delayed by ~3 minutes (Table 1). Finally, Cin8-3A cells exhibited deformed, bent and asymmetrical spindles (Fig. 3B). Thus our data indicate that phosphorylation of Cin8 controls anaphase spindle elongation.

The higher spindle elongation rate in cells expressing the phosphorylation-deficient Cin8-3A is likely to cause the shorter iMT overlapping region in these cells (Table 1). Because this region serves as a binding site for proteins that control spindle stabilization and iMT plus-end dynamics during anaphase (Buvelot et al., 2003; Fridman et al., 2009; Higuchi and Uhlmann, 2005; Khmelinskii et al., 2007; Schuyler et al., 2003; Thomas and Kaplan, 2007), the decreased size of this region is likely to reduce the stabilization of the iMT plus-ends and thus cause the deformed and asymmetrical spindle morphologies observed in these cells. Hence, our results indicate that the decreased amount of Cin8 at the midzone region at the end of anaphase is required for slowing down the rate of spindle elongation and maintaining the correct structure of the midzone and anaphase spindle.

To summarize, we propose that the phosphorylation–dephosphorylation of Cdk1 sites in Cin8 motor domain controls the binding of a correct amount of Cin8 to the spindle during mitosis. Before the onset of anaphase, Cin8 is dephosphorylated to promote its attachment to the spindle and facilitate its function in spindle assembly. At early–mid anaphase, the activation of Cdc14 phosphatase by the FEAR (Cdc14 early anaphase release) pathway (Chiroli et al., 2009; Ross and Cohen-Fix, 2004; Stegmeier et al., 2002) dephosphorylates Cin8, thus inducing binding of Cin8 to the short anaphase spindle and promoting fast spindle elongation. At late anaphase, when Cdc14 is no longer present at the spindle midzone (Stegmeier et al., 2002) (supplementary material Fig. S4), Cin8 phosphorylation becomes dominant and it detaches from the spindle. This detachment is

Table 1. Effect of Cin8 phosphorylation variants on anaphase spindle elongation

Cin8 variant	Fast-phase rate (μm/minute)	Slow-phase rate (μm/ minute)	Maximal spindle length (μm)	Time before spindle breakage (minutes)	Midzone size (% of spindle length)
WT Cin8	0.82±0.04 (20)	0.22±0.01 (28)	7.72±0.20 (20)	10.24±1.14 (17)	18.9±0.9 (62)
Cin8-3A	0.89±0.03 (43)	0.33±0.02*** (50)	8.31±0.20 (41)	13.83±1.07* (18)	12.9±0.3*** (173)
Cin8-3D	0.41±0.04*** (10)	0.16±0.01** (12)	ND	ND	17.8±1.2 (34)

Values represent mean±s.e.m.; the number of cells analyzed is shown in brackets. *P<0.05; **P<0.01; ***P<0.001, compared with WT cells. The maximal spindle length was determined following spindle disassembly. The time before spindle breakage is the time between the end of slow phase and spindle disassembly. The midzone size was measured for long spindles (>5.5 μm). ND, not determined; few cells were observed in anaphase B.

required for slowing down the rate of spindle elongation to maintain the correct structure of the spindle.

Materials and Methods

Yeast strains

S. cerevisiae strains used are listed in supplementary material Table S1.

Cell growth, cell-cycle arrest and western blot analysis

Cell cycle was arrested after overnight growth to mid-log phase and transfer into the arrest conditions for 3-4 h. The following reagents were applied: for G1 arrest, 5 μg/ml α-factor (Sigma); for S phase, 0.1 M hydroxyurea (Sigma); for metaphase, 10 μg/ml nocodazole (Sigma). To release cells from the S-phase arrest, cells were washed and resuspended in fresh medium lacking hydroxyurea. For late anaphase arrest, cells expressing the *ts* mutations *cdc15-2*, *cdc14-1* or *cdc5-7* were shifted to 37°C. The ability of Cin8 phosphorylation mutants to complement a *CIN8* deletion allele was tested in a strain with deletions in both *CIN8* and *KIP1*. Since *cin8*Δ *kip1*Δ double mutants are not viable, the viability of the tester strain was maintained by a *CIN8* plasmid carrying *CYH2* as well. Despite the presence of a recessive cycloheximide resistance allele *cyh2* in the genome, this strain is sensitive to cycloheximide because of the dominant *CYH2* allele carried on the plasmid (Gheber et al., 1999). At 26°C, transformation with a second plasmid carrying *CIN8*, *cin8-3A* or *cin8-3D*, however, allowed cells to live without the *CIN8-CYH2* plasmid and therefore to grow on cycloheximide-containing medium (supplementary material Fig. S1). Treatment of extracts with CIP phosphatase was previously described (Cohen-Fix and Koshland, 1997). Whole-cell protein extracts were obtained as desribed (Hildebrandt et al., 2006). For 3HA-Cdc14 overexpression during anaphase arrest, *cdc15-2* cells expressing Cin8-13Myc and P_{GAL1}-HA-Cdc14 were grown in 2% raffinose medium to mid-log phase and shifted to 37°C. 2% galactose was added to the medium for 1 or 2 hours, whilst holding the cells at 37°C. The total incubation time at 37°C was 6 hours. For the band-retardation assay, to visualize the slow-migrating band of Cin8 (Fig. 1H), whole-cell extracts were fractionated on a 10% SDS PAGE for 7–9 hours and subjected to western blot analysis. The antibodies used were mouse anti-HA (12CA5, Abcam), mouse anti-Myc (9E10, Santa Cruz) and goat anti-mouse IgG HRP conjugate (W402B, Promega).

In vitro phosphoryalation assay

The TAP method was applied for purification of cyclin Cdc28 complexes from *sic1*ΔP_{GAL}-*CLB2-TAP*, *sic1*ΔP_{GAL}-*CLB3-TAP*, and *sic1*ΔP_{GAL}-*CLB5-TAP* strains, as described previously (Puig et al., 2001; Ubersax et al., 2003). Purification of CIN8-590–TEV–EGFP–6xHIS and Cin8-3A-590–TEV–EGFP–6xHIS was performed using standard cobalt affinity chromatography, and the elution was accomplished with 200 mM imidazole. For the phosphorylation assays of Cin8, about 0.1 mg/ml purified Cin8 was used. The basal composition of the assay mixture contained 50 mM HEPES, pH 7.4, 150 mM NaCl, 80 mM imidazole, 2% glycerol, 2 mM EGTA, 0.25 mg/ml BSA, 80 μg/ml Cks1, and 500 μM ATP [with added [γ-^{32}P]ATP (Perkin Elmer)]. Around 50 nM of the purified kinase complex was used and the reaction was stopped with SDS-PAGE sample buffer.

Imaging and anaphase spindle elongation kinetics

Real-time imaging of GFP-tagged proteins and measurements of the spindle elongation kinetics were previously described (Fridman et al., 2009; Gerson-Gurwitz et al., 2009; Movshovich et al., 2008). In brief, time-lapse *z*-stacks images were acquired with a Zeiss Axiovert 200M-based Nipkow spinning-disc confocal microscope (UltraView ESR, Perkin Elmer, UK) equipped with an EMCCD camera, with 0.2 μm separation between planes and 4–60 second time intervals. Image processing and spindle length measurements (in 3D) were performed using ImageJ and MetaMorph software.

Statistical analysis

The significance of the differences between the average values was determined using Student's *t*-test. **P*<0.05; ***P*<0.01; ****P*<0.001.

DNA manipulation

The N-terminal 6-Myc tag and the C-terminal 3HA tags of Cin8 were previously described (Hildebrandt et al., 2006). A PCR-generated strategy was used to replace *CDC55* open reading frame with SpHIS5 (Delneri et al., 2000) using (f) 5′-TCGAT-TACGTCAATTAGGCTCTCTTCTATATTTTAGTTCAGATCCGCTAGGGATAA-CAGG-3′ and (r) 5′-TTTCAATTAAAACAGTAGTAGTATGTGGGGAAGATA-TGGGTCATCGATGAATTCGAGCTC-3′ primers and the pKT101 plasmid from EUROSCARF collection (Sheff and Thorn, 2004) as a template. The C-terminal 13-Myc tag was constructed using the pKT233 plasmid from EUROSCARF collection (Sheff and Thorn, 2004). The *13Myc* and *ADH1* termination region were PCR-amplified from the pKT233 plasmid using (f) 5′-CGCGAGCGGCCGCCCCGGGT-TAATTAACGGTGAAC-3′ and (R) 5′-GAGCGTCTAGACCCTAGCGGATCTGC-CGGTAGAG-3′ primers and replacing the *Not*I-*Xba*I fragment of pTK47, creating pRM39. Then, the *Pst*I-*Xba*I fragment was transferred from pRM39 to pTK49 (Hildebrandt et al., 2006), creating pRM40. Finally, the *Sal*I-*Xba*I fragment of *CIN8-13Myc* was transferred to the pRS316 vector, resulting in pJKY1. The C-terminal 3GFP tag of Cin8 was constructed by PCR amplification of the 3GFP sequence using the pB1963 plasmid as a template [a gift from David Pellman's lab (Buttery et al., 2007)] and the (f) 5′-TCAAGCGGCCGCTCCTGCAGCCCGGGGGATCCA-3′ and (r) 5′-ACCGCGGTGGCGGCCGCT-3′ primers. The PCR-amplified sequence was introduced into the C-terminal *Not*I site of *CIN8* (Hildebrandt et al., 2006). For genomic integration of *CIN8-3GFP*, *CIN8-3GFP* was subcloned into the pRS305 vector (Sikorski and Hieter, 1989) and integrated into the *LEU2* locus. Plasmid containing the mutations of the five Cdc28 phosphorylation sites (*cin8-5A*) in pTK49 (Hildebrandt et al., 2006), pYLCIN8 1-5, was a gift from Gavin Sherlock (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY). To construct *6Myc-cin8-5A*, *Aat*II-*Sph*1 fragment of *cin8-5A* sequence was transferred from the pYLCIN8 1-5 plasmid to pTK103 (Hildebrandt et al., 2006). The separation between the Cdc28-kinase phosphorylation sites in the motor domain and the tail of Cin8 was done using the unique *Pst*I site within the *CIN8* sequence. The phosphomimic *cin8-3D* mutations (D277, D285, D493) were generated by PCR site-directed mutagenesis (Ko and Ma, 2005), the following primers were used: (f) 5′-ATGCAAGCTTTGTCGACGTTTTCGCC-CCAGGTT-3′, (r) 5′-ATGCGGTCTCAATGACCTAGGGTCACTGTTCCTAGAAT-TACTCCTGGAACTACTAGC-3′, (f) 5′-ATGCGGTCTCATCATTAAATGATCTG-GATCCTAAAGCTGCTCTATTAAGAAAAAGG-3′, (r) 5′-ATGCCTGCAGG-TTTCTTCAGAAGTTACCTTTGCAGGATCGATAGTAGCAATTAGTGCGGTTT-TCGT-3′. The pRM59 plasmid containing the *3HA-CDC14* coding sequence under the *PGAL1* promoter was constructed by PCR-amplifying the *3HA-CDC14* sequence from the p194 plasmid (a gift from Angelica Amon, MIT Cambridge, MA), using (f) 5′-CGTCGTCTAGACGCTTATAAAAAAAAAAAAAAAAATGCG-3′ and (r) 5′-CGGTGAAGTTATTCCTAGGTACCAGG-3′ primers and sub-cloning into the *Kpn*I-*Xba*I sites of p416-GAL1 (Mumberg et al., 1994). For bacteria expression of Cin8 motor domain, first 590 residues of (CIN8-WT) and (cin8-3A) were PCR amplified and cloned into *Nde*I and *Sph*I sites of modified pRSET-B plasmid that contain TEVEGFP–6HIS at the C-terminus, creating pAG26 and pJKY34, respectively. The following primers were used: (f) 5′-AGTCCATATGGTATGGCCAGAAAG-TAACGTTGAG-3′, (r) 5′-CTCGAGGCGGCCGCGCGCATGCCTTGCATTTTTC-GATGTCAAACTTTCAAT-3′.

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Dynamics of Cdk1 substrate specificity during the cell cycle.
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Dynamics of Cdk1 Substrate Specificity during the Cell Cycle

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SUMMARY

Cdk specificity is determined by the intrinsic selectivity of the active site and by substrate docking sites on the cyclin subunit. There is a long-standing debate about the relative importance of these factors in the timing of Cdk1 substrate phosphorylation. We analyzed major budding yeast cyclins (the G1/S-cyclin Cln2, S-cyclin Clb5, G2/M-cyclin Clb3, and M-cyclin Clb2) and found that the activity of Cdk1 toward the consensus motif increased gradually in the sequence Cln2-Clb5-Clb3-Clb2, in parallel with cell cycle progression. Further, we identified a docking element that compensates for the weak intrinsic specificity of Cln2 toward G1-specific targets. In addition, Cln2-Cdk1 showed distinct consensus site specificity, suggesting that cyclins do not merely activate Cdk1 but also modulate its active-site specificity. Finally, we identified several Cln2-, Clb3-, and Clb2-specific Cdk1 targets. We propose that robust timing and ordering of cell cycle events depend on gradual changes in the substrate specificity of Cdk1.

INTRODUCTION

The rise or fall of different cyclin levels is an important switching mechanism triggering the major events of the cell division cycle. The three major switch points that are controlled by cyclin-Cdk activity can be distinguished as Start (G1/S), mitotic entry, and the metaphase-anaphase transition (Morgan, 2007). The general principle of sequential cyclin signals as a periodic driving force of the cell cycle is conserved throughout the eukaryotes. In the budding yeast *Saccharomyces cerevisiae*, cyclins Cln1–3 are triggers for G1 and G1/S, Clb5 and Clb6 drive S phase, Clb3 and Clb4 are specific for early mitotic events, and Clb1 and Clb2 complete the progression to mitosis. A single Cdk, Cdk1, associates with these cyclins to mediate all major cell cycle transitions. However, despite extensive studies in multiple model organisms, there is still no general model of the functional specificity of cyclins, which would explain how they provide temporal separation for cell cycle transitions.

In the course of the cell cycle, cyclins appear and disappear as periodic and partly overlapping waves. Quantitative analysis of cycling cultures of budding yeast suggests that the abundance of different cyclins is relatively similar, with only a few-fold variance (Cross et al., 2002). This finding suggests that one can consider the period from G1 to mitotic exit as a state of relatively unchanging net levels of activated Cdk1. Therefore, it is unlikely that thresholds of total cyclin levels are responsible for triggering cell cycle transitions; instead, it is more likely that precise control of cell cycle transitions depends on differential substrate recognition by different cyclin-Cdk complexes. Cyclin-dependent substrate specificity of Cdk1 has been addressed in several previous studies (Cross et al., 1999; Ikui et al., 2007; Loog and Morgan, 2005), but the general dynamics of Cdk specificity in the cell cycle remain unclear. If differences in target recognition among the cyclin-Cdk complexes exist, then to what extent are these complexes able to provide exclusively specific signals, and to what extent is the specificity overlapping? Clearly, some mechanism must exist that, in addition to providing high specificity toward some substrates, also eliminates activity toward substrates associated with other cell cycle stages. Why, for example, does the activation of Cdk1 in the early cell cycle by G1/S cyclins not trigger premature S or M phase processes? On the other hand, there should exist a common specificity among different complexes toward some Cdk targets. To prevent rereplication, for example, components of the prereplicative complex should be kept in their phosphorylated state throughout the cycle by all B-type cyclin-Cdk1 complexes (Nguyen et al., 2001).

In vivo evidence hints that a filtering mechanism might prevent early cyclins from acting prematurely on later triggers. A number of experiments with cultured cells and transgenic mice suggest that some mammalian cyclins can functionally compensate for the absence of others. However, of all the different cyclins tested, the later mitotic cyclins A2 and B1 appeared to be the most nonredundant, suggesting that the early cyclins cannot perform their function (Satyanarayana and Kaldis, 2009). Genetic evidence from budding yeast has indicated that the major cyclin genes are not fully interchangeable, as replacement of the budding yeast S phase cyclin Clb5 with the mitotic cyclin Clb2 causes replication defects (Cross et al., 1999; Cross and Jacobson, 2000; Donaldson, 2000). Similarly, early cyclins appear less capable of performing later Cdk functions, as the deletion of both Clb5 and Clb6 results only in an S phase delay (Schwob and Nasmyth, 1993), whereas the deletion of both mitotic cyclins is lethal (Mendenhall and Hodge, 1998). In fission yeast, early cyclins can be deleted without severe consequences, while the mitotic cyclin is essential for division (Fisher and Nurse, 1996).

Our goal in the present study was to analyze the dynamics of Cdk1 specificity during the cell cycle of budding yeast. Operating with a single Cdk1 molecule makes the yeast cell cycle an ideal model for comparative studies of the differential impact of cyclins on Cdk function. We carried out a detailed specificity study of the four major classes of cyclin-Cdk1 complexes and outlined the general mechanisms underlying changes in Cdk1 specificity during the cell cycle. We propose that the later appearing cyclins gradually increase the specificity of Cdk1 toward mitotic targets by improving its ability to recognize the optimal Cdk consensus site. On the other hand, at the early stages of the cell cycle, Cdk1 is able to target selected substrates using docking sites and cyclin-specific consensus motifs. This model explains the paradox raised by the in vivo studies referred to above, answering the question of why the early cyclins are incapable of triggering prematurely the later stages of the cycle. Also, quite surprisingly, we found that cyclins may differentially modulate the optimal consensus motif of Cdk1, revealing a previously unappreciated cyclin function. Our results suggest that the robust ordering of cell cycle events depends on gradual changes in cyclin specificity.

RESULTS

The Cyclins Gradually Change the Specificity of Cdk1

To study how different cyclins modulate the substrate specificity of Cdk1, we performed a quantitative analysis of yeast Cdk1 specificity in complex with each of four cyclins: the G1/S cyclin Cln2, the S phase triggering cyclin Clb5, the G2/M cyclin Clb3, and the mitotic cyclin Clb2. The cellular levels of these four cyclins peak in sequence over the course of the cell cycle (Figure 1A). We purified the four cyclin-Cdk1 complexes from yeast (Figure 1D) and analyzed their kinetic properties with a commonly used optimal model substrate for Cdk1, a peptide derived from the general substrate Histone H1 (PKTPKKAKKL). We showed previously that this substrate has about 20-fold higher specificity for the mitotic Clb2-Cdk1 complex as compared with the S phase Clb5-Cdk1 complex (Loog and Morgan, 2005). We extended this work in the current study, finding that each of the four major cyclin-Cdk1 complexes exhibited different specificity toward this substrate (Figures 1B and 1C). Remarkably, these specificity differences were gradual and correlated with the order of appearance of the cyclins in the cell cycle. This difference was mostly manifested in K_M values, while the k_{cat} values showed much smaller variation (Figure 1C).

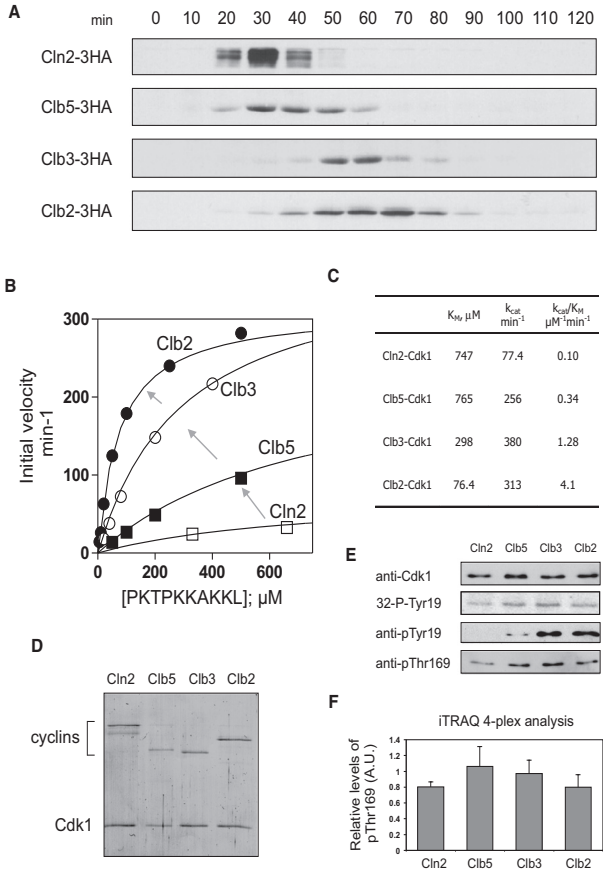
Next, we asked if this gradually changing ability to phosphorylate the substrate could be due to differences in posttranslational modifications of Cdk1. We analyzed two known regulatory phosphorylation sites of Cdk1: the inhibitory site at Y19 and the activating site at T169. First, we performed an active site titration by determining the concentration of unphosphorylated Y19 sites in our enzyme preparations, using an excess of purified Swe1 to radiolabel these sites (Figure 1E). The amount of Y19 labeling was similar for the four cyclin-Cdk1 complexes. Next, we estimated the relative levels of inhibitory phosphorylation already present in the enzyme preparations. Western blotting analysis revealed that pY19 levels increased gradually in the cyclin series, being lowest in the case of Cln2-Cdk1 and highest in the case of Clb2-Cdk1. These data are in agreement with previously reported observations that Cln2-Cdk1 is a poor substrate for Swe1 (Booher et al., 1993), and that Clb5-Cdk1 is less susceptible than Clb2-Cdk1 to inhibition by Swe1 (Hu and Aparicio, 2005; Keaton et al., 2007). However, the different levels of inhibitory phosphorylation at Y19 cannot be responsible for the activity differences observed for individual cyclin-Cdk1 complexes, since these changes occur in the opposite direction. Thus, differences in the western blotting experiment represent a trace fraction of the total enzyme in each case.

Using specific antibodies against the phosphorylation site pT169, we found that activating phosphorylation was present at equal levels in all Cdk1 preparations tested (Figure 1E). To confirm this result, we used a quantitative mass-spectrometry-based iTRAQ four-plex analysis of pT169 levels in the four cyclin-Cdk1 preparations (see the Supplemental Experimental Procedures, available with this article online, for details). This technique indicated that the levels of pT169 were equal for the four kinase complexes (Figure 1F).

Cyclin-Specific Docking Sites Are Able to Compensate for the Gradually Decreasing Specificity of Early Cyclin-Cdk1 Complexes

Our data reveal that cyclins are not just activators of Cdk1 but are also modulators of the catalytic specificity of the kinase active site. This gradual increase in the intrinsic activity toward the optimal substrate motif in the course of the cell cycle could provide an important delay in the accumulation of mitosis-promoting activity, preventing the premature initiation of mitotic processes by Cdk1. On the other hand, the fact that the early cyclin-Cdk1 complexes have very low intrinsic activity raises the question of how these complexes initiate such important events as Start and S phase. In our previous studies, we found that Clb5-Cdk1 can compensate for its low intrinsic activity by using a docking site on its cyclin surface—the hydrophobic patch—that binds selectively to an R/KXL motif in the substrate (Loog and Morgan, 2005). In contrast, the intrinsically potent Clb2-Cdk1 does not seem to utilize extra specificity from this docking site.

We therefore suspected that the weak intrinsic activity of early cyclin-Cdk complexes is compensated by docking sites. However, a hydrophobic patch is not apparent in the primary sequence of Cln1, -2, or -3, and so it is not clear how Cln-Cdk1 complexes are able to efficiently phosphorylate their G1 targets. To address this question, we analyzed Cln-Cdk1 activity toward Sic1, a Cdk1 inhibitor and well-established target of Cln-Cdk1 at the G1/S transition. We constructed a version of Sic1 lacking the inhibitory C-terminal region (Sic1ΔC), and mutated the Cdk consensus motifs (S/TP) to alanines, except for the functionally essential site T33 (Nash et al., 2001). This construct allowed us to analyze the phosphorylation of a single site and quantitatively characterize individual specificity elements of the kinase. Studies of phosphorylation of the T33-Sic1ΔC protein and its derivatives revealed numerous important features of cyclin-Cdk1 specificity (Figures 2A–2E, Table S1). To allow direct comparison with specificity for the H1 control peptide motif, we also analyzed a control protein in which the H1 substrate sequence PKTPKKAKKL replaced the T33 substrate site (T33H1-Sic1ΔC).



	K_M, μM	k_{cat}, min^{-1}	k_{cat}/K_M, $\mu M^{-1}min^{-1}$
Cln2-Cdk1	747	77.4	0.10
Clb5-Cdk1	765	256	0.34
Clb3-Cdk1	298	380	1.28
Clb2-Cdk1	76.4	313	4.1

Figure 1. Intrinsic Specificity of Cyclin-Cdk1 Complexes toward the Histone H1-Based Model Substrate PKTPKKAKKL Increases Gradually in the Series Cln2-Clb5-Clb3-Clb2, Correlating with Cell Cycle Progression

(A) Western blotting analyses of endogeneous cyclin levels in the course of the cell cycle. The time points were taken after the release of cells from α factor-induced G1 arrest. The strains carried the indicated cyclin with a C-terminal 3HA tag.

(B) Michealis-Menten curves of Histone H1 peptide (PKTPKKAKKL) phosphorylation by four different cyclin-Cdk1 complexes. Arrows indicate the change of Cdk1 kinetic properties during cell cycle progression.

(C) Steady-state kinetic parameters determined for four representative cyclin-Cdk1 complexes using Histone H1-based peptide PKTPKKAKKL as a substrate.

(D) Silver-stained SDS gel showing the purified preparations of cyclin-Cdk1 complexes used in this study.

(E) Analyses of the levels of activating phosphorylation at T169 and inhibitory phosphorylation at Y19 of cyclin-Cdk1 preparations. Equal protein amounts of each cyclin-Cdk1 preparation were loaded and blotted using specific antibody for Cdk1 (upper panel). The second panel shows the active site titration of cyclin-Cdk1 preparations using an excess of purified Swe1. Equal protein amounts of cyclin-Cdk1 preparations were phosphorylated with Swe1 until Cdk1 was totally inactivated. The third panel shows the relative levels of pY19 initially present in the preparations, determined using western blotting with specific antibody. The lower panel shows western blotting to determine the relative levels of activating phosphorylation at T169 (see the Supplemental Experimental Procedures for details).

(F) iTRAQ four-plex analysis of the relative levels of activating phosphorylation at T169 in cyclin-Cdk1 preparations. Cyclin/Cdk1 complexes were separated by 10% PAGE, and the Cdk1 band was excised and digested with trypsin. Eluted peptides were labeled with iTRAQ reagents and analyzed by tandem mass-spectrometry (see the Supplemental Experimental Procedures for details). Intensities of four reporter ions at m/z 114, 115, 116, and 117 are presented. Analyses were performed in triplicate, and error bars indicate standard error of the mean.

First, we found that T33 in Sic1 is efficiently phosphorylated by Clb5-Cdk1 and that this specificity depends on the hydrophobic patch of the cyclin. Thus, T33-Sic1ΔC belongs to the Clb5-specific subset of Cdk1 targets that we previously described (Loog and Morgan, 2005). We also found that T33-Sic1ΔC was a relatively specific substrate for Cln2- and Clb3-Cdk1 when compared with the gradual H1 peptide phosphorylation profile (Figures 2A, 2B, and 2E). When the hydrophobic patch was mutated in the B-type cyclins (Figures 2B–2E, black bars) or when the four RXL motifs in Sic1ΔC (in positions 13–15, 89–91, 114–116, and 147–149) were mutated to alanines (T33-Sic1ΔC-*1234rxl*), the rate of phosphorylation by Clb5 and Clb3 decreased considerably, producing a rising specificity pattern for sequential Clb-Cdk1 complexes that is similar to that observed with the model peptide (Figure 2E). Remarkably, this rising specificity is accompanied by declining hydrophobic patch-dependent docking of B-type cyclins. This effect was calculated by dividing the k_{cat}/K_M values for wild-type and hpm versions of the enzyme complexes (Figures 2B and 2C; dark gray bars). As we observed with peptide specificity, Clb3-Cdk1 showed an intermediate effect of the docking interaction as compared to Clb5- and Clb2-Cdk1. These data indicate that the early B-type cyclin-Cdk1 complexes can specifically phosphorylate Sic1 using the RXL-hp docking motif, while still being weak kinases for the optimal consensus site. Thus, a blend of increasing intrinsic activity and decreasing docking-site dependence results in roughly equal rates of T33-Sic1ΔC phosphorylation by all B-type cyclin-Cdk complexes, as expected given that the levels

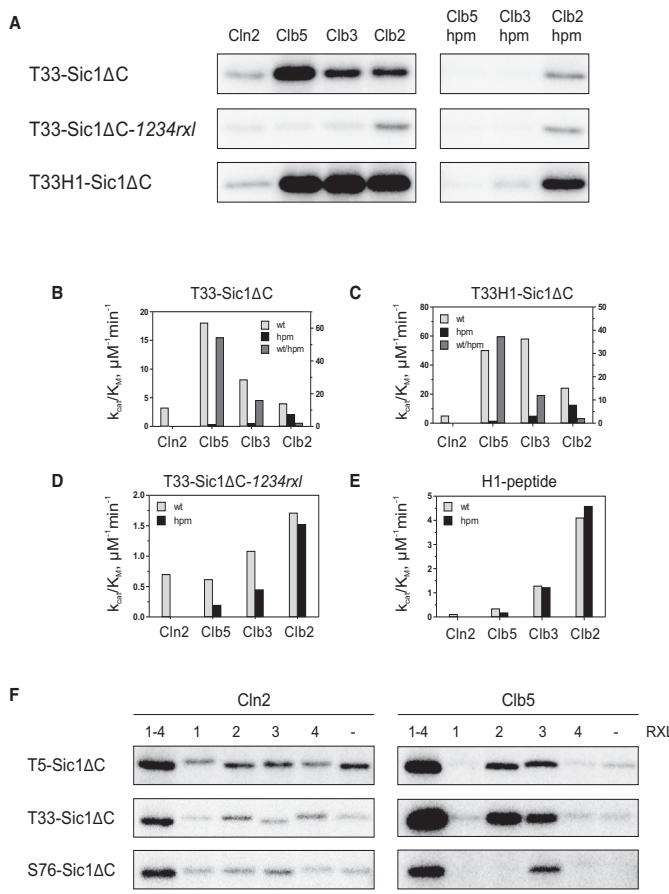


Figure 2. Analysis of Cyclin Specificity with Respect to RXL Docking Sites

(A) Equal amounts of cyclin-Cdk1 complexes were used in a phosphorylation assay with purified T33-Sic1ΔC, T33-Sic1ΔC-*1234rxl*, and T33H1-Sic1ΔC. The first two mutant proteins contained the original sequence of T33, QA^{33}TPQKPSQNL, while the last contained the Histone H1 peptide sequence PK^{33}TPKKAKKL in place of the T33 site of T33-Sic1ΔC.

(B–D) Quantified specificity profiles showing the k_{cat}/K_M values obtained from the experiments shown in (A), using the indicated cyclin-Cdk1 complexes to phosphorylate T33-Sic1ΔC (B), T33H1-Sic1ΔC (C), or T33-Sic1ΔC-*1234rxl* (D). In the case of Clb cyclins, activity was also measured with hydrophobic patch-mutated cyclins (hpm, black bars). The dark gray bars in (B) and (C) denote the relative hp-dependent docking effect, and these values are bound to the right-hand scale of the Y axes.

(E) The k_{cat}/K_M profiles for the control substrate, the H1-based peptide PKTPKKAKKL.

(F) The impact of different RXL motif-bearing docking sites on the phosphorylation specificity of T5, T33, and S76 was studied with Cln2-Cdk1 and Clb5-Cdk1, using substrate constructs with a single RXL motif left unmutated: Sic1ΔC, Sic1ΔC-*234rxl*, Sic1ΔC-*134rxl*, Sic1ΔC-*124rxl*, Sic1ΔC-*123rxl*, and Sic1ΔC-*1234rxl*.

of Sic1 must be kept to a minimum from G1/S to mitotic exit. In contrast, for mitotic targets, whose specificity profile is exemplified by the model peptide (Figure 2E), the gradually increasing Cdk1 specificity peaks in mitosis, creating temporal separation of the phosphorylation relative to T33-Sic1ΔC-like targets.

In the case of Cln2-Cdk1, which does not seem to possess a conventional hydrophobic patch, mutation of the docking motifs in the Sic1 substrate caused about a 3- to 4-fold decrease in activity (compared with a 50-fold effect in the case of Clb5; Figures 2B and 2D, and Table S1).

To further understand the importance of the docking mechanism, we studied the impact of different potential RXL motifs on the phosphorylation of sites T5, T33, and S76 in Sic1. We used T5-Sic1ΔC, T33-Sic1ΔC, and S76-Sic1ΔC constructs with all the RXL motifs mutated to alanines, or with one motif left unmutated (Figure 2F). The results indicated that for Clb5 there are different designated RXL motifs for each phosphorylation site. RXL2 and to a lesser extent RXL3 enhance the phosphorylation of T5 and T33, while S76 is connected exclusively to RXL3. Cln2-Cdk1, in contrast, profits weakly but almost equally from each of the docking sites. This assumption is supported by the fact that a similar exclusive designation of the RXL docking site for Clb5-specific S phase targets has been shown previously for Fin1 (Loog and Morgan, 2005) and Orc6 (Archambault et al., 2005). The potential importance of the distance between the RXL docking site and the active site of the Cdk has been addressed previously in structural studies with cyclin A-Cdk2 (Cheng et al., 2006).

Since the RXL motifs in T33-Sic1ΔC, when compared with the H1 peptide specificity profile, were only partly responsible for the relative Cln2 specificity, we searched for additional potential Cln2-specific docking sites by truncating the Sic1 molecule from its C terminus. We identified a 10 amino acid stretch that considerably enhanced the rate of Cln2-dependent phosphorylation, but not that of the Clbs (Figures 3A and 3B). The stretch contained a row of hydrophobic residues, and mutating the first five of them, VLLPP, to alanines (Sic1ΔC-*vllpp*) caused a considerable loss of the Cln2-dependent phosphorylation rate (Figure 3C). A synthetic peptide based on the 10 amino acid stretch was a competitive inhibitor of the docking, confirming that the effect of the deletion mutation was likely not due to a conformational anomaly in the mutant but a docking interaction between Cln2 and the substrate (Figure 3C). The specificity of the interaction was further confirmed by the observation that both the *vllpp* mutation and the peptide had an effect on Cln2, but not on Clb5. Cln1, which is closely related to Cln2, showed a similar LP peptide effect (data not shown). In contrast to the RXL docking sites used by Clb5, the LP interaction enhanced phosphorylation at all three sites in Sic1 almost equally (Figure 3D), suggesting that this is not a strictly distance-dependent docking but rather a general enhancement of the interaction between the substrate and the cyclin.

To locate the potential hydrophobic docking pocket in Cln2, we introduced *hpm*-like triple mutations into several candidate sites bearing some resemblance to the *hp* of B-type cyclins, as well as into the predicted *hp* itself. None of these mutations changed the specificity profile (data not shown), suggesting that there are several hydrophobic regions on the surface of Cln2 that contribute to the interaction. We also considered the possibility that the mild effect on Cln2 specificity that we observed with mutations in the RXL motifs (Figure 2F) was due to the removal of hydrophobic leucine residues. To test this, we compared the effects on Cln2 activity of a synthetic peptide based on the RXL2 motif of Sic1 (RVNRILFPT) with a similar peptide in which the arginine of the RXL motif was replaced with alanine (''AXL peptide''). Both peptides caused a 3- to 4-fold effect on Cln2-dependent phosphorylation of Sic1ΔC, whereas only the peptide with the intact RXL motif had an effect on Clb5-dependent phosphorylation (Figure 3E). A summary of docking interactions for Cln2-Cdk1 and Clb5-Cdk1 is presented in Figure 3F.

Cyclins Modulate the Consensus Site Specificity Profile of Cdk1, Creating Distinct Optimal Profiles for Cln2- and Clb2-Cdk1

Next, we asked if, in addition to the observed docking interaction, some other mechanism might further enhance the specificity of Cln2. We noticed that many known physiological Cdk target sites, and also the H1 peptide, contain multiple P and K residues (e.g., PKTPKKAKKL). We wondered if these residues, while being important constituents of the Cdk1 consensus motif S/T-P-X-R/K, might also have a role in recognition and specificity when present in other nearby positions. To address the importance of proline and lysine residues in Cln2 specificity, we introduced these residues at different positions around T33 in the T33-Sic1ΔC construct. This system provides an advantage over traditional random peptide library techniques used for specificity studies of kinases (Mok et al., 2010), as it enables the study of docking effects and phosphorylation site primary structure requirements simultaneously in the context of a physiological protein target.

First, we analyzed the effects of adding lysine in different positions within the sequence motif QA^{33}TPQAPSQ in the context of Sic1ΔC (Figure 4A), using Cln2-Cdk1 or Clb2-Cdk1. We found that Clb2 had a strong requirement for the lysine at position +3, the conventional position belonging to the consensus motif, while Cln2 exhibited specificity for lysine both at position +2 and +3. Importantly, the Lys+2 was exclusively specific for Cln2 over Clb2 and the two other B-type cyclins (Table S1). Variation of proline in the same manner, within a template sequence motif QA^{33}TPQAASQ, revealed a positive element for Cln2 and Clb2: a proline at position −2 (Figure 4B).

Data from comparative analyses (Table S1) suggest that in the case of B-type cyclins lacking the hydrophobic patch, the overall substrate phosphorylation efficiency toward the T-P motif changes gradually as described above for the model peptide, and exhibits little variation among Clb5, Clb3, and Clb2. Clb specificity relative to Cln2 can be attained by simultaneously introducing multiple basic residues on the right-hand side of the T-P motif as well as a proline in position −2. Substrates containing a combination of these specificity elements follow the pattern of gradually rising mitotic specificity described in Figure 1, with the specificity for Cln2-Cdk1 being the lowest and for Clb2-Cdk1 the highest (e.g., H1 motif, PKTPQKKKK, PKTPKK; Table S1). On the other hand, the specificity of Cln2-Cdk1 relative to B-type cyclins can be enhanced by introducing lysine at position +2 and avoiding it at position +3. Figure 4C provides examples of the sharp differences that can be obtained in Cln2 versus Clb5 specificity. The *1234rxl* mutation or the LP competitor peptide was used to assess the docking site-independent fraction of the specificity of Cln2- or Clb5-Cdk1. This abrupt change of specificity may be used in switches after the G1/S transition, when Cln2 is degraded and the system needs to be adjusted from G1 to S phase mode.

To demonstrate that the specificity profiles we determined above are also reflected in phosphorylation of physiologically important Cdk1 sites, we determined the differential specificity

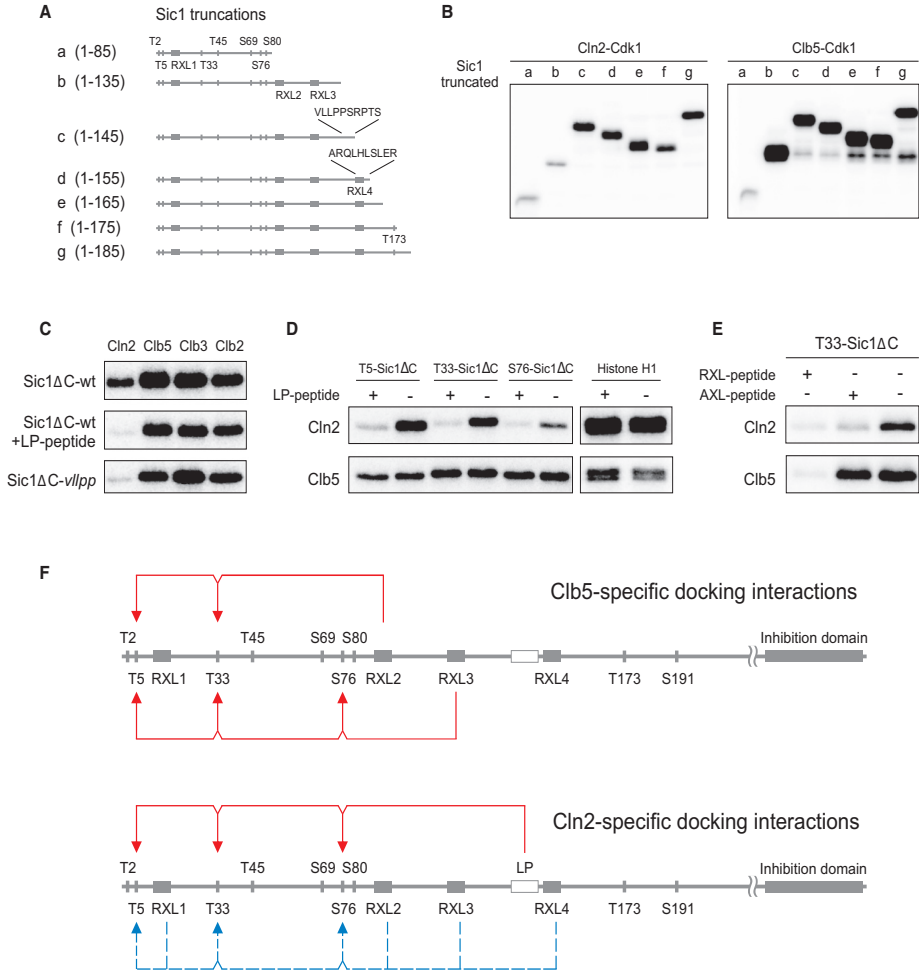


Figure 3. A Hydrophobic Stretch in Sic1 Enhances Cln2-Specific Phosphorylation Relative to Clb-Cdk1

(A and B) A 10 amino acid stretch that enhances the Cln2 specificity was located by systematic truncation of Sic1.

(C) A five alanine mutation in the docking site of Sic1 (*vllpp* mutation) reduced the phosphorylation specificity for Cln2-Cdk1 but not for Clb5-Cdk1. Similar results were obtained using a competitor peptide based on the 10 amino acid stretch (the LP peptide, VLLPPSRPTS) between residues 136 and 145 as indicated in (A).

(D) The LP peptide is equally effective in the inhibition of Cln2 specificity for sites T5, T33, and S76, as demonstrated with Sic1ΔC-based constructs described in Figure 2F.

(E) The rate of Cln2-dependent phosphorylation of Sic1Δ can be reduced by addition of either RXL- or AXL-containing competitor peptides (based on the docking sequence of RVNRILFPT of Sic1: RVNRILFPT and RVNAIAFPT), whereas only the RXL peptide reduces the specificity of Clb5.

(F) A scheme showing the observed docking interactions between Sic1 and Clb5 or Cln2. The dashed arrows indicate the mild cooperative effect of the leucines of the RXL motifs on Cln2 specificity.

of Cln2-Cdk1 and Clb5-Cdk1 for eight sites of Sic1 (Figure 4D). Indeed, the four most Cln2-specific sites (T5, T45, T173, and S191) contained Cln2-specific motifs.

Cyclin Specificity Determinants Are Important In Vivo

To test the validity of the gradual cyclin specificity model in vivo, we set up a system to follow the cyclin-specific phosphorylation

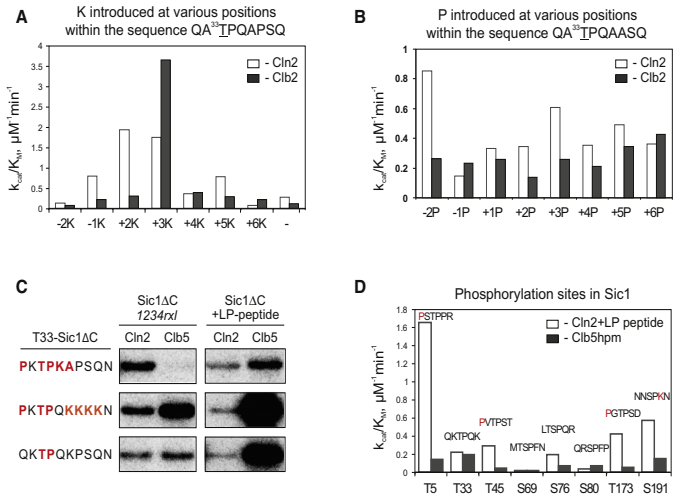


Figure 4. Cyclins Differentially Modulate the Phosphorylation Site Consensus Sequence of Cdk1

(A) Differential effect of lysines on the specificity profiles of Cln2-Cdk1 (open bars) or Clb2-Cdk1 (dark bars), using T33-Sic1ΔC variants with lysine introduced at various positions within the sequence QA^{33}TPQAPSQ.

(B) Differential effect of proline on the specificity profiles of Cln2-Cdk1 (open bars) or Clb2-Cdk1 (dark bars), using T33-Sic1ΔC variants with proline introduced at various positions within the sequence QA^{33}TPQAASQ.

(C) Examples of Cln2 versus Clb5 cyclin specificity, using different T33-Sic1ΔC and T33-Sic1ΔC-*1234rxl* variants bearing the motifs with highest differential specificity. The impact of LP motif-dependent specificity of Cln2 was determined with the LP competitor peptide. Red letters indicate the amino acid substitutions into different positions of the T33 site.

(D) Cln2 versus Clb5 phosphorylation consensus site specificity profiles showing the docking-site-independent k_{cat}/K_M values for eight different physiological phosphorylation sites of Sic1. Sic1ΔC constructs in which only the indicated single phosphorylation site is left unmutated were used in the phosphorylation assays. Cln2-Cdk1 combined with the LP competitor peptide and Clb5hpm-Cdk1 were used to eliminate the impact of the docking sites.

of nondestructible versions of Sic1ΔC in cells released from a G1 arrest. Cells expressing one of two different Sic1 mutants were studied. Either the consensus motif P-X-T-P-X-K, specific for both Cln2 and Clbs, or the Cln2-specific motif P-X-T-P-K-A was introduced at all four of the Cdk sites T5, T33, T45, and S76. The rest of the Cdk1 sites were mutated to alanines, and site T2 was left unchanged. We examined the dynamics of phosphorylation of these proteins in vivo by measuring phosphorylation-dependent mobility shifts on Phos-Tag SDS-PAGE gels (Figure S1A). We also confirmed that the two constructs had the predicted cyclin specificity in vitro (Figure S1D). Using a Sic1ΔC construct in which all Cdk sites were mutated (Sic1ΔC-9A), and also by specific chemical inhibition of Cdk1, we confirmed that increasing phosphorylation of Sic1 as cells entered the cell cycle was caused by Cdk1 activity (Figures S1A–S1C). The non-Cdk1-dependent fraction of the shifts did not change over the cell cycle (Figure S1C), and the kinase(s) responsible for these shifts remains to be identified. Several kinases other than Cdk1 (Pho85, Hog1, and CK2) may phosphorylate Sic1 (Coccetti et al., 2006; Escote et al., 2004; Nishizawa et al., 1998).

Profiles of total Sic1 phosphorylation in vivo, plotted in Figures 5A and 5B, revealed that the phosphorylation status of the construct bearing the P-X-T-P-X-K motifs reached half-maximal levels at early time points, compared to the construct with the same consensus motifs but containing the *1234rxl/vllpp* mutations in the docking sites. The latter construct showed a delayed accumulation of phosphorylated forms with a half-maximum at 40–50 min, corresponding roughly to the onset of mitosis. This result is in agreement with the kinetic data presented earlier. The docking sites, however, compensated for the low activity in the early cycle. As shown in our earlier results in vitro, the relative effect of the docking sites decreased at the late time points, indicating that Clb2 gains relatively less from the docking sites than the earlier complexes. However, since the cyclin peaks exhibit considerable overlap (Figure 1A), the changing effects of the docking sites in the time course experiments are not as sharp as the differences in kinetic constants of individual cyclin-Cdk1 complexes.

The construct bearing the Cln2-specific motif P-X-T-P-K-A showed a different profile in which an early maximal activity was followed by a slight decline as the cells progressed toward mitosis. Analogously to the previous case, the *1234rxl/vllpp* mutations lowered the phosphorylation equilibrium, and the relative effect of these mutations decreased with time. These experiments outline the physiological relevance of the major elements of the gradual cyclin specificity model in vivo.

Further demonstrating the physiological importance of substrate docking sites, we found that overexpression of full-length Sic1-*23rxl* or Sic1-*1234rxl* severely reduced the viability of yeast cells, while the overexpression of Sic1-wt had little effect (Figure 5C). In contrast, the *vllpp* mutation in Sic1 did not affect the growth of cells. However, Cln2-Cdk1 and Clb5-Cdk1 still seem to cooperate in the phosphorylation of Sic1, as the *vllpp* mutation was found to affect the growth of *cln2Δ* cells (Figure 5D), probably because the reduced Cdk1 activity in these cells makes them more sensitive to decreased Cln-dependent Sic1 phosphorylation.

To further confirm that the change in Sic1 phosphorylation specificity is accompanied by a change of Sic1 function in vivo, we demonstrated that expression of a version of Sic1 with mutated cyclin docking sites resulted in delayed Sic1 degradation and entry into S phase (Figures 5E and 5F). Furthermore, overexpression of a Sic1 mutant with mutated docking sites caused DNA rereplication (Figure 5G), suggesting that the docking-site-enhanced Cdk1 substrate specificity toward the Cdk1 inhibitor Sic1 has been, at least partially, evolved to ensure the irreversibility of G1/S transition by promoting potent and continuous suppression of Sic1. We propose that this irreversibility is important throughout S phase, as any erroneous bursts of late Sic1 expression would inhibit Clb-Cdk1 complexes and allow origin relicensing and rereplication.

A Screen for Cyclin-Specific Cdk1 Targets Reveals Proteins with Different Specificity for Each of Four Representative Cyclin-Cdk1 Complexes

Next, we explored how cyclin-dependent changes in Cdk1 specificity are reflected in other physiological targets of Cdk1. We developed methods for a quantitative Cdk1 substrate screen, with a special emphasis on searching for Cln2- and Clb2-specific physiological targets. Interestingly, when examining the reported targets of Cln-Cdk1, we noted that they are all multiphosphorylated proteins. We therefore analyzed Cln2-Cdk1 activity with several candidate multisite Cdk1 targets that were chosen with an emphasis on their potential functional connection to Cln2 (Horak et al., 2002; Sundin et al., 2004), and on the condition that they contain at least five Cdk sites. We included a number of known Cln2 and Clb targets as well as several uncharacterized ORFs with at least five Cdk sites. Potential targets were expressed and purified from bacterial expression systems, and those with reasonable yields were submitted to specificity analyses with the four cyclin-Cdk1 complexes. Relative k_{cat}/K_M values obtained from the phosphorylation experiments (Table S2), with examples presented in Figure 6, revealed several types of cyclin specificity profile. We grouped targets into four types.

Type I substrates are proteins with high Cln2 specificity or Cln2 and Clb(3)2 specificity (Figure 6A). Among these targets was Whi5, the repressor of the G1-specific SBF transcription factor and the analog of mammalian pRB in budding yeast. The screen revealed a number of other Cln2-specific targets involved in G1-specific transcriptional control, including Xbp1, Xhp1, and Tos8 (Figure 6A and Table S2). Strikingly, we demonstrated that the Cln2 specificity for several targets was largely dependent on the LP docking site, as the presence of the LP competitor peptide reduced the phosphorylation signal for Cln2 but not for Clbs. These results suggest that the LP docking interaction may have broad significance and can be used by many Cln-Cdk1 targets. The amino acid sequences of these proteins contain several hydrophobic regions whose sequence is reminiscent of the LP site in Sic1.

The second substrate type was defined as Clb5-specific targets whose specificity depends on hydrophobic patch docking as described by us previously (Loog and Morgan, 2005). These proteins have specificity for Clb5, but they may also be specific for Clb3, exhibiting an intermediate level of hp dependency as described above in Figure 2A for T33-Sic1ΔC. This group includes the spindle-stabilizing protein Fin1, which must be fully phosphorylated early in the cell cycle to prevent it from binding to the spindle (Woodbury and Morgan, 2007). In addition, we found one target (the Cdk1 inhibitor Far1, Table S2) that showed specificity for Cln2 and Clb5, but not for Clb3 and Clb2.

The third type of Cdk target was defined as those showing hp-dependent specificity for Clb3 and no specificity for Clb5 and/or Clb2 (Figure 6C). This was a surprising finding, as there are no previous reports of Clb3-Cdk1-specific substrates or functions. This group included the putative transcription factor Tos4, the transcription factor Ash1, and a protein of unknown function, YPR174C. Among these targets was the replication factor and pre-RC component Cdc6, which shared the characteristics of type II and III substrates and showed a sharp specificity difference between Clb3 and Clb2. We found that this specificity was due to the efficient inhibitory interaction of Cdc6 with Clb2-Cdk1, but not with Clb5- and Clb3-Cdk1 (our unpublished data).

The fourth type of Cdk target was defined as "Clb2-specific" or mitotic substrates. These proteins followed the characteristic gradual cyclin specificity pattern outlined in the model peptide studies above. One example of this type was Ndd1, a component of the transcription factor complex controlling the expression of G2/M-specific genes, including *CLB2* itself (Darieva et al., 2006). Ndd1 is known to be phosphorylated and thereby activated by the polo kinase Cdc5, which uses phosphorylated Cdk sites as docking elements (Asano et al., 2005; Snead et al., 2007). We predict that Clb2-specific phosphorylation might accelerate the Cdc5-dependent secondary phosphorylation, thus creating positive feedback in G2-specific gene transcription. Additionally, the important cell cycle-related transcription factor Swi6 belongs to the type IV category.

Since the screen was performed on multisite Cdk targets, we wondered if the specificity profiles might reflect the consensus site motifs of the phosphorylation sites in these proteins. We found that the targets showing the strongest gradual mitotic pattern (hp-independent) (Ndd1, Cdc6, Orc2, Plm2, Swi6, and Fir1) all contained one to two sites with the H1-like mitotic consensus sequence P-X-S/T-P-X-$[K/R]_{n>1}$, which contains more than one K/R residue within the six C-terminal positions from the phosphorylation site. On the other hand, in the Cln2-specific targets, the positive determinants +2K/R, −2P, and the prolines in other C-terminal positions were found to be frequent. For example, one of the most potent Cln2-specific targets, Stb1, contains four strong Cln2-specific sites bearing the motifs P-X-S/T-P-K/R-X, P-X-S/T-P-X-X, P-X-S/T-P-P-X, or X-X-S/T-P-K/R-X, but no sites with the mitotic motif P-X-S/T-P-$[K/R]_{n>1}$. It is

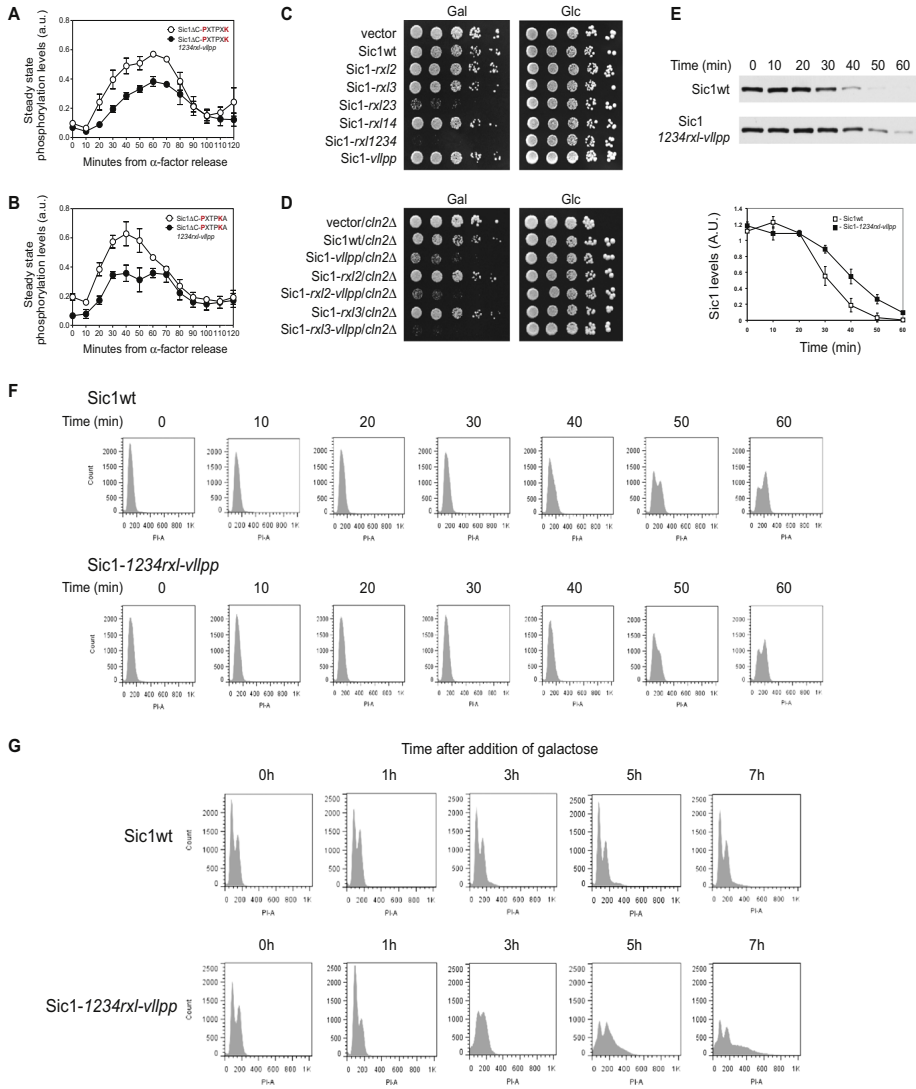


Figure 5. Analysis of Changes in Cdk1 Substrate Specificity during the Cell Cycle and of the Physiological Importance In Vivo of Different Substrate Docking Sites

(A and B) Cells constitutively expressing the indicated Sic1ΔC-3HA construct were released from α factor arrest, and at the indicated time points Phos-Tag SDS-PAGE and western blotting were used to assess phosphorylation state (see Figure S1). The constructs contained either the Clb2-specific P-X-T-P-X-K motif (A) or the Cln2-specific P-X-T-P-K-X motif (B) at positions T5, T33, T45, and T76. Other Cdk1 sites were mutated to alanines, and T2 was left unchanged. As controls, we also tested the same constructs with mutations of the LP and the RXL docking sites. Sic1 bands on the western blot were scanned using the GelDoc (GE), and each phosphorylated species was quantified. The relative steady-state phosphorylation at each time point was calculated as a ratio of pS/S_T, where S_T is the total signal of Sic1ΔC-3HA and pS is the total signal of phosphorylated bands multiplied by the number of phosphates each band contains. See also Figure S1. Analyses were performed in duplicates, and error bars indicate standard error of the mean.

important to remember, however, that it is difficult to extrapolate our studies of consensus motifs to multisite substrates, because different phosphorylation sites may not be similarly accessible. In such cases, a few accessible sites may largely determine the net specificity despite the presence of other motifs in the primary sequence of the protein.

DISCUSSION

In this study, we sought a quantitative understanding of dynamic changes in Cdk1 specificity over the budding yeast cell cycle. We found that a gradual change of specificity is an intrinsic feature of the cyclin-Cdk1 system, and it seems to have evolved to prevent Cdk1 from prematurely triggering mitosis using the built-in delay mechanisms created by the weaker activity of the earlier cyclin-Cdk1 forms toward Clb2-specific mitotic targets. This weak activity, however, does not prevent the phosphorylation of G1 and S phase Cdk substrates, whose targeting is accomplished by RXL-hydrophobic patch interactions (Clb5, Clb3), by a hydrophobic LP docking site (Cln2), or by different consensus sites (Cln2) (Figure 7A). Thus, as a general conclusion, we can state that Cdk1 specificity is periodically changing in the course of the cell cycle.

Throughout the eukaryotes, mitotic Cdk1 activity is regulated in part by inhibitory phosphorylation by the tyrosine kinase Wee1. The homolog of Wee1 in budding yeast, Swe1, has been shown to exhibit a differential ability to phosphorylate and inactivate different cyclin-Cdk1 complexes, with the highest inhibitory potency toward Clb2 and gradually lower for the earlier cyclin-Cdks (Hu and Aparicio, 2005; Keaton et al., 2007). We determined that the relative differences in Swe1 specificity are about the same order of magnitude as the gradual change in the intrinsic specificities of Cdk1 (Figure S2). These data reveal another remarkable gradual phenomenon in the cyclin-Cdk1 system in yeast. The identity of the cyclins in complex with Cdk1 is sensed by the Swe1 kinase, which then applies inhibitory pressure in proportion to the intrinsic specificity of the complex. In fact, the same structural elements may control the accessibility of the active site of different cyclin-Cdk1s for substrate and for the kinase domain of Swe1.

The cyclin specificity model is an alternative to the quantitative model of the cyclin response, according to which different levels of accumulating Cdk1 activity trigger different cell cycle events (Coudreuse and Nurse, 2010; Stern and Nurse, 1996). The major weakness of a system behaving solely according to the quantitative model is that the temporal resolution of events depends entirely on the use of substrates with wide differences in specificities toward cyclin-Cdk1. According to this model, early events of the cell cycle would be switched on by optimal substrates that are extensively phosphorylated at low Cdk1 activity levels, while later events must be triggered by suboptimal substrates that are phosphorylated only at high kinase levels. For this model to work, the differences between the cyclin levels triggering S phase and M phase must be very large, with S phase triggered by a small fraction of the mitotic Cdk1 activity levels. This would apparently make S phase very vulnerable to even mild deviations and fluctuations of the cyclin signal, which could lead to premature initiation of later events.

While the quantitative model is apparently not sufficient to describe the function of cyclins, it also appears that the other extreme, according to which docking mechanisms are used throughout the cycle, is not correct either. Instead, each cyclin-Cdk1 complex in the sequence has improved intrinsic specificity culminating with the mitotic complex, which relies almost entirely on the intrinsic consensus-site specificity and minimally on docking sites. Thus, Cdk1 broadens its specificity gradually for wider and wider fractions of the proteome.

With several hundred Cdk1 targets in the cell, most of which contain multiple Cdk1 consensus sites, the total substrate concentration for Cdk1 could be hundreds of micromolar or even low millimolar. This target pool is unphosphorylated in early G1 phase, and because multiple targets of an enzyme act as competitive inhibitors relative to one another, the higher K_M values prevent early cyclin-Cdk1 complexes from being inhibited by the pool of unphosphorylated targets, as illustrated in Figure 7B. The highlighted inhibition term ($1 + S_{TOT}/K_{M,TOT}$) in the modified Michaelis-Menten equation raises the apparent K_M for any given substrate and thereby decreases its phosphorylation rate. The panel on the left side of Figure 7B schematically describes a simplified system following the cyclin specificity model, with three cyclins synthesized in sequence. As the concentration of the bulk unphosphorylated Cdk1 substrate pool decreases in correlation with the $K_{M,TOT}$ values (phosphorylated residues have considerably lower affinity for the active site), the inhibition term is kept at a constant low level, allowing each complex to perform its specific function using the cyclin-specific docking sites, while being unhindered by the bulk substrate pool, at the point of the cycle where it is assigned. The panel on the right side of Figure 7B shows the system behaving according to the quantitative model, based on

(C and D) The importance of Clb5-specific RXL motifs (C) and the Cln2-specific LP motif (D) in the degradation of Sic1 was tested by overexpressing different Sic1 constructs under the *GAL1* promoter. To assess the effect of the LP docking site, the strain used in (D) was sensitized by reducing Cln-Cdk1 activity in the cell by deleting Cln2.

(E) Western blotting analysis of Sic1 levels after the release of cells from a G1 arrest. Strains carrying CEN vectors with *GAL-SIC1* or *GAL-SIC1-1234rxl -vllpp* were arrested in G1 with α factor for 2.5 hr. *SIC1* expression was induced in the arrested cells by addition of galactose for 45 min. Both galactose and α factor were removed and the cells were released into glucose-containing medium. Sic1-3HA levels were analyzed at different time points using western blotting. In the lower panel the combined quantified Sic1 profiles from two independent experiments are presented. The error bars indicate standard error of the mean.

(F) To demonstrate how altered Cdk1 specificity toward Sic1 affects the timing of DNA replication, we performed flow cytometry of DNA content for cells taken at different time points of the α factor release experiment presented in (E).

(G) To explore further how altered Cdk1 specificity toward Sic1 affects the control of DNA replication, we performed flow cytometry of DNA content in asynchronous cells expressing the Sic1 mutant lacking cyclin docking motifs. Strains carrying CEN vectors with *GAL-SIC1* or *GAL-SIC1-1234rxl-vllpp* were grown to log phase, and the expression of Sic1 was initiated by addition of galactose. Cells expressing Sic1 with mutations in the docking sites caused DNA rereplication (DNA > 2N). Cells expressing wild-type Sic1 did not exhibit any signs of rereplication.

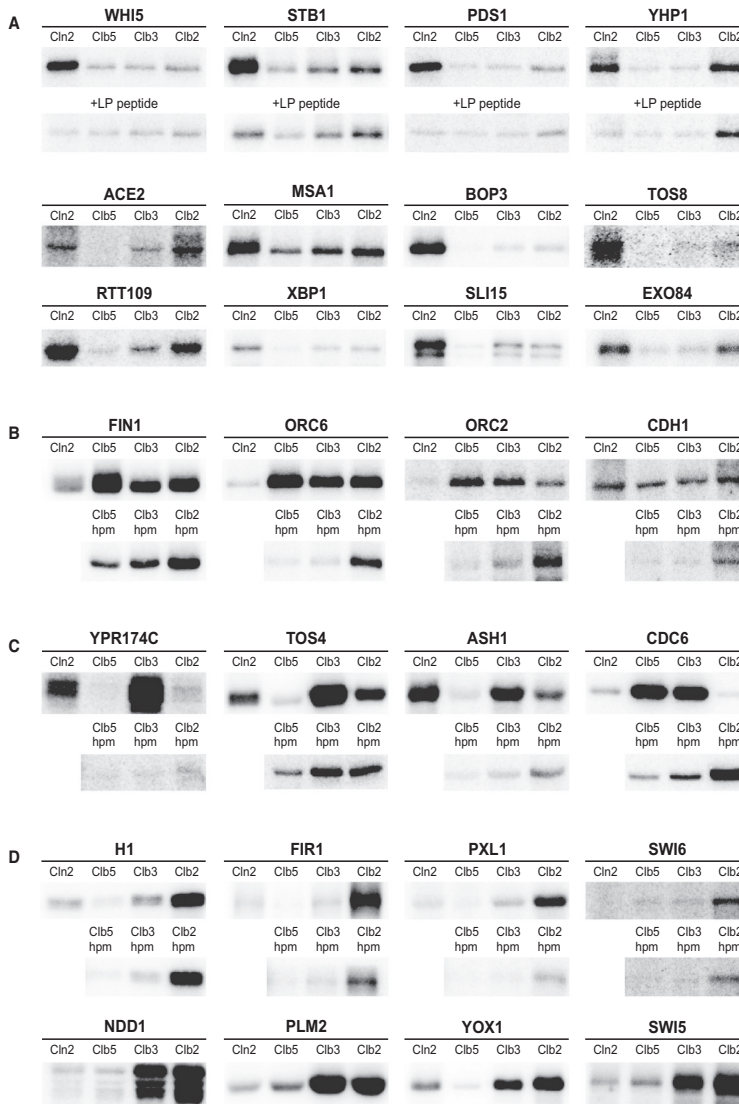


Figure 6. Phosphorylation Screen of Cyclin-Specific Cdk1 Targets

Purified protein substrates were tested in a standard kinase assay using four representative complexes of cyclin-Cdk1. The apparent k_{cat}/K_M values are provided in Table S2.

(A) Type I substrates specific for Cln2-Cdk1. Lower panels indicate activity in the presence of the competitor peptide based on the LP docking site of Sic1.

(B) Type II substrates specific for Clb5- and Clb3-Cdk1. Lower panels indicate activity with the hpm versions of each Clb.

(C) Type III substrates showing hp-dependent specificity for Clb3 and no specificity for Clb5 and/or Clb2.

(D) Gradual specificity profile of the "mitotic targets" (Type IV substrates) presented together with selected examples of Clb-hpm-Cdk1 profiles. See also Table S2.

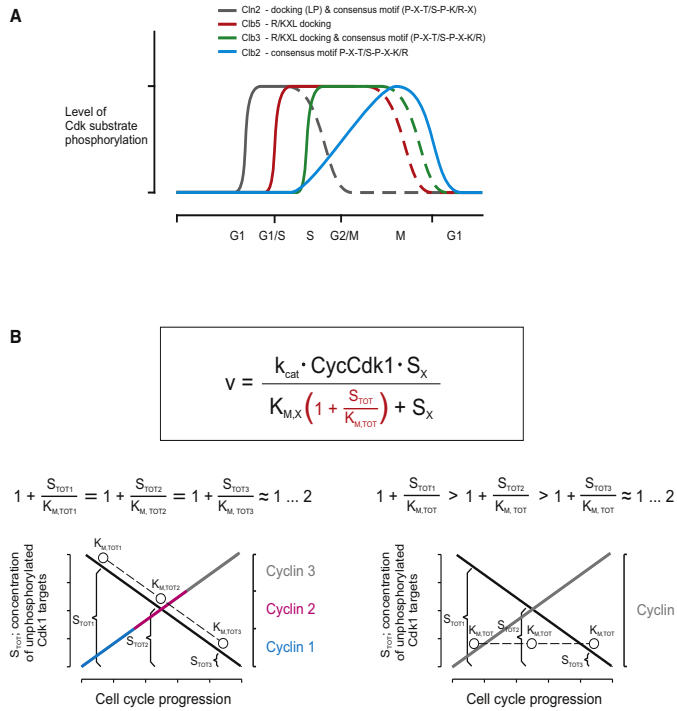


Figure 7. Dynamics of Cdk1 Substrate Specificity during the Cell Cycle

(A) A schematic view of the dynamic changes of Cdk1 specificity during the cell cycle.

(B) Modified Michaelis-Menten formula with the highlighted inhibitory term describing how the Cdk1 substrate pool (S_{TOT}) can act as a competitive inhibitor relative to any particular substrate (S_X). The lower panel on the left side presents a schematic view of a hypothetical system based on sequential accumulation of three cyclins with gradually changing specificity, and the panel on the right side presents a similar system with a single mitotic cyclin (rising linear lines). The gradual phosphorylation and decrease of the unphosphorylated Cdk1 substrate pool during the progression of the cell cycle is depicted as the declining black line. In the three-cyclin system, the inhibitory terms ($1 + S_{TOT}/K_{M,TOT}$) are kept relatively low and equal throughout the course of the cyclin synthesis, because as the substrate pool gets gradually phosphorylated S_{TOT} for each cyclin decreases in correlation with $K_{M,TOT}$. In the system with a single cyclin, the inhibitory term is high in the early stages of the cell cycle, potentially delaying timely phosphorylation of premitotic targets.

accumulation of a single cyclin similar to mitotic cyclin 3 with respect to specificity. In such a system, a full phosphorylation rate of any target is achieved only after substantial accumulation of cyclin when a large part of the total Cdk substrate pool is phosphorylated and the inhibition term is minimal.

We speculate that cyclin specificity evolved as follows. Early eukaryotic cell cycle control depended on a single cyclin system, in which S phase was switched on by low levels of kinase activity, using substrates bearing the optimal consensus motifs (S/TPXK/R), after which higher kinase levels triggered M phase by phosphorylation of suboptimal motifs (S/TP). Due to a relatively low complexity of regulation, there were few substrates and little substrate competition. Eventually, evolution of cyclin docking sites (LP and/or RXL) provided additional affinity for S phase targets carrying cyclin docking motifs in addition to the optimal consensus phosphorylation site. At this stage, more regulatory complexity also evolved, requiring more substrate sites and therefore more competition and mutual inhibition by substrates in S phase. This competition limited the possibilities for further complexity, until multiple cyclins appeared with weaker earlier complexes to reduce the competition at the active site level. As earlier cyclins became weaker activators of Cdk1 (higher K_M), early targets with docking motifs, like Sld2 and Sic1, would be more rapidly phosphorylated as competition from optimal substrates decreased. Thus, as a result of cyclin docking interactions, suboptimal intrinsic specificity became an advantage, allowing greater complexity in Cdk-triggered processes and control systems. Such a system can survive (with some difficulty) using a single mitotic cyclin, which will hit the S phase targets (containing S/TPXK/R motifs)

earlier than the later targets (containing S/TP motifs), because the former ones have lower K_Ms and are able to outcompete the latter ones.

The most surprising outcome of our work is that both Clb- and Cln2-Cdk1 possess some strikingly different elements in their phosphorylation site consensus sequence. While the general activity of a protein kinase has been shown to be regulated in a wide variety of cases, the modulation of the primary structure specificity profile by a regulatory subunit has to our knowledge not been reported. Additionally, Cln2 specificity was found to be strongly enhanced by a previously unreported docking interaction involving a hydrophobic stretch in Sic1. While the precise structural motifs of this docking interaction are yet to be established, we speculate that a hydrophobic pocket on the cyclin could serve as a docking site for this motif.

The concept of dynamically changing Cdk1 specificity could be used to explain previously reported phenotypes obtained by genetic manipulations of cyclin genes in yeast. For example, it may explain why yeast cells lacking both Clb5 and Clb6 experience only an S phase delay (Schwob and Nasmyth, 1993), while double mutants of Clb2 and Clb3 or Clb2 and Clb1 are inviable and arrest prior to mitosis (Mendenhall and Hodge, 1998). In the first case, after a delay, the intermediate Clb3-Cdk1 and the intrinsically most potent Clb2-Cdk1 can phosphorylate the S phase targets at a reasonable rate. In the two last-mentioned cases, however, the poor ability of early B-type cyclins to promote the phosphorylation of Clb2-specific targets apparently makes the cells incapable of initiating mitosis. Perhaps for the same reason, partly stabilized Clb5 is unable to block mitotic exit (Wasch and Cross, 2002), when compared with a strain overexpressing stabilized Clb2 (Surana et al., 1993). Interestingly, our cyclin specificity model also fits well with the observations that fully stabilized Clb5 is still capable of mitotic exit but incapable of S phase initiation, which we propose is due to constant Clb5-specific phosphorylation of S phase targets of the preRC, preventing origin licensing (Sullivan et al., 2008). We also propose that blocking Clb2 activity toward Clb5 targets may be important at certain stages of mitosis (e.g., for the dephosphorylation switch of "Fin1-like" targets [Woodbury and Morgan, 2007]) and could be accomplished, again, by the inability of Clb2 to use the RXL-hp docking mechanism.

It will be important to determine if higher eukaryotes possess a similar dynamic specificity scheme. The general conservation of the gradual model remains to be shown, but our studies of budding yeast shed light on somewhat puzzling and unexpected cyclin knockout studies in mice: if a cyclin is deleted, the process it was meant to trigger is delayed until the activated Cdk, through the synthesis of other accumulating cyclins, reaches the levels where the net value of $(k_{cat}/K_M)^*[E]$ corresponds to the threshold of the trigger. If one deletes the weaker early cyclins, compensation by the later, stronger ones is more likely than the opposite situation, as mice lacking cyclins E and D have been shown to be viable, while cyclins B1 and A2, for example, are the most nonredundant of all cyclins and are required for embryo viability (Satyanarayana and Kaldis, 2009).

In conclusion, we have shown that in the course of the cell cycle, different cyclins gradually change the substrate specificity of Cdk1 at the active-site level. This modulation of specificity, when combined with docking site interactions, reveals the dynamic nature of continuous specificity changes of Cdk1 in the course of the cell cycle and provides a wide range of selective switchpoints for different cell cycle transitions.

EXPERIMENTAL PROCEDURES

The TAP method was applied for purification of cyclin-Cdk1 complexes and Swe1 as described previously for Clb5-TAP-Cdk1 and Clb2-TAP-Cdk1 (Puig et al., 2001; Ubersax et al., 2003). 3HA-Cln2-Cdk1 was purified according to published protocols (McCusker et al., 2007). 6His-tagged recombinant T33-Sic1ΔC constructs and substrates were purified by cobalt affinity chromatography. GST-tagged substrates were purified on glutathione agarose columns.

For the quantitative phosphorylation assays of T33-Sic1ΔC constructs and recombinant substrates, substrate concentrations were kept in the range of 0.5–2 μM (in the linear [S] versus v_0 range, several-fold below the estimated K_M value), and initial velocity conditions were defined as an initial substrate turnover of up to 10% of the total turnover. For the steady-state peptide kinetics of the Histone peptide PKTPKKAKKL, a similar assay composition was used as for protein substrates, and phosphocellulose paper was used for the quantification of the phosphorylated substrate.

For the western blotting experiments using the Phos-Tag SDS-PAGE, the Sic1ΔC-3HA versions were cloned into vector pRS315 and constitutively expressed under the *ADH* promoter. The cells were treated for 2.5 hr with 1 μg/ml α factor and released by washing. After the 50 min time point, α factor was readded to collect the cells in the next G1. The cells were lysed by bead beating in lysis buffer containing urea. Blotting of Phos-Tag SDS-PAGE gels was performed using a dry system iBlot (Invitrogen).

For viability assays on galactose plates and for galactose-induced expression time courses, Sic1 mutants were cloned into vector pRS416 under the *GAL1* promoter. For flow cytometry experiments, the DNA was stained with propidium iodide and the analysis was performed on a Becton Dickinson BD LSRII flow cytometer.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures, four tables, Supplemental Discussion, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.molcel.2011.05.016.

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LETTER

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Cascades of multisite phosphorylation control Sic1 destruction at the onset of S phase

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Multisite phosphorylation of proteins has been proposed to transform a graded protein kinase signal into an ultrasensitive switch-like response[1–4]. Although many multiphosphorylated targets have been identified, the dynamics and sequence of individual phosphorylation events within the multisite phosphorylation process have never been thoroughly studied. In *Saccharomyces cerevisiae*, the initiation of S phase is thought to be governed by complexes of Cdk1 and Cln cyclins that phosphorylate six or more sites on the Clb5–Cdk1 inhibitor Sic1, directing it to SCF-mediated destruction[1,5–8]. The resulting Sic1-free Clb5–Cdk1 complex triggers S phase[9]. Here, we demonstrate that Sic1 destruction depends on a more complex process in which both Cln2–Cdk1 and Clb5–Cdk1 act in processive multiphosphorylation cascades leading to the phosphorylation of a small number of specific phosphodegrons. The routes of these phosphorylation cascades are shaped by precisely oriented docking interactions mediated by cyclin-specific docking motifs in Sic1 and by Cks1, the phospho-adaptor subunit of Cdk1. Our results indicate that Clb5–Cdk1-dependent phosphorylation generates positive feedback that is required for switch-like Sic1 destruction. Our evidence for a docking network within clusters of phosphorylation sites uncovers a new level of complexity in Cdk1-dependent regulation of cell cycle transitions, and has general implications for the regulation of cellular processes by multisite phosphorylation.

To study the multiphosphorylation of Sic1, we used a non-inhibitory truncated version of Sic1 (Sic1ΔC) as a substrate for purified Cln2–Cdk1 and Clb5–Cdk1 complexes (Supplementary Fig. 1a; Cdk1 is also known as Cdc28). Intriguingly, both Cln2–Cdk1 and Clb5–Cdk1 generated phosphorylation patterns with abruptly accumulating hyperphosphorylated species (Fig. 1a, b, d, e and Supplementary Fig. 1b). This pattern depended on Cks1, the Cdk1 subunit that binds phosphate groups[10]. Mutation of the phosphate-binding site of Cks1 reduced the accumulation of multiphosphorylated forms (Fig. 1a and Supplementary Fig. 2a). Similarly, a phosphorylated competitor phosphopeptide reduced phosphorylation (Fig. 1b). Cks1 mutation and the peptide had little effect on the phosphorylation of a Sic1ΔC version containing a single Cdk site (Fig. 1c).

Cks1 is essential for Cdk1 function[11,12], with roles at the G_1/S and G_2/M transitions[13,14]. We found that the Cks1:Cdk1 stoichiometry *in vivo* was about 1:1 for Cln2–Cdk1 and at least 0.5:1 for Clb5–Cdk1, confirming that Cks1-dependent multiphosphorylation is the prevalent mode of Cdk1 action *in vivo* (Supplementary Fig. 1a–c). An isothermal calorimetry binding assay of fully phosphorylated Sic1ΔC (pSic1ΔC) and Cks1 measured a K_D of 11 ± 2 μM, whereas the non-phosphorylated version showed no detectable binding (Supplementary Fig. 1d). Approximately three to four molecules of Cks1 bound each molecule of pSic1ΔC, suggesting that several phosphorylated sites can bind Cks1 independently. Finally, we found that the phospho-binding pocket of Cks1 is required for phosphorylation and degradation of Sic1 *in vivo* (Supplementary Fig. 1e, f).

To understand the Cks1-dependent mechanism, we analysed Sic1ΔC multiphosphorylation over time (Fig. 1d, e). We did not observe significant accumulation of intermediate phosphorylated forms, indicating that phosphorylation was processive. When we performed kinase reactions at Sic1ΔC concentrations higher than apparent K_M, multiphosphorylation patterns remained constant despite the increase in the inhibition term $1 + [S_{0P}]/K_M$ (where S_{0P} is unphosphorylated substrate) (Supplementary Fig. 2b–e). Thus, the enzyme displays processivity; that is, it is able to transfer two or more phosphates to the substrate during a single association event. This conclusion was additionally confirmed using different enzyme concentrations

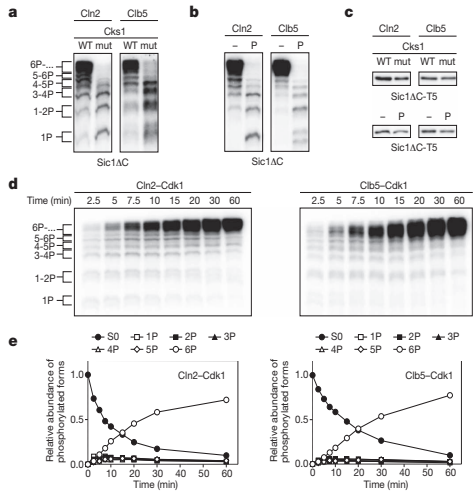

Figure 1 | The phospho-adaptor subunit Cks1 provides processivity for the multiphosphorylation of Sic1 by Cln2–Cdk1 and Clb5–Cdk1. a, Cln2–Cdk1 and Clb5–Cdk1 complexes were incubated with Sic1ΔC and [γ-^{32}P]ATP. The reactions also included wild-type Cks1 (WT) or a version with a mutated phosphate-binding site (mut; see Supplementary Methods). Phosphorylated substrates were separated using Phos-tag SDS–PAGE gels. **b**, Reactions were performed in the presence of a phosphopeptide competitor (P) based on the sequence surrounding T45 in Sic1. **c**, The phosphorylation of a Sic1ΔC version containing a single Cdk site (Sic1ΔC-T5, with other Cdk consensus sites mutated to alanines) was not affected by the Cks1 mutant (mut) or the phosphopeptide. Standard SDS–PAGE was used. **d**, Time courses of Sic1ΔC multiphosphorylation were followed by Phos-tag SDS–PAGE. **e**, The quantified data from **d**. The intensities of ^{32}P-labelled proteins were divided by the number of phosphates as indicated to obtain the levels of different phosphoforms (see also Methods). In the experiments presented in Fig. 1, enzyme concentrations were chosen to obtain roughly equal substrate labelling.

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in the assay (Supplementary Fig. 2f) and in mathematical simulations (Supplementary Fig. 3). This processive pattern argues against the current model of ultrasensitivity in the Sic1 phosphorylation switch, which is based on the assumption of a distributive mechanism with equal specificity of different sites[1,15].

To dissect the mechanism of the processive multiphosphorylation cascade, we first studied the impact of potential docking interactions between Sic1 and cyclins. In previous studies, we found that rapid Sic1 phosphorylation by Clb5–Cdk1 depends on an interaction between RXL motifs in Sic1 and the hydrophobic patch docking site (hp) in Clb5; a triple mutation in this site (Clb5hpm) decreases the net phosphorylation rate (Supplementary Fig. 4a–h)[16]. Further, we found here that a version of Sic1ΔC with mutations at its four RXL motifs (Sic1ΔC-1234rxl) showed less abrupt production of multiphosphorylated species by Clb5–Cdk1, showing that processive multiphosphorylation requires both Cks1-dependent and hp-dependent docking (Supplementary Fig. 4i). Cln2–Cdk1 had only a mild RXL effect on the phosphorylation pattern, probably because Cln2 does not contain a conventional hp like that in the B-type cyclins. In recent studies, we also located a ten-amino-acid stretch in Sic1, 136VLLPPSRPTS145, which confers Cln2 specificity[16]. Here we found that a five-alanine mutation of the first five hydrophobic residues in this stretch, or a synthetic competitor peptide containing the docking site, reduced the abrupt multiphosphorylation pattern for Cln2 (Supplementary Fig. 4j). In conclusion, both Clb5–Cdk1 and Cln2–Cdk1 use docking mechanisms, in addition to Cks1, to achieve processive multiphosphorylation of Sic1.

Using Sic1ΔC mutants carrying only one Cdk site (Fig. 2a, b), we found that Clb5–Cdk1 rapidly phosphorylated just four sites (T5, T33, S76 and S80), and this specificity depended on the RXL2 and RXL3 docking sites in Sic1 (Fig. 2b; note that in all figure labels, the indicated Cdk sites are those left unmutated, unless otherwise indicated). Cln2–Cdk1, on the other hand, showed a preference for the N-terminally located site T5 (Fig. 2b). Thus, docking interactions direct the associated kinase to a small number of primary phosphorylation sites. We speculate that these primary sites interact with Cks1 to drive processive phosphorylation of additional sites.

With these primary specificities in mind, we set out to map the pathways along which Cln2–Cdk1 and Clb5–Cdk1 catalyse the phosphorylation of the critical sites required for Sic1 degradation. The original model of Sic1 regulation proposed that six or more sites must be simultaneously phosphorylated *in vivo* to facilitate binding of phospho-Sic1 to the SCF subunit Cdc4 (ref. 1). On the other hand, later binding studies revealed that closely positioned pairs of phosphorylation sites (pT5/pS9, pT45/pT48, or pS76/pS80; see Fig. 2a) each present separate entities with a strong affinity for Cdc4, indicating that just two phosphorylation sites, in the right positions, might be sufficient for Sic1 degradation[17]. Our results provided a way to reconcile these findings: we proposed that the requirement for six or more sites *in vivo* reflects a requirement for priming phosphorylation events that direct processive phosphorylation of critical phosphodegrons. To test this possibility, we first measured phosphorylation of a Sic1ΔC mutant with all Cdk sites changed to alanine except for the triple cluster S69/S76/S80, which contains two potential paired degrons (S69/S76 and S76/S80). There was no processive multiphosphorylation of the cluster S69/S76/S80 (Fig. 2c, lane 2 in each panel), but processivity could be induced by adding back single Cdk1 sites to

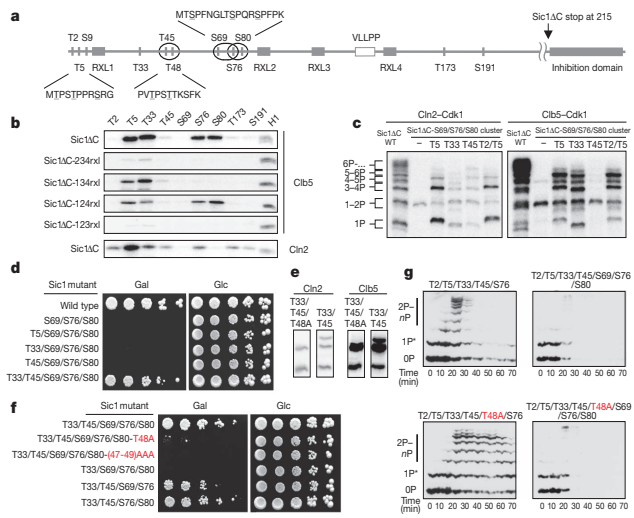

Figure 2 | Phosphorylated priming sites provide docking interactions for efficient phosphorylation of suboptimal sites in phosphodegrons. **a**, Schematic view of phosphorylation sites, docking motifs (Clb5- and Cln2-specific), phosphodegrons (ovals[17]) in Sic1. **b**, Phosphorylation specificity of Clb5–Cdk1 and Cln2–Cdk1 towards different Cdk sites was studied using Sic1ΔC constructs containing a single fixed Cdk site. For Clb5–Cdk1, the dependence of the site-specificity profile on RXL docking sites was assessed using Sic1ΔC constructs containing a single Cdk site and a single fixed RXL motif. **c**, The impact of different priming phosphorylation sites on cooperative phosphorylation of the degron cluster S69/S76/S80. Phospho-site mutants of Sic1ΔC carrying the intact S69/S76/S80 cluster and the indicated sites left unmutated were used in a kinase assay with Cln2–Cdk1 and Clb5–Cdk1 using Phos-tag SDS–PAGE. **d**, Full-length Sic1 versions containing the combination of sites described in **c** were overexpressed under the galactose promoter to assay the ability of cells to degrade Sic1. Gal, galactose; Glc, glucose. **e**, Comparison of the *in vitro* phosphorylation profiles of Sic1ΔC versions containing only the phosphorylation sites T33/T45 or T33/T45 with mutation T48A. **f**, The nonconsensus Cdk1 site T48 is important for viability of cells overexpressing Sic1. The same assay as **d** was used. In panels **d** and **f**, the labels indicate unmutated amino acids, and all other consensus Cdk sites are mutated; mutations in the nonconsensus Cdk sites are highlighted in red. **g**, The phosphorylation and degradation dynamics of Sic1 were followed after the release of cells from α-factor in a system constitutively expressing mutated versions of noninhibitory haemagglutinin-tagged Sic1ΔC–3HA. The asterisk indicates a G_1-specific phosphorylation by an unknown kinase.

the amino-terminal side of the cluster. The rate of the appearance of multiphosphorylated species correlated with the site specificity data for Cln2 and Clb5 (Fig. 2b). Addition of the most Cln2-specific site, T5, caused a much greater effect in Cln2 reactions than the less Cln2-specific sites, T33 and T45. Addition of the Clb5-specific sites T5 and T33, but not the poor Clb5 site T45, greatly increased processivity in Clb5 reactions. These results indicate that sites T5, T33 and T45 are able to serve as priming sites for Cks1-dependent phosphorylation of the S69/S76/S80 degron cluster. The phosphopeptide-dependence was confirmed for these mutants as described for Sic1ΔC-wild type in Fig. 1b (data not shown).

Cells overexpressing Sic1 containing only the triple cluster S69/S76/S80 were inviable (Fig. 2d). Addition of T5, T33 or T45 did not prevent this lethal effect, indicating that phosphorylation of the S69/S76/S80 cluster alone is not sufficient for degradation of Sic1. However, viability improved when both T33 and T45 were added. Notably, the addition of T45, together with a nonconsensus Cdk site T48 (Fig. 2a), creates a double degron, T45/T48, as predicted previously[17]. T48 is known to be phosphorylated *in vivo*[7]. We proposed that T33 serves as a docking site for both the T45/T48 and S69/S76/S80 clusters, and that T45 serves both as a constituent site of the T45/T48 degron as well as a Cks1-dependent docking site for the degron cluster S69/S76/S80. Indeed, T48 alone (the Sic1-9A mutant with all Cdk sites changed to alanine) was a very poor substrate for Clb5 and Cln2 (data not shown), but the addition of T33 or the T33/T45 pair made it a specific site (Fig. 2e, Supplementary Fig. 5 and Supplementary Table 1), implying that T33 phosphorylation allows bypass of the consensus motif requirement of a +1 proline at T48. Notably, we found that the alanine mutation in T48 had a strong growth-suppressing effect in the galactose assay within the context of sites T33/T45/S69/S76/S80 and also had a weaker effect in the background containing all the Cdk sites (Fig. 2f and Supplementary Fig. 4k). Our results indicate that the benefit of multisite phosphorylation of Sic1, compared to a system with a single phosphorylated site with high affinity for Cdc4 (for example, based on an optimal degron site of cyclin E[1]), is likely to be the ability of this mechanism to provide docking-dependent kinase specificity for paired degrons. Thus, phosphorylation sites in Sic1 can be divided into three categories: (1) paired degron sites that are spaced 3–7 amino acids from each another; (2) N-terminally positioned priming sites for each paired degron; and (3) sites that serve as both priming and degron sites (for example, T45).

It is unclear why a single paired degron with a priming site is insufficient for degradation of Sic1, as suggested by our observation that viability in the galactose plate assay requires both T45/T48 and either S69/S76 or S76/S80 (Fig. 2f). To test if the simultaneous presence of both paired degrons is required for degradation of Sic1, we used western blotting to measure phosphorylation and degradation of mutated versions of Sic1ΔC. Remarkably, a Sic1ΔC construct (equivalent to the Sic1-5p mutant of ref. 1) containing only the degron T45/T48, but missing the intact degron around S76, was rapidly degraded after the release of cells from G_1 (Fig. 2g). This degradation was abolished by mutation of the single nonconsensus Cdk site at T48. A construct also containing the S69/S76/S80 degron (Sic1-7p of ref. 1) was more rapidly degraded, and mutation of T48 in this background did not influence the degradation rate. We conclude that the T45/T48 degron is sufficient to promote some degradation of Sic1 *in vivo*, but this rate of degradation is not sufficient to prevent the lethality of overexpressed Sic1.

Our model assumes differential roles of Cln2 and Clb5 in the order of Sic1 phosphorylation events. To explore this possibility, we developed methods for determining the apparent rate constants, which we termed k_{dock}, for individual Cks1-enhanced phosphorylation steps (Fig. 3a, b and Supplementary Table 1). The results revealed considerable differences between Clb5 and Cln2. Clb5–Cdk1 was much more effective than Cln2–Cdk1 in taking shortcuts to the critical degron pair of S76/S80, using T5 and T33 as priming sites for Cks1, and with assistance from RXL-mediated docking (Supplementary Tables 1 and 2, Fig. 3a–c and Supplementary Fig. 6). Notably, in the case of Clb5, different RXL motifs supported different Cks1-dependent docking events (Supplementary Table 2).

We propose that in late G_1, Clb5–Cdk1 is inhibited by Sic1, and the cascade of phosphorylation events begins with T5 phosphorylation by

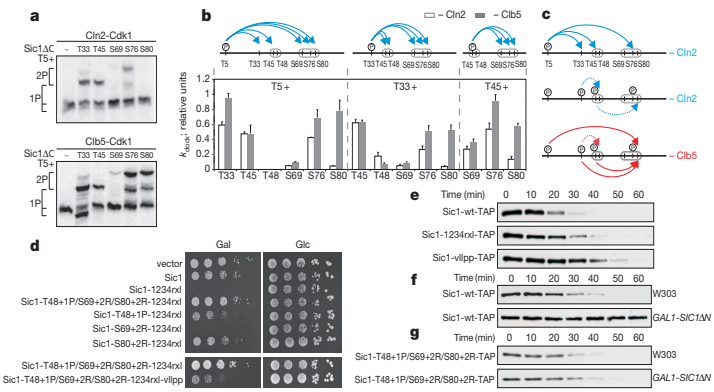

Figure 3 | Differential roles of Cln2 and Clb5 in Sic1 multiphosphorylation and degradation. a, Pair-wise mapping of the docking connections underlying Sic1 multiphosphorylation, using purified Sic1ΔC mutants containing just two of the Cdk phosphorylation sites per mutant. Representative examples of autoradiographs of phosphorylation assays, showing different docking specificities between Cln2–Cdk1 and Clb5–Cdk1. **b**, The specificity profiles for different pair-wise docking connections. The error bars indicate standard errors of the means of at least two independent experiments. (See Supplementary Information and Supplementary Table 1). **c**, Schematic view of fast and slow docking-dependent phosphorylation steps for Cln2–Cdk1 and Clb5–Cdk1. **d**, Sic1 mutants with improved Cdk recognition determinants in suboptimal phosphodegrons rescue the inviability of cells overexpressing Sic1-1234rxl. The Cln2-dependent docking site becomes essential under these conditions. **e**, Cells carrying *SIC1wt-TAP* or versions with docking site mutations at the endogeneous *SIC1* locus were released from an α-factor arrest and the degradation pattern of Sic1–TAP protein was followed by western blotting, using standard SDS–PAGE. **f**, A wild-type strain (*SIC1wt-TAP*) and a strain also expressing the nondegradable inhibitory domain of Sic1 (*SIC1ΔN*) under the *GAL1* promoter were arrested in α-factor, followed by the addition of galactose. After 45 min, the cells were washed into galactose media lacking α-factor and Sic1–TAP levels were followed by western blotting (see also Supplementary Fig. 7). **g**, Sic1 mutants with improved Cln–Cdk recognition determinants in suboptimal phosphodegrons trigger rapid degradation of Sic1 in the presence of *SIC1-ΔN*. Experiment was performed as in panel **f**.

Cln2–Cdk1. This priming event is followed by docking-enhanced phosphorylations leading to a phosphorylated chain of sites pT5/pT33/pT45/pS76 but no fully phosphorylated paired degrons, as phosphorylation by Cln2–Cdk1 of suboptimal sites in the degrons (T48 and S69, or S80) is slow (Fig. 3a, b). However, the phosphorylated cluster pT5/pT33/pT45/pS76 serves as a powerful Cln2–Cdk1-dependent docking platform for emerging Clb5–Cdk1. As Cln2 levels rise, such priming forces would create a synergistic effect between Cln2 and Clb5, greatly amplifying the impact of low emerging levels of free Clb5–Cdk1 complexes and defining the point of no return for Clb5-dependent positive feedback. A prediction of this model is that changing the limiting suboptimal degron sites to optimal Cdk sites will rescue the lethality of Sic1-1234rxl (Supplementary Fig. 4f), as the degradation in this case should be driven primarily by Cln2. Indeed, changing T48 to a Cdk1 site by introducing a proline at position +1 partially rescued the lethal phenotype of Sic1-1234rxl (Fig. 3d). A similar rescue was attained by introducing a positive determinant for Cln2–Cdk1, an arginine at position +2 (ref. 16), to the site S80. Optimization of the S69 site had no effect. Almost complete rescue was gained by a triple mutation with all three limiting degron sites (T48, S69 and S80) changed to optimal Cdk sites. Importantly, these effects are unlikely to be due to improved binding of phosphodegrons to Cdc4, as the basic residues on the carboxy-terminal side of pS/pT are known to disrupt the Cdc4 interaction[1]. Finally, to confirm that degradation of these Sic1 mutants is driven by Cln–Cdk1, instead of Clb5–Cdk1, we additionally mutated the Cln2-specific docking site VLLPP in the triple mutant background (Fig. 3d, lower panel). The *vllpp* mutation abolished the rescue effect of the triple mutant. These data indicate that Cln2 alone does have the potential to drive Sic1 degradation, but the Cln2-driven phosphorylation cascade is terminated at the rate-limiting final steps. However, this mechanism allows creation of the Clb5 docking platform containing the chain of optimal sites pT5/pT33/pT45/pS76.

Finally, to compare further the functions of Clb5 and Cln2, we analysed the degradation of endogeneous Sic1. We found that mutation of either the Cln-specific *vllpp* docking motif or Clb5-specific RXL docking sites delayed Sic1 degradation (Fig. 3e), confirming that both Cln2 and Clb5 have a role in the timing of Sic1 degradation. However, when all Clb–Cdk1 activity in the cell was specifically inhibited by overexpression of nondegradable Sic1, endogenous Sic1 was completely stabilized (Fig. 3f and Supplementary Fig. 7), indicating that the key trigger for Sic1 degradation and the G_1/S transition is the emerging free Clb5–Cdk1, after its levels exceed those of the inhibitory complex.

The inability of Cln alone to cause Sic1 degradation could be attributed to the slow phosphorylation rate of sites in the S76/S80 degron. Indeed, by introducing optimal Cln consensus motifs into the slow degron sites, analogously to the experiment described in Fig. 3d, the Sic1 degradation pattern was restored to normal despite the absence of Clb–Cdk1 activity (Fig. 3g). The processive multiphosphorylation cascades, composed of a set of fast and slow steps and different docking specificities, enable this discrimination between the signal outputs of different cyclin–Cdk1 complexes. Furthermore, the Cln output state acts as a primer state for the second complex, creating a potential 'AND gate' in which Cln2 is not allowed to trigger the G_1/S transition until sufficient levels of Clb5 activity accumulate.

In conclusion, our model provides novel insights into the multisite phosphorylation mechanism of Sic1 and, potentially, of Cdk1 targets in general. The multiple sites create a network of docking connections that exploit Cks1-dependent and cyclin-specific docking interactions to process Cdk1 signals to achieve proper tuning of the timing of the G_1/S transition (Supplementary Fig. 8). As most Cdk1 targets in the cell contain clusters of multiple sites[18], the regulation of cell cycle switchpoints by Cdk1-dependent multiphosphorylation might prove to be far more complex than generally anticipated, and it is possible that beneath the seemingly random constellations of phosphorylation sites, an intricate signal processing logic may be hidden.

METHODS SUMMARY

Yeast strains were in the W303 background and are listed in Supplementary Table 3. Plasmid constructs are listed in Supplementary Table 4. Phos-tag Acrylamide AAL-107 (ref. 19) was purchased from NARD Institute.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions M.K., E.V., R.V., A.I., M.Le. and M.Lo. designed and performed the experiments, except for the isothermal calorimetry experiments, performed by E.R.M.B. and S.M.R.; M.Lo. coordinated the project and wrote the manuscript with assistance from D.O.M. and S.M.R.

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METHODS

Protein purification. TAP-purification of cyclin–Cdk1 complexes was performed as described previously[20,21] using C-terminally TAP-tagged cyclin constructs cloned into 2 micron vectors and overexpressed from the *GAL1* promoter. For purification of 3HA–Cln2–Cdk1, a yeast strain (a gift from D. Kellogg) with the *GAL1* promoter introduced along with the N-terminal 3HA tag in the chromosomal locus of the *CLN2* gene was used. The overexpressed 3HA–Cln2–Cdk1 complex was purified as described[22], using immunoaffinity chromatography with a rabbit polyclonal antibody against the haemagglutinin epitope (purchased from Labas). Purification of N-terminally 6His-tagged recombinant Sic1 constructs was performed using cobalt affinity chromatography. Cks1 was purified as described previously[12].

Phosphorylation assays. For quantitative phosphorylation assays, the substrate concentration was kept in the range of 0.5–2 μM (in the linear [*S*] versus v_0 range, several-fold below estimated K_M) and the initial velocity conditions were defined as a substrate turnover ranging up to 10%/*N* of the total concentration of *N* Cdk sites. The general composition of the assay mixture was as follows: 50 mM HEPES pH 7.4, 5 mM $MgCl_2$, 150 mM NaCl, 0.1% NP-40, 20 mM imidazole, 2% glycerol, 2 mM EGTA, 0.2 mg ml^{-1} BSA, 500 nM Cks1 and 500 μM ATP (with added [γ-^{32}P]ATP (Perkin Elmer)). Around 1–10 nM of the purified kinase complex was used, the amount depending on the setup of the experiment. The optimal working concentration for purified Cks1 was taken as 500 nM according to the optimization performed for cyclin–Cdk1 preparations using Sic1ΔC as a substrate. For the phosphorylation assay with mutant Cks1, purified kinase complexes were preincubated for 45 min with Cks1 wild type or mutant to compensate for differences in the amounts of Cks1 already present in the preparations. The composition of the preincubation mixture was: 50 mM HEPES pH 7.4, 5 mM $MgCl_2$, 150 mM NaCl, 0.2 mg ml^{-1} BSA and 500 μM ATP. Kinase assay was initiated by adding preincubation mixture and [γ-^{32}P]ATP to the substrate. Aliquots were taken at least at two different time points and the reaction was stopped by SDS–PAGE sample buffer. For separating the phosphorylated versions of Sic1, 10% SDS–PAGE was used, supplemented with the Phos-tag reagent[19] according to the instructions from the manufacturer. For quantitative analysis of the dynamics of multisite phosphorylation (in Fig. 1d, e and Supplementary Fig. 2a–d, f) the six major bands separated in Phos-tag SDS–PAGE were quantified as 1- to 6-phosphate species.

For the phosphorylation assay of full-length Sic1 as part of the inhibitory complex, Clb5–TAP–Cdk1 was isolated from yeast cell extract containing overexpressed Clb5–TAP with IgG beads (Supplementary Fig. 4b). The stoichiometric Sic1–Clb5–Cdk1 complex was formed by incubating an excess amount of purified Sic1 with the beads, and unbound Sic1 was removed by washing (50 mM HEPES pH 7.4, 0.5 M NaCl, 0.1% NP-40). The phosphorylation reaction was performed according to the standard kinase assay protocol and was initiated by adding purified cyclin–Cdk1 complexes to the washed beads. The Clb5-dependent phosphorylation of full-length Sic1 as part of the stoichiometric inhibitory complex revealed a similar RXL specificity profile (Supplementary Fig. 4b). This result also suggests that Sic1ΔC is a valid model substrate, which was chosen for large scale analysis instead of the hard-to-adjust assay with the stoichiometric complex. It is reasonable to consider the N-terminal region of Sic1 as an independent polypeptide entity, given that only a short C-terminal part is required for high-affinity inhibition and that Sic1 is an intrinsically disordered protein[23].

The mutant Cks1 used in our experiments was designed to disrupt the phosphate-binding site and contained the combination of mutations R33E, S82E and R102A (ref. 24). The triple mutants in the hydrophobic patch (hpm) of Clb5 and Clb2 were described previously[25], and the hpm of Clb3 (F201A, L205A, T208A) was designed according to sequence homology with other B-type cyclins.

The relative rate constants for different phospho-docking enhanced steps (k_{dock}) were determined using mutated Sic1ΔC versions containing different pair-wise combinations of Cdk1 sites (the rest of the serine and threonine residues in S/TP motifs were mutated to alanines). It is impossible to estimate the effects of docking on the reaction rates directly, as it is hard to produce a version of substrate protein where the primed docking sites are fully phosphorylated and the secondary sites are unphosphorylated. Therefore, we used an indirect approach by estimating the relative formation rate of doubly phosphorylated species. The k_{dock} was defined as the ratio of the observed fraction of the doubly phosphorylated form and the estimated kinase activity towards the single N-terminal priming site present in the pair-wise Sic1ΔC construct. The latter parameters were estimated using quantified values for single site specificities from the experiment in Fig. 2b (in the absence or presence of different docking sites). Thus, the k_{dock} values are independent of the rates of the priming steps and reflect only the rates of the secondary steps. The phosphorylation of the substrate was followed in a conventional kinase assay and singly and doubly phosphorylated species were resolved using Phos-tag SDS–PAGE and quantified by PhosphorImager. The k_{dock} was calculated from two consecutive time points (that is, 8 and 16 min, in the low initial range of total substrate consumption) from at least two independent experiments. We applied a condition of a minimal ratio of 0.7 for the k_{dock} values from these two time points, to ensure that the singly phosphorylated species had not reached the temporary quasi-steady state. The obtained values are 'apparent constants' as it is impossible to precisely determine the relative contributions of processive and distributive mechanisms. Nevertheless, the obtained k_{dock} values provide very good estimates of how much the phosphorylation of a site is enhanced when another site is present.

Western blotting and viability assays. For viability assay, log-phase cultures were equalized in density and spotted as serial dilutions on selective synthetic complete (SC) plates. The plates were incubated for 48–60 h at 30 °C. For the western blotting experiments, cells were grown to $OD_{600\,nm}$ = 0.3 and treated for 2.5 h with 1 μg ml^{-1} α-factor or for 2.5 h with 15 mg ml^{-1} hydroxyurea and released by washing. In the experiments presented in Fig. 3f, g and Supplementary Fig. 7 the *GAL1-SIC1ΔN* was integrated into the *URA3* locus. In Fig. 2g the Sic1ΔC–3HA versions were cloned into the pRS315 vector and constitutively expressed under the *ADH* promoter. In the experiments presented in Fig. 3e, f, g the endogeneous *SIC1* was C-terminally TAP-tagged. The cells were lysed by bead-beating in lysis buffer containing urea. Blotting of Phos-tag SDS–PAGE gels was performed using a dry system iBlot (Invitrogen). The antibody used for western blotting of 3HA-tagged proteins was HA.11 clone 16B12 from Covance and the antibodies used for western blotting of Cdk1 (Cdc28 (yC-20)) and of TAP-tagged proteins (c-Myc (A-14)) were from Santa Cruz. The rabbit anti-Cks1 antibody was from Labas.

Isothermal calorimetry. Recombinant Sic1ΔC was phosphorylated with purified Clb2–Cdk1, and complete phosphorylation was confirmed by following the phosphorylation shift by Phos-tag SDS–PAGE. Recombinant Cks1 was expressed in *Escherichia coli* from a pET vector and purified with anion exchange and size-exclusion chromatography. Calorimetry experiments were performed with a VP-ITC system (MicroCal). 0.3-0.4 mM Cks1 was titrated into a 30 μM solution of phospho-Sic1ΔC. Experiments were carried out at 25 °C in a buffer containing 25 mM Tris and 150 mM NaCl (pH 8.0). Data were analysed with the MicroCal Origin software package. The reported binding constant and stoichiometry are the average from 2 experiments, and the reported errors are the standard deviation of these measurements.

Quantitative mass spectrometry. For quantitative determination of Cks1-dependent phosphorylation of T48, equal amounts of Sic1ΔC-wt protein were phosphorylated by Clb5–Cdk1 supplemented with normal isotopic ATP ([^{16}O]ATP) or heavy ATP ([^{18}O]ATP) (Cambridge Isotope Laboratories). Kinase assays were incubated at room temperature for 60 min then pooled together in a 1:1 ratio (v/v) in SDS–PAGE sample buffer. The proteins were separated by 10% SDS–PAGE and the gels were stained with Coomassie brilliant blue G-250 (Sigma) and protein bands were excised from the stained gels. Proteins were in-gel digested by LysC/P (10 ng μl^{-1}) (Wako) and peptides were purified by using C18 StageTips.

Peptides were separated with an Agilent 1200 series nanoflow system (Agilent Technologies) connected to a LTQ Orbitrap classic mass-spectrometer (Thermo Electron) equipped with nanoelectrospray ion source (Proxeon). Purified peptides were loaded on a fused silica emitter (75 μm × 150 mm) (Proxeon) packed in-house with Reprosil-Pur C18-AQ 3-μm particles (Dr. Maisch HPLC GmbH). Peptides were separated with 30 min 3–40% B gradient (A, 0.5% acetic acid; B, 0.5% acetic acid/80% acetonitrile) at a flow-rate of 200 nl min^{-1}, eluted peptides were sprayed directly into LTQ Orbitrap mass-spectrometer with a spray voltage of 2.2 kV. The mass spectrometry scan range was *m/z* 300–1,800 and the top 5 precursor ions were selected for subsequent MS/MS scans. A lock-mass was used for the LTQ-Orbitrap to obtain constant mass-accuracy during the gradient analysis. Peptides were identified with the Mascot 2.3 (http://www.matrixscience.com) search engine. A peptide mass tolerance of 7 p.p.m. was used and a fragment ion mass tolerance of 0.6 Da. Two missed cleavage sites for LysC/P were allowed. The oxidation of the methionine and the phosphorylation of serine, threonine were set as variable modifications.

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Cdk1
A kinase with changing substrate specificity

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Cyclin-dependent kinases (Cdks) are master regulators triggering the major events of the cell cycle.[1] In the course of the cell cycle, the activating subunits of Cdks, the cyclins, appear and disappear as periodic and partly overlapping waves. This general principle of sequential cyclin signals as a periodic driving force of the cell cycle is conserved throughout the eukaryotes. However, despite extensive studies in several model organisms, there is still no general model, which would explain how the cyclins provide temporal separation for cell cycle transitions.

In the budding yeast *Saccharomyces cerevisiae*, cyclins Cln1–3 are triggers for G_1 and G_1/S, Clb5, 6 drive S phase, Clb3, 4 are specific for early mitotic events, and Clb1, 2 complete the progression to mitosis. A single Cdk, Cdk1, associates with these cyclins to mediate all major cell cycle transitions. Quantitative analysis of cycling cultures of budding yeast suggests that the abundance of different cyclins is relatively similar, with only a few-fold variance.[2] This finding suggests that one can consider the period from G_1 to mitotic exit as a state of relatively unchanging net levels of activated Cdk1. However, such a pattern raises questions of how the temporal Cdk1 signaling specificity is achieved and hints to the existence of a cyclin-directed substrate targeting specificity profile of Cdk1. Why, for example, does the activation of Cdk1 in the early cell cycle by G_1/S cyclins not trigger premature S- or M-phase processes? On the other hand, there should exist a common specificity among different complexes toward some Cdk targets. To prevent rereplication, for example, components of the pre-replicative complex should be kept in their phosphorylated state throughout the S- and M phase by all B-type cyclin-Cdk1 complexes.[3]

In vivo evidence suggests that some mechanism might prevent early cyclins from acting prematurely on later triggers. Experiments with cultured cells and transgenic mice suggest that some mammalian cyclins can functionally compensate for the absence of others. However, of all the different cyclins tested, the later mitotic cyclins A2 and B1 appeared to be the most nonredundant, suggesting that the early cyclins cannot perform their function.[4] In budding yeast, the major cyclin genes are not fully interchangeable, and the early cyclins appear less capable of performing later Cdk functions, as the deletion of both S-phase cyclins results only in an S-phase delay,[5] whereas the deletion of both mitotic cyclins is lethal.[6] In fission yeast, early cyclins can be deleted without severe consequences, while the mitotic cyclin is essential for division.[7]

Although the cyclin-dependent substrate specificity of Cdk1 has been addressed in several previous studies,[8,9] the general dynamics of Cdk1 specificity in the cell cycle has remained unclear. We recently presented a detailed study on the specificity of budding yeast cyclin-Cdk1 complexes and outlined the general mechanisms underlying changes in Cdk1 specificity during the cell cycle.[10] We found that the sequentially appearing cyclins gradually increase the specificity of Cdk1 toward the optimal Cdk consensus site (**Fig. 1**). The increasing intrinsic specificity explains the paradox raised by the in vivo studies referred to above, answering the question of why the early cyclins are incapable of triggering prematurely the later stages of the cycle. On the other hand, for selected G_1- and S-phase substrates at the early stages of the cell cycle, Cdk1 is able to compensate for the poor specificity on the phosphorylation site level by using cyclin-specific docking sites including a novel hydrophobic docking motif for G_1-specific Cln1 and Cln2-Cdk1 complexes. In addition, the G_1 complexes showed distinct phosphorylation consensus site specificity, further suggesting that cyclins do not merely activate Cdk1 but also modulate its active-site specificity. Finally, we identified several Cln2-, Clb3- and Clb2-specific Cdk1 targets and presented a classification system for Cdk1 targets. Based on the obtained data, we propose that robust timing and ordering of cell cycle events depends on gradual changes in the substrate specificity of Cdk1.

The cyclin specificity model is an alternative to the purely quantitative model of the cyclin response, according to which different levels of accumulating cyclin-Cdk1 activity trigger different cell cycle events.[11] We propose that while the cyclin levels may play some role in the ordering of the phosphorylation events during the onset of individual cyclins, the resolution span of such a system would remain too narrow for temporal separation of all the Cdk1-driven cell cycle transitions. To overcome this disadvantage, the gradually changing pattern of cyclin specificity has been evolved to facilitate proper resolution of the phosphorylation switches and thereby also to increase the robustness of the cell cycle oscillator.

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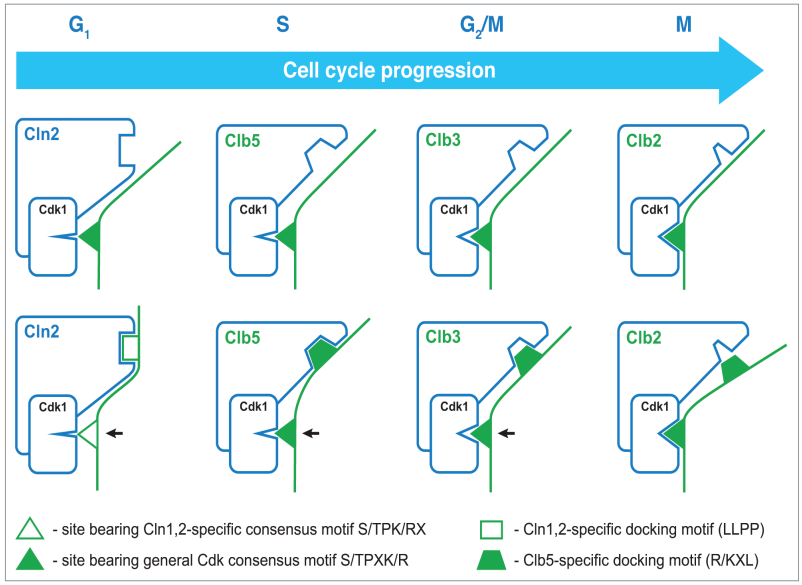

Figure 1. Schematic representation explaining the changes of Cdk1 substrate specificity during the cell cycle. The kinase-substrate complexes presented in the upper row show the gradually changing intrinsic specificity toward the general Cdk consensus motif. The triangle-formed pocket represents the active site of Cdk1. The further the cell cycle progresses, the better active-site fit is gained. The lower row presents the possibilities of how the G_1- and S phase-specific Cdk1 target proteins can become efficiently phosphorylated despite the poorly fitting active site by using cyclin-specific docking interactions. The G_1-specific cyclins Cln1- and Cln2-Cdk1 may additionally use the S/TPK/RX consensus motif to gain specificity relatively to Clb-Cdk1s.

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Multisite phosphorylation networks as signal processors for Cdk1

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Abstract

The quantitative model for cyclin-dependent kinase (CDK) control of the cell cycle states that different CDK activity thresholds control the order and timing of cell cycle events. However, it is not understood how a single protein kinase can trigger hundreds of switches in a sufficiently time-resolved fashion. We show that the output signal of cyclin-Cdk1-Cks1-dependent phosphorylation of multisite targets is controlled by key set of parameters including distances between phosphorylation sites, the distribution of serines and threonines as phospho-acceptors, the positioning of cyclin-docking motifs, processivity factors, and the number of sites in the network. We propose that variation of these parameters within the networks of multiple phosphorylation sites in different targets provides a wide range of possibilities for the differential amplification of a Cdk1 input signal, providing a mechanism to generate a wide range of well-defined thresholds in the cell cycle.

Introduction

Cyclin-dependent kinases (CDKs) are the master regulators of the eukaryotic cell division cycle. Together with a set of periodically expressed cyclin subunits they form a central control unit responsible for the cell cycle clock[1]. CDKs catalyze an ordered phosphorylation of hundreds of targets that trigger a sequence of coordinated molecular events that drive the cell through the stages of cell division[2-5]. Several individual CDK-driven switches have been identified[6], but our understanding of CDK function as a general coordinator of the entire process of cell division is not clear.

The existing model states that as levels of CDK activity rise during the cell cycle, specific molecular events are executed at each of a series of activity thresholds[7-11]. This model requires that substrates are phosphorylated with a wide range of efficiencies, such that very good substrates are phosphorylated early, at a low CDK activity threshold, while poor substrates are phosphorylated only when much higher CDK activity is achieved, later in the cell cycle. CDK targets contain both optimal consensus phosphorylation motifs (S/T-P-x-K/R)[12, 13] and suboptimal consensus sites (S/T-P) that are phosphorylated much less efficiently[12, 14]. Therefore, a mechanism in which optimal motifs are used for the 'early' switches and suboptimal motifs for the 'later' switches is conceivable, but it would require a very wide range of CDK activity, providing both a low and high threshold. However, total cyclin accumulation is not more than 2–3 times higher in mitosis compared with S phase in budding yeast [15]. Furthermore, cells can be arrested before mitosis with a CDK inhibitor concentration that is only a fraction of the estimated peak cyclin levels in mitosis[8, 16]. In budding yeast, the estimated peak nuclear concentration of mitotic cyclins is about 2–3 μM[15, 17], while the inhibitor concentration required for G2/M arrest is 500 nM[16]. Similarly, in fission yeast, the CDK inhibitor concentration required for arrest in G2 was 300 nM [8]. Thus, only a relatively small increase in CDK levels must be able to trigger discrete temporally resolved events. In addition, if the system relied entirely on the distinction between optimal and suboptimal consensus motifs, there could be, in principle, only two robustly resolved thresholds. However, several studies have shown that even within the narrow window of time at the onset of mitosis there is a finely resolved order of discrete events that are triggered in response to different levels of accumulating mitotic cyclins[18-20]. How do relatively small changes in total CDK levels provide temporally resolved triggering of these events?

Cyclin specificity in substrate recognition has provided some insight into the mechanism by which CDKs coordinate cell cycle events[21-25]. In budding yeast, the intrinsic activities of different cyclin-Cdk1 complexes increase during the cycle, and the low activity of early complexes is compensated by cyclin-dependent docking sites[14, 26]. This setup helps to enhance the abrupt phosphorylation of selected early targets in late G1 and early S phases without interference from later targets (due to their higher K_M values with the early cyclin-Cdk1 complexes). Although this specificity allows early cyclins to

efficiently target early targets, they cannot drive the later stages of the cell cycle in budding yeast[27]**.** This difference in cyclin specificities provides a coarse mechanism to separate early and late events but it still fails to explain how relatively small changes in CDK activity can trigger the ordered sequence of discrete events observed on a finer time scale.

The complexity of CDK function is further increased by the multisite nature of most of its targets. The input CDK signal is often processed through several phosphorylation steps to yield a multi-phosphorylated output state of the target [28-32]**.** Recently, we demonstrated that a network of phosphorylation sites in a budding yeast Cdk1 target, Sic1, is phosphorylated in semi-processive cascades. This processivity is determined by a phospho-adaptor subunit of the cyclin-Cdk1 complex, Cks1, together with cyclin-substrate docking sites[25]. The output of the cascade is the phosphorylation of two diphosphodegrons that direct Sic1 to degradation via the SCF-proteasome pathway[33, 34]. The degron phosphorylation sites are not specific enough to be efficiently phosphorylated by Cdk1 directly, because they contain suboptimal sets of recognition elements. Instead, the suboptimal degron sites become phosphorylated due to docking-dependent amplification of Cdk1 specificity via sequential steps of priming phosphorylations. Once the Sic1 molecule has accumulated several phosphates, the CDK complex binds to these phosphates via Cks1, and is held at high local concentration with respect to the suboptimal sites, allowing suboptimal sites to be efficiently phosphorylated.

These semi-processive cascades, whose net phosphorylation output does not depend solely on the consensus motifs of the critical sites, but also on other parameters, lead us to a hypothesis that may provide a solution to the mechanistic problems of the threshold model described above. According to our hypothesis, the positions and the distances of phosphorylation sites and cyclin-dependent docking sites within these clusters encode signal processors that govern the net phosphorylation rate through the cascades and allow a large dynamic range in the possible input-output relationship – thereby generating thresholds for phosphorylation of functional sites at any CDK activity level. Variation of these parameters provides a wide range of possibilities for the differential amplification of outputs in response to moderate changes of CDK input strengths.

The central element defining the output signal strength of these multisite processor systems is the phospho-adaptor Cks1. Cks1 was first discovered in *S. pombe* as a high-copy suppressor of a defective Cdk1 mutant[35]. In budding yeast, Cks1 is essential for both G1/S and G2/M transitions[36, 37] and associates with cyclin-Cdk1 complexes at close to stoichiometric ratios *in vivo*[38]. An *in vitro* study with the *Xenopus* version of Cks1 demonstrated its possible role in promoting the multisite phosphorylation of the CDK regulators Wee1, Myt1, and Cdc25, as well as the APC[39, 40], while in budding yeast the G1-specific complex Cln1,2-Cdk1 requires the Cks1 subunit for activity[41]. However, the function of Cks1 as a phospho-adaptor protein in the mechanism of CDK-driven switches has been largely overlooked. Also, recent large-scale proteomic

screens have identified hundreds of CDK targets[2, 4], but these studies have not touched on the level of complexity that the multisite nature of these targets may present for the mechanism of CDK signal processing.

In the present study we analyzed the biochemical parameters that control Cdk1 signal flux through multisite phosphorylation networks. We explored how different combinations of these parameters influence the net output of the signal, and we analyzed the mechanism of Cks1-dependent multiphosphorylation cascades and how processivity can be used for differential amplification of output signals. Based on these data, we propose a new mechanism that provides a solution to the question of how accumulating CDK activity triggers the correct timing and sequence of cell cycle events.

Results

Phosphorylation of multisite targets by Cdk1 shows different levels of Cks1-dependent processivity. Because nearly all known Cdk1 targets contain multiple phosphorylation sites, it was of interest to obtain an overview of the relative importance of Cks1 in promoting processive phosphorylation cascades in different targets. We analyzed the multisite phosphorylation dynamics of a set of Cdk1 targets in Cdk1 phosphorylation assays involving either the wild type Cks1 or a version of Cks1 with a mutated phosphate-binding pocket (Cks1*mut*) [25, 42]. The autoradiographs of the phosphorylation patterns, presented in Figure 1a, reveal that there are wide variations in Cks1 dependence among the targets. In some cases, the abrupt appearance of multi-phosphorylated patterns is highly dependent on Cks1. In other cases, phosphorylation is not affected by Cks1. Also, there are targets that display intermediate effects, suggesting that only a subset of sites is enhanced by Cks1. We also found that different cyclin-Cdk1 complexes exhibit different rates of Cks1-dependent hyperphosphorylation: for example, compare phosphorylation of the G1 transcriptional regulator Whi5 by G1-specific Cln2-Cdk1 and M-phase specific Clb2-Cdk1 complexes. However, Cks1 dependence seems to be a general mechanism throughout the cell cycle. Note that in control experiments, Cks1 had little effect on the phosphorylation of a substrate containing a single Cdk1 site (T5-Sic1ΔC; Fig. 1b).

The differences among the targets indicate that the multisite networks may have functionally different patterns. What are the parameters that determine Cdk1 processivity through the networks? We aimed to study these parameters using the Cdk1 target Sic1 as a model system. The specificity of cyclin-Cdk1 complexes is controlled at three different levels: first, by the active site specificity of Cdk1, second, by cyclin-specific docking interactions, and third, by the specificity of Cks1 (Fig. 1c). Using a non-inhibitory form of Sic1 (Sic1ΔC) we designed several sets of combinatorial mutants to study how these three factors control the Cdk1-dependent phosphorylation of multisite networks.

The phospho-binding pocket of Cks1 binds phospho-threonines but not phospho-serines. One source of variation among the targets of Cdk1 presented in Figure 1 is the ratio of threonines to serines as phospho-acceptor residues. Interestingly, we noticed that three of the targets that do not display Cks1 dependence (Bop3, Sld2, and Ypr174) had only serine residues within optimal consensus motifs (S-P-x-K/R). Therefore, we questioned if Cks1 is able to dock via both pSer and pThr residues, or whether it prefers one over the other. Of the nine CDK consensus motifs in Sic1, five are Thr and four are Ser residues (Fig. 2a). The N-terminal residues T2, T5, and T33 were shown previously to serve as phospho-docking sites for Cks1, thereby promoting fast phosphorylation of the C-terminal phospho-degrons [25]. To test if Ser residues are equally able to mediate Cks1-dependent phosphorylation, we replaced the threonine residues in the CDK consensus sites of Sic1 with serine residues. Strikingly, the abrupt accumulation of multiply phosphorylated species was severely suppressed (Fig. 2b). The effect was as strong as that of the Cks1*mut* presented in Figure 1a, indicating that the entire phospho-binding capacity of Cks1 was lost. The effect was entirely due to Cks1 specificity because the Cks1-independent phosphorylation sites in the all-serine mutant was not affected (Supplementary Fig. 1a,b).

To directly confirm the inability of a serine to prime a single Cks1-dependent phosphorylation step we constructed a version of Sic1ΔC containing only two sites: the optimal Cdk1 target T33 (or S33) served as a priming site and a suboptimal site T48, with very weak direct specificity towards Cdk1[25], served as a secondary site. Indeed, the threonine was required for the accumulation of the doubly phosphorylated species (Fig. 2c).

Cells overexpressing the all-Ser form of Sic1 are inviable, unlike cells overexpressing wild-type Sic1 (Fig. 2d). This result indicates that Cdk1 is not able to phosphorylate Sic1-Ser to a sufficient level to cause its proper degradation. It is most likely that inviability is caused by weak binding of pSer sites to Cks1 because no pSer versus pThr specificity has been observed for SCF-Cdc4 phospho-degrons[34]. Additionally, a single serine substitution in the crucial priming site at position 33 caused a partial loss of viability (Fig. 2d). Thus, it is possible to disrupt the docking connections of the Cks1-dependent cascade by replacing threonine residues in CDK sites with serine residues. The resulting construct follows a distributive phosphorylation mode (Fig. 2b) in which phosphorylation of one site is not dependent on previous phosphorylation of the other. In fact, such a distributive mode was the basis of an earlier model of Sic1 phosphorylation [43], and these results provide an additional argument supporting the processive Cks1-dependent cascade model [25].

To measure the direct binding affinity between the phosphorylated sites of Sic1 and Cks1 we used an isothermal calorimetry (ITC) assay with purified proteins. We produced stoichiometrically phosphorylated versions of Sic1ΔC containing single phosphorylation sites. The data obtained further confirmed the exclusive preference of pThr over pSer in Cks1 binding (Fig. 2e, Supplementary Table 1).

Finally, we demonstrated the requirement for threonine as the crucial priming residue by directly following the appearance of multi-phosphorylated forms of Sic1 *in vivo*. The replacement of the threonine at the crucial N-terminal priming site T5 resulted in a severe reduction in the multi-phosphorylated forms of an artificial Sic1 substrate construct containing five evenly-paced serine consensus motifs downstream of the priming site (Fig. 2f).

We also performed a set of positional variations by introducing basic and hydrophobic residues around the T33 site. We found that a proline residue at position -2 relative to pThr enhanced the interaction of the phospho-epitope with Cks1 (Table 1, Supplementary Fig. 1c,d; see also cosubmitted paper[44]).

Cks1-dependent phosphorylation depends on a well-defined distance between the priming site and a C-terminal secondary site.

The second parameter that is likely to control signal flux through multisite cascades is the distance and relative positioning between the priming phosphorylation site and the secondary phosphorylation site. To analyze the impact of this parameter on the rate of Cks1-dependent phosphorylation steps, we created a series of Sic1ΔC-based substrate constructs containing two phosphorylation sites at different distances from each other. Due to its intrinsically disordered nature, [45, 46] the Sic1 polypeptide is an excellent system to study such distance requirements. By varying the distance between the two phosphorylation sites in the Sic1 constructs we aimed to measure the optimal distances between the sites in terms of the number of amino acids in the polypeptide chain.

We constructed a series of Sic1 proteins carrying the site T33, which contains the optimal CDK consensus motif (TPQK), as the primary phosphorylation site. The secondary site was a short sequence bearing a suboptimal CDK motif (TPQA), which was placed at various distances from T33 (see Supplementary Methods for details). The priming site T33 was efficiently phosphorylated by cyclin-Cdk1 complexes, while the suboptimal motif used in the secondary site was not phosphorylated in the kinase assay conditions used (data not shown). Therefore, when doubly-phosphorylated species were detected, the sequence of the two-step cascade was always primary phosphorylation of T33 first, followed by the phosphorylation of the secondary site (Fig. 3a).

A striking distance dependence in the rates of Cks1-dependent secondary steps was observed (Fig. 3b). Furthermore, a surprisingly sharp change in rates was observed in the step from 10 to 12 amino acids from the T33 priming site: a distance of 10 amino acids yielded no secondary phosphorylation, while a distance of 12 amino acids showed very rapid accumulation of the doubly phosphorylated form. This drastic distance cut-off was similar for all three cyclin-Cdk1 complexes tested (Fig. 3c-e), indicating that the Cks1-Cdk1 module has a similar architecture and functional capability that does not depend on cyclin specificity. In all three enzyme complexes tested, a sharp peak value of 12-16 amino acids in the secondary rates was followed by rapid decline around the distance of 20-30 amino acids downstream from the priming site.

A surprising feature of the Cks1-dependent secondary step was that the docking-dependent cascade operates exclusively in the N-to-C direction (Fig. 3c-e). We did not observe any docking-enhanced secondary phosphorylation even when the priming site T33 was moved further downstream, yielding distances of -40, -60 and -80 amino acids (Fig. 3c–e).

Interestingly, the relatively sharp distance optimum was broadened when the Cks1 specificity was improved by introducing a proline residue at position -2 from the T33 priming site. This suggests that the negative effect of above-optimal distances can be compensated by stabilizing the enzyme substrate complex via improved binding of the phosphorylated site to Cks1. The improved Cks1 specificity, however, did not improve activity with a distance shorter than the 10 amino acid minimal cut-off. Additionally, it is possible to increase the effective distance window for secondary phosphorylation by introducing the optimal CDK consensus site in the secondary site (Supplementary Fig. 2b-d). Also, we confirmed that the distance relationship was not specific for the particular sequence context downstream from the site T33. For this we created a control set of similar constructs with the priming site T5 bearing an optimal motif PSTPPR (Supplementary Fig. 2a).

To test if the distances between sites are also important for multisite phosphorylation *in vivo*, we varied the positions of phosphorylation sites in a non-destructible version of Sic1ΔC and analyzed phosphorylation by western blotting, as in the experiment presented in Figure 2f. We made one artificial substrate with 5 phosphorylation sites in their original positions, but lacking the intermediate sites T33 and T45 to remove the possibility for Cks1-dependent docking. This substrate was compared to a construct that was identical, except that three serine Cdk1 sites were repositioned to be 16 amino acids apart, as indicated in the scheme in Figure 3f (d16-Sic1ΔC). These constructs contain a single optimal priming site T5, while the other sites were serine-based sub-optimal sites. The first construct, which has sites either amino-terminal of T5 or greater than 64 amino acids downstream, showed almost no change in phosphorylation profiles after entry into S phase. In contrast, the construct with the sites repositioned at accessible distances from the T5 priming site displayed mobility shifts of singly- and doubly-phosphorylated species. The phosphorylation pattern of the construct with repositioned sites fits well within the distance relationship observed in the case of the optimal priming site containing a proline in position -2 (Fig. 3c-e, Supplementary Fig. 2a). The sites at distances of +16 and +32 are within the range of relatively fast rates while the longer distances apparently fail to gain any enhancement from Cks1-dependent docking.

These experiments reveal a strikingly sharp minimal distance cut-off and strict N-to-C-directionality both *in vitro* and *in vivo*. From this we can conclude that the underlying mechanism involves simultaneous binding of the phosphorylated priming site to the Cks1 pocket and the secondary site to the active site pocket of Cdk1 (Fig. 3g).

Distances between phosphorylation sites are critical for Cdk1 biological function. To further confirm that the distances between sites encode biological information in multisite phosphorylation networks, we performed a series of viability assays as described in Figure 2d. To increase the sensitivity of the critical distance variations we used a version of Sic1 with a minimally viable set of 5 phosphorylation sites (Fig. 4). Surprisingly, moving the priming site T33 in these constructs by only 2 amino acids upstream or downstream caused inviability. Apparently, in these constructs a docking distance that perfectly fits the optimum of 12-16 amino acids (Fig. 3c-e) is required for efficient phosphorylation of both sites of the diphosphodegron (T45 and T48). One of the acceptor sites of the diphosphodegron is a 'non-CDK' consensus site (T48) whose phosphorylation could be even more sensitive to the docking distance compared with the consensus sites. We also moved the position of the diphosphodegron T45/T48 by 10 amino acids in a version of Sic1 containing all physiological sites. Similarly, this moderate shift of the position caused a severe reduction of viability. These data indicate that site positioning in multisite networks in Cdk1 targets is not random, but involves critical distances between primer and acceptor sites that must fit the distance between Cks1 phosphate binding pockets and the active site of Cdk1.

Docking to B-type cyclin positions phosphorylation at a specific C-to-N distance. Next we studied the effect of altering the distance of phosphorylation sites from cyclin-specific docking sites. In the model substrate Sic1 there are two Clb5-specific docking sites (RXL)[21-23, 47-52], and a single Cln2-specific docking motif (LLPP)[14, 53]. We analyzed constructs containing only one of the RXL motifs and the LLPP motif (Fig. 5a). The position of an optimal CDK consensus motif based on site T5 was varied over a wide range in both C- and N-terminal directions. We tested phosphorylation of these constructs with wild-type Clb5-Cdk1 or Clb2-Cdk1, as well as with kinase complexes in which the cyclin carried a mutation in the hydrophobic patch docking site (hpm) [21, 22]. In Figure 5b and c the distance profiles are plotted as ratios of the phosphorylation rates obtained with wild-type and hpm kinase complexes. For Clb5, a striking increase of docking-enhanced phosphorylation rate was observed when the phosphoacceptor site was 16-20 amino acids N-terminal from the RXL motif. No strong potentiation was observed when the phospho-acceptor site was C-terminal to the RXL (Fig. 5b, e). For Clb2, only a small increase in activity was observed at the same distance. These data are consistent with our previous results that Clb2 has much weaker hydrophobic patch specificity towards its substrates compared with Clb5 [23].

Cln2-dependent docking can potentiate phosphorylation of sites both N- and C-terminal from the docking site. In Figure 5d the specificity profile is plotted for the Cln2-Cdk1-Cks1 complex in the absence and in the presence of a competitor peptide containing the Cln2-specific LLPP docking site. A gradual increase in docking-dependent enhancement of the phosphorylation of N-

terminally located sites was observed, as well as, quite surprisingly, a large potentiation towards a distant C-terminal site. These data suggest that the LLPP binding site, whose location in Cln2 is not known, is less directionally constrained than the Cks1 phospho-docking or hydrophobic patch/RXL docking interactions (Fig. 3e, Fig. 5d,e).

Suboptimal sites are not phosphorylated in the absence of priming phosphorylation and Cks1-dependent docking. We also tested the ability of cyclin docking mechanisms to promote phosphorylation of a suboptimal Cdk site. When the optimal CDK consensus motif was replaced with a suboptimal motif, the potentiation of phosphorylation due to cyclin docking was lost, as illustrated for a construct with 44 amino acid distance from the RXL motif, chosen from the set of constructs used in experiments presented in Figure 5b-d (Fig. 5f). Since suboptimal sites were efficiently phosphorylated in the experiments addressing Cks1–dependent docking (in which the cyclin docking motifs were not mutated, Fig. 3), we also analyzed cyclin docking effects for three different optimal Cks1-distance mutants from the set of constructs analysed in Figure 3b-e. In the case of Clb5-Cdk1, the hydrophobic patch stimulated Cks1-dependent phosphorylation (Fig. 5g). A slightly lesser effect was observed with Cln2-Cdk1 and the competitor peptide. However, in the case of Clb2-Cdk1, cyclin docking had almost no effect. These data suggest that both Cks1-dependent docking via a priming phosphorylation site and cyclin-dependent docking are required for phosphorylation of suboptimal CDK sites by earlier cyclin-Cdk1 complexes (Fig. 5h, see also Supplementary Fig. 2b and 3). The mitotic complex Clb2-Cdk1 can phosphorylate suboptimal sites with the help of Cks1 alone, presumably due to its higher intrinsic activity.

The net output rate of the multi-phosphorylation cascade depends on its degree of processivity. Our observations suggest that abrupt multisite phosphorylation of many Cdk1 substrates depends on multiple interactions between the substrate and kinase. It is likely that these interactions enable the processive or semi-processive attachment of multiple phosphates during a single substrate-binding event (Fig. 6a).

In the case of Cks1-dependent multisite cascades, there are two mechanisms by which the processive step could take place. The first mechanism allows that the phosphorylated priming site withdraws from the active site of Cdk1 and subsequently reassociates with the phosphate-binding pocket of Cks1 without dissociation from the enzyme (Fig. 6b). Support from cyclin docking sites would be required to maintain the complex during such a displacement. To explore the existence of such a mechanism we used two Sic1ΔC-based constructs, containing pairs of physiological phosphorylation sites (T5/T33 or T33/T45) (Fig. 6c, d). We performed kinase assays in which we measured the accumulation of singly and doubly phosphorylated forms, which were resolved in Phos-Tag gels. The enzyme was used at two-fold different concentrations at subnanomolar range. We did not see any significant change in the ratios of

doubly and singly phosphorylated species, arguing that the reactions were independent of enzyme concentration and thus processive (see Fig. 6a). Interestingly, we observed a considerable degree of processivity for both substrate constructs when either Clb5- or Cln2-Cdk1 was used. The processivity factors suggest that after the phosphorylation of the first site there is about a 30-40 % chance to add the secondary phosphate without dissociation. Intriguingly, in the case of the hpm version of Clb5 the secondary phosphate was almost undetectable. Thus, support from the cyclin docking site is necessary for processivity, as depicted in Figure 6b. Furthermore, while Cln2-Cdk1 showed only a slightly lower level of processivity compared with Clb5-Cdk1, the mitotic Clb2-Cdk1 complex showed almost no processivity. This finding correlates with the fact that the hydrophobic patch of Clb2 is unable to strongly potentiate phosphorylation (Fig. 5b). Thus, the earlier cyclin complexes show higher processivity because they can use cyclin docking sites.

The second possible mechanism for Cks1-dependent processivity is the sequential addition of phosphates without dissociation of the primed phosphate from Cks1 pocket (Fig. 6e). To analyze this mechanism we used a substrate construct containing T33 as a priming site and a triple-serine cluster S69/S76/S80 as secondary sites. These experiments showed that this type of processivity exists as well. The processivity factors at subsequent steps showed a 20–30% probability to continue with the next phosphorylation step without dissociation (Fig. 6f).

Thus, the degree of processivity may be an additional factor that can differentiate the output signals of networks in different Cdk1 substrates. We tested this possibility on two additional physiological targets: Whi5 and Fin1. Our results (Supplementary Fig. 4) suggest that various multisite phosphorylation networks can provide different degrees of processivity for each cyclin-Cdk complex, illustrating the potential for broad dynamic range in the processing of Cdk1 signals by different substrates.

Discussion

In this study we analyzed parameters that control the phosphorylation of multisite targets of Cdk1. We propose that the overall spatial pattern of the multisite cluster, the distances between the sites, the direction of docking connections, the composition of the network with respect to serine versus threonine residues, Cks1-phosphoepitope specificity, and the processivity at each step determine the ability of Cdk1 to produce the multiphosphorylated output form of its targets. This model changes the prevailing understanding that the strength of a response to a Cdk1 signal is defined simply by the specificity of phosphorylation consensus motifs (and cyclin-specific docking consensus motifs).

The network parameters together form a pattern or a code that is read by the cyclin-Cdk1-Cks1 complex. The fixed architecture of the cyclin-Cdk1-Cks1 complex defines the rate and processivity of each step. The model in Figure 7a,

constructed from crystal structures of domains in complex with substrate peptides, shows the positioning of the three key substrate pockets in the complex. In addition, the directionality requirements observed for the Cks and hydrophobic path-docking enhancement are consistent with the directionality of the peptides in the crystal structures. As shown, one can model a continuous polypeptide chain that has an N-terminal Cks-docking phosphate, a middle phosphor-acceptor site, and a C-terminal RxLxF cyclin-docking sequence. Assuming a substrate in an extended conformation, the minimum sequence lengths between these elements roughly correspond to the minimum distance requirements found in our kinetics experiments.

One way these phosphorylation cascades can progress is along an intrinsically disordered peptide stretch. The cascade starts at specific N-terminal priming site(s) and proceeds, in a Cks1-dependent manner and with help from cyclin docking sites, towards C-terminal output sites (e.g. diphosphodegrons) (Fig. 7b, top). Interestingly, we have observed that in several cases (Sic1, Far1, and Cdc6; data not shown), one of the sites in the diphosphodegrons is a suboptimal CDK site. This setup prevents short-circuiting of the cascade and ensures that the signal flux is directed through the network. This enables the overall signal flux and the timing of the switches to be controlled because different networks present different resistance to the signal current (using an electronic circuit analogy). On the other hand, when the distances are too short or too long, or alternatively, if there are only serines in the phosphorylation sites, then the network has no connections (Fig. 7b, bottom). In this case, the multisite output state is achieved via independent phosphorylation events. We note that such networks of sites could also exist in globular protein, with analogous connections drawn between the disordered loops containing the phosphorylation sites. In multidimensional space, it is conceivable that closed circles, in which docking sites become docking-dependent sites, and sites with branching connections could exist.

In a simplified cell cycle model with rising CDK activity, the window on the specificity scale that could provide thresholds for temporal resolution of switches falls between the specificity for optimal and suboptimal sites. Within this window the specificity can be varied by changing the distances, Cks1 specificity or other network parameters. While the bulk phosphorylation of optimal sites in the networks, and substrates having only optimal sites, takes place roughly at the same time early in the cell cycle, the substrates with connected networks containing the steps with limiting rates (e.g. steps between sites connected by longer than optimal distance, but still having a docking-potentiated effect) must have a delayed accumulation rate of the multi-phosphorylated output forms. The limiting steps in these targets link the timing of the cell cycle switch to a certain Cdk1 activity threshold. As demonstrated by us recently, the rate-limiting Cks1-dependent phosphorylation steps in Sic1 determine the timing and the cyclin-Cdk1 activity threshold of the G1/S switch [25].

Finally, there is also a possibility that dephosphorylation specificity contributes to the timing of CDK-dependent phosphorylation, with earlier targets being more resistant to phosphatases. Gradually changing phosphatase specificity has been shown to play role in ordered dephosphorylation of Cdk1 targets in mitotic exit by Cdc14 [54]. However, it is not clear if the same is true for phosphatases counteracting the rising Cdk1 activity before mitotic exit. Instead, a phosphatase was shown to have highly specific interaction with a mammalian early CDK target, the pRb protein [55]. Indeed, different phosphatase specificity at each step would further increase the possible complexity of the networks.

Similar principles of stepwise modulation of the output signal of Cdk1 targets can be extended to other kinases that use phosphorylated Cdk sites as priming sites (e.g. GSK, Cdc7 and Cdc5), as demonstrated recently [56, 57]. Future studies of the mechanisms of Cdk1-dependent multisite processor systems will hopefully uncover the intricate complexity of Cdk1 switches and will finally provide a full understanding of the general mechanism of the Cdk-controlled cell cycle clock.

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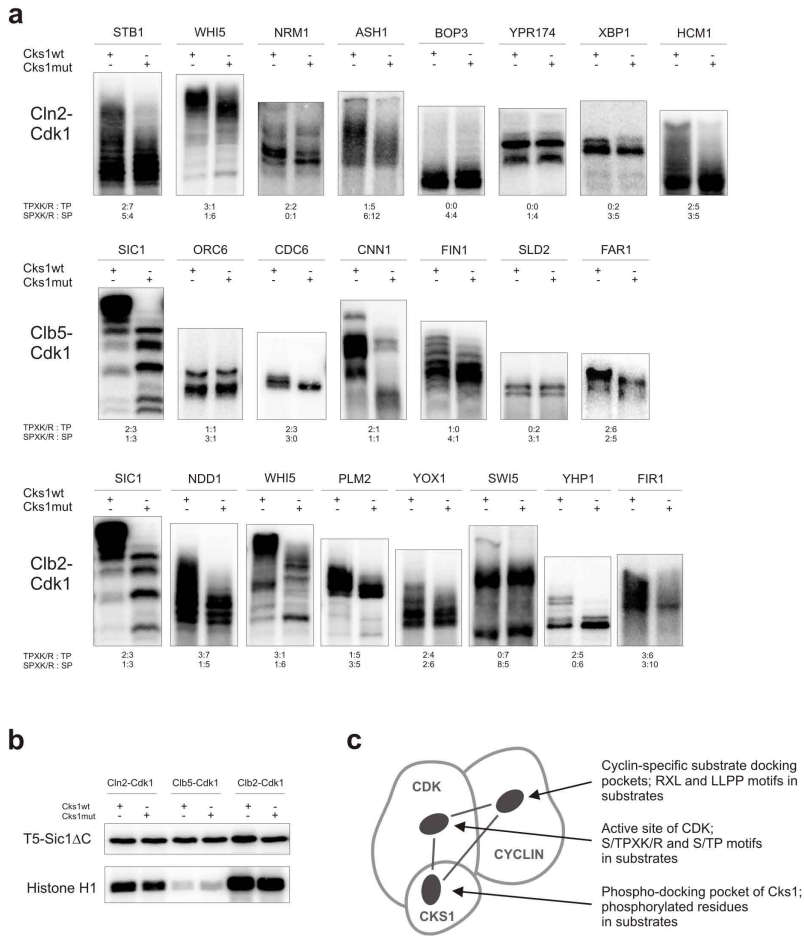

Figure 1. Cks1-dependent multisite phosphorylation of Cdk1 targets. (a) Demonstration of Cks1-dependent accumulation of multi-phosphorylated forms in selected targets of Cdk1. The kinase assays were performed using purified Cln2-, Clb5-, and Clb2-Cdk1 complexes, which were preincubated with either wild-type Cks1 or Cks1*mut*. The radioactively labeled multi-phosphorylated forms were separated using Phos-Tag SDS PAGE. The number of optimal and suboptimal consensus motifs, together with an indication if the sites have Ser or Thr residue as the phospho-acceptor, are provided below the panels; (b) Phosphorylation of a substrate construct containing a single CDK consensus phosphorylation site (T5-Sic1ΔC) is not influenced by Cks1-dependent phospho-docking. Additionally, the phosphorylation assays using a standard substrate histone H1 are shown; (c) A schematic diagram indicating three pockets in cyclin-Cdk1-Cks1 complex whose local site-specificity and positioning geometry could potentially control the phosphorylation dynamics of multisite targets.

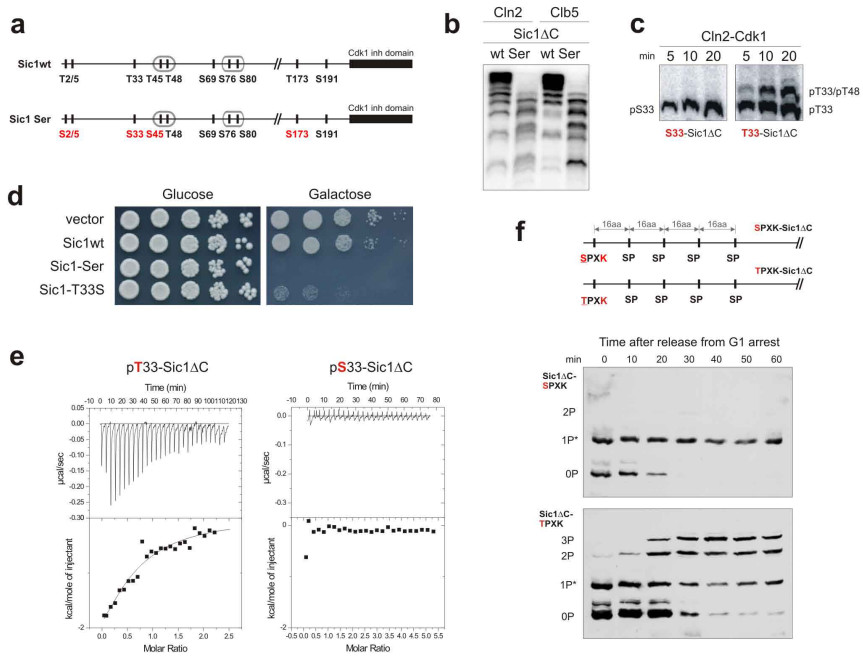

Figure 2. Exclusive preference of threonine over serine residues as the priming sites for Cks1-dependent docking and phosphorylation steps. (a) A diagram indicating the Thr-to-Ser substitutions made in Sic1 for the experiments in panels 'b' and 'd'; (b) The kinase assay demonstrating the loss of abrupt multisite phosphorylation caused by Thr-to-Ser substitution of the Cdk1 phosphorylation sites in Sic1ΔC; (c) The kinase assays demonstrating the Thr-to-Ser substitution effect at position T33 of T33/T48-Sic1ΔC on the accumulation rate of doubly phosphorylated forms; (d) Full-length Sic1 versions were overexpressed under the galactose promoter to assay the ability of cells to degrade Sic1; (e) Representative ITC experiment to analyze the binding of pT33-Sic1ΔC and pS33-Sic1ΔC to Cks1. No binding signal was detected in the case of pS33-Sic1ΔC; (f) The *in vivo* demonstration of the requirement of Thr as a priming site of multiphosphorylation using western blotting of Phos-Tag SDS-PAA gels. Schematic diagrams (above) showing different constitutively expressed and non-destructible Sic1ΔC -derived substrate constructs that were used to follow the dynamics of phosphorylation shifts after the release of cells from α-factor arrest. These constructs contained a single N-terminal threonine-based priming site at position 5 surrounded by an optimal CDK consensus motif (PSTPPR). The serine-based secondary sites were introduced C-terminally from the priming site at 16 aa intervals by replacing the original sequence at these sites with a short sequence fragment containing a suboptimal CDK consensus site (see Supplementary Methods section). Additionally, in these constructs lacked the cyclin specific docking sites. *Partial phosphorylation of sites by an unknown kinase activity in G1.

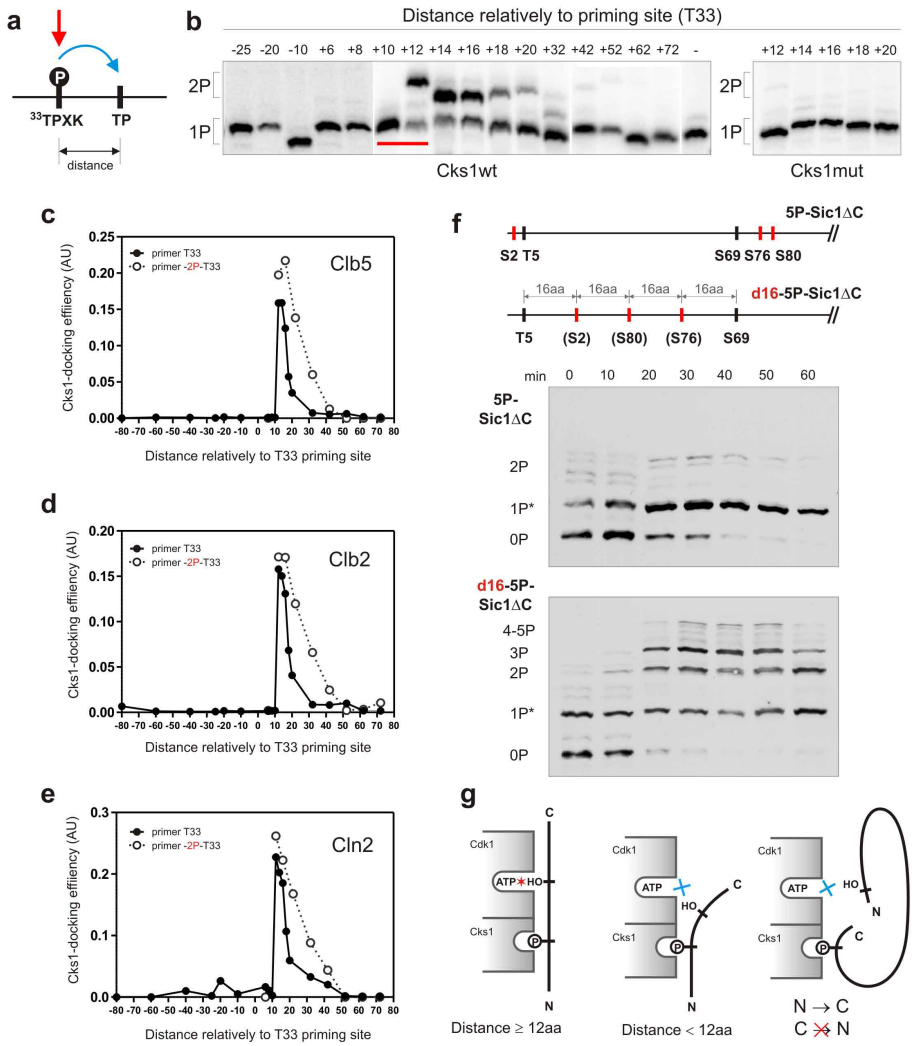

Figure 3. Analysis of the influence of distance between the priming phosphorylation site and the secondary phosphorylation site. (a) A scheme explaining the positional variation of the secondary site along the Sic1 polypeptide chain; (b) The autoradiograph of Phos-Tag gels showing the phosphorylation of constructs with varied distances between the priming site (T33) and the secondary site using Clb5-Cdk1. The Cks1-dependence of the abrupt appearance of doubly phosphorylated forms in these assay conditions was confirmed by assaying the constructs with the most efficient docking distances in the presence of Cks1mut (right panel). Within the whole set of tested constructs some variations in the mobility were detected, which is likely caused by the specific primary structure surroundings of particular phosphorylation sites in these constructs. The red bar indicates where the abrupt increase of the rate of the secondary step was observed. The last lane on the right side of the left panel shows the construct containing only the priming site T33; (c-e) The quantified profiles that show the relative accumulation rate of doubly phosphorylated forms at different distances between the

priming sites and the secondary sites using Clb5-Cdk1 (c), Clb2- Cdk1 (d), and Cln2-Cdk1 (e). For quantification details see the Supplementary Methods section; (f) The demonstration of the requirement of optimal distances between the phosphorylation sites for efficient multi-phosphorylation *in vivo* using western blotting of Phos-Tag SDS-PAA gels. Schematic diagrams above the blots show the Sic1ΔC-derived substrate constructs used to follow the dynamics of phosphorylation shifts after the release of cells from α-factor arrest. The construct with either too long or too short distances between the sites was comprised of a selection of 5 sites at their original positions in Sic1 (see also the scheme in Fig. 2a). The second construct was designed by removing 3 of the 5 sites as indicated. The only optimal site in this constructs was the T5. The R in the position +3 of S76 consensus motif was changed to A in both constructs; (g) A scheme explaining the optimal distance requirements and the N-to-C-directionality in the Cks1-dependent phosphorylation step.

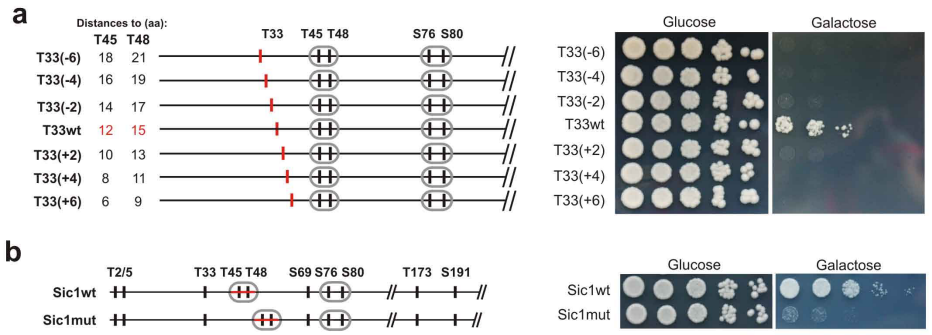

Figure 4. The optimal distances between the sites of Sic1 are critical for its degradation *in vivo*. (a) The viability assay overexpressing Sic1 versions containing a set of phosphorylation sites T33/T45/T48/S76/S80. The position of the priming site T33 was shifted by 2 amino acid steps upstream and downstream of its original position. Because the strain expressing T33/T45/T48/S76/S80-Sic1 grows slightly slower compared with the strain expressing wild type Sic1, the colonies were grown for a longer time compared with the assay presented below in panel 'b' involving the wild type version of Sic1; (b) Similar viability assay as in 'a' showing the effect of shifting the position of a critical di-phosphodegron T45/T48 in context of wild type Sic1.

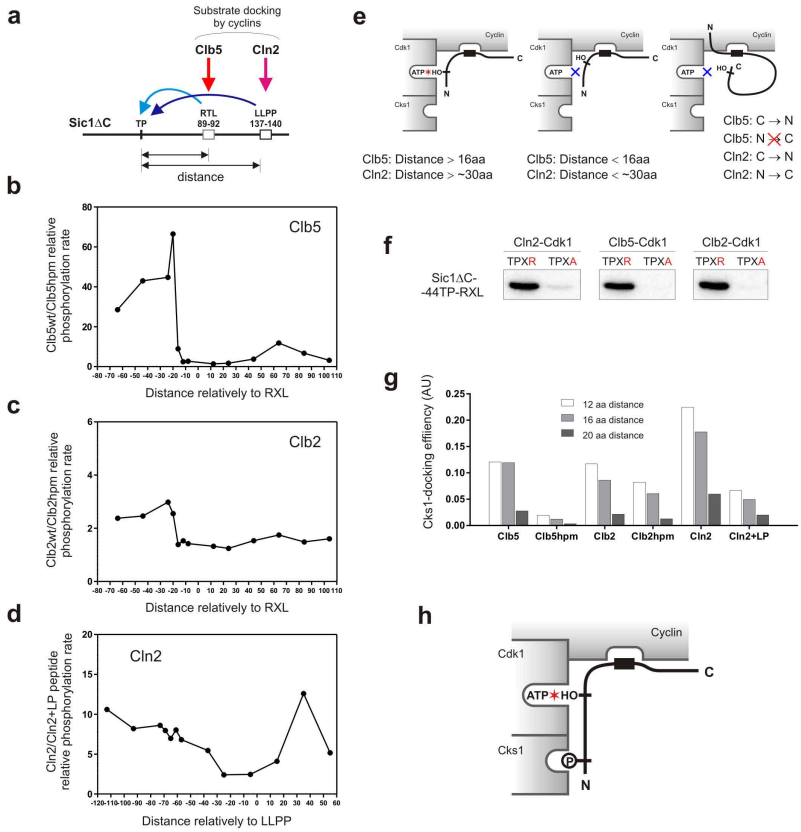

Figure 5. Analysis of the influence of distance between the phosphorylation site and the cyclin-specific docking sites. (a) The scheme of varied phosphorylation site position along the polypeptide chain of constructs based on Sic1ΔC with all CDK sites mutated to alanines and containing a single RXL motif and a Cln2-specific LLPP motif. In these constructs a single CDK consensus motif based on T5 was placed at various distances from the Clb5 docking site RTL and Cln2 docking site LLPP; (b) The specificity profiles obtained by using substrate constructs with different distances from the RXLF motif are plotted as a ratio of phosphorylation rates of wild type and hpm version of Clb5-Cdk1 complex; (c) Analogous specificity profile as in 'b' plotted for Clb2; (d) Analogous specificity profile as in 'b' plotted for Cln2-Cdk1 when assayed in the absence and in the presence of the competitor peptide containing the cyclin docking motif 'LLPP'; (e) Schematic representation of the cyclin-dependent docking modes; (f) The effect of the replacement of optimal CDK consensus motif with a suboptimal motif on the phosphorylation of a construct (constructs of the type described in panel 'a'), in which there are 44 and 92 residues between the phosphorylation site and the RXL and LLPP motifs, respectively; (g) The same constructs, with three different distances, as used in experiments presented in Figure 3a-e were used to determine the effect of cyclin docking interactions on Cks1-dependent phosphorylation (as in Fig. 3c-e), using wild-type cyclin complexes or the hpm versions (Clb5, Clb2), or when the competitor peptide (+LP) was included (Cln2); (h) Schematic depiction of the triple docking mechanism used by the cyclin-Cdk1-Cks1 complex.

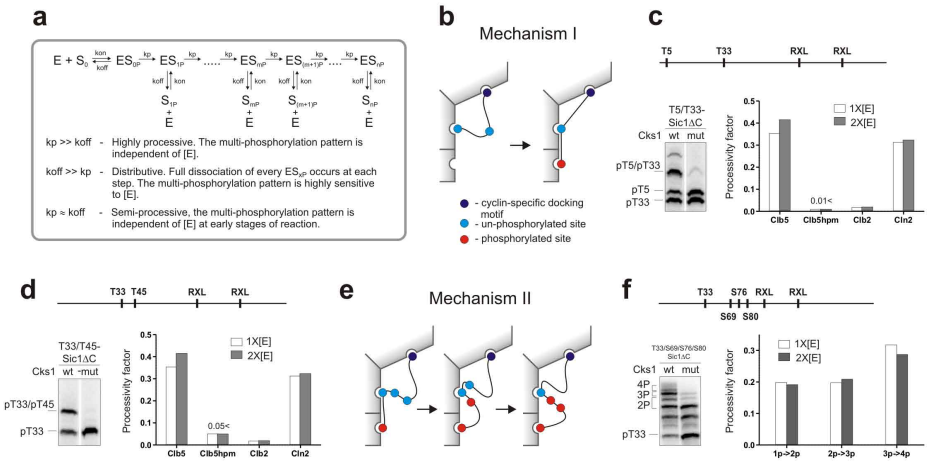

Figure 6. Analysis of the processivity of Cdk1-catalysed multi-phosphorylation. (a) A general kinetic scheme of a sequential multiphosphorylation process. For the sake of clarity, each form of substrate containing a certain number of phosphates is considered as kinetically equal; (b) A depiction of the mechanism of a processive step in which the phosphorylation of a priming site is followed by the phosphorylation of the secondary site directly, without dissociation of the substrate intermediate from the enzyme; (c) Different phosphorylated forms of substrate construct T5/T33-Sic1ΔC were used to test Mechanism I (separated in Phos-Tag SDS-PAGE). The bar graph on the right shows the patterns of processivity for different cyclin-Cdk1 complexes. The y-axis shows the ratio of 2P/(1P+2P), where 2P is the quantified amount of the doubly phosphorylated species, and 1P is the singly phosphorylated form pT5/T33-Sic1ΔC. Because this ratio does not change at early time points upon lowering the enzyme concentration, the observed ratio represents a processivity factor that indicates the probability for the reaction to proceed without dissociation relative to the probability of dissociation of the singly phosphorylated species from the enzyme; (d) An analogous experiment as in 'b' for the analysis of Mechanism I using a substrate construct with a different pair of sites, T33/T45; (e) A schematic depiction of a second major processive mechanism. The phosphorylated priming site when docked to the Cks1 pocket can processively phosphorylate several secondary sites without dissociation; (f) Analysis of Mechanism II using a substrate construct T33/S69/S76/S80-Sic1ΔC, in which T33 is a priming site. The bar graph shows the processivity factors at each step.

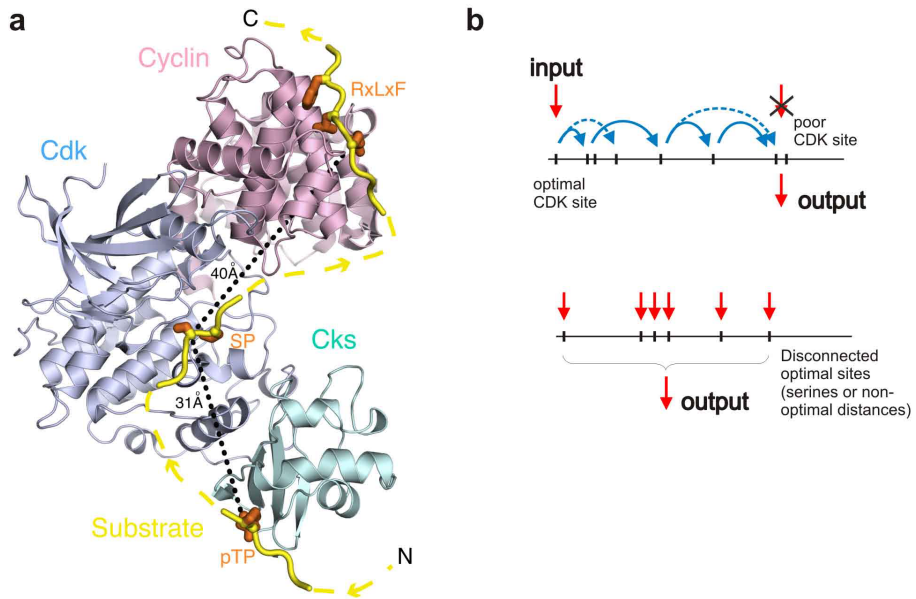

Figure 7. The three interaction sites in the cyclin-Cdk1-Cks1 complex are the key for processing of Cdk1 signal through different multisite phosphorylation networks; (a) A structural model showing the arrangement of the three key pockets in the complex. The model was created by superimposing domains from crystal structures (PDB codes: 1BUH, 2CCI, in submission) each solved in the presence of the relevant substrate peptide bound to the pocket [44, 48, 58]; (b) The upper scheme shows an example of a network on a stretch of disordered peptide chain. The cascade starts at specific N-terminal priming site(s) and proceeds, in a Cks1-dependent manner with assistance from cyclin docking sites, towards C-terminal output sites. The lower panel shows a cluster of sites with disconnected sites with either too short or too long distances, or alternatively, with only serines in the phosphorylation sites.

Online Methods

Protein purification. TAP-purification of cyclin-Cdk1 complexes (Clb5- and Clb2-Cdk1)was performed as described previously [2, 59] using C-terminally TAP-tagged cyclin constructs cloned into 2 micron vectors and overexpressed from the *GAL1* promoter. For purification of 3HA-Cln2-Cdk1, a yeast strain was used (a kind gift from Dr. Doug Kellogg, UCSC) with the *GAL1* promoter introduced along with the N-terminal 3HA tag in the chromosomal locus of the *CLN2* gene. The overexpressed 3HA-Cln2-Cdk1 complex was purified as previously described [60], using immunoaffinity chromatography with a rabbit polyclonal antibody against the HA epitope (purchased from Labas, Estonia). N-terminally 6His-tagged recombinant Sic1ΔC constructs and 6His-tagged substrates in Figure 1 were purified by standard cobalt affinity chromatography with 200 mM imidazole used for elution. GST-tagged substrates used in the kinase assay presented in Figure 1 were purified on glutathione agarose columns.

Kinase assays. For the phosphorylation assays of Sic1ΔC constructs, substrate concentrations were kept in the range of 0.5–2 μM (in the linear [S] versus v_0 range, several-fold below the estimated K_M value). About 0.1–10 nM of purified kinase complex was used, reaction aliquots were taken at two or more time points, and the reaction was stopped using an SDS-PAGE sample buffer. The basal composition of the assay mixture contained 50 mM Hepes, pH 7.4, 180 mM NaCl, 5 mM $MgCl_2$, 20 mM imidazole, 0.1 mg/ml 2HA peptide, 2% glycerol, 2 mM EGTA, 0.2 mg/ml BSA, 500 nM Cks1, and 500 μM ATP (with added γ-^{32}P-ATP (Perkin Elmer)). For the phosphorylation assay with mutant Cks1 (Cks1mut), purified kinase complexes were preincubated for 45 minutes with Cks1wt or Cks1mut to compensate for differences in the amounts of Cks1 already present in the preparations. The optimal working concentration for Cks1 (500 nM) was based on optimizations decribed previously [25]. The general composition of the preincubation mixture was: 50mM Hepes pH 7.4, 5 mM $MgCl_2$, 150 mM NaCl, 0.4 mg/ml BSA, 500 μM ATP, 1 mM Cks1. In the assays with LP peptide (VLLPPSRPTS), 4 mM peptide was used. To separate the phosphorylated versions of Sic1, 10% SDS-PAGE was used, supplemented with the Phos-tagTM reagent according to the instructions from the manufacturer.

The kinase assays for determination of docking efficiencies (Fig. 3b–e, Fig. 5g) were performed under conditions below 10-30% of initial substrate turnover. The phosphorylation of the substrate was followed in a conventional kinase assay and singly and doubly phosphorylated species were resolved using Phos-Tag SDS-PAGE and quantified by ImageQuant. The quantified values expressing the docking efficiencies (Fig. 3c-e) were calculated as the ratio of observed fraction of doubly phosphorylated form and the sum of observed fraction of doubly phosphorylated form and of the form with a single phosphate. The obtained value was divided by enzyme concentration. In this way,

quantitative and comparable indicators showing the ability of Cks1-dependent docking steps were obtained. The determination of real kinetic constants in this system would become complicated due to the mixed mechanism of the two-step process. The reaction aliquot was taken at two consecutive time points (i.e. 8 and 16 minutes) and the average value for the docking efficiency of the two time points was calculated. For processivity analysis lower enzyme concentrations were used to minimize the impact of the distributive/cooperative mechanism in the formation of the multi-phosphorylated form(s). The range of enzyme concentration for observation of purely processive process was chosen so that the difference in relative ratios of multiply phosphorylated forms at two different enzyme concentrations (dilution factors as indicated in the figure panels) was lower than 20%. The processivity factors were calculated similarly to docking efficiencies, except that the obtained value was not divided by enzyme concentration. For all the phosphorylation assays presented in the paper the concentration of the Phos-Tag in 10% SDS-PAGE was 100μM for Sic1, Fin1, Ypr174c, or 20μM for Stb1, Whi5, Nrm1, Bop3, Xbp1, Hcm1, Orc6, Cdc6, Cnn1, Sld2(1-185aa N-terminal fragment), Ndd1, Plm2, Yox1 and Swi5. Ash1, and Fir1 phosphorylation assays were separated in 8% SDS-PAGE, supplemented with 20μM Phos-Tag reagent. 7% SDS-PAGE supplemented with 100μM Phos-Tag reagent was used for Far1 protein.

Isothermal calorimetry. Dissociation constants were obtained using ITC binding experiments. Recombinant 6His-Sic1ΔC-based constructs were phosphorylated with purified Clb2-Cdk1 (no Cks1 was added). Stoichiometric phosphorylation was confirmed by following the phosphorylation shift by Phos-tag SDS-PAGE. Phosphorylated constructs were repurified on chelating chromatography like described above with the exception that the elution was performed using 10mM EDTA. Purified proteins were dialyzed overnight at 4 °C in 25 mM Tris (pH 8.0) and 150 mM NaCl. Cks1 at a concentration of ~ 0.2–1 mM was titrated into a 20–90 μM solution of phospho-Sic1. Experiments were performed at 25 °C using a VP-ITC instrument (Microcal). When binding was detected, experiments were performed in duplicate. Binding constants were calculated by averaging the Kds, and the error is the standard deviation of the Kds.

Western blotting and viability assays. For western blotting experiments, versions of Sic1ΔC-3HA were cloned into vector pRS315 and constitutively expressed under the *ADH1* promoter. The cells were treated for 2.5 hr with 1 μg/ml α factor and released from the arrest by removing the α factor by washing. The cells were lysed by bead beating in lysis buffer containing urea. Blotting of Phos-Tag SDS-PAGE gels was performed using a dry system iBlot (Invitrogen). For the viability assays the *SIC1* versions were cloned into the pRS413 vector under the control of *GAL1* promoter.

Sic1-based substrate constructs. For studies of docking distances we used substrate constructs based on a non-inhibitory version of Sic1 with truncation of

the inhibitory domain at position 216-284 (Sic1ΔC). For the constructs used for experiments presented in Figure 3b-e the sequence motif QATPQAAAQ (based on the surroundings of T33 with the lysines, prolines and serines mutated to alanines) was introduced in positions 8, 13, 23, 39, 41, 43, 45, 47, 49, 51, 53, 65, 75, 85, 95 and 105 (the number designates the position of phospho-acceptor threonine). The priming site T33 was left unchanged in this set of the constructs. At the positions of other physiological phosphorylation sites the double alanine replacements were introduced at the S/TP motifs in case of all Sic1ΔC constructs. In order to analyze the potential N-terminally directed docking-enhanced phosphorylation steps over longer distances the T33 motif (QKTPQKPSQ) was replaced into the positions 47, 67, and 87, while the secondary site motif was introduced at position 8. For studies of distance requirements of cyclin-dependent docking sites the substrate constructs contained only a single RXL motif (positions 89-91), while the other motifs were mutated to alanines as described previously (ref). The Cln2-specific LLPP motif (in positions 137-140) was left intact. In these constructs the sequence motif PSTPPRSRG based on the site T5 was introduced at positions 25, 45, 67, 69, 73, 77, 81, 103, 115, 135 and 155. In case of the construct used in Figure 5f QATPQAAAQ phosphorylation site was introduced at position 45.

The substrate constructs used in the western blotting experiments presented in Figure 2f were based on non-inhibitory version of Sic1 containing a 3HA tag in its C-terminus (Sic1 ΔC-3HA). The sequence fragments AMSPSA, LTSPQA, and QRSPFP, based on the primary structure surrounding the physiological sites T2, S76, and S80 (with exceptions that the lysine in position +3 from the site S76 was mutated to alanine and the threonine in position 2 was mutated to serine), were replaced into positions 21, 37, and 53 (indicating the new positions of the phospho-acceptor residue). The sites T5 and S69 were left in their original positions. These replacements yielded constructs with 5 CDK consensus sites with 16 amino acids intervals between the sites. For testing serine as the priming site, the T5S mutation was introduced. In case of these constructs the RXL motifs and the LLPP motif was mutated to alanines as described in [25], due to the observed slight RXL/LLPP-dependent preference of serine residues over the threonine in the phosphorylation efficiencies *in vivo*. In the western blotting experiment presented in Figure 3f the construct with the five physiological sites in their original positions was made by mutating the sites T33, T45, T173, and S191 to alanines; the site T2 to serine; the arginine in position +3 from the site S76 to alanine. The construct with the changed distances between the sites was made by similar rearrangements of sequence motifs as described above for the constructs used in Figure 2f. For the western blotting experiments presented in the Supplementary Figure 3 the constructs with suboptimal sites had similar positioning of the sequence fragments as the constructs with equal distance interval shown in Figures 2f and 3f with the exception that the arginine in position +3 from the phosphorylation site T5 or S5 was mutated to alanine.

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II Avunie-Masala R, Movshovich N, Nissenkorn Y, Gerson-Gurwitz A, Fridman V, Kõivomägi M, Loog M, Hoyt MA, Zaritsky A, Gheber L. (2011) Phospho-regulation of kinesin-5 during anaphase spindle elongation. J Cell Sci. 15;124(Pt 6):873–8
III Kõivomägi M, Valk E, Venta R, Iofik A, Lepiku M, Morgan DO, Loog M. (2011) Dynamics of Cdk1 substrate specificity during the cell cycle. Mol Cell 10;42(5):610–23
IV Kõivomägi M, Valk E, Venta R, Iofik A, Lepiku M, Balog ER, Rubin SM, Morgan DO, Loog M. (2011) Cascades of multisite phosphorylation control Sic1 destruction at the onset of S phase. Nature 12;480(7375):128–31
V Kõivomägi M, Loog M (2011) Cdk1: a kinase with changing substrate specificity. Cell Cycle 1;10(21):3625–6

VI Venta R, Valk E, Kõivomägi M, Loog M. (2012) Double-negative feedback between S-phase cyclin-CDK and CKI generates abruptness in the G1/S switch. Front Physiol 3:459

VII Kõivomägi M, Iofik A, Örd M, Valk E, Venta R, Faustova I, Kivi R, Balog ERM, Rubin SM, Loog M. (2013) Multisite phosphorylation networks as signal processors for Cdk1 (Submitted)

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Publikatsioonide loetelu:

I Ord T, Ord D, Kõivomägi M, Juhkam K, Ord T. (2009) Human TRB3 is upregulated in stressed cells by the induction of translationally efficient mRNA containing a truncated 5'-UTR. Gene 1;444(1–2): 24–32.
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SA Archimedes

Muu teaduslik organisatsiooniline ja erialane tegevus:
Stendiettekanne 33. FEBS Kongressil

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