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LIST OF ORIGINAL PUBLICATIONS

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

1. 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).

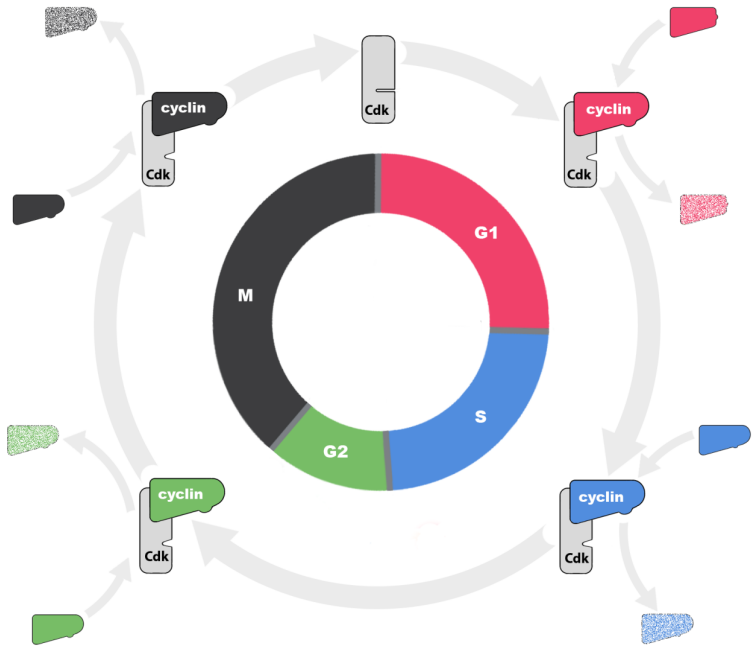

Figure 1. The mitotic cell cycle. The mitotic cell cycle is a sequence of coordinated events that leads to the reproduction of the cell. The cell cycle is divided into 4 phases: G1 →S→G2→M. DNA replication takes place in S phase and the separation of sister chromatids occurs in M phase (mitosis). These 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).

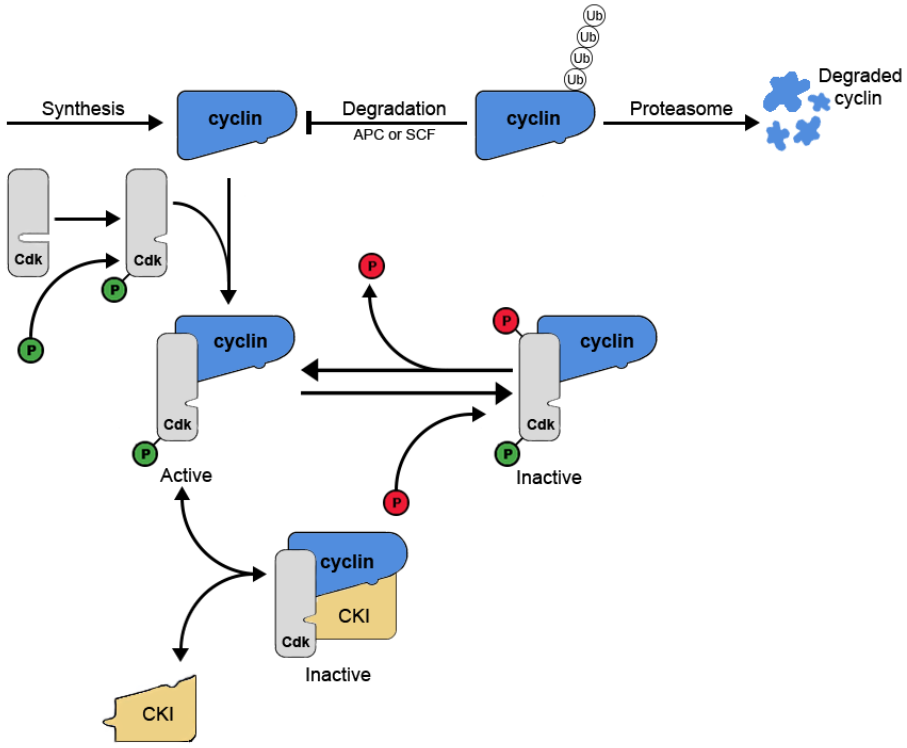

Figure 2. Cyclin-dependent kinase (CDK) activity is regulated at multiple levels. Monomeric CDK lacks activity until it is phosphorylated by CDK-activating kinase (CAK) and associates with a cyclin. The availability of cyclins is controlled by the rates of their synthesis and degradation. Cyclins are 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).

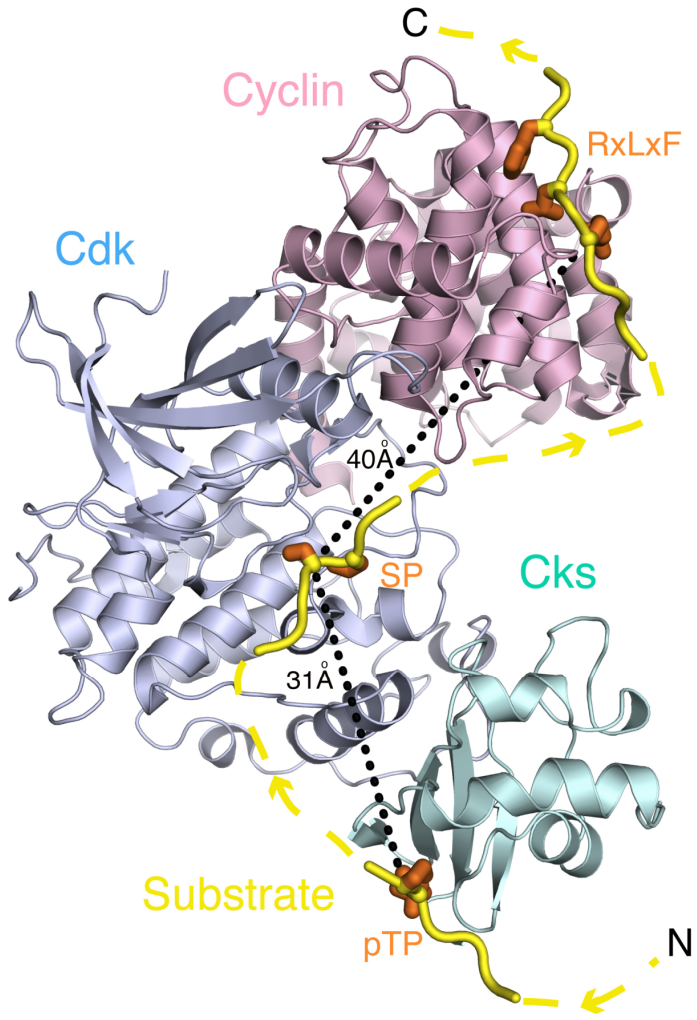

Figure 3. The three substrate interaction sites of the cyclin-CDK-Cks complex. A structural model showing the arrangement of the three key pockets in the cyclin-Cdk-Cks complex that are important for substrate recognition. The substrate specificity of CDK is determined by the active site of the kinase, 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).

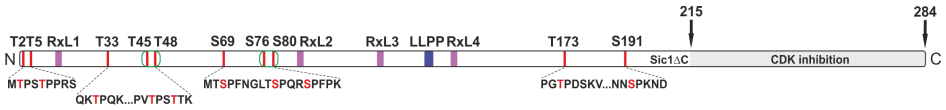

Figure 4. Schematic representation of the CDK phosphorylation sites and the interaction sites of the cyclin-dependent kinase inhibitor Sic1. Sic1 has nine CDK consensus sites: T2, T5, T33, T45, S69, S76, S80, T173, and S191, and one non-CDK consensus site T48, known to be phosphorylated by Cdk1, all 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*.

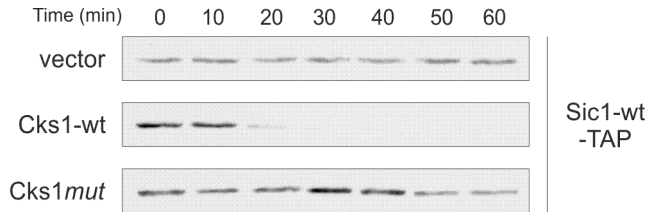

Figure 5. The effect of the phosphate-binding pocket of Cks1 on the degradation of endogenous Sic1. Cks1wt or Cks1mut were expressed from a CEN vector under a constitutive *pADH1* promoter. The endogenous *CKS1* was under a *pGALL* promoter, and its expression was repressed by growing the cells in media 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).

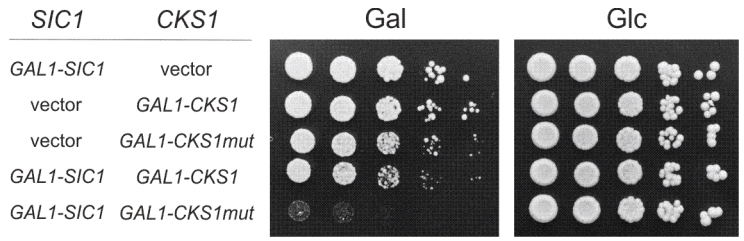

Figure 6. The importance of the Cks1 phosphate-binding pocket for suppression of Sic1-dependent inhibition of cell cycle progression. Viability assay using overexpression of both Sic1 (in CEN vector) and Cks1wt or Cks1*mut* from a pGAL1 promoter (in 2-micron vector). The expression of Cks1*mut* severely 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 üleminekul. 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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