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This dissertation was accepted for the commencement of the degree of Doctor of Philosophy in biomedical engineering on August 15th, 2018 by the Council of the Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia.

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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 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.

II 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.

III 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.

IV 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. Nat Struct Mol Biol 20(12):1415–24.

V Venta R, Valk E, Örd M, Koshik O, Shtaida N, Lepiku M, Möll K, Maljavin A, Kivi R, Pääbo K, Faustova I, Doncic A, Kõivomägi M, Loog, M. (2018) A processive phosphorylation circuit with multiple kinase inputs and mutually diversional routes controls the G1/S decision (Manuscript).

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

I I optimised the experimental methods for quantitative cyclin specificity screening. Assisted with the *in vitro* experiments, and with data analysis. I contributed with constructing plasmids, purifying enzymes and substrate proteins. I assisted with proofreading of the manuscript.

II I generated and purified Sic1 phosphoproteins for the ITC study. I performed part of the *in vitro* experiments and data analysis. I assisted with constructing plasmids, purifying proteins, and with proofreading of the manuscript.

III I designed and performed the experiments. I analyzed the data and assisted in writing the manuscript.

IV I assisted with purifying enzymes and proteins. I generated and purified Sic1 phosphoproteins for the ITC study. I assisted with proofreading of the manuscript.

V I partially designed the experiments. I performed the majority of the experiments, except the kinase assay with Fus3 and western blot in Supplementary Fig. 3 with Cln3 deletion strain. I analyzed the data and assisted in writing the manuscript.

LIST OF ABBREVIATIONS

APC	Anaphase-promoting complex, ubiquitin ligase complex at the end of mitosis
Cak	Cdk-activating kinase
Cdc	Cell division cycle, Cdc2 is *S. pombe* cyclin-dependent kinase, Cdc28 is *S. cerevisiae* cyclin-dependent kinase, the same as Cdk1, and Cdc4 is a substrate recognition subunit of the ubiquitin ligase E3 complex
Cdk	Cyclin-dependent kinase
CIP/KIP	Cdk-interacting protein/kinase inhibitory protein, a mammalian Cki family that contains the $p21^{cip1}$, $P27^{kip1}$, and $p57^{kip2}$ proteins
Cip1	Cyclin-dependent kinase inhibitor 1, Cki in *S. cerevisiae*
Ck2	Casein kinase 2
Cki	Cyclin-dependent kinase inhibitor
Cks	Cdc28 kinase subunit, *S. cerevisiae* Cks1 is a phospho-adaptor subunit of Cdk
Clb	Cyclin B, cyclins of S and M phase in *S. cerevisiae*
Cln	G1-phase type (G1-type) cyclin in *S. cerevisiae*
CTD	C-terminal domain
D1	Domain 1 in p27 KID
D2	Domain 2 in p27 KID
Far1	Pheromone-activated response 1, Cki in *S. cerevisiae*
HP	Hydrophobic patch, the substrate docking site of a certain subset of cyclins
IDP	Intrinsically disordered protein
INK4	Inhibitor of Cdk4, a mammalian Cki family protein that contains the $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$ proteins
KID	Kinase inhibitory domain, a region in Cki that is responsible for Cdk inhibition
MAPK	Mitogen-activated protein kinase
MRAIL helix	Helix in the subset of cyclins; the acronym is derived from the amino acid consensus sequence
NTD	N-terminal domain
NMR	Nuclear magnet resonance
Pho	Phosphate metabolism-related, Pho85 is a cyclin-dependent kinase in *S. cerevisiae*
pRb	Retinoblastoma protein, a mammalian transcription repressor
PSTAIRE helix	Helix in Cdks, a structural element at the cyclin-Cdk contact surface
SBF/MBF	Protein complexes regulating transcription in S. cerevisiae during G1, a metazoan E2F homolog

SCF	Skp, Cullin, F-box-containing complex, E3 ubiquitin ligase in *S-cerevisiae*
Sic1	Substrate inhibitor of cyclin-dependent protein kinase, *S. cerevisiae* Cki
Skp1	S-phase kinase-associated protein 1, adaptor protein for E3 ubiquitin ligase
Whi5	Whiskey 5, transcription repressor protein associated with the SBF/MBF complex, metazoan pRb functional homolog

1. INTRODUCTION

The cell division cycle is a process where cells duplicate their chromosomes and segregate them by forming two new daughter cells. To sustain the subsequent life of daughter cells, an errorless copy of the genome as well as adequate resources of other cellular contents must be synthesized and passed on upon division.

In all eukaryotic organisms, the cell cycle can be divided into three main phases: growth phase, S phase and mitosis. The growth phase and S phase together are also called interphase. All microscopically well distinguishable events, such as centrosome duplication, chromosomal condensation, nuclear envelope breakdown, spindle formation, chromosome separation and finally cytokinesis, were first described during the 19th century.

Cell biology reached a truly molecular level after the 1950s with the discovery of the DNA structure and its regular duplication in the cell division cycle; the interphase was additionally divided into distinct G1 and S phases, and the pause between DNA doubling and the first mitotic event was termed the G2 phase, separating the cell cycle into four timely distinct phases (Howard & Pelc, 1951; Watson & Crick, 1953; Meselson, 1958) (Fig. 1). Further studies revealed that the transitions between the phases are irreversible and unidirectional (Rao & Johnson, 1970). Additionally, in yeast and metazoan cells, G1 phase contains a certain moment where further progression toward S phase becomes independent of external (mating or mitogenic) signals, which was coined as the START-point in yeast and the restriction point in metazoans (Hartwell, et al., 1970; Pardee, 1974). Therefore, G1 phase has an early stage and a late G1 stage when the commitment decision to enter the cell division cycle is made (Fig. 1). All the main events of the cell cycle are periodically intercepted at certain points called cell-cycle checkpoints. These points are placed between the G1/S and G2/M phase and to the end of mitosis and from which further progression is impossible if progression through the previous phase is delayed or impaired (Weinert & Hartwell, 1988; Hartwell & Weinert, 1989; Hoyt, et al., 1991; Li & Murray, 1991) (Fig. 1).

The molecular guardians and assistants of this elaborate and precise mechanism have been at the main stage of molecular and cellular biology ever since these discoveries, as understanding the regulation of the cell division process has been one of the key challenges of life sciences.

2. LITERATURE REVIEW

2.1. The coordination of the cell cycle

To ensure sufficient preparedness for the cell division cycle and to carry through its main events unimpaired, the decision to enter the cell division cycle is a tightly controlled process (Morgan, 2007). At the checkpoints, the cells check whether previous cell cycle phases have produced sufficient results to trigger irreversible transition to the next cell cycle phase (Morgan, 2007). The complexity of environmental and cellular conditions and the irreversible nature of complex cell division mechanisms place considerable stress on the decision-making process and necessitate high degrees of robustness (Zhu & Yanlan, 2015).

In eukaryotic organisms, cell cycle coordination is achieved via controlling enzyme activity, protein stability, and localization (Morgan, 2007). The master regulators of the cell cycle are cyclin-dependent kinases (Cdks) (Hartwell, et al., 1970; Hartwell, et al., 1974; Lohka, et al., 1988), their interaction partner cyclins (Evans, et al., 1983; Swenson, et al., 1986; Murray & Kirschner, 1989) and cell cycle-dependent phosphatases (Nurse, 1975; Fantes, 1979; Gould & Nurse, 1991).

The general course of the cell cycle is controlled by an overall increase in Cdk activity, leading to concurrent increases in protein phosphorylation (Bloom & Cross, 2007) (Fig. 1). The number of substrates that are phosphorylated reaches its maximum at the end of mitosis during the metaphase-to-anaphase transition, after which Cdk activity is downregulated and the phosphatases dephosphorylate Cdk substrates (Fantes, 1979; Russell & Nurse, 1986; Gould & Nurse, 1991; Swaffer, et al., 2016) (Fig. 1). Cdks govern the beginning and the transition through the cell cycle, while finishing off the cell cycle is triggered by ubiquitin ligase APC, ubiquitin-directed proteolysis and phosphatase that counteract Cdk (Morgan, 2007).

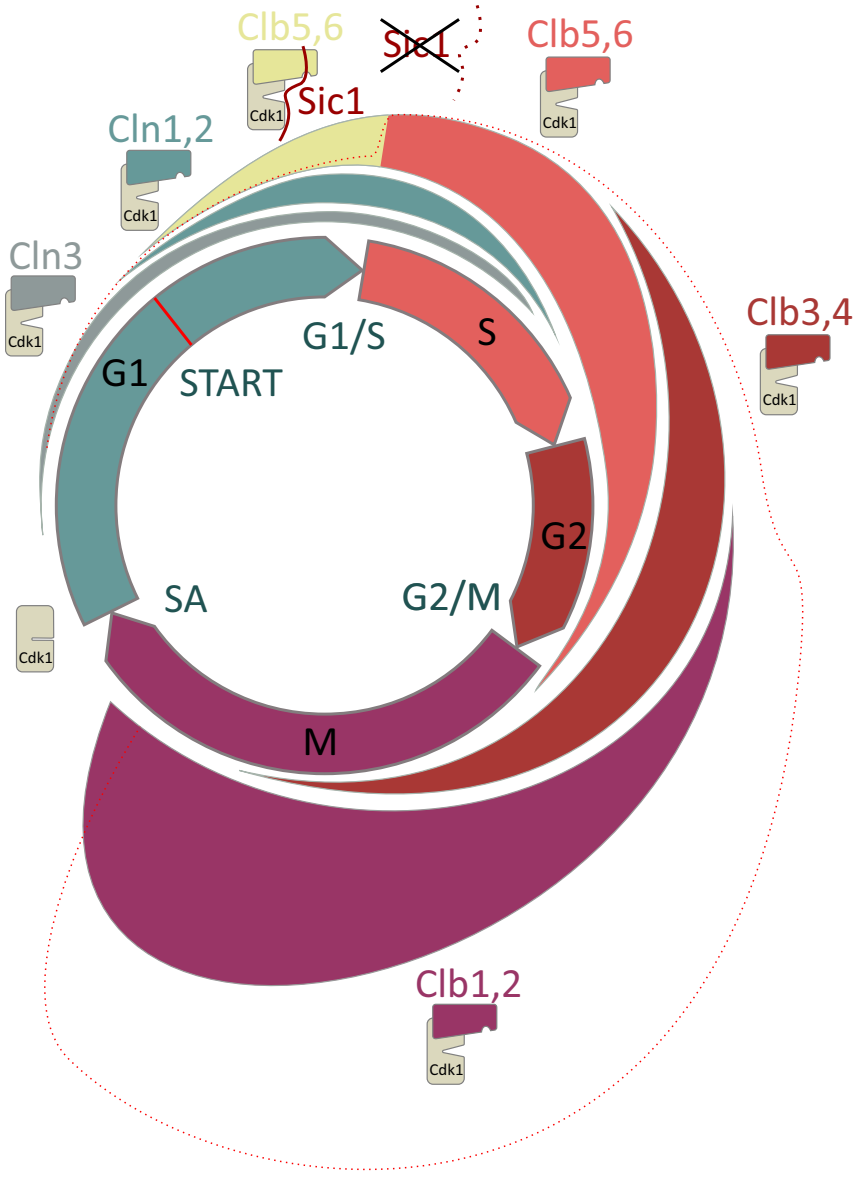

Figure 1. A generalized diagram of *S. cerevisiae* cell cycle control. Cell division progress through four phases (inner circle): G1, S, G2 and M. Each cell cycle phase is separated by irreversible transitions (arrow shapes) governed by cell cycle checkpoints (START, G1/S, G2/M and SA – spindle assembly). Each cell cycle phase is governed by different cyclin subunits activating Cdk1. The temporal order of the cyclins changes the specificity and total activity of Cdk1 (colored areas). The total Cdk1 kinase activity is rising throughout the cell cycle (dotted line, red). During the G1-phase the Cb5,6-Cdk1 activity is kept inhibited by Sic1 (yellow area). After G1/S transition Sic1 is degraded and active Clb5,6-Cdk1 released (light red area).

2.2. The Cdk holoenzyme in the cell cycle

The components of the eukaryotic cell division control system are largely conserved, from yeast to humans (Morgan, 2007). The central actuators of the cell division process are cyclins, proteins with temporal expression waves that generate oscillatory control over the activity of Cdks (Morgan, 2007) (Fig. 1). Since the initial discovery of cyclin proteins in sea urchin eggs (Evans, et al., 1983) the behavior of these proteins, including their expression, localization, and degradation, is firmly regimented in a cell cycle-dependent manner (Morgan, 2007). The catalytic partner of cyclins, cyclin-dependent kinase (Cdk), initially discovered in yeast (Nurse, 1975; Hartwell, et al., 1974) is an approximately 34 kDa protein that possesses phosphorylation activity only upon binding with its heteromeric cyclin partner (Lohka, et al., 1988; Moreno, et al., 1989). The third essential heteromeric subunit, a small protein (9–18 kDa) called Cks (Cdc28 kinase subunit), is a highly conserved binding partner to Cdk (Pines, 1996) (Fig. 3). Initially discovered in fission yeast (Hayles, et al., 1986), Cks1 is essential for cell cycle progression (Pines, 1996). Required for the assembly of some cyclin-Cdk heteromeric complexes and exhibiting a phosphate-binding pocket, Cks1 contributes to the recognition of phosphorylated substrates by the cyclin-Cdk complex (Bourne, et al., 1996; Harper, 2001; Balog, et al., 2011).

Nine Cdk interacting cyclins that have temporal cell cycle-related roles are expressed in yeast (Malumbres, 2014). In humans, at least 29 proteins are members of the cyclin protein family, and 15 of the proteins are interacting partners for the Cdks, as established by sequence similarity (Malumbres, 2014). Centrally important cyclins can be divided into two subsets: G1-and S/M-phase cyclins. The yeast G1 family contains cyclins Cln1-3, and the S/M-phase cyclins are Clb1-4. The corresponding mammalian D, E (G1), A, and B cyclin (S) families are partly interchangeable with the yeast cyclins (Morgan, 1997) (Table 1).

For cell cycle regulation in yeast, there is only one essential Cdk, a prototypical Cdk1 alternatively known as Cdc28 (Morgan, 2007). Another cell cycle regulator, Pho85, is an auxiliary nutrient sensitive Cdk (Malumbres, 2014). Four additional yeast Cdks, Kin28, Srb10, Bur1 and Ctk1, play a role in transcriptional regulation and are involved in the cell cycle only through secondary processes (Malumbres, 2014).

Although there are 20 Cdk genes in human cells, only Cdk1-6 are directly involved in cell cycle regulation, and Cdk1 is the only essential and sufficient Cdk for *in vitro* cell culture and for early embryo development (Santamaria, et al., 2007; Malumbres, 2014). Other mammalian Cdk-encoding genes either play auxiliary cell cycle roles or are not involved in cell cycle regulation (Morgan, 2007; Malumbres & Barbacid, 2005). The central human Cdk genes can be replaced by the baker's yeast Cdk1 gene or the fission yeast Cdc2 gene, and the cell-cycle regains complete regulation (Lee & Nurse, 1987; Wittenberg & Reed, 1989; Malumbres & Barbacid, 2005).

Cdk activity differentiates over time and space of the cell as it is combined with different cyclins to form an active holoenzyme complex (Fig. 1, Table 1). *Saccharomyces cerevisiae* is one of the most important prototypical model for understanding cell cycle regulation. The transition from early to late G1 in yeast is dependent on growth-dependent accumulation of Cln3-Cdk1 activity (Polymenis & Schmidt, 1997). The accumulation of the early cyclin-Cdk complex results in phosphorylation of Whi5, the transcription repressor (mammalian pRb homolog) that represses SBF-/MBF-dependent gene expression (Costanzo, et al., 2004; de Bruin, et al., 2004). The phosphorylation of Whi5 results in the derepression of its associated promoters, resulting in transcription of late G1- and S-phase-related genes (Costanzo, et al., 2004). At this point, coined as a restriction point or START, the accumulation of G1-specific Cln1,2-Cdk1 begins (Wittenberg, et al., 1990; Tyers, et al., 1993; Stuart & Wittenberg, 1995; Doncic, et al., 2011) (Fig. 1). In addition, S-phase-specific Clb5,6-Cdk1 starts to accumulate (Nasmyth & Dirick, 1991; Schwob & Nasmyth, 1993) but unlike Cln1-3-Cdk1, Clb5 and 6-Cdk1 are immediately inhibited by the yeast Cki (cyclin-dependent kinase inhibitor) Sic1 (Mendenhall, 1993; Schwob & Nasmyth, 1993) (Table 1). S phase is initiated by phosphorylation-dependent abrupt degradation of Sic1 and the subsequent release of Clb5,6-Cdk1 activity (Nughoro & Mendenhall, 1994; Schwob, et al., 1994; Donovan, et al., 1994). The progression through S phase is assisted by Clb3,4-Cdk1, and the following mitotic events are coordinated by the activation of Clb1,2-Cdk1 accumulated during S phase (Tachibana, et al., 1987; Richardson, et al., 1992; Kuczera, et al., 2010) (Table 1).

Table 1. Central cell cycle regulating Cdks, associated cyclins and Ckis. *S. cerevisiae* and metazoan core drivers of early G1 (or G0-to-G1 transition), late G1 and early S phase, S phase and G2/M phase. Adapted from (Malumbres & Barbacid, 2005; Morgan, 1997; Sherr & Roberts, 1999).

<table>
<tr><th rowspan="2">Cell cycle phase</th><th colspan="3">Yeast cells (Saccharomyces cerevisiae)</th><th colspan="3">Mammalian cells</th></tr>
<tr><th>Cdk</th><th>Cyclin partners</th><th>Cki partners</th><th>Cdk</th><th>Cyclin partners</th><th>Cki partners</th></tr>
<tr><td>G0 / G1</td><td rowspan="4">Cdk1</td><td>Cln3</td><td>Far1, Cip1</td><td>Cdk6 Cdk4</td><td>D1, D2, D3</td><td>INK4 family, CIP/KIP family</td></tr>
<tr><td>G1/S</td><td>Cln1, Cln2</td><td>Far1</td><td rowspan="2">Cdk2</td><td>E1, E2</td><td rowspan="3">CIP/KIP family</td></tr>
<tr><td>S</td><td>Clb5, Clb6</td><td>Sic1</td><td>E1, E2, A1, A2</td></tr>
<tr><td>G2/M</td><td>Clb1, Clb2, Clb3, Clb4</td><td>Sic1, Cdc6</td><td>Cdk1</td><td>A1, A2, B1, B2, B3</td></tr>
</table>

Although metazoan Cdk protein families are diverse, the general involvement of different cyclin-Cdk complexes in basic cell cycle regulation follows a prototypical model that was first described in yeast (Morgan, 2007) (Table 1). The G0 or early G1 progression to the restriction point is driven by mitogenic signals that induce the production and gradual activation of early cyclin D-Cdk4,6 complexes (Ortega, et al., 2002; Kato, et al., 1994). The activation of cyclin D-Cdk4,6 includes mitogen-dependent modulation of Cdk inhibitory proteins (Ckis) from INK4 and CIP/KIP families to release early kinase activity (Sherr & Roberts, 1999) (Table 1). The activated cyclin D-Cdk4,6 phosphorylates a transcription repressor from the retinoblastoma protein (pRb) family, which in turn relieves the E2F-dependent transcriptional program (Kato, et al., 1993; Connell-Crowley, et al., 1997). Subsequent transcription of late G1- and S-phase genes results in the production and accumulation of cyclin E-Cdk2, cyclin A-Cdk2 and cyclin A-Cdk1 complexes (Ortega, et al., 2002; Sherr & Roberts, 1999) (Table 1). During its accumulation in middle G1, cyclin E,A-Cdk2 kinase activity is tightly inhibited by binding a Cki protein belonging to the CIP/KIP family and becomes gradually active during late G1 (Galea, et al., 2008; Galea, et al., 2008). The phosphorylation-dependent degradation of the CIP/KIP family inhibitors results in the abrupt release of cyclin E,A-Cdk2 activity that initiates DNA replication and S phase (Jackson, et al., 1995; Ohtsubo, et al., 1995; Woo & Poon, 2003). After completing S phase, cells progress through mitosis by using the activity of the accumulated cyclin B-Cdk1 (Gavet & Pines, 2010; Hara, et al., 2012; Lindqvist, et al., 2009).

In both yeast and humans, the first cyclin accumulates over time under conditions of cell growth and persistent mitogenic signals (Ortega, et al., 2002; Santamaria & Ortega, 2006; McInerny, et al., 1997) (Fig. 1). This accumulation finally triggers the removal of transcriptional repression of hundreds of genes by which the other components of the cyclic oscillator of the cell cycle control system are produced. The generation of cyclic activity waves or the oscillation of different cyclin-Cdk activities following the START event of the cell cycle requires positive and negative feedback loops generated by rapid activation or inhibitory activities controlling cyclin-Cdk activity.

2.3. Structural properties of Cdk holoenzyme activation

Cyclin-dependent kinases are minimalistic protein kinases that share common structures with most serine or threonine protein kinases (Morgan, 1997). Mammalian Cdk1-6 share 41–65% of their sequence identity with prototypical yeast Cdk1, while human Cdk2 and Cdk3 share over 62% of their identity. The extensive crystallographic studies of mammalian Cdk2 (De Bondt, et al., 1993; Russo, et al., 1996; Jeffrey, et al., 1995; Davies, et al., 2001; Honda, et al., 2005; Bourne, et al., 1996; Brown, et al., 1999), Cdk1 (Brown, et al., 2015), Cdk4 (Day, et al., 2009; Takaki, et al., 2009) or Cdk6 (Brotherton, et al., 1998)

complexed with cyclin or Cks subunits provide detailed information about common structural features that are most likely conserved in yeast Cdk1.

The human Cdk2 protein kinase subunit is a bean-shaped molecule containing two lobes, the N-terminal and C-terminal, and a cleft between the lobes (De Bondt, et al., 1993) (Fig. 2). The N-terminal lobe is smaller and contains a beta sheet and PSTAIRE helix (De Bondt, et al., 1993). The C-terminal lobe is larger and contains a number of helixes (De Bondt, et al., 1993). The cleft between the lobes contains residues to accommodate ATP and a substrate and to catalyze phosphate transfer (De Bondt, et al., 1993). The N-terminal lobe is a binding surface for cyclins (Jeffrey, et al., 1995) and the C-terminal lobe is a binding surface for the Cks subunit (Bourne, et al., 1996). Before binding to a cyclin subunit, a large flexible region called the activation loop protrudes from the C-terminal lobe and blocks substrate entry (De Bondt, et al., 1993). The activation loop also contains a small L-helix that nudges toward the PSTAIRE helix, dispositioning the amino acids in the active site and preventing kinase activity (De Bondt, et al., 1993) (Fig. 2).

Cdk activation combines two steps: a fast occurring T-loop phosphorylation (T160 in Cdk2 or T169 in Cdc28) and a rate-limiting binding of cyclin (Morgan, 2007). Both modifications are important to transition the Cdk-subunit from an inactive closed conformation to an active open conformation (Jeffrey, et al., 1995). Phosphorylated cyclin complexed Cdk has an open conformation, meaning that the N- and C-terminal domains are bent to open the cleft of the active site, the T-loop is displaced away to unblock the active site, and the L-helix is completely disrupted or nudged away from the PSTAIRE-helix to arrange active site residues for catalysis (Jeffrey, et al., 1995; Brown, et al., 2015) (Fig. 3).

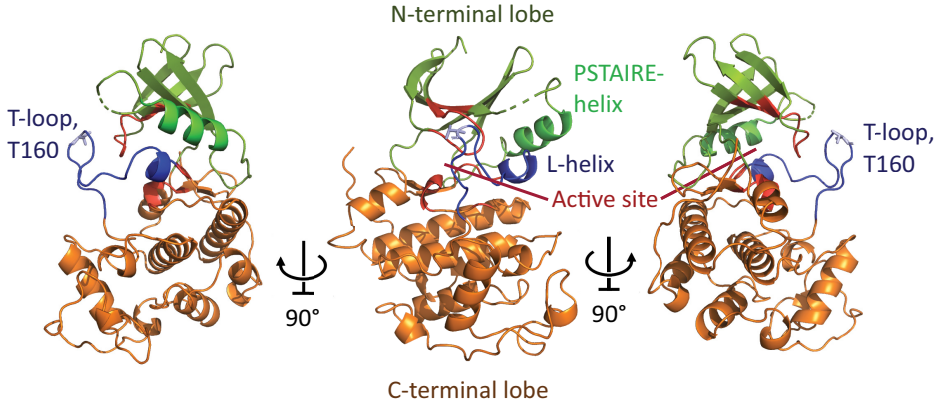

Figure 2. Ribbon diagram of the crystal structure of human Cdk2 without cyclin or the Cks subunit (PDB 1HCL). The N-terminal domain (dark green) contains the PSTAIRE-helix (light green). The C-terminal lobe is dark orange. The Cdk2 here represents the inactive conformation. The activator loop (blue) containing the L-helix and T-loop is blocking the entrance of the substrates into the active site (dark red) between the N- and C-terminal lobes. The activator phosphorylation site T160 in the T-loop is distinguished by stick-mode rendering.

The T-loop (or activator segment) is specifically phosphorylated by Cdk-activating kinase (Cak) (Lolli & Johnson, 2005). In yeast, this step is carried out by a single monomeric Cak1 protein, and in mammals, it is carried out by a heterotrimeric cyclin-dependent kinase cyclin H-Cdk7-Mat1 (Kaldis, 1999). In both yeast and mammalian cells, Cak protein levels (Espinoza, et al., 1996), kinase activity (Sutton & Freiman, 1997) and localization (Kaldis, et al., 1998) are not affected by the cell cycle (Kaldis, 1999). Interestingly, the T-loop phosphorylation in human cells follows, and in yeast precedes, the binding of the cyclin subunit (Kaldis, 1999). The phosphorylation of the T-loop triggers its conformational loosening, which, in combination with cyclin binding, results in its displacement and release of the steric obstruction (Jeffrey, et al., 1995; Brown, et al., 2015) (Fig. 3). In addition, the phosphorylated T-loop offers a surface for the substrate recognition cleft and thereby determines the ability to recognize the optimal substrate consensus motif S/T-P-X-R/K via a pocket offered for +1 positioned proline and a contact between the positively charged amino acid of the substrate consensus motif and the activator phosphate of the Cdk loop (Brown, et al., 1999).

In addition, cyclin binding to Cdk affects the conformation of the active site and its vicinity (Lolli, 2010). The surface on cyclin proteins interacting with Cdk is called the cyclin box, and it occupies a notable area around the PSTAIRE helix of the Cdk N-terminal lobe (Jeffrey, et al., 1995; Lolli, 2010) (Fig. 3). The cyclin box contains a bundle of five helixes within a region of 100 amino acids and is conserved between all the major cyclins (Lolli, 2010). The binding of the cyclin box induces large conformational changes in the Cdk subunit by shifting the PSTAIRE-helix, bending the open active site of the cleft and aligning the catalytic amino acids of the active site to be able to catalyze phosphate transfer (Jeffrey, et al., 1995) (Fig. 3). As the cyclin subunit experiences almost no conformational changes during the binding event, the Cdk subunit is virtually molded against the rigid cyclin surface (Jeffrey, et al., 1995).

The sequence composition and structural features flanking the cyclin box are variable between different cyclins (Lolli, 2010). This divergence generates the cyclin-specific activation pattern of individual cyclin-Cdk complexes. For example, G1 cyclins are weak in forming contacts with the T-loop and controlling its conformation, whereas S-phase cyclins actively mold the T-loop by melting its L-helix and redirecting it via direct binding (Brown, et al., 2015; Day, et al., 2009; Takaki, et al., 2009) (Fig. 3). Additionally, G1-, S- and M-phase cyclins provide structural differences to the MRAIL motif required for substrate recruitment for Cdk (Jeffrey, et al., 1995; Brown, et al., 2015; Day, et al., 2009; Brotherton, et al., 1998; Takaki, et al., 2009; Petri, et al., 2007). Finally, different cyclins are unique sites for posttranslational modifications and therefore allow different regulation by the cellular machinery (King, et al., 1996; Clurman, et al., 1996; Won & Reed, 1996; Gallant, et al., 1995; Diehl, et al., 1997; Lim & Kaldis, 2013).

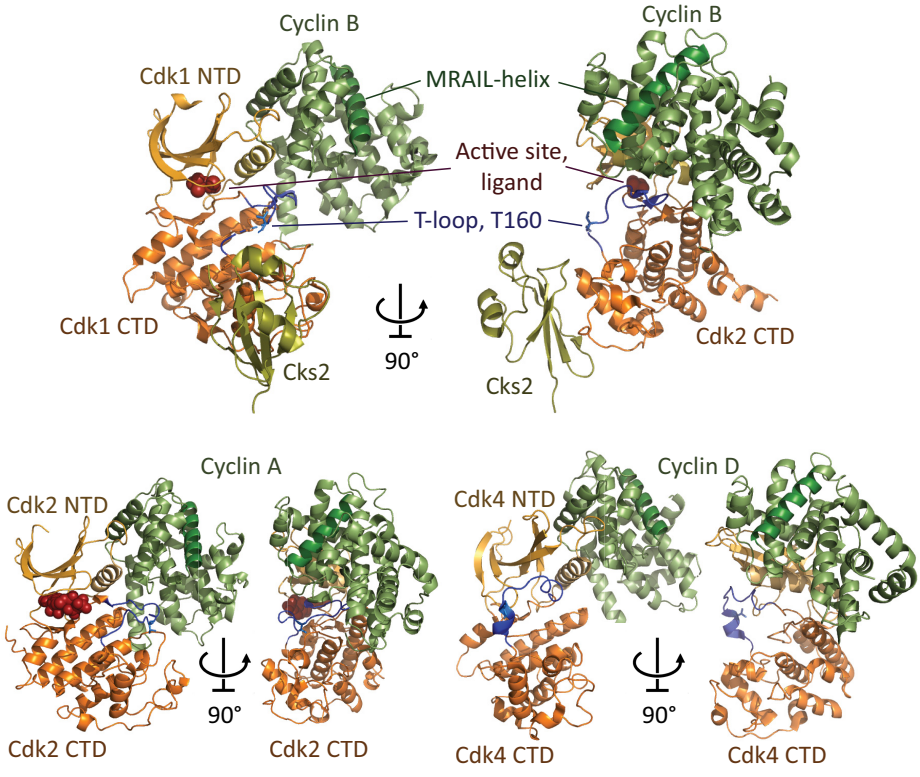

Figure 3. Crystal structure of the activated cyclin-Cdk-Cks holoenzyme. Upper panel: human cyclin B-Cdk1-Cks2 holoenzyme with a ligand in the open Cdk conformation active site (PDB 4YC3, (Brown, et al., 2015)). Cyclin (green) binds to the Cdk N-terminal lobe (light orange) and its PSTAIRE-helix (dark orange), whereas Cks (olive green) binds to the C-terminal lobe (orange) of Cdk. The cyclin MRAIL-helix forms a floor for the hydrophobic substrate docking cleft (HP). Both the cyclin and Cks substrate docking pockets are accessible in the same direction to the Cdk active site. Bottom panel: crystal structures of human S-phase cyclin A-Cdk1 (PDB 1FIN, (Jeffrey, et al., 1995)) and early-G1-phase cyclin D3-Cdk4 (PDB 3G33, (Takaki, et al., 2009)) positioned in similar projections. Cyclin A and D fold the Cdk subunit to different extents. The active site of cyclin A-Cdk2 (contains dark red ligand) is more accessible for outer substrates than is cyclin D3-Cdk4 (no ligand present). The L-helix (blue) is weakly disturbed in Cdk4, and the T-loop (blue) is obstructing the entrance of the active site of the cleft. Cyclin A and B disturb the Cdk L-helix completely and displace the T-loop.

2.4. The cyclin-dependent kinase inhibitors (Ckis)

The function of the cyclin-dependent kinase inhibitors is to inhibit cyclin-dependent kinase activity, and they act in an oscillatory manner, supporting the overall temporal organization of the cell cycle (Morgan, 2007). Ckis quench Cdk activity while finishing the previous cell cycle, maintaining the inhibited state for G0 arrest and the G1 phase, and regulating the release of Cdk activity upon entry into S phase (Morgan, 2007).

The first Cki was discovered in yeast. *S. cerevisiae* Sic1 protein was found to be a tightly bound substrate of the cyclin-Cdk1-Cks1 complex (Reed, et al., 1985). At the same time, Sic1 also acts as a nanomolar inhibitor for S-phase-specific cyclin-Cdk1 kinase activity, but not for G1-specific cyclin-Cdk1 activity (Mendenhall, 1993). The detectable expression and inhibitory activity of Sic1 protein is limited to G1 (Mendenhall, et al., 1987; Wittenberg & Reed, 1988). The gene was named after *s*ubstrate *i*nhibitor of *c*yclin-dependent protein kinase *Sic1* (Nughoro & Mendenhall, 1994).

Although Sic1 transcripts can be detected at low levels across the cell cycle (Schwob, et al., 1994; Amoussouvi, et al., 2018) its expression is still cell-cycle regulated, peaking in late mitosis (Donovan, et al., 1994). In parallel, Sic1 protein disappears at the G1/S transition and begins to reaccumulate in late mitosis (Donovan, et al., 1994; Schwob, et al., 1994). Sic1 contains 9 optimal and suboptimal Cdk sites and its degradation in G1/S is related to its hyperphosphorylation by Cdk kinases (Verma, et al., 1997) (Fig. 4).

The analysis of deletion or overexpression phenotypes for Sic1 in yeast reveal defects in chromatin integrity and mitotic entry, suggesting a role in coordinating S- and M-phase Cdk activity at M/G1 and, more importantly, in G1/S transitions (Nughoro & Mendenhall, 1994; Schwob, et al., 1994; Donovan, et al., 1994). The presence of Sic1 in G1 is associated with inhibiting Clb5,6-Cdk1 activity and allowing its accumulation in the inhibited form prior to S-phase initiation (Schwob, et al., 1994; Schneider, et al., 1996). As discussed later, the basic function of Sic1 is to control the timely release of S-phase Cdk activity to facilitate coordinated and irreversible initiation of DNA replication (Lengronne & Schwob, 2002; Schwob, et al., 1994). Sic1 is suggested to be a mammalian $p21^{cip1}$ or $p27^{kip1}$ functional homolog (Peter & Herskowitz, 1994; Barberis, et al., 2005).

In mammalian cells, there are two families of Cki proteins that regulate the activity of Cdks: INK4 and CIP/KIP. The INK4 (*Inhibitors of CDK4*) family consists of four proteins: $p16^{INK4a}$ (Serrano, et al., 1993), $p15^{INK4b}$ (Hannon & Beach, 1994), $p18^{INK4c}$ (Guan, et al., 1994; Hirai, et al., 1995), and $p19^{INK4d}$ (Chan, et al., 1995; Hirai, et al., 1995). They specifically modulate Cdk4- and Cdk6-dependent kinase activity at G0 or during the early stage of G1 (Sherr & Roberts, 1999). The CIP/KIP family (*C*dk *i*nteracting *p*rotein/*K*inase *i*nhibitory *p*rotein) contains three proteins: $p21^{cip1/waf1}$ (Gu, et al., 1993; Xiong, et al., 1993; Harper, et al., 1993), $p27^{kip1}$ (Toyoshima & Hunter, 1994; Slingerland, et al., 1994; Polyak, et al., 1994), and $p57^{kip2}$ (Matsuoka, et al., 1995; Lee, et al.,

1995). These inhibitors modulate cyclin-Cdk activity during G1, the G1/S transition and at the onset of G1 after completion of the previous mitosis (Sherr & Roberts, 1999). The INK4 family of inhibitors is robust and specifically targets the Cdks early in the cell cycle to attain a complete inhibition of G0 or early G1, while the CIP/KIP family of inhibitors contains more versatile modulators of cyclin-Cdk activities across the cell cycle (Sherr & Roberts, 1999).

Contrary to mammalian cells, in *S. cerevisiae* cells, G1 Cdk activity is conditionally inhibited upon mating (pheromone pathway activation), starvation or extreme stress. In haploid yeast cells, the presence of the mating pheromone α-factor induces Far1, which inhibits G1-cyclin Cdk activity (Chang & Herskowitz, 1990). Additionally, if cells experience phosphate starvation, a nutrient-sensitive inhibitor Pho81 will target a particular Cdk called Pho85 in the G1 phase (Schneider, et al., 1994). A stress-activated Cln1-3 inhibitor Cip1 has been discovered (Ren, et al., 2016; Chang, et al., 2017). Of these, only the Pho81 inhibitory domain is considered similar to the mammalian INK4 family of inhibitors (Schneider, et al., 1994). With no stress or pheromones present, the yeast cell cycle uses Sic1 as the main regulatory inhibitor of Cdk1 activity (Mendenhall, 1993).

2.5. Structural properties of Sic1

Sic1 (Brocca, et al., 2009; Mittag, et al., 2008; Liu, et al., 2014; Mittag, et al., 2010) and the mammalian KIP/CIP family proteins $p21^{cip1}$ (Kriwacki, et al., 1996) and $p27^{kip1}$ (Bienkiewicz, et al., 2002) are intrinsically disordered proteins (IDPs) that do not adopt a stable secondary or tertiary fold in the free solution state. As an isolated molecule in solution, Sic1 adopts a variety of extended and compacted forms that alternate dynamically in the timescale of molecular diffusion (Liu, et al., 2014). Such intrinsic disorder is a common property of proteins involved in signal transduction (Dunker, et al., 2005; Daughdrill, et al., 2008). Structural disorder in solution or a dynamically volatile structure characterize a broad class of regulatory proteins or loops of otherwise structured proteins, including many inhibitory proteins (Lee, et al., 2014; Wright & Dyson, 2009; Galea, et al., 2008).

In its primary structure, Sic1 contains 284 amino acids. The kinase inhibitory domain (KID) of Sic1 is located in a 70 amino acid-long stretch in its C-terminus (Hodge & Mendenhall, 1999) (Fig. 4). The N-terminal region of 159 amino acid comprises a multiphosphorylation pathway together with 7 optimal Cdk phosphorylation sites and is necessary for Sic1 Skp1/Cdc4-dependent destruction mediated by the SCF^{Cdc34} ubiquitin ligase during the G1/S transition (Verma, et al., 1997; Verma, et al., 1997). Another two optimal Cdk sites together with a casein kinase 2 (Ck2) consensus site were located in the C-terminal region right before the Cdk inhibitory domain (T173, S191 and S201, respectively, Fig. 4). The role of the secluded Cdk sites at the C-terminal has been addressed recently and will be discussed later, whereas the S201 site in

Sic1 is a target for Ck2, and its phosphorylation is related to Clb5,6-Cdk1 accumulation in G1, suggesting a role in the functioning of the Sic1 KID (Coccetti, et al., 2004).

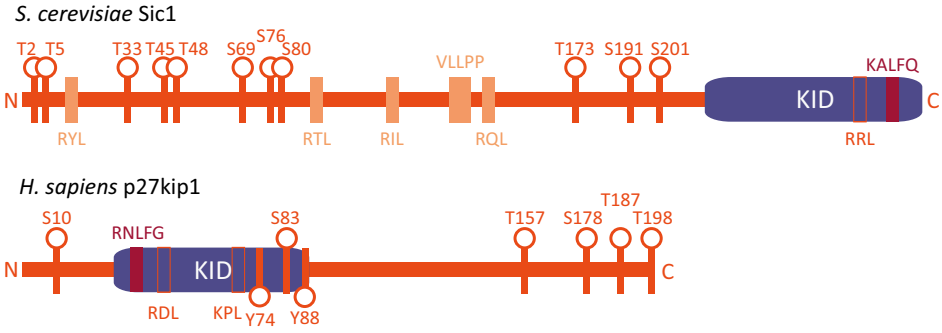

Figure 4. Domain organization of *S. cerevisiae* Sic1 and human p27 relative to primary amino acid sequences. Both proteins contain a kinase inhibitory domain (KID, blue) and a regulatory domain (orange) containing multiple phosphorylation input sites (sticks with circles). A conserved K/RXLF sequence motif is located at one end of the KID (red). Other R/KXL motifs are located in the KID and are marked as open boxes (orange). Sic1 contains at least 10 identified phosphorylation input sites, whereas p27 contains at least 8 (Lu & Hunter, 2010). In addition to phosphorylation sites, several cyclin docking motifs (R/KXL and VLLPP) have been noted for Sic1 (light orange). The cyclin docking sites in the regulatory part (if existent) are left unmarked in p27. If present, they have different sequence motifs than the *S. cerevisiae* RXL and VLLPP docking motifs.

The KID of Sic1 was mapped by using Sic1 9-AP version with all optimal Cdk sites mutated to alanine (Hodge & Mendenhall, 1999). The overexpression of such mutations causes yeast cells to arrest in G1 with elongated multiple bud morphology, indicating that Clb5,6-Cdk1 activity is inhibited while Cln1-3-Cdk1 activity is unperturbed (Dirick, et al., 1995; Mendenhall, 1993). By generating a series of truncations of stable Sic1, cellular arrest was maintained only if the C-terminal region between 215 and 284 was intact (Hodge & Mendenhall, 1999). Deletions exceeding position 215 and deletion of most of the C-terminal stretch of 11 amino acids starting from position 273 lead to a loss of cell cycle arrest (Hodge & Mendenhall, 1999).

2.5.1. Inhibitory domains of Sic1 and human p27

The mechanism of how Cki proteins inhibit their targets is revealed in detail for mammalian p27 (Russo, et al., 1996; Bienkiewicz, et al., 2002; Lacy, et al., 2004; Sivakolundu, et al., 2005; Otieno & Kriwacki, 2012). In p27, the KID is mapped onto the N-terminal of the protein (region at positions 22–104, Fig. 4)

(Russo, et al., 1996). The domain contains an unstructured N-terminal domain 1 (D1) and a C-terminal domain 2 (D2) spaced by a linker helix (LH), that constitute the only distinguishable secondary structure of the protein (Bienkiewicz, et al., 2002; Lacy, et al., 2004) (Fig. 5).

Binding to cyclin A-Cdk2 takes place in a multistep manner (Lacy, et al., 2004). The first interaction forms rapidly between the small and highly dynamic D1 and the cyclin subunit (Lacy, et al., 2004). The RXLFG sequence motif from D1 binds into the substrate recruitment cleft of cyclin A at the MRAIL-helix (Russo, et al., 1996; Lacy, et al., 2004) (Fig. 5). The interaction results in almost no conformational changes to the cyclin-Cdk complex but is necessary to fold the linker helix of KID, which in turn directs D2 to bind Cdk2 (Lacy, et al., 2004). Only after this step do slow but comprehensive structural changes result, forming a ternary inhibitory complex (Lacy, et al., 2004). In this complex, D1 blocks the cyclin substrate recruitment site, and the small 3_{10}-helix from D2 occupies the Cdk active site by substituting ATP and shielding the activator tyrosine residue for Cak (Russo, et al., 1996; Lacy, et al., 2004) (Fig. 5).

The RNLFG sequence in p27 is important for the recruitment of the inhibitor to initiate its folding (Lacy, et al., 2004; Ou, et al., 2011). The multiple sequence alignment of Sic1 KID with the corresponding region of the fission yeast *S. pombe* functional homolog Rum1 (Sánchez-Díaz, et al., 1998) shows a conserved similar R/KXL sequence at the C-terminal end (Fig. 5). Additionally, the Sic1 KID contains one more R/KXL motif in its C-terminal region. Hodge and Mendenhall tested both of them, as the KXL at position 260 was disturbed by introducing point mutations and the C-terminal KALF was removed by the truncation starting from position 274, and they found that both mutations result in the loss of arrest in overexpressed Sic1-9AP (Hodge & Mendenhall, 1999). *S. pombe* Rum1 only contained the conserved C-terminal R/KXLF motif (Fig. 5).

Another similarity to the p27 KID is that the middle of the KID Sic1 in solution exhibited a similar short α-helical preorganized secondary structure with a similar amphiphilic profile (Brocca, et al., 2009; Barberis, et al., 2005; Bienkiewicz, et al., 2002). The p27 linker helix, despite having almost no sequence homology to the other CIP/KIP family members, demonstrated a conserved secondary structure and dynamic properties (Sivakolundu, et al., 2005). The structure of this helix is crucial to the correct folding of the inhibitor to the cyclin-Cdk complex (Otieno & Kriwacki, 2012). Therefore, it has been suggested that the Sic1 KID could bind the cyclin-Cdk complex in a manner similar to that of p27 (Barberis, et al., 2005).

The positioning of the KID in proteins and the order of the KID components in *S. cerevisiae*, *S. pombe* and p27 are relatively different. In Sic1, the KID is C-terminally located (Hodge & Mendenhall, 1999), whereas in Rum1, it is located in the center of the protein (positions 87–147) (Sánchez-Díaz, et al., 1998). In p27, the KID is located in the N-terminal part of the protein (Russo, et al., 1996). In Sic1 or Rum1, the positioning of potential conserved recruitment of the RXL motif with respect to the linker helix is reciprocal compared with that of p27 (Fig. 5).

At its C-terminal end, the p27 KID contains phosphorylation sites (Y74 and Y88) that affect its function (Grimmler, et al., 2007; Chu, et al., 2007; Ou, et al., 2012) (Fig. 4 and 5). In Sic1, no such phosphorylation sites have been identified yet, although a casein kinase 2 (Ck2) target (S201) in the N-terminal vicinity of the KID (Fig. 4) affected its inhibitory function for S-phase cyclin activity *in vivo* (Coccetti, et al., 2004). The site is missing in *S. pombe* Rum1. A Ck2 target site (S83) is positioned in D2 of the p27 KID domain, and thus, its phosphorylation could possibly alter its inhibitory function (Tapia, et al., 2004; Rath & Senapati, 2016).

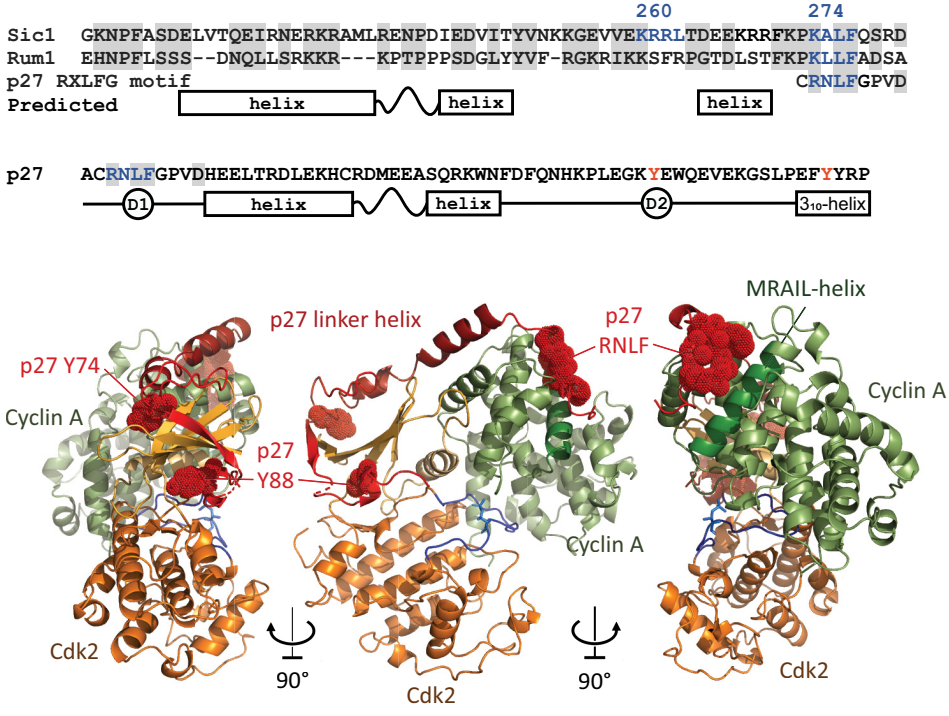

Figure 5. Yeast KID is similar to mammalian p27 but has reciprocal domain architecture. Upper panel: multiple sequence alignment of *S. cerevisiae* Sic1 KID (positions 215–281) and *S. pombe* Rum1 KID (positions 89–149) produced by MUSCLE (3.8) using default options. Similar amino acid positions are highlighted in gray. The conserved R/KXLF region indicated in blue and the corresponding sequence from human p27 were aligned. PHD and PSIDRED predicted α-helical structures, marked by rectangles. Middle panel: p27 kinase inhibitory domain from positions 28 to 91. The RXLFG sequence is highlighted in blue. Domain D1 and domain D2 are noted by a line with a circle. A helical secondary structure assessment from (Bienkiewicz, et al., 2002; Lacy, et al., 2004). The RXLFG motif binds the cyclin subunit HP at MRAIL-helix and the 3_{10}-helix binds to the Cdk catalytic site. Tyrosine residues Y74 and Y88 are indicated in orange. Bottom panel: ternary inhibitory complex of the p27 KID with cyclin A-Cdk2, crystal structure (1JSU, (Russo, et al., 1996)). Cyclin subunit colored in green, Cdk2 in orange and the p27 KID in red. The RNLF motif and Y74 and Y88 sites are represented as dotted spheres. The surrounding amino acids at Y74 are part of the 3_{10}-helix in D2.

Attempts have been made to inhibit human cyclin A-Cdk2 with Sic1, but the measured Kd values for cyclin A-Cdk2 binding (Barberis, et al., 2005) are two orders of magnitude weaker than those with p27 (Bienkiewicz, et al., 2002; Lacy, et al., 2004) (Table 2). In accordance with Ki measurements using H1 as a model substrate, Sic1 acts on cyclin A-Cdk2 as a competitive inhibitor forming much looser contacts than p27 does (Barberis, et al., 2005; Lacy, et al., 2004).

Table 2. Kd values of Sic1 and p27 binding to the human cyclin A-Cdk2 complex.

Cdk component	Kd (nM), SPR*	Kd (nM), ITC*	Kd (nM), SPR*
Cdk2	19000	70	248
cyclin A	5100	25	50
cyclin A-CDK2	200	3,5	4,4
Reference	(Barberis, et al., 2005)	(Lacy, et al., 2004)	(Grimmler, et al., 2007)

* SPR – Kd measured by surface plasmon resonance with an immobilized inhibitor; ITC – Kd measured in solution by isothermal titration calorimetry.

It is doubtful whether Sic1 is able to trigger similar conformational changes in cyclin A-Cdk2 as p27 does. However, despite the lack of strong binding to cyclin A-Cdk2, nanomolar binding with the native interaction partner of Sic1, Clb5-Cdk1, takes place (Mendenhall, 1993). The common unstructured feature and cryptic helical region of the KID and the tight inhibition suggest that upon binding to Clb5-Cdk1, Sic1 induces similar comprehensive structural folding of its Cdk partner. Internal disorder itself is one of the common mechanisms related to the folding of cyclin-Cdk complexes, despite the differences in specific docking contacts.

2.6. The N-terminal phosphorylation pathway and Sic1 destruction

During the G1/S transition, Sic1 protein is degraded via SCF^{Cdc34}-mediated proteolysis (Schwob, et al., 1994). The proteolysis of Sic1 is orchestrated by phosphorylation-dependent recognition of Sic1 by the Cdc4-Skp1 phospho-adaptor subunit of the SCF^{Cdc34} complex (Verma, et al., 1997; Verma, et al., 1997; Feldman, et al., 1997; Skowyra, et al., 1997). The multiphosphorylation mechanism of Sic1 protein leading to binding of Cdc4 as an output has led to grown interest in the dynamics of Sic1 phosphorylation and the binding specificity of Cdc4 and the dynamic relationship between these two processes.

2.6.1. Sic1 phosphorylation and degradation

Achieving irreversibility of the G1/S transition while simultaneously maintaining control over the timing of this process is an essential requirement of cell cycle regulation (Nasmyth, 1995; Tyson, et al., 1995). The phosphorylation and subsequent degradation of Sic1 is a key event in the G1/S transition and, as demonstrated, requires Cdk1 kinase activity (Dirick, et al., 1995; Tyers, 1996; Schneider, et al., 1996). As Clb5,6-Cdk1 activity is blocked in G1 by Sic1 inhibition, Cln1-3-Cdk1 has been suggested to be mainly responsible for phosphorylating Sic1 and sending it into degradation (Dirick, et al., 1995; Tyers, 1996; Schneider, et al., 1996). On the other hand, Cln1-3 activity is available far before Sic1 degradation and initiation of S phase (Tyers, et al., 1993). Therefore, how cells delay Sic1 degradation, and more importantly, how they manage the abrupt and timely degradation of Sic1 for the proper initiation of S phase (Lengronne & Schwob, 2002; Schwob, et al., 1994) with relatively slow accumulating G1 enzymes is controversial.

The hypothesis that Cln1-3 are responsible for Sic1 degradation was tested by genetic studies with yeast strains lacking Cln1-2. In such strains, either Cln3 overexpression or Sic1 deletion is necessary for rescue from G1 arrest (Dirick, et al., 1995; Tyers, 1996; Schneider, et al., 1996). Minimal attention has been paid to the fact that the rescue of Sic1 on a Cln1-3 deletion background is offered by ectopically overexpressing the S-phase cyclin Clb5 (Epstein & Cross, 1992; Schwob & Nasmyth, 1993). In addition, native expression of Clb5 is Cln3-dependent through the activation of MBF-related transcription (Tyers, et al., 1993; Dirick, et al., 1995; Stuart & Wittenberg, 1995). All that Cln3 overexpression offers is early activation of Clb5, which at some point simply leads to levels of Clb5 that exceed those of Sic1 and therefore promote Sic1 phosphorylation and release from the arrest. Thus, Cln3 overexpression might rescue Sic1 by changing its activity on Clb5 transcription. In addition, Cln3 enhances the transcription of Pcl1,2 cyclins of Pho85, which, when overexpressed, contribute significantly to Sic1 degradation (Nishizawa, et al., 1998).

An elegant biochemical demonstration of Sic1 poly-ubiquitination is presented by Verma, Feldman, Skowyra and colleagues who successfully reconstituted SCF^{Cdc34}-dependent ubiquitin ligase activity *in vitro* (Verma, et al., 1997; Verma, et al., 1997; Feldman, et al., 1997; Skowyra, et al., 1997). They demonstrate that for Sic1 ubiquitination *in vitro*, extensive phosphorylation by purified Cln2-Cdk1, Clb2-Cdk1 (Verma, et al., 1997; Feldman, et al., 1997) or Clb5-Cdk1 (Feldman, et al., 1997; Skowyra, et al., 1997) complexes is necessary. However, the kinetic parameters of the phosphorylation reactions performed did not allow the quantitative assessment of cyclin specificity in connection with the Sic1 ubiquitination reaction. Verma, Feldman, Skowyra and colleagues demonstrated that for Sic1 ubiquitination, phosphorylation at multiple N-terminal sites in the region of 1–105 are necessary (Verma, et al., 1997), especially the sets containing T33, T45 and S76 (Verma, et al., 1997) or T5, T45 and S76 (Feldman, et al., 1997) and that these sites are crucial for binding

Cdc4-Skp1 (Feldman, et al., 1997). Moreover, ubiquitination of Sic1 does not depend upon its binding to the Clb5-Cdk1 inhibitory complex (Verma, et al., 1997), and ubiquitination successfully dislodges Sic1 from the Clb5-Cdk1 inhibitory complex (Feldman, et al., 1997) by releasing the functional Clb5-Cdk1 complex (Verma, et al., 2001).

Albeit these studies prove that Sic1 degradation is phosphorylation-dependent and requires the SCF^{Cdc34} pathway, it remains an open question through which kinases and by which dynamics the Sic1 multiphosphorylation process leads to its destruction. These questions are addressed in detail in the Results and discussion section.

2.6.2. Sic1 binding specificity to the $Cdc4^{SCF}$ subunit

The important question in the regulation of Sic1 degradation is related to the binding specificity of Cdc4 and has been a much-debated subject during the past decade. No single Cdk site in Sic1 possesses the optimal consensus sequence for Cdc4 binding (Nash, et al., 2001) and is insufficient for Sic1 degradation *in vivo* (Verma, et al., 1997; Nash, et al., 2001). For *in vivo* Sic1 destruction, a set of multiple sites must be phosphorylated (Nash, et al., 2001). Initially, Cdc4 binding was expected to take place through single optimal TP or SP sites (Nash, et al., 2001; Orlicky, et al., 2003) but the lack of optimal binding sites in Sic1 and its multiphosphorylation suggested an allovalent binding model. This model states that Cdc4 is able to multiply six nonoptimal sites in phosphorylated Sic1 into high affinity dynamic binding equilibrium: the allovalent interaction (Klein, et al., 2003; Nash, et al., 2001). The allovalency of Cdc4 was soon challenged by structural studies of phosphorylated substrates bound to the human Cdc4 homolog Fbw7 and later to Cdc4 itself (Wu, et al., 2003; Hao, et al., 2007; Csizmok, et al., 2017) and by detailed analyses of Sic1 phosphorylation pathways (reviewed in the Results and discussion section).

The main confusion about Cdc4 binding to Sic1 arises from the propeller-shaped WD40 substrate recruitment domain of Cdc4 that recruits substrates via many different binding modes. For example, optimal degron in human cyclin E at T380 can interact both via single phosphorylated threonine (LL**T**PPQSG) (Orlicky, et al., 2003) and via a doubly phosphorylated form of the same motif (LL**T**PPQ**S**G) (Hao, et al., 2007). As shown by Hao and colleagues, the first interaction generated a Kd of approximately 1 μM and the second interaction generated a Kd of approximately 0,07 μM (70 nM) (Hao, et al., 2007). The main phosphorylated threonine, pT380, inserts itself into a positively charged pocket in the central part of the WD40 domain whereas auxiliary S384 makes additional contact through the distal positively charged surfaces (Hao, et al., 2007; Welcker & Clurman, 2007; Csizmok, et al., 2017). Moreover, in the case of a single phosphorylated threonine in human cyclin E, the dimerization of Fbw7 improves the binding affinity of the protein over the monomeric form, whereas doubly phosphorylated degron restricts Fbw7 dimerization (Welcker &

Clurman, 2007). Similarly, yeast Cdc4 forms dimers, and although dimerization do not appear to enhance the binding affinity of phosphorylated Sic1, it is indispensable for its ability to process Sic1 for destruction *in vivo* (Tang, et al., 2007).

With Sic1, the binding affinity (Kd) of single site phosphorylated forms of Cdc4 fall into the range of 10–100 μM (Orlicky, et al., 2003). In contrast, Sic1 peptides with certain diphosphorylated sites, called double degrons, at either combinations of pT5/pT9, pT45/pS48 or pS76/pS80, bind Cdc4 with an affinity below 4 μM (Hao, et al., 2007). Further, full-length Sic1 phosphorylated from multiple Cdk sites bind Cdc4 with a Kd of approximately 1 μM, which is sufficient for Sic1 degradation *in vivo* (Orlicky, et al., 2003; Hao, et al., 2007; Tang, et al., 2012; Csizmok, et al., 2017). It is important to add that full-length phosphorylated Sic1 preparations used in binding assays (Orlicky, et al., 2003; Mittag, et al., 2008; Csizmok, et al., 2017; Hao, et al., 2007) contain the largest populations of Sic1 with 6-phosphates (T5, T33, T45, S69, S76, and S80). As these preparations exclude T9 or T48 from the assay, they are limited to only one fully phosphorylated double degron S76/S80 (Mittag, et al., 2008).

NMR (nuclear magnet resonance) studies using a p69/pS76/pS80 triply phosphorylated peptide demonstrate that Sic1 binds Cdc4 through two phosphates, although S69 might negatively interfere with the positioning of S76 in the main phosphate binding pocket (Csizmok, et al., 2017). Unfortunately, there are no available affinity or structural studies for Sic1 that simultaneously encompass both pairs (pT45/pS48 and pS76/pS80) of high-affinity degrons to form contacts with a Cdc4 monomer or dimer. Therefore, how Cdc4 exposes Sic1 for fast ubiquitination *in vivo* remains a question open for discussion.

3. STUDY OBJECTIVES

Although cyclins and Cdks have been known as master regulators of cell cycle for decades, we still do not understand how they coordinate the complex events of cell division involving temporal resolution in the regulation of hundreds of substrates. One of the major enigmas in this research field has been the phenomenon of multisite phosphorylation. The vast majority of Cdk targets are phosphorylated at multiple sites at disordered regions, but the mechanism and logic of the signal processing is a mystery. Moreover, it is unknown whether the linear patterns of phosphorylation sites and kinase docking sites carry a certain code for Cdk signal processing and the temporal ordering of the cell cycle events. An intrinsically disordered protein, Sic1, a multisite target of Cdk and a G1/S transition point, serves as one of the central model systems for studying Cdk function and multisite phosphorylation. The general objective of this study is to unveil the Sic1 multisite phosphorylation mechanism and its role in the G1/S switch. The specific objectives of this study are listed below.

1) To map the phosphorylation specificity in yeast cyclin-Cdk complexes with respect to the specificity of the Cdk active site and cyclin-dependent docking (I).
2) To investigate the dynamics of multisite phosphorylation of Sic1 and to analyze the specific roles of G1- and S-Cdk complexes in Sic1 phosphorylation and degradation in G1/S. Critical evaluation of the distributive multisite phosphorylation model as s general basis of ultrasensitivity of the cell cycle switches (II, III, IV).
3) To perform a systems level analysis of Sic1 degradation at the G1/S switch based on biochemical evidence gathered in paper II.
4) To define the biochemical parameters controlling the multisite phosphorylation of target proteins by yeast cyclin-Cdk1-Cks1 complexes (III, IV).
5) To study the role and mechanism of intracomplex phosphorylation in controlling the dynamics of Sic1 degradation and the G1/S transition (V).

4. MATERIALS AND METHODS

Recombinant cyclin-dependent kinase holoenzyme complexes for *in vitro* characterization were purified from *S. cerevisiae* carrying an Δ*sic1* allele by using ectopic galactose promoter-driven overexpression of tagged cyclin as in Ref. I and as described for the Clb1-5 cyclins and for Cln2(Puig, et al., 2001; Ubersax, et al., 2003) (McCusker, et al., 2007). The presence of the Cks1 subunit in the complex and the optimal Cks1 concentrations for the *in vitro* kinase assay are described in Ref. II Supplementary Fig. 1, and purification was carried out as described in (Reynard, et al., 2000). Sic1, Sic1ΔC and other substrate proteins were purified as 6xHis tagged recombinant proteins from *E. coli* as described in Ref. I.

The composition and setup of *in vitro* kinase assays for assessing Michaelis-Menten kinetics in Ref. I–III is described in Ref. II. The intracomplex phosphorylation assay for Clb5-Cdk1-Cks1 used in Ref. IV–V is described in Ref. V. For quantifying the phosphorylation signals, trace amounts of [γ-^{32}P]ATP (Perkin Elmer) were used, with a final activity of 0,1 mCi/ml for Michaelis-Menten kinetics and 1 mCi/ml for the intracomplex phosphorylation assay. For signal quantification, a Typhoon 9410 GE Healthcare phosphoimager and its software was used.

For the quantitative titration of the intramolecular phosphorylation signal to reveal the number of phosphates added per molecule of bound Sic1 in Ref. V, the parallel full phosphorylation of the Cdk1 subunit from its inhibitory site, Y19, was used as a standard. The full phosphorylation was assessed by using excess amounts of Swe1-TAP purified kinase (1 μM) cross-checked by the residual activity of a targeted Clb5-Cdk1 pool (Ref. V, Supplementary Fig. 1b,c).

For running denaturing PAA gel electrophoresis, standard methods were used. To separate phosphorylated isoforms by using denaturing PAA gels, a phosphate-binding ligand (PhosTag, 100 μM final concentration) with Mn^{2+} was used to supplement the separation of the gel according to the manufacturer's instructions. Gels were run at 15 mA per 0,75 mm for 3 hours.

Mass spectrometry analysis for intramolecularly phosphorylated Sic1 was carried out as described in Ref. II. For the assay, the intracomplex phosphorylation mixture was separated on a denaturing SDS-PAA gel, and the Sic1 band was excised for further in-gel protease digestion.

An isothermal calorimetry assay was performed in collaboration with Seth Rubin's group as described in Ref. II and Ref. III. To phosphorylate the Sic1ΔC protein, Clb2-Cdk1 enzyme without added Cks1 was used, as described in Ref. II.

For yeast culturing for Western blot or viability assays, standard procedures were followed. For pheromone treatment, the synthetic pheromone peptide was dissolved in DMSO in 10 mg/ml (approximately 5,9 mM) concentration as a stock solution and stored at –80°C. For yeast strains carrying the *bar1*$^{-}$ allele, a

final concentration of 0,59 μM was used. For yeast strains with the *Bar1*$^{+}$ allele, a final concentration of 5,9 μM was used. The strains and plasmids used in these experiments are all listed in the supplementary information of Ref. I – V.

The viability spotting assay is described in the Materials and Methods sections of Ref. I – III. The process of yeast FACS analysis is described in Ref. I. The microfluidics assay is described in Ref. V and by (Doncic, et al., 2011). Experimental conditions for Cln1-3 shutdown strains are described in Ref. V. Fluorescent image quantification was performed as described in (Doncic, et al., 2011; Doncic, et al., 2013) and in Ref. V.

5. RESULTS AND DISCUSSION

5.1. Characterization of cyclin-CDK substrate specificity in *S. cerevisiae* (Ref. I)

As demonstrated by Loog and Morgan, yeast Clb5-Cdk1 and Clb2-Cdk1 complexes have differences in overall intrinsic kinase activity and substrate specificity (Loog & Morgan, 2005). Although Clb5-Cdk1 has a lower intrinsic kinase activity (lower kcat and higher K_m values toward peptide substrates) than that of Clb2-Cdk1, it benefits over the latter from substrate recruitment by the cyclin hydrophobic patch (HP) region to bind substrate RXL motifs (Loog & Morgan, 2005). The hydrophobic patch motif is located in the MRAIL-helix region of Clb5 (Bazan, 1996) and is attributed to the S-phase-specific activity of Clb5, the activity of which is not replaceable by ectopically expressing Clb2 instead (Cross, et al., 1999). Similarly, the G1- and S-phase cyclins Cln2 and Clb5 have divergent roles, as Cln1-3 activity is able to initiate budding but is unable to initiate the replication; the latter function is attributed to Clb5-Cdk1 (Dirick, et al., 1995; Mendenhall, 1993). Differences in enzyme specificities might underlie the temporal resolution and timing of different cell cycle events, including the multiphosphorylation of Sic1.

In this study, we assessed the enzyme specificity of representatives of all four classes of the yeast cell cycle-related Cdk complexes: Cln2-, Clb5-, Clb3- and Clb2-Cdk1 complexes, toward Sic1. Each of the cyclins activates the Cdk1 active site to a different extent. Strikingly, the intrinsic activity toward short optimal model peptides gradually rose in the order of the appearance of the cyclins in the cell cycle (Ref. I, Fig. 1). The early cell cycle cyclins, Cln2 and Clb5, provided relatively low Cdk1 activity compared with that of the mitotic cyclin Clb2 (Ref. I, Fig. 1). The gradual increase in the means of the k_{cat}/K_m value was most pronounced in the K_m component and was the result of Cdk1 activation by different cyclin subunits and not the result of different posttranslational modifications of Cdk1 (Ref. I, Fig. 1 and Supplementary Fig. S2).

However, the noninhibitory Sic1ΔC protein demonstrates an almost mirrored profile of the different cyclin-Cdk1 activities, with the highest activity originating from Clb5-Cdk1 (Ref. I, Fig. 3C). We found that in the case of Sic1, early cyclin-Cdk1 complexes compensate their gradually lower intrinsic activity toward consensus phosphorylation by specific docking motifs (Ref. I, Fig. 2, Fig. 3, Supplementary Fig. S1). Most importantly, we discovered a docking motif for G1-Cdk complexes, a short hydrophobic motif starting at position 136 in Sic1 (Ref. I, Fig. 3). The VLLPP region was simultaneously identified by the Pryciak group (Bhaduri & Pryciak, 2011) and later shown to interact with a region in Cln2 not homologous to the substrate recruitment pocket found in Clb5 (Bhaduri, et al., 2015). As demonstrated by us, Cln2-Cdk1 preferentially

targets the T5, T33 and S76 sites in Sic1 and for this substrate recruitment through the VLLPP motif is crucial (Fig. 6; Ref. I, Fig. 3).

Moreover, we demonstrated that different cyclin-Cdk1 complexes diverged in the requirements for the amino acid composition of the consensus substrate motif (Ref. I, Fig. 4, Supplementary Table S1). The differences are particularly pronounced between Cln2 and Clb5-Cdk1. Cln2-Cdk1 strongly preferred a +2 positioned lysine and a –2 positioned proline from the targeted S/T consensus, whereas Clb5 was much more tolerable to the surrounding amino acids (Ref. I, Fig. 4, Supplementary Table S1). Therefore, the intrinsic Cln2-Cdk1 kinase activity toward different S/TP sites in Sic1 is much more divergent than it is by Clb5-Cdk1 (Ref. I, Fig. 4D).

In agreement with the gradual compensation of early cyclins by docking motifs, Clb5-Cdk1 activity toward the noninhibitory version of Sic1 was largely led through the R/KXL substrate motifs (Fig. 6; Ref. I, Fig. 2, Fig. 3). Different RXL sites on Sic1 led to site-specific engagement of Clb5 docking, as T33 phosphorylation was dependent on the 2nd and 3rd R/KXL motifs (positions 89–91 and 114–116, respectively), and S76 phosphorylation was exclusively dependent on the 3rd R/KXL motif (Fig. 6; Ref. I, Fig. 2). Moreover, the 2nd and 3rd RXL motifs are crucial for rescue from cell cycle arrest induced by Sic1 overexpression (Ref. I, Fig. 5). In addition, RXL-mutated Sic1 has a 10 min degradation timing delay in release from pheromone-induced G1 arrest. (Ref. I, Fig. 5).

Finally, we screened a set of proteins containing multiple potential Cdk target phosphorylation sites with all four cyclin-Cdk1 kinases *in vitro*. Most of the substrates were regulated in a cell cycle-dependent manner. The groups of substrates with vast differences in phosphorylation profiles among the cyclin-Cdks were identified (Ref. I, Fig. 6). The early cell cycle cyclins Cln2 and Clb5 are most dependent on VLLPP or K/RXL docking respectively. Clb3 has an intermediate effect, and Clb2 is relatively independent of RXL docking. A group of substrates follow a gradual specificity profile among different cyclin-Cdks similar to the model substrate histone H1.

Our results demonstrate that during the progression of the cell cycle, different cyclins gradually add the intrinsic kinase activity of Cdk1. However, additional mechanisms exist to target substrates via cyclin docking motifs. This aspect is especially important for early cell-cycle events where low intrinsic Cdk activity copes with vast amounts of potential targets and selectively phosphorylates a subset of substrates. The gradually changing specificity model in combination with cyclin-specific docking and substrate consensus preferences uncovered in this study lay the groundwork for the general unified model of Cdk function in the cell cycle that combines both elements of the quantitative threshold model (Stern & Nurse, 1996; Hochegger, et al., 2008; Swaffer, et al., 2016) and the cyclin specificity model (Bloom & Cross, 2007; Kõivomägi & Skotheim, 2014).

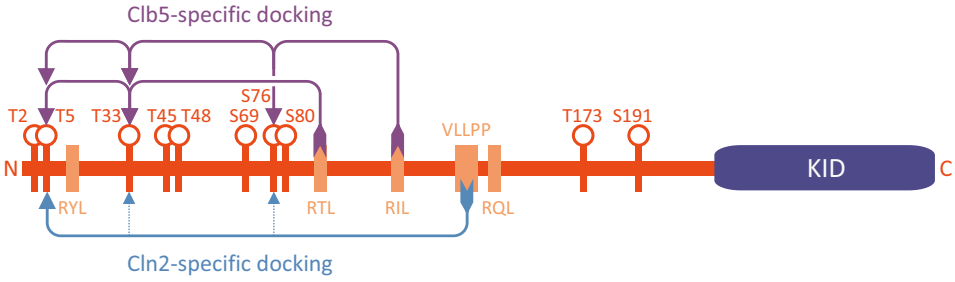

Figure 6. Cln2 and Clb5 provide different docking sites for Cdk1 activity. Sic1 N-terminal domain (orange) contains a multiple phosphorylation input sites (sticks with circles, targeted amino acid position noted by a number) and several cyclin docking motifs (R/KXL and VLLPP, light orange boxes). Cln2 uses a single VLLPP consensus motif to target N-terminal phosphorylation sites of Sic1. Docking-phosphorylation routes of Cln2 are marked with light blue lines and for Clb5 dark blue lines. The herringbone-shaped end is for docking and arrowhead end for target phosphorylation, bold arrowhead for main target site, dotted line for auxiliary target sites. Clb5 mainly uses two specific K/RXL motifs (RTL and RIL sequences) in Sic1 to target N-terminal phosphorylation sites. Note that for Clb5 such docking interactions are mainly available if KID is removed or if Clb5 is in excess of Sic1 (see figure 8).

5.2. The cyclin and Cks1 double docking mediates the sequential mechanism of the multisite phosphorylation of Sic1 (Ref. II and IV)

As previously proposed, phosphorylation of a random selection of 6 phosphorylation sites in Sic1 by the G1-Cdk complex leads to the ultrasensitive degradation dynamics of Sic1 at the G1/S transition (Nash, et al., 2001). The model was based on the assumption that the apparent affinity of the multiply-phosphorylated Sic1 with SCF-Cdc4 is increased via a multivalent interaction involving dynamic association and dissociation of all six sites with a single pocket on Cdc4. Although the side of the model addressing the interaction with Cdc4 had already received some criticism from structural biologists (Hao, et al., 2007), the element of Cdk phosphorylation dynamics was entirely ignored by Nash et al (Nash, et al., 2001). The main problem with it was that the assumed ultrasensitive dose-response was only possible in the case when the multisite phosphorylation was entirely distributive, meaning that each site is phosphorylated in a random order and independent of each other (Klein, et al., 2003).

To bridge the gap of missing kinase mechanism in the model proposed by Nash et al., we performed a detailed study of the multisite phosphorylation dynamics and specificity of Sic1 by different cyclin-Cdk complexes. By using a noninhibitory version of Sic1 (the KID at positions 215–284 was deleted, designated Sic1ΔC) encompassing all of its optimal Cdk1 sites, we first demonstrated that both Cln2-Cdk1 and Clb5-Cdk1 were phosphorylating Sic1

processively. It appeared that the processivity of the Cdk holoenzyme complex was simultaneously mediated by Cks1 and the cyclin subunits (Ref. II: Fig. 1, Fig. 2 and 3, Supplementary Fig. 1, 2 and 4; Ref. IV; Fig. 1, Fig. 2, Supplementary Fig. 1).

Including the Cks1 subunit in purified preparations of cyclin-Cdk1 the hyperphosphorylated forms of Sic1 appeared without notable accumulation of the intermediate phosphoforms (Ref. II, Fig. 1 and Supplementary Fig. 1). Most importantly, we demonstrated that Cks1 mediated priming had its own determinants of specificity. The distance between the priming site and the target site is important. Cks1 mediated Cdk activity to its target sites over a sharp optimum peak at a distance 12–16 amino acids from the priming site. Priming by Cks1 was unidirectional, working from the N-terminus to the C-terminus (Ref. IV, Fig. 3, Supplementary Fig. 3 and 8, Supplementary Table 1, Fig. 4). Moreover, Cks1 binding has its own consensus requirements. Cks1 only binds phosphorylated threonine followed by proline and discriminates phosphoserine residues in that context (Ref. IV, Fig. 2, Supplementary Fig. 1) (McGrath, et al., 2013). Additionally, specific proline residues in position (–2) enabled better Cks1 docking and widened the effective distance to 18–25 amino acids (Ref. IV: Fig. 3, Fig. 4, Supplementary Fig. 3 and Supplementary Table 1).

The processive phosphorylation manner is enhanced by cyclin-specific docking sites. The processivity of Clb5 is dependent on the R/KXL motifs of the substrate (Ref. II, Fig. 2, Fig. 3), and the processivity of Cln2 is dependent on the VLLPP motif of the substrate (Ref. II, Fig. 3, Supplementary Fig. 4). Similar to Cks1, cyclin subunit-mediated docking had an optimal distance for enhancing Cdk1 activity. For hydrophobic patch docking, the optimal distance was 16–20 amino acids between the R/KXL motif and the target phosphorylation site (Ref. IV, Fig. 5). B-type cyclins enhance docking in a strictly unidirectional manner but reciprocal to that of Cks1, that is, from the C-terminus to the N-terminus (Ref. IV, Fig. 5). However, Cln2 docking enhanced phosphorylation in both directions (Ref. IV, Fig. 5). Substrate recruitment by Cln2 is assisted by abundant and not very specific hydrophobic contacts over the Sic1 molecule and therefore has more plasticity.

The specificity requirements of different cyclin-Cdk complexes all manifest in Sic1 phosphorylation. We demonstrated that through the VLLPP motif, Cln2 effectively primed the Sic1 T5 and T33 sites and much less effectively primed the T45, S76, T173 and S191 sites (Fig. 7). The phosphorylation of the latter sites is only improved if the N-terminal T5 or T33 are already phosphorylated. Moreover, Cln2-Cdk1-Cks1 is rather inefficient in phosphorylating the T48, S69 or S80 sites (Ref. II, Fig. 2), the sites that form double degrons of Sic1 (Fig. 7). In contrast, the Clb5-Cdk1-Cdk1 holoenzyme used priming avidly from the T5 to the T33 sites to mediate the phosphorylation of the T45, S69, S76 and S80 sites (Ref. II, Fig. 2; Ref. IV: Fig. 2, Supplementary Fig. 2). Additionally, Clb5 was able to prime T5 and T33 by using the 2nd and 3rd R/KXL motifs of Sic1 (Fig. 7). For targeting S76 and S80, Clb5 exclusively used the 3rd R/KXL motif (Ref. II, Fig. 2). Additionally, phosphorylated T45

improved the targeting of the S69 and S80 sites by the Cln2-Cdk1-Cks1 holoenzyme (Ref. IV, Fig. 6).

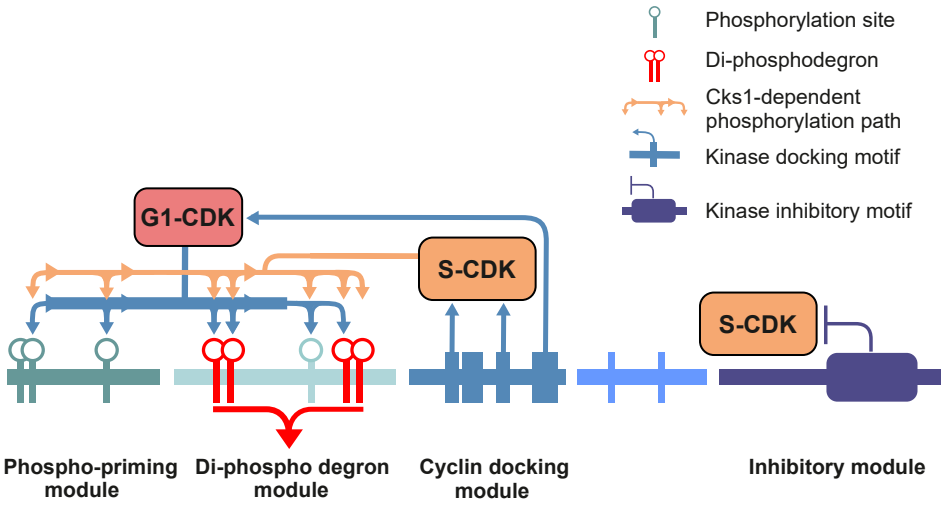

Figure 7. Processive or semiprocessive pathways of Cln2 and Clb5 in Sic1. The ribbon diagram of Sic1 is separated into different modules. Most N-terminal is phosphopriming module (greenish blue). A module containing the phosphorylation sites necessary for Sic1 degradation follows this (light blue). In the central part of Sic1 the RXL and VLLPP sites form cyclin docking module (blue). Most C-terminal part contains KID that forms inhibitory module for Clb5,6-Cdk1. Phosphorylation sites are marked with circles, cyclin docking sites with rectangular boxes. G1-specific cyclin-Cdk1 (Cln2) is able to provide priming (blue lines originating from red box "G1-CDK") by using cyclin-docking module (blue lines entering red box "G1-CDK"). Cln2 is able to progress in N-to-C terminal direction by using generated priming phosphorylation to target a subset of phosphorylation sites in Sic1, excluding the last degradation output sites in degron module. Clb5-Cdk1 (beige, "S-CDK") is inhibited by KID module. If in excess it phosphorylates Sic1 N-terminal modules by benefiting from the cyclin docking module (blue lines entering the box "S-CDK"). Clb5-Cdk1-Cks1 complex processively phosphorylates all degron module sites by using priming and docking module.

The T45, S76, S80 sites, and more importantly the nonoptimal site T48, are responsible for high-affinity interactions between Sic1 and Cdc4, thus generating Sic1 degradation (Hao, et al., 2007). We demonstrated *in vivo* that both degron pairs T45/T48 and S76/S80 contributed to Sic1 degradation and that the T45/T48 degron pair was indispensable (Ref. II, Fig. 2, Supplementary Fig. 2 and Fig. 4). As our results demonstrated, access to these sites was different between the Cln2- and Clb5-mediated Cdk1 (Fig. 7). The priming resulting from the T33/T45 sites and the R/KXL motifs were crucial for the rescue from cell-cycle arrest in the Sic1 overexpression system (Ref. II, Fig. 2 and 4), suggesting that Clb5 is crucial to phosphorylating Sic1 degron output sites. We

further corroborated this by inhibiting Clb5-Cdk1-specific activity by using the KID of Sic1 expressed from an exogenous source and monitored the behavior of Sic1-wt expressed from an endogenous source. If Clb5-Cdk1 activity was completely inhibited, the endogenous Sic1-wt was stabilized (Ref. II, Fig. 3).

Our results explain why T48 was reported to be missing from most of the previous *in vitro* studies if the main phosphorylation driver was Cln2-Cdk1 without the Cks1 subunit (Nash, et al., 2001). In the Cln1-3 shutdown yeast strain arrested in G1, Verma et al. were unable to recover sufficient amounts of detectable Sic1 phospho-T48 or phospho-S76 peptides, although they were able to recover all six optimal Cdk-consensus motif-containing phosphopeptides (Verma, et al., 1997).

Our study presents a new model for Sic1 phosphorylation and G1/S transition in budding yeast (Fig. 8). We found that Sic1 degradation output was exclusively generated by Clb5-Cdk1 whereas Cln2-Cdk1 activity served as a priming event. The Sic1 N-terminal pathway was phosphorylated in a processive or semiprocessive manner by using dual docking via Cks1 and a cyclin subunit (Fig. 7). All the components have their own output specificities and tunable ranges of Cdk1 activation. The assembly of such components in the form of an organized pathway enables the generation of finely tuned output efficiency.

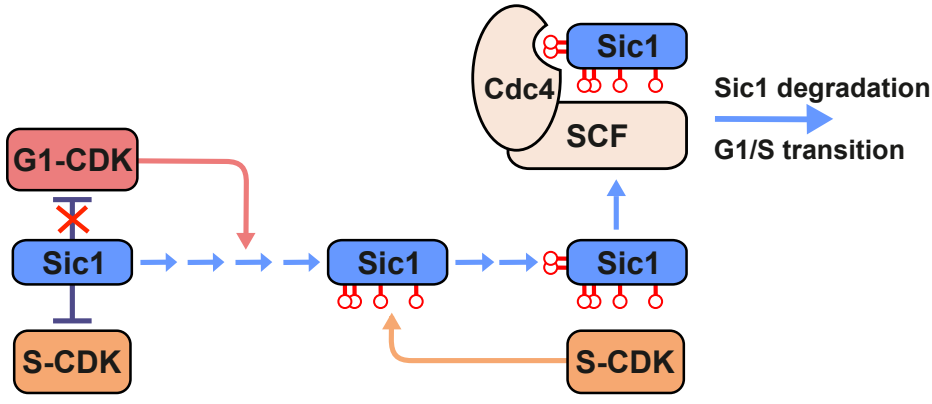

Figure 8. A model diagram of the improved G1/S switch including a positive feedback component. Left: Sic1 (blue box) stoichiometrically inhibits S-Cdk1 (beige box) but do not inhibit G1-CDK (red box). Through semiprocessive phosphorylation (blue arrows from left to right), Sic1 is primed by G1-Cdk1 (arrow originating from red box). Primed Sic1 (red circles) is further phosphorylated by S-Cdk if latter is exceeding Sic1 concentration (beige arrow). This phosphorylation step finishes Sic1 phosphorylation pathway by S-Cdk triggering Cdc4-dependent degradation of Sic1 (light pink complex at right side). Note that S-Cdk phosphorylation step generates double negative feedback loop for Sic1 destruction (see next chapter for details).

5.3. Abruptness of the G1/S switch is generated by feedback loops between Sic1 and the Clb5-Cdk1-Cks1 holoenzyme (Ref. III)

Our new model of Sic1 degradation at the G1/S transition (Fig. 8) presented in Ref. II proposes a double-negative feedback loop between Sic1 and S-Cdk with G1-Cdk assuming the role of a priming kinase and a trigger. This is in stark contrast to the previous model by Nash et al that proposes an ultrasensitive response to Sic1 degradation likely resulting from the allovalency of Cdc4 binding, which in turn is a response to the G1-Cdk signal. We aimed to create a quantitative framework for the new G1/S model using mathematical modeling and numerical simulation of ordinary differential equations (ODE) systems.

Sic1 degradation and release of Cdk1 activity in S phase is followed by a bistable switch-like response (Cross, et al., 2002). In the case of multisite substrates, a high degree of processivity might lose ultrasensitive behavior and therefore bistability (Takahashi, et al., 2010; Salazar & Höfer, 2009). The bistable nature of the input-output conversion in processive systems can be restored by adding a feedback loop (Salazar & Höfer, 2009). Moreover, highly switch-like bistable behavior is achievable with systems combining distributive and processive mechanisms with a feedback loop (Salazar & Höfer, 2009).

First, we performed a quantitative Sic1 dose response analysis for the Clb5-Cdk1-Cks1 and Cln2-Cdk1-Cks1 complexes (Ref. III, Fig. 1). The calculated IC50 for the Clb5 holoenzyme is approximately 1 nM whereas the Cln2 holoenzyme is not inhibited (Ref. III, Fig. 1). Therefore, we proposed that the Sic1 off-rate from the Clb5-Cdk1-Cks1 complex was extremely low. The stoichiometric inhibition was an important feature in generating bistability, as we saw later in this study.

We further tested whether the extracomplex phosphorylation rates toward Sic1 engaged in the inhibitory complex of Clb5-Cdk1-Cks1 were similar to the noninhibitory Sic used in Ref. I – III. By titrating the Clb5 holoenzyme concentration over the concentration of the inhibitory Sic1, the results indicated that Sic1 binding to the Cdk1 complex did not affect its substrate qualities for extracomplex phosphorylation (Ref. III, Fig. 2). As expected, the Clb5 holoenzyme was processing both the complexed and noninhibitory Sic1 with higher rates than was Cln2 (Ref. III, Fig. 2). Simultaneously, the phosphorylation rates between the full length Sic1 and Sic1ΔC were similar except that Clb5-Cdk1 had slightly higher rates toward the complexed Sic1 (Ref. III, Fig. 3). This result demonstrated that the Sic1 N-terminal region, although complexed with the Clb5 holoenzyme, was freely accessible for outer enzyme activity, which is also in agreement with the results obtained by assaying Cdc4 binding and ubiquitination *in vitro* (Verma, et al., 1997). Therefore, during the G1/S transition, the initial release of the Clb5-Cdk1-Cks1 complex could further accelerate Sic1 degradation and its own release and generate a double-negative feedback loop for Sic1 destruction (Fig. 8).

We generated an ODE model to simulate the concentrations of free Sic1, free Clb5 and complexed Sic1-Clb5-Cdk1 in the G1/S transition (Fig. 9). Our model contains three ODE equations (Ref. III, Table 1). First equation describes free Sic1 levels, second describes free Clb5 levels and third equation describes the concertation of Sic1-Clb5-Cdk1 inhibitory complex. The concentrations of free Sic1 and Sic1-Clb5-Cdk1 complex were set to depend on the complex formation K_d (or principally IC50 in this case) and Cln2 basal degradation activity toward complexed Sic1. The concentration of free Clb5 was dependent on the complex K_d and the sum of the destruction rates of the complexed Sic1 (two rates: Cln2 basal, Clb5 outer). The observed processivity in Ref. I – III enabled us to simplify the model by excluding phosphorylation intermediates. The phosphatase rate was summarized in the net phosphorylation rate, and the degradation of Sic1 was considered a nonlimiting step.

As expected, in the case of tight inhibition and positive feedback generated by fast extracomplex Clb5 activity, the emergence of free Clb5 followed a steep hysteretic curve with a robust bistable property, where one stable state corresponded to high Sic1 and low free Clb5 and the other to high free Clb5 and low Sic1 (Fig. 9; Ref. III, Fig. 4). Importantly, the steepness of hysteresis was dependent on the IC50 value of the inhibitory complex (Ref. III, Fig. 5). Increasing the IC50 to 100 nM caused the hysteretic behavior and bistability of the system to be lost (Ref. III, Fig. 5). Therefore, only the low nanomolar Ki values were required to inhibit Clb5 activity during its accumulation and subsequent release during the G1/S transition (Fig. 9).

Moreover, the hysteretic behavior of Clb5 release was dependent on the limited activity of Cln2 towards the Sic1 degron output sites (Ref. III, Fig. 6–8). When increasing Cln2 activity from 0,01 to 0,3 fraction units of Clb5 activity, the hysteresis and sufficient Sic1 threshold levels were lost, resulting in the loss of bistability (Ref. III, Fig. 6–8). Strikingly, this model presented an option to tune the fractional Cln2 activity between 0,01 and 0,1 units without a significant loss in hysteresis but with the ability to shift the timing of when the G1/S transition occurred. Therefore, weak Cln2 activities toward Sic1 degradation might be important for controlling and fine-tuning the timing of the G1/S transition.

Another important property revealed is that by tuning Cln2 extracomplex activity, it is possible to prime Sic1 for emerging free Clb5, which in turn would enable to fine-tune the Sic1 threshold levels for emerging free Clb5 before the G1/S takes place. This behavior suggests the AND gate type decision process where sufficient activities of both G1 and S phase cyclins must be present to trigger the switch. Omitting Cln2 from the model resulted in a delayed but less abrupt G1/S transition (Ref. III, Fig. 7 and 9 and 10).

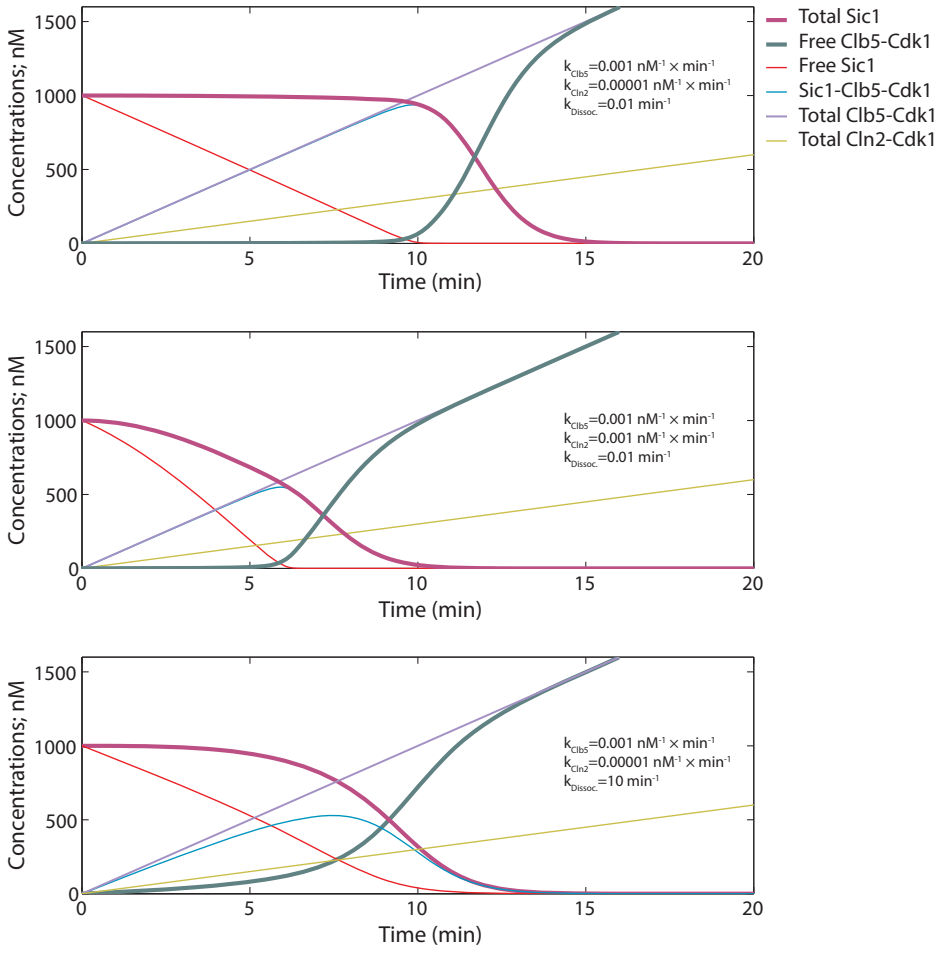

Figure 9. Simulation of the G1/S transition by the ODE model. The ODE system describes three variables: free Sic1 levels (red line), free Clb5 levels (greenish-blue line) and levels of Sic1-Clb5-Cdk1 inhibitory complex (blue line). Total Clb5 and Cln2 levels are set to accumulate by constant rates (pink and beige lines respectively) and initial total Sic1 levels are set steady (pink line). The ODE is defining three main variables by using the phosphorylation rates of Cln2 and Clb5-Cdk1 and K_d of Sic1-Clb5-Cdk1 inhibitory complex. The bistability of the switch is dependent on abrupt Sic1 degradation, which in turn is dependent on a positive feedback mechanism generated by Clb5-Cdk1 activity (upper panel). The abruptness is lost if rising Cln2-Cdk1 levels are able to degrade Sic1 with comparable rates to Clb5 itself (center panel). A similar situation can be observed if the tight inhibition of Clb5-Cdk1 is lost (bottom panel).

Our results were independently corroborated by an elegant single-cell study observing the *in vivo* dynamics of Sic1 during the G1/S transition by the Tang group (Yang, et al., 2013). First, they demonstrated that Clb5,6, and not Cln1,2, are primarily responsible for the degradation profile of Sic1. Second, Cln1,2 affects the variability and median of Sic1 degradation timing from Whi5 nuclear exit (defined as START) and does not affect the Sic1 degradation half-time (Yang, et al., 2013). They concluded that the abruptness of the G1/S transition is generated by a double-negative feedback loop provided by Clb5 itself and that the robust timing is maintained by Cln1,2 via Sic1 priming (Yang, et al., 2013). In addition, the presence of Clb5,6 is crucial to dampening the noise generated by environmental perturbations that affect G1 cyclin expression levels, which under Clb5,6 deletion generate a large variability in the Sic1 half-life (Yang, et al., 2013). The ODE presented by them proposes that the feedback loop, and not the multisite option, is responsible for the abruptness of the G1/S switch (Yang, et al., 2013). The limited activity of the primer kinase functions as a timer of the switch (Yang, et al., 2013). The bistability is a result achieved through double-negative feedback (Yang, et al., 2013).

The conclusion of our study is that the G1-Cdk priming model combined with the S-Cdk double-negative feedback system proposed by us and Tang is kinetically possible. Our study found that the key parameter for ensuring ultrasensitivity is the stoichiometric inhibition of S-Cdk by Sic1, limited degradation activity of G1-Cdk1 and a positive feedback loop provided by S-Cdks. Differentiated tasks of G1 and S-type cyclin Cdk activities enable fine-tuning and the sharpness of the G1/S switch and provides filtering mechanisms to dampen the effects of noise. It is likely that evolutionarily fine-tuning biological systems uses simultaneously mixed but highly balanced mechanisms that include distributive, processive and feedback components.

5.4. Intracomplex phosphorylation step process input signals (Ref. V)

We outlined a new model for Sic1 degradation and G1/S transition in budding yeast (Fig. 8). Here, we address a still unsolved question of how S-Cdk, while being inhibited by Sic1, could at the same time, be the major driving force in Sic1 phosphorylation and degradation. We observed that, despite the tight nanomolar inhibition of S-Cdk by Sic1, Sic1 was still phosphorylated within the inhibitory complex. Our hypothesis was that intramolecular phosphorylation provides an intermediate step between Sic1 priming by G1-Cdk and the final abrupt destruction by release of S-Cdk.

This idea was partly derived from studies on mammalian Cki p27 where the priming phosphorylation releases an intramolecular phosphorylation activity of otherwise inhibited cyclin-Cdk complexes, providing an intermediate step of Cdk activity before the abrupt G1/S transition. The silent ternary G1 and S-type

cyclin-Cdk complexes are awakened by regulated unfolding which includes multistep phosphorylation of the KID of p27 (Grimmler, et al., 2007; Ou, et al., 2011; Ou, et al., 2012). The priming step is generated by promitogenic tyrosine kinases that sequentially target specific residues of p27 KID, which in turn triggers the initial low rate of Cdk activity (Ou, et al., 2012). The low rate of Cdk activity leads to the intracomplex phosphorylation of the flexible CTD tail to phosphorylate T187 of p27 (Grimmler, et al., 2007; Galea, et al., 2008). This step initiates ubiquitin-dependent degradation of p27 and the release of the active cyclin-Cdk complex (Montagnoli, et al., 1999).

5.4.1. The extent of intracomplex phosphorylation is reciprocally dependent on the status of Sic1 T5 and T173

We observed that during the formation of the Sic1-Clb5-Cdk1-Cks1 inhibitory complex, limited, rapid, intramolecular phosphorylation independent of the Clb5:Sic1 ratio if Sic1 was taken in excess occurred (Ref. V, Fig. 1d,e, Supplementary Fig. 1c).

Surprisingly, some fraction of Sic1 was highly hyperphosphorylated to contain 8–10 phosphates per Sic1 molecule, which *in vivo* could lead to its destruction (Ref. V, Fig. 1e and 2a). The abundance of the hyperphosphorylated form appears to be dependent on the Cdk1 subunit Cks1 and the Sic1 N-terminal T5 and C-terminal T173 sites (Ref. V, Fig. 2a, Supplementary Fig. 1d). In the case of the Sic1 T173A or T173S sites, extensive accumulation of the hyperphosphorylated forms of the complexed Sic1 were observed *in vitro* (Ref. V, Fig. 2a,b; Supplementary Fig. 1d,f). In the case of the Sic1-wt, an average level of hyperphosphorylation of Sic1 was observed. In the case of the T5 mutants, the extent of the hyperphosphorylation was reduced (Ref. V, Fig. 2a,b; Supplementary Fig. 2f).

The intramolecular phosphorylation process is entirely dependent on the Sic1 KID and Cks1. If the KID is removed (Sic1ΔC), the phosphorylation dynamic between Sic1-wt and Sic1-T173A is indistinguishable, demonstrating that only the intramolecular phosphorylation step could generate differences in Sic1 phosphorylation patterns for the T173 site (Ref. V, Supplementary Fig. 1g). Cks1 removal leads to uncontrollable accumulation of the hyperphosphorylated forms, similar to Sic1 T173S or Sic1 with all the Cdk sites mutated to serine residues (Ref. V, Fig. 2a,b, Supplementary Fig. 1f).

Our previous studies have identified T5 and T33 as priming sites for the N-terminal degradation pathway, whereas phosphorylation of T173 was previously shown to generate a G1/S delay (Zinzalla, et al., 2007; Escoté, et al., 2004; Moreno-Torres, et al., 2015). Additionally, phosphorylated T173 was proposed to turn Sic1 from substrate to inhibitor for Clb5-Cdk1 by using its phospho-adaptor subunit Cks1 (Moreno-Torres, et al., 2017). However, the Sic1 T173A, Sic1 T173S or Sic1 with all the Cdk sites mutated to alanine residues

had no variations in their IC50 values for inhibiting Clb5-Cdk1-Cks1 activity against the external substrate H1 compared with the Sic1-wt (data not shown).

Therefore, we hypothesized that T5 and T173 might serve as reciprocal regulators setting limits to the extent of hyperphosphorylation of Sic1 by Clb5-Cdk1-Cks1 intramolecular process.

5.4.2. Intramolecular process regulates Sic1 threshold levels *in vivo* in response to Fus3 and Cln2 activity

We discovered that in addition to previously identified input kinases, pheromone-activated kinase Fus3 was targeting Sic1 T173 *in vivo* and *in vitro* (Ref. V, Supplementary Fig. 2b,d,f). Surprisingly, Fus3 also phosphorylated T45 *in vitro* (Ref. V, Supplementary Fig. 2d). Most importantly, the addition of Fus3 enabled us to observe the effect of T173 phosphorylation on the dynamics of Sic1 *vivo* by using a mating pheromone and single-cell fluorescence microscopy (Ref. V, Fig. 3). If the cells were treated with a short high-concentration (240 nM) pheromone pulse followed by a flow of low-concentration (3 nM) (Ref. V, Fig. 3a), the exit of Whi5 was delayed and G1 was prolonged (Doncic & Skotheim, 2013; Doncic, et al., 2015). Simultaneously, Clb5 was accumulating (Ref. V, Fig. 3f) opening up the possibility of testing the effects of Fus3 on Clb5 intramolecular priming *in vivo*.

Strikingly, under these conditions, the phosphorylation status of T173 was decisive. The Sic1 T173A or T173S site was degraded 40 minutes earlier (Ref. V, Fig. 3d,c,e,h, Supplementary Fig. 3a,b) than the Sic1-wt was (Ref. V, Fig. 3d,b,e,g), and that is far earlier than the Whi5 nuclear exit (Ref. V, Fig. 3h). Sic1 with all the N-terminal Cdk sites mutated to serine residues (Sic1-9SP) rescued the phenotype of early degradation (Ref. V, Fig. 3e,i). Therefore, early Sic1 degradation most likely occurs due to intramolecular turnover by Clb5-Cdk1 dependent on Cks1. Moreover, to cause such an effect, the previous input signal to Sic1 T173 must have been present, because in asynchronously dividing cells no differences in dynamics between full length Sic1-wt and Sic1-T173S were observed (data not shown). Asynchronously growing cultures of cells lack the pheromone (or other stressor) input to Sic1, and therefore, they progress quickly to the G1/S switch. For cells to sense the status T173 phosphorylation, the KID was crucial. The deletion of the KID (Sic1ΔC) caused all differences between Sic1-wt and Sic1-T173S *in vivo* to be lost (Ref. V, Supplementary Fig. 3c), confirming that only the intracomplex phosphorylation step is sensitive to the status of the T173 site.

Most importantly, the N-terminal (T5) and C-terminal (T173) input sites regulated Sic1 levels via intramolecular phosphorylation by Clb5-Cdk1-Cks1. If the N-terminal input T5 or VLLPP motif was mutated, the Sic1 level in prolonged G1 arrest was elevated (Ref. V, Fig. 3j, Supplementary Fig. 3d). This result demonstrates that Cln1-3 was responsible for generating a signal affecting the Sic1 threshold in prolonged G1. We further attempted to influence the Sic1

levels by connecting Cln2 to the T173 site by optimizing the T173 site to be Cln2-Cdk1-specific (–2P+2K+3A). As expected, this resulted in elevated Sic1 levels in G1 arrest, suggesting an elevated T173 input (Ref. V, Fig. 3k). In addition, we altered the N-terminal to C-terminal priming balance by upgrading the N-terminal pathway with three additional Cdk sites and a potential double-site degron to compete with T173 phosphorylation and, as expected, this resulted in a lowered Sic1 level (Ref. V, Fig. 5a).

In addition, we were curious about whether Clb5 affected Sic1 levels through the N-terminal 2nd and 3rd RXL motifs. Our previous results suggested that mutating the RXL motifs would not affect the Sic1 levels because first, no extramolecular Clb5 process would be present due to high affinity binding to Sic1 and second, the intramolecular process would be independent of the cyclin hydrophobic patch that was likely already occupied by the KID. Unexpectedly, the RXL mutations caused the Sic1 level to drop (Ref. V, Fig. 3k). Mutations in the RXL motifs did not cause considerable effects to the intramolecular phosphorylation patterns *in vitro* (Ref. V, Supplementary Fig. 1f). This finding confirms that the intramolecular process itself was not responsible for the effect. We found that Fus3 was dependent on the docking site overlapping with the 2nd RXL motif and that this affected T173 phosphorylation by Fus3 (Ref. V, Supplementary Fig. 3e). Moreover, the docking site identified did not affect the Fus3 phosphorylation rate toward T45 (Ref. V, Supplementary Fig. 3e), which is a component of the Sic1 T45/T48 degron and serves as priming site of Cln2 for S76/S80 degron. Therefore, it is likely that Fus3 generated a dual input signal for Sic1, one for diversions and one for degradation. If the diversional signal was reduced by altering the docking site, the input balance would shift toward the degradation signal and the Sic1 levels would drop.

Therefore, we have demonstrated that T5 and T173 create a priming balance that is processed via the Clb5-Cdk1-Cks1 intramolecular phosphorylation mechanism into steady-state threshold levels of the G1/S inhibitor.

5.4.3. G1/S timing is dependent on the Clb5-Cdk1-Cks1 intramolecular process

Upon the Whi5 nuclear exit (START), which marks the shutdown of the Fus3 signal (Oehlen & Cross, 1994; Strickfaden, et al., 2007; Repetto, et al., 2018), the situation of input signals changes. Our data revealed that after Fus3 activity was discontinued the rising Cln2 levels took over and kept the Sic1 levels steady.

Together with Clb5 the Cln2 levels began an accelerated accumulation after START whereas the Sic1-wt protein levels stayed steady (Ref. V, Fig. 3f,g). Strikingly, the Sic1 levels started to increase when the Cln1, 2 input sites were mutated (Ref. V, Fig. 3j, Supplementary Fig. 3d). In this case, the growth rate in Cln1-3 activity was unable to compensate for the loss of Fus3 activity toward the Sic1 T45 site and, therefore, unable to compensate for the accumulation rate

of Sic1. According to previous results presented by the Tang group (Yang, et al., 2013) the altered Cln1-3 input in Sic1 affects the timing of the Whi5 exit but does not affect the degradation half-time during the G1/S transition once the external Clb5 double-negative feedback is activated. According to the Tang group (Yang, et al., 2013), the Cln2 input is unimportant if the double-negative feedback loop of free Clb5-Cdk1 is not activated.

In case of defective Cln2 input sites, the delay in the G1/S switch was detected only in full-length Sic1 (Ref. V, Fig 4a,b, Supplementary Fig. 3d). This result suggests that the lack of Cln2 priming was important only if the inhibitory threshold was determined via following the intramolecular phosphorylation process and is relatively unimportant if no additional intramolecular process is followed in the case of KID truncation. In the case of defective Cln2 input, to reach the G1/S transition, ever-higher Clb5 accumulation rates were necessary. The truncation of the KID in Sic1-wt generated a 4 minutes delay in the G1/S transition (Ref. V, Fig. 4a,b). This observation suggests that although Sic1-wt with a truncated KID contains a fully available Cln2 input, it lacks an intra-molecular step and therefore has to wait for higher amounts of free Clb5 to achieve abrupt degradation of Sic1-wt. Therefore, Cln2 priming is important to the intracomplex phosphorylation mechanism.

The Clb5-Cdk1 double-negative feedback loop is dependent in RXL motifs. Mutations in these motifs affected timing of both the full length and truncated Sic1 (Ref. V, Fig. 4a,b). In the case of KID truncation, the effect in timing was more pronounced. This effect can be explained by omitting the intracomplex step that resulted in limited phosphopriming and that caused additional effect in timing. It appears that the extra complex Clb5 is highly dependent on RXL docking but is also fine-tuned by an intracomplex priming step to initiate a double-negative feedback loop.

In this study, we resolved the missing connection between single-site input and double-negative feedback phosphorylation events. The processor appears to be Clb5-Cdk1-Cks1 itself, its intramolecular phosphorylation mode, which is able to turn the multisite phosphorylation option of Sic1 into a relay that fine-tunes the initiation of its own double-negative feedback loop. Previously, the T173 site was identified as an input of starvation related signals from the target of rapamycin complex 1 (TORC1) pathway and phosphorylation of the site caused Sic1 stabilization (Zinzalla, et al., 2007; Moreno-Torres, et al., 2015; Moreno-Torres, et al., 2017). Our study demonstrates that the stress signal via the Sic1 T173 site is processed only if the Clb5-Cdk1-Cks1 intracomplex phosphorylation mechanism is followed.

6. CONCLUSIONS

The core elements of the *S. cerevisiae* cell cycle control mechanism have been known for more than two decades. The past decade has focused on dynamic relationships between these elements to unveil quantitative mechanisms of cell cycle regulation. Our present study began with the quantitative characterization of different cyclin-Cdk activities. From simple active site specificities, we quickly moved on to characterize docking effects and to quantitatively measure substrate phosphorylation dynamics in a multiphosphorylated system. This investigation led us to unveil the elements of signal processing in G1 and the G1/S transition related to the cell cycle inhibitor Sic1 and the regulatory cyclin-Cdks. We identified three different Cdk activity modes in Sic1 phosphorylation: a priming step (Ref. I), an intracomplex phosphorylation step (Ref. V) and an extracomplex double-negative feedback (Ref. II–IV). The resulting modified G1/S model enabled us to assess the dynamic relationship between different input signals and properties of the switch. The results of this study are listed below.

1. We identified the specificity determinants at the level of the primary structure of the substrate for the Cdk1 active site associated with different cyclins and quantitatively characterized the effect of these determinants on cyclin-Cdk activity.
2. We identified docking requirements for the processive multistep phosphorylation mechanism for different cyclin-Cdk complexes and quantitatively characterized the effect of these determinants on cyclin-Cdk distributive and processive activities. More specifically, we characterized the effect of the Cks1 phospho-adaptor subunit (for phosphorylated threonines) and the cyclin hydrophobic docking sites (for RXL and VLLLPP motifs) and identified optimal distances in the multiphosphorylating network of the Sic1 protein.
3. We demonstrated the double-negative feedback loop to trigger the G1/S transition.
4. We resolved the multisite phosphorylation pathway of Sic1 into different modules responsible for different tasks, i.e., priming sites, specific output sites and a diversional module of T173.
5. We quantitatively assessed the effect of each module in connection with Cdk activity and the G1/S transition.
6. We successfully used the elements of the Sic1 pathways to remodulate the signal processing properties.

SUMMARY IN ESTONIAN

Kõikide päristuumsete elusorganismide rakkude jagunemise tsüklit saab jagada kaheks etapiks: interfaasiks ja mitoosiks (M-faasiks). Selline jaotus tekkis 19. sajandi keskel ajal, mil rakkude jagunemist uuriti peamiselt kasutades algelist valgusmikroskoopi. Interfaasis rakk kasvab või lihtsalt elutseb, mitoosis aga jagab organellid, tuuma ja tsütoplasma kaheks, lõpetades kahe eraldiseisva tütarrakuna.

Alles 1950.ndate aastate alguses, mil rakubioloogia jõudis tõeliselt molekulaarse tasandini, märgati, et interfaasi käigus toimub raku pärilikkusaine (DNA) kahekordistamine. Aega interfaasis, mis kulub DNA kahekordistamisele, hakati edaspidi nimetama S-faasiks. Lisaks märgati, et S-faasi ja sellele järgneva mitoosi vahel on ajaline paus. Seda hakati nimetama G2 faasiks. S faasile eelnevat aega, kus rakud kasvavad, hakati nimetama G1 faasiks. Sellega eristusid raku jagunemistsüklis järjestikku paiknevad G1, S, G2 ja M faas.

1970.ndatel aastatel tehti kindlaks, et raku jagunemise tsüklit iseloomustab alati ühesuunalisus ning täielik pöördumatus. Nii ei ole peale S faasi sisenemist enam võimalik eelmisesse, see on G1 faasi, tagasi pöörduda. Samuti ei ole võimalik pöörduda peale M-faasiga alguse tegemist enam tagasi eelmise, G2 faasi, tegevuste juurde. Neid momente raku jagunemise etappide vahel, kus on justkui seatud ette vaid ühte pidi läbitav vahesein, nimetatakse raku jagunemise kontrollpunktideks.

1980.ndatel aastatel, seoses geenitehnoloogiliste meetodite jõudsa arenguga, hakati iseloomustama raku jagunemise tsüklit mõjutavate geenide produkte. Geenid ise olid suures osas 1970.ndatel aastatel juba kaardistatud süstemaatiliste mutatsioonide abil, kasutades mudelorganismina pärmseeni.

Peamiste regulaatoritena avastati tsükliinidest sõltuvad kinaasid. Need on valgud, mis lisavad teistele valkudele fosfaatseid jääke, mis toimivad signaalmärgistena sihtmärkvalgu saatuse määramisel. Ka kinaasid ja neid aktiveerivad tsükliinid on valgud. Fosforüleerimise teel muudavad need sihtmärkvalkude aktiivsust, suunavad nende paigutamist rakus või määravad need lagundamisele. Rakutsüklit reguleerivate kinaaside aktiivsus on määratud eelkõige tsükliinide poolt, mis annavad kinaasi osaühikule töövõime ja määravad ka tema sihtmärkide eelistused. Tsükliinid on rakutsüklis väga rangelt reguleeritud – nad tekivad täpselt siis, kui nende aktiivsust on tarvis ja nii kui nad on oma töö lõpetanud, nad lagundatakse. Iga rakutsükli etapi jaoks on oma kindel või kindlad tsükliinid. Seejuures iga tsükliin käivitab endale järgneva tsükliini tootmise, mis on üks olulisi lisamehhanisme, kuidas on tagatud rakutsükli ühesuunaline kulgemine. Nende valkude tekkimise ja lagundamise perioodilisusest tuletati ka nende üldine nimetus – tsükliin.

Lisaks tsükliinsõltuvatele kinaasidele avastati nende aktiivsust tugevasti pidurdavad valgud (inhibiitorid), mille roll on hoida kinaaside aktiivsust kontrolli all. Sarnaselt tsükliinidele töötavad ka inhibiitorid rangelt reguleeritud lainetena. Nii on pärmis *S. cerevisiae* G1 faasis tuvastatud inhibiitor Sic1. Selle

valgu peamiseks ülesandeks on lukustada S-faasi käivitava tsükliinsõltuva kinaasi aktiivsus ajal, kui seda tarvis ei lähe, ehk G1 faasi ajaks. Sellega aitab Sic1 rakkudel toota ja koguda S-faasi kinaasi piisaval hulgal ette, mis on vajalik, et hiljem tagada S-faasi veatu ja kiire läbiviimine. S-faas käivitatakse rakus plahvatusliku kinaasi aktiivsuse vabastamisega, mis on oluline selle tõrgeteta käivitamiseks. Plahvatusliku alguse tagab Sic1 lagundamise järsk dünaamika. Lisaks näitavad varasemad uuringud, et Sic1 on oluline S-faasi kinaasi vabastamise täpseks ajastamiseks, mis on oluline osa raku kaalutletud otsusest jagunema hakata. On oluline märkida, et samal ajal, kui Sic1 blokeerib S-faasi tsükliinsõltuva kinaasi aktiivsust, ei tee ta seda G1-faasi tsükliinsõltuva kinaasiga.

Nagu märgitud, selleks et S-faasi käivitada, peab rakk vabanema inhibiitorist Sic1. See käib inhibiitorvalgu järsu lagundamise teel, mis omakorda on käivitatud selle fosforüleerimisega. Sic1 valgule lisatakse järgemööda kokku kuni 10 fosforhappe jääki. Fosforijääke lisavad rakutsüklit reguleerivad kinaasid, näiteks G1 kinaasid, mille aktiivsust Sic1 ei pidurda. Selleks, et käivitada Sic1 lagundamine, on sellele vaja lisada mitu fosforhappe jääki. Varasemad uuringud on jäänud kahetisele seisukohale selles osas, kas Sic1 lagundamiseks tuleb lisada mingi piirarv fosfaate suvalistesse kohtadesse, või viib järgemööda toimuv fosfaatide lisamine Sic1 molekulil välja kindlatesse piirkondadesse, kuhu jõudmine käivitab lagundamise. Kuni viimase ajani ei olnud teada, kuidas täpselt nende paikadeni jõutakse, kas seda teeb G1-tsükliinsõltuv kinaas üksi või võtavad selle protsessi mingis etapis üle esmased vabanevad S-faasi kinaasi molekulid. Seetõttu ei olnud ka teada, mis tagab järsu plahvatusliku dünaamika Sic1 lagunemisel ning kuidas seda reguleeritakse nii, et rakutsükli S-faas ei käivituks liiga vara, ega ka mõttetult hilja. Seejuures on huvitav fenomen, et Sic1 on ühteaegu S-faasi kinaasile nii substraat kui ka inhibiitor, mis keemilisest loogikast lähtudes, on vastanduvad rollid.

Käesoleva doktoritöö käigus mõõdeti G1 ja S-faasi tsükliinsõltuvate kinaaside erisusi Sic1 valgule fosforhappejääkide lisamisel. Meie töö tulemustena selgus, et G1 ja S-faasi tsükliinid mängivad väga erinevat, kuid omavahel täiesti seotud rolli Sic1 lagundamisel. Me leidsime, et Sic1 fosforüülimine on suunatud ning kindlas järjestuses toimuv protsess, mis tipneb lagundamiseks vajalike paikade fosforüülimisega. Eraldiseisvalt nendesse kindlatesse piirkondadesse fosforhappejääkide lisamine on väga vaevaline protsess, seda eriti G1 tsükliinsõltuvale kinaasile. Me avastasime, et Sic1 valgule fosforhappejääkide lisamine on kolmeastmeline protsess, mis koosneb rakutsükli G1 faasis toimuvast kaheastmelisest fosforüülimisest ning viimaks Sic1 lagundamisele suunavast fosforüülimisest G1/S üleminekul.

Täpsemalt, G1 faasis toimub Sic1 osaline fosforüülimine mitteinhibeeritud kinaaside poolt. Seda kutsutakse ka praimerfosforüülimiseks. Siin fosforüülivad Sic1 valku mitmed kinaasid, mis tajuvad raku „tervislikku“ seisundit ning ümbritsevat keskkonda. Varasemalt on teada, et need kinaasid võivad raku jagunemist edasi lükata või ka varasemaks tuua, kuid seni puudus arusaam, kuidas nad seda teevad. Järgmises etapis tekivad S-faasi kinaasi kompleksid,

mis inhibeeritakse Sic1 poolt. Me avastasime, et inhibitoorse kompleksi moodustamise käigus fosforüülib inhibeeritav S-faasi kinaas Sic1 teatud tasemeni kuni viimane pidurdab kinaasi katalüütilise aktiivsuse täielikult. Lõpuks, olles jõudnud S-faasi käivitamiseks sobiva hetkeni, toimub Sic1 viimase etapi fosforüülimine, mis viib tema lagundamiseni. Me avastasime, et viimane Sic1 fosforüülimise etapp sisaldab topelt-negatiivset tagasisidet vabaneva S-faasi kinaasi enda poolt, põhjustates G1/S lülituseks vajaliku aktiivsuse vabanemise plahvatusliku iseloomu.

Erakordselt huvitavaks kujunes aga avastus, kuidas Sic1 kaudu reguleeritakse G1/S lülituse ajastamist. Nimelt, me leidsime, et esimese ja kolmanda etapi vahel toimuv inhibitoorse kompleksi sisene Sic1 fosforüülimine toimub mööda kahte erinevat rada. Üks rada suunab Sic1 lagundamiseks vajalike piirkondadeni, kuid teine rada viib tupikusse, kus Sic1 lagundamist ei järgne. S-faasi kinaas, mille aktiivsust Sic1 pidurdab, ei oska ise kahe raja vahel valida, ning liigub kahe raja vahel juhuslikult. Samas, kui eelnevalt on tehtud praimer-kinaaside poolt algust ühe või teise rajaga, valib S-faasi kinaas inhibitoorse kompleksi moodustumise käigus eelistatult selle raja. Mida rohkem on lagundamisele viivat rada ette märgitud, seda tõenäolisemalt valib ka S-faasi kinaas inhibiitoriga kompleksi moodustades oma jääk-aktiivsuse väljendamiseks selle raja ja seda raskem on Sic1 valgul S-faasi kinaasi aktiivsust pidurdada. Kui aga G1-faasis on ülekaalus kinaasid, mille sihtmärgiks on tupikraja alustamine, valib S-faasi kinaas selle raja ning jääb inhibitoorset kompleksi moodustades pidama tupikus. Selle mehhanismiga fikseeritakse eelseisvaks G1/S lülituseks Sic1 tase ning selle kaudu omakorda määratakse ette S-faasi alustamise aeg. Ühtlasi tagatakse, et stressiolukorras oleks võimalik hoida piisavat ajavaru olukorraga kohanemiseks.

Sellega lahendasime me pärmi rakutsükli G1/S lülituse mudeli kvantitatiivsetel alustel. Oma viimases töös näitasime me õnnestunult, et tänu uudsele teadmisele on võimalik pärmi G1/S lülitust soovitud viisil ümber kujundada, avades sellega uudse võimaluse sünteetilise mikrobioloogia rakendusteks.

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PUBLICATIONS

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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.

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.

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Publikatsioonide loetelu:

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.

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.

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