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Ordering the phosphorylation of cyclin-dependent kinase Cdk1 substrates in the cell cycle

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Ordering the phosphorylation of cyclin-dependent kinase Cdk1 substrates in the cell cycle

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ORIGINAL PUBLICATIONS

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II **Örd M.**, Puss K.K., Kivi R., Möll K., Ojala T., Borovko I., Faustova I., Venta R., Valk E., Kõivomägi M., and Loog M. (2020). **Proline-Rich Motifs Control G2-CDK Target Phosphorylation and Priming an Anchoring Protein for Polo Kinase Localization.** Cell Rep. 2020 Jun 16;31(11):107757.

III Faustova I., Bulatovic L., Matiyevskaya F., Valk E., **Örd M**., and Loog M. (2021). **A new linear cyclin docking motif that mediates exclusively S-phase CDK-specific signaling.** EMBO J. 2021 Jan 15;40(2):e105839

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V Bandyopadhyay, S., Bhaduri, S., **Örd, M.**, Davey, N.E., Loog, M., and Pryciak, P.M. (2020). **Comprehensive Analysis of G1 Cyclin Docking Motif Sequences that Control CDK Regulatory Potency In Vivo.** Curr Biol. 2020 Nov 16;30(22):4454–4466.

Author's contributions to the listed articles are as follows:

REF I: I partially conceived the study and performed the experiments, interpreted the results and co-wrote the manuscript.

REF II: I partially conceived the study, performed the experiments, analyzed the data and co-wrote the manuscript.

REF III: I contributed to designing and performing the experiments, interpreting the data and writing the manuscript.

REF IV: I contributed to experiment design, performed the experiments, analyzed the data and co-wrote the manuscript.

REF V: I performed and analyzed the fluorescence microscopy experiments.

ABBREVIATIONS

APC	Anaphase-promoting complex
CAK	Cyclin-dependent kinase activating kinase
CDK	Cyclin-dependent kinase
CKI	Cyclin-dependent kinase inhibitor
Cks	Cdc28 kinase subunit
E2F	Factor activating adenovirus E2 promoter
FEAR	Cdc Fourteen Early Anaphase Release
GINS	Complex of Sld5, Psf1, Psf2, and Psf3
Hp	Cyclin hydrophobic patch
Hpm	Hydrophobic patch mutant
k_{cat}	Catalytic constant, turnover number
k_{cat}/K_M	Specificity constant
K_d	Dissociation constant
K_M	Michaelis constant
LP	Leucine- and proline-rich docking motif for yeast G1 cyclins
MBF	MCB-binding factor
MAPK	Mitogen-activated protein kinase
MCB	MluI cell cycle box
MEN	Mitotic exit network
ORC	Origin Recognition Complex
PEST motif	Protein degradation motif rich in proline, glutamic acid, serine and threonine
PP2A	Protein phosphatase 2A
Pre-RC	Pre-replication complex
Rb	Retinoblastoma protein
RxL	Cyclin docking motif with consensus R/K-x-L-x{0,1}-Φ
SAC	Spindle assembly checkpoint
SBF	SCB-binding factor
SCB	Swi4 cell cycle box
SCF	Skp1/Cullin/F-box ubiquitin-protein ligase complex
SLiM	Short linear interaction motif
SPB	Spindle pole body

1. INTRODUCTION

Cell division is the basis of growth and reproduction. In eukaryotes, cell division is part of a network of coordinated events called the cell cycle. The intent of mitotic cell cycle is flawless transfer of genetic material and other cellular components from one cell to two daughter cells. This requires an ordered progression through a series of events, by which first the organelles and DNA are precisely duplicated and then segregated between the daughter cells. Errors in the coordination of cell cycle with environmental signals or in cell cycle processes such as DNA replication or chromosome segregation can often lead to uncontrolled cell division and cause cancer.

Most cell cycle events are controlled by phosphorylation of proteins that carry out specific processes. Phosphorylation is the most wide-spread post-translational modification that is highly reversible and it can affect the stability, activity, localization and interactions of the target protein. The central regulators of cell cycle processes are cyclin-dependent kinases (CDKs), which phosphorylate hundreds of target proteins to trigger most cell cycle events. In human cells, a set of different cyclin-CDK complexes are activated during the cell cycle, whereas in fungi a single CDK – Cdk1 – that is activated by different cyclins, drives the cell cycle.

Importantly, it has been shown that the timing of DNA replication, mitosis, and other cell cycle events, is directly connected to Cdk1 activity. This has raised a question that how a single kinase promotes different events during the cell cycle. Cdk1, and several other central cell cycle regulators are highly conserved in eukaryotes. This, in combination with the easy genetic manipulation, has made the unicellular fungi popular model organisms for the eukaryotic cell cycle.

Saccharomyces cerevisiae Cdk1 is activated by nine different cyclins and phosphorylates about 500 substrate proteins during the cell cycle. The Cdk1 substrate recognition takes place on at least three levels. First, the kinase active site targets a variety of phosphorylation motifs. Secondly, in addition to activating Cdk1 and directing it to specific subcellular locations, cyclins can bind linear docking motifs on substrate proteins and direct the kinase to phosphorylate specific substrates. Thirdly, Cdk1 complex contains a third protein – Cks1 – that binds to phosphorylated proteins and promotes multisite phosphorylation.

The aim of this study was to analyze the mechanisms that enable CDKs to phosphorylate hundreds of target proteins in a timely-resolved manner during the cell cycle. For this, the cyclin-specific interactions of S, G2, and M phase cyclin-Cdk1 complexes were analyzed with the aim to describe the cyclin-specific targeting mechanisms and to understand the functions and regulation of these complexes. Further, the study aimed to analyze how the properties and patterns of different linear motifs on substrate proteins affect the timing of phosphorylation.

2. LITERATURE OVERVIEW

The literature overview first gives an introduction to the eukaryotic cell cycle and then focuses on the cyclin-dependent kinases that regulate cell cycle. The overview explains the regulation of CDK activity and describes the substrate proteins and processes governed by CDK. Finally, the mechanisms ordering cell cycle phosphorylation, including CDK substrate targeting and counteracting phosphatases, are addressed. The literature overview focuses on *Saccharomyces cerevisiae*, but information from metazoans and fission yeast is also included.

2.1. Eukaryotic cell cycle

The cell cycle is a coordinated series of events leading to first duplication and later segregation of chromosomes and organelles to produce two daughter cells. As cells are the minimal independent units of life, cell cycle is the basis for both reproduction of unicellular organisms and the development of a mature organism from a fertilized egg.

The key events of cell cycle must occur in a strict order to ensure robust renewing and partitioning of the cellular components. The cell cycle can be divided to distinct phases: DNA is replicated in S phase (synthesis), which is followed by division of chromatids and cytoplasm in M phase (mitosis). S and M phases are often separated by two gap phases G1 and G2, occurring before S and M phases, respectively. The gap phases provide additional time for cell growth and preparation for either DNA replication or mitosis. Further, the gap phases function as regulatory points to integrate extra- and intracellular information into cell cycle progression. For example, G1 phase is also a decision point for the cell whether to enter the cell cycle, stay in G1 phase for longer, or exit the cycle and differentiate.

There is significant variability in the structure of cell cycle depending on the organism and cell type. The length of the cell cycle can range from 8 minutes in early embryos of *Drosophila melanogaster* to around 24 hours in fast-dividing mammalian cells. The early embryonic cell cycle of *D. melanogaster* consists of only S and M phases, lacking both gap phases and cytokinesis, and leads to creation of a multinucleate cell (Morgan, 2007). Mammalian HeLa cells grown in tissue cultures, on the other hand, allocate around 10 hours to both G1 phase and S phase, and 1–3 hours to G2 and M phase (Hahn et al., 2009). Importantly, despite the variability in cell cycle organization, the core regulatory network controlling the cell cycle events has remained conserved in eukaryotes (Morgan, 2007). For this reason, simple model systems such as budding yeast *Saccharomyces cerevisiae* and fission yeast *Schizosaccharomyces pombe* and frog *Xenopus laevis* embryo are widely used to study the core cell cycle control machinery. Due to being easily genetically modifiable, studies in budding yeast have provided much of the fundamental understanding of cell cycle regulation.

At the center of cell cycle control machinery are the cyclin-dependent kinases (CDKs) that phosphorylate hundreds of target proteins to initiate most cell cycle

events, including DNA replication and mitosis (Enserink and Kolodner, 2010). Through phosphorylation, CDKs control the stability, localization, activity and interactions of cell cycle proteins, leading to initiation of specific events at different phases of the cell cycle (Fisher et al., 2012).

2.2. Cyclin-dependent kinases – the master regulators of cell cycle

CDKs were first described as proteins that are essential for both DNA replication and mitosis (Lörincz and Reed, 1984; Nurse and Bissett, 1981; Reed and Wittenberg, 1990). Importantly, the cyclin-dependent kinase *CDK1* gene was found to be homologous in yeasts and mammalians and it was shown that the human *CDK1* gene is able to replace the endogenous gene in budding yeast (Beach et al., 1982; Lörincz and Reed, 1984; Ninomiya-Tsuji et al., 1991). This was the first indication that the regulatory network controlling the cell division is highly conserved in eukaryotes.

The human proteome contains 20 CDKs and budding yeast 6 CDKs that can be divided based on function to cell-cycle related and transcriptional CDKs (Malumbres, 2014). Importantly, only one CDK – Cdk1 – is essential for cell cycle progression in yeasts and mammalian cells (Morgan, 1997; Santamaría et al., 2007). In budding yeast, along with Cdk1 also Pho85 contributes to cell cycle regulation, whereas the four other CDKs – Kin28, Ctk1, Bur1 and Srb10 – regulate transcription (Malumbres, 2014). In humans, Cdk1, Cdk2, Cdk4 and Cdk6 are directly involved in cell cycle regulation (Malumbres, 2014).

CDKs are serine/threonine kinases that govern the progression of cell cycle events by phosphorylation of hundreds of target proteins (Enserink and Kolodner, 2010). For example, Cdk1 in budding yeast is estimated to phosphorylate 500–700 proteins, which is roughly 10% of the proteome (Holt et al., 2009; Ubersax et al., 2003). In addition to triggering the core cell cycle processes such as DNA replication and chromosome segregation, CDKs coordinate transcription, metabolism and other processes with cell division. As CDKs regulate a wide range of events, they are often referred to as the master regulators of cell cycle.

2.2.1. Cyclin-CDK-Cks complex

CDKs are active only in complex with a regulatory protein – a cyclin. The CDK catalytic subunit consists of the protein kinase domain, which has an N-terminal lobe rich in β-sheet and a mainly helical C-terminal lobe (De Bondt et al., 1993). The kinase active site lies between the two lobes, however, solving the structure of human Cdk2 revealed that in monomeric Cdk2 the accessibility of protein substrates to active site is limited and the key catalytic site residues are oriented in a way that does not enable efficient catalysis (De Bondt et al., 1993) (**Fig. 1**). Cyclin binding induces extensive conformational changes in CDK opening up the

active site for protein substrates and orienting ATP to enable transfer of the γ-phosphate (Jeffrey et al., 1995) (**Fig. 1**). The binding of cyclin A to Cdk2 increases the kinase activity by five orders of magnitude (Connell-Crowley et al., 1993).

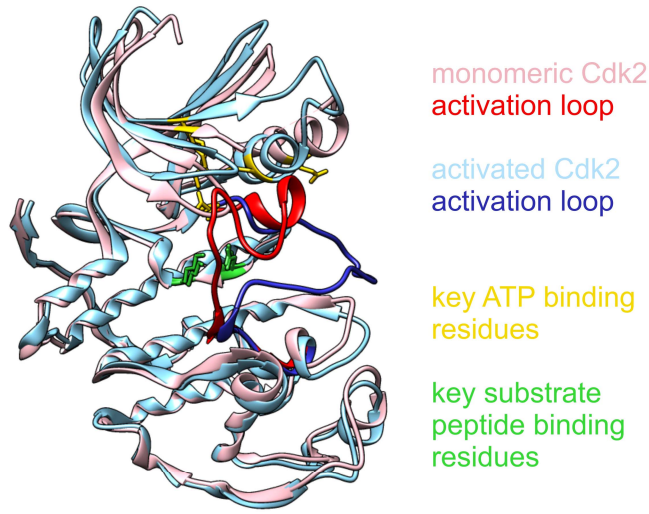

Figure 1. Structure of Cdk2 showing the cyclin-dependent activation of CDK. The structure of monomeric Cdk2 (1HCK (Schulze-Gahmen et al., 1996)) is presented in pink, with the activation loop shown in red. Aligned with the monomeric structure is the structure of Cdk2 that is activated by cyclin A binding, shown in light blue with the activation loop in blue (1JST (Russo et al., 1996a)). The kinase active site lies between the β-sheet and the α-helical domain. The key ATP orienting residues (K33, E51 and D145) are shown in yellow and the Cdk2 residues interacting with the phospho-acceptor residue in the substrate peptide (D127, K129) are highlighted in green (Andzelm et al., 1995; Brown et al., 1999). In the monomeric Cdk2, the activation loop blocks access of substrate peptide to the active site, whereas in the cyclin-bound Cdk2, the activation loop moves away from the active site and forms a platform that contributes to substrate recognition (Brown et al., 1999; Jeffrey et al., 1995). Upon cyclin binding, a small helix in the activation loop melts and this enables proper orientation of ATP (Jeffrey et al., 1995; Wood and Endicott, 2018). In addition, cyclin binding induces minor conformational changes in N- and C-terminal lobes of Cdk2 (Jeffrey et al., 1995).

Cyclins are proteins that activate CDKs and that have a cyclin box, which is around 100 amino acids and forms a structure of 5 α-helixes (Morgan, 1997). Except for the conserved cyclin box, cyclins are quite diverse in amino acid sequence (Lees and Harlow, 1993). Most cyclins have two cyclin boxes, but only one interacts with CDK (Malumbres, 2014). Upon cyclin-CDK binding, CDK undergoes significant conformational changes, however, the cyclin structure remains unchanged, thus, cyclin functions as a rigid structure that shapes the

CDK to an active conformation (Brown et al., 1995; Jeffrey et al., 1995). In addition to CDK activation, cyclins can directly interact with substrate proteins to promote phosphorylation of a specific set of CDK targets (Kõivomägi and Skotheim, 2014). Also, cyclins often have N- or C-terminal disordered domains that contain regulatory motifs such as localization and destruction signals (Brown et al., 1995; Edgington and Futcher, 2001; Salama et al., 1994; Zachariae, 2004). Thus, cyclins are not merely activators of CDK, they confer specificity to the kinase by governing its localization, substrate interactions and timing of activity.

The CDK complex also contains a third protein – Cks (Cdc28 kinase subunit). Budding yeast has one Cks protein, Cks1, whereas animal cells express two, Cks1 and Cks2 (Arvai et al., 1995). The 9–18 kDa Cks proteins bind to the C-terminal lobe of CDK, but unlike cyclins, are not essential for activating CDK, although budding yeast Cks1 has been found to stabilize G1-Cdk1 complexes (Bourne et al., 1996; Reynard et al., 2000). Instead, Cks proteins have a phosphate binding pocket and function as adaptors (Arvai et al., 1995). For example, Cks proteins promote multisite phosphorylation, mediate degradation of cyclins A and B and CDK inhibitor $p27^{Kip1}$ by interacting with E3 ubiquitin-protein ligases anaphase-promoting complex/cyclosome (APC) and Skp1/Cullin/F-box protein complex (SCF), respectively (Ganoth et al., 2001; Kõivomägi et al., 2011a; McGrath et al., 2013; Spruck et al., 2001; Wolthuis et al., 2008; Van Zon et al., 2010). Similarly to CDKs, Cks proteins are highly conserved and human Cks1 and Cks2 are able to substitute the endogenous Cks1 in budding yeast (Richardson et al., 1990).

CDKs initiate specific cell cycle events, however, the levels of CDKs are constant throughout the cell cycle (Morgan, 2007). The activities of CDKs are regulated via cyclin expression, phosphorylation of CDKs and binding of inhibitor proteins.

2.2.2. Different cyclin-CDK complexes are active in different cell cycle phases

The first layer in regulation of CDK activity is based on expression of cyclins, as cyclins are essential for activation of CDKs. Most cell cycle CDKs are activated by multiple cyclins whose levels oscillate during the cell cycle. This is in contrast to transcriptional CDKs that bind a single cyclin with constant levels in the cell cycle (Malumbres, 2014).

Budding yeast Cdk1 is sequentially activated by nine different cyclins, whose expression is limited to specific cell cycle stages mainly by transcriptional and post-translational mechanisms (Bloom and Cross, 2007). Cdk1 cyclins can be divided to five groups based on expression profiles (**Fig. 2**). The most upstream G1 cyclin is Cln3, which contributes to cell cycle entry and is present at very low levels throughout the cell cycle (Bállega et al., 2019; Cross and Blake, 1993; Tyers et al., 1993). The eight following cyclins form pairs of closely related cyclins that are expressed simultaneously in the cell cycle (Fitch et al., 1992a; Hadwiger et al., 1989; Schwob and Nasmyth, 1993). In late G1,

cyclins *CLN1* and *CLN2* are transcribed (Hadwiger et al., 1989; Wittenberg et al., 1990), followed by appearance of S phase cyclins Clb5 and Clb6 that initiate S phase (Epstein and Cross, 1992; Schwob and Nasmyth, 1993). During S phase the transcription of G2 cyclins *CLB3* and *CLB4* is activated, followed by expression of *CLB1* and *CLB2* in mitosis (Fitch et al., 1992a; Surana et al., 1991).

A similar sequential expression of cyclins occurs during the mammalian cell cycle, however, there is an additional level of complexity as different cyclins can bind different CDKs. At least three CDKs – Cdk4, Cdk6 and Cdk2 – govern the G1 events in mammals. Cdk4 and Cdk6 are activated by cyclin D, whereas Cdk2 is activated by cyclin E in late G1. To initiate S phase, Cdk2 forms a complex with cyclin A, whereas cyclin A and cyclin B with Cdk1 mediate the progression through G2 and M phase (Malumbres, 2014; Morgan, 2007).

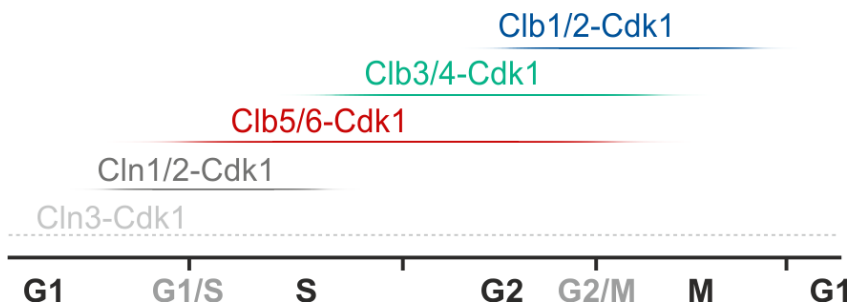

Figure 2. The expression profiles of cyclin-Cdk1 complexes in *S. cerevisiae* cell cycle.

2.2.2.1. Regulation of cyclin transcription

The transcription of cyclin genes is cell-cycle-regulated and follows similar principles in yeast and higher eukaryotes. The transcription of the G1 cyclins, *CLN1-3* in yeast and cyclin D1 in mammals, is regulated by extracellular signals such as mitogenic growth factors, nutrients or different stress conditions (Bállega et al., 2019; Flick et al., 1998; Klein and Assoian, 2008). Once committed to cell cycle, sequential transcriptional waves promote the expression of following cyclins so that each cyclin-CDK complex induces transcription of the next cyclin (Bertoli et al., 2013; Enserink and Kolodner, 2010). Following the commitment to cell cycle, the transcription of cyclins is no longer dependent on mitogenic signals (Bertoli et al., 2013).

2.2.2.2. Regulation of cyclin degradation

In addition to the periodic transcription of cyclin genes, the abundance of cyclins is governed by ubiquitin ligases that target cyclins for degradation by the proteasome.

Ubiquitination is a 3-step process that involves an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme and an E3 ubiquitin-protein ligase and results in attaching 76-amino-acid long ubiquitin proteins to the target protein, tagging it for destruction (Zheng and Shabek, 2017). The specificity in ubiquitination rises from the E3 ubiquitin-protein ligases that target substrate protein through degron motifs or domain interactions (Zheng and Shabek, 2017). There are two key E3 ubiquitin-protein ligase complexes controlling the cell cycle: SCF and APC (Vodermaier, 2004). The SCF complexes are active throughout the cell cycle and have a variety of functions in addition to cell cycle regulation (King et al., 1996; Zhou and Howley, 1998). The activity of APC, on the other hand, is restrained to mitosis and G1, where its only essential functions are degradation of mitotic cyclins and anaphase inhibitor securin (Thornton and Toczyski, 2003). In both yeasts and metazoans, the SCF complexes mediate degradation of early cyclins, whereas APC is necessary for degradation of S and M phase cyclins.

The SCF complexes target budding yeast G1 cyclins Cln1, Cln2 and Cln3 and S cyclin Clb6 (Deshaies et al., 1995; Jackson et al., 2006; Skowyra et al., 1997). As a result, these cyclins are short-lived proteins and their levels drop shortly after the inactivation of their transcription (Deshaies et al., 1995; Jackson et al., 2006). The substrate specificity of the SCF complexes is governed by over 20 F-box proteins that function as substrate adaptors (Willems et al., 2004). Cyclins are targeted by two F-box proteins – Cdc4 and Grr1 – that have different localization and degron motif specificity. While Grr1 is present both in the nucleus and cytoplasm, Cdc4 is found only in the nucleus (Blondel et al., 2000). Likewise, SCF^{Cdc4} has been found to be responsible for degradation of nuclear cyclins Cln3 and Clb6, whereas SCF^{Grr1} destabilizes cytoplasmic Cln2 (Deshaies et al., 1995; Jackson et al., 2006; Landry et al., 2012). In many cases, F-box proteins interact with phosphorylated substrates, thus linking phosphorylation and protein degradation (Willems et al., 2004). The consensus phosphodegron motif for SCF^{Cdc4} was defined based on a peptide from cyclin E1 as I/L/P-I/L-pS/pT-P-{RKY}4, where pS/pT is phospho-serine or -threonine and {RKY} refers to disfavored residues (Nash et al., 2001). Later, it was found that the presence of another phosphate in position +3 or +4 from the first increases the degron binding affinity and that the doubly phosphorylated degrons function as optimal Cdc4 degrons (Bao et al., 2010; Hao et al., 2007). A well-defined degron motif for SCF^{Grr1} has not been defined, however rapid turnover of Cln cyclins has been shown to depend on their intrinsically disordered C-terminal regions that contain CDK phosphorylation sites and proline, glutamate, serine and threonine rich PEST sequences (Deshaies et al., 1995; Skowyra et al., 1997).

The stability of G1 cyclins is also regulated by extracellular signals, as G1-CDK activity determines the entry to cell cycle. Interestingly, Pho85, a CDK that is regulated by environmental conditions, affects Cln3 stability and cell cycle entry in two opposite ways, depending on the cyclin that activates Pho85. First, in conditions with enough phosphate, Pho80-Pho85 complex

phosphorylates Cln3 at specific sites in the PEST region, leading to stabilization of Cln3 and promotion of cell cycle entry, whereas phosphate starvation results in Pho80-Pho85 inhibition and decrease in Cln3 levels (Menoyo et al., 2013). On the other hand, nitrogen starvation or mating pheromone stimulation leads to activity of Pho85 with cyclins Clg1 and Pcl2 that hinder Cln3 accumulation through phosphorylating Hsp70 chaperone Ssa1 that then binds and destabilizes Cln3 (Truman et al., 2012).

The degradation of S and M phase cyclins Clb1-5 is initiated in mitosis by the APC, a large E3 ubiquitin-protein ligase consisting of 13 subunits in budding yeast (McLean et al., 2011). APC induces both the onset of anaphase and exit from mitosis, with securin and mitotic cyclins as the essential substrates (Thornton and Toczyski, 2003). The activity of APC is tightly regulated by phosphorylation and binding of inhibitor and activator proteins (McLean et al., 2011). There are two differentially regulated activators – Cdc20 and Cdh1 – that contribute to ordered ubiquitination of APC targets (Dawson et al., 1995; Zachariae et al., 1998). APC^{Cdc20} is active in mitosis, where the Cdk1 activity is high, whereas dephosphorylation of Cdh1 in anaphase results in activation of APC^{Cdh1} in late mitosis and G1 phase (Kramer et al., 2000). APC substrates are ubiquitinated in strict order: for example, APC^{Cdc20} targets S phase cyclin Clb5 prior to anaphase, followed by ubiquitination of securin, and APC^{Cdh1} finalizes the destruction of mitotic cyclin Clb2 in anaphase (Lu et al., 2014). Similarly, in metazoans, cyclin A is degraded in prometaphase about 30 minutes before degradation of cyclin B (Elzen and Pines, 2001). The ordering of APC substrate degradation is determined by a variety of mechanisms including the degron motif specificity, co-operativity of multiple motifs, phosphorylation of degron motifs and localization (Arnold et al., 2015; Davey and Morgan, 2016; Lu et al., 2014). Activation of APC leads to destruction of mitotic cyclins and loss of Cdk1 activity, thereby resetting the cell cycle in G1. Therefore, the cell cycle can be viewed as a CDK/APC oscillator.

2.2.3. Regulation of CDK activity by phosphorylation

Cdk1 is subjected to both activating and inhibitory phosphorylation (**Fig. 3**). The activating phosphorylation in the T-loop on T169 in yeast Cdk1 or T160 in human Cdk2 increases the kinase activity about 100-fold (Cismowski et al., 1995; Connell-Crowley et al., 1993). Phosphorylation of T160 in Cdk2 causes a small movement in the T-loop that leads to enhanced interaction with the substrate peptide (Russo et al., 1996a). Activating phosphorylation is carried out by CDK activating kinases (CAK): Cdk7 in mammals (Fesquet, 1993; Poon, 1993; Solomon et al., 1993) or by monomeric Cak1, a distant relative of CDKs, in budding yeast (Espinoza et al., 1996; Kaldis et al., 1996). Cak1 activity is constant during the cell cycle, and different cyclin-Cdk1 complexes had similar levels of pT169, indicating that the activating phosphorylation of

Cdk1 is not altered during the cell cycle (Espinoza et al., 1996; Kõivomägi et al., 2011b).

In contrast to the activating phosphorylation, the inhibitory phosphorylation is regulated during the cell cycle and in response to checkpoint signaling. Phosphorylation in the glycine-rich G-loop, on residue Y19 in *S. cerevisiae* Cdk1 or on T14 and Y15 in human Cdk2 results in decrease in kinase activity by interfering with protein substrate binding and ATP alignment for phosphorylation (Bártová et al., 2004; Endicott et al., 1999).

The inhibitory phosphorylation of Cdk1 has been shown to play a key part in timing the onset of mitosis in animals and fission yeast, but not budding yeast (Gould and Nurse, 1989; Lew and Kornbluth, 1996; McNulty and Lew, 2005). Mitotic entry requires full activity of mitotic Cdk1 that is regulated by inhibitory phosphorylation of Cdk1 carried out by kinase Wee1 (Swe1 in budding yeast) and removed by phosphatase Cdc25 (Mih1 in budding yeast) (Malumbres, 2014; Mendenhall and Hodge, 1998; Russell and Nurse, 1986). The Wee1/Cdk1/Cdc20 form a bistable system, where Wee1 is active prior to mitosis and Cdk1/Cdc20 are fully active in mitosis (Pomerening et al., 2003; Sha et al., 2003). The bistable system is based on two feedback loops. Wee1 and Cdk1 form a double negative feedback loop, where Wee1 phosphorylation by Cdk1 results in Wee1 inactivation (Harvey et al., 2005; Tang et al., 1993). Additionally, Cdc25 and Cdk1 form a positive feedback loop, where Cdc25 activity leads to dephosphorylation of Cdk1 and subsequent Cdk1-dependent multiphosphorylation of Cdc25 further increases the phosphatase activity (Hoffmann et al., 1993; Izumi and Maller, 1993; Kumagai and Dunphy, 1992).

In budding yeast, Wee1 homolog Swe1 has a minor role in timing the mitotic entry in unstressed cells, but has been reported to function in morphogenesis checkpoint to delay nuclear division in case of actin perturbations (Harvey and Kellogg, 2003; McNulty and Lew, 2005). Importantly, only mitotic cyclin-Cdk1 complexes are targeted by Swe1 for inhibitory phosphorylation (Hu and Aparicio, 2005; Keaton et al., 2007). In addition, Cdk1 Y19 phosphorylation increases in response to DNA damage, however, inhibitory phosphorylation of Cdk1 is not essential for DNA damage induced G2 arrest (Amon et al., 1992).

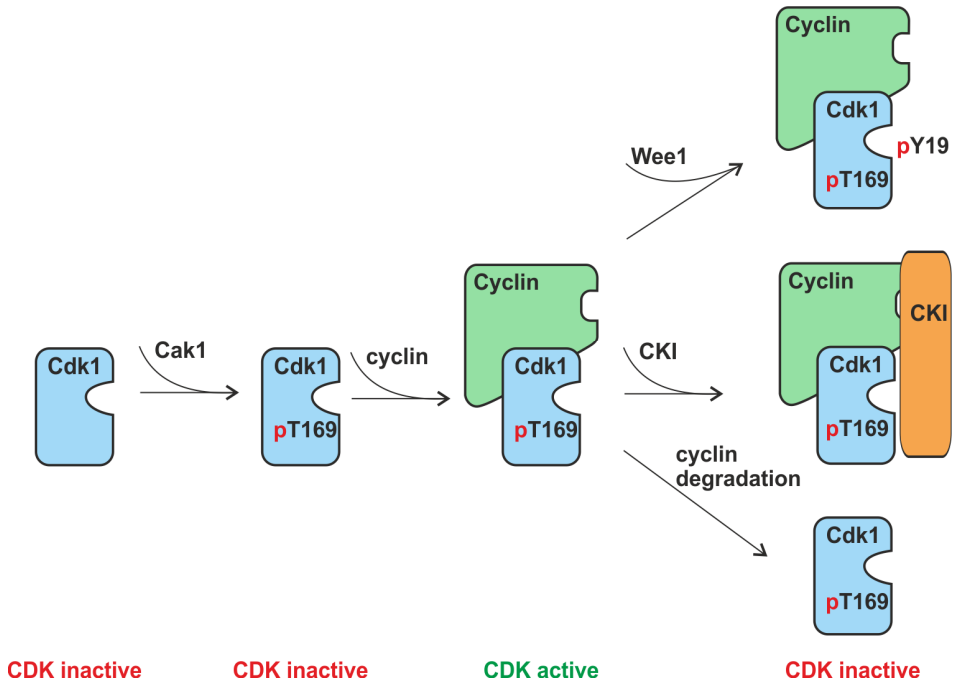

Figure 3. Regulation of CDK activity. Full activation of CDK requires cyclin binding and activating phosphorylation by CAK, which can target either monomeric CDK or cyclin-CDK complex (Kaldis et al., 1998). The cyclin-CDK complex can be inactivated either by inhibitory phosphorylation by Wee1, binding of an inhibitory protein (CKI), or by degradation of the cyclin.

2.2.4. Regulation of CDK activity by inhibitor proteins

To ensure tight control of kinase activity, Cdk1 is also targeted by several stoichiometric inhibitor proteins (**Fig. 3**). The cyclin-dependent kinase inhibitors (CKIs) are crucial to prevent leakage of Clb-Cdk1 activity in G1 and to inhibit Cln-Cdk1 activity in response to environmental stress or mating pheromone.

The G1/S transition requires activity of Clb-Cdk1 complexes that are inhibited in G1 by Sic1 (Mendenhall et al., 1993; Schwob et al., 1994). The inhibitory domain of Sic1 is structurally homologous to mammalian $p27^{KIP1}$, an inhibitor of cyclin A-Cdk2 (Barberis et al., 2005). Sic1 is expressed from late M-phase to G1/S, where it is phosphorylated by Cln- and Clb-Cdk1 complexes, creating a feedback-driven abrupt release of Clb-Cdk1 activity necessary for the S phase (Donovan et al., 1994; Kõivomägi et al., 2011a; Schwob et al., 1994; Venta et al., 2012; Verma et al., 1997).

Cdc6, a replication protein, interacts specifically with mitotic Clb2-Cdk1 complex and contributes to inactivation of Clb2-Cdk1 in mitotic exit along with Sic1 (Archambault et al., 2003; Calzada et al., 2001; Mimura et al., 2004). In

addition to Cdk1 inhibition, the Clb2-Cdk1-Cdc6 interaction could prevent the origin licensing function of Cdc6 prior to the destruction of mitotic cyclins, thus contributing to replication control (Mimura et al., 2004).

Mating pheromone induces cell cycle arrest in G1 that is mediated by inhibition of Cln-Cdk1 by CKI Far1 (Chang and Herskowitz, 1990; Peter and Herskowitz, 1994). Far1 slightly delays cell cycle entry also during vegetative growth (Alberghina et al., 2004), but the presence of pheromone increases both Far1 expression and inhibitory potential through Far1 phosphorylation by MAPK (mitogen-activated protein kinase) Fus3 (Chang and Herskowitz, 1990; Gartner et al., 1998; Tyers and Futcher, 1993). As Sic1, following commitment to cell cycle, Far1 is phosphorylated by Cdk1, leading to Far1 degradation (Gartner et al., 1998).

Finally, Cip1 is a Cln-Cdk1 inhibitor whose expression and activity is increased in response to environmental stress, similarly to mammalian CKI $p21^{CIP1}$ (Chang et al., 2017; Ren et al., 2016). Similar to Far1 that is phosphorylated and activated by MAPK Fus3 (Tyers and Futcher, 1993), Cip1 is phosphorylated by osmotic stress MAPK Hog1, increasing the binding affinity of Cip1 with Cln-Cdk1 (Chang et al., 2017). Cip1 is stable throughout the cell cycle and although it is phosphorylated by Cdk1, the regulation of Cip1 is not understood yet (Chang et al., 2017).

2.3. Cdk1-controlled processes

In addition to the fundamental cell cycle processes like replication and segregation of the genetic material, many other molecular processes, such as morphogenesis, metabolism and transcription, are coordinated with cell cycle progression. Budding yeast Cdk1 is estimated to phosphorylate around 500 proteins, which is about 10% of the proteome (Ubersax et al., 2003). Importantly, the function of these phosphorylation events has been characterized for over 100 Cdk1 targets and this has revealed that Cdk1 regulates many proteins in addition to the core cell cycle control machinery (Enserink and Kolodner, 2010). The following chapters will give a brief overview of Cdk1-controlled events in different cell cycle phases.

2.3.1. Cdk1 functions in G1 phase

The key functions of Cdk1 in G1 phase are initiation of cell cycle transcriptional program, induction of bud formation and regulation of metabolism to fuel cell cycle. In G1, Cdk1 is activated by cyclins Cln1, Cln2 and Cln3 that have overlapping but also distinct functions. Cln3 is an activator of Cln1 and Cln2 expression, whereas Cln1 and Cln2 are responsible for other G1 functions of Cdk1 (Dirick et al., 1995). The early G1 phase is a decision point for the cell, as it can stay in G1 for a longer period or commit to cell cycle or differentiation

(Morgan, 2007). The commitment to cell cycle is decided at Start point in yeast (restriction point in animal cells), defined as the point after which the cell will progress through cell cycle independently of extracellular signals (Johnston et al., 1977).

Commitment to cell cycle is initiated by the most upstream G1 cyclin Cln3, whose activity determines the time of Start (Cross and Blake, 1993; Tyers et al., 1993). In early G1, Cln3-Cdk1 is sequestered to the endoplasmic reticulum by Whi3, Whi7 and chaperones Ssa1/2, where it cannot phosphorylate the nuclear targets necessary for activation of G1/S transcription (Vergés et al., 2007; Wang et al., 2004; Yahya et al., 2014). In late G1, Cln3 is released from the endoplasmic reticulum and accumulates to the nucleus via two mechanisms (Moreno et al., 2019; Vergés et al., 2007; Yahya et al., 2014). First, phosphorylation of Whi7, potentially by Cln3-Cdk1, decreases Whi7 association with endoplasmic reticulum and induces Whi7 degradation, releasing Cln3-Cdk1 (Yahya et al., 2014). Secondly, a co-chaperone Ydj1 competes with Cln3 in Ssa1 binding and displaces Cln3 from Ssa1 in late G1 (Vergés et al., 2007). Importantly, the chaperone Ydj1 availability links growth rate and stress with timing of cell cycle entry by modulating the accumulation of Cln3 in the nucleus (Moreno et al., 2019).

Nuclear Cln3-Cdk1 activates the G1/S transcriptional regulon that consists of over 200 genes including G1 and S phase cyclins *CLN2* and *CLB5* and many proteins necessary for budding and DNA replication (de Bruin et al., 2004; Costanzo et al., 2004; Dirick et al., 1992; Iyer et al., 2001; Tyers et al., 1993). The G1/S transcription wave is regulated by two transcription factor complexes: SBF (SCB-binding factor) and MBF (MCB-binding factor) (Dirick et al., 1992; Spellman et al., 1998). SBF is a complex of DNA-binding protein Swi4 and Swi6 that regulates transcription from promoters with SCB (Swi4 cell cycle box) elements. MBF, a complex of DNA-binding Mbp1 and Swi6, on the other hand, is necessary for inhibiting expression of G1/S genes with MCB (MluI cell cycle box) motifs outside G1 (de Bruin et al., 2006; Koch et al., 1993). Prior to Start, SBF is inhibited by Whi5 and Whi7, which are one of the earliest Cdk1 targets in G1 (de Bruin et al., 2004; Costanzo et al., 2004; Gomar-Alba et al., 2017). Phosphorylation of Whi5 by G1-Cdk1 (Cln1/2/3-Cdk1) causes dissociation of Whi5 from SBF and nuclear export of Whi5, freeing SBF to active G1/S transcription (de Bruin et al., 2004; Costanzo et al., 2004; Palumbo et al., 2016). Activation of SBF leads to increase in Cln1/2 expression, which further phosphorylate and inactivate Whi5, creating a positive feedback loop (Skotheim et al., 2008). The Start point has been defined as the nuclear export of over 50% of Whi5, showing the importance of Whi5 inactivation as a switch for cell cycle commitment (Doncic et al., 2011). In addition to Whi5 inactivation, Cln-Cdk1 may also phosphorylate and activate Swi6, as mutation of both Whi5 and Swi6 phosphorylation sites causes lethality (Costanzo et al., 2004). Interestingly, the G1/S transcription in animal cells is controlled by similar regulatory mechanisms, although the participating proteins are not conserved (Cooper, 2006). In animal cells, the G1/S transcriptional wave is controlled by E2F (factor activating adenovirus E2

promoter) transcription factor complexes and their inhibitor Rb (retinoblastoma protein), that is inactivated by cyclin D-Cdk4/6 (Bertoli et al., 2013). In addition to regulating SBF, Cdk1 localizes at a subset of genes and has been found to phosphorylate the C-terminal domain of RNA polymerase II, thereby activating the expression of these genes (Chymkowitch et al., 2012).

Start checkpoint has been defined as the point after which haploid cells do not respond to mating pheromone. Cell cycle and mating pathway are opposing paths, therefore, at Start, Cln-Cdk1 complexes inactivate mating pathway (Oehlen and Cross, 1994). Mating pheromone activates a MAPK cascade that after Start is repressed by Cln1/2-Cdk1 by phosphorylating scaffold protein Ste5 and kinase Ste20 (Oehlen and Cross, 1998; Strickfaden et al., 2007). Further, Far1, the CKI in pheromone response, is phosphorylated by Cln-Cdk1, targeting Far1 to degradation by SCF^{Cdc4}-mediated ubiquitination (Gartner et al., 1998).

Cell cycle is coordinated with changes in metabolism, as over 50% of metabolites change in abundance during the cell cycle (Ewald et al., 2016). From 309 Cdk1 targets identified in a phosphoproteomic screen, 127 proteins were found to function in metabolism (Zhao et al., 2016). Cln-Cdk1 induces global changes in metabolism to provide the necessary metabolites for cell cycle (Ewald et al., 2016; Kurat et al., 2009). This includes utilization of storage carbohydrates trehalose and glycogen to generate glucose and is mediated by Cdk1-dependent phosphorylation and activation of enzymes Nth1 and Gph1 (Ewald et al., 2016; Zhao et al., 2016). Also, Cdk1 promotes lipolysis by phosphorylating and activating triacylglycerol lipase Tgl4, which is necessary to provide lipids for membrane synthesis and budding (Kurat et al., 2009).

Activation of Cln1/2-Cdk1 brings about major changes in cell morphogenesis, as they trigger the formation of a bud (Lew and Reed, 1993). Cln-Cdk1 phosphorylates septin Cdc3, thus promoting disassembly of the old inherited septin ring (Tang and Reed, 2002). Key players in growth polarization that are regulated by Cdk1 are GTPases Cdc42 and Rho1 (Enserink and Kolodner, 2010). Before Start, Cdc42 is inhibited by two mechanisms that are reversed by Cdk1. First, Cdc24 is sequestered to the nucleus by CKI Far1, however, after Cdk1-induced degradation of Far1, Cdc24 localizes to the presumptive bud site (Nern and Arkowitz, 2000). Secondly, in early G1, Cdc42 is kept in inactive GDP-bound state by GTPase activating proteins Rga1, Rga2, Bem2 and Bem3, but it has been shown that Rga2, Bem2 and Bem3 are phosphorylated and inactivated by Cdk1 (Knaus et al., 2007; McCusker et al., 2007; Sopko et al., 2007). Cln2-Cdk1 also phosphorylates Tus1, an exchange factor for Rho1, thus activating Rho1 that controls actin organization (Kono et al., 2008). Following establishment of polarity, Cdk1-dependent phosphorylation of adaptor proteins Boi1 and Boi2 is necessary to maintain polarized growth (McCusker et al., 2007). In addition, Cdk1 directly controls vesicle transport to target exocytic vesicles to the bud (Duan et al., 2019; McCusker et al., 2007, 2012).

The segregation of chromosomes in anaphase is mediated by the mitotic spindle that originates from spindle pole bodies (SPBs), the functional analogues of centrosomes in budding yeast. The spindle pole body undergoes a duplication

cycle that is regulated by Cdk1 in multiple stages (Haase et al., 2001). In late G1, the Cln-Cdk1 complexes initiate SPB duplication by at least two mechanisms. Cln2-Cdk1 phosphorylates SPB component Spc42 to activate assembly of Spc42 into SPB (Jaspersen et al., 2004; Jones et al., 2018). Secondly, Cdk1 phosphorylation stabilizes Mps1, a kinase that regulates SPB duplication cycle (Jaspersen et al., 2004; Winey et al., 1991).

The final event in G1 is the degradation of Clb-Cdk1 inhibitor Sic1, as this leads to release of Clb5/6-Cdk1 activity and the onset of S phase (Schneider et al., 1996; Schwob et al., 1994). Cdk1 phosphorylates two di-phosphodegrons in Sic1, leading to SCF^{Cdc4}-mediated ubiquitination and proteolysis (Feldman et al., 1997; Hao et al., 2007; Kõivomägi et al., 2011a; Nash et al., 2001). Importantly, multisite phosphorylation and degradation depends on both Cln- and Clb-Cdk1 complexes, leading to a feedback-amplified release of Clb-Cdk1 activity necessary for abrupt G1/S switch (Kõivomägi et al., 2011a; Venta et al., 2012; Yang et al., 2013).

2.3.2. Cdk1-controlled processes in S phase

The key event in S phase is replication of the genome, which must occur only once per cell cycle. Cdk1 is essential for both initiation of DNA replication and inhibition of re-replication (Dahmann et al., 1995; Diffley, 2004). The replication process is divided to two stages to ensure that replication is initiated from each origin only once per cell cycle. Prior to replication, pre-replication complexes (pre-RCs) must assemble on the replication origins (Sclafani and Holzen, 2007). This process is called licensing and can occur only in G1, when Cdk1 activity is low. Then, the increase in Cdk1 activity at G1/S initiates origin firing, but also inhibits formation of pre-RCs (Parker et al., 2017; Sclafani and Holzen, 2007).

The licensing of replication origins in G1 is initiated by binding of ORC (Origin Recognition Complex) to the replication origins. ORC, a complex of six subunits (Orc1-6), recruits Cdc6, Cdt1 and the Mcm2-7 helicase complex, leading to formation of the pre-RC (Chen et al., 2007; Randell et al., 2006). Following the assembly of pre-RC during low Cdk1 activity, Cdk1 and Dbf4-dependent kinase Cdc7 are needed to recruit firing factors (Sld2, Sld3, Sld7, Dpb11, Cdc45, GINS (complex of Sld5, Psf1, Psf2, and Psf3) and DNA polymerase epsilon) that activate the helicase to unwind dsDNA (Heller et al., 2011; Yeeles et al., 2015). Cdc7 phosphorylates the Mcm2-7 helicase complex, leading to interaction of Sld3/Sld7 and Cdc45 with the helicase. The only essential Cdk1 substrates in replication initiation are Sld2 and Sld3, which function as adaptor proteins to recruit other firing factors including Dpb11 and DNA polymerase epsilon, finally leading to initiation of DNA replication (Masumoto et al., 2002; Tanaka et al., 2007; Yeeles et al., 2015; Zegerman and Diffley, 2007).

At the same time, Cdk1 phosphorylates the pre-RC components to inactivate licensing. Clb5-Cdk1 inhibits loading of Cdt1 to ORC by competitively interacting with Orc6 and by phosphorylating Orc2 and Orc6 (Chen and Bell, 2011; Nguyen et al., 2001; Wilmes et al., 2004). Also, Cdk1 phosphorylates Cdc6, triggering its SCF^{Cdc4}-mediated degradation (Drury et al., 1997, 2000). Finally, Clb-Cdk1-mediated phosphorylation of Mcm2 and Mcm3 results in nuclear export of the Mcm2-7 helicase (Liku et al., 2005; Nguyen et al., 2000). Therefore, along with triggering initiation of replication, Clb-Cdk1 inhibits licensing by multiple mechanisms to avoid re-replication.

DNA replication is accompanied by establishment of cohesion between sister chromatids, necessary for their bipolar segregation in anaphase (Uhlmann, 2009). Cdk1 modulates cohesin dynamics (Srinivasan et al., 2019) and, along with Cdc7, restricts generation of cohesion to S phase by triggering degradation of a cohesion-promoting acetyltransferase Eco1 after replication (Lyons and Morgan, 2011; Seoane and Morgan, 2017). Also, S phase Clb5/6-Cdk1 complexes promote telomere replication by phosphorylating telomere-binding protein Cdc13, whereas M phase Clb2-Cdk1 complex inactivates telomerase by phosphorylating Stn1 (Gopalakrishnan et al., 2017).

Cdk1 also regulates DNA damage response and checkpoint activation. DNA double-stranded breaks can be repaired either by homologous recombination or non-homologous end-joining (Trovesi et al., 2013). Replication in S phase provides a template for homologous repair and it has been shown, that Cdk1 phosphorylates many proteins in DNA repair to promote repair of double-stranded breaks by homologous recombination in S, G2 and M phases (Aylon et al., 2004; Ira et al., 2004). The homologous repair is activated by Cdk1 through phosphorylation of Sae2 (Huertas et al., 2008), Fun30 (Chen et al., 2016), Dna2 (Chen et al., 2011), Srs2 (Saponaro et al., 2010). Also, later in the cell cycle, Rad51 and Rad52 are phosphorylated by Cdk1 to further activate homologous recombination (Lim et al., 2020). Contrarily, Cdk1 modulates non-homologous end-joining by phosphorylating Mre11, Xrs2, Xbp1 and Lif1 (Matsuzaki et al., 2012; Simoneau et al., 2014; Tao et al., 2011). In addition, Cdk1 has been found to promote DNA damage checkpoint activation by phosphorylating checkpoint protein Rad9 (Granata et al., 2010; Wang et al., 2012).

S phase brings about the second wave of cell cycle regulated transcription containing around 180 genes, including the transcription factors Fkh1, Ndd1 and many spindle regulators (Pramila et al., 2006). This wave is activated by transcription factor Hcm1, that is subject to Cdk1-dependent activation and inactivation, but the cyclin specificity of this regulation is not understood yet (Landry et al., 2014; Pramila et al., 2006). Replication creates a need for histone synthesis, and it has been found that Cdk1 activates histone gene transcription by phosphorylation-dependent release of Yta7 from the chromatin at histone genes (Kurat et al., 2011). The G1/S regulon is inactivated during S phase, partially by phosphorylation of Swi6 by Clb6-Cdk1 that leads to nuclear export and inactivation of SBF (Geymonat et al., 2004). Cdk1 promotes tRNA synthesis in late S phase, as Clb5-Cdk1 localizes to tRNA genes, where it

activates RNA polymerase III, potentially to manage the translational needs for bud growth (Herrera et al., 2018).

In G1 phase, high APC/Cdh1 activity constrains the accumulation of mitotic, but not G1 and S phase cyclins (Zachariae et al., 1998). At the onset of S phase, the early Cln1/2-Cdk1 and Clb5-Cdk1 complexes phosphorylate Cdh1, leading to inactivation of APC/Cdh1 and SCF^{Cdc4}-mediated degradation of Cdh1, thus enabling the expression of mitotic cyclins (Jaspersen et al., 1999; Nagai et al., 2018; Ondracka et al., 2016; Zachariae et al., 1998). In addition, Acm1, a pseudosubstrate inhibitor of APC/Cdh1, is expressed in S phase and is stabilized by Cdk1-dependent phosphorylation, resulting in complete inhibition of APC, necessary for build-up of G2 and M cyclins (Enquist-Newman et al., 2008; Martinez et al., 2006; Ostapenko et al., 2008).

2.3.3. Cdk1 substrate proteins in G2 phase

Following SPB duplication in late G1 phase, the two SPBs stay side-by-side connected by a bridge that is broken upon expression of cyclins Clb3 and Clb4 in late S phase, triggering spindle assembly (Ear et al., 2013; Richardson et al., 1992). Separation of SPBs requires kinesins Cin8 and Kip1 that accumulate after Cdk1-mediated inhibition of APC/Cdh1 activity and that mediate formation of short spindle that connects the two SPBs that are localized on the opposite poles of the nucleus (Crasta et al., 2006). Clb3-Cdk1 was also found to phosphorylate Tub4 in the γ-tubulin complex, thus promoting proper formation of interpolar microtubules (Ear et al., 2013; Nazarova et al., 2013). An important switch in SPB cycle is phosphorylation of SPC component Sfi1 by Cdk1 that serves two functions: promoting SPB separation and inhibiting SPB reduplication in mitosis (Avena et al., 2014; Elserafy et al., 2014). As with DNA replication, SPB duplication must occur only once per cell cycle and the duplication cycle also consists of a licensing phase with low Cdk1 activity and along with separation in high Cdk1 activity, the duplication is inhibited.

After SPB separation, the spindle is aligned with the mother-bud axis and placed at the bud neck (Kusch et al., 2003). This is dependent on Kar9, a protein that localizes asymmetrically to only one SPB and the plus ends of microtubules originating from it, where it binds myosin Myo2 that then pulls the SPB to the bud neck (Liakopoulos et al., 2003; Maekawa et al., 2003). The asymmetric localization of Kar9 is dependent on its phosphorylation by Clb4-Cdk1 (Liakopoulos et al., 2003; Maekawa et al., 2003). Interestingly, Clb4 is also asymmetrically localized to the SPBs, preferentially on the mother-bound SPB, opposite to Kar9, suggesting that Clb4-Cdk1 inhibits localization of Kar9 on mother-bound SPB (Liakopoulos et al., 2003). The S phase Clb5-Cdk1 complex has been found to contribute to Kar9 asymmetry by regulating spindle assembly and to target Kar9, potentially at different phosphorylation sites than Clb4-Cdk1 (Hotz et al., 2012; Huisman et al., 2007; Moore and Miller, 2007).

Yeast undergoes closed mitosis, where the nuclear envelope remains intact throughout mitosis, creating a need for nuclear membrane growth before anaphase (Takemoto et al., 2016). Clb3-Cdk1 activates phospholipid synthesis that is necessary for nuclear membrane growth by inactivating Smp2, a transcriptional repressor of genes involved in lipid biosynthesis (Santos-Rosa et al., 2005). Further, Clb3-Cdk1 enhances the energetic activity of mitochondria by promoting import of proteins to mitochondria through phosphorylation of translocase Tom6 (Harbauer et al., 2014). Interestingly, a large change in metabolite concentrations, especially those related to cell wall synthesis and lipid metabolism, has been detected in G2, however, the regulatory mechanisms behind these changes are not fully understood (Ewald et al., 2016).

In G2, the transcription of 33 mitotic genes, including mitotic cyclin *CLB2*, is activated by transcriptional regulators Ndd1, Fkh2 and Mcm1 (Gefeng et al., 2000; Loy et al., 1999; Spellman et al., 1998). Activation of this transcriptional wave requires phosphorylation of Ndd1 T319 by Cdk1 (Reynolds et al., 2003) and there is evidence that Clb3 promotes *CLB2* transcription (Linke et al., 2017). However, Cdk1 was also found to destabilize Ndd1 in mitotic arrest (Edenberg et al., 2015), but the mechanism of such differential regulation has not been described yet. Interestingly, transcription factors Tos4 and Plm2 are efficiently phosphorylated by Clb3-Cdk1 *in vitro* (Kõivomägi et al., 2011b), and Cdk1-mediated phosphorylation has been shown to destabilize Tos4 (Landry et al., 2014).

2.3.4. Mitotic processes regulated by Cdk1

Progression through mitosis is coordinated by mitotic Clb1- and Clb2-Cdk1 complexes (Eluère et al., 2007; Rahal and Amon, 2008; Surana et al., 1991). Mitotic Cdk1 activity regulates cell growth, spindle dynamics and it is essential for the metaphase-anaphase transition.

The activity of Clb1/2-Cdk1 triggers a switch from polarized bud growth to isotropic, where the bud grows uniformly in all directions (Lew and Reed, 1993). This is mediated by redistribution of Cdc42 from the bud tip to the bud cortex and rearrangement of actin (Lew and Reed, 1993; Richman et al., 1999). Interestingly, several actin binding proteins (Sac6, Bni1, Crn1) are Clb2-Cdk1 targets and their phosphorylation regulates actin cable assembly (Miao et al., 2013, 2016). Further, Clb2-Cdk1 phosphorylates Swi4 to inactivate SBF and *CLN2* transcription, possibly contributing to growth depolarization, as Cln2-Cdk1 activity maintains the polarized growth (Amon et al., 1993; McCusker et al., 2007). Finally, the growth rate decreases in mitosis prior to anaphase, possibly by Clb2-mediated phosphorylation of Exo84 that inhibits exocytosis (Duan et al., 2019; Goranov et al., 2009).

In mitosis, the chromatin is condensed into chromosomes to facilitate efficient segregation in anaphase (Baxter and Aragón, 2012). Recently, it was found that condensin complex subunit Smc4 is a Cdk1 target in early mitosis

and that phosphorylation of Smc4 promotes chromosome condensation (Robellet et al., 2015).

An essential function of Clb1-Cdk1 and Clb2-Cdk1 is triggering anaphase by activation APC^{Cdc20} (Rahal and Amon, 2008). Before anaphase, sister chromatids are held together by cohesin, however, APC^{Cdc20} triggers degradation of securin (Pds1), releasing separase (Esp1) that cleaves cohesin subunit Scc1, allowing separation of chromatids (Uhlmann et al., 1999). Cdk1 regulates anaphase onset on multiple levels, including phosphorylation of three APC subunits, Cdc16, Cdc23 and Cdc27 to promote APC^{Cdc20} activity (Rudner and Murray, 2000). Phosphorylation of APC subunits stimulates binding of the activator, Cdc20 (Fujimitsu et al., 2016; Zhang et al., 2016). Additionally, Cdk1-dependent phosphorylation protects degradation of securin before anaphase (Holt et al., 2008; Lu et al., 2014), and Cdk1 phosphorylates and activates separase (Lianga et al., 2018). APC is inhibited by DNA damage response and spindle assembly checkpoint (SAC).

Exit from mitosis is dependent on dephosphorylation of Cdk1 targets mainly by phosphatases Cdc14 and protein phosphatase 2A (PP2A) (Touati et al., 2019). In addition to degrading securin, APC triggers degradation of mitotic cyclins, however, many anaphase processes are still regulated by both phosphorylation and dephosphorylation of Cdk1 sites.

Before anaphase, the phosphatase Cdc14 is sequestered to the nucleolus by Net1, but upon anaphase entry, Cdc14 is released and dephosphorylates a subset of Cdk1 targets (Visintin et al., 1999). Cdc14 is released by two sequential pathways: first, FEAR (Cdc Fourteen Early Anaphase Release) pathway causes a transient release of Cdc14 to the nucleus, and later, MEN (Mitotic Exit Network) is needed for retaining the activity of Cdc14 and distributing Cdc14 to the cytoplasm (Shou et al., 1999; Stegmeier et al., 2002; Visintin et al., 1999). Cdk1 stimulates FEAR pathway, but inhibits MEN through multiple substrates in both cases. First, FEAR pathway, along with many other mitotic processes, is regulated by Polo-like kinase Cdc5 that is activated in a step-wise manner by Cdk1 (Rodriguez-Rodriguez et al., 2016; Simpson-Lavy and Brandeis, 2011). Secondly, FEAR is triggered by Cdk1-dependent Net1 phosphorylation, that is counteracted by phosphatase $PP2A^{Cdc55}$ (Queralt et al., 2006). Sufficient Net1 phosphorylation in anaphase is achieved by down-regulation of $PP2A^{Cdc55}$ by separase and also direct phosphorylation by Cdk1 (Játiva et al., 2019; Queralt et al., 2006). Thirdly, Clb2-Cdk1 activates FEAR through phosphorylation of Spo12 (Tomson et al., 2009). MEN, on the other hand, is inhibited by high Cdk1 activity, as MEN kinases Cdc15 and Mob1-Dbf2 are inactivated by Cdk1-dependent phosphorylation (Campbell et al., 2019; König et al., 2010). In addition, MEN is only activated once the SPB has reached the bud, ensuring that mitotic exit occurs only after successful segregation of chromatids (Campbell et al., 2019). Therefore, Cdk1 and APC activity in early anaphase trigger transient release of Cdc14 to the nucleus, followed by Cdk1-mediated inhibition of MEN and APC-dependent degradation of mitotic cyclins, creating an ultrasensitive switch for mitotic exit (Campbell et al., 2019).

Successful anaphase requires stabilization of the spindle and regulation of motor proteins. Interestingly, Cdk1 promotes spindle destabilization in metaphase, but spindle stability in anaphase. This is because S-phase Clb5-Cdk1 complex, that is active until anaphase onset, inhibits spindle stabilizers Fin1 and Ase1, but upon Clb5 destruction, Fin1 and Ase1 are dephosphorylated by Cdc14 and promote spindle stability (Khmelinskii et al., 2007; Loog and Morgan, 2005; Woodbury and Morgan, 2007). Further, Cdk1 phosphorylates motor protein Cin8 and the chromosomal passenger complex (Ipl1, Sli15, Bir1, Nbl1) that contribute to spindle elongation (Avunie-Masala et al., 2011; Goldstein et al., 2017; Pereira and Schiebel, 2003; Widlund et al., 2006).

Cell cycle is finalized by cytokinesis, which depends on degradation of mitotic cyclins and dephosphorylation of Cdk1 targets (Stegmeier and Amon, 2004). Cdc14-mediated dephosphorylation of Cdk1 targets regulates actin and septin dynamics, septum formation and actomyosin ring contraction (Jakobsen et al., 2013; Meitinger et al., 2010; Naylor and Morgan, 2014; Palani et al., 2012; Sanchez-Diaz et al., 2012). Therefore, Cdk1-dependent phosphorylation induces various events during the cell cycle, but also inhibits some events, such as cytokinesis, that occur upon inactivation of Cdk1 in late mitosis.

2.4. Ordering cell cycle events

A fundamental question in cell cycle research is that what governs the orderly progression of cell cycle events. On one hand, checkpoints could inhibit later events before completion of earlier events (Barnum and O'Connell, 2014; Hartwell and Weinert, 1989). For example, a defect in bud formation triggers the morphogenesis checkpoint that delays mitotic progression (Lew, 2003). Delays in DNA replication can lead to stalled replication forks that activate the replication checkpoint, which inhibits mitosis (Giannattasio and Branzei, 2017). Finally, the spindle assembly checkpoint monitors the chromosome biorientation and inhibits APC until proper orientation is achieved (Musacchio, 2015). However, the checkpoints are not essential for orderly progression of cell cycle events in yeast (Cross et al., 2002). Also, delayed replication due to insufficient origin licensing can lead to anaphase entry before replication is completed, suggesting that ongoing DNA replication does not inhibit mitosis in yeast (Lengronne and Schwob, 2002). Furthermore, recent studies have found that DNA replication is ongoing in anaphase in a substantial part of unperturbed yeast cells, but that in human cells, DNA replication restricts the activity of Cdk1 and Plk1, connecting replication with the timing of mitosis in animal cells (Ivanova et al., 2020; Lemmens et al., 2018).

On the other hand, there is significant evidence that timely phosphorylation of cell cycle proteins by CDK governs the cell cycle progression (Bloom and Cross, 2007; Coudreuse and Nurse, 2010; Stern and Nurse, 1996; Swaffer et al., 2016; Uhlmann et al., 2011). Importantly, it has been shown that different level of Cdk1 activity is needed for S and M phase, and that simple manipulations

with Cdk1 activity can lead to reordering of cell cycle events (Coudreuse and Nurse, 2010; Swaffer et al., 2016). For example, inhibition of Cdk1 in G2-arrested fission yeast cells, and subsequent release of Cdk1 activity is sufficient to promote another round of replication (Swaffer et al., 2016). Therefore, progression through cell cycle is directly governed by Cdk1 activity, raising a question that how does Cdk1 phosphorylate different proteins at different cell cycle stages, thus ensuring the optimal order of cell cycle events.

Two models have been proposed on how Cdk1 mediates orderly phosphorylation of around 500 targets during the cell cycle (Holt et al., 2009; Ubersax et al., 2003; Uhlmann et al., 2011). Different cyclin-CDK complexes are active in different cell cycle phases where they activate different processes (Bloom and Cross, 2007), leading to a hypothesis of cyclin specificity, which states that cyclins direct CDK to phosphorylate specific substrates. While cyclins do mediate specific substrate targeting of Cdk1 (Archambault et al., 2005; Bhaduri and Pryciak, 2011; Kõivomägi et al., 2011b; Loog and Morgan, 2005), none of the nine *S. cerevisiae* Cdk1 cyclins is essential, indicating that there is significant overlap between functions of different cyclins (Bloom and Cross, 2007). Furthermore, in fission yeast, a single mitotic cyclin-CDK complex (Cdc13-Cdc2) is sufficient to mediate ordered cell cycle progression (Fisher and Nurse, 1996). Alternative to the cyclin specificity is the quantitative model, which states that different events require different levels of Cdk1 activity (**Fig. 4**). This was inspired by studies in fission yeast, where one cyclin-CDK complex could sequentially initiate S phase and mitosis (Stern and Nurse, 1996). It was proposed and later experimentally confirmed that early cell cycle events, such as replication, require low levels of CDK activity, whereas late events, like mitosis, need high kinase activity (Coudreuse and Nurse, 2010; Stern and Nurse, 1996). Therefore, progression through cell cycle is mediated by increasing CDK activity, being low in G1 and peaking in mitosis, where activation of APC leads to degradation of cyclins and decrease in activity (Uhlmann et al., 2011).

Importantly, in addition to the different CDK activity thresholds for S and M phase, various events are triggered at increasing kinase activity thresholds within a single cell cycle phase as well. For example, increasing activity of Clb2-Cdk1 orders mitotic events, as growth depolarization, followed by spindle formation and spindle elongation, each require higher Clb2-Cdk1 activity (Oikonomou and Cross, 2011). Similarly, in animal cells, gradual accumulation of cyclin B-Cdk1 governs sequential progression of mitotic events (Deibler and Kirschner, 2010; Gavet and Pines, 2010). Hence, combination of differential cyclin expression and the quantitative model could enable more precise timing of cell cycle events compared to the uniform increasing CDK activity model that discriminates chromosome replication and segregation (**Fig. 4**). Dephosphorylation of CDK targets regulates mitotic events such as spindle elongation and cytokinesis. Interestingly, CDK substrates were found to be dephosphorylated in an orderly manner in mitotic exit, and this was also partially dependent on cyclin specificity (Bouchoux and Uhlmann, 2011; Touati et al., 2018).

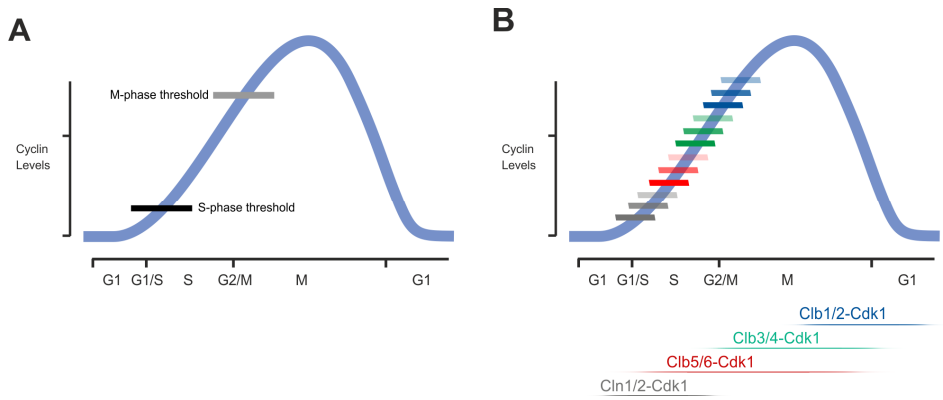

Figure 4. Cyclin specificity and the quantitative model. (A) According to the quantitative model of CDK function, a gradual increase in CDK activity during the cell cycle governs orderly phosphorylation of CDK substrates (Stern and Nurse, 1996; Swaffer et al., 2016). Thus, early (S phase) substrates require less CDK activity for phosphorylation compared to late (M phase) substrates. (B) The cyclin specificity model states that the expression of different cyclins during the cell cycle leads to phosphorylation of different substrate proteins at different times. Combination of the two models would allow finetuning of phosphorylation events in the period of each cyclin expression.

2.5. Substrate targeting by Cdk1

The basis of the quantitative model is that CDK phosphorylates substrates with very different rates, to enable separation of phosphorylation timing in the cell cycle. A phosphoproteomics study in fission yeast revealed that CDK substrates can be divided to early, mid and late substrates depending on their timing of phosphorylation in the cell cycle (Swaffer et al., 2016). Importantly, the phosphorylation timing was found to correlate with the substrates' sensitivity to CDK activity, so that early substrates require lower kinase activity to be phosphorylated (Swaffer et al., 2016). This raises a question that what mechanisms govern the differential phosphorylation of hundreds of targets by Cdk1.

In addition to the differential sensitivity of substrates to generic CDK activity (Swaffer et al., 2016), there are many examples of diverse phosphorylation by different cyclin-CDK complexes and of differential regulation of a CDK target during the cell cycle. For example, transcription factors Hcm1 and Ndd1 are first activated and later targeted to proteolysis by Cdk1-mediated phosphorylation (Edenberg et al., 2015; Landry et al., 2014; Reynolds et al., 2003). The mechanism of Hcm1 and Ndd1 regulation is not fully understood, it could be either different activity thresholds for different phosphorylation sites or cyclin-specific targeting of specific sites. Further, G1-Cdk1 and G2/M-Cdk1 activities have opposing outcomes on SPB duplication cycle, as G1-Cdk1 promotes SPB duplication whereas the later complexes inhibit the duplication (Haase et al., 2001).

Similarly, telomere replication is initiated by S-Cdk1, but later suppressed by M-Cdk1 by differential phosphorylation of the Cdc13-Stn1-Ten1 complex (Gopalakrishnan et al., 2017). Many more examples of cyclin specificity have been described (Bloom and Cross, 2007; Enserink and Kolodner, 2010) and this highlights the importance of understanding the role of cyclins in CDK substrate targeting.

The following chapters will give an overview of substrate targeting interactions of the CDK complex. All three components of the cyclin-CDK-Cks complex participate in substrate targeting. First, the CDK active site recognizes the phosphorylation motifs and catalyzes the transfer of phosphoryl group. Secondly, Cks1 binds to phosphorylated sites, promoting multisite phosphorylation. Thirdly, cyclins can interact directly with substrates and direct CDK to phosphorylate a specific set of substrates.

2.5.1. Cdk1 active site specificity

The first level of substrate specificity originates sequence preferences of the phospho-acceptor residue and in its flanking positions that constitute the consensus phosphorylation motif (Mok et al., 2010). CDKs are proline-directed serine/threonine kinases and the minimal consensus phosphorylation motif has been defined as S/T-P (Nigg, 1993; Songyang et al., 1994). A positively charged residue in position +3 from the phospho-acceptor site increases the phosphorylation rate significantly, thus S/T-P-x-K/R is the optimal phosphorylation motif for Cdk1 (Holmes and Solomon, 1996; Songyang et al., 1994). The proline in +1 position is strongly favored as it fits to a hydrophobic pocket formed by the CDK activation segment (Brown et al., 1999). The basic residue in +3 position makes hydrogen bonds with the T160 phosphate in Cdk2 (the site for activating phosphorylation) and also makes contacts with the cyclin (Brown et al., 1999). Other positions flanking the phospho-acceptor site have smaller effects, for example additional positively charged residues in positions +4 to +6 and proline in position -2 has been shown to stimulate phosphorylation (Kõivomägi et al., 2011b; Suzuki et al., 2015). Importantly, Cdk1 can also phosphorylate sites without +1 proline, referred to as non-S/TP sites, and in this case, the other specificity determinants such as additional basic residues and -2 proline are of greater importance and provide discrimination from random serines and threonines (Kõivomägi et al., 2011a; Suzuki et al., 2015).

Cyclins stimulate the intrinsic activity of CDK towards a peptide with consensus phosphorylation motif differently. It has been found in both yeast and mammalian cells that earlier cyclin-CDK complexes have lower intrinsic activity compared to later cyclins (Kõivomägi et al., 2011b; Loog and Morgan, 2005; Topacio et al., 2019). For example, the specificity constant (k_{cat}/K_M (catalytic constant k_{cat} divided by Michaelis constant K_M)) of S phase Clb5-Cdk1 complex and a substrate peptide is about 20 times lower than that of mitotic Clb2-Cdk1 (Loog and Morgan, 2005). Interestingly, structural studies of

mammalian CDKs have revealed that the phosphorylated activation loop is more flexible in Cdk1 and this allows phosphorylation of a wider range of phosphorylation motifs (Brown et al., 2007, 2015). The lower intrinsic activity of earlier cyclin-CDK complexes could be a safeguard mechanism that holds back phosphorylation of late targets prior to expression of late CDK complexes.

2.5.2. Cks1 – phospho-adaptor for Cdk1

In addition to the kinase subunit and cyclin, the Cdk1 complex contains Cks1 that binds phosphorylated TP motifs, thus promoting multisite phosphorylation (Arvai et al., 1995; Kõivomägi et al., 2013; McGrath et al., 2013) (**Fig. 5**). The mechanism of Cks1-mediated multisite phosphorylation has been studied in detail in *S. cerevisiae* (Kõivomägi et al., 2011a, 2013; McGrath et al., 2013), however, there is evidence of such mechanism functioning also in vertebrates (Patra and Dunphy, 1998; Patra et al., 1999; Van Zon et al., 2010).

Cks1 binds to pre-phosphorylated CDK phosphorylation motifs with around 10-100 μM dissociation constant (K_d) (McGrath et al., 2013). Importantly, Cks1 only binds phosphorylated TP sites, but not SP sites, and the binding is enhanced by the presence of a large hydrophobic residue in position -2 from the phospho-threonine (Kõivomägi et al., 2013; McGrath et al., 2013). Therefore, the minimal motif for Cks1 binding is pT-P, and the optimal is F/I/L/P/V/W/Y-x-pT-P, where pT is phosphorylated threonine (Kõivomägi et al., 2013; McGrath et al., 2013).

Budding yeast Cks1 was found to be essential for multisite phosphorylation and degradation of CKI Sic1 at G1/S transition (Kõivomägi et al., 2011a). Sic1 phosphorylation sites T45/T48 and S76/S80 form two di-phosphodegrons, that upon full phosphorylation are recognized by SCF ubiquitin ligase (Hao et al., 2007; Kõivomägi et al., 2011a; Nash et al., 2001). Importantly, it was revealed that only a subset of phosphorylation sites, those bearing the full consensus motif, are phosphorylated in the absence of Cks1 (Kõivomägi et al., 2011a). Binding of Cks1 to the phospho-substrate leads to more efficient phosphorylation of secondary sites, which turned out to be critical for phosphorylation of the non-S/TP site T48 (Kõivomägi et al., 2011a). This showed that there are connections between phosphorylation sites, and the presence of TP sites can increase phosphorylation of other sites via Cks1 docking.

Importantly, the efficiency of Cks1 docking dependent phosphorylation of secondary sites is extremely dependent on the positioning of the two sites along the disordered protein (Kõivomägi et al., 2013). Cks1 docking was found to only promote phosphorylation in case the secondary site is at least 12 amino acid residues C-terminal of the Cks1 binding site, and the docking efficiency was found to decrease as the distance between the sites is increased further (Kõivomägi et al., 2013). Therefore, the distances between the sites could be a key determinant on how efficiently the cyclin-Cdk1-Cks1 complex multi-phosphorylates the substrate.

In addition to stimulating substrate phosphorylation, Cks1 interactions can contribute to inhibition of CDK activity. For example, Cks1 docking to phosphorylated Swe1 mediates inhibitory phosphorylation of Cdk1 by Swe1 (McGrath et al., 2013). Also, phosphorylation of T173 in Sic1 by MAPK Slt2 creates an inhibitory Cks1 docking site that contributes to tight inhibition of S-Cdk1 (Moreno-Torres et al., 2017).

2.5.3. Cyclins bind to linear motifs in substrates

Although the later cyclin-CDK complexes have higher intrinsic activity, early CDK complexes phosphorylate some targets more efficiently (Kõivomägi et al., 2011b; Loog and Morgan, 2005; Topacio et al., 2019). The specific phosphorylation of selected targets is mediated by cyclin-substrate interactions that occur through short linear interaction motifs (SLiMs) on substrates (**Fig. 5**). Similarly to Cks1-mediated phosphorylation, cyclin-substrate docking increases the binding affinity that leads to increased phosphorylation rate and specificity (Takeda et al., 2001).

Cyclin-substrate docking was initially found to be critical for targeting of S phase substrates by cyclin A/E-Cdk2 and Clb5-Cdk1 in animal and yeast cells, respectively (Adams et al., 1996; Chen et al., 1996; Loog and Morgan, 2005; Wilmes et al., 2004). These complexes bind to RxL motifs with the consensus R/K-x-L-F/L/M/P or R/K-x-L-x-F/L/M/P, an interaction that is conserved between yeasts and human (Adams et al., 1996; Chen et al., 1996; Loog and Morgan, 2005; Lowe et al., 2002; Wilmes et al., 2004; Wohlschlegel et al., 2001). The linear RxL motif docks to the cyclin hydrophobic patch (*hp*), which contains a conserved MRAIL sequence in many cyclins, and the interaction increases the binding affinity up to 100-fold (Schulman et al., 1998; Takeda et al., 2001). Mutation of the *hp* (M210A L214A W217 in human cyclin A, denoted as *hpm* (*hp* mutant)) decreases the phosphorylation rate of RxL-containing substrates significantly, but does not affect phosphorylation of other substrates, such as histone H1 (Schulman et al., 1998). While the presence of an RxL motif greatly enhances phosphorylation by S phase complexes, it only weakly promotes phosphorylation by mitotic Cdk1, in both yeast and mammalian cells (Brown et al., 2007; Kõivomägi et al., 2011b; Loog and Morgan, 2005; Petri et al., 2007). In addition to stimulating phosphorylation, RxL motifs also mediate CDK inhibition in CKIs $p21^{Cip1}$ and $p27^{Kip1}$ (Chen et al., 1996; Russo et al., 1996b).

Budding yeast G1 cyclins Cln1, Cln2 and Cln3 do not interact with RxL motifs, however, Cln1 and Cln2 dock to leucine and proline rich LP motifs to promote phosphorylation in G1 (Bhaduri and Pryciak, 2011; Kõivomägi et al., 2011b). The consensus for LP motif is not defined as well as for RxL motif, it is present as LLPP sequence in Sic1 and Ste5, but as LDDPIQF in Ste20 (Bhaduri and Pryciak, 2011; Kõivomägi et al., 2011b). The LP motif binds to a region on the cyclin that is adjacent to, but not overlapping with the *hp* (Bhaduri et al., 2015). LP docking is necessary for timely phosphorylation of G1 transcriptional

repressor Whi5, CKI Sic1, mating pathway proteins Ste5 and Ste20 and the motif is present in many proteins involved in bud morphogenesis (Bhaduri and Pryciak, 2011; Bhaduri et al., 2015; Kõivomägi et al., 2011b). Interestingly, Far1, the inhibitor of Cln-Cdk1 complexes that is activated upon pheromone treatment, inhibits both the kinase activity and docking interactions of Cln-Cdk1, which is necessary for proper cell cycle arrest (Pope et al., 2014).

As with Cks1 docking, the effect of cyclin-substrate docking on phosphorylation also depends on the relative positions of the phosphorylation site and the docking motif (Kõivomägi et al., 2013). To promote phosphorylation, the cyclin docking motif and phosphorylation site must be simultaneously bound to the CDK complex (Takeda et al., 2001). Mapping of the distance requirements revealed that RxL motif promotes phosphorylation of sites that are at least 16 residues N-terminal of the motif (Kõivomägi et al., 2013). LP motif, however, was found to promote phosphorylation of sites that are at least 30 residues in either N- or C-terminal direction (Kõivomägi et al., 2013). Therefore, the cyclin docking motifs direct CDK to phosphorylate specific sites and in combination with Cks1 docking that direct N-to-C-terminal multisite phosphorylation, these docking connections can lead to ordered and processive multisite phosphorylation (Kõivomägi et al., 2013).

Two cyclin docking motifs that promote phosphorylation of Rb and cell cycle entry have been found for mammalian cyclin D-Cdk4/6 complex. While RxL and LP motifs are intrinsically disordered segments that bind to the cyclin box fold, cyclin D contains a disordered LxCxE motif that binds to the pocket domain of Rb (Dick and Rubin, 2013; Dowdy et al., 1993). Disruption of the LxCxE docking, however, had little effect on Rb phosphorylation by cyclin D-Cdk4/6 (Topacio et al., 2019). Instead, it was found that a hydrophobic face of an α-helix was critical for specific Rb phosphorylation (Topacio et al., 2019).

There are examples of cyclin-specific docking motifs only for early (G1 and S) CDK complexes. This, combined with the higher intrinsic activity and the more relaxed phosphorylation motif specificity of late CDK complexes, has led to a model by which cyclin docking compensates for the lower intrinsic activity of early CDK complexes in phosphorylation of the limited set of motif-containing substrates, whereas the later CDK complexes are not dependent on docking to carry out their functions (Brown et al., 2007; Kõivomägi et al., 2011b; Loog and Morgan, 2005; Topacio et al., 2019). However, there are reports of cyclin-specific targets for late CDK complexes, such as Ypr174c and Tos4 for Clb3-Cdk1 (Kõivomägi et al., 2011b), Kar9 for Clb4-Cdk1 (Liakopoulos et al., 2003; Maekawa et al., 2003), and of specific inhibition of Clb2-Cdk1 by Cdc6 (Archambault et al., 2003; Calzada et al., 2001). Also, recent studies have revealed that human cyclin B1 binds to an acidic helical motif in kinetochore protein Mad1 (Allan et al., 2020; Jackman et al., 2020).

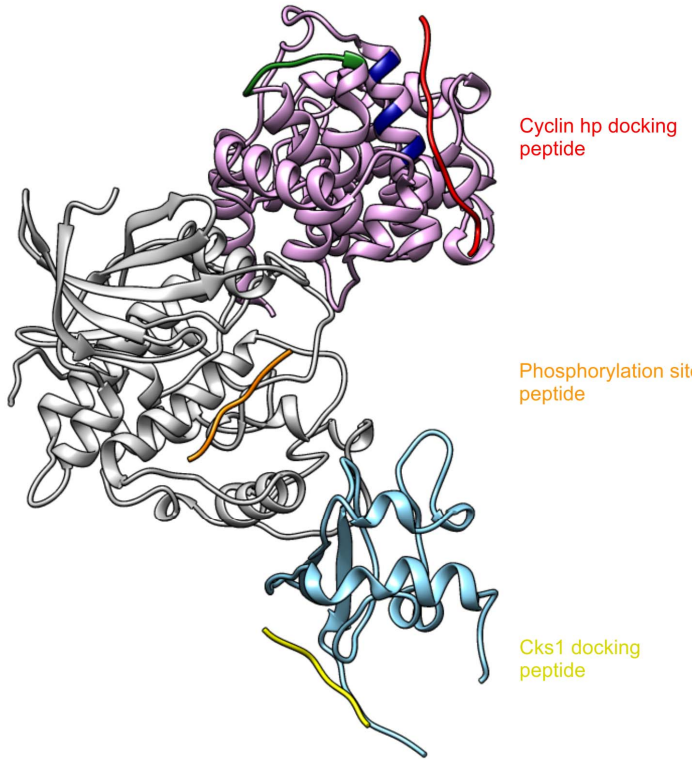

Figure 5. CDK active site, Cks1 phosphate-binding pocket and cyclin surface contribute to substrate targeting by CDK. The figure shows the ribbon diagram of the crystal structure of cyclin-CDK-Cks complex with substrate peptides interacting with Cks1 (yellow), CDK active site (orange) and cyclin hydrophobic patch (red) (Kõivomägi et al., 2013). Cks1 is presented in light blue, CDK in gray, and cyclin in magenta. The key residues in cyclin hydrophobic patch are colored blue, whereas the LP docking interface in G1 cyclins is in green.

2.5.4. Cyclins determine the localization of CDK complex

Another level of cyclin specificity in substrate targeting arises from different subcellular localization of cyclin-CDK complexes. The G1 cyclins Cln1 and Cln3 are primarily nuclear, whereas Cln2 is mainly cytoplasmic, where it promotes bud formation (Edgington and Futcher, 2001; Miller and Cross, 2000). Deletion of Cdk1 cyclins *CLN2*/*CLN1* and Pho85 cyclins *PCL1*/*PCL2* disrupts bud formation, and this could be rescued by expression of stabilized Cln3 only when it is fused with a nuclear export signal, indicating that localization affects the functions of G1 cyclins (Edgington and Futcher, 2001; Miller and Cross, 2000). Cyclins Clb1-6 are mainly nuclear, but also localize to spindle and SPBs (Bailly et al., 2003; Jacobson et al., 2000). Clb4 can also be detected at the cytoplasmic

microtubule tips, and only Clb2 localizes to the bud neck in addition to nucleus (Bailly et al., 2003; Maekawa and Schiebel, 2004; Maekawa et al., 2003). In mammalian cells, cyclins D, E and A are primarily nuclear, whereas cyclin B accumulates in the cytoplasm (Gladden and Diehl, 2005; Ohtsubo et al., 1995; Pines and Hunter, 1994).

Interestingly, the *hp* of mitotic cyclins mediates their localization, as it recruits Clb2 to bud neck and cyclin B to centrosomes (Bailly et al., 2003; Basu et al., 2020; Bentley et al., 2007). It was recently found that in fission yeast, mutation of mitotic cyclin *hp* (Cdc13(hpm)) causes a global decrease in phosphorylation of mitotic substrates and that Cdc13(hpm) is not capable of executing mitosis (Basu et al., 2020). This indicates that *hp* interactions also contribute to the functions of late cyclins.

2.6. Phosphatases counteracting CDK activity

Phosphorylation can be reversed by protein phosphatases, making it a dynamic modification. The median phosphorylation half-life for CDK sites was found to be 2.2 minutes in fission yeast (Swaffer et al., 2016), indicating that CDK targets go through many cycles of phosphorylation and dephosphorylation during the cell cycle. The high counteracting phosphatase activity could contribute to translating the gradual increase in CDK activity to switch-like changes in phosphorylation (Goldbeter and Koshland, 1981). Furthermore, phosphatases contribute to the ordering of CDK phosphorylation events by preferentially targeting a subset of substrates and phosphorylation sites (Godfrey et al., 2017; Queralt et al., 2006). The two major phosphatases counteracting CDK activity are PP2A and Cdc14. PP2A opposes CDK phosphorylation throughout the cell cycle, whereas Cdc14 is activity is limited to late mitosis and G1 (Touati et al., 2019).

2.6.1. PP2A antagonizes CDK and contributes to ordering cell cycle phosphorylation

PP2A is a major group of phosphatases that regulate cell cycle and cell growth, among other processes (Wlodarchak and Xing, 2016). PP2A is a trimeric complex consisting of catalytic subunit (Pph21/Pph22), a scaffold (Tpd3), and a regulatory subunit (Cdc55/Rts1/Rts3) that governs the specificity and localization of PP2A (Moyano-Rodriguez and Queralt, 2020). All three regulatory subunits confer localization to the cytoplasm and nucleus, however, Cdc55 can also be detected in the bud cortex and bud neck, whereas Rts1 accumulates to the SPBs (Gentry and Hallberg, 2002).

PP2A contributes to ordering cell cycle phosphorylation both on a global level and by targeting specific proteins. Importantly, deletion of Cdc55 leads to accelerated cell cycle, whereas overexpression causes a delay in cell cycle

progression (Godfrey et al., 2017). In budding yeast, $PP2A^{Cdc55}$ was found to oppose threonine phosphorylation more compared to serine phosphorylation, leading to later phosphorylation of TP sites compared to SP sites (Godfrey et al., 2017). This suggests that dephosphorylation rate governs the timing of phosphorylation in the cell cycle. However, a study in fission yeast did not find differences in dephosphorylation rate between early and late CDK targets (Swaffer et al., 2016). In addition, PP2A regulates several specific processes, such as FEAR pathway and anaphase onset by restraining Net1, Scc1 and APC phosphorylation (Lianga et al., 2013; Queralt et al., 2006; Yaakov et al., 2012). $PP2A^{Cdc55}$ is inactivated in anaphase by separase and Cdk1-mediated phosphorylation, leading to an anaphase-specific phosphorylation pattern (Játiva et al., 2019; Queralt et al., 2006; Touati et al., 2019). The absence of Cdc55 leads to premature phosphorylation of Net1 and release of Cdc14 in metaphase (Queralt et al., 2006; Yellman and Burke, 2006). PP2A also dephosphorylates Cdk1 targets in mitotic exit, regulating septin dynamics (Dobbelaere et al., 2003; Touati et al., 2019).

2.6.2. Cdc14 opposes Cdk1 activity in late mitosis

Mitotic exit requires inhibition of Cdk1 activity and dephosphorylation of CDK targets (Visintin et al., 1998). Mitotic exit brings about changes in phosphorylation of over 300 proteins because of changes in both kinase and phosphatase activities (Touati et al., 2018). Importantly, dephosphorylation of CDK sites in mitotic exit is an ordered process mediated by the increase in phosphatase to kinase activity ratio (Bouchoux and Uhlmann, 2011). Phosphatase Cdc14 inactivation in budding yeast results in cell cycle arrest with high Cdk1 activity in late mitosis, leading to a model that Cdc14 is necessary for dephosphorylation of Cdk1 targets late mitosis (Hartwell et al., 1973; Visintin et al., 1998). However, it has also been argued that Cdc14 dephosphorylates only a small fraction of Cdk1 sites and that depletion of Cdc14 does not cause mitotic exit delay and that the essential function might arise from a few specific substrates (Powers and Hall, 2017). Also, Cdc14 does not regulate dephosphorylation of CDK substrates in mitotic exit in animal cells, where PP1 and PP2A have been shown to oppose CDK activity in mitosis (Grallert et al., 2015; Mocciaro and Schiebel, 2010; Mochida et al., 2009).

Cdc14 is a dual-specificity protein phosphatase that dephosphorylates phosphoserine, -threonine and -tyrosine (Taylor et al., 1997). Importantly, Cdc14 has a similar phosphorylation site specificity as Cdk1, as it preferentially targets sites with +1 proline and the dephosphorylation rate is increased by the presence of basic residues in +2 to +4 positions (Bremmer et al., 2012; Gray et al., 2003). Furthermore, Cdc14 has higher activity towards phosphoserine compared to -threonine (Bremmer et al., 2012). Despite this, a larger fraction of phosphothreonine is dephosphorylated in mitotic exit compared to phosphoserine, suggesting that threonine-specific phosphatases such as $PP2A^{Cdc55}$ also

contribute to mitotic exit (Powers and Hall, 2017; Touati et al., 2018, 2019). In addition to selectivity at the active site level, Cdc14 binds to a linear docking motif, the PxL motif, in a subset of substrates to advance their dephosphorylation (Kataria et al., 2018). This multi-level substrate recognition mediates the orderly dephosphorylation of Cdk1 sites in mitotic exit (Bremmer et al., 2012; Kataria et al., 2018; Touati et al., 2019).

3. AIMS OF THE STUDY

The aim of this thesis was to study the mechanisms that enable differential phosphorylation of Cdk1 targets in the cell cycle. Phospho-proteomic studies have shown that Cdk1 targets differ in their sensitivity to kinase activity, so that more sensitive substrates are phosphorylated earlier and less sensitive later in the cell cycle. However, it is still unclear what is the mechanism behind the different specificity.

The more specific aims were as follows:

First (Ref I), to study the mechanisms that enable specific regulation and substrate targeting of the mitotic Clb2-Cdk1 complex.

- To describe the inhibitory interactions in Cdc6-Clb2-Cdk1-Cks1 complex.
- To characterize phosphorylation of Cdc6 by Clb5-Cdk1.
- To study the specific substrate targeting by Clb2-Cdk1.

Second (Ref II), to study the substrate docking interactions of Clb3-Cdk1 and analyze the *hp* docking specificity of major cyclin-Cdk1 complexes.

- To study the functions of Clb3-Cdk1 and their dependence on *hp* docking.
- To define the docking motif that governs Clb3-specific phosphorylation of Ypr174c and analyze the function of Ypr174c phosphorylation.
- To study *hp* docking specificity of both yeast and human cyclin-CDK complexes.

Third (Ref III), to describe the Clb5 docking motif in Far1 and analyze the impact of different cyclin docking motifs and Cks1 on the timing of Far1 phosphorylation.

- To analyze the determinants that affect phosphorylation of Far1 N terminus using time-lapse microscopy.
- To study the functional differences between RxL and the Far1 Clb5 docking motif.

Fourth (Ref IV), to investigate how the presence and the pattern of different docking sites and phosphorylation sites affect the timing of substrate phosphorylation in the cell cycle.

- To create Cdk1 activity threshold sensor based on protein degradation for precise measurement of phosphorylation in time-lapse microscopy experiments.
- To study how Cks1 and cyclin docking affect the timing of Cdk1-mediated phosphorylation in the cell cycle.
- To analyze the impact of different cyclins in Cdk1 threshold encoding.

Fifth (Ref V), to analyze in detail the specificity determinants in LP motifs.

- To investigate how LP motifs of different strength affect the timing of Cdk1 phosphorylation switches.

4. MATERIALS AND METHODS

For *in vitro* kinase assays, yeast Cdk1 complexes were purified from yeast cells, where the cyclin of interest had been overexpressed using galactose induction. The overexpressed cyclin was tagged with TAP tag in case of Clb1-5 and 3HA tag in case of Cln2 and the Cdk1 complex was purified using TAP or HA immunoaffinity purification as described previously (McCusker et al., 2007; Puig et al., 2001). Assays containing mammalian CDK complexes were carried out with cyclin E-Cdk2 (Millipore, 14-475), cyclin A-Cdk2 (Millipore, 14-448) and cyclin B-Cdk1 (Millipore 14-450). Cks1 was extracted from *E. coli* BL21 cells as described previously (Reynard et al., 2000). 6xHis- or GST-tagged substrate proteins were expressed and purified from *E. coli* BL21 cells as described in Ref. I-IV.

The kinase assays were performed in initial velocity conditions at substrate protein concentrations below the estimated K_M values, unless noted otherwise. Trace amounts of γ-^{32}P-ATP was added to the kinase reactions to enable detection of phosphorylation using Typhoon Trio imager (GE Healthcare). The reactions were stopped by addition of SDS-PAGE sample buffer and were subjected to SDS-PAGE. For analysis of multisite phosphorylation, Phos-tag SDS-PAGE was used as described in Ref. I-IV.

Yeast strains were in W303 or BY4741 background and are described in Ref. I–V. Gene deletions and replacements, promoter substitutions and epitope tagging were performed using PCR-based homologous recombination (Janke et al., 2004; Longtine et al., 1998).

Time-lapse fluorescence microscopy was carried out using Zeiss Observer Z1 microscope with an automated stage as described in Ref I-V. Briefly, cells were grown in liquid culture to logarithmic growth phase and were placed under 1.5% agarose in synthetic complete media. The experiments were up to 8 hours long and cells were imaged every 3 minutes. In every cycle, images from phase-contrast and fluorescent channels were taken. Image segmentation, cell tracking and fluorescent signal quantification were done in MATLAB (The MathWorks, Inc.) as described in (Doncic et al., 2013).

For Western blot, yeast cells were snap frozen and lysed by bead beating in urea lysis buffer containing protease and phosphatase inhibitors. Cell cycle synchronization and Western blotting is described in more detail in Ref. I-IV. For co-immunoprecipitation, the cells were lysed in buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP40, 1 mM DTT and the same buffer was used for washing the beads.

The search for linear motifs from the disordered regions of the proteome was performed using SlimSearch4 (Krystkowiak and Davey, 2017), with IUPRED cutoff score set to 0.3. The secondary structure predictions of the studied proteins were carried out with PSIPred 4.0 (http://bioinf.cs.ucl.ac.uk/psipred/).

5. RESULTS AND DISCUSSION

This section provides an overview of the main results of the five research articles of this study. Here, an overview of the main findings is provided, more detailed information can be found in the publications.

5.1. Cyclin-specific docking mediates the functions of M-Cdk1 complex (Ref I)

Prior studies had revealed the importance of specific linear motifs in substrates that interact with G1 or S phase cyclins to promote the timely phosphorylation of these targets. However, there are also proteins that are phosphorylated specifically by either G2- or M-phase cyclin-Cdk1 complexes, suggesting that cyclin-substrate docking could promote orderly phosphorylation of Cdk1 substrates throughout the cell cycle. In this study, we aimed to understand the cyclin-specific regulation of mitotic Cdk1 activity and the importance of cyclin-specific substrate targeting on Cdk1-controlled mitotic processes.

5.1.1. Cdc6 inhibits Clb2-Cdk1 by docking to Cks1 and cyclin *hp*

Cdc6, a protein that controls replication origin loading, was previously found to be a specific substrate for Clb5- and Clb3-Cdk1, however, for Clb2-Cdk1, Cdc6 has been proposed to function as an inhibitor (Archambault et al., 2003; Calzada et al., 2001; Kõivomägi et al., 2011b; Mimura et al., 2004). The strong cyclin-specific differences in Cdc6 function prompted us to investigate the mechanisms of this specificity. Mutation of the cyclin *hp* decreased the phosphorylation rate of Cdc6 by Clb5- and Clb3-Cdk1, but had the opposite effect in case of Clb2-Cdk1 (Ref I, Fig. 1A). This suggested that in case of Clb5 and Clb3, *hp* docking promotes phosphorylation, as shown previously for other substrates, however, with Clb2, the *hp* interaction could be inhibitory. The inhibition of Clb2-Cdk1 by Cdc6 was tested in a kinase assay using histone H1 as a substrate protein and this confirmed that Cdc6 is a tight inhibitor of Clb1 and Clb2-Cdk1 and that strong inhibition is lost upon mutation of the *hp* (Ref I, Fig. 1B, C).

Next, we aimed to describe the inhibitory interactions. The intrinsically disordered N terminus of Cdc6 has been shown to bind Clb2-Cdk1 in a phosphorylation-dependent manner (Mimura et al., 2004). Studying the interaction in more detail, we found that the Cdk1 phosphorylation sites T7 and T23 in Cdc6 bind to the phosphate-binding pocket of Cks1 (Ref I, Fig. 1D-F). Disruption of the Cks1-Cdc6 interaction either by mutation of the Cks1 phospho-threonine binding pocket or by mutation of T7 and T23 caused around 10-fold decrease in the inhibitory potential. However, the deletion of the N-

terminal disordered region in Cdc6 caused over 100-fold reduction (Ref I, Fig. 1D-F). We then mutated a conserved ^{47}LxF motif, which resulted in strong reduction in inhibition (Ref I, Fig. 1G). Importantly, mutation of the LxF motif showed very little effect in inhibition of Clb2(*hpm*)-Cdk1, indicating that the LxF motif binds to the *hp*. The protein fragment containing only the Cdc6 disordered N terminus including Cks1 binding sites T7 and T23 and Clb2 *hp* binding motif LxF, however, was not sufficient for low nanomolar inhibition of Clb2-Cdk1. Search for additional inhibitory motifs revealed that the sequence ^{126}FQSLP, located in a disordered loop and showing the same extent of conservation, was also necessary for tight inhibition (Ref I, Fig. 1G). Thus, Clb2-Cdk1 first phosphorylates the N-terminal sites T7 and T23, which then occupy the Cks1 pocket and in combination with interactions with LxF and FQSLP motifs result in inhibition of Clb2-Cdk1 complex.

5.1.2. Differential regulation of Cdc6 by Clb5- and Clb2-Cdk1

To restrict origin licensing to G1, Cdc6 is synthesized in late mitosis and is degraded at G1/S by Cdk1-dependent phosphorylation of two di-phospho-degrons (Calzada et al., 2000; Drury et al., 2000). Interestingly, the N-terminal degron (T39 S43) partially overlaps with the LxF motif, indicating that the degron could be shielded by Clb2 in the inhibitory complex. To test if the Cdc6-Clb2 interaction affected the stability of Cdc6 in mitosis, we measured the accumulation of Cdc6-Citrine in time-lapse microscopy. While mutation of the N-terminal degron caused only a slight increase in Cdc6 abundance in G1, mutation of the C-terminal degron (T368A S372A) resulted in greatly accelerated accumulation of Cdc6 already in mitosis (Ref I, Fig. 3A-D). This suggests that the stability of Cdc6 in mitosis is controlled via the C-terminal degron. Mutation of the LxF motif caused a slight decrease in Cdc6 abundance in wild-type background, but reversed the effect of C-terminal degron inactivation when combined with T368A S372A (Ref I, Fig. 3C-D). Furthermore, stabilization of Clb2 by deletion of *CDH1*, an APC subunit necessary for Clb2 destruction, also blocks cell cycle dependent degradation of Cdc6(T368A S372A) (Ref I, Fig. 3I), confirming that the LxF-Clb2 interaction shields and inactivates the N-terminal degron in mitosis.

S phase Clb5-Cdk1 complex, however, utilizes both Cks1 and *hp*-docking with an RxL motif to multiphosphorylate Cdc6 to trigger its degradation (Ref I, Fig. 3E-G). Importantly, while Clb5-Cdk1 efficiently multiphosphorylates Cdc6, Clb5-Cdk1 does not phosphorylate the Cdc6 in the inhibitory complex, providing further evidence that Clb2 binding shields the phosphorylation sites (Ref I, Fig. 3H). Therefore, the different docking interactions enable Clb5- and Clb2-Cdk1 to prompt opposing outputs in Cdc6 regulation, as Clb5-Cdk1 promotes degradation of Cdc6, whereas Clb2-Cdk1 stabilizes Cdc6 (**Fig. 6**).

FACS analysis revealed that cdc6(lxf) strain has fewer cells in S phase (Ref I, Fig. S3H), indicating that Clb2 binding could also shield Cdc6 from inter-

action with replication proteins, as suggested previously (Mimura et al., 2004). This could be especially important in meiosis, where the mitotic cyclin Clb1 is not degraded between transition from meiosis I to meiosis II and the origin licensing is inhibited (Carlile and Amon, 2008; Phizicky et al., 2018).

In addition, the Cdc6-Clb2 interaction has been proposed to contribute to inhibition of Cdk1 activity in late mitosis and G1 (Archambault et al., 2003; Calzada et al., 2001). To measure the inhibition of Cdk1 in mitotic exit, we set up a time-lapse microscopy experiment to follow the dephosphorylation of a Cdk1-regulated nuclear localization module in mitotic exit (Ref I, Fig. 2B-D). This revealed that Cdc6 was not necessary for Cdk1 inactivation in mitotic exit, but it contributed to Cdk1 inhibition along with Sic1 and Cdh1 (Ref I, Fig. 2E-G). Interestingly, although mutation of LxF in Cdc6 did not significantly affect dephosphorylation of the localization module, mutation of Clb2 *hp* caused a profound delay in dephosphorylation. This suggests that Clb2 *hp* docking mediates additional functions.

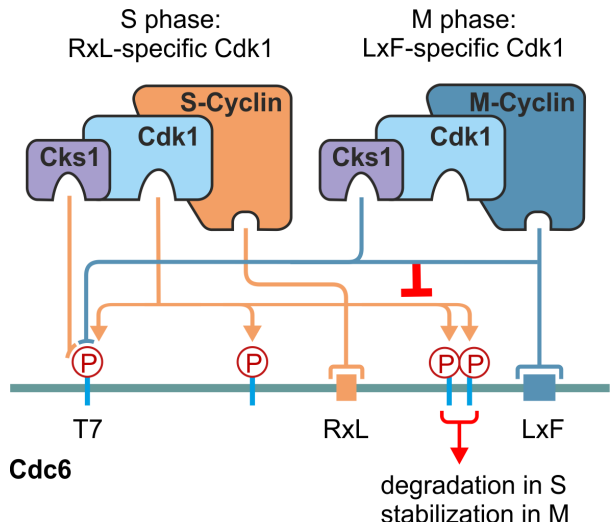

Figure 6. Differential regulation of Cdc6 by Clb5- and Clb2-Cdk1. Clb5-Cdk1 docks to the RxL in Cdc6, leading to phosphorylation of T7, which then promotes phosphorylation of other sites, including the di-phosphodegron to trigger Cdc6 degradation. Clb2-Cdk1, however, interacts with the LxF motif and with the T7-Cks1 interaction, an inhibitory complex is formed. This leads to shielding of the T39/S43 di-phosphodegron and protection of Cdc6 from degradation.

5.1.3. LxF interaction promotes phosphorylation of Spo12 and other M-Cdk1 substrates

To test if the delay in Cdk1 substrate dephosphorylation in anaphase seen in *clb2(hpm)* strain (Ref I, Fig. 4A) could be caused by loss of LxF docking dependent substrate phosphorylation by Clb2-Cdk1, an extra copy of wild-type *CLB2* was added to the strain. This restored the nuclear accumulation of the Cdk1-inhibited nuclear localization module. Clb2-Cdk1 is known to activate phosphatase Cdc14 through FEAR pathway by phosphorylation of Spo12, thus promoting timely release of Cdc14 (Tomson et al., 2009). Interestingly, the intrinsically disordered C terminus of Spo12 contains a potential LxF motif downstream of the phosphorylation sites regulating Cdc14 release (Ref I, Fig. 4D). Mutation of the predicted LxF motif caused a similar delay in nuclear accumulation of the Cdk1 activity sensor as *SPO12* deletion and *clb2(hpm)* mutation (Ref I, Fig. 4A-C). Furthermore, analysis of Spo12 phosphorylation showed that the LxF motif is necessary for Spo12 phosphorylation both *in vivo* and *in vitro* (Ref I, Fig. 4E-F). Thus, in addition to mediating inhibitory interactions, the *hp* of Clb2 promotes specific substrate phosphorylation, similarly to the *hp* of S phase cyclins.

To estimate the importance of LxF motif in M-Cdk1 substrate targeting, potential LxF motifs in the intrinsically disordered regions of *S. cerevisiae* proteins were predicted. Potential LxF motifs were found in 72 proteins, including 14 previously identified Cdk1 targets (Ref I, Fig. 6A). We purified two of these, Bud3 and Bni1, and found that they are highly Clb2-specific Cdk1 substrates and that their phosphorylation rate is greatly reduced by mutation of either *hp* of Clb2 or the predicted LxF motif (Ref I, Fig. 6B, C). This indicates that the cyclin-specific substrate targeting can have a wider role in regulation of mitosis. Further, this shows that cyclin-substrate interactions are not merely to compensate for the lower intrinsic activity of early CDK complexes, but they mediate specific substrate targeting throughout the cell cycle.

5.1.4. LxF docking governs the specific regulation of M-Cdk1 by Swe1

Only the mitotic complexes Clb1- and Clb2-Cdk1 are subjected to inhibitory phosphorylation by Swe1 (Hu and Aparicio, 2005; Keaton et al., 2007). Furthermore, as with Cdc6, both the hydrophobic patch of Clb2 and Cks1 were necessary for this regulation (Hu et al., 2008; McGrath et al., 2013). This led us to investigate whether the LxF motifs mediate this interaction. The intrinsically disordered N terminus of Swe1 contains three potential LxF motifs (Ref I, Fig. 5A). Clb2-Cdk1 and Swe1 form a double-negative feedback loop, where phosphorylation of both kinases leads to their inactivation. Thus, the docking interactions could mediate Swe1-dependent phosphorylation of Cdk1, Cdk1-mediated phosphorylation of Swe1 or both.

To understand the mechanism of this regulation, we first studied the effects of predicted LxF motif mutations on Cdk1-mediated phosphorylation of the N-terminal enzymatically inactive fragment of Swe1. This showed that mutation of ^{50}LKF greatly reduced phosphorylation of Swe1, while the other two LxF motifs did not have a significant effect (Ref I, Fig. 5B). Next, we analyzed the contribution of different motifs to inhibition of Cdk1 by Swe1. An analysis of Cdk1-Y19 inhibitory phosphorylation by different Swe1 mutants revealed that mutation of LxF motifs ^{229}LNF and ^{241}LLF significantly decreased Cdk1-Y19 phosphorylation, while ^{50}LKF had a smaller effect (Ref I, Fig. 5D, E). Interestingly, an optimal Cks1 binding site, T196, lies around 30 residues N-terminal of the inhibitory LxF motifs (Ref I, Fig. S5B). Mutation of T196 to serine decreased the inhibitory phosphorylation of Swe1 to the same extent as mutation of the ^{229}LNF and ^{241}LLF motifs (Ref I, Fig. 5D, E).

These results suggest that the N-terminal ^{50}LKF promotes phosphorylation of Swe1 sites, including T196, which then interacts with Cks1 and in combination with the C-terminal LxF motifs triggers inhibitory phosphorylation of Cdk1 (Ref I, Fig. 5F). This model is also supported by previously published results that Swe1 phosphorylation takes place in two steps, and that the initial phosphor. ylation is necessary for activation of Swe1 (Asano et al., 2005; Harvey et al., 2005).

5.2. *hp*-substrate interactions of Clb3 govern progression to mitosis (Ref II)

Similarly to S phase cyclins, G2 cyclin Clb3 utilizes RxL motifs for substrate targeting, however, although some RxL-dependent substrates are efficiently phosphorylated by both Clb5- and Clb3-Cdk1, some substrates are specific only to Clb3-Cdk1 (Kõivomägi et al., 2011b). The aim of this study was to describe the mechanisms that enable Clb3-specific phosphorylation and to analyze the functions of Clb3 in cell cycle regulation.

5.2.1. *hp* docking directs Clb3-Cdk1 to regulate spindle formation and mitotic gene expression

G2 cyclins Clb3 and Clb4 are expressed at the time of spindle formation and previous studies suggest that these cyclin-Cdk1 complexes promote spindle assembly (Fitch et al., 1992b; Richardson et al., 1992; Segal et al., 2000). To study the contribution of Clb3 and Clb4, and analyze the importance of *hp* docking in this process, we set up a time-lapse microscopy experiment to follow the timing of spindle formation relative to G1/S transition in different cyclin mutant strains (Ref II, Fig. 1B). Deletion of *CLB3* led to a median 12-minute delay in spindle formation, whereas deletion of *CLB4* caused a spindle assembly delay only in *clb3* deletion strain, but not in wild-type strain (Ref II,

Fig. 1C). This confirms that Clb3 promotes spindle assembly and reveals a functional difference between Clb3 and Clb4. Furthermore, mutation of Clb3 *hp* caused a similar delay as *clb3* deletion (Ref II, Fig. 1C), indicating that the docking specificity is critical in governing this function of Clb3.

The sequentially expressed cyclins have been proposed to coordinate the cell cycle transcriptional waves (Linke et al., 2017). To test the possible impact of Clb3 specificity on the transcriptional waves, we analyzed the expression of both Clb3 and mitotic cyclin Clb2 in time-lapse microscopy experiments. Interestingly, a 10-minute delay from Start to accumulation of 50% of peak Clb3- or Clb2-Citrine was detected in *clb3(hpm)* strain (Ref II, Fig. 1E-F). In case of Clb2-Citrine, the deletion of clb3 caused a similar delay as mutation of Clb3 *hp*. These results indicate that the *hp* docking is essential for these functions of Clb3.

5.2.2. A linear PxF motif promotes phosphorylation by Clb3-Cdk1

To understand the mechanism of Clb3 specificity, we set out to map the specificity determinants in phosphorylation of Ypr174c, a previously identified highly Clb3-specific Cdk1 target (Kõivomägi et al., 2011b). Ypr174c is predicted to contain an intrinsically disordered C terminus with 4 Cdk1 phosphorylation sites (Ref II, Fig. 2A). We analyzed the phosphorylation of C-terminally truncated variants of Ypr174c and found that deletion of residues in position 192-198 caused a sharp drop in Clb3-dependent phosphorylation rate, while this region had no effect on Clb2-dependent phosphorylation (Ref II, Fig. 2B). This indicated that the region could be part of a Clb3 docking SLiM.

Next, we made single amino acid substitution to the identified region and found that mutations P190A, P193A and F195A greatly decreased the Clb3-Cdk1-mediated phosphorylation, while other positions had either no or minor effect (Ref II, Fig. 2C). This indicates that a SLiM with consensus PxxPxF could function as Clb3-specific docking motif. To test whether it is a modular and linear docking motif, we introduced segments from Ypr174c containing the PxxPxF with different length of flanking sequences to a minimal Cdk1 model substrate Sic1(1-33) containing a single minimal consensus phosphorylation site (Ref II, Fig. 2D). Analysis of Clb3-Cdk1-mediated phosphorylation of these substrates revealed that while the minimal PKGPNF enhanced phosphorylation rate about 25-fold, longer segments PPKGPNF and PPKGPNFYAK increased phosphorylation rate by 65- and 160-fold, respectively (Ref II, Fig. 2E). These experiments show that the PxxPxF is a modular Clb3-docking SLiM and that the flanking positions also contribute to the interaction. Importantly, the PxxPxF motif only promoted phosphorylation by Clb3-Cdk1 and not Cln2-, Clb5- or Clb2-Cdk1 (Ref II, Fig. S2E).

To search for additional targets whose phosphorylation is promoted by the PxxPxF motif, we performed a prediction of PxxPxF motifs in intrinsically

disordered regions of *S. cerevisiae* proteome. We searched for motif with consensus [PILVM]PxxPxFxx[KR], where the key residues are fixed, but a set of similar residues are allowed in the flanking positions. This motif is present in four proteins: Ypr174c, Sen1, Boi1 and Tgl5 (Ref II, Fig. 3A). We purified Sen1 and the disordered fragments containing the motif and Cdk1 phosphorylation sites from Boi1 and Tgl5, and analyzed their phosphorylation by different cyclin-Cdk1 complexes. Tgl5 and Boi1 were most rapidly phosphorylated by Clb3-Cdk1, whereas Sen1 showed high specificity for Cln2-Cdk1 as well (Ref II, Fig. 3B). Importantly, rapid phosphorylation of Ypr174c, Tgl5, Boi1 and Sen1 by Clb3-Cdk1 was dependent on the Clb3 *hp* and the predicted PxxPxF motif in the substrate (Ref II, Fig. 3B).

We identified additional Clb3-specfic Cdk1 targets – Ndd1, Plm2 and Spc29 – that do not have an exact match to the PxxPxF consensus (Ref II, Fig. 3B). This indicates that there could be further variations in the PxxPxF motif or additional Clb3 docking motifs.

5.2.3. Clb3-Cdk1 targets Ypr174c *in vivo*

Ypr174c is a protein of unknown function that localizes to the nuclear envelope and SPBs (Sundin et al., 2004). First, we aimed to study how Ypr174c is phosphorylated *in vivo*. We performed Western Blot of asynchronous cultures using Phos-tag SDS-PAGE to detect multisite phosphorylation of Ypr174c-6HA. Deletion of *CLB3*, mutation of Clb3 *hp* or Ypr174c PxF motif reduced the multisite phosphorylation of Ypr174c-6HA (Ref II, Fig. 4A-B). Further analysis suggested that sites S158, S170 and S175 are phosphorylated *in vivo*, and that Clb3-Cdk1 promotes phosphorylation of minimal consensus sites S170 and S175, while Clb3 is not necessary for phosphorylation of the optimal site S158 (Ref II, Fig. 4C).

Next, we analyzed the effect of phosphorylation sites on Ypr174c phosphorylation, and found that mutation of all Cdk1 consensus sites abolished the nuclear envelope localization of Ypr174c-GFP (Ref II, Fig. 4D). This indicates that Ypr174c localization could be phospho-regulated, however, no single site was essential for the localization and the localization was uniform throughout the cell cycle.

We found that GFP-tagged Clb3 with an added nuclear export signal accumulates to the nuclear envelope and SPBs, like Ypr174c, and as discrete points in the cytoplasm (Ref II, Fig. 4E, G). Mutation of Clb3 *hp* or Ypr174c PxxPxF decreased the nuclear envelope localization of NES-Clb3 (Ref II, Fig. 4E-F). Further, the cytoplasmic puncta of NES-Clb3 colocalize with mCherry-Tgl5 (Ref II, Fig. 4G), which has been shown to accumulate to lipid droplets (Athenstaedt and Daum, 2005). As with Ypr174c, the strong co-localization of NES-Clb3 and mCherry-Tgl5 was lost upon mutation of Tgl5 PxF motif or Clb3 *hp* (Ref II, Fig. 4G-H). Analysis of localization of endogenous Clb3-Citrine indicated that Clb3 is predominantly nuclear, but a significant cyto-

plasmic fraction was also detected (Ref IV, Fig. S3A). We note that the nuclear Clb3-Citrine signal could obscure detection of the Clb3 nuclear envelope localization using wild-type Clb3. Although requiring further investigation, the results obtained with NES-Clb3 suggest that the *hp*-PxF interaction could direct localization of Clb3 to the nuclear envelope, SPBs and lipid droplets.

5.2.4. Clb3-Cdk1 substrate Ypr174c anchors Cdc5 to SPBs

A search for additional SLiMs in the disordered C terminus of Ypr174c revealed the presence of potential Polo-box binding motif at S170 and a PxL docking site for the mitotic exit phosphatase Cdc14 (Ref II, Fig. 5A) (Elia et al., 2003; Kataria et al., 2018). Polo kinase Cdc5 localizes to the SPBs, however, it is not known how Cdc5 is recruited to the SPBs prior to anaphase (Botchkarev et al., 2014, 2017; Song et al., 2000). To uncover the function of Ypr174c in Cdc5 localization, we analyzed the localization of Cdc5-Citrine during the cell cycle, using Spc42-mCherry as a marker for SPBs. Cdc5-Citrine localized to the nucleus and SPBs in the wild-type strain, but mutation of the Polo-box binding motif S170 in Ypr174c caused a loss of SPB accumulation of Cdc5-Citrine (Ref II, Fig. 5B-C). Mutation of PxF motif in Ypr174c caused only a minor decrease in SPB accumulation of Cdc5-Citrine (Ref II, Fig. 5C, S5A). This suggests that Clb3-Cdk1, and possibly other Cdk1 complexes, promote phosphorylation of S170, which recruits Cdc5 to the SPB.

We performed a pull-down assay to test whether Ypr174c and Cdc5 are direct interactors. For this, purified Ypr174c was phosphorylated with Clb3 and used to pull down Cdc5-6HA from yeast lysate. These experiments confirmed that Ypr174c interacts with Cdc5 directly and that phosphorylation of S170 is necessary for the interaction (**Fig. 7**) (Ref II, Fig. 5F). Therefore, we proposed to rename Ypr174c as Csa1 – Cdc5 SPB anchor.

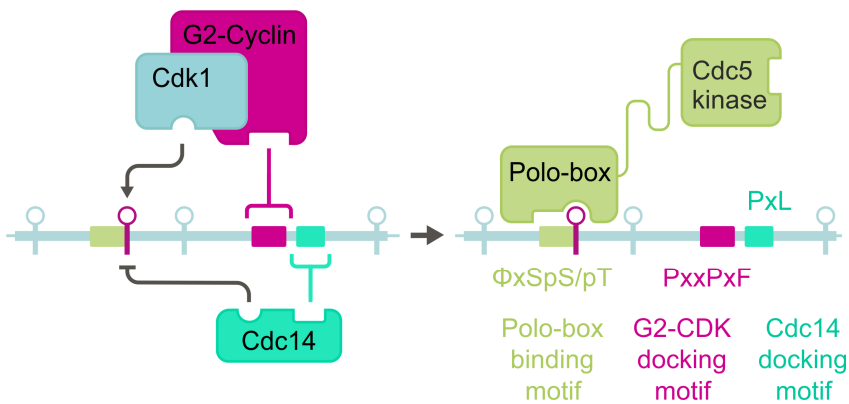

Figure 7. Scheme of the disordered C terminus of Ypr174c showing how different SLiMs lead to cell cycle dependent recruitment of Cdc5.

5.3. S-CDK specific signaling via NLxxxL motif (Ref III)

Early studies on cyclin specificity revealed that S-CDK recognizes its targets by an RxL motif, an interaction that is conserved from yeasts to humans (Chen et al., 1996; Loog and Morgan, 2005; Schulman et al., 1998; Wilmes et al., 2004). The conventional RxL motif, however, is not only specific for S phase cyclins Clb5 in budding yeast and cyclin A in human cells, as it is also utilized by yeast cyclin Clb3 and human cyclin E and to some extent also cyclin B (Kõivomägi et al., 2011b; Takeda et al., 2001; Topacio et al., 2019; Wohlschlegel et al., 2001). Thus, whereas the LxF and PxxPxF motifs are specific for a single or a pair of paralogous cyclins, the RxL motif is a more general cyclin docking motif. However, the N terminus of Far1 was found to contain a region that enables Clb5-specific phosphorylation, while having no effect on other tested cyclins-Cdk1 complexes (Ref III, Fig. 2A, EV2B, C). The aim of this study was to define and analyze the properties of the orthogonal S-CDK docking motif.

5.3.1. A SLiM with consensus NLxxxL promotes phosphorylation of Far1 degron by Clb5/6-Cdk1

A potential Clb5 docking motif was initially mapped to positions 130-139 in Far1 (Ref III, Fig. 1B). Upon Cdk1-dependent phosphorylation, Far1 is ubiquitinated by SCF^{Cdc4} and degraded (Gartner et al., 1998). To map the docking motif, we used time-lapse fluorescence microscopy, where we could follow the timing of Far1 degron phosphorylation by measuring the levels of GFP fusion protein in the cell cycle. As full-length Far1 is an inhibitor of Clb5-Cdk1 and we aimed to map the docking motif in the N terminus, for simplicity, we used only the N-terminal positions 1-150 that contain the degron and the potential docking motif in these experiments. As a reference for cell cycle progression, we used the nuclear export of 50% of Whi5-mCherry, which defines the Start point in late G1 (Doncic et al., 2011).

Far1(1-150)-GFP is degraded at around 14 minutes after Start (Ref III, Fig. 1D-E), which is around the same time as Cdc6 and Sic1 at the onset of S phase (Ref I, Fig. S3A, (Venta et al., 2020)). Mutation of the phosphorylation sites that comprise the degron, S87 and S91, leads to stabilization of the protein over the cell cycle. This confirms that measuring Far1(1-150)-GFP degradation provides an indirect way to measure phosphorylation of the degron sites. Next, we analyzed the effect of mutations in the 130-139 region and found that mutation of N130A, L131A and L135A delayed the degradation by 10-30 minutes, while other single mutations did not have a significant effect (Ref III, Fig. 1F-G). The direct effect of these mutations on Clb5-Cdk1-mediated phosphorylation was also confirmed by *in vitro* kinase assay, where the N130A, L131A and L135A mutants were phosphorylated with 20-50 times lower rate than the wild-type substrate (Ref III, Fig. 1H). Importantly, mutation of these residues greatly decreased phosphorylation by S-phase complexes Clb5 and Clb6-Cdk1, but did not affect phosphorylation by Cln1/2-, Clb3/4- or Clb1/2-Cdk1 (Ref III, Fig.

2A, EV2A-C). Therefore, the results suggest that a novel SLiM with the consensus NLxxxL promotes S-CDK-specific phosphorylation. Also, like other substrate docking motifs of Clb cyclins, the NLxxxL interacts with the cyclin *hp* (Ref III, Fig. 2A, EV2D-F).

5.3.2. NLxxxL targets Clb5-Cdk1 activity more efficiently than RxL motif

To study whether NLxxxL and RxL motifs have other functional differences in addition to the cyclin specificity, we replaced the NLxxxL motif in Far1 with an RxL motif from Sic1 (Ref III, Fig. 3A-B). This caused a minor 2-3-minute delay in Far1(1-150)-GFP degradation (Ref III, Fig. 3C). Interestingly, Michaelis-Menten kinetics analysis of the two substrates showed that the wild-type with NLxxxL motif had 13-fold higher specificity (k_{cat}/K_M) compared to the mutant with RxL motif (Ref III, Fig. 3D). This shows that the NLxxxL motif is more efficient than RxL in promoting Clb5-dependent phosphorylation. However, as the 13-fold change in kinase specificity results in only a minor change in phosphorylation timing, it also indicates that a very rapid increase in Cdk1 activity must take place during this period.

Processive multisite phosphorylation of Sic1 has been shown to depend on both cyclin and Cks1 docking interactions (Kõivomägi et al., 2011a, 2013). To study the role of Cks1 in phosphorylation of Far1 N terminus, we mutated the TP phosphorylation sites to SP, as only phospho-threonines bind Cks1 (Kõivomägi et al., 2013; McGrath et al., 2013). Mutation of the Cks1 binding sites caused a 5-minute delay in Far1(1-150)-GFP degradation (Ref III, Fig. 4A-B). However, the same mutation in Far1(1-150 RxL) resulted in a greater 20-minute delay (Ref III, Fig. 4A-B). This shows that the Cks1-dependency of multisite phosphorylation is affected by the cyclin docking motif, and that the higher affinity of NLxxxL enables efficient degron phosphorylation even without Cks1 docking.

To test the modularity of the NLxxxL motif, we introduced 9- and 13-residue segments containing the NLxxxL from Far1 to Sic1. While both segments promoted Clb5-dependent phosphorylation, the 13-residue ATNLTTSLLRESI was much more effective than the 9-residue ATNLTTSLL (Ref III, Fig. 5). This shows that the defined NLxxxL motif is a functional SLiM and that the flanking residues can also contribute to the interaction. Also, as in Far1, the NLxxxL motif was more efficient in promoting Sic1 phosphorylation than the wild-type RxL motif (Ref III, Fig. 5). Thus, in addition to the exclusive S-CDK specificity, the NLxxxL is a stronger potentiator of phosphorylation and could make the NLxxxL-containing substrates less dependent on priming phosphorylation on Cks1 sites.

5.3.3. Homology of the NLxxxL and RxL motifs

To see if additional proteins could be targeted by Clb5-Cdk1 using the NLxxxL motif, we performed a prediction of potential NLxxxL motifs. The consensus sequence for NLxxxL motif is present in the disordered regions of 300 yeast proteins, 50 of which have been previously identified as Cdk1 targets (Ref III, Table EV1). We purified two of the predicted targets, Lif1 and Slx4, and found that they are phosphorylated specifically by Clb5-Cdk1 in an NLxxxL-dependent manner (Ref III, Fig. 5B-C). This indicates that the NLxxxL motif could have a more global function in targeting Clb5-Cdk1 activity.

Surprisingly, the predicted NLxxxL motif overlapped with RxL motif in two Clb5-specific substrates – Fin1 and Spc110 (Ref III, Fig. 6A) (**Fig. 8A**). An analysis of the conservation of NLxxxL motifs in Far1, Fin1 and Lif1 revealed that while some homologs contain an NLxxxL motif, some homologs carry only the consensus for RxL motif, and some species have an overlap of both motifs (Ref III, Fig. EV4B-D). This indicates that the two motifs are homologous and that extreme changes in cyclin specificity can emerge from key mutations in the motif.

5.3.4. Cyclin docking motifs can affect the substrate dephosphorylation rate in anaphase

In addition to ordering Cdk1 phosphorylation events during the accumulation of Cdk1 activity, the different docking motifs could also affect Cdk1 target dephosphorylation in anaphase, as Clb5 is degraded before anaphase, much earlier than Clb2 (Lu et al., 2014). To gain a detailed understanding of cyclin degradation timing, we followed the degradation of cyclin-Citrine fusion proteins in late mitosis. The analysis showed that Clb5 is degraded before anaphase, followed shortly by degradation of Clb4 and finally degradation of Clb3, Clb2 and Clb1 about 20 minutes later compared to Clb5 (Ref III, Fig. 7A-B). The sequential degradation of cyclins creates a possibility of cyclin docking SLiMs to also regulate the ordering of substate dephosphorylation in anaphase (**Fig. 8B**).

We found that several Cdk1 targets that are dephosphorylated in early anaphase – Fin1, Spc110, Cnn1 (Bock et al., 2012; Liang et al., 2013; Woodbury and Morgan, 2007) – bear NLxxxL motifs and are phosphorylated most efficiently by Clb5-Cdk1 (Ref III, Fig. 7D-E). Fin1 is a filament protein that is phosphorylated by Clb5-Cdk1 and dephosphorylated in early anaphase to enable Fin1 accumulation to the spindle and SPBs (Woodbury and Morgan, 2007). To test how the cyclin specificity affects Fin1 dephosphorylation, we replaced the Fin1 NLxxxL motif with a conventional RxL motif (PKKLQF) that is also utilized by Clb3- and Clb2-Cdk1. We measured the SPB accumulation of Fin1-GFP relative to the onset of anaphase as an indicator for dephosphorylation timing. Wild-type Fin1-GFP localized rapidly to the SPBs at

the time of anaphase onset, but the SPB accumulation of Fin1(PKKLQF)-GFP was slightly delayed (Ref III, Fig. 7F-G). Fin1 is dephosphorylated by Cdc14 (Bokros et al., 2016; Woodbury and Morgan, 2007). To analyze the effect of kinase specificity in the absence of specific phosphatase, we also measured Fin1 localization in *spo12Δ* strain, where there is no Cdc14 activity in early anaphase (Stegmeier et al., 2002). Interestingly, even in the absence of Cdc14 activity, wild-type Fin1 was partially recruited to SPBs at the onset of anaphase, followed by a slower accumulation period. Fin1(PKKLQF), however, showed a gradual slow SPB recruitment throughout anaphase (Ref III, Fig. 7F-G). These data indicate that both kinase and phosphatase specificities contribute to the timing of Cdk1 substrate dephosphorylation. Thus, the specificity of the cyclin docking motif in a substrate can affect the timing of phosphorylation during the accumulation of cyclins and the reversal of phosphorylation in mitosis when the cyclins are sequentially degraded (**Fig. 8B**).

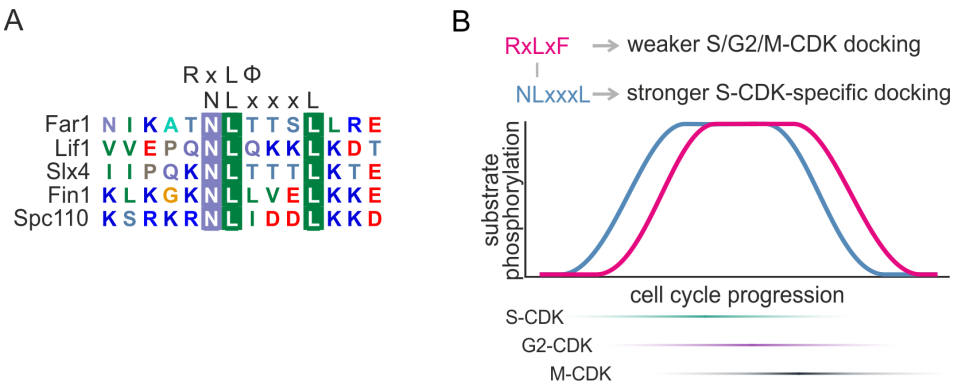

Figure 8. The homologous NLxxxL and RxL motifs fine-tune the timing of substrate phosphorylation. (A) Alignment of NLxxxL motifs. In case of Fin1 and Spc110, the NLxxxL motif overlaps with RxL motif. (B) Minor changes in the cyclin docking motif sequences can have a profound impact on docking specificity, which can affect both timing of substrate phosphorylation in the S-phase and dephosphorylation in mitosis.

5.4. Diversity of SLiMs docking to cyclin *hp* (Refs I, II, III)

The cyclin *hp* had been described as a conserved hydrophobic pocket that binds RxL motifs, an interaction that is conserved in yeast and humans and that targets S-CDK to phosphorylate specific substrates (Schulman et al., 1998; Takeda et al., 2001; Wilmes et al., 2004). Our work has revealed that a variety of motifs can bind to the *hp* and that minor mutations either in the motif or the *hp* can lead to differences in cyclin specificity of the interaction. Thus, *hp* docking could be a central mechanism that directs CDK activity throughout the cell cycle.

Different cyclin docking SLiMs can be partially overlapping. For example, some RxL and LxF motifs differ in only one position (Ref II, Fig. 6A), and the NLxxxL and RxL motifs overlap in Fin1 and Spc110 (Ref III, Fig. 6A). To gain a better understanding of specificity determinants of *hp* docking SLiMs, we performed a systematic mutational analysis of the motifs using a minimal model substrate containing a consensus phosphorylation site, a disordered linker sequence and the docking motif (Ref I, Fig. 6G).

5.4.1. Specificity of *hp* docking SLiMs for yeast cyclins

As some RxL and LxF motifs differ in only the -2 position from the L and yeast B-type cyclins have different residues in the *hp* position that interacts with the motif in L-2 position ((Archambault et al., 2005), Ref I, Fig. S7C-D), we started by analyzing the importance of this position. Addition of an LxF motif from Cdc6 (PEKLQF) greatly increased phosphorylation of the minimal substrate construct by Clb2-Cdk1, but had no or a minor effect with Clb5- and Clb3-Cdk1 (Ref II, Fig. 6B). However, when the -2E from LQF was replaced with K or R, creating a consensus for RxL, the motif was utilized by Clb5-, Clb3 and Clb2-Cdk1. Therefore, the L-2 residue is a key specificity determinant of these motifs. Interestingly, Clb5-Cdk1, but not the other two tested complexes, showed a strong preference for -2R over -2K (Ref II, Fig. 6B).

Next, we studied the effect of N-terminal flanking residues, and found that negative residues could have a strong negative effect on docking to Clb5 and Clb3 (Ref II, Fig. 6C). Another variation in different RxL motifs is the identity of the hydrophobic residue C-terminal of the L. To test the effect of this position, we mutated the F in L+2 position to L. While this mutation decreased the phosphorylation rate in case of both RxL and LxF motifs for the tested complexes, the motif KKLQL was still utilized by Clb5-Cdk1 (Ref II, Fig. 6D). Importantly, the loss of docking efficiency caused by the replacement of F with L in L+2 position could be compensated by introduction of optimal N-terminal flanking residues for Clb5-Cdk1 (Ref II, Fig. 6D). This motif, LKGKKLQL, promoted Clb5-Cdk1-mediated phosphorylation slightly less efficiently than KKLQF motif, but it had increased specificity for Clb5, compared to Clb3 and Clb2.

The C-terminal hydrophobic residue can be in either L+1 or L+2 position. Finally, we tested the effect of position of the C-terminal F and found that Clb3- and Clb2-Cdk1 have strong preference for F in L+2 position, while Clb5-Cdk1 also utilizes the L+1F motifs efficiently (Ref II, Fig. 6E).

These data show that variations within the core RxL motif and the flanking regions have profound effects on docking potential and cyclin specificity. This has revealed that there are RxL motifs with strong specificity for Clb5-Cdk1 (such as RKLF and LKGKKLQL), but also RxL motifs that are utilized by Clb5-, Clb3- and Clb2-Cdk1 (with the consensus R/KxLxF). Furthermore, our studies have uncovered novel cyclin docking motifs – NLxxxL for Clb5-Cdk1, PxxPxF for Clb3-Cdk1, and LxF for Clb2-Cdk1 – that enable exclusive tar-

geting by the specific cyclin (**Fig. 9**). Thus, cyclin-specific substrate targeting controls Cdk1 target phosphorylation throughout the cell cycle, and as variations within the motifs also affect the docking efficiency, the variations could contribute to fine-tuning of phosphorylation events within expression periods of each cyclin as well.

The effect of docking on phosphorylation potentiation also depends on the phosphorylation site specificity. For example, while LxF motif was much more efficient than an RxL motif (VNRILFP) in directing Clb2-Cdk1 to phosphorylate a minimal consensus phosphorylation site, the difference was marginal in case of a full consensus site (Ref I, Fig. 6H-I).

5.4.2. Cyclin *hp* docking specificity of human cyclin-CDK complexes

As yeast cyclins exhibit diverse *hp* docking specificity, we aimed to test whether human cyclin-CDK complexes also have differential motif preferences. Human cyclins E, A, and to a lesser extent also cyclin B all dock to RxL motifs, whereas only the G1 cyclin D-Cdk4/6 has unique docking motifs (Brown et al., 2007; Chen et al., 1996; Dowdy et al., 1993; Topacio et al., 2019; Wohlschlegel et al., 2001). Therefore, the cell cycle events from G1/S to M phase are controlled by CDK complexes that seemingly utilize the same substrate docking specificity. However, as we found for yeast cyclin-CDK complexes, differences in specificity for human cyclins E and A have also been found with different RxL variants (Wohlschlegel et al., 2001).

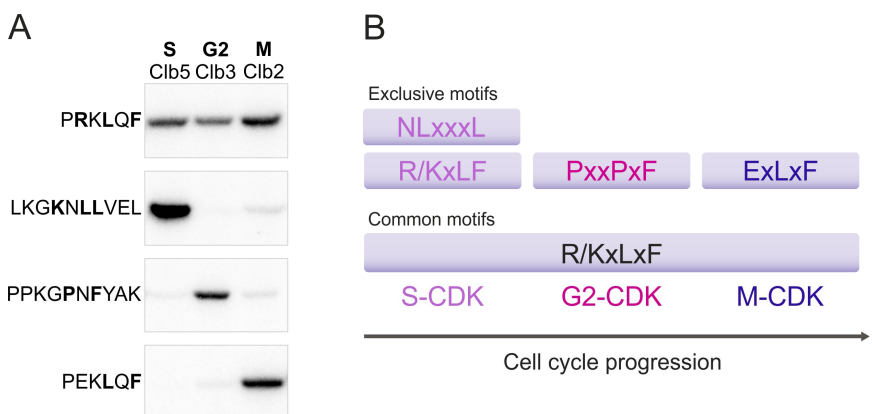

Figure 9. *Hp* docking sites enable both overlapping and orthogonal phosphorylation specificity for major yeast S-, G2-, and M-Cdk1 complexes. (A) Autoradiograph showing the phosphorylation of minimal substrate constructs with a single consensus phosphorylation site and the cyclin docking motif indicated on the left. (B) Exclusive and common cyclin docking motifs enable different phosphorylation dynamics over the cell cycle.

To explore the diversity in docking specificity of human cyclins, we performed phosphorylation experiments using the same minimal model substrate described above (Ref II, Fig. 2D). As the starting point, we used the Cdc6 LxF motif PEKLQF, which only slightly promoted phosphorylation by cyclin B-Cdk1 and had no effect for cyclin E-Cdk2 and cyclin A-Cdk2 (Ref II, Fig. 7A). Replacement of the -2E with K or R increased phosphorylation significantly by all tested complexes, with cyclins E and A exhibiting a substantial preference for R in L-2 (Ref II, Fig. 7A).

Next, we tested the effect of different N-terminal flanking sequences using the motif SVNRILFP as the basis. This RxL motif was very efficiently used by all tested CDK complexes, however, replacement of the SVN flanking sequence with EEE completely abolished the docking by cyclin E and A complexes, but retained considerable docking potential by cyclin B-Cdk1 (Ref II, Fig. 7B). Further, while cyclin E and B complexes preferred the SVN flanking sequence, cyclin A-Cdk2 preferred KKK.

Further mutational analysis revealed that as yeast cyclins, human cyclins also prefer F to L in L+2 position and that cyclin E has a strong preference for F in L+1 position compared to L+2 (Ref II, Fig. 7C). This indicates that cyclin E efficiently docks to a limited selection of RxL motifs, preferring those with R in L-2 and F in L+1, while cyclins A and B dock to a wider range of RxL motifs. Thus, both the flanking sequences and variations in the core motif affect the docking specificity also for human CDK complexes.

5.5. Linear encoding of Cdk1 thresholds (Ref III, IV, V)

The quantitative model of CDK function states during the cell cycle, the increasing CDK activity reached certain activity thresholds necessary for specific phosphorylation events (Stern and Nurse, 1996; Swaffer et al., 2016). This creates a requirement that CDK substrates from different cell cycle phases must be phosphorylated with very different rates. Despite extensive studies on CDK substrate interactions, we still do not have an answer to what governs the timing of phosphorylation in the context of the cell cycle. This has been difficult to answer likely because of the complexity of CDK substrates, as they often contain multiple phosphorylation sites, cyclin docking sites, they are differentially regulated by phosphatases and have different output signals of the phosphorylation (Fisher et al., 2012; Godfrey et al., 2017; Holt et al., 2009; Touati et al., 2019).

In this study, we investigate how patterns of phosphorylation sites and docking sites affect the timing of phosphorylation. As most phosphorylation networks are in intrinsically disordered regions of proteins, according to our hypothesis, the different linear motifs and importantly the pattern of these motifs form a barcode that determines the phosphorylation timing of the network.

To test this hypothesis, we asked if we could rationally design CDK threshold sensors with different phosphorylation timings. For this, we used the non-inhibitory part of Sic1 as a chassis for a disordered protein with two di-phosphodegrons as an output for phosphorylation. As the output of phosphorylation is protein degradation and the limiting step in degradation is degron phosphorylation (Kõivomägi et al., 2011a; Zhou and Howley, 1998), we could measure the degradation of the threshold sensor fused to GFP using time-lapse microscopy to precisely measure the timing of degron phosphorylation in the cell cycle.

The N terminus of Sic1 contains 8 CDK phosphorylation sites, two RxL motifs and an LP motif (Ref IV, Fig. 1C). To first focus on the CDK and Cks1 modules and not cyclin docking, we mutated the RxL and LP motifs in the wild-type threshold sensor (Ref IV, Fig. 1H). As a reference point for cell cycle progression we used the point of 50% nuclear export of Whi5-mCherry to define the Start point in late G1 (Doncic et al., 2011). The wild-type threshold sensor was degraded to 50% at 29 minutes from Start and mutation of phosphorylation sites in either of the degrons (T45/T48 and S76/S80) caused stabilization of the sensor over the cell cycle, confirming that phosphorylation of the degron sites regulates the degradation of the threshold sensor (Ref IV, Fig. 1G).

5.5.1. The pattern of phosphorylation sites can affect the phosphorylation timing by Cks1 docking

One key parameter that could affect the phosphorylation dynamics is Cks1 docking, which has been shown to be critical in phosphorylation of the non-proline site T48 in the degron (Kõivomägi et al., 2011a). Cks1 binds to only phosphorylated TP sites and primes phosphorylation of secondary sites in the C-terminal direction. Importantly, the Cks1 docking efficiency is highly dependent on the distance between the two sites, with 12-20 residues as the optimal distance (Kõivomägi et al., 2013; McGrath et al., 2013).

To test how the distance between the priming site T33 and the degron T45/T48 affects the phosphorylation timing, we introduced linkers of 4 and 8 residues between the sites (Ref IV, Fig. 1H). Increasing the distance between T33 and T45/T48 by 4 and 8 residues delayed the degradation extensively to 67 and 79 minutes, respectively (Ref IV, Fig. 1I-J). Completely removing Cks1 priming by mutating the primer sites to serines abolished the sensor degradation. These data show that Cks1 docking connections and thus also the distances between the phosphorylation sites is a crucial parameter in encoding of phosphorylation timing.

The importance of Cks1 docking in encoding early thresholds was also seen with Far1(1-150)-GFP (Ref III, Fig. 4A-B). Far1(1-150)-GFP degradation was delayed to the same extent by mutation of Cks1 priming sites and by replacement of NLxxxL motif with an RxL motif (Ref III, Fig. 4B). Interestingly,

however, *in vitro* phosphorylation experiment revealed that substitution of NLxxxL with RxL caused over 15-fold reduction in phosphorylation rate, whereas mutation of Cks1 docking did not decrease the overall phosphorylation rate (Ref III, Fig. 4C-D). The combination of RxL and Cks1 docking leads to ordered N- to C-terminal multisite phosphorylation, but mutation of Cks1 abrogates the ordered process (Ref III, Fig. 4C-D). This highlights the importance of proper docking connections in achieving timely phosphorylation of multisite phosphorylation networks.

5.5.2. Encoding of CDK thresholds by serine-threonine swapping

Simply swapping serines and threonines in CDK sites could affect the phosphorylation threshold by at least three mechanisms. First, only phosphorylated threonines bind to Cks1 (Kõivomägi et al., 2013; McGrath et al., 2013), so presence of threonines N-terminal of other phosphorylation sites could decrease the kinase activity threshold. Secondly, threonines are preferentially dephosphorylated by $PP2A^{Cdc55}$ (Godfrey et al., 2017), thus threonine sites create a higher threshold. Thirdly, we found that serines have about 2-3 times higher specificity (k_{cat}/K_M) for Cdk1 compared to threonines (Ref IV, Fig. 2A), thus threonines could also be phosphorylated later due to decreased kinase specificity. However, the relative effect of priming phosphorylation by TP sites is much more prominent than the small differences in kinase specificity (Ref IV, Fig. 2A-B). Threonine-serine swapping could enable 2^N (N is number of phosphorylation sites) different thresholds without changing the linear structure of the phosphorylation network.

To test the potential of serine-threonine swapping in threshold encoding, we used a Sic1-based threshold sensor without cyclin docking sites and where all phosphorylation sites had been mutated to minimal consensus sites (including T48, a non-proline site in wild-type sensor) (Ref IV, Fig. 2C). The sensor with all 8 N-terminal phosphorylation sites as minimal consensus SP sites (8SP) was degraded about 27 minutes after Start (Ref IV, Fig.2D). Next, when 3 C-terminal sites were mutated to threonines (T69 T76 T80), a 12-minute delay in degradation was observed (Ref IV, Fig. 2D). This is likely due to the decreased Cdk1 specificity of the TP sites, and not due to increased phosphatase $PP2A^{Cdc55}$ specificity, as a similar effect was seen in *CDC55* deletion strain (Ref IV, Fig. S2D-E). Mutation of other sites to threonine, however, did not delay the degradation further, but instead moved the degradation earlier. This is because when the N-terminal sites are mutated to threonines, they can function as Cks1 priming sites for the C-terminal sites, and this compensates for the loss of active site specificity due to SP to TP mutations. Indeed, the earliest sensor contained TP sites in the N-terminal positions and SP sites in C-terminal positions. Importantly, the sensors with an identical linear pattern of motifs with differences only in serine-threonine distribution in phosphorylation sites were

degraded over a period of 20 minutes, a considerable part of the cell cycle. Combination of serine-threonine swapping with modifying the distances between the sites, could possibly enable encoding of different thresholds with high resolution over the whole cell cycle.

5.5.3. Cyclin docking SLiMs bring the phosphorylation timings forward

Recent studies have revealed cyclin-specific substrate docking motifs for G1, S, G2 and M phase Cdk1 complexes (Bhaduri and Pryciak, 2011; Kõivomägi et al., 2011b) (Ref I–III). Therefore, cyclin docking SLiMs could contribute to threshold encoding over the cell cycle. To study how cyclin docking motifs affect the phosphorylation timing of different threshold sensors, we added two copies of either LP, RxL, PxxPxF, or LxF motifs to the sensors.

Addition of LP motifs to the wild-type sensor brought the degradation timing 10 minutes earlier (Ref IV, Fig. 3B, E). To bring the sensor degradation even earlier, we introduced a single optimal di-phosphodegron (LLTPPRSP) to the sensor or alternatively, increased the T45-T48 degron Cdk1 specificity by mutating T48 to a minimal consensus site by addition of +1P. These sensors were degraded very early, 12 and 16 minutes after Start, respectively (Ref IV, Fig. 3E). However, addition of LP motif to these sensors had a smaller impact on their degradation compared to the wild-type sensor (Ref IV, Fig. 3B, E). These data show that the poor specificity of output site can be compensated by additional docking interactions.

Next, we analyzed how addition of RxL, PxxPxF and LxF motifs affect the degradation timing of later threshold sensors. Importantly, addition of either RxL or LxF shifted the degradation of the sensor forward to the time window of either Clb5 or Clb2 expression (Ref IV, Fig. 1I, 3C, H). Therefore, the combination of threshold encoding by active site specificity and Cks1 docking with cyclin-specific docking motifs enables establishment of a set of Cdk1 thresholds in the expression window of each cyclin. Further, addition of different RxL motifs to Far1(1-150) resulted in different degradation timings over a span of 20 minutes (Ref III, Fig. 6G-H). This highlights the versatility of cyclin docking motifs in Cdk1 threshold encoding.

Addition of PxxPxF motifs to late threshold sensors had a smaller impact on sensor degradation than addition of LxF motifs, although Clb3 is expressed earlier than Clb2 (Ref IV, Fig. 3H). Quantitative analysis of the nuclear levels of Clb cyclins revealed that Clb5 and Clb2 are much more abundant than other Clb cyclins (Ref IV, Fig. 3H). This indicates that Clb5 and Clb2 provide the majority of Cdk1 activity, whereas Clb3 and Clb4 contribute less to the increase in Cdk1 activity, but phosphorylate specific targets by cyclin-substrate docking. This hypothesis is also supported by the finding that *CLB3* deletion and mutation of the Clb3 *hp* results in a similar defect in several functions of Clb3 (Ref II, Fig 1).

5.5.4. The potency of LP motifs affects the timing of phosphorylation in G1 phase

There is considerable heterogeneity in different LP motifs from G1-Cdk1 targets (Bhaduri and Pryciak, 2011; Kõivomägi et al., 2011b). To better understand the key features of LP motifs and the effects of variations in the motifs, we undertook a detailed study based on competitive growth assay and deep mutational scanning. The presence of pheromone in the growth medium arrests the cell cycle of haploid yeast cells in G1 phase. However, this arrest can be overrun by overexpression of *CLN2* from *GAL1* promoter (Oehlen and Cross, 1994). The MAPK pathway has been rewired by fusion of Ste20 with Ste5 membrane binding domain ($Ste20^{Ste5PM}$), which contains Cdk1 phosphorylation sites and the LP motif (Bhaduri and Pryciak, 2011). In $Ste20^{Ste5PM}$ cells, the Cln2-mediated repression of mating pathway depends on LP-mediated phosphorylation of $Ste20^{Ste5PM}$ (Bhaduri and Pryciak, 2011). This laid the basis for a competitive growth assay, as in the presence of pheromone and overexpression of *CLN2*, the efficiency of LP-mediated suppression of mating pathway is connected to growth rate (Ref V, Fig. 3A).

We introduced five different wild-type LP motifs to $Ste20^{Ste5PM}$ and analyzed their relative frequencies at different time points in a mixed population from the competitive growth assay via deep sequencing. Motifs from Ste5 and Lam5 became greatly enriched, whereas the motifs from Whi5 and Sic1 became depleted (Ref V, Fig. 3C). Interestingly, the most potent Ste5 and least potent Sic1 motifs both contain the core LLPP sequence, indicating that residues outside the core greatly affect the potency of the docking motif.

To better understand the determinants of LP motifs, we made libraries with single randomized substitutions at eight positions of the five LP motifs. These libraries were transformed to yeast and analyzed in the competitive growth assay. The results revealed that the leucine and proline in p1 and p4 from LLPP sequence were strongly preferred, whereas the middle positions p2 and p3 were more tolerant to substitutions (Ref V, Fig. 4A). Interestingly, a strong preference for hydrophobic residues was detected at p5 and p7, and these substitutions greatly enhanced the Sic1 motif potency (Ref V, Fig. 4A).

To test how the docking motif potency affects the timing of phosphorylation in the cell cycle, we measured the phosphorylation-induced degradation of non-inhibitory Sic1ΔC-GFP. Mutation of LP motif caused 3-minute delay in timing of Sic1ΔC degradation (Ref V, Fig 7B, C). However, when the LP motif was improved by introduction of phenylalanine to p5, the degradation was advanced by 10 minutes, and Sic1ΔC levels began to decrease already simultaneously with Whi5-mCherry nuclear export (Ref V, Fig. 7B, C). This data shows that in addition to the presence or absence of specific cyclin docking motifs, also the potency of motifs can greatly affect the timing of phosphorylation switches. Sic1 degradation controls the onset of S phase and has been shown to require the activities of both G1- and S-Cdk1 complexes (Kõivomägi et al., 2011a; Nash et al., 2001; Venta et al., 2020; Yang et al., 2013). The experiments with

Sic1ΔC indicate that the weak LP motif delays the timing of Sic1 degradation and could possibly limit G1-Cdk1 to being a primer of Sic1 phosphorylation, creating a requirement for both G1- and S-Cdk1 in Sic1 degradation.

5.5.5. Cyclin rearrangements change the order of thresholds

To gain a deeper understanding of how different cyclins contribute to ordering of phosphorylation events, we substituted *CLB5* at its locus with *CLB2* (Cross et al., 1999). Further, we deleted *SWE1* in this strain, as Swe1 specifically inhibits the Clb2-Cdk1 complex (Hu and Aparicio, 2005; Keaton et al., 2007). In the *clb5::CLB2* strain, addition of LxF motifs to a late threshold sensor advanced the degradation timing by 40 minutes, over 20 minutes more than in the wild-type strain (Ref IV, Fig. 4A-D). This confirms that phosphorylation of sensors with specific cyclin docking motifs is correlated with the expression timing of each cyclin.

In addition, the degradation of a late sensor without cyclin docking motifs occurred also 25 minutes earlier in the *clb5::CLB2* strain (Ref IV, Fig. 4B-D). This provides an important *in vivo* evidence of the principle of increasing intrinsic activity of the sequentially expressed cyclin-Cdk1 complexes (Kõivomägi et al., 2011b). The sensor with RxL motifs was degraded with similar kinetics in wild-type and *clb5::CLB2* strain (Ref IV, Fig. 4B–D), likely because the loss in RxL docking is compensated by the higher intrinsic activity of Clb2-Cdk1. These data explain the previous findings that Clb2 can compensate the absence of Clb5 in triggering replication initiation (Hu and Aparicio, 2005).

The increasing intrinsic activity has been proposed to contribute to the ordering of cell cycle events by restraining early phosphorylation of late targets (Kõivomägi and Skotheim, 2014). We propose that the increasing activity profile is also critical to achieve better resolution for late targets with high Cdk1 threshold. In case of uniform accumulation of Cdk1 activity, a high fold-change in activity would occur in the early stages of cell cycle, whereas the fold-change would be modest in late phases (Ref IV, Fig. 4E-G). However, the increasing intrinsic activity of sequential cyclin-Cdk1 complexes helps to keep a significant fold-change throughout the cell cycle, which could be necessary for switch-like responses upon expression of each cyclin (Ref IV, Fig. 4G-I). Therefore, cyclin specificity contributes to threshold encoding on both the level of docking specificity and on the level of intrinsic kinase activity.

5.5.6. The filter principle and helper networks

In the studied threshold sensors, the requirement for degradation is full phosphorylation of the four sites in two di-phosphodegrons (Ref IV, Fig. 1C, G). Importantly, the limiting step in this output is the phosphorylation of the non-proline site T48 in the N-terminal di-phosphodegron, as improving T48 to a

consensus site greatly advanced the degradation (Ref IV, Fig. 3B). Decreasing the efficiency of T48 phosphorylation by weakening or prohibiting Cks1 docking resulted in a profound delay in sensor degradation, which could be rescued to different extent by addition of cyclin docking motifs (Ref IV, Fig. 1H-J). Therefore, the threshold on T48 phosphorylation without the docking connections is above the Cdk1 activity during the cell cycle, but by adding different helper networks consisting of Cks1 and cyclin docking sites, the threshold can be brought down to any level, including those that are reached in the very early stages of the cell cycle (Ref IV, Fig. 6C-D) (**Fig. 10**).

In addition to filtering Cdk1 activity, the non-proline sites could separate Cdk1 activity from the activities of other-proline directed kinases, as only Cdk1 can utilize the helper networks. Non-proline sites as the output sites have now been identified in several CDK targets (Huis In 't Veld et al., 2016; Kõivomägi et al., 2011a; Suzuki et al., 2015) and in addition, the filter principle and the limiting step could also be achieved by consensus sites with poor accessibility, which could be due to close proximity to structural domains.

Due to the complexity of CDK substrates with multiple phosphorylation sites and different phosphorylation output signals, here, we used the "build to understand" principle to study the linear encoding of kinase thresholds. This study describes the principles that enable differential phosphorylation of hundreds of Cdk1 targets. As cyclin and Cks1 docking connections have been found in many Cdk1 substrates now (Bhaduri and Pryciak, 2011; Bhaduri et al., 2015; Kõivomägi et al., 2011b; Loog and Morgan, 2005; McGrath et al., 2013; Schulman et al., 1998, Ref I–III), these principles could be important in assigning the phosphorylation timing of Cdk1 substrates on a global scale.

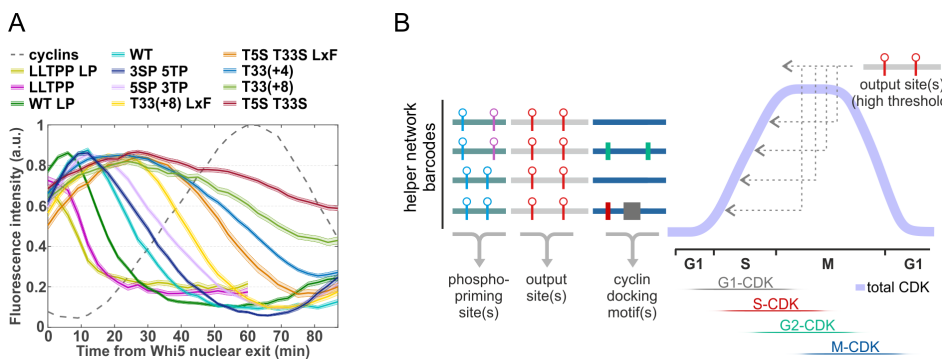

Figure 10. Linear encoding of Cdk1 thresholds over the cell cycle. (A) Plot showing the degradation profiles of selected Sic1-based Cdk1 threshold sensors. The accumulation of cyclins is shown by the sum of nuclear fluorescence levels of B-type cyclin-Citrine fusion proteins. For explanations of the sensors, please see Ref IV. (B) Helper networks of Cks1 binding sites and cyclin docking motifs enable to bring the Cdk1 activity threshold for otherwise high threshold output sites down to any necessary level.

6. CONCLUSIONS

Cell cycle is an ordered series of molecular events that guarantees the reproduction of cells. Flawless cell cycle requires specific order of events, whereby the cellular contents are first duplicated and later segregated in mitosis. CDKs are the central regulators of eukaryotic cell cycle and the discovery that oscillation in the activity of a single kinase – Cdk1 – is necessary and sufficient to initiate DNA replication and mitosis has raised a question that what mechanisms govern the order of cell cycle events.

Two models have been proposed to answer this question. The quantitative model states that different substrate proteins are phosphorylated at different CDK activity thresholds and thus the order of events is governed by the increase in kinase activity in the cell cycle. Alternatively, the cyclin specificity model proposes that different cyclins expressed during the cell cycle direct CDK to phosphorylate stage-specific substrate proteins. In this study, the substrate targeting mechanisms of the Cdk1 complex were studied with the aim to understand how Cdk1 can differentially phosphorylate hundreds of proteins. We found that Cdk1 thresholds can be encoded both based on the increase in CDK activity in the cell cycle and also based on cyclin-specific docking interactions. Importantly, we show that the pattern of linear motifs, including phosphorylation sites and docking motifs, can determine the timing of CDK substrate phosphorylation throughout the cell cycle.

The key findings of the study are:

- Identification of novel docking motifs specific for S (NLxxxL motif for Clb5), G2 (PxxPxF motif for Clb3) and M phase (LxF motif for Clb2) cyclin-Cdk1 complexes. These findings show that cyclin-substrate docking is an important mechanism governing timely substrate phosphorylation in all cell cycle phases.
- Analysis of the M-CDK inhibitors Cdc6 and Swe1 revealed a critical role for both Cks1 docking and cyclin interaction with the LxF motif in Cdk1 inhibition. Therefore, depending on the context, the docking motifs that promote phosphorylation of many Cdk1 substrate proteins, can also lead to inhibition of Cdk1.
- The three key mechanisms – CDK active site specificity, Cks1, and cyclin docking – can all contribute significantly to the timing of Cdk1 phosphorylation events. For example, conversion of a non-consensus output phosphorylation site to a consensus site can greatly advance the timing of phosphorylation, but also smaller differences, like serine/threonine swapping of the output site can fine-tune the phosphorylation events. The Cks1 binding sites and cyclin docking sites function as helper networks that can promote phosphorylation of output sites by specific cyclin-CDK complexes and advance the timing of a phosphorylation switch to any specified time point in the cell cycle. The contribution from distal docking interactions is predicted to be greater on output sites with lower CDK active site specificity.

SUMMARY IN ESTONIAN

Uurimus tsükliinist sõltuva kinaasi Cdk1 substraatide fosforüleerimise ajastamisest rakutsükli jooksul

Rakkude jagunemine on organismi kasvamise ja paljunemise aluseks. Eukarüootse raku jagunemise aluseks on kindlalt järjestatud sündmuste jada, mida nimetatakse rakutsükliks. Rakutsükli eesmärgiks on tagada veatu pärilikkusaine ja organellide duplikatsioon ja nende võrdne jaotamine kahe tütarraku vahel. Probleemid rakutsüklis, näiteks vead DNA replikatsioonil või kromosoomide lahknemised viivad sageli rakkude kontrollimatu jagunemiseni ja kasvajate tekkeni.

Enamik rakutsükli sündmusi on kontrollitud nendes protsessides osalevate valkude fosforüleerimise kaudu. Fosforüleerimine on kõige arvukam post-translatsiooniline modifikatsioon, mis võib mõjutada sihtmärkvalkude aktiivsust, stabiilsust, paiknemist rakus ning seondumist teiste valkude või nukleiinhapetega. Rakutsükli kesksed regulaatorid on tsükliinist sõltuvad kinaasid (CDK-d), mis fosforüleerivad sadu substraatvalke, et käivitada enamik rakutsükli protsesse. Erinevate rakutsükli sündmuste, nagu näiteks DNA replikatsiooni või kromosoomide lahknemise, ajastus on otseselt sõltuv Cdk1 aktiivsusest. Näiteks on leitud, et manipuleerimine Cdk1 aktiivsusega võib põhjustada mitu järjestikkust DNA replikatsiooni tsüklit ilma mitoosita või ka samaaegset replikatsiooni ja mitoosi. Sellest lähtuvalt on püstitatud küsimus, et kuidas üks kinaas saab erinevatel hetkedel käivitada erinevaid sündmusi.

Sellele küsimusele on pakutud vastuseks kaks mudelit. Esiteks, rakutsükli jooksul toimub CDK aktiivsuse pidev tõus ning kvantitatiivse mudeli kohaselt põhjustab just CDK aktiivsuse tõus erinevate valkude fosforüleerimise erinevatel ajahetkedel. Lisaks on teada, et CDK seondub rakutsükli jooksul erinevate tsükliinidega, mis võivad suunata kinaasi fosforüleerima kindlaid valke. Praeguseni ei ole aga vastust küsimusele, et mis eristab varaseid ja hiliseid substraate ning et kui oluline on tsükliin-spetsiifiline substraatide fosforüleerimine.

Cdk1 ja ka mitmed teised kesksed rakutsükli regulaatorid on konserveerunud eukarüootides. Seetõttu on pärmid populaarsed mudelorganismid rakutsükli uuringuteks. Pärmi *Saccharomyces cerevisiae* Cdk1 moodustab rakutsükli jooksul kompleksi üheksa tsükliiniga ning fosforüleerib hinnanguliselt umbes 500 erinevat valku. Kinaasi Cdk1 substraatide valik toimub põhiliselt kolmel tasemel. Esiteks, kinaasi aktiivtsenter seondub erinevate fosforüleerimismotiividega. Teiseks, tsükliinid, mis on hädavajalikud CDK aktiveerimiseks, võivad seonduda ka lineaarsete motiividega substraatvalkudes, suunates CDK kompleksi kindlaid valke fosforüleerima. Kolmandaks, CDK kompleksi kuulub Cks1 valk, mis seondub fosforüleeritud valkudega ja stimuleerib nende multifosforüleerimist.

Käesoleva doktoritöö eesmärgiks oli analüüsida, millised mehhanismid võimaldavad CDK-del rakutsükli jooksul erineva ajastusega fosforüleerida sadu substraatvalke. Selleks uuriti detailselt tsükliin-spetsiifilisi interaktsioone nii S-,

G2-, kui ka M-faasi tsükliin-Cdk1 kompleksidega. Lisaks analüüsiti, kuidas erinevate lineaarsete motiivide omadused ja muster substraatvalkudes mõjutab fosforüleerimissündmuste ajastust rakutsüklis.

Selle doktoritöö peamised tulemused ja järeldused olid järgmised:

- Kirjeldati kolm uut tsükliin-substraat seondumismotiivi: NLxxxL motiiv S-faasi Cdk1 kompleksile, PxxPxF motiiv G2-faasi kompleksile ja LxF motiiv M-faasi Cdk1 kompleksile. Need avastused näitavad, et tsükliin-spetsiifiline substraatide seondumine on oluline mehhanism valkude fosforüleerimise ajastamiseks kogu rakutsükli vältel. Lisaks, uute seondumismotiivide kirjeldamisega leiti ka mitmeid uusi substraatvalke nende Cdk1 komplekside jaoks, avardades teadmisi Cdk1 funktsioonidest rakutsükli regulatsioonis.
- Mitootilise Cdk1 kompleksi inhibiitorite uurimisel leiti, et nii Cks1 kui ka tsükliin-substraat seondumise LxF vahendusel on kriitilised M-Cdk1 reguleerimiseks inhibiitorvalkude poolt. See näitab, et interaktsioonid, mis mitmetes teistes valkudes stimuleerivad substraatide fosforüleerimist, võivad sõltuvalt kontekstist olla ka inhibitoorsed.
- Kõik kolm keskset CDK substraatide äratundmise mehhanismi – aktiivtsentri spetsiifika, Cks1 ja tsükliin-substraat seondumine – võivad olla kriitilised määramaks valgu fosforüleerimise aega rakutsüklis. Leiti, et fosforüleerimismotiivi optimaalsusega manipuleerides on võimalik mõjutada selle fosforüleerimise ajastust nii väga suurtes piirides kui ka täpsemalt vähem oluliste modifikatsioonide kaudu. Lisaks, fosforüleerimise ajastust võivad stimuleerida substraatvalgu seondumine nii Cks1 valgu kui spetsiifiliste tsükliinidega. Need interaktsioonid võivad tuua fosforüleerimise ajastuse varasemaks, sõltuvalt motiivide olemusest ja omavahelisest paiknemisest.
- Erinevad lineaarsed fosforüleerimis- ja seondumismotiivid ja nende muster substraatvalgus määravad valgu fosforüleerimise ajastuse rakutsüklis. Erinevate valkude fosforüleerimise ajastamisel on oluline nii CDK aktiivsuse üldine tõus rakutsükli jooksul kui ka tsükliin-spetsiifiline substraatide äratundmine. Need kaks mehhanismi kombineeritud võimaldavad väga täpselt järjestatud valkude fosforüleerimist nii erinevate faaside lõikes kui kogu rakutsükli jooksul.

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