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**Design of Clb5-specific Cdk1 substrates for cell
cycle independent signalling**

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Abstract

Cyclin-dependent kinases (Cdks) are master regulators of the cell cycle with Cdk1 driving most cell cycle events in budding yeast. Phosphorylation enables the regulation of protein stability, localization and interactions, thus, the ability to predictably program phosphorylation regulation could have a strong impact on the design of synthetic pathways. Cdk1 has a complex, but well-studied substrate targeting mechanisms, making it potentially suitable for such applications. In this work a minimal phosphodegron module was created to show, that phosphorylation efficiency can be manipulated. By inducing Clb5 it was possible to enhance phosphorylation, leading to degradation of the phospho-degron modules. Also it was found that the activity of Clb5 can be targeted specifically to the degron module by addition of a cyclin docking motif.

Keywords: Cell cycle, phosphorylation, docking motifs

CERCS: B230 (Microbiology, bacteriology, virology, mycology)

Rakutsüklist sõltumatute Clb5-Cdk1 substraatide disainimine

Lühikokkuvõte

Tsükliinist sõltuvad kinaasid (Cdk-d) on rakutsükli peamised regulaatorid, mille hulgast vastutab Cdk1 enamiku rakutsükli sündmuste läbi viimise eest. Fosforüleerimine reguleerib valkude stabiilsust, lokaliseerimist ja interaktsioone, seega, võime programmeerida fosforüleerimist võib avaldada suurt mõju sünteetilisele bioloogiale. Kinaasil Cdk1 on mitmekesine ning hästi uuritud substraatide äratundmismehhanismid, võimaldades kasutamist sünteetilise bioloogia rakendustes. Antud töös loodi minimaalse fosfodegroni moodul ning näidati, kuidas on fosforüleerimise efektiivsusega võimalik manipuleerida. Tsükliini Clb5 indutseerides suurendati fosforüleerimist, millest tulenevalt suurenes ka fosfodegroni moodulite lagundamine. Lisaks leiti, et Clb5 aktiivsust saab suunata degroni mooduli juurde, lisades tsükliinspetsiifilise seondumismotiivi.

Võtmesõnad: Rakutsükkel, fosforüleerimine, seondumismotiivid

CERCS: B230 (Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia)

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ABBREVIATIONS

APC – Anaphase-promoting complex

ATP – Adenosine triphosphate

CAK – Cdk-activating kinase

Cdk – Cyclin-dependent kinase

CSM – Complete supplement mixture

D-box – Destruction box

DMSO – Dimethyl sulfoxide

GFP – Green fluorescent protein

MAPK – Mitogen-activated protein kinase

NES – Nuclear export signal

NLS – Nuclear localization signal

SCF – Skp1-Cul-F-box

SC-URA – Synthetic complete media lacking uracil

Sic1 – Subunit inhibitor of Cyclin-dependent protein kinase 1

SS-DNA – Salmon sperm single-stranded DNA

YPD –Yeast extract, Peptone, Dextrose

INTRODUCTION

All life is based on cell division, which is divided into distinct cell cycle events, such as duplication of the cell's biological material and equal spreading of it into a pair of daughter cells. The eukaryotic cell contains a robust regulatory network, that controls the correct order and timing of the cell cycle events. The eukaryotic cell cycle can be divided into 4 main stages based on the chromosomal events and the gap-phases G1 and G2 during which the cell prepares for further events. The cell grows and prepares for replication in G1 phase; DNA replication occurs in S phase; in G2 phase the cell prepares for mitosis; nuclear division and cell division between the daughter cells take place in M phase (Chen et al., 2002; Morgan, 2007).

The activation of different cell cycle events is mediated by phosphorylation of specific proteins by the master regulator of cell division, the cyclin-dependent kinase (Cdk). As Cdk has to phosphorylate hundreds of proteins in an ordered manner during the cell cycle, such regulation is based on changing substrate specificity and different activity thresholds of the cyclin-dependent kinases. The activity of Cdk rises during the cell cycle, causing the execution of specific molecular events at certain activity thresholds. Cyclin specificity in substrate recognition has helped researchers to understand how the Cdk regulation of the cell cycle functions as different cyclins catalyze certain cell cycle events. However, it still fails to explain how relatively small changes in Cdk activity can trigger the ordered sequence of discrete events in a sufficiently time-resolved fashion.

The birth of synthetic biology field has revealed, that it is possible to design and construct biological modules or re-design existing biological systems. However, as the field is yet very novel, a lot of research needs to be carried out in order to push the boundaries of synthetic biology and tackle widespread issues facing the world. Despite the understanding, that a significant part of cellular regulation occurs through phosphorylation, unfortunately, we are not yet able to use this knowledge for creation of synthetic phospho-regulation modules. Subsequently, such riddle has inspired the idea and development of the thesis.

The aims of the thesis were to analyze the possibilities of using Cdk1 for cell cycle independent signalling. For this phosphorylation-dependent degraon-modules with different docking sites were constructed. Degradation of these modules was studied in unperturbed cell cycle as well as with Clb5 induction.

1 LITERATURE REVIEW

1.1 Cyclin-dependent kinases

Cell cycle events are driven by an independent control system, which is based on oscillations in the activities of serine/threonine protein kinases called the cyclin-dependent kinases (Cdks). Their main function is catalyzing the covalent attachment of phosphoryl groups derived from ATP to protein substrate (phosphorylation), leading to changes in substrate protein functions. Cdk activities increase and decrease as the cell cycle progresses, although concentrations of Cdk proteins are constant throughout the cell cycle. Oscillations in Cdk activities result in cyclical changes in the phosphorylation of cell cycle components, determining the ordered initiation of cell cycle events (Morgan, 2007). The enzymatic activation of Cdks requires the binding of a regulatory subunit – cyclin. Usually, full activation also requires phosphorylation of a threonine residue near the kinase active site (Morgan, 2007). As levels of Cdk activity rise during the cell cycle, specific molecular events are executed at certain activity thresholds. This model requires that substrates are phosphorylated with a wide range of efficiencies, such that very good substrates are phosphorylated early, at a low Cdk activity threshold, while poor substrates are phosphorylated only when higher Cdk activity is achieved later in the cell cycle (**Figure 1**) (Miller and Turk, 2018; Swaffer et al., 2016).

Although there are six Cdks in the budding yeast *S. cerevisiae*, Cdk1 (also known as Cdc28) will be in the main focus as it is essential to drive the cell cycle in budding yeast (Liu and Kipreos, 1999). Cdk function has remained fundamentally unchanged over hundreds of millions of years of eukaryotic evolution. For example, the cell cycle of yeast functions even if yeast Cdk1 is replaced with human Cdk1 or Cdk2 (Elledge and Spottswood, 1991). Cdks direct the cell cycle events by phosphorylating a large number of proteins in the cell. During mitosis in particular, Cdks phosphorylate hundreds of distinct proteins: for instance, yeast has approximately 600-700 proteins that are Cdk1 substrates, which makes up about 10% of all proteins (Ubersax et al., 2003).

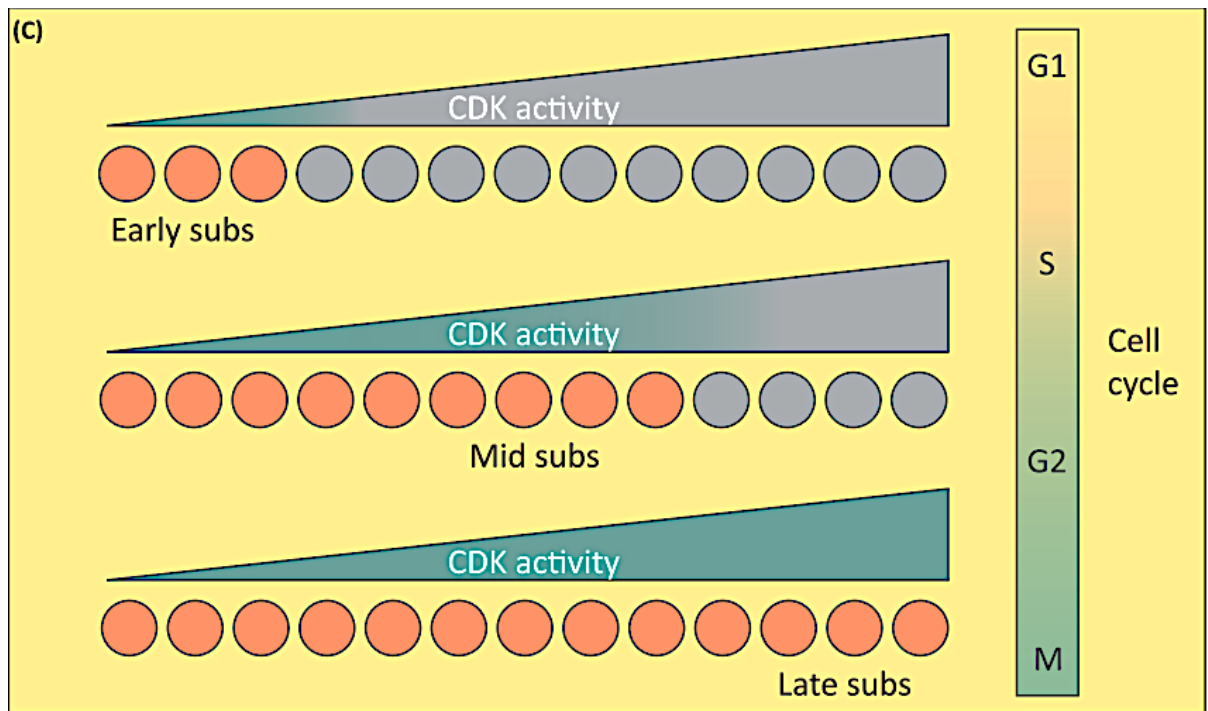


Figure 1. Differential substrate phosphorylation by the Cdk during the cell cycle. Increasing Cdk activity during the cell cycle contributes to the timing of substrate phosphorylation: high-quality substrates are phosphorylated early (in G1/S phase), when Cdk activity is low, and lower quality substrates are phosphorylated later in cell cycle (in G2/M phase), when Cdk activity reaches a higher activity threshold (Miller and Turk, 2018).

1.2 Cyclins

Cyclins are proteins, that determine the Cdk activity with its defining feature of binding and activating Cdks. Most cyclins go through vast changes in concentration during the cell cycle, leading to the oscillations in Cdk activity – the foundation of the cell cycle control system. Therefore, the regulation of cyclin concentration, mainly by changes in cyclin gene expression and destruction of cyclins by proteolysis is highly important in cell cycle control.

Cyclins are involved in numerous processes besides cell cycle control, however, the cyclins that directly regulate Cdks controlling cell cycle progression will further remain in focus. Cyclins can be arranged in four classes, based on the timing of their expression as well as their functions in the cell cycle. G1/S, S and M cyclins make up three classes and these cyclins are directly involved in the control of cell cycle events (**Figure 2**). The fourth class, the G1 cyclins, contributes to the control of cell cycle entry in response to extracellular factors.

In the budding yeast *S. cerevisiae* the G1/S cyclins Cln1 and Cln2 oscillate during the cell cycle, rising in late G1 and falling during S phase (**Figure 2**). The main role of G1/S cyclin - Cdk complexes is to trigger progression through the Start checkpoint and activating the process leading to DNA duplication by shutting down numerous braking systems that suppress S-phase Cdk activity in G1. For example, the activity of S phase cyclins is inhibited in G1 phase by an inhibitor protein Sic1 (Schwob et al., 1994). This is important for preventing initiation of DNA replication at low S cyclin levels. As cyclins accumulate in late G1 phase, the G1/S and S cyclins cooperatively phosphorylate Sic1, leading to degradation of Sic1 and further release of S cyclin-Cdk complexes (Kõivomägi et al., 2011a). Furthermore, G1/S cyclins initiate other early cell cycle events, for example duplication of spindle pole body, the functional analogue of centrosome in higher eukaryotes, and activation of bud morphogenesis in yeast. S cyclins Clb5 and Clb6 accompany the rise of G1/S cyclins and direct the stimulation of DNA replication.

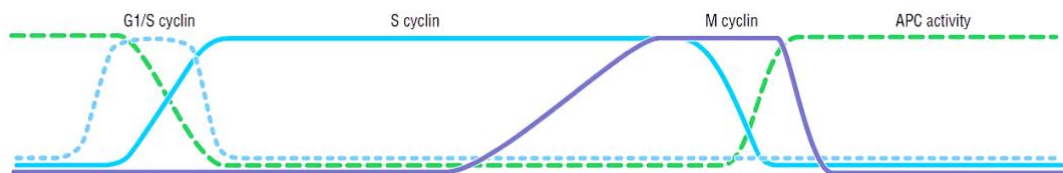


Figure 2. A simplified view of the cell cycle control system. Scheme showing how levels of the three major cyclin types (G1/S, S, M) oscillate during the cell cycle. The increase of APC activity causes the destruction of S and M cyclins and thus the inactivation of Cdks, which promotes the completion of mitosis and cytokinesis (Morgan, 2007). Therefore, cell cycle is an oscillation of Cdk and APC activities, with the levels of cyclins and Cdk activity being high in S and early M phase and APC activity peaking in late M phase and G1.

The cyclins of M phase (Clb1, 2, 3 and 4) are the last in sequence to appear. Their concentrations are rising as the cell approaches mitosis and culminate in metaphase. M cyclin-Cdk complexes are responsible for such cellular changes that lead to assembly of the mitotic spindle and the alignment of sister-chromatid pairs on the spindle in metaphase. M-Cdks stimulate activation of the anaphase-promoting complex (APC), which triggers the metaphase-anaphase transition (Bloom and Cross, 2007). APC is a ubiquitin-protein ligase complex that stimulates the destruction of proteins that hold the sister chromatids together (Davey and Morgan, 2016). Additionally, the APC activity oscillates in the opposite pattern compared to S and M cyclins and causes their destruction. The destruction of cyclins takes

place in anaphase followed by mitotic exit and cytokinesis. The G1 cyclins, known as Cln3 in budding yeast and cyclin D in vertebrates, support the coordination of cell growth with entry into a new cell cycle (Start checkpoint in yeast, restriction point in animals). The cyclins of G1 phase differ from the rest of cyclins as their levels do not oscillate in a set pattern during the cell cycle, instead, they increase gradually throughout the cycle in response to cell growth and external growth-regulatory signals (Morgan, 2007).

Proteolysis is critical at the metaphase-to-anaphase transition, where sister-chromatid separation and mitotic exit are triggered by the irreversible destruction of mitotic cyclins and proteins that control sister-chromatid cohesion. Destruction of cyclins also helps establish the state of low Cdk activity in G1, while the destruction of Cdk inhibitor proteins at the end of G1 helps drive the irreversible activation of S-Cdks (Morgan, 2007). Additionally, after metaphase-anaphase transition, phosphatase Cdc14 is activated in order to restore the initial state of G1 (Stegmeier and Amon, 2004).

In addition to cyclin binding, a complete activation of a Cdk also requires phosphorylation of a threonine residue adjacent to the kinase active site, which is catalyzed by enzymes called Cdk-activating kinases (CAKs). The activity of CAK is maintained at a constant high level throughout the cell cycle. CAKs have been highly conserved during evolution and are not regulated by any known pathway.

1.3 Targeting of substrates by cyclin-Cdk complexes

The specificity of cyclin-Cdk1 complexes is controlled at three different levels: first, by the active site specificity of Cdk1; secondly, by cyclin-specific docking interactions; and thirdly, by the specificity of phospho-adaptor Cks1 (**Figure 3**). The Cdk active site phosphorylates full consensus motifs [S/T*]PX[K/R], where S/T* stands for the phosphorylated serine or threonine, P for proline, X represents any amino acid and K/R represents the basic amino acid lysine (K) or arginine (R), and minimal consensus ([S/T*]P) motifs (Nigg, 1993). The phosphorylation rate of a site can be increased by two docking interactions: Cks1 can bind to phosphorylated TP sites and cyclins can interact with substrates via specific short linear docking sequences. On both occasions, the effect of docking depends on the relative positioning of docking sites and phosphorylation sites along the disordered substrate (Kõivomägi et al., 2013; Örd and Loog, 2019).

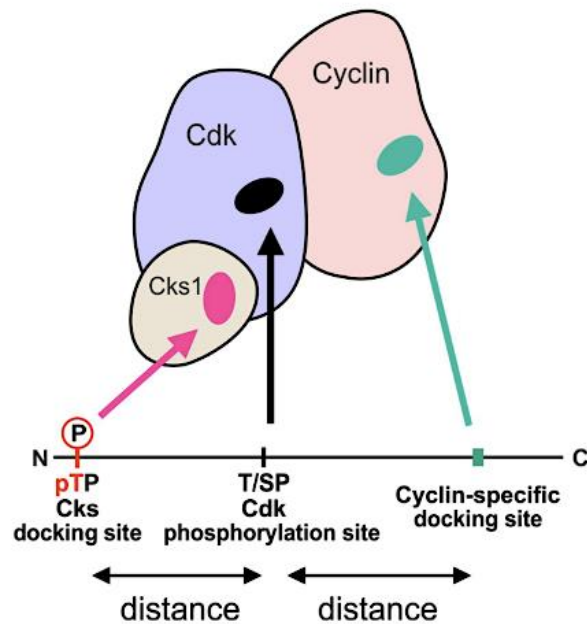


Figure 3. Cyclin-Cdk1-Cks1 complex. Schematic diagram showing the main interactions between substrate proteins and the Cdk complex determining the phosphorylation rate and specificity (Örd and Loog, 2019).

1.3.1 Phospho-adaptor Cks1 promotes multiphosphorylation of Cdk substrates

In *S. cerevisiae* the cyclin-Cdk1-Cks1-dependent phosphorylation of multisite targets is controlled by such key substrate parameters, as distances between phosphorylation sites, the distribution of serines and threonines as phospho-acceptors, and the positioning of cyclin-docking motifs (Kõivomägi et al., 2013). The element mediating the key interactions in this process is Cks1, the phospho-adaptor subunit of the cyclin-Cdk1-Cks1 complex.

It has been discovered that Cks1 binds only to phospho-threonines and not phospho-serines (Kõivomägi et al., 2013; McGrath et al., 2013) (**Figure 3**). By binding to phosphorylated threonine sites, Cks1 stabilises the enzyme-substrate interaction, increasing the phosphorylation efficiency of secondary sites (Kõivomägi et al., 2011a, 2013; McGrath et al., 2013) (**Figure 4**). Therefore, Cks1 enhances multisite phosphorylation. Cks1 is especially important in phosphorylation of low quality Cdk sites, for example the di-phosphodegron sites in Cdk inhibitor protein Sic1 (Kõivomägi et al., 2011a). To prove this hypothesis, the threonines in the Cdk consensus sites of Sic1 had been replaced with serines and as a result, the abrupt accumulation of multiply phosphorylated species was severely suppressed (Kõivomägi et al., 2013). Thus, it is possible to disrupt the docking connections

of the Cks1-dependent cascade by replacing threonines in Cdk sites with serines (Kõivomägi et al., 2013).

The second important parameter influencing the Cks1-mediated phosphorylation is the distance and relative positioning between the priming site and the secondary phosphorylation site (**Figure 4**). Observations in distance dependence in the rates of Cks1-dependent secondary steps have led to the conclusion, that the distance of 12-16 amino acids between the phosphorylation sites is the most optimal (Kõivomägi et al., 2013).

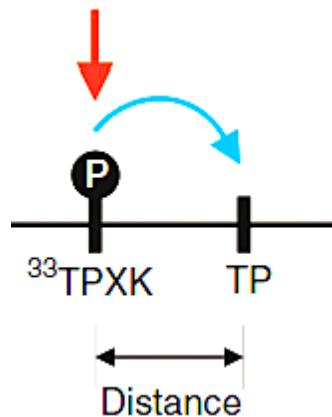


Figure 4. Amino acid distance between the priming site and the secondary phosphorylation site affects Cks1-mediated phosphorylation. A scheme explaining the mechanism of Cks1-mediated phosphorylation. First, Cdk1 phosphorylates a priming site, that then binds to the phosphate-binding pocket of Cks1. This increases the binding affinity of the substrate and Cdk complex and promotes phosphorylation of secondary sites, leading to multisite phosphorylation. Importantly, the efficiency of Cks1-mediated phosphorylation depends on the positioning of primer and secondary sites (Kõivomägi et al., 2013).

1.3.2 G1 cyclin specificity

As cell cycle events have to occur in a specific order, different cyclins must stimulate different cell cycle processes, meaning there is a molecular basis of the cyclin specificity. Considerable evidence has supported the belief that cyclins are not simply activators of the associated Cdk subunit but also help direct the Cdk to specific substrates, either by directly binding the substrate or by taking the Cdk to a subcellular compartment where the substrate is found (Bloom and Cross, 2007). Functional specialization of cyclins may not be absolutely essential in all species, particularly in fission yeast (Coudreuse and Nurse, 2010). Cyclin specificity is achieved on at least two levels. First, cyclins modulate the active site activity of Cdk, as the early (G1 and S) cyclins confer lower activity to the Cdk complex and later cyclins confer higher activity (Kõivomägi et al., 2011b; Loog and Morgan, 2005; Topacio

et al., 2019). This could be a mechanism that prevents early phosphorylation of late Cdk targets, ensuring that mitotic events are not initiated before the expression of mitotic cyclins. Secondly, cyclins can bind to specific substrates, directing Cdk activity to a subset of targets. Recent studies have found that a subset of substrates contain a short linear docking motif that is recognized only by G1/S cyclins Cln1 and Cln2, and not by Cln3 or later S or M phase cyclins (Das et al., 2011; Kõivomägi et al., 2011b).

Despite some functional overlap among Cln1–Cln3, they have important differences (Bloom and Cross, 2007; Levine et al., 1996) that contribute to a two-stage commitment process: Cln3 plays an early priming role initiating expression of Cln1 and Cln2, which further enhance their own expression via a positive feedback loop, leading to a sharp increase in Cln1/2 activity that triggers a decisive entry into the cell cycle (Cross and Tinkelenberg, 1991; Skotheim et al., 2008). G1 cyclins show distinct subcellular distributions, while Cln3 and Cln1 are nuclear, Cln2 is concentrated in the cytoplasm (Edgington and Futcher, 2001; Miller and Cross, 2000).

During the G1 phase the decision to enter cell cycle is responsive to such cues as nutrient availability, cell size, and inhibitory signals. Ultimately, these cues affect the function of Cln1–Cln3, which then drive the Cdk phosphorylation events that commit cells to division, followed by the G1/ S transition (Cross, 1995; Jorgensen and Tyers, 2004). Key Cdk substrates in this period are inhibitors of cell cycle entry, such as Whi5, a repressor of G1/S transcription (Bruin et al., 2008; Costanzo et al., 2004) as well as Cdh1 and Sic1, which prevent the expression and activity of later cyclins, respectively (Morgan, 2007).

Reduction in ability to fully phosphorylate the G1/S transcriptional repressor Whi5 has been observed after blocking the Cln2 docking function (Bhaduri et al., 2015). Furthermore, disruption of Cln2 docking disturbs the coordination between cell size and division resulting in a delay of G1/S transition (Bhaduri et al., 2015).

1.3.3 S cyclin specificity

The conducted analysis of yeast cyclin mutants has revealed notable overlap between S and M cyclins: M cyclins are able to stimulate S phase to some extent, for example. In fission yeast, progression through S and M phase can be achieved in mutant cells lacking S cyclins and expressing only the M cyclin Cdc13 (Coudreuse and Nurse, 2010). One possibility of how a single cyclin drives the correct sequence of S and M phase events is that the substrate phosphorylation of the cyclin-Cdk complex is dependent on concentration, and a complex

that promotes phosphorylation of S phase substrates at one concentration is also capable of promoting M phase substrate phosphorylation, when it accumulates to higher levels (**Figure 1**) (Coudreuse and Nurse, 2010; Stern and Nurse, 1996). Another possibility is that some Cdk substrates become available for phosphorylation only during a specific stage in the cell cycle.

In some situations, it is clear that the functional specialization of cyclins is due to a direct interaction between the cyclin and a certain subset of Cdk substrates. The S cyclins in particular (Clb5 in budding yeast) interact with numerous substrates involved in early cell cycle events. Clb5-Cdk1 complexes, but not mitotic Clb2-Cdk1 complexes, bind and rapidly phosphorylate various proteins involved in DNA replication. (Kõivomägi et al., 2011b; Loog and Morgan, 2005; Schulman et al., 1998).

The substrate specificity of S cyclins depends on a region called the hydrophobic patch, which lies on the surface of the cyclin protein (Loog and Morgan, 2005; Schulman et al., 1998). The hydrophobic patch binds with moderate affinity to substrates that contain a linear docking motif known as the RXL motif, which is a conserved mechanism from yeast to humans. The interaction increases kinase-substrate affinity and thereby enhances the rate of substrate phosphorylation. Mutation of the hydrophobic patch on the cyclin, or the RXL motif on the substrate, leads to reduction in the interaction between the kinase and substrate, decreasing the rate of phosphorylation (Archambault et al., 2005; Loog and Morgan, 2005). In addition to binding substrates, the hydrophobic patch also interacts with proteins that inhibit Cdk activity (Gawlin´ski et al., 2007).

In order to understand how different cyclins promote distinct cell cycle events, the specificity of two budding yeast cyclins, the S phase cyclin Clb5 and the M phase cyclin Clb2 were compared in the phosphorylation of 150 Cdk1 substrates. It was discovered, that about 24% of these proteins were phosphorylated more efficiently by Clb5-Cdk1 than Clb2-Cdk1. The Clb5-specific targets include several proteins (Sld2, Cdc6, Orc6, Mcm3 and Cdh1) involved in early S phase events and Clb5 specificity depends on an interaction between the hydrophobic patch in Clb5 and an RXL motif in the substrate. By replacing Clb5 with Clb2 or by mutating the substrate RXL motif, phosphorylation of Clb5-specific targets during S phase was reduced, hence, confirming the importance of cyclin specificity *in vivo*. Therefore, Clb5 and Clb2 use different mechanisms to enhance the phosphorylation of S phase and M phase substrates (Loog and Morgan, 2005).

1.3.4 Clb5-specific NPF motif and its influence on phosphorylation

In earlier studies, the C-terminal inhibitory domain of Sic1 has been researched for possible binding motifs that would positively regulate phosphorylation. A short linear motif that stimulates phosphorylation specifically by Clb5-Cdk1 was discovered. The motif was found from the Sic1 sequence ²¹⁶KNPFAS²²¹, where the NPF sequence is the most critical in the interaction (NPF motif) (Schilf, 2018). Kinase assays have proven that addition of NPF motif can enhance substrate phosphorylation rate by Clb5-Cdk1 by over 25-fold. Experiments with mutated hydrophobic patch on cyclin Clb5 using Clb5(m1) indicated that it did not affect degradation efficiency of mutants containing NPF motif. Therefore, NPF motif was found to be the first docking motif of B-type (S and M phase) cyclins that does not bind to cyclin's hydrophobic patch. Clb5(m1) contains mutations in four positions close to the hydrophobic patch, making Clb5(m1) unable to bind to RXL motifs and also the inhibitory domain of Sic1 (Schilf, 2018). This makes Clb5(m1) potentially useful in cell cycle independent signalling, as without Sic1 inhibition it can be active throughout the cell cycle and the lack of RXL specificity decreases the phosphorylation of replication substrates by Clb5(m1)-Cdk1.

1.4 Protein degradation in cell cycle control

Transitions from one cell cycle phase to the next have to be unidirectional and irreversible, which is achieved through irreversible Cdk activation or inactivation. Irreversibility is achieved by the proteolytic destruction of regulatory proteins. Cyclins, Cdk inhibitor proteins and other cell cycle regulators are targeted for degradation by the attachment of multiple copies of a small protein, ubiquitin, resulting in a process called ubiquitination. Ubiquitinated proteins are recognized and destroyed by giant protease complexes called proteasomes. The process is carried out in a series of reactions - ubiquitin activation, ubiquitin conjugation and ubiquitin-protein ligation - by a corresponding series of enzymes known as E1, E2 and E3 enzymes (Zheng and Shabek, 2017). Substrate recognition depends on degrons, that are the binding sites for E3 enzymes.

Two large, multisubunit ubiquitin-protein ligases are crucial for the G1/S and metaphase/anaphase transitions. For the ubiquitination and proteolysis of targets such as Cdk inhibitors at the G1/S transition, the key ubiquitin-protein ligase is an enzyme called SCF, which is a multisubunit complex consisting of a core (Skp1, Cul1 and Rbx1), together with

an F-box protein that binds to substrate molecules. There are various F-box proteins, and the majority of these recognize phosphorylated degron motifs (Skaar et al., 2013).

The metaphase/anaphase transition is promoted by an even larger ubiquitin-protein ligase known as the anaphase-promoting complex (APC). SCF and APC are distantly related members of the same enzyme family, however, they use quite different mechanisms of substrate recognition and regulation of enzyme activity (Holt, 2012; Morgan, 2007).

1.5 Phospho-regulatable modules

1.5.1 The phosphodegron module

Two modules that link phosphorylation to protein stability have been discovered. The first is the phosphodegron, a short linear motif that is activated by the addition of one or more phosphate groups and upon phosphorylation generates a binding surface that interacts with a ubiquitin ligase. In this module, phosphorylation drives instability: the protein is stable in the absence of phosphorylation but is degraded upon phosphorylation (**Figure 5A**). SCF is the main ubiquitin ligase that recognizes phosphodegrons in the cell cycle (Holt, 2012).

One of well-studied phosphodegrons is that of Sic1. Sic1 binds to the cyclin-Cdk1 complex stoichiometrically keeping it tightly inhibited, thereby preventing progression to S phase of the cell cycle (Schwob et al., 1994; Venta et al., 2012). However, the G1 cyclin-Cdk1 complex does not bind to Sic1 and hence is not inhibited. The levels of G1-phase Cln1/2-Cdk1 accumulate as the cell grows and starts to phosphorylate the Cdk1 consensus motifs present at the N-terminus of Sic1 (Schwob et al., 1994).

The phosphorylated Cdk1 consensus motifs in Sic1 are specifically recognized by the F-box protein Cdc4, which recruits Sic1 for ubiquitination by the Cdc34 - SCF complex (Chen et al., 2002). SCF conjugates ubiquitin chains onto Sic1, thus targeting Sic1 for destruction at the proteasome (Holt, 2012; Verma et al., 1997). Cdc4 binds with high affinity to a consensus phosphopeptide motif (the Cdc4 di-phosphodegron), yet Sic1 itself has two sub-optimal degron motifs that act in concert to mediate Cdc4 binding (Kõivomägi et al., 2011a; Nash et al., 2001). The weak degron sites in Sic1 establish a phosphorylation threshold that delays degradation *in vivo*, and thereby establishes a minimal G1 phase period needed to ensure proper DNA replication (Nash et al., 2001). As each molecule of Sic1 is destroyed, an active Clb-Cdk1 complex is released, that is capable of further phosphorylation of Sic1. Phosphorylation of Sic1 by cyclin-Cdk1 leads to further Sic1 destruction and therefore the

release of more cyclin-Cdk1 in a positive feedback loop, ensuring commitment to S phase (Kõivomägi et al., 2011a; Venta et al., 2012).

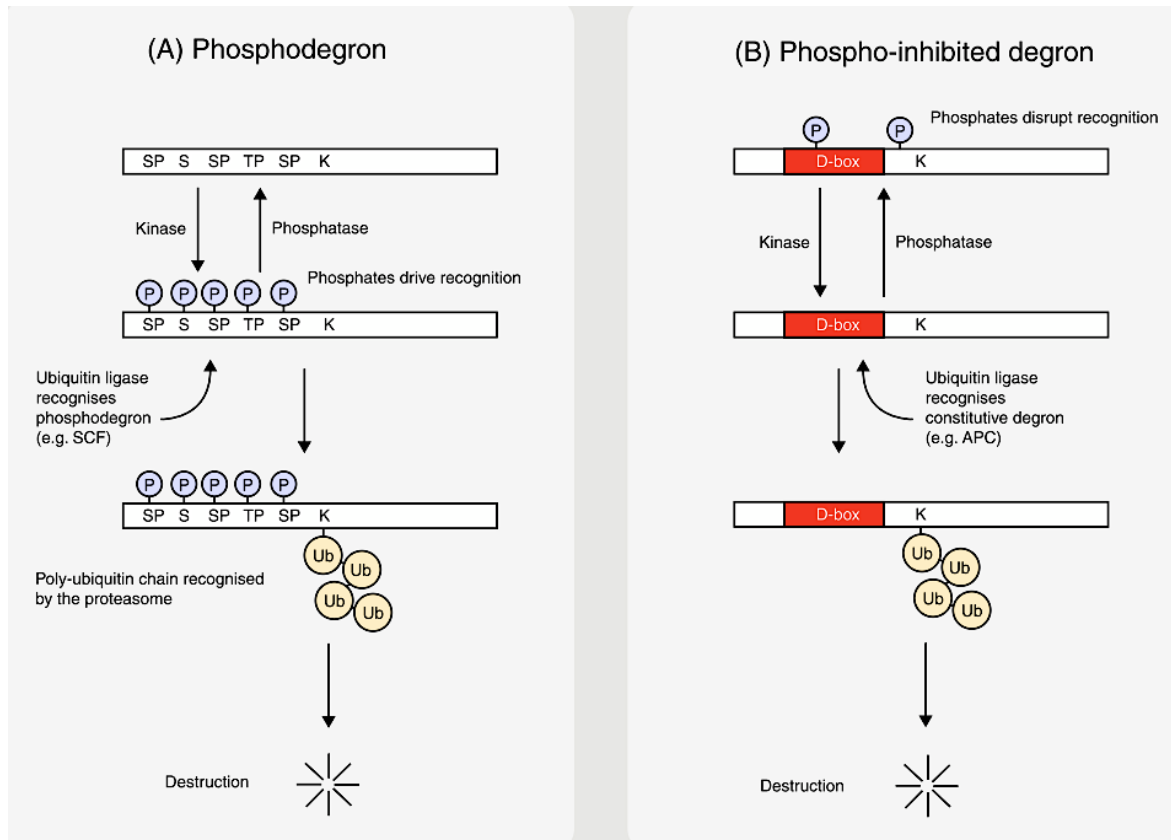


Figure 5. Regulatory modules coupling phosphorylation to protein stability. (A) The phosphodegron: a kinase phosphorylates residues to generate a phosphopeptide that binds to a ubiquitin ligase. The protein is degraded upon phosphorylation. (B) A degradation signal (degron) is recognized by a ubiquitin ligase. Phosphorylation of residues within and around the degron disrupts the interaction between degron and ubiquitin ligase. Thus, the protein is stabilized upon phosphorylation (Holt, 2012).

Overexpression of stabilized forms of Sic1 that lack Cdk1 phosphorylation sites cause an arrest in the G1 phase, whereas deletion of *SIC1* causes premature DNA replication and genome instability (Lengronne and Schwob, 2002). Cdc4 recruits several other substrates to the SCF complex in a phosphorylation-dependent manner, including the Cln – Cdk1 inhibitor protein Far1, the replication protein Cdc6 and the transcription factor Gcn4 (Blondel et al., 2000). In the mammalian cell cycle, SCF complexes target phosphorylated forms of cyclin E1 and the CDK inhibitor p27Kip1. The important role of SCF pathways is shown by the G1 phase arrest caused by non-phosphorylatable forms of p27Kip1, and by the genome instability caused by expression of stabilized forms of cyclin E1 (Chen et al., 2002).

1.5.2 The phospho-inhibited degron module

The second module is the combination of a constitutively active destruction motif with phosphorylation motifs such that phosphorylation masks the destruction signal. This module, known as the phospho-inhibited degron, has the opposite logic: phosphorylation stabilizes an otherwise unstable molecule (**Figure 5B**) (Holt, 2012). The second important cell cycle ubiquitin ligase is the APC, which recognizes a vast set of short linear interaction motifs (Davey and Morgan, 2016).

The best described degradation motif, which is recognized by the APC enzyme is known as the Destruction box (D-box) with its complete consensus sequence being R-x-x-L-x-x-x-[N/D/E] (Davey and Morgan, 2016). The D-box motif is necessary for the degradation of S and M phase cyclins in anaphase. As the APC is a highly regulated module, the stability of APC substrates is found to be a reflection of the activity state of the particular ubiquitin ligase.

1.5.3 Phospho-regulation in synthetic biology

The advances in genome sequencing has led to annotation of many biosynthesis pathways, making it possible to introduce the pathways to microbial cell factories for production of the natural products. To achieve the highest yield, the expression of the proteins in the pathway and also in the competing pathways have to be optimized (Pearsall et al., 2015). Recently, an example of production of a complex mixtures of flavor determinants was reported (Denby et al., 2018). Such approaches require not just the highest expression of pathway proteins, but precisely controlled levels of the proteins, as the relative concentrations of the products have to be in a specific range. Until now, main synthetic biology methods controlling heterologous pathways have been based on using transcriptional regulation (Engstrom and Pflieger, 2017). In cellular processes, however, in addition to transcriptional control, post-translational regulation is widely used to modulate protein levels, activity and localization. Despite the understanding of how phosphorylation functions, not enough research has been conducted regarding creation of synthetic phospho-regulation modules with predictable and programmable behavior. One of the studies, however, has reported the development of a modular approach for flexibly engineering phosphorylation circuits using designed phospho-regulon motifs. Potentially, these phospho-regulon tools open up the possibility to engineer cells with customized dynamical control. For example, in this particular study the creation of different phospho-modules for MAPKs (mitogen-activated protein kinase) was demonstrated in order to show how that phosphorylation is able to affect a wide range of

cellular parameters, such as intracellular localizations, interactions as well as protein degradation (Gordley et al., 2016).

A simple way of using protein degradation to improve production of a biosynthetic pathway is to lower the expression of enzymes from a competing pathway that is using the same precursor molecules. Targeting protein degradation can be made by tagging the proteins with specific degron modules. For example, a squalene synthase protein degradation method for improved production of chemicals with utility as fragrances, flavors, pharmaceuticals, and potential biofuels has been reported (Peng et al., 2017). Overall, the approach of protein degradation might be more widely applied to improve metabolic engineering outcomes (Peng et al., 2017). This creates a need for different degradation modules that enable controlling protein levels on a wide scale.

2 THE AIMS OF THE THESIS

To regulate the biosynthetic pathways in synthetic biology applications, mainly transcriptional regulation has been applied so far. In cellular processes, however, the stability, localization, activity and interactions of proteins is often controlled by protein phosphorylation. Therefore, we aim to test the possibilities of using Cdk1 activity for signalling protein degradation. For the use of phospho-regulation, there is a need for well-defined phospho-modules and the ability to predictably program the phosphorylation of these modules. Cdk1 is a potentially suitable kinase for such applications, because it has a complex, but well-studied substrate targeting mechanisms that enable differential phosphorylation of hundreds of targets.

The aims of the thesis are:

- First, to create a minimal phosphodegron module based on Sic1, that would enable regulated degradation.
- Secondly, the research aimed to control degradation efficiency of the module by manipulating the Cks1-dependent phosphorylation of the degrons.
- The third goal of the thesis included controlling degradation of phosphodegron module by induced expression of Clb5.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Constructs

For conducting the experimental part, the backbone plasmid pRS306 and pRS405 were used in order to serve as a vector that expresses genes of interest. These plasmids include ampicillin resistance gene, which is used for selection in bacteria. Another marker gene present in pRS306 is URA3 and in pRS405 LEU2 which are expressed in yeast and enable selection of cells carrying the plasmid. The Sic1-based substrate constructs were cloned into pRS306 vector containing *CYC1* promoter and EGFP for C-terminal tagging. For Clb5 expression pRS405 plasmid was used, containing the LexA promoter.

3.1.2 PCR, restriction and ligation

Cloning was performed using PCR, restriction enzyme and ligation based approach. For PCR, Phusion High Fidelity DNA Polymerase (Thermo Fisher Scientific) was used according to the manufacturers' instructions. To ligate inserts with vectors, both of them need to be restricted with the same enzymes. For restriction, 1.5 µg of plasmid DNA or about 100 ng of PCR product was mixed with 0.5 µl of each enzyme (FastDigest™ restrictases, Thermo Fisher Scientific), 2 µl of 10x FastDigest Green buffer and water was added to make up to 20 µl. For vector digestion, 1 µl of FastAP alkaline phosphatase (Thermo Fisher Scientific) was also added in order to prevent vector from recircularization without the insert during ligation. The samples were placed into 37 °C incubator for 15-30 minutes.

PCR products as well as restricted plasmids were loaded on gel. For visualizing the products on gel, 3-5 µl of Thermo Scientific *6X Orange Loading Dye* was mixed into samples before loading, while for reading the resulting lengths of the products ZipRuler Express DNA Ladder 1 (Thermo Fisher Scientific) was used.

For purifying the products from gel FavorPrep™ *GEL/PCR Purification Kit* was used and the steps were proceeded according to the protocol.

For ligation, insert and vector were mixed in 1:3 molar ratio. The ligation was performed in 10 µl, where 1 - 3 µl of linearized vector (20-50 ng), 3 - 5 µl of insert, 1 µl of 10x ligation buffer, 1 - 4.5 µl of water and 0.5 µl of T4 DNA ligase (Thermo Fisher Scientific) were mixed. The ligation reaction was carried out at 18 °C for 1-16 hours.

3.1.3 Bacterial transformation

Transformation is the process by which foreign DNA (ligation mixture) is introduced into cells (*Escherichia coli* strain DH5 α). Transformation of plasmids into bacteria is desired because bacteria are used as means for both storing and replicating plasmids. Because of this, almost all plasmids carry a bacterial origin of replication as well as an antibiotic resistance gene for use as a selectable marker in bacteria.

The procedure started with taking competent cells out of -80 °C refrigerator, letting them thaw on ice (approximately 10-20 minutes). Next, 2 μ l of ligation mixture was gently mixed into 50 μ l of competent cells in a microcentrifuge tube. After keeping the competent cells and DNA mixture on ice for 30 minutes, heat shock at a 42 °C thermostat for 1 minute was performed. This step is crucial for introducing foreign DNA into bacterial cells. Next, the tubes were kept on ice for 2 minutes. Subsequently, 500 μ l of LB media (10 g/l tryptone (BD Biosciences), 5 g/l yeast extract (Formedium), 10 g/l NaCl) was added to the bacteria and the cells incubated at 37 °C shaking incubator for 40 minutes. This step allows the bacteria to generate the antibiotic resistance protein, which enables growth once plated on the antibiotic containing agar plate. Also, LB agar plates with 100 μ g/mL ampicillin (Sigma) were taken out of 4 °C storage in order to warm up before plating the cells onto it. Then the mixture was centrifuged at 6000 rpm for 1 minute and plated onto an LB agar plate containing ampicillin. Finally, the plates were left at 37 °C overnight.

3.1.4 Extracting and sequencing of the plasmid DNA

For extracting the ligated plasmid from bacteria FavorPrep™ Plasmid Extraction Mini Kit (Favorgen) was used. The procedures were carried out according to the manual.

For determination of the sequence of nucleotides, Sanger sequencing was implemented. The sequencing was carried out by the core facility in the Estonian Biocentre.

3.1.5 Yeast transformation

Before transformation the pRS306-based plasmids needed to be linearized with Eco147i and pRS405-based plasmids with BshTI. This step enables the plasmid to integrate into either URA3 (pRS306) or LEU2 (pRS405) locus of *S. cerevisiae*. 3-5 μ l of purified plasmid (1-2 μ g), 1 μ l of 10x FastDigest buffer, 0.5 μ l of FastDigest Eco147i or BshTI (Thermo Fisher Scientific) and water up to 10 were mixed in the microcentrifuge tubes and placed into 37 °C incubator for 15-30 minutes.

Yeast cells, which have grown for 5 - 8 hours in YPD media (10 g/l yeast extract (Formedium), 20 g/l peptone (Formedium), 20 g/l glucose (Oriola)) in a 37 °C shaking incubator, were centrifuged for 1 minute at 3100 rpm. The supernatant was then removed, and the cell pellet was resuspended in 1 ml of sterile 100 mM lithium acetate (LiAc) in 0.5xTE (5 mM Tris-HCl (pH 8), 0.5 mM EDTA). Next, the mixture was centrifuged at 3600 rpm for 1 minute. After removing the supernatant two times of cell volume of 100 mM LiAc in 0.5xTE was added and the mix was incubated at room-temperature for 10 minutes. Simultaneously, the Salmon Sperm DNA (SS-DNA) was being prepared by boiling it for 10 minutes at 100 °C and chilling immediately on ice before use. This is crucial to ensure that the DNA will be single stranded. After preparation of beforementioned substances, linearized plasmid DNA was mixed with 10 µl of SS-DNA and 100 µl of yeast competent cells. Next, 700 µl of sterile freshly made PEG/LiAc (40% PEG 3350, 100 mM LiAc in 1xTE) solution was added to the mixture. Afterwards, 48 µl of DMSO was added and mixed well by pipetting up and down. The samples were then incubated at 42 °C for 40 minutes and chilled on ice for 2 minutes. Before plating, the samples were centrifuged for 1 minute at 6000 rpm, the supernatant was removed, and cells were resuspended in 1 ml of sterile 1xTE buffer. The cells were centrifuged again for 1 minute at 3600 rpm, the supernatant was removed, and cells were resuspended in 200 µl of sterile 1xTE buffer. Finally, the total volume of samples was plated on the selection plates. In case of transformation of substrate constructs in pRS306 vector, the cells were plated on SC-URA (synthetic complete lacking uracil) plates (7 g/l yeast nitrogen base (BD Biosciences), 1.9 g/l -URA powder (MP Biomedicals), 20 g/l glucose (Oriola)). When pRS405-based Clb5 expression plasmids were transformed to the cells, the cells were plated on SC-LEU (synthetic complete lacking leucine) plates (7 g/l yeast nitrogen base (BD Biosciences), 1.9 g/l -LEU powder (MP Biomedicals), 20 g/l glucose (Oriola)). The plates were incubated upside down (for 2-4 days) at 30 °C until colonies appeared.

Table 1. Yeast strains used in this study. All strains are based on haploid MATa strain from w303 background. All strains were *bar1* deletion, had FRP880 plasmid integrated to *his3* locus and NLS-NES-mCherry construct with *TRP1* marker gene integrated to *ADHI* promoter locus. FRP880 is a plasmid expressing the LexA-ER-AD transcription factor necessary for estradiol-induced expression of Clb5 (Ottoz et al., 2014).

Strain	Description	Background strain	Reference
MÖ467	<i>MATa bar1::hisG his3::FRP880::HIS3 Padh::Padh-NLSmodule(wt)-mCherry::TRP1</i>	w303	Mihkel Örd
AA1	<i>ura3::PCYC1-Sic1(dC)-EGFP-URA3</i>	MÖ467	This study
AA2	<i>ura3::PCYC1-Sic1(short)-EGFP-URA3</i>	MÖ467	This study
AA3	<i>ura3::PCYC1-Sic1(min)-EGFP-URA3</i>	MÖ467	This study
MÖ487	<i>ura3::PCYC1-Sic1(min-NPF)-EGFP-URA3</i>	MÖ467	This study
MÖ488	<i>ura3::PCYC1-Sic1(min-T33S-NPF)-EGFP-URA3</i>	MÖ467	This study
MÖ489	<i>ura3::PCYC1-Sic1(min-T5S-T33S-NPF)-EGFP-URA3</i>	MÖ467	This study
MÖ490	<i>ura3::PCYC1-Sic1(min-T33S)-EGFP-URA3</i>	MÖ467	This study
MÖ491	<i>ura3::PCYC1-Sic1(min-T5S-T33S)-EGFP-URA3</i>	MÖ467	This study
MÖ492	<i>ura3::PCYC1-Sic1(min)-EGFP-URA3</i>	MÖ467	This study
MÖ484	<i>leu2::PLEXA-NLS-3HA-Clb5(m1)-LEU2</i>	MÖ488	This study
MÖ486	<i>leu2::PLEXA-NLS-3HA-Clb5(wt)-LEU2</i>	MÖ488	This study
AA4	<i>leu2::PLEXA-NLS-3HA-Clb5(m1)-LEU2</i>	MÖ489	This study
AA5	<i>leu2::PLEXA-NLS-3HA-Clb5(wt)-LEU2</i>	MÖ489	This study
AA6	<i>leu2::PLEXA-NLS-3HA-Clb5(m1)-LEU2</i>	MÖ490	This study
AA7	<i>leu2::PLEXA-NLS-3HA-Clb5(wt)-LEU2</i>	MÖ490	This study

3.1.6 Time-lapse microscopy

In order to measure the fluorescence of proteins and visualize the cell division process, time-lapse microscopy experiments were performed. Before setting up the experiment the strains were grown from 4 - 10 h in the 30°C shaking incubator in complete supplement mixture (CSM, 7 g/l yeast nitrogen base (BD Biosciences), 0.79 g/l CSM powder (Formedium), 20 g/l glucose (Oriola)). Cells were grown to OD 0.4 to 0.8. If the density was lower, samples were centrifuged at 3600 rpm for 1 min, the supernatant was removed and the cell pellet resuspended to obtain the desired density. Then, 0.3 µl of each sample was loaded onto a 24 x 50 x 0.08 mm micro cover glass and covered with the CSM agarose (CSM with 1.5% NuSieve GTG agarose (Lonza)), which had previously been heated up in a microwave and solidified for 10 minutes at room temperature. For Clb5 induction, CSM agarose with estradiol was prepared. Normally 3 - 8 samples were included in the experiment. After the cells were in place the leftover of agarose gel was placed on the edges of the glass to prevent samples from drying. The Zeiss Axio Observer Z1 microscope with an automated stand and ZEN software was used for time-lapse imaging. The temperature was kept at 30 °C during the experiment using Tempcontrol 37-2 (Pecon). Up to 16 positions were followed in one experiment. 2 - 3 positions of each sample were chosen to ensure sufficient amount of data. Three images were made from each position during each time point: phase-contrast (25 ms), GFP (15 ms) and mCherry (400 ms). Fluorescent imaging was done using Colibri LED module at 25% intensity. Experiment was generally set for 8 hours with the image capturing interval of 3 minutes resulting in 161 time points. The data was then exported, cells were segmented and the fluorescence intensities quantified using appropriate ready-made MATLAB programs (Doncic et al., 2013).

3.2 RESULTS

3.2.1 Time-lapse fluorescence microscopy for measuring phosphorylation-dependent degradation

The conducted experiments can be divided into multiple stages: the first stage included cloning and transforming of plasmids to yeast, followed by time-lapse fluorescence microscopy experiments and quantification of the gathered experimental data. To evaluate the phosphorylation of di-phosphodegrons *in vivo*, the Sic1-based constructs were fused to GFP and were expressed from a constitutively active *CYC1* promoter (**Figure 6A, B**). To analyse the phosphorylation-dependent degradation of the phospho-modules during the cell cycle, an NLS-NES-mCherry construct was used as a reference point for cell cycle progression (Liku et al., 2005) (**Figure 6C**). The time-lapse microscopy provided a visual understanding of how the GFP (Sic1-based constructs tag) and mCherry (NLS-NES construct tag) signals appear throughout the cell cycle (**Figure 6D**). The data in microscopy experiments is gathered from single cells without synchronizing the cell cycle. The mCherry signal plays an important role as it enables to define the moment of G1/S transition in the cell cycle: when 50 % of NLS-NES-mCherry sensor is exported from nucleus to cytoplasm.

3.2.2 Constructing a minimal Cdk1-dependent phospho-degradation module based on Sic1

Several versions of Sic1 mutants were made with the aim to decrease the size of the protein without substantially affecting its phosphorylation or degradation. The full-length Sic1 contains a C-terminal domain (215-284) (**Figure 6A**), mediating inhibition of Clb-Cdk1 complexes (Hodge and Mendenhall, 1999). In all constructs studied here, the inhibitory domain has been deleted (**Figure 6B**) and the RXL motifs were mutated in order to make it unable to bind to the hydrophobic patch of Clb cyclins. A shorter version of Sic1(dC) called Sic1(short) (**Figure 7A**) has previously been made resulting in the length of 180 amino acids (Schilf, 2018). In Sic1(short) mutant the distances between the phosphorylation sites of T5-T33 and T45-S69 have been decreased to 12 amino acids. In previous studies it has been observed that a distance of 12 amino acids between the priming and the secondary phosphorylation site is the most optimal (Kõivomägi et al., 2013).

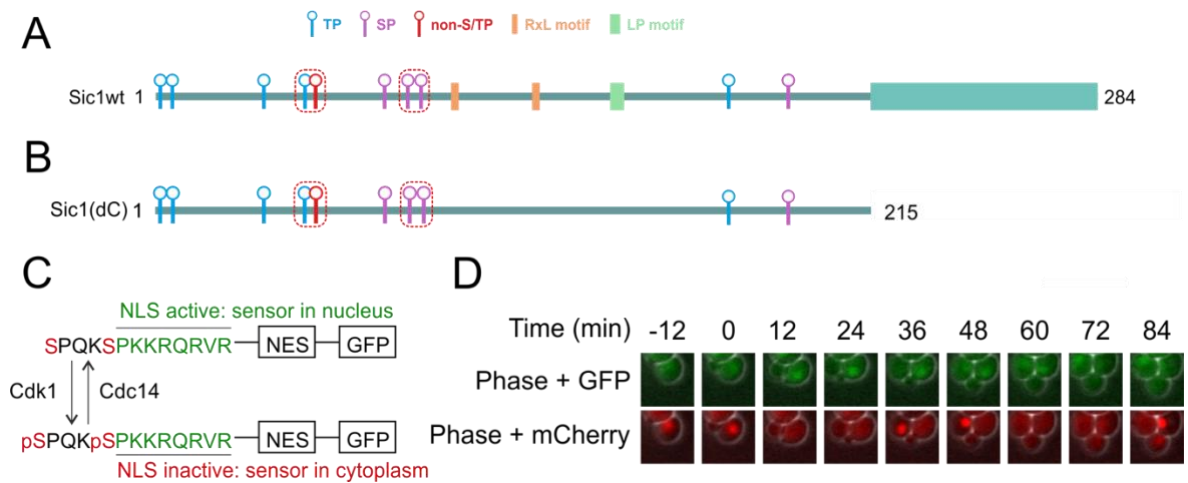


Figure 6. Time-lapse microscopy experiment enables to measure GFP-tagged substrate levels in undisturbed cell cycles of single cells. (A) The scheme of Sic1(wt) illustrating distances between the phosphorylation sites, inhibitory domain and the total length of protein (284 amino acids). (B) The scheme of Sic1(dC) indicating mutated RXL and LP motifs, the deleted inhibitory domain, resulting in the length of 215 amino acids. The schemes in A and B show the positioning of phosphorylation sites and docking motifs. The two pairs of sites surrounded by the red dotted oval are the di-phosphodegrons (C) Shown is the simplified scheme explaining phospho-regulatable NLS-NES Cdk1 activity sensor, which is based on the mechanism that Cdk1-dependent phosphorylation of the SP sites adjacent to the nuclear localization signal (NLS) leads to inactivation of the NLS and nuclear export of the sensor. The SP-sites are dephosphorylated in late anaphase by phosphatase Cdc14, leading to the sensor returning to the nucleus. Figure adapted from Örd et al., 2019. (D) Images of an exemplary cell from the time-lapse microscopy experiments expressing Sic1(dC)-GFP and NLS-NES-mCherry. The images are merged from phase-contrast and fluorescent channels. Time 0 stands for G1/S transition, which is determined by nuclear export of 50% of NLS-NES-mCherry Cdk1 activity sensor. The time-series presented covers one cell cycle, as the NLS-NES-mCherry is nuclear in time points -12 and 0, after which it is exported (G1/S) and the sensor returns to the nucleus at 84 minutes, in late anaphase.

Sic1(min) mutant is a version of Sic1(short) mutant, where the C-terminal domain from the 90th amino acid position has been deleted (**Figure 7A**). Also, to serve as a linker region between the phosphorylation sites and the NPF docking motif, sequence from Sic1 positions 200-215 was added to Sic1(min). This results in a construct with the length of 80 amino acids (**Figure 7A**).

Observations showed that Sic1(dC) level started decreasing immediately after the G1/S transition, whereas the level of Sic1(short) dropped faster than that of Sic1(dC) (**Figure 7B**). This might be caused by an increase of Cks1-dependent phosphorylation in case of Sic1(short), because of the optimal distances between phosphorylation sites. The

phosphorylation efficiency of Sic1(min), on the other hand, decreases, however, it is still rather similar to the phosphorylation and degradation efficiency of Sic1(dC). This suggests that Sic1 positions 90-215 might have a slight positive effect on degradation. Although, as the result in degradation level of Sic1(min) is similar to that of Sic1(dC) for further research Sic1(min) mutant has been chosen due to its relatively high phosphorylation activity and short length, making it flexible to manipulate with.

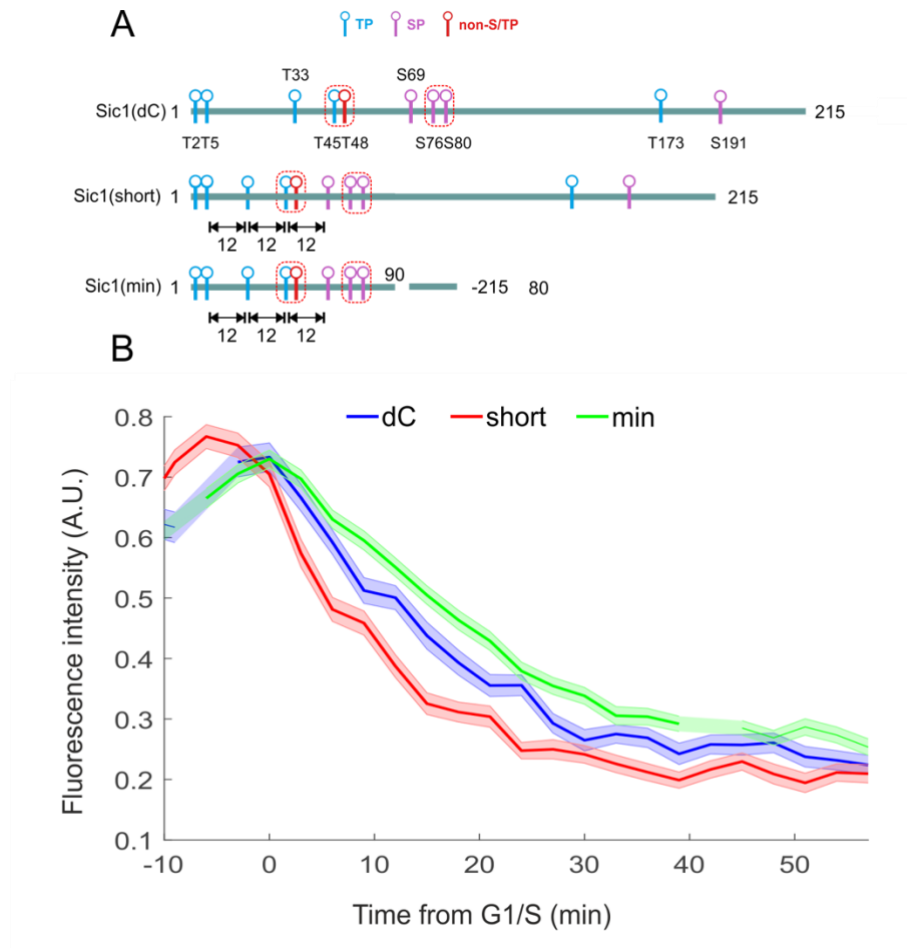


Figure 7. An 80 amino acid fragment of Sic1 is degraded with similar dynamics as the non-inhibitory Sic1 in the cell cycle. (A) The scheme showing Sic1(dC) mutant, Sic1(short) mutant with decreased distances between the phosphorylation sites and Sic1(min) with deleted C-terminal region resulting in the length of 80 amino acids. (B) Graph showing the fluorescence levels of Sic1(dC), Sic1(short) and Sic1(min) fused to GFP in the cell cycle. The time is from G1/S, determined by the nuclear export of NLS-NES-mCherry. The plot shows mean values with \pm SEM error bars.

3.2.3 Mutation of Cks1 priming sites leads to stabilization of Sic1

One of the goals was to find a substrate, which is not fully phosphorylated and degraded during the cell cycle. The phosphorylation of Sic1 di-phosphodegrons has been shown to be dependent on Cks1 (Kõivomägi et al., 2011a), which binds to only phosphorylated threonine sites (Kõivomägi et al., 2013; McGrath et al., 2013). Therefore, the disruption of Cks1-specific docking was desired to decrease the efficiency of degron phosphorylation. To disrupt the Cks1 docking, threonines were replaced with serines resulting in six mutants: WT (+/-NPF), T33S (+/-NPF), T5S T33S (+/-NPF) (**Figure 8A**). By analysing the fluorescence levels of these constructs, it was detected that T33S and T5S T33S mutants in Sic1(min) became more stable, meaning that the di-phosphodegrons were not phosphorylated (**Figure 8B, 8C**). By substituting only one threonine site with serine (T33S) in Sic1(min) a vast decrease in degradation was observed, while with two serine mutations (T5S T33S) even greater decrease in degradation was detected. This confirms that Cks1 does not bind phospho-serines, resulting in severely suppressed phosphorylation efficiency. The degradation dynamics of constructs with or without the NPF docking motif were similar, however, with the T33S mutant more active degradation was seen with the module containing the NPF docking motif (**Figure 8B, C**). That indicates that the NPF motif increases the phosphorylation. With the wild-type phosphorylation sites no difference in degradation was detected, possibly due to strong Cks1-mediated potentiation of degron phosphorylation. In the case of T33S, however, Cks1 docking efficiency is decreased, creating a need for cyclin substrate docking. When all the Cks1 binding sites are mutated (T5S T33S), the module is stable even upon the addition of the NPF motif, indicating that the cyclin docking interaction is not sufficient to promote full phosphorylation of the degrons in context of the cell cycle Cdk1 activity.

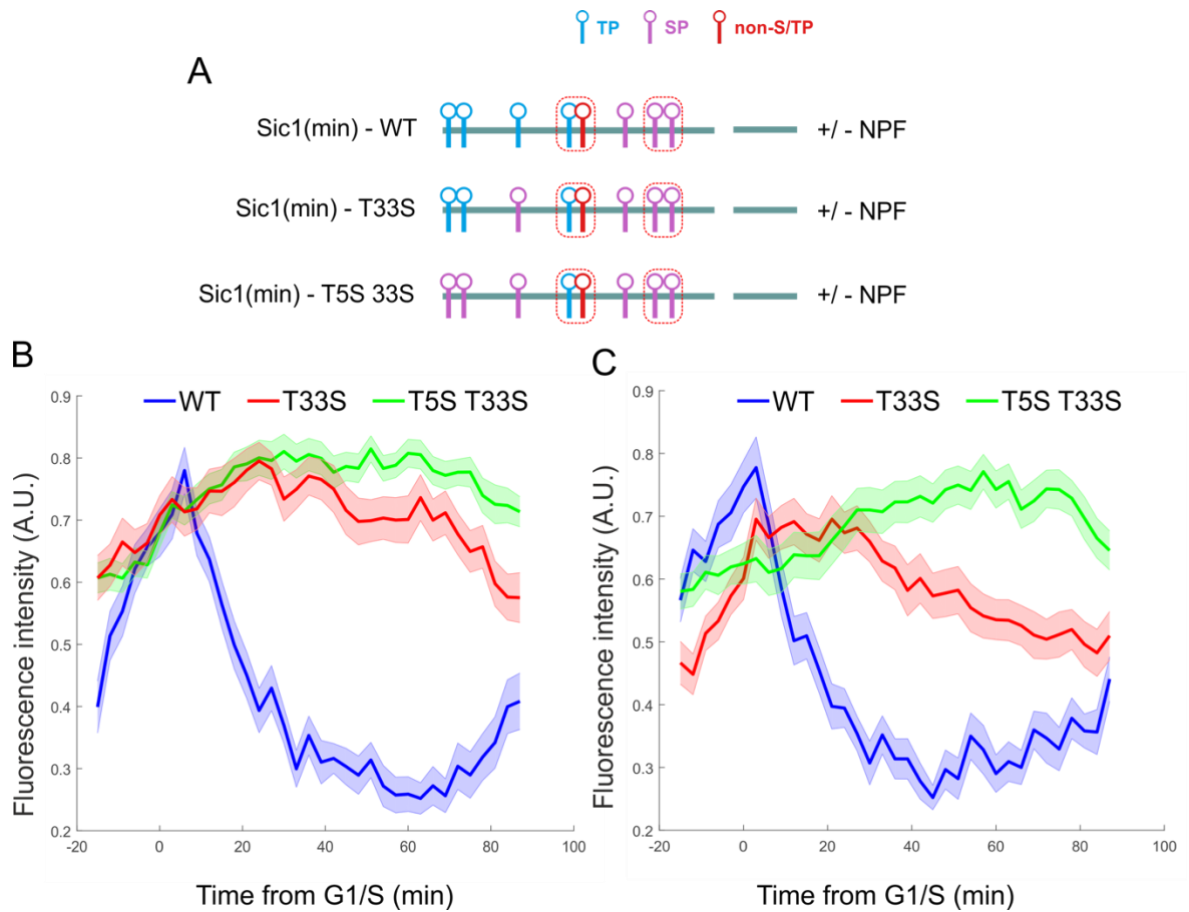


Figure 8. Threonine-to-serine mutations in Cks1 primer sites lead to stabilization of Sic1 constructs. (A) The scheme explaining Thr-Ser mutants Sic1(min)-wt, Sic1(min)-T33S and Sic1(min)-T5S T33S. (B) Graph showing the fluorescence levels of Sic1(min)-wt, Sic1(min)-T33S and Sic1(min)-T5S T33S in unperturbed cell cycle. (C) Plot showing the fluorescence levels of Sic1(min)-wt-NPF, Sic1(min)-T33S-NPF and Sic1(min)-T5S T33S-NPF in unperturbed cell cycle.

3.2.4 Induced overexpression of Clb5 enhances degradation of the Sic1(min) mutants

The next aim included controlling degradation of the phospho-degron modules. For this, Clb5 expression was induced at different levels in order to see its influence on degradation of the Sic1(min) constructs. Additional copy of *CLB5* was integrated to the genome under the control of a promoter that can be induced using a synthetic transcription factor LexA-ER-AD (Ottoz et al., 2014). The LexA-ER-AD transcription factor is activated in the presence of estradiol and the strength of expression depends on the concentration of estradiol.

The Clb5(m1) mutant was used, that has 4 mutations near the hydrophobic patch, making it unable to bind a complementary hydrophobic RXL motif. As Clb5(m1) lacks the RXL interaction used by S cyclin-Cdk complexes for substrate targeting, overexpression of this mutant could lead to lower phosphorylation of RXL-containing substrates compared to wild-type Clb5. This would reduce the off-target activity of the induced kinase. Clb5(m1) is, however, capable of phosphorylating Sic1 mutants that have the NPF motif. Hence, the Clb5-specific docking motif NPF does not bind to the cyclin hydrophobic patch (Schilf, 2018).

First, we tested the effects of differential overexpression of Clb5(m1) and Clb5(wt). For this, estradiol was added to the media at three concentrations: 40 nM, 300 nM, and 3000 nM. As a substrate construct, GFP-tagged Sic1(min)-T33S-NPF was expressed and the levels of the substrate were followed during an 8-hour period of Clb5 induction (**Figure 9**).

With the smallest prepared concentration of 40 nM estradiol, a slight decrease on the levels of Sic1(min)-T33S-NPF were seen, possibly indicating that the induced Clb5-Cdk1 leads to phosphorylation and degradation of a fraction of the substrate. With 300 nM estradiol concentration it was seen, that protein phosphorylation increased as its fluorescence intensity is lower in comparison with 40 nM estradiol, meaning that higher concentration of estradiol increased the speed of degradation, however, Sic1(min) was not fully degraded. As Clb5 was degraded after metaphase/anaphase transition (Lu et al., 2014), the residual Sic1(min)-T33S-NPF-GFP signal could partially be from G1-phase cells lacking Clb5 expression.

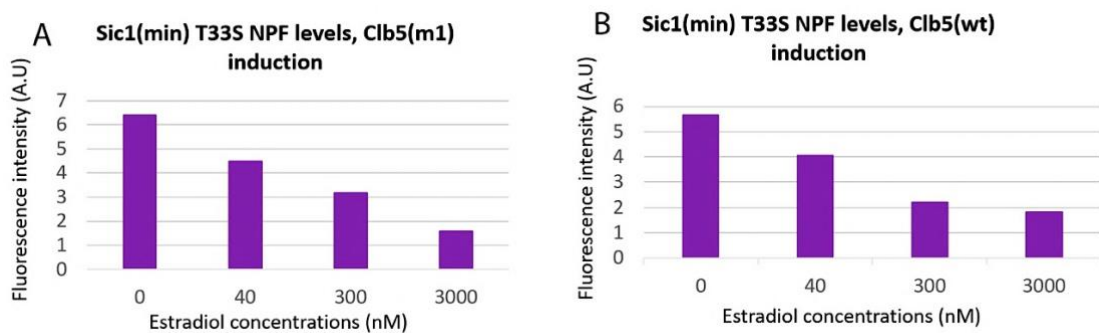


Figure 9. Differential induction of Clb5 leads to different levels of Sic1(min)-T33S-NPF. Graphs showing the fluorescence intensities of GFP-tagged Sic1(min)-T33S-NPF in cells with induced overexpression of (A) Clb5(m1) and (B) Clb5(wt) at four estradiol concentrations: 1) 0 nM, 2) 40 nM, 3) 300 nM, 4) 3000 nM. The GFP levels were followed in an 8-hour time-lapse experiment with 3-minute time points. The graphs show intensity values at 25th percentile of the time points.

Although induction with 3000 nM estradiol indicated the highest rate of degraded protein and, thus, the strongest induced Clb5 signal, time-lapse microscopy revealed, that the highest estradiol concentration caused an increase in proportion of mitotic cells leading to cell cycle arrest. In order to decrease the effect caused by induced Clb5 on the cell cycle progression, 300 nM estradiol was chosen for further research.

3.2.5 Induced Clb5-mediated degradation of Sic1(min) constructs is dependent on the NPF docking motif

Next, we wanted to test whether it is possible to promote degradation of different Sic1(min) constructs by overexpression of either wild-type Clb5 or Clb5(m1). Based on the experiment presented in Figure 8, we selected three Sic1(min) mutants (**Table 2**). Sic1(min) with wild-type phosphorylation sites was not studied here, because it is degraded during the cell cycle and therefore cannot be used for cell cycle independent signalling. The T33S and T5S T33S mutants, however, were mostly stable throughout the cell cycle (**Figure 8B, 8C**). In case of T33S, constructs both with and without the NPF motif were included in the Clb5 induction experiment to test, whether the induction-dependent degradation is specifically mediated through NPF docking.

Table 2. Clb5-inducible Sic(min) mutants. The scheme visualizing elements and combinations of mutants. Clb(m1) mutant with mutated hydrophobic patch as well as its wild-type version Clb5(wt) were tested with three combinations of phospho-degron modules: T33S indicates one Thr-Ser substitution at position 33, T33S-NPF contains Thr-Ser substitution at position 33 and the NPF motif C-terminal of the phosphorylation sites, whereas T5S T33S-NPF includes two Thr-Ser substitutions as well as the NPF motif.

Clb5	Sic1(min)
m1	T33S
m1	T33S-NPF
m1	T5S T33S-NPF
wt	T33S
wt	T33S-NPF
wt	T5S T33S-NPF

First, the substrate Sic1(min)-T33S was analysed (**Figure 10**). Gathered results indicated that Clb5(m1) expression at 300 nM estradiol is capable of inducing some phosphorylation of the GFP-tagged substrate, as during the experiment the level of Sic1(min)-T33S dropped slightly (**Figure 10B**). However, the fluorescence intensities averaged over a population of cells are in the same range with and without Clb5(m1) induction, indicating that the majority of the substrate is unaffected.

Interestingly, the induction of Clb5(wt) revealed that the level of Sic1(min)-T33S protein decreased the most (**Figure 10C**). Hypothetically, this data suggests that by induction of Clb5(wt) the proportion of mitotic cells increases, resulting in cell cycle arrest, as the levels of Sic1(min)-T33S showed a slight decrease in late cell cycle phase (**Figure 8B, 8C**). It is possible that when Clb5(wt) is overexpressed, it restricts dephosphorylation of substrates necessary for finishing mitosis. Therefore, it is possible that accumulation of mitotic cells is caused by relatively high Cdk1 activity.

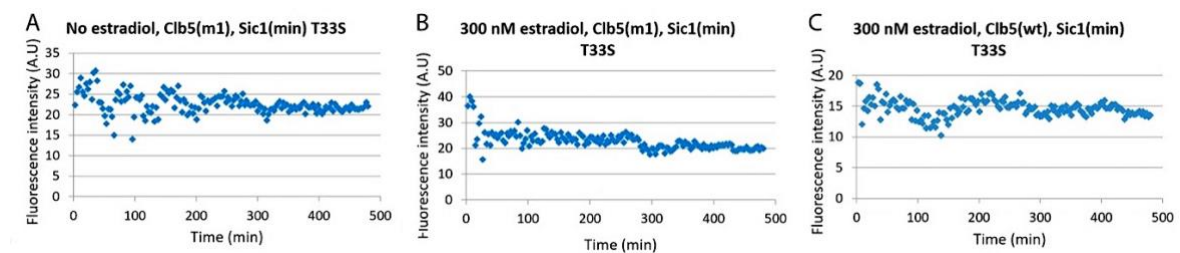


Figure 10. The levels of Sic1(min)-T33S during 8-hour period of Clb5 induction. Shown are average fluorescence levels of GFP-tagged Sic1(min)-T33S. Sic1(min)-T33S fluorescence levels were measured in three conditions: (A) without Clb5(m1) induction, (B) induced Clb5(m1) and (C) induced Clb5(wt). Clb5 was induced using 300 nM estradiol.

Next, the effect of T33S mutation in combination with the NPF motif was studied. The induction of Clb5 leads to the average Sic1(min)-T33S-NPF levels dropping considerably in the early stages of experiment, but the intensities level off at around 100-minute time point (**Figure 11**). As shown in Figure 11, the protein levels dropped compared to the corresponding mutants lacking the NPF docking motif. This shows that the NPF motif is necessary for targeting Clb5-Cdk1 to phosphorylate the substrate. Similarly, the biggest degradation difference appeared at the induction of Clb5(wt) in Sic1(min)-T33S-NPF (**Figure 11C**), where a rapid decrease in the protein level was detected, supporting the previous statement of increased proportion of mitotic cells. Another possible reason of not a remarkable rate of induced Clb5(m1) on Sic1(min)-T33S-NPF (**Figure 11B**) degradation is the delay of experimental start, as the degradation slope in the first 50 minutes is more abrupt

than that of further experiment. This proposes, that some of the protein could have been degraded during the setting up of the experiment, thus, before the actual start of fluorescence imaging.

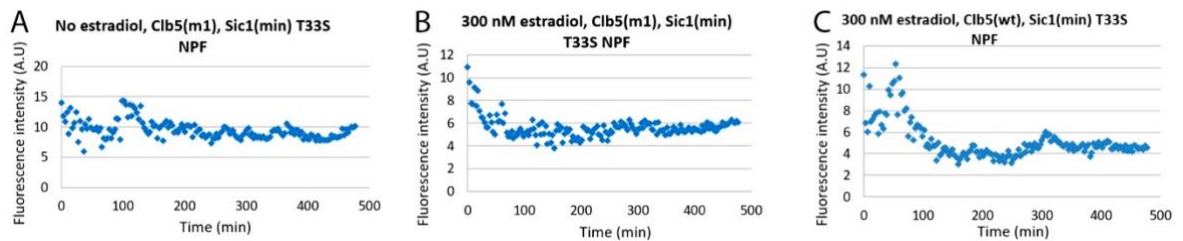


Figure 11. Induced expression of Clb5 leads to decrease in Sic1(min)-T33S-NPF levels. Shown are degradation dynamics of Sic1(min)-T33S-NPF under three conditions: (A) uninduced Clb5(m1), (B) induced Clb5(m1) and (C) induced Clb5(wt). Clb5 was induced using 300 nM estradiol. The GFP-tagged substrate levels were followed in a time-lapse microscopy experiment for 8 hours after induction of Clb5 by addition of estradiol.

Finally, we analysed the degradation of Sic1(min)-T5S-T33S-NPF. This substrate lacks all Cks1-mediated phosphorylation of the di-phosphodegrons and is stable throughout the cell cycle (**Figure 8**). An increase in the rate of phosphorylation by Clb5(m1) overexpression led to a smooth decrease in Sic1(min)-T5S-T33S-NPF protein levels (**Figure 12B**), resulting in lower levels at the end of the experiment compared to uninduced cells and induced Clb5(wt) (**Figure 12A, 12C**). Based on the average Sic1(min)-T5S-T33S-NPF-GFP fluorescence levels, about 50% of the substrate is degraded at 100 minutes after Clb5(m1) induction. This shows that a phospho-degradation module, that is stable in the unaffected cell cycle, can be partially degraded by induction of Clb5(m1).

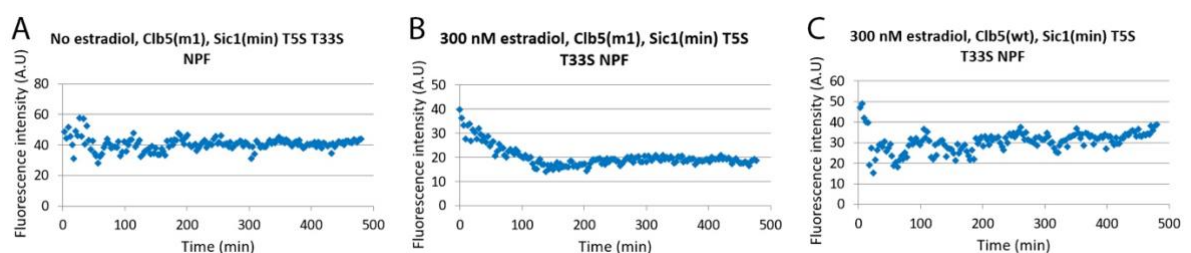


Figure 12. The induction of Clb5(m1) leads to degradation of the Sic1(min)-T5S-T33S-NPF construct. Shown are degradation profiles of Sic1(min)-T5S T33S-NPF under three conditions: (A) uninduced Clb5(m1), (B) induced Clb5(m1) and (C) induced Clb5(wt). Clb5 was induced using 300 nM estradiol. The GFP-tagged substrate levels were followed in a time-lapse microscopy experiment for 8 hours after induction of Clb5 by addition of estradiol.

3.3 DISCUSSION

In the experimental part of the thesis, Sic1(min) mutant was created, indicating that it is possible to construct a short module that contains two di-phosphodegrons, which can be used for tagging different proteins.

Mutants with Thr - Ser mutations were created in order to disrupt the phosphorylated TP-Cks1 docking, as one of the goals was to find a substrate, which is not phosphorylated by Cdk1 without additional cyclin expression, hence making it stable throughout the cell cycle. This effect is especially recognizable in the degradation of Sic1(min)-T5S T33S mutant, where in comparison with Sic1(wt) it is clearly seen (**Figure 8**), that the mutant was not fully phosphorylated, making it independent of the cell cycle phosphorylation networks.

We showed that by inducing Clb5 expression, it is possible to promote phosphorylation and degradation of the otherwise stable phospho-degron modules. For induction, either wild-type Clb5 or Clb5(m1) was used. In case of Sic1(min)-T33S and Sic1(min)-T33S-NPF, induction of wild-type Clb5 led to more degradation of the substrate compared to Clb5(m1) (**Figures 10, 11**). One possible explanation of this could be that overexpression of wild-type Clb5 leads to an accumulation of mitotic cells, where the Cdk1 activity is high. Importantly, in mitotic cells, most Cdk1 activity likely arises from mitotic cyclins and not the induced Clb5, because of the higher activity of mitotic cyclin-Cdk complexes (Loog and Morgan, 2005). Interestingly, in case of Sic1(min)-T5S-T33S-NPF, Clb5(m1) promoted stronger degradation of the substrate than wild-type Clb5 (**Figure 12**). Wild-type Clb5 has a functional hydrophobic patch that binds to RXL motifs in many Cdk1 substrates (Loog and Morgan, 2005; Schulman et al., 1998). These substrates bind to Clb5 with around 1 μ M affinity and therefore can lead to high substrate competition for the Clb5-Cdk1 complex (Loog and Morgan, 2005). Clb5(m1) however, lacks RXL binding specificity and is possibly less affected by substrate competition, which could enable more efficient phosphorylation of substrates with NPF docking motif.

Addition of the NPF motif (**Figures 11, 12**) resulted in the decrease of protein levels compared to the corresponding mutants lacking the NPF presence (**Figure 10**), proving its strong effect on phosphorylation efficiency. By applying two thr-ser substitutions well as adding the NPF motif the effect on phosphorylation appeared to be the most notable. As

expected, an increase in the rate of phosphorylation by 300 nM estradiol induced Clb5(m1)-Sic1(min)-T5S T33S-NPF overexpression lead to a smooth decrease in protein levels, resulting in lowest levels at the end of the experiment (**Figure 12B**). The important principle of cell cycle independent signalling by Cdk1 is that the phosphorylation efficiency of the substrate has to be decreased so that it would not be phosphorylated during a normal cell cycle. Then, the phosphorylation of an otherwise low quality substrate can be improved by addition of specific cyclin docking motifs, like the NPF motif for example. This motif would then enable specific targeting of the substrate by the induced cyclin-Cdk1 complex.

Thus, by specifying substrate recognition, varying phosphorylation efficiency or applying overexpression of different cyclins it is possible to reach different protein levels and to control discrete cell cycle events on a finer time scale.

SUMMARY

In this work it was shown that 80 amino acid domain from Sic1 could function as a phospho-degion module. It was found that the distance of 12 amino acids between the Cdk1 phosphorylation sites leads to an increase in the phosphorylation efficiency of Sic1. The deletion of the C-terminal region did not affect degradation significantly, leading to a minimal Sic1-based degradation module.

Next Cks1-mediated phosphorylation of the degions was manipulated with the aim to create differentially phosphorylated versions of the degion module. It was found that phosphorylation decreases by mutating the threonine-based primer sites to serines, which leads to a loss of Cks1 binding, degion modules were stabilized. This shows that Cks1 is critical in Cdk1-dependent phosphorylation of Sic1 degions and by decreasing the efficiency of Cks1-dependent phosphorylation, it is possible to design Sic1 mutants that are stable throughout the cell cycle.

Next the possibility of induced degradation of the otherwise stable degion modules was analyzed. For this expression of Clb5 was induced to different levels using estradiol-inducible expression. With estradiol concentration of 3000 nM the highest rate of degradation was seen, however, time-lapse microscopy revealed, that the highest estradiol concentration caused an increase in proportion of mitotic cells leading to cell cycle arrest. Consequently, to decrease such inhibiting effect on the cell cycle progression, 300 nM estradiol was chosen for further research.

It was shown that by inducing overexpression of Clb5 it is possible to degrade the otherwise stable Sic1 constructs. Importantly, the presence of the NPF motif strongly promoted the module degradation upon Clb5 induction, indicating that via specific docking interactions it is possible to phosphorylate the otherwise stable low quality substrates. Hence, it is possible to use Cdk1 in cell cycle independent signalling networks. Based on the results it can be concluded, that it is possible to create phospho-regulatable modules, that are independent of cell cycle. By changing the efficiency of Cks1-dependent phosphorylation, which can be done either by mutating the primer sites or by varying the distances between primer and secondary sites, it is possible to design Cdk1 substrates with differential phosphorylation during the cell cycle.

REFERENCES

- Archambault, V., Ikui, A.E., Drapkin, B.J., and Cross, F.R. (2005). Disruption of Mechanisms That Prevent Rereplication Triggers a DNA Damage Response †. *Mol. Cell. Biol.* 25, 6707–6721.
- Bhaduri, S., Valk, E., Winters, M.J., Gruessner, B., Loog, M., and Pryciak, P.M. (2015). A docking interface in the cyclin Cln2 promotes multi-site phosphorylation of substrates and timely cell-cycle entry. *Curr. Biol.* 25, 316–325.
- Blondel, M., Galan, J.M., Chi, Y., Lafourcade, C., Longaretti, C., Deshaies, R.J., and Peter, M. (2000). Nuclear-specific degradation of Far1 is controlled by the localization of the F-box protein Cdc4. *EMBO J.* 19, 6085–6097.
- Bloom, J., and Cross, F.R. (2007). Multiple levels of cyclin specificity in cell-cycle control. *8*, 149–160.
- Bruin, R.A.M. De, McDonald, W.H., Kalashnikova, T.I., Iii, J.Y., Wittenberg, C., and Jolla, L. (2008). Cln3 Activates G1-Specific Transcription via Phosphorylation of the SBF Bound Repressor Whi5. *117*, 887–898.
- Chen, Q., Orlicky, S., Tang, X., Pawson, T., Tyers, M., Sicheri, F., Mendenhall, M.D., Gertler, F.B., and Nash, P. (2002). Multisite phosphorylation of a CDK inhibitor sets a threshold for the onset of DNA replication. *Nature* 414.
- Costanzo, M., Nishikawa, J.L., Tang, X., Millman, J.S., Schub, O., Breitkreuz, K., Dewar, D., Rupes, I., Andrews, B., and Tyers, M. (2004). CDK Activity Antagonizes Whi5 , an Inhibitor of G1 / S Transcription in Yeast. *117*, 899–913.
- Coudreuse, D., and Nurse, P. (2010). Driving the cell cycle with a minimal CDK control network. *Nature* 468, 1074–1079.
- Cross, F.R. (1995). Starting the cell cycle: what's the point? *Curr. Opin. Cell Biol.* 7, 790–797.
- Cross, F.R., and Tinkelenberg, A.H. (1991). A potential positive feedback loop controlling CLN1 and CLN2 gene expression at the start of the yeast cell cycle. *Cell* 65, 875–883.
- Das, M., Bhowmik, A. Das, Bhaduri, N., Sarkar, K., Ghosh, P., Sinha, S., Ray, A., Chatterjee, A., and Mukhopadhyay, K. (2011). Role of gene–gene/gene–environment

interaction in the etiology of eastern Indian ADHD probands. *Prog. Neuro-Psychopharmacology Biol. Psychiatry* 35, 577–587.

Davey, N.E., and Morgan, D.O. (2016). Building a Regulatory Network with Short Linear Sequence Motifs: Lessons from the Degrons of the Anaphase-Promoting Complex. *Mol. Cell* 64, 12–23.

Denby, C.M., Li, R.A., Vu, V.T., Costello, Z., Lin, W., Chan, L.J.G., Williams, J., Donaldson, B., Bamforth, C.W., Petzold, C.J., et al. (2018). Industrial brewing yeast engineered for the production of primary flavor determinants in hopped beer. *Nat. Commun.* 9, 965.

Doncic, A., Eser, U., Atay, O., and Skotheim, J.M. (2013). An Algorithm to Automate Yeast Segmentation and Tracking. *PLoS One* 8, e57970.

Edgington, N.P., and Futcher, B. (2001). Relationship between the function and the location of G1 cyclins in *S. cerevisiae*. 3.

Engstrom, M.D., and Pflieger, B.F. (2017). Transcription control engineering and applications in synthetic biology. *Synth. Syst. Biotechnol.* 2, 176–191.

Gawlin'ski, P.G., Nikolay, R., Goursot, C., Lawo, S., Chaurasia, B., Herz, H.-M., Kuler-Schneider, Y., Ruppert, T., Mayer, M., and Grohans, J. (2007). The *Drosophila* mitotic inhibitor Frühstart specifically binds to the hydrophobic patch of cyclins. *EMBO Rep.* 8, 490–496.

Gordley, R.M., Williams, R.E., Bashor, C.J., Toettcher, J.E., Yan, S., and Lim, W.A. (2016). Engineering dynamical control of cell fate switching using synthetic phospho-regulons. *Proc. Natl. Acad. Sci. U. S. A.* 113, 13528–13533.

Hodge, A., and Mendenhall, M. (1999). The cyclin-dependent kinase inhibitory domain of the yeast Sic1 protein is contained within the C-terminal 70 amino acids. *Mol. Gen. Genet.* 262, 55–64.

Holt, L.J. (2012). Regulatory modules: Coupling protein stability to phosphoregulation during cell division. *FEBS Lett.* 586, 2773–2777.

Jorgensen, P., and Tyers, M. (2004). How Cells Coordinate Growth and Division. 14, 1014–1027.

Kõivomägi, M., Valk, E., Venta, R., Iofik, A., Lepiku, M., Balog, E.R.M., Rubin, S.M., Morgan, D.O., and Loog, M. (2011a). Cascades of multisite phosphorylation control Sic1

destruction at the onset of S phase. *Nature* 480, 128–131.

Kõivomägi, M., Valk, E., Venta, R., Iofik, A., Lepiku, M., Morgan, D.O., and Loog, M. (2011b). Dynamics of Cdk1 substrate specificity during the cell cycle. *Mol. Cell* 42, 610–623.

Kõivomägi, M., Örd, M., Iofik, A., Valk, E., Venta, R., Faustova, I., Kivi, R., Balog, E.R.M., Rubin, S.M., and Loog, M. (2013). Multisite phosphorylation networks as signal processors for Cdk1. *Nat. Struct. Mol. Biol.* 20, 1415–1424.

Lengronne, A., and Schwob, E. (2002). The Yeast CDK Inhibitor Sic1 Prevents Genomic Instability by Promoting Replication Origin Licensing in Late G1. *Mol. Cell* 9, 1067–1078.

Levine, K., Huang, K., and Cross, F.R. (1996). *Saccharomyces cerevisiae* G 1 Cyclins Differ in Their Intrinsic Functional Specificities. *16*, 6794–6803.

Liku, M.E., Nguyen, V.Q., Rosales, A.W., Irie, K., and Li, J.J. (2005). CDK Phosphorylation of a Novel NLS-NES Module Distributed between Two Subunits of the Mcm2-7 Complex Prevents Chromosomal Rereplication. *Mol. Biol. Cell* 16, 5026–5039.

Liu, J., and Kipreos, E.T. (1999). Evolution of Cyclin-Dependent Kinases (CDKs) and CDK-Activating Kinases (CAKs): Differential Conservation of CAKs in Yeast and Metazoa. 1061–1074.

Loog, M., and Morgan, D.O. (2005). Cyclin specificity in the phosphorylation of cyclin-dependent kinase substrates. 434.

Lu, D., Hsiao, J.Y., Davey, N.E., Van Voorhis, V.A., Foster, S.A., Tang, C., and Morgan, D.O. (2014). Multiple mechanisms determine the order of APC/C substrate degradation in mitosis. *J. Cell Biol.* 207, 23–39.

McGrath, D.A., Balog, E.R.M., Kõivomägi, M., Lucena, R., Mai, M. V., Hirschi, A., Kellogg, D.R., Loog, M., and Rubin, S.M. (2013). Cks confers specificity to phosphorylation-dependent CDK signaling pathways. *Nat. Struct. Mol. Biol.* 20, 1407–1414.

Miller, C.J., and Turk, B.E. (2018). Homing in : Mechanisms of Substrate Targeting by Protein Kinases. *Trends Biochem. Sci.* 43, 380–394.

Miller, M.E., and Cross, F.R. (2000). Distinct Subcellular Localization Patterns Contribute to Functional Specificity of the Cln2 and Cln3 Cyclins of *Saccharomyces cerevisiae*. *20*, 542–555.

- Morgan, D.O. (2007). (London: New Science Press Ltd.).
- Nash, P., Tang, X., Orlicky, S., Chen, Q., Gertler, F.B., Mendenhall, M.D., Sicheri, F., Pawson, T., and Tyers, M. (2001). Multisite phosphorylation of a CDK inhibitor sets a threshold for the onset of DNA replication. *Nature* *414*, 514–521.
- Nigg, E.A. (1993). Cellular substrates of p34cdc2 and its companion cyclin-dependent kinases. *Trends Cell Biol.* *3*, 296–301.
- Örd, M., and Loog, M. (2019). How the cell cycle clock ticks. *Mol. Biol. Cell* *30*, 169–172.
- Ottoz, D.S.M., Rudolf, F., and Stelling, J. (2014). Inducible, tightly regulated and growth condition-independent transcription factor in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* *42*.
- Pearsall, S.M., Rowley, C.N., and Berry, A. (2015). Advances in Pathway Engineering for Natural Product Biosynthesis. *ChemCatChem* *7*, 3078–3093.
- Peng, B., Plan, M.R., Chrysanthopoulos, P., Hodson, M.P., Nielsen, L.K., and Vickers, C.E. (2017). A squalene synthase protein degradation method for improved sesquiterpene production in *Saccharomyces cerevisiae*. *Metab. Eng.* *39*, 209–219.
- Schilf, R. (2018). Mapping of CDK inhibitory docking motifs on Sic1. University of Tartu.
- Schulman, B.A., Lindstrom, D.L., and Harlow, E. (1998). Substrate recruitment to cyclin-dependent kinase 2 by a multipurpose docking site on cyclin A. *Proc. Natl. Acad. Sci. U. S. A.* *95*, 10453–10458.
- Schwob, E., Böhm, T., Mendenhall, M.D., and Nasmyth, K. (1994). The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in *S. cerevisiae*. *Cell* *79*, 233–244.
- Skaar, J.R., Pagan, J.K., and Pagano, M. (2013). Mechanisms and function of substrate recruitment by F-box proteins. *Nat Rev Mol Cell Biol* *14*.
- Skotheim, J.M., Talia, S. Di, Siggia, E.D., and Cross, F.R. (2008). Positive feedback of G1 cyclins ensures coherent cell cycle entry. *454*.
- Stegmeier, F., and Amon, A. (2004). Closing Mitosis: The Functions of the Cdc14 Phosphatase and Its Regulation. *Annu. Rev. Genet.* *38*, 203–232.
- Stern, B., and Nurse, P. (1996). A quantitative model for the cdc2 control of S phase and mitosis in fission yeast. *Trends Genet.* *12*, 345–350.
- Swaffer, M.P., Jones, A.W., Flynn, H.R., Snijders, A.P., and Nurse, P. (2016). CDK

Substrate Phosphorylation and Ordering the Cell Cycle. *Cell* 167, 1750-1761.e16.

Topacio, B.R., Zatulovskiy, E., Cristea, S., Xie, S., Tambo, C.S., Rubin, S.M., Sage, J., Kõivomägi, M., and Skotheim, J.M. (2019). Cyclin D-Cdk4,6 Drives Cell-Cycle Progression via the Retinoblastoma Protein's C-Terminal Helix. *Mol. Cell* 74, 758-770.e4.

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

Verma, R., Chi, Y., and Deshaies, R.J. (1997). Cell-free ubiquitination of cell cycle regulators in budding yeast extracts. *Methods Enzymol.* 283, 365–376.

Zheng, N., and Shabek, N. (2017). Ubiquitin Ligases: Structure, Function, and Regulation. *Annu. Rev. Biochem.* 86, 129–157.

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