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**Cell cycle independent signaling by modified Clb5-
Cdk1 complex**

Master's Thesis (30 ECTS)

Curriculum Bioengineering

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Tartu 2021

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Abstract:

A eukaryotic cell contains a robust regulatory network that controls the ordered sequence and timing of distinct cell cycle events. In budding yeast this is mainly regulated by the growing activity of cyclin-dependent kinases (Cdks) during the cell cycle. Post-translational modifications, particularly protein phosphorylation is a powerful mechanism regulating stability, localization, activity and interactions of proteins. Building synthetic circuits based on protein phosphorylation has remained a riddle due to the overlapping specificity of protein kinases. To tackle this issue it was aimed to obtain a cell cycle independent cyclin-Cdk1 input that could target substrates created specifically for this particular cyclin-Cdk1 complex. By manipulating the Cdk1 specificity of phospho-degron and -localization modules it was anticipated to design orthogonal Cdk circuits that respond to the modified Clb5-Cdk1 complex.

Keywords: Cell cycle, phosphorylation, docking motifs

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

Rakutsüklist sõltumatu signaalimine modifitseeritud Clb5-Cdk1 kompleksiga

Lühikokkuvõte:

Eukarüootsel rakul on tugev reguleeriv võrgustik, mis tagab erinevate rakutsükli sündmuste korrektset järjestust ja kulgemist. Pärmsis on see enamasti reguleeritud tsükliinist sõltuvate kinaaside (Cdk-de) kasvava aktiivsuse vahendusel rakutsükliis. Posttranslatsioonilised modifikatsioonid, eelkõige valgu fosforüülimine, on võimas mehhanism, mis reguleerib valkude stabiilsust, lokaliseerimist, aktiivsust ja interaktsioone. Valkude fosforüülimisel põhinevate sünteetiliste võrgustike disainimine on proteiinikinaaside kattuva spetsiifilisuse tõttu jäänud mõistatuseks. Selle probleemi lahendamiseks on püüeldud leida rakutsüklist sõltumatu tsükliin-Cdk1 sisend, mis saaks sihtida neid substraate, mis on spetsiifiliselt loodud konkreetse tsükliin-Cdk1 kompleksi jaoks. Fosfodegroni ja lokaliseerimismoodulite Cdk1 spetsiifilisuse kavandamisega püütakse luua ortogonaalseid Cdk võrgustikke, mis reageerivad modifitseeritud Clb5-Cdk1 kompleksile.

Võtmesõnad: Rakutsükkel, fosforüülimine, 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 discrete cell cycle events. In eukaryotes the cell cycle consists of four main phases: G1, S, G2 and M. The cell grows and prepares for replication in G1 phase; DNA replication occurs in S phase; the cell prepares for mitosis in G2 phase; nuclear division and cell division between the daughter cells is carried out in M phase. A robust regulatory network of the eukaryotic cell controls the correct order and timing of the cell cycle events, which is crucial for the accurate cell proliferation.

The activation of separate molecular events is triggered by phosphorylation of specific proteins by the master regulator of cell division, the cyclin-dependent kinase (Cdk). The ability of Cdks to phosphorylate hundreds of proteins in an ordered manner depends on changing substrate specificity and reaching different Cdk activity thresholds. However, there is still no mechanistic explanation how relatively small changes in Cdk activity can initiate the ordered sequence of distinct events in a sufficiently time-resolved fashion.

Deciphering the mechanisms of the cell cycle regulation and ability to manipulate them could open a slew of new opportunities in both medicine as well as synthetic biology. However, despite the understanding that a significant portion of cellular regulation occurs through phosphorylation, we have yet to figure out how to apply this knowledge to the development of synthetic phospho-regulation modules. Subsequently, there is a need for well-defined phospho-modules and the ability to predictably program the phosphorylation of these modules.

Another factor that confers to the correct substrate targeting is subcellular localization. Proteins are synthesized in cytosol, however, some of them have to function in the nucleus. Such nucleocytoplasmic shuttling is mediated by the NLS (nuclear localization signal) and NES (nuclear export signal) motifs present in the target protein. Differential location of various cyclin-Cdk1 complexes impacts the fate of protein degradation, as target protein may be restrained by one cyclin-Cdk1 complex, while isolated from the other cyclin-Cdk1 complex. Controlling protein localization by decreasing or increasing exposure of enzyme to the target protein strongly influences the degradation pattern, therefore the potential to manipulate it could become a vigorous tool in biology. In this work, the impact of Cdk1 sites and phosphodegrons on the regulation of protein localization is studied on the example of protein Dna2.

It has been difficult to achieve efficient models of synthetic phosphorylation-based signaling circuits due to overlapping substrate specificities of protein kinases. One implied approach to prevent this is to design orthogonal signaling circuits using a combination of different kinase docking modules from known substrates and inhibitors. Subsequently, within this work it was desired to manipulate the Cdk1 specificity of phospho-degron and -localization modules to design Cdk circuits that are unaffected by native cell cycle machinery, but that respond to the induced S phase Cdk1 complex.

1 LITERATURE REVIEW

1.1 Cyclin-dependent kinases

The events of cell cycle are mediated by an independent control system, which is based on oscillations in the activities of serine/threonine protein kinases called the cyclin-dependent kinases (Cdks). These regulators are present in all known eukaryotes, and their main function is catalyzing the phosphorylation of specific serine and threonine residues of the target proteins that are determined by the associated cyclin. Phosphorylation is the process involving covalent attachment of phosphoryl groups derived from ATP to protein substrate, which leads to changes in substrate protein functions. Cdk activities increase and decrease as the cell cycle progresses, despite the fact that Cdk protein concentrations remain constant. Fluctuations in Cdk activities trigger cyclical changes in the phosphorylation of cell cycle components, determining the order in which cell cycle events are initiated (Morgan, 2007; Swaffer *et al.*, 2016a). Namely, such oscillations serve important functions in restricting DNA replication, repair and segregation to specific phases of the cell cycle and ensures irreversibility of the discrete phases of the cell cycle.

For the enzymatic activation of Cdks the binding of a regulatory subunit - cyclin - is necessary. Normally, full activation also requires phosphorylation of a threonine residue near the kinase active site (Morgan, 2007). In other terms, the cyclin ties the target protein for the Cdk to phosphorylate it, thereby changing the specificity of each target protein. As the levels of Cdk activity increase during the cell cycle, relevant molecular processes are executed at specific 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 can be phosphorylated only later in the cell cycle, when higher Cdk activity is achieved (**Figure 1**) (Miller and Turk, 2018; Swaffer *et al.*, 2016b).

Cdk function has remained fundamentally unchanged over millions of years of eukaryotic evolution. Six different Cdks exist in *S. cerevisiae*, however, Cdk1 (also known as Cdc28) will be in the main focus as it is vital to drive the cell cycle in budding yeast (Liu and Kipreos, 1999). Cyclins and Cdks are remarkably conserved between *S. cerevisiae* and mammals. For example, the cell cycle of yeast operates even if budding yeast cyclins are replaced with human cyclins (Lew *et al.*, 1991), and if Cdk1 of *S. cerevisiae* is replaced with

with human Cdk1 or Cdk2 (Elledge and Spottswood, 1991), confirming the evolutionary conservation of cell cycle control.

Cdks drive the cell cycle progression by phosphorylating a large number of proteins in the cell. Due to low cyclin concentrations and the involvement of the cyclin dependent kinase inhibitors (CKIs) Sic1 and Far1, Cdk1 is inactive in early G1 (Schwob *et al.*, 1994). Its activity increases at late G1, when cyclin concentrations rise and the CKIs are degraded. Cdk1 activity remains high until anaphase, afterwards it drops because cyclins are destroyed and CKIs are expressed again (Amon *et al.*, 1994). The drop in Cdk1 activity is a signal for the cell to exit from mitosis, leading to the reset of the cell cycle to an initial G1 state of low Cdk1 activity. In particular, during mitosis, Cdks phosphorylate hundreds of distinct proteins: for example, yeast has approximately 600-700 proteins that are Cdk1 substrates, which makes up about 10% of all proteins (Ubersax *et al.*, 2003).

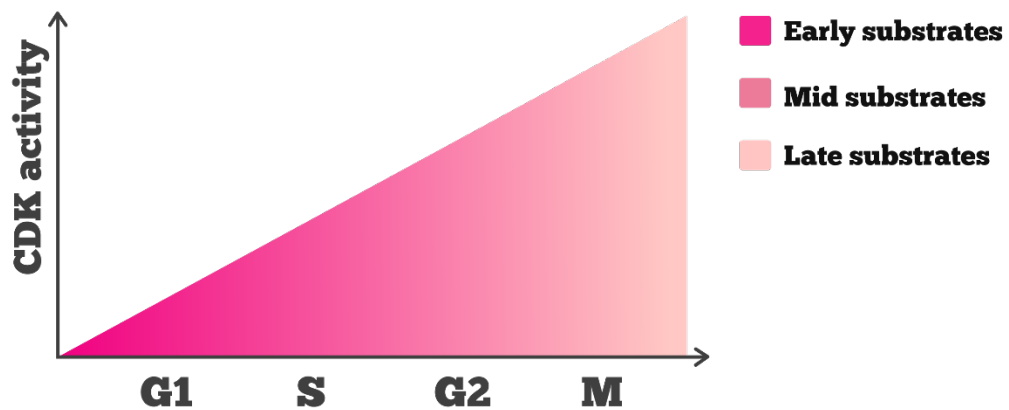


Figure 1. Differential substrate phosphorylation by the Cdk during the cell cycle. Rising Cdk activity during the cell cycle contributes to the timing of substrate phosphorylation: high-quality substrates are phosphorylated in G1/S phase at a low Cdk activity, whereas lower quality substrates are phosphorylated later in G2/M phase, when Cdk activity reaches a higher activity threshold.

1.2 Cyclins

Cyclins are proteins, that determine the Cdk activity with its distinctive feature of binding and activating Cdks. Most cyclins go through vast changes in concentration during the cell cycle, mainly by changes in cyclin gene expression and destruction of cyclins by proteolysis, which leads to the oscillations in Cdk activity. Throughout the cell cycle, *S. cerevisiae* expresses nine cyclins that interact with Cdk1. Cln1, Cln2, and Cln3 are the three G1 cyclins involved in cell cycle entry. Clb1-6 are the B-type cyclins, that function after the G1 cyclins

in the cell cycle. Upon transcriptional activation in G1/S (late G1) by Cln3, the G1 cyclins Cln1 and Cln2 are able to bind Cdk1 to complete progression through the Start checkpoint - due to that they can altogether be called G1/S cyclins. In *S. cerevisiae* these G1/S cyclins 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 activate the process leading to DNA duplication by shutting down numerous braking systems that suppress S-phase Cdk activity in G1.

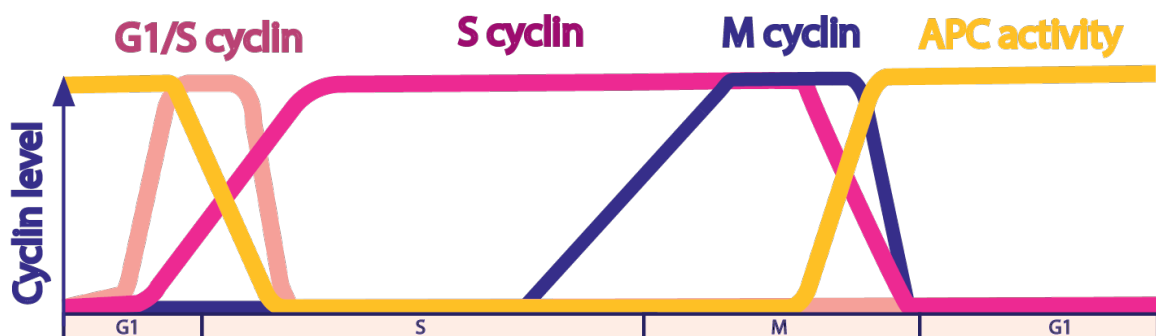


Figure 2. A simplified view of the cell cycle control system. Scheme showing how levels of the three major cyclin types (G1/S, S, M) oscillate during the cell cycle. The increase of APC activity causes the destruction of S and M cyclins and, hence, the inactivation of Cdks, which promotes the completion 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, while APC activity is peaking in late M phase and G1. Figure adapted from Morgan, 2007.

For example, the activity of S phase cyclins is inhibited in G1 phase by an inhibitor protein Sic1 (Schwob *et al.*, 1994), which is important for preventing premature 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 activate 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. S cyclins Clb5 and Clb6 accompany the rise of G1/S cyclins and direct the stimulation of DNA replication. Clb6 is degraded at the G1/S border as it is targeted for destruction by the SCF^{Cdc4} (*Skp1/Cullin/F-box*) (Schneider *et al.*, 1996) upon phosphorylation by Cdk1 and Pho85. On the contrary, Clb5 is stable until mitosis due to the presence of an anaphase-

promoting complex (APC^{Cdc20}) destruction box, which causes it to be degraded by the APC^{Cdc20} (Jackson *et al.*, 2006). APC^{Cdc20} is a ubiquitin-protein ligase complex that stimulates the destruction of proteins that hold the sister chromatids together (Davey and Morgan, 2016).

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 APC, triggering the metaphase-anaphase transition (Bloom and Cross, 2007). As a direct consequence, 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 cyclin 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 manner 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 is helpful in establishing 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). Finally, to restore the initial state of G1 after metaphase-anaphase transition, phosphatase Cdc14 is activated (Stegmeier and Amon, 2004).

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 active site of Cdk 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). Two docking interactions can increase phosphorylation rate of the site: Cks1 can bind to phosphorylated TP sites, and cyclins can interact with substrates through specific short linear

docking sequences. In both cases, the effect of docking depends on the relative positioning of docking sites and phosphorylation sites along the disordered protein (Kõivomägi *et al.*, 2013a; Örd and Loog, 2019).

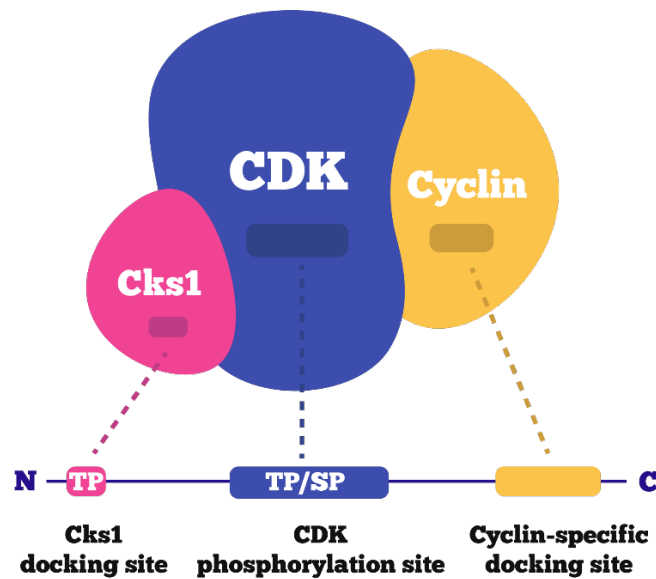


Figure 3. Cyclin-Cdk1-Cks1 complex. The scheme showing the main interactions between substrate proteins and the Cdk complex determining the phosphorylation rate and specificity (Örd and Loog, 2019).

1.3.1 Phospho-adaptor Cks1 promotes multiphosphorylation of Cdk substrates

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

Cks1 has been discovered to bind only phospho-threonines and not phospho-serines (Kõivomägi *et al.*, 2013a; McGrath *et al.*, 2013) (**Figure 3**). By binding to phosphorylated threonine sites, Cks1 stabilises the enzyme-substrate interaction, enhancing the phosphorylation efficiency of secondary sites (Kõivomägi *et al.*, 2011a, 2013a; McGrath *et al.*, 2013) (**Figure 4**). 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). This hypothesis was supported by the finding that mutation of threonines to serines in the Cdk consensus sites of Sic1 led to a severe suppression of multisite phosphorylation (Kõivomägi *et al.*, 2013a). Therefore, it is possible to disrupt the Cks1-dependent cascade

by replacing threonines in Cdk sites with serines (Kõivomägi *et al.*, 2013a). The second factor strongly impacting the Cks1-mediated phosphorylation is the distance and relative positioning between the priming site and the secondary phosphorylation site (**Figure 4**). First of all, Cdk1 phosphorylates a priming site, that then binds to the phosphate-binding pocket of Cks1. This increases the substrate-Cdk complex binding affinity and facilitates phosphorylation of secondary sites, resulting in multisite phosphorylation. Therefore, the efficiency of Cks1-mediated phosphorylation is highly dependent on the placement of the primary and secondary sites. (Kõivomägi *et al.*, 2013a). Latest observations have led to the conclusion, that the distance of 12 amino acids between the phosphosites is the most optimal (Kõivomägi *et al.*, 2013a; Örd *et al.*, 2019).

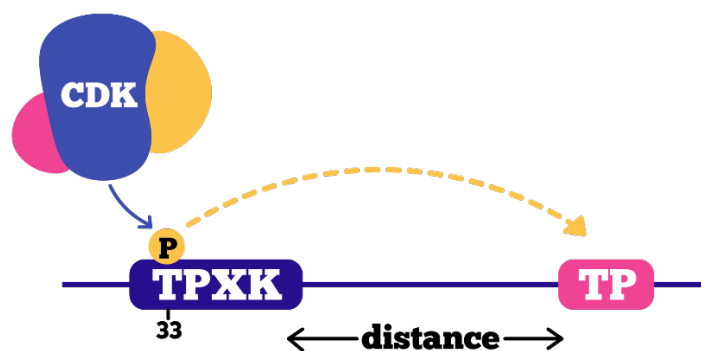


Figure 4. Cks1-mediated phosphorylation is influenced by the amino acid distance between the priming site and the secondary phosphorylation site. A scheme explaining the mechanism of Cks1-mediated phosphorylation, wherein phosphorylation of the secondary site occurs only after Cdk1 phosphorylation of the primary site and its binding to the phosphate-binding pocket of Cks1 (Kõivomägi *et al.*, 2013a).

1.3.2 G1 cyclin functions and specificity

Considerable evidence supports the idea that cyclins are not simply activators of the Cdk subunit, but also help guide the Cdk to specific substrates, either by directly binding the substrate or by transporting the Cdk to a subcellular compartment where the substrate is located (Bloom and Cross, 2007).

Cyclin specificity is controlled 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 embody a mechanism that prevents early phosphorylation of late Cdk targets, assuring that mitotic events are not triggered 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 called LP motif that is recognized only by G1/S cyclins Cln1 and Cln2, and not by Cln3 or later S or M phase cyclins (Bhaduri and Pryciak, 2011; Kõivomägi *et al.*, 2011b).

Despite the fact that Cln1–Cln3 share some functional similarities, they have significant differences (Bloom and Cross, 2007; Levine *et al.*, 1996) that contribute to a two-stage commitment process: Cln3 is necessary for initiation of Cln1 and Cln2 expression, which further amplify their own expression via a positive feedback loop. This results in a sharp Cln1/2 activity increase that prompts a decisive entry into the cell cycle (Cross and Tinkelenberg, 1991; Skotheim *et al.*, 2008). G1 cyclins exhibit differential subcellular localization, while Cln3 and Cln1 are nuclear, Cln2 is concentrated in the cytoplasm (Edgington and Futcher, 2001; Miller and Cross, 2000).

The function of Cln1–Cln3 is determined by such cues as nutrient availability, cell size, and inhibitory signals. Ultimately, the Cdk phosphorylation events in G1 are driven by Cln1–Cln3, committing cells to division, followed by the G1/ S transition (Cross, 1995; Jorgensen and Tyers, 2004). Key Cdk substrates in this term are inhibitors of cell cycle entry, such as a repressor of G1/S transcription Whi5, (Bruin *et al.*, 2008; Costanzo *et al.*, 2004) as well as Cdh1 and Sic1 that prevent the expression and activity of later cyclins, respectively (Morgan, 2007). An interesting observation has shown an inability to fully phosphorylate the G1/S transcriptional repressor Whi5 upon blocking the Cln2 docking function. Consequently, it agitates 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 notable overlap between the functions of S and M cyclins has been detected. For example, M cyclins are able to stimulate S phase to some extent. One possibility of how a single cyclin drives the correct order 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). Alternatively, some Cdk substrates become available for phosphorylation only during a specific stage in the cell cycle.

In some cases, 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) are known to 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 is determined by a region called the hydrophobic patch, lying on the surface of the cyclin (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 the reduced 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).

The specificity of two budding yeast cyclins, the S phase cyclin Clb5 and the M phase cyclin Clb2, in the phosphorylation of 150 Cdk1 substrates was compared to better understand how different cyclins facilitate discrete cell cycle events. Clb5-Cdk1 was discovered to phosphorylate approximately 24% of these proteins more efficiently than Clb2-Cdk1. The Clb5-specific targets include several proteins (Sld2, Cdc6, Orc6, Mcm3 and Cdh1) involved in early S phase events, whereas Clb5 specificity is based on an interaction between the hydrophobic patch in Clb5 and an RXL motif on the substrate protein. By replacing Clb5 with Clb2 or by mutating the substrate RXL motif, phosphorylation of Clb5-specific targets during S phase was reduced, thus, affirming the importance of cyclin specificity *in vivo*. This serves as an evidence, that Clb5 and Clb2 use different mechanisms to enhance the phosphorylation of S phase and M phase substrates (Loog and Morgan, 2005).

A recent study found that an NLxxxL motif, that is homologous to the RxL motif but has different specificity determinants, promotes phosphorylation exclusively by S phase Clb5/6-Cdk1 complexes, whereas the RXL motif is also used by later Clb-Cdk1 complexes to some extent (Faustova and Loog, 2021). This highlights the multitude of cyclin docking interactions and their importance in finetuning the timing of Cdk phosphorylation events.

1.3.4 Clb5-specific NPF motif and its influence on phosphorylation

The C-terminal inhibitory domain of Sic1 has been widely researched for possible binding motifs that would promote phosphorylation. Recently, a short linear motif that stimulates phosphorylation specifically by Clb5-Cdk1 was discovered. The motif was found from the Sic1 sequence $^{216}\text{KNPFAS}^{221}$, where the NPF sequence (NPF motif) is the most critical in the interaction (Schilf, 2018). Kinase assays have shown that addition of the NPF motif increases the rate of substrate phosphorylation by Clb5-Cdk1 by over 25-fold. Experiments with mutated hydrophobic patch on cyclin Clb5 (further as Clb5(m1)) showed that it had no impact on the degradation efficiency of mutants containing the NPF motif. Therefore, the NPF motif was discovered to be the first B-type (S and M phase) cyclin docking motif that does not bind to the cyclin's hydrophobic patch. Instead, the data has indicated that the NPF motif binds to the region containing F284, which is distant from the RXL binding pocket (**Figure 5**). Also, as the NPF motif was found from an inhibitory interaction in Sic1, it is not known to mediate phosphorylation of native S-Cdk targets.

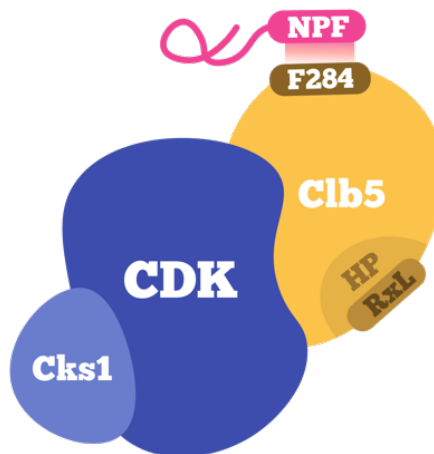


Figure 5. Substrate docking pockets on Clb5. The NPF motif binds to F284 away from the conserved RXL binding pocket.

1.4 Clb5 degradation and inhibition

The main drivers of the transition from G1 into S phase are S-phase cyclins Clb5 and Clb6, which are transcribed in a pulse in late G1 (Basco *et al.*, 1995). However, it has been shown that deletion of *CLB6* has little or no effect, while *CLB5* deletion greatly extends S phase (Schwob and Nasmyth, 1993). Therefore, Clb5 plays a more predominant role in regulating timely progression through S phase. Although *CLB5* is transcribed in G1, Clb5-Cdk1

complex is not activated until reaching the G1/S threshold when the Clb-specific Cdk inhibitor, Sic1, is degraded by the the ubiquitin ligase complex, SCF^{Cdc4} (Schneider *et al.*, 1996). Multiple studies have proven that Clb5 persists throughout S phase and is marked for destruction only in mitosis by the action of the ubiquitin ligase complex APC^{Cdc20} (Irniger and Nasmyth, 1997). On the other hand, a deletion of the Clb5 mitotic destruction box has shown only little influence on cell cycle progression (Sari *et al.*, 2007). High Clb-Cdk1 activity blocks the assembly of pre-replicative complexes (pre-RC) by various mechanisms acting on Cdc6, the minichromosome maintenance (MCM) complex and origin replication complex (ORC) (Labib *et al.*, 1999; Wilmes *et al.*, 2004). Only when Cdk activity vanishes, after mitotic exit, new competent origins are allowed to assemble. The proper control of replication origins, both in timely onset and in preventing aberrant initiation, is crucial to avoiding DNA damage and guaranteeing DNA integrity (Lengronne and Schwob, 2002). Recently, a novel Clb5 mutant has been identified that is not inhibited by Sic1. This Clb5 version has mutations close to the hydrophobic patch and does not bind the RxL motifs common in S-Cdk1 targets (Pääbo, 2017). Therefore, this modified Cdk complex could potentially be used for cell cycle independent signaling.

1.5 Protein degradation in cell cycle control

The unidirectionality of transitions from one cell cycle phase to the next is achieved through irreversible activation or inactivation of Cdk. As stated before, this 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 immense protease complexes called proteasomes. The process is carried out by a series of enzymes known as E1, E2, and E3 catalyzing ubiquitin activation, ubiquitin conjugation, and ubiquitin-protein ligation, respectively (Zheng and Shabek, 2017). In fact, substrate recognition depends on degrons acting as the binding sites for E3 enzymes.

The G1/S transition and metaphase/anaphase transition in mitosis require two massive multisubunit ubiquitin-protein ligases. The central ubiquitin-protein ligase essential for the ubiquitination and proteolysis of targets such as Cdk inhibitors at the G1/S transition is SCF, a multisubunit complex consisting of a core (Skp1, Cull1, and Rbx1) and an F-box protein that binds to substrate molecules. SCF is constitutively active throughout the cell cycle and the

majority of its various F-box proteins recognize phosphorylated degron motifs, triggering ubiquitin-mediated proteolysis (Berset *et al.*, 2002; Holt, 2012). Cln1, Cln2, Far1 and Sic1 are the SCF targets and their degradation depends on the Cln1/2-Cdk1 activity in the G1 phase, therefore, they participate in regulation of the G1/S switch (Yang *et al.*, 2013)

Depending on the F-box protein present in the complex, SCF recognizes different targets. For example, in *S. cerevisiae* two different F-box protein, Cdc4 and Grr1 regulate the SCF specificity: SCF^{Cdc4} recognizes Sic1 and Far1 (Henchoz *et al.*, 1997), while SCF^{Grr1} targets Cln1,2 (Barral *et al.*, 1995). Such behaviour is governed to some extent by the subcellular localization. It has been demonstrated that the SCF^{Grr1} pathway degrades both Cln1 and Cln2, however, it was also concluded that the degradation of Cln2, but not Cln1, is also dependent on the SCF^{Cdc4} pathway (Quilis and Igual, 2017). Cdc4 is usually found within the nucleus, but studies have detected its ability to drive Cln2 degradation when forced to locate in the cytoplasm, supporting the Cdc4's ability to mediate cytoplasmic Cln2 degradation (Landry *et al.*, 2012). In mammals G1 cyclins are controlled similarly, because cyclin E (G1/S cyclin in mammals) carries a phosphodegron that is recognized by SCF^{Cdc4} (Orlicky *et al.*, 2003).

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, but their substrate recognition and control mechanisms are somewhat different (Holt, 2012; Morgan, 2007).

1.5.1 The phosphodegron module

The phosphodegron module is 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 case, phosphorylation module drives instability: the protein is stable in the absence of phosphorylation but is degraded upon phosphorylation (**Figure 6**). 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 S 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, thus, 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-terminal domain of Sic1 (Schwob *et al.*, 1994).

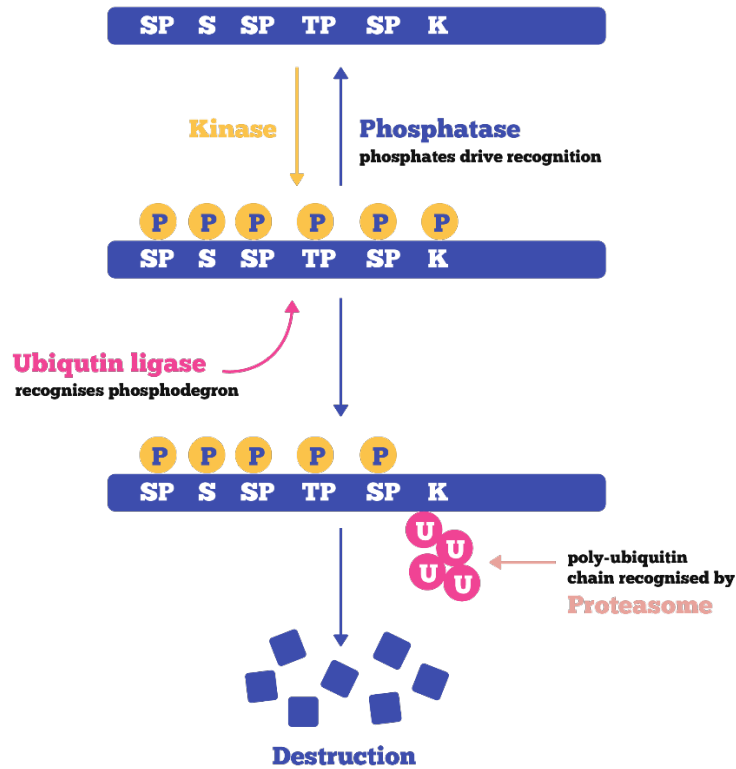


Figure 6. The phosphodegron module. A kinase phosphorylates residues to generate a phosphopeptide that binds to a ubiquitin ligase. The protein is degraded upon phosphorylation. Figure adapted from Holt, 2012.

The phosphorylated Cdk1 consensus motifs in Sic1 are recognized by the F-box protein Cdc4, which recruits Sic1 for ubiquitination by the Cdc34 - SCF complex (Chen *et al.*, 2002). SCF conjugates ubiquitin sequence 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 facilitate Cdc4 binding (Kõivomägi *et al.*, 2011a; Nash *et al.*, 2001). The weak degron sites in Sic1 set phosphorylation threshold that delays degradation *in vivo*, and thereby establishes a minimal G1 phase period needed to secure proper DNA replication (Nash *et al.*, 2001). Phosphorylation of Sic1 by G1 cyclin-Cdk1 leads to further Sic1 destruction and therefore the release of more S cyclin-Cdk1 in a positive feedback loop, ensuring commitment to the S phase (Kõivomägi *et al.*, 2011a; Venta *et al.*, 2012). Interestingly, it was found that the additional phosphorylation sites and cyclin docking motifs around the two di-phosphodegrons in Sic1 form a helper network that determines the timing of the degron phosphorylation in the cell cycle (Örd *et al.*, 2019). Without the N-terminal threonine

sites that function as Cks1-dependent priming sites for the degrons and the cyclin docking motifs, the degron sites were not phosphorylated over the cell cycle and manipulation of the helper network led to very different phosphorylation timings of the degron sites. Thus, as the phosphorylation of the Sic1 degrons can be predictably programmed, it has the potential to be used in various synthetic phospho-regulation circuits.

1.6 Subcellular localization of proteins

The conditions in which proteins operate are determined by their subcellular localizations. As a result, protein function is influenced by subcellular localization, which regulates access to and availability of various interaction partners. Therefore, understanding protein localization is crucial to determine the cellular function of hypothetical and newly discovered proteins (Scott *et al.*, 2005). Large macromolecules (greater than 60 kDa) are not able to diffuse in and out of the nucleus passively and must be actively transported in a controlled manner (Lim, 2015).

In eukaryotic cells, internal membrane-bound structures sequester all genetic material. The nucleus, which is surrounded by a double membrane known as the nuclear envelope (NE), is the most notable of these structures. Though the NE isolates the nucleoplasm and genetic material inside the nucleus from the surrounding cytoplasm, it embeds the nuclear pore complexes (NPCs), regulating the bidirectional transport of macromolecules, including proteins, between the nucleus and cytoplasm (Wente and Rout, 2010). Protein import into the nucleus is a multistep process that begins with a cognate receptor recognizing a nuclear localization signal (NLS), followed by attachment of the complex at the nuclear pore, and GTP-dependent translocation into the nucleoplasm (Seedorf and Silver, 1997).

Karyopherins are an evolutionarily conserved family of transport proteins regulating nuclear import and export and, therefore, can be further divided into importins and exportins (Mosammaparast and Pemberton, 2004). Importins act as the receptors for the canonical NLS and are composed of an NLS-binding subunit α and the NPC docking subunit β (Adam and Geracet, 1991). In addition to binding the cargo, α subunit binds β 1, which then binds NPC. Exportins, on the other hand, recognize nuclear export signal (NES). To translocate in and out of the nucleus proteins must bear both NLS and NES sequences (**Figure 7**).

Nucleocytoplasmic shuttling via the nuclear pore is driven by GTP hydrolysis, which is catalyzed by the GTP-binding protein Ran and its regulators (Melchior *et al.*, 1995). In other words, importins require absence of RanGTP to bind NLS, which is present in cytoplasm,

and the release of cargo occurs in the nucleus wherein RanGTP is in abundance. Contrarily, exportins require RanGTP to bind NES, which is released in conditions where RanGTP is turned to RanGDP, hence, leading to dissociation of the substrate in cytoplasm (Fried and Kutay, 2003).

Some of the proteins that exhibit such cell cycle dependent nucleocytoplasmic shuttling are Whi5, Mcm3, and Dna2. Dna2 is an ATP-dependent nuclease involved in DNA replication and the repair of DNA double-strand breaks in nucleus and mitochondria (Stewart *et al.*, 2008). The NLS of Dna2 is activated by Cdk activity and is imported to the nucleus during the cell cycle. Dna2 is active in S phase as it is also involved in the processing of Okazaki fragments.

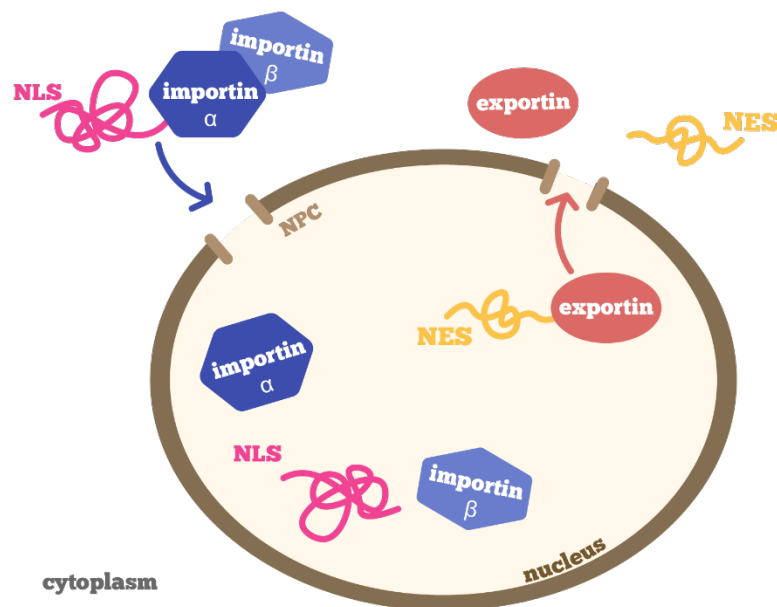


Figure 7. Nucleocytoplasmic transport of proteins. In cytoplasm, importins bind a protein carrying NLS amino acid sequence and transfer it into nucleus. Proteins with NES amino acid sequence are brought back to cytoplasm by exportins.

1.6.1 NLS and NES sequences

The classical nuclear import pathway incorporates importin α as an adaptor protein, recognizing the NLS on the protein intended for transfer inside the nucleus (Lange *et al.*, 2007). These classical NLSs (cNLSs) can be divided into two distinct classes: monopartite NLSs, containing one cluster; bipartite NLSs, containing two clusters of basic residues attached by

a linker region of ~10–12 residues [KR(X)_{10–12}K(K/R)X(K/R)] (Dingwall and Laskey, 1991).

In addition to having a short importin β binding domain (Kutay *et al.*, 1997), importin α also comprises a large NLS binding domain (Conti *et al.*, 1998), containing major and minor NLS binding sites. For the binding of bipartite NLS both binding sites are essential (Fontes *et al.*, 2003), whereas for the monopartite NLS binding to only one site, mainly major site, is necessary (Conti and Kuriyan, 2000). Monopartite cNLSs can be exemplified by the SV40 large T antigen NLS (¹²⁶PKKKRRV¹³²) being the first ever NLS motif to be identified (Kalderon *et al.*, 1984). The NLS of the nucleoplasmin (¹⁵⁵KRPAATKKAGQAKKK¹⁷⁰) is the prototype of the bipartite signal (Dingwall *et al.*, 1988).

In budding yeast nuclear export signals are recognized by exportins Crm1 and Msn5 (Ossareh-Nazari *et al.*, 2001). Crm1 recognizes a short leucine-rich amino acid sequence on the NES (Fornerod *et al.*, 1997), typical containing the consensus motif: Lx(2-3)[L/I/V/F/M]x(2-3)Lx[L/I], where X(2,3) represents any two or three amino acids (Bogerd *et al.*, 1996). Although Msn5 has been studied for decades, no NES sequence able to bind the exportin has been characterized (Bakhrat *et al.*, 2008). Myriad export cargoes for the mammalian Crm1 protein have now been established, for instance the protein kinase A inhibitor PKI, and the human immunodeficiency virus protein Rev. In *Saccharomyces cerevisiae*, however, only a few NES-containing proteins have been described at the molecular level.

Cdks utilize several mechanisms to inhibit the reassembly of prereplicative complexes (pre-RCs) to avoid improper rereplication, which in *S. cerevisiae* triggers the DNA damage response and cell cycle arrest (Archambault *et al.*, 2005). In budding yeast, one of such mechanisms has been found to stimulate the net nuclear export of a pre-RC subunit - the Mcm2–7 complex. Two partial NLSs on Mcm2 and Mcm3 have been identified to be necessary for nuclear localization of the Mcm2–7 complex. Similarly, Crm1-dependent NES adjacent to the Mcm3 NLS has been defined (Liku *et al.*, 2005a). Together, the Mcm2-Mcm3 NLS and the Mcm3 NES, configure a transport module iterating the cell cycle-regulated localization of the whole Mcm2–7 complex.

1.7 Phospho-regulation in synthetic biology

The advance 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 different 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 Pfleger, 2017). For these reasons inducible promoter systems have been developed and widely used in biological research. One of the most successful examples is the use of the synthetic transcription factor LexA-ER-AD for estradiol-induced expression (Ottoz *et al.*, 2014). Another example includes the development of the blue light inducible promoter complex using a small (222-residue) bacterial transcription factor (TF) EL222 (Nash *et al.*, 2011) as the basis of the expression system (Motta-Mena *et al.*, 2014).

In cellular processes, however, in addition to transcriptional control, post-translational regulation is vital 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 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). Specifically, there is a growing interest towards improvement of betulinic acid (BA) biosynthesis. This plant-derived small molecule can increase apoptosis specifically in cancer and not in normal cells, which makes it an attractive anti-cancer agent (Reiner *et al.*, 2013). 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. However, in cellular processes posttranslational modifications, particularly protein phosphorylation, control the stability, localization, activity and interactions of proteins. Until now, there are no convenient examples of designing synthetic circuits based on protein post-translational modifications. Orthogonality and specificity of the Cdk are the main factors limiting efficient engineering of the synthetic signaling circuits. To fill this research niche, one of the goals was to obtain the cell cycle independent cyclin-Cdk1 input that targets various substrates designed specifically for the particular Clb5-Cdk1 module.

Secondly, the ability to control protein localization during the cell cycle would be beneficial for both the research and industry. This could be used to control protein levels by their activation and inactivation during the cell cycle. Studying Dna2 NLS, putative NES and Cdk consensus motifs opens up possibilities to develop the localization modules that would allow regulated degradation when fused with the proteins of interest. Combining motifs of different proteins could lead to creation of the diverse localization and degradation profiles.

The aims of the thesis are:

- Design cyclin-Cdk1 modules for cell cycle independent signaling that target its specific substrates without affecting the progression of cell cycle.
- Design cell cycle independent modules to enable transport of tagged proteins from cytoplasm to nucleus for use in synthetic biology.
- Create phosphodegron modules facilitating regulated degradation.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Constructs

Experimental part consisted of 3 panels of constructs:

- 1) Light-inducible (VP-EL222) Clb5 panel: different constructs of Clb5 expressed under control of EL222 promoter and tagged with Citrine.
- 2) β -estradiol-inducible Clb5 panel: Clb5 variants expressed under control of estradiol-inducible promoter (Ottoz *et al.*, 2014) and tagged with hemagglutinin (HA) epitope.
- 3) Phospho-activated Dna2-NLS panel: a nuclear import module based on Dna2 expressed from the ACT1 promoter and fused with C-terminal sfGFP.

For VP-EL222 and Clb5-3HA constructs the backbone plasmid pFA6a-HIS was used, whereas Dna2 constructs were cloned into pRG203MX vector. The vectors were used for genomic integration of the expression cassette in yeast. These plasmids also include ampicillin resistance gene, which is used for selection in bacteria.

3.1.2 PCR

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. The total volume of the PCR reaction mixture was 25 μ l in case of the following restriction and ligation procedure, and 50 μ l for the preparation of the Clb5 expression cassette for transformation into yeast. The reaction contained: 1 μ l (around 15 ng) of the template, 5x Phusion Green HF Buffer (Thermo Fisher Scientific) with final concentration being 1x, 200 μ M dNTPs (stock concentration 10 mM), 0.75 μ M of forward and reverse primers, 0.04 U/ μ l high-fidelity Phusion DNA Polymerase (Thermo Fisher Scientific), Milli-Q H₂O to adjust the mixture volume to 25 μ l.

The three-step PCR program was used for the amplification, as shown in **Table 1**. The annealing temperature was estimated and set according to each set of primers, whereas the extension time (indicated as * in **Table 1**) was determined based on the synthesis rate of Phusion polymerase (1kb per 30s).

Following the program execution, the PCR mixture was loaded on to 1% Agarose TAE gel (40 mM Tris-acetate (pH 8.3), 1 mM EDTA, 1% agarose, 0.05 μ l/ μ l Atlas ClearSight DNA Stain (BioAtlas)) together with the ZipRuler Express DNA Ladder 1 (Thermo Fisher Scientific). The gel electrophoresis was completed in 1x TAE buffer (40 mM Tris-acetate (pH 8.3), 1 mM EDTA). PCR products were excised from gel under the UV light and purified from gel using Favorgen GEL/PCR Purification Kit.

Table 1. PCR program. Extension time, indicated by *, was determined by the length of the amplified fragment according to the speed of polymerase: 1000 bp per 30 s.

Cycle step	Temperature	Time	Number of cycles
Initial Denaturation	98°C	3 min	1
Denaturation	98°C	15 s	33
Annealing	56-60°C	15 s	
Extension	72°C	*	
Final Extension	72°C	5 min	1

3.1.3 Restriction and ligation

Prior to ligating inserts with vectors, both of them were restricted with the same enzymes. For insert restriction, 1.5 μ g of plasmid DNA or around 100 ng of PCR product was mixed with 0.5 μ l of each enzyme (FastDigest™ restrictases, Thermo Fisher Scientific), 2 μ l of 10x FastDigest™ Green buffer and water was added to make up to 20 μ l. For vector digestion, 1 μ l of FastAP alkaline phosphatase (Thermo Fisher Scientific) was also added to prevent vector from recircularization without the insert during ligation. The samples were incubated at 37°C for 15-30 minutes.

Next, the restricted plasmids were loaded on gel. For visualizing the products on TAE-agarose gel, 3-5 μ l of Thermo Scientific *6X Orange Loading Dye* was mixed into samples before loading, while for reading the DNA fragment lengths ZipRuler Express DNA Ladder 1 (Thermo Fisher Scientific) was used. For purifying the PCR and restriction 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 μ l, where 1 - 3 μ l of linearized vector (20-50 ng), 3 - 5 μ l of insert, 1 μ l of 10x ligation buffer, 1 - 4.5 μ l of water and 0.5 μ l of T4 DNA ligase (Thermo Fisher Scientific) were mixed. The ligation reaction was carried out at 18 °C for 1-16 hours.

3.1.4 Bacterial transformation

Transformation of plasmids into bacteria is desired because bacteria are used as means for both storing and replicating plasmids. Due to that, almost all plasmids carry a bacterial origin of replication as well as an antibiotic resistance gene for use as a selectable marker in bacteria. Bacterial transformation was performed with NEB Turbo competent cells. The procedure started with taking competent cells out of -80 °C refrigerator and 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 30 s was performed. This step is crucial for introducing foreign DNA into bacterial cells. The tubes were again kept on ice for 5 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 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.

3.1.5 Extracting and sequencing of the plasmid DNA

2-4 colonies from the bacterial transformation plate were resuspended in 4 ml of LB media with 100 μ g/ml ampicillin. After 6 to 9 hours of growth in a shaker at 37 °C, NEB Turbo cells were collected by centrifugation. For extracting the plasmid from bacteria FavorPrep™ Plasmid Extraction Mini Kit (Favorgen) was used. The procedures were carried out according to the manual. The concentration of the purified DNA (ng/ μ l) was measured with Thermo Fisher's NanoDrop 1000 Spectrophotometer. To validate the length of the insert and vector backbone, the plasmid was restricted again using Thermo Fisher's FastDigest™ enzymes

and visualized with UV light. For determination of the sequence of the insert, Sanger sequencing was implemented. The sequencing was carried out in the Institute of Genomics Core Facility.

3.1.6 Yeast transformation

First of all, a basis for the desired strains needed to be assembled. For the light-inducible panel, a following strain has been prepared: NLS-NES-(MCM2/3)-mCherry (Liku *et al.*, 2005a) with natNT2 marker was transformed into MÖ546, a strain expressing the light-inducible transcription factor VPEL222 under control of ACT1 promoter and CYC1 terminator in the *LEU2* locus. Consequently, upon insertion of *HIS3* homologous sequences, the Clb5 expression cassettes were transformed into the prepared strain MÖ813 (*LEU2::P_{ACT1}-VPEL222-T_{CYC1}(pDB58) P_{ADHI}::P_{ADHI}-Mcm2/3NLS-NES-mCherry-natNT2*). For the second panel, HA-tagged constructs with estradiol-inducible promoter were transformed into the *HIS3* locus of strain MÖ789, expressing estrogen receptor transcription factor LexA-ER-AD (Ottoz *et al.*, 2014) necessary for estradiol-induced expression of Clb5 (*MATa bar1::hisG trp1::pRS304-ER-TF-TRP1*).

Thirdly, yeast strains expressing the Dna2-based nuclear import modules were obtained by transformation of SgsI linearized Dna2 constructs into strain MÖ172, carrying an Mcm2/3-based NLS-NES module tagged with mCherry (*MATa bar1::hisG P_{ADHI}::P_{ADHI}-NLSmodule(WT)-mCherry-TRP1*); all the strains used in this study are listed in the **Table 2**. Linearization step enables the plasmid to integrate into *HIS3* locus of *S. cerevisiae*. For that, 3-5 µl of purified Dna 2 plasmid (1-2 µg), 1 µl of 10x FastDigest™ buffer, 0.5 µl of FastDigest™ SgsI (Thermo Fisher Scientific) and water up to 10 were mixed in the microcentrifuge tubes and placed into 37°C incubator for 15-30 minutes.

The yeast culture was 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 the 30 °C shaker. When the optical density reached 0.8-1, cells 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. Meanwhile, 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 aforementioned constituents, 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 gentle pipetting. 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 SC-HIS (synthetic complete lacking histidine) glucose plates (7 g/l yeast nitrogen base (BD Biosciences), 1.9 g/l -HIS powder (MP Biomedicals), 20 g/l glucose (Oriola)). The plates were incubated upside down (for 2-4 days) at 30 °C until colonies appeared.

The yeast strains used in this study were constructed by Alissa Agerova, Mihkel Örd, Ilona Faustova, Viacheslav Kiselev, Dmytro Fedorenko, Juli Mukhadze and Avishan Aghayari.

Table 2. Yeast strains used in this study.

Light-inducible (VP-EL222) Clb5 panel:

Strain	Description	Background strain
MÖ813	<i>LEU2::P_{ACT1}-VPEL222-T_{CYC1}(pDB58)</i> <i>P_{ADH1}:: P_{ADH1}-Mcm2/3NLS-NES-mCherry-natNT2</i>	BY4741
MÖ825	<i>his3::P_{EL222}-NLS-Sic1(minWT)-Clb5(dell-100, m1)-Citrine-HIS3</i>	MÖ813
MÖ827	<i>his3::P_{EL222}-Clb5-Citrine-HIS3</i>	MÖ813
MÖ828	<i>his3::P_{EL222}-SV40 NLS Clb5 dell-133 m1-Citrine-HIS3</i>	MÖ813
MÖ829	<i>his3::P_{EL222}-Clb5(NLS-to-NES m1)-Citrine-HIS3</i>	MÖ813
MÖ830	<i>his3::P_{EL222}-Clb5(FL m1)-Citrine-HIS3</i>	MÖ813

β-estradiol-inducible Sic1-Clb5-3HA panel:

Strain	Description	Background strain
MÖ789	<i>MATa bar1::hisG trp1::pRS304-ER-TF-TRP1</i>	w303
MÖ794	<i>his3::P_{LEXA}-SV40 NLS Clb5 del1-133 m1-3HA-HIS3</i>	MÖ789
MÖ795	<i>his3::P_{LEXA}-Clb5 NES-3HA-HIS3</i>	MÖ789
MÖ796	<i>his3::P_{LEXA}-NLS-Sic1(min WT)-Clb5(del1-100, m1)-3HA-HIS3</i>	MÖ789
MÖ806	<i>his3::P_{LEXA}-SV40 NLS Clb5 del1-133-3HA-HIS3</i>	MÖ789
MÖ807	<i>his3::P_{LEXA}-NLS-Sic1(min T33S)-Clb5(del1-100, m1)-3HA-HIS3</i>	MÖ789
MÖ808	<i>his3::P_{LEXA}-Clb5(FL m1)-3HA-HIS3</i>	MÖ789
MÖ809	<i>his3::P_{LEXA}-Clb5(FL NES m1)-3HA-HIS3</i>	MÖ789
MÖ810	<i>his3::P_{LEXA}-Clb5(delN NES WT)-3HA-HIS3</i>	MÖ789
MÖ811	<i>his3::P_{LEXA}-Clb5(delN NES m1)-3HA-HIS3</i>	MÖ789
MÖ812	<i>his3::P_{LEXA}-Clb5(FL WT)-3HA-HIS3</i>	MÖ789
MÖ823	<i>his3::P_{LEXA}-NES-Sic1(min WT)-Clb5(del1-100, m1)-3HA-HIS3</i>	MÖ789
MÖ824	<i>his3::P_{LEXA}-NES-Sic1(min T33S)-Clb5(del1-100, m1)-3HA-HIS3</i>	MÖ789

Phospho-activated Dna2-NLS panel:

Strain	Description	Background strain
MÖ172	<i>MATa bar1::hisG P_{ADHI}::P_{ADHI}-NLSmodule(WT)-mCherry-TRP1</i>	w303
MÖ593	<i>his3::PACT1-Dna2(1-100 SPAQQ)-HIS3</i>	MÖ172
MÖ594	<i>his3::Dna2(1-100 LLTPPRSP N-terminal)-HIS3</i>	MÖ172
MÖ598	<i>his3::Dna2(1-100 LLAPPRAP C-terminal)-HIS3</i>	MÖ172
MÖ600	<i>his3::Dna2(1-100 wt + Sic1 1-130 S69+2R S80+2R RxL2/3)-HIS3</i>	MÖ172
MÖ601	<i>his3::Dna2(1-100 wt + Sic1 1-130 T33+4 S69+2R S80+2R RxL2/3)-HIS3</i>	MÖ172
MÖ607	<i>his3::Dna2(1-100 LLTPPRSP N-terminal T4S)-HIS3</i>	MÖ172
MÖ611	<i>his3::Dna2(1-100 T4S SPAQQ + NPF)-HIS3</i>	MÖ172
MÖ612	<i>his3::Dna2(1-100 LLTPPRSP N-terminal T4S SPAQQ + NPF)-HIS3</i>	MÖ172
MÖ613	<i>his3::Dna2(1-100 SPAQQ + NPF)-HIS3</i>	MÖ172
MÖ614	<i>his3::Dna2(1-100 LLTPPRSP N-terminal SPAQQ + NPF)-HIS3</i>	MÖ172
MÖ615	<i>his3::Dna2(1-100 LLTPPRSP N-terminal SPAQQ)-HIS3</i>	MÖ172
MÖ814	<i>his3::Dna2(1-100 PKI NES)-HIS3</i>	MÖ172
MÖ639	<i>his3::Dna2(1-100 LLTPPRSP C-terminal)-HIS3</i>	MÖ172
MÖ569	<i>his3::Dna2(1-100 wt)-HIS3</i>	MÖ172

3.1.7 Time-lapse microscopy

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 24x50x0.08 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 oven and solidified for 10 minutes at room temperature. For Clb5 induction, CSM agarose with estradiol was prepared. 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. Strains expressing GFP fusion proteins were exposed for 15 ms using a Colibri 470 LED module.

Cyclin constructs fused with yeCitrine (YFP) (P_{EL222}-Clb5-Citrine) were imaged by using Colibri 505 LED for 100 ms. NLS-NES-mCherry imaging was performed using Colibri 555 LED module for 450 ms. The EL222 promoter was induced by using a Colibri 470 LED module for 150 ms and 1 s. Colibri LED modules were used at 25% intensity. For the light induction experiment of VP-EL222 constructs two types of induction settings were used:

- 1) constant induction for studying the effect on cell cycle: phase-contrast (25 ms), 470 nm LED for induction (150 ms), YFP (10 ms), mCherry (450 ms);
- 2) pulses of induction for determining protein levels, as shown in **Table 3** together with excitation wavelengths.

Table 3. VP-EL222 microscopy panel setup. The scheme represents the light induction setup for Pel222 constructs, wherein monitoring was carried out for 8 h in case of constant induction, whereas for the pulse induction yeast cells were monitored for 3 h per block and blue light pulse induction lasted for ~12 s per block (1 s per 12 positions). The table shows the light excitation wavelengths and timing of channels.

Constant Induction (161 cycles)				
Channels	Phase-contrast	mCherry	YFP	GFP (for induction)
Excitation time	25 ms	450 ms	10 ms	150 ms

M Monitoring	P Pulse	M Monitoring	P Pulse	M Monitoring
15 cycles	1 cycle	40 cycles	1 cycle	40 cycles

Block	Channel	Excitation wavelength	Excitation time
M	Phase-contrast		25 ms
M	mCherry	555 nm	450 ms
M	YFP	505 nm	10 ms
P	GFP	470 nm	1 s

The second microscopy panel aimed to investigate the influence of Clb5m1 induction on the nuclear import Dna2-based modules, for which three images were made from each position during each time point: phase-contrast (25 ms), GFP (15 ms) and mCherry (450 ms). Experiment was generally set for 8 hours with the image capturing interval of 3 minutes resulting in 161 time points.

All the obtained pictures were converted to grayscale prior to exporting, cells were segmented and the fluorescence intensities quantified using appropriate ready-made MATLAB programs (Doncic *et al.*, 2013).

3.1.8 Flow cytometry

The effect of induction of different Clb5 variants on the cell cycle was analyzed by using DNA labeling and fluorescence-activated cell sorting (FACS) analysis. For that, yeast cells were grown 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)). First, cells were grown until an OD of 0.3 - 0.7 was reached, following with 250 nM estradiol induction for 3h in 2 ml CSM. Next, the samples with the OD of 0.5 to 1 were pelleted by centrifuging at 13000 rpm for 30 s, the supernatant was removed and the cell pellet resuspended in 70% ethanol for fixation overnight at 4°C. Then, the cells were vortexed vigorously, washed with 1 ml of 50 mM Tris-HCl (pH 8) and pelleted again. The pellet was resuspended in 200 µl of 50 mM Tris-HCl as well as 0.5 µl of 10 mg/ml Rnase A (Thermo Fisher Scientific) and the samples were vortexed. After sonication for 5 s at 25% intensity, the samples were incubated at 37°C for 1 h. The cells were then pelleted and washed once with 1 ml of 50 mM Tris-HCl and 0.5 µl of 20 mg/ml Proteinase K (Thermo Fisher Scientific). The samples were dyed with 50 µl of 1:1000 dilution of Invitrogen™ SYBR™ Green I in 50 mM Tris-HCl followed by incubation for 15 minutes at room temperature in dark. Yeast cells were pelleted again and resuspended in 300 - 1000 µl (depending on the amount of cells) of 50 mM Tris-HCl. 200 µl of each sample mixture was moved to 96 well plate for analysis, which was carried out with Attune™ NxT Flow Cytometer. The probes were read using approximate dilutions in Tris-HCl to achieve ~1000 events per second.

3.2 RESULTS AND DISCUSSION

As it was aimed to design the cell cycle independent Cdk1 input, three different panels of constructs were created. Firstly, it was studied on the basis of Dna2 import modules by including bipartite NLS, putative NES motifs, and phosphodegrons. The effect of differential phosphorylation on protein localization dynamics was investigated using time-lapse fluorescence microscopy, during which the intensity of nuclear and cytoplasmic GFP fluorescence was measured. For the rapid regulation of input expression of various Clb5 mutants VP-EL222 light-inducible system was used. To test the effect of overexpression of Clb5 mutants on DNA replication and the cell cycle, estradiol-regulated promoter was induced, and its effect on the cell cycle was monitored using flow cytometry. Additionally, the degradation profiles of Dna2 and Clb5 versions fused with Sic1 mutants were also tested.

3.2.1 Phospho-activated nuclear import of the Dna2 module

Phosphorylation of the bipartite NLS sequences inside Dna2 leads to its nuclear import. However, an earlier study has found Dna2 localized in cytoplasm during G1 and in nucleus during S and M phases, demonstrating that Dna2's cytoplasmic localization is G1 specific (Kosugi *et al.*, 2009).

This study uses the intrinsically disordered Dna2 region containing 1-100 amino acids of the N-terminus as a basis module for Cdk1-activated nuclear import. Previous experiments have proven that this fragment is sufficient to enable regulated localization as shown in the unpublished data by Irina Borovko. Two overlapping bipartite NLS motifs and two Cdk1 phosphorylation sites are found in the Dna2 1-100 segment (**Figure 8A**): T at the 4th position and S at the 17th position. Both sites are optimal Cdk1 targets due to the presence of a K positioned 3 amino acids (+3) after the priming sites. Past work demonstrated that mutation of a serine to alanine (S17A) results in a loss of phosphorylation and, thus, solely cytoplasmic localization of Dna2 (Kosugi *et al.*, 2009).

The linker region contains an optimal phosphorylation site S17 (SPAKK) in the wild type module of Dna2, which is needed for the activation of NLS located downstream. However, in previous work the K21A mutation did not influence the nuclear localization (Kosugi *et al.*, 2009). The first aim was to obtain a phospho-localization module that was unaffected by native cell cycle machinery. For this, both lysinies following S17 were mutated into glutamines leading to suboptimal consensus site SPAQQ (**Figure 8A**) with the disrupted middle region of NLS. Also, the NPF motif that is known to promote phosphorylation specifically by Clb5-Cdk1 was added to the C-terminal domain of Dna2.

To analyze the phosphorylation-dependent degradation of the Dna2 modules during the cell cycle, an Mcm2/3-based NLS-NES module tagged with mCherry was used as a reference point for cell cycle progression, as its nuclear export denotes the G1/S switch (Liku *et al.*, 2005b). The time-lapse microscopy provided a visual understanding of how the GFP (Dna2-based constructs tag) and mCherry nuclear signals appear over the cell cycle (**Figure 8B**). 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 Mcm2/3-NLS-NES-mCherry sensor is exported from nucleus to cytoplasm. As the cells in the experiment were growing asynchronously, the nucleocytoplasmic shuttling of Mcm2/3-NLS-NES-mCherry was also monitored and used to synchronize the cell cycles of individual cells during the analysis.

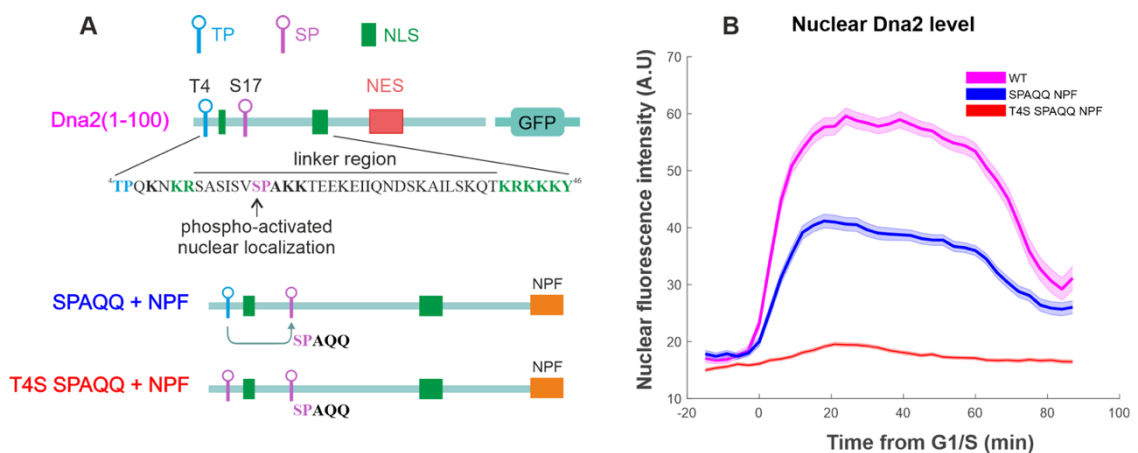


Figure 8. Mutations of optimal Dna2 motifs leads to stability of the constructs also in the presence of NPF motif. (A) Schemes showing wild type Dna2(1-100) and two mutants: Dna2(1-100)-SPAQQ-NPF, Dna2(1-100)-T4S-SPAQQ-NPF. (B) The plot demonstrating the levels of the constructs from B. Time 0 stands for G1/S transition, which is determined by nuclear export of 50% of Mcm2/3-NLS-NES-mCherry Cdk1 activity sensor.

Compared to the wild type, the nuclear accumulation of SPAQQ + NPF variant (**Figure 8A**) was much lower, as the maximal nuclear fluorescence intensity was nearly twice weaker (**Figure 8B**). As T4 is positioned 13 amino acids upstream from S17, it is highly likely that upon phosphorylation of T4 it binds to the phosphate-binding pocket of Cks1 increasing the binding affinity and promoting phosphorylation of S17. To disrupt the Cks1 docking interaction, T4 was mutated to serine, as Cks1 recognizes only phosphothreonines (Kõivomägi *et al.*, 2013b) (**Figure 8A**). As a result, an expectedly lower nuclear intensity

was achieved (T4S + SPAQQ + NPF) compared to the other Dna2 versions, whereas the time of reaching the maximal nuclear intensity is almost identical to the wild type. As illustrated, the mutant containing both mutations is the most stable and, therefore, the most independent of the cell cycle progression. Thus, the Dna2(1-100)-T4S+SPAQQ+NPF module is largely unaffected by cell cycle even with the NPF motif, likely because the Clb5 cyclin that docks to NPF motifs is localized to the nucleus.

3.2.2 Combining cyclin E degron with Dna2 leads to stable levels of Dna2 constructs

One possibility to achieve regulated degradation and different protein expression levels is to fuse phosphodegrons to the proteins of interest, targeting them for degradation. The degradation mechanism of nuclear phosphodegrons via SCF^{Cdc4} is better understood than the cytoplasmic degradation driven by SCF^{Grr1}. Therefore, within this study it was desired to induce phosphodegron degradation by regulated nuclear import. For that purpose bipartite Dna2 NLS module was used, which translocates from cytoplasm to the nucleus, where it is targeted for SCF^{Cdc4} specific degradation.

A modified version of mammalian cyclin E phosphodegron (LLTPPRSP) was tested in the N-terminus as well as the C-terminus of the Dna2(1-100) wild type module (**Figure 9A**). The inactivated degron (LLAPPRAP) was also used, serving as the control for the active degron. As shown (**Figure 9B**) the nuclear concentration of the construct with C-terminal degron was the lowest, as the lack of nuclear accumulation indicates that it is degraded as soon as it is imported. The N-terminal degron also exhibited higher rate of degradation compared to the inactivated controls and wild type, however, its accumulation in nucleus at the start of DNA replication is still noticeable. Analysis of the total GFP concentration in the cell showed much lower level of the module with the C-terminal degron and a slightly lower level of the construct with the N-terminal degron compared to the controls (**Figure 9C**). These results show that the phospho-localization and degradation modules can be combined to achieve controlled degradation of cytoplasmic proteins by a two-step mechanism. First, phosphorylation of the Dna2 module triggers the nuclear import of the protein and then the cyclin E based degron module mediates degradation by SCF^{Cdc4}.

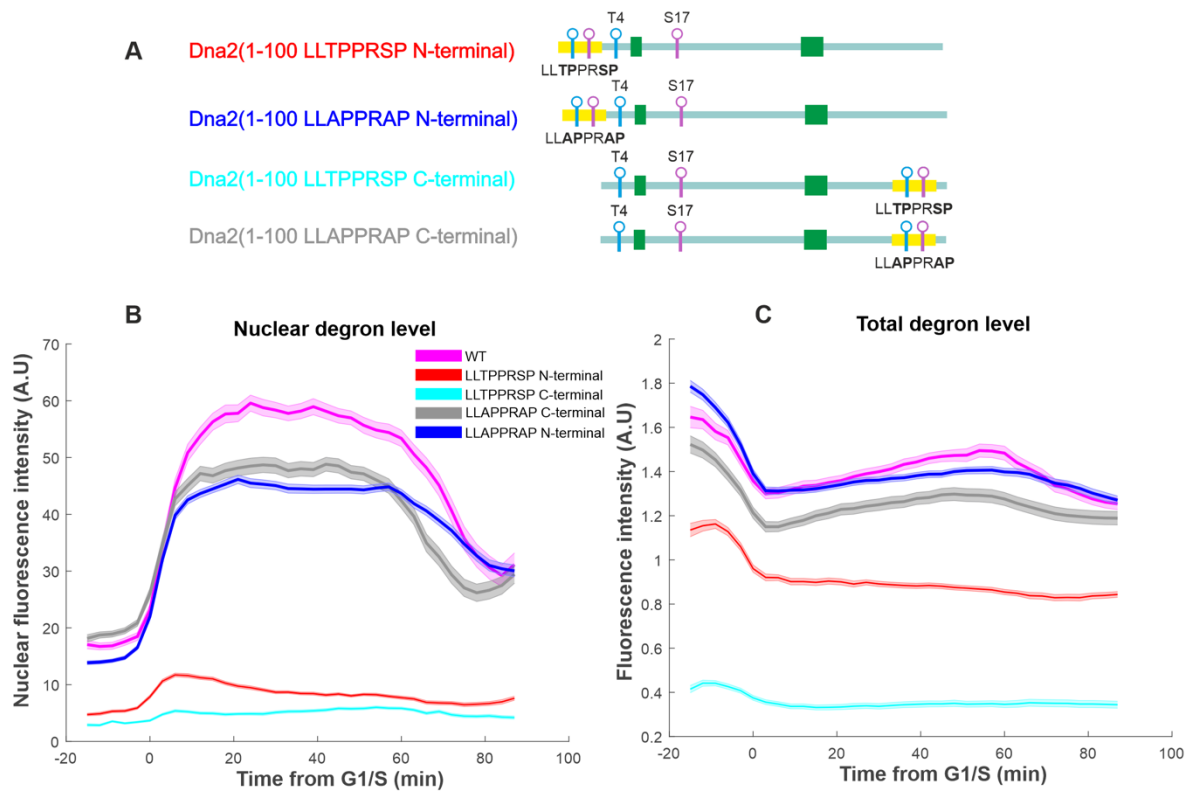


Figure 9. Fusion of phospho-localization and degradation modules enables rapid degradation of cytoplasmic proteins. (A) The scheme showing activated (LLTPPRSP) and inactivated (LLAPPRAP) N-terminal degnon mutants; activated and inactivated C-terminal degnon mutants. (B) Graph showing nuclear fluorescence intensity of the wild type and degnon constructs in unperturbed cell cycle. Time 0 stands for G1/S transition, which is determined by nuclear export of 50% of Mcm2/3-NLS-NES-mCherry Cdk1 activity sensor. (C) Plot showing total cellular fluorescence intensity of the constructs in unperturbed cell cycle.

3.2.3 Sic1 fusions with Dna2 result in slow degradation

Sic1 possesses two suboptimal di-phosphodegnon motifs that facilitate Cdc4 binding (Kõivomägi *et al.*, 2011a; Nash *et al.*, 2001). Phosphorylation of two di-phosphodegrons in Sic1 (sites T45–T48 and S76–S80) is a vital requirement for Sic1 degradation (**Figure 10A**). While the cyclin E degnon is an optimal and rapid degnon, the Sic1 degrons have been designed with many different phosphorylation thresholds (Örd *et al.*, 2019). Combination of the Dna2 localization modules and Sic1-based degnon modules, that are activated at higher Cdk thresholds could result in nuclear accumulation of the protein in a short time period in the cell cycle. To test their impact on degradation of Dna2 constructs, two versions of Sic1 were used in combination with Dna2(1-100) (**Figure 10B, 10C**). The first mutant includes

Sic1(1-130) with two RXL motifs facilitating S-cyclin binding. The second mutant also included the additions of 4 extra amino acids downstream of the Cks1 priming site T33 (T33+4) on Sic1, to decrease the phosphorylation rate of the di-phosphodegrons (Örd *et al.*, 2019).

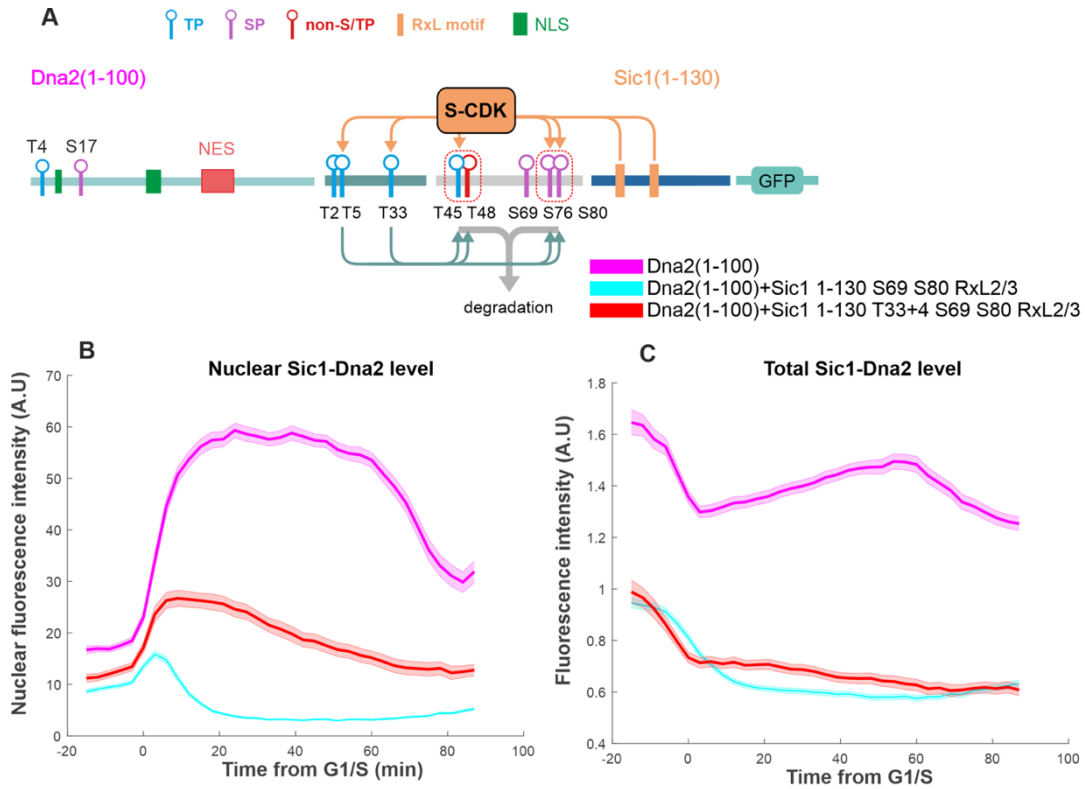


Figure 10. Increasing the distance between T33 and the di-phosphodegrons lead to the slower rate of phosphorylation. (A) The scheme explaining the fusion constructs of Sic1(1-130) to Dna2(1-100). (B) The graph demonstrating nuclear Sic1-Dna2 levels on the example of wild type Dna2, and two Sic1-Dna2 fusions. (C) The plot showing total cellular concentration of the constructs.

As the graph demonstrates degradation with fused Sic1s is slower (**Figure 10B**) than the previous example with cyclin E degrons (**Figure 9B**). The tendency of Dna2-Sic1 constructs to accumulate in the nucleus is clearly seen immediately after the G1/S (**Figure 10B**). The first mutant exhibited smaller nuclear accumulation and faster degradation compared to the second mutant, that was degraded considerably slower. This highlights the influence of the increased distance (T33+4) on Sic1-Dna2 degradation (**Figure 10B**). As shown in **Figure 10C**, the difference between the degradation of wild type Dna2 module and of Sic1-Dna2 constructs is significant. It is clearly seen that at G1/S the cyclins start degrading Sic1-Dna2

construct, whereas the wild type Dna2 after G1/S oscillates in an opposite manner compared to the degron modules. This data shows that different kinase activity thresholds can be encoded into a single protein and highlights the versatility of Cdk1-regulated phosphorylation switches.

3.2.4 Optimized NES sequence decreases the nuclear accumulation of Dna2

To decrease the background level of nuclear accumulation as observed with previous examples (**Figure 8B, 9B, 10B**) an optimized NES sequence (PKI NES) was introduced to the place of the putative NES of Dna2 (**Figure 8A**). This optimized NES motif is known to facilitate the rapid nuclear export (Wen *et al.*, 1995). Therefore, two constructs were created: the Dna2(1-100) with optimized NES (in magenta); Dna2(1-100) with N-terminal degron LLTPPRSP, suboptimal S17 (SPAQQ) and optimized NES (in blue), as shown in **Figures 11A, 11B, 11C**.

The optimized NES motif strongly decreases the background nuclear level compared to the wild type Dna2(1-100) (**Figure 11A**). A decrease in nuclear level was also seen in G1, indicating that the wildtype module is not fully exported during G1. To further stabilize Dna2 degradation the N-terminal degron with suboptimal SPAQQ and optimized NES sequence was also tested. As illustrated in **Figure 11B** compared to the optimized NES Dna2 construct (PKI NES) addition of degron decreased the level nearly 3 times, whereas compared to the wild type the protein level was nearly 8 times smaller in case of the degron mutant. This indicates that it is possible to decrease the nuclear accumulation of the Dna2 module by introducing an optimal NES.

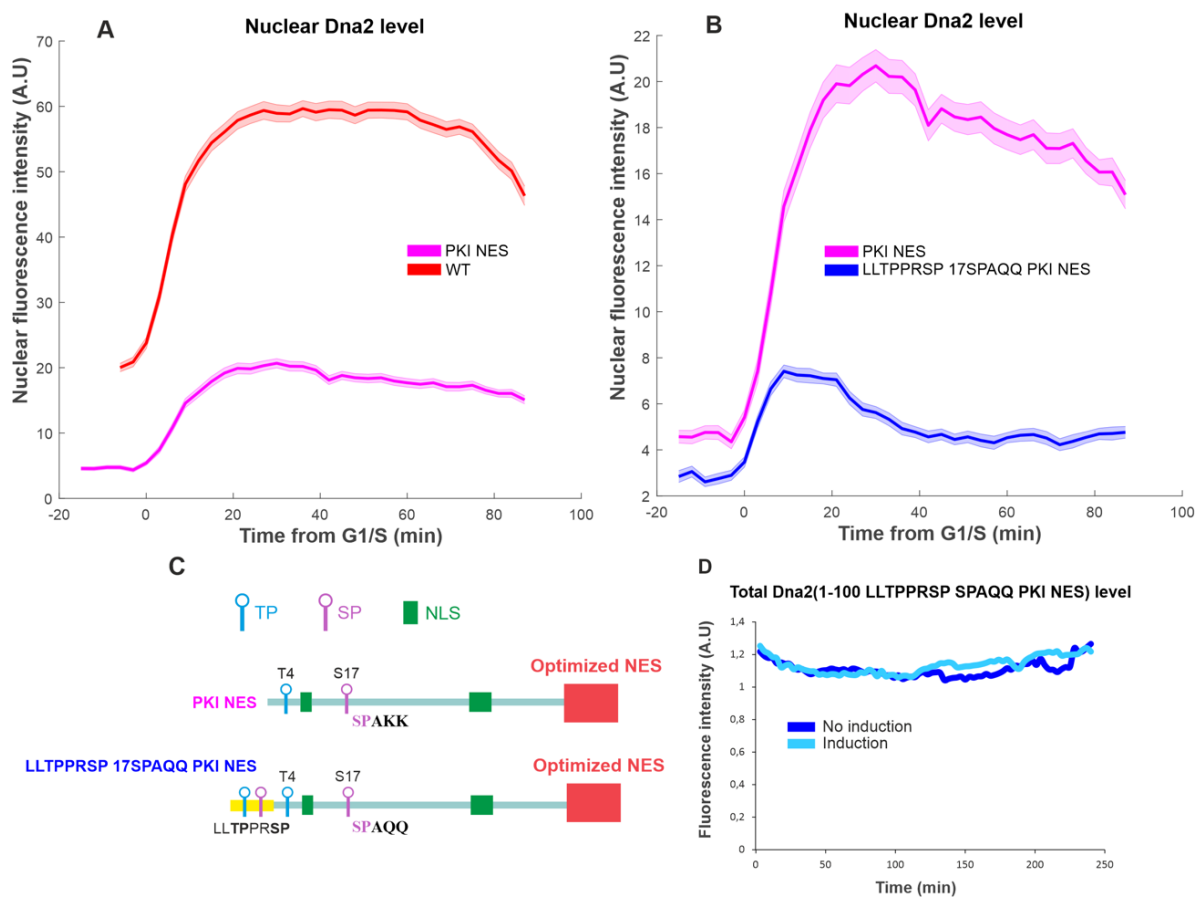


Figure 11. Addition of the optimized NES sequence decreases the nuclear accumulation. (A) The plot showing the nuclear levels of Dna2(1-100 PKI NES) and Dna2(1-100), termed as WT. (B) The plot showing the nuclear levels of Dna2(1-100 PKI NES) and Dna2(1-100 LLTPPSR 17SPAQQ PKI NES). (C) The scheme illustrating the constructs plotted in A, B, D. (D) The graph demonstrating levels of Dna2(1-100 LLTPPSR 17SPAQQ PKI NES) upon Clb5m1 induction.

To test the effect of induced Clb5 expression on the degradation of Dna2(1-100 LLTPPSR 17SPAQQ PKI NES), a microscopy experiment with and without NES-Clb5m1-mCherry induction was carried out. For that the Clb5 with mutated hydrophobic patch was used (termed Clb5m1), which is expressed under control of the β -estradiol-inducible promoter P_{LEXA} . As shown in **Figure 11D** no significant effect on the level of the Dna2 construct was detected between uninduced and induced cells. This supports the belief that in the absence of NPF motif Clb5m1 induction does not influence the degradation of constructs, as there are no motifs to bind to Clb5m1 to facilitate degradation, therefore such Clb5-Cdk1 activity can be used for orthogonal signaling.

3.2.5 Rapid regulation of input expression

Inducible promoter systems are vital for various applications in biological research (Motta-Mena *et al.*, 2014). Nevertheless, a huge drawback of these tools often is their dependence on small-molecule inducers as it limits their efficacy when localized induction is aspired (Motta-Mena *et al.*, 2014). Compared to that, light is harmless and relatively fast stimulus that is able to control diverse cellular processes under different conditions (Briggs and Spudich, 2005). That is why, for the rapid regulation of input expression the blue light inducible promoter complex using a small (222-residue) bacterial transcription factor (TF) EL222 (Nash *et al.*, 2011) was used in this work (**Figure 12A**).

To test the effect of overexpression of different Clb5 mutants on DNA replication and the cell cycle two methods were used: time-lapse fluorescence intensity and flow cytometry. First of all, it was desired to check how different Clb5 variants are expressed during the cell cycle, using microscopy and a cell cycle reference sensor based on Mcm2/3 helicase. As the level of wild type Clb5 is the lowest in late G1, the time 0 in **Figure 12C** is taken closer to the late G1 - when 50% of Mcm2/3-NLS-NES-mCherry Cdk1 activity sensor is imported into the nucleus (**Figure 12D**).

3.2.6 Clb5 constructs designed for rapid regulation of input expression

Five different Clb5 mutants were studied using the light-inducible gene expression system (**Figure 12B**). Firstly, the wild type full length Clb5 (Clb5 FL WT) that includes the APC degron, NLS motif and native cyclin domain. Based on that the full length Clb5 was created with mutated hydrophobic patch (Clb5 FL m1) to reduce interaction between the Cdk1 complex and its specific substrates. Because full-length Clb5 is degraded by APC^{Cdc20} in mitosis (Shirayama *et al.*, 1999), it cannot be used for kinase signaling in G1. To address this problem, the N-terminal intrinsically disordered domain of Clb5 was truncated, as it contains the APC degron and fused to the minimal Sic1(min WT)-based phospho-degron module. This was done with the hypothesis that Clb5-Cdk1 would phosphorylate the di-phosphodegron of Sic1 in the fusion protein, resulting in cell cycle independent auto-regulation of Clb5 expression level. Also, the similar Sic1(min)-Clb5 mutant was designed, but with T33S mutation to disrupt the Cks1 docking and potentially partially stabilize the degron.

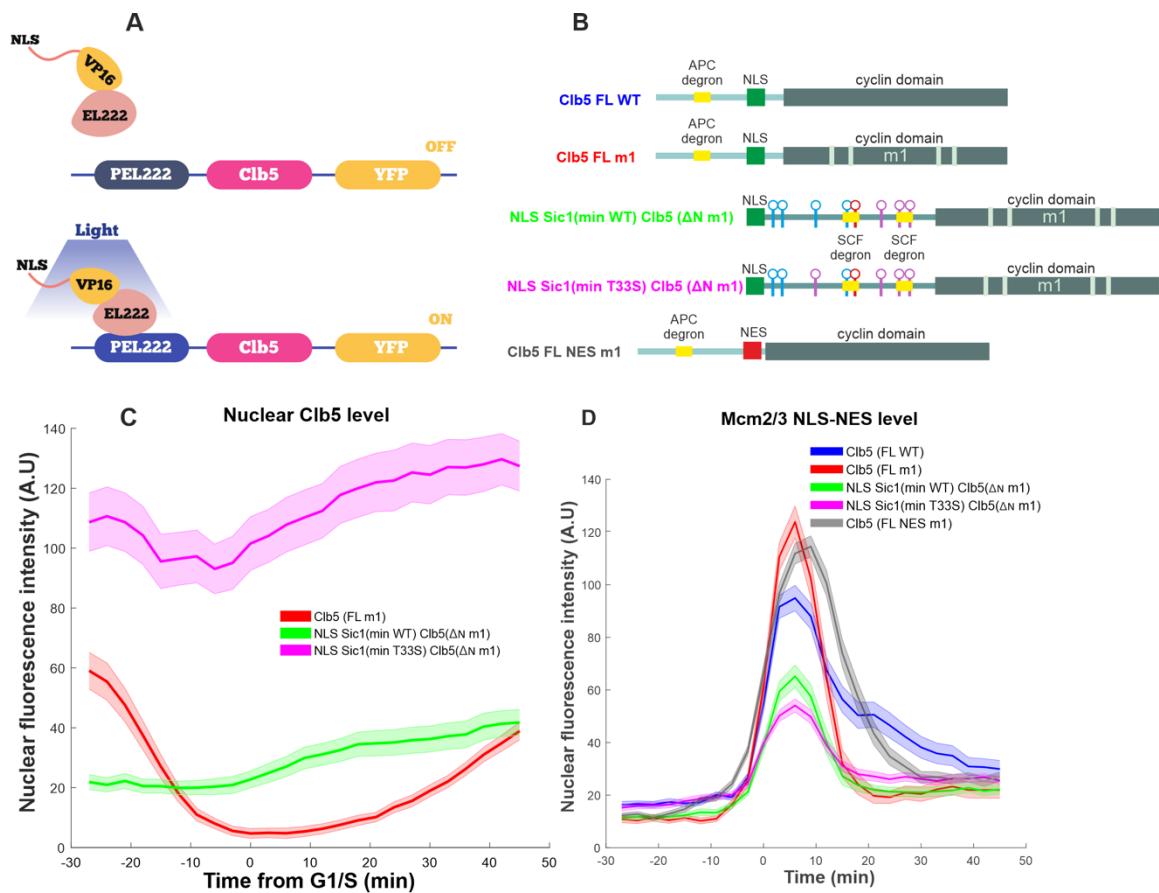


Figure 12. EL222-based light-inducible gene expression system. (A) In the dark, VP-EL222 is unable to bind to the TF binding site located a few amino acids before promoter, whereas exposure to blue light (450 nm) allows EL222 dimerization and binding to the TF binding site resulting in activation of promoter region and the following gene transcription. (B) The scheme illustrating Clb5 mutants used in C and D. (C) The graph showing expression profiles of different Clb5 constructs under constant induction by blue light. The cells are synchronized by the point of Mcm2/3-mCherry sensor nuclear import, indicating G1 entry. (D) The plot showing Mcm2/3-NLS-NES level at time 0, which is used as a reference point for G1/S switch in C.

3.2.7 Effect of the constant Clb5 induction on protein levels

To test the constant induction on the degradation profile the constructs were exposed to 150 ms of blue light every 3 minutes for 8 hours. It can be clearly seen (**Figure 12C**) that Clb5 (FL m1) mutant exhibits the fastest degradation as it incorporates APC recognition motifs that are targeted by ubiquitin ligase complex APC. NLS Sic1-Clb5 fusions, on the other hand, include the deleted N-terminus from Clb5, therefore there are no degrons to be targeted and the levels are high and, indicating its independence of the Cdk1 activity. The Sic1-Clb5 variant with T33S shows the highest level as the rate of phosphorylation is very low due to

the suboptimal Cks1 priming site. The slight decrease at G1/S occurs due to division of the nucleus. This data shows that by replacing the APC degron in Clb5 with the SCF^{Cdc4} degron from Sic1, Clb5 can be expressed constitutively throughout the cell cycle, enabling CDK signaling in G1 as well. Also, manipulation of the Sic1 degrons in the Sic1-Clb5 fusion proteins allows tuning of the Clb5 expression level.

Clb5 phosphorylates Mcm2/3-NLS-NES and as a result it exits the nucleus, thus mutants with higher Clb5 level in G1 demonstrate elevated Mcm2/3-NLS-NES levels (**Figure 12D**). The Mcm2/3-NLS-NES levels of Sic1-Clb5 fusions are notably lower, because shortly after G1/S the SCF degrons of Sic1 are being targeted and constructs degraded.

3.2.8 Pulse induction of Clb5 mutants illustrates accumulation dynamics

Two blue light pulses for activation of the VP-EL222 promoter were made for 10s: at 90th minute and at 330th minute. As shown in **Figure 13** Clb5-Citrine levels are low before the pulse induction, after what the levels increase and before the second pulse their levels decrease to the initial level due to Clb5 degradation. The second pulse illustrates even more effective dynamics of the Clb5 level magnification especially in the cases of Clb5 (FL m1) and NLS Sic1(min T33S)-Clb5(Δ N m1). However, NLS Sic1(min WT)-Clb5(Δ N m1) level is relatively low throughout the experiment. This can be explained by the fact that its levels are low due to higher phosphorylation rate compared to the T33S mutant.

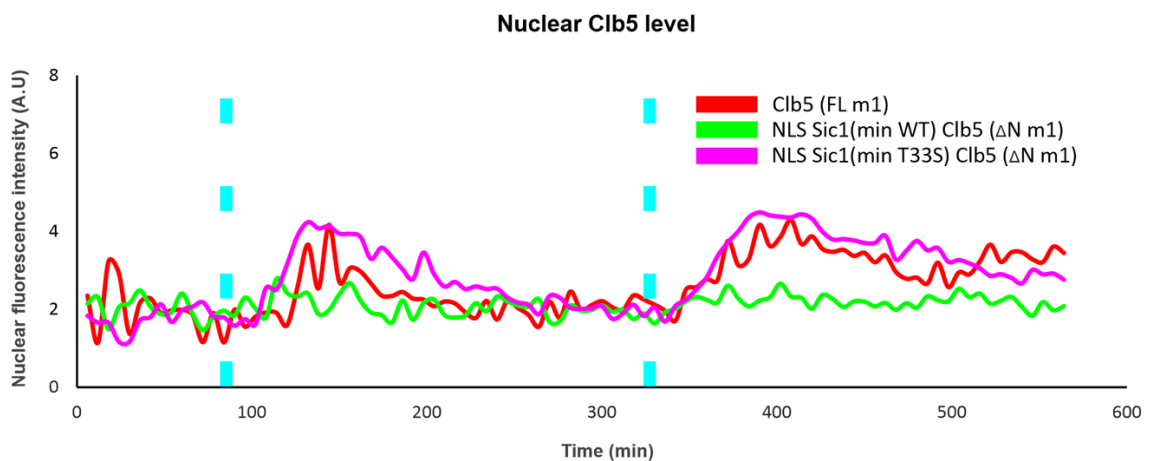


Figure 13. Influence of pulse induction on Clb5 levels. The graph showing levels of 3 different Clb5 mutants in an 8 hour time-lapse microscopy experiment, where 10s long pulses were made at 90th minute and at 330th minute.

3.2.9 Optimal Clb5-Cdk1 input does not affect the cell cycle

To test the influence of Clb5 overexpression in various mutants on the cell cycle, Clb5 expression was induced from estradiol-regulated promoter for 3 hours and the effect on the cell cycle was monitored using SYBR-Green staining and flow cytometry (**Figures 14, 15**).

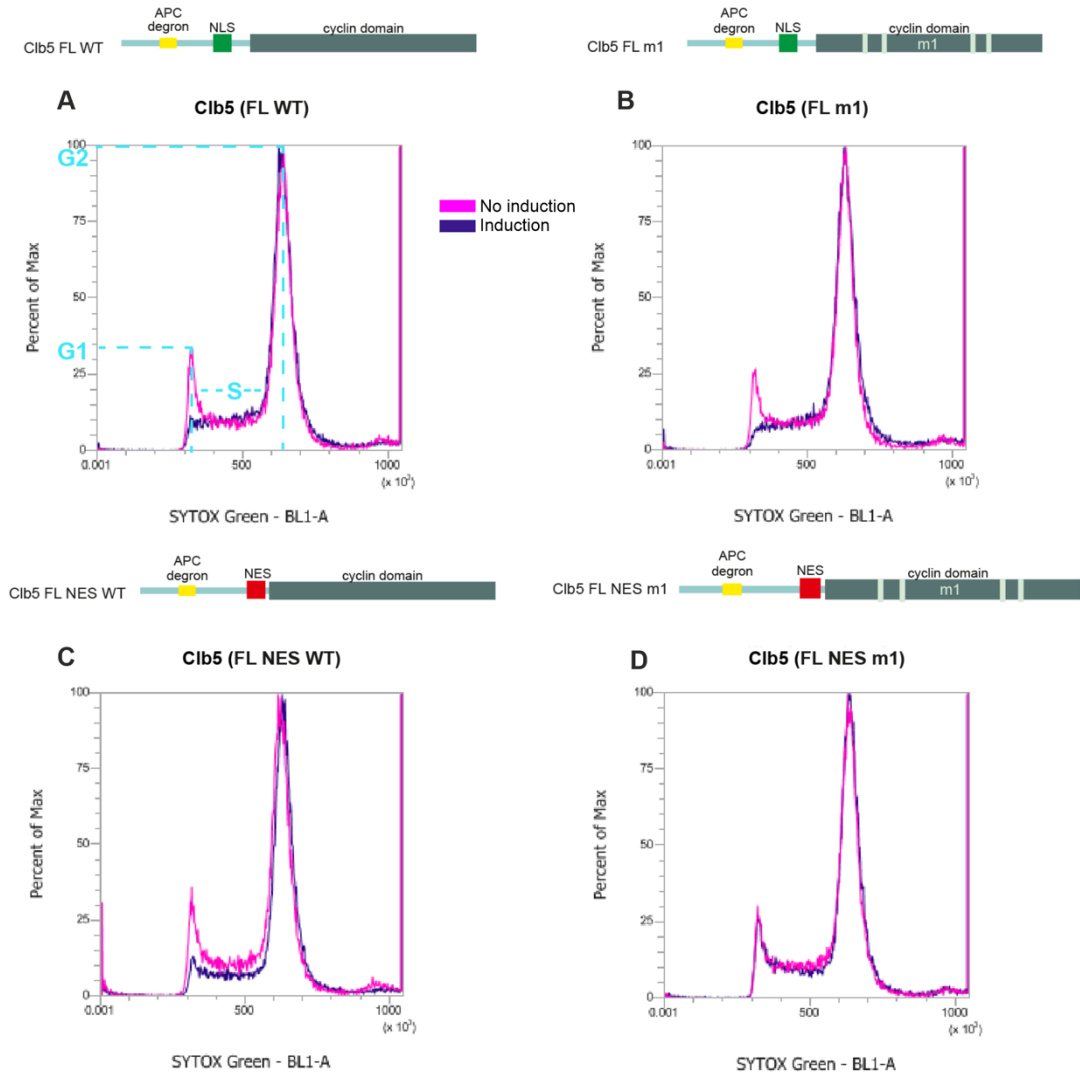


Figure 14. Expression of cytoplasmic Clb5 has lesser impact on cell cycle progression compared to nuclear Clb5. The graphs showing the effect of overexpression of different Clb5 overexpression on yeast cell cycle. The histogram represents the content of DNA along the x-axis and the fraction of cells detected at that intensity along the y-axis.

Induced overexpression of wild type Clb5 resulted in a decrease in G1 cells and increase in G2 cells and a similar effect was detected with Clb5m1 mutant (**Figure 14A, 14B**). Next, the cytoplasmic Clb5 was created by replacing the native NLS with a NES. Interestingly, while a decrease in G1 cells was detected with wild-type NES-Clb5 overexpression, no decline occurred in the culture where NES-Clb5m1 was overexpressed (**Figure 14C, 14D**). This suggests that cells are less sensitive to increased Clb5 activity in the cytoplasm, which might be because most of the substrates related to DNA replication are localized in the nucleus. Because the Cdk1 background activity in cytoplasm is around 10 times weaker than in nucleus it is more convenient to create orthogonal signaling networks that operate in cytoplasm. Also, most metabolic proteins are localized to the cytoplasm, creating a demand for phospho-regulation opportunities in the cytoplasm.

As in the light induction panel, the effect of overexpression on NLS Sic1(min WT) and NLS Sic1(min T33S) fused to the truncated Clb5 Δ Nm1 were also tested (**Figure 15A, 15B**). It is clearly seen that the amount of cells of NLS Sic1-Clb5 fusion was very small in G1, indicating that these constructs are very sensitive to increased Clb5-Cdk1 activity in the nucleus. This could be due to their high level in G1 (Figure 12C) and because the Clb5m1 is not inhibited by Sic1. Corresponding to the data obtained in **Figure 14C, 14D** replacement of the NLS with NES led to an unaffected cell cycle, emphasizing the benefit of using cytoplasmic constructs for orthogonal signaling. Therefore, the data obtained from the Mcm2/3-NLS-NES plot supports the data obtained from the flow cytometry analysis (**Figures 14, 15**).

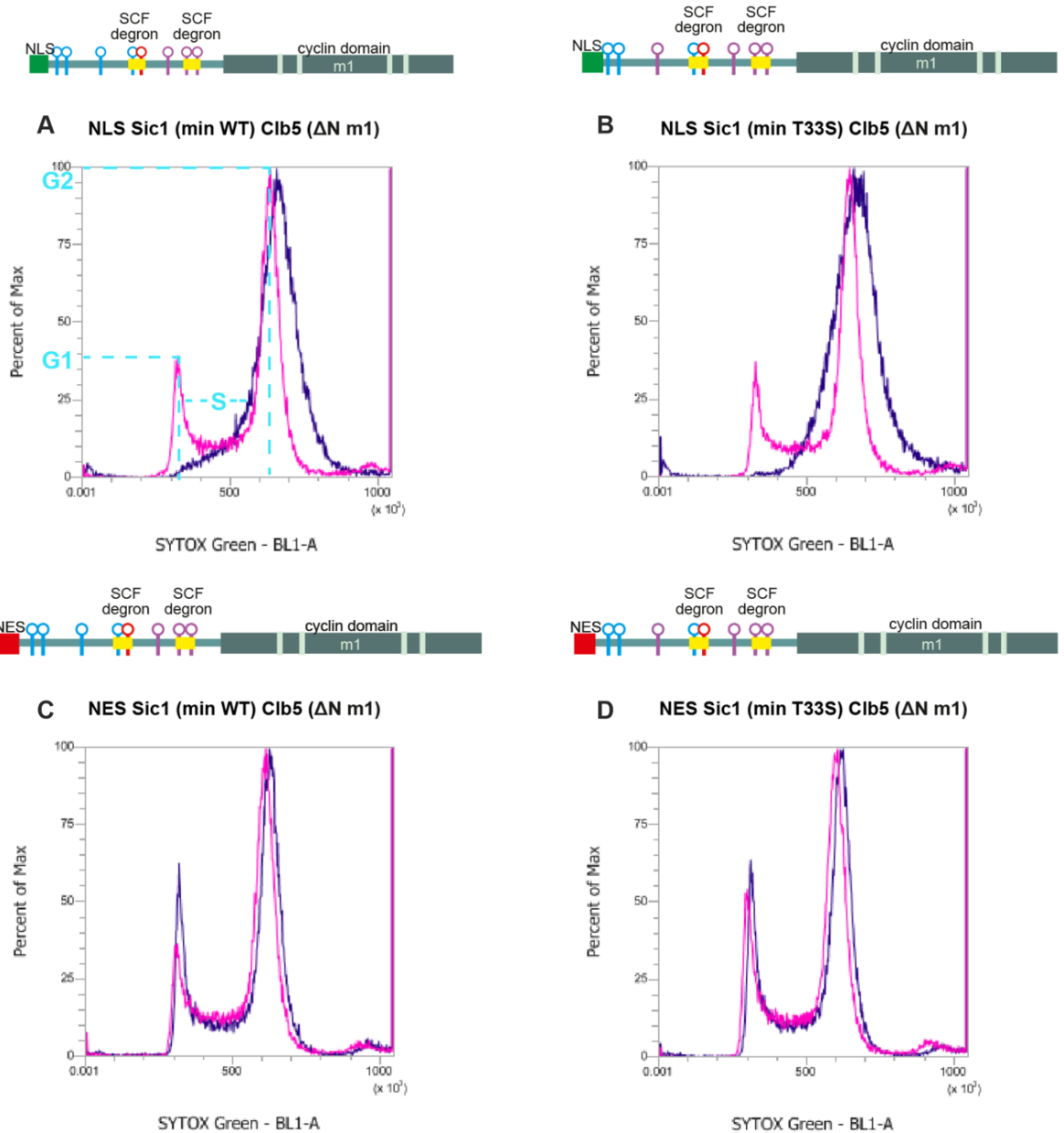


Figure 15. Expression of cytoplasmic Sic1-Clb5 has lesser impact on cell cycle progression compared to nuclear Sic1-Clb5. The graphs showing the effect of overexpression of different Sic1-Clb5 fusions on yeast cell cycle. The histogram represents the content of DNA along the x-axis and the number of cells detected at that intensity along the y-axis.

SUMMARY

Cell cycle independent signaling by Cdk1 requires phosphorylation modules that are unaffected by the cell cycle and a kinase input that does not disrupt cell cycle progression. In this work phospho-localization modules that are unaltered by the endogenous cell cycle machinery were designed. These modules were based on the phospho-activated nuclear localization signal in Dna2. The decreased Cdk specificity could be rescued by addition of cyclin docking motifs and it was found that the modules were constantly cytoplasmic even in the presence of Clb5-Cdk1 specific NPF motif. This is likely because wild type Clb5 is localized in nucleus and it lays the basis for regulation by modified Clb5-Cdk1.

Precise enzyme abundance is important for production in cell factories. However, controlled stability of cytoplasmic proteins is limited due to lack of well characterized degrons. For this reason, the nuclear import modules were combined with nuclear degrons, thus allowing a two-step degradation mechanism. Fusion of the Dna2 module with cyclin E degron resulted in rapid protein degradation upon nuclear import. In contrast, coupling Dna2 localization modules with Sic1-based degrons that are phosphorylated less efficiently lead to longer period of nuclear accumulation. Thus, multiple Cdk activity thresholds can be encoded into a protein, highlighting the many possibilities to create versatile phosphorylation switches for synthetic biology applications.

The modified Clb5 mutant (Clb5m1) has shown to possess the properties necessary for cell cycle independent signaling: it lacks binding of S-Cdk1 targets and it is not inhibited by Sic1. Cdk activity is regulated by cyclin degradation, and to achieve a stable kinase input, the degradation of Clb5 was manipulated. Substitution of the APC degron with the constantly active SCF degron resulted in Clb5 constructs that were stably expressed throughout the cell cycle, but at different levels. To study the impact of these Clb5 variants on the cell cycle, the effect of their overexpression on cell cycle distribution was analyzed in flow cytometry. These experiments revealed that while nuclear Clb5 variants had a greater effect in triggering DNA replication, the cytoplasmic cyclins had lower impact. Importantly, the cytoplasmic Clb5m1 variants did not affect the cell cycle progression, showing that they function as orthogonal cyclin-Cdk1 complexes. When combined with the described phospho-localization and -degradation modules, this approach could allow precisely controlled activities of multiple cytoplasmic proteins. Therefore, such approach can be used for optimization and development of phospho-regulation mechanisms to target various metabolic proteins as they are mostly found in cytoplasm.

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