UNIVERSITY OF TARTU
Faculty of Science and Technology
Institute of Technology

Valentina Mikhailova

Analysis of the docking interaction between Sld2 and Clb5 in budding yeast

Master's Thesis (30 ECTS)

Curriculum Bioengineering

Supervisor:
MSc, Ervin Valk

Tartu 2021

Analysis of the docking interaction between Sld2 and Clb5 in budding yeast

Abstract:

The cell cycle of *Saccharomyces cerevisiae* is governed by cyclin dependent kinases (Cdks), which are activated by periodically synthesized and degraded cyclins. The substrate targeting by a cyclin-Cdk complex is mediated by the active site on the Cdk and the cyclin docking pocket. Docking interactions between short linear motifs (SLiMs) in target proteins and cyclin-dependent kinases are shown to be critical regulators of different cell cycle events. SLiMs function in almost every pathway due to their role in regulatory function and signal transduction.

S-phase Cdk specificity is known to be governed by RxL motifs with the consensus R/Kx-L-F/L/M/P or R/K-x-L-x-F/L/M/P sequence.

In this work we analyzed the docking interaction between the cyclin Clb5 and the Clb5-specific substrate Sld2 in Saccharomyces cerevisiae at the G1/S stage. It was revealed that the Sld2 substrate targeting by Clb5-Cdk1 complex in the early S phase was mediated not only by conventional cyclin docking motifs. We analyzed Sld2 specificity using both *in vivo* and *in vitro* methods, and specified the precise location of the potential docking site.

Keywords:

Budding yeast, cell cycle, Cdk, Clb5, SLiM, Sld2

CERCS:

Sld2 ja Clb5 vahelise dokkimise vastasmõju analüüs tärkavas pärmis

Lühikokkuvõte:

Pagaripärmi rakutsüklit juhib tsükliinsõltuv kinaas (Cdk1), mis aktiveeritakse perioodiliselt sünteesitavate ja lagundatavate tsükliinide poolt. Tsükliin-Cdk1 kompleksi poolt substraadi ära tundmine on vahendatud Cdk1 aktiivsaidi ja tsükliini poolt. Taolised tsükliini ja sihtmärkvalgus leiduvate lühikeste lineaarmotiivide vahelised interaktsioonid on olulise tähtususega erinevate rakutsükli jooksul aset leidvate sündmuste reguleerimisel. S-faasi tsükliinid kasutavad selleks R/K-x-L-F/L/M/P or R/K-x-L-x-F/L järjestusega motiive. Käesolevas töös analüüsiti, millised teised lühikesed lineaarmotiivid peale eelpool mainitute

tagavad Clb5-Cdk1 kompleksti poolt valgu Sld2 ülimalt spetsiifilise fosforüleerimise S-faasi alguses. Kasutades nii in vitro kui in vivo lähenemist kirjeldati käesoleva töö raames uudne tsükliin Clb5 poolt spetsiifiliselt ära tuntav järjestus Sld2 valgus.

Võtmesõnad:

Pagaripärm, rakutsükkel, Cdk, Clb5, SLiM, Sld2

CERCS:

TABLE OF CONTENTS

TERMS, ABBREVIATIONS AND NOTATIONS

AA – amino acid

ATP – Adenosine triphosphate

Cdk – Cyclin-dependent kinase

DMSO – Dimethyl sulfoxide

SCF – Skp, Cullin, F-box containing complex

EGFP – Enhanced green fluorescent protein

IPTG – Isopropyl β- d-1-thiogalactopyranoside

LB – Luria-Bertani media

NLS – nuclear localization signal

ORC – origin recognition complex

RxL – Short linear Clb5-docking motif with consensus sequence of R/Kx-L-F/L/M/P or R/K-x-L-x-F/L/M/P

pSIC1 – Sic1 promoter

SLiM – short linear motif

SS-DNA – Salmon sperm DNA

WT – wild type

INTRODUCTION

The cell cycle of *Saccharomyces cerevisiae* is governed by cyclin dependent kinases (Cdks), which are activated by periodically synthesized and degraded cyclins. The substrate targeting by a cyclin-Cdk complex is mediated by the active site on the Cdk and the docking pocket on the cyclin subunit, the hydrophobic patch (hp) (Kõivomägi, 2013). These docking pockets recognize short linear motifs (SLiMs) in target proteins. By previous studies (Faustova et al., 2021) it was shown that hp docking may be a key regulator of Cdk activity during the cell cycle, since even small mutations in either the motif or the hp can result in variations of the cyclin specificity and thus affect the interaction with the substrate. Importantly, the variations within the motifs may influence Cdk phosphorylation of different targets. Consequently, cyclins have a clear biochemical role in activation of cyclin-dependent kinase catalytic subunits, and a less well-defined role in directing kinase activity to particular substrates or regions of the cell.

Docking interactions between SLiMs and cyclin-dependent kinases are shown to be critical regulators of eukaryotic DNA replication (Wilmes et al., 2004).

In this work we analyzed the docking interaction between the cyclin Clb5 and the Clb5-specific substrate Sld2 in *Saccharomyces cerevisiae* at the G1/S stage. Sld2 is an essential substrate: phosphorylation of Sld2 promotes its binding to the replication protein Dpb11, and the Sld2-Dpb11 complex recruits DNA polymerases to origins of replication.

As Sld2 is an essential gene, in order to study its specificity we developed and adjusted several systems.

Time-lapse fluorescent microscopy was used as the one of the tools to analyze docking potency of several regions within the C-terminus of Sld2. For this study we designed the construct which consisted of four parts fused together: the nuclear localization signal (NLS), the degron part of Far1(85-129 AAs), the part of Sld2 (without 200 AAs from the N-terminus) and EGFP. This method was based on the finding that specificity between the cyclin and the docking site within the substrate can affect phosphorylation-dependent degradation of the target protein. The prerequisite for using this system was the recent study (Faustova et al., 2021), where the authors successfully implemented the same strategy to uncover the NLxxxL motif that contributed to phosphorylation-dependent degradation of Far1 at the G1/S stage.

The other system which could be used for addressing this research question was based on the yeast cells that contained a temperature-sensitive mutation. Temperature-sensitive mutants defective in the essential cell-cycle gene Sld2 could be rescued by the introduction of the same gene with particular pre-designed mutations. This approach allowed studying how the essential gene function and how different mutations contribute to the cell cycle progression.

1 LITERATURE REVIEW

1.1 The cell cycle engine

A cell cycle is a highly-regulated process of duplication and division of cell components and their distribution into two daughter cells (Morgan, 2007).

In general, the cell cycle is divided into four discrete phases - these are G1, S (DNA synthesis), G2 and M (mitosis) (**Figure 1**). Interphase occupies the majority of the cell cycle and consists of S phase, preceded by a gap called G1 and followed by a gap called G2 (Murray, 1993). These gaps ensure that the vital processes of each stage are carried out before the cell moves onto or leaves the S phase and thus provide the integrity of the genome. Feedback from the cell cycle's intracellular activities, as well as signals from the cell's environment, determine whether the cycle will progress beyond certain checkpoints (Alberts, 2008).

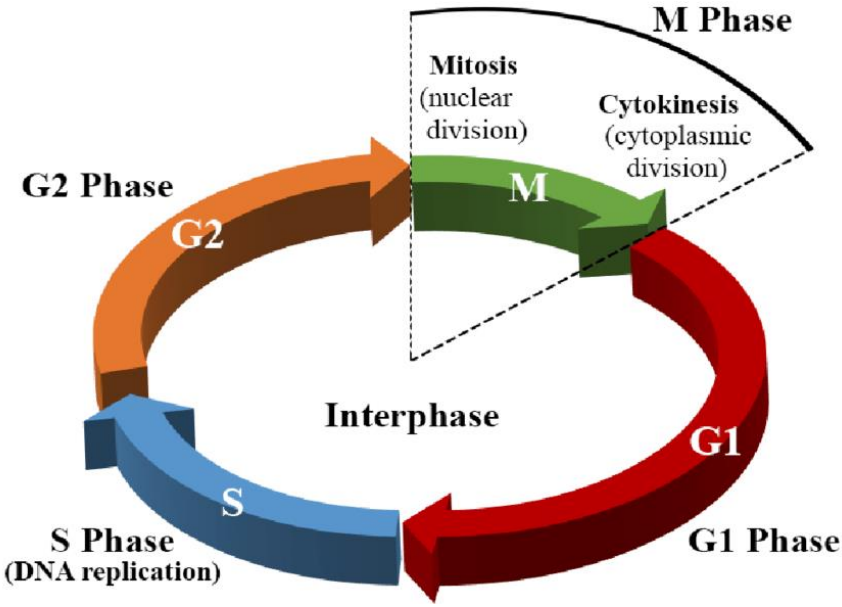

Figure 1. The cell cycle mechanism. The cell cycle is a process four discrete phases: the cell increases in size (gap 1, or G1-phase), copies its DNA (synthesis, or S, phase), prepares to divide (gap 2, or G2-phase), and divides (mitosis, or M-phase) (Alberts, 2008).

Cdks are one of the most essential proteins involved in cell cycle regulation because of their ability to temporally coordinate the molecular events (Örd and Loog, 2019). Protein kinases are enzymes that catalyze a phosphoryl transfer from an ATP molecule to a serine, threonine, and tyrosine residue of a substrate. For a Cdk to be active, it must be phosphorylated at one site and dephosphorylated at two other sites. The cyclical changes in cyclin concentrations aid in the cyclic assembly and activation of the cyclin–Cdk complexes, which in turn acti-

vates various cell-cycle events such as S phase or M phase entry (Alberts, 2008). Additionally, cyclins, while periodically synthesized and degraded, can activate Cdk subunits and thus affect Cdk specificity (Faustova et al., 2021). **Figure 2** illustrates substrate-targeting by the cyclin–Cdk-Cks1 complex. Cks1 is a small adaptor protein which promotes and facilitates phosphorylation of substrates with multiple Cdk phosphorylation sites (Morgan, 2007).

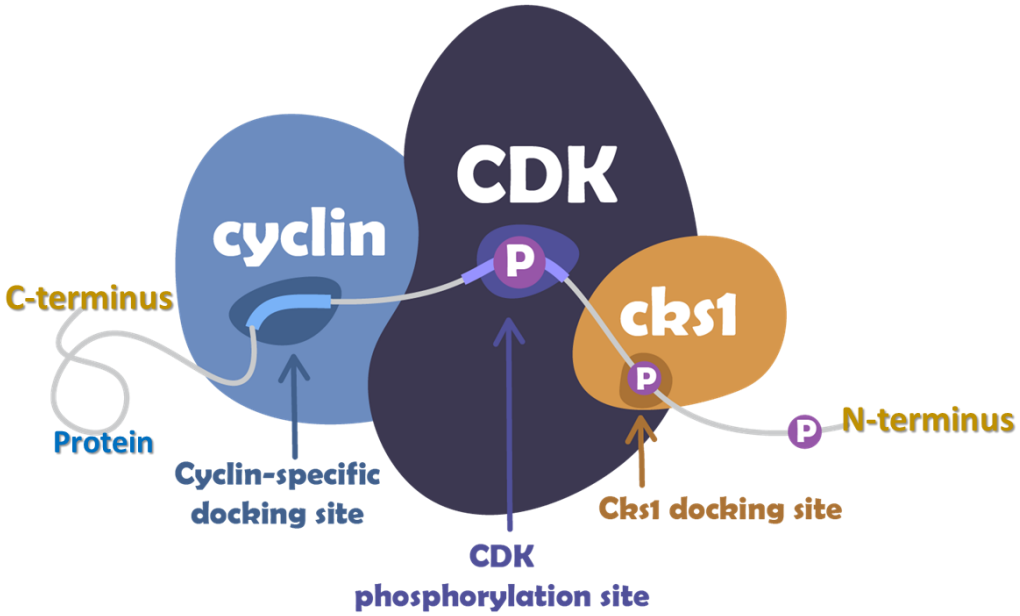

Figure 2. Substrate-targeting by the cyclin–Cdk-Cks1 complex. Cyclins bind to linear docking motifs on substrate proteins, prompting phosphorylation of particular substrates by the kinase. Cks1 facilitates multisite phosphorylation by binding to phosphorylated proteins (Örd and Loog, 2019).

As it is shown in (Örd and Loog, 2019), the intrinsic activity of cyclin–Cdk complexes was found to increase in correlation with the appearance of the particular cyclin in the cell cycle. That is, the G1 and G1/S cyclins produce complexes with the lowest activity, which is represented by the highest Km value – an intrinsic parameter representing the characteristics of the enzyme-catalyzed reactions. The Km value steadily decreases in the following S-, G2-, and M-phase complexes, indicating a strong affinity between enzyme and substrate. The poor specificity of the early complexes can be compensated for by cyclin-specific docking interactions in G1- and S-phase specific Cdk targets, which explains how effective phosphorylation switches can be activated at very low Cdk activity in the early stages of the cell cycle (Örd and Loog, 2019).

Yeasts, which are unicellular fungi, are commonly used in the research of the cell cycle process. The most studied yeast is Saccharomyces cerevisiae, which divides by forming a small bud that steadily develops until it splits from the mother cell (Alberts, 2008). Cell

growth and division of *S. cerevisiae* is a highly controlled process which is governed by a set of regulatory networks. This makes this species an ideal model eukaryotic system for the analysis of gene functions within the cell cycle due to the ease and precision with which its genome can be manipulated. For example, PCR-based approaches enable delete and mutate yeast genes, create fusions to epitope tags and fluorescent proteins because of the ability of budding yeast to undergo efficient homologous recombination with short stretches of sequence homology (Gardner et al., 2014).

Here is a short overview of the cell-cycle control system of budding yeast *S. cerevisiae*. Start (G1/S), mitotic entry, and the metaphase-anaphase transitions are the three main switch points that are regulated by cyclin-Cdk activity (Morgan, 2007). In budding yeast, CDC28 encodes Cdk which is activated at each stage of the cell cycle by binding of stage-specific cyclins. The entry into the cell cycle is induced by Cln3-Cdk1, which targets Whi5, the repressor of G1/S transcription. During G1 and the G1/S transition, Cln1 and Cln2 activate Cdk1 and thus provide bud emergence, spindle pole body duplication, and subsequent DNA replication (Kõivomägi, 2013). In the late G1 phase, G1 cyclin–Cdk complexes phosphorylate and inactivate transcriptional repressor Whi5 to unleash the transcription of the genes required for cell cycle entry and S phase (Örd and Loog, 2019). Clb5 and Clb6 levels rise at the beginning of S phase, and these proteins function primarily in the control of DNA replication (Morgan, 2007). In G2/M phase Clb3 and Clb4 are expressed which are involved in the process of the spindle pole bodies separation. Cyclins that promote mitotic entry are Clb1 and Clb2.

1.2 Short linear motifs (SLiMs)

The process of phosphorylation events catalyzed by Cdks involves recognition of short linear motifs (SLiMs) in target proteins by cyclin docking pockets (Örd et al., 2019). Different cyclins can modulate the intrinsic active site specificity against the phosphorylation motifs by interacting with cyclin-specific short linear motifs within the substrates. (Loog & Morgan, 2005; Kõivomägi et al, 2011a; Topacio et al, 2019). According to the recent study (Tatum & Endicott, 2020), cyclins can present various pocket configurations to dock different SLiMs in target proteins. Most of the SLiMs are usually located in intrinsically disordered regions of proteins and thus the interaction with a structured partner can promote formation of the protein's secondary structure (Örd et al., 2019).

According to some studies (Songyang et al., 1994) in the context of Cdk specificity, the protein docking motifs may be even more important than the specificity of phosphorylation sites themselves.

In mammalian cells, E, A, and B-type cyclins possess a so-called hydrophobic patch region in the cyclin box that contains an Met-Arg-Ala-Ile-Leu (M-R-A-I-L) sequence conserved among a number of mammalian and yeast cyclins (Adams, Sellers et al., 1996; Kelly, Wolfe et al., 1998; Schulman, Lindstrom et al., 1998; Cross and Jacobson, 2000). Thus the cyclins interact with the RxL motif of Cdk substrates and inhibitors. However, as the hydrophobic patches in different cyclin families are divergent, there is a whole family of similar RxL-like docking motifs with a hydrophobic center. This hydrophobic core can be preceded by either positively charged (RxLF or RxLxF) or hydrophobic/negatively charged residues (LxF, PxF, LP). Thus, although the motif is often called RxL, there are actually four core binding residues, with only the Leucine being fully conserved (Kõivomägi, 2013).

The specificity and affinity of SLiM binding is derived from both intrinsic and extrinsic binding determinants. Intrinsic determinants are the core residues of the motif that are usually in direct contact with the surface of the domain partner. Computational studies suggest that on average the core of the motif contributes about 80% of the energy of binding. These core residues (the required positions) are used to describe the specificity of the binding pocket (Van Roey et al., 2014).

Docking interactions between SLiMs and cyclin-dependent kinases are shown to be critical regulators of eukaryotic DNA replication. One example is the interaction between the S-phase cyclin Clb5 and the origin recognition complex (ORC). According to (Wilmes et al., 2004), the Clb5 hydrophobic patch mediates an interaction with an RxL sequence in the Orc6 subunit of ORC in budding yeast. However, mutation of the Orc6 RxL motif prevents stable binding and strongly increases the vulnerability of cells to induction of lethal re-replication.

SLiM functions in almost every pathway due to their role in regulatory function and signal transduction. Depending on the partner proteins that recognize them, these sites can facilitate a diverse set of functions including targeting a protein to a specific subcellular location, determining the modification state of a protein, controlling the stability of a protein, and regulating the context-dependent activity of a protein (Van Roey et al., 2014).

For example, the recent study (Örd et al., 2019) identified a conserved motif, LxF, which plays a critical role in Cdk function during mitosis. The LxF motif was proved to be crucial

for targeting M-Cdk to phosphorylate several mitotic regulators. For example, Spo12 is targeted via LxF to release the phosphatase Cdc14 and thus the LxF motif stimulates mitotic exit. Also the LxF motif mediates binding of replication factor Cdc6 to mitotic cyclin, which inhibits M-Cdk via Cks1 and the LxF motif.

New methods to identify SLiMs and their docking surface are constantly emerging. For example, the authors in (Bandyopadhyay et al., 2020) developed an *in vivo* method to measure LP docking by exploiting the ability of Cln2-Cdk to inhibit pheromone signaling. This study helped to reveal how variations in docking strength can tune the degree and timing of regulatory modifications.

For now, a full set of highly specific and selective motifs have been defined for each of the four major cyclins in budding yeast: the LP, RxL, PxF, and LxF motifs for G1-, S-, G2-, and M-phase cyclins, respectively (Bhaduri & Pryciak, 2011; Kõivomägi et al, 2011a; Örd et al, 2019a; Örd et al, 2020). Nevertheless, the authors in (Faustova et al., 2021) uncovered a new specific SLiM (NLxxxL motif) for docking by S-phase Clb5/6-Cdk1. NLxxxL motif was proved to be more effective than conventional RxL motif in directing Clb5-Cdk1 activity and thus substitution of the NLxxxL motif with an RxL resulted in partial loss of function. However it was revealed that NLxxxL motifs overlap with previously described RxL motifs in the Clb5-specific targets Fin1 and Spc110. This could promote an idea that cyclins can present different various pocket configurations to dock different SLiMs and to facilitate substrate and inhibitor protein binding with a wide range of affinities.

1.3 The process of protein degradation

Cdk function is linked to another important regulatory mechanism, protein degradation. Cells possess specialized pathways to enzymatically break proteins down first to short peptides and then into their constituent amino acids by proteases (Alberts, 2008).

The majority of proteins are targeted for degradation by a special mechanism of attaching several ubiquitin molecules to the substrate. This process is referred to ubiquitylation and can be considered as a post-translational modification that forms an isopeptide bond between a lysine residue on the substrate and the carboxyl terminus of ubiquitin.

The ubiquitylation system consists of four different classes of enzymes: E1–E4. As it is shown in **Figure 3** (Morgan, 2007), first, ubiquitin is covalently conjugated to the E1 (ubiquitin-activating enzyme) in an ATP-dependent reaction, and then it is transferred to the E2 (ubiquitin-conjugating enzyme). The E3 (ubiquitin-protein ligase) transfers the ubiquitin

from the E2 to the substrate protein. After the first ubiquitin has been attached, the E3 can elongate the ubiquitin chain by creating ubiquitin-ubiquitin isopeptide bonds. The E4 enzymes (chain elongation factors) are a subclass of E3-like enzymes that only catalyse chain extension (Tai, et al., 2008). After the covalent attachment of multiple ubiquitin molecules to the protein substrate the targeted protein is degraded by the 26S proteasome complex.

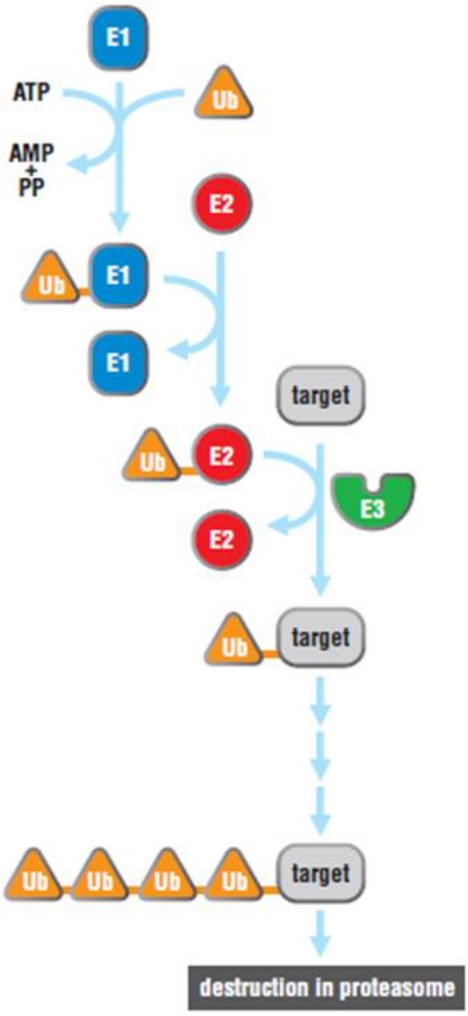

Figure 3. The ubiquitylation system. Ubiquitination is a three-steps series of events, that requires the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme, and the E3 ubiquitin-protein ligase to bind ubiquitin proteins to target proteins, thus tagging them for degradation (Morgan, 2007).

A multi-protein E3 ubiquitin ligase complex is one of the most important within the process of protein degradation. The largest family of E3 ligases is the Skp1–Cullin–F-box ubiquitin ligase (SCF). The F-box subunit of SCF complex provides specific recognition of phosphorylated motifs called phosphodegrons. The phosphodegron can be described as a short amino acid sequence which upon phosphorylation directly interacts with a ubiquitin ligase and thus triggers many important cell-cycle processes (Ang et al., 2005).

In yeast several substrates phosphorylated by Cdk can be subsequently recognized by the F-box protein Cdc4. By previous studies it was shown that Cdc4 prefers peptides containing a phosphoserine or phosphothreonine followed by a proline and preceded by hydrophobic residues (Lyons et al., 2013). Cdc4 recruits the substrates to the SCF core complex in a phosphorylation-dependent manner and this leads to the degradation of the substrates. Examples of such substrates are the Cdk inhibitor protein Far1, the replication protein Cdc6, and the transcription factor Gcn4 (Patton et al., 1998). In the case of Far1, by a systematic mutational analysis it was revealed (Fausova et al., 2021), that Clb5-dependent phosphorylation of the degron part of Far1 (85–150) and its subsequent degradation required the presence of two phosphosites: S87 and S91.

Sic1, a budding yeast inhibitor of the Clb-Cdk complex, is another example of phosphorylation-induced degradation. Degradation is driven by the interaction between RxL motifs in Sic1 and the hydrophobic patch docking site of Clb5 (Barberis, 2012). G1/S–Cdks phosphorylate Sic1 at six or more sites, resulting in its SCF-dependent ubiquitination and destruction, once G1/S-Cdk activity reaches high levels in late G1. The S-Cdks are released when Sic1 is destroyed, thus starting chromosome duplication in S phase (Morgan, 2007).

1.4 The replication protein Sld2

DNA replication begins at origins of replication, which recruit specific proteins that control the initiation and completion of DNA replication.

In budding yeast, the initiation of DNA replication is under the control of the essential Clb5-Cdk1 targets Sld2 (also known as Drc1) (Wong et al., 1999) and Sld3, which are components of a massive protein assembly which is referred to as the preinitiation complex. Formation of the preinitiation complex results in activating the Mcm helicase, leading to unwinding of the DNA helix.

It has been shown (Wong et al., 1999) that Cdk-mediated phosphorylation of Sld2 promotes binding to the replication protein Dpb11 (**Figure 4**). Dpb11 binds directly to one of the replicative DNA polymerases – polymerase ε (Pol ε), which further interacts with the DNA and extends the primers to start synthesis of the two leading strands.

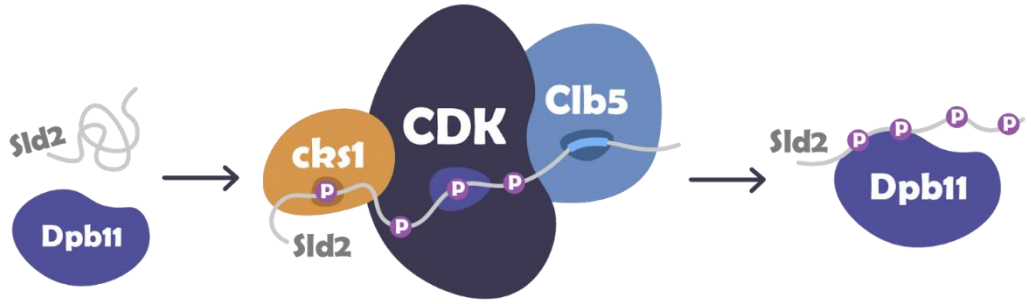

Figure 4. The Clb5-Cdk1-Cks1 complex phosphorylates Sld2 leading to its interaction with the replication protein Dpb11 (Wong et al., 1999).

The Sld2 protein has a cluster of 11 Cdk phosphorylation motifs (Ser/Thr–Pro), six of which match the canonical sequences Ser/Thr–Pro–X–Lys/Arg, Lys/Arg–Ser/Thr–Pro and Ser/Thr–Pro–Lys/Arg. As it is shown in (Tak et al., 2006), simultaneous substitution of alanine for serine or threonine in all the canonical motifs severely reduces complex formation between Sld2 and Dpb11 and the initiation of DNA replication is prevented. However, studies of Sld2 mutants with alanine substitutions suggest that not all Cdk phosphorylation motifs affect cell growth. The key residues, which are necessary for the formation of the Sld2-Sld3-Dpb11 complex, are T84 (Masumoto et al. 2002; Zegerman and Diffley 2007; Tanaka, Umemori et al. 2007) and S100 (Masumoto et al., 2002).

1.5 Temperature-sensitive mutants

For essential proteins, some conditional method is needed to inactivate the protein, and the most widespread genetic method is to use temperature-sensitive mutants (Kearsey, 2009).

The use of conditional alleles is a powerful approach for functional analysis of essential genes since it allows investigating not only the loss of function, but also provides data for the development of further genetic approaches (Stark et al., 2007).

Along with temperature-sensitive alleles, other types of mutant alleles that perturb essential gene functions and thus makes it possible to study their roles throughout the cell cycle, include cold-sensitive, temperature-inducible degron, tetracycline-regulatable promoter-replacement and decreased abundance by mRNA perturbation alleles (Li et al., 2011).

The study of mutant phenotypes has proven to be a fundamental approach for obtaining a detailed understanding of gene function and deciphering its functional role. However, since

temperature-sensitive alleles are rare and difficult to produce and classify, their use in most multicellular organisms has been limited.

Temperature-sensitive mutations are typically missense, conditional mutations, which retain the function of a specific essential gene at standard low temperature (the permissive condition, typically room temperature) and lack that function at a defined high (nonpermissive, the restrictive condition, typically 37 °C)) temperature. After the temperature is increased, such mutants continue to progress through the cell cycle until they arrest at the point at which the gene is required for further progress. As it is shown in **Figure 5** (Alberts, 2008), the protein carries an amino acid substitution (red) that prevents its proper folding at 37ºC but permits proper folding at 25ºC. Thus, it is possible to analyze physiologic changes that follow controlled inactivation of a gene or gene product by shifting cells to a nonpermissive temperature (Kearsey et al., 2009).

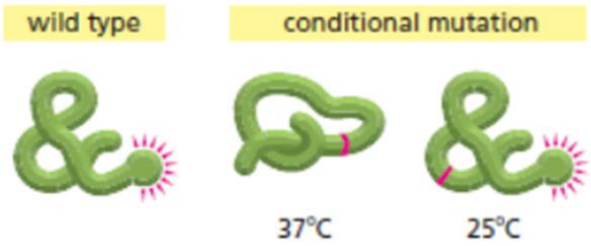

Figure 5. The concept of a temperature-sensitive mutation. Here, the wild-type protein serves a particular cellular role, as shown by the red rays. In this figure, the allele produces active protein under 25°C, while under the higher temperature, it produces inactive protein. The protein has an amino acid substitution (red) that prevents it from folding properly at 37°C but allows it to fold properly at 25°C (Alberts, 2008).

2 THE AIMS OF THE THESIS

S-phase Cdk specificity is known to be governed by RxL motifs with the consensus R/Kx-L-F/L/M/P or R/K-x-L-x-F/L/M/P sequence, however, our recent results (unpublished data) revealed that the S-Cdk (Clb5) specific substrate Sld2 C-terminus may contain a motif responsible for high Clb5-specificity, but it does not contain the aforementioned conventional consensus sequence.

Thus, we can state the following hypothesis: the Sld2 substrate targeting by Clb5-Cdk1 complex in the early S phase is mediated not only by conventional cyclin docking motifs.

The following are the aims of this work:

- To determine specificity and define docking potency within the C-terminus region (268-453 amino acids) of the S-Cdk (Clb5) specific substrate Sld2.
- To analyze the effect of different mutations within the potent docking region on specific phosphorylation of Sld2 protein *in vivo*.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Media

The LB (Luria-Bertani) medium for culturing bacteria: 10 g/L tryptone (BD Biosciences), 5 g/L yeast extract (Formedium), 10 g/L NaCl.

For bacteria selection with an ampicillin-resistance plasmid, agar plates with ampicillin were used: 15 g/L of Bacto agar (Formedium) and 100 μg/mL of ampicillin were added to 1 L of LB medium.

YPD media for culturing yeast: 20 g/L peptone (Formedium), 10 g/L yeast extract (Formedium), 20 g/L D-Glucose anhydrous.

Agar plates with YPD medium were used for growing yeast. For making them 15 g/L Bacto agar (Formedium) was added to 1 L of YPD medium.

For making the media which allowed plasmid selection, the appropriate amino acid was omitted: for -URA media uracil was omitted.

2xYT microbial growth medium: 16 g of Bacto Tryptone, 10 g of Bacto yeast extract, 5 g of NaCl and dis-tilled H2O adjusted to 1 L.

3.1.2 The list of yeast strains

The yeast strains are described in Table 1.

Table 1. The list of strains used in this research.

Strain	Genotype	Source
RV298	MATa, bar1::hisG *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]* WHI5-mCherry-SpHIS5	Rainis Venta
Drc1-1	*MAT*a *drc1-1 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*	Araki et al.,2010

EV638	MATa, bar1::hisG *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]* WHI5-mCherry-SpHIS5 *ura3*::pEV1138::*ura3*	This study
EV638	MATa, bar1::hisG *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]* WHI5-mCherry-SpHIS5 *ura3*::pEV1139::*ura3*	This study
EV638	MATa, bar1::hisG *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]* WHI5-mCherry-SpHIS5 *ura3*::pEV1186::*ura3*	This study
EV638	MATa, bar1::hisG *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]* WHI5-mCherry-SpHIS5 *ura3*::pEV867::*ura3*	This study
EV638	MATa, bar1::hisG *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]* WHI5-mCherry-SpHIS5 *ura3*::pEV868::*ura3*	This study
EV638	MATa, bar1::hisG *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]* WHI5-mCherry-SpHIS5 *ura3*::pEV869::*ura3*	This study
EV638	MATa, bar1::hisG *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]* WHI5-mCherry-SpHIS5 *ura3*::pEV870::*ura3*	This study
EV638	MATa, bar1::hisG *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]* WHI5-mCherry-SpHIS5 *ura3*::pEV871::*ura3*	This study

EV638	MATa, bar1::hisG *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]* WHI5-mCherry-SpHIS5 *ura3*::pEV1192::*ura3*	This study
EV638	MATa, bar1::hisG *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]* WHI5-mCherry-SpHIS5 *ura3*::pEV1193::*ura3*	This study
EV638	MATa, bar1::hisG *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]* WHI5-mCherry-SpHIS5 *ura3*::pEV1194::*ura3*	This study
EV638	MATa, bar1::hisG *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]* WHI5-mCherry-SpHIS5 *ura3*::pEV1195::*ura3*	This study
EV638	MATa, bar1::hisG *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]* WHI5-mCherry-SpHIS5 *ura3*::pEV1196::*ura3*	This study
EV638	MATa, bar1::hisG *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]* WHI5-mCherry-SpHIS5 *ura3*::pEV1197::*ura3*	This study
EV638	MATa, bar1::hisG *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]* WHI5-mCherry-SpHIS5 *ura3*::pEV1198::*ura3*	This study
EV638	MATa, bar1::hisG *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]* WHI5-mCherry-SpHIS5 *ura3*::pEV1199::*ura3*	This study

3.1.3 Molecular cloning

Molecular cloning is a technique which is used for producing multiple copies of a DNA fragment by using PCR and restriction-ligation-transformation steps.

Here is the list of the plasmids which were obtained by molecular cloning and used in this research (Table 2). All the plasmids are ampicillin-resistant and have markers that allow selection for successful yeast transformants (URA3). Besides that, all the inserts which were ligated with the plasmid DNA vectors contain EGFP sequence at the C-terminus.

Table 2. The list of plasmids used in this research.

Plasmid	Backbone	Description	Source
pEV1138	pRS306	pSic1-Far1(RxL)-EGFP	This study
pEV1139	pRS306	pSic1-Far1(AxA)-EGFP	This study
pEV1186	pRS306	pSic1-Far1(mini)-Sld2-EGFP	This study
pEV867	pGEX-4T-1	GST-Far1-Sld2-EGFP	This study
pEV868	pGEX-4T-1	GST-Far1-Sld2	This study
pEV869	pGEX-4T-1	GST-Far1(RxL)-EGFP	This study
pEV870	pGEX-4T-1	GST-Far1(AxA)-EGFP	This study
pEV871	pGEX-4T-1	GST-Far1WT-EGFP	This study
pEV1192	pRS306	pSic1-Far1(mini)-Sld2dC50-EGFP	This study
pEV1193	pRS306	pSic1-Far1(mini)-Sld2dC100-EGFP	This study
pEV1194	pRS306	pSic1-Far1(mini)-Sld2dC150-EGFP	This study
pEV1195	pRS306	pSic1-Far1(mini)-Sld2mut1-EGFP	This study
pEV1196	pRS306	pSic1-Far1(mini)-Sld2mut2-EGFP	This study

pEV1197	pRS306	pSic1-Far1(mini)-Sld2mut3-EGFP	This study
pEV1198	pRS306	pSic1-Far1(mini)-Sld2mut1&3-EGFP	This study
pEV1199	pRS306	pSic1-Far1(mini)-Sld2mut2&3-EGFP	This study

3.1.4 PCR

The PCR reaction was used to amplify a DNA template and produce specific DNA fragments. The total reaction mixture volume was 50 µL and it consisted on 10 µL of 5xHF buffer (Thermo Fisher Scientific), 10 ng of the template, 250 µM of dNTPs, 0.5 µM of the forward and reverse primers and 1 U of high-fidelity Phusion DNA Polymerase (Thermo Fisher Scientific). Milli-Q water was added up to the final volume.

The thermocycling conditions for a PCR reaction are listed below in Table 3.

Table 3. Thermocycling conditions for a PCR reaction.

Cycle step	Temperature	Time	№ of cycles
Initial Denaturation	98° C	5 min	1
Denaturation	98° C	20 s	25
Annealing	55° C - 58° C (based on the Tm of the primer pair)	20 s	25
Extension	72° C	30 sec/kb	25
Final Extension	72° C	5 min	1
Hold	15° C		

After the PCR reaction the Orange DNA Loading Dye of the final concentration 1X (Thermo Fisher Scientific) was added to the PCR products. After that the mixtures were loaded on 1% agarose gel in 1X TAE buffer (Thermo Scientific™) and pulled through a gel matrix by an electric current in order to separate them according to the size. The PCR products were visualized under the UV light by the dye used in the gel mixture, cut out of the agarose gel and purified using FavorPrep GEL/PCR Purification Kit (Favorgen) according to the manufacturer's protocol.

3.1.5 Restriction

Restriction enzymes selectively cut the amplified DNA segments at specific "recognition" sequences thus allowing them to be subsequently ligated to the backbone plasmid.

In this research several specific pairs of restriction enzymes were used depending on the desired DNA product characteristics and purposes. The recombinant DNA constructs and corresponding restriction enzymes were summarized in Table 2.

The 50 µL mixture for insert restriction consisted of 5 µL of 10x FastDigest™ Buffer, 40 µL of PCR product, 1 µl of each FastDigest™ restriction enzyme and milli-Q water which was added up to the final volume. The circular plasmid which served as a vector should have been linearized by the same enzymes. Both the insert and the vector which were treated by the restriction enzymes should have been incubated at 37°C heat block for 1 hour and then be separated on the agarose gel. The DNA bands with proper size were cut out of the gel and purified using FavorPrep GEL/PCR Purification Kit (Favorgen).

3.1.6 Ligation

During the ligation step the digested insert is combined with a compatible digested vector backbone. The ratio of the vector and the insert in the mixture was 1:3. The volume of the mixture was 20 µl and it included the vector and the insert, 2 µL 10X T4 DNA Ligase buffer (Thermo Fisher Scientific) (final concentration 1X), T4 DNA Ligase (Thermo Fisher Scientific) (5 U), and Milli-Q water which was added up to the final volume. If we used an enzyme which generated fragments with blunt ends (SmaI), 2 µl of 50% PEG 4000 (Polyethylene Glycol) was also added in order to enhance the proper DNA ligation. The ligation reactions were carried out at a room temperature (22°C) for 1 hour.

3.1.7 Bacterial transformation

The last step in molecular cloning was bacterial transformation, which resulted in formation of multiple copies of the recombinant DNA molecule. The transformation was performed with NEB® Turbo Competent E. coli cells. The competent cells (50 µL) firstly were thawed on ice for 10 minutes. Then the ligated product (3 µL) was added to the recombinant cells and the transformation mixture was placed on ice for 30 minutes. This step was followed by the short (40 seconds) heat shock at 42 °C and subsequent keeping the mixture on ice for an additional 5 minutes. After that 500 µL of LB media was added and the cells were placed at a shaking incubator at 37 °C for 1 hour and then centrifuged at 6000 RPM for 2 minutes. Then the cell suspension was plated onto the agar plates containing ampicillin and the plates were incubated at 37 °C for 14 hours (overnight).

3.1.8 Recovering Plasmid DNA from Bacterial Culture

After bacterial transformation a single colony from an agar plate was used to inoculate a liquid bacterial culture (LB media and appropriate antibiotic) for subsequent plasmid DNA purification. After 4-6 hours of incubation, plasmid DNA from the cells was purified using FavorPrep Plasmid DNA Extraction Mini Kit (Favorgen) according to the protocol provided by the manufacturers. The DNA concentration was measured using the NanoDrop 1000 Spectrophotometer (Thermo Fisher) and after that the samples were sent to the Estonian Biocentre core laboratory for Sanger DNA sequencing.

3.1.9 Site Directed Mutagenesis by PCR

In this research most of the plasmids were constructed by introducing specific, targeted nucleotide substitutions in the plasmid DNA (Table 2). Point-mutations could be introduced to the plasmids using the pair of primers which contained the desired mutation. This technique required two steps: PCR of the whole plasmid with the pre-designed primers and ligation of the product after performing the PCR reaction. The 50 µL PCR reaction was performed according to the same protocol as the one which was previously described, however it was important to adjust the extension time according to the length of the plasmid. After the PCR reaction, 3 µl of the PCR product was mixed with 1 µl of DpnI (FastDigest™) in order to destroy the plasmid template, 1 µL of T4 polynucleotide kinase (PNK) (Thermo Fisher Scientific) to phosphorylate the 5'-ends, 2 µL of T4 DNA Ligase buffer and with

Milli-Q water. The mixture of total volume 10 μL was incubated at 37 °C for 40 min, then cooled to room temperature. After that 2 μL of 50% PEG 4000 solution and T4 DNA Ligase (Thermo Fisher Scientific) (5 U) were added and left at room temperature for 10 minutes.

After circularization of the PCR product and removal of the template DNA, the final reaction was transformed into the bacterial competent cells.

3.1.10 Yeast transformation

Before the pRS306-based vectors were transformed to S. cerevisiae strain RV298, they were digested with FastDigest Eco147I restriction enzyme to provide integration to URA3 locus. This step was done in accordance with the aforementioned protocol for restriction of a DNA construct.

For transformation, yeast cells had to be grown for 5 - 8 hours in YPD media. For the experiments with the temperature-sensitive strains, -URA media was used for yeast growth depending on the selection marker used in the particular strain.

The culture for transformation was grown in the 30 °C shaking incubator until the optical density (OD) reached the value between 0.4 and 0.7. The OD was measured at 600 nm wavelength by the spectrophotometer Ultrospec 10 (Amersham Biosciences). After the incubation time, the yeast culture was transferred into a 50 mL tube and centrifuged for 2 min at 3000 RPM. After the centrifugation the cell pellet was resuspended in 1 mL of sterile buffer I [100 mM lithium acetate in 1X TE (10 mM Tris-HCl (pH 8), 0.51 mM EDTA)] and centrifuged for 30 seconds at 3600 RPM. Then the supernatant was removed, and the pellet was resuspended again in the same buffer (the buffer volume was two times the volume of the cell pellet). Then the cells were left at room temperature for 10 minutes. At the same time, salmon sperm DNA (SS-DNA) was boiled for 10 minutes at 100° C and then was immediately placed on ice. Once the incubation period was over, 100 μL of yeast competent cells were mixed with 10 μL SS-DNA.

After that, 700 μL of sterile PEG/lithium acetate solution (40 % PEG 3350, 100 mM lithium acetate, 10 mM Tris-HCl (pH 8), 1 mM EDTA) was applied to the reactions and thoroughly mixed together because the solution was viscous. Also 48 μL of DMSO were added and the mixture was resuspended. After that, it was held at 42 °C for 40 minutes. The mixture was then chilled for 2 minutes on ice before being centrifuged for 1 minute at 6000 RPM, and the cell pellet was resuspended in 1 mL 1X TE buffer (10 mM Tris-HCl (pH 8), 1 mM

EDTA) and again centrifuged for 1 minute at 3600 RPM. At the last step, the supernatant was removed, and the cells were resuspended in 200 µL of 1X TE buffer before being plated on the agar plates.

3.1.11 Time-lapse quantitative microscopy

Time-lapse microscopy experiments were used to quantify EGFP and MCherry fluorescence at the single-cell level. The Zeiss Axio Observer Z1 microscope was used to monitor the changes in EGFP levels in the cells in real time. In one experiment it was possible to monitor 18-24 positions during 9 hours with the image capturing interval of 3 minutes.

For this experiment the special molecule was designed which consisted of four parts fused together: the nuclear localization signal (NLS), the degron part of Far1, the C-terminus part of Sld2 and EGFP. EGFP signals were continuously measured in a live-cell fluorescent microscopy experiment and the changes in EGFP levels quantitatively responded to the changes in Far1-Sld2 expression. At the same time our system utilized mCherry fluorescent protein fused to the Whi5 transcriptional repressor that inhibited Start of the cell cycle in late G1. When phosphorylated by Cdk1, Whi5 was exported from the nucleus. Therefore, the Start point was defined by the time point when the Whi5 nuclear level was dropping to 50% of the level in G1 (Doncic et al., 2011). In the analysis of microscopy experiments, different cells were synchronized by the Start point and Far1-Sld2-EGFP levels were followed for 90 minutes after Start, covering the time of an average cell cycle.

The data was then exported and the images were processed using MATLAB software developed by Doncic et al. (2013) and updated in our lab to analyze the data.

3.1.12 Protein purification

After the plasmid sequences were confirmed by sequencing, we did bacterial transformation for subsequent expression of eukaryotic proteins using Rosetta™ competent cells. After the colonies appeared, the starter culture for protein expression was made (2 mL of LB media, the appropriate antibiotic, one selected bacterial colony) and incubated in a shaker (250 RPM) at 37 °C until the OD reached the value 1.0. After this, we prepared bacterial growth media: 1.5 L of 2xYT media and the appropriate antibiotic. This culture was grown at 37 °C 170 RPM until OD reached the value 0.8. Then we induced the culture to express protein by

adding IPTG in final concentration 1 mM and placed the cultures to the 16 °C 170 RPM shaking incubator overnight. After induction, we centrifuged the whole volume of the bacterial culture for 12 minutes at 5,000 RPM and resuspended the pellet in 50 mL 1X PBS buffer (ph 7.4; NaCl 58 g/mol, KCl 74 g/mol, Na2HPO4 142 g/mol, KH2PO4 136 g/mol). Then we again centrifuged the bacteria for 12 minutes at 5,000 RPM and the cell pellet was then frozen in liquid nitrogen and stored at -80 °C.

Prior to purification of bacteria, the samples were thawed on ice and then resuspended in 30 ml of the lysis buffer. The lysis buffer was consisted of 50 mM Tris-HCl pH 7.4, 5% glycerol, 600 mM NaCl, 1% Triton-X100, DNase, protease inhibitors (1 mM PMSF, 1 µg/ml pepstatin A, 0.6 µg/ml aprotinin), 2 mM DTT, and lysozyme with concentration 1 mg/mL (Sigma). Then the samples were rotated at 4 °C for 30 minutes. Afterwards, the suspension was sonicated to shear the DNA for 3 rounds of 40 seconds with an ice chill in between the sessions and centrifuged for 30 minutes at 16000 g. The supernatant was saved and loaded to the falcon tube with 400 µl Glutathione Sepharose Beads (GE Healthcare), which were previously washed with 4 ml of lysis buffer. The falcon tubes were rotated for 3 hours. Then the supernatant was flown through the columns. The unspecific binding of untagged proteins was first washed off with lysis buffer with 2 mM DTT, and remaining nonspecific proteins were further eliminated with washing buffer (50 mM Tris-HCl pH 7.4, 5% glycerol, 150 mM NaCl, 0.1% NP-40)) with 1 mM of DTT. In the final step target proteins were subsequently eluted 3 times with 400 µL elution buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% NP-40, 5% glycerol) and 1 mM DTT for 5 minutes resulting in 3 aliquots. The aliquots were then frozen in liquid nitrogen and stored at -80 °C.

3.1.13 SDS-PAGE

SDS-PAGE is an analytical technique to separate proteins based on their molecular weight. After purification it was important to analyze the samples using SDS-PAGE in order to confirm their presence and to assess their purity. For this 5 µL of eluted protein was mixed with 5 µL of 6x SDS loading buffer (375 mM Tris-HCl, 9% SDS, 50% Glycerol, 0.03% Bromophenol blue) and incubated at 72°C for 5 minutes. Then we applied the samples together with PageRuler™ Prestained Protein Ladder (Thermo Scientific™) to their respective wells. Electrophoresis was carried out at constant currency 15 mA per one gel in 1x running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) for 1.5 hours. For protein visualization Coomassie blue staining solution was applied to the gels. The staining solution

contained 0.1% Coomassie Blue R-250 in 40% ethanol and 10% acetic acid. It was necessary to incubate the gels in the staining solution for 20 minutes on an orbital shaker until the bands appeared. After that we rinsed the gel with MilliQ water and kept the gels in the destaining solution (10% methanol, 7% acetic acid) on the orbital shaker for another 20 minutes. Heating up both the staining and destaining solutions was accelerating both processes.

3.1.14 Kinase assay

Kinase assays were performed in order to analyze designed constructs *in vitro* and determine the docking and phosphorylation efficiency of different docking and phosphorylation sites respectively. Clb5-Cdk1 phosphorylation rate of substrates was measured using a kinase assay with γ-32P ATP. As histone H1 was an effective substrate for a number of serine/threonine kinases, it was used as a control reference substrate (Sigma-Aldrich). The general composition of the assay mixture contained 50 mM TRIS-HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl2, 2% glycerol, 0.2 mg/ml BSA, 500 nM Cks1 and 500 μM ATP [(with added [g-32P]-ATP (Hartmann Analytic)].

For the phosphorylation assays, substrate protein concentrations were in the range of 1-2 mM, while the concentrations of kinase complexes were 0.2-2 nM. Reactions were separated using SDS–PAGE. Protein phosphorylation was detected using Typhoon Trio (Amersham Biosciences).

3.2 RESULTS AND DISCUSSION

S-phase Cdk specificity is known to be governed by RxL motifs with the consensus R/Kx-L-F/L/M/P or R/K-x-L-x-F/L/M/P sequence, however, our recent results (unpublished data) revealed that the S-Cdk (Clb5) specific substrate Sld2 C-terminus conserved region contains a motif responsible for high Clb5-specificity, but it does not contain the aforementioned conventional consensus sequence. This fact clearly indicates that Sld2 substrate targeting could be mediated by additional cyclin docking motifs.

To analyze the specificity and docking potency of the S-Cdk (Clb5) specific substrate Sld2, we used several methods.

For real-time imaging of living cells by time-lapse fluorescence microscopy, we designed the construct which consisted of four parts fused together: the nuclear localization signal (NLS), the degron part of Far1(85-150 AAs), the part of Sld2 (without 267 AAs from the N-terminus) and EGFP. EGFP signals were continuously measured in a live-cell fluorescent microscopy experiment and the changes in EGFP levels quantitatively responded to the changes in Far1-Sld2 expression.

As Sld2 is an essential gene, we utilized a system of integration of plasmids with Sld2 mutations to the strain with the temperature-sensitive allele (conditional mutant of SLd2), which allowed us to analyze interaction pathways of Sld2. Quantitative analysis of protein phosphorylation was performed using protein purification and subsequent kinase assay.

3.2.1 The replication protein Sld2 is a Clb5-specific substrate

Nine different cyclins, which are divided to five groups (**Figure 6B**) based on their expression profiles, activate Cdk1 in budding yeast, and their expression is controlled at particular cell cycle stages primarily via transcriptional and post-translational mechanisms. (Bloom and Cross, 2007). According to the cyclin specificity model, different cyclins expression during the cell cycle causes phosphorylation of different substrate proteins at different times.

As it is shown in **Figure 6B**, cyclins Clb5 and Clb6 in *Saccharomyces cerevisiae* mediate Cdk activity and thus contribute to initiation of S phase. By previous studies (Masumoto et al. 2002), it was shown that Sld2 replication protein was required for replication initiation. In a kinase assay (**Figure 6A**), the effect of the cyclin-Cdk1 complexes specificity on the

phosphorylation of Sld2 was analyzed. The results revealed that Sld2 is most efficiently phosphorylated by Clb5-Cdk1.

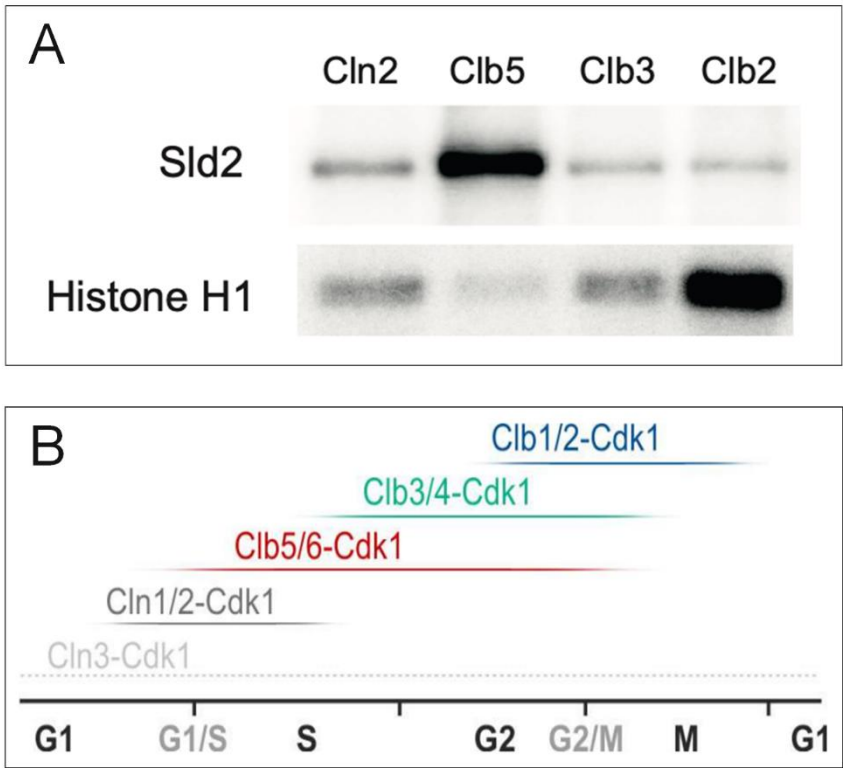

Figure 6. Sld2 as an S-phase cyclin substrate. (**A**) Kinase assay specificity panel shows that Sld2 is a highly-specific substrate towards Clb5-Cdk1. Histone H1 kinase activity was used as a control. H1 phosphorylation is not dependent on cyclin docking but relies on the consensus phosphorylation site motif. (**B**) The expression profiles of cyclin-Cdk1 complexes in *S. cerevisiae* cell cycle (Örd, 2019).

3.2.2 Detection of the docking interaction *in vivo* between Clb5 and C-terminus of Sld2

In this work, we aimed to determine docking interaction within the C-terminus region (268-453 amino acids) of the S-Cdk (Clb5) specific substrate Sld2. For this, we used time-lapse fluorescence microscopy - a powerful quantitative tool allowing us to track single cells for several (8-10) cell cycles (90 minutes each). To analyze the results,we used a completely automated algorithm (Doncic et al., 2013) for segmentation and monitoring of the cells within strains of interest. As a tool for defining the phase throughout the cell cycle progression, the algorithm used the reference to the nuclear export of 50% of Whi5-mCherry in the cells (Doncic et al., 2013). Whi5 was used as a reporter of cell cycle dynamics since it was exported from the nucleus during G1 (prior to DNA replication) and then imported again at the end of the cell cycle.

During the experiment we followed the timing of phosphorylation of the di-phosphodegron in Far1 by measuring the levels of EGFP fusion protein throughout the cell cycle. Far1 was phosphorylated at the S87 and S91 residues by Clb5-Cdk, leading to ubiquitination by SCF-Cdc4 and subsequent degradation (Gartner et al., 1998). The construct of Far1 (85-150)-EGFP was expressed under SIC1 promoter. EGFP fluorescent marker was used to determine dynamics of the proteins of interest. Only the N-terminal part of Far1 (85-150 amino acids), which comprised the degron and the Far1 docking motif (Faustova et al., 2021), was used in this experiment.

The role of Far1 phosphosites in terms of the degradation of the protein via the ubiquitin pathway was observed by introducing alanine mutations to S87 and S91 (**Figure 7**). By previous work (Faustova et al., 2021) the Clb5 docking motif within the N-terminus of Far1 was mapped using the same system, thus confirming the applicability of the suggested approach.

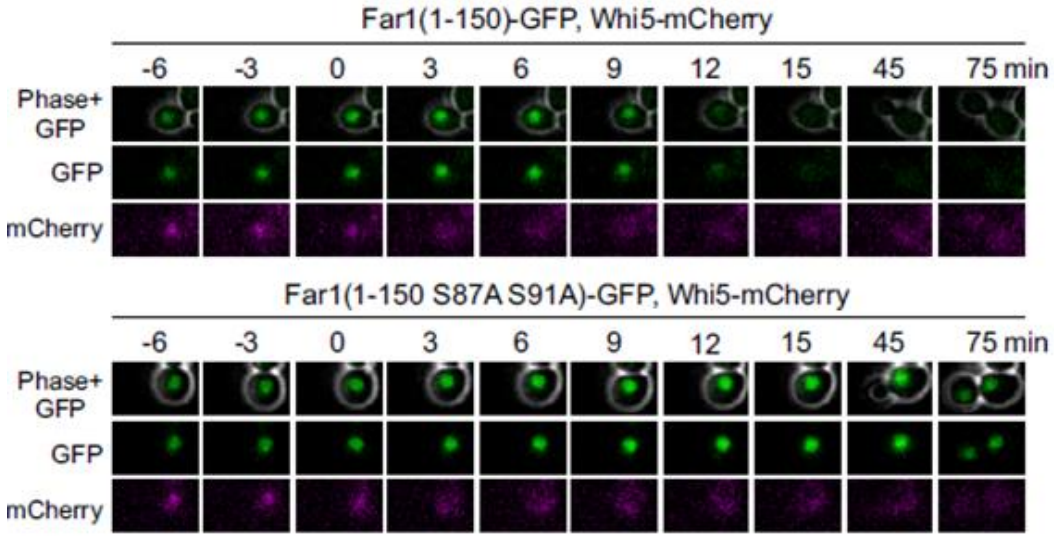

Figure 7. Time-lapse fluorescence microscopy images which show the wild-type Far1(85-150)-GFP and S87A S91A mutant Far1(85–150)-GFP. Introducing alanine mutations to Far1 di-phosphodegron sites leads to the degradation of the construct with Far1. It is indicated by the loss of GFP signal after 10 minutes of the cell cycle. This confirms the importance of the aforementioned phosphorylation sites in terms of the degradation of the protein via the ubiquitin pathway and provides the base for further experiments with the use of the degron part of Far1 (Faustova et al., 2021).

In our experiment, we designed four constructs in order to estimate the docking potency of the known RxL motif within the N-terminus of Sld2 and the unknown docking motif within the C-terminus of Sld2. First, the fragments were generated via PCR with a unique set of primers and inserted into the vectors. Afterwards, these yeast integrative plasmids carrying

the desired DNA sequence, were introduced into the yeast genome in linearized form via homologous recombination into *URA3* locus. The constructs are shown in **Figure 8**. Principally, they consist of four parts fused together: the nuclear localization signal (NLS) (not shown at the picture), the SIC1 promoter, the desired DNA construct and the EGFP.

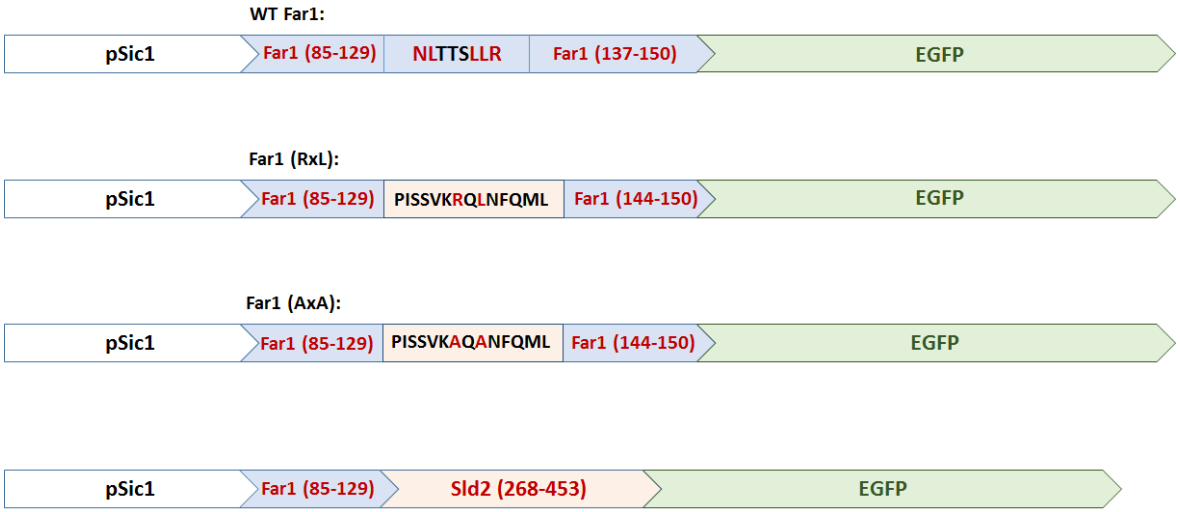

Figure 8. The constructs which enable detection of the docking interaction *in vivo* between Clb5 and C-terminus of Sld2 by the time-lapse fluorescent microscopy experiment.

First, we analyzed the effect of WT Far1 (85-150) degradation caused by the specificity between Far1 docking motif NLxxxL and Clb5. The degradation pattern determined by the fluorescent intensity was correlated with the one obtained in the previous study (Fausova et. Al, 2021) thus making our data reliable.

Introducing the RxL consensus motif of Sld2 to Far1 leads to the degradation of the protein, however the specificity is weaker compared with the Far1 WT consensus motif. Alanine mutations within the RxL consensus motif of Sld2 introduced to Far1 stabilizes the level of the protein. Further we investigated the degradation pattern of the part of Sld2 (without 267 AAs from the N-terminus) and the degron part of Far1(85-150 AAs). Fusion of the truncated part of Far1 (85-129) without its docking site and the C-terminus of Sld2 leads to the protein degradation thus confirming the presence of the unknown docking site within the Sld2 C-terminus (268-453) (**Figure 9**).

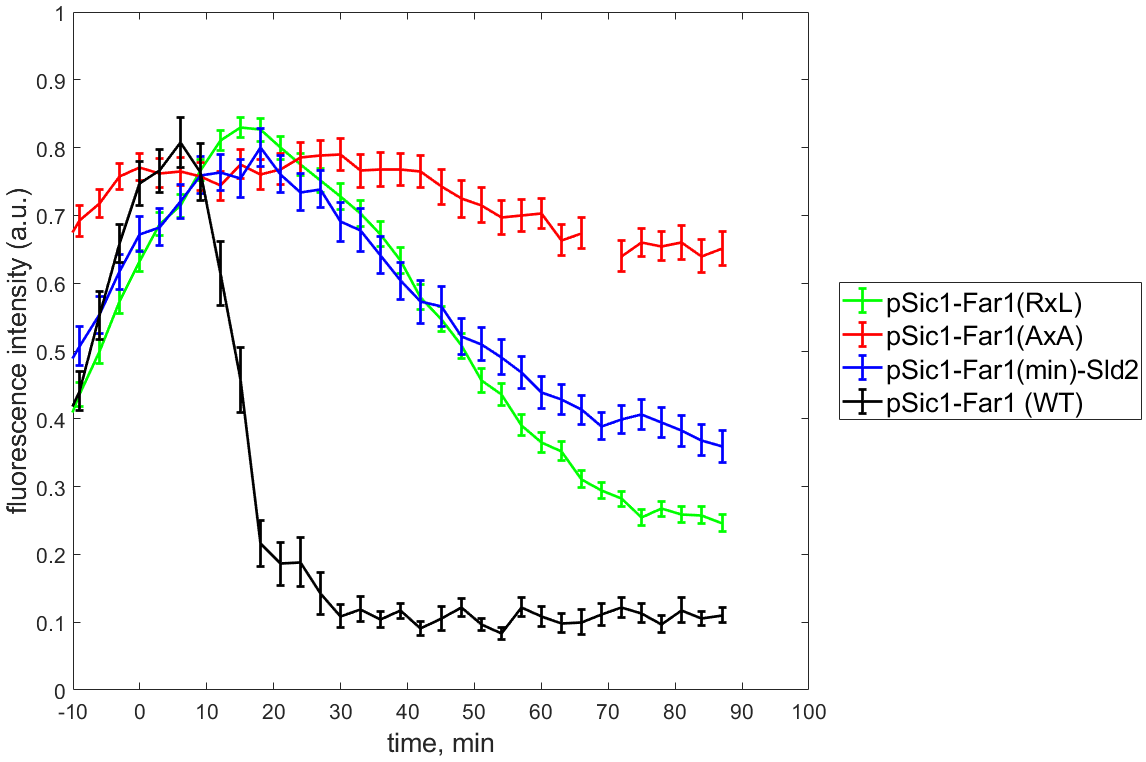

Figure 9. The C-terminus of Sld2 is responsible for triggering degradation.

3.2.3 *In vitro* kinase assays showed specificity of Sld2 to Cdk1-Clb5

In the previous experiment we utilized the *in vivo* system which was generated by fusion of the di-phosphodegron part of Far1 and the C-terminus region of Sld2, which was shown to be important in the terms of triggering the degradation of the whole construct. However, it still remaines unclear if this mechanism of degradation specifically related to Clb5 or the ohter cyclins are also involved in this process.

To address this question, in this part of work the following constructs (**Figure 10**) were designed and analyzed *in vitro* using kinase assay. For this, the constructs were inserted by restriction cloning into the pGEX-4T-1 vector - the expression vector permitting production of a GST-tagged fusion protein.

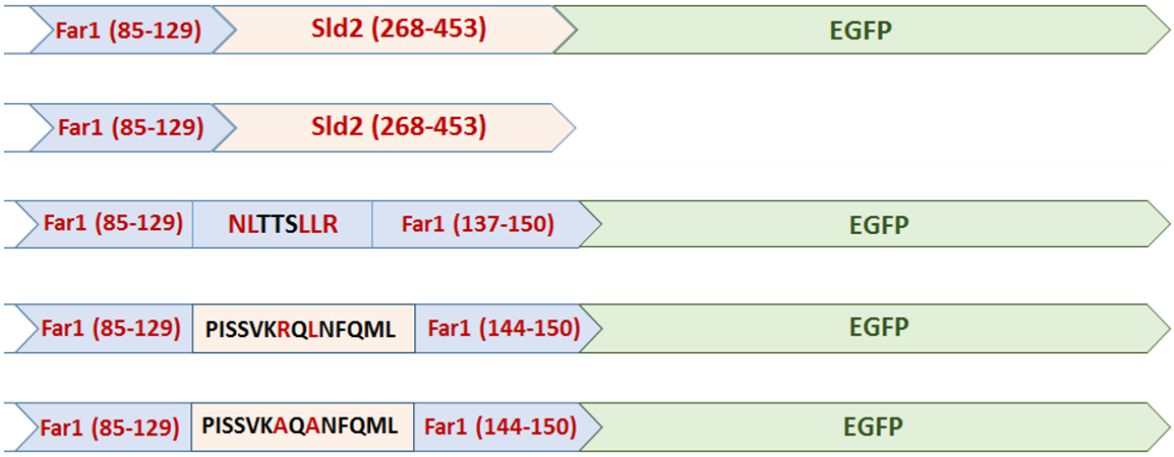

Figure 10. The constructs generated for *in vitro* studies. The DNA constructs were fused with GST-tag for subsequent purification and kinase assay. The last three constructs were described above (**Figure 8**) as they were studied by *in vivo* fluorescent microscopy experiment. The ohter two constructs were generated by fusion of the di-phosphodegron part of Far1 with the C-terminus region of Sld2. One construct had the C-terminus EGFP, while the other did not.

The result of the kinase assay of purified proteins (**Figure 11 A, B**) revealed that phosphorylation of the N-terminus part of Far1 (85-150 AAs), phused with the C-terminus part of Sld2 and the EGFP molecule does not dependent on the presence of EGFP protein. The construct is phosphorylated in the same way without the EGFP, thus confirm that degradation pattern that we got by our previous results (*in vivo* time-lapse fluorescent microscopy) was dependent only on C-terminus of Sld2 and fused to the construct EGFP molecule does not influence on phosphorylation.

The C-terminus part of Sld2 construct is phosphorylated specifically by Clb5. As it is shown in **Figure 11 C**, Cln2, Clb5, Clb3 and Clb2 are not specific to Sld2. According to the result in **Figure 11 D**, both SLiMs in Far1 (NLxxxL and RxL motif from Sld2) possess high specificity to Clb5.

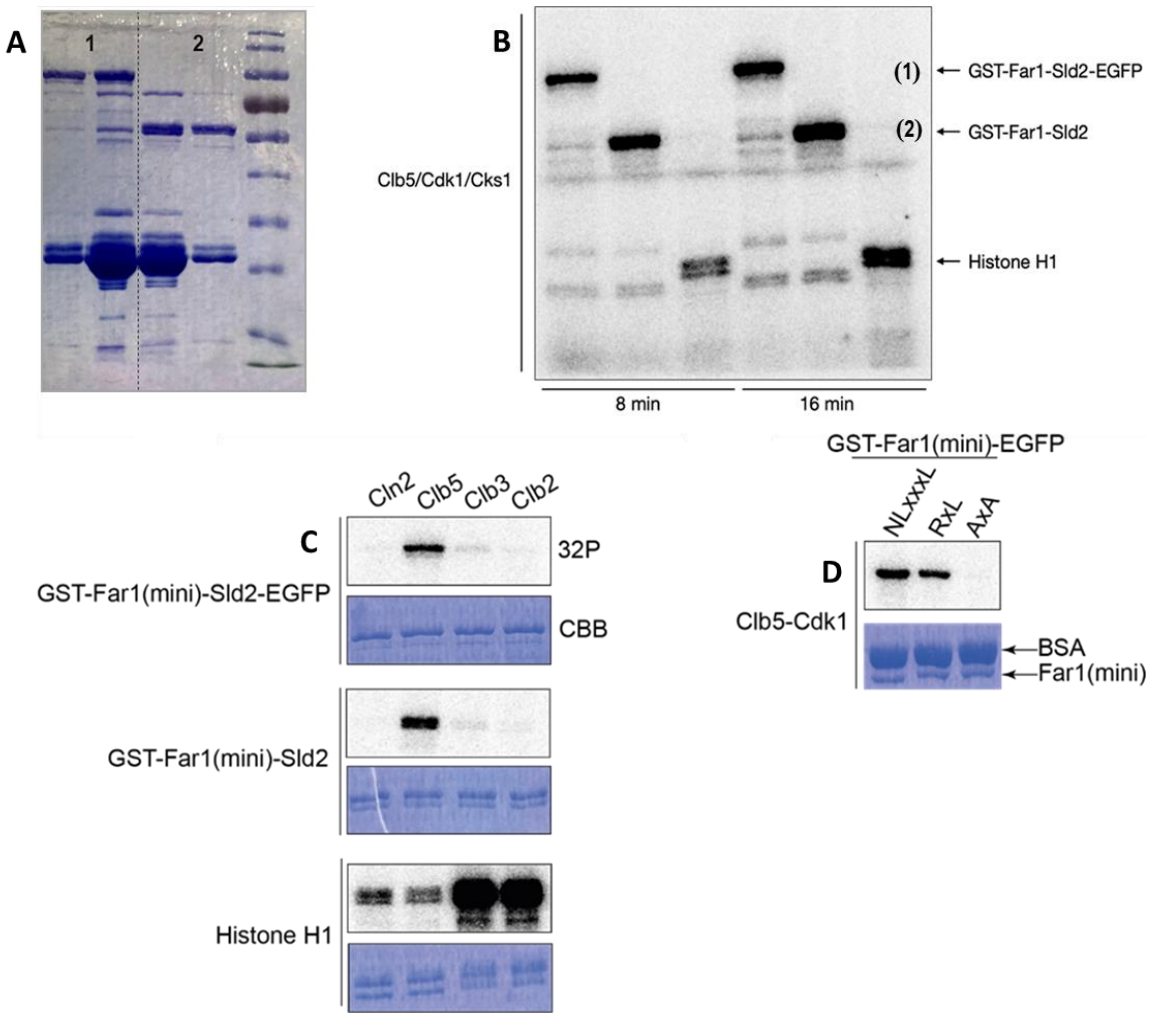

Figure 11. Protein purification and kinase assays. (**A**) Purification of proteins. (**B**) Phosphorylation of the construct is not affected by presence of the EGFP molecule. (**C**) The construct is phosphorylated specifically by Clb5. (**D**) Specificity panel towards Clb5. Both SLiMs in Far1 (NLxxxL and RxL motif from Sld2) possess high specificity to Clb5.

3.2.4 Introducing truncations and mutations within the Sld2 C-terminus part help localize the potential CLb5 docking site

As the Sld2 C-terminus specificity was confirmed both *in vivo* and *in vitro*, we started to search for a potential Clb5-specific docking motif by truncating the Sld2 molecule from its C-terminus. Every truncation within the region of interest was done by eliminating 50 AAs from the Sld2 C-terminus part thus minimizing the search area (**Figure 12**). Truncations were introduced to the region of interest by generating special pairs of primers allowing to get a truncated version of the protein. Afterwards, the plasmid carrying the truncated DNA sequence, was introduced into the yeast genome.

Figure 12. The untruncated construct with the Sld2 potential docking site and the truncated versions of the Sld2 C-terminus part. Every truncation within the region of interest was done by eliminating 50 AAs from the Sld2 C-terminus part thus minimizing the search area for the Clb5-specific docking site. Fusion with the degron part of Far1 (85-129) without its docking site was done to analyze the degradation rate of the whole construct using the microscopy experiment.

These constructs include the short version of Far1 which comprises only the di-phosphodegron part. To analyze the influence of the Sld2 shortened regions on the docking interaction with Clb5 we analyzed timing of degradation by means of time-lapse fluorescence microscopy (**Figure 13**). The results indicate that the third truncation stabilizes the level of the protein of interest meaning that in this case the Far1 phosphorylation-dependent degradation was not induced by the Clb5-Cdk1 complex. Hence, the potential Clb5 docking site was localized within the truncated 150 AAs of Sld2.

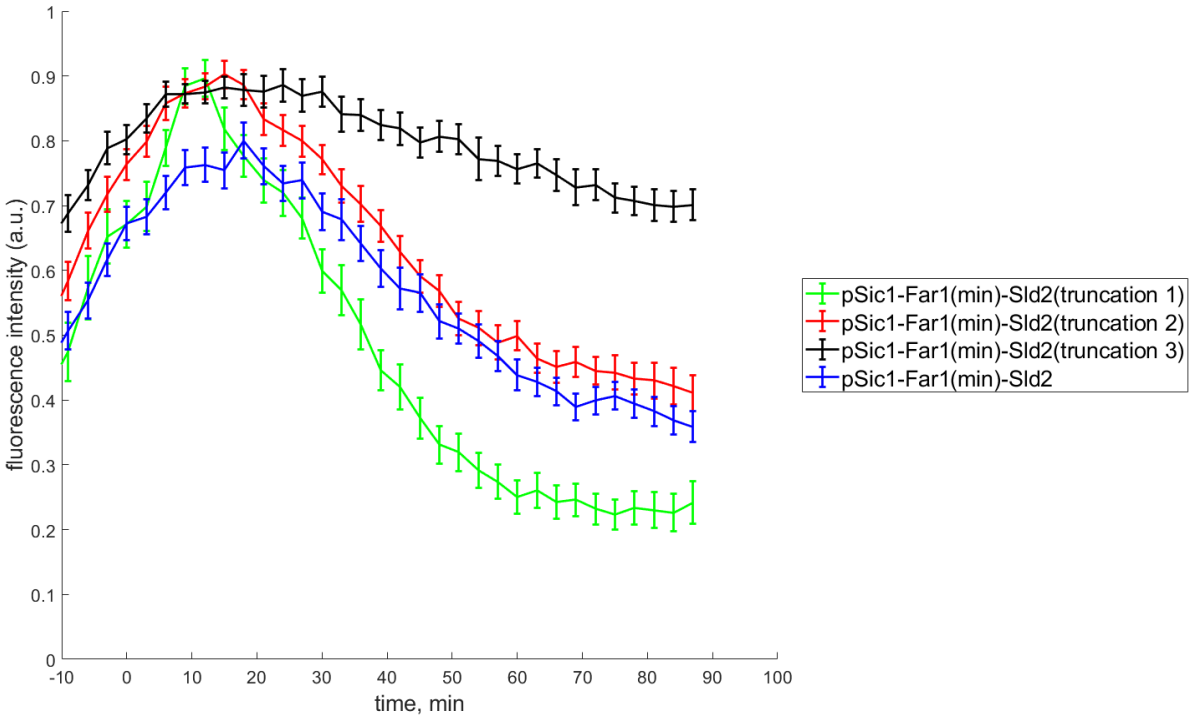

Figure 13. The effect of truncations on the degrafation. Introducing truncations to the Sld2 C-terminus part helped localize the potential Clb5 docking site. Truncation 3 stabilizes the level of the protein hence indicating that the potential Clb5 docking site was removed. Hence, the potential Clb5 docking site was localized within the truncated 150 AAs of Sld2.

To further map the potential docking site, we decided to introduce mutations in the region of Sld2 (303-353 AAs). This region of 50 AAs was localized by the 100 AAs truncation and claimed to be the most important in terms of Clb5 specificity since it contributed to the stabilization of the protein over the cell cycle.

To compare the Sld2 amino acid sequencies from diferent species we used a web-based visualization tool ProViz (**Figure 14**). Allignment within several yeast species showed us highly conserved region between the 345 and 358 AAs. This conserved regioon contains positive amino acids (Lys, K; Arg, R) and based on this finding we decided to introduce point mutations which potentially can have an impact on the interaction with Clb5. Point mutations with charged amino acids influence protein structure and folding therefore they could modulate binding affinity and specificity.

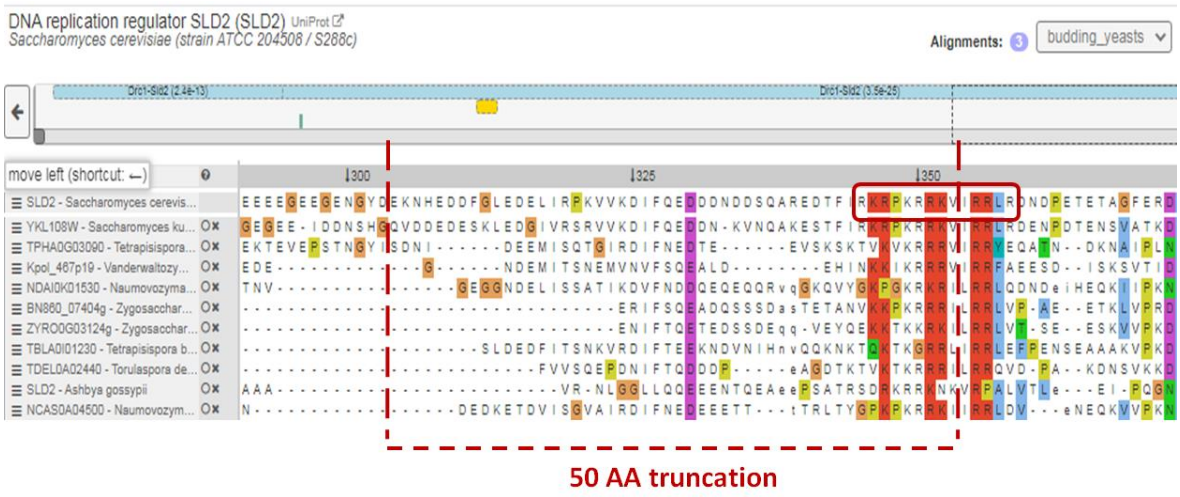

Figure 14. Localization of the Sld2 potential docking site by 50 AA truncation (303-353 AAs).

The first mutation (**Figure 15**) altered positive amino acids (Lys, K; Arg, R) within the conserved region of Sld2 to the uncharged alanines (Ala, A). The second mutation altered positive amino acids to the negative ones (Glu, E) within the same region. The third mutation includes both conserved and non-conserved residues and alters negative amino acids (Asp, D; Glu, E) to the uncharged alanines (Ala, A).

Figure 15. Introducing mutations to the region of 50 AAs of Sld2. Mutation 1 and 3 provide substitution of AAs to alanines, whereas mutation 2 provides substitution to negative AAs.

To analyze the effect of mutations we used the time-lapse fluorescence microscopy (**Figure 16**). Designed mutations 1, 2 and 3 within the region of interest did not considerably change specificity toward Clb5-Cdk1. Degradation difference was insignificant as the degradation curves were nearly identical in comparison with the degradation pattern of the WT Sld2.

Thus, to further investigate the effect of mutations, new constructs were designed which contained the combination of mutations within the framework of Sld2 50 AAs (mitation 1 was combined with mutation 3 and mutation 2 was combined with mutation 3).

The combination of mutation 2 and mutation 3 caused the delay in degradation (**Figure 16**). Hence, the combination of amino acids substitutions within the two regions affects the Clb5 specificity preventing the degradation.

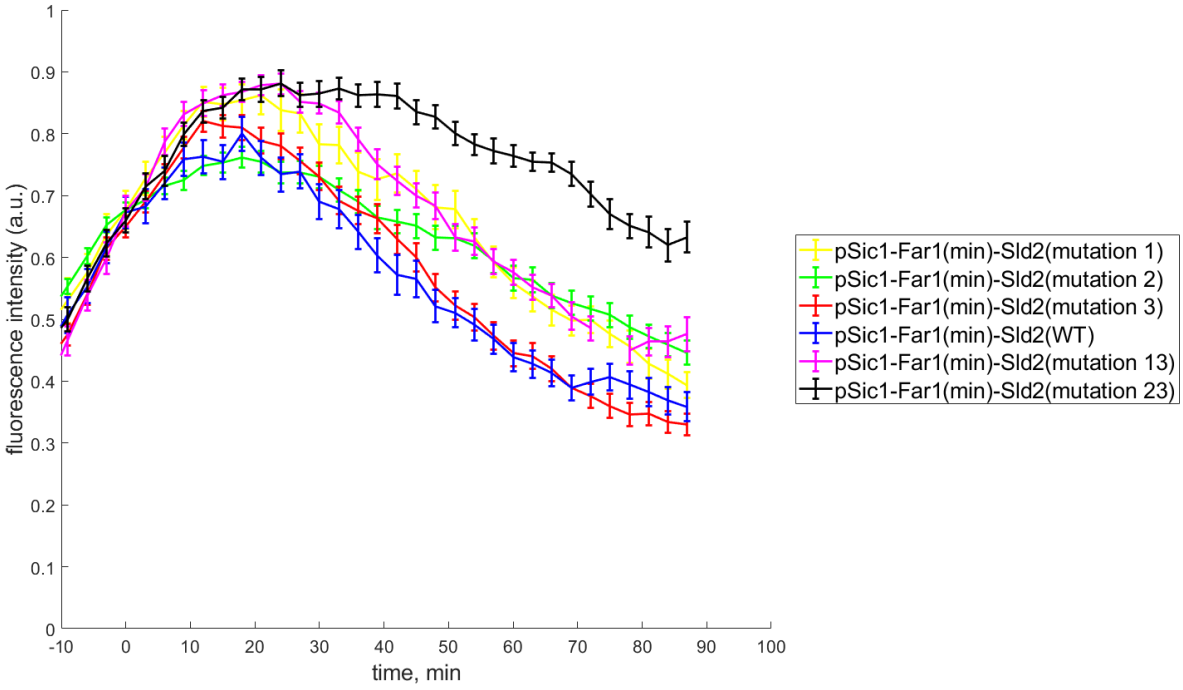

Figure 16. The effect of mutations on the degrafation. Simultaneous introduction of mutation 2 and mutation 3 causes the delay in degradation.

3.2.5 Use of the temperature-sensitive mutants in terms of investigating the role of Sld2 mutations *in vivo*

In this part of the research, we were interested in getting results from *in vivo* experiments and comparing them with *in vitro* data, since the *in vitro* processes sometimes may poorly reflect *in vivo* performance, for example, due to the influence of the cellular environment or because of the unexpectable limitations of recombinant systems.

Sld2 is an essential gene, since the Cdk-dependent pre-LC formation requires Sld2. [25]. Thus, a complete loss of Sld2 function is lethal to the yeast cell.

In this work, we are planning to use a temperature-sensitive strain with allele of *SLD2* (*drc1-1)*, to abolish the interaction between Sld2 and Dpb1. The *drc1-1* has four amino acid alterations (K59R, P85S, G282C, and R358Q) in the Sld2 protein. [25]. These mutations, introduced into the targeted essential gene locus, makes the allele functional under permissive conditions (+25°C) and nonfunctional under restrictive conditions (+37°C).

According to [22], these mutations directly affect formation of the Sld2–Dpb11 complex under the restrictive conditions (+37°C) by making it defective.

First of all, we analyzed the thermosensitive growth of the aforementioned strain *drc1-1* by performing a yeast spotting assay (**Figure 17**) to compare the cell growth rate under the permissive and restrictive growth conditions. The result clearly reflects the growth defect phenotype which serves as an indicator of the loss of interaction between the Sld2–Dpb11 complex thus preventing proper replication and limiting the cells' viability.

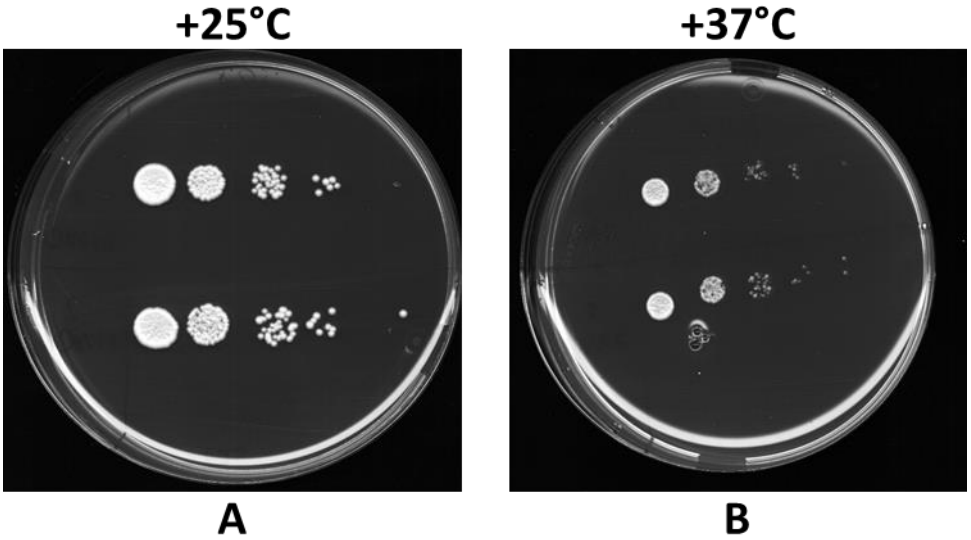

Figure 17. The yeast growth assay of two colonies of *drc1-1 (sld2)* strain under the permissive (**A**) and restrictive (**B**) growth conditions.

All our studies of Sld2 were done by implementing the artificial system, since Sld2 is an essential gene, however, the use of the strain with the temperature-sensitive allele of Sld2 provides the possibility of studying the full length Sld2 under the native promoter and therefore get the more representative data. By now, we constructed several plasmids containing mutations within the full length of Sld2 under its native promoter, and we expect that these mutations will influence the growth pattern promoting the further understanding of control mechanisms under the replication initiation.

SUMMARY

Docking interactions between SLiMs and cyclin-dependent kinases are shown to be critical regulators of eukaryotic DNA replication (Wilmes et al., 2004). S-phase Cdk specificity is known to be governed by RxL motifs with the consensus R/Kx-L-F/L/M/P or R/K-x-L-x-F/L/M/P sequence, however, our recent results (unpublished data) revealed that the S-Cdk (Clb5) specific substrate Sld2 C-terminus may contain a motif responsible for high Clb5-specificity, since it remains highly specific even in the absence of all short [RxL] motifs it has.

In this work we analyzed the docking interaction between the cyclin Clb5 and the Clb5-specific substrate Sld2 in Saccharomyces cerevisiae at the G1/S stage. We determined specificity within the C-terminus region (268-453 amino acids) of the S-Cdk (Clb5) specific substrate Sld2 both *in vitro* and *in vivo* and further mapped this region by truncations. To analyze more precisely the effect of different mutations *in vivo* by time-lapse fluorescence microscopy we utilized the system of the di-phosphodegron part of Far1 and EGFP fused to the constructs of interest. It was revealed that within a truncated region of Sld2 (303-353 AAs) there were two motifs, responsible for Clb5 specificity, - one was in the conserved region while another one was found among non-conserved residues.

One aspect of our next work will include study of the temperature-sensitive mutant of Sld2, *drc1-1*. We are planning to perform an analysis of the thermosensitive growth in terms of investigating the role of different Sld2 mutations *in vivo* (alanine mutations of phospho-sites, alanine mutations of RxL motifs, as well as introducing the pre-designed mutations to the region of Sld2 (303-353 AAs), which seems most prominent).

Considering all of these, in the current work we mapped the potencial docking site which is responsible for Sld2 specific phosphorylation by Clb5-Cdk1 complex.

REFERENCES

Alberts, Bruce. "Molecular biology of the cell." (2008).

Ang, Xiaolu L., and J. Wade Harper. "SCF-mediated protein degradation and cell cycle control." Oncogene 24.17 (2005): 2860-2870.

Ardito, Fatima, et al. "The crucial role of protein phosphorylation in cell signaling and its use as targeted therapy." International journal of molecular medicine 40.2 (2017): 271-280.

Bandyopadhyay, S., Bhaduri, S., Örd, M., Davey, N. E., Loog, M., & Pryciak, P. M. (2020). Comprehensive analysis of G1 cyclin docking motif sequences that control CDK regulatory potency *in vivo*. Current Biology, 30(22), 4454-4466.

Barberis, Matteo. "Sic1 as a timer of Clb cyclin waves in the yeast cell cycle–design principle of not just an inhibitor." The FEBS journal 279.18 (2012): 3386-3410.

Faustova, Ilona, et al. "A new linear cyclin docking motif that mediates exclusively S-phase CDK-specific signaling." The EMBO Journal 40.2 (2021): e105839.

Gardner, Jennifer M., and Sue L. Jaspersen. "Manipulating the yeast genome: deletion, mutation, and tagging by PCR." Yeast Genetics. Humana Press, New York, NY, 2014. 45-78.

Harper, Sandra, and David W. Speicher. "Expression and purification of GST fusion proteins." Current protocols in protein science 52.1 (2008): 6-6.

Kearsey, Stephen E., and Juraj Gregan. "Using the DHFR heat-inducible degron for protein inactivation in Schizosaccharomyces pombe." DNA Replication. Humana Press, 2009. 483-492.

Li, Yinyin, Frederick R. Cross, and Brian T. Chait. "Method for identifying phosphorylated substrates of specific cyclin/cyclin-dependent kinase complexes." Proceedings of the National Academy of Sciences 111.31 (2014): 11323-11328.

Li, Zhijian, et al. "Systematic exploration of essential yeast gene function with temperature-sensitive mutants." Nature biotechnology 29.4 (2011): 361-367.

Loog, Mart, and David O. Morgan. "Cyclin specificity in the phosphorylation of cyclin-dependent kinase substrates." Nature 434.7029 (2005): 104-108.

Lyons, Nicholas A., et al. "Sequential primed kinases create a damage-responsive phosphodegron on Eco1." Nature structural & molecular biology 20.2 (2013): 194.

Majumdar, Abir, et al. "Allostery governs Cdk2 activation and differential recognition of CDK inhibitors." Nature Chemical Biology 17.4 (2021): 456-464.

Mardo Kõivomägi. Studies on the substrate specificity and multisite phosphorylation mechanisms of cyclin-dependent kinase Cdk1 in Saccharomyces cerevisiae (2013).

Masumoto, Hiroshi, et al. "S-Cdk-dependent phosphorylation of Sld2 essential for chromosomal DNA replication in budding yeast." Nature 415.6872 (2002): 651-655.

Morgan, David Owen. The cell cycle: principles of control. New science press, 2007.

Muramatsu, Sachiko, et al. "CDK-dependent complex formation between replication proteins Dpb11, Sld2, Pol ε, and GINS in budding yeast." Genes & development 24.6 (2010): 602-612.

Murray, Hunt. The cell cycle (1993).

Nishi, Hafumi, Kosuke Hashimoto, and Anna R. Panchenko. "Phosphorylation in protein-protein binding: effect on stability and function." Structure 19.12 (2011): 1807-1815.

Örd, Mihkel, and Mart Loog. "How the cell cycle clock ticks." Molecular biology of the cell 30.2 (2019): 169-172.

Örd, Mihkel, et al. "Cyclin-specific docking mechanisms reveal the complexity of M-CDK function in the cell cycle." Molecular cell 75.1 (2019): 76-89.

Örd, Mihkel, et al. "Proline-rich motifs control G2-CDK target phosphorylation and priming an anchoring protein for Polo kinase localization." Cell reports 31.11 (2020): 107757.

Stark, Michael JR. "5 Studying Essential Genes: Generating and Using Promoter Fusions and Conditional Alleles." Methods in Microbiology 36 (2007): 79-102.

Tai, Hwan-Ching, and Erin M. Schuman. "Ubiquitin, the proteasome and protein degradation in neuronal function and dysfunction." Nature Reviews Neuroscience 9.11 (2008): 826-838.

Tak, Yon-Soo, et al. "A CDK-catalysed regulatory phosphorylation for formation of the DNA replication complex Sld2–Dpb11."

Van Roey, Kim, et al. "Short linear motifs: ubiquitous and functionally diverse protein interaction modules directing cell regulation." Chemical reviews 114.13 (2014): 6733-6778.

Wilmes, Gwendolyn M., et al. "Interaction of the S-phase cyclin Clb5 with an 'RXL'docking sequence in the initiator protein Orc6 provides an origin-localized replication control switch." Genes & development 18.9 (2004): 981-991.

Wong, H. & Elledge, S. J. DRC1, DNA replication and checkpoint protein 1, functions with DPB11 to control DNA replication and the S-phase checkpoint in Saccharomyces cerevisiae. Proc. Natl Acad. Sci. USA 96, 3824–3829 (1999)

Zegerman, Philip, and John FX Diffley. "Phosphorylation of Sld2 and Sld3 by cyclin-dependent kinases promotes DNA replication in budding yeast." Nature 445.7125 (2007): 281-285.

Non-exclusive licence to reproduce thesis and make thesis public

I, Valentina Mikhailova,
(*author's name*)

1. herewith grant the University of Tartu a free permit (non-exclusive licence) to:

1.1. reproduce, for the purpose of preservation, including for adding to the DSpace digital archives until the expiry of the term of copyright, and

1.2. make available to the public via the web environment of the University of Tartu, including via the DSpace digital archives, under the Creative Commons licence CC BY NC ND 3.0, which allows, by giving appropriate credit to the author, to reproduce, distribute the work and communicate it to the public, and prohibits the creation of derivative works and any commercial use of the work from ***20/05/2024*** until the expiry of the term of copyright,

"Analysis of the docking interaction between Sld2 and Clb5 in budding yeast**"**,
(*title of thesis*)

supervised by Ervin Valk,
(*supervisor's name*)

2. I am aware of the fact that the author retains the rights specified in p. 1.

3. I certify that granting the non-exclusive licence does not infringe other persons' intellectual property rights or rights arising from the personal data protection legislation.

Valentina Mikhailova
29/05/2021