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**Construction and analysis of Far1 based
synthetic inhibitor protein for Cln2-Cdk1**

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Controlling the yeast cell cycle G1/S phase transition by synthetic inhibitory protein

Abstract:

The complex cell divides the process into distinct and more easily attainable events, allowing it to reproduce itself with exceptional precision. This highly regulated series of events is known as the cell cycle. The enzymes called cyclin-dependant kinases (Cdks) are the chief constituents of the cell cycle control system and are activated by binding to regulatory proteins called cyclins. Cell cycle progression requires accurately ordered inhibition of cyclins and Cdk inhibitor proteins (CKIs) by ubiquitination. Far1: an inhibitor of G1/S cyclin-Cdk complexes in response to mating pheromones can arrest the cell cycle in the G1 phase. In this study, a docking site from Sic1 (an inhibitor of S and M Cdk complexes) responsible for phosphorylation was put to N-Far1(1-170aa) to make a synthetic Cln2 specific inhibitor and test its ability to cause cell cycle arrest in the G1 phase.

Keywords:

Cyclin-dependent kinase, cyclins, Cdk inhibitor proteins, cell cycle arrest

CERCS:

Proteins, Enzymology

Pagaripärmi Far1 valgul põhineva Cln2 spetsiifilise sünteetilise inhibiitorvalgu konstrueerimine ja analüüsimine.

Lühikokkuvõte:

Rakutsükkel on tuntud kui täpselt reguleeritud keerukate sündmuste jada, mis võimaldab rakkudel paljuneda. Selle kontrolli eest vastutavad tsükliinsõltuvad kinaasid (Cdk), mis aktiveeritakse rakutsükli erinevatele faasidele omaste tsükliinvalkude poolt. Sellise süsteemi veatu toimimine vajab lisaks aktiveerivatel tsükliinidele ka inhibeerivaid Cdk inhibiitor valke (CKI). Üheks selliseks on pagaripärmi valg Far1, mis keskkonnas leiduva feromooni korral peatab rakutsükli G1 faasis. Käesolevas uuringus tõsteti tsükliinvalgule Cln2 spetsiifiline

äratundmismotiiv valgust Sic1 valgu Far1 N-terminaalsesse ossa, et luua sünteetiline inhibiitor Cdk'le ja uurida selle võimet inhibeerida Cln2 valku feromoonist sõltumatult.

Võtmesõnad:

Tsükliinsõltuv kinaas, tsükliinid, Cdk inhibiitorvalk, rakutsükli arest.

CERCS:

Proteiinid, ensümolooia

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TERMS, ABBREVIATIONS AND NOTATIONS

aa - Amino Acids

CAK - Cyclin-Dependent Kinase Activating kinase

CDK - Cyclin-Dependent Kinase

CKI - Cyclin-Dependent Kinase Inhibitor

clonNAT - Nourseothricin

DMSO - Dimethyl Sulfoxide

EDTA - Ethylenediaminetetracetic acid

EGFP - Eukaryotic Green Fluorescent Protein

GFP - Green Fluorescent Protein

LB - Luria-Bertani media

LP - Leucine- and Proline-rich docking motif

MAP - Mitogen-Activated Protein

MBF - MCB Binding Factor

MUT - Mutant version

OD600 - Optical Density at 600nm

PCR - Polymerase Chain Reaction

RxL - S-CDK docking motif

SBF - SCB Binding Factor

SC-URA - Synthetic Complete Media without Uracil

SDM – Site-Directed Mutagenesis

SDS - PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SLiM - Short Linear Motif

SS-DNA - Salmon Sperm DNA

TE - Tris-EDTA

WT - Wild Type version

YPD - Yeast extract, Peptone, Dextrose media

INTRODUCTION

Cells are the smallest unit of life and are frequently referred to as the building blocks of life. All cells emerge from the division of pre-existing cells making cell division a vital process for the function and development of all life. This highly regulated and complex series of events of cell growth and division is termed the cell cycle. The cell cycle can be divided into four stages, namely G1, S, G2 and M, wherein the cell grows, replicates its contents, and then distributes them among two daughter cells. The dysregulation of the cell cycle has severe consequences; for example, cancer is caused by unregulated cell division during the cell cycle. Thus, knowing the molecular pathways governing cell cycle control is essential for developing therapeutics.

The yeast *Saccharomyces cerevisiae* can be considered a model organism to study the cell cycle. Despite being single-celled, yeasts have cellular organisation analogous to that of higher-order organisms. Since it is highly crucial that the events of the cell cycle occur in the correct order, the cell cycle control system regulates the timing and coordination of the cell cycle events. The main components of the cell cycle control system are enzymes known as cyclin-dependent kinases (Cdks), which are activated by binding to regulatory proteins known as cyclins. The key regulator in budding yeast is Cdk1 which controls all of the cell cycle events. The level of cyclins oscillates in the cell cycle, and so different cyclins promote different cell cycle events. The expressed cyclins alter Cdk1 substrate targeting by binding to linear motifs on the substrate. Recognising unique docking motifs in substrate allows for cyclin-specific phosphorylation.

Cdk inhibition by Cdk inhibitor proteins (CKIs) is essential for cell cycle progression. In budding yeast, the two CKIs are Sic1 and Far1 proteins which suppress the activity of Cdks in the G1 phase bringing about a stable transition during which extracellular factors can administer entry into the next cycle.

In our lab, it was found that in N-terminus Far1, the phosphorylation of a phospho-dependant docking site by Cln2-Cdks is needed for inhibition. But if this site is replaced with VLLPP docking site from Sic1, the inhibition is stronger. Thus, we decided to experiment with how a docking site that has specificity for phosphorylation in one protein can be used to make a specific inhibitor for another protein.

1 LITERATURE REVIEW

1.1 The Cell Cycle

All cells emerge from the division of pre-existing cells. Cell reproduction is crucial for the development and function of all life. In unicellular organisms, the entire new organism is a result of cell division. In multicellular organisms, numerous divisions of a single founder cell lead to different types of cells that make up the tissues and organs.

The complex cell reproduces itself with exceptional precision by dividing the process into distinct and more easily attainable events. The highly regulated series of events leading to eukaryotic cell division is termed the **cell cycle**, wherein the duplication of the cell's contents followed by their equal distribution into daughter cells takes place.

G1/ Growth1/ Gap1 is the longest phase of the cell cycle. Most of the cell's components like cytoplasmic organelles (ribosomes, lysosomes etc.), membrane, structural proteins and RNAs are replicated continuously, resulting in the progressive doubling of cell size by the end of the cycle. G1 is a crucial regulatory period because this is where the cell decides to either continue division or exit the cell cycle to enter the non-dividing stage called G0 (e.g., neurons don't undergo cell division) (Morgan, 2007).

The synthetic or S phase of the cell cycle is where DNA replication and chromosome duplication takes place. The centrosome/spindle pole body (in yeast) is responsible for organising microtubules, which form the spindle fibers during mitosis and is duplicated in the S phase. The chromosomes and centrosome are present as only one copy per cell and must be doubled only once per cycle. The duplicated chromosomes are known as sister chromatids.

G2/ Growth2/ Gap2 is another growth phase where the cell prepares itself for mitosis. The gap phases provide extra time for growth and serve as regulatory transitions where various intracellular or extracellular signals can control advancement to the next stage.

Following G2 is the M phase where nuclear division (mitosis) followed by cell division (cytokinesis) occurs. Mitosis distributes the duplicated chromosomes equally to a pair of daughter nuclei and involves the following processes: prophase, metaphase, anaphase and telophase.

During prophase, the nuclear membrane starts to disintegrate, and the centrosomes start migrating towards the opposite poles of the cell (in higher eukaryotes). However, in yeast, closed mitosis takes place wherein the nuclear envelope remains intact. At metaphase, the centrosomes are already at the opposite poles of the cell. The sister chromatids in each chromosome pair are attached to microtubules coming from opposite poles of the spindle to the cell's middle at the metaphase plate (in budding yeast, chromosomes don't align at the metaphase plate). Sister chromatid segregation occurs during anaphase, where they are pulled away from each other to the opposite ends of the cell. In telophase, the nuclear membrane and nuclei start reappearing such that there are new nuclei, one for each set of chromosomes (for higher eukaryotes).

The cell itself divides by cytokinesis, and the process varies greatly among different organisms. Completion of the cell cycle is when the cell pinches into two. These individual daughter cells can again enter the cell cycle to further divide into two, and so on.

In most cells, the cell cycle's different events occur against the background of continuous cell growth. This means that the cell's mass doubles in each cycle, but the average size remains the same. But on the other hand, cell growth and division are not coordinated in many types of cells. E.g., neurons and muscle cells are capable of considerable growth without cell division (Morgan, 2007).

There are three critical regulatory checkpoints in the cell cycle where cell cycle events are commenced: Start or G1/S, G2/M, and metaphase - to - anaphase transition. The Start is a vital regulatory transition at entry into the cell cycle in mid to late G1; G2/M controls entry into mitosis. Metaphase-to-anaphase transition is where the cell cycle's final events are initiated.

(Morgan, 2007)

1.2 Yeast as a model organism

Yeast is a unicellular eukaryotic microorganism belonging to kingdom fungi. It typically grows via asexual reproduction - by budding (*Saccharomyces cerevisiae*) or binary fission (*Schizosaccharomyces pombe*). There are various practical applications of yeast, e.g., baking, brewing, molecular genetics etc. But what makes yeast a model organism for the study and research of cell cycle control?

Among several advantages, the most prominent one is the ease of genetic analysis. They can multiply quickly in culture conditions and have comparatively small, fully defined genomes. Both budding and fission yeast can proliferate in a haploid state, thereby avoiding complications caused by a second copy of the gene while generating mutations (that inactivate a gene) and analysing them. Yeast have a high rate of homologous recombination, so various methods can manipulate the expression of specific genes. Even though yeasts are single-celled, their cellular organisation is comparable with higher-order organisms. Which is why they are important organisms to study in order to gain a better understanding of basic molecular processes in humans.

In budding yeast, the bud first appears at the end of the G1 phase and grows throughout the S and M phase consistently till its size is slightly smaller than that of the mother cell. Following the distribution of one set of chromosomes to the bud after mitosis completion, pinching off of bud takes place from the mother cell - (bud is) now called the daughter cell. Consequently, bud size is a helpful marker of cell cycle position. The metaphase - anaphase transition is a more critical regulatory checkpoint in budding yeast. (Morgan, 2007)



Figure 1. The cell cycle of budding yeast. The G1 phase in *Saccharomyces cerevisiae* is more prolonged, and there is a vague G2 phase between S and M phase. Mitosis is not regulated as extensively compared to other eukaryotic organisms. *Figure adapted from Cell Cycle: Principles of Control (Morgan,2007)*

1.3 Cell Cycle Control System

It is exceptionally vital that the events of the cell cycle occur in the correct order. The eukaryotic cell contains a complex regulatory network - the cell cycle control system that controls cell cycle events' timing and coordination.

The cell cycle control system's chief constituents are the enzymes called cyclin-dependant kinases (Cdks) activated by binding to regulatory proteins called cyclins. Cdks are a family of serine/threonine protein kinases whose members are small proteins. The level of cyclins oscillates in the cell cycle, thereby causing fluctuation in Cdk activities (**Figure 2**). The active site of Cdks is blocked in the absence of cyclins. Different cyclins stimulate different cell cycle processes. Functional specialisation of cyclins is due to direct interaction between the cyclin and a specific subset of Cdk substrates.

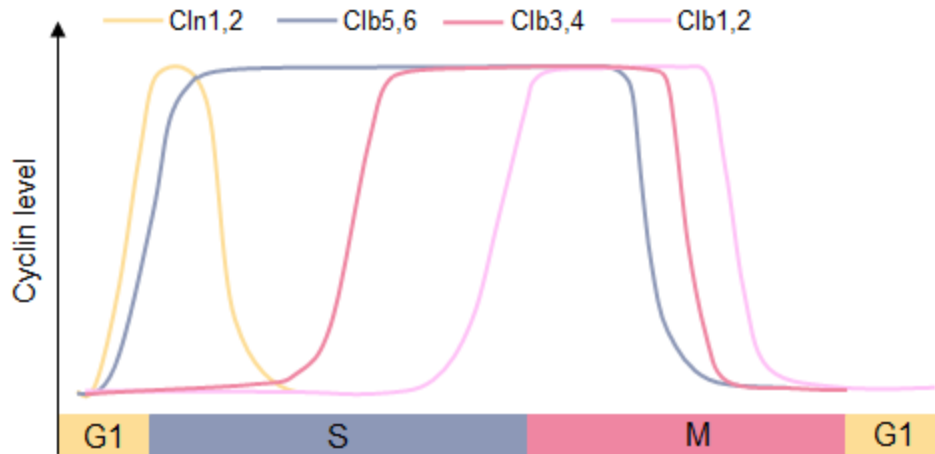


Figure 2. Expression of cyclins during the cell cycle. The graph represents the accumulation of cyclins during different phases of the cell cycle. The level of cyclins oscillates in the cell cycle, thereby causing fluctuation in Cdk activities. *Figure adapted from Cell Cycle: Principles of Control (Morgan, 2007)*

Kinase-substrate specificity is of utmost importance. Protein kinases form tight complexes with their substrate which is either bridged by a third protein forming a scaffold or by direct high-affinity interaction between a part of the kinase and a short sequence on substrate known as a docking site. Docking sites increase the efficiency of substrate phosphorylation. A single substrate can have multiple docking sites thereby increasing affinity for a kinase (Holland and Cooper, 1999). Several kinases recognize docking motifs which are Short Linear Motifs (SLiMs) often in disordered regions (Bandyopadhyay *et al.*, 2020). Intrinsically disordered proteins are also important components of cellular signalling machinery, while some proteins are completely disordered, some contain intrinsically disordered regions which play a key role in the regulation of signalling pathways and cellular processes (Wright and Dyson, 1999; Wright and Dyson, 2014)

Control of all cell cycle events in the budding yeast is by a solitary fundamental Cdk called Cdk1 (Cdc28). Different cyclins stimulate different cell cycle processes many of which recognize unique docking motifs in substrates that allow them to be phosphorylated in a

cyclin-specific manner. The timing and expression of the function of the cell cycle events depend upon gradual changes in the substrate specificity of Cdk1 (Kõivomägi *et al.*, 2011).

Cyclins that regulate Cdks controlling cell cycle progression can be divided into four classes. G1/S, S and M cyclins are directly involved in the control of distinct cell cycle events, whereas the G1 cyclins contribute to cell cycle entry with regards to extracellular factors.

G1 cyclin - Cln3 helps in cell growth coordination with entrance into the new cell cycle. It differs from other cyclins because its level doesn't oscillate in a fixed pattern during the cell cycle but gradually increases throughout the cycle in response to cell growth and external growth regulatory signals.

G1/S cyclins - Cln1 and Cln2 oscillate during the cell cycle, rising in late G1 and falling in the early S phase. Functions mostly include triggering progression through Start and initiating progress leading to DNA replication and initiating duplication of the spindle pole body.

An upsurge of G1/S cyclins culminates in the appearance of S cyclins - Clb5 and Clb6, which form S cyclin-Cdk complexes directly responsible for stimulating DNA replication. Their levels remain high throughout the S phase, G2 and early mitosis. S cyclins' substrate specificity depends upon the region called the hydrophobic patch, which lies on the cyclin surface. This patch binds to substrate protein containing complementary hydrophobic sequence known as RXL (or Cy) motif. The interaction increases kinase-substrate affinity and enhances the rate of substrate phosphorylation.

M cyclins - Clb1, 2, 3, 4, form M cyclin-Cdk complexes responsible for cellular changes leading to the assembly of mitotic spindle and alignment of sister chromatid pairs on the spindle at metaphase. Their destruction in anaphase paves the way for mitotic exit and cytokinesis.

Specifically, the late G1 cyclins Cln1 and Cln2 and not the other G1, S or M cyclins recognise a SLiM called an "LP" motif (for its enrichment in Leu and Pro residues). These LP docking interactions are found to increase the efficiency, specificity, and multiplicity of substrate phosphorylation (Kõivomägi *et al.*, 2011; Bhaduri and Pryciak, 2011).

Phosphorylation of a threonine residue beside kinase active site is required for the complete activation of a Cdk and normal Cdk function in the cell in addition to cyclin binding. The enzymes Cdk-activating kinases (CAKs) catalyse phosphorylation at this point.

The cell cycle control has evolved to promote clear and irreversible transitions so that the initiation of the cell cycle sets in motion mechanisms such that each event proceeds to completion. This is achieved by Cdks' behaviour as molecular switches. This allows the cells to prevent the damage that might occur if the events were only partially initiated. (Morgan, 2007)

Cell cycle progression requires accurately ordered inhibition of cyclins and Cdk inhibitor proteins (CKIs) by ubiquitination. (Tyers and Jorgensen, 2000) In yeast, Start requires a threshold level of G1 cyclins to activate Cdk1 in the late G1 phase. As the cells pass Start, S cyclin-Cdk complexes are activated. The prime function of Cln-Cdk activity is to phosphorylate an inhibitor, thereby targeting it for destruction to move towards the S phase. (Schwob *et al.*, 1994)

1.4 What happens during the G1 phase

Nutrient deficiency or the presence of mating pheromones may cause cells to be arrested in G1 prior to the execution of Start. However, once G1 is started, these factors can't prevent progression until the following G1 phase, and therefore the cells are committed to dividing. Cln3 regulates CLN1, and CLN2 RNAs as the accumulation of these requires Cln associated kinase activity. Cln genes regulate the size and morphology of cells. SWI4 RNA levels rise dramatically during late G1 and peak just before or at the same time as CLN1, CLN2 RNA levels (Di Como *et al.*, 1995).

The transcription factors SBF and MBF (bind to SCBs (activated by Swi4-Swi6 complex) and MCBs (activated by Mbp1-Swi6 complex) respectively-promoter regions upstream of key genes expressing G1/S proteins which signal transition from G1 to S phase) drive the G1 to S

transition of the cell cycle. Cln3-Cdk is primarily responsible for activating SBF (SCB binding factor) and MBF (MCB binding factor) at Start. This is accomplished by phosphorylation and subsequent shuttling out of the nucleus of the repressor Whi5, which frees SBF and MBF from inhibition (**Figure 3**). MBF targets are mostly involved in DNA replication and associated processes, whereas SBF targets regulate cell morphology (Teufel *et al.*, 2019).

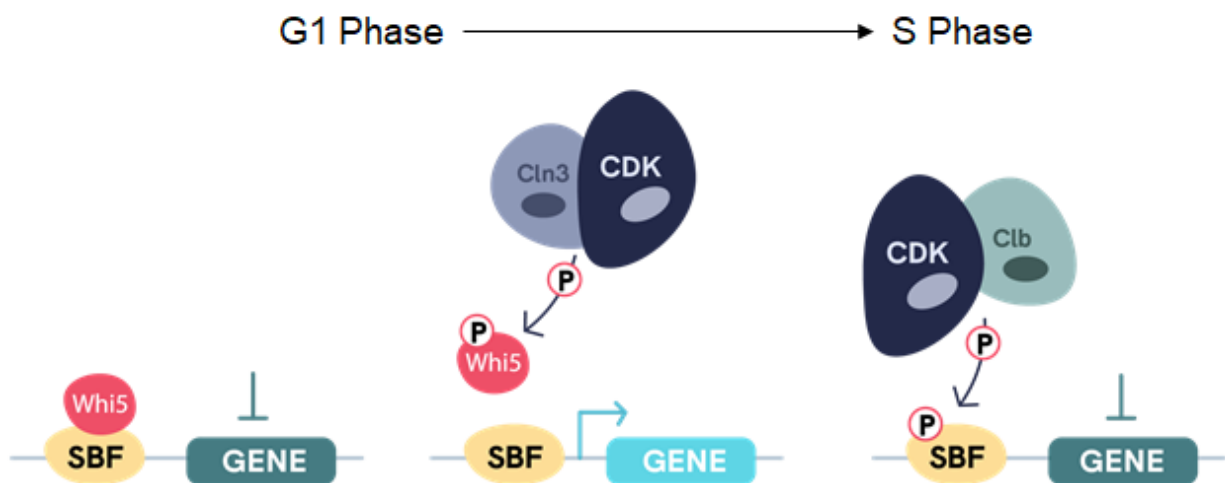


Figure 3. Whi5 regulation during cell cycle. Whi5 is the transcriptional regulator in G1 phase for key regulatory genes controlling transition from G1 to S phase. It binds to SBF and suppresses its transcription. Cln3-Cdk complex via phosphorylation promotes the dissociation of Whi5 from SBF at Start which results in activation of SBF and progression to S-phase. *Adapted from Anastasiya Kishkevich.*

The knockout of G1 cyclins alters the size and transcriptional timing. Loss of 2 of the three cyclins causes cell size increase relative to the wild type before division. The amount of Cln3 protein per unit of cell protein is constant, and since Cln3 appears to be nuclear, Cln3 concentration in the nucleus increases with increasing cell size (Sillje *et al.*, 1997). Cln3 loss as a single deletion results in a cell size increase equivalent to double deletions (dramatic increase in cell size). It also delays the onset of the cell cycle. Cln1 or Cln2 deletion results in

wild type-like size, though Cln2 deletion has been shown to have a more drastic effect on cell cycle timing. Additionally, SBF and MBF target expression were shown to be delayed more prominently (Teufel *et al.*, 2019). Moreover, distinct G1 cyclins regulate cell cycle timing in mother and daughter cells in *S. cerevisiae*. On one hand, Cln3 is crucial for mother cells in the absence of which the G1 phase is much longer. And on the other hand, in daughter cells, the normal timing of cell cycle initiation is dependent on Cln1 and Cln2 transcription (induced in size dependent manner) (Lew *et al.* 1992).

1.5 Inhibition of Cdks

In actively multiplying cells, Cdk activity is subdued during G1 bringing about a stable transition period during which cell growth and other regulatory influences, for example, extracellular factors, can administer entry into the next cell cycle.

A combination of three processes suppresses Cdk activity in G1. They are: increased cyclin destruction, decreased cyclin gene expression, and Cdk activity inhibition by CKIs that bind and inactivate cyclin-CDK complexes. CKIs are also responsible for encouraging cell cycle arrest in G1 in response to unfavourable conditions or intracellular conditions like DNA damage. The hydrophobic patch interacts with CKIs where several CKIs bind to it on cyclin, thus preventing binding and phosphorylation of Cdk substrates.

Sic1 is a CKI contributing to the establishment of a stable G1 in budding yeast. It is an inhibitor of S and M-CDK complexes and is expressed in the G1 phase at a high level to ensure that no S and M-CDK activity exists in those cells. Sic1 does not inhibit G1/S CDKs, so these kinases are active at the Start transition. Upon phosphorylation by G1/S CDKs, Sic1 is targeted for destruction in late G1, allowing S-CDK activation at the beginning of the S phase. This essentially means that Sic1 inactivation is essential for the transition to the S phase (Morgan, 2007).

When the Sic1 molecule was truncated at its C-terminus, a 10-amino-acid stretch was identified that significantly increased the rate of Cln2-dependent phosphorylation but not that of the Clbs. The loss of Cln2 dependent phosphorylation rate was significant when the first half of the hydrophobic residues: VLLPP were changed to Alanines. The consequence of the deletion mutation was possibly due to a docking interaction between Cln2 and the substrate, as a synthetic peptide based on the 10 amino acid stretch was a competitive inhibitor of docking. The fact that both the vllpp mutation and the peptide affected Cln2 but not Clb5 further demonstrated the interaction's specificity (Kõivomägi *et al.*, 2011).

The Far1 protein is another CKI that can inactivate G1/S cyclin-Cdk (Cdc28-Cln) complex in response to mating pheromone (α factor), thereby stopping the cell cycle at Start in G1 (Peter and Herskowitz, 1994; Mendenhall, 1998). This means that the docking interactions between the G1/S cyclins and Cdk1 are inhibited by pheromone signalling, and Far1 is needed for this inhibition. Fus3: a mitogen-activated protein kinase (MAP) homolog is activated in the presence of pheromones which phosphorylates Far1 at T306 and starts the cyclin inhibitory action (Pope *et al.*, 2014) (**Figure 4**). In mating, the haploid a and α cells in budding yeast synchronise their cell cycles by arresting at Start to fuse and become diploid. Ubiquitin-mediated proteolysis controls the level of Far1, which is unstable throughout the cell cycle except during G1. A null mutation in the Cln2 gene (codes for G1 cyclin) reverses the effect of far1 mutant so far1-cln2 strains arrest in response to α factor (Chang and Herskowitz, 1990). It appears that Far1 might be a multimode inhibitor separately, disrupting both Cdk activity and substrate docking (Pope *et al.*, 2014).

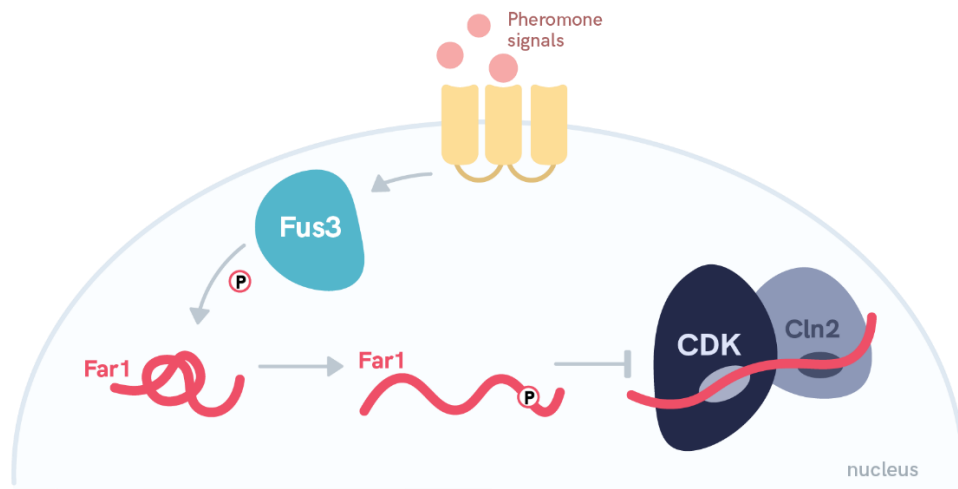


Figure 4. Inhibitory activity of Cyclin Kinase Inhibitor protein Far1. In response to mating phormone signals in the G1 phase, Far1 can be activated by Fus3 mediated phosphorylation of T306 in Far1. This leads to the inactivation of the G1/S cyclin-Cdk complex causing cell cycle arrest at Start in G1.

Far1 has a length of 830 amino acids. Considering the fragment comprising of the first 150 N-terminal amino acids of Far1 (Far1(1-150)), it was seen that this fragment contains S87 Cdk1 phosphorylation site, which is necessary for cell-cycle dependent degradation of Far1 (Gartner *et al.*). <https://pubmed.ncbi.nlm.nih.gov/9632750/> SCF^{Cdc4} ubiquitinates Far1 and S87-S91 match the consensus for Cdc4 di-phosphodegron. It was experimentally shown that Clb5 dependent phosphorylation of Far1(85-150) and the presence of both S87 and S91 was necessary for interaction with Cdc4. Mutation of phosphorylation sites S87 and S91 were found to disrupt the degradation of Far1, implying that phosphorylation of degron is needed to tag the protein for degradation (Faustova *et al.*, 2020).

Inhibition of Cdks is of particular importance for cancer treatment as dysregulation of cyclins and Cdks in the cell cycle are often found elevated in human tumour cells associated with the unregulated proliferation of cells (Malumbres and Barbacid, 2009). CDK dysregulation may result from cyclin overexpression or the loss of CDK1 expression due to mutations or

epigenetic alterations. As a result, CDKs are important targets for anticancer drugs (Ali *et al.*, 2009).

In humans, CDK4 and CDK6, two closely related family members that control cell cycle transition from G1 to S, are well-known oncogenic drivers in a variety of solid tumours (O'Leary *et al.*, 2016). There are three dual FDA approved CDK4/6 inhibitors to treat cancer, namely abemaciclib, palbociclib, and ribociclib. On analysing the live cell engagement potency for CDK4/6 probes, the IC50 was reported as $<1\mu\text{M}$ ($<1000\text{nM}$) for several experiments (Wells *et al.*, 2020).

The duration of G1 has been linked to the amount of carbohydrates stored, and budding has been linked to a decrease in storage carbohydrates (Ewald *et al.*, 2016). This indicates that arresting cells in the G1 phase is advantageous and has synthetic biology applications.

1.6 Controlling gene expression and yeast promoter comparison

Control of gene expression is an essential tool for the design and optimisation of cell factories. In yeast, the promoter is the most critical tool for controlling gene expression. Two classes of promoters are constitutive and inducible promoters. Constitutive promoters give stable expression levels across varying culture conditions (e.g.: P_{ADH1} , P_{TEF1} , P_{GAP1} , P_{CYC1}), whereas inducible promoters (e.g.: P_{GAL1}) show dramatic changes in response to external stimuli. (Peng *et al.*, 2015)

For cell cycle progression, it is vital to cross the threshold level to move on to the next phase. But, in certain circumstances, CKIs or phosphorylation introduce a delay between the formation and activation of cyclin-Cdk complexes (Morgan, 2007). Green fluorescent protein (GFP) is used to examine the activity of a variety of promoters.

2 THE AIMS OF THE THESIS

Far1 protein can inactivate Cln-Cdk complexes in response to mating pheromone. Fus3 phosphorylates Far1 at T306 and starts the cyclin inhibitory action. But, in N-Terminus Far1 (1-170 aa), since there is no T306 phosphorylation site, the study conducted was to check if Far1 can cause cell cycle arrest independent of Fus3.

This thesis's objectives are as follows:

1. To take a SLiM responsible for phosphorylation in Sic1 and put it to N-Far1 (1-170 aa) where it acts as a specific docking site for Cln2 inhibition
2. Design an *in-vivo* Cln2 specific inhibitor
3. Use different promoters to modulate the levels of the synthetic inhibitor created.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Materials

Table 1. DNA cloning

S. Nr.	Materials/Media	Components
1.	1% agarose TAE gel	1mM ethylenediaminetetraacetic acid (EDTA), 4µl/mL Atlas ClearSight DNA Stain (BioAtlas), 40mM Tris-Acetate pH 8.3 and 1% agarose
2.	TAE buffer	40mM Tris-Acetate pH=8.3 and 1mM EDTA
3.	LB media	10g/L tryptone (Formedium), 5g/L Yeast Extract (Formedium), 10g/L Sodium Chloride (Chempur)
4.	LB agar plates with ampicillin	LB media, 15g/L Bacto agar (Formedium); 100µg/mL ampicillin (Sigma)
5.	Competent <i>Escherichia coli</i> Turbo cells	Competent <i>E. coli</i> cells

Table 2. Plasmids constructed in this study

S.Nr.	Plasmid	Backbone	Description	Source
1.	pEV1361	pRS306	pCYC1-NFar1(1-170) WT-EGFP	This source
2.	pEV1387	pRS306	pADH1-NFar1(1-170) WT-EGFP	This source
3.	pEV1390	pRS306	pTEF1-NFar1(1-170) WT-EGFP	This source
4.	pEV1362	pRS306	pGAP1-NFar1(1-170) WT-EGFP	This source
5.	pEV1386	pRS306	pCYC1-NFar1(1-170) VLLPP-EGFP	This source
6.	pEV1374	pRS306	pADH1-NFar1(1-170) VLLPP-EGFP	This source
7.	pEV1391	pRS306	pTEF1-NFar1(1-170) VLLPP-EGFP	This source
8.	pEV1363	pRS306	pGAP1-NFar1(1-170) VLLPP-EGFP	This source
9.	pEV1400	pRS306	pCYC1-NFar1(1-170) nlxxx1-EGFP	This source
10.	pEV1401	pRS306	pADH1-NFar1(1-170) nlxxx1-EGFP	This source
11.	pEV1404	pRS306	pTEF1-NFar1(1-170) nlxxx1-EGFP	This source
12.	pEV1407	pRS306	pGAP1-NFar1(1-170) nlxxx1-EGFP	This source
13.	pEV1398	pRS306	pCYC1-NFar1(1-170) nlxxx1 VLLPP-EGFP	This source
14.	pEV1402	pRS306	pTEF1-NFar1(1-170) nlxxx1 VLLPP- EGFP	This source

Table 3. Yeast strains used in this study

S. Nr.	Name	Description
1.	RV298	<i>leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+] bar1::hisG WHIS-mCherry-SpHIS5</i>

2.	EV509	<i>leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG</i> <i>WHI5-mCherry-SpHIS5 ura3:: pEV1361::ura3</i>
3.	EV510	<i>leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG</i> <i>WHI5-mCherry-SpHIS5 ura3:: pEV1387::ura3</i>
4.	EV511	<i>leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG</i> <i>WHI5-mCherry-SpHIS5 ura3:: pEV1390::ura3</i>
5.	EV512	<i>leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG</i> <i>WHI5-mCherry-SpHIS5 ura3:: pEV1362::ura3</i>
6.	EV513	<i>leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG</i> <i>WHI5-mCherry-SpHIS5 ura3:: pEV1386::ura3</i>
7.	EV514	<i>leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG</i> <i>WHI5-mCherry-SpHIS5 ura3:: pEV1374::ura3</i>
8.	EV515	<i>leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG</i> <i>WHI5-mCherry-SpHIS5 ura3:: pEV1391::ura3</i>
9.	EV516	<i>leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG</i> <i>WHI5-mCherry-SpHIS5 ura3:: pEV1363::ura3</i>
10.	EV517	<i>leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG</i> <i>WHI5-mCherry-SpHIS5 ura3:: pEV1400::ura3</i>
11.	EV518	<i>leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG</i> <i>WHI5-mCherry-SpHIS5 ura3:: pEV1401::ura3</i>

12.	EV519	<i>leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG</i> <i>WHI5-mCherry-SpHIS5 ura3:: pEV1404::ura3</i>
13.	EV520	<i>leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG</i> <i>WHI5-mCherry-SpHIS5 ura3:: pEV1407::ura3</i>
14.	EV521	<i>leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG</i> <i>WHI5-mCherry-SpHIS5 ura3:: pEV1398::ura3</i>
15.	EV522	<i>leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG</i> <i>WHI5-mCherry-SpHIS5 ura3:: pEV1402::ura3</i>

Table 4. Yeast transformation

S. Nr.	Materials/Media	Components
1.	YPD media	20g/L Peptone (Formedium), 10g/L Yeast Extract (Formedium), 20g/L Glucose
2.	1xTE buffer	10mM Tris-hydrochloride (Tris-HCl) pH=8.0, 1mM EDTA
3.	PL1 buffer	100mM Lithium Acetate (LiAc) in 1xTE buffer
4.	PL2 buffer	40% Polyethylene glycol (PEG) 3350, 100mM

		LiAc, 1xTE buffer
5.	YPD plates	YPD media and 20g/L Bacto agar (Formedium)
6.	SC-URA glucose agar plates	20g/L glucose, 20g/L Bacto agar (Formedium), 2g/L SC-URA powder (MP Biomedicals), 7g/L yeast nitrogen base without amino acids (BD Biosciences)

Table 5. Western blotting

S. Nr.	Materials/Media	Components
1.	Urea lysis buffer	20mM Tris pH=7.4, 8M Urea (Sigma), 2M Thiourea (Sigma), 4% CHAPS (Sigma), 1% DTT (Sigma), 50mM NaF, 89mM BGP, 1mM Na ₃ VC ₄
2.	6xSDS buffer	0.3M Tris-HCl, 0.6M DTT, 12% SDS (Sodium Dodecyl Sulphate), 50% Glycerol, 0.06% Bromophenol blue
3.	Semi-dry buffer	25mM Tris-HCl, 192mM glycine, 0.1% SDS
4.	1xTBS-T buffer	20 mM Tris-HCl, 150 mM NaCl, 0.1%, Tween20 (Bio-Rad)

5.	Blocking solution	5% milk powder, 1xTBS-T buffer
6.	Primary antibody solution	3% milk powder, 1xTBS-T buffer, 1:500 GFP (B-2) [mouse monoclonal IgG _{2a}](Santa Cruz Biotechnology)
7.	Secondary antibody solution	3% milk powder, 1xTBS-T buffer, 1:7500 anti-mouse antibody (LabAS)

Table 6. Time-lapse Microscopy

S. Nr.	Materials/Media	Components
1.	CSM-URA and 2% glucose media	7 g/L yeast nitrogen base (BD Biosciences, 0.79 g/L CSM powder (Formedium), 20 g/L Glucose (Oriola), 1.5% NuSieve GTG agarose (Lonza).
2.	1.5% glucose/agarose gel patch	20g/L peptone (Formedium), 10g/L CSM (Formedium), 20g/L glucose, 1.5% NuSieve GTG agarose

3.1.2 Methods

3.1.2.1 DNA cloning

Table 2 lists the plasmids that were constructed during this research. The initial step for constructing the plasmids was to fuse the promoter with N-terminus FAR1. This was carried out via fusion PCR. Two target fragments (promoter and N-Far1) were initially amplified separately using overlapping primers in PCR. The PCR products were purified and combined together in a third PCR where they were spliced into a single fragment using complementary primer sequences. PCR plasmids were created in silico in SnapGene before plasmid cloning.

These plasmid inserts containing SacI and SmaI restriction sites were restricted and cloned into a vector containing C-terminus EGFP (Eukaryotic Green Fluorescent Protein). The resulting plasmids were then integrated (via homologous recombination) into the yeast genome and the cells carrying this mutation were analysed by carrying out fluorescence microscopy experiments.

3.1.2.1.1 PCR

PCRs were carried out using Thermo Scientific High-Fidelity DNA Polymerase and oligonucleotides from Microsynth and Thermo Fisher. Reactions were performed as directed by the manufacturers. Completion of PCRs was followed by gel electrophoresis, wherein the PCR products were separated based on their size. This was accomplished by loading the PCR mixture on 1% agarose gel. The Atlas ClearSight DNA Stain which was added while preparing 1% agarose gel was used to visualize the DNA under UV light, followed by the excision of the DNA products and their subsequent purification using the FavorPrep GEL/PCR Purification Kit (Favorgen) according to the manufacturer's protocol.

NEB Site-Directed Mutagenesis (SDM) was done to create specific, targeted changes in double stranded plasmid DNA.

Table 7. PCR program for Site-Directed Mutagenesis

Phase	Temperature	Time	Number of cycles
Initial denaturation	98°C	5 minutes	1
Denaturation	98°C	20 seconds	30
Annealing temperature	58-68°C (varies according to the annealing temperature of oligonucleotides)	20 seconds	
Extension	72°C	30 seconds per kilo base	
Final extension	72°C	5 minutes	1
Hold	15°C	∞	1

3.1.2.1.2 Restriction

The DNA fragment and the backbone vector need to be restricted for the PCR product to be inserted into the backbone vector. This was done by using FastDigest restriction enzymes according to the manufacturer's instructions. The restriction enzymes were deactivated after incubation by either running the restriction mixture sample on the gel (gel electrophoresis) or by heat inactivation (according to enzyme specifications).

3.1.2.1.3 Ligation

The linearised vector and restricted insert are ligated to form the recombinant plasmid. For this, the concentration of insert and vector (is taken) in 3:1 ratio, 2µl of T4 Ligase Buffer, 1µl of T4 DNA Ligase (Thermo Scientific) and milli-Q H₂O up to a final volume of 20µl were taken in the reaction mixture. The reaction mixture was incubated at room temperature (20°C) for at least 2 hours before transforming into bacteria.

For Site-Directed Mutagenesis, the PCR product, 2µl T4 Ligase Buffer, 1µl of T4 Polynucleotide Kinase (PNK) (Thermo Scientific), 1µl of DpnI enzyme (FastDigest) and water up to a final volume of 20µl were taken in the reaction mixture, which was incubated at 37°C for 30 minutes. After incubation, 2µl of Polyethylene Glycol (PEG) 4000 and 1µl of T4 DNA Ligase was added to the reaction mixture, followed by 10 minutes of incubation at room temperature before the bacterial transformation.

3.1.2.1.4 Bacterial Transformation

The *E. coli* Turbo cells were taken from the -80°C freezer and thawed on ice for 10 minutes. 2-3µl ligation mix was added to 50µl of competent cells, and this mixture was chilled on ice for 30 minutes. The cells were then exposed to 42°C for 45 seconds and then immediately placed on ice for approximately 5 minutes for heat shock. Subsequently, 500µl of LB media was added to the mixture, which was then placed in the 37°C 220 rpm (rotations per minute)-shaker for 40-60 minutes for cell recovery. The cells were centrifuged for 1 minute at 6000 rpm, and most of the supernatant was discarded, leaving only 150µl. The cells were resuspended in the remaining 150µl of supernatant and plated on LB agar plates with ampicillin using glass beads. The plates were incubated at 37°C for 12-16 hours.

3.1.2.1.5 Plasmid Extraction

Individual colonies from the transformation plates were selected and incubated for 4-6 hours in 5mL LB media and 100µg/mL ampicillin in 37°C 220 rpm-shaker. After incubation, the plasmid DNA was isolated from the bacterial cells using the FavorPrep Plasmid DNA Extraction Mini Kit (Favorgen) according to the manufacturer's protocol. The concentration of the extracted plasmid DNA was measured using NanoDrop 1000 Spectrophotometer (ThermoFisher) according to the manufacturer's instructions. The extracted DNA was also restricted with FastDigest restriction enzymes to see whether the amplified plasmid

corresponded to the correct sizes of cloning vector and insert. The restricted plasmid mixture was run on 1% agarose gel and visualised under UV light after incubation. Samples were sent for Sanger DNA sequencing (Estonian Biocentre) to confirm gene mutations.

3.1.2.2 Lithium-Acetate mediated yeast transformation

The vectors were transformed into *S. cerevisiae* RV298 strain.

Cells from the RV298 strain were streaked on a YPD plate and incubated overnight in the 30°C incubator. The next day, cells from the plate was transferred to 50mL of YPD media and grown in the 30°C 160 rpm-shaker until sufficient cell density (measured OD600 = 0.6-0.8) after which the culture was centrifuged at 3500 rpm for 2 minutes. The supernatant was discarded, and cells were resuspended in 1mL of PL1 solution followed by centrifugation at 3600 rpm for 1 minute. Following the removal of the supernatant, PL1 buffer was added in the ratio of 2:1 to the cell volume, and the mixture was incubated for 10 minutes at room temperature. Simultaneously, Salmon Sperm DNA (SS-DNA) was boiled at 100°C for 10 minutes and then chilled on ice.

After this, 10µL linearised plasmid, 10µL of SS-DNA and 100µL of the yeast competent cells were mixed together in a separate 1.5mL tube. To this mixture, 700µL of PL2 buffer and 48µL of Dimethyl Sulfoxide (DMSO) were added. Following careful resuspension, the mixture was incubated for 40 minutes at 42°C and then placed on ice (heat shock). The cells were pelleted by centrifugation at 6000 rpm for 1 minute. The supernatant was discarded, and 1mL of 1xTE buffer was added. After centrifuging the mixture again and discarding the supernatant, 200 µL of 1xTE buffer was added to the cells, and after resuspending the cells, the solution was plated on SC-URA glucose agar plates with the help of sterile glass beads and incubated at 30°C for two days.

For plasmid integration yeast transformation, the constructed plasmids containing the promoter, truncated FAR1 and EGFP were linearized with StuI restriction enzyme and integrated into the URA locus of RV298 following this yeast transformation protocol.

3.1.2.3 Detection of proteins

3.1.2.3.1 Sample preparation

The yeast cells were put to grow for approximately 6-8 hours, after which they were transferred to 15mL falcon tubes for centrifugation. After centrifuging for 5 minutes at 6000 rpm, the supernatant was removed, and tissue paper was used to dry the tubes and remove the excess supernatant droplets. The tubes were put to liquid nitrogen to snap freeze and then stored in the -80°C freezer.

3.1.2.3.2 Western Blotting

The 15mL falcon tubes were taken from the freezer and thawed on ice. In separate Eppendorf tubes, 200µL of glass beads were added. 200µL of urea lysis buffer was used to resuspend the cells, and this suspension was transferred to Eppendorf tubes with glass beads. Following this, the cells were disrupted in FastPrep-24 bead beater (MB Biomedicals) at 4 metres/seconds for 40 seconds.

The Eppendorf tubes were centrifuged at 13,200 rpm for 10 minutes. The lysate obtained was transferred to new Eppendorf tubes. To have similar concentration of lysates while loading them on 10% acrylamide gel, the lysate concentration was measured where 3µL of the lysate was mixed with 200µL of Protein Assay Dye Reagent Concentrate (BIO-RAD).

12µl of the sample was mixed with 3µL of 6xSDS buffer, and the remaining lysate was stored at -80°C. The mixture of sample and 6xSDS buffer was loaded on 10% acrylamide gel along with 3µl of PageRuler™ Prestained Protein Ladder loaded with 3µl of 6xSDS buffer.

Proteins were separated on the gel by running the SDS-PAGE (Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis) for 1 hour at 15mA (per gel). After completion of electrophoresis, the gels were placed in Semi-Dry buffer for 15 minutes. On the side, 4 pieces of filter paper (Blotting-Papier MN 827 B, Macherey-Nagel, Germany) and a piece of 0.45µm nitrocellulose blotting membrane (Amersham, Germany) were cut according to the size of the gel pieces. The filter papers and membrane were moistened in water and Semi-dry buffer. The sandwich was assembled in this order (from bottom to top)- 2 layers of filter paper - nitrocellulose membrane - gel pieces - 2 layers of filter paper; for transferring the proteins from the gel on to the membrane. The transfer was done by placing the sandwich in Pierce G2 Fast Blotter (Thermo Scientific) and running the Standard semi-dry transfer Program for 1 hour. After the transfer was complete, the membrane was stained using Ponceau S solution (Sigma) to visualise the proteins on the membrane. After staining, the membrane was washed with water and 1xTBS-T buffer till the stain was removed completely. Subsequently, the membrane was placed in the blocking solution for an hour at room temperature or overnight at 4°C on a tilting shaker.

After blocking, the membrane was washed once with 1xTBS-T buffer and then transferred to the primary antibody solution and incubated for 1 hour on the tilting shaker. The membrane was then washed in the 1xTBS-T buffer for 15 minutes firstly and then thrice for 5 minutes. Following this, the membrane was transferred to the secondary antibody solution and incubated for 30 minutes on the tilting shaker. Washing with 1xTBS-T buffer once for 15 minutes and thrice for 5 minutes was done again after the incubation was completed.

In order to visualise the protein bands on X-ray film, the surface of the membrane was completely covered with a 1:1 ratio of SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) solution and covered entirely in between cellophane layers.

The membrane was exposed to autoradiography film (AGFA Medical X-Ray film blue, Belgium) in a dark room for 15 seconds to 10 minutes (depending upon protein expression level) for the final visualising step. After exposure, the film was dipped into G150 developer (AGFA) for 30 seconds, dried for 5 seconds, dipped into water for 10 seconds, and finally dipped into the G354 fixer (AGFA) for 20 seconds, after which it was washed with water. The bands of the PageRuler™ Prestained protein Ladder were marked on the dried film.

To obtain the quantified image of the Western Blot, the Image Studio version 5.2 program and LI-COR machine were used.

3.1.2.4 Time-Lapse Microscopy

Colonies were selected from SC-URA plates and streaked onto fresh plates, and incubated at 30°C one day before the experiment. On the day of the experiment, fresh CSM-URA with 2% glucose mixture media was prepared, and the cells were resuspended in this media and grown for 3-4 hours in the 30°C 160 rpm shaker. Following incubation, the yeast cells were screened for levels of EGFP fluorescence signal. The cells showing fluorescence were selected, and 0.3µl of the mixture was taken for the time-lapse experiment. The cells were mounted on a 24x50mm glass plate and covered with CSM-URA media and 1.5% glucose/agarose gel patches. A 20x20mm cover glass and a small plastic cover were placed on the top to prevent gel pieces from moving during the experiment or drying out.

Zeiss Observer Z1 microscope equipped with AxioCam 506 mono-camera and 63x/1.4NA objective was used to perform Fluorescent Imaging. The average time for each experiment was 8 hours, during which no more than 8 positions were imaged every three minutes using automated ZEN software and 3x3 binning. To eliminate focus drift in the images, Definite Focus was employed. Reporter proteins EGFP and mCherry were excited with Colibri 470 LED and Colibri 540-580 LED modules set at 15ms and 750ms exposure times respectively. The temperature of agarose gel during the whole experiment was maintained at 30°C. The images were analyzed using MATLAB software (The MathWorks, Inc.). Cell tracking, image segmentation and quantification were performed with supplemented MATLAB code by Doncic *et al.*, 2013. The cells were synchronized by the time of nuclear import and export of 50% of Whi5-mCherry in the analysis. The plots show mean fluorescence values with SEM error bars from the population of cells over the time of the cell cycle. To avoid errors caused by plasmid copy number differences between strains, the results were normalized to the overall signal.

3.2 RESULTS AND DISCUSSION

3.2.1 VLLPP motif for FAR1

Far1 contains an inhibition site at T306. But, in our lab, it was found that in N-terminus Far1, there is a phospho-dependant docking site that gets phosphorylated by Cln2 Cdk and is needed for inhibition. The inhibition constant (IC₅₀) for Far1 was approximately 48nM for inhibiting 50% of the Cln2-Cdks. If a VLLPP motif replaces this docking site, it will provide a stronger inhibition bringing down the inhibition constant to approximately 7nM (**Figure 5**). This was seen in *in-vitro* experiments signifying that in N-ter Far1, the inhibition is Fus3 independent. We also know that Cln1 and Cln2 recognise LP docking motifs, and these docking interactions increase the efficiency, specificity, and multiplicity of substrate phosphorylation.

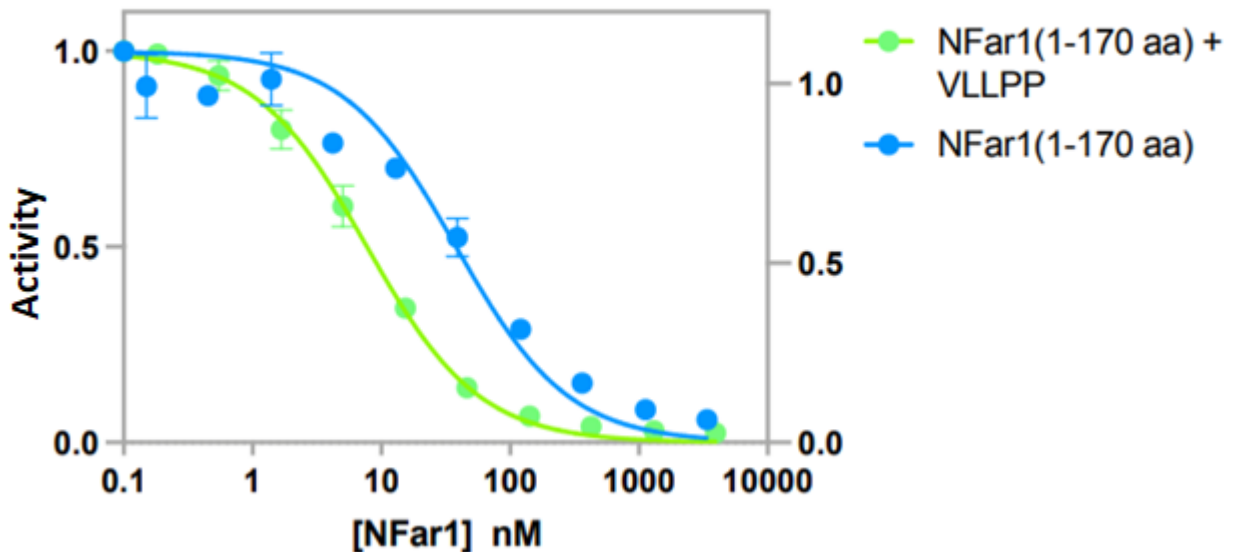


Figure 5. Inhibition curve for NFar1(1-170aa)+VLLPP and NFar1(1-170 aa). The x-axis shows the concentration (nM) and the y-axis shows the fractional activity. The plot depicts the concentration of Far1 needed to inhibit 50% of the Cln2-Cdks within the presence and absence of the VLLPP motif.

There is a possibility to take docking interaction from one protein and put it into another protein. Since the VLLPP motif is not inherently present in Far1 protein, it was taken from Sic1 protein (responsible for phosphorylation) and put to Far1 (responsible for inhibition here) to design an *in-vivo* experiment (**Figure 6**). We wanted to examine if Far1 (without Fus3) sets the Cln2 inhibition threshold and if it can be raised.

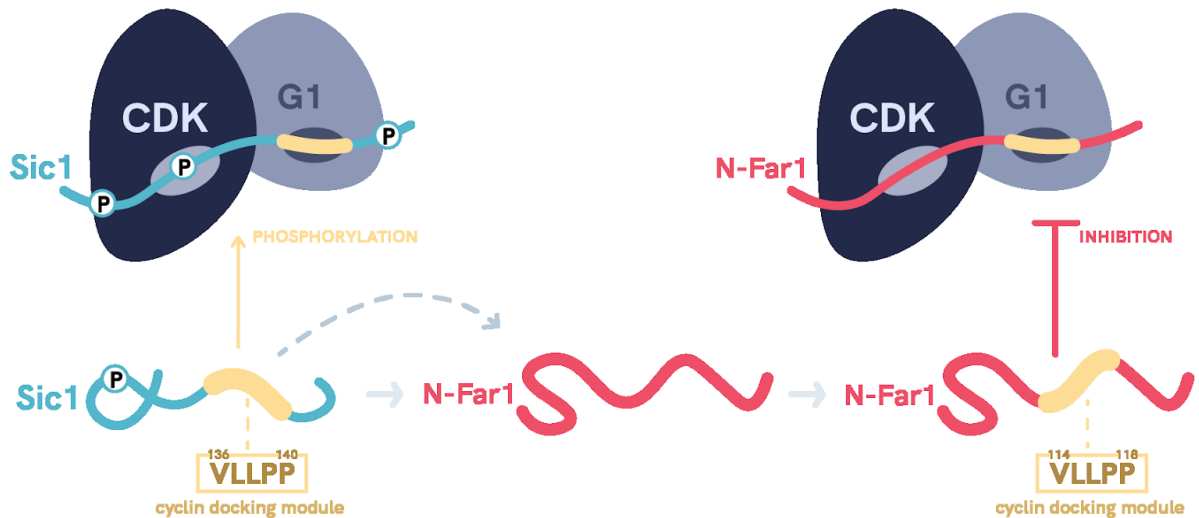


Figure 6. Substituting phospho-dependent docking site in N-Far1(1-170 aa) with VLLPP docking motif taken from Sic1 protein. VLLPP docking motif responsible for phosphorylation in Sic1 is taken and put N-Far1(1-170 aa) where it is responsible for inhibition.

The wild type (WT) and mutant (MUT) plasmid strains were constructed wherein the mutant versions had VLLPP docking motif present (**Figure 7**). The construct was in the following order:

Promoter → N-Terminus FAR1 → EGFP.

EGFP was used to examine the activity of a variety of promoters.

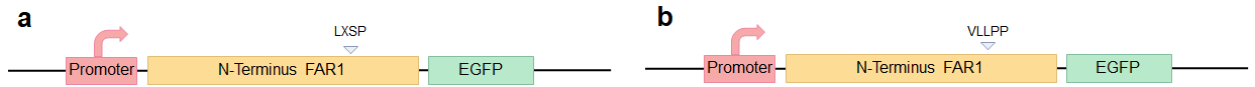


Figure 7. Wild Type (WT) and Mutant (MUT) plasmid constructs. **a)** At position S114, N-Far1 has LXSP motif inherently (wild type). **b)** This docking site was replaced with the VLLPP motif in the mutant construct.

3.2.2 Examining the strength of different promoters

We worked with four promoters, namely, P_{CYC1} , P_{ADH1} , P_{TEF1} , P_{GAP1} . To analyse the strength of these different promoters, Western Blot was performed. We wanted to get different expression levels to inhibit the cell cycle as the promoter shouldn't be too weak or too strong, so western blot was useful for measuring protein expression. The obtained results are represented in Figure 8.

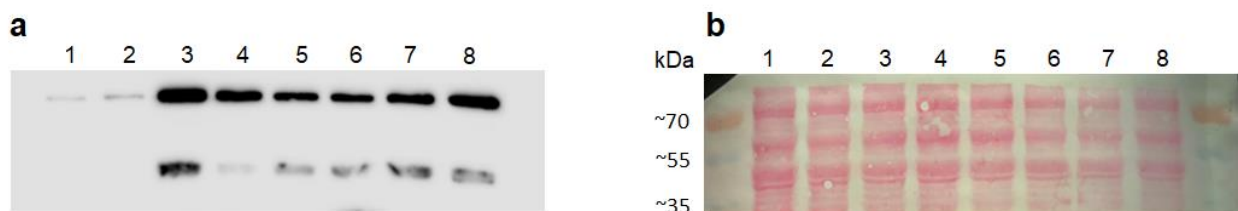


Figure 8. Western blot analysis for analysing promoter strength.

1 – CYC1 WT; 2 – CYC1 MUT; 3 – ADH1 WT; 4 – ADH1 MUT; 5 – TEF1 WT; 6 – TEF1 MUT; 7 – GAP1 WT; 8 – GAP1 MUT **a)** shows different expression levels of constructs **b)** image shows the membrane after staining with Ponceau S solution. The stain binds to positively charged amino groups and non-polar regions of proteins and is used to visualise the proteins on the nitrocellulose membrane.

From the Western Blot analysis results, it was seen that CYC1 is the weakest promoter compared to the rest of the promoters and ADH1 and GAP1 are strong promoters. Taking these results into consideration, the further results were inspected.

3.2.3 EGFP and Whi5 analysis for Wild Type and Mutant Far1 constructs

The time-lapse microscopy experiments provide a means to measure the degradation of the inhibitor over the cell cycle. 0 min (Start Point) is where the nuclear export of Whi5-mCherry takes place. This was utilised to synchronise the cells so that quantitative analysis could be carried out.

To obtain the graphs, daughter cells were selected. For Whi5 analysis, 50% of Whi5-mCherry nuclear import was selected, whereas, for EGFP and analysis of cell size, 50% of Whi5-mCherry nuclear export was selected.

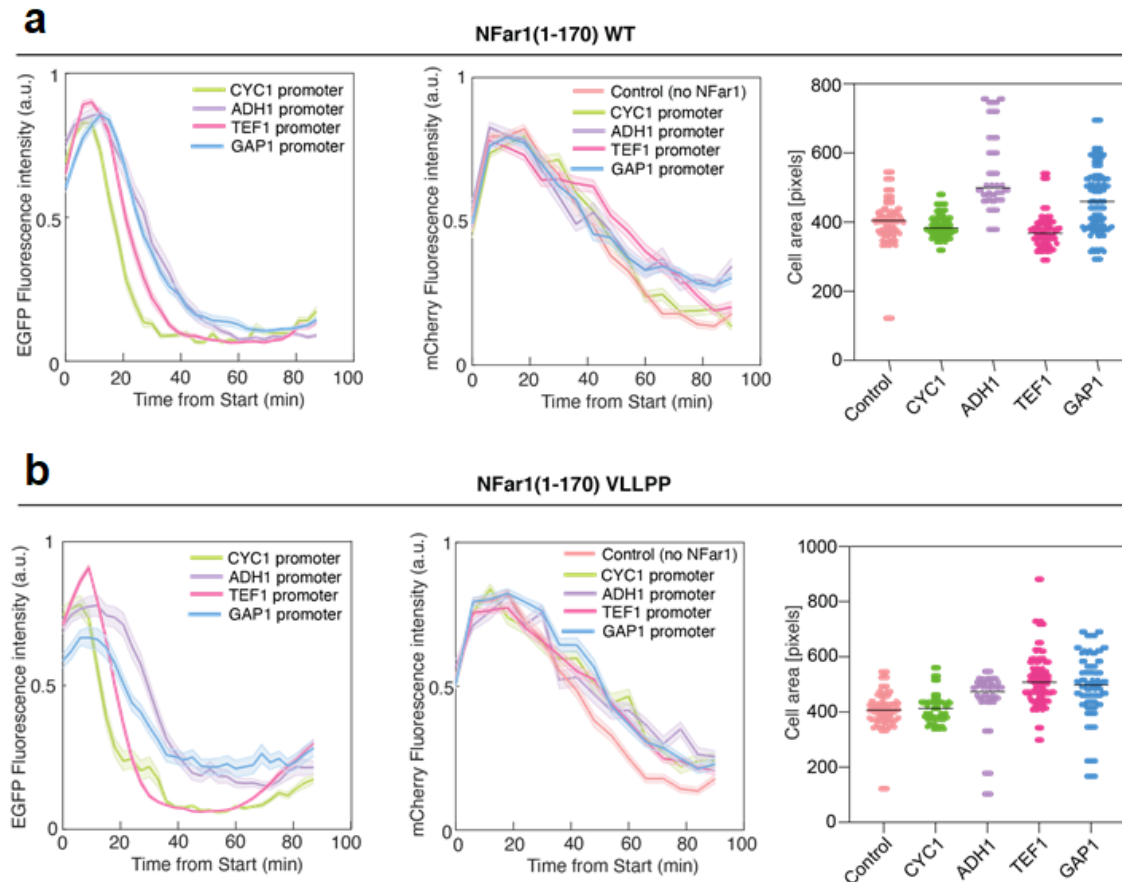


Figure 9. Mean \pm SEM fluorescence intensities and cell size of NFar1(1-170) WT and NFar1(1-170) VLLPP versions of NFar1(1-170aa). Mean fluorescence values with SEM shaded error bars from the population of cells over the time of the cell cycle as well as the cell area in pixels is shown. The time it takes for the degradation of inhibitor and how different promoters

affect this delay as well as cell size is depicted in the graphs. CONTROL is RV298 strain with only mCherry and no EGFP.

On examining the EGFP analysis graphs, it was seen that the behaviour of all the promoters was quite similar i.e. EGFP was degraded rapidly irrespective of the promoter. The EGFP degradation signifies the degradation of the protein; in our case: the inhibitor.

It seems that expressing the NFar1(1-170) WT under a stronger promoter already causes huge variability inside the yeast population as can be seen in the cell size graph in Figure9. The stronger promoter has an effect on cell size at Start making cells bigger when compared to CONTROL.

Surprisingly, the mutant version expressed under a strong promoter doesn't seem to change the cell size at the moment of Start. But TEF1 and GAP1 promoter increase the size at Start in NFar1(1-170) VLLPP.

From the Whi5 analysis graphs, it can be seen that the VLLPP mutants in this context don't cause the delay. There is almost no effect. In the CONTROL cells, however, the delay is slightly less compared to the mutant versions signifying that the G1 phase is shorter. This may be due to the intervention of Clb5 cyclin which phosphorylates Far1 and sends it to degradation. We decided to mutate the NLxxxL motif in N-Far1(1-170 aa) which is the docking motif for Clb5 which phosphorylates S87/S91 phosphodegron in turn targeting Far1 for degradation.

These graphs signified that the inhibition wasn't strong enough because there was negligible effect and delay when comparing the wild type and mutant strains. The NLxxLmotif was mutated for further experiments.

3.2.4 EGFP and Whi5 analysis in NLxxxL mutated N-Far1 (1-170 aa)

NLxxxL mutant (nlxxxl) constructs were made with wild type N-Far1(1-170aa) and NFar1(1-170)VLLPP. The graphs shown below depict the degradation of N-Far1 and the delay in time from Start.

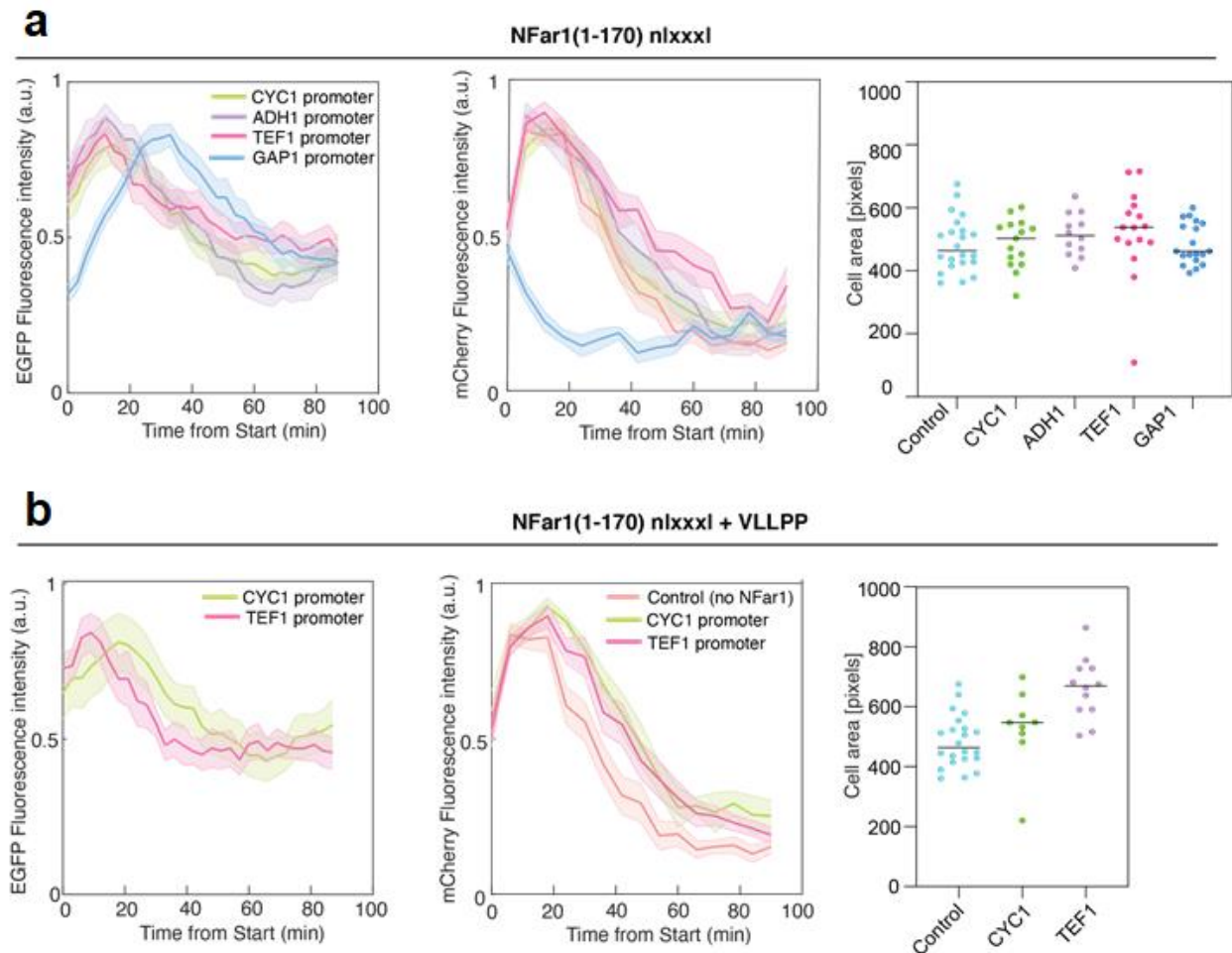


Figure 10. Mean \pm SEM fluorescence intensities and cell size of nlxxxl mutant and nlxxxl mutant+VLLPP versions of NFar1 (1-170aa). Mean fluorescence values with SEM shaded error bars from the population of cells over the time of the cell cycle as well as the cell area in pixels is shown.

In these graphs, degradation of N-Far1 was stabilised compared to Figure 9. In contrast to N-Far1(1-170) WT and N-Far1(1-170) VLLPP mutants (Figure 9), the half-life of N-Far1 degradation in (N-Far1) nlxxxl and (N-Far1) nlxxxl + VLLPP was almost double (Figure10).

The cells containing the construct with a stronger promoter show a lower growth rate under the microscope leaving a small number of cells to analyse. The integration into the yeast genome for ADH1 and GAP1 constructs did not work. But, based on the data we have, we saw that the cells containing the construct with stronger promoter show a lower growth rate under the microscope leaving a small number of cells to analyse.

It seems that the VLLPP mutation with NLxxxL mutation increases the cell size which is not the case if cells are carrying only the NLxxxL mutation. But definitely more cells need to be analysed to make the conclusion. It is unclear what is behind this increase in cell size because Whi5-mCherry acts in the same way as the CONTROL.

Since the NLxxxL deletion wasn't enough to arrest the cell cycle in the G1 phase, we decided to mutate the S87/S91 phosphodegron to Alanine.

3.2.5 Effect of [S87A/S91A] on yeast cells

Upon mutation of S87/S91 to Alanine, abnormality in the cell morphology was observed in cells under the microscope. Under the agar patches, even though the cell volume was increasing, there was no cell division which led to the cells looking extremely large. However, in liquid culture and SC-URA plate, the cells growth was normal i.e., the cells were budding and dividing.

3.2.6 Discussion and Future plans

While looking at the degron mutants, it was seen that the cells are still growing. This means that the other cyclins are playing a role. Based on *in-vitro* experiments it was seen that VLLPP docking motif is highly specific for Cln2 but it is hard to confirm from *in-vivo* experiments on the basis of the preliminary experiments because single cyclin deletions should be done so there is only one G1 cyclin present for confirmation.

In a strain, if Cln1 and Cln3 are deleted and only Cln2 is present, and constitutive promoters manage to inhibit Cln2 causing cell cycle arrest, then this construct can be tested under the strong P_{GAL1} inducible promoter. This strong promoter may inactivate Cln2 based on the levels of induction. This can be beneficial so as to arrest the cell cycle at certain time points when needed.

3.3 SUMMARY

All cells emerge from the division of pre-existing cells. Cell reproduction is crucial for the development and function of all life. In the cell cycle, the duplication of the cell's contents followed by their equal distribution into daughter cells takes place. There are three critical regulatory checkpoints in the cell cycle where cell cycle events are commenced: Start or G1/S, G2/M, and metaphase - to - anaphase transition.

The cell cycle control system controls cell cycle events' timing and coordination. The cell cycle control system's chief constituents are the enzymes called Cdks, which are activated by binding to regulatory proteins called cyclins. Control of all cell cycle events in the budding yeast is by Cdk1. Kinase-substrate specificity is of utmost importance. High-affinity interaction between a part of the kinase and a short sequence on substrate known as a docking site. Docking sites increase the efficiency of substrate phosphorylation. Cell cycle progression requires accurately ordered inhibition of cyclins and Cdk inhibitor proteins (CKIs) by ubiquitination.

In budding yeast, Sic1 and Far1 are the Cdk inhibitor proteins that inhibit S and M-CDK complexes and G1/S cyclin-Cdk complexes in response to mating pheromones, respectively. The phosphorylation of a phospho-dependant docking site by Cln2-Cdks in N-Far1(1-170 aa) is needed for inhibition. But if this site is replaced with the VLLPP docking site from Sic1, the inhibition is stronger as was seen in the in-vitro experiment in our lab.

The effect of different promoters to test if we can stop the cell cycle in the G1 phase or prolong the G1/S transition was also one of the goals of the thesis. Western Blot was

performed to see the strength of the promoters, which showed that CYC1 is the weakest and ADH1 is the strongest out of the four promoters analysed.

Upon performing time-lapse microscopy with yeast strains having wild type and VLLPP mutation constructs, it was seen that there was barely any difference in delay in moving onto the next phase of the cell cycle. This result was seen when 50% of Whi5-mCherry nuclear import was selected. The EGFP analysis by taking 50% of Whi5-mCherry nuclear export also revealed that the behaviour of all the promoters was quite similar, and EGFP was degraded rapidly. This led us to believe that this might be due to Clb5 intervention, which phosphorylates Far1 and sends it to degradation.

For this reason, we decided to mutate the NLxxxL motif in N-Far1 (1-170 aa) which is the docking motif for Clb5 which phosphorylates S87/S91 phosphodegron in turn targeting Far1 for degradation. In these mutants, the EGFP signal (signifying Far1 degradation) was more stabilised as compared to wild type and VLLPP mutants. The inhibition wasn't strong enough which is why we mutated the S87/S91 degrons to Alanines.

The degron mutants showed abnormalities as the cells were huge in size. Instead of dividing like in normal circumstances, the cells increased in volume under the microscope. Whereas in liquid culture and plates for culturing yeast cells, they grew normally.

These results suggest that it's not so easy to construct such an inhibitor even with a cyclin specific site. This may be because there are so many key players involved in inhibition.

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