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The impact of Cln2 degron and Cln2 promoter on protein expression levels

Bachelor's Thesis (12 ECTS)

Curriculum Science and Technology

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Abstract:

Precise regulation of the cell cycle events is essential for correct DNA replication and successful cell reproduction. Progression through the cell cycle is tightly controlled over a multisite phosphorylation network. Phosphorylation of specific amino acids (phosphorylation sites, or phosphosites) might either activate a protein or send it for degradation if phosphorylation occurs inside the destruction box called degrons. In this work, C-terminal degron from Saccharomyces cerevisiae Cln2 cyclin was used to generate a set of multisite phosphorylation protein tags. The protein tags were tested for their ability to regulate protein expression by their fusion with GFP and monitoring its fluorescence. GFP fusion with a longer degron containing 5 phosphosites resulted in the stronger drop in the fluorescence, in comparison to a four phosphosites degron. When the distance between two phosphosites in the Cln2 degron was shortened from 21 to 12-18 amino acids, GFP fluorescence significantly dropped. Generated degron can be potentially used for the regulation of target protein levels. In addition, the contribution of the Cln2 promoter on Cln2 oscillation waves during the cell cycle was analyzed. Analysis of the expression patterns of Cln2 and GFP with a short half-life under the control of Cln2 promoter showed that Cln2 fall in the early S phase is likely due to phosphorylation of protein degron, while promoter role is to provide a sufficient number of transcripts to ensure adequate levels of Cln2 in G1 phase.

Keywords:

Phosphorylation site, phosphodegron, degradation, cyclin, protein tag

CERCS: P310 Proteins, Enzymology

Cln2 degroni ja promootori mõju valgu ekspressiooni tasemele

Lühikokkuvõte:

Rakutsükli sündmuste täpne regulatsioon on vajalik täpseks DNA replikatsiooniks ja rakkude jagunemiseks. Rakutsükkel on reguleeritud valkude multifosforüleerimise kaudu. Kindlate positsioonide – fosforüleerimissaitide – fosforüleerimine võib valke aktiveerida või ka suunata lagundamisele, kui fosforüleerimine toimub degradatsioonimotiivides. Käesolevas töös kasutati *Saccharomyces cerevisiae* tsükliini Cln2 C-terminaalset degradatsioonimotiivi selleks, et luua multifosforüleeritavaid valgumärgiseid. Vastavad märgised liideti GFP valguga ning mõõtes fluorestsentsi analüüsiti nende mõju liitvalgu ekspressioonile. Viie fosforüleerimissaidiga märgise lisamine GFP valgule alandas fluorestsentssignaali tugevamalt kui nelja saidiga märgise lisamine. Lisaks, fosforüleerimissaitide vahelise distantsi vähendamisel 21 aminohappejäägi pealt 15-18 jäägini langes oluliselt ka GFP liitvalgu fluorestsents. Seega, loodud degradatsioonimotiive saab kasutada valkude tasemete langetamiseks. Järgnevalt analüüsiti ka Cln2 promootori mõju Cln2 ekspressiooniprofiilile rakutsüklis. Cln2 promootorilt ekspresseeritud tsükliini Cln2 ja ebastabiilse GFP ekspressiooniprofiilide analüüsil selgus, et Cln2 taseme langus varajases S faasis toimub ilmselt degradatsioonimotiivi fosforüleerimise tõttu, samas kui promootori roll on kindlustada piisav *CLN2* transkriptide arv saavutamaks vajalikku valgu taset G1 faasis.

Võtmesõnad:

Fosforüleerimissait, fosfodegron, degradatsioon, tsükliin, valgumärgis

CERCS: P310 Proteiinid, ensümoloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

Terms:

Cyclin – a protein, activating the CDK that regulate the progression of the cell through the cell cycle.

Degron – the part of the protein, regulating its degradation rate.

Protein phosphorylation – post-translational modification of the protein in which phosphate group is added via covalent bound to one or several amino acid residues in protein sequence. It usually performs regulatory function.

Abbreviations:

APC/C – the anaphase-promoting complex/cyclosome.

Cdc28 – Cell Division Cycle protein 28. The catalytic subunit of CDK. Yeast homolog of mammalian CDK1.

Cdc4 – cell-division control protein 4.

CDK – cyclin-dependent kinases.

Cki – cyclin-dependent kinase inhibitor

Cks1 – Cdc28 subunit 1.

Clb1, Clb2, Clb3, Clb4, Clb5, Clb6 - G2/mitotic-specific cyclin (CLN) B proteins.

Cln1, Cln2, Cln3 – G1/S-specific cyclin (CLN) proteins.

Ctk1 - carboxy-terminal domain kinase.

Far1 – factor arrest, inhibits CDK.

FACS – fluorescence-activated cell sorting

Grr1 – glucose repression-resistant protein.

GFP – green fluorescent protein

Kin28 – serine/threonine-protein protein kinase 28.

MBF - MluI cell cycle box binding factor. **NES** – Nuclear export signal.

OD – optical density

Pho85 – phosphate metabolism-involved cyclin-dependent kinase.

SBF – Swi4/6 cell cycle box binding factor.

SCF – Skp, Cullin, F-box containing complex.

Sic1 - substrate/subunit inhibitor of cyclin-dependent protein kinase.

Ssn3 – suppressor of SNf1.

YPD - Yeast extract peptone dextrose

INTRODUCTION

The proper cell cycle ordering is essential for the correct DNA replication and cell reproduction. It is a tightly regulated process controlled by complexes consisting of cyclins and cyclin-dependent kinases (CDKs). Cyclins are one of the key components of the cell cycle regulatory mechanism, they govern both kinase activity and substrate specificity. *Saccharomyces cerevisiae* is an attractive model organism for biological research, including the cell cycle studies. In budding yeast, cell cycle progression is controlled by a single kinase, CDC28, homologous to CDK1 in mammalian cells, in combinations with cyclins. Unlike constitutively expressed CDC28, cyclins expression is subjected to oscillations during the cell cycle. Oscillation waves of G1/S cyclins are generated due to coordinated action of transcriptional and post-translational regulation. Protein phosphorylation is one of the best-studied post-translational modifications, and it plays an essential role in the cell cycle control. Being phosphorylated in the so-called phosphodegron regions, cell cycle control proteins are degraded. Thus, phosphodegron-containing proteins can potentially serve as a source of multisite phosphorylation protein tags to regulate target protein expression.

In the current work, we aimed at developing a set of multisite phosphorylation protein tags, which are based on the degron sequence of Cln2 cyclin. In addition to that, we wanted to analyze the impact of transcriptional regulation at the promoter level on the oscillation dynamics of Cln2 during the cell cycle.

1 LITERATURE REVIEW

1.1 CELL CYCLE

The cell cycle is a complex sequence of events through which a cell reproduction occurs. The correct order of these events is needed for the successful replication and reproduction of cells (Fig. 1). It consists of four main phases: genome duplication (S phase) and the mitotic phase (M phase), which are separated by 2 gap phases (G1 and G2). Gap phases provide more time for cell growth and serve as regulatory transitions, in which progression to the next stage can be controlled by a variety of different signals (Morgan, 2007). Three phases, G1, S, and G2, are called interphase. During interphase, the cell is growing and replicating its DNA. During the mitotic phase, replicated DNA and cytoplasmic contents are separated and the cell divides. The first gap phase, G1, is important because it can determine if a cell should continue its division or exit from the cell cycle. If there are some unfavorable growth conditions or inhibitory signals from the surrounding environment, a cell may be arrested in G1 for a longer period (or sometimes enter a G0 phase, which is a long-term non-dividing state). The S phase, in turn, is responsible for the duplication of the cell's genetic material. In this phase, the DNA is replicated and chromosomes are duplicated. When the S phase is over, the cell cycle moves to the G2 phase, where the cell continues to grow. To ensure correct ordering and timely transition from one phase to another, cells go through several checkpoints in the cycle where cell state is controlled. The major checkpoints are the G1/S or Start checkpoint, the metaphase-to-anaphase transition checkpoint, and the G2/M checkpoint. The G1/S checkpoint is essential for the G1 phase progression as it controls the transition from the G1 phase to the DNA synthesis. G2 phase is characterized as the last step in preparation for the mitosis. If DNA is damaged, the cell should be prevented from entering mitosis. Therefore, the G2/M checkpoint deals with repairing and stopping the proliferation of damaged cells. Finally, during the M phase, the chromosomes must be equally divided between two daughter cells to ensure the correct transfer of genetic material to progeny (Morgan, 2007).

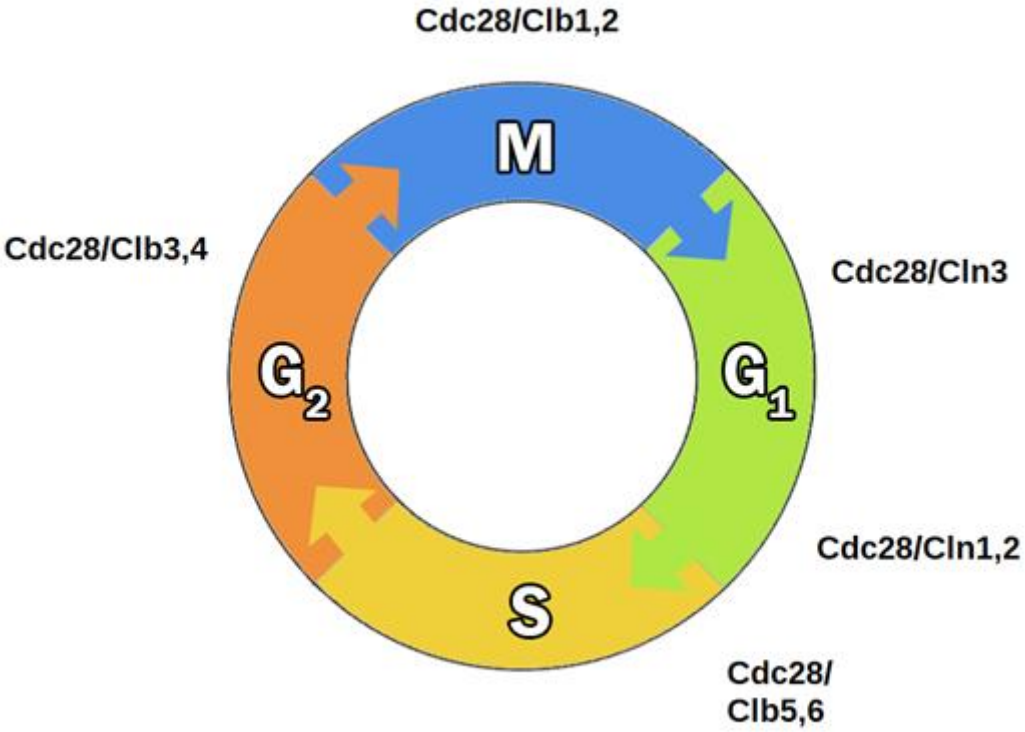

Figure 1. **Representation of the cell cycle.** The cell cycle is a complex series of events that are necessary to control cellular growth and reproduction. Cyclins, regulatory subunits of CDK/cyclin complexes, play a major role in the cell cycle by driving its progression through the cell-cycle checkpoints.

Cell cycle events are tightly controlled. The main regulatory elements of the cell cycle are cyclin-dependent kinases (CDKs). CDKs are serine/threonine kinases accompanied by cyclins (regulatory subunits), which control kinase activity and substrate specificity (Pines, 1995). These CDK/cyclin complexes are assembled and active at specific stages of the cell cycle. They are required for successful progression through mitotic and S phases (Morgan, 2007). While CDKs are constitutively expressed in cells, cyclins' abundance undergoes oscillations. Different cyclins peak at different stages of the cell cycle. (Morgan, 2007). Hence, cell cycle progression is regulated by different CDK/cyclin complexes.

1.2 CDK1 FROM BUDDING YEAST

The budding yeast *Saccharomyces cerevisiae* used for this research is one of the most attractive model organisms for a comprehensive study of the cell cycle. *S. cerevisiae* possesses 5 different CDKs (CDC28, Pho85, Kin28, Ssn3, and Ctk1), but CDC28 (corresponds to CDK1 in mammalian cells and CDK2 in fission yeast; often designated as CDC28/CDK1, or just CDK1) is the best-studied so far because it is the only CDK enzyme with a major role in the regulation of the cell cycle progression in budding yeast (Reed and Wittenberg, 1990). CDC28 is required for the regulation of different cell-cycle events. It contains activating and inhibitory phosphorylation sites, cyclin-binding domain, and Cks1 binding domain, which is essential for proper CDC28 function. In addition to activating cyclins, a small CDK-interacting protein, Cks1, serving as a phosphor-adaptor subunit, targets CDKs to initially phosphorylated priming site in a protein substrate. This binding promotes the phosphorylation of other sites in the target protein. It binds directly to Cdk1 with high affinity (Hao et al., 2005). One of the main substrate parameters for the cyclin-Cdk-Cks1 multisite phosphorylation is distances between phosphorylation sites. The sites positioning in multiple targets of Cdk1 is critical for its phosphorylation rates and it might be the major important mechanism in the regulation of the cell cycle (Kõivomägi et al., 2013). In the G1-specific cyclins the Cks1 activates the ability of Cln2/CDC28 complexes to phosphorylate multiple substrates (Reynard *et al*., 2000).

1.3 CYCLINS AND CELL CYCLE ORDERING

Cyclins, the activating partners of the CDKs are proteins that are synthesized and degraded during each cycle of cell division (Fig. 2). In addition to activating the CDK, cyclins determine its' specificity via association with selected proteins, bringing together CDK1 active site and protein substrates (Morgan, 2007). The structure of all the cyclins is similar at the primary sequence level in the so-called "cyclin-box" region (approximately 100 amino acids), which mediates binding to CDKs. Outside of the cyclin box amino acid sequences of different cyclins are very diverse (Kobayashi et al., 1992).

Unlike CDC28, which is constitutively expressed throughout the cell cycle, cyclins expression has oscillatory behavior (Fig. 2). There are nine cyclins in budding yeast. Their expression patterns have a maximum at the different stages of the cell cycle: G1 cyclins (Cln3), S cyclins (Clb5, Clb6), M cyclins (Clb1, Clb2, Clb3, Clb4) and G1/S cyclins (Cln1, Cln2) (Morgan, 2007). The oscillation waves in the cyclins' expression are governed by

various transcription factors with periodical expression (Andrews and Herskowitz, 1989). For example, the SBF transcription factor complex is involved in the regulation of mRNA abundance of Cln2 cyclin in G1 (Stuart and Wittenberg, 1994). Multisite phosphorylation inside the degron sequence followed by proteasomal degradation is another key mechanism in generation cell-cycle-depend oscillatory dynamics of G1/S cyclins.

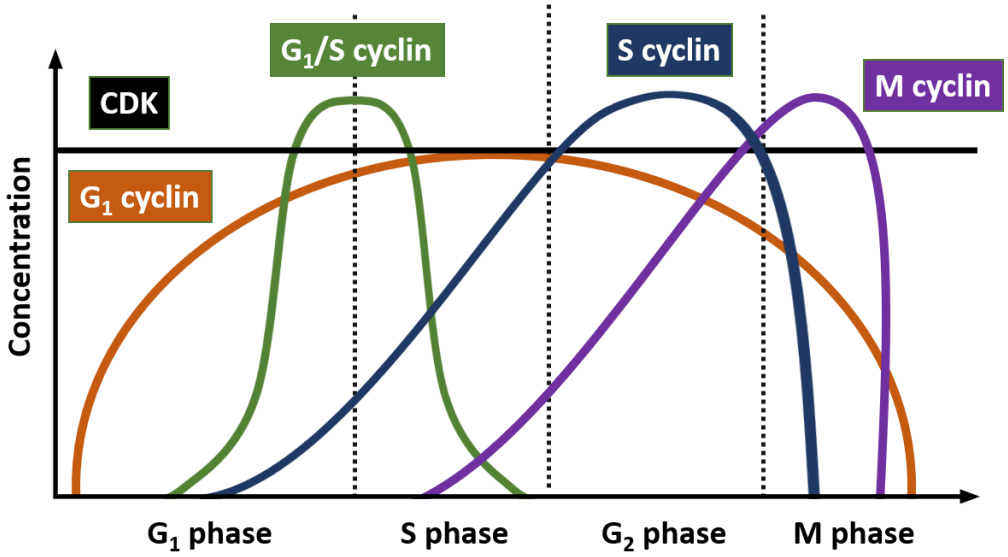

Figure 2. Periodical expression of the cyclins. The different cyclins have different peaks of their expression levels at different stages of the cell cycle while CDK1 is constitutively expressed in cells (Morgan, 2007). G1 cyclins G1/S cyclins rise in late G1 and their activity drops at the beginning of S-phase.

In budding yeast, in early G1, Cln3 activates CDK1 that promotes transcription of other G1-specific genes, and, finally, leads to Cln1 and Cln2 expression. Cln1-CDC28 and Cln2-CDC28 complexes initiate progression through START (Harris et al., 2013). It is a G1/S transition checkpoint at the end of G1. START is a no-return point. After passage through the START, the cell is committed to undergo cell division, it initiates bud formation, duplication of the spindle pole body, and DNA replication. CDC28 forms the complex with Cln1 or Cln2, which possess high sequence similarity. These complexes initiate progression through START, phosphorylate of an inhibitor of the Clb-CDC28 kinases called Sic1, successful DNA replication, and other G1 events. After Sic1 is phosphorylated by Cln-CDC28 complexes and, therefore, targeted for degradation, the activation of Clb5 and Clb6-CDC28 complexes takes place (Schneider et al., 1996). Cln1 and Cln2 cyclins also promote the simultaneous activation of regulatory proteins called SBF or Swi4/6 cell cycle box binding fac-

tor (Swi4-Swi6) and MBF or MluI cell cycle box binding factor (Mbp1-Swi6). These transcription factors regulate the expression of over 200 genes, dependent on the cell-cycle: those are largely non-overlapping sets of G1/S genes. For example, these sets include the genes encoding S-phase cyclins that initiate DNA synthesis. (Harris et al., 2013). Activation of SBF and MBF transcription factors accompanied by Sic1 destruction results in the activation of Clb5 and Clb6 B-type cyclins in the late G1 phase, which is essential for the S-phase transition (Schwob and Nasmyth, 1993).

1.4 REGULATION BY PROTEIN DEGRADATION

During the cell cycle, various regulatory elements (cyclins, adaptor subunits, inhibitors) are involved in the regulation of CDK activity. Therefore, the periodical degradation of proteins (e.g cyclins or CDK inhibitors) governing CDK activity and affinity towards different substrates serves as a key mechanism in the control of cell cycle events. Generally, there are 2 major pathways for protein degradation in eukaryotic cells: ubiquitin-proteasome proteolysis pathway and the lysosomal proteolysis In the lysosomal proteolysis pathway proteins are degraded by proteolytic enzymes, the cathepsins (Cooper, 2000).

The ubiquitin-proteasome-mediated pathway plays a crucial role in the regulation of the cell cycle by the destruction of different regulatory proteins including cyclins and CDK inhibitors (Cooper, 2000). Before degradation in proteasomes, target protein needs to be ubiquitinated (Fig. 3).

Ubiquitination is a post-translational modification mechanism providing the bonding of ubiquitin to lysine residues of the protein substrate. This process is catalyzed by 3 types of enzymes (E1, ubiquitin-activating enzymes; E2, ubiquitin-conjugating enzymes; E3, ubiquitin-protein ligases).

As the initial step, ubiquitin molecules are activated by binding to cysteine residues of the E1 enzyme. The E1 then passes the ubiquitin protein to a cysteine residue in the active site of the ubiquitin-conjugating enzyme or E2. Following this step, one of the E3 ubiquitin ligases can bind to the ubiquitin-conjugated E2 to form an E3/E2 complex. This complex is in charge of transferring ubiquitin to a target protein by forming an isopeptide bond between the lysine residue of the substrate and the C-terminus of the ubiquitin molecule. The E3 ligase is responsible for determining the substrate specificity of the entire complex. A series of E3 enzymes can bind to the substrate forming a polymerized ubiquitin chain. Polyubiquitinated protein can then be recognized and degraded by proteasomes.

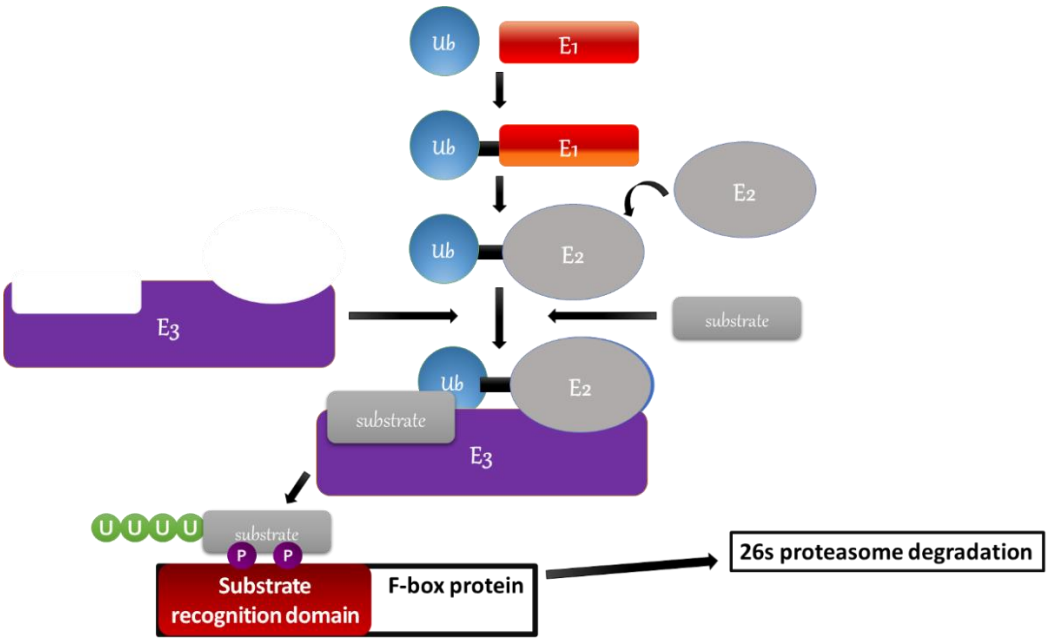

Figure 3. Diagram showing the ubiquitin-proteasome proteolysis steps. The ubiquitin-activating enzyme (E1) starts the ubiquitination process. The E1 enzyme along with ATP binds to the ubiquitin protein. The E1 enzyme then passes the ubiquitin protein to a second protein, called Ubiquitin carrier or conjugation protein (E2). The E2 protein complexes with a Ubiquitin protein ligase (E3). This Ubiquitin protein ligase recognizes which protein needs to be tagged and catalyzes the transfer of ubiquitin to that protein. This process repeats itself until the polyubiquitin chain is formed and the protein can be degraded.

There are two major E3-ubiquitin-ligase complexes: APC/C complex and SCF complex. They both play an important role in cell cycle regulation. The APC/C, or the anaphase-promoting complex, is a large ubiquitin-protein ligase that acts as a trigger of the metaphase-to-anaphase transition. Skp1-Cullin-F-box protein, or SCF complex, is involved in the degradation of cell cycle regulators at the other stages of the cell cycle.

SCF complex consists of three core protein subunits (Rbx1, Cul1 - Cullin, and Skp1) and a variable F-box protein. Rbx1 binds to the E2 ubiquitin-conjugating enzyme. Cullin (CDC53 in yeast) is a scaffold protein, which brings together the Rbx1 domain with the Skp1 domain. Skp1, in turn, is an adaptor protein that identifies and binds the F-box protein, which serves as a substrate recognition component of the SCF complex. F-box protein first identifies the substrate, binds to it, and then binds to the Skp1 subunit of the SCF complex. It brings together the substrate protein and E2 component of the complex. In S. cerevisiae, two F-box proteins, Grr1 and Cdc4, have been widely studied. Grr1, localized to the cytosol, mediates the interaction of SCF complex with phosphorylated forms of the G1/S cyclins

Cln1 and Cln2. The SCFGrr1 targets the G1/S cyclins Cln1 and Cln2 for ubiquitination (Barral, et. al. 1995). Nuclear-localized Cdc4 in turn is required for the ubiquitination of the CDK inhibitors Sic1 and Far1 (Henchoz, 1997; Morgan, 2007), which inhibit the cell cycle progression by binding to Clb/CDK complexes (Nasmyth, 1993).

Protein substrates have to be phosphorylated usually on multiple sites (multisite phosphorylation) in a region called degron (or phosphodegron). Degron or phosphodegron is a protein region, which, upon phosphorylation of certain phosphosites, generates a binding surface that interacts with an SCF complex.

Different F-box proteins require the presence of certain phosphorylation sites in their substrates. It was shown that in addition to phosphosites, Grr1 substrates often contain the so-called PEST region (Rogers, et. al. 1986). PEST is a sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T). The deletion of PEST domain in S. cerevisiae leads to the stabilization of Cln2 (Salama, et. al. 1994). PEST sequence is a destruction signal in the protein. In Cln2, PEST can be identified in the 373-409 aa region of C-terminal degron (Fig. 4).

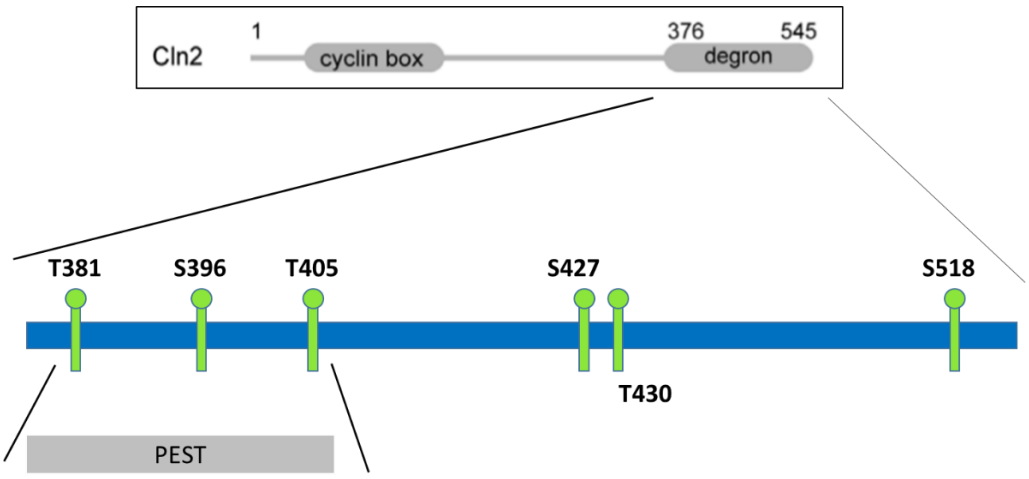

Figure 4. Domain organization of Cln2. Primary structure of Cln2 cyclin. In the degron region, there are 6 phosphorylation sites including the PEST region. Elements in this construct: Cdc28 binding domain, cyclin-box; degron, including 6 different phosphorylation sites; and the PEST sequence located in the degron region. PEST region serves as a signal for proteolytic degradation. The PEST domain, originally found in all 3 yeast G1 cyclins, was first described as an indicator of protein instability based on the frequent occurrence in unstable proteins but its functions are still undetermined.

2 THE AIMS OF THE THESIS

Proteins containing multisite phosphorylation degrons potentially could be a source of tags to regulate target protein expression. As a result of the phosphorylation of degron tags, the whole fusion protein is sent to degradation.

The current work can be divided into two main parts. In the first part, we wanted to analyze the impact of Cln2 C-terminal degron on protein stability, using GFP as a reporter. GFP is highly stable and has a long protein half-life (from approx. 3 h in cultures with inhibited protein synthesis (Halter et al., 2007) to 15 h in cultures with active protein synthesis (Jiang et al., 2004). GFP fusion with multisite phosphorylation protein tags leads to much faster protein degradation and, as a result, to lower GFP fluorescence levels.

The specific aims were:

1) To analyze the levels of fluorescence of GFP fused to Cln2 C-terminus containing either 4 or 5 phosphorylation sites.
2) To study the impact of the distances between phosphorylation sites on the level of GFP fluorescence.

In the second part of the work, we wanted to analyze the impact of Cln2 promoter in protein expression during the cell cycle. To be able to monitor the correlation between gene expression and protein levels, GFP protein with a short half-life (shlGFP, short-half-life GFP) was used.

The third aim was:

3) To analyze the oscillations of Cln2 and shlGFP proteins whose transcription is driven by pCln2 promoter during the cell cycle.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Media

Bacterial media:

LB (Lysogeny broth) medium: 800mL/L H2O, 10 g/L tryptone (Formedium), 5 g/L microgranulated yeast extract (Formedium), 10 g/L NaCl (Chempur). To prepare agar plates 15 g/L of bacto agar (Formedium) was added to the mixture before autoclaving. For plasmid selection, 100 mg/L of sterile ampicillin solution was added to the medium prior plate preparation or in a liquid medium when bacterial cells were grown for miniprep.

Yeast media:

YPD medium: 800 mL/L H2O, 20g/L Peptone (Formedium), 10g/L yeast extract (Formedium), 20g/L Dextrose (Formedium). To prepare YPD plates, 15g/L of Bacto agar (company) was added to the medium.

CSM medium: CSM medium: 2% Glucose, CSM powder (Formedium). For the selection of insert after yeast transformation, G-418 antibiotic was added to the medium at the concentration of 200 mg/L.

3.1.2 Bacterial strains

Escherichia coli Dh5α strain competent cells were used for transformation.

3.1.3 Plasmids

Different variants of Cln2-degron C-terminal sequences were ordered as synthetic DNA strings from GenScript. All gene strings contained *BamHI* and *SgsI* restriction sites at their 5'- and 3'-ends, respectively, for subsequent cloning into pFA6a plasmid vectors. The synthetic DNA fragments and pFA6A vector were restricted with *BamHI*/*SgsI* restriction enzymes (1 µl of alkaline phosphatase was added in the case of plasmid digestion to prevent self-ligation). After purification, the ligation reactions were set. 1 µl of ligation mixture was used for bacterial transformation. Two colonies from each transformation were grown in 3 ml of liquid LB to perform plasmid Miniprep. Insertion was confirmed by sequencing. All plasmids generated or used in this study are listed in **Supplementary Table 2**.

The resulted plasmids were used as a template in PCR to amplify DNA fragments for yeast transformation. The pFA6a contains a KanMX selection marker which confers resistance of yeast cells to the G418 antibiotic (Bähler et al., 1998). NS229 yeast strain was transformed with PCR products containing degron sequence followed by KanMX. In addition to that, fragments contained regions corresponding to DNA sequences of NES and *leu2* locus at 5'- and 3'-ends, respectively for homologous recombination in yeast.

3.1.4 Yeast strains

All the strains created in this work are based on NS229 which was created by introduction of pADH1-GFP-NES in LEU2 locus by using CRISPR/CAS9 method. pADH1 – expression of foreign genes with this constitutive promoter results in high protein production levels. pADH1 sequence located 716bp upstream of ADH1 gene start codon, and therefore, referred as ADH1 promoter. The NES stands for nuclear export signal serving for protein transportation from the cell nucleus to the cytoplasm. There are also several variants of Cln2 phosphodegron: Cln2_4WT region is located in the range from 393 to 445 aa of Cln2 protein sequence; 5WT region is located in the range from 373 to 445 aa of Cln2 protein sequence; Cln2_4A (with 4 phosphorylation sites mutated to alanine); In addition to the length, the distance between 2 phosphosites was shortened from 21 (in Cln2_WT) to 18 (4WT_18aa; 5WT18aa), 15 (4WT_15aa; 5WT_15aa) or 12 (4WT_12aa; 5WT_12aa) amino acids respectively (Fig. 5B).

NS228 was used as a background strain (ordered from EuroScarf): CEN.PK-2-1C: MATa ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2.

Saccharomyces Cerevisiae strains used in this work are listed below in the **Supplementary Table 1**.

3.1.5 Extraction of genomic DNA from yeasts

To confirm the transformation of yeast, a small part of the colony (at the end of a pipet tip) was suspended in lysis solution (100 μL of 200 mM lithium 17 acetate (LiOAc) with 1% SDS), vortexed and incubated for 5 minutes at 70°C. At the next step, 300 μL of 96% ethanol was added for DNA precipitation followed by vortexing. DNA was collected by centrifugation at 13200 rpm for 3 minutes. Precipitated DNA was washed with 500 μL

of 70% ethanol by turning Eppendorf tube up-side-down 5-10 times and spun down by centrifugation at 13200 rpm for 1 minute. The supernatant was removed and the pellet was left for air drying for 2 minutes under the fume hood. The pellet was dissolved in 1x TE buffer (10mM Tris-KOH, pH=8.0; 1mM EDTA) followed by centrifugation at 15000 rpm for 1 minute. The supernatant was transferred into a new 1.5 ml tube and used as a template for PCR to amplify CYC1 and PDA1 promoters (Marko Lõoke et al., 2011).

3.1.6 LiAc-Mediated Yeast transformation protocol

Fresh yeast plate with NS229 strain was used to inoculate 50 ml of YPD medium. The culture was grown at 160 rpm in the shaker at 30 °C. Cells were grown until cultures reached 0.6-0.8 OD_{600} (optical density at 600 nm). Then cell culture was transferred into 50 ml falcon conical tubes, centrifuged at 3600 rpm for 2 minutes, and the supernatant was removed. The cell pellet was resuspended in 1 ml of 100mM LiAc in 1x TE solution (10mM Tris-KOH, pH=8.0; 1mM EDTA) and centrifuged at 3600 rpm for 1 minute. The supernatant was removed and two times of cell volume of 100mM LiAc in 1x TE was added. Salmon Sperm DNA (SS-DNA) was boiled at 100 °C for 10 minutes to make sure that the DNA will be single-stranded. 1 µL of plasmid DNA and 10 µL of SS-DNA were mixed in a separate 1.5 mL tube with 100 µL of yeast competent cells (those left for 10 min at room temperature in 100mM LiAc in 1x TE buffer). 700 µL of sterile PEG/LiAc (40% PEG 3350 + 100 mM LiAc in 1x TE) solution and 48 µL of DMSO were added and everything was mixed by pipetting. Cells were incubated for 40 minutes at 42 °C and then were centrifuged for 1 minute at 6000 rpm at RT. 1 ml of sterile 1x TE buffer (10mM Tris-KOH, pH=8.0; 1mM EDTA) was added to the cells and centrifuged for 2 minutes at 3600 rpm at RT. Once the supernatant was removed, the cells were resuspended in 200 µL of 1x TE buffer and then plated on the selection plates, which were incubated for at 30 °C until colonies appeared (approx. 2 days).

3.1.7 Bacterial transformation protocol

Escherichia coli Dh5α strain was used for propagation and construction of the plasmids. For each transformation, 50 µL of competent cells were mixed with 2 µL of DNA and incubated on ice for 25 minutes, followed by heat shock at 42 °C for 30 seconds. 600-700 18 µL of LB (distilled water, agar, tryptone, NaCl, yeast extract) or SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM MgSO4, and 20 mM glucose) media was added to recover the cells. They were incubated for 1 hour at 37 °C shaking

incubator (200 rpm) and then centrifuged for 1 minute at 4000 rpm. Cells were plated on LB-agar plate containing ampicillin and incubated at 37 °C overnight.

3.1.8 Phusion polymerase PCR protocol

Phusion polymerase was used to amplify all fragments for cloning into pFA6a plasmid and fragments for yeast transformation. PCR mix was prepared according to the instruction manual.

Reaction mix for Phusion DNA polymerase:

Component	50 µL Reaction	Final Concentration/Amount
Distilled water	To 50 µL	
5x Phusion HF Buffer	10 µL	1x
10mM dNTPs	0.4 µL	200 µM
10 µM Forward Primer	2 µL	0.5 µM
10 µM Reverse Primer	2 µL	0.5 µM
DNA template	Variable	< 250 ng
DMS (optional)		2-5%
Phusion DNA Polymerase	0.5 µL	1.0 unit/50 µL PCR

*It is important to add Phusion DNA Polymerase last to prevent any primer degradation caused by the 3' to 5' exonuclease activity.

Thermocycling conditions for Phusion DNA Polymerase

Step	Temperature	Time
Initial Denaturation	98 °C	30 sec
Denaturation	98 °C	5-10 seconds
Primers annealing	58 °C (optional)	10-30 seconds
Elongation	72 °C	15-30 seconds per kb
Final Extension	72 °C	5 minutes
Hold	15 °C	∞

*The annealing temperature depends directly on composition and length of the primers (Rychlik et al., 1990). The sequences and melting temperatures of the primers are listed in the **Supplementary Table 3**.

3.1.9 Taq Polymerase PCR protocol

For colony PCR, Taq polymerase was used. PCR mix was prepared according to the instruction manual.

Reaction mix for Taq Polymerase

Component	**Final Concentration/amount**	**Final Concentration/amount**
Distilled water	to 20 µL	
10x Direct Load PCR Buffer, Yellow	2 µL	1x
25 mM DNTP mix	0.25 µL	200 µM
$MgCl_2$	1 µL	0.1-0.5 mM
10 µM Forward Primer	2 µL	0.5 mM
10 µM Reverse Primer	2 µL	0.5 mM
DNA template	variable	< 250 ng
Taq Polymerase	0.3 µL	0.05 units/µL

Thermocycling conditions for Taq DNA Polymerase

Step	**Temperature**		**Time**
Initial Denaturation	95 °C		5 minutes
Denaturation Primers annealing Elongation	95 °C 52 °C (55-72) 72 °C	40 cycles	30 seconds 35 seconds 15-30 seconds per kb
Final Extension	72 °C		5 minutes
Hold	15 °C		∞

3.1.10 Gel Electrophoresis protocol

Agarose gel components: 1x TAE Buffer, 1.5% agarose. To run DNA on the gel, 6x Loading Dye (Solys BioDyne) was added to the DNA samples and they were loaded on the gel (40 mM Tris-acetate with pH 8.3, 1 mM EDTA, 1.5% agarose, 5 µL/L Atlas ClearSight DNA Stain, BioAtlas). Samples were loaded into the wells and gel was run at 150V for 30 min. Gels were visualized under UV light and, if required, bands of expected sizes were cut

out and purified (the FavorPrep GEL/PCR Purification Mini Kit and the manufacturer's protocol were used).

3.1.11 Plasmid Extraction Miniprep

The FavorPrep Plasmid Extraction Mini Kit and the manufacturer's protocol were used.

3.1.12 Ligation with T4 DNA ligase

The following reaction mix should be kept on ice. T4 DNA Ligase (New England BioLabs) was added at last. Vector to insert had a molar ratio of 1:3.

Mix

Component	**20 µL Reaction:**
10x T4 DNA Ligase Buffer	2 µL
PEG4000 (was added for blunt-end ligation only)	2 µL
Vector DNA	1 µL
Insert DNA	5 µL
T4 DNA Ligase	1 µL
Distilled water	To 20 µL

The reaction was vortexed, spun down by centrifugation, and incubated at 18 °C overnight.

*Depending on the DNA ends (blunt or sticky) the incubation time may change. For sticky ends, the incubation period is 10 minutes (at room temperature) or 16 °C overnight. For blunt ends, the incubation period is 2 hours (at room temperature) or 16 °C overnight.

3.1.13 DNA Concentration measurements

NanoDrop 3300 (Thermo Scientific) was used for nucleic acid quantification

3.1.14 Alpha-factor mediated cell cycle arrest

To synchronize the *S. cerevisiae* cell cycle we used the following protocol:

Cells were grown in 3 ml YPD medium at 30 °C overnight, diluted to 0.1 OD, incubated once again until the OD level reached 0.25-0.35, and then harvested by brief centrifugation. α-factor was added to a final concentration of 10 mg/ml and the mixture was incubated for 2.5-3 hours at 30 °C. The cell pellet was washed with 45 ml of YPD medium in

the 50 ml falcon conical tube, briefly vortexed, and centrifuged at 4000 rpm for 1 minute. The washing steps were repeated 3 times, followed by mixing cells with 60 ml of YPD medium to the new flasks. The α-cells were examined microscopically to ensure that they reached the necessary stage. The samples were collected and frozen 8 times for every 10 minutes since the α-factor was added to the medium.

3.1.15 Yeast growth conditions and sample collection for Western Blotting

NS343, NS344, NS345, NS346, RV1110, and RV1111 yeast strains were grown in 3 ml YPD medium in the shaker at 30 °C overnight and diluted to 0.1 OD_{600} in 50 ml YPD. When cultures reached OD 0.25-0.35, 5 ml of cultures were collected by centrifugation at 4000 RPM for 1 min (asynchronous culture time point, As), the supernatant was discarded and the pellet was frozen in liquid nitrogen. Then α-factor was added to the cultures a final concentration of 1 μg/ml, and cultures were grown for about 3 hours at 30 °C (to make sure that all cells in the culture are synchronized). After that, cultures were centrifuged in 50 ml falcon tubes at 4000 RPM for 1 min. The supernatant was discarded and 45 ml of YPD medium was added to the cell pellet, briefly vortexed, and centrifuged at 4000 rpm for 1 minute. The washing steps were repeated 3 times. At the next step, cells were resuspended in 60 ml of YPD medium in the new flasks. Samples were collected immediately (0 min) and every 10 min of growth during the following 70 min. Samples were stored at -80 ^{o}C until further analysis.

3.1.16 Western blot

To analyze the levels of shlGFP and Cln2 proteins after the release from α-factor arrest, Western blotting was used. Collected samples were resuspended in 200 μl of urea lysis buffer. 200 μl of the glass bead was added to the tube and cells were disrupted in a bead beater at for 40 s. Lysates were centrifuged and transferred into new Eppendorf tubes. Protein concentration in the samples was measured using Biorad Protein Assay in 96-well microplates according to the instruction manual. Each sample was measured in technical triplicates. Equal amounts of proteins were loaded on the 10% acrylamide gel (separating gel: 0.375 M Tris-HCl (pH 8,8), 10% acrylamide (29:1 acrylamide:bis-acrylamide), 0,1% SDS; stacking gel: 28 0.125 M Tris-HCl (pH 6,8), 5% acrylamide [29:1 acrylamide:bis-acrylamide], 0,1% SDS)). PageRuler™ Prestained Protein Ladder was used to estimate protein size. Proteins were separated using SDS-PAGE for about 90 min at 12 mA. The gel was soaked in the semi-dry buffer (25 mM Tris, 192 mM glycine, 0,1% SDS – we have methanol

in the buffer). After that, proteins were transferred to nitrocellulose membranes with Pierce G2 Fast Blotter (Thermo Scientific) for 60 min using the standard semi-dry transfer program. The reversible staining with Ponceau dye was used to assess the quality of the transfer and relative amount of total proteins. The membrane was incubated at 4 ^{o}C on the shaker (slow agitation) overnight in a blocking solution (5% Milk powder in TBS-T buffer). Then the solution was replaced with the 3% Milk powder in TBS-T buffer containing 1:500 dilution anti-MYC tag antibodies and left on the shaker at room temperature for 60 min. Afterward, the membrane was thoroughly washed with TBS-T (1 wash for 15 min and 3 washes for 15 min, shaker, high speed), and probed with the secondary antibody solution (anti-mouse, HRP-conjugated antibodies, 1:7500 dilution in TBS-T with 3% milk) for 60 min. After that, the membrane was washed again 4 times (see above). The results were visualized on the film using SuperSignal West Pico PLUS Chemiluminescent substrate (Thermo Fisher Scientific).

3.1.17 Flow Cytometry

The yeast strains with GFP fused to different degrons (**Supplementary Table 1**) were analyzed using FACS. The CSM medium (See Media and Culture conditions) was prepared and filtered into the 50 ml falcon conical tubes. The cells from fresh plates were grown in the 3 ml of filtered CSM medium for 2 h at 30oC in the shaker incibator. Then, 200 μL of resuspended cells were added per well of the 96-well plate and loaded to the autosampler of the Attune NxT FACS machine. FACS results were visualized using Microsoft Excel.

3.2 RESULTS AND DISCUSSION

3.2.1 The fusion of GFP with multisite phosphorylation degron tags resulted in different levels of GFP fluorescence

In this work, the effect of multisite phosphorylation protein tags on GFP fluorescence was studied. C-terminal degron of yeast cyclin Cln2 was used to generate a set of different protein tags (Fig. 5B). Cln2 C-terminus includes 6 phosphosites, which being phosphorylated send protein for degradation (Fig. 5A). It was shown that shortening the distance and relative positioning between phosphorylation sites of the Cks1 priming sites and the degron in Cln2 might have an impact on its degradation rates. However, a distance shorter than 10-aa yielded no secondary phosphorylation (Kõivomägi et al., 2013).

To test this hypothesis, we decided to compare the relative GFP expression of several mutants. Several strains with GFP-degron tags fusion under the control of the ADH1 promoter were generated. Degron tags of different length, containing either 4 or 5 phosphorylation sites (4WT region is located in the range from 393 to 445 aa of Cln2 protein sequence; 5WT region is located in the range from 373 to 445 aa of Cln2 protein sequence), were tested for their ability to degrade GFP (Fig. 5B). In addition to the length, the distance between 2 phosphosites was shortened from 21 (in Cln2_WT) to 18 (4WT_18aa; 5WT_18aa), 15 (4WT_15aa; 5WT_15aa) or 12 (4WT_12aa; 5WT_12aa) amino acids respectively (Fig. 5B). Generated strains were analyzed by FACS for its GFP fluorescence intensities. The results were compared to the GFP fluorescence in the strain with 4A mutant tag (protein tag where serine or threonine of 4 phosphosites was replaced with alanine, which cannot be phosphorylated) (Fig. 6).

The fluorescence signal of positive control strain containing only EGFP without any phosphodegron tags under the same promoter was expressed and showed 186% of relative fluorescence compared to the 4A. Potential reasons for higher fluorescence of GFP fused to 4A tag in comparison to GFP-NES alone: 1) the presence of any relatively long tag might decrease GFP fluorescence; 2) nevertheless phosphorylation sites in 4A degron are mutated to alanine, it contains about half of initial PEST sequence that might somehow enhance protein degradation. Further research is needed to understand the precise mechanism.

The 4WT_15aa and 4WT_18aa mutants show around 50% of relative fluorescence in comparison to the 4WT, while 4WT_12aa seems to be an artifact and should not be taken into account (it is very unlikely that the distance between phosphosites can result in nearly

0% of the relative GFP expression). On the other hand, change in the distance between phosphosites from 21 to either 12, 15, or 18 amino acid did not lead to a further decrease in fluorescence level in comparison to 4WT or 5WT degrons. At the same time, 15 amino acid distance between phosphosites seemed to promote degradation of GFP compared to both 4WT or 5WT constructs. These results also require further clarification using more precise methods.

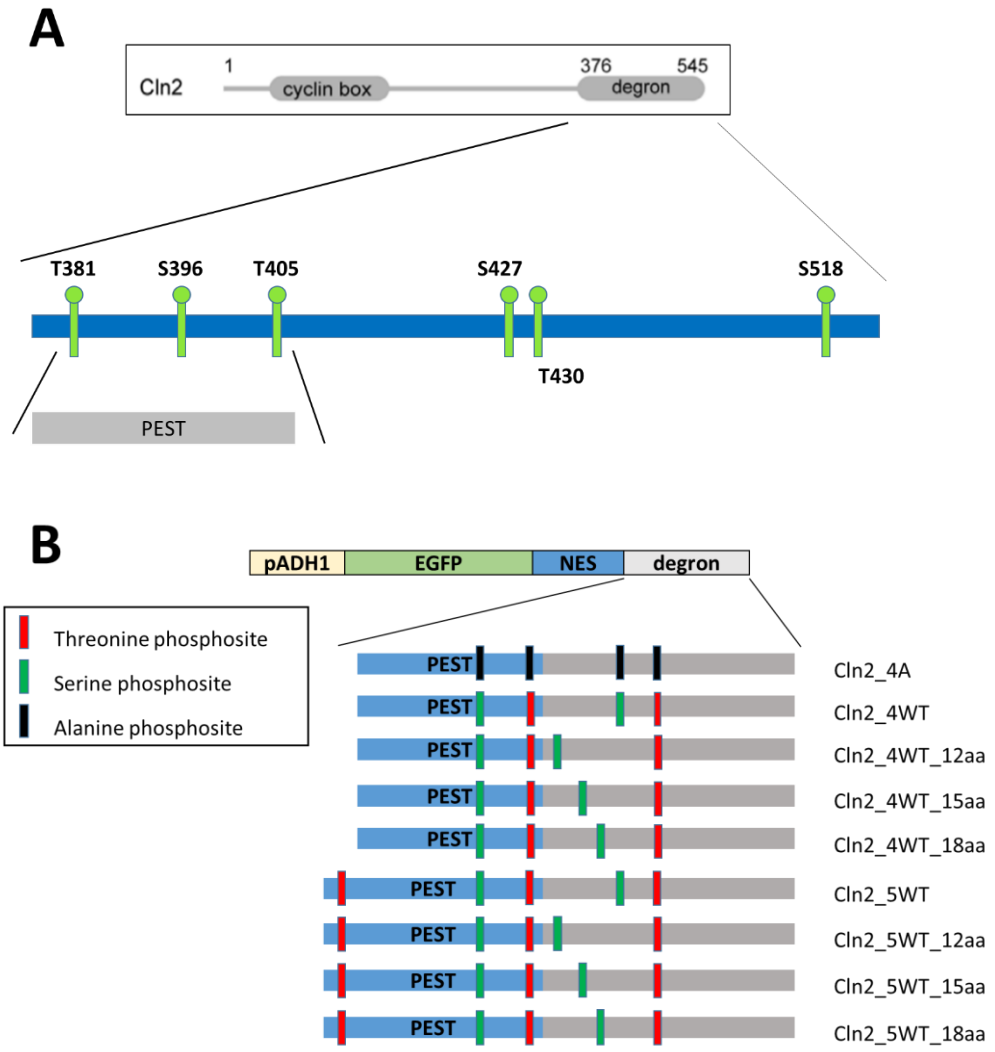

Figure 5. Domain organization of Cln2 (A) and constructs designed in this work (B). (A) Primary structure of Cln2 cyclin. In the degron region, there are 6 phosphorylation sites including the PEST region. Elements from this construct: Cdc28 binding domain, cyclin-box; degron, including 6 different phosphorylation sites; and the PEST sequence located in the degron region. PEST region serves as a signal for proteolytic degradation. The PEST domain, originally found in all 3 yeast G1 cyclins, was first described as an indicator of protein instability based on the frequent occurrence in unstable proteins but its functions are still undetermined. **(B)** EGFP-NES sequence under the ADH1 promoter with different degradation promoting fragments constructed in this work.

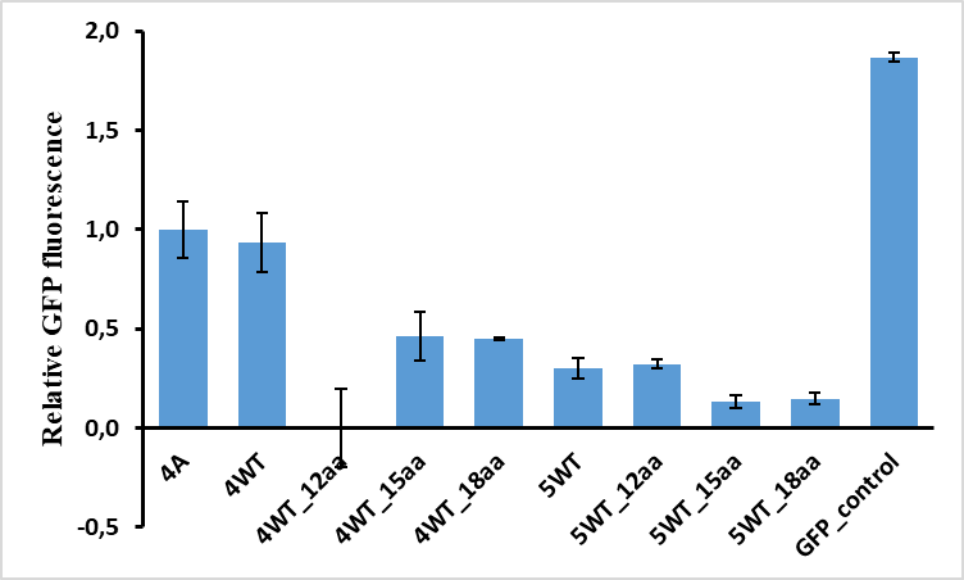

Figure 6. Relative GFP fluorescence of yeast strains with different Cln2 degron tags. The Y-axis of the bar chart represents a relative GFP fluorescence (4A strain was used as a reference strain) whereas the X-axis shows different strains and related degrons.

4WT_12aa, 4WT_15aa, 4WT_18aa, 5WT_12aa, 5WT_15aa, and 5WT_18aa degrons were made in this study (every bar represents an average result from several strains taken in two biological replicates). The chart is divided into 10 parts (each represents an average relative GFP fluorescence with EGFP-NES without any degron tags as a control). 12aa, 15aa, 18aa numbers demonstrate the distance between 2 phosphorylation sites (in amino acids).

3.2.2 The analysis of the impact of promoter region in Cln2 oscillation behavior during the cell cycle

In order to understand the contribution of promoter region in oscillation waves of Cln2 we compared the behavior of Cln2 and shlGFP under the control of Cln2 promoter during the cell cycle. NS343-NS346 strains were created at the background of RV1110 (NS343, NS344) and RV1111 (NS345, NS346). In NS343-NS346 yeast strains Cln2 ORF was replaced with shlGFP-13Myc containing the short-half-life GFP (shlGFP) instead of Cln2 region, Sic1_WT or Sic1_9SP (all 9 pairs of TP phosphorylation sites mutated to SP), and 13Myc tag with two strains expressing either the Sic1_WT-13Myc or Sic1_9SP (yeast strains are listed in Table 1).

To analyze the phosphorylation of the proteins of interest, SDS-page Western Blot experiments were performed with 6 different strains (The strains are listed in the **Supplementary Table 1**) containing *13Myc* tags. The cells were arrested in G1 by α-factor, released after 2-2.5 hours, and collected in the following time points: 0, 10, 20, 30, 40, 50, 60, 70 minutes after the release (Fig. 7).

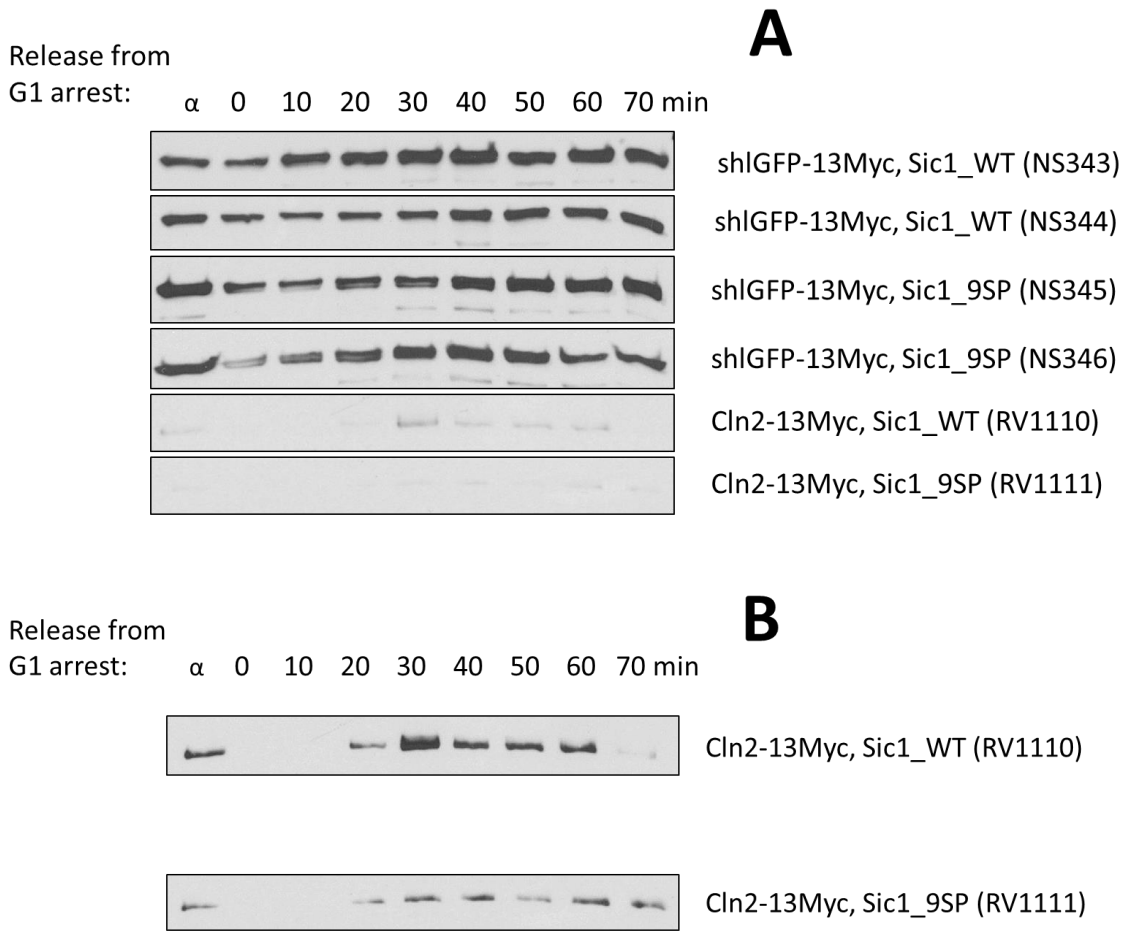

Figure 7 Analysis of the influence of the Cln2 promoter in Cln2 protein levels. Time courses showing different protein levels of cln2::ShlGFP-13MYC; *Cln2-13MYC*, *Sic1_WT*; and *Cln2-13MYC*, *Sic1_9SP* with 5 seconds exposure **(A)** and *Cln2-13MYC*, *Sic1_WT*; *Cln2-13MYC*, *Sic1_9SP;* at the 30 seconds exposure **(B)**.
(A) Strains containing short-half-life-GFP (shlGFP) under the control of Cln2 promoter show high protein levels at 0 and 10 time points probably due to the longer sequence compared to the Cln2. It is clear that the protein levels of strains lacking Cln2_WT are at least 10 times higher compared to the background RV1110 and RV1111 strains at the same 5 seconds exposure.
(B) RV1110 (*Cln2-13MYC*, *Sic1_WT*) strain shows normal Cln2 behavior during the cell cycle. RV1111 demonstrates the loss of protein levels caused by threonine-proline to serine-proline substitution of the Sic1_WT phosphorylation sites.

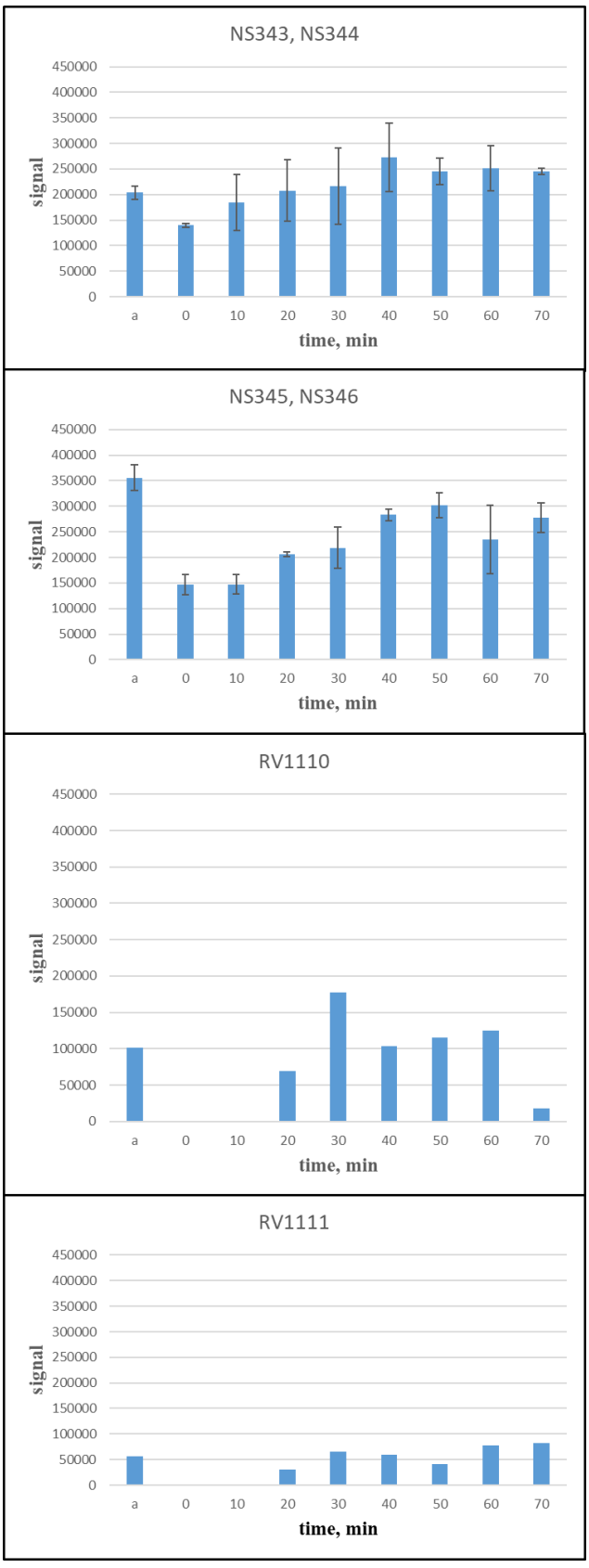

Figure 8. Band intensities of the Cln2 protein region in different mutants. comparison of protein levels in cln2::ShlGFP-13MYC; *Cln2-13MYC, Sic1_WT*; and *Cln2-13MYC, Sic1_9SP*.

The Cln2 promoter region has SBF and MBF transcription factors which activate the expression of native Cln2 and forming a positive feedback loop for Cln2 expression (Cross and Tinkelenberg, 1991). Cln2 also phosphorylates Sic1 and, therefore, targets it for degradation which leads to Clb5 activation (Schneider et al., 1996). The Cln2 is unable to phosphorylate yeast strain containing Sic1_9SP at the proper cell cycle stage which makes the Clb5 rapid activation impossible. Clb5, in turn, is responsible for Whi5 phosphorylation inhibiting SBF and MBF transcription factors. Our reference strain with Sic1_9SP shows that the promoter activation takes place at much lower rates. Nevertheless, the strains with the Cln2 region replaced by short-half-life-GFP were able to stabilize the protein levels.

SUMMARY

The current work can be divided into two parts. In the first part, we generated a set of multisite phosphorylation protein tags based on the Cln2 degron for the regulation of protein expression in yeast cells. Based on the levels of fluorescence of GFP (was used as a reporter) in different strains, we can conclude that our phosphodegron tags can significantly reduce protein expression. Changing the length of the degron tags and the distance between the phosphosites it is possible to achieve the desired protein levels.

In the second part, we wanted to analyze the impact of transcriptional regulation in the oscillation waves of Cln2 protein during the cell cycle. To do that, we compared the behavior of Cln2 and short-half-life-GFP (shlGFP) proteins under the control of Cln2 promoter during the cell cycle. Based on the results of the Western blot, we can conclude that the reason for Cln2 cell-cycle oscillations is lying in the protein sequence itself, while promoter activity is likely responsible for the synthesis of a sufficient number of transcripts. Further research of this subject will help to reveal the exact mechanism by which the interaction between transcriptional and translational regulation of Cln2 oscillations occurs during the cell cycle.

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SUPPLEMENTARY MATERIALS

Table 1. Yeast strains

NS228 was used as a background strain (ordered from EuroScarf): CEN.PK-2-1C: MATa ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2.

№	Genotype	Short description/transformation	Source
NS228	CEN.PK-2-1C: MATa ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2	SBY27	Euroscarf
NS229	leu2::pADH1-EGFP-NES	Constitutive expression of EGFP fused with NES/CRISPR-Cas9 mediated integration of a PCR fragment	Nastassia Shtaida
NS262	pADH1-GFP-NES-4A-KanMX	Based on NS229, transformed using PCR product from pNS112, col8	Nastassia Shtaida
NS266	pADH1-GFP-NES-5WT-15aa-KanMX	Based on NS229, transformed using PCR product from pNS101, col17	This study
NS267	pADH1-GFP-NES-5WT-15aa-KanMX	Based on NS229, transformed using PCR product from pNS101, col18	This study
NS276	pADH1-GFP-NES-5WT-KanMX	Based on NS229, transformed using PCR product from pNS97, col 1	Nastassia Shtaida
NS277	pADH1-GFP-NES-5WT-KanMX	Based on NS229, transformed using PCR product from pNS97, col 4	Nastassia Shtaida
NS278	pADH1-GFP-NES-5WT-KanMX	Based on NS229, transformed using PCR product from pNS97, col 5	Nastassia Shtaida
NS279	pADH1-GFP-NES-4WT-KanMX	Based on NS229, transformed using PCR product from pNS98, col 7	Nastassia Shtaida
NS280	pADH1-GFP-NES-4WT-KanMX	Based on NS229, transformedusing PCR product from pNS98 col 9	Nastassia Shtaida
NS289	pADH1-GFP-NES-4A-KanMX	Based on NS229, transformed using PCR product from pNS112, col 33	This study

NS292	pADH1-GFP-NES-4A-KanMX	Based on NS229, transformed using PCR product from pNS112, col 6	This study
NS295	pADH1-GFP-NES-5WT-12aa-KanMX	Based on NS229, transformed using PCR product from pNS100, col 1	This study
NS296	pADH1-GFP-NES-5WT-18aa-KanMX	Based on NS229, transformed using PCR product from pNS102, col 2	This study
NS299	pADH1-GFP-NES-4WT-15aa-KanMX	Based on NS229, transformed using PCR product from pNS104, col 7	This study
NS300	pADH1-GFP-NES-4WT-15aa-KanMX	Based on NS229, transformed using PCR product from pNS104, col 8	This study
NS343	*cln2::SHL_GFP-13MYC_TAG, Sic1_WT*	*Based on RV1110*	Nastassia Shtaida
NS344	*cln2::SHL_GFP-13MYC_TAG, Sic1_WT*	*Biological replica of NS343*	
NS345	*cln2::SHL_GFP-13MYC_TAG, Sic1_SP*	*Based on RV1111*	
NS346	*cln2::SHL_GFP-13MYC_TAG, Sic1_SP*	*Biological replica of NS345*	
NS348	pADH1-GFP-NES-4wt-12aa	Based on NS229, transformed using PCR product from pNS103, col 1	This study
NS349	pADH1-GFP-NES-4wt-18aa	Based on NS229, transformed using PCR product from pNS105, col 1	This study
RV1110	*Cln2-13MYC, Sic1_WT*		Rainis Venta
RV1111	*Cln2-13MYC-Sic1_9SP*	Based on RV1110	

Table 2. Plasmids used as PCR templates to amplify fragments for yeast transformation

№	Description:	Strain:	Primers (F/R):	Reference:
pNS89	Cln2_5WT-15aa	NS274, NS275	4731/4826	This study
pNS100	Cln2_5WT-12aa	NS295	4731/4826	
pNS101	Cln2_5WT-15aa	NS267	4731/4826	
pNS102	Cln2_5WT-18aa	NS296	4731/4826	
pNS103	Cln2_4WT-12aa	NS348	4732/4826	
pNS105	Cln2_4WT-18aa	NS349	4732/4826	
pNS108	Cln2_5WT-KKKK	NS284, NS285	4724/4729	
pNS109	Cln2_4WT-KKKK	NS286, NS287	4719/4729	

Table 3. Primers, used in this work.

Primer	Sequence	Tm (°C)
864	CCT TGT TTC TTT TTC TGC	48
2032	TAA TACTTT CAA CATTTT CGGTTT G	50.6
2175	GTT ACT TAT GAC AAA CAA TGC	49.6
2176	CAA GTG AAA GAA TAT CAC TTG G	51.9
2248	GTA TGT GGT CTC TCT TTT CCC G	57.7
3237	ACT TGG ATC CAT GGC TAG TGC TGA ACC AAG AC	73.8
4084	ACC TAA GAG TCA CTT TAA AAT TTG TAT AC	62.1
4302	CAA TGT TAT TGC TCA ATC ATG	53.4
4719	CAG AGG ATC CAT TTC GAG AAA GCT TAC CAT ATC AAC CCC	76.7
4724	CAG AGG ATC CTC CAT TCC TTC GCC CGC TTC	76.2
4729	TGT ATG GCG CGC CTC ATT TCT TCT TCT TAC ATA TAC TGT TTG ACT GCT G	79.8
4731	TGA ATT GGC TTT GAA ATT GGC TGG TCT GGA CAT TAA CAA AAT TTC GAG AAA GCT TAC CAT ATC	81.1
4732	TGA ATT GGC TTT GAA ATT GGC TGG TCT GGA CAT TAA CAA ATC CAT TCC TTC GCC CGC TTC	83.7

4826	GAG GCT TCA TCG GAG ATG ATA TCA CCA AAC ATG TTG CTG GGC AGA TCC GCG GCC GCA TAG	87.8
5312	CAA GCT GGG GTT GCT ATA GCA AGC TTT CTA GCA ATG TTC AAG TTG GAT GCA ATT TGC AG	83
5315	TTA AAA AAG GAC CGT GGT CTT GAT TGG TGA ATT GAT TGA TAA TGG	75.4
5316	TGA GCC TCC ACC TCC AGA AC	62.5
5318	GGG TAG AAC ACC ATT GAC CGT TTT ATA CAC TTC C	72.8
5419	CCT CCA CCT CCA GAA CCT CCG CCG CCT GAA CCG CCA CCA CCT ATT ACT TGG GTA TTG CCC	90.7
5667	GCA TTG CAT TTA AGC TTA CAC CTG	62
5689	GTA TAC ATG CTA AGG CAA AAA GTT TC	61.7
5697	GGA TAG CCC GCA TAG TCA GGA ACA TCG TAT GGG TAT GAG CCT CCA CCT CCA GAA C	86.5
5700	TTT TAC ACA ATA ATT CGT TTT CAT TTA ATC G	61.9
5701	TAG TGA GGC TAA ACA GTT TTG CGG TTT CCT TTA TAC TAA GAA GGT CTA TAA TGC GTA CGC TGC AGG TCG AC	85.4
5702	TCA GGT GGG TGC AGC CTA TTG CTG TCA TTG TGG TTG TTG TTA TCC TGA TCC ATC GAT GAA TTC TCT GTC G	86.5
5764	TTT CTT CTT ATA TAC AGC ATT GCT AG	51.1
5788	TGT TGT GGT TGA AAT GTC AGC	54
5789	TAT CAC CTG ACC AGA TGA GG	53.2
5869	TTT TCG TGA TTT TCA CCG AGC	54
5870	CAT TGT TAA TAA TGA TAC TGA GGT TC	50.2
5871	CCT CCC TAA CAT GTA GGT GG	54.4

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