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**Degradation of budding yeast protein Far1 during
G1/S phase transition**

Bachelor's Thesis (12 ECTS)
Curriculum Science and Technology

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Tartu 2021

Degradation of budding yeast protein Far1 during G1/S phase transition

Abstract:

Cell cycle events must be precisely controlled for the cell to proliferate. Cyclin-dependent kinases (Cdks) upon activation by cyclins control cell cycle events. Cyclin-Cdk complexes govern events of cell cycle by binding their substrate proteins at specific docking motifs and phosphorylating them, thus changing the proteins' activity. Far1 is a cyclin-dependent kinase inhibitor, that causes cell cycle arrest in response to mating pheromones. However, if mating pheromones are not present, Far1 is degraded. Clb5-Cdk1 complex phosphorylates the protein at S87/S91 residues, which triggers Far1 degradation. A Clb5-Cdk1 docking motif in N-terminal part of Far1 was recently discovered. This sequence, however, serves as docking motif only in truncated NFar1(1-170 aa). In this work it was shown that another possible motif involved in Far1 degradation might be located between 170th and 190th amino acids in the protein, Far1 does not contain another degron besides S87/S91 and Pho85 kinase does not affect Far1 degradation.

Keywords: cell cycle, cyclin, cyclin-dependent kinase, phosphorylation, protein degradation

CERCS: P310 Proteins, enzymology

Valgu Far1 lagundamine pagaripärmi rakutsükli G1 faasist S faasi üleminekul

Lühikokkuvõte:

Rakkude jagunemise aluseks on rakutsükli sündmused, mis peavad olema hästi kontrollitud. Rakutsükli peamise regulaatorina käsitletakse tsükliinsõltuvat kinaasi (Cdk) ja sellega seonduvaid tsükliine. Tsükliin-Cdk kompleksid tunnevad ära sihtmärk valgud tänu nendes leiduvatele spetsiifilistele aminohappelistele järjestustele – motiividele. Pagaripärmi valg Far1 on Cdk inhibiitor ning vahendab rakutsükli seiskumist G1 faasis, kui keskkonnas leidub feromooni. Feromooni puudumisel Far1 fosforüülitakse aminohapetest S87 ja S91 ning saadetakse lagundamisse ja rakutsükkel saab jätkuda tavalisel viisil. Hiljuti kirjeldati meie töögrupi poolt Far1 valgus leiduv spetsiifiline aminohappeline järjestus, mis võimaldab enne S-faasi algust Clb5-Cdk1 kompleksil sellist fosforüleerimist läbi viia. Huvitaval kombel pääseb uude motiivi mõju esile ainult, kui vaadelda Far1 valgu esimest 170 aminohapet. Täispika valgu korral motiivi muutmine või eemaldamine ei avalda mõju lagundamise kiirusele. See viis meid mõttele, et täispikas Far1 valgus (1-830 aminohapet) peab olema veel

midagi, mis võimaldab lagundada Cdk inhibiitorvalku enne S-faasi alustamist. Käesolevas töös lokaliseeriti 20 aminohappeline piirkond aminohapete 170 ja 190 vahele. Samas näidati, et eelnevalt kirjeldatud spetsiifilist äratundmisjärjestusest sõltumatule lagundamisel aitab kaasa sellesse piirkonda jääv Cdk1 poolt fosforüleeritav aminohape S174.

Võtmesõnad: rakutsükkel, tsükliin, tsükliinsõltuvat kinaas, fosforüülimine, valkude lagunemine

CERCS: P310 Proteiinid, ensümoloogia

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ABBREVIATIONS

aa – amino acids

APC – Anaphase-promoting complex

BGP – beta-Glycerophosphoric acid, disodium salt pentahydrate

Cdk – Cyclin-dependent kinase

CKI – Cyclin-dependent kinase inhibitor

DMSO – Dimethyl sulfoxide

EDTA – Ethylenediaminetetraacetic acid

eGFP – eukaryotic green fluorescent protein

Far1 – Inhibitor of Cyclin-dependent protein kinase 1

GFP – Green fluorescent protein

LB – Luria-Bertani media

MAPK – mitogen-activated protein kinase

NLxxxL - Short linear Clb5-specific docking motif

OD – Optical density

pSic1 – Sic1 promoter

SCF – Skp, Cullin, F-box containing complex

SC-Ura – Synthetic complete media lacking uracil

SDS – Sodium dodecyl sulfate

SDS-PAGE – sodium dodecyl sulphate–polyacrylamide gel electrophoresis

Sic1 – Inhibitor of Cyclin-dependent protein kinase 1

SLiM - Short linear interaction motif

SS-DNA – Salmon sperm DNA

TAE buffer - Tris-acetate-EDTA buffer

TAP tag – Tandem Affinity Purification tag

TBS-T – Tris-buffered saline – Tween buffer

TE buffer – Tris-EDTA buffer

Whi5 – Transcriptional repressor in G1 phase

YPD – Yeast extract, Peptone, Dextrose

INTRODUCTION

The life span of the cell consists of growth, DNA replication and division. All the events throughout the cell's lifespan must be extensively controlled in order for the cell to proliferate successfully. A term that describes these events has fit into two words: cell cycle. Disturbance of the flow of events in the cell cycle can result in the accumulation of mutations, cell death or uncontrolled division, which leads to cancer (Elledge, 1996).

Research on the topic of cell cycle is extremely important to understand the mechanisms that control timing and frequency of DNA duplication and cell division. So far, scientists still have a long way ahead in understanding the processes that control the cell cycle.

The most popular model organism to study the cell cycle is budding yeast *Saccharomyces cerevisiae*. There are several reasons for this: firstly, budding yeast is easy to manipulate in the laboratory. Also, it belongs to the eukaryotic domain, and cell cycle regulatory machinery is highly conserved among eukaryotes, making yeast an ideal model organism to study the cell cycle. Enzymes that control the events of the cell cycle are called cyclin-dependent kinases (Cdks). As might be seen from the name, Cdks must be activated by cyclins in order to govern cell cycle events. Upon association with cyclins, Cdk-cyclin complexes bind and phosphorylate their substrates, thus changing their properties (Russo, Jeffrey and Pavletich, 1996).

Plenty of proteins must be phosphorylated by Cdks in order for the cell cycle events to move forward. One important family of proteins on the cell cycle is a family of cyclin-dependent kinase inhibitors (CKIs). They play a crucial role in the process of cell cycle arrest. Upon phosphorylation of these proteins, Cdk activity is inhibited, and the cell cycle cannot progress (Henchoz *et al.*, 1997).

One of such proteins is Far1, a CKI that is activated in response to the activation of yeast mating pathway. It inhibits the cyclin-Cdk1 complex, thus causing cell cycle arrest. However, if the mating pathway is not activated, Far1 is triggered for degradation by the Clb5-Cdk1 complex. It is known that the Clb5-Cdk1 complex phosphorylates Far1 at S87/S91 residues. A docking motif in NFar1(1-150 aa) specific for the Clb5-Cdk1 complex has been recently identified (Faustova *et al.*, 2021). However, it was later also shown that this motif has effect only in truncated NFar1(1-170 aa). This work aims to identify other factors behind full-length Far1 degradation.

1. LITERATURE REVIEW

1.1. Yeast as a model organism

Yeasts are a large group of eukaryotic, single-celled microorganisms, which belong to the fungus kingdom. Several yeast species are widely used as model organisms. One of these species is the well-known budding yeast *Saccharomyces cerevisiae*. Since ancient times, it has been widely used in winemaking and baking due to its ability to convert carbohydrates to carbon dioxide and alcohol (Legras *et al.*, 2007). Nowadays, it is also a popular model organism for biotechnology research. There are, of course, several reasons for it and they are listed below. To begin with, yeast has a significant number of genes that are homologous to those in humans. Its chromosome segregation is extensively homologous to those in plant and animal cells. This property is extremely important as it allows the study of fundamental processes such as cell cycle, DNA replication, recombination, cell division, and metabolism (Botstein, Chervitz and Cherry, 1997). *S. cerevisiae* is considered non-pathogenic, widely available, and has a short generation time. It can proliferate in a haploid state and its genome is fully sequenced (Goffeau *et al.*, 1996). Therefore, it can be easily manipulated in the laboratory, allowing adding or deleting specific genes through homologous recombination. Finally, the availability of the complete set of yeast gene-deletion strains and other functional genomics tools for the comprehensive analysis of gene and protein function makes yeast an increasingly accessible model for delineating mechanisms of cell cycle control (Ma *et al.*, 1987).

1.2. Yeast cell cycle

Cell cycle is a highly controlled series of events leading to cell division. Typically, there are four phases in eukaryotic cell cycle. Two main phases are S and M phases. Throughout the S phase, DNA is replicated, and chromosomes are duplicated. M phase stands for nuclear and cell division (mitosis and cytokinesis). Mammalian cell cycle contains two additional phases - G1 and G2, which represent gaps between the main phases. G1 follows cytokinesis, whereas G2 occurs before the M phase. Gap phases are essential to provide time for cell growth. Additionally, they serve as checkpoints to control progression through the cell cycle (Ma *et al.*, 1987). The basic scheme of cell cycle events varies between species, even though the S phase and mitosis are present in every cell cycle. G phases for some organisms might not exist at all or be very short, while for other organisms, gap phases are quite long (Hartwell and Weinert, 1989).

Saccharomyces cerevisiae cell cycle is a subject of numerous studies. It has a long G1 phase but lacks clearly defined G2 between S and M phases. The reason for it is that preparation for mitosis can sometimes begin before the S phase is complete, i.e., before the completion of DNA synthesis. As was already mentioned above, *S. cerevisiae* can proliferate in a haploid state. The cells exist in 2 types – α and a type; α - and a-factors are special pheromones secreted by these cells accordingly. These mating factors allow cells of opposite mating types to bind each other and fuse to form a diploid cell, triggering arrest in the G1 phase. If the diploid cells encounter unfavorable conditions, they can undergo sporulation. Spores consist of 4 haploid cells, 2 of each type, formed by meiosis and packed together under a protective covering (Figure 1). As soon as conditions again become favorable, cells return to a haploid state (Morgan, 2007).

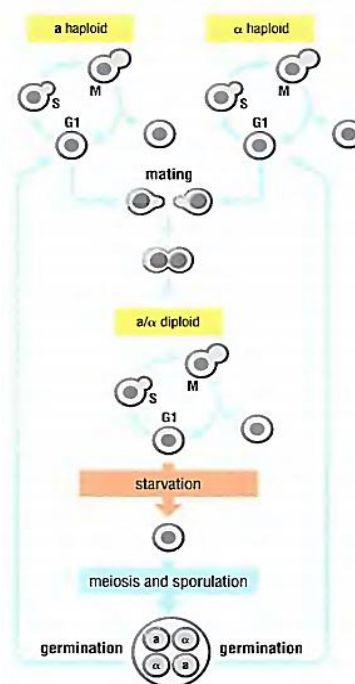


Figure 1. A simplified scheme of *S. Cerevisiae* life cycle. Cells can proliferate both in haploid and diploid states. Cells of opposite cell types (a and α) can mate to form a diploid. If it undergoes unfavorable conditions it forms a spore with 4 haploid cells, 2 of each type. Figure is taken from Morgan, 2007.

1.3. Cell cycle control

It is no wonder that cell cycle events must be extensively controlled in order for the cell to proliferate successfully. For example, cell is able to pause the cell cycle if the extracellular conditions are hostile. If cell cycle arrest happens in G1, the cell will enter a special G0 phase, a prolonged nondividing state, where the cell will stay until conditions normalize. Two types of mechanisms govern the cell cycle events – extrinsic and intrinsic. Extrinsic mechanisms are

generally not induced, except for the case when the defect is detected. Intrinsic mechanisms are, on the contrary, usually active in cell cycle to order events (Hartwell and Weinert, 1989).

If DNA damage, replication, or mitotic errors occur during cell cycle, as already stated above, it will be arrested to prevent cell from proliferation. Cell cycle contains several checkpoints, inhibitory biochemical pathways, that control the defined order and timing and prevent cell from proliferation if one of the stated above errors occur. First, G/S checkpoint, or Start, is essential for the cell to start proliferating. If conditions are favorable, specific mechanisms are activated inside the cell; they initiate DNA replication, centrosome duplication and other early cell-cycle processes. In addition to unfavorable conditions, yeast cell cycle is also arrested if cells start mating. In this case, yeast mating pathway is activated, and cell cycle is arrested (Elledge, 1996; Wang and Dohlman, 2004). The other two checkpoints are G2/M and M (metaphase-to-anaphase transition). These pathways regulate cell cycle progression at specified points of the cycle and are able to induce cell cycle arrest upon, for example, DNA damage (Lydall and Weinert, 1997).

1.4. Protein kinases and phosphorylation

Protein kinases are defined as enzymes that phosphorylate amino acid residues in a substrate protein. Depending on the protein site that is being phosphorylated, it can have a stimulatory or inhibitory effect (Holt *et al.*, 2009). Eukaryotic protein kinases have different specificity. Some of them phosphorylate the hydroxyl group of serine and threonine residues, others are tyrosine specific meaning that they phosphorylate tyrosine amino acid residues (Hanks, Quinn and Hunter, 1988). Protein phosphorylation itself is a type of post-translational modification which lies in removing a phosphate group from ATP and attaching it to the specific amino acid residue (Figure 2). Phosphorylation changes a protein conformation, which results in a change in protein's activity (Cohen, 2002). Therefore, it is the most important post translational modification for the progression of cell cycle. One of the many examples of phosphorylation in cell cycle is the phosphorylation of Whi5 protein. Whi5 is a transcription cofactor that represses transcription during the G1/S transition. Full phosphorylation of its functional sites results in activation of the inhibitory activity, thus activating G1/S transcription (Quilis and Igual, 2012).

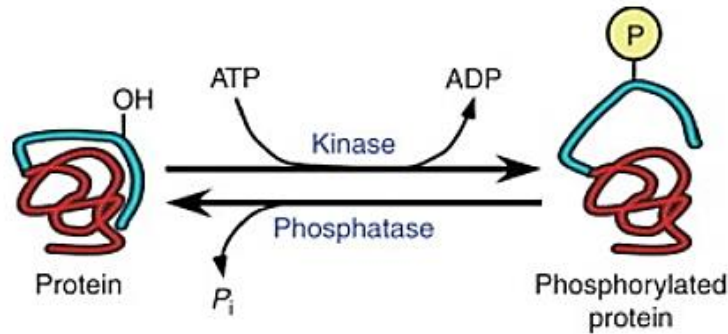


Figure 2. A scheme of the phosphorylation process. Protein kinases catalyze the addition of γ -phosphate molecule from ATP to proteins, resulting in formation of ADP and activation or deactivation of a protein. Phosphorylation is a reversible process. Phosphatase is an enzyme that removes phosphate molecule from a protein. Figure is taken from Weber, 2010.

1.5. Cyclin-dependent kinases

A high number of proteins and genes are involved in the regulation of the cell cycle. Cdks play a key role in cell cycle control. Cdks are proline-directed serine/threonine protein kinases, as they can phosphorylate their substrates only at serine or threonine residues and only if proline is following the serine or threonine sites. In other words, Cdks phosphorylate proteins at minimal consensus motifs S/T-P (Suzuki *et al.*, 2015). Cdks must be activated by regulatory proteins, cyclins, at specific points of the cell cycle. Budding yeast *S. cerevisiae* contains six different Cdks: Cdk1 (also known as Cdc28), Pho85, Ssn3, Bur1, Ctk1 and Kin28. Cdk1 is the main cell cycle control machinery, progression of the cell cycle without Cdk1 is impossible. Cdk1 and Pho85 kinases can be activated by several cyclins, while other kinases are specific only for one cyclin. Ssn3, Bur1, Ctk1 and Kin3 kinases function mainly in regulation of transcription (Enserink and Kolodner, 2010).

Cyclins that regulate Cdk1 are divided into four groups: G1, G1/S, S and M cyclins. G1 cyclin controls entry to the cell cycle in response to extracellular factors, while others are directly involved in the control of cell cycle events. Cyclin-Cdk1 complexes are mainly regulated by phosphorylation on threonine and tyrosine residues. Cyclin levels, in turn, are regulated by a wide range of mechanisms, resulting in a complex network forming the core of cell cycle control system (Morgan, 2007). In order to successfully control cell cycle, levels of different types of cyclins oscillate throughout the cell cycle progression. This causes the oscillation in cyclin-Cdk1 complexes' levels and thus drives cell cycle events (Figure 3). G1/S cyclins in

Saccharomyces cerevisiae are Cln1 and Cln2. These cyclins' concentration rises in late G1 and decreases in the early S phase. G1/S – Cdk complex is formed with the rise in cyclins concentration. This complex's primary function is to initiate the progression through Start and prepare cell for DNA replication (Quilis and Igual, 2012). S-phase cyclins – Clb5 and Clb6 – function in stimulating DNA replication. Cyclins Clb2, Clb3 and Clb4 are M-cyclins, they control events, that lead to mitotic spindle assemble and alignment of sister-chromatid pairs ((Morgan, 2007).

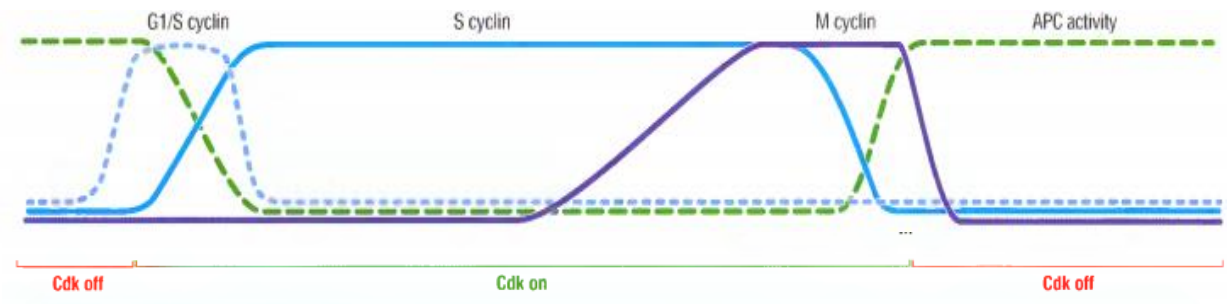


Figure 3. A simplified scheme of the cyclins' oscillations throughout the cell cycle. Levels of G1/S, S and M cyclins oscillate during cell cycle. When G1/S cyclin level rises, and the G1/S-Cdk complex is formed, the cell's new division cycle begins at the Start checkpoint. G1/S-CDK activates S-Cdk complex, which then initiates DNA replication at the beginning of S phase. As the S phase is completed, M-Cdk complex is formed. After the second checkpoint is successfully passed the assembly of mitotic spindle begins. The sister-chromatid separation characterizes metaphase to anaphase transition checkpoint. Anaphase promoting complex (APC) activity is held on the high level until the beginning of a new cell cycle, i. e. the rise in G1/S cyclin concentration. The figure is adapted from Morgan, 2007.

1.6. Pho85 kinase

Pho85 cyclin-dependent kinase is found in yeast *Saccharomyces cerevisiae* and is responsible for several processes during cell cycle depending on the cyclin it is activated by. There are two subfamilies of cyclins specific for this Cdk. The first, Pho80 subfamily includes Pho80, Pcl6, Pcl7, Pcl8 and Pcl10 cyclins. The second, Pcl1,2 subfamily, includes Pcl1, Pcl2, Pcl9, Clg1 and Pcl5 cyclins. Pho80 subfamily mainly acts in sensing environmental changes, while genes of the Pcl1,2 subfamily are expressed in G1 phase. Pcl1 and Pcl2 cyclins contribute to Start - the G1/S phase transition (Measday *et al.*, 1997). For instance, in the presence of nitrogen starvation or pheromone stimulation, Pho85 phosphorylates chaperon Ssa1 at Thr36, destabilizing Cln3 to delay the onset of the S phase (Truman *et al.*, 2012). Studies have shown that deletion of Pho85 results in, among other defects, slow growth with a delay in G1 phase (Timblin, Tatchell and Bergman, 1996). Pho85 is also related to the degradation of one of the CKIs. Namely, Sic1,

an inhibitor of Clb-Cdk1 kinases, must be phosphorylated and degraded in G1 for the cell to initiate DNA replication. Sic1 controls both exit from mitosis and the transition from G1 to S phase, hence preventing premature S phase and ensuring genome integrity. In case of the decreased activity of Cln-Cdk1 (e.g., DNA damage, nutrient starvation or pheromone presence), Pho85 is the Cdk in charge of phosphorylating Sic1 and allows the cell cycle to resume. It was shown that Pcl1-Pho85 complex can phosphorylate Sic1 in vitro, thus targeting it for degradation (Nishizawa *et al.*, 1998).

1.7. Protein degradation

Proteolysis is a process of breaking down proteins into amino acids or smaller polypeptides. Two main protein degradation pathways in eukaryotes are the ubiquitin-proteasome pathway and lysosomal proteolysis. The ubiquitin-proteasome pathway, as the name states, utilizes ubiquitin molecules as markers for degradation. This process includes enzymes E1, E2 and E3, which respectively activate ubiquitin and catalyze its transfer to a substrate protein. SCF complex is a key ubiquitin-protein ligase in G1/S control, consisting of Skp1, cullin, RING finger protein and F-box. Here Skp1 is an adaptor protein essential for recognition and binding of F-box proteins, cullin forms a scaffold of the complex and F-box protein contributes to the substrate specificity of SCF complex; RING finger is a binding domain that carries E2 and ubiquitin. (Figure 4; Morgan, 2007). The main function of SCF complex is to catalyze the addition of ubiquitin to the proteins targeted for degradation. As a result of ubiquitination, several ubiquitin molecules are added to the protein, making it a target for proteasome complex (Freemont, 2000). Ubiquitin-dependent degradation is a critical mechanism in regulating cell cycle transitions, which allows the achievement of the irreversibility of cell cycle (Henchoz *et al.*, 1997). Some proteins throughout the cell cycle must be phosphorylated in order to be recognized by ubiquitination machinery. Sites on the protein that must be phosphorylated in order to trigger its degradation are called phosphodegrons. An example is Cdc6 protein, that is a part of complex essential for DNA replication. Cdc6 must be phosphorylated by cyclin-Cdk1 complex in order to be sent to degradation and prevent DNA re-replication. It contains two consensus sites with three amino acids in between. Double phosphorylation of these sites allows the recognition by ubiquitination machinery, thus they serve as phosphodegrons (Al-Zain *et al.*, 2015).

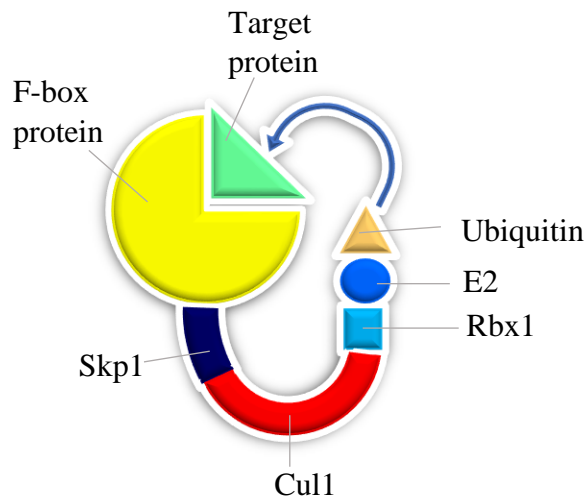


Figure 4. SCF enzyme catalyzing ubiquitination. SCF consists of RING protein Rbx1, cullin Cul1, Skp1 and F-box protein, which specifically binds to the target protein. Rbx1 carries E2 and ubiquitin. After F-box protein is bound to the target protein, ubiquitin is transferred from E2 to the lysine side chain of the target protein. Figure is adapted from Morgan, 2007.

1.8. Far1 protein

Protein Far1 (stands for "factor arrest") is the first identified CKI. It functions in cell cycle arrest in G1, namely by inhibiting the Cln-Cdk1 kinase. G1 arrest is mediated upon mating of a and α cell types (Pope, Bhaduri and Pryciak, 2014) that activates yeast mating pheromone response pathway. Only the presence of Far1 in large amounts is not enough for the cell cycle arrest. The mating factor response pathway triggers activation of Far1, Far1, in turn, inhibits Cln-Cdk1 kinase to trigger the cell cycle arrest (Peter and Herskowitz, 1994). Mitogen-activated protein kinase (MAPK) Fus3 phosphorylates Far1 at the T306 site, thus triggering it for cyclin-Cdk1 complex inhibition and pheromone-dependent cell cycle arrest (Figure 5). Far1 protein contains N-terminal intrinsically disordered regions. These regions contain short linear motifs (SLiMs), which serve as docking motifs for protein kinases. They bind SLiMs of Far1 and phosphorylate the protein (Örd and Loog, 2019).

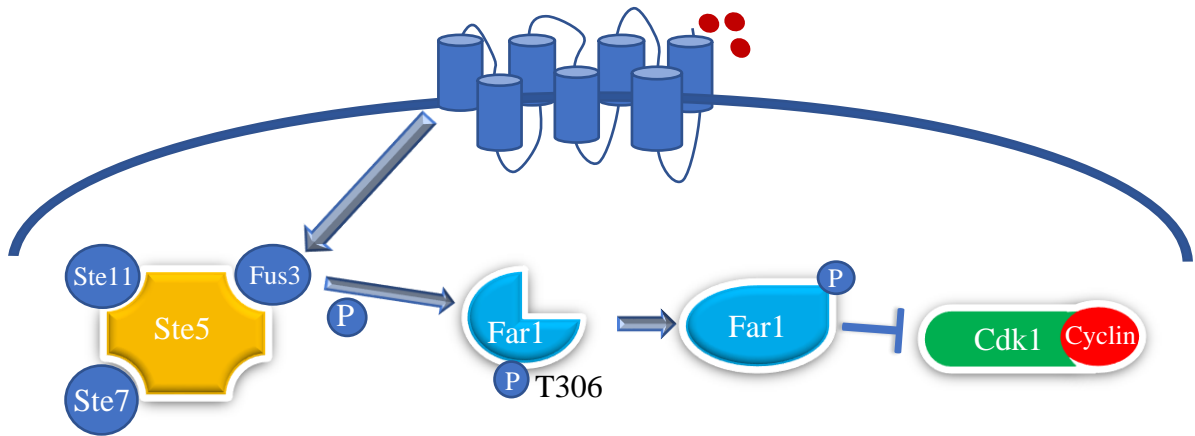


Figure 5. Far1 activation in response to pheromone signals. Far1 is activated for inhibition in G1 phase if mating pheromones (red circles) are present in the environment. Fus3 mediates Far1 phosphorylation at T306 site. Phosphorylation causes Far1 activation, that inhibits Cdk1-cyclin complex and causes cell cycle arrest.

1.9. Far1 protein degradation

Far1 phosphorylation may result in several outcomes. Firstly, as mentioned above, phosphorylation at the T306 site is involved in the Far1 activation process for cell cycle arrest. In contrast, S87/S91 site phosphorylation is involved in Far1 degradation (Gartner *et al.*, 1998). In the absence of mating pheromones, Far1 does not arrest the cell cycle. In this case, Far1 is sent to degradation. Clb5-Cdk1 phosphorylates S87/S91 sites, thus triggering Far1 degradation (Figure 6). S87/S91 site is known as di-phosphodegron – a part of the protein that must be phosphorylated in order to regulate protein degradation.

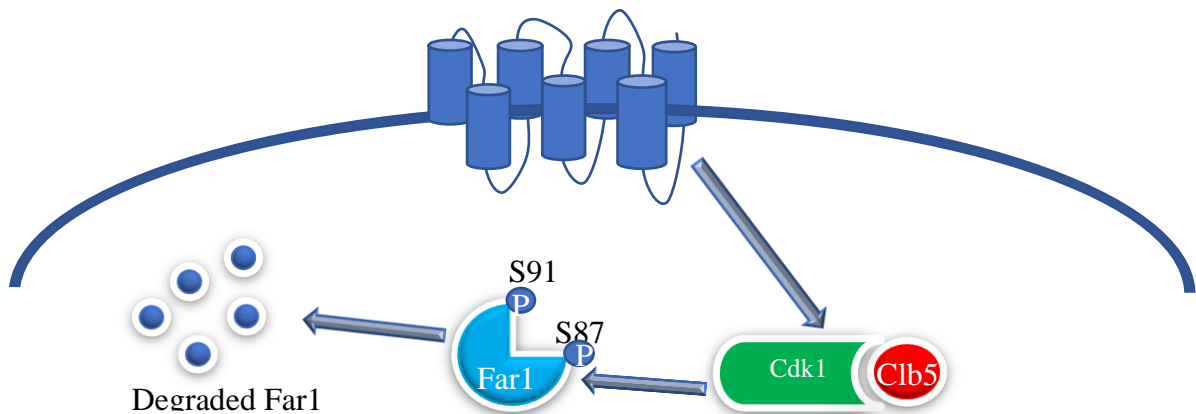


Figure 6. Far1 degradation in absence to pheromone signals. If pheromone pathway is not activated, Clb5-Cdk1 phosphorylates Far1 at S87 and S91 sites, thus targeting Far1 to degradation.

S87 P91 site in Far1 is a consensus SP motif, that can be directly phosphorylated by Cdk1, however S91 site is not followed by proline. In order for Cdk1 to phosphorylate this site, kinase must firstly phosphorylate other consensus T/S-P sites in N-terminal part of the protein. These sites are situated at positions T3, T15, S26, T43, T63 (Faustova *et al.*, 2021).

Recent studies have identified NLxxxL short linear motif specific for Clb5 in Far1 N-terminal disordered region. This motif mediates phosphorylation of the above-mentioned di-phosphodegron in truncated Far1(1-150 aa) (Faustova *et al.*, 2021).

2. THE AIMS OF THE THESIS

It has been shown that mutation of the NLxxxL motif causes a delay in the degradation of NFar1(1-150). If this motif is mutated in full-length Far1, protein is degraded in the same way as wild type. In this work, we are trying to identify other mechanisms involved in full-length Far1 degradation.

The aim of this work is to:

- Identify the region involved in Far1 degradation in full-length Far1.

3. EXPERIMENTAL PART

3.1. Materials and methods

3.1.1. Materials

1. Media:

- a. LB media (5 g/l yeast extract (Formedium), 10 g/L NaCl (Chempur), 10 g/l tryptone (Formedium))
- b. YPD media (20 g/l glucose (Oriola), 10 g/l yeast extract (Formedium), 20 g/l peptone (Formedium))
- c. YPR media (10 g/l yeast extract (Formedium), 20 g/l peptone (Formedium), 2% raffinose)
- d. Synthetic complete media (SC) with 2% glucose (10 g/l CSM (Formedium), 20 g/l glucose (Oriola), Yeast nitrogen base without amino acids (BD Biosciences))

2. Buffers:

- a. 1x TAE buffer (40 mM Tris-acetate pH 8.3, 1 mM EDTA)
- b. 1x TE buffer (10 mM Tris hydrochloride (Tris-HCl) pH 8, 1 mM EDTA)
- c. PL1 buffer (100 mM Lithium Acetate, 0.5 x TE buffer)
- d. PL2 buffer (40% Polyethylene glycol (PEG) 3350, 100 mM Lithium Acetate, 1x TE buffer)
- e. 6xSDS buffer (375 mM Tris-HCl, 9% SDS, 50% Glycerol, 0.03% Bromophenol blue, 600 mM DTT)
- f. Semi-dry buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS)
- g. T-BST buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20 (Bio-Rad))
- h. Urea lysis buffer (20 mM Tris (pH 7.4), 8 M Urea (Sigma), 2M Thiourea (Sigma), 4% CHAPS (Sigma), 1% DTT (Sigma), 50 mM NaF (Sigma), 89 mM beta-glycerol phosphate (Acros Organics), 1 mM Na₃VO₄ (Sigma))
- i. Lysis buffer for TAP purification (25 mM Tris-HCl, pH 7.4, 1 M NaCl, 0.1% NP-40, 1 mM EDTA, 1 mM PMSF (Sigma), 1 ug/ml Leupeptin (Sigma), 80 mM beta-glycerol phosphate (Sigma), 50 mM NaF, 1 mM Na₃VO₄ (Sigma))
- j. TEV cleavage buffer (10 mM TRIS-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 1 mM DTT)
- k. Coomassie staining fixating solution (30% ethanol, 10% acetic acid)
- l. Blue silver micellar solution (0.12% Coomassie Blue G-250, 10% ammonium sulfate, 10% phosphoric acid, 20% methanol)

3. Agar plates:

- a. LB agar plates with ampicillin: (LB media, 15 g/l agar (Formedium), 100 µg/ml ampicillin (Sigma))
- b. SC-URA glucose agar plates (20 g/l glucose (Oriola), 20 g/l agar (Formedium), 2 g/l SC-URA powder (MP Biomedicals), 7 g/l yeast nitrogen base without amino acids (BD Biosciences))
- c. YPD plates (10 g/l yeast extract (Formedium), 20 g/l peptone (Formedium), 20 g/l dextrose (Formedium), 20 g/l agar (Formedium))

1% agarose TAE gel (1% agarose in 1x TAE buffer, 5 µl/l Atlas ClearSight DNA Stain (BioAtlas))

1.5% SC/glucose-agarose gel pad (20 g/l yeast nitrogen base without amino acids (Formedium), 10 g/l complete synthetic media (Formedium), 20 g/l glucose (Oriola))

3.1.2. Plasmids used in the study

Table 1. PCR program for introducing truncations

Plasmid	Backbone	Description	Source
pEV1140	pRS306	pSic1-NFar1(1-393) NLxxxL-EGFP	This study
pEV1141	pRS306	pSic1-NFar1(1-350) NLxxxL-EGFP	This study
pEV1152	pRS306	pSic1-NFar1(1-329) NLxxxL-EGFP	This study
pEV1172	pRS306	pSic1-NFar1(1-300) NLxxxL-EGFP	This study
pEV1174	pRS306	pSic1-NFar1(1-250) NLxxxL-EGFP	This study
pEV1170	pRS306	pSic1-NFar1(1-200) NLxxxL-EGFP	This study
pEV1199	pRS306	pSic1-NFar1(1-200) NLxxxL/S87AS91A-EGFP	This study
pEV1182	pRS306	pSic1-NFar1(1-200) NLxxxL/S174A-EGFP	This study
pEV1178	pRS306	pSic1-NFar1(1-190) NLxxxL-EGFP	This study
pEV1180	pRS306	pSic1-NFar1(1-180) NLxxxL-EGFP	This study
pEV1089	pRS306	pSic1-NFar1(1-170) WT-EGFP	This study
pEV1090	pRS306	pSic1-NFar1(1-170) NLxxxL-EGFP	This study

3.1.3. Strains used in the study

Table 2. PCR program for introducing truncations

Plasmid	Genotype	Source
pEV200	leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG WHI5-mCherry-SpHIS5 ura3::pEV1140::ura3	This study
pEV201	leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG WHI5-mCherry-SpHIS5 ura3::pEV1141::ura3	This study
pEV202	leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG WHI5-mCherry-SpHIS5 ura3::pEV1152::ura3	This study
pEV203	leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG WHI5-mCherry-SpHIS5 ura3::pEV1172::ura3	This study
pEV204	leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG WHI5-mCherry-SpHIS5 ura3::pEV1174::ura3	This study
pEV205	leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG WHI5-mCherry-SpHIS5 ura3::pEV1170::ura3	This study
pEV206	leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG WHI5-mCherry-SpHIS5 ura3::pEV1199::ura3	This study
pEV207	leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG WHI5-mCherry-SpHIS5 ura3::pEV1182::ura3	This study
pEV208	leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG WHI5-mCherry-SpHIS5 ura3::pEV1178::ura3	This study
pEV209	leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG WHI5-mCherry-SpHIS5 ura3::pEV1180::ura3	This study
pEV210	leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG WHI5-mCherry-SpHIS5 ura3::pEV1189::ura3	This study
pEV211	leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15 [phi+] bar1::hisG WHI5-mCherry-SpHIS5 ura3::pEV1190::ura3	This study
pEV212	leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+] bar1::hisG WHI5-mCherry-SpHIS5 pho85::kanMX ura3::pEV1170::ura3	This study

pEV213	leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+] bar1::hisG WHI5-mCherry-SpHIS5 pho85::kanMX ura3::pEV1182::ura3	This study
pEV214	leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+] bar1::hisG WHI5-mCherry-SpHIS5 pho85::kanMX ura3::pEV1189::ura3	This study
pEV215	leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+] bar1::hisG WHI5-mCherry-SpHIS5 pho85::kanMX ura3::pEV1190::ura3	This study
RV298	leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+] bar1::hisG WHI5-mCherry-SpHIS5	Rainis Venta

3.1.4. NEB site-directed mutagenesis

NEB site-directed mutagenesis allows introduction of truncations by PCR reactions. PCRs for DNA truncations were performed with Thermo Scientific Phusion High-Fidelity DNA Polymerase and oligonucleotides by Integrated DNA technology (IDT). For 50 µl reaction 0.5 µl of Phusion DNA polymerase, 2.5 µl of 100 µM oligonucleotides (IDT), 1 µl of DNA fragment, 0.5 µl of dNTPs, 10 µl of Thermo Scientific 5 x Phusion Green HF Buffer and 33 µl water were mixed on ice. According to the fragment length and oligonucleotides' melting temperatures, elongation time and annealing temperature were adjusted in the PCR program (Table 3). After the PCR program completion, samples were loaded on the 1% agarose gel with Atlas ClearSight DNA Stain (BioAtlas) added to allow visualization of DNA bands. DNA fragments were separated by electrophoresis. visualized under UV light, excised from the gel and purified using FavorPrep GEL/PCR Purification Kit (Favorgen) according to the manufacturer's protocol.

In the case when DNA fragments were truncated with PCR reaction, truncated samples were ligated back together in 20 µl. 7 µl of PCR product, 1 µl T4 polynucleotide kinase (PNK), 1 µl DPN (Thermo Scientific), 2 µl 10xT4 ligation buffer 9 µl and water were mixed. Samples were incubated at 37 °C for 30 minutes. After incubation 2 µl of 50% polyethylene glycol (PEG) 4000 and 1 µl of 5 U/µl T4 DNA ligase (Thermo Scientific) were added. Samples were incubated at room temperature for 10 minutes and used for transformation.

Table 3. PCR program for introducing truncations

Phase	Temperature	Time	Number of cycles
Initial Denaturation	98 °C	5 minutes	1
Denaturation	98 °C	20 seconds	30
Annealing	58-68 °C, according to oligonucleotides annealing temperatures	20 seconds	
Extension	72 °C	30 seconds/kilobase	
Final extension	72 °C	5 minutes	1

3.1.5. PCR

PCR to amplify pGAL1 was performed using the same protocol as for NEB site-directed mutagenesis PCR. Difference lies in annealing temperature that is 51 °C.

Yeast colony PCR was performed using 5 µl of FIREPol DNA polymerase (Solis Biodyne), 1.5 µl of DNA, 0.7 µl of oligonucleotides and water up to 25 µl of final volume. Prior to mixing reactions yeast cells were lysed in 20mM NaOH by heating for 10 minutes at 100 °C and centrifuging down at 13200 rpm for 20 seconds. Supernatant was used for PCR reaction.

All plasmids constructed in the study are listed in Table1

3.1.6. Bacterial transformation

Ligated plasmids were transformed into *E. Coli* competent Turbo cells (NEB). The competent cells are stored at -80°C. For transformation cells were thawed on ice, 2 µl of plasmid were mixed with 50 µl of competent cells and incubated on ice for 20 minutes. Next, heat shock was performed at 42 °C for 45 seconds and samples were incubated on ice for 5 minutes. Samples were mixed with 500 µl of LB media and incubated in the 200-rpm shaker at 37 °C for 1 hour. After incubation, samples were centrifuged at 6000 rpm for 1 minute. 350 µl of supernatant discarded, leaving 200 µl in the tube. Precipitated cells were resuspended in the media and plated on the ampicillin agar plates. Plates were incubated at 37°C for 12-16 hours.

3.1.7. Plasmid extraction

For plasmid extraction, individual colonies were picked from the agar plates. Cells in 5 mL of LB media with 100 µg/mL ampicillin added were incubated in 200-rpm shaker at 37 °C for 4-5 hours. According to the manufacturer's protocol, after incubation, plasmid DNA was extracted using FavorPrep Plasmid DNA Extraction Mini Kit (Favorgen). DNA concentrations were measured using in the NanoDrop 1000 Spectrophotometer (Thermo Fisher) following the manufacturer's instructions. To validate gene mutations, samples were sent for Sanger DNA sequencing (Estonian Biocentre).

3.1.8. Plasmid linearization and Lithium-Acetate-mediated yeast transformation

For plasmid integration into the yeast genome, plasmid was linearized beforehand. For linearization 2 µg of plasmid was mixed with 1µl (10U/ µl) of *StuI* FastDigest enzyme (Thermo Scientific) and 10xFD Buffer (Thermo Scientific). Reactions were incubated at 37 °C for 1 hour. 20 µl of reaction was used directly for yeast transformation.

For the microscopy experiments, linearized vectors were transformed to *S. cerevisiae* strains RV298 (*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+] bar1::hisG WHI5-mCherry-SpHIS5*) and ΔPHO85 (*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+] bar1::hisG WHI5-mCherry-SpHIS5 pho85::kanMX*). All strain used in the study are listed in Table 2.

Strains were streaked out on YPD plates and incubated overnight at 30 °C. After incubation cultures were resuspended in 50 ml YPD media and left in the 30°C 160 rpm shaker until OD600 reached 0.6-0.8. When desired optical density was reached, cultures were centrifuged at 3600 rpm for 1 minute. Supernatant was discarded and cells were resuspended in 1 ml PL1 solution and transferred into 1.5 ml Eppendorf tube. In 1.5 ml reaction tube cells were centrifuged at 3600 rpm for 1 minute, supernatant discarded. Cell pellet was resuspended in PL1 solution at the amount if two volumes of the cell pellet and incubated at room temperature for 10 minutes. Meanwhile salmon sperm DNA (Sigma) was boiled at 100 °C for 10 minutes, after 10 minutes it was immediately placed on ice. DNA fragment was mixed with 10 µl of ssDNA, 100 µl of yeast competent cells, 700 µl of PL2 solution and 48 µl of DMSO (Fisher chemical) and gently resuspended. Heat shock was performed at 42 °C for 40 minutes. After incubation samples were cooled on ice for 5 minutes and centrifuged at 3600 rpm for 1 minute, supernatant was discarded. Samples were resuspended in 1 ml of sterile 1 x TE buffer and pelleted by

centrifugation at 3600 rpm for 1 minute, supernatant was discarded. Cells were resuspended in 200 μ l of sterile 1 x TE buffer and plated on the agar plates (YPD plates for pGAL1, they were plated on selective media (Geneticin) the next day; SC-URA glucose agar plates, for integration). Plates incubation was performed at 30 °C for 2 days or until colonies appear.

3.1.9. Western Blot

3.1.9.1. Culture growth

Colonies are grown at 30 °C overnight on fresh YPD plates. The following day cells are put to grow in 18 ml YPR media + 2 ml raffinose for 2-3 hours at 30 °C. In 2-3 hours, cultures are split in two. 1 ml of 20% glucose is added to the first set and 1 ml of 20% galactose is added to the second set. Cultures are put to grow for 2.5 hours at 30 °C. Cultures are centrifuged at 4000 rpm for 5 minutes, supernatant is discarded, pellet is used for lysis.

3.1.9.2. Cells' lysis

Tubes with frozen pellet are thawed on ice. Cells were resuspended in 200 μ l of Urea lysis buffer. Glass beads are added to the 1.5 ml tubes, Urea lysis buffer with cells is added on top of the glass beads (MP Biomedicals). Samples are put into FastPrep-24 disruptor (MB Biomedicals) for 40 seconds 4 meters/second. Samples are then centrifuged at 13200 rpm for 10 minutes and supernatant is transferred to the 1.5 ml tubes.

3.1.9.3. Western blot

Protein concentration of lysates was measured, and samples were diluted according to the concentrations in order to be equally loaded on the 10% acrylamide gel. 3 μ L of 6xSDS buffer were added to the 12 μ L of samples and heated at 72 °C for 5 minutes. Samples were loaded on the 10% acrylamide gel. First well of the gel were loaded with 2 μ L of PageRuler™ Prestained Protein Ladder with 3 μ L of 6xSDS buffer. SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) ran for 1.5 hour with constant current of 15mA per gel. After this, gel, filter paper and nitrocellulose membrane (Amersham, Germany) were soaked in the semi-dry buffer. Gel, 4 pieces of filter paper and nitrocellulose membrane were assembled in the following order from bottom to top: 2 pieces of filter paper, membrane, gel, 2 pieces of filter paper. Excess of liquid was removed. Transfer was performed in the standard semi-dry transfer program in Pierce G2 Fast Blotter (Thermo Scientific). In one hour, "sandwich" was disassembled, membrane put into a container, Ponceau S solution (Sigma) added in order to visualize protein

bands on the membrane. Dye was poured away. Membrane was washed with 1x T-BST in order to remove red dye. 5% milk solution in 1xT-BST was added to the membrane. Left for blocking at 4 °C on the rocking platform overnight. The next day milk solution was removed, membrane washed once with 1xT-BST. 5000 times diluted primary antibody (IgG from rabbit serum (Sigma)) in 3% milk solution were added to the membrane and incubated on the rocking platform for 1 hour at room temperature. In 1 hour membrane was washed 1 time for 15 minutes and 3 times for 5 minutes with 1x T-BST. 7500 times diluted secondary antibody (anti-rabbit IgG) in 3% milk solution were added to the container with membrane and incubated on the rocking platform for 30 minutes at room temperature. Membrane was washed 1 time for 15 minutes and 3 times for 5 minutes with 1x T-BST, 1x T-BST discarded. SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) solutions were mixed in proportion 1:1, membrane was covered with substrate and wrapped in cellophane. Membrane in cellophane was exposed to autoradiography film (AGFA Medical Xray film blue, Belgium). Film was developed in G150 developer (AGFA) afterwards the film was washed with water and dipped in the G354 fixer (AGFA). After washing the film with distilled water and drying it, results were analyzed.

3.1.10. Time-lapse microscopy

Colonies were grown in 3 ml SC media and grown for 2-4 hours in 30 °C 160 rpm shaker. In 2-4 hours, upon reaching by cells exponential growth phase (OD600 0.2-0.4), tubes were vortexed to prevent clumping of the cells. 0.5 µl of culture was used for microscopy experiment. 0.5 µl of each culture were placed next to each other on the 24 x 50 mm glass plate and covered with individual 1.5% SC/glucose-agarose gel pads. Samples were additionally covered with 20 x 20 mm micro-glass and a plastic chamber to prevent drying out. Chamber with cells was placed under the Zeiss Observer Z1 microscope with AxioCam 506 mono-camera and 63x/1.4NA objective. 3 positions on each sample were chosen for observation and fixed using ZEN software. These positions were imaged every 3 minutes for 181 cycles, during imaging constant temperature of 30 °C was kept. Reporter proteins mCherry and EGFP were excited during experiment with wavelengths 590 nm and 490 nm accordingly. Upon completion of experiment, results were analyzed using MATLAB software. Image was segmented and data quantified using MATLAB code by (Doncic et al., 2013). Cells were synchronized by 50% Whi5-mCherry nuclear export time. The plots show mean GFP fluorescence values with error bars from a population of cells over the cell cycle time.

3.1.11. Tandem affinity purification (TAP)

Samples were firstly prepared in the Retsch MM400 disruptor machine. Firstly, half of the chamber was filled with metal ball, frozen cell pellet with lysis buffer. Chamber is cooled down in liquid nitrogen and placed in the disruptor machine. Machine is run 3 minutes with speed of 30 Hz and 1 minute off. Procedure is repeated for 3 times. Cell powder was extracted from the chamber, room temperature lysis buffer was added, and centrifuged for 10 minutes 10000 rpm. Supernatant was poured to the ultracentrifuge tubes and centrifuged for 45 minutes at 50000 rpm. Ultracentrifuge was cooled down to 4 °C prior to usage.

In 45 minutes 350-400 µl of IgG agarose beads suspension was poured to a column and washed with 10 ml of lysis buffer. Supernatant was poured to Falcon tube and mixed with washed beads. Falcon tube was rotated for 1 hour at 4 °C. In 1 hour beads were transferred to the column and eluted by gravity flow. Next, beads were washed 3 times with 10 ml of lysis buffer and once with 10 ml of TEV cleavage buffer, bottom of the column was closed. Cleavage was done by adding 1 ml of TEV cleavage buffer and 100 units of TEV protease (10 mg/ml 10-20 ul). Top of the column was closed. Beads were rotated for 90 minutes at 16 °C. In 90 minutes, elute was recovered by gravity flow and collected to the new Falcon tubes, snap frozen in liquid nitrogen and stored at -80 °C.

3.1.12. Coomassie staining

Samples purified by TAP purification were mixed with 6xSDS buffer and run on the SDS-PAGE for 1 hour at constant current of 15mA per gel. After separation of proteins in the polyacrylamide gel, it was placed into fixating solution for 1 hour on the shaker. The solution was then removed and gel was covered with Blue silver micellar solution. Gel was left on the shaker for 1 hour and then washed with distilled water several times until band became clearly visible.

3.2. RESULTS AND DISCUSSION

It was shown that NLxxxL motif mutation has no effect in full-length Far1. In order to find the explanation of this, several constructs described below were constructed. Far1 degradation rates were then tracked under the microscope. To track Far1 degradation timing, Far1 was tagged with EGFP sequence in the C-terminus. Sic1 promoter was inserted in the N-terminal end of the protein. Time-lapse microscopy experiments were conducted to identify the time of Far1 degradation. pSic1 – FAR1 – EGFP construct was integrated into the pRS306 vector and transformed into the RV298 yeast strain. The strain contained the Whi5-mCherry construct in the genome. Whi5 is a transcriptional repressor that inhibits the Start of the cell cycle in late G1. The presence of mCherry signal allowed us to track Whi5 localization. It was important in the context of identifying time passed from the beginning of the cell cycle as Whi5 is exported from the nucleus when phosphorylated by Cdk1 thus 50% drop in Whi5 levels indicate the Start point of cell cycle (Doncic, Falleur-Fettig and Skotheim, 2011). After identification of the Start of cell cycle, EGFP signal was tracked for 90 minutes.

3.2.1. Far1 region 200 – 393 is not responsible for degradation

In order to identify the region responsible for Far1 degradation, several truncations were introduced in the protein. Full-length Far1 is 830 amino acids long, however, it was shown that it is possible to shorten its sequence while maintaining the functions necessary for cell cycle arrest. Minimal functioning version of Far1 is 393 amino acids long (Gartner *et al.*, 1998). Therefore, with the first truncation the protein was shortened to its minimal version. Next two truncations removed docking sites for cyclins Clb5 and Cln2. These truncations were made at residues 350, 329. Three plasmids containing truncated Far1 proteins were obtained. The backbone plasmid contained functional URA3 locus. Plasmid with pSic1-Far1-EGFP was linearized in URA3 gene and integrated into the URA3 locus in the RV298 yeast strain genome. Time-lapse microscopy results for these three truncations showed similar rates in Far1 degradation (Figure 7).

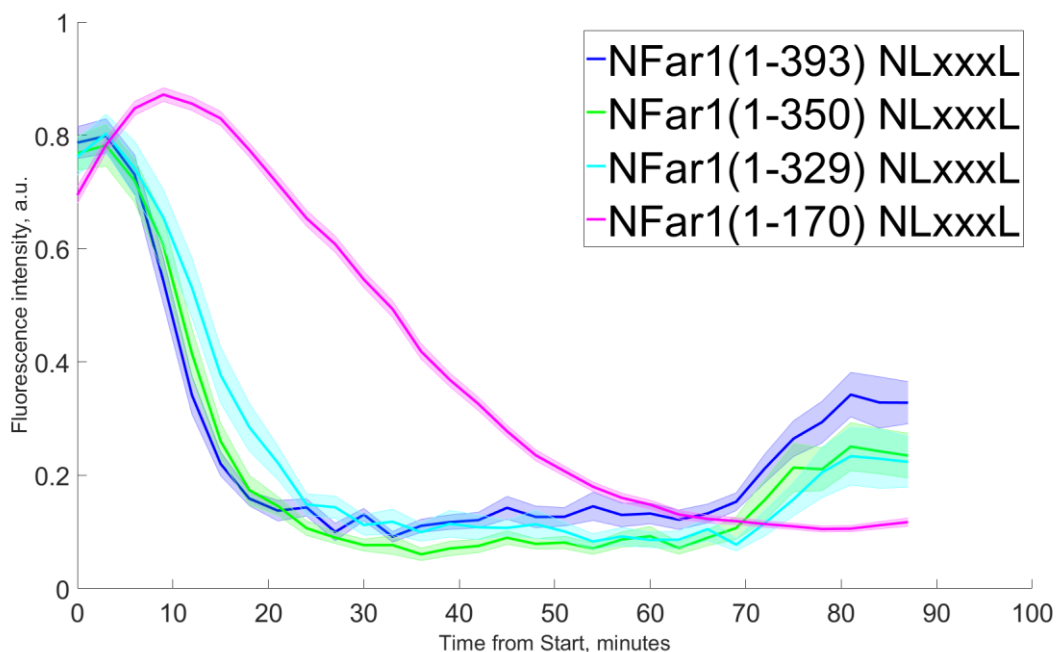


Figure 7. Mean fluorescence intensities of the Far1 - GFP mutants over the cell cycle. Constructs NFar1(1-393), NFar1(1-350) and NFar1(1-329) were analyzed. All constructs contained mutated NLxxxL motif.

Next three truncations were made at positions 300, 250 and 200. Plasmids were constructed and integrated into the RV298 strain in the same way as previous truncations. Strains with truncated Far1 were checked under the microscope. Time-lapse microscopy revealed that no site affecting degradation of Far1 protein is present after the 200th amino acid (Figure 8). As previously it was shown that NLxxxL motif takes place in Far1 degradation only if Far1 is truncated to 170 amino acids and our experiments identified that truncations up to 200 amino acids do not cause any effect on degradation, the next intention was to truncate Far1 further in the region 170-200.

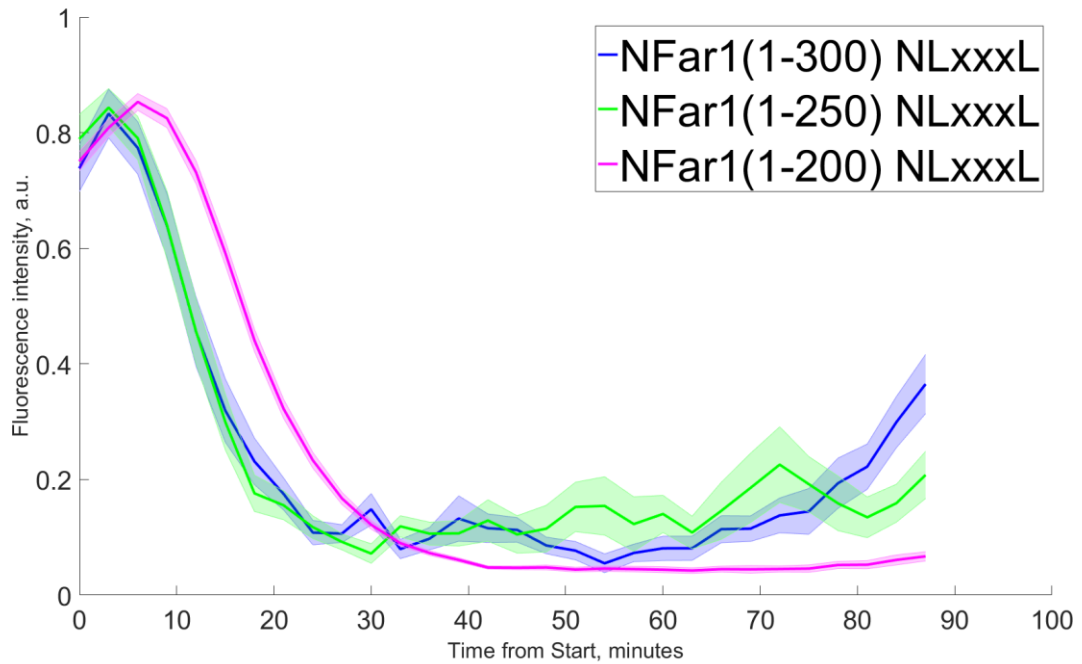


Figure 8. Mean fluorescence intensities of the Far1 - GFP mutants over the cell cycle. Constructs NFar1(1-300), NFar1(1-250) and NFar1(1-200) were analyzed. All constructs contained mutated NLxxxL motif.

3.2.2. Far1 region 170 – 200 might contain motif responsible for degradation.

In the region 170 – 200 Far1 contains a conserved KR region at residues 195 and 196 and a consensus SP motif at residues 174, 175 (Figure 9). Conserved regions are maintained by natural selection among species and often play a role in the regulation of the protein. SP motif is important because it is a sub-optimal site that can be phosphorylated by Cdk.

offset	↓175	180	190	↓200
≡ FAR1 - <i>Saccharomyces cerevisiae</i> ...	Y Y E E D S P T F M I S L E R N I K K C N S Q F S F K R Y I G E K C			
≡ KAFR0F01840 - <i>Kazachstania</i> ...	Y L E E S S P T F L A T V N R N M K K Y N S N H G F K K F V G E R C			
≡ TDEL0C03630 - <i>Torulaspota d...</i>	Y L E E D S P T Y L R S I S Q N I S K V N A K A G F R R F I G T Q C			
≡ TBLA0A06240 - <i>Tetrapisispora</i> ...	F I L E H S K K R s s M H S R N T S T - F S S S G L R K F V T E Q C			
≡ CAGL0I06138g - <i>Candida glabr...</i>	L S L D T V P S Q R E S L L S G - - - S T c s l K K Y N N Q K C			
≡ KLLA0_B07469g - <i>Kluyveromy...</i>	Y D E V S S P T G M M Q M Q R N - - - I - R K a a K R Y N T C T C			
≡ FAR1 - <i>Candida albicans</i>	- - - - - - - - - t - K L E S - - - I L P F p e K K Y N N I K C			

Figure 9. Alignment of Far1(169–202 aa) with homologs from other yeast species. Far1 segment 195-196 (marked with yellow frame) is conserved among species. At positions S174 and P175 consensus motif is situated (marked with red frame). Alignment is taken from <http://slim.icr.ac.uk/proviz>.

Therefore, the truncation at residue 190 was made in order to check the effect of the region on stability of the protein. Another truncation was made at residue 180. Thus every 10 amino acids were truncated in 170-200 region of Far1. The microscopy results revealed that NFar1(1-180 aa) degradation rate has a delay in comparison to NFar1(1-200 aa), while NFar1(1-190 aa) is degraded in the same way as NFar1(1-200 aa) (Figure 10). Thus, we can conclude that KR conserved region is not involved in Far1 degradation.

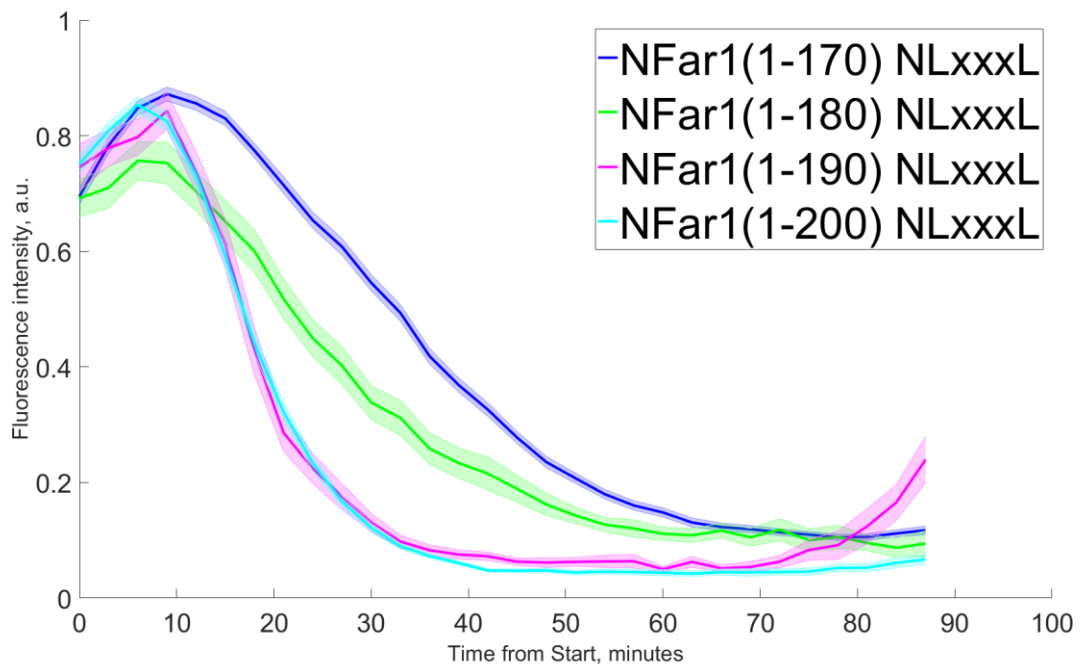


Figure 10. Mean fluorescence intensities of the Far1 - GFP mutants over the cell cycle. Constructs NFar1(1-170), NFar1(1-180), NFar1(1-190) and NFar1(1-200) were analyzed. All constructs contained mutated NLxxxL motif.

We have also made the S174A mutation in order to check the effect of the SP consensus motif on degradation. It was seen from the microscopy results that mutation in the motif causes a delay in degradation (Figure 11). This may indicate that the S174P175 motif is involved in the phosphorylation of the S87/S91 di-phosphodegron. It is possible that along with N-terminal consensus motifs situated at residues T3, T15, S26, T43, T63, Cdk1 also phosphorylates S174 site prior to phosphorylation of S91 degron in NFar1(1-200 aa). The possible interaction between above mentioned sites and S174 site must be tested later. The effect of S174A mutation and NFar1(1-180 aa) truncation with NLxxxL mutated, however, is not as significant as the effect in NFar1(1-170 aa) NLxxxL mutation. Therefore, region between 170th and 190th amino acids should be investigated further.

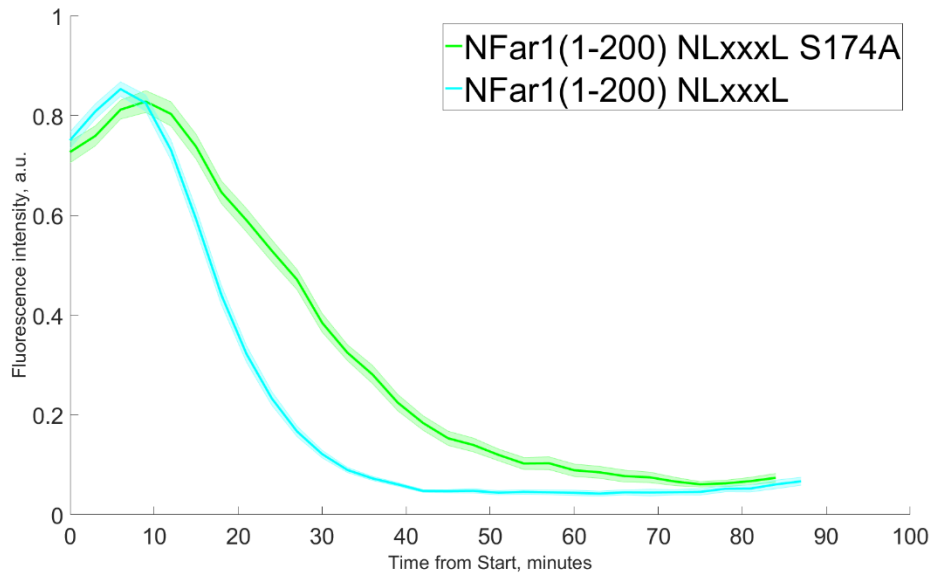


Figure 11. Mean fluorescence intensities of the Far1 - GFP mutants over the cell cycle. Constructs NFar1(1-200) and NFar1(1-200) S174A were analyzed. All constructs contained mutated NLxxxL motif.

3.2.3. NFar1(1-200) does not contain alternative degron besides S87/S91

It was important to check the presence of an alternative to the S87/S91 degron. It might have been possible that another site in the sequence of the protein is being phosphorylated. Separate degron could have been independent of NLxxxL motif. In order to check such possibility, di-phosphodegron was mutated in NFar1(1-200). Protein already contained the mutations in NLxxxL motif. Therefore, if an alternative degron independent of the motif was present in NFar1(1-200), the protein would be degraded regardless of the mutation in the known di-phosphodegron. However, microscopy revealed that mutations in S87/S91 sites cause the protein stability, which indicates that there is no alternative degron present in the protein (Figure 12).

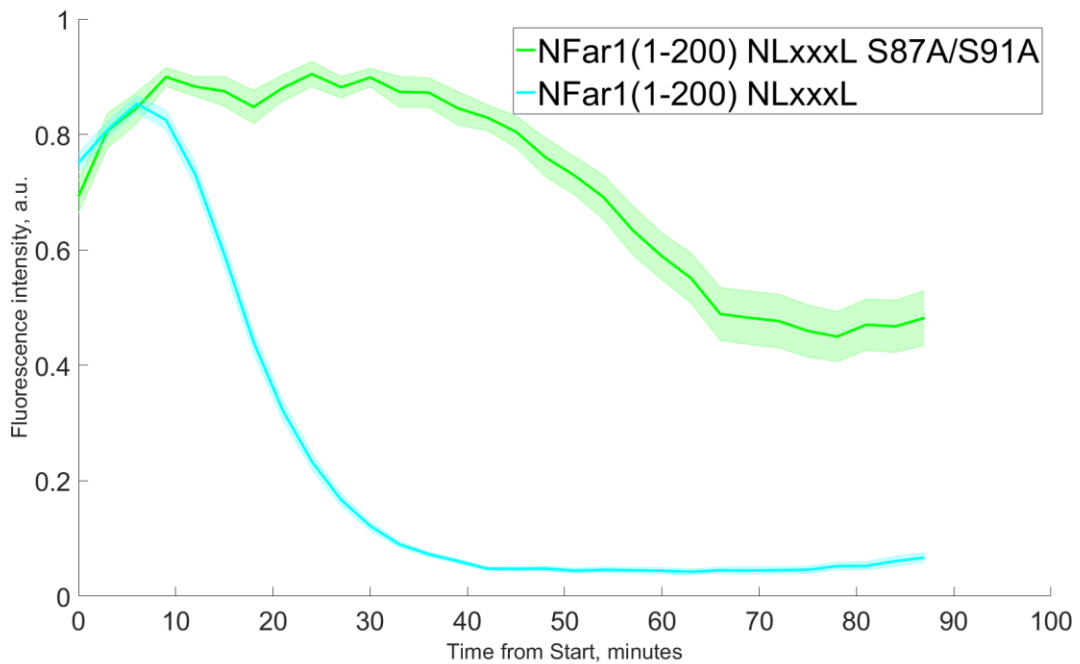


Figure 12. Mean fluorescence intensities of the Far1(1–200) - GFP mutants over the cell cycle. Far1(1–200) contains NLxxxL mutation. The graph indicates that mutation of the di-phosphodegron causes stability of the protein.

3.2.4. Pho85 kinase is not involved in the degradation of Far1.

Pho85 cyclin-dependent kinase is found in yeast *Saccharomyces cerevisiae* and is responsible for several processes during cell cycle depending on the cyclin it is activated by. Deletion of Pho85 results in, among other defects, slow growth with a delay in G1 phase (Timblin, Tatchell and Bergman, 1996). Pho85 is also related to the degradation of one of the CKIs – Sic1, an inhibitor of Clb-Cdk1 kinases, that must be phosphorylated and degraded in G1 for the cell to initiate DNA replication. Pcl1-Pho85 complex phosphorylates Sic1 and triggers its degradation. Here we checked the assumption that Pho85 might also be involved in the degradation of Far1.

It is possible to check if Pho85 phosphorylates Far1 with kinase assay. To conduct the experiment, TAP-tagged Pho85 was used. TAP (Tandem Affinity Purification) tag library allows the purification of the proteins. TAP tag consists of a calmodulin binding peptide, a TEV cleavage site, two IgG binding domains of *Staphylococcus aureus* protein A, and a selectable marker. Pho85 kinase tagged with TAP-tag is available as a part of the TAP-library, that allows purification of the proteins (Ghaemmaghami *et al.*, 2003).

As the expression of Pho85 was rather low, Gal1 promoter was added upstream of the TAP-tagged PHO85 gene. pGal1 was chosen, because it is a strong inducible promoter that has high

expression when induced with galactose. The promoter presence in the strain was checked initially with colony PCR reaction. Positive colonies were used for western blot. Total concentration of the proteins in the samples was measured prior to loading the gel in order to achieve equal total protein concentration from all samples in the gel. The expression of proteins with induced (with galactose) and uninduced (with glucose) pGal1 were checked. The results showed that the expression of pGal1 – Pho85 complex induced with galactose has higher expression than the uninduced one (Figure13). Colony number 2 from western blot was used for TAP protein purification. Purification was then validated with Coomassie staining. However, there were no bands on the gel, suggesting that purification either did not work or the concentration of the protein was too small to see the bands. As a next step, kinase assay was performed. It, however, did not show positive results.

As western blot results showed mild expression of the protein induced with galactose and purification did not work, the next step in attempting to purify Pho85 kinase would be to integrate it into 2-micron yeast vector. These vectors are present in the cells in around 100 copies that may help to increase the expression of the protein for successful purification (Murray, 1987).

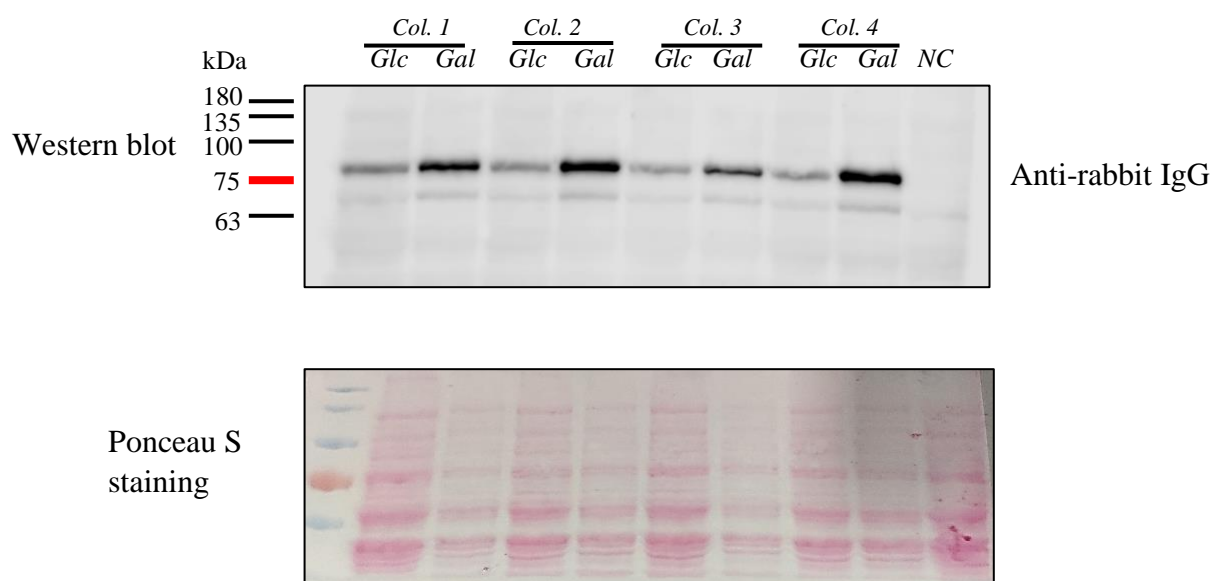


Figure 13. Western blot results. Figure on the top depicts the TAP-tagged Pho85 protein with uninduced (Glc) and induced (Gal) promoter. The expression of protein with induced promoter is higher than the one with uninduced promoter. Negative control (NC) does not contain TAP-tagged Pho85 protein. Image on the bottom shows staining with Ponceau S solution. Protein concentration in each pair is equal.

As an alternative method to check the influence of Pho85 on Far1 degradation, microscopy experiment was conducted. Pho85 was deleted from the yeast genome. Degradation of Far1 was compared in the strains with and without Pho85 kinase. Four constructs were analyzed in the experiment. Wild type NFar1(1-170), NLxxxL mutant NFar1(1-170), NLxxxL mutant Far1(1-200) and NLxxxL mutant NFar1(1-200) with additional S174A mutation. Microscopy experiments, however, showed that Pho85 does not change the degradation rates of Far1 (Figure 14).

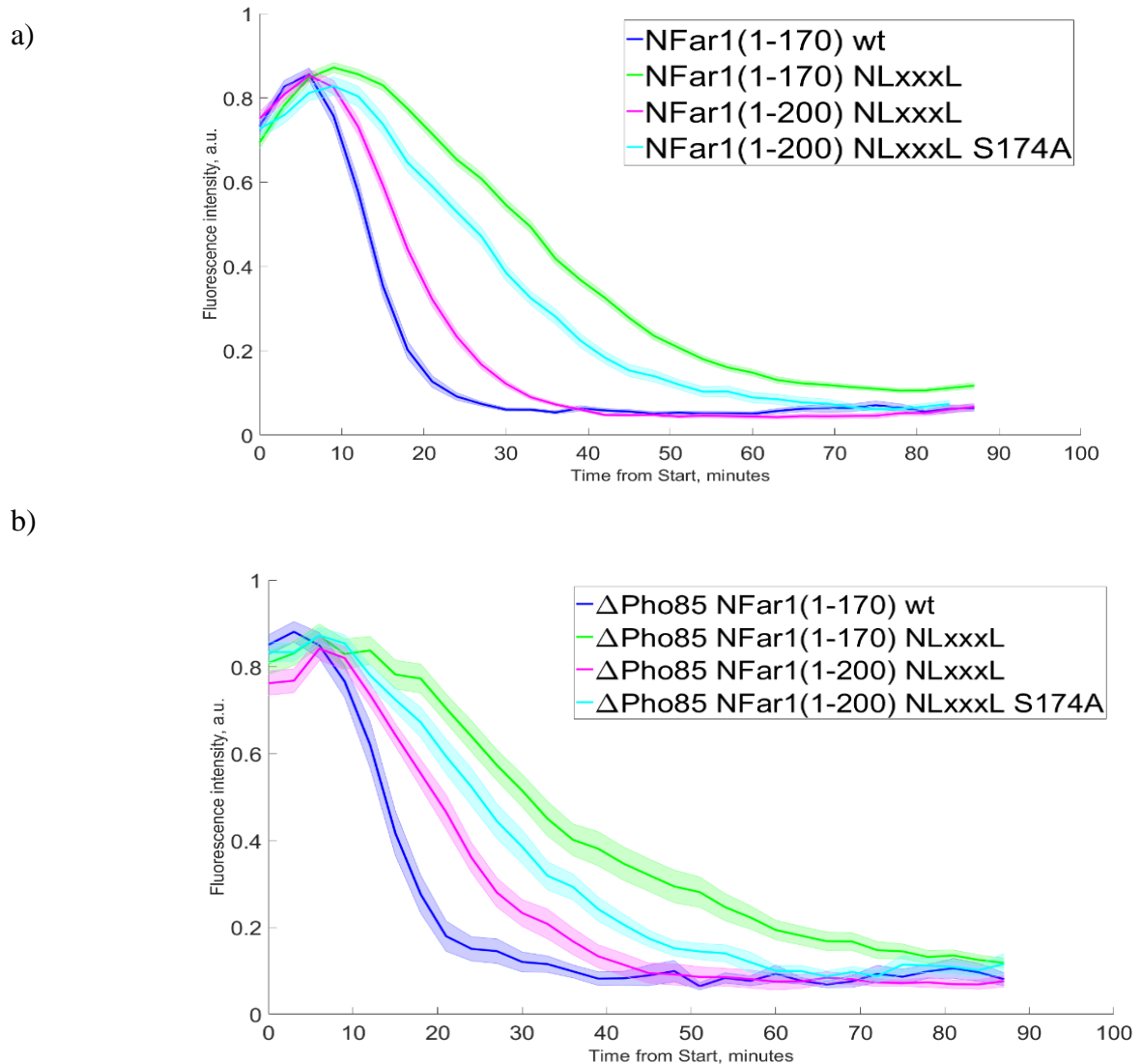


Figure 14. Mean fluorescence intensities of the Far1 - GFP mutants over the cell cycle. 4 constructs were analyzed: NFar1(1-170), NFar1(1-170) NLxxxL mutation, NFar1(1-200) NLxxxL mutation, NFar1(1-200) NLxxxL and s174a mutations. Half-lives of the proteins in both graphs were compared. a) Figure shows Far1 degradation rates in the strain with PHO85 gene. b) Figure shows Far1 degradation rates in the strain with deletion of PHO85 gene.

SUMMARY

Cdks direct the cell cycle events in complex with different cyclins. There are specific cyclins in each phase of the cell cycle. They bind the substrate proteins, phosphorylate them and change their activity. However, if certain conditions are present the Cdk-cyclin complex itself must be inhibited in order to arrest the cell cycle. CKIs, proteins that cause cell cycle arrest, are activated if cell encounters unfavorable conditions or if, for example, mating pheromones signals have occurred. A protein that serves as CKI if mating pathway is activated is Far1. It inhibits Cdk1-cyclin complex thus triggering cell cycle arrest. However, if there is no need for the cell cycle arrest, Far1 gets degraded. Far1 is phosphorylated at residues S87/S91 by Clb5-Cdk1 complex in order to be degraded. Far1 also contains SLiMs in its N-terminal disordered region. SLiMs serve as docking motifs for kinases, as in order to phosphorylate the substrate, Cdks must bind it first. An NLxxxL SLiM for Clb5-Cdk1 complex in Far1(1-170 aa) N-terminal region has been recently discovered. This motif, however, loses its function in the longer version of Far1.

The attempt to identify the region that ousts the NLxxxL effect in longer versions of Far1 has been taken in this work. The minimal NFar1(1-393 aa) has been truncated several times in order to narrow down the region responsible for degradation. All analysed constructs contained NLxxxL mutation. The truncations up to 200 amino acids did not show the effect on Far1 degradation. Therefore, the region was narrowed down to 30 amino acids at positions 170-200.

Looking deeper into this region, the possibility that another degron besides S87/S91 is situated there was checked. However, it was also proven, that no alternative degron is present in the region. Another assumption considered Pho85 kinase, which might be responsible for degradation of the protein. This particular kinase was chosen as it is involved in the degradation of another CKI – Sic1. However, this assumption was proven to be wrong. Truncations in the region 170-200 aa revealed that region 190-200 aa is not involved in degradation, while truncation up to 180 aa and S174A mutation causes a delay in degradation. This delay is, however, smaller than delay caused by mutation of NLxxxL motif in NFar1(1-170). Thus, there is a possibility that region that would cause similar effect to the one in NFar1(1-170) is situated in the region 170-190 aa.

All in all, further mapping of region 170-190 is necessary to identify the exact motif involved in the degradation of Far1.

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