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Description of the cyclin Clb5 docking motif in Far1

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Description of the cyclin Clb5 docking motif in Far1

Abstract:

Cell cycle is a timely ordered, multistep event during which a cell's chromosomal DNA is first replicated and later divided into two identical daughter cells. Cyclin-dependent kinases (Cdks) regulate the events of the cell cycle through phosphorylation of different substrates. Cdks are activated by binding to an activating partner – a cyclin. Cyclins are also substrate specific, i.e. they bind to a substrate and promote its phosphorylation by the Cdk complex. In this work, we mapped a newly discovered Clb5 cyclin docking motif present in the N – terminal region of a Cdk inhibitor protein, Far1. We found that mutations in the motif drastically delay phosphorylation-dependent degradation rates of Far1. In addition to this, we showed that Far1 docking region functions as an independent linear docking motif. Interestingly, our data also suggests that the motif does not bind to the hydrophobic patch of Clb5, suggesting that there might be some other, not yet discovered, binding pocket on the cyclin.

Keywords:

Cell cycle, Phosphorylation, Cyclin-dependent kinase, Cyclin, Kinase specificity

CERCS: B230 Microbiology, bacteriology, virology, mycology

Far1 valgus paikneva tsükliini Clb5 seondumismotiivi kirjeldamine

Lühikokkuvõte:

Rakutsükkel on mitmeosaline järjestikkune protsess, mille käigus raku DNA esmalt paljundatakse ja seejärel jagatakse kahe tütarraku vahel. Tsükliinist sõltuvad kinaasid (Cdk) reguleerivad rakutsükli sündmusi erinevate substraatide fosforüleerimise kaudu. Cdk on aktiivne ainult kompleksis tsükliiniga, kusjuures tsükliinid suunavad kinaasi kindlaid valke fosforüleerima. Käesolevas töös leiti, et Cdk inhibiitorvalgu Far1 N-terminaalses alas paikneb tsükliini Clb5 seondumismotiiv. Selle motiivi muteerimine põhjustab suure hilinemise Far1 fosforüleerimisest sõltuvas lagundamises rakutsükli käigus. Lisaks näidati, et Far1 seondumismotiiv toimib iseseisva lineaarse motiivina, mis toimib tsükliini Clb5 hüdrofoobsest taskust sõltumatult, vihjates, et tsükliini pinnal on teisigi substraatide sidumistaskuid.

Võtmesõnad:

rakutsükkel, fosforüleerimine, tsükliinist sõltuv kinaas, tsükliin, kinaasi spetsiifika

CERCS: B230 Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

APC - Anaphase promoting complex

Cdk – Cyclin-dependent kinase

CKI - Cyclin-dependent kinase inhibitor

CSM - Complete supplement mixture

SCF - Skp, Cullin, F-box containing complex

EDTA - Ethylenediaminetetraacetic acid

eGFP – Eukaryotic green fluorescent protein

LLPP – Short linear Cln2-dependent motif

MAPK - Mitogen-activated protein kinase

OD – Optical density

P_{SIC1} – Sic1 promoter

RXL - Short linear Clb5-dependent motif with consensus sequence of K/R-x-L- φ or K/R-x-L-x-φ

TAE buffer - Tris-acetate-EDTA buffer

TE buffer - Tris-EDTA buffer

YPD – Yeast extract peptone dextrose

INTRODUCTION

Cell cycle is a highly regulated, timely ordered set of cellular events during which the chromosomal DNA is first duplicated and later divided into two identical daughter cells. The cycle proceeds in four different phases: G1, S, G2, and M phase. The cell cycle control system regulates the events of the cell cycle and ensures correct timing of different phases, i.e. makes sure that the previous phase has finished before the beginning of the next phase. A central part of the cell cycle control system is the cyclin-dependent kinase (Cdk). The main role of Cdks is to phosphorylate target substrates, thereby, either promoting their activation or inactivation. During the cell cycle, the budding yeast Cdk1 is estimated to phosphorylate about 600 proteins. This leads to a question that how timely phosphorylation of hundreds of proteins is achieved.

In order for a Cdk protein to be catalytically active, it has to bind to its activating partner protein, a cyclin. In *S. cerevisiae* there are nine different cyclins that are expressed during different phases of the cell cycle, resulting in a formation of different cyclin – Cdk1 complexes. In addition to activating Cdks, cyclins are also substrate specific, meaning they bind to a specific substrate, thereby, promoting its phosphorylation by a particular cyclin – Cdk1 complex. Some proteins, such as Sic1, contain a short linear motif (RXL) that docks to Clb5 (S phase cyclin) by binding to its hydrophobic patch. This docking, in turn, enhances phosphorylation of Sic1 by the Clb5 – Cdk1 complex. Alongside cyclins, a phosphoadaptor protein Cks1 binds to the Cdk1 protein and promotes multisite phosphorylation of substrates.

Through truncation experiments it has previously been shown that a Cdk inhibitor protein, Far1, has a Clb5-specific docking motif in the N-terminal region. In this work, we plan to map the Clb5-dependent motif in Far1 and gain an understanding of how mutations in this region might affect the phosphorylation and degradation rates of the protein. We also hope to be able to find the functional length of the motif and test its modularity by introducing it into a different protein. Lastly, we wish to examine the importance of Cks1-mediated multisite phosphorylation in degradation of Far1 1 – 150.

1 LITERATURE REVIEW

1.1 Cell cycle overview

A cell is the smallest self-functional and structural unit of an organism. In order for a new cell to emerge it has to develop from an already existing living cell. This process of cellular reproduction serves as an essential part of the functional and developmental regulatory system in all living organisms. In a eukaryotic cell, the process of cell division is commonly referred to as cell cycle. The cell cycle is a tightly controlled, multi-step process during which chromosomes and other cellular components are first, duplicated, and later segregated to two daughter cells. The cell cycle proceeds in four different phases that are not only highly regulated but also timely triggered. The four main phases are G1, S, G2, and M phase **(Figure 1)**.

The two gap phases, known as G1 and G2 phase, separate the S and M phase and serve as important transition points that control the progression of the upcoming stages of the cell cycle. During G1, the cell acquires more biomass and prepares for DNA replication, while during G2 the cell organises a newly replicated genome and prepares for its segregation. In addition, the G1 phase serves as an important regulatory phase during which a cell chooses to either commit to the progression of the cell cycle or initiate its exit from the same. The decision to exit the cycle depends on the presence of unfavourable signals, arising either from the environment or neighbouring cells, which cause the cell to enter a prolonged non-dividing state, known as the G0 phase (Morgan, 2007).

On the other hand, the first major phase of the cell cycle is the S phase. During this phase, the cell's DNA is replicated and the duplicated pair of chromosomes that arises after the replication is called the sister chromatids. The sister chromatids will later be equally divided into the new set of daughter nuclei during the second major phase of the cell cycle, known as the M phase. This phase is itself divided into two sub-phases, the first of which is called mitosis or the nuclear division phase, and the second called cytokinesis or the actual cell division phase. The progression through different phases of the cell cycle is tightly governed by the cell's in-built system known as the cell cycle control system (Morgan, 2007).

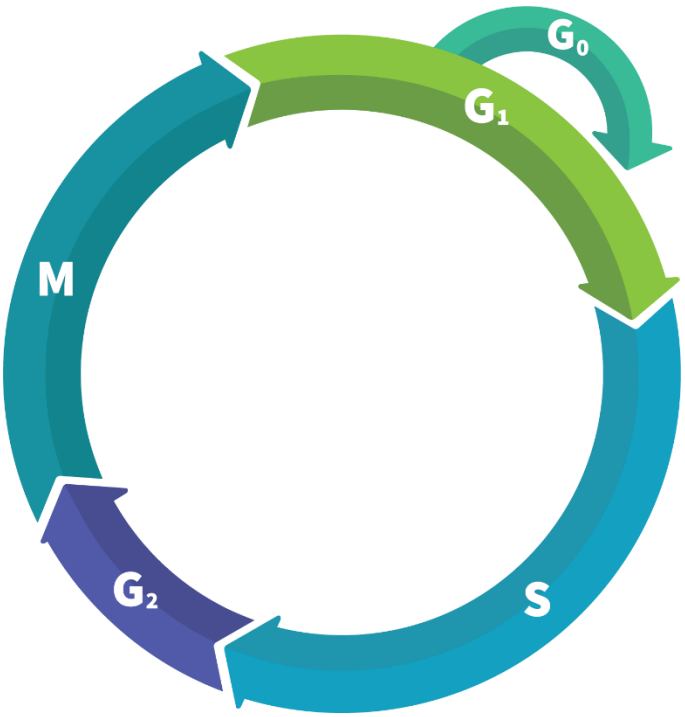

Figure 1. Cell cycle overview. The cell cycle is a multistep process taking place in an ordered manner. The four main phases of the cell cycle are G1, S, G2, and M phase. Due to unfavorable conditions a cell may enter a prolonged non-dividing state, G0. During G1 a cell acquires more biomass and prepares for the start of the cycle; S phase is when the chromosomes are duplicated into two identical copies; during G2 a cell prepares for the mitosis; M phase is when a cell divides into two identical cells (Morgan, 2007).

1.2 Cell cycle control system

The main role of the cell cycle control system is to allow a smooth progression of the cell cycle whilst ensuring that the events of the previous phases have taken place before the following phase. The control system timely triggers a series of biochemical switches that enable a cell to go through three major checkpoints in the cycle. The central components of the control system are proteins called cyclin-dependent kinases (Cdks). Due to their ability to transfer a phosphate group from an ATP molecule to a substrate protein (phosphorylation), the Cdks can alter the activity of cell cycle driving proteins, thereby, initiating new events of the cell cycle (Morgan, 2007). Cdks regulate the start of the cell cycle, the initiation of DNA replication, the transition between the G2 and M phase as well as the initiation of various mitotic events (Enserink and Kolodner, 2010).

However, in order for a Cdk protein to become catalytically active, it has to form a complex with its activating partner – a cyclin (Pines, 1995). Many different cyclins are expressed during different stages of cell cycle. This results in a formation of different cyclin-Cdk complexes which interact with each other in a way that the activation of the upcoming cyclin-Cdk complex is promoted by the currently functioning one. These interactions ensure an ordered unfolding of the cell cycle (Morgan, 2007).

Overall, the cell cycle control system is a highly adoptable and robust system which employs different feedback loops and regulatory networks in order to ensure that every phase of the cell cycle is executed in a timely and ordered manner (Morgan, 2007). The system also appears to be very well evolutionary conserved, in a sense that a human Cdk1 can be expressed in place of Cdk1 in *S. cerevisiae*, while still successfully proceeding with the cell cycle events (Wittenberg and Reed, 1989).

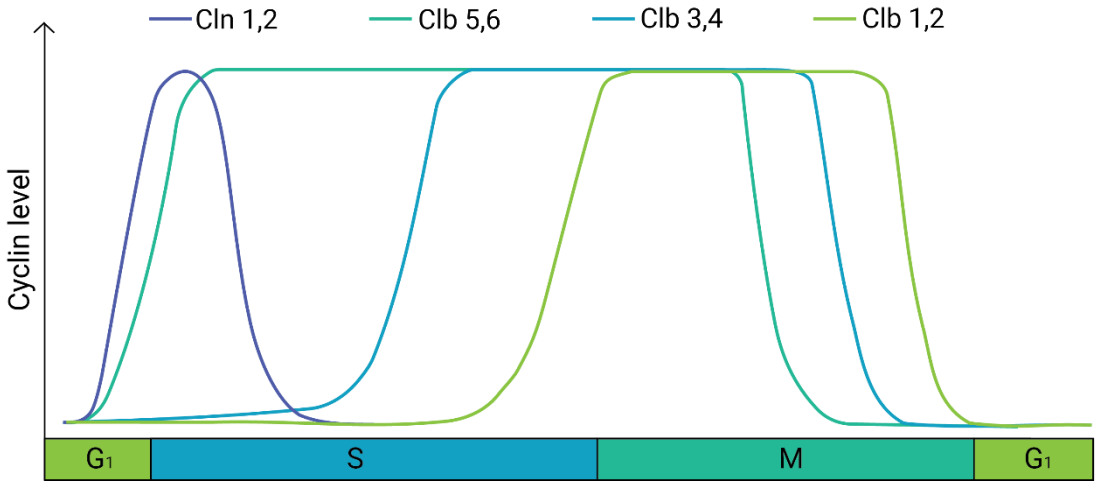

Figure 2. Expression of cyclins during the cell cycle of *S. cerevisiae*. Different cyclins are expressed at different phases of the cell cycle. Cln1, 2 are G1/S cyclins whose rise is accompanied by the rise of S-cyclins Clb5, 6 which promote DNA replication in S phase and stay active until anaphase. Late S phase cyclins Clb3, 4 support the cell's transition to M phase and M phase cyclins, Clb1, 2 initiate important mitotic events (Morgan, 2007). (The figure was adopted and modified from Pihelgas 2013).

1.3 Cyclin-Dependent Kinase (Cdk) Protein and Cyclins

The activity of the Cdk proteins is best described in a model organism *S. cerevisiae*, where only a single Cdk (Cdk1) protein is necessary to regulate the cell cycle. Cdk1 is a proline-directed serine/threonine protein kinase which mainly phosphorylates the full consensus phosphorylation sequence T/S-P-x-K/R, where T/S stands for threonine or serine, P for proline, x for any amino acid, and K/R for positively charged amino acids lysine and arginine,

respectively (Songyang et al., 1994). Nonetheless, Cdk1 can also phosphorylate the minimal consensus S/T-P sequence, though with a lot lower efficiency (Kõivomägi et al., 2011). Interestingly, the recent studies have found that Cdk1 can phosphorylate non-proline sites with a lot lower efficiency compared to the minimal consensus S/T-P sequence, though the efficiency can further be enhanced in presence of lysine and arginine residues (Kõivomägi et al., 2011; Suzuki et al., 2015).

During the cell cycle, Cdk1 forms complexes with different cyclins. Cyclins are a group of proteins which bind to the Cdks and promote their catalytic activity. Different cyclins are expressed during different phases of the cell cycle with most notable ones being expressed during G1/S, S, and M phase **(Figure 2)** (Morgan, 2007). The formation of cyclin-Cdk1 complexes not only promotes the activation of the kinase but also enables the enlisting and more efficient binding of different substrates (Enserink and Kolodner, 2010). *S. cerevisiae* expresses nine different cyclins that activate and associate with the Cdk1 protein: three are known as G1 cyclins and the remaining six are known as B-type cyclins (Enserink and Kolodner, 2010).

The G1 cyclins, Cln1, Cln2, and Cln3, initiate the beginning of the cell cycle. The research has shown that only a triple *cln1Δ cln2Δ cln3Δ* mutant is inviable, further indicating that any of the three cyclins is capable of starting the cell cycle on its own (Richardson et al., 1989). However, despite their apparent similarities, the G1 cyclins are functionally very different. Cln3 acts as a transcriptional regulator and it is believed to function upstream of Cln1 and Cln2 and promote the transcription of *CLN1* and *CLN2* genes during the G1 phase (Marini and Reed, 1992; Tyers et al., 1993). Nonetheless, the cyclin-Cdk1 complexes are mainly inactivated during the G1 phase due to a low level of cyclins being expressed and an active presence of Far1 and Sic1 proteins that both act as Cdk1 inhibitors (CKIs) (Alberghina et al., 2004; Schwob et al., 1994).

Cln1 and Cln2, known as G1/S phase cyclins, are essential in the initiation of the bud morphogenesis and spindle pole body duplication (Enserink and Kolodner, 2010). They are also important in shutting down the inhibitory system which supresses the S phase Cdk1 activity during the G1 phase (Schwob et al., 1994). As levels of these cyclins start to rise in late G1 phase, the activity of the Cdk1 increases while CKIs proteins are phosphorylated by the cyclin-Cdk1 complex and directed for ubiquitin-mediated proteolysis (Mendenhall and Hodge, 1998). Subsequently, the activity of the Cdk1 keeps increasing until the beginning

of anaphase, where cyclins are degraded and CKIs are re-expressed (Amon et al., 1994; Schwob et al., 1994).

Similarly, the stability and therefore activity of G1 cyclins is regulated through post-translational modifications; Cln1, 2, 3 promote their own phosphorylation and are thereafter targeted for SCF-mediated destruction (Lanker et al., 1996; Tyers et al., 1992).

Clb1-6, the B-type cyclins, are expressed after the G1 cyclins, with the exception of Clb5 and Clb6 whose expression is already induced in late G1 phase (Enserink and Kolodner, 2010). Clb5, 6 are the main S phase cyclins and they are believed to first allow the cell to timely enter the S phase (Schwob et al., 1994), and later initiate DNA replication and prevent re-replication of a single origin, i.e. ensure that a single origin of replication is only replicated once per cell cycle (Dahmann et al., 1995).

In addition to CKIs, the activity of anaphase promoting complex (APC) starts to rise during the M phase. APC is a giant protein ligase which catalyses the ubiquitination of different proteins, most importantly securin and S and M cyclins (McLean et al., 2011). APC is activated by two different activator proteins during the cell cycle. First, APC^{Cdc20} complex is formed at the transition between metaphase and anaphase which allows APC to degrade securin. The destruction of securin releases a protease, called separase, which lyses the cohesion complex, thereby, allowing the sister chromatids to be taken to the opposite ends of the dividing cell (Morgan, 2007).

In the end of mitosis APC degrades S and M cyclins. For example, Clb5 and Clb6 are degraded at very different stages of the cell cycle. Clb5 contains the APC destruction box and lacks Cdc4 degrons which makes it stable until anaphase where it is sent for degradation by the APC complex; Clb6, on the other hand, has a Cdc4 degron and it is phosphorylated by Cdk1 and Pho85 complexes and sent for SCF-mediated destruction at the beginning of the S phase (Jackson et al., 2006). Clb3, 4 are late S phase cyclins which are expressed at the S/G2 border. They are responsible for the initiation of the spindle assembly and the mitotic entry as they stay active until anaphase where they get degraded (Mendenhall and Hodge, 1998; Richardson et al., 1992).

Clb1, 2 are the M phase cyclins that are expressed during the transition between the G2 and M phase and are later degraded by the APC complex at the end of M phase. (Mendenhall and Hodge, 1998; Seufert et al., 1995). They are involved in the initiation of mitotic events as well as the prolongation of bud morphogenesis (Lew and Reed, 1993). Interestingly, APC has to be phosphorylated by M-Cdk1 complex before it can be activated by Cdc20. As

APC^{Cdc20} complex degrades M cyclins, it will promote its own inactivation in a way that there will be fewer M-Cdk1 complexes to phosphorylate APC, thus, leading to decrease in phosphorylation of APC and dissociation of APC^{Cdc20} complex at the end of M phase (Morgan, 2007).

The sudden drop in the activity of Cdk1, at the end of M phase, is of huge importance for the continuation of the cell cycle, as it enables the cell to exit mitosis and start G1 phase with a low Cdk1 activity (Enserink and Kolodner, 2010). Additionally, in order to ensure low concentrations of S and M cyclins during G1, APC still needs to be active. The second activator subunit, Cdh1, is capable of binding un-phosphorylated APC and forming and active APC^{Cdh1} complex. The complex prevents a premature entry into the cell cycle, and as it is not specific for G1/S cyclins, it will stay active until late G1 where G1/S-Cdk1 complex will phosphorylate Cdh1, hence, inactivating the APC (Morgan, 2007).

Therefore, it can be inferred that throughout the cell cycle the activity of Cdk1 and APC oscillates in a negative feedback loop manner, i.e. when the activity of APC is high, the activity of Cdk1 is low (G1 phase), and vice versa, when the activity of Cdk1 is high the activity of APC is low (rest of the cell cycle) (Lin and Scott, 2012).

1.4 Substrate targeting by cyclin-Cdk1 complex

Cyclin-Cdk1 complexes play an essential role in ordered progression of the cell cycle, and Cdk1 alone is believed to have over 300 potential target substrates (Holt et al., 2010). A large variety of substrates is mainly due to the presence of cyclins which are believed to direct Cdk1 to specific substrates. This can be done either by direct binding to the substrate, or by taking Cdk1 to a part of the cell where the substrate is found. S cyclins, in particular, are known to interact with many different substrates predominantly involved in the early events of the cell cycle (Loog and Morgan, 2005; Schulman et al., 1998). Their substrate specificity depends on the presence of the region called hydrophobic patch. The patch, which is located on the surface of the cyclin protein, binds substrates that contain a complementary hydrophobic sequence, commonly referred to as the RXL motif **(Figure 3)** (Morgan, 2007). RXL is a short linear motif with consensus sequence of K/R-x-L- φ or K/R-x-L-x-φ, where K is lysine, R is arginine, x is any amino acid, L is leucine, and φ is a large hydrophobic amino acid (Lowe et al., 2002).

For example, in order to initiate S phase, Clb5-Cdk1 complex needs to phosphorylate Sic1 protein which is then directed for ubiquitin-mediated proteolysis (Schneider et al., 1996).

Clb5, being an S cyclin, contains a hydrophobic patch which binds the substrates containing the RXL motif. The research has also shown that mutations present in either the cyclin's hydrophobic patch or the RXL motif of a substrate, greatly reduce the rate of phosphorylation (Loog and Morgan, 2005; Wilmes et al., 2004).

On the other hand, a G1/S cyclin Cln2 specific LLPP motif increases the binding between the substrate and cyclin in Cln2-Cdk1 complex, and the research has found that an alanine mutant of the LLPP motif considerably decreases the Cln2-mediated phosphorylation of Sic1 (Kõivomägi et al., 2011). This was further supported in the example of Ste5 protein, where it was shown that the LLPP motif was needed in Cln2-mediated phosphorylation of Ste5, suggesting that LLPP motif could act as a Cln2 docking site (Bhaduri and Pryciak, 2011).

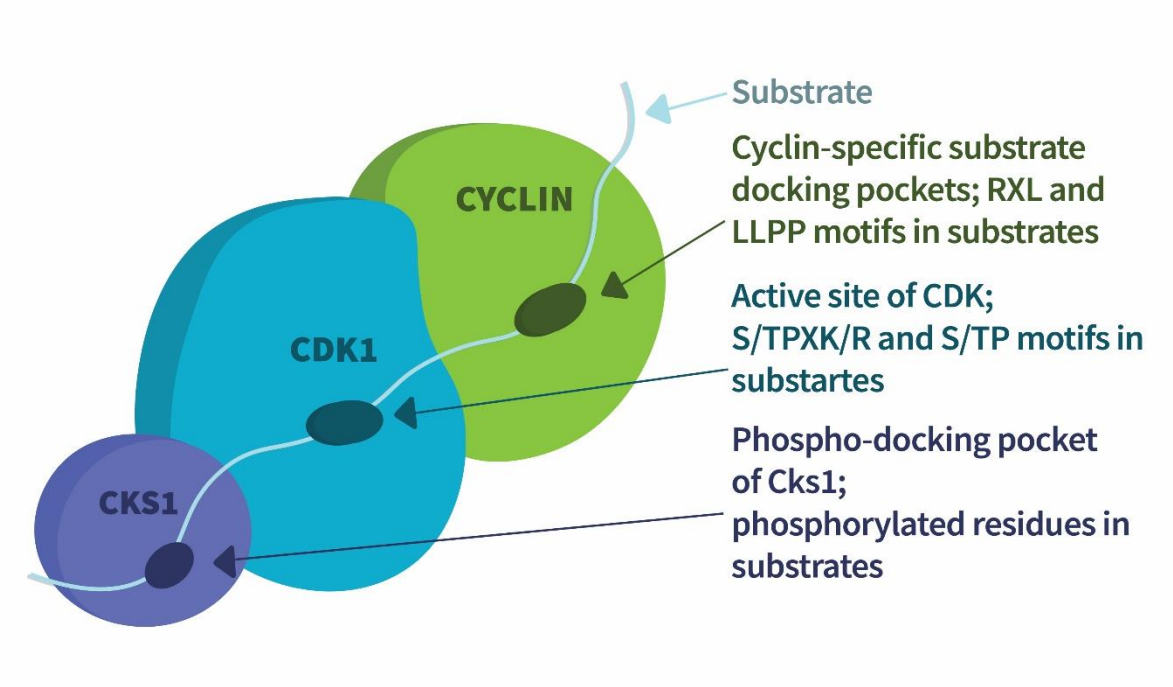

Figure 3. A schematic diagram of cyclin-Cdk1-Cks1 complex. Cdk (Cdk1 in *S. cerevisiae*) phosphorylates a substrate's full consensus phosphorylation sequence (T/S-P-x-K/R), or the minimal consensus sequence (S/T-P). Cyclins are Cdk's activators and enable substrate specific phosphorylation though binding the substrates with RXL and LLPP motifs. Cks1 is a phospho-adaptor which binds to a priming phospho-threonine site and promotes multisite phosphorylation of a substrate. (The figure was modified from Kõivomägi et al. 2013)

In addition to cyclins, another protein, Cks1, binds to the Cdk1 and acts as a phospho-adaptor subunit (**Figure 3**). The binding of Cks1 promotes the multisite phosphorylation of substrates. In *S. cerevisiae*, for example, the binding of Cks1 subunit is essential for the activity of Cn1, 2-Cdk1 complexes (Reynard et al., 2000). The research has shown that the multisite phosphorylation of Sic1 by Cdk1 is greatly reduced once the Cks1 is mutated. In addition to this, Cks1 seems to bind phospho-threonines but not phospho-serines and uses them as priming sites (Kõivomägi et al., 2013; McGrath et al., 2013). Once bound to a priming site, the Cks1 protein promotes the multisite phosphorylation of the substrate in N to C direction, with the optimal distance between the priming phosphorylation site and the secondary phosphorylation site being 12-16 amino acids (Kõivomägi et al., 2013).

1.5 Far1 protein

Eukaryotic cells constantly receive different signals from the environment which can significantly alter the cell cycle. In *S. cerevisiae,* the presence of mating pheromones can arrest the cells in G1 phase, effectively stopping the cell cycle (Bardwell, 2005). In presence of mating pheromone, a Cdk1 inhibitor protein, Far1, is believed to inactivate Cln-Cdk1 complexes during G1 phase. Far1 does this by outcompeting the substrates for association with G1/S cyclins due to its high affinity binding with the substrate binding site on G1 cyclins. Therefore, Far1 is a multi-mode inhibitor which inhibits the kinase activity as well as substrate recognition and phosphorylation by Cln1/2-Cdk1 complexes (Pope et al., 2014).

However, in the absence of mating pheromones the concentration of Far1 is a lot lower, and Far1 can be phosphorylated both in the nucleus and cytoplasm (Blondel et al., 2000) and sent for degradation. The research has shown that during the G1 phase of the cell cycle, Far1 is phosphorylated at S87 minimal consensus site by Cln2-Cdk1 complex. A mutant containing alanine mutation at serine 87 prevented the degradation of Far1 during the cell cycle (Gartner et al., 1998). Once phosphorylated, the nuclear Far1 is then ubiquitinated by SCF^{Cdc4} complex and degraded by proteasomes (Blondel et al., 2000).

Furthermore, it was shown that in a *clb5Δclb6Δ* mutant Far1 arrests cells for a lot longer period of time than in the wild type strain (Doncic and Skotheim, 2013) suggesting that Clb5 might play a role in the phosphorylation of Far1. This piece of evidence is further supported by the unpublished experimental data of E. Valk, where through truncation experiments it was discovered that there is a Clb5-dependent motif in 130-150 amino acid region of Far1. It is postulated that this motif binds the hydrophobic patch of Clb5, thereby, enhancing the phosphorylation of Far1 by Cdk1. Furthermore, in the unpublished data of I. Faustova it has

been shown that mutations in this region affect the function of Far1 during pheromone arrest and that this region might work as a short linear motif. This short linear motif might affect cyclin binding to substrate and can be further used to modulate substrate affinity towards Cdk-cyclin complex and therefore regulate phosphorylation rate. This research represents the basis of my work.

2 THE AIMS OF THE THESIS

It has been previously shown that the Clb5-specific RXL motifs are important in the S phase substrate targeting. Recently, however, it has been found that the N-terminal region of a Cdk inhibitor protein, Far1, is a highly Clb5-specific Cdk1 substrate. Nonetheless, as there is no RXL motif present in this region, there ought to be another, so far undescribed, Clb5-specific motif which mediates the docking interaction between Far1 and the cyclin. In this work, we aim to better understand the motif, its modularity, and its role in phosphorylation and degradation of Far1 protein.

The aims of this work are:

- Mapping of Clb5-specific motif in Far1
- Testing the modularity of the motif
- Importance of Cks1-mediated phosphorylation in Far1 degradation
- Evaluation of the importance of the cyclin hydrophobic patch in the docking interaction

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 A table of strains

Table 1. A list of strains used in this work. The list includes the strain's name, background strain, short description of a strain, and the source.

Strain	Background strain	Description	Source
RV298	w303	*MATa, bar1::hisG WHI5-mCherry-SpHIS5*	Rainis Venta
MO371	RV298	*clb5::clb5 hpm clb6::clb6 hpm*	Mihkel Örd
LB1	RV298	*ura3:: pRS306(pSIC1-Far1(1-150)-eGFP)*	This study
LB2	RV298	*ura3::pRS306(pSIC1- Far1 [1-150 LTTSAAA (L135A L136A R137A)]-eGFP)*	This study
LB3	RV298	*ura3::pRS306(pSIC1- Far1 [1-150 ATTSAAR (L131A L135A L136A)]-eGFP)*	This study
LB4	RV298	*ura3::pRS306(pSIC1-Far1 [1-150 LAAALLR (T132A T133A S134A)]-eGFP)*	This study
LB5	RV298	*ura3::pRS306(pSIC1- Far1 [1-150 degron mutant (S87A S91A)]- eGFP)*	This study
LB6	RV298	*ura3::pRS306(pSIC1- Far1 (1-150 RXL) -eGFP)*	This study
LB7	RV298	*ura3::pRS306(pSIC1- Far1 (1-150 AxA) -eGFP)*	This study
LB8	RV298	*ura3::pRS306(pSIC1- Far1 [1-150 (T3S, T15S, T43S, T63S)] -eGFP)*	This study
LB9	RV298	*ura3::pRS306(pSIC1- Far1 [1-150 (T3S, T15S, T43S, T63S, L135A, L136A, R137A)] -eGFP)*	This study
LB10	RV298	*ura3::pRS306(pSIC1- Far1 [1-150 (T3S, T15S, T43S, T63S, RXL)] -eGFP)*	This study
LB11	RV298	*ura3::pRS306(pSIC1- Far1 (85 – 150) -eGFP)*	This study
LB12	RV298	*ura3::pRS306(pSIC1- Far1 [1-150 (L135A)]-eGFP)*	This study

LB13	RV298	*ura3::pRS306(pSIC1- Far1 [1-150 (E138A)]-eGFP)*	This study
LB14	MO371	*ura3:: pRS306(pSIC1- Far1(1-150)-eGFP)*	This study
LB15	MO371	*ura3::pRS306(pSIC1- Far1 (1-150 RXL) -eGFP)*	This study
MO211	RV298	*ura3::pRS306(pSIC1- Sic1 (1-219)-eGFP)*	Mihkel Örd
MO175	RV298	*ura3::pRS306(pSIC1- Sic1 [1-219 (AxA2 AxA3)]-eGFP)*	Mihkel Örd
LB16	RV298	*ura3::pRS306(pSIC1- Sic1 [1-219 (AxA2 Far1 docking motif)]-eGFP)*	This study
LB17	RV298	*ura3::pRS306(pSIC1- Far1[1-150 (102 – 109 linker)] eGFP)*	This study
LB18	RV298	*ura3::pRS306(pSIC1- Far1[1-150 (112 – 119 linker)] eGFP)*	This study

3.1.2 Cloning of the plasmid constructs

The backbone plasmid used for the ligation of the Far1 (1-150) inserts is a pRS306 yeast integrative vector. The plasmid contains an ampicillin resistance gene used for the selection in bacteria and URA3 gene which acts as a yeast selectable marker. The mutants that were inserted into the plasmid, were expressed under the Sic1 promoter and had an eGFP protein attached to their C-terminus. The inserts in strains *LB2, LB3, LB4, LB5, LB6, LB7, LB12, LB15* were amplified by PCR of templates provided by Ilona Faustova, and the ones used in strains *LB1, LB8, LB9, LB14* were provided by Ervin Valk **(Table 1).**

3.1.2.1 PCR

The inserts were obtained by PCR of templates containing different Far1 mutants. The PCR mixture had a total volume of 50 µL. The mixture contained 1 µL of template DNA, 5x Phusion HF Buffer (Thermo Fisher Scientific) in a final concentration of 1x, 10 mM dNTPs in a final concentration of 200 µM, forward and reverse primers in a final concentration of 0.5 µM each, and high-fidelity Phusion DNA Polymerase (Thermo Fisher Scientific) in a final concentration of 0.02 U/µL. Milli-Q H2O was added to the mixture to make up to 50 µL. The PCR mixture was then taken to the PCR machine following a three-step programme **(Table 2)**. The annealing temperature depends on length and composition of primers and it can be calculated by entering primers' sequences into different online sources (Benchling in

case of this study). The extension time has been determined by the amplicon's length (around 500 bp), and the synthesis rate of Phusion polymerase 1kb/30s.

Table 2. PCR cycle. A three step PCR programme

Cycle step	Temperature	Time	Cycle
Initial Denaturation	98 °C	5 min	1
Denaturation	98 °C	20 s	
Annealing	54 °C	20 s	34
Extension	72 °C	20 s	
Final Extension	72 °C	5 min	1

Once the PCR programme finished, the mixture was stained with 6x Orange DNA Loading Dye (Thermo Fisher Scientific) and loaded on to 1% Agarose gel (40 mM Tris-acetate (pH 8.3), 1 mM EDTA, 1% agarose, 0.05 µL/µL Atlas ClearSight DNA Stain (BioAtlas)). The gel electrophoresis took place in 1x TAE buffer (40 mM Tris-acetate (pH 8.3), 1 mM EDTA). The fragments were visualised using GeneRuler DNA Ladder 1 (Thermo Fisher Scientific). Afterwards, a picture of the gel under the UV light was taken, and appropriately sized bands were cut out of the gel and placed in separate Eppendorf tubes. The gel pieces were then purified using FavorPrep™ GEL/PCR Purification Kit (Favorgen) according to the manufacturers' instructions. Once purified, the concentration of amplified DNA segments was measured using Thermo Fisher's NanoDrop 1000 Spectrophotometer.

3.1.2.1 Enzyme Restriction

The forward primer that was used to amplify the insert has a BamHI cutting site and the reverse primer has a SmaI cutting site. Thus, it was possible to restrict the amplified DNA segments with SmaI and BamHI enzymes. In order to perform the restriction Termo Fisher's FastDigest™ enzymes were used. The restriction mixture contained 100 ng of amplified DNA segment, 3 µL of 10x FastDigest™ Buffer, 1 µL of FastDigest™ enzymes SmaI and BamHI, and Milli-Q H_2O was added to the rest of the mixture to make up to 30 µL. The restriction mixture was then gently re-suspended and span down. Afterwards, the mixture was incubated in a heat block for 30 minutes at 37 °C. Once the incubation was over the mixture was further incubated at 80 °C in order to inactivate the enzymes. The inactivation is important as otherwise the restriction enzymes will be present in the ligation mixture and

will re-digest the ligation. Once the inactivation was finished, the restricted DNA fragments were ready to be inserted into the pRS306 (P_{SIC1}-eGFP) vector.

However, in order for the inserts to be ligated with the vector, the circular pRS306 (P_{SIC1}-eGFP) plasmid had to be linearised by both SmaI and BamHI enzymes. The vector was digested with FastDigest™ enzymes. The restriction mixture contained 2 µg of pRS306 (P_{SIC1}-eGFP) plasmid, 3 µL of 10x FastDigest™ Green Buffer, 1 µL of FastDigest™ Enzymes SmaI and BamHI, 1 µL of FastAP (Thermo Fisher Scientific), and Milli-Q H_2O was added to the mixture to make up to 30 µL. FastAP is a thermo-sensitive alkaline phosphatase which catalyses the removal of phosphate groups from the areas where the restriction enzymes made cuts, in order to prevent the vector from ligating back together. The mixture was incubated in a heat block for 30 minutes at 37 °C. The mixture containing the linearised vector was loaded on to 1% agarose gel (40 mM Tris-acetate (pH 8.3), 1 mM EDTA, 1% agarose, 0.05 µL/µL Atlas ClearSight DNA Stain (BioAtlas)). The gel electrophoresis took place in 1x TAE buffer (40 mM Tris-acetate (pH 8.3), 1 mM EDTA). The fragments were visualised using GeneRuler DNA Ladder 1 (Thermo Fisher Scientific). A picture of the gel under the UV light was taken, and appropriately sized band was cut out of the gel and placed in separate Eppendorf tubes. The gel piece was then purified using FavorPrep™ GEL/PCR Purification Kit (Favorgen) according to the manufacturers' instructions and had its concentration measured with Thermo Fisher's NanoDrop 1000 Spectrophotometer.

3.1.2.2 Ligation

Once the vector and the insert were restricted with SmaI and BamHI enzymes, they were ligated together. The ligation mixture contained around 20 ng of linearised vector, 5:1 molar ratio over vector of insert DNA, 2 µL of 10x T4 DNA Ligase buffer, T4 DNA Ligase (Thermo Fisher Scientific) in a final concentration of 5 U/µL, and Milli-Q H_2O up to 20 µL. As a SmaI enzyme is a blunt-end cutter, 2 µL of 50% PEG 4000 solution was added to the ligation mixture. PEG increases the chance of correct ligation as it is a hydrophobic molecule which takes up space in the reaction, thereby, concentrating the reaction components. After the mixture was re-suspended, it was incubated at 16 °C overnight.

3.1.2.3 Bacterial transformation

Bacterial transformation was performed with *E. coli* DH5α competent cells in order to produce multiple copies of the plasmid. The cells were first taken out of -80 °C refrigerator and left on ice to thaw. In the meantime, 1 µL of the ligation mixture was transferred to a new

tube. Once thawed, 50 µL of the cell solution was transferred to the tube already containing the ligation mixture. After re-suspension, the tube containing the transformation mixture was placed on ice for 30 minutes. After 30 minutes, the transformation mixture was heat shocked at 42 ℃ for 60 seconds. Afterwards, the mixture was put back on ice for another minute. 500 µL of LB media (10 g/L tryptone (BD Biosciences), 5 g/L yeast extract (Formedium), 10 g/L NaCl) was added and the mixture was incubated in a 37 ℃ shaker for 45 minutes. After the incubation, the tube was centrifuged for 60 seconds at 6000 rpm in order to concentrate cells. The cells were plated on LB plates containing 100 µg/mL ampicillin (Sigma) and incubated for 12 hours at 37 ℃.

3.1.2.4 Colony control

The bacterial colonies that grew after the plating on LB ampicillin plates were inoculated in a flask containing 5mL of LB media, in presence of 100 µg/mL ampicillin. The flasks containing the cells were incubated in a shaker at 37 ℃ for 12-16 hours. Once the incubation finished, FavorPrep™ Plasmid DNA Extraction Mini Kit (Favorgen) was used to extract the plasmids. After the extraction, the DNA concertation (ng/µL) was measured with Thermo Fisher's NanoDrop 1000 Spectrophotometer. In order to check if the plasmid had the correct insert, the plasmid was again restricted with SmaI and BamHI enzymes. The restriction mixture contained 2-3 µg of plasmid DNA, 3 µL of 10x FastDigest™ Green Buffer, 1 µL of FastDigest™ enzymes SmaI and BamHI, and Milli-Q H_2O up to 20 µL. The mixture was incubated in a heat block at 37 ℃ for 30 minutes. Afterwards, the mixture was loaded to 1% agarose gel (40 mM Tris-acetate (pH 8.3), 1 mM EDTA, 1% agarose, 0.05 µL/µL Atlas ClearSight DNA Stain (BioAtlas)). The fragments were visualised using GeneRuler DNA Ladder 1 (Thermo Fisher Scientific). The gel electrophoresis was performed in 1x TAE buffer (40 mM Tris-acetate (pH 8.3), 1 mM EDTA). A picture of the gel under the UV light was taken and the plasmids containing the insert were sent for Sanger sequencing to the Estonian Biocentre core laboratory.

3.1.3 Yeast transformation

The *S. cerevisiae* strains used in the study were based on strain RV298 obtained from Rainis Venta. RV298 is a haploid derivative of w303 with bar1 deletion and Whi5 tagged with mCherry. The genotype of RV298 is *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi$^+$] bar1::hisG WHI5-mCherry-SpHIS5*). The constructs were transformed in RV298 strain. One day before the transformation, a tip of a streaking stick was cover with colonies

from a fresh YPD (10 g/L yeast extract (Formedium), 20 g/L peptone (Formedium), 20 g/L glucose (Oriola)) plate containing RV298 cells and transferred to a tube containing 5 mL of YPD media. The inoculum was incubated at 30 °C shaker overnight. In the morning the optical density of the culture was measured at 600 nm wavelength using Ultrospec 10 cell density meter (Amersham Biosciences). The culture was then diluted by calculating the ration of the over-night inoculum and YPD medium needed to yield a final OD of 0.2 in 25 mL of broth placed in a 50 mL Erlenmeyer flask. The diluted culture was incubated at 30 °C shaker until the optical density reached 0.8-1 (approximately 5 hours).

Around 30 minutes before the beginning of transformation the plasmid construct was linearised in order for the plasmid to be able to integrate in to the yeast's genome. The restriction mixture contained 1 µg of plasmid DNA, 1 µL of 10x FastDigest™ Buffer, 1 µL of FastDigest™ enzyme Eco147I (Thermo Fisher Scientific), and Milli-Q H_2O up to 10 µL. The mixture was incubated in a heat block for 30 minutes at 37 °C. Eco147I linearises the plasmid by cutting through URA3 gene present in the plasmid. This will allow the integration to take place through homologous recombination in the URA3 locus.

Once ready, the yeast culture was transferred to 50 mL centrifuge tubes and centrifuged for 1 minute at 3600 rpm. After centrifugation, the supernatant was discarded and the cells were re-suspended in 25 mL of Milli-Q H_2O and centrifuged for 1 minute at 3600 rpm. The Milli-Q H_2O was discarded and the cell pellet was re-suspended in 1 mL of sterile buffer I (100 mM lithium acetate in 0.5 x TE (5 mM Tris-HCl (pH 8), 0.5 mM EDTA)) buffer. The mixture was centrifuged at 3600 rpm for 60 seconds. The supernatant was removed after centrifugation and two times the cell volume of buffer I was added. The mixture was left to incubate for 10 minutes at room temperature.

The tube containing plasmid restriction was taken to the table and mixed with 10 µL of pre-boiled Salmon Sperm DNA (SSDNA, kept at 100 °C for 10 minutes). Once the incubation period was over, 100 µL of yeast competent cells were transferred to the tube containing the linearised plasmid and SSDNA. After re-suspension 700 µL of sterile PEG/lithium acetate solution (40% PEG 3350, 100mM lithium acetate, 10 mM Tris-HCl (pH 8), 1 mM EDTA) was added. Afterwards, 48 µL of DMSO were added and the mixture was slowly pipetted up and down. The mixture was then incubated at 42 °C for 40 minutes.

After the incubation, the cells were left to chill on ice for 2 minutes. The cells were later centrifuged for 60 seconds at 6000 rpm at room temperature. Once the centrifugation was over, the supernatant was removed and the cells were re-suspended in 1 mL 1x TE buffer

(10 mM Tris-HCl (pH 8), 1 mM EDTA). The mixture was re-centrifuged for 2 minutes at 3000 rpm at room temperature. The supernatant was removed and the cells were re-suspended in 200 µL of 1x TE buffer. There were then plated on synthetic complete –URA glucose (SC-URA) plates (7 g/L yeast nitrogen base without amino acids (BD Biosciences), 2 g/L SC-URA powder (MP Biomedicals), 20 g/L glucose (Oriola), 20 g/L agar) and incubated at 30 ℃ for 2-3 days.

3.1.4 Microscopy

In order to measure and quantify the GFP fluorescence in unperturbed cell cycles, time-lapse microscopy experiments were performed. These experiments allowed to see the growth of the cells in real time as well as changes in GFP levels present in the cell. To study the phosphorylation and degradation dynamics of the N-terminus of Far1, the first 150 amino acids of Far1 protein were fused N-terminally to SV40 nuclear localization signal (PKKKRKVG) and C-terminally to GFP. The proteins were expressed under the Sic1 promoter. The changes in GFP level correspond to changes in levels of Far1 protein which is being phosphorylated and degraded during the cell cycle.

Before starting the microscopy experiment the transformed colonies were checked under the microscope for the presence of GFP fluorescence. If a colony had a nuclear GFP signal that meant that the insert had successfully been transformed, and such colony could later be used in a time-lapse experiment. One day prior to the experiment the colonies were streaked from the transformation plate onto a new SC–URA glucose plate and left overnight at 30 ℃. In the morning a tip of a streaking stick was covered with cells from a fresh plate and the colonies were transferred to 3 mL of SC–URA 2% glucose medium. The culture was incubated on a 30 ℃ shaker for 8-10 hours. After incubation the cells were taken out of the incubator and vortexed. 0.3 µL of the cell culture was pipetted on a 24 x 50 x 0.08 mm micro cover glass, and covered with a small piece of complete supplement mixture (CSM) with 2% glucose 1.5 % agarose gel (7 g/L yeast nitrogen base (BD Biosciences, 0.79 g/L CSM powder (Formedium), 20 g/L glucose (Oriola), 1.5% NuSieve GTG agarose (Lonza)). The same procedure was repeated, on the same micro cover glass, for the remaining colonies. Later, longer pieces of CSM glucose agar slabs were cut out and placed around the pieces covering the cells; a smaller 20 x 20 mm micro cover glass was placed over the cell-covering gel pieces. This is done in order to prevent the cell-covering gel pieces from drying out during the experiment as well as moving out of place. On the top of this, a small plastic cover was

placed, effectively covering the whole 24 x 50 mm micro cover glass piece, thereby further preventing the gel slices from drying out.

The microscopy experiment was performed using Zeiss Axio Observer.Z1 microscope with 63x/1.4 Oil M27 Plan-Apochromat objective (Zeiss). The fluorescence imaging was performed using Colibri LED modules at 25% power. The GFP channel had an exposure time of 15 ms at the wavelength of 470nm; mCherry had a higher exposure time of 750 ms at 555 nm. The experiments were 8 hours long, containing 161 time points with 3 minutes between each. During the experiment, the temperature of the agarose gel was kept at 30 °C with Tempcontrol 37-2 digital (Pecon). 8-12 positions were followed in one experiment using the automated stage and ZEN software. The positions were kept in focus using Definite Focus. Once the experiment was over, the programme would give 3 different pictures per one-time point (phase-contrast, GFP, mCherry). These were later converted into a greyscale images and the quantified as per (Doncic et al., 2013). In the end, the graphs presenting the GFP fluorescence were plotted using MatLab. The fluorescence values were also normalised so that it would be easier to compare different mutants.

3.2 RESULTS

In the region of Far1 N-terminal of position 140, there is a Clb5 specificity determinant that enhances phosphorylation of Far1 by the Clb5-Cdk1 complex (unpublished data by E. Valk). The disordered N-terminus of Far1 also contains 8 Cdk1 phosphorylation sites, two of these (S87 and S91) make up a di-phosphodegron. For simplicity, I chose to work with inserts of Far1, which contained only the first 150 amino acids, excluding the structured C-terminal domain.

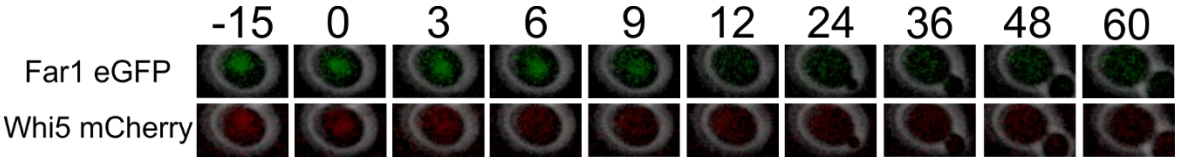

Figure 4. Microscopy experiment for studying Far1 degradation during the cell cycle. Time series showing a cell expressing Far1 1-150 eGFP and Whi5 mCherry. The images are merged from phase contrast and fluorescence channels. The numbers represent time in minutes. The Start point of the cell cycle is at time 0.

3.2.1 Leucine mutations lead to more stable concentrations of Far1 (1–150)

To begin with, we tested whether the N-terminus of Far1 (positions 1-150) fused to EGFP is degraded during the cell cycle similarly as reported previously for the full-length Far1 (Blondel et al., 2000). For this, we set up a time-lapse microscopy protocol, where the cell cycle progression can be measured using Whi5-mCherry and phosphorylation-dependent degradation of Far1 can be analysed by measuring Far1(1-150)-EGFP levels. Live cell microscopy enables quantitative measurement of Far1 (1-150)-EGFP levels on a single cell level with high temporal precision in unperturbed cell cycle. Whi5 is a G1 transcriptional repressor that is exported from the nucleus in late G1 (Costanzo et al., 2004). The time point, when the nuclear levels of Whi5 have decreased to 50% of the G1 peak, has been defined as the Start point of cell cycle (Doncic et al., 2011). We analysed Far1 (1-150)-EGFP levels for 90 minutes from Start, covering the whole yeast cell cycle **(Figure 4).**

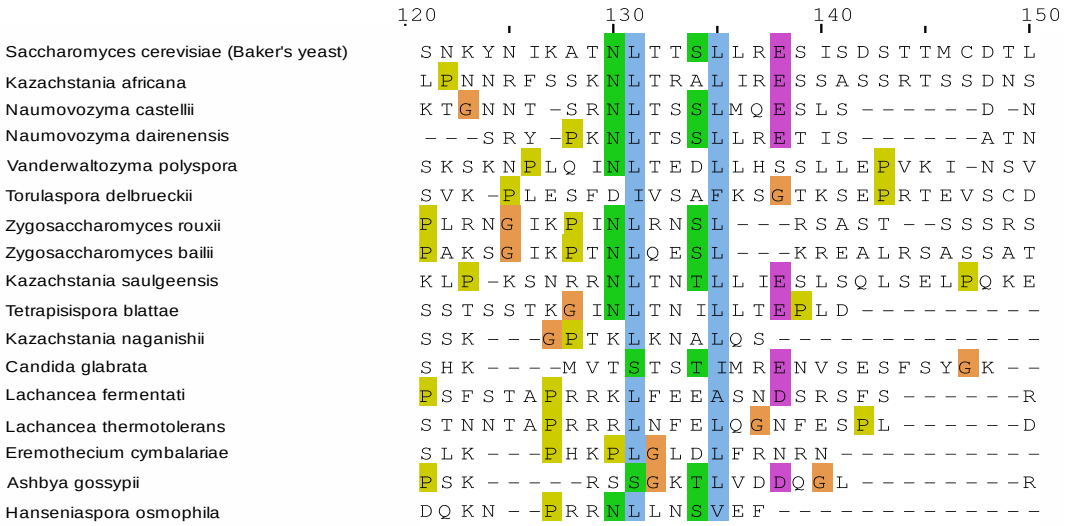

Figure 5. Alignment of homologues of Far1 in different yeast species shows conservation of leucines L131 and L135. Using virtual alignment programme (ProViz) we were able to observe conserved 131 and 135 leucine sequences in different homologues of Far1.

The wild type Far1(1-150) is degraded quite rapidly in the nucleus; it takes on average 12 minutes after Start for the protein levels to halve and only 6 minutes for the protein to degrade, defined by the fluorescence intensities dropping from 80% to 20%. In a similar experiment, the Cdk1-dependent phosphorylation of a replication protein Cdc6 leads to degradation of Cdc6 about 10-12 minutes after Start (Örd et al., 2019). Therefore, as the G1/S transition occurs about 10-12 minutes after nuclear export of Whi5-mCherry, the Far1 (1-150)-EGFP is phosphorylated and degraded very rapidly after the Start of the cell cycle.

As the cyclin docking motifs reported so far (RXL and LLPP) are mainly of hydrophobic residues and leucines show the highest conservation in the Far1 130-140 region (**Figure 5**), we firstly, wanted to see how the mutations of a non-polar, hydrophobic amino acid leucine would, if at all, affect the degradation rate of Far1 protein. We chose to mutate leucines as it seemed to be the most conserved amino acid present in this region in different homologues of Far1 **(Figure 5)**.

As the first step of initial mapping of the interaction, we made combinations of mutations of three positions. The degradation of LTTS**AAA** mutant (L135A L136A R137A) was almost 4 times slower compared to the wild type; it takes 40 minutes for the protein levels to halve and 30 minutes for the protein to degrade **(Figure 6. A-C)**. The data shows that these leucines are important likely because they increase the docking interaction between Far1 and Clb5, thereby, promoting the phosphorylation of Far1.

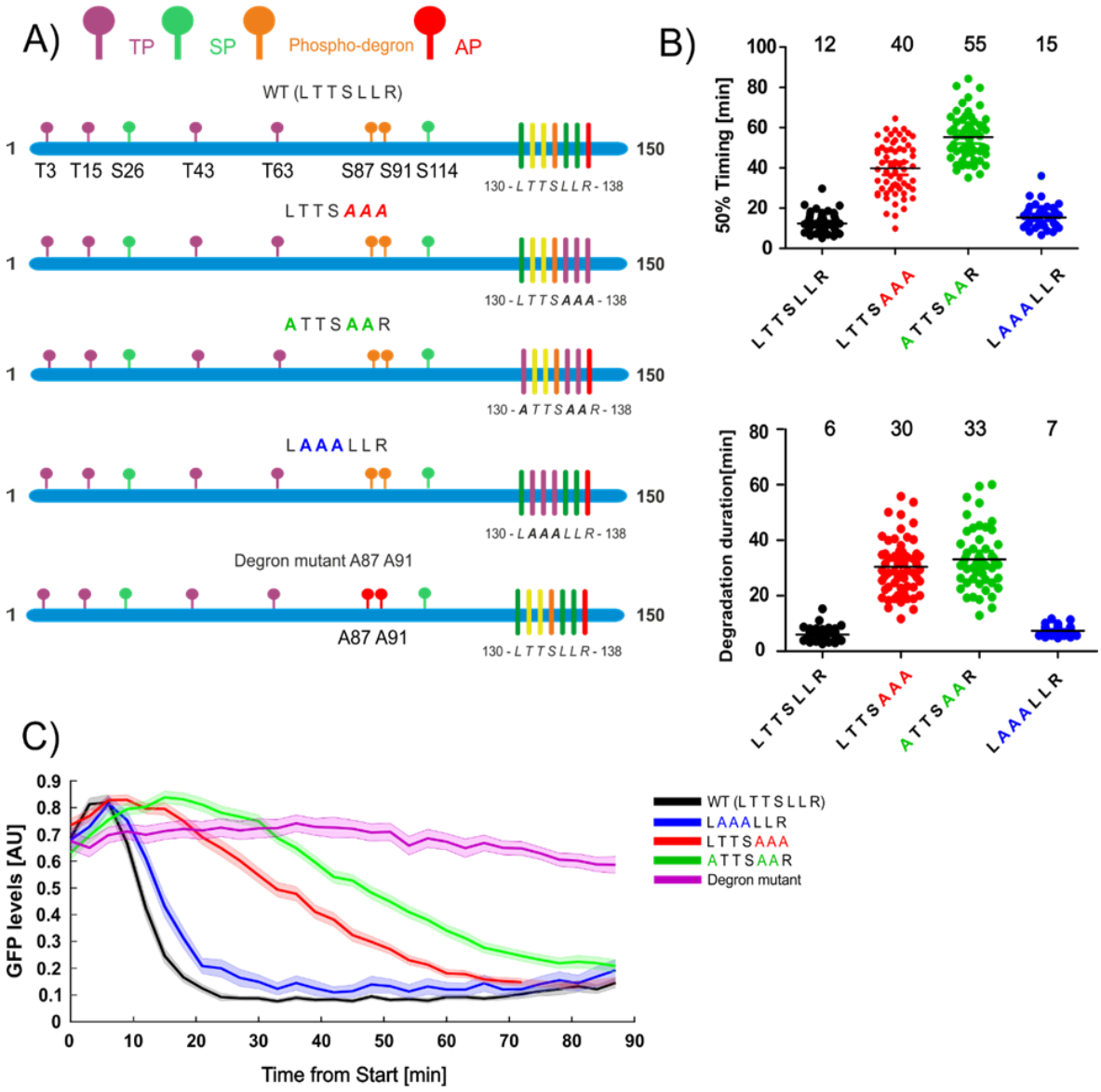

Figure 6. Mutations in Clb5-dependent motif lead to more stable concentrations of Far1 (1-150) during the cell cycle. A) The diagrams show Far1 (1-150) wild type, LTTS**AAA** (L135A L136A R137A), **ATTSAA**R (L131A L135A L136A), L**AAA**LLR (T132A T133A S134A), and degron (S87A S91A) mutants. The N - terminal TP, SP, and degron sites are also shown on the diagrams. B) The graphs obtained from single cell data show the average time for 50% timing and degradation duration. 50% timing shows the time from the start of cell cycle needed for the concentration of Far1 to drop to 50%; degradation duration shows the time needed for the concentration of Far1 to drop from 80% to 20%. Degron mutant is not included in the plot as levels do not reach 50% during the cycle. C) The graph shows the average levels of different Far1 mutants after the start of cell cycle. The error bars are ±SEM.

However, once mutated, they lead to weaker docking interactions between Far1 and Clb5 which drastically delays the phosphorylation and degradation of the Far1 protein. This piece of evidence is further supported by the data obtained from **A**TTS**AA**R mutant (L131A L135A L136A). As another leucine was mutated, the time it took for proteins levels to halve increased to 55 minutes; interestingly, the degradation time still seemed to stay almost unchanged compared to LTTS**AAA** mutant **(Figure 6. A-C)**. In order to test whether the threonines and serine between the leucines also contribute to the docking, we constructed the L**AAA**LLR mutant. The L**AAA**LLR mutant was degraded almost as fast as the wild type Far1, indicating that these positions do not affect the interaction **(Figure 6. A-C)**. On the other hand, constant levels of degron mutant (S87A S91A) support literature suggesting that phosphorylation of S87 and S91 is of crucial importance in degradation of Far1 **(Figure 6. A, B)**. The data obtained in these experiments correlated to the unpublished work of I. Faustova, where she arrived to similar conclusion by using a different method. This verifies that the decrease in Far1 (1-150)-EGFP levels during the cell cycle is caused by phosphorylation and activation of the di-phosphodegron. This led us to postulate that phosphorylation and later degradation of Far1 (1–150) is mainly dependent on the docking interaction with Clb5 which promotes direct phosphorylation of the degron sites by the Cdk1.

In order to gain a more detailed understanding of the docking motif, we performed single mutation experiments. The L135A mutant was observed to behave similarly as LTTS**AAA** and **A**TTS**AA**R mutants; it takes from 40 to 45 minutes for levels of three mutants to halve **(Figure 7. A-C)**. This suggests that even a single leucine mutation in Far1 docking motif leads to loss of Clb5-Far1 interaction.

Another amino acid which appeared to be conserved in different homologues of Far1, though not as widely as L131 and L135, is glutamic acid E138. Also, in the conventional Clb5 docking motif, the RXL motif, the hydrophobic residues are followed by negatively charged residue (Lowe et al., 2002). However, it came to our surprise that E138A mutant was identical to the wild type; both took on average 12 minutes for the protein levels to halve and 6 for proteins to be degraded **(Figure 7. A-C)**. This piece of data further suggests that the leucines play an essential role in regulation of docking interaction between Far1 and Clb5. In addition to this, it would be interesting to see how L131A, L135A, R137A, and N130A mutants would affect the degradation of Far1 protein. The aim of our future experiments will be to further map Far1 docking region through the use of single mutants.

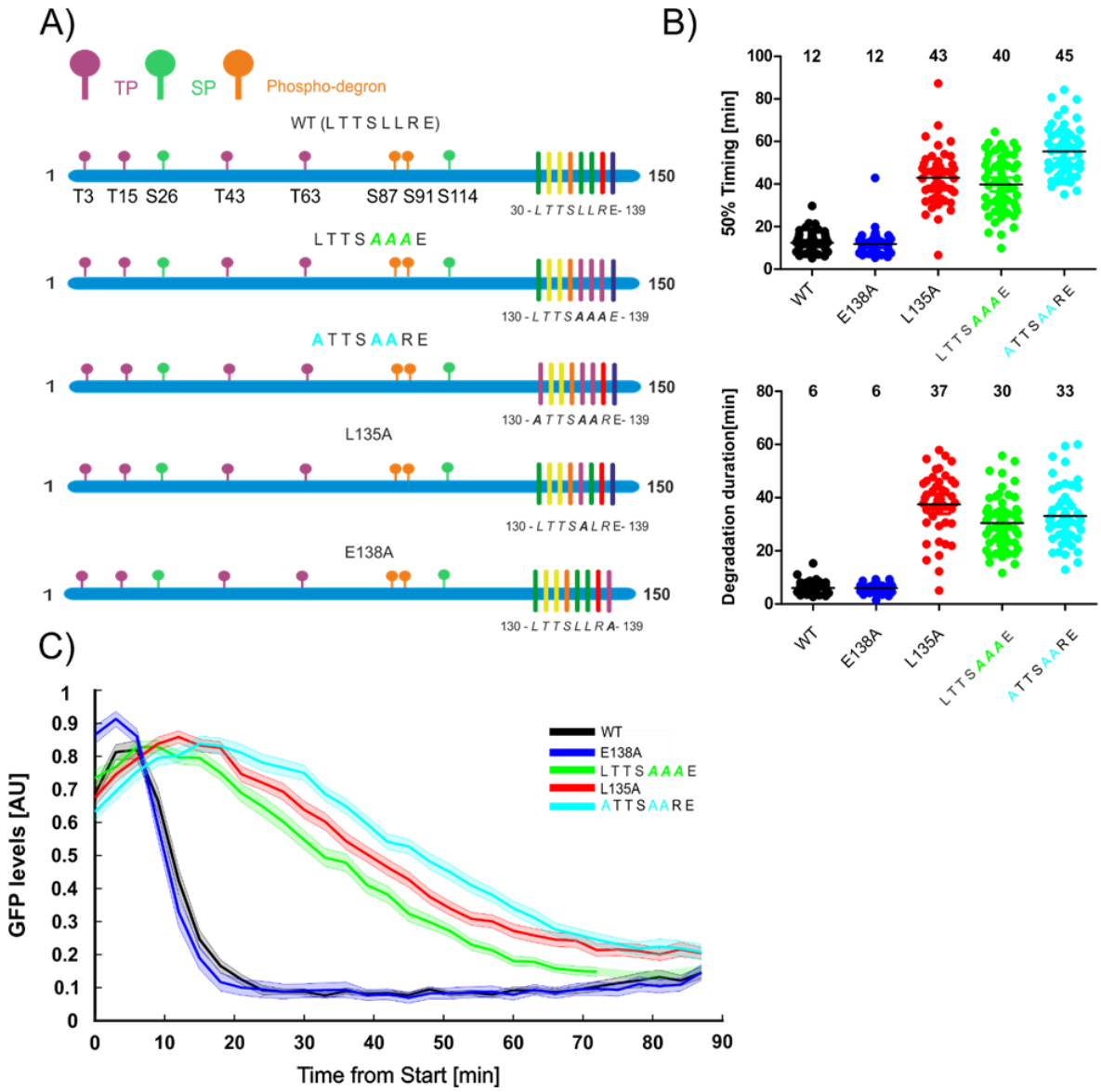

Figure 7. L135A mutant stabilises Far1 1 – 150 concentrations while E138A mutant has no effect. A) The schematics show Far1 1-150 wild type, LTTS**AAA**E (L135A L136A R137A), **A**TTS**AA**RE (L131A L135A L136A), L135A, and E138A mutants. The N - terminal TP, SP, and degron sites can also be seen on the schematics. B) The graphs obtained from single cell data show the average time for 50% timing and degradation duration. 50% timing shows the time from the start of cell cycle needed for the concentration of Far1 to drop to 50%; degradation duration shows the time needed for the concentration of Far1 to drop from 80 to 20 percent. C) The graph shows the average levels of different Far1 mutants after the start of cell cycle. The error bars are ±SEM.

3.2.2 Far1 (128-140) functions as a linear docking motif

RXL is a Clb5-specific short linear motif that is shown to function independently. We wanted to see if RXL could replace Far1 docking motif and still enable phosphorylation of Far1 by Clb5 – Cdk1 complex and later degradation. To do this we inserted 14 amino acids including the RXL motif in Sic1, into 129 – 142 amino acid region of Far1, thereby, replacing the Far1 docking motif with the RXL motif. As a control we used AxA, a mutated version of RXL **(Figure 8. A)**.

WT (RXL) mutant seemed to be a bit more stable compared to the wild type; it took 17 minutes for the protein level to halve which was 5 minutes longer than in the wild type. The protein degradation took 9 minutes which was a bit slower than the degradation of the wild type (6 minutes) **(Figure 8. B-C)**. The data suggests that RXL can indeed replace the Far1 docking motif, and still bind to Clb5 and promote phosphorylation and degradation of Far1. This was earlier shown by using a different method in the unpublished work of I. Faustova. However, due to a delay between the WT and WT (RXL) mutant, it can be hypothesised that Far1 docking motif has a higher Clb5 binding affinity which leads to faster phosphorylation and degradation of Far1 protein.

After we were able to successfully introduce the RXL motif in Far1 protein, we wanted to check if Far1 docking motif could replace the RXL motif in Sic1. The phosphorylation of Sic1 degron sites is efficiently promoted by Cks1-mediated multisite phosphorylation, and it does not rely too much on the docking of RXL motif to the hydrophobic patch in Clb5 (Kõivomägi et al., 2013). Therefore, in order to make the phosphorylation of degron sites more dependent on the docking interaction, we used a Sic1 construct which has 16 amino acids between T33 and T45 phospho-sites (the distance is 12 amino acids in WT Sic1). This 4 amino acid insertion decreases the effect of Cks1-mediated multisite phosphorylation, thereby, giving enough time to for the docking interactions to influence the phosphorylation of the degron sites (Örd et al., 2019). To measure the effect of cyclin docking, we used Sic1 AxA mutant as a control and inserted 13 amino acids of the Far1 docking motif in region of 111 – 124, thereby, replacing AxA3 with a Far1 docking motif **(Figure 9. A)**.

We were able to observe that Far1 docking motif was just as effective as the RXL motifs in Sic1 WT; it took 23 minutes for the levels of both proteins to halve and around 15 minutes for them to be degraded **(Figure 9. B-C)**. This suggests that Far1 docking motif, similar to RXL motifs, is a fully functional linear motif that displays modularity. However, more experiments need to be performed in order to obtain a minimal length of the motif. We now

know that the motif consisting of only 13 amino acids (ATNLTTSLLRESI) can function independently, however, it would be interesting to see if a shorter one would give a similar result. Once we gather more single mutant data it will be easier to estimate the length of the motif as we will have an understanding of amino acids which affect the docking interaction.

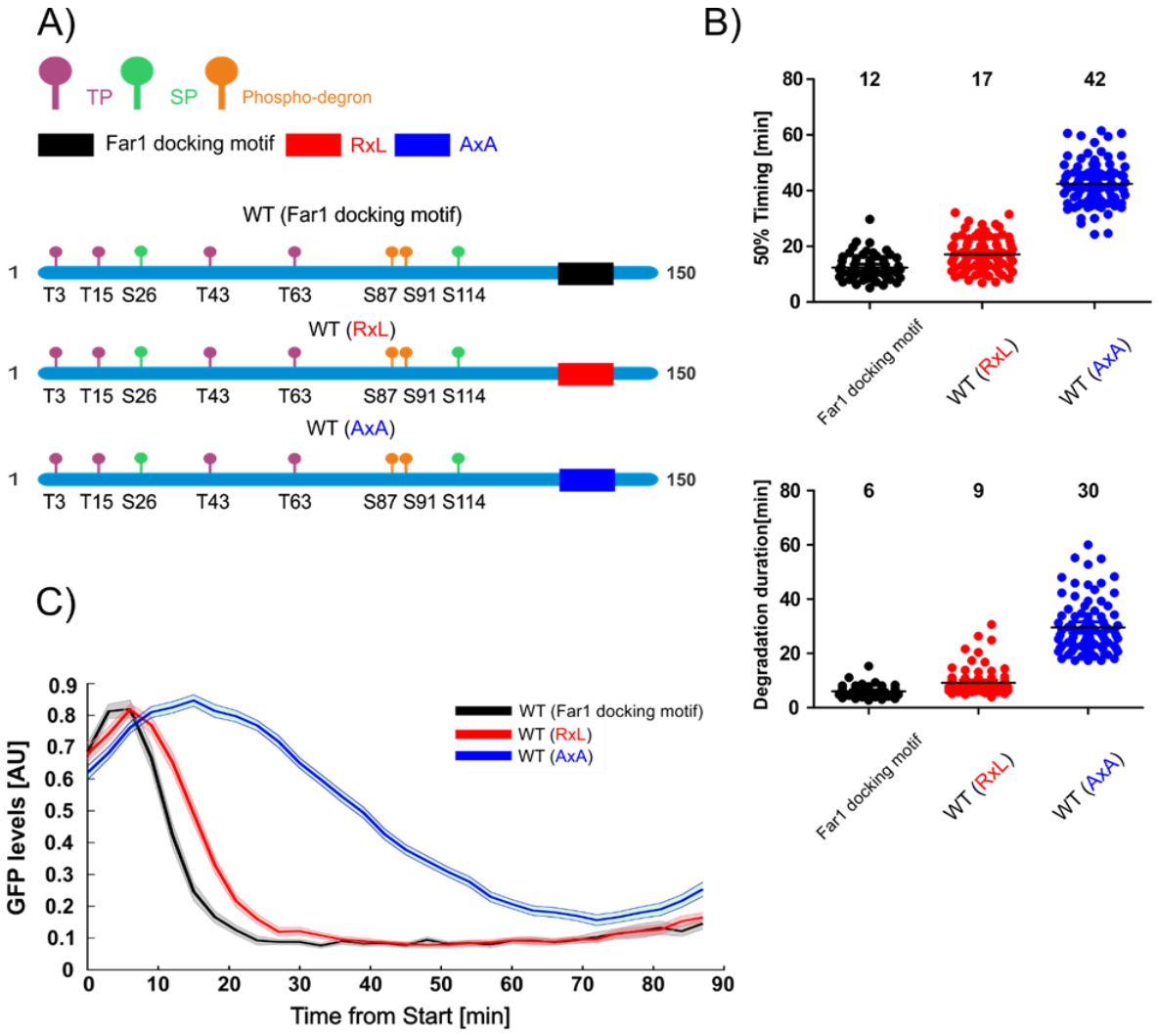

Figure 8. RXL motif can replace Far1 docking motif in Far1 though with a lower efficiency. A) The schematics show Far1 1-150 wild type, WT(RXL) containing 14 amino acid sequence from Sic1 RXL motif (PKSSVK**RTL**FQFET) integrated in 129 – 142 region of Far1 1 – 150, WT(AxA) containing a 14 amino acid sequence of mutated RXL (PKSSVK**A**T**A**FQFET) integrated in 129 – 142 region of Far1 1 – 150, and N - terminal TP, SP, and degron sites. B) The graphs obtained from single cell data show the average time for 50% timing and degradation duration. 50% timing shows the time from the start of cell cycle needed for the concentration of Far1 to drop to 50%; degradation duration shows the time needed for the concentration of Far1 to drop from 80 to 20 percent. C) The graph shows the average levels of different Far1 mutants after the start of cell cycle. The error bars are ±SEM.

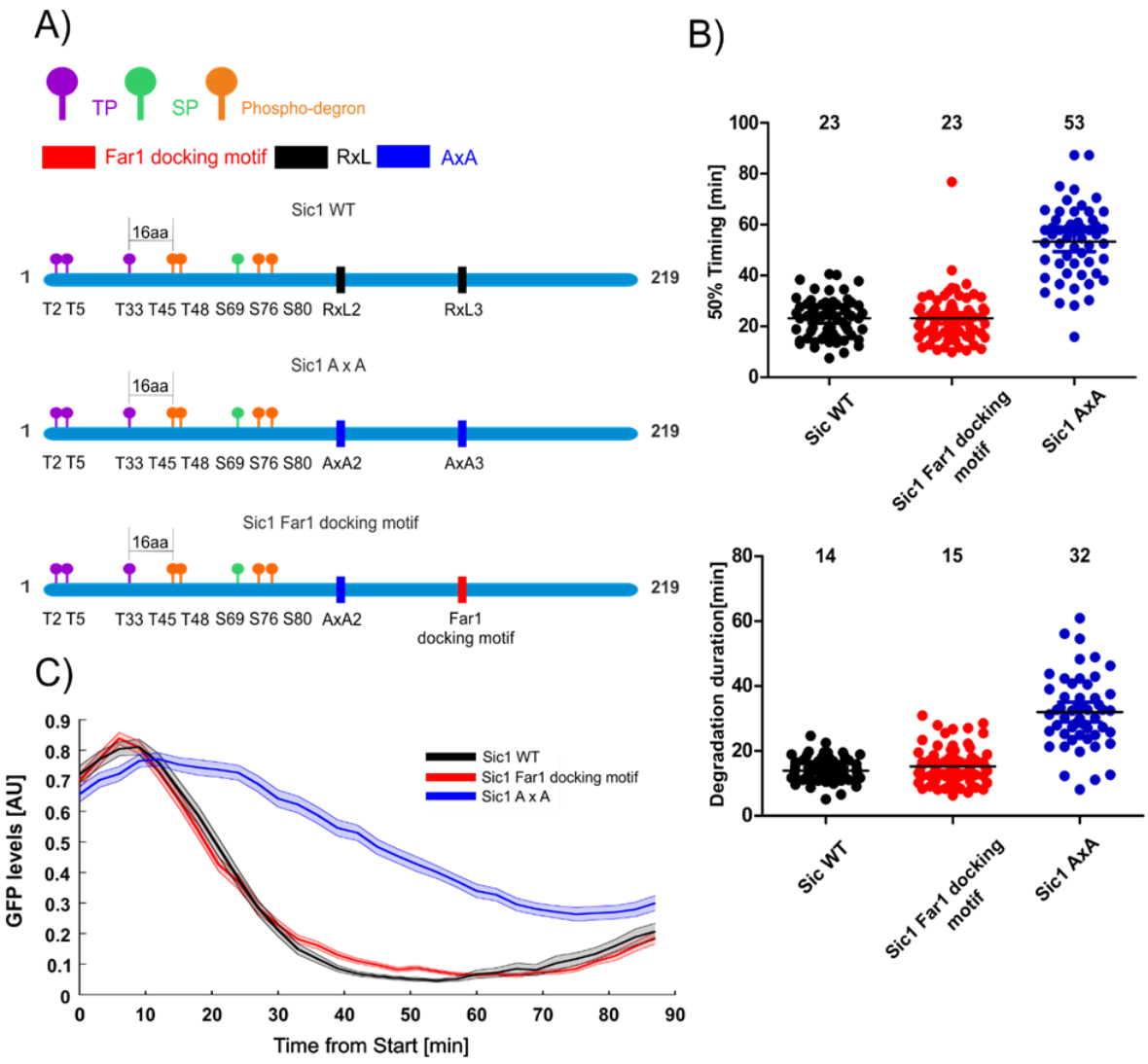

Figure 9. Far1 docking motif is just as effective in degradation of Sic1 as its native RXL motif. A) The diagrams show Sic1 WT (including 2 RXL motifs), Sic1 AxA (RXL motifs mutated to AxA), and Sic1 Far1 docking motif mutant which has RXL3 replaced with 13 amino acids from Far1's docking motif (AxA2, Far1 docking motif (ATNLTTSLLRESI) 111 – 124). The diagram also shows Sic1 N - terminal TP, SP, and degron sites. B) The graphs obtained from single cell data show the average time for 50% timing and degradation duration. 50% timing shows the time from the start of cell cycle needed for the concentration of Far1 to drop to 50%; degradation duration shows the time needed for the concentration of Sic1 to drop from 80 to 20 percent. C) The graph shows the average levels of different Sic1 mutants after the start of cell cycle.

3.2.3 Degron sites (S87 S91) can be phosphorylated without Cks1

As previously mentioned, the phosphorylation of degron sites in Sic1 depends on the Cks1-mediated multisite phosphorylation. In this experiment, we wanted to understand how important Cks1 is in phosphorylation of two degron sites (S87 S91) in Far1 (1 – 150).

The data showed that it took 17 minutes for the levels of SP (T3S, T15S, T43S, T63S) mutant to halve which was 5 minutes later compared to the WT. The degradation of the SP mutant was similar to the one of the WT, lasting 8 and 6 minutes, respectively **(Figure 10. A-C)**. This suggest that Cks1-medated phosphorylation is not essential in phosphorylation of degron sites in Far1 (1 – 150), though it might speed up the process. However, it was interesting to see that WT (RXL) mutant behaved the same as the SP mutant; it took 17 minutes for protein levels to halve in both cases and around 8 minutes for them to get degraded **(Figure 10. A-C)**. Interestingly, in case of mutation of both Cks1 priming sites and the Far1 docking motif (SP Far1 docking motif mutant), there is very little degradation, as the profile is similar to the degron mutant (**Figure 10. C, 5. C**).

Furthermore, the SP (T3S, T15S, T43S, T63S) RXL mutant displayed a huge delay in both halving time and degradation (39 and 36 minutes, respectively) **(Figure 10. A-C)**. This data suggests that in order for degron sites to be phosphorylated, the Cks1-mediated docking is needed in case of no cyclin docking or with RXL docking. However, Far1 docking motif seems to be able to promote the phosphorylation of the degron sites without the need for Cks1. This is further supported by the data obtained from Far1 (85 – 150) where the N – terminus was truncated. Though the halving (23 minutes) and degradation (17 minutes) time were delayed compared to the SP mutant, Far1 85 – 150 was still phosphorylated suggesting that Cks1-medated phosphorylation in not essential in phosphorylation of degron sites in Far1 1 – 150 **(Figure 10. A-C)**.

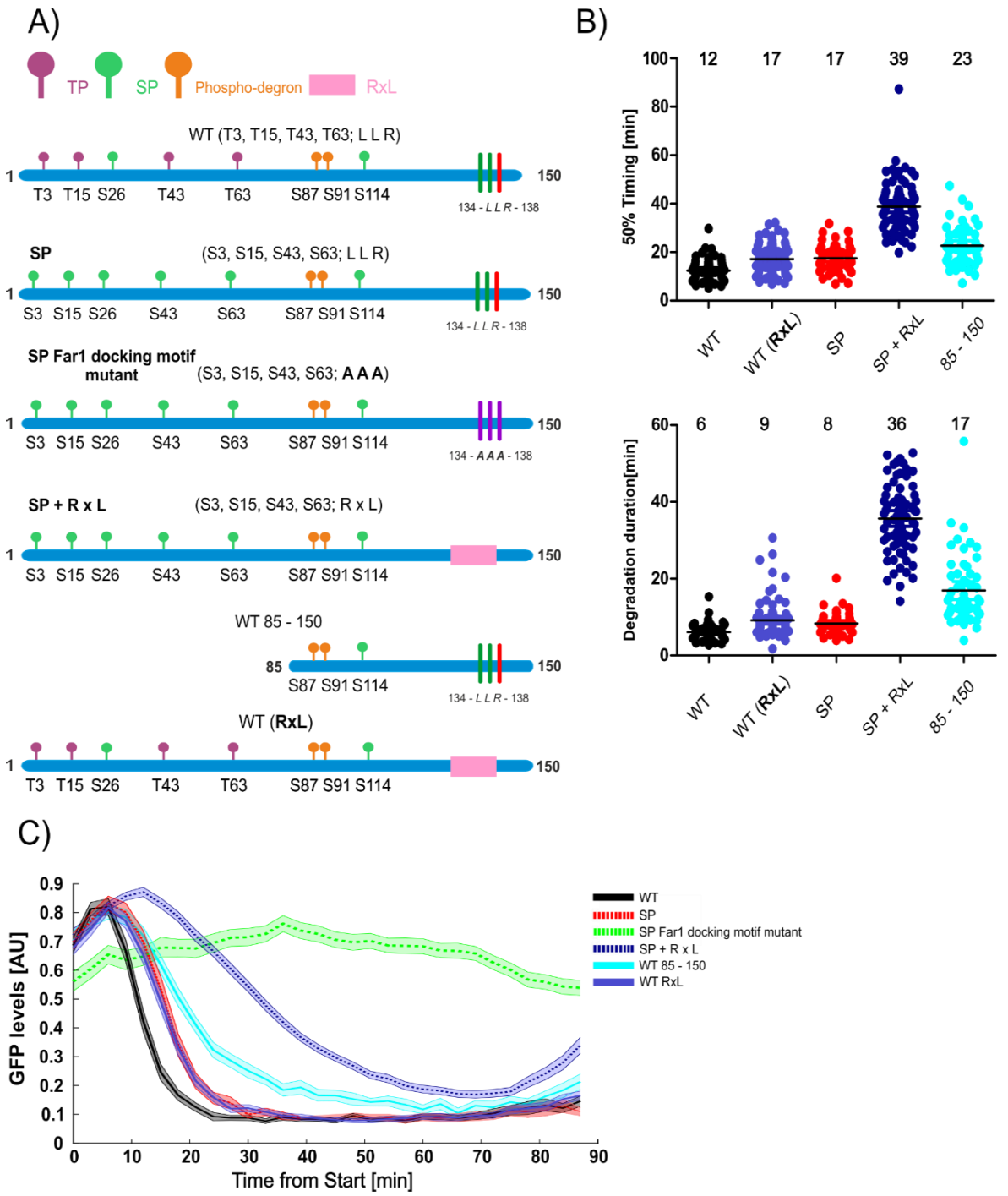

Figure 10. Cks1-mediated phosphorylation is not essential in degron S87 S91 site phosphorylation. A) The diagram showing Far1 (1 – 150) WT, SP mutant (T3S, T15S, T43S, T63S), SP Far1 docking motif (T3S, T15S, T43S, T63S, L135A, L136A, R137A), SP (T3S, T15S, T43S, T63S) + RXL (containing 14 amino acid sequence from Sic1 RXL motif (PKSSVK**R**T**L**FQFET) integrated in 129 – 142 region of Far1 1 – 150), WT 85 – 150, and WT(RXL) containing 14 amino acid sequence from Sic1 RXL motif (PKSSVK**R**T**L**FQFET) integrated in 129 – 142 region of Far1 1 – 150. The diagram also shows Sic1's N - terminal

TP, SP, and degron sites. B) The graphs obtained from single cell data show the average time for 50% timing and degradation duration. 50% timing shows the time from the start of cell cycle needed for the concentration of Far1 to drop to 50%; degradation duration shows the time needed for the concentration of Sic1 to drop from 80 to 20 percent. SP Far1 docking mutant is not included in the graph as its concentration never falls below 50%. C) The graph shows the average levels of different Far1 mutants after the start of cell cycle.

3.2.4 Linker region Far1 (102 – 119) does not play a role in phosphorylation of the degron

We postulated that the linker region between the degron sites S87 S91 and the Far1 docking domain might play a role in phosphorylation of degron sites by the Clb5 – Cdk1 complex. Linker 102 – 109 had GGGSGGSGS sequence introduced in 102 – 109 region of Far1 (1 – 150) and linker 112 – 119 had the same sequence introduced in 112 – 119 region of Far1 (1 – 150) **(Figure 11. A).** There was very little difference between the linkers and the WT **(Figure 11. B),** suggesting that this region does not play an essential role in phosphorylation of the degron sites. This provides additional evidence that the Far1 docking motif is a short linear motif.

3.2.5 Far1 docking motif does not seem to bind to the hydrophobic patch in Clb5

As cyclins have a highly conserved hydrophobic patch for substrate interactions**,** we wanted to know if Far1 docking motif also binds to it. For this, we transformed Far1 1 – 150 WT (WT hpm) and Far1 1 – 150 RXL (RXL hpm) into a strain with mutated hydrophobic patches on Clb5 and Clb6. As a control we used Far1 1 – 150 WT (WT) and Far1 1 – 150 RXL (RXL) constructs transformed in a wild type strain **(Figure 12. A).** As RXL binds to the hydrophobic patch we expected a delay in degradation rate in RXL hpm compared to RXL. Indeed, we were able to observe a 15-minute delay in halving time and a 9-minute delay in degradation time of RXL hpm compared to RXL **(Figure 12. B-C).** Interestingly, WT hpm and WT showed no difference in halving and degradation time which suggests that Far1 docking motif does not bind to Clb5 hydrophobic patch, but to some other binding pocket.

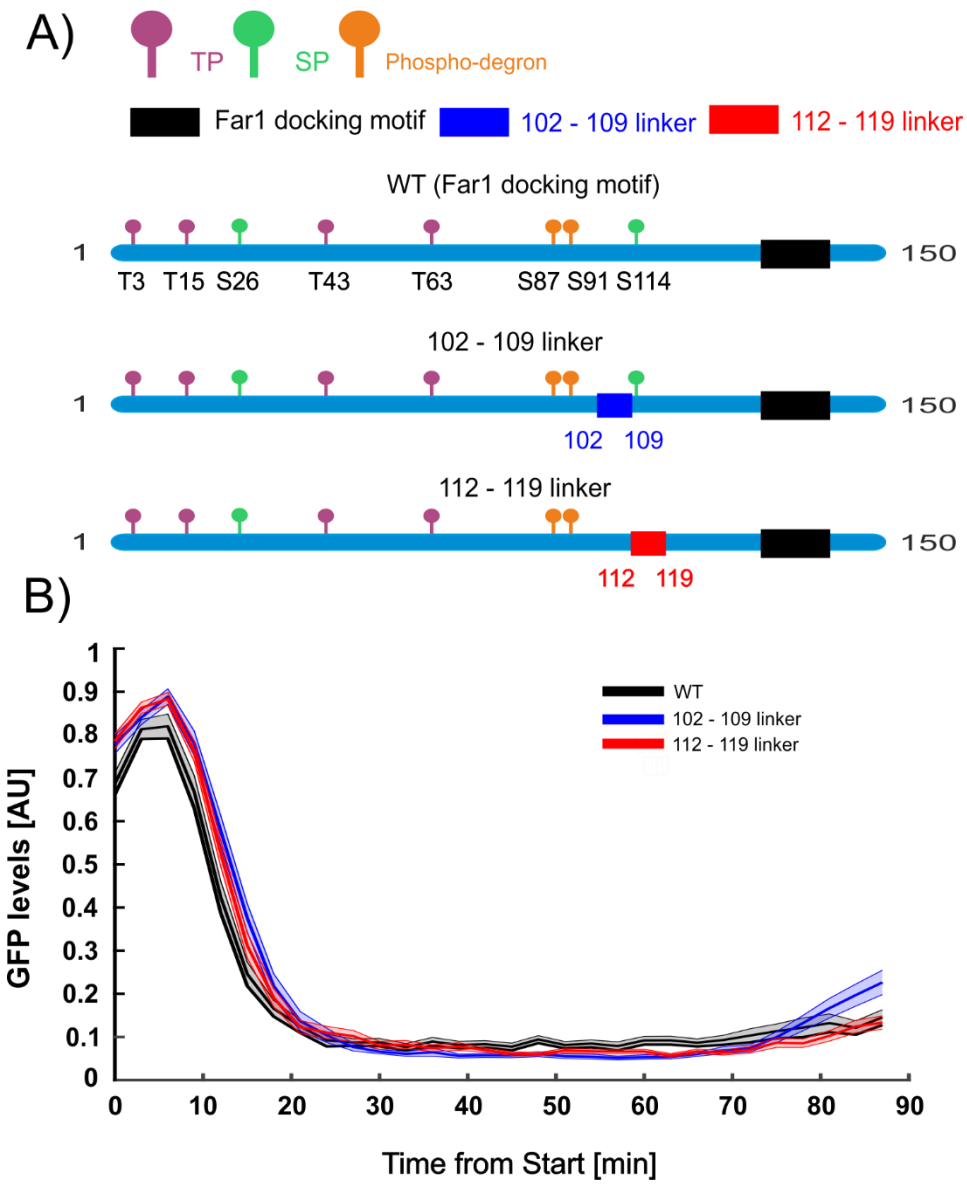

Figure 11. The region between Far1 docking site and phospho-degrons does not affect the degron site phosphorylation. A) The diagram shows Far1 1 – 150 WT, linker 102 – 109 with GGGSGGSGS sequence introduce in 102 – 109 region of Far1 (1 – 150), and linker 112 – 119 with GGGSGGSGS sequence introduced in 112 – 119 region of Far1 (1 – 150). B) The graph shows the average levels of different Far1 mutants after the start of cell cycle. The error bars are ±SEM.

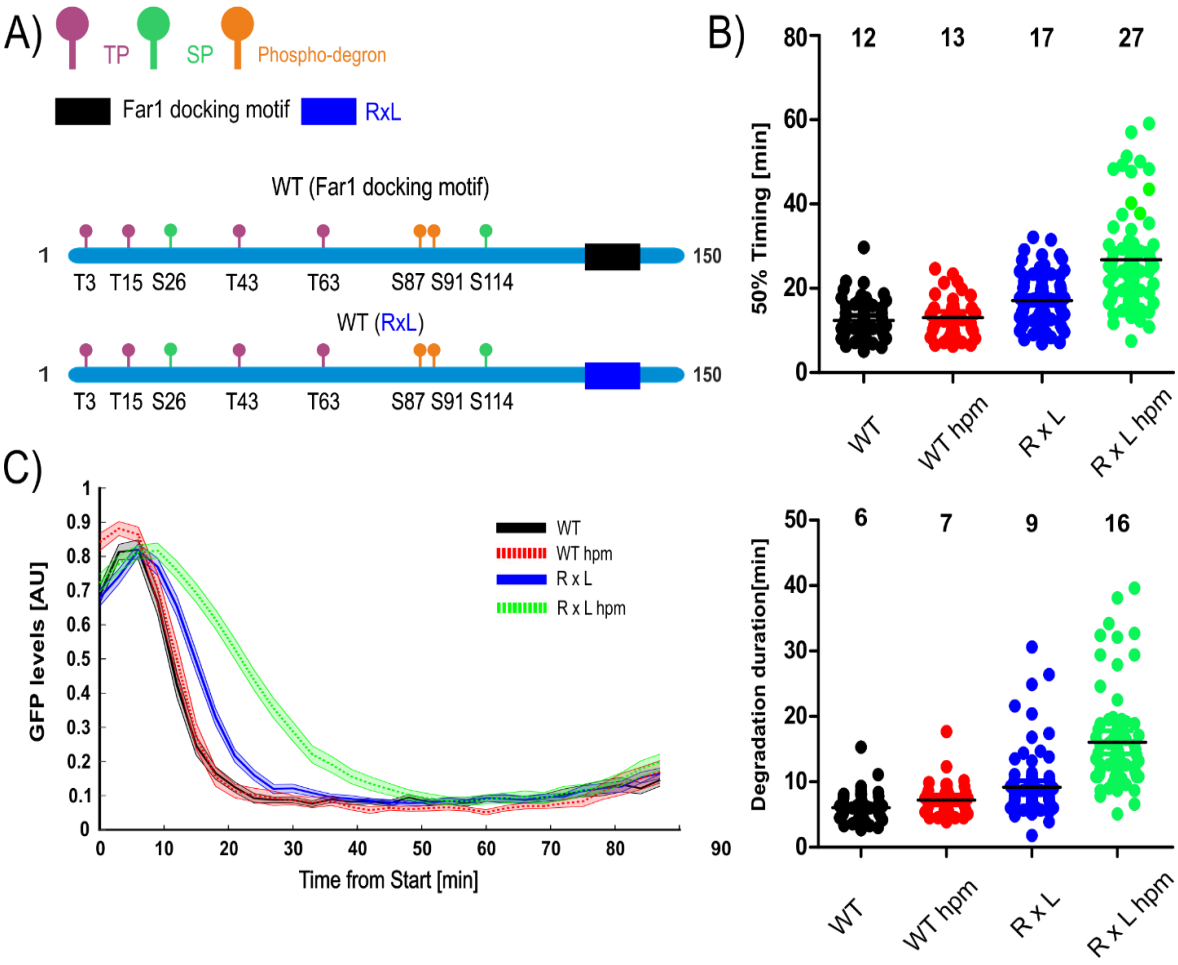

Figure 12. Far1 docking motif does not seem to bind to Clb5 hydrophobic patch. A) Far1 WT 1 – 150 and Far1 WT (RXL - containing 14 amino acid sequence from Sic1 RXL motif (PKSSVK**R**T**L**FQFET) integrated in 129 – 142 region of Far1 1 – 150) are show. WT hpm and RXL hpm stand for WT and RXL constructs that were transformed into a strain with mutated hydrophobic patches on Clb5 and Clb6. B) The graphs obtained from single cell data show the average time for 50% timing and degradation duration. 50% timing shows the time from the start of cell cycle needed for the concentration of Far1 to drop to 50%; degradation duration shows the time needed for the concentration of Sic1 to drop from 80 to 20 percent. C) The graph shows the average levels of different Far1 mutants after the start of cell cycle. The error bars are ±SEM.

3.3 DISCUSSION

We have found a Clb5 linear docking motif from Far1 that enables rapid phosphorylation and degradation of Far1 in the cell cycle. Our data has shown that leucines are the most important amino acids present in the Far1 docking region as mutations in these amino acids dramatically decreased the phosphorylation and degradation rates of Far1 1-150. However, in order to have a better understanding of how other amino acids might influence the docking interaction between Far1 and Clb5 we need to perform more single mutant experiments. At this point we cannot conclude if the motif is unique to Far1, or if it could have a wider importance in the S phase substrate targeting. A better definition of the motif would enable prediction of the motif in other Cdk targets.

On the other hand, Far1 docking motif seems to promote a higher phosphorylation of targets than RXL motif **(Figures 7, 8).** Far1 docking motif appears to promote phosphorylation of degron sites independent of Cks1-mediated multisite phosphorylation, whereas in case of RXL the phosphorylation is dependent on Cks1 interaction. These observations could come from two different mechanisms. First, the Far1 docking motif could lead to higher binding affinity compared to RXL motif. Secondly, as we have observed that the Far1 docking motif binds to a different region on the Clb5 surface it could be more effective at directing the disordered substrate to Cdk active site. It has been shown that cyclin docking motifs not only increase the substrate binding affinity but also direct Cdk to phosphorylate specific phosphorylation sites. For example, the RXL motif promotes phosphorylation of sites that are at least 16 amino acids N-terminal of the docking motif (Kõivomägi et al. 2013). The difference between the two docking motifs suggests that Far1 docking motif might be critical in the early S phase docking interactions. This could enable differential phosphorylation of Cdk targets at the onset of the S phase. As Far1 is an inhibitor of Cdk and RXL motifs are shown to be present in many replication proteins (Loog and Morgan, 2005; Wilmes et al., 2004) we can hypothesise that the inhibitors are targeted before other substrates, possibly contributing to the irreversibility of the cell cycle entry.

Our research can be further applied in a synthetic context by constructing a phospho-degron module. Previously, a phospho-dependent regulation motif was constructed where the phosphorylation of the module was mediated by the Fus3 MAPK (Gordley et al., 2016). However, in order for the kinase to be active the cells had to be exposed to an α-factor which effectively arrested them in a G1 phase. By understanding the specificity of Cdk phospho-

regulation modules that are differentially activated during the cell cycle could be constructed. This would enable to use phospho-regulation without affecting the cell cycle.

SUMMARY

Docking motifs found in substrates serve as recognition sites that are identified by different cyclins which, in turn, promote substrates' phosphorylation by the cyclin – Cdk1 complex. These docking motifs are located in the disordered parts of a protein and some of them are proven to be modular and function as short linear motifs. Such region has been found in the N-terminal region of a Cdk inhibitor protein, Far1. The region is Clb5 cyclin specific and the aims of our work were to gain a better understanding of the region and its importance in the docking interaction between the Far1 and Clb5. Moreover, we wanted to check the modularity of this region and its role in phosphorylation and degradation of Far1.

To summarise, by introducing different mutations in 130 – 140 amino acid region of Far1, we were able to map the docking motif and examine the changes that took place as a result of these mutations. Our experimental data strongly suggests that leucines present in this region are important in mediating the docking interaction between the motif and the cyclin. Furthermore, we observed Far1 docking motif's modularity by using Far1 (129-142) to replace an RXL motif in Sic1. The data showed that Far1 (129-142) can function as a linear docking motif and it would be interesting to see in future, if a docking motif of a smaller length would give similar results. Furthermore, it appears that Far1 docking motif is more effective in targeting substrate phosphorylation compared to the RXL motif, further suggesting that these interactions might be critical in the early S phase substrate phosphorylation.

On the other hand, we were able to observe that Cks1-mediated multisite phosphorylation of the N – terminal sites in Far1 does not play an important role in the phosphorylation of the degron sites. Thus, we have postulated that Far1 docking motif can independently promote the phosphorylation of the phospho-degron without relying on Cks1. Lastly, our experimental data suggests that Far1 docking motif does not bind Clb5's hydrophobic patch, suggesting the presence of an unknown binding pocket on the cyclin.

Overall, we were able to observe the importance of Far1 docking motif in mediating the interaction between Clb5 and Far1 as well as to test its modularity. Our data also suggests that the docking motif directly promotes the phosphorylation of the degron sites without a need for a Cks1-mediated phosphorylation. As a described docking motif enables a very specific substrate targeting, it could be used in construction of a synthetic phoshorylation circuit.

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