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Mapping of Swe1 region affecting its dephosphorylation by PP2A and Cdc14

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Abstract:

The eukaryotic cell cycle is a tightly regulated sequence of events. The master regulator of the cell cycle in yeast – cyclin-dependent kinase 1 (Cdk1) drives these events by phosphorylating hundreds of different protein substrates. At the same time, phosphatases counteract kinases by dephosphorylating proteins. Recent studies have shown that different mechanisms define the substrate's specificity to protein phosphatases. The importance of enzyme-substrate interactions outside the phosphatase catalytic domain have been shown, and several docking motifs on the substrates that affect the phosphatase's activity have been identified. However, the full spectrum of phosphatases' substrate recognition principles has remained to be discovered.

Opposing actions of Cdk1 and the two key cell cycle phosphatases Cdc14 and PP2A were shown to control Swe1, a protein kinase that regulates Cdk1 activity in mitosis. In this study, the N-terminal regulatory part of Swe1 (that lacks the catalytic domain) was used as a substrate to determine the regions that are important for its dephosphorylation by Cdc14 and PP2A. The *in vitro* dephosphorylation assay results indicate that regulatory elements are located in both Swe1 1-180 and 300-450 regions. Additional experiments are required to precisely map the linear motifs that interact with Cdc14 and PP2A.

Keywords:

cell cycle, cyclin-dependent kinase, dephosphorylation, docking motifs, phosphorylation

CERCS: P310 Proteins, Enzymology

Fosfataaside PP2A- ja Cdc14-vahendatud defosforüülimist mõjutavate regioonide kaardistamine Swe1 valgus

Lühikokkuvõte:

Eukarüootne rakutsükkel on väga täpselt kontrollitud sündmuste jada, mis on pagaripärmis reguleeritud tsükliinsõltuva kinaasi 1 (Cdk1) poolt. Cdk1 koordineerib rakutsükli olulisemaid protsesse sadade märklaudvalkude fosforüülimise kaudu. Fosfataasid, kinaaside funktsionaalsed oponendid, on ensüümid, mis defosforüülivad valke. Uuringud on näidanud, et erinevad mehhanismid reguleerivad fosfataaside substraatide valikut. Erinevad seondumismotiivid substraatvalkudes võimaldavad fosfataasidel tuvastada kindlaid substraate, suurendades nende defosforüülimise efektiivsust. Hiljutised uuringud on toonud palju selgust rakutsükli kinaaside seondumismotiivide kohta, kuid fosfataaside substraatide tuvastamise mehhanismidest ei ole veel täielikult aru saadud.

Kinaasi Cdk1 ja fosfataaside suhteline aktiivsus määrab mitoosi inhibiitorkinaasi Swe1 aktiivsuse, kusjuures Swe1 valku defosforüülivad mõlemad kesksed rakutsükli fosfataasid Cdc14 ja PP2A. Käesolev töö uurib Swe1 valgu N-terminaalset regulatoorst osa eesmärgiga leida regioonid, mis vahendavad Swe1 defosforüülimist fosfataaside Cdc14 ja PP2A poolt. *In vitro* defosforüülimiskatsetega leiti, et fosfataaside aktiivsust mõjutavad regioonid on nii positsioonides 1-180 kui ka 301-450. Täiendavad katsed on vajalikud, et täpselt määrata lineaarsed motiivid, mis seonduvad fosfataasidega Cdc14 ja PP2A.

Võtmesõnad:

rakutsükkel, tsükliinsõltuv kinaas, defosforüülimine, seondumismotiivid, fosforüülimine

CERCS: Proteiinid, ensümoloogia

TABLE OF CONTENTS

TERMS, ABBREVIATIONS AND NOTATIONS 6

INTRODUCTION 7

1 LITERATURE REVIEW 8

1.1 Cell cycle 8

1.2 Mechanisms of substrate phosphorylation by Cdk1 8

1.3 Phosphatases and their role in timing cell cycle events. 10

1.3.1 Substrate specificity of PP2A and Cdc14 12

1.3.1.1 Protein phosphatase 2A 12

1.3.1.2 Cdc14 13

1.3.2 Net1 and Swe1 regulation by phosphatases PP2A and Cdc14. 15

2 THE AIMS OF THE THESIS 17

3 EXPERIMENTAL PART 18

3.1 MATERIALS AND METHODS 18

3.1.1 Materials 18

3.1.2 Plasmid construction 19

3.1.2.1 Amplification of the Swe1 variants with PCR 19

3.1.2.2 Restriction and Ligation 21

3.1.2.3 Bacterial transformation, plasmid extraction and sequencing. 22

3.1.3 Protein purification 24

3.1.3.1 Culture growing conditions 24

3.1.3.2 His tag protein purification 24

3.1.4 Dephosphorylation assay 25

3.2 RESULTS AND DISCUSSION 27

3.2.1 N-Swe1 may have several distinct regions that mediate dephosphorylation by $PP2A^{Cdc55}$ and Cdc14. 28

3.2.2 Stepwise truncations of the Swe1 N-terminus cause a gradual decrease in dephosphorylation rate 30

3.2.3 Cks1 may block dephosphorylation of T196 32

SUMMARY 34

REFERENCES 35

NON-EXCLUSIVE LICENCE TO REPRODUCE THESIS AND MAKE THESIS PUBLIC 39

TERMS, ABBREVIATIONS AND NOTATIONS

ADP – Adenosine diphosphate

AP – Mutated Cdk phosphorylation site where serine or threonine are substituted with alanine

ATP – Adenosine triphosphate

BPR – Bipartite Polybasic Recognition determinant

BSA – Bovine serum albumin

Cdk – Cyclin-dependent kinase

CV – Column volume

DTT – Dithiothreitol

EDTA – Ethylenediaminetetraacetic acid

FEAR – Cdc-Fourteen Early Anaphase Release

hp – Hydrophobic patch

IPTG – Isopropyl ß-D-1-thiogalactopyranoside

KB – Kinase buffer

LB – Luria-Bertani media

MEN – Mitotic Exit Network

PBS – Phosphate-buffered saline

PMSF – Phenylmethylsulphonyl fluoride

PP2A – Protein phosphatase 2A

SDS – Sodium Dodecyl Sulfate

SDS-PAGE - Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SLiMs – Short Linear Motifs (also referred to as docking motifs)

TAE – Tris-acetate-EDTA

WT – Wild-type

YT – Microbial growth medium

INTRODUCTION

The eukaryotic cell cycle is a precisely controlled series of events. In yeast, cyclin-dependent kinase 1 (Cdk1) drives the progression through the cell cycle by phosphorylating hundreds of target proteins. Phosphatases, another family of enzymes, counteract the actions of kinases by removing phosphate group from the substrate proteins and contribute greatly to the regulation of the protein phosphorylation state. In yeast, protein phosphatase 2A (PP2A) and phosphatase Cdc14 play a major role in the cell cycle control.

PP2A functions during the interphase and is inactivated in the anaphase. PP2A sets the phosphorylation thresholds for Cdk1, since the higher levels of Cdk1 activity are needed to overcome PP2A-mediated dephosphorylation and shift the protein into the phosphorylated state. These thresholds define the order of the various substrate phosphorylation events and regulate the correct progression through the cell cycle. Cdc14, on the other hand, gets activated during anaphase, dephosphorylates Cdk1 target sites in various protein substrates, facilitates cyclin destruction, and is responsible for regulating mitotic exit.

It was demonstrated that the amino acid sequences (further referred to as docking motifs) in the phosphatases' substrates promote enzyme-substrate complex formation via docking interactions outside the enzyme's active site leading to the enhanced levels of dephosphorylation. The presence or abcence of these docking motifs may, therefore, affect the timing and/or efficiency of dephosphorylation.

Swe1, a protein regulating mitotic entry, has been previously shown to be tightly controlled by opposing actions of Ckd1 kinase and the phosphatases PP2A and Cdc14. It was hypothesised that it may contain docking motifs in its disordered N-terminal part that modulate its dephosphorylation efficiency.

To identify regions in the N-terminal part of the Swe1 (N-Swe1) that are important for its dephosphorylation by PP2A and Cdc14, set of His-tagged truncated N-Swe1 variants was constructed. Purified variants were further used as the substrates in the *in vitro* dephosphorylation assays, and regions affecting the dephosporylation were defined.

1 LITERATURE REVIEW

1.1 Cell cycle

The eukaryotic cell cycle consists of G1, S, G2 and M phases (**Figure 1**). In G1, cells are growing and preparing for DNA replication, that happens in the S phase. In the G2 phase, cells prepare to divide. Cell cycle completes when cellular and nuclear components are divided between two daughter cells during mitosis (M phase) (Morgan, 2007). Cyclin-dependent kinases (Cdk) play a central role in controlling the timely progression of the cell cycle events by phosphorylating hundreds of target proteins in the precise order (Barnum & O'Connell, 2014; Enserink & Kolodner, 2010; Örd & Loog, 2019).

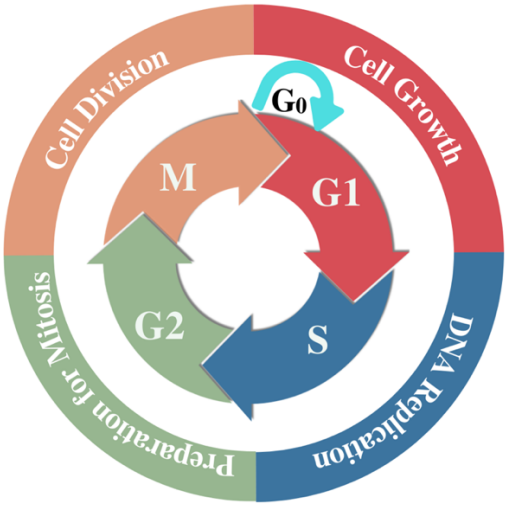

Figure 1. Phases of the eukaryotic cell cycle. In G1, cells grow and prepare for the DNA replication. In S phase, DNA is replicated. In G2, cells prepare to divide. At last, cellular content is divided between two daughter cells in mitosis (M phase). If growth conditions are unfavourable, cells may enter the dormant G_0 phase (Morgan, 2007).

1.2 Mechanisms of substrate phosphorylation by Cdk1

Cyclin dependent kinase 1 (Cdk1) catalyses the covalent binding of phosphoryl group to the substrate proteins. Phosphorylation is shown to affect target proteins by changing their activity, localisation, or marking them for degradation (Morgan, 2007).

Cdk1 belongs to the family of serine/threonine kinases that recognises and phosphorylates a full consensus motif S/T-P-x-K/R and a minimal motif S/T-P (x stands for any amino acid) (Örd & Loog, 2019).

The activity of the Cdk1 depends on the cyclin bound to it. There are 9 cyclins in yeast that bind Cdk1. They are produced and degraded at different phases of the cell cycle (**Figure 2**), target Cdk1 to different substrates, regulate its intrinsic activity, and ensure correct order of the cell cycle events (Morgan, 2007; Örd & Loog, 2019).

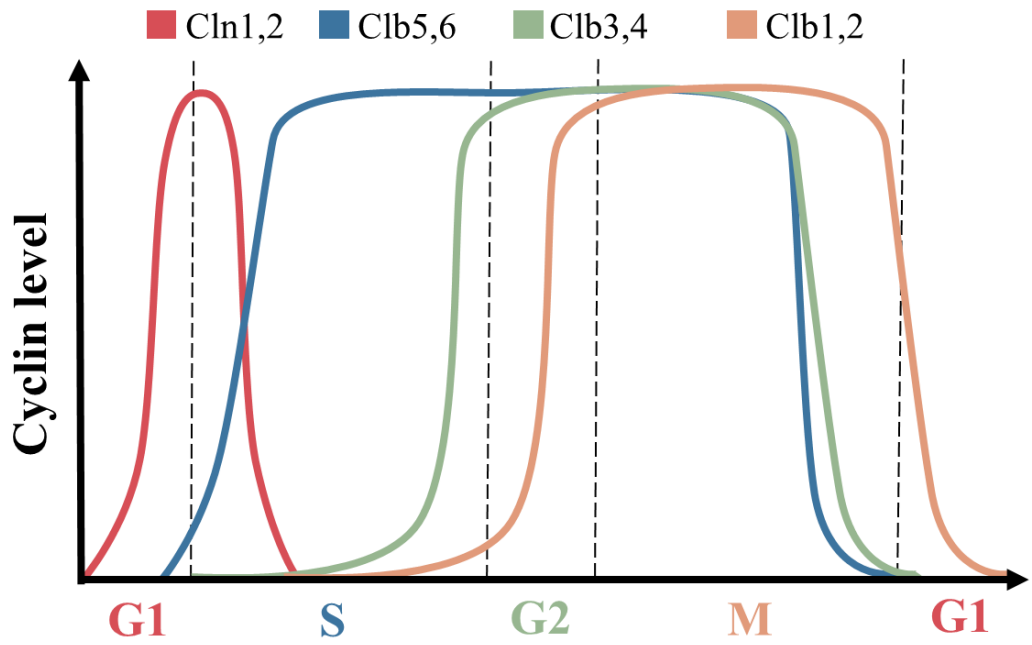

Figure 2. Cyclins levels during different cell phases. Different cyclins are expressed in the cells during different phases of the cell cycle and are divided into groups depending on their expression profiles (Morgan, 2007).

Cyclins have a hydrophobic patch pocket (*hp*) that allows them to recognize short linear motifs (SLiMs, also referred to as docking motifs) in the disordered regions of substrates, bind them and increase the affinity between substrate and cyclin-Cdk1 complex by up to 100 times. Different cyclins recognise different motifs. For example, S phase cyclin Clb5 binds NLxxxL motif, M phase cyclin Clb2 recognises LxF motif, and Clb1-6 cyclins bind common RxL motif (Faustova *et al*., 2021; Loog & Morgan, 2005; Örd *et al*., 2020).

In addition to cyclin-specific docking motifs, the phosphorylation is enhanced by phosphoadaptor Cks1. It binds to phosphorylated threonines but not serines, and promotes phosphorylation of secondary sites. Moreover, the distances between Cks1 docking site, Cdk1 phosphorylation site, and cyclin-specific docking sites greatly affect the phosphorylation rate (Kõivomägi *et al*., 2013). A schematic representation of these interactions is shown in

Figure 3. Altogether, these mechanisms allow Cdk1 to phosphorylate substrates in the precise order and ensure timely progression through the cell cycle.

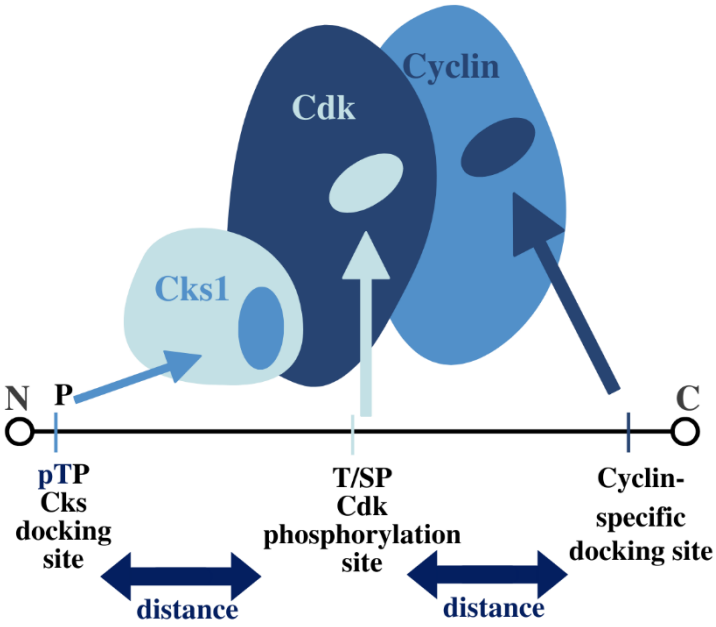

Figure 3. Cdk complex consisting of cyclin-dependent kinase, cyclin and Cks1 and their interactions with substrate. Cdk binds its substrates via multiple ways. First, Cdk recognises consensus motif on the substrate. Cdk activity is regulated by cyclins that bind specific linear docking motifs on the substrates. Cks1, a phosphoadaptor subunit, binds phosphorylated threonines and promotes multisite phosphorylation. The distance among Cks1 binding, phosphorylation site, and the cyclin docking site also contributes to the phosphorylation efficiency (Örd & Loog, 2019).

1.3 Phosphatases and their role in timing cell cycle events.

Phosphatases catalyse cleavage of phosphorylated protein into inorganic phosphate and free protein (**Figure 4**). Phosphatases are divided into two distinct families: serine/threonine and tyrosine phosphatases. They are important enzymes that affect cell cycle progression and regulation through counteracting kinases by dephosphorylating protein substrates (Godfrey *et al*., 2017; Visintin *et al*., 1998). There are approximately four times less phosphatase genes present in eukaryotic genomes compared to kinase genes. This leads to an idea that phosphatases should recognise a broader spectrum of substrates (Lim *et al*., 2015).

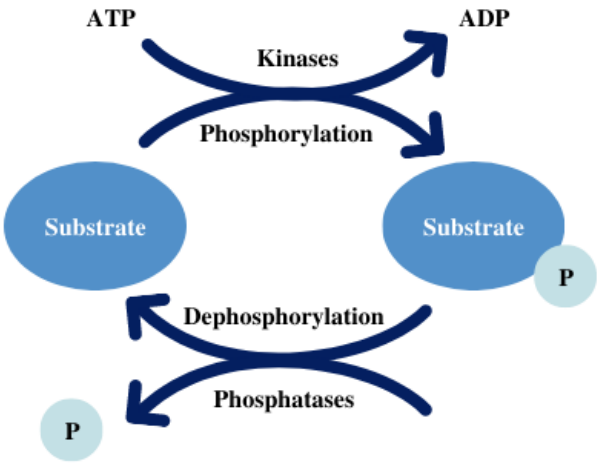

Figure 4. Schematic representation of phosphorylation/dephosphorylation reactions. Kinases catalyse the attachment of phosphoryl group from ATP to the substrate leading to formation of ADP and phosphorylated protein. Phosphatases catalyse the release of phosphate group and formation of the non-phosphorylated protein. (Ha *et al*. 2019).

Two key phosphatases, protein phosphatase 2A (PP2A) and Cdc14, were shown to counteract Cdk1 phosphorylation in yeast (Godfrey *et al*., 2017; Visintin *et al*., 1998).

PP2A plays an essential role in the cell growth, proliferation, and cell cycle progression (Godfrey *et al*., 2017; Moyano-Rodriguez & Queralt, 2020). It is active during the interphase and is inactivated during anaphase (Játiva *et al*., 2019). PP2A directly dephosphorylates Cdk substrates and delays their further phosphorylation until Cdk activity reaches a certain level, sufficient for shifting substrate into the phosphorylated state (Uhlmann *et al*., 2011).

Cdc14 is a key phosphatase that controls mitotic exit. Cdc14 is kept in inhibited state until anaphase (Visintin *et al*., 1999), when it is activated by Cdc-Fourteen Early Anaphase Release (FEAR) network and kept active by mitotic exit network (MEN) throughout mitotic exit (Rock & Amon, 2009). It dephosphorylates Cdk1 target sites in various protein substrates, facilitates cyclin destruction, and promotes accumulation of the Cdk1 inhibitor Sic1 (Manzano-López & Monje-Casas, 2020; Visintin *et al*., 1998). It has been shown that Cdc14 role extends from control of DNA and centrosome duplication cycle and the maintenance of genome stability, rDNA transcription and condensation, cytokinesis, to defence against cellular stress. This makes Cdc14 one of the central regulatory elements in the cell (Manzano-López & Monje-Casas, 2020).

Therefore, these two phosphatases are active during different phases of the cell cycle, counteract Cdk1-mediated phosphorylation, and are important for the correct progression through the cell cycle.

1.3.1 Substrate specificity of PP2A and Cdc14

Studies have shown that phosphatases may prefer one type of phosphosites over another, recognise and bind specific motifs on the substrates with increased affinity (Godfrey *et al*., 2017; Hertz *et al*., 2016; Kataria *et al*., 2018). This introduces level of substrate specificity and opens questions regarding how this substrate recognition is achieved.

1.3.1.1 Protein phosphatase 2A

PP2A phosphatase belongs to a serine/threonine family of phosphatases (Janssens & Goris, 2001). PP2A is a heterotrimeric holoenzyme that consists of three subunits: subunit A (core dimeric scaffold), B (regulatory subunit) and C (catalytic subunit). In *S. cerevisiae*, scaffold protein Tpd3 (A subunit), catalytic subunits Pph21-22 (C subunit), and one of the three regulatory subunits: B subunit Cdc55 (homolog to human B55), B' subunit Rts1 (homolog to human B56), or predicted B subunit Rts3 (Moyano-Rodriguez & Queralt, 2020; Seshacharyulu *et al*., 2013). It is worth noting that the regulatory subunits play a major role in substrate specificity (Shi, 2009). $PP2A^{Cdc55}$ mainly counteracts Cdk1 in the interphase, while $PP2A^{Rts1}$ regulates the cell size at the G1/S transition and participates in the correct chromosome biorientation in mitosis (Godfrey *et al*., 2017; Peplowska *et al*., 2014; Zapata *et al*., 2014).

It has been shown that PP2A prefers phosphothreonines over phosphoserines. This leads to threonines being kept in dephosphorylated state by PP2A until Cdk1 activity reaches certain threshold. For example, Ndd1 contains 10 phosphothreonine sites and it is known to be a late Cdk1 substrate. When threonines are substituted with serines, Ndd1 is phosphorylated much earlier in the cell cycle. In combination with the higher Cdk1 activity towards serines, this explaines how the time of substrate phosphorylation may be defined (Godfrey *et al*., 2017).

Both regulatory subunits (Cdc55 and Rts1) share the pool of the target subtstrates to some extent, where Rts1 has more unique substrates compared to Cdc55 (Touati *et al*., 2019). Since both PP2A versions share the same catalytic subunit, the difference in the substrate targeting must be regulated through the substrate recruitment regions outside the enzyme active site.

PP2A has been shown to recognise and bind specific motifs outside the enzyme active site. Cundell *et al* found that in humans, PP2A-B55 (human homolog of Cdc55) recognises bi-partite polybasic recognition determinant (BPR), located around the Cdk phosphorylation site, and that binding to it leads to more efficient dephosphorylation (Cundell *et al*., 2016). However, no such motifs were determined in yeast $PP2A^{Cdc55}$ substrates (Godfrey *et al*., 2017), and the exact docking motif that is recognised by the Cdc55 is still unknown.

Human B56, another regulatory subunit of PP2A, was reported to have conserved surface-exposed pocket that recognises the LxxIxE linear motif in the disordered regions of the substrates. Glutamic acid E at position 6 (P6) was found to be crucial since its mutation to even very similar aspartic acid (D) led to loss of binding. Either leucine (L) at P1 or isoleucine (I) at P4 was enough to provide sufficient binding in the presence of E at P6. Moreover, phosphorylated amino acid at P2 or acidic amino acids at P7-9 also increased the binding affinity of PP2A-B56. This motif is a good example, how a relatively flexible, but conserved motif may affect dephosphorylation (Hertz *et al*., 2016).

Therefore, while substrate docking interactions have been characterised for other B subunits, there are no known mechanisms that mediate specific dephosphorylation by $PP2A^{Cdc55}$ in yeast.

1.3.1.2 Cdc14

Cdc14 is considered to be a serine/threonine phosphatase. However, it has been shown to dephosphorylate phosphotyrosines as well, making it a dual specificity phosphatase (Cho *et al*., 2005; Kataria *et al*., 2018). Cdc14 is a dimer in *S. cerevisiae*, (Krissinel & Henrick, 2007) and its dimerization is crucial for Cdc14 function (Kataria *et al*., 2018).

Cdc14 prefers dephosphorylation of phosphoserines within consensus motif S-P-x-K/R (Bremmer *et al*. 2012), that is at the same time a full consensus motif for Cdk1 (Örd and Loog 2019). Phosphothreonines, on the other hand, are poorly dephosphorylated by Cdc14 due to the structure of the enzyme's active site. Alanine residue containing methyl group is located at the edge of active site cleft and restricts access to threonine residues, giving the phosphatase an ability to distinguish serines from threonines (Bremmer *et al*. 2012).

Recently, PxL docking motif has been described that binds to hydrophobic patch of the Cdc14. The motif consensus sequence is Φ-x-x-φ-**P**-**x**-**L**-x-Φ, where Φ is aromatic or large hydrophobic amino acid, φ is amino acid with a small hydrophobic side chain, a x is any amino acid. The presence of PxL docking motif on the substrate enhances dephosphorylation

efficiency. Moreover, early Cdc14 substrates contain PxL motif, while late substrates are lacking it (Kataria *et al*., 2018). This suggest that this docking interaction may define the order of substrates' dephosphorylation and provides mechanistic explanation for substrate recognition.

Kataria *et al*. proposed a model how PxL docking motif may promote dephosphorylation (**Figure 5**). The hydrophobic patch on the surface of Cdc14 has relative position that may allow both *cis*- and *trans*-dephosphorylation. This means that PxL motif may bind the hydrophobic patch of one protomer and utilise the active site of either the same or adjacent protomer. Even though structural analysis suggests *trans*-dephosphorylation preferences, it is believed that both types are used. This leads to an idea that PxL motif might promote dephosphorylation of the multiple sites by both protomers of the Cdc14 dimer (Kataria *et al*., 2018).

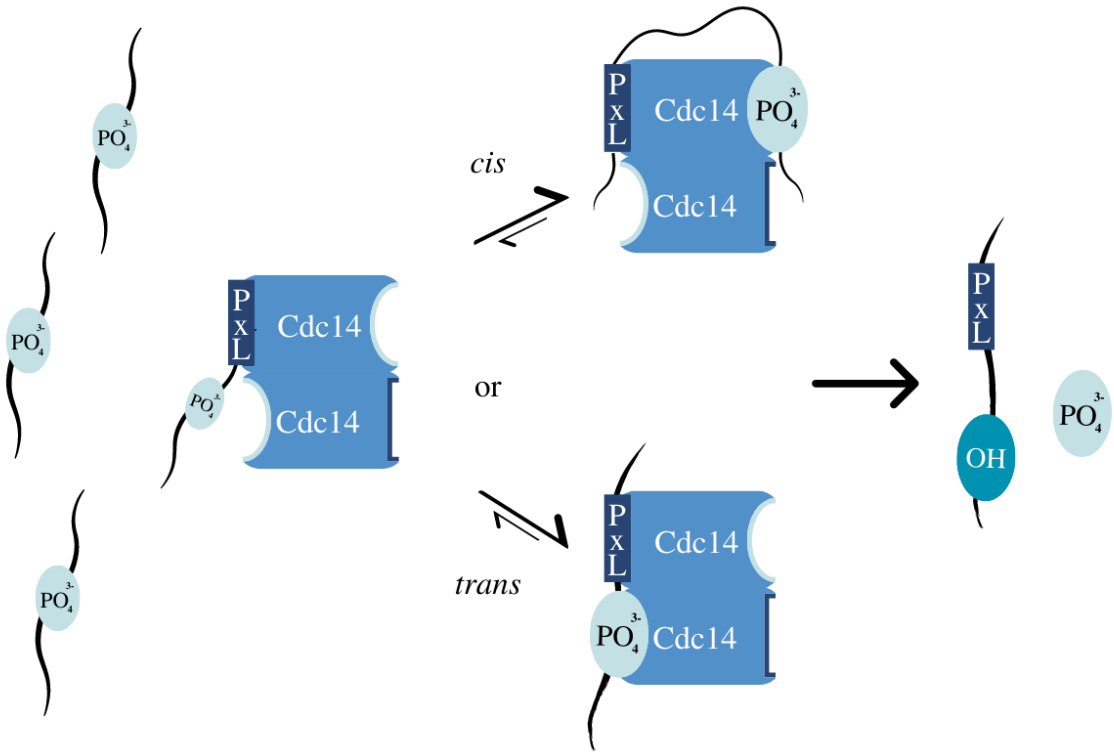

Figure 5. A model of PxL motif driven Cdc14 substrate dephosphorylation. Phosphorylated substrate binds to Cdc14 hydrophobic patch via PxL docking motif. This interaction leads to dephosphorylation by the same (*cis*) or adjacent (*trans*) Cdc14 protomer. Adapted from Kataria *et al*. 2018.

1.3.2 Net1 and Swe1 regulation by phosphatases PP2A and Cdc14.

Net1 plays an important role in Cdc14 and PP2A activity regulation. PP2A counteracts Net1 phosphorylation by Cdk1 and keeps Net1 in underphosphorylated state throughout the interphase (Queralt *et al*., 2006). In underphosphorylated state, Net1 binds Cdc14 via PxL docking motif and acts like a pseudosubstrate inhibitor (Kataria *et al*., 2018). Increase of Cdk1 activity and downregulation of PP2A shifts Net1 to fully phosphorylated state, leading to release of the Cdc14 (Azzam *et al*., 2004). Moreover, Cdc55 gets phosphorylated by Clb2-Cdk1 upon downregulation of PP2A, and phosphorylated Net1 physically interacts with $PP2A^{Cdc55}$ to act as a substrate trap (Játiva *et al*., 2019). Therefore, Net1 may inhibit both Cdc14 and $PP2A^{Cdc55}$, depending on its phosphorylation state. This shows the interplay among these phosphatases, their substrates and inhibitors. The molecular details of these interactions, however, are not fully understood.

Swe1 protein kinase plays an important role in the cell cycle progression, it is phosphorylated by Clb2-Cdk1 and dephosphorylated by $PP2A^{Cdc55}$ and Cdc14 in yeast (Harvey *et al*., 2011; Raspelli *et al*., 2015). The regulatory N-terminal part of Swe1 has 8 phosphorylation sites (T45, S111, T121, S133, T196, S201, S263, and T373) and an LxF Clb2 docking motif (Harvey *et al*., 2011; Örd *et al*., 2019). The schematic diagram of the N-terminal part of Swe1 is shown in **Figure 6**.

Figure 6. Diagram of phosphorylation sites and Clb2 docking motif of the N-terminal Swe1. TP and SP phosphorylation sites are shown as yellow and blue pinpoints, respectively. Clb2 LxF docking motif is marked with a light blue box.

It has been shown that $PP2A^{Cdc55}$ regulates Swe1 phosphorylation state by counteracting Clb2-Cdk1 mediated phosphorylation (Harvey *et al*., 2011). Phosphorylation of Swe1 activates it and leads to the formation of Cdk1-Swe1 complex, phosphorylation of Cdk1 tyrosine at position 19 (Y19) by Swe1, which leads to Cdk1 inhibition (Harvey *et al*., 2005; Hu & Aparicio, 2005). Cdk1 inhibition delays transition into mitosis and acts as a cell size and morphogenesis checkpoint (Harvey & Kellogg, 2003). Mih1 phosphatase dephosphorylates

Cdk1 Y19, reactivates Cdk1 and promotes transition to the M phase (Harvey *et al*., 2005; Pal *et al*., 2008).

Harvey *et al*. proposed the following model that explains how $PP2A^{Cdc55}$ regulates Swe1 activity. At early *CLB2* expression stages, $PP2A^{Cdc55}$ helps Clb2-Cdk1 escape Swe1-mediated inhibition by dephosphorylating Swe1. This allows low levels of Clb2-Cdk1 to trigger early mitotic events. When Clb2-Cdk1 concentration passes a threshold, Swe1 gets phosphorylated by Clb2-Cdk1. It, in turn, results in Swe1-Clb2-Cdk1 complex formation, phosphorylation of Cdk1 Y19, and Cdk1 inhibition. When cells are ready to enter mitosis, Y19 is dephosphorylated by phosphatase Mih1, and Cdk1 is reactivated. Swe1 is then hyperphosphorylated by Cdk1 that blocks Swe1 inhibitory function and promotes mitotic events (Harvey *et al*., 2005, 2011).

Apart from the Cdk1 inhibition, Swe1 has been shown to be involved in controlling spindle elongation. Moreover, its phosphorylation levels determined its localisation and distribution between mother and daughter cells. Interestingly, the above mentioned functions of Swe1 are dependent on Cdc14 that has been shown to dephosphorylate Swe1 in late anaphase, when Cdc14 is fully activated by MEN. Even though Swe1 does not contain the Cdc14 docking motif PxL, it is a critical substrate and an interactor of Cdc14 (Raspelli *et al*., 2015). This suggests that Swe1 might contain additional determinants that mediate its dephosphorylation by Cdc14.

2 THE AIMS OF THE THESIS

The highly coordinated action of Cyclin-Cdk complexes and phosphatases ensures precise control over the cell cycle progression. It was found that multiple mechanisms are involved in the control of Cdk1 activity and substrate specificity. However, the mechanisms driving phosphatase activity are not fully understood.

In this work, two yeast phosphatases $PP2A^{Cdc55}$ and Cdc14 were analysed for their ability to dephosphorylate Swe1, its natural substrate whose activity depend on the phosphorylation state.

The aim of the thesis was:

- To identify regions in the N-terminal intrinsically disordered part of the Swe1 important for dephosphorylation by $PP2A^{Cdc55}$ and Cdc14.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Materials

Buffers, gels, growth media, and plasmids used in this work are presented in **Tables 1-3**.

Table 1. Buffers and gels used in this work.

Name	Composition
1x TAE buffer	40 mM Tris-acetate pH 8.3 and 1 mM ethylenediaminetetraacetic acid (EDTA)
1% agarose TAE gel	1 mM EDTA, 5 µl/l Atlas ClearSight DNA Stain (BioAtlas), 40 mM Tris-acetate pH 8.3 and 1% agarose
1x PBS buffer	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4
Lysis buffer	25 mM Hepes-KOH pH 7.4, 300 mM NaCl, 10% glycerol Protease inhibitors: 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 µg/ml pepstatin A, 1 µg/ml aprotinin, 1 µg/ml DnaseI
Elution buffer	25 mM Hepes-KOH pH 7.4, 300 mM NaCl, 10% glycerol, 200 mM imidazole
6xSDS buffer	0.375 M Tris-HCl pH 6.8, 0.6 M DTT, 12% SDS (Sodium Dodecyl Sulphate), 60% Glycerol, 0.06% Bromophenol blue
SDS–polyacrylamide (SDS-PAGE) separating gel	0.375 M Tris-HCl (pH 8.8),12% acrylamide [29:1 acrylamide:bis-acrylamide], 0.1% SDS
SDS-PAGE stacking gel	0.125 M Tris-HCl (pH 6.8), 5% acrylamide [29:1 acrylamide:bis-acrylamide], 0.1% SDS)
5x kinase buffer (5xKB)	250 mM Hepes-KOH (pH 7.4), 500 mM NaCl, 25 mM $MgCl_2$, 2.5 mM ATP
Coomassie R250 staining solution	0,1 % Coomassie Blue R250 (w/w), 30 % methanol, 5 % acetic acid
Destain solution	30 % methanol, 5 % acetic acid

Table 2. Growth media and plates used in this work.

Name	Composition
LB media	5 g/l yeast extract (Formedium), 10 g/l NaCl (Chempur) and 10 g/l tryptone (Formedium)
LB agar plates with ampicillin or kanamycin	LB media, 15 g/l bacto agar (Formedium), 100 µg/ml ampicillin (Sigma) or 100 µg/ml kanamycin (Sigma)
LB agar plates with kanamycin and chloramphenicol	LB media, 15 g/l bacto agar (Formedium), 100 µg/ml kanamycin (Sigma) and 50 µg/ml chloramphenicol (Sigma).
2x YT media	16 g/l Tryptone, 10 g/l Yeast Extract, 5 g/l NaCl, 20 g/l Glucose

Table 3. Plasmids used in this work.

Name	Backbone	Description	Source
pMO415	pET28a	N-Swe1(1-450 WT)	Loog Lab
pAM103	pET28a	N-Swe1(181-450 WT=	This Study
pAM104	pET28a	N-Swe1(1-300 WT)	This Study
pAM117	pET28a	N-Swe1(121-300 WT)	This Study
pAM110	pET28a	N-Swe1(151-300 WT)	This Study
pAM111	pET28a	N-Swe1(181-300 WT)	This Study
pAM115	pET28a	N-Swe1(105-300 AP T196)	This Study
pAM116	pET28a	N-Swe1(115-300 AP T196)	This Study
pAM118	pET28a	N-Swe1(121-300 AP T196)	This Study

3.1.2 Plasmid construction

Plasmids used in this work are listed in **Table 3**.

3.1.2.1 Amplification of the Swe1 variants with PCR

To obtain different Swe1 variants for phosphorylation assay experiments, different variants of the Swe1 were cloned into pET28a plasmid with NdeI/BamHI or NheI/BamHI FastDigest™ restriction enzymes. First, Swe1 variants were PCR amplified from the *S. cerevisiae*

w303 strain genomic DNA or synthetic DNA (Swe1 1-450 amino acids where all S/TP sites are mutated to AP sites except for T196; later referred to as Swe1 AP T196) by Thermo Scientific Phusion High-Fidelity DNA polymerase. The annealing temperature for primers were calculated according to the primer specifications, and the extension time was estimated according to the synthesis rate of the polymerase (1 kb/30 s). The exact composition of the PCR reaction mixture and 3-step PCR program are shown in **Tables 4** and **5**. To confirm the correct length of the product, samples were stained with 6x Orange DNA Loading Dye (Thermo Fisher Scientific) and loaded onto 1% Agarose gel along with GeneRuler DNA Ladder 1 (or 2) (Thermo Fisher Scientific). The electrophoresis chamber was filled with 1x TAE buffer where the Agarose gel was placed and operated for 20 minutes at 180V. Next, the gel was placed under the UV light and the PCR product size was estimated by comparing sample bands and the ladder. Later, the correct PCR products were cut out of the gel, and DNA was extracted with FavorPrep™ GEL/PCR Purification Kit (Favorgen) according to the instructions provided by the manufacturer.

Table 4. PCR mixture.

Component	µl	Final Concentration
DNA template (yeast genomic DNA)	1	1-10 ng/µl
5x Phusion HF Buffer	10	1x
Forward Primer	2.5	0.5 µM
Reverse Primer	2.5	0.5 µM
dNTPs	0.5	200 µM
High-fidelity Phusion DNA polymerase	0.5	0.02 U/µl
Milli-Q H_2O	33	
Total	50	

Table 5. Three-step PCR program.

Step	Temperature	Time	Number of Cycles
Initial Denaturation	98°C	5 min	1
Denaturation	98°C	20 s	32
Annealing	X°C	20 s	
Extension	72°C	Y s	
Final Extension	72°C	5 min	1

3.1.2.2 Restriction and Ligation

Before ligating Swe1 variants and pET28a vector, both must be digested with the FastDigest™ (Thermo Fisher Scientific) enzymes to create compatible single stranded DNA overhangs. In this work, either NdeI/BamHI or NheI/BamHI restriction enzymes were used to treat both PCR products and pET28a vector. Around 1.5 µg of plasmid and 80-120 ng of PCR product were cut with the restriction enzymes. The composition of the restriction digestion mixtures is shown in **Tables 6** and **7**. Restriction digestion was carried out at 37°C for 30 min. Restriction enzymes in the mix with PCR products were heat-inactivated at 80°C for 10 minutes. The mixture containing digested plasmid pET28a were loaded onto 1% Agarose gel together with GeneRuler DNA Ladder 1 (or 2). The electrophoresis chamber was filled with 1x TAE buffer where the Agarose gel was placed and operated for 20 minutes at 180 V. Next, the gel was placed under the UV light and the correct DNA sample was cut out from the gel. Plasmid backbone DNA was then extracted from the gel using FavorPrep™ GEL/PCR Purification Kit (Favorgen) according to the instructions provided by the manufacturer.

Next, the pET28a and the Swe1 variants were ligated together using T4 DNA ligase (Thermo Fisher Scientific). In this work, molar ratio of 1:3 vector to insert was used. Around 20-50ng of the plasmid vector was used. The exact composition of the ligation mixture is shown in **Table 8**. Ligation mixtures were incubated at 16°C for 3-20 hours.

Table 6. Restriction mixture for PCR product

Component	µl
PCR Product	8
10x FD Buffer	2
NdeI or NheI	1
BamHI	1
Milli-Q H_2O	8
Total	20

Table 7. Restriction mixture for plasmid.

Component	µl
Plasmid (ca. 1.5 µg)	y
10x Green FD Buffer	2
NdeI or NheI	1
BamHI	1
Milli-Q H_2O	y
Total	20

Table 8. Ligation mixture.

Component	µl
Restricted PCR product	x(according to molar ratio)
Linearized vector	y (20-50 ng)
10x T4 DNA ligase buffer (Thermo Fisher Scientific)	1
T4 DNA ligase (Thermo Fisher Scientific)	1
Milli-Q H_2O	Up to 10

3.1.2.3 Bacterial transformation, plasmid extraction and sequencing.

Ligated plasmids were amplified in *E. coli* NEB Turbo cells. For that, chemically competent *E. coli* NEB Turbo cells were taken out of the -80°C freezer and thawed on ice. 50 µl of the chemically competent cells and 2 µl of the ligation mixture were mixed and incubated on ice

for 30 minutes. After incubation, cells were heat-shocked at 42°C for 30 seconds, followed by 5-minute cool down on ice. As the next step, 400 µl of LB media was added to every transformation tube, and transformation mixtures were incubated for 45-60 minutes at 37°C with shaking for recovery.

After recovery, transformation mixtures were centrifuged at 6000 rpm for 1 minute, 400 µl supernatant was removed from the tube, cell pellets were resuspended in the remaining supernatant and transferred to LB plate containing kanamycin (100 µg/ml), where the transformation mix was spread using glass beads. Plates were then incubated at 37°C overnight.

After bacterial colonies appeared on the plate, several of them were picked and incubated in 3 ml liquid LB medium with kanamycin (100 µg/ml) at 37°C with shaking for 4-8 hours. Plasmid DNA was extracted from cells with FavorPrep™ Plasmid DNA Extraction Mini Kit (Favorgen) according to the manufacturer instructions. The concentration and purity of the extracted plasmid DNA was assessed with Thermo Fisher's NanoDrop 1000 Spectrophotometer.

To validate that extracted plasmids contain the cloned fragment, restriction analysis was performed. Around 1.5 µg of the plasmid was restricted with NheI/NdeI and BamHI. The restriction reaction mixture is shown in **Table 9**. Restriction reactions were incubated for 30 minutes at 37°C followed by agarose gel electrophoresis. In case the restricted plasmid had 2 DNA fragments of the correct size, plasmids were sequenced to determine nucleotide sequence. The Institute of Genomics Core Facility provided the Sanger sequencing service.

Table 9. Restriction analysis mixture.

Component	µl
Plasmid (ca. 1.5 µg)	x
10x Green FD Buffer	2
NdeI or NheI	1
BamHI	1
Milli-Q H_2O	y
Total	20

3.1.3 Protein purification

For the *in vitro* dephosphorylation assay, purified N-Swe1 variants were obtained. *E. coli* cells expressing N-Swe1 variant were in cubated in the presense of the inducer for 10-14 h, cell were collected, lysed, and protein was purified using affinity chromatography method.

3.1.3.1 Culture growing conditions

BL21 *E. coli* cells were transformed with pET28a plasmids containing Swe1 variants for protein expression and purification. Transformation procedure was the same as described above. Transformed cells were plated out to LB plates containing kanamycin (100 µg/ml) and chloramphenicol (32 µg/ml) and incubated at 37°C for 12-16 hours.

On the next day, 3 ml of LB liquid media supplemented with kanamycin (100 µg/ml) and chloramphenicol (32 µg/ml) was inoculated with single colony of the BL21 *E. coli* containing plasmid and incubated at 37°C for 2-3 hours. Next, the cell culture was diluted in 250 ml of 2xYT liquid media with kanamycin (100 µg/ml) and chloramphenicol (32 µg/ml) to OD600 0.1-0.2. When OD600 reached the 0.6-0.8, cell cultures were cooled down to 16°C and the expression of the Swe1 proteins was induced by addition of Isopropyl ß-D-1-thio-galactopyranoside (IPTG) to the final concentration of 500 nM. Afterwards, cells were incubated at 16°C with shaking (200 rpm) for 10-14 hours.

Cells were collected by centrifugation (4000 rpm, 10 minutes, 4°C), resuspended in 1x PBS buffer and centrifuged again (4000 rpm, 10 minutes, 4°C). Supernatant was removed and cells were frozen in liquid nitrogen and stored at -80°C prior to protein purification.

3.1.3.2 His tag protein purification

E. coli cells expressing polyhistidine-tagged Swe1 protein variants, collected as described above, were resuspended in lysis buffer containing protease inhibitors and lysozyme (1 mg/ml) and left on end-over-end mixing at 4°C for 30 min. Afterwards, cells were sonicated 3 times for 20 seconds at ca. 40% power with 1 minute cool-down on ice between sonications. Next, lysates were centrifuged at 14800 rpm at 4°C for 20 minutes. To prepare a column, 100 µl of Chelating Sepharose beads (GE Healthcare) were first washed with 1 ml (10 column volumes (CV)) of Milli-Q H_2O, then with 200 µl of 200 mM $CoCl_2$. Lastly the columns were equilibrated with the 10x CV of the lysis buffer. After centrifugation, supernatant of the lysates was loaded to the column. The column was washed 2 times with 10x CV lysis buffer and 1 time with 10x CV lysis buffer containing 50 mM imidazole. Proteins

of interest were eluted 3 times with elution buffer. The concentration of the purified proteins was estimated using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and bovine serum albumin (BSA) standard curve. Eluted proteins were frozen in the liquid nitrogen and stored at -80°C before experiments.

3.1.4 Dephosphorylation assay

To measure the dephosphorylation efficiency of the Swe1 variants the dephosphorylation assays were performed. First, 5 µl of substrate (final concentration of ca. 1 µM) were mixed with 15 µl of Kinase pre-mix that contained Clb2-Cdk1 and incubated for 1 hour to ensure sufficient phosphorylation of the substrate in the presence of the radioactive ATP ([γ-^{32}P]-ATP (PerkinElmer). Then, 0 time point was collected (4 µl of reaction mixture + 4 µl 2x SDS buffer) to have the time point with maximal level of the phosphorylation. Next, 5 µl of the phosphatase mix with phosphatase Cdc14 (final concentration ca. 525 nM) or PP2A^{Cdc55} (final concentration ca. 10.89 nM) was added to the reaction mixture. Phosphatase mixture also contained Cyclin-Cdk1 inhibitor Sic1 to stop further phosphorylation of the substrate. 5.33 µl aliquots were collected at 4, 8, and 16 minutes after addition of the phosphatase. For all time points, the dephosphorylation reaction was stopped by mixing the aliquot with the equal amount of 2x SDS buffer and heating the samples at 80°C for 10 minutes.

Then, SDS-PAGE was performed, polyacrylamide gels were stained in Coomassie R250 staining solution, destained in destaining solution and dried.

To analyse the results, Amersham Typhoon 5 biomolecular Imager (GE Healthcare Life Sciences) was used to detect γ -^{32}P phosphorylation signals. The quantification of signals was done using ImageQuant TL (Amersham Biosciences), and Microsoft Excel was used for data analysis.

Table10. Kinase pre-mix.

Component	µl
5xKB (Table 1)	4
BSA (200 µg/ml)	0.2
Cks1 (30 µM)	0.25
Cyclin-Cdk1 (ca. 8 nM)	0.5
ATP ([γ-^{32}P]-ATP (PerkinElmer) 100 µCi/ml	0.2
Milli-Q H_2O	9.85

Table11. Phosphatase mix.

Component	µl
Phosphatase	0.8
Sic1 (1µM)	0.2
5xKB (Table 1)	1
Milli-Q H_2O	3

3.2 RESULTS AND DISCUSSION

Enzyme-substrate interactions outside the enzyme's active site affect substrate specificity and phosphorylation rate of cyclin-dependent kinases. The biological role of these docking interactions is defining the timing of the phosphorylation and, hence, ordering cell cycle events (Örd & Loog, 2019). Since the number of protein phosphatase genes in the cells is ca. four times fewer than protein kinases, the spectrum of their substrates should be broader (Lim *et al*., 2015). In recent years, specific docking motifs responsible for the recognition by phosphatases were identified in the disordered regions of protein substrates. The presence of these motifs determines binding affinity and affects dephosphorylation efficiency of the target protein (Godfrey *et al*., 2017; Hertz *et al*., 2016; Kataria *et al*., 2018) that resembles protein kinase regulation. Although some mechanisms regulating substrate specificity and efficiency of protein dephosphorylation were identified, a lot remains unknown.

In this work, N-terminal Swe1 lacking catalytic domain (N-Swe1) was used as a model substrate to map regions that positively affect the dephosphorylation. The regulatory N-terminal part of Swe1 has 8 phosphorylation sites (T45, S111, T121, S133, T196, S201, S263, and T373) and an LxF Clb2 docking motif (Harvey *et al*., 2011; Örd *et al*., 2019).

To identify regions of Swe1 important for dephosphorylation, a set of Swe1 truncated mutants was created. Swe1 variants were fused to polyhistidine protein tag, expressed in *E. coli* cells, and purified. Dephosphorylation efficiency was tested via *in vitro* dephosphorylation assay. First, N-Swe1 variants were phosphorylated by Clb2-Cdk1. The initial time point was collected after 1-hour incubation with Clb2-Cdk1. At this time point (in **Figures 7-9** it is referred to as 0 time point), Swe1 possesses a maximal level of phosphorylation. Next, Clb2-Cdk1 inhibitor Sic1, and either $PP2A^{Cdc55}$ or Cdc14 were added to initiate a dephosphorylation reaction. Aliquots were collected at 4, 8, and 16 minutes after the dephosphorylation start point. Enzymatic reactions were terminated by mixing aliquots with SDS buffer. SDS-PAGE was performed, and the dephosphorylation efficiency was quantified from autoradiographs.

3.2.1 N-Swe1 may have several distinct regions that mediate dephosphorylation by PP2A^{Cdc55} and Cdc14.

First, the ability of PP2A^{Cdc55} and Cdc14 to dephosphorylate *1-450*, *181-450, 1-300*, and *181-300* amino acids (aa) truncated variants of Swe1 was tested. In general, Cdc14 was more efficient in dephosphorylating N-Swe1 variants than PP2A^{Cdc55} (**Figure 7**).

In the case of Cdc14, truncations of either the first 180 or last 150 amino acids of N-Swe1 did not affect the dephosphorylation efficiency compared to the *1-450* variant. However, dephosphorylation of *181-300* variant was drastically impaired (about 70% of the initial phosphorylation level maintained after 16 minutes) (**Figure 7C**, right part). This leads to the hypothesis that N-Swe1 may contain two distal regulatory motifs that promote dephosphorylation. One is located in the 1-181, and another is located in the 300-450 amino acid regions of Swe1 protein. These motifs seem to be redundant, as the removal of one may be compensated by another. The removal of both, however, leads to a decreased dephosphorylation efficiency as in the case of *181-300* N-Swe1.

A similar effect was observed in the case of PP2A^{Cdc55}. Interestingly, PP2A^{Cdc55} more efficiently dephosphorylates variants that have the first 180 amino acids (*1-300* and *1-450*). This might suggest that the Swe1 regions responsible for the dephosphorylation by PP2A^{Cdc55} and Cdc14 may partially overlap.

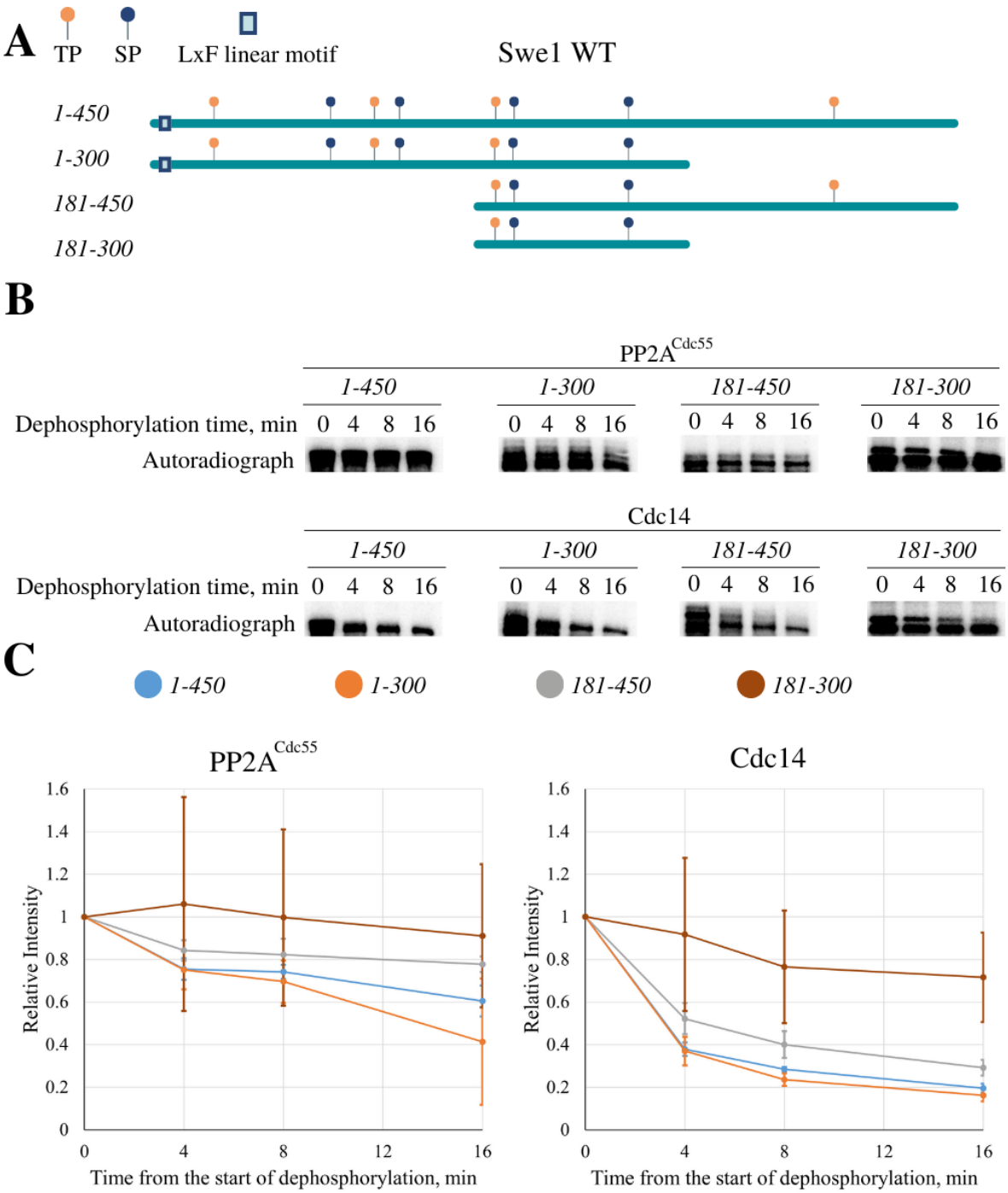

Figure 7. Dephosphorylation assay of *1-450*, *181-450, 1-300*, and *181-300* variants of N-Swe1. A. The schematic representation of N-Swe1 substrate variants used in the assay. TP and SP phosphorylation sites are shown as yellow and blue pinpoints, respectively. Clb2 LxF docking motif is marked with a light blue box. **B.** The autoradiographs of the dephosphorylation assays. **C.** Quantified dephosphorylation time courses of the *1-450*, *181-450, 1-300*, and *181-300* truncated variants of N-Swe1. Dephosphorylation levels are quantified relative to the phosphorylation intensity at 0 time point. Substrate Swe1 variants were incubated with either PP2A^{Cdc55} (left) or Cdc14 (right) phosphatases. Three repeats of the assay were performed. Error bars indicate standard deviations.

3.2.2 Stepwise truncations of the Swe1 N-terminus cause a gradual decrease in dephosphorylation rate

Since the dephosphorylation rate of N-Swe1 by both phosphatases was dependent on the presence of the first 180 amino acids, another set of truncated variants was created to map the regulatory region more precisely. Truncated variants *1-300*, *121-300*, *151-300*, and *181-300* were purified, and dephosphorylation assays were performed (**Figure 8**). In the case of Cdc14, *1-300*, *121-300*, *151-300*, and *181-300* variants showed a gradual decrease in dephosphorylation efficiency. Although $PP2A^{Cdc55}$ showed a similar dephosphorylation pattern, no difference between *121-300* and *151-300* was observed after 16 min (**Figure 8B**, top part). These results provide additional evidence of the importance of the first 180 amino acids of Swe1, although, they do not shed light on the exact location of the regulatory motif.

Interestingly, in case of Cdc14, electrophoretic shift of Swe1 dissappeared rapidly in *151-300*, but not the *181-300* variant (**Figure 8B and C**, Cdc14 panel**)**. The electrophoretic shift has been previously shown to indicate the highly phosphorylated forms of the protein (Venta *et al*., 2020). In the *151-300* variant, this shift disappeared already after 4 minutes after addition of the phosphatase. On the contrary, the phosphorylation shift remained relatively unchanged until 16 minutes in the *181-300* variant. It has been shown that docking motifs promote the dephosphorylation of multiple phosphosites in a processive manner during a single enzyme-substrate binding event (Kataria *et al*., 2018).Therefore, the rapid disappearance of the high electrophoretic shift in *151-300* variant compared to 181-300 variant indicates the presense of a Cdc14 docking motif in the 151-180 region of the Swe1.

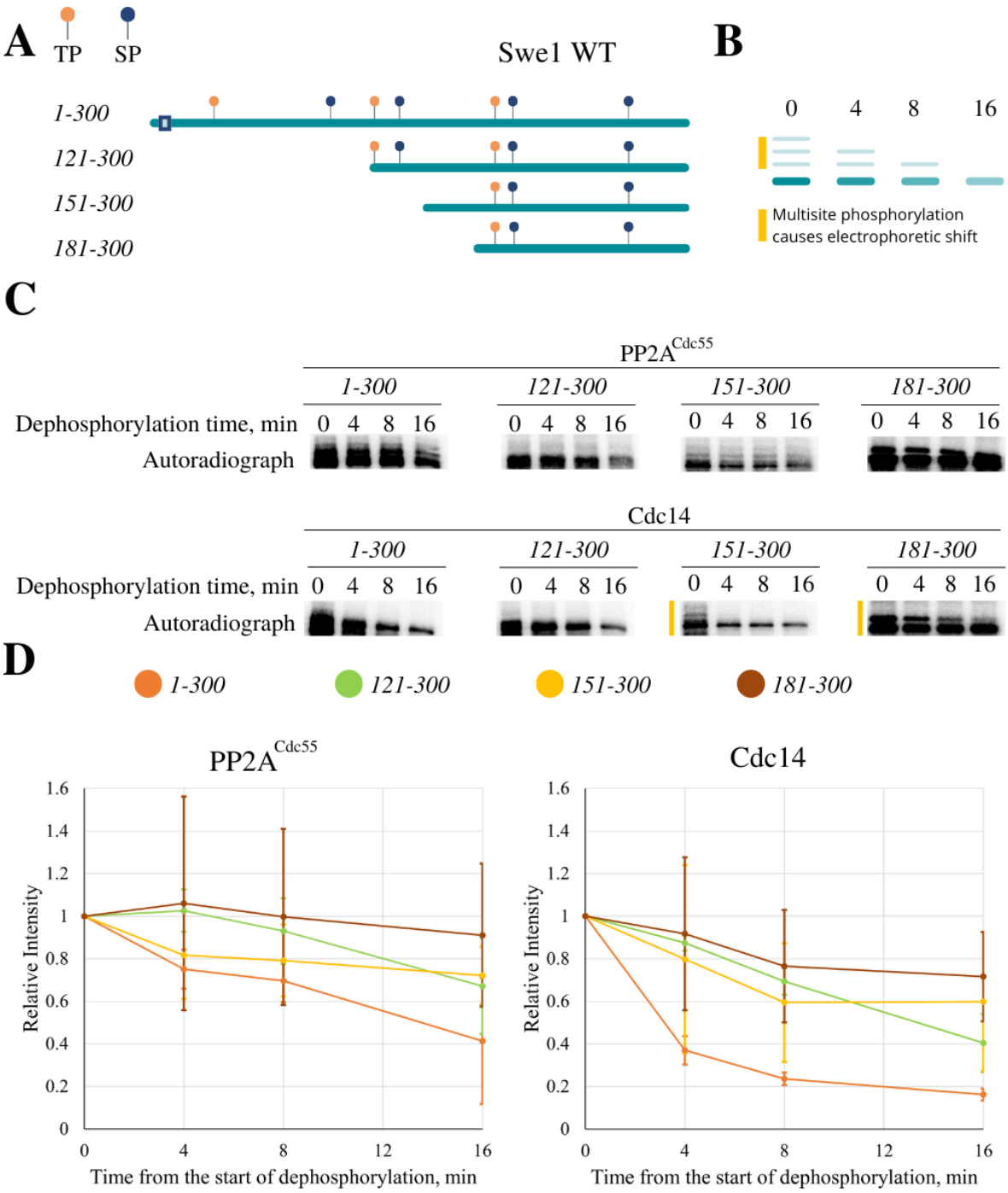

Figure 8. Dephosphorylation assay of *1-300*, *121-300*, *151-300*, and *181-300* variants of N-Swe1. A. The schematic representation of N-Swe1 variants used in the assay. TP and SP phosphorylation sites are shown as yellow and blue pinpoints, respectively. Clb2 LxF docking motif is marked with a light blue box. **B**. Schematic representation of the electrophoretic shift caused by the hyperphosphorylation. **C.** The autoradiographs of the dephosphorylation assays. **D.** Quantified dephosphorylation time courses of the of *1-300*, *121-300*, *151-300*, and *181-30* truncated variants of N-Swe1. Dephosphorylation levels are quantified relative to the phosphorylation intensity at 0 time point. Swe1 variants were incubated with either PP2A^{Cdc55} (left) or Cdc14 (right) phosphatases. Two repeats of the assay were performed. Error bars indicate standard deviations.

3.2.3 Cks1 may block dephosphorylation of T196

Truncated N-Swe1 variants used in this study have a different number, combinations, and accessibility of the phosphorylation sites. This inconsistency may cause phosphorylation/dephosphorylation fluctuations and hide the effects of the regulatory motifs. To eliminate possible signal noise from the multisite phosphorylation, a set of mutated versions of N-Swe1 containing a single phosphosite was created (referred to as Swe1 AP T196). In these variants, all serines (S) and threonines (T) of Cdk1 phosphorylation sites, except for T196, were substituted with alanines (A), which can not be phosphorylated. T196 plays an important biological role in regulating Swe1 function. It is an optimal consensus site for Clb2-Cdk1, and has been shown to be dephosphorylated by $PP2A^{Cdc55}$ (Harvey *et al*., 2011; Örd *et al*., 2019).

Swe1 AP T196 truncated variants *105-300*, *115-300*, *121-300* were purified, and dephosphorylation assays were performed (**Figure 9**). The results of the assays indicate that the dephosphorylation of the Swe1 AP T196 variants was practically absent (except for *105-300* Swe1 AP T196 variant, which lost around 50% of initial phosphorylation intensity after 16 min incubation with $PP2A^{Cdc55}$). However, the high intensity of the phosphorylation signals (**Figure 9B**) indicates that Clb2-Cdk1 efficiently phosphorylates Swe1 AP T196. In case of Cdc14 the lack of dephosphorylation of Swe1 may be caused by consensus site specificity since Cdc14 has been previously shown to dephosphorylate phoshoserines better than phosphothreonines (Bremmer *et al*. 2012).

Another possible explanation would be the binding of the phoshoadaptor Cks1 to the phosphothreonine that would lead to shielding T196 from the action of the phosphatase. However, this blocking is not absolute, as shown by the partial dephosphorylation observed in case of $PP2A^{Cdc55}$. To overcome this effect *in vitro*, a mutant version of Cks1 (which cannot bind phosphothreonines) could be used in the dephosphorylation assays, and further experiments are needed to determine whether the observed Cks1 shielding effect has an important biological role.

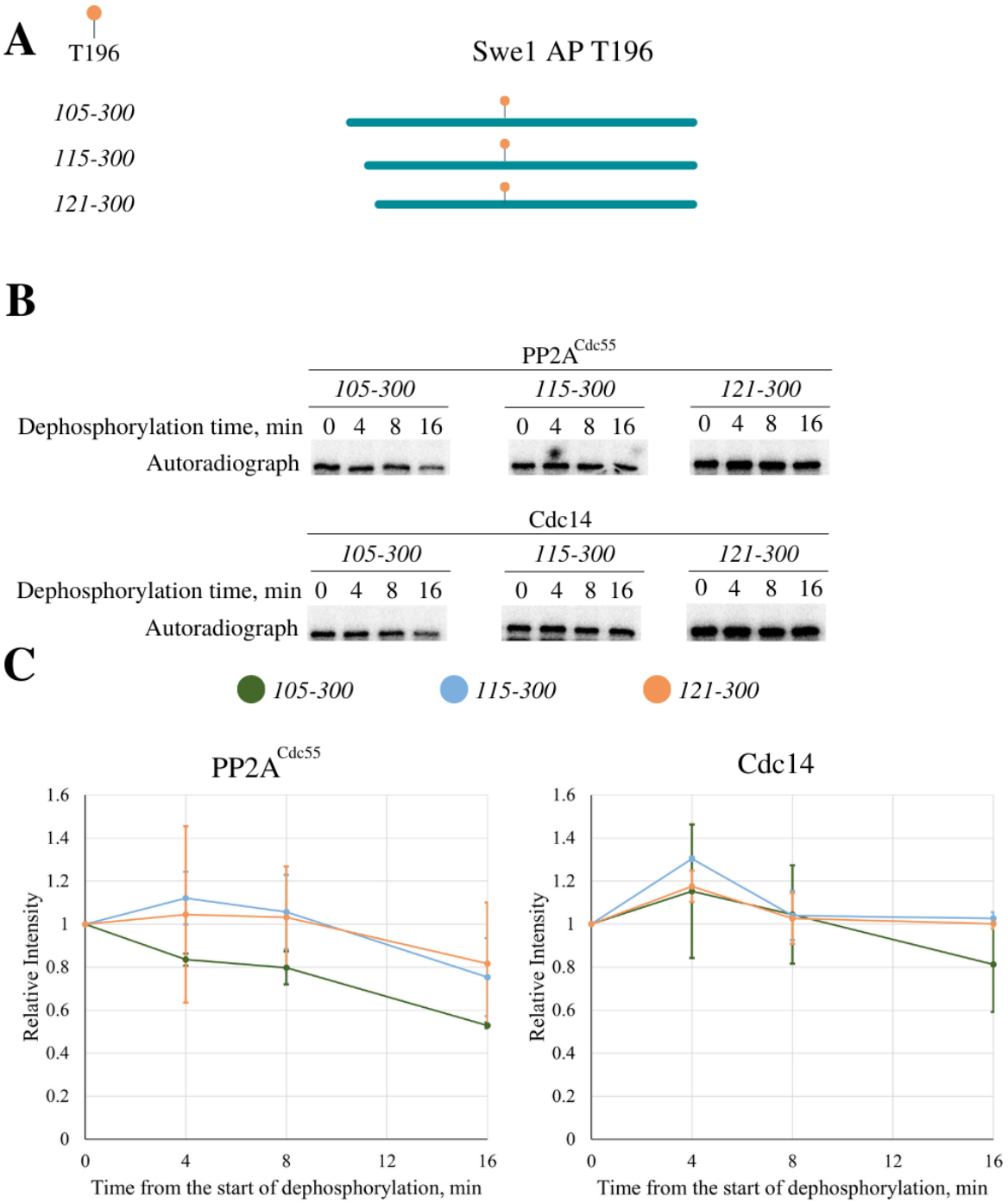

Figure 9. Dephosphorylation assay of *105-300*, *115-300*, and *121-300* AP T196 variants of N-Swe1. A. The schematic representation of N-Swe1 variants used in the assay. TP and SP phosphorylation sites are shown as yellow and blue pinpoints, respectively. Clb2 LxF docking motif is marked with a light blue box. **B.** The autoradiographs of the dephosphorylation assays. **C.** Quantified dephosphorylation time courses of the *105-300*, *115-300*, and *121-300* AP T196 variants of N-Swe1. Dephosphorylation levels are quantified relative to the phosphorylation intensity at 0 time point. Swe1 variants were incubated with either PP2A^{Cdc55} (left) or Cdc14 (right) phosphatases. Two repeats of the assay were performed. Error bars indicate standard deviations.

SUMMARY

Cdk1 inhibitor Swe1, important for the timing of cell cycle progression, is phosphorylated by Clb2-Cdk1 and dephosphorylated by $PP2A^{Cdc55}$ and Cdc14 in yeast. The mechanisms of Swe1 targeting by phosphatases are not yet fully understood. In this work, N-terminal Swe1 (lacking catalytic domain) was used as a model substrate to identify regulatory regions that affect its dephosphorylation by $PP2A^{Cdc55}$ and Cdc14 phosphatases *in vitro*.

We found at least two regions that are important for Swe1 dephosphorylation. These are located between positions 1-180 and 300-450 in Swe1, respectively. Moreover, these putative regulatory elements may play a redundant role in the case of Cdc14. $PP2A^{Cdc55}$ showed a greater dependence on the 1-180 region of the Swe1, since deletion of this region led to decreased dephosphorylation of the substrate. Multiple regulatory elements might be present in the Swe1 1-180 region since its stepwise truncations caused a gradual decrease in the dephosphorylation efficiency. In addition, the region from positions 151-180 of Swe1 was shown to be important for dephosphorylation of multiple sites by Cdc14.

In further experiments, the use of truncated mutated forms of Swe1 with a single phosphosite in combination with a mutated Cks1 subunit that cannot bind phosphothreonine would be beneficial in revealing precise mechanisms of Swe1 dephosphorylation.

Current work provides the first steps in discovering the mechanism of Swe1 dephosphorylation. This can be used as a basis for further research that would aim at shedding more light on the coordinated action of kinases and phosphoatases in the cell cycle control. Ultimately, phosphatase docking motifs may be utilized in the synthetic biology and used as additional level of control of metabolic enzymes in the cell factories.

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