Qualitative and Quantitative Cdk Control of the Budding Yeast Cell Cycle

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Declaration

I, Deniz Pirinççi Ercan, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Timely and ordered progression through the cell cycle is crucial for error-free proliferation of cells. In eukaryotes, cell division cycle is controlled by the master regulator cyclin-cyclin dependent kinase (Cdk) complexes. However, it is still unclear how cyclin-Cdk complexes ensure the order in the cell cycle so that DNA replication in S phase always precedes chromosome segregation in mitosis.

Two models have been put forward to explain cell cycle ordering by cyclin-Cdk complexes. The qualitative model suggests that distinct substrate specificities of the different cyclins at successive cell cycle stages order substrate phosphorylation. In contrast, the quantitative model for Cdk control of the cell cycle suggests that the overall gradual increase in Cdk activity from G1 to mitosis orders cell cycle progression. In line with the quantitative model, a single cyclin-Cdk complex is sufficient to order the fission yeast cell cycle. However, the relative contributions of qualitative and quantitative Cdk control in other organisms is incompletely understood.

In this project, I investigate cyclin specificity and redundancy in the budding yeast *S. cerevisiae*, which encodes three G1 (Cln1-3), two S (Clb5 and Clb6) and four G2/M (Clb1-4) cyclins that are orthologous to those found in many metazoans, including humans. With an aim to identify the minimal set of cyclins required to drive the ordered cell cycle progression in budding yeast, I have removed seven of the nine cyclins, establishing a strain harbouring one G1 cyclin, Cln2, and a mitotic cyclin, Clb2, that is expressed from an S phase cyclin *CLB5* promoter in addition to its own promoter. In this strain, expressing a third copy of Clb2 under control of *CLN2* promoter is sufficient to order DNA replication and chromosome segregation in the absence of Cln2. However, these cells cannot polarise or form buds. My findings indicate that the budding yeast G1 cyclin Cln2 has evolved to carry unique crucial functions to couple cell cycle progression to morphogenetic events.

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I was just a teenager at high school when I decided to study molecular biology & genetics at university. Not many people around me knew what it meant to be a molecular biologist & geneticist, nor did they understand my choice of this unknown future over a “canonical pathway”, studying medicine or engineering. My loving parents, Nurten & Bülent, however, were the only exception to those people. Since the day I made my mind to pursue this career, they have always been beside me, supporting me in any way that one can think of. Therefore, first and foremost, I would like to thank my parents for their infinite love and trust in me. I would not be able follow my dreams without your endless support. “Sizi çok seviyorum canım ailem.”

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... In memory of my loving grandma, Nurten

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Abbreviations

AEBSF	4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride
APC/C	Anaphase promoting Complex/Cyclosome
BSA	Bovine serum albumin
CAK	Cdk activating kinase
Cdk	Cyclin dependent kinase
Cks	Cdc28 kinase subunit
Cdc	Cell division cycle
DAPI	4',6-Diamidino-2- phenylindole
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetraacetic acid
FEAR	Fourteen early anaphase release
GAP	GTPase activating protein
GEF	Guanine exchange factor
GTP	Nucleotide guanosine triphosphate
h	Hour
HA	Hemagglutinin
HRP	Horseradish peroxidase
HU	Hydroxyurea
kDa	Kilodalton
MAP	Mitogen activated protein
MBF	MluI cell cycle box binding factor
MEN	Mitotic exit network
min	Minute
mM	Millimolar
ml	Millilitre
Ndd	Nuclear division defective
NEBD	Nuclear envelope breakdown

NES	Nuclear export signal
Net	Nucleolar silencing establishing factor & telophase regulator
NLS	Nuclear localization signal
nM	nanomolar
ORC	Origin recognition complex
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PI	Propidium iodide
PPP	Phospho protein phosphatase
PSP	Protein serine phosphatase
PTP	Protein threonine phosphatase
RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	Revolutions per minute
Rts	Rox three suppressor
s	Seconds
SBF	Swi4/6 cell cycle box binding factor
SCF	Skp, Cullin, F-box
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sld	Synthetically Lethal with Dpb11-1
SLiMs	Short linear motifs
TCEP	Tris (2-carboxyethyl) phosphine
WT	Wild type
YP	Yeast peptone
YPD	Yeast peptone dextrose
µl	Microlitre
µm	Micrometer
µM	Micromolar
Zds	Zillion different screens

Chapter 1. Introduction

It was as early as the 1600's when a cell was described after Robert Hooke observed "pores" in a thin slice of bottle cork under a microscope. Hooke did not know that those pores were the empty cell walls of a plant tissue, hence not alive. However, his findings triggered the curious minds of his time to further investigate the cells. In 1676, Anton van Leeuwenhoek, for the first time, observed living organisms under a microscope. Named by Leeuwenhoek as "animalcules", the discovery of protozoa and bacteria added a layer of complexity to Hooke's observations because they were motile, thus alive.

Later, in 1838, Theodor Schwann and Matthias Schleiden postulated the cell theory that the cells are the basic unit of life and all living organisms are composed of cells. When Rudolf Virchow proposed "Omnis cellula e cellula", the cells arise from existing cells, in 1855 the three tenets of the traditional cell theory together laid the foundations of modern cell biology as we now know.

Following Virchow's famous statement, Hugo von Mohl was first to observe cell division in green algae, in 1835. Furthermore, Walther Flemming was also a pioneer in cell biology with his remarkable hand-drawn illustrations describing mitosis in salamander fins and gills, in 1878.

Today, almost 350 years after humankind first observed cells under a microscope, we are still far from the complete understanding of cell cycle control. In this chapter, I introduce our current knowledge on the mitotic cell division cycle and its regulation by the master regulators. I next provide insights into the budding yeast cell cycle and discuss the use of budding yeast as a model organism to study cell division. I end by presenting the findings on the two models that have been proposed to explain the ordered cell cycle progression and how these models allowed me to formulate my research questions.

1.1 The eukaryotic cell division cycle

The cell division cycle is an intricately ordered series of molecular events during which a mother cell gives rise to two genetically identical daughter cells. During cell division, the chromosomal events must be orchestrated such that the duplication of genomic material in synthesis, S phase is always completed prior to its segregation into the daughter cells in mitosis, M phase. These main events are separated by gap phases, G1 and G2, during which cells grow. Gap phases are also important to link cell growth to cell cycle progression.

The period between the two M phases is known as interphase and each M phase can also be divided into further stages, namely, metaphase, anaphase and cytokinesis. After completion of S phase, replicated DNA condenses to form sister chromatids. In metaphase, the sister chromatids are positioned in the middle of the cell and are attached to the microtubules emerging from spindle poles. Anaphase is marked by the separation and movement of sister chromatids towards the opposite spindle poles. When the sister chromatids are fully segregated and distributed equally into two nuclei, the contractile actomyosin ring that is formed at the division site starts constricting in cytokinesis. The cell division is then completed upon physical separation of the mother cell into two.

Whilst the cells that are actively proliferating are going through the main cell cycle stages, some cells can be present at a non-dividing stage called G0. Most of the time the cells enter G0 under restrictive growth conditions. Therefore, once the environmental factors become favourable, the cells can re-enter the cell division cycle. However, if the cells are terminally differentiated or have entered senescence, they stay arrested in G0.

Although some elements of the cell cycle slightly differ between organisms such as the length of each cell cycle phase, the process of nuclear division in mitosis (open vs. closed mitosis) and whether the cell breaks symmetry during division (symmetric vs. asymmetric division), the order in eukaryotic cell division is fundamentally conserved throughout evolution.

1.1.1 Phosphorylation and dephosphorylation events drive the cell cycle

The cell division is a process driven by the activity of protein kinases which "phosphorylate" their substrates by catalysing transfer of a phosphate from an ATP molecule to an amino acid with a hydroxyl group such as serine (S), threonine (T) or tyrosine (Y). These substrates can then act in many different signalling networks and perform diverse tasks in a cell.

Cyclin-cyclin dependent kinase (Cdk) complexes are the key players in cell division. The cyclin-Cdks allow ordered and accurate cell cycle progression by controlling phosphorylation of their targets in time and space. For instance, substrates involved in early events such as DNA replication in S phase will always get phosphorylated before the late mitotic events such as spindle formation and elongation. Similarly, in budding yeast, for example, the cyclin-Cdk targets to be phosphorylated at the spindle pole bodies (SPBs; yeast centrosomes) will always precede phosphorylation of bud neck proteins which is required to allow cytokinesis.

Phosphorylation is a post translational modification in eukaryotic cells which is very stable, but can be reversed by phosphatases. The reversibility of phosphorylation is vital, in particular for the cell cycle control, because dephosphorylation events bring about mitotic exit during which the cell cycle is reset in preparation for the next round of cell division. The interplay between cell cycle kinases and phosphatases determines the net phospho-state of a target protein (Queralt and Uhlmann, 2008b; Uhlmann et al., 2011). Furthermore, the kinase to phosphatase activity ratio allows each substrate to be dephosphorylated at a specific time during mitotic exit (Bouchoux and Uhlmann, 2011; Touati et al., 2019). Other kinases such as Plk (Polo like kinase) and Aurora kinase also play a role during mitotic exit (Afonso et al., 2017; Botchkarev and Haber, 2018; Gelens et al., 2018). Recently, Plk, Aurora and NDR (nuclear Dbf related) kinases were shown to trigger as many phosphorylation events as dephosphorylation events in yeast using a time resolved phosphoproteome analysis (Swaffer et al., 2018; Touati et al., 2018). Altogether, the dynamic relationship between the kinases and phosphatases support temporal order of the cell cycle.

1.1.2 Resetting the cell cycle requires protein degradation

The cyclin-Cdk complexes are the main drivers of the cell cycle and their activity is tightly regulated. Ubiquitin mediated degradation of cyclins is one of the mechanisms controlling the cyclin-Cdk activity. There are two main E3 ubiquitin ligases in eukaryotes that are implicated in the cell cycle control, namely, APC/C (anaphase promoting complex/cyclosome) and SCF (Skp/cullin/F-box-containing). The APC/C plays a role in G2/M transition and subsequent G1 phase and the SCF complex acts in G1/S transition.

The APC/C is a multi-subunit protein complex which has two coactivators, Cdc20 and Cdh1. Both Cdc20 and Cdh1 are important for the recognition of short linear motifs (SLiMs) in APC/C substrates. Located in the disordered parts of target proteins, SLiMs are composed of less than ten amino acids long stretches with three to four essential residues promoting interactions with APC/C coactivators. There are three SLiMs that have been identified to date in APC/C targets, namely, the destruction box (D box), RxxLxx(I/L/V/K), the KEN box and the ABBA motif, (I/L/V)(F/H/Y)x(D/E) (Figure 1.1.). Whilst APC/C^{Cdc20} recognises D box and ABBA motif, APC/C^{Cdh1} can bind to all three SLiMs (Di Fiore et al., 2015; Glotzer et al., 1991; Pfleger and Kirschner, 2000).

Cdc20 starts accumulating in cells during S phase and APC/C^{Cdc20} is activated upon phosphorylation by mitotic cyclin-Cdk complexes in metaphase (Fang et al., 1998; Prinz et al., 1998). The APC/C^{Cdc20} then ubiquitinates and targets S and M cyclins for degradation (Kraft et al., 2003; Rudner and Murray, 2000). Activation of the APC/C^{Cdc20} also causes degradation of securin which is an inhibitor of protease separase. Following securin degradation, separase cleaves cohesin and allows sister chromatid segregation towards the opposite poles of the cell (Ciosk et al., 1998; Jager et al., 2001; Uhlmann et al., 1999; Waizenegger et al., 2000). At this point, Cdk counteracting phosphatases contribute to the reversal of Cdk mediated substrate phosphorylation and APC/C^{Cdc20} is inactivated as the cells exit mitosis. From late mitosis through the G1 phase of the cell cycle, APC/C^{Cdh1} completes degradation of mitotic cyclins and help maintenance of low kinase activity (Zachariae et al., 1998). During the G1/S transition, the rise in cyclin-Cdk activity phosphorylates Cdh1 and inactivates APC/C^{Cdh1} (Jaspersen et al., 1999; Lukas et al., 1999).

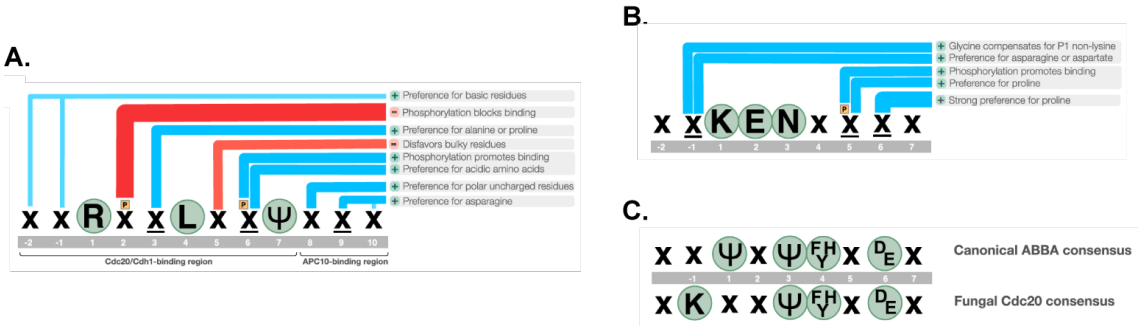


Figure 1.1. APC/C binding motifs

Preference of the **A.** D box **B.** KEN box **C.** ABBA motif binding pocket residues. Note the variation between the canonical and yeast ABBA motif. "ψ" depicts a leucine, isoleucine, or valine residue. "x" can be any amino acid unless marked as disfavoured. Underlined "x" can be any residue with a preference as pointed out for each motif. "P" indicates phosphorylation of the residue and the residues in green circles are the consensus residues of the motifs. Adapted from (Davey and Morgan, 2016).

Simultaneously with the inactivation of APC/C^{Cdh1}, the SCF complexes start promoting the G1/S transition. Similar to the APC/C, the SCF is also a multi-subunit complex. SCF is composed of three core proteins, Skp1, Cul1 and Rbx1, and an adapter F-box protein which is crucial for substrate binding. The F-box proteins recognise phosphorylated SLiMs called phosphodegrons in their targets (Skowyra et al., 1997). There are three main F-box proteins in higher eukaryotes, namely, Skp2, Fbw7 and β-Trcp (Cardozo and Pagano, 2004). Although the SCF is mainly responsible for the regulation of cyclin-Cdk kinase activity by degrading Cdk inhibitors and G1/S cyclins (Ganoth et al., 2001; Koepp et al., 2001; Watanabe et al., 2004), in the form of SCF^{Skp2}, it can also regulate cell division cycle by ubiquitinating the licencing factors, Orc1 and Cdt1, and preventing re-replication (Li et al., 2003; Mendez et al., 2002).

1.1.3 Cell cycle checkpoints

Cell cycle checkpoints are required to delay cell cycle progression to allow correction of the errors that may occur throughout the cell cycle. Faithful genome duplication and chromosome segregation can be accomplished only when these errors are sensed and corrected. The checkpoints are highly conserved from yeast to human and are essential for the maintenance of genome integrity. There are three major checkpoints during cell

division, namely, G1/S, G2/M and metaphase to anaphase transition (also known as the mitotic checkpoint).

The G1/S transition marks the cell cycle entry. When the cell reaches to a certain size, if the environmental signals and mitogens are supportive of growth and proliferation, cells commit to divide. The passage through G1/S checkpoint is irreversible and supported by the activation of transcriptional programmes and positive feedback loops. Therefore, cells will complete division regardless of the environmental conditions upon cell cycle entry.

The purpose of G2/M checkpoint is to ensure that replication is completed and DNA damage is repaired before mitotic onset. During S phase, replication stress and DNA crosslinks can cause single-stranded DNA formation hence the activation of DNA damage repair (DDR) signalling. There are many proteins implicated in DDR pathways. ATR/Chk1 signalling, for example, can stall replication forks, prevent mitotic entry by inhibiting cell cycle kinases Cdk and Plk and promote lesion repair as part of their role in G2/M checkpoint (Lemmens and Lindqvist, 2019; Sancar et al., 2004). When DDR is completed, cells can enter mitosis.

Following the passage through G2/M checkpoint, the activity of M cyclin-Cdk complexes allow mitotic spindle assembly and drive cells to metaphase. At this stage, together with the APC/C activity, bi-oriented attachment of all sister chromatids to the spindles from opposite poles bring about the metaphase to anaphase transition. Spindles are attached to the centromeres of sister chromatids through a protein complex called the kinetochore. The microtubule-kinetochore attachments as well as the tension at kinetochores are monitored in metaphase. In case the spindle attachments are defective and/or the kinetochores lack tension, the mitotic checkpoint is activated. This checkpoint activation delays degradation of M cyclins by inhibiting APC/C^{Cdc20} and halts the cell cycle in metaphase with high cyclin-Cdk activity (Musacchio, 2015; Pines, 2011). Interestingly, S cyclins can be degraded even in the presence of the active mitotic checkpoint. This is because Cdk regulatory subunit Cks1 can still direct S cyclin-Cdk complexes to the APC/C^{Cdc20}, and the ABBA motif in S cyclin facilitates its interaction with the APC/C^{Cdc20} (Di Fiore et al., 2015; Di Fiore and Pines, 2010). The mitotic checkpoint is

satisfied when microtubule-kinetochore attachments are corrected. APC/C^{Cdc20} can then degrade securin and M cyclins to allow the metaphase to anaphase transition.

In addition to the abovementioned checkpoints, there are two additional checkpoints in budding yeast, the morphogenesis checkpoint and the spindle position checkpoint (SPOC). Whilst the former postpones nuclear division in the absence of a bud, the latter inhibits mitotic exit if the mitotic spindle is not oriented accurately along the mother–daughter axis (Caydasi et al., 2010; Lew, 2003). Altogether, these safeguarding mechanisms prevent uncoupling of bud formation and nuclear division as well as the generation of binucleated cells.

1.2 The master regulators of the cell cycle: Cyclin-Cdk complexes

1.2.1 Cyclin-Cdk activity drives the cell cycle

The precision in timing and directionality of the cell cycle events is crucial for cell proliferation and maintenance of genomic integrity. Cyclin-Cdk complexes are the major controllers of timely and ordered cell cycle progression as well as transcription. Discovered in yeast genetic screens in 1970's, the kinase component of cyclin-Cdk complexes has been shown to be indispensable for the progression of the cell cycle (Hartwell et al., 1970; Hartwell et al., 1973; Nurse and Thuriaux, 1980; Nurse et al., 1976). Subsequent studies demonstrated the possibility of complementing yeast Cdk mutants with human Cdk homologs (Draetta et al., 1987; Elledge and Spottswood, 1991; Lee and Nurse, 1987), highlighting the conserved role of Cdk throughout evolution.

Cdks, serine/threonine protein kinases, require activatory phosphorylation and pairing with non-enzymatic cell cycle regulated proteins, cyclins, to become fully functional. No kinase can be called a Cdk unless its kinase activity depends on its interaction with cyclin-like regulatory subunits. Cdks contain a conserved PSTAIRE motif in their N terminal lobe which interacts with cyclins (see 1.2.2. Structural insights into the activation of cyclin-Cdk). In addition to PSTAIRE Cdks, there are other Cdk related kinases. Although these Cdk related kinases show sequence similarity to bona fide Cdks, they differ in their

PSTAIRE motif. When Cdk related kinases were first discovered, it was unknown whether they interact with cyclins. Therefore, the Cdk related kinases could not be called Cdk and named after their unique PSTAIRE motifs: PCTAIRE 1-3, PFTAIRE 1-2, PITAIRE, KKIALRE, PISSLRE, NKIAMRE and PITSLRE (Meyerson et al., 1992). When cyclin binding partners of the Cdk related kinases were identified (e.g. PCTAIRE3 interacts with Cyclin K (Rual et al., 2005), PFTAIRE1 is activated by Cyclin D3 and Cyclin Y (Jiang et al., 2009; Shu et al., 2007)), the use of Cdk for all Cdk family proteins were proposed in 2009 (Malumbres et al., 2009). Currently, in mammals, there are twenty Cdks (Cdk1-20) which are implicated in diverse cell cycle regulatory and tissue specific roles.

Table 1 Mammalian cyclin-Cdk complexes (Adapted from (Malumbres, 2014))

Related to cell cycle	
Cdk1,2,3	Cyclin A,B,E and Cyclin C (?)
Cdk4,6	Cyclin D
Cdk5,14,15,16,17,18,	Cdk5R1, Cdk5R2, Cyclin D,Y
Related to transcription	
Cdk7,20	Cyclin H
Cdk8,19	Cyclin C
Cdk10,11	Cyclin M,L
Cdk9	Cyclin T
Cdk12,13	Cyclin K

Cell cycle related Cdks are expressed constitutively in throughout the cell cycle. Thus, Cdk activity is not regulated by the quantity of Cdk in the cells. In contrast, changes in the concentration and the identity of cyclins allow oscillations in kinase activity during cell cycle. Altogether, Cdk phosphorylation, changes in subcellular cyclin-Cdk localisation, binding of stoichiometric inhibitors to Cdk and cyclin expression and degradation cycles regulate cyclin-Cdk activity (King et al., 1996; Mendenhall and Hodge, 1998; Pines, 1995; Sherr and Roberts, 1999; Solomon and Kaldis, 1998). The

differentially activated cyclin-Cdk complexes can then drive specific cell division processes.

In higher eukaryotes, the cells encode a number of different cell cycle related Cdks and cyclins. Among Cdks, the most important ones are Cdk1, Cdk2, Cdk4 and Cdk6 because of their stage-specific involvement in cell cycle progression. Cyclins, on the other hand, can be categorised in four classes based on their expression patterns and the roles during the cell cycle, namely, G1, G1/S, S and M cyclins. Whilst the G1 cyclins are responsible for coupling extracellular stimuli, cellular growth and cell cycle entry, the G1/S cyclins promote progression through the restriction point (R in higher eukaryotes or "Start" in budding yeast) and help initiation of DNA duplication. After passing through R, the cells are fully committed to divide unless they encounter perturbations in the coming stages of the cell cycle. Next, the S cyclins help completion of replication and the M cyclins bring about mitotic events. The levels of M cyclins are the highest in metaphase. In anaphase, degradation of S and M cyclins by the APC/C allows mitotic exit and concludes the cell division cycle. In mammalian cells, G1 cyclins activate Cdk4 or Cdk6, G1/S cyclins bind to Cdk2, S cyclins form complexes with Cdk1 or Cdk2 and M cyclins act together with Cdk1 (Morgan, 1997; Morgan, 2007; Nigg, 1995).

The cyclin-Cdk complexes phosphorylate their substrates at the minimal serine/threonine-proline (S/T-P) consensus motifs, with a preference for a basic residue, lysine (K) or arginine (R), at the +3 position, (S/T)Px(K/R) (Alexander et al., 2011; Errico et al., 2010; Holmes and Solomon, 1996; Songyang et al., 1994) and exhibit specificity towards a set of targets by employing SLiMs in substrate recognition and docking (Figure 1.2.). The presence of SLiMs in substrates can potentiate their phosphorylation by a cyclin-Cdk complex that would otherwise possess low intrinsic kinase activity towards this target. Therefore, it is natural for distinct cyclin-Cdk complexes to present differential kinase activity towards the same substrates (Bhaduri and Pryciak, 2011; Faustova et al., 2020; Koivomagi et al., 2011b; Loog and Morgan, 2005; Ord et al., 2020; Ord et al., 2019b; Schulman et al., 1998; Wilmes et al., 2004). Substrate phosphorylation by cyclin-Cdk complexes can be activatory or inhibitory, can cause changes in cellular localisation of their targets or can mark substrates for proteasomal degradation.

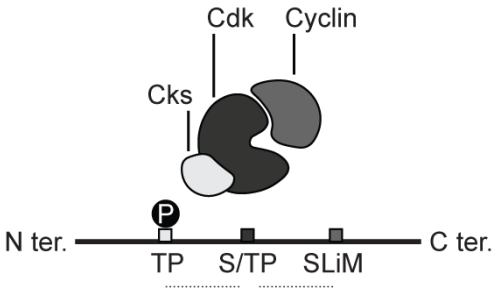


Figure 1.2. Cyclin substrate docking interactions

Schematic indicating the interactions between cyclin-Cdk complexes and their substrates that regulate the substrate phosphorylation rate and specificity. Cyclin-Cdk complexes recognise and phosphorylate full (S/T)Px(K/R) and minimal (S/T)P consensus motifs. Phosphorylation of a substrate can be potentiated by docking interactions such as Cks binding to phosphorylated TP sites (see 1.2.5. Cks: Phospho-adaptor of cyclin-Cdk complexes) and/or by cyclin-Cdk docking to SLiMs. The relative positioning of docking sites and phosphorylation sites along the disordered regions of substrate is essential in determining the effect of docking as indicated by dashed lines. Adapted from (Örd M., 2018).

1.2.2 Structural insights into the activation of cyclin-Cdk

Cdk is inactive in its free form. Cyclin binding and activatory phosphorylation of Cdk are the prerequisites for complete activation of cyclin-Cdk complexes. How cyclin binding can activate Cdk and why activatory phosphorylation is necessary for the formation of an active cyclin-Cdk complex have been a mystery for decades. Only after the structural studies could we explain the mechanistic insights into cyclin-Cdk activation.

The very first Cdk crystal structure solved in 1993 revealed the properties of monomeric Cdk2. The structure of Cdk2 is comprised of N terminal β-sheets and C terminal α-helices as well as a catalytic cleft for ATP binding. Moreover, a regulatory loop (T-loop) predicted to contain the phosphorylation site for Cdk activation and an α1-helix with a conserved amino acid sequence PSTAIRE are the peculiarities of Cdks compared to the other protein kinases. In the structure of monomeric Cdk2, the T-loop is oriented in a way to block the entrance of the ATP binding cleft. In addition, the position of major residues at the active site is not compatible with kinase reactions. Therefore, Cdk was anticipated to undergo conformational changes upon cyclin binding and activatory phosphorylation (De Bondt et al., 1993).

Although all Cdks share smaller N terminal and larger C terminal lobes together with an activation loop and a conserved PSTAIRE helix, cyclins are diverse in their sequence except for their Cdk binding domain. The structural studies of the S cyclin, cyclin A, monomer revealed that the Cdk binding domain is composed of approximately a hundred amino acid long stretch, known as the conserved cyclin box. Cyclin A has two tandem cyclin boxes each containing five α-helices. The first five of these α-helices define the conserved cyclin fold and are implicated in binding to Cdk. The latter five α-helices, on the other hand, display negligible homology to the main cyclin box and cannot bind to Cdk (Brown et al., 1995).

Only 2 years after the monomeric Cdk2 structure had been solved, the crystal structure of the cyclin A-Cdk2 complex was elucidated (Figure 1.3.A). Upon cyclin binding, the Cdk α1-helix containing PSTAIRE residues moves towards the ATP catalytic cleft and lock onto the cyclin box. Furthermore, the αL12 helix positioned N terminally to the T-loop melts to become a β-strand. Melting of αL12 helix is of great importance because it allows movement of the T-loop away from the ATP binding pocket which would otherwise block the catalytic cleft in monomeric Cdk and prevent the motion of PSTAIRE helix (Figure 1.3.C and D) (Jeffrey et al., 1995). In addition, comparative structural analysis of the bovine cyclin A monomer and human cyclin A-Cdk2 complex showed that cyclin A does not undergo conformational changes upon binding to Cdk (Figure 1.3.B) (Brown et al., 1995). These findings revealed the role of cyclin binding in Cdk activation, but how does activatory Cdk phosphorylation fit in this mechanism?

Cdk activatory phosphorylation contributes to the complete activation of cyclin-Cdk complexes in two ways, by stabilising the complex and helping with the substrate binding. Upon cyclin binding, the Cdk T-loop interacts with the cyclin. This interaction makes the Cdk activatory phosphorylation site present on the T-loop more accessible for Cdk activating kinases. Phosphorylation of the T-loop can then allow formation of additional contacts between the T-loop and cyclin which is believed to stabilise cyclin-Cdk interactions. In addition, T-loop phosphorylation was proposed to affect the presumptive substrate binding site (Russo et al., 1996b).

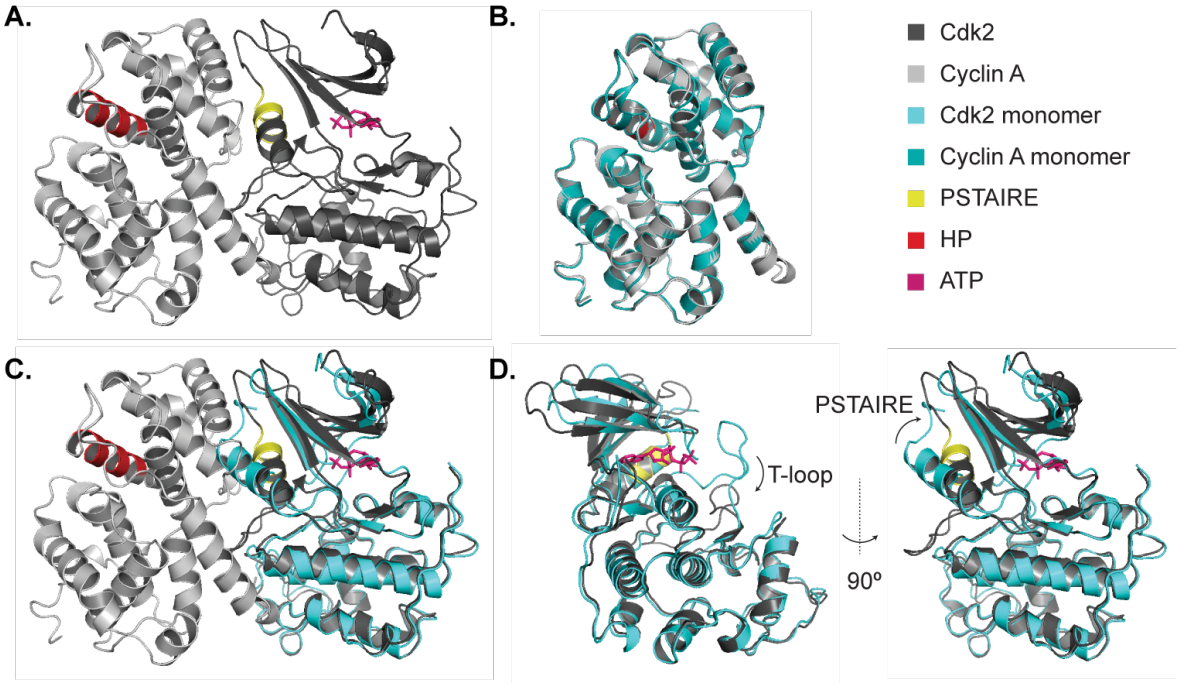


Figure 1.3. Crystal structure of cyclin A-Cdk2

A. Human Cdk2 bound to cyclin A2 (PDB ID: 1FIN). PSTAIRE helix, cyclin hydrophobic patch (HP) and ATP are highlighted. **B.** Overlay of cyclin A2 from (A) with a monomeric bovine cyclin A3 (PDB ID: 1VIN). Cdk2 is not shown for simplicity. Note that cyclin A does not change confirmation upon binding to Cdk. **C.** Overlay of Cdk2 from (A) with a monomeric human Cdk2 (PDB ID: 1HCL). Note the movement of PSTAIRE helix towards the cyclin when cyclin binds to Cdk. **D.** As in (C). Cyclin A2 is not shown for simplicity. Upon cyclin binding, melting of αL12 helix (see below the T-loop) allows T-loop movement away from the ATP binding pocket. This movement makes the catalytic cleft accessible and allows PSTAIRE helix-cyclin interaction.

The structural analysis of mammalian G1, S and M cyclin-Cdk complexes (cyclin D1 or D3-Cdk4, cyclin A2-Cdk2 and cyclin B1-Cdk1, respectively) suggested that not all Cdks follow the same conformational changes upon cyclin binding. While cyclin A2 and B1 binding to Cdk2 and Cdk1, respectively, can modify the Cdk active site to activate cyclin-Cdk complexes, cyclin D binding does not trigger structural changes in Cdk4 (Brown et al., 2015). Interestingly, the activation loop in the cyclin D-Cdk4 structure was found to be disordered or conflicting substrate binding. Therefore, it is anticipated that the activation of cyclin D-Cdk4 complex is achieved after the interaction between cyclin D and a substrate triggered conformational change in Cdk4 (Takaki et al., 2009).

1.2.3 Phosphorylation of Cdk regulates cyclin-Cdk activity

Cdk activation requires both cyclin binding and phosphorylation of Cdk. Cdk activating kinases (CAKs) target the Cdk T-loop (T160 in Cdk2, T161 in Cdk1 and T169 in budding

yeast Cdk) for activatory phosphorylation (Espinoza et al., 1996; Fisher and Morgan, 1994; Kaldis et al., 1996). In metazoans, CAK consists of a catalytic subunit Cdk7, a regulatory subunit Cyclin H and a RING finger protein Mat1 (Fisher and Morgan, 1994; Tassan et al., 1995). The cyclin H-Cdk7-Mat1 complex forms part of a general transcription factor TFIIH and has dual specificity for Cdk phosphorylation and RNA polymerase II C-terminal domain (Pol II CTD) phosphorylation (Larochelle et al., 2006; Serizawa et al., 1995; Shiekhattar et al., 1995). In budding yeast, on the other hand, a monomeric kinase Cak1 is responsible for T-loop phosphorylation and it is not implicated in Pol II CTD phosphorylation (Espinoza et al., 1996; Kaldis et al., 1996). Instead, Cak1 activates Kin28, Bur1 and Ctk1 which can then regulate transcription by phosphorylating Pol II CTD (Espinoza et al., 1998; Ostapenko and Solomon, 2005; Yao and Prelich, 2002). Although Cak1 phosphorylates Cdk prior to cyclin binding in budding yeast (Ross et al., 2000), in higher eukaryotes cyclin binding precedes and stimulates Cdk phosphorylation (Desai et al., 1992; Fisher and Morgan, 1994; Kaldis et al., 1998). In the case of the S cyclin-Cdk, cyclin A-Cdk2, complex for instance, cyclin binding to Cdk results in conformational changes in the Cdk T-loop. This change in structure makes T160 in Cdk accessible for phosphorylation (Russo et al., 1996b).

In contrast to the activatory Cdk phosphorylation, the S phase and M phase cyclin-Cdk complexes can be phosphorylated by Wee1 kinase (tyrosine (Y) 15 in humans, Y19 in budding yeast) to block their activity (Booher et al., 1993; Gould and Nurse, 1989; McGowan and Russell, 1993, 1995). This phosphorylation reduces Cdk kinase activity by causing adverse interactions between the ATP gamma-phosphate residue and the catalytic site of Cdk (Welburn et al., 2007). Absence of Wee1 was shown to cause premature entry into mitosis and production of particularly small daughter cells in both fission and budding yeast due to loss of inhibitory Cdk phosphorylation (Harvey and Kellogg, 2003; Nurse, 1975). Therefore, the role of Wee1 is to coordinate growth and mitotic entry. In *Schizosaccharomyces pombe*, in addition to Wee1, Mik1 can also phosphorylate Y15. Although absence of Mik1 does not show any phenotype, loss of Wee1 in fission yeast cells lacking Mik1 results in mitotic catastrophe (Lundgren et al., 1991), highlighting the importance of inhibitory Cdk phosphorylation in cell cycle progression. In vertebrates, an additional kinase Myt1, is also involved in inhibition of

Cdk by phosphorylating T14 which is adjacent to the Wee1 phosphorylation site (Booher et al., 1997; Liu et al., 1997). Because structural analysis of Cdk suggested that both T14 and Y15 inhibitory phosphorylation sites are located above the ATP binding pocket, inhibition of Cdk by phosphorylation can be explained by the steric hindrance of kinase reaction (De Bondt et al., 1993; Russo et al., 1996b).

When the inhibitory Cdk phosphorylations are reversed by the phosphatase activity (Cdc25 in humans, Mih1 in budding yeast), cells can progress through G2 phase and mitosis (Dunphy and Kumagai, 1991; Lee et al., 1992; Strausfeld et al., 1991). Interestingly, activity of both Wee1 and Cdc25 is controlled by their target, M cyclin-Cdk complexes (Hoffmann et al., 1993; Watanabe et al., 2004). By inhibiting their inhibitor Wee1, and activating their activator Cdc25 through phosphorylation, M cyclin-Cdk complexes take part in complex, bistable switch-like feedback loops in order to promote mitotic entry (Deibler and Kirschner, 2010; Lindqvist et al., 2009; Pomerening et al., 2003). A bistable switch implies that the cell division programme is robust and irreversible (Morgan, 2007; Pomerening et al., 2003).

1.2.4 Stoichiometric Cdk inhibitors

The activity of cyclin-Cdk complexes are also regulated by the stoichiometric Cdk inhibitors (CKIs). As a general rule, CKIs are present in most eukaryotic cells and are needed to establish a stable G1 stage in which the cells can grow and prepare for the upcoming cell division cycle (Morgan, 2007).

In budding yeast, Far1, Sic1 and Cdc6 are the three stoichiometric CKIs. Far1 is implicated in mating pathway and arrests cells prior to Start in response to environmental mating pheromone (Chang and Herskowitz, 1990; Peter et al., 1993). Sic1 is an inhibitor of S and M cyclin-Cdk complexes and is expressed in G1 as well as in late mitosis (Nugroho and Mendenhall, 1994; Schwob et al., 1994). Cdc6 is responsible for inhibition of M cyclin-Cdk during mitotic exit in addition to its role in origin licencing for DNA replication (Calzada et al., 2001; Elsasser et al., 1996). In fission yeast, the only CKI Rum1 is responsible for uncoupling replication from mitosis (Correa-Bordes and Nurse, 1995; Martin-Castellanos and Moreno, 1996; Moreno and Nurse, 1994). Moreover, in

mammals, there are two cyclin-Cdk inhibitor families, namely, inhibitors of Cdk4 (INK4; $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, $p19^{INK4d}$) and Cdk interacting protein/kinase inhibitory proteins (Cip/Kip; $p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$) (Pennycook and Barr, 2020; Sherr and Roberts, 1999).

The INK family CKIs specifically bind to Cdk4 or Cdk6 monomers and inhibit cyclin binding (Brotherton et al., 1998; Guan et al., 1994; Hirai et al., 1995; Russo et al., 1998), Cip/Kip family members, on the other hand, can bind to all cyclin-Cdk complexes with a preference for G1 and S cyclin-Cdk complexes (Harper et al., 1993; Lee et al., 1995; Toyoshima and Hunter, 1994; Xiong et al., 1993). Interestingly, even though the Cip/Kip family is known as CKIs, in the case of G1 cyclin-Cdk complexes, cyclin D-Cdk4 or Cdk6, they can undertake both inhibitory and activatory roles (Cheng et al., 1999; Guiley et al., 2019; LaBaer et al., 1997; Zhang et al., 1994). An example to an activatory Cip/Kip is binding of phosphorylated $p27^{Kip1}$ (at Y74) to cyclin D-Cdk4. This interaction increases the catalytic efficiency of cyclin D-Cdk4. Therefore, cyclin D-Cdk4-phospho $p27^{Kip1}$ trimeric complex does not require docking interactions to efficiently phosphorylate its substrate retinoblastoma protein (Rb) (Guiley et al., 2019). In contrast, in confluent culture of contact arrested cells phosphorylation of $p27^{Kip1}$ Y74 is lost as a result of reduced tyrosine kinase activity, hence $p27^{Kip1}$ is inhibitory (James et al., 2008).

In order to promote the G1/S transition, CKIs must be inactivated in late G1. In budding yeast, for example, Sic1 is primed for degradation as cells pass Start. Accumulation of G1 cyclin Cln2-Cdk complexes, which are not subject to Sic1 inhibition, trigger phosphorylation of Sic1 phosphodegrons. This allows Sic1 recognition by SCF^{Cdc4} (orthologue of mammalian SCF^{Fbw7}) which can then target Sic1 for proteasomal degradation (Feldman et al., 1997; Skowyra et al., 1997). Simultaneously with Sic1 destruction, S phase cyclin Clb5-Cdk complexes get activated. The cooperative effort of Cln2-Cdk and Clb5-Cdk can then result in processive multisite phosphorylation of Sic1 and its complete degradation (Koivomagi et al., 2011a). Similarly, in mammalian system, $p27^{Kip1}$ is also primed for destruction by phosphorylation. In late G1, $p27^{Kip1}$ is phosphorylated at the T187 residue by the activity of G1/S and S cyclin-Cdk complexes, cyclin E-Cdk2 and cyclin A-Cdk2 (Montagnoli et al., 1999). This allows its recognition

by SCF^{Skp2} which in turn targets $p27^{Kip1}$ for proteasomal degradation (Carrano et al., 1999).

1.2.5 Cks: Phospho-adaptor of cyclin-Cdk complexes

Cks is a highly conserved accessory subunit of cyclin-Cdk complexes that is bound to the C terminal lobe of Cdk (Bourne et al., 1996). Isolated as a suppressor of temperature sensitive Cdk alleles, Cks (Cks1 in budding yeast, Suc1 in fission yeast) is an essential gene for cell viability in yeast (Hadwiger et al., 1989a; Hayles et al., 1986). The budding yeast cells carrying a temperature sensitive Cks1 allele accumulate as unbudded cells with unreplicated or replicated DNA under restrictive conditions. This indicates a role for Cks1 at both G1/S and G2/M transitions (Tang Y, 1993). On the other hand, the cells lacking Suc1 arrest with long spindles and condensed chromosomes in fission yeast, indicating a role for Suc1 in mitosis (Moreno et al., 1989). Cks allows processive phosphorylation of substrates that have multiple phosphorylation sites. Studies in budding yeast have put forward two mechanisms to explain how Cks1 works. The first mechanism involves continuous association of Cks1 with the primed phosphorylated site on the substrate while cyclin-Cdk is sequentially phosphorylating the sites in the vicinity. In the second mechanism, cyclin-Cdk primes a site by phosphorylation which is then removed from the Cdk catalytic site and is bound by Cks1. This requires cyclin-substrate docking because the substrate needs to stay in contact with the cyclin-Cdk-Cks1 complex during the translocation of the primed site from the Cdk catalytic cleft to the Cks1 binding pocket (Koivomagi et al., 2013). Studies in budding yeast also revealed that Cks1 binds to the phosphorylated threonine residues in cyclin-Cdk substrates, and directs cyclin-Cdk to a neighbouring phospho-acceptor site that resides 12-16 amino acids C terminal to the Cks1 site (Koivomagi et al., 2013; McGrath et al., 2013).

A well-known example of the role of Cks1 is the multisite phosphorylation of budding yeast CKI Sic1, which is critical for the G1/S transition. To prime Sic1 for degradation, G1- and S-Cdk complexes cooperatively phosphorylate Sic1 phosphodegrons thereby leading to Sic1 destruction and progression through S phase (Koivomagi et al., 2011a). Insights into the roles of Cks multisite phosphorylation in mitosis were obtained from the studies on the Cks homolog p9 in *Xenopus laevis* egg extracts. Immunodepletion of p9 in

interphase extracts causes mitotic entry defects due to the accumulation of inhibitory Y15 phosphorylation on M cyclin-Cdk. In contrast, the absence of p9 in mitotic extracts inhibits mitotic exit because M cyclin-Cdk cannot be degraded. Therefore, it was proposed that p9 regulates both mitotic entry and exit by promoting phosphorylation of the cell cycle regulators Cdc25, Myt1, and Wee1 and activation of APC/C subunits Cdc27 and Apc1 via modulating M cyclin-Cdk activity for these targets (Patra and Dunphy, 1996, 1998; Patra et al., 1999).

In budding yeast, Cks1 was shown to be involved in the transcriptional control which does not require Cdk activity. Instead, in this context, Cks1 and Cdk are needed to recruit the proteasome to a gene locus in order to promote transcription (Yu et al., 2005). An example to this comes from the studies on investigating the role of Cks1 in Cdc20 expression regulation. The budding yeast cells carrying a temperature sensitive Cks1 allele were found to be defective in promoting the transcription of APC/C coactivator subunit Cdc20 under restrictive conditions. Here, Cdc20 transcription was shown to rely on the recruitment of Cdk and Cks1 to the Cdc20 promoter as well as the replacement of Cdk later by the proteasome (Morris et al., 2003) suggesting that Cks is also implicated in transcriptional control.

1.2.6 Cyclins direct cyclin-Cdk complexes to specific sites

In addition to their role in activating Cdks and mediating the interaction between cyclin-Cdk complexes and the substrates, cyclins can also recruit Cdks to specific subcellular locations. Differences in cyclin-Cdk localisation can then impose spatial regulation of substrate phosphorylation. Localisation signals can be encoded in the sequence of cyclins such as nuclear localisation signal (NLS) or nuclear export signal (NES). Moreover, cyclins can also localise to a specific location in cells using their hydrophobic patch (HP) which is implicated in recognition of SLiMs in substrates.

Mammalian S and M cyclins, cyclin A, cyclin B1 and B2, show distinct localisation in cells. Cyclin A shows nuclear localisation from S phase until its degradation in metaphase (Pines and Hunter, 1991), whereas cyclin B1 continuously shuttles between nucleus and cytoplasm during interphase due to its N terminal NES (Hagting et al., 1998; Pines and

Hunter, 1994; Toyoshima et al., 1998). At prophase, cyclin B1 localises to centrosomes and nucleus prior to nuclear envelope breakdown (NEBD) and interacts with the unattached kinetochores, spindle and condensed chromatin (Bentley et al., 2007; Pines and Hunter, 1991). On the other hand, Cyclin B2 is cytoplasmic; localises to the Golgi apparatus in interphase and stays in contact with the disassembled Golgi apparatus during mitosis (Draviam et al., 2001; Jackman et al., 1995). Swapping the N terminal fragment of cyclin B1 with of B2 directed cyclin B1 to the Golgi apparatus such that it could not localise at nucleus to trigger DNA condensation and NEBD any longer (Draviam et al., 2001). This highlights the relevance of subcellular localisation of cyclin-Cdk complexes to their function.

The HP is also an important determinant for localising cyclin-Cdk to specific locations in cells. For instance, in fission yeast, the HP on the mitotic cyclin Cdc13 is needed for its localisation to the SPB and phosphorylation of SPB related substrates for mitotic entry (Basu et al., 2020). Similarly, Cyclin B1 localisation to centrosome in human cell lines also relies on its HP (Basu et al., 2020; Bentley et al., 2007). Moreover, in budding yeast, the HP mutant version of M cyclin Clb2 is not able to localise to the bud neck because Clb2 bud neck localisation requires the HP mediated docking interactions between Clb2 and its substrate Bud3 (Bailly et al., 2003; Ord et al., 2019b). Altogether, these studies suggest a conserved role of the HP and docking mechanisms in mediating cyclin localisation and substrate phosphorylation in eukaryotes.

1.3 Budding yeast is a tractable model organism for cell cycle studies

The budding yeast, *Saccharomyces cerevisiae*, is a unicellular fungal eukaryote with 16 chromosomes that has been used to study the fundamentals of cell division since the 70's (Hartwell et al., 1970). *S. cerevisiae* is an appealing research tool to study cell cycle not only because of its short generation time and suitability for genomic modifications, but also for the similarities between the budding yeast master regulator of the cell cycle and their counterparts in higher eukaryotes. For instance, the budding yeast harbours all major cyclin types that exist in humans which are structurally and functionally homologous to their human partners to a great extent (Morgan, 2007). The budding yeast grows and

divides asymmetrically. It undergoes a closed mitosis meaning that instead of NEBD in prometaphase, genomic material is transferred to a newly born cell by nuclear migration during mitosis. Exponentially growing budding yeasts double approximately every 100 minutes at 25°C.

In *S. cerevisiae*, there are six different Cdks which can be further separated into two categories. Cdc28 and Pho85 form the first category. These Cdks are capable of binding to multiple cyclins and are implicated in cell cycle regulation. The second category involves four Cdks, Kin28, Srb10, Bur1 and Ctk1, which can only be activated by a single specific cyclin. The activity of these Cdks are not cell cycle regulated. Their main role is to regulate gene transcription (Malumbres, 2014).

A single Cdk molecule, Cdc28, in budding yeast triggers the entry into both S and M phases. Budding yeast Cdk associates with the G1, S, and M phase specific cyclins sequentially at the respective stages of the cell cycle. The G1 phase cyclins (CLNs), Cln1-3, are crucial for progression through Start, a commitment point for the cell cycle entry. S phase cyclins, Clb5,6, on the other hand, are important for DNA replication, whilst M phase cyclins, Clb1-4, play a role in spindle formation, chromosome segregation, completion of the cell cycle and formation of two genetically identical daughter cells (Figure 1.4.). S and M phase cyclins are together also known as B type cyclins or CLBs.

Budding yeast cells commit to an irreversible cell cycle entry by passing through Start which marks the activation of G1/S transcriptional programme, nuclear export of the G1/S transcriptional programme inhibitor Whi5 and the positive feedback loop initiated by Cln1/2-Cdk complexes (Doncic et al., 2011; Skotheim et al., 2008) (see 1.3.1.1. Transcriptional control of CLNs and the G1/S transcriptional programme). Therefore, Start is a major checkpoint in budding yeast. It not only responds to nutrient availability and presence of mating factor, but also to cellular stress and DNA damage. For instance, DNA damage induced in G1 by exposing cells to ionising radiation delays bud emergence and SPB duplication. These cells also remain sensitive to mating pheromone for a longer period (Gerald et al., 2002). Additionally, Start is a control point for cell size because

large mother cells spend less time in pre-Start stage compared to smaller daughter cells (Hartwell and Unger, 1977; Johnston et al., 1977).

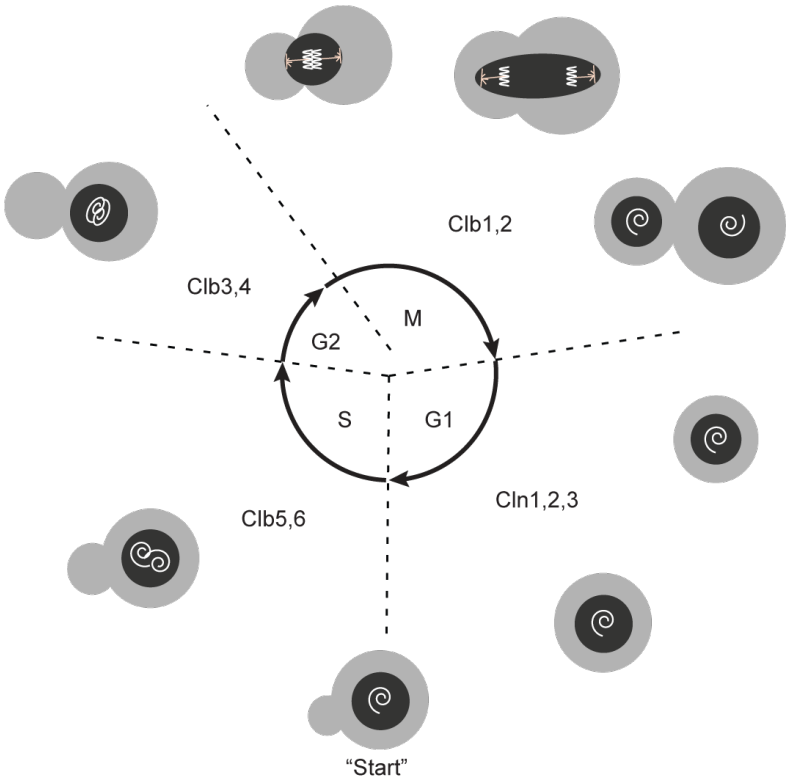


Figure 1.4. Budding yeast cell cycle

Cells grow to a critical size in G1 and reach to the cell cycle entry commitment point called "Start". After passing through start, DNA is duplicated in S phase, chromosomes are aligned and segregated into two daughter cells in mitosis.

Loss of mating pheromone response, duplication of the SPB and onset of DNA replication (see 1.3.3. S cyclin-Cdk controls replication initiation and inhibition of re-replication) point out exit from G1. In budding yeast, SPBs are the equivalent of centrosomes and are embedded in the nuclear envelope. The SPB duplicates once per cell division cycle in S phase, alongside DNA replication. Because the kinetochores are also assembled in S phase, sister chromatids attach to the mother SPB during DNA replication. SPB duplication is triggered by Cln1/2 at G1/S transition and promoted by Clb5/6 in S phase. The spindle forms in the nucleus and is kept intact during mitosis, in contrast to the spindle formation in animal cells which occurs in mitosis (Morgan, 2007).

The budding yeast does not have the G2/M checkpoint as in mammalian cells. Instead, in response to DNA damage, budding yeast cells rely on a mitotic checkpoint and are arrested at the metaphase to anaphase transition to prevent unfaithful chromosome segregation. This requires the activity of the Mec1 (ATR in mammals), Chk1, Rad53 and Dun1 kinases. In response to DNA damage, Chk1 and Rad53 are linked to Mec1 via the same mediator Rad9 (Melo and Toczyski, 2002). One of the mechanisms to arrest cells prior to anaphase involves phosphorylation and stabilisation of securin by Chk1 kinase (Wang et al., 2001). Another mechanism comprises of the maintenance of Bub2/Bfa1 activity. In an unperturbed cell cycle Cdc5 inhibits Bub2/Bfa1 to promote Tem1 activation, hence anaphase initiation (see 1.3.7.3. Cdc14 and mitotic exit) (Hu et al., 2001). Because Cdc5 is inhibited through Rad53 activity when cells encounter with DNA damage (Sanchez et al., 1999), Cdc5 can no longer trigger anaphase by inhibiting Bub2/Bfa1 activity. Although both Chk1 and Rad53 are activated in response to DNA damage, Rad53 can also respond to replication block (Allen et al., 1994; Gardner et al., 1999; Liu et al., 2000; Sanchez et al., 1999; Sanchez et al., 1996; Weinert et al., 1994). In response to perturbations of DNA replication, Rad53 activation is initiated by another Mec1 mediator protein, Mrc1. Mrc1 is a replication fork component which recruits Rad53 to Mec1 for phosphorylation (Alcasabas et al., 2001; Osborn and Elledge, 2003). Phosphorylated and activated Rad53 can then inhibit firing of the late replication origins (Shirahige et al., 1998; Tercero and Diffley, 2001) and regulate dNTP pools, replication fork integrity and transcription of the DNA damage response genes (Pardo et al., 2017).

1.3.1 CLNs, CLBs and transcription

1.3.1.1 Transcriptional control of CLNs and the G1/S transcriptional programme

During the G1/S transition, budding yeast cells need to transcribe more than 200 genes (Ferrezuelo et al., 2010; Spellman et al., 1998). The two well-characterised heterodimer transcription factors Swi4/6 cell cycle box (SCB) binding factor, SBF, and MluI cell cycle box (MCB) binding factor, MBF, drive the G1/S transcriptional programme (Figure 1.5.). The SBF consists of Swi4 and Swi6 proteins. Swi4 ensures SBF binding to DNA, whereas Swi6 is a transcription cofactor and cannot bind to DNA alone. When in complex with another DNA binding protein Mbp1, Swi6 also forms the MBF. Both SBF and MBF act through cis-acting sequence elements SCB and MCB which are recognised by Swi4 and

Mbp1, respectively. Recognising different sequences allow division of labour between SBF and MBF, therefore, they can control transcription of a different set of genes. For instance, SBF genes involve *CLN1*, *CLN2* and *SWE1*, while the examples of MBF activated genes are *CLB5* and *CLB6*, and the genes implicated in replication such as *CDC45* and *POL1* (Iyer et al., 2001). Although the loss of Swi4, Swi6 and Mbp1 are not lethal individually, lack of Swi4 and Swi6 or Swi4 and Mbp1 results in cell death (Koch et al., 1993; Nasmyth and Dirick, 1991).

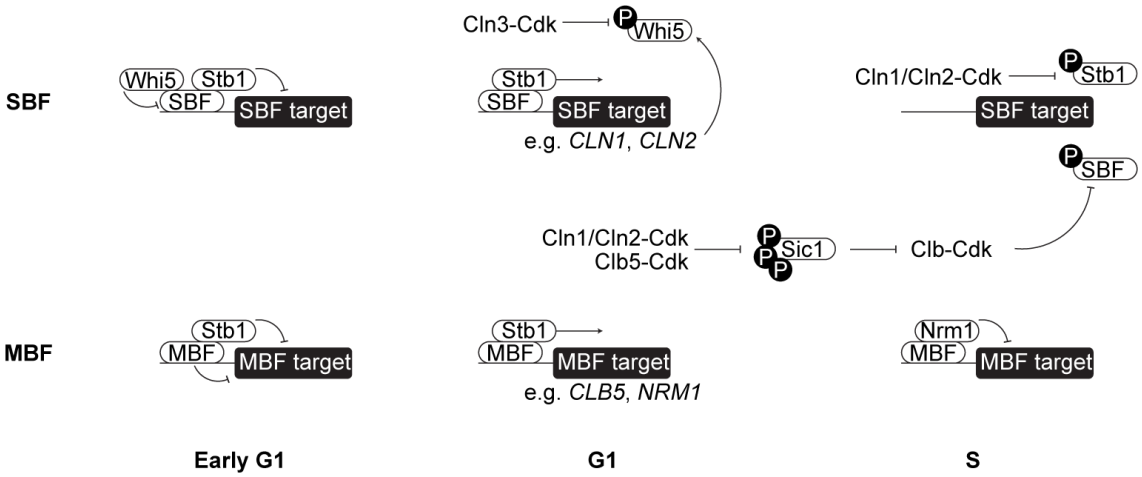


Figure 1.5. G1/S transcriptional programme

SBF is an activator of G1/S transcription. In early G1, transcription of SBF genes is repressed by Whi5 binding. Phosphorylation of Whi5 by Cln3/Cdk dissociates it from SBF promoters and turns on transcription. In S phase Clb/Cdk phosphorylates and inactivates SBF. In addition, Cln1/Cln2 phosphorylate and negatively regulate Stb1. MBF represses its targets in early G1 and late S phase. During S phase MBF target gene Nrm1 binds MBF to repress transcription. Adapted from (de Bruin et al., 2008).

The vast changes in gene expression at the G1/S boundary are tightly regulated by the transcriptional activators and repressors which are also under control of the activity of cyclin-Cdk complexes. Whi5 and Stb1 are the two main SBF and MBF regulators responsible for the repression of the G1/S transcriptional programme is G1 (Costanzo et al., 2004; de Bruin et al., 2008). Whi5 is a specific SBF inhibitor, loss of which accelerates the G1/S transition (Costanzo et al., 2004; de Bruin et al., 2004). In a typical cell division cycle, Cln3-Cdk complexes phosphorylate and inhibit Whi5 in order to overcome SBF inhibition (Costanzo et al., 2004; Wittenberg and Reed, 2005). Activation of SBF then allows accumulation of Cln1/Cln2-Cdk which in turn contributes to the phosphorylation of Whi5 in a positive feedback loop to facilitate cell cycle entry (Skotheim et al., 2008).

Although the proteins implicated in this feedback loop are not conserved, a similar regulatory mechanism for G1/S transcriptional activation exists in mammalian cells (Figure 1.6.). Unlike Whi5, Stb1 is not specific to SBF or MBF, and it can repress transcription from both SBF and MBF promoters prior to Cln3-Cdk dependent transcriptional activation (de Bruin et al., 2008). In the absence of Cln1 and Cln2, Stb1 is retained at the SBF and MBF controlled gene promoters (e.g. *SVS2* and *RNR1*, respectively) and can increase the G1 transcripts (de Bruin et al., 2008), suggesting that Cln1/Cln2-Cdk activity negatively regulates Stb1. In line with this, phosphorylation of Stb1 by Cln1/Cln2-Cdk was shown to disrupt the interaction between Stb1 and Swi6 (Costanzo et al., 2003; de Bruin et al., 2008; Ho et al., 1999). Analogous to Stb1, Mbp1 acts as a transcription repressor during late G1. MBF genes are derepressed in cells lacking Mbp1, indicating that Mbp1 is responsible for limiting transcription of MBF targets to G1 phase (Bean et al., 2005; Koch et al., 1993).

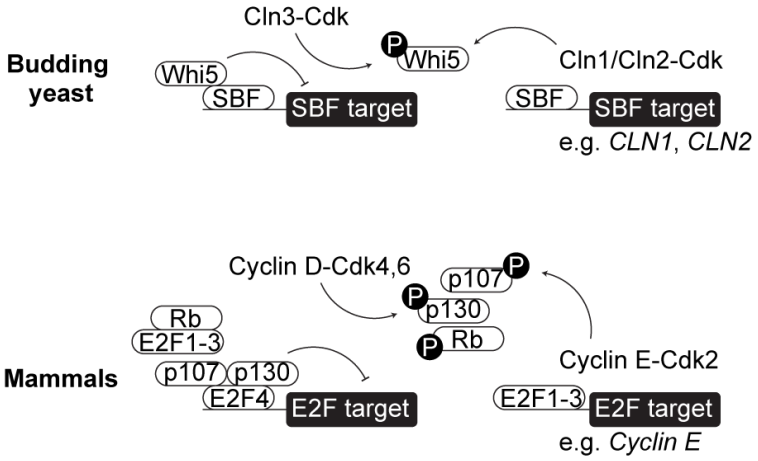


Figure 1.6. G1/S transcriptional control is similar in budding yeast and mammalian cells

The G1/S transcriptional programme is subject to a positive feedback loop both in budding yeast and mammals. In budding yeast, Whi5 inhibits SBF in early G1. Following phosphorylation of Whi5 by Cln3-Cdk, Whi5 is exported from the nucleus. This allows SBF activation hence accumulation of Cln1 and Cln2. Cln1/Cln2-Cdk can then further inactivate Whi5 through phosphorylation. This mechanism ensures passage through Start reinforced with a positive feedback loop. Similarly, in mammals, pocket proteins RB, p107 and p130 repress transcription in early G1 by binding to E2F activator (E2F1-3) and repressor (E2F4) proteins. Phosphorylation of the pocket proteins by Cyclin D-Cdk4/6 releases inhibition on the E2F transcription factors and activates G1/S transcriptional programme. As a result, Cyclin E is encoded. This allows initiation of a feedback loop whereby Cyclin E-Cdk2 phosphorylates and inhibits pocket proteins further. Adapted from (Bertoli et al., 2013).

The SBF and MBF transcription factors are also subject to regulation by negative feedback. For instance, in late G1, Cln1/2-Cdk complexes phosphorylate and target S and M-Cdk inhibitor Sic1 for proteolytic degradation (Koivomagi et al., 2011a). This leads to the activation of CLBs (Schneider et al., 1996; Schwob et al., 1994) which in turn inhibit SBF driven transcription by phosphorylating and releasing SBF from gene promoters (Amon et al., 1993; Geymonat et al., 2004). Yet another example is an MBF target gene, Nrm1, which can act as a corepressor in late G1 to inhibit G1 specific transcription by associating with MBF after it is own expression (de Bruin et al., 2006).

As opposed to the *CLN1* and *CLN2* transcription which are SBF regulated and oscillatory, *CLN3* transcription is evident in most of the cell cycle phases and peaks in the late M/G1 transition (McInerny et al., 1997). At the M/G1 transition, *CLN3* transcription is controlled by the binding of a transcription factor Mcm1 to a cis-acting element early cell cycle box (ECB) in the *CLN3* promoter (Mai et al., 2002). Transcription of the genes containing the ECB in their promoters is restricted to the M/G1 transition because of the inhibitory binding of Mcm1 repressors, Yox1 and Yhp1, to Mcm1 and ECB (Pramila et al., 2002). Lastly, because presence of glucose in the growth medium was shown to promote *CLN3* transcription, it is believed that *CLN3* transcription is also subject to nutrient availability (Parviz and Heideman, 1998).

1.3.1.2 Transcriptional control of CLBs

Following the G1/S transition, increasing kinase activity of S and M-Cdk complexes allow phosphorylation of the Fkh2 transcription factor and its coactivator Ndd1. Fkh2 in complex with Mcm1 and Ndd1 can then bind to the G2/M gene promoters and trigger the CLB cluster expression which is associated with late cell cycle events such as sister chromatid separation and cytokinesis (Althoefer et al., 1995; Koranda et al., 2000; Reynolds et al., 2003; Spellman et al., 1998). The mitotic cyclin Clb2 is a CLB cluster gene which also provides a positive feedback to the system to maximise CLB cluster transcription. This positive input allows M cyclin-Cdk activity to peak at mitotic entry before the cyclins are targeted for proteasomal degradation during mitosis. For instance, Clb2-Cdk kinase activity is indeed needed for transcription of *CLB1* and *CLB2* (Amon et al., 1993). Moreover, phosphorylation of Ndd1 by Clb2-Cdk is needed for Ndd1 *in vivo*

function and is responsible from the recruitment of Ndd1 to the CLB cluster gene promoters (Reynolds et al., 2003). The CLB cluster transcription, in particular transcription of *CLB2*, also gives feedback to the G1/S transcriptional programme. Clb2-Cdk interaction with Swi4 is believed to counteract SBF transcription via Swi4 through a mechanism yet to be explored (Amon et al., 1993; Siegmund and Nasmyth, 1996).

1.3.2 Molecular mechanism of polarisation and bud formation

In every division cycle, budding yeast cells grow, polarise its actin cytoskeleton and form a bud at the cell cycle entry. There are two forms of budding, axial or bipolar. Haploid budding yeast cells use axial budding which allows both the mother and the daughter cells to form a bud near the previous division site. Bipolar pattern, on the other hand, is used by diploid budding yeast cells. In diploid mothers the bud can form near the former division site or around the opposite pole, whereas in diploid daughters the bud forms near the pole distant to birth scar (Chant and Herskowitz, 1991; Chant and Pringle, 1991; Freifelder, 1960). Budding pattern is controlled by a group of landmarks (Chant and Herskowitz, 1991; Chant et al., 1995) which are integral plasma membrane proteins (Chen et al., 2000; Halme et al., 1996; Harkins et al., 2001; Kang et al., 2004). The persistent interaction of the landmarks with the preceding division site guarantees axial budding in haploid cells (Chant and Herskowitz, 1991). Moreover, the landmarks determine the incipient bud site where the polarity regulator protein, a small Rho GTPase, Cdc42 will be recruited (Adams et al., 1990; Johnson and Pringle, 1990).

The landmark proteins bind to Bud5, a guanine nucleotide exchange factor (GEF) for the Ras related Rsr1 GTPase (Kang et al., 2001). This allows incipient bud site localisation of Rsr1-GTP that is otherwise distributed all over the cell surface (Michelitch and Chant, 1996; Park et al., 1997). Rsr1-GTP associates with both Cdc42-GDP and its GEF Cdc24, thereby localising the actin cytoskeleton regulators to the presumptive bud site (Kozminski et al., 2003; Zheng et al., 1995). The GEF Cdc24 activates Cdc42 by converting it to Cdc42-GTP and stimulates its concentration at the polarisation site. Finally, Cdc42-GTP, together with its effector proteins, promote bud emergence (Figure 1.7.) (Howell and Lew, 2012).

Although Rsr1 plays an important role in the recruitment of Cdc24 and Cdc42 near the landmarks, loss of Rsr1 is not lethal. In fact, the cells lacking Rsr1 can still polarise and bud timely and efficiently, but at a random site (symmetry breaking) (Bender and Pringle, 1989; Chant and Herskowitz, 1991). In symmetry breaking mode of polarisation, cells can still form a single bud. The molecular mechanism behind this involves recruitment of a complex composed of the scaffold protein Bem1, the GEF Cdc24 and the p21 activated kinases (PAKs), Cla4 or Ste20, to the small Cdc42-GTP clusters at the cell cortex. This complex can then promote enlargement of Cdc42-GTP clusters by converting nearby Cdc42-GDP to Cdc42-GTP (Bose et al., 2001; Irazoqui et al., 2003; Kozubowski et al., 2008). Modelling symmetry breaking revealed that forming multiple Cdc42-GTP clusters can lead to depletion of cytoplasmic Bem1 pools. Consequently, the competition between clusters increases and the site with largest Cdc42-GTP pool is chosen as the bud site (Goryachev and Pokhilko, 2008; Howell et al., 2009).

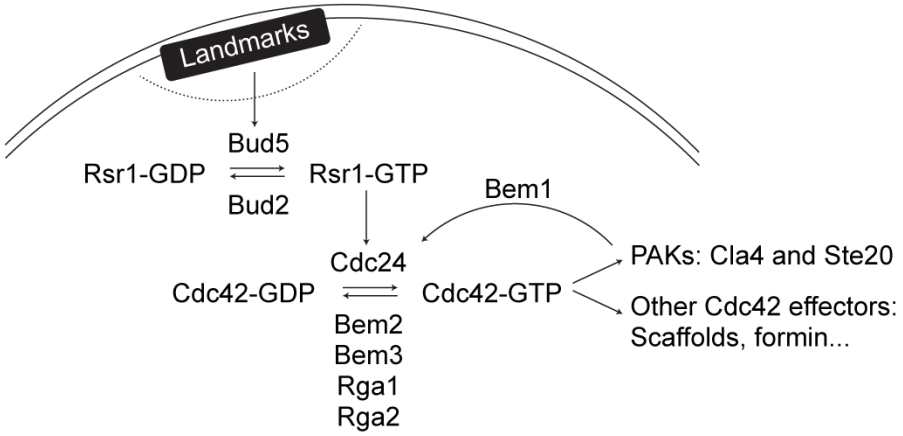


Figure 1.7. Polarisation pathway

Landmarks define incipient bud site and stimulate GTP loading of Rsr1. Rsr1-GTP can then recruit Cdc24 from cytoplasm to the cell cortex. Cdc24 in turn activates Cdc42. Cdc42-GTP signals to its effector proteins which are implicated in various events such as the assembly of the septin ring, reorganisation of the actin cytoskeleton, exocytosis. Positive feedback from Cdc42 effectors PAKs concentrates Cdc42-GTP pools further at the presumptive bud site and maintains polarity. Finally, a bud is produced with the cooperative effort of Cdc42 effectors.

The canonical polarisation pathway does not operate when budding yeast cells are exposed to a mating pheromone. During mating, the cells polarise to form a mating projection tip called shmoo towards the highest concentration of the pheromone and bypass the presumptive bud site polarisation (Segall, 1993). Binding of mating

pheromone to a G protein coupled receptor at the plasma membrane dissociates Gα subunit of the receptor from the Gβγ subunits. Gβγ subunits in turn recruit a CKI Far1 as well as the polarity establishment factors Bem1, Cdc24 and Cdc42 to allow mating tip polarisation (Butty et al., 1998; Nern and Arkowitz, 1999).

1.3.2.1 G1 cyclin-Cdk regulates polarisation and budding

Cdc42 has a central role in polarisation and bud formation as the cells that cannot activate Cdc42 fail to produce a bud (Adams et al., 1990). Polarisation and bud formation is also tightly regulated by the activity of G1 cyclin-Cdk complexes because the cells lacking G1 cyclins cannot polarise (Lew and Reed, 1993). Therefore, it is not surprising that G1 cyclin-Cdk activity promotes polarisation and subsequent bud formation by regulating Cdc42 (Figure 1.8.). In fact, the activity of Cdc42 is controlled by G1 cyclin-Cdk complexes through regulation of the Cdc42 GEF Cdc24 and Cdc42 GTPase activating proteins (GAPs), Bem2, Bem3, Rga1 and Rga2. Cdc24 is an *in vivo* and in *vitro* target of G1 cyclin-Cdk complexes (McCusker et al., 2007; Moffat and Andrews, 2004). However, the importance of Cdc24 phosphorylation for its function is debatable because substituting Cdc24 cyclin-Cdk phosphorylation sites with nonphosphorylatable residues does not cause changes in Cdc24 localisation, cell growth, polarisation and morphology (Wai et al., 2009). Nevertheless, Cln1/Cln2-Cdk activity is still required for Cdc24 localisation to the polarisation site. In haploid cells, the CKI Far1 sequesters the GEF Cdc24 in the nucleus. Once activated, Cln1/Cln2-Cdk complexes phosphorylate Far1 and target it for degradation. This allows Cdc24 release from the nucleus to cytoplasm (Henchoz et al., 1997; Nern and Arkowitz, 2000; Shimada et al., 2004). Cdc24 can then localise at the polarisation site through its interaction with Rsr1-GTP (Park et al., 1997; Park et al., 2002; Zheng et al., 1995). Interestingly, a mutant version of Cdc24 which cannot bind to Far1 localises to the cytoplasm, yet still cannot accumulate at the presumptive bud site (Gulli et al., 2000; Shimada et al., 2004). This indicates that G1-cyclin Cdk activity is not only needed for dissociation of Cdc24 and Far1, but also for Cdc24 recruitment to the polarisation site.

Cdc42 GAPs are also targeted by G1 cyclin-Cdk complexes. For instance, Bem2 and Bem3 are phosphorylated by Cln1/Cln2-Cdk at bud emergence. Loss of Bem2 results in

premature Cdc42 activation and formation of multiple buds. This suggests that Bem2 activity is needed to inhibit spontaneous Cdc42 activation in G1 and phosphorylation of Bem2 at bud emergence may be needed to allow Cdc42 activation at a single site. Bem3 overexpression, on the other hand, is lethal as cells arrest without a bud. Nonphosphorylatable Bem3 is hyperactive and also results in accumulation of unbudded cells, suggesting that Bem3, similar to Bem2, needs to be phosphorylated to inhibit its GAP activity and to promote Cdc42 activation for budding (Knaus et al., 2007). Another Cdc42 GAP, Rga2, is also a target of Cln2-Cdk complex that is phosphorylated at bud emergence (McCusker et al., 2007; Sopko et al., 2007). Overexpression of Rga2 in cells lacking CLNs is lethal due to inhibition of polarisation. Expression of nonphosphorylatable Rga2 results in growth defects and accumulation of unbudded cells indicating that phosphorylation of Rga2 is inhibitory (Sopko et al., 2007). Together, these results imply a general role for G1 cyclin-Cdk in inhibition of GAPs to activate Cdc42 at bud emergence.

Cdc42 downstream effectors are also targeted by G1 cyclin-Cdk complexes in order to regulate Cdc42 activity. For instance, Boi1 and Boi2, proteins involved in fusion of secretory vesicles with plasma membrane at bud sites, are Cln2-Cdk targets which interact with Cdc24 (McCusker et al., 2007). In particular, the nonphosphorylatable Boi1 shows defects in polarisation and localisation to bud site suggesting that phosphorylation of Boi1 is essential for promoting polarised growth (McCusker et al., 2007).

G1 cyclin-Cdk complexes must have additional roles to activating Cdc42 since the cells expressing GTP locked version of Cdc42 can polarise, but are not able to produce buds in the absence of G1 cyclin-Cdk activity (Gulli et al., 2000). It is believed that G1 cyclin-Cdk complexes play a role in the regulation of an essential Rho type GTPase, Rho1, that is implicated in bud growth at G1/S transition, cell wall biogenesis and actin cytoskeleton organisation. Although Rho1 GAP Sac7 is a known Cdk target (Ubersax et al., 2003) and Rho1 GEF Tus1 phosphorylation by Cln2-Cdk was proposed to be necessary for Rho1 activation (Kono et al., 2008), further studies are needed to determine the contribution of G1 cyclin-Cdk mediated Rho1 activation to budding as well as the other potential targets of CLNs in the budding pathway.

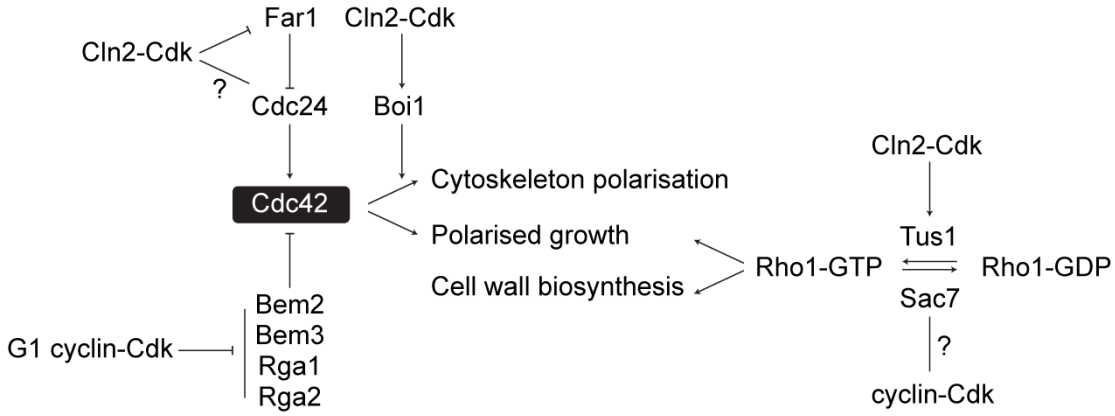


Figure 1.8. Regulatory network of Cdc42 activity

G1 cyclin-Cdk complexes activate Cdc42 in multiple ways. Briefly, phosphorylation of Cdc42 GAPs are inhibitory, whereas phosphorylation of Rho1 GEF and the adaptor protein Boi1 is activatory. Far1 phosphorylation by Cln2-Cdk targets it for proteasomal degradation and allows Cdc24 release from nucleus to cytoplasm. Rho1 GAP Sac7 is an *in vitro* Cdk substrate. However, the importance of this phosphorylation is unknown. Similarly, Cln2-Cdk can phosphorylate Cdc24, but implications of this phosphorylation need to be studied further. For detailed information see 1.3.2.1. G1 cyclin-Cdk regulates polarisation and budding. Adapted from (Pedraza et al., 2019).

Once the bud is formed, first apical, then isotropic growth facilitates bud development. During apical growth, cells expand linearly from the bud tip, whereas isotropic growth allows uniform radial bud enlargement. Bud growth is also controlled by the cyclin-Cdk complexes which have distinct roles in apical to isotropic switch. For instance, overexpression of Cln2-Cdk results in hyperpolarisation and elongated buds suggesting that Cln2-Cdk is implicated in apical bud growth. In contrast, Clb1/Clb2-Cdk overexpression fastens the transition from apical to isotropic growth indicating their role in opposing polarisation and promoting uniform bud development. Although overproduction of Clb3 allows cells to keep the apical growth pattern longer than wild type, Clb4 was shown to have no effect (Lew and Reed, 1993).

1.3.3 S cyclin-Cdk controls replication initiation and inhibition of re-replication

Simultaneously with the bud formation, budding yeast cells start replicating their genome, a process that is tightly controlled by the activity of cyclin-Cdk complexes. Cyclin-Cdk complexes have two distinct roles in replication, namely, activation of origins and

inhibition of the origin re-licencing before the next cell division cycle. The two step control mechanism ensures licencing of replication origins only in G1 when Cdk activity is low, and activation of origins in S phase when S cyclin-Cdk is active.

In eukaryotes, origin licencing precedes origin activation. In G1, when the cyclin-Cdk complex activity is low, origins are licenced when the two hexameric AAA+ ATPase mini chromosome maintenance helicases (MCM, consists of Mcm2-7 subunits) are loaded onto DNA with the help of the origin recognition complex (ORC; a hexameric complex with subunits Orc1-6) together with a licencing factor Cdt1 and an ATPase Cdc6 (Abid Ali et al., 2017; Evrin et al., 2009; Miller et al., 2019; Remus et al., 2009). In budding yeast, ORC selectively binds to an autonomously replicating sequence (ARS) consensus sequence and remains bound to the replication origins throughout the cell cycle (Bell and Labib, 2016; Bell and Stillman, 1992; Theis and Newlon, 1997).

The pre-replicative complex (pre-RC) that is composed of ORC, Cdc6, Cdt1 and MCM is inactive in G1. After the sequential loading of two MCM hexamers onto DNA, Cdt1 and Cdc6 dissociate. In S phase, Clb5-Cdk and Dbf4 dependent kinase (DDK) collaborate to phosphorylate numerous substrates to establish an active CMG (Cdc45, MCM, GINS) helicase (Bousset and Diffley, 1998; Donaldson et al., 1998; Zou and Stillman, 1998). To convert inactive MCM double hexamer to an active CMG helicase, firstly, phosphorylation of the Mcm4 and Mcm6 subunits by DDK is needed to allow recruitment of Sld3/7 and a helicase activator protein Cdc45 to the pre-RC (Deegan et al., 2016). Following that, Clb5-Cdk driven phosphorylation of the two key replication targets, Sld2 and Sld3, creates a binding site for another replication initiation factor Dpb11 and promotes recruitment of further components known as firing factors (DNA Polymerase ε, Mcm10 and the GINS complex) which altogether form the minimal set of proteins needed for an active helicase (Tanaka et al., 2007; Yeeles et al., 2015; Zegerman and Diffley, 2007). Active CMG helicase can then start melting DNA for replication. Next, Mcm10 promotes ATP hydrolysis function of the MCM double hexamer helicases so that they can unwind DNA, move towards each other, cross paths and establish a replication bubble while exposing a single stranded DNA as a template for replicative polymerases (Douglas et al., 2018; Looke et al., 2017).

S phase cyclin Clb5-Cdk mediated phosphorylation of Sld2 and Sld3 is a key event and a minimal requirement for the S phase entry (Masumoto et al., 2002; Zegerman and Diffley, 2007). In budding yeast, replication can be artificially triggered in cells harbouring a Sld3-Dpb11 fusion protein and a phosphomimetic Sld2 T84D protein in the absence of cyclin-Cdk kinase activity. However, these cells are not able to prevent DNA re-replication (Zegerman and Diffley, 2007), suggesting that cyclin-Cdk activity is not only needed to promote DNA replication, but also necessary to ensure that replication occurs only once per cell division cycle. In budding yeast, three different mechanisms which involve CLB-Cdk activity cooperate to prevent re-assembly of pre-RC complexes outside of the G1 phase (Nguyen et al., 2001). Firstly, phosphorylation of Cdc6 targets it for the SCF mediated ubiquitination and proteasomal degradation (Drury et al., 1997, 2000; Elsasser et al., 1996). Also, phosphorylation of the transcriptional activator Swi5 by CLBs excludes Swi5 from nucleus, and hence prevents Cdc6 transcription (Moll et al., 1991; Piatti et al., 1996; Piatti et al., 1995). Secondly, phosphorylation of the MCM hexamer by CLBs leads to its exclusion from the nucleus and thereby inhibits origin re-licencing (Labib et al., 1999; Liku et al., 2005; Nguyen et al., 2000). Lastly, phosphorylation of Orc2 and Orc6 prevent new MCM loading onto origins. Clb5-Cdk driven phosphorylation of Orc6 disrupts the interaction between the Orc6 N terminus and the Cdt1/MCM complex, therefore inhibiting recruitment of new MCM to the origins. Furthermore, a Clb5-Cdk docking interaction with the RxL motif on Orc6 is believed to inhibit Cdt1/MCM recruitment to the origins through steric hindrance (Chen and Bell, 2011; Nguyen et al., 2001). However, it is worth revisiting the interaction between Orc6 and Cdt1 as well as the consequence of ORC phosphorylation. This is because Orc6 and Cdt1 interaction could not be observed in a recent structural study addressing MCM loading by ORC-Cdc6 and Cdt1 (Yuan et al., 2017), and Cdk phosphorylated ORC was shown to recruit Cdc6, Cdt1 and MCM, but inhibit ORC-Cdc6-MCM complex formation which is the platform for MCM double hexamer formation (Fernandez-Cid et al., 2013).

1.3.4 Mitotic entry in budding yeast

In an unperturbed cell cycle progression, completion of S phase is followed by the mitotic entry. In the end of S phase, the cells readily contain duplicated DNA pairs, called sister

chromatids, which are held together by cohesin complexes. During mitotic entry, two major events occur simultaneously, namely, preparation of sister chromatids for segregation and the assembly of mitotic spindle. The former involves compaction of DNA and removal of DNA catenanes so that the resolved sister chromatids are ready for segregation. For the mitotic spindle assembly, separated SPBs need to nucleate microtubules while moving towards the opposite poles of the nucleus. In vertebrate cells NEBD occurs at this point. In budding yeast, because the cells undergo closed mitosis, nuclear envelope does not disassemble. Accordingly, the budding yeast mitotic spindle forms inside the nucleus while it is present in cytoplasm in vertebrates. Next, microtubules from the opposite poles of the cell attach to the kinetochores prior to metaphase to anaphase transition (Morgan, 2007).

In budding yeast CLBs are needed for mitotic entry. Although none of the CLBs are essential, deleting more than one of the CLBs can result in an inviable phenotype. In particular, Clb2-Cdk complexes among the others are more important because Clb2 alone can drive mitotic entry in the absence of Clb1/3/4 and loss of Clb2 results in delayed mitotic entry (Fitch et al., 1992; Richardson et al., 1992). Another regulator of mitotic entry in budding yeast is Swe1. Swe1 can inhibit mitotic entry by phosphorylating Cdk at the Y19 residue. The phosphatase Mih1, on the other hand, counteracts Y19 phosphorylation and promotes mitotic entry (Russell et al., 1989). In contrast to higher eukaryotes and fission yeast, the budding yeast cells lacking Swe1 or Mih1 can continue cell cycle progression normally in an unperturbed cell division cycle, indicating that Cdk Y19 phospho regulation is dispensable in budding yeast (Amon et al., 1992). However, Cdc28 Y19 phosphorylation turns out to be vital upon activation of the morphogenesis checkpoint, when cells encounter with problems in bud morphology and actin cytoskeleton (McMillan et al., 1998). As a consequence, Swe1 remains active and delays mitotic entry (Sia et al., 1998).

Because G2 phase is relatively short in budding yeast compared to other eukaryotes, budding yeast cells do not have a distinct G2/M checkpoint control prior to mitotic entry. Instead, in response to incomplete DNA replication or DNA damage, budding yeast cells arrest in metaphase. The mechanism behind this process involves the Chk1 kinase.

Phosphorylation of securin by Chk1 prevents securin degradation by the APC^{Cdc20} (Wang et al., 2001). Stabilised securin keeps separase inactive, and hence prevents sister chromatid separation. Since separase is also implicated in inactivation of Cdk during mitotic exit, stabilisation of securin also impedes mitotic exit (Cohen-Fix and Koshland, 1999; Queralt et al., 2006; Queralt and Uhlmann, 2008b).

1.3.5 Cyclins are ubiquitinated and targeted for proteasomal degradation

In later stages of the cell cycle, in order to bring about mitotic exit, CLBs are targeted for proteasomal degradation by the APC/C through recognition of their degradation motifs. In budding yeasts, all CLBs are believed to arise from a common ancestor CLB which contained a D box that would allow its degradation in metaphase. While the G2 cyclins Clb3 and Clb4 preserved their D box, Clb1 and Clb2 diverged to gain a KEN box, and both Clb5 and Clb6 established an ABBA motif during cyclin evolution (Davey et al., 2015; Gunbin et al., 2011). Interestingly, later in evolution, Clb6 substituted its D box and ABBA box with a phosphodegron motif, (L/I)(L/I/P)phosphoTP, which rendered it a target for early degradation by the SCF^{Cdc4} complex (Nash et al., 2001). Overall, variability of the CLB N terminal disordered region containing these motifs establishes the temporal control of cyclin degradation (Amon et al., 1994; Baumer et al., 2000; Hendrickson et al., 2001; Jackson et al., 2006; Lu et al., 2014; Thornton and Toczyski, 2003).

In early anaphase, the APC/C^{Cdc20} ubiquitinates S phase cyclin Clb5 and causes its degradation (Shirayama et al., 1999; Wasch and Cross, 2002). At this point, only a small pool of the mitotic cyclin Clb2 is targeted by the APC/C^{Cdc20}. The complete Clb2 degradation is achieved only after the APC/C subunit Cdc20 is replaced by Cdh1 in late anaphase (Baumer et al., 2000). Simultaneously with Clb2 degradation, Cdk activity is gradually reduced and the cell cycle is completed upon cytokinesis. When the mitotic checkpoint is active, Clb2 and the main APC/C^{Cdc20} target securin, which needs to be cleaved for sister chromatid separation, are stabilised (Baumer et al., 2000; Lu et al., 2014). In contrast, S phase cyclin Clb5 can still be degraded by APC/C^{Cdc20} when the mitotic checkpoint is active (Keyes et al., 2008; Lu et al., 2014). The mitotic checkpoint independent Clb5 degradation is facilitated by a multivalent binding of Clb5 to

APC/C^{Cdc20} via a D box, an ABBA motif and a mechanism involving Cks1 which increases the binding affinity of Clb5 to the APC/C^{Cdc20} (Lu et al., 2014). The APC/C ubiquitinates all CLBs (Wasch and Cross, 2002) except the S phase cyclin Clb6 because it lacks canonical APC/C recognition motifs as discussed above. Instead, the N terminal region of Clb6 contains three Cdk phosphosites, mutation of which to non-phosphorylatable amino acids stabilises Clb6 during G1/S transition. This confirms that phosphorylation of the Clb6 phosphodegron is needed for Clb6 recognition and ubiquitination by the the SCF^{Cdc4} (Jackson et al., 2006).

The SCF complex also recognises phosphorylated CLNs and targets them for degradation (Landry et al., 2012; Lanker et al., 1996; Skowyra et al., 1997; Skowyra et al., 1999). Cln1 is ubiquitinated by the SCF^{Grr1} (Barral et al., 1995; Skowyra et al., 1999), but Cln2 and Cln3 can be targeted for degradation by both SCF^{Grr1} and SCF^{Cdc4} (Landry et al., 2012; Quilis and Igual, 2017). Notably, as in most other SCF targets, CLNs contain a C terminal destabilising PEST domain which controls their high turnover rate (Hadwiger et al., 1989b; Nash et al., 1988; Salama et al., 1994).

1.3.6 Differences in cellular localisation of CLNs and CLBs

The spatial regulation of cyclins is functionally relevant and contributes to the temporal order of the cell cycle (Bailly et al., 2003; Eluere et al., 2007; Miller and Cross, 2000, 2001). In budding yeast, G1 cyclins Cln2 and Cln3 have distinct localisation patterns. While Cln1 and Cln2 are mainly cytoplasmic proteins with a small nuclear fraction (Miller and Cross, 2000; Quilis and Igual, 2012), Cln3 is mainly nuclear and accumulates in large budded cells (Miller and Cross, 2000). Interestingly, in small unbudded cells Cln2 is not phosphorylated and mostly nuclear. Moreover, mutation of the Cdk phosphorylation sites in Cln2 allows accumulation of Cln2 in nucleus. These two observations suggest that the maintenance of Cln2 cytoplasmic pool relies on Cln2 phosphorylation (Miller and Cross, 2000). On the other hand, Cln3 localisation is not regulated by phosphorylation. Instead, nuclear localisation of Cln3 depend on its C terminal NLS signal. Forced localisation of Cln3 to cytoplasm impairs its function to compensate for loss of the other CLNs highlighting the importance of Cln3 nuclear localisation (Edgington and Futcher, 2001; Miller and Cross, 2001).

The G2/M cyclins exhibit mostly nuclear localisation and a small cytoplasmic fraction (Bailly et al., 2003). In particular, localisation of CLBs to the SPBs was implicated in many different roles during cell division. For instance, the Clb3 HP dependent localisation to the SPBs was recently shown to phosphorylate a Clb3 specific target Csa1 which in turn recruits Cdc5 to the nuclear side of SPBs in metaphase (Ord et al., 2020). Furthermore, Clb4-Cdk localised to the SPB in the mother cell was shown to phosphorylate and prevent spindle positioning factor Kar9 loading onto this SPB. Phosphorylated Kar9 could then asymmetrically localise to the bud directed SPB to ensure the mitotic spindle alignment and establishment of the mother-daughter division axis (Liakopoulos et al., 2003; Maekawa et al., 2003).

Although Clb5 and Clb2 are mainly nuclear, they also show distinct localisation in different stages of the cell cycle. For example, Clb5 is nuclear at bud emergence and co-localises with the SPB. Identification of the SPB inner plaque component Spc110 as a specific target of Clb5-Cdk suggests a role for Clb5-Cdk in spindle dynamics and integrity through phosphorylation of substrates at the nuclear side of the SPB (Huisman et al., 2007; Loog and Morgan, 2005). Later in the cell cycle Clb5 concentrates at the short mitotic spindle until spindle elongation (Huisman et al., 2007). Likewise, Clb2 localises as a spot nearby the SPBs before nuclear division, co-localises with the SPBs during anaphase, and shows a HP dependent localisation to the bud neck (Bailly et al., 2003; Eluere et al., 2007; Hood et al., 2001). Presence of a central NLS as well as N and C terminal NESs in Clb2 could explain its cell cycle dependent localisation differences (Eluere et al., 2007; Hood et al., 2001).

1.3.7 Phosphatases in budding yeast

Counteracting phosphorylation events is as important as phosphorylation of hundreds of substrates for timely and ordered cell cycle progression. This essential task is performed by the phosphatases in cells. In *S. cerevisiae*, there are at least 43 protein phosphatases which can be categorized in 5 different families among which phosphoprotein phosphatase (PPP), protein phosphatase metal dependent (PPM) and phospho tyrosine phosphatase (PTP) families are the most well-known. PPP family involve protein

phosphatase 1 (PP1) and protein phosphatase 2A (PP2A). Cdc14 and Mih1, on the other hand, belong to PTP family (Offley and Schmidt, 2019).

1.3.7.1 Protein phosphatase 1

PP1 is a holoenzyme which is composed of a catalytic subunit and a regulatory subunit. In budding yeast, an essential catalytic subunit Glc7 has at least 20 regulatory binding partners and is implicated in many processes such as carbon metabolism, transcription regulation, actin organisation and cell cycle progression (Cannon, 2010). In mammals, PP1 binds to PP1 interacting proteins (PIPs) using an RVxF, SILK and MyPhoNE SLiMs (Hendrickx et al., 2009), whereas in *S. cerevisiae* the PP1 docking motif is (K/R)xVRF (Offley and Schmidt, 2019). Mutations in this substrate docking motif were shown to impair Glc7 interaction with its targets (Bharucha et al., 2008a; Bharucha et al., 2008b; Chang et al., 2002).

Under restrictive conditions, a temperature sensitive mutant of Glc7 causes cell cycle arrest in cells with short spindle. Bypass of the mitotic checkpoint in these cells allows anaphase which indicates a role for Glc7 in controlling accurate microtubule-kinetochore attachments (Bloecher and Tatchell, 1999). Glc7 regulates this process at kinetochores by counteracting Aurora B (Ipl1 in budding yeast) mediated phosphorylation and stabilises correct microtubule-kinetochore attachments (Akiyoshi et al., 2009; Pinsky et al., 2006; Sassoon et al., 1999). Moreover, Glc7 is also involved in replication checkpoint deactivation and recovery from replication fork stalling (Bazzi et al., 2010).

PP1 was shown to be implicated in sequential phosphatase activation during mitotic exit in fission yeast by a mechanism that is not conserved in budding yeast. In fission yeast, activity of Dis2 (fission yeast PP1 isoform) is inhibited by M cyclin-Cdk in early mitosis which is reversed when M cyclin-Cdk levels are reduced as a result of APC/C activation. Activated Dis2 can then reactivate $PP2A^{B55}$ which in turn dephosphorylates Dis2 docking sites on $PP2A^{B56}$. Next, recruitment of Dis2 to $PP2A^{B56}$ activates $PP2A^{B56}$ and cooperative activity of PP1 and PP2A phosphatases allow exit from mitosis (Grallert et al., 2015).

1.3.7.2 Protein phosphatase 2A

PP2A is a heterotrimeric complex formed of a scaffold subunit Tpd3, a catalytic subunit, Pph21 or Pph22, and a regulatory subunit, Cdc55, Rts1 (B55 and B56 in humans, respectively) or Rts3, in budding yeast (Jiang, 2006). Time resolved analysis of the phosphoproteome dynamics during mitotic exit revealed a great cooperation between $PP2A^{Cdc55}$ and $PP2A^{Rts1}$ substrates (Touati et al., 2019). However, these phosphatases also regulate distinct processes at different stages of the cell cycle. For instance, $PP2A^{Cdc55}$ was shown to counteract not only cyclin-Cdk activity but also other proline directed kinases such as Cdc5 (budding yeast Plk), glycogen synthase kinase (GSK3), casein kinase I and II (Baro et al., 2018; Godfrey et al., 2017; Touati et al., 2019).

From interphase until metaphase, $PP2A^{Cdc55}$ specifically targets proline directed threonine residues. Therefore, phosphosites with a serine residue are phosphorylated earlier than the sites containing a threonine residue (Godfrey et al., 2017). This ensures temporal order in interphase phosphorylation events. $PP2A^{Cdc55}$ has also multiple roles in mitosis. Absence of Cdc55 results in elevated levels of inhibitory Cdk phosphorylation at Y19 and a delay in early mitosis which can be rescued by the deletion of Swe1 (Minshull et al., 1996; Yang et al., 2000). This indicates a role for $PP2A^{Cdc55}$ in counteracting inhibitory phosphorylation of Cdk at Y19. Initial phosphorylation of Swe1 by Clb2-Cdk triggers a negative feedback loop because activated Swe1 can then inhibit Clb2-Cdk by phosphorylating it at Y19. $PP2A^{Cdc55}$ counteracts this initial activatory Swe1 phosphorylation to allow a period where a low constant level Clb2-Cdk escapes from Swe1 inhibition and initiates early mitotic events (Harvey et al., 2011). $PP2A^{Cdc55}$ is also implicated in the activatory dephosphorylation of Mih1. Activated Mih1 can then contribute to the activation of Clb2-Cdk by dephosphorylating Y19 (Pal et al., 2008). These findings suggest that at the G2/M transition, $PP2A^{Cdc55}$ promotes mitotic entry by modulating the activity of Clb2-Cdk regulators Swe1 and Mih1 in a pathway that is specific to budding yeast. At anaphase onset, downregulation of $PP2A^{Cdc55}$ aids activation of the mitotic exit network by facilitating the inactivation of Bfa1 (see 1.3.7.3. Cdc14 and mitotic exit) (Baro et al., 2013). $PP2A^{Cdc55}$ also counteracts phosphorylation of the kleisin subunit of cohesin Scc1 by Cdc5. Thus, downregulation of $PP2A^{Cdc55}$ by separase in early anaphase facilitates Scc1 cleavage and sister chromatid separation (Yaakov et al., 2012).

Lastly, $PP2A^{Cdc55}$ is important for the mitotic checkpoint as it counteracts Clb2-Cdk dependent phosphorylation of APC/C subunits Cdc16 and Cdc27 and prevents formation of the active APC/C^{Cdc20} complex (Rossio et al., 2013; Vernieri et al., 2013).

Loss of Rts1 prolongs G1, causes a delay in *CLN2* transcription and increases cell size (Artiles et al., 2009). Moreover, Cln3 transcriptional repressor protein Ace2 is hyperphosphorylated in cells lacking Rts1, which makes Ace2 a more potent repressor of *CLN3* transcription (Zapata et al., 2014). These findings suggest a role for $PP2A^{Rts1}$ at cell cycle entry, particularly in transcriptional control of G1 cyclins and cell size regulation. In addition, $PP2A^{Rts1}$ is important for modulating pericentromeric region for establishment of accurate bipolar spindle attachments. For this, $PP2A^{Rts1}$ works together with Sgo1 to recruit condensin to the pericentromere (Peplowska et al., 2014; Sherwin and Wang, 2019). Lastly, $PP2A^{Rts1}$ also plays a role in septin ring re-organisation during cytokinesis by localising to the bud neck and dephosphorylating septin Shs1 (Dobbelaere et al., 2003).

$PP2A^{Rts3}$ is expressed in stationary cells (Touati et al., 2019), but its function is yet to be explored.

1.3.7.3 Cdc14 and mitotic exit

Cdc14 was first discovered in a yeast genetic screen as an essential gene because its temperature sensitive mutant caused cell cycle arrest in cells with a long mitotic spindle at restrictive temperatures (Culotti and Hartwell, 1971). After its identification as a phosphatase (Taylor et al., 1997), Cdc14 was characterized as a major phosphatase in budding yeast implicated in mitotic exit (Queralt and Uhlmann, 2008a; Stegmeier and Amon, 2004; Visintin et al., 1998). In budding yeast mitotic exit, increasing the phosphatase to kinase ratio governs sequential substrate dephosphorylation. *In vitro* analysis of Cdc14 mediated dephosphorylation events revealed that early dephosphorylated proteins are better substrates for Cdc14 than cyclin-Cdk (Bouchoux and Uhlmann, 2011). It is also known that Cdc14 shows *in vivo* and *in vitro* specificity towards SPx(K/R) full Cdk motif (Bremmer et al., 2012; Powers and Hall, 2017) and

employs a PxL motif docking interaction to dephosphorylate its targets (Kataria et al., 2018).

Until anaphase, Cdc14 is sequestered in nucleolus by Net1. There are two mechanisms, namely, the Cdc fourteen early anaphase release (FEAR) and the mitotic exit network (MEN) that trigger stepwise Cdc14 release in anaphase (Figure 1.9.). Firstly, activation of separase lowers $PP2A^{Cdc55}$ activity via Zds1/Zds2 (Queralt and Uhlmann, 2008b). This allows Net1 phosphorylation by cyclin-Cdk and Cdc5 lifting Cdc14 inhibition. Subsequently, Cdc14 is released into the nucleus (Azzam et al., 2004; Shou et al., 2002; Stegmeier et al., 2002; Visintin et al., 2003; Yoshida and Toh-e, 2002). In early anaphase, Clb2-Cdk activity restrains the MEN pathway by inhibiting Cdc15 and Dbf2-Mob1 at the mother SPB (Konig et al., 2010), whereas the GTPase activating protein complex Bub2-Bfa1 of Ras-like GTPase Tem1 prevents the MEN signalling at the daughter SPB until Bfa1 is phosphorylated by Cdc5 (Hu et al., 2001). Early release of Cdc14 triggers a positive feedback loop by dephosphorylating Cdc15 which can then promote the MEN activation (Jaspersen and Morgan, 2000). Next, activation of the MEN pathway permits Cdc14 release into the cytoplasm.

The MEN pathway signalling cascade consists of a GTPase Tem1 and kinases Cdc15 and Dbf2-Mob1. Prior to the MEN activation, Tem1 is inhibited by Bub2-Bfa1. Cdc5 phosphorylates Bfa1, and hence inhibits the Bub2-Bfa1 complex. Inhibition of Bub2-Bfa1 in turn releases inhibition on Tem1 (Hu et al., 2001). Next, Tem1 activates its downstream kinase Cdc15 which can also activate Dbf2-Mob1 (Lee et al., 2001; Mah et al., 2001; Visintin and Amon, 2001). Additionally, Dbf2-Mob1 phosphorylate sites nearby Cdc14 NLS to contribute to Cdc14 release by inactivating its NLS (Mohl et al., 2009). Altogether, Net1 phosphorylation and Cdc14 activity are maintained until the end of mitotic exit during which Cdc14 orders substrate dephosphorylation by counteracting cyclin-Cdk activity (Uhlmann et al., 2011). In late anaphase, Cdc14 dephosphorylates and activates APC/C^{Cdh1} which in turn completes mitotic cyclin degradation and targets Cdc5 for degradation (Charles et al., 1998; Visintin et al., 2008; Visintin et al., 1998). Moreover, Cdc14 dephosphorylates Bfa1 to reactivate the Bub2-Bfa1 inhibitory complex

(Pereira et al., 2002). Consequently, Cdc14 returns back to the nucleolus following the inhibition of MEN pathway.

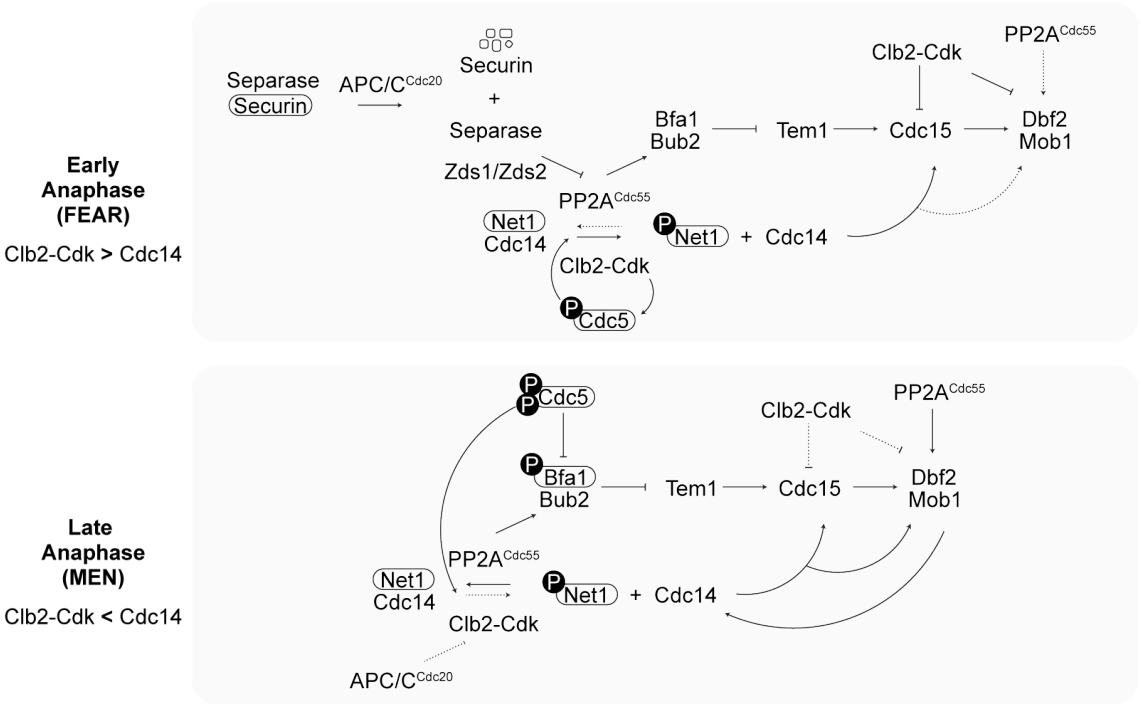


Figure 1.9. FEAR and MEN pathways

In early anaphase, activation of separase allows $PP2A^{Cdc55}$ inhibition via Zds1/Zds2 so that Clb2-Cdk can phosphorylate Net1. Cdc5, which is also activated by the Clb2-Cdk, promotes early Cdc14 release by contributing to the Net1 phosphorylation. At this point, the MEN components Cdc15 and Dbf2-Mob1 are inhibited by the Clb2-Cdk. However, early release of Cdc14 counteracts Clb2-Cdk inhibition on Cdc15 and facilitates the MEN activation. In late anaphase, Cdc5 phosphorylates the Bfa1-Bub2 complex to remove inhibition on Tem1, thereby activating the MEN pathway. Meanwhile, additional phosphorylation of Cdc5 further activates Cdc5 to maintain Net1 phosphorylation and Cdc14 release. Finally, increased Cdc14 activity and decreased Clb2-Cdk activity remove inhibition on Dbf2-Mob1 resulting in the fully active MEN signalling. Adapted from (Baro et al., 2013; Rodriguez-Rodriguez et al., 2016).

1.4 Qualitative and quantitative models of the cell cycle

Cyclin-Cdk not only phosphorylate many substrates implicated in various tasks in cells, but also ensures ordered and timely cell cycle progression to prevent genomic instability. There are two cell cycle models to explain how cyclin-Cdk executes temporal regulation during cell division, namely, a qualitative model and a quantitative model (Figure 1.10.). The qualitative model anticipates the presence of cyclin substrate specificity in different

stages of the cell cycle, whereas the quantitative model relies on gradual increase in cyclin-Cdk kinase activity from G1 to M in ordering the cell cycle.

1.4.1 The qualitative model

Cyclins are expressed in waves and form differential cyclin-Cdk complexes throughout the cell cycle (Bloom and Cross, 2007; Mendenhall and Hodge, 1998; Morgan, 2007). In the qualitative model of the cell cycle, these successive cyclin waves are believed to allow phosphorylation of subsets of substrates, hence temporally ordering the cell cycle events. However, how do the different cyclin-Cdk complexes recognise specific substrates to order the critical steps during the cell division cycle?

Cyclins recognise SLiMs on the cyclin-Cdk substrates and promote phosphorylation of these targets by Cdk. An RxL motif is the first SLiM identified as a specific recognition site by the S phase cyclin-Cdk complex, cyclin A-Cdk2. Cyclin A interacts with the RxL motif (RxLΦ or RxLxΦ, Φ depicting a hydrophobic amino acid) through its HP which consists of a highly conserved motif, xMRxILxDWLV, from yeast to human (Adams et al., 1996; Schulman et al., 1998). In budding yeast, the (K/R)xL motif is recognised by the S phase cyclin Clb5-Cdk complexes. Point mutations of the conserved M197, L201 and W204 residues of the Clb5 HP (SMRTILVDWLV [196-206]) to alanine, or mutations of the (K/R)xL motif disrupts the interaction between Clb5 and its targets. This results in alleviated Clb5-Cdk substrate phosphorylation (Loog and Morgan, 2005). An *in vitro* study comparing phosphorylation levels of a set of substrates by Clb5-Cdk and Clb2-Cdk has led to the identification of Clb5-Cdk specific substrates which are enriched in S phase functions. When tested *in vivo*, Clb2-Cdk could not phosphorylate these targets efficiently in the absence of Clb5. This confirms the biological relevance of the *in vitro* data revealing a Clb5 specific set of substrates (Loog and Morgan, 2005). NLxxxL is another motif recognised specifically by the HP of Clb5-Cdk and Clb6-Cdk complexes. This SLiM was shown to better potentiate phosphorylation of S phase cyclin-Cdk targets compared to the RxL motif, indicating that SLiMs can show functional differences even though they are recognised by the same cyclin-Cdk complexes (Faustova et al., 2020).

The M phase cyclin Clb2-Cdk complexes also contain a slightly different HP (QNRDILVNWLV [259-269]) compared to Clb5-Cdk and they are not able to recognise the RxL motif. Instead, Clb2 uses its HP to dock substrates containing an LxF motif (Ord et al., 2019b). This explains why Clb2-Cdk could not phosphorylate Clb5 targets *in vivo*, even though it has an intrinsically higher kinase activity towards a generic substrate compared to its counterparts (Koivomagi et al., 2011b; Loog and Morgan, 2005). Moreover, Clb2-Cdk complexes are less dependent on the docking interactions despite their specificity for the LxF motif due to their innately greater kinase activity (Koivomagi et al., 2011b; Ord et al., 2019b). Recently, studies on the role of mammalian M phase cyclin, cyclin B1, as a scaffold protein during mitotic checkpoint activation showed that cyclin B1 interacts with an N terminal acidic patch on checkpoint protein Mad1. However, it remains elusive if cyclin B1 uses its HP to interact with this acidic patch (Allan et al., 2020; Jackman et al., 2020).

The G2 cyclin Clb3 also presents specificity towards a different SLiM known as a PxF motif, (P/I/L/V/M)PxxPxFxx(K/R). Although a conservative SLiM search resulted in a list of 4 substrates containing this motif in budding yeast, a candidate based *in vitro* screen revealed more proteins that rely on the Clb3 HP to be specifically phosphorylated by Clb3 (Ord et al., 2020). This implies presence of an additional Clb3 specific SLiM on substrates or a possibility to accommodate modifications in the canonical PxF motif.

In addition to the motifs recognised by CLBs, the budding yeast G1 cyclins Cln1/Cln2-Cdk also employs an LP, minimally LxxP and most favourably LLPPΦxΦ, motif docking interactions to phosphorylate their substrates (Bandyopadhyay et al., 2020; Bhaduri and Pryciak, 2011; Koivomagi et al., 2011b). In contrast to CLBs which interact with their motif via the HP, Cln1/2 uses a distinct region near their HP to dock their substrates (Bhaduri et al., 2015). LP docking motif interactions are also implicated in polarisation because the cells expressing the docking mutant version of Cln2 ($Cln2^{lpd}$) showed reduced polarised growth (Bhaduri et al., 2015). Furthermore, variations in LP motif affects cyclin docking potency and fine tunes substrate phosphorylation timing. For example, presence of a less potent LP motif in Sic1 delays its phosphodegron phosphorylation by Cln1/Cln2-Cdk, hence the initiation of its degradation (Bandyopadhyay et al., 2020). Finally, LP

motif recognition by G1 cyclins are conserved among fungi suggesting that this docking interaction existed in ancestral fungi (Bandyopadhyay et al., 2020).

The interaction between cyclins and SLiMs does not always allow substrate phosphorylation, but can also cause inhibition of the cyclin-Cdk complexes. For instance, a structural study of cyclin A2-Cdk and the CKI $p27^{Kip1}$ revealed that $p27^{Kip1}$ binding to the cyclin A2 HP allows subsequent $p27^{Kip1}$ binding to Cdk2 which inhibits Cdk2 active site by mimicking ATP (Russo et al., 1996a). Similarly, Clb2 HP interaction with Cdc6 LxF motif inhibits Clb2-Cdk kinase activity (Ord et al., 2019b). This inhibition has been suggested to reduce M cyclin-Cdk activity to allow mitotic exit (Calzada et al., 2001).

The abovementioned studies address cyclin specificities by means of substrate docking motifs using *in vitro* approaches. However, the expressions of stage specific substrates and cyclins are also essential for the timely and ordered cell cycle progression as demonstrated by the identification of cyclin E1/A2/B1-Cdk interactors *in vivo*, in human cell lines. Dependence of cyclin-Cdk and substrate interactions to the type of cyclin and the cell cycle stage exemplifies the importance of expressing multiple cyclins throughout the cell cycle (Pagliuca et al., 2011).

1.4.2 The quantitative model

The qualitative model of the cell cycle suggests that S and M phases are temporally ordered by the biochemical specificity of distinct cyclin-Cdk complexes for their substrates. Although many studies have provided plausible evidence in favour of the qualitative model, it is intriguing that cyclins and Cdks can be eliminated in a variety of eukaryotes without having detrimental effects on the order of cell cycle (Brandeis et al., 1998; Epstein and Cross, 1992; Haase and Reed, 1999; Santamaria et al., 2007; Schwob and Nasmyth, 1993). This implies that the qualitative model cannot serve as an only explanation to the question how the cell cycle is ordered.

According to the quantitative model of the cell cycle, a quantitative increase in cyclin-Cdk kinase activity from G1 to M is critical to drive the cell cycle in an order. In this model, increasing cyclin-Cdk activity can initiate S phase when a low kinase activity

threshold is reached; mitosis can be then triggered as the kinase activity peaks (Stern and Nurse, 1996). The fission yeast *S. pombe* is the utmost example in which oscillation of a single cyclin-Cdk complex, Cdc13-Cdc2, can promote ordered progression into both S and M phases and allow successful completion of cell division and proliferation (Fisher and Nurse, 1996). Another study on fission yeast revealed that an increasing concentration of a Cdc13-Cdc2 fusion protein over the course of the cell cycle alone is sufficient to order S and M phases (Coudreuse and Nurse, 2010). The same study also illustrated that replication and segregation can be inhibited using distinct concentrations of a Cdk inhibitor even though the fusion protein is expressed at constant levels. This supports the notion that cycles of differential Cdk activity from low to high would allow temporal order of the S and M phases. In line with the previous observations in fission yeast, a global phosphoproteomics analysis of the strain harbouring Cdc13-Cdc2 fusion protein demonstrated that a monotonic increase in a Cdc13-Cdc2 activity allows sequential phosphorylation of cyclin-Cdk targets when the substrate specific kinase activity thresholds are met (Swaffer et al., 2016).

In support of the quantitative model, some degree of similarity and interchangeability of the cyclins also exist in budding yeast. For instance, the mitotic cyclin Clb2 is not capable of activating the late origins when expressed earlier in the cell cycle in the absence of S phase cyclin Clb5-Cdk complexes (Donaldson, 2000). However, this can be overcome by deletion of the Cdk inhibitory kinase Swe1 (Hu and Aparicio, 2005) as removing the inhibitory Cdk phosphorylation would allow increasing Clb2-Cdk activity earlier in the cell cycle. Another example is the G1 cyclin-Cdk complexes which are crucial in a wild type strain background for viability, but can become dispensable in the absence of the CKI Sic1 or upon overexpression of the S phase cyclin (Epstein and Cross, 1992; Schwob and Nasmyth, 1993; Tyers, 1996). Furthermore, Cln2 can trigger G1/S transcriptional programme when expressed under control of the *CLN3* promoter highlighting the importance of G1 kinase activity rather than cyclin identity (Stuart and Wittenberg, 1995). Lastly, cells overexpressing the destruction box mutant Clb2 in the absence of CLNs could replicate, form spindles and segregate chromosomes until they arrest in telophase with long spindles. However, the destruction box mutant Clb2 could not compensate for the roles of G1 cyclins in budding and activation of the SBF (Amon et al., 1994).

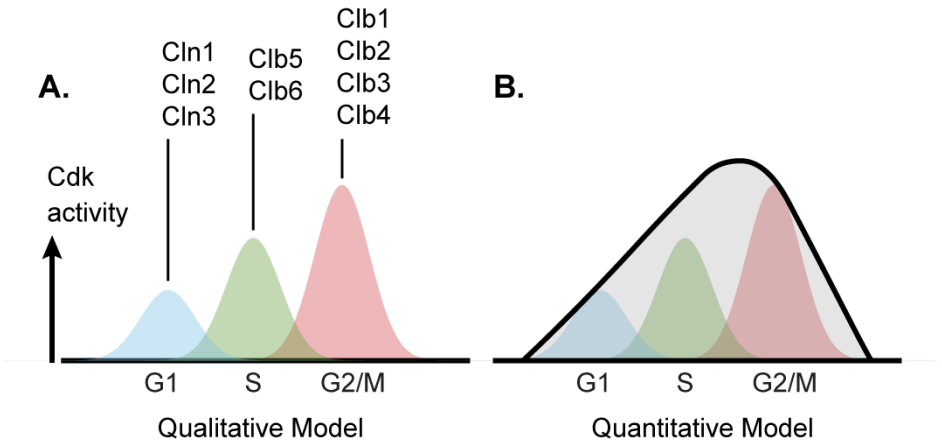


Figure 1.10. Qualitative and quantitative Cdk control orders the cell cycle

Two models have been proposed to explain how the cell cycle is ordered by the master regulators cyclin-Cdk complexes. **A.** The qualitative model stems from the specificity of cyclins for a certain set of substrates in different stages of the cell cycle. **B.** In the quantitative model, continuous increase in cyclin-Cdk activity from G1 to mitosis sets substrate phosphorylation thresholds to order the cell cycle events.

Lastly, the budding yeast cyclins can activate Cdk to different levels due to their unique and intrinsic capacity. For instance, Clb2-Cdk complexes present higher kinase activity towards a generic substrate Histone H1 than Clb5-Cdk complexes, the latter also being more active than Cln2-Cdk complexes (Koivomagi et al., 2011b; Loog and Morgan, 2005). Therefore, there is an overall trend of increasing cyclin-Cdk activity in budding yeast provided by the expression of different cyclins from G1 to M, supporting the presence of a quantitative component in this organism.

Table 2 Summary of phenotypes in the absence of CLNs and CLBs

Genetic background	Phenotype
Single deletion of CLNs, *CLN1-3*	Viable
*cln2*Δ	Passage through Start is delayed (Lew et al., 1992)
*cln3*Δ	Cells are larger at Start, duration of the G1 phase is increased (Cross, 1988; Nash et al., 1988)

*cln1*Δ *cln2*Δ *cln3*Δ	Inviable Rescued by *sic1*Δ or *CLB5* overexpression (Epstein and Cross, 1992; Schwob and Nasmyth, 1993; Tyers, 1996)
Single deletion of CLBs, *CLB1-6*	Viable
*clb2*Δ	The bud is elongated, mitotic onset is delayed relative to bud formation and cell size is increased (Surana et al., 1991; Watanabe et al., 2009)
*clb1*Δ *clb3*Δ *clb4*Δ	Viable Asynchronous culture of these cells has a similar DNA content flow cytometry profile to wild type cells with a slightly reduced peak for unreplicated DNA (Epstein and Cross, 1992)
*clb1*Δ *clb2*Δ *clb2*Δ *clb3*Δ *clb1*Δ *clb2*Δ *clb3*Δ *clb1*Δ *clb2*Δ *clb4*Δ *clb1*Δ *clb2*Δ *clb3*Δ *clb4*Δ	Inviable (Richardson et al., 1992)
*clb1*Δ *clb3*Δ *clb1*Δ *clb4*Δ *clb2*Δ *clb4*Δ *clb3*Δ *clb4*Δ *clb1*Δ *clb3*Δ *clb4*Δ	Viable (Richardson et al., 1992)
*cln1*Δ *cln2*Δ *cln3*Δ *clb5*Δ *clb6*Δ	Inviable Rescued by destruction box mutant Clb3 expression (Pecani and Cross, 2016)

1.4.3 Lessons from higher eukaryotes: plasticity of the cell cycle

Cell cycle plasticity and the redundancy of master cell cycle regulators are also widely studied in higher eukaryotes, particularly in murine models, leading to the identification of Cdk1 as the only essential cell cycle Cdk. Overall, loss of individual G1/S cyclins (D1, D2, D3, E1, E2) or Cdks (Cdk2, Cdk4, Cdk6) is not lethal in mice (Berthet et al., 2003; Fantl et al., 1995; Geng et al., 2003; Malumbres et al., 2004; Ortega et al., 2003; Parisi et al., 2003; Rane et al., 1999; Sicinski et al., 1995). Mice lacking Cdk2 or Cdk4 are infertile and are smaller compared to wild type mice, but otherwise healthy (Berthet et al., 2003; Ortega et al., 2003; Rane et al., 1999).

Loss of more than one cyclin or Cdk, on the other hand, prevents development of a full organism. For instance, mouse embryos without any cyclin D isoforms or Cdk4 and Cdk6 develop until late gestation and lose viability due to anaemia indicating a role for cyclin D in haematopoiesis (Kozar et al., 2004; Malumbres et al., 2004). Similarly, Cdk1 can compensate for the absence of Cdk2, Cdk3, Cdk4 and Cdk6 in mouse embryos during early development until mid-gestation. In these embryos, Cdk1 can interact with the G1/S cyclins, cyclin D and cyclin E, but embryos eventually die after encountering with heart defects and reduction in the number of common myeloid progenitors (Santamaria et al., 2007). In human epithelial cell lines, depletion of neither cyclin B1 nor B2 alone affects cell cycle progression. However, the cells lacking both cyclins are defective in mitotic progression even though they can trigger chromosome condensation, NEBD and spindle formation, indicating that cyclin B1 and B2 can compensate for each other during mitotic progression (Hegarat et al., 2020).

Although in human epithelial cell lines cyclin B1 and B2 can compensate for each other, absence of cyclin B1, but not cyclin B2, is lethal in mice (Brandeis et al., 1998). The mouse embryos lacking cyclin B1 arrest after the second division, at the four cell stage, in G2 phase following DNA replication (Strauss et al., 2018) indicating a crucial role for cyclin B1 during development. Furthermore, the cells lacking cyclin A2 cannot initiate mitosis. Induction of nuclear cyclin B1 expression can partially rescue this phenotype, highlighting the importance of nuclear cyclin-Cdk localisation for its function (Gong et al., 2007; Hegarat et al., 2020).

1.5 Aim of this project

To date, aspects of both cyclin specificity and cyclin redundancy have been documented in budding yeast. However, no systematic attempt has yet been made to study whether a single source of cyclin-Cdk activity might be sufficient to order the budding yeast cell cycle, or else, what the minimal essential set of cyclins might be. In this project, I challenged the two cell cycle models once again and addressed how qualitative cyclin-Cdk specificity and quantitative Cdk activity cooperate to orchestrate the cell division cycle.

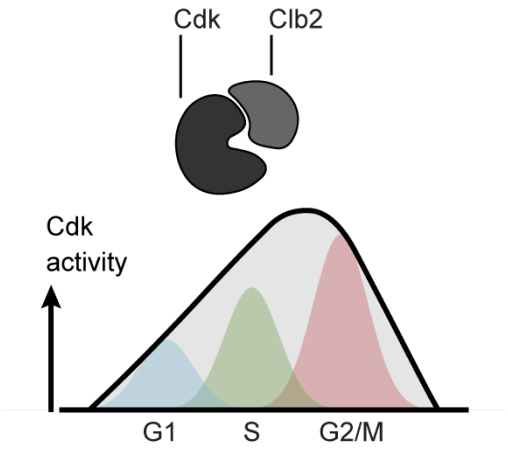


Figure 1.11. Driving the cell cycle with a single cyclin-Cdk complex in budding yeast

A hypothetical budding yeast strain I aimed to establish which would drive the cell cycle from G1 to M using Clb2-Cdk as the sole source of cyclin-Cdk activity.

I used the budding yeast *S. cerevisiae* as a tool to address if a mitotic cyclin Clb2-Cdk complex can compensate for the absence of remaining G1, S and M cyclin-Cdk complexes. In chapter 3, I explore the potential of Clb2-Cdk in triggering S and M phases in the absence of other CLBs. In chapter 4, I extend this question to G1 and ask whether a mitotic cyclin-Cdk complex can replace CLNs. Finally, in chapter 5, I discuss the relevance and implications of my findings to the current view of the cell cycle control.

Chapter 2. Materials & Methods

2.1 Yeast techniques

2.1.1 Yeast strains and culture

All budding yeast strains used in this study were of W303 background. The cells were grown in YP (yeast peptone) media supplemented with 2% w/v glucose (YPD) or in methionine dropout formulation of complete supplement mixture (CSM-Met) supplemented with 2% w/v glucose at 25°C. In Cln2 shut off experiments, 130 µg/ml methionine was added to CSM-Met with 2% w/v glucose.

For selection of transformants, yeast nitrogen agar plates were supplemented with 60 µg/ml of adenine, leucine, uracil, histidine, tryptophan, methionine, amino acid mix (arginine, isoleucine, lysine, phenylalanine and threonine) except for the one used for selection. Drug resistance selection plates contained 180 µg/ml hygromycin (Invitrogen), 200 µg/ml clonNAT (Discovery Fine Chemicals), 160 µg/ml G418 (Sigma). Cells were counter selected on 5-fluoroorotic acid (FOA) plates to allow recycling of the CRISPR-Cas9 plasmid for further editing.

Budding yeast *MAT***a** cells were synchronised in G1 by the addition of mating pheromone α-factor. To arrest cells, early log phase cultures were treated with α-factor at final a concentration of 5 µg/ml. The same amount of α-factor was added to cultures every hour for 2.5 h. Cells were checked under the phase contrast microscope. If more than 90% of cells accumulated as unbudded cells with a shmoo, cells were released by filtration and washed with 5 times culture volume of YP or CSM media and released into the appropriate media. Upon bud formation (approximately 60 minutes following the release) α-factor was re-added to the culture so that cells arrested again following complete passage through one cell cycle. This step is not required in experiments when cells are released from G1 into media containing 0.2 M hydroxyurea (Sigma) to induce replication stress, 8 µg/ml nocodazole (Sigma) to activate the mitotic checkpoint or methionine to shut off Cln2.

Table 3 Media composition

Media	Composition
YP	For 1 L use 20 g peptone (BD Biosciences), 10 g yeast extract (BD Biosciences), 0.05 g Adenine-HCl, autoclave before use
YNB	For 1 L use 1.7 g YNB (Sigma), 5 g ammonium sulfate (Sigma), 0.05 g Adenine-HCl, autoclave before use
CSM-Met	For 1 L use 6.7 g YNB, 0.76 g CSM-Met (Formedium), filter sterile before use
YP agar	As YP and mix with 2% w/v agar
YNB agar	As YNB and mix with 2% w/v agar
Sporulation	100 mM CH_3COONa, 20 mM NaCl, 25 mM KCl, 1.5mM $MgSO_4$ and 1.5% w/v agar
FOA	For 100 ml use use 0.675 g YNB, 0.1 g FOA (Thermo Fisher),120 μg/ml adenine, leucine, histidine, tryptophan, methionine, amino acid mix, 49.5 μg/ml uracil, 2% w/v glucose filter sterilise and mix with 2% w/v agar

Table 4 Strain list

Name	Genotype	Mating type
5046	*CLB1,6/clb1,6D::kanMX6 CLB3/clb3Δ::TRP1 CLB4/clb4Δ::HIS3 CLB5/clb5Δ::CLB2 SWE1/swe1Δ::URA3*	*MAT***a**/α
5090	*swe1Δ::HIS3 clb5Δ::TRP1*	*MAT***a**
DP27	*CLB5::CLB2 swe1Δ::URA3*	*MAT***a**
DP91	*clb1,6Δ::kanMX6 clb3Δ::TRP1 clb4Δ::HIS3 swe1Δ::LEU2 CLB5::CLB2-HA6 sic1Δ cln3Δ cln1Δ pCLN2::URA3-pMET3*	*MAT***a**
DP184	*clb1,6Δ::kanMX6 clb3Δ::TRP1 clb4Δ::HIS3 swe1Δ::LEU2 CLB5::CLB2-HA6 sic1Δ cln3Δ cln1Δ pCLN2-CLB2-HA3::ADE2 pCLN2::URA3-pMET3*	*MAT***a**

DP185	*clb1,6Δ::kanMX6 clb3Δ::TRP1 clb4Δ::HIS3 swe1Δ::LEU2 CLB5::CLB2-HA6 sic1Δ cln3Δ cln1Δ CLN2-MYC9::URA3*	*MAT***a**
DP187	*swe1Δ::HIS3 sic1Δ::LEU2 CLN2-MYC9::URA3*	*MAT***a**
DP193	*clb1,6Δ::kanMX6 clb3Δ::TRP1 clb4Δ::HIS3 swe1Δ::LEU2 CLB5::CLB2-HA6 sic1Δ cln3Δ cln1Δ pCLN2-CLB2-HA3::ADE2 CLN2-MYC9::URA3*	*MAT***a**
DP200	*clb1,6Δ clb3Δ clb4 swe1Δ*	*MAT***a**
DP266	*swe1Δ*	*MAT***a**
DP269	*clb1,6Δ clb3Δ clb4Δ swe1Δ CLB5::CLB2-HA6*	*MAT***a**
DP400	*clb1,6Δ::kanMX6 clb3Δ::TRP1 clb4Δ::HIS3 swe1Δ::LEU2 CLB5::CLB2-HA6 sic1Δ cln3Δ cln1Δ pCLN2-CLB2-HA3::ADE2 whi5Δ::hphMX6 pCLN2::URA3-pMET3*	*MAT***a**
DP402	*swe1Δ CDC28-PK3::URA3*	*MAT***a**
DP404	*clb1,6Δ clb3Δ clb4Δ swe1Δ CLB5::CLB2-HA6 CDC28-PK3::URA3*	*MAT***a**
DP457	*clb1,6Δ::kanMX6 clb3Δ::TRP1 clb4Δ::HIS3 swe1Δ::LEU2 CLB5::CLB2-HA6 sic1Δ cln3Δ cln1Δ pCLN2-CLB2-HA3::ADE2 whi5Δ::natMX6 pCLN2::URA3-pMET3*	*MAT***a**
DP460	*clb1,6Δ::kanMX6 clb3Δ::TRP1 clb4Δ::HIS3 swe1Δ::LEU2 CLB5::CLB2-HA6 sic1Δ cln3Δ cln1Δ pCLN2-CLB2-HA3::ADE2 stb1Δ::hphMX6 pCLN2::URA3-pMET3*	*MAT***a**
DP538	*swe1Δ CDC5-MYC9::TRP1*	*MAT***a**
DP539	*clb1,6Δ clb3Δ clb4Δ swe1Δ CLB5::CLB2-HA6 NET1-MYC9::TRP1*	*MAT***a**
DP541	*clb1,6Δ clb3Δ clb4Δ swe1Δ CLB5::CLB2-HA6 CDC5-MYC9::TRP1*	*MAT***a**
DP543	*clb1,6Δ clb3Δ clb4Δ swe1Δ CLB5::CLB2-HA6 CDC14-PK9::TRP1*	*MAT***a**
DP544	*swe1Δ NET1-MYC9::TRP1*	*MAT***a**

DP545	*swe1Δ CDC14-PK9::TRP1*	*MAT***a**
DP546	*swe1Δ KAR9-PK3::LEU2*	*MAT***a**
DP547	*swe1Δ clb5Δ::TRP1 CDC5-MYC9::URA3*	*MAT***a**
DP551	*clb1,6Δ clb3Δ clb4Δ swe1Δ CLB5::CLB2-HA6 KAR9-PK3::LEU2*	*MAT***a**
DP556	*clb1,6Δ::kanMX6 clb3Δ::TRP1 clb4Δ::HIS3 swe1Δ::LEU2 CLB5::CLB2-HA6 sic1Δ cln3Δ cln1Δ pCLN2-CLB2-HA3::ADE2 mbp1Δ::hphMX6 pCLN2::URA3-pMET3*	*MAT***a**
DP567	*clb1,6Δ clb3Δ clb4Δ swe1Δ CLB5::CLB2-HA6 sic1Δ cln3Δ cln1Δ pCLN2::URA3-pMET3 pCLN2-Chimera2-HA3::ADE2*	*MAT***a**
DP568	*clb1,6Δ clb3Δ clb4Δ swe1Δ CLB5::CLB2-HA6 sic1Δ cln3Δ cln1Δ pCLN2::URA3-pMET3 pCLN2-Chimera5-HA3::ADE2*	*MAT***a**
DP570	*clb1,6Δ clb3Δ clb4Δ swe1Δ CLB5::CLB2-HA6 sic1Δ cln3Δ cln1Δ pCLN2::URA3-pMET3 pCLN2-Chimera4-HA3::ADE2*	*MAT***a**
DP572	*clb1,6Δ clb3Δ clb4Δ swe1Δ CLB5::CLB2-HA6 sic1Δ cln3Δ cln1Δ pCLN2::URA3-pMET3 pCLN2-Chimera3-HA3::ADE2*	*MAT***a**
DP573	*clb1,6Δ clb3Δ clb4Δ swe1Δ CLB5::CLB2-HA6 sic1Δ cln3Δ cln1Δ pCLN2::URA3-pMET3 pCLN2-Chimera1-HA3::ADE2*	*MAT***a**
DP598	*clb1,6Δ clb3Δ clb4Δ swe1Δ CLB5::CLB2-HA6 sic1Δ cln3Δ cln1Δ pCLN2::URA3-pMET3 pCLN2-CLN2^{lpd}-HA3::ADE2*	*MAT***a**
DP607	*clb1,6Δ clb3Δ clb4Δ swe1Δ CLB5::CLB2-HA6 sic1Δ cln3Δ cln1Δ pCLN2::URA3-pMET3 pCLN2-CLB2$^{\Delta NLS}$::ADE2*	*MAT***a**
DP615	*clb1,6Δ clb3Δ clb4Δ swe1Δ CLB5::CLB2-HA6 sic1Δ cln3Δ cln1Δ pCLN2::URA3-pMET3 pCLN2-CLB2^{CLB5HP}-HA3::ADE2*	*MAT***a**
DP617	*clb1,6Δ clb3Δ clb4Δ swe1Δ CLB5::CLB2-HA6 sic1Δ cln3Δ cln1Δ pCLN2::URA3-pMET3 pCLN2-CLB2-Linker-CDC28::ADE2*	*MAT***a**
DP623	*clb1,6Δ::kanMX6 clb3Δ::TRP1 clb4Δ::HIS3 swe1Δ::LEU2 CLB5::CLB2-HA6 sic1Δ cln3Δ cln1Δ pCLN2::URA3-pMET3 pCLN2-CLN2-HA3::ADE2*	*MAT***a**

FC1	*clb1,6Δ clb3Δ clb4Δ swe1Δ CLB5::CLB2-HA6 SLI15-PK3::LEU2*	*MAT***a**
FC3	*clb1,6Δ clb3Δ clb4Δ swe1Δ CLB5::CLB2-HA6 NDD1-PK3::LEU2*	*MAT***a**
FC5	*swe1Δ SLI15-PK3::LEU2*	*MAT***a**
FC7	*swe1Δ NDD1-PK3::LEU2*	*MAT***a**
FC9	*swe1Δ PDS1-PK3::LEU2*	*MAT***a**
FC11	*swe1Δ MAD1-PK3::LEU2*	*MAT***a**
FC13	*clb1,6Δ clb3Δ clb4Δ swe1Δ CLB5::CLB2-HA6 PDS1-PK3::LEU2*	*MAT***a**
FC17	*clb1,6Δ clb3Δ clb4Δ swe1Δ CLB5::CLB2-HA6 MAD1-PK3::LEU2*	*MAT***a**

2.1.2 Yeast transformation

Gene deletions were performed using either PCR based methods (Wach et al., 1994) or CRISPR-Cas9 targeted genome editing technology (Laughery et al., 2015). For the latter, gene specific gRNAs were designed and cloned into the pML104 vector as described (Laughery et al., 2015). The vector was then co-transformed with a double stranded DNA fragment consisting of 150 base pairs upstream and downstream of the targeted gene. Positive transformants were then selected on YNB agar plates lacking uracil and counter selected on FOA plates to allow recycling of the CRISPR-Cas9 plasmid for further editing. Epitope tagging of endogenous gene loci and promoter substitutions were performed using PCR based methods (Knop et al., 1999).

For transformation, 25 ml of mid-exponential phase cells (OD600 = 0.2 - 0.4) were pelleted and washed firstly with 1 ml of distilled water and then with 1 ml of TEL (10 mM Tris HCl pH 7.5, 100 mM EDTA, 100 mM lithium acetate). Next, the cells were resuspended in 50 µl TEL. For plasmid integration 1 µg of linearised plasmid DNA, for CRISPR-Cas9 genome editing 700 ng Cas9-gRNA plasmid and 8 µl double strand template DNA, and for gene deletions or epitope tagging 8 µl PCR product were mixed with 2 µl of 10 mg/ml single stranded salmon sperm DNA. 300 µl of TELP (TEL

containing 40% PEG 4000) was added to the mixture and vortexed briefly. The cells were incubated at 25°C for 4 h, heat shocked for 15 min at 42°C washed once with 1 ml 1 M sorbitol and plated on selective media. Positive clones were checked by PCR, Western Blotting or Sanger sequencing.

2.1.3 Yeast mating and tetrad dissection

Mating was induced by incubation of the opposite mating type yeast strains on YPD or CSM-Met plates at 25°C for 8 hours. Diploids were selected on appropriate selective media and grown again on appropriate plate overnight at 25°C. Diploid cells were streaked on sporulation plates and incubated at 30°C for at least 3 days before spores were visible under the microscope. Spores were then resuspended in 1 M sorbitol and treated with lyticase at 30°C for 10 minutes to digest the walls of their asci. The four spores from each ascus were dissected using a Singer-MSM micromanipulator and incubated at 30°C until colony formation.

2.2 Molecular & Cell biology

2.2.1 Genomic DNA preparation

Yeast genomic DNA for PCR genotyping was prepared from freshly streaked patches of the strain to be tested. Cells were lysed in 100 µl 200 mM lithium acetate, 1% SDS solution at 70°C for 10 min. After incubation, 300 µl of 100% ethanol was added and DNA was precipitated by centrifugation at 13,000 rpm for 3 min. Pellets were dried for 10 minutes at 37°C and dissolved in 100 µl water. Cell debris was spun down by centrifugation at 6,000 rpm for 2 min and 1 µl supernatant was used for the PCR.

2.2.2 Polymerase Chain Reaction

PCR reactions were carried out in 25 or 50 µl reactions containing Taq (Qiagen) or CloneAmp (Takara) polymerases with buffers supplied by the manufacturers, 0.2 µM of each primer, and 0.2 mM dNTPs for Taq polymerase reaction. All PCRs were performed on a Peltier Thermal Cycler (MJ Research). PCR products were resolved by agarose gel electrophoresis to confirm the size of the fragments.

2.2.3 Agarose gel electrophoresis

DNA samples were resolved in a 0.8-1% agarose gel. Agarose gels were prepared in 1x Tris acetate EDTA buffer (TAE) (40 mM Tris base, 1 mM EDTA pH 8.0, 0.115% v/v acetic acid) to which GelRed (Biotium) was added to a final concentration of 0.5 µg/ml. Electrophoresis was carried out in TAE buffer at 110 V for at least 30 min in electrophoresis tanks (Anachem).

2.2.4 Cloning

All except Cas9-gRNA plasmids were generated using the In-Fusion (Takara) or Q5 Mutagenesis (New England Biolabs) kits according to the manufacturer's instructions. Cas9-gRNA plasmids were generated using restriction enzymes as described (Laughery et al., 2015). DNA fragments to be used for cloning were resolved in an agarose gel and purified using Macherey-Nagel Nucleospin kit according to the manufacturer's instructions. Following In-Fusion reaction or Q5 Mutagenesis ligation reaction, 5 µl of sample from these reactions were used to transform 50 µl of chemically competent *Escherichia coli* (DH5α) cells. Briefly, DNA and DH5α cells were mixed and incubated on ice for 30 min. Following a heat shock at 42°C for 45 s and cool down of cells on ice for 2 min, 950 µl of lysogeny broth (LB) medium was added. Cells were allowed to recover on a shaking incubator for 10 min at 37°C and then plated on LB agar plates containing the appropriate antibiotic. Colonies were obtained after an overnight incubation at 37°C.

Plasmids were purified from bacteria using the Qiagen Miniprep kit according to the manufacturer's instructions and sent for Sanger sequencing. In brief, each sequencing reaction was set up using 200 ng of DNA, 3.2 pmol of sequencing primer, 8 µl of BigDye Terminator Cycle Sequencing Ready Reaction Mix, made up to 20 µl with water. Following thermal cycling, unincorporated dideoxynucleotides were removed by ethanol precipitation and the sequencing reactions were loaded on to automated sequencing machines (Applied Biosystems) at the Crick Equipment Park.

Table 5 Plasmid list

Name	Details
626	*K. lactis URA3* flanked by 2 hisG repeats (*URA3* can be popped-out on 5'FOA) Reid et. al., Yeast, 2002 (plasmid pWJ1077)
DP1	pWJ1077 - Clb2-HA6 Clb2-HA6 cloned prior to *K. lactis URA3* cassette
DP3	pRSII426 - *pTDH3*-Cas9 with a single gRNA expression cassette from pSNR52 Laughery et. al., Yeast, 2015 (plasmid pML104), a kind gift from F. Caudron
DP4	Cloned into DP3: gRNA matches *CLN1* at position 57 within ORF gRNA sequence: CTGCATTGGACAATTCAATTGGG
DP5	Cloned into DP3: gRNA matches *CLN3* at position 20 within ORF gRNA sequence: TGCATTAGCGTATCTAATTATGG
DP6	Cloned into DP3: gRNA matches *SIC1* at position 26 within ORF gRNA sequence: CGCAAGGTACCTAGTCCCTCTGG
DP32	Cloned into DP3: gRNA matches *CLB1* at position 36 within ORF gRNA sequence: CATTAATAGTAATGAAGAGAAGG
DP35	Cloned into DP3: gRNA matches *CLB6* at position 81 within ORF gRNA sequence: AACTTTCTCTTGGAATGGTATGG
DP40	Cloned into DP3: gRNA matches *CLB4* at position 45 within ORF gRNA sequence: GATAGGTGACATTGAAATTCAGG
DP19	pRSII402 - Clb2-HA3 flanked by 500 bp *CLN2* promoter and terminator
DP102	pRSII402 - Cln2-HA3 flanked by 810 bp *CLN2* promoter and 500 bp *CLN2* terminator
DP119	pRSII402 - Cln2^{lpd}-HA3 R109A, L112A, R113A flanked by 810 bp *CLN2* promoter and 500 bp *CLN2* terminator

DP90	pRSII402 - Clb2^{CLB5HP}-HA3 Clb2[259-270] QNRDILVNWLVK replaced with Clb5[196-207] SMRTILVDWLVE flanked by 810 bp *CLN2* promoter and 500 bp *CLN2* terminator
DP81	pRSII402 - Clb2-Linker-Cdc28 flanked by 810 bp *CLN2* promoter and 500 bp *CLN2* terminator, linker sequence: AAAGGGSGGGSGGGS
DP112	pRSII402 - Chimera 1-HA3 construct: Cln2[1-339]-Clb2[446-491] flanked by 810 bp *CLN2* promoter and 500 bp *CLN2* terminator
DP113	pRSII402 - Chimera 2-HA3 construct: Clb2[1-224]-Cln2[36-339]-Clb2[446-491] flanked by 810 bp *CLN2* promoter and 500 bp *CLN2* terminator
DP114	pRSII402 - Chimera 3-HA3 construct: Clb2[1-224]-Cln2[36-545] flanked by 810 bp *CLN2* promoter and 500 bp *CLN2* terminator
DP115	pRSII402 - Chimera 4-HA3 construct: Clb2[1-445]-Cln2[340-545] flanked by 810 bp *CLN2* promoter and 500 bp *CLN2* terminator
DP111	pRSII402 - Chimera 5-HA3 construct: Cln2[1-35]-Clb2[225-445]-Cln2[340-545] flanked by 810 bp *CLN2* promoter and 500 bp *CLN2* terminator
DP82	pRSII402 - Clb2$^{\Delta NLS}$ [176-213] deletion introduces a single leucine residue flanked by 810 bp *CLN2* promoter and 500 bp *CLN2* terminator

2.2.5 Immunofluorescence

Indirect immunofluorescence was performed on formaldehyde fixed cells. Briefly, 1 ml of cells were resuspended in 1 ml ice-cold fixation buffer (100 mM KH_2PO_4 pH 6.4, 0.5 mM $MgCl_2$) containing 3.7% formaldehyde, and fixed overnight at 4°C. The cells were then washed in the same buffer lacking formaldehyde. Next, the cells were washed in 1 ml wash buffer (100 mM KH_2PO_4 pH 7.4, 0.5 mM $MgCl_2$, 1.2 M Sorbitol) and resuspended in 200 µl wash buffer containing 2 µl 2-mercaptoethanol (Sigma) and 2 µl lyticase (Sigma; stock: 20000 units/ml in 20% glycerol, 50 mM Tris HCl pH 8.0, stored at -20 °C) per ml of solution. Spheroplasted cells were washed once and subsequently resuspended in wash buffer. 8 µl of cells were seeded on polylysine coated wells on 15-

well slides (MP Biomedicals). The slides were blocked with a blocking buffer (0.5% Bovine Serum Albumin, BSA, in PBS) after fixing cells in methanol and acetone.

For spindle and Cdc14 tagged with a Pk epitope, α-Tubulin (Abcam, clone YOL 1/34, 1:200), α-Pk (Bio-Rad, clone SV5-Pk1, 1:200) primary antibodies were used, respectively. FITC and Cy3 conjugated secondary antibodies (Sigma and Chemicon, respectively) were used at a final concentration of 1:200. Cells were also counterstained with the DNA binding dyes 4',6-diamidino-2-phenylindole (DAPI), or Hoechst 33342, present in the mounting media ProLong Gold at a concentration of 100 ng/ml.

For F-actin staining cells were fixed with formaldehyde, washed with PBS and incubated for 1h in the dark in a solution containing 0.66 μM rhodamine phalloidin (Thermo Fisher) dissolved in methanol. Cells were then washed and resuspended in a drop of mounting media containing DAPI.

Fluorescent images were acquired as serial sections along the z-axis on a DeltaVision microscope system (Applied Precision) and processed using the quick projection algorithm in SoftWoRx.

2.2.6 Flow cytometry

1 ml of cells were fixed in 70% ethanol overnight at 4°C. The next day, the cells were treated with 0.1 mg/mL RNase in 50 mM Tris HCl pH 7.5 overnight at 37°C. The cells were then stained with propidium iodide containing buffer (200 mM Tris HCl pH 7.5, 210 mM NaCl, 78 mM $MgCl_2$, 50 μg/ml propidium iodide) and sonicated prior to sorting in an LSR Fortessa (BD Biosciences) flow cytometer. 10,000 cells per sample were counted and analysed in FlowJo.

2.3 Biochemistry

2.3.1 SDS-PAGE and Western Blotting

Protein extracts for western blotting were prepared after cell fixation using 20% trichloroacetic acid (TCA) and bead beating. Extracts were then separated by SDS-PAGE on 8-10% acrylamide/bis-acrylamide gels (37.5:1, 30% solution) with 375 mM Tris HCl pH 8.8 and 0.1% SDS. Proteins were migrated at 50 mA per gel using SDS-PAGE running buffer (25 mM Tris base (Sigma), 250 mM glycine (Sigma) and 0.1% SDS) in electrophoresis tanks from CBS scientific, CA. Precision Plus Protein Standard Dual Colour (Bio-Rad) was added to determine protein migration. To detect phosphorylated forms of Rga2 and Boi1, 0.144 w/v bis-acrylamide and 9% w/v acrylamide 375 mM Tris HCl pH 8.8 containing separating gels were run at constant current 20 mA in SDS-PAGE running buffer until the 75 kDa ladder band escaped the gel. Separated proteins were then transferred to nitrocellulose membranes using a wet-transfer tank (Bio-Rad). Transfer buffer contained 3.03 g/l Tris base, 14.1 g/l glycine, 0.05% SDS and 20% v/v methanol, and was carried out either at 400 mA for 2 hours at 4°C.

Membranes were blocked in 5% milk PBS-T at room temperature for 1h, incubated with the following primary antibodies diluted in 5% milk PBS-T overnight for detection: α-Clb5 (Santa Cruz, sc20170, 1:5000), α-Clb2 (Santa Cruz, sc9071, 1:1000), α-Sic1 (Santa Cruz, sc50441, 1:1000), α-Orc6 (clone SB49, 1:500), α-Orc2 (a kind gift from Stephen P. Bell, 1:10000), α-Rga2 and α-Boi1 (a kind gift from Derek McCusker, 2 μg/ml and 1:10000, respectively) α-Cdc24 (a kind gift from Matthias Peter, 1:1000), α-myc (clone 9E10, 1:3000), α-HA (clone 12CA5, 1:3000), α-Pk (Bio-Rad, clone SV5-Pk1 and Abcam, ab15828, both 1:3000), and α-Tubulin (clone TAT-1, Crick services, 1:10000). The next day, membranes were washed 3 times for 5 min in PBS-T. Incubated in secondary antibodies horse radish peroxidase coupled mouse, rabbit or goat (GE Life Sciences, 1:5000) diluted in 5% milk PBS-T. After secondary antibody incubation, for 1h, membranes were washed thoroughly before incubating them in ECL solution (Amersham) according to the manufacturer's instructions. Finally, membranes were either exposed to a film or proteins were detected using an Amersham ImageQuant CCD imager.

Table 6 Proteins studied in this project and their molecular weight

Protein	Molecular weight (kDa)	Detection
Clb5	50.4	Just below 50 kDa mark
Clb2	56.2	Endogenous Clb2 runs at ~50 kDa mark. HA3 and HA6 tagged Clb2 run above the endogenous Clb2 and below 75 kDa mark
Cdk	34	PK3 tagged ~50 kDa mark
Orc2	71.2	~75 kDa mark
Orc6	50.2	Just below 50 kDa mark
Sic1	32.2	Just below 37 kDa mark
Tubulin	50.8	~50 kDa mark
Kar9	74.2	PK3 tagged ~75 kDa mark
Ndd1	60.5	PK3 tagged ~75 kDa mark
Mad1	87.6	PK3 tagged Between 75 – 100 kDa marks
Rad53	91.9	Between 75 – 100 kDa marks
Securin	41.8	PK3 tagged Just above 50 kDa mark
Net1	128.5	MYC9 tagged ~250 kDa mark
Cdc5	81	MYC9 tagged ~50 kDa mark

Cdc14	61.9	PK9 tagged Between 50 – 75 kDa marks, closer to 75 kDa
Cln2	61.6	MYC9 tagged Between 75 – 100 kDa marks
Cdc24	96.9	Between 75 – 100 kDa marks, closer to 100 kDa
Rga2	113.2	~150 kDa mark
Boi1	109.3	Between 100 – 150 kDa marks
Bovine Histone H1	32	Just below 37 kDa mark

2.3.2 Immunoprecipitation and *in vitro* kinase assay

CDC28 Pk epitope tagged cells were grown and synchronised by the addition of α-factor. Samples were collected at regular intervals by centrifugation following the release and rapidly frozen in liquid nitrogen. Proteins were extracted by bead beating in lysis buffer (50 mM HEPES/KOH pH 7.5, 150 mM NaCl, 0.2% Triton X-100, 2.5 mM $MgCl_2$, 10% glycerol, 0.5 mM TCEP, 120 µg/mL AEBSF including benzonase and RNase A and supplemented with a cOmplete protease inhibitor tablet). Extracts were cleared by centrifugation and 800 µg extract per time point was incubated with 1 µg α-PK (Bio-Rad, clone SV5-Pk1) antibody for 30 min on a wheel at 4°C. 20 µl Dynabeads Protein A per sample were then added for further 30 min on a wheel at 4C. Beads were extensively washed in lysis buffer and equilibrated in kinase buffer (50 mM HEPES/KOH pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 0.05% Triton X-100, 0.25 mg/ml BSA) before performing the kinase reaction. Histone H1 phosphorylation reactions were carried out in kinase buffer containing 0.33 mg/ml of Histone H1 and 660 mM ATP, including 11 nM γ-^{33}P-ATP 15 minutes at 30°C. Reactions were terminated by the addition of SDS-PAGE loading buffer and boiling at 95°C for 5 min. Proteins were resolved in 4–15% Criterion™ TGX™ precast midi gels and transferred to a nitrocellulose membrane. Membranes were exposed to a Phosphorimager screen (GE Healthcare) and

phosphorylation of Histone H1 was quantified using ImageQuant. To control immunoprecipitation efficiency, the membranes were then processed for western blotting, probed with α-Pk (Abcam, ab15828) primary antibody (1:3000) and anti-rabbit secondary antibody (1:5000).

2.4 Tandem mass tag proteomics

The mass spectrometry experiment was performed once. However, because the same phosphosites could be detected in multiple time point samples which were run at the same time, each time point sample could potentially be considered as technical replicates.

2.4.1 Mass spectrometry sample preparation

Cells were synchronised in G1 by the addition of α-factor for 2.5 h and were released by filtration and washed with 5 times culture volume of YP media and released into the YPD media. Ten samples were collected at 10 min intervals, spanning from G1 to 90 min, when the control cells reach to mitosis. Samples were processed as described previously (Jones et al., 2020). Briefly, cells were pelleted and resuspended in 20% TCA and kept on ice for at least 1 h. Following cold acetone washes, pellets were air-dried to remove residual acetone and resuspended in 400 µl lysis buffer (50 mM ammonium bicarbonate, 5 mM EDTA pH 7.5, 8 M Urea). Proteins were extracted by bead beating and 200 µg of protein per sample was used for phosphoproteome analysis. Extracts were trypsin digested, concentrated. Peptides were then labelled using 10 isobaric tandem mass tags (TMT10plex, Thermo Fisher). Each pair of two sequential time points received the same label, then sets of five alternating time points of control and mutant were combined to yield two TMT10plexes. Phosphopeptides were enriched using sequential metal oxide affinity chromatography (TiO_2 and Fe-NTA) and liquid chromatography tandem mass spectrometry (LC-MS/MS) data were acquired (with the help of H. Flynn from the Crick Proteomics STP).

2.4.2 Phosphoproteome data analysis

The data were filtered to contain phosphorylated peptides with a single phosphorylated site and a localisation probability score above 0.75. The majority of phosphosites were

detected in both TMT10plexes, covering all 10 time points per strain. Phosphosites that were detected in only one of the TMT10plex were also included in the analysis after the phosphosite intensities for the missing alternating time points were calculated by imputation. Missing values were imputed by calculating the mean of the two adjacent time points. Smoothing was then performed by replacing each technical or imputed value by the mean of the two adjacent time points. For both imputation and smoothing of the 0 and 90 minute time points, only the 10 and 80 minute values, respectively, were used. All phosphosite intensities were then transformed to a linear scale.

For the identification of phosphorylated phosphosites, the following rules were applied to the control sample. For each site, phosphosite intensities were normalised to the lowest phosphosite intensity. Sites with normalised phosphosite intensity >1.5 in two consecutive time points after the time point with the lowest intensity are categorised as phosphorylated. Sites with higher intensity in time point 0 compared to 90 min were strictly eliminated to avoid dephosphorylated and some of the stable sites. Sites with the lowest intensity at time point 30 min or later were further analysed. Among these, sites with intensities that were >1.5 times of the time point 0 intensities in two consecutive time points were also included in the phosphorylated category. All phosphorylated sites were then filtered for the minimum Cdk motif (S/TP) to create the dataset covering all phosphorylated Cdk sites.

In order to identify Cdk phosphorylation waves additional analysis of the control in the dataset containing all phosphorylated Cdk sites was performed. Firstly, for each site, time points with the highest intensity after the time point with the lowest intensities were identified. The average for these two values were calculated and considered as a phosphorylation threshold. The sites were then categorised based on the time point that they pass the average value for the first time. The sites with the lowest intensity at time point 30 min or later were also further analysed. For these sites, again the time point with the highest intensity before the time point with the lowest intensity was identified. The phosphorylation threshold was then calculated by averaging the intensities of time point 0 and the maximum value at previous step. Similar to above, the sites were categorised based on the time that they pass the average value for the first time.

Data were always presented by normalising intensities of all the time points (including mutant) to the time point with the highest intensity in the control. For each phosphosite category, I plotted the median value. To identify phosphosites nearby SLiMs in predicted unstructured protein regions, I used an SlimSearch4 tool using a IUPRED disorder score >0.3 (http://slim.icr.ac.uk/slimsearch/) (Krystkowiak and Davey, 2017).

To assign phosphorylation midpoints to groups of phosphosites, I plotted the median values at each time. I then determined the average of minimum and maximum median values and identified at which time point this average was passed. Next, I fitted a trend line using two intensities and time points (the median value of the time point that the average was passed and the median value for the time point prior to this) and calculated the phosphorylation midpoint using this formula.

Heat maps were generated using phosphosites grouped by their phosphorylation timing in the control. Intensity values per phosphosite represented by rows were clustered using a Euclidean distance matrix and McKuitty clustering, based on phosphosite behaviour in the Clns Clb2(S-M) strain. Rows were divided into 3 groups using K means. Heatmaps were generated using the ComplexHeatmap package in R (version 3.6.0) (with the help of P. Chakravarty from the Crick Bioinformatics & Biostatistics STP).

Chapter 3. S phase can be driven by a mitotic cyclin-Cdk

The quantitative model of cell division cycle relies on an increasing Cdk activity from G1 to mitosis (Stern and Nurse, 1996). The model suggests that S phase can be initiated with low cyclin-Cdk activity while a higher kinase activity threshold needs to be reached for mitotic phosphorylation events. Differences between the Cdk activity thresholds for replication and mitosis prevent premature mitotic entry in cells with lower Cdk activity and ensure ordered cell cycle progression (Stern and Nurse, 1996). In line with the quantitative model, in fission yeast, cells harbouring a single M cyclin-Cdk complex, Cdc13-Cdc2, can meet stage specific activity thresholds to drive both S and M phases in an order (Coudreuse and Nurse, 2010; Fisher and Nurse, 1996; Swaffer et al., 2016). In budding yeast, sequential rise in the activity of G1, S, G2 and M cyclin-Cdk complexes towards a generic substrate (Koivomagi et al., 2011b) also supports the quantitative model. However, differential cyclin-Cdk complexes in budding yeast clearly show specificity towards distinct substrates (see 1.4.1. The qualitative model), arguing for a qualitative component that needs to be added to the quantitative model of the cell cycle.

With an aim to dissect the relative contributions of the two cell cycle models to the cell cycle progression in budding yeast, firstly, I attempt to drive S and M phases by a single M cyclin-Cdk complex. In this chapter, I test the potential of Clb2-Cdk in promoting both S and M phases and address if Clb2-Cdk is sufficient to sustain timely substrate phosphorylation in the absence of all other CLBs. I also explore the impact of losing S and G2/M cyclin specificity to the cyclin-Cdk substrate phosphorylation and timely cell cycle progression. My findings demonstrate that Clb2-Cdk can drive entry into both S and M phases when expressed earlier in the cell cycle, albeit with a delay. I also show *in vivo* evidence for the importance of cyclin specificity for timely and efficient cyclin-Cdk substrate phosphorylation. Lastly, I discuss the characteristics of S phase and mitosis driven by Clb2-Cdk in detail.

3.1 Early expressed Clb2 can promote S phase entry with a delay

Expression of mitotic cyclin Clb2 from an S phase cyclin *CLB5* promoter, hereafter S-Clb2, promotes S phase entry in the absence of Cdk inhibitor Swe1. However, phosphorylation of the replication initiation factor Sld2, whose phosphorylation is a minimal requirement for DNA replication, is delayed (Hu and Aparicio, 2005; Loog and Morgan, 2005; Masumoto et al., 2002; Zegerman and Diffley, 2007). To determine the consequences of driving the S and M phases with a mitotic cyclin-Cdk in the absence of remaining CLBs, I established a strain expressing two copies of Clb2, under control of *CLB5* and its own promoters, as a sole source of CLB-Cdk activity, hereafter Clns Clb2(S-M) (Figure 3.1.). This strain also lacks the Cdk inhibitor Swe1.

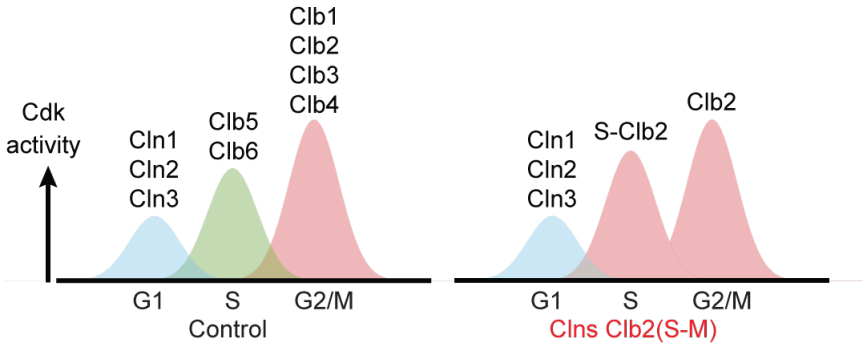


Figure 3.1. Schematics of the control and Clns Clb2(S-M)

Control cells harbour all nine budding yeast cyclins in different stages of the cell cycle. Clns Clb2(S-M) cells contain G1 cyclins as in control, but express only two copies of Clb2, under the *CLB5* promoter (S-Clb2) and an endogenous *CLB2* promoter (Clb2). Both strains lack the Cdk inhibitor Swe1. Note the gradual increase in kinase activity from G1 to M in the control. Accumulation of higher kinase activity due to the early expression of Clb2 in Clns Clb2(S-M) cells was also illustrated.

Next, Clns Clb2(S-M) cells were synchronised in G1 using mating pheromone α-factor and released to observe their cell cycle progression in a time course experiment compared to a control strain. The control strain also lacks Swe1, but is wild type for all budding yeast cyclins. Control and Clns Clb2(S-M) cells formed buds with similar timing as expected because budding is controlled by CLNs which are present in both strains (Benton et al., 1993; Lew and Reed, 1993). Moreover, S-Clb2 was detected 30 min after G1 release, coinciding with the timing of Clb5 expression in control cells (Figure 3.2.A). This indicates that replacing *CLB5* with *CLB2* still allows timely expression from the

CLB5 promoter. The Clns Clb2(S-M) cells showed a 15 min delay in S phase entry as assessed by flow cytometry analysis of DNA content, suggesting that Clb2 has a lower potential than Clb5 in promoting DNA replication. This observation could be explained by the notion that Clb5-Cdk has specific substrates, some of which are directly implicated in DNA replication, that cannot be efficiently phosphorylated by Clb2-Cdk (Loog and Morgan, 2005). Next, I performed *in vitro* kinase assays to determine the kinase activity levels in Clns Clb2(S-M) cells in comparison to the control strain. In these assays the Clns Clb2(S-M) cells showed higher kinase activity towards a generic substrate Histone H1 (Figure 3.2.B). This result was in line with the findings that Clb2-Cdk has higher intrinsic kinase activity than the Cln2-, Clb3- and Clb5-Cdk complexes (Koivomagi et al., 2011b; Loog and Morgan, 2005). Yet, having higher kinase activity earlier in the cell cycle was still not enough for these cells to trigger S phase entry on time, hinting the importance of cyclin specificity.

In the time course experiment, the residual Clb2 could be detected throughout the cell cycle (Figure 3.2.A). This can be a consequence of poor cell synchrony because approximately 10-15% of Clns Clb2(S-M) cells were budded in the beginning of this experiment, at the time of G1 arrest. Another reason for the detection of persistent Clb2 can be altered APC/C activity or checkpoint activation. It is also worth noting that Clns Clb2(S-M) cells struggle to complete mitotic exit because even after 180 min from G1 release, not all of the cells were re-arrested in G1. I will return to these arguments later in this chapter. Of note, it was difficult to synchronise Clns Clb2(S-M) cells in G1 using the mating pheromone α-factor, the nature of which remains elusive.

Overall, these results suggest that Clb2 has a lower potential than Clb5 in promoting DNA replication regardless of its higher intrinsic kinase activity. In the absence of remaining CLBs, Clb2 drives S phase with a delay.

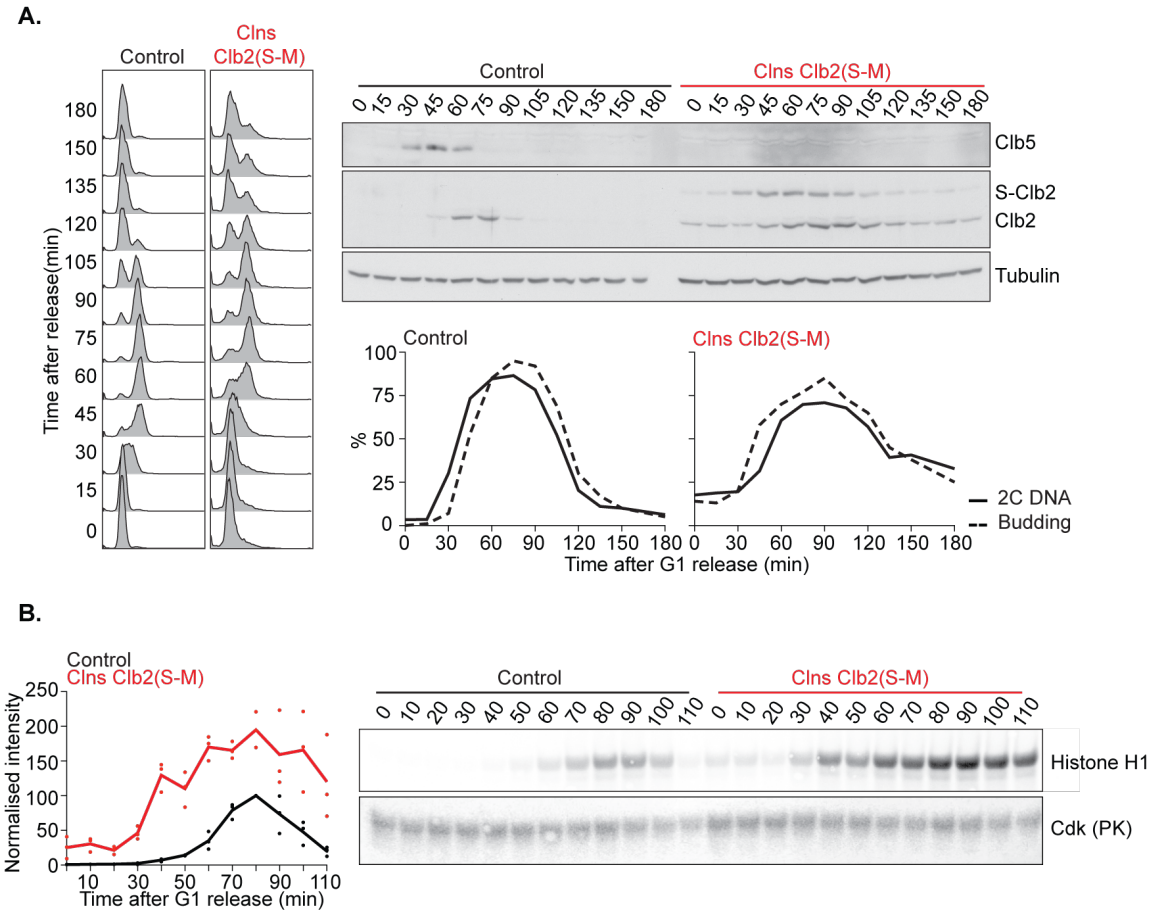


Figure 3.2. Clns Clb2(S-M) promotes S phase with a delay

A. Cells were arrested by α-factor in G1 and released to a synchronous cell cycle progression through one cell cycle before re-arrest in the following G1. Flow cytometry analysis of DNA content was performed after staining cells with propidium iodide. Clb5 and Clb2 antibodies were used for western blotting. S-Clb2 was fused to 6xHA epitope tag, hence it migrates slower than endogenous Clb2. Tubulin served as a loading control. Budding was scored by the presence of a small bud, one hundred cells per time point was counted in aliquots from the experiment. In flow cytometry profiles, propidium iodide signal after 1C DNA peak was quantified and plotted as 2C DNA content. A representative of three biological replicates was shown. **B.** Cells were arrested by α-factor in G1 and released. Cdk was fused to 3xPK epitope tag and was immunoprecipitated using a PK antibody at indicated times. Cdk associated kinase activity against histone H1 was measured by an *in vitro* kinase assay. Western blotting was used to control the amount of immunoprecipitated Cdk per time point. Quantification of histone H1 phosphorylation corrected for the Cdk amount was normalised to the 80 min time point in the control sample and three independent experiments were presented. The lines represented the mean of three independent experimental values. A representative autoradiogram was shown. The experiment in panel (A) was performed with the help of my summer student Florine Chretien.

3.2 Phosphoproteome analysis reveals importance of cyclin specificity

3.2.1 Phosphorylation dynamics in interphase

To determine the effect of driving S and M phases with a single mitotic cyclin Clb2-Cdk complex in the absence of the remaining CLBs to the phosphorylation landscape, I performed time resolved phosphoproteome analysis using tandem mass tag mass spectrometry. Control and Clns Clb2(S-M) cells were arrested in G1 using mating pheromone α-factor and released to synchronous cell cycle progression. Samples were collected at 10 min intervals from G1 until 90 min, when the control cells reached mitosis. Protein extracts were trypsin digested and peptides were labelled using 10 isobaric tandem mass tags. Following phosphopeptide enrichment liquid chromatography tandem mass spectrometry (LC-MS/MS) data were acquired (Figure 3.3.) (see 2.4.1. Mass spectrometry sample preparation). Overall, 9909 phosphosites were identified by mass spectrometry 3578 of which showed an increase in phosphorylation and categorised as phosphorylated phosphosites. Among 3578 phosphorylated phosphosites, 1091 sites contained minimal Cdk motif (S/T)P and were further categorised as phosphorylated cyclin-Cdk phosphosites (see 2.4.2. Phosphoproteome data analysis). The mass spectrometry analysis allowed me to study not only the contribution of cyclin specificity to phosphoproteome dynamics in *S. cerevisiae*, but also it expanded the current knowledge on the changes in phosphorylation dynamics from G1 to mitosis (Godfrey et al., 2017).

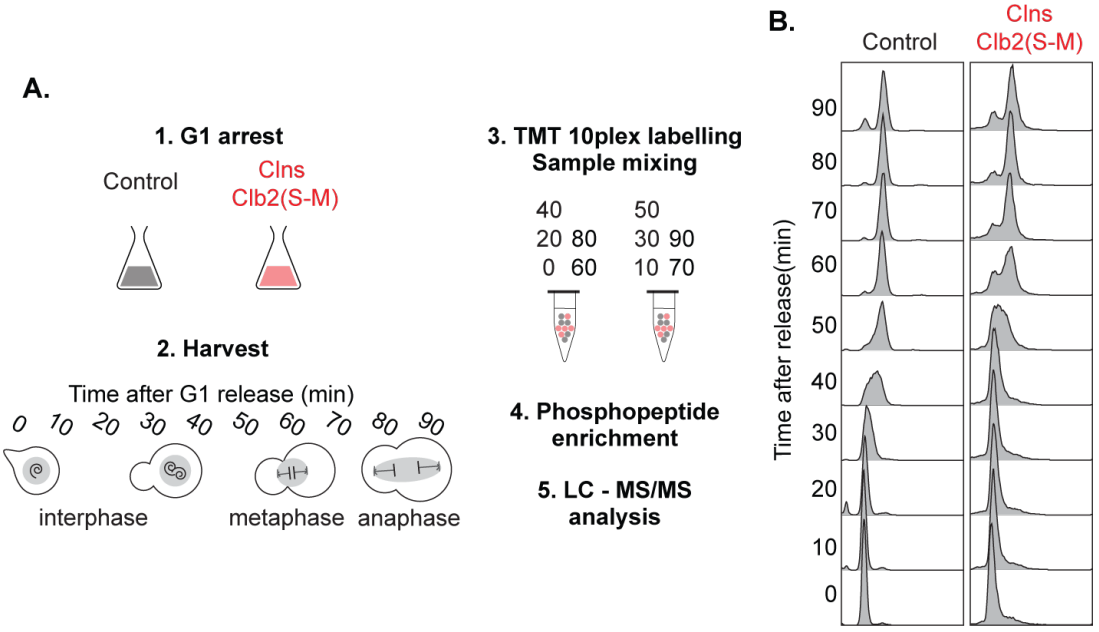


Figure 3.3. Mass spectrometry experimental design

A. Schematics showing the experimental design of tandem mass tag phosphoproteome analysis (see 2.4.1. Mass spectrometry sample preparation for details). **B.** Cells were arrested by α-factor in G1 and released to a synchronous cell cycle progression. Samples were collected every 10 min for mass spectrometry and DNA content analysis. Flow cytometry analysis of DNA content was performed after staining cells with propidium iodide.

3.2.2 Cdk phosphorylation landscape in Clns Clb2(S-M) cells

To gain further insights into the changes in the cyclin-Cdk phosphorylation landscape in cells lacking all CLBs except Clb2, I studied phosphorylated Cdk phosphosites further. In interphase, $PP2A^{Cdc55}$ counteracts cyclin-Cdk phosphorylation of proline directed threonine residues (TP) and allows earlier phosphorylation of serine residues (SP) (Godfrey et al., 2017). Therefore, I first compared the phosphorylation timing of Cdk sites containing SP or TP (Figure 3.4.A). Although the phosphosites with SP residues were phosphorylated earlier compared to TP in the control as expected, the difference between SP and TP phosphorylation timing vanished in Clns Clb2(S-M) cells. Next, I aimed to determine the differences in phosphorylation timing of phosphosites containing full or minimal Cdk motifs (Figure 3.4.B). In both strains, phosphosites with full Cdk motifs were phosphorylated efficiently and earlier than the ones with minimal motifs. However, phosphorylation of the Cdk sites involving minimal motifs was drastically affected in the absence of CLBs, suggesting that sites containing minimal Cdk motif might rely on cyclin docking interactions to facilitate substrate phosphorylation.

Cyclin-Cdk complexes sequentially phosphorylate substrates during the cell division cycle (Godfrey et al., 2017; Koivomagi et al., 2011b; Loog and Morgan, 2005; Swaffer et al., 2016; Touati et al., 2019; Touati et al., 2018). Sub-categorisation of phosphorylated Cdk sites based on their phosphorylation timing in the control cells (see 2.4.2. Phosphoproteome data analysis) allowed me to confirm successive cyclin-Cdk substrate phosphorylation waves. I then plotted the same phosphosites from these sub-categories for the Clns Clb2(S-M) strain to monitor if sequential phosphorylation of the cyclin-Cdk substrates was maintained (Figure 3.4.C). Timing of the early phosphorylations observed from 10 to 30 min was similar in the control and Clns Clb2(S-M) cells. This was expected because of the presence of CLNs in both strains. Phosphosites phosphorylated from 40 to 70 min, on the other hand, lost the temporal resolution in Clns Clb2(S-M) cells. In particular, 50 min and 70 min categories showed advanced phosphorylation in these cells. This suggests that the 50 min and 70 min groups might involve Clb2 specific sites, timing of which was affected from the early Clb2 expression. In addition, phosphorylation of the sites which exist in the 80 min category was completely lost in Clns Clb2(S-M) cells. These findings highlight the importance of cyclin specificity and the presence of a full set of cyclins for establishing Cdk phosphorylation landscape in budding yeast. Finally, a hierarchical clustering analysis allowed me to determine fine details of phosphosite behaviour in each category. Phosphosites containing S/TP Cdk minimal motif which showed an increase in phosphorylation during cell cycle progression were grouped by the time of phosphorylation. Heat maps were then generated based on phosphosite behaviour in the Clns Clb2(S-M) strain and the respective sites in control were plotted. Heat maps were separated into three subcategories which included phosphorylated phosphosites to a lesser, to approximately equal and to a greater extent in Clns Clb2(S-M) cells compared to the control. This indicates that the phosphorylation of Clns Clb2(S-M) targets is differentially affected, some sites being efficiently phosphorylated by Clb2-Cdk and some sites not (Figure 3.5.).

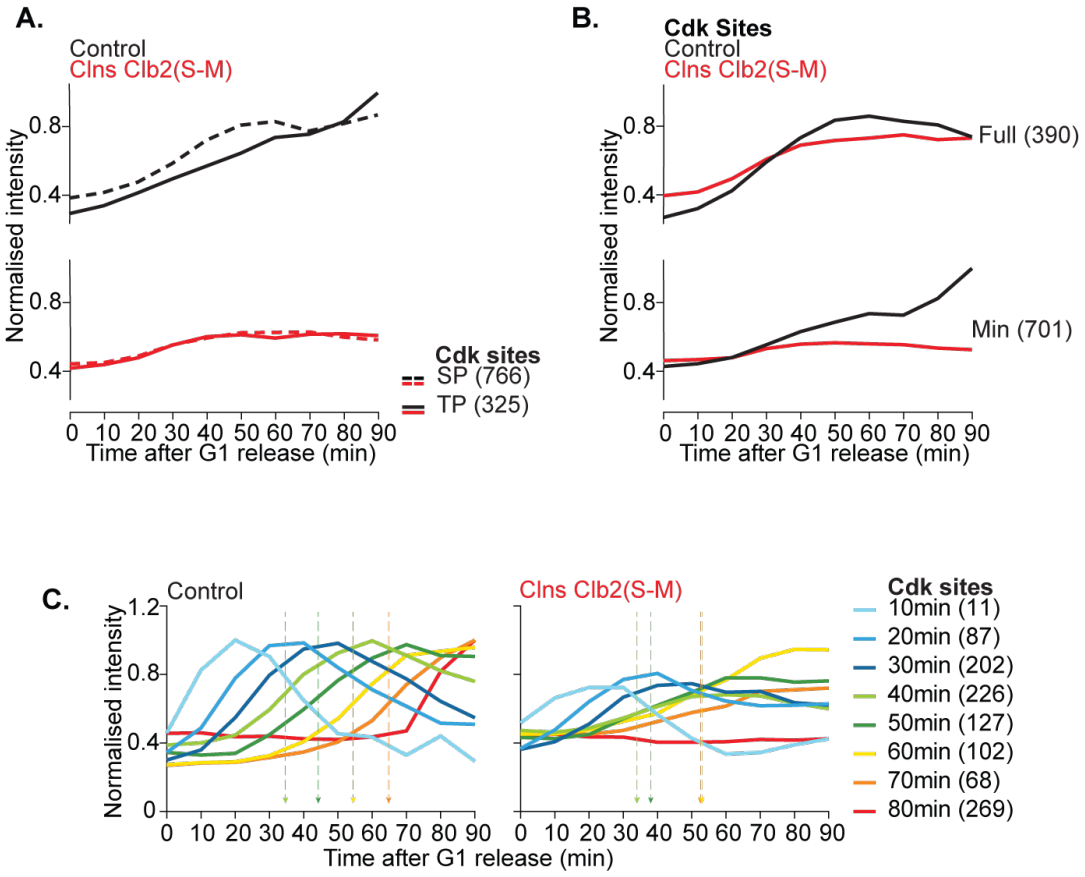
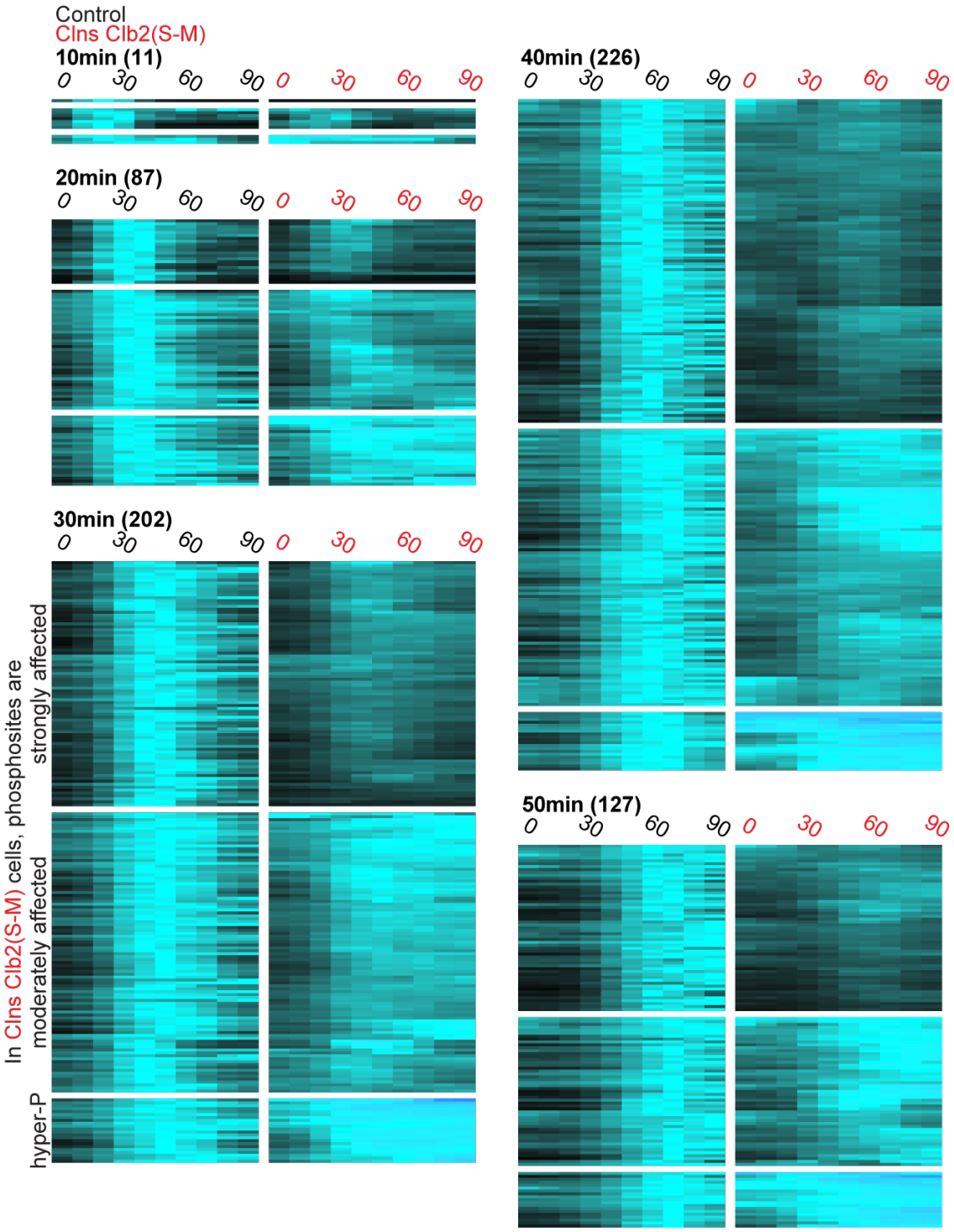


Figure 3.4. Phosphoproteome analysis of the phosphorylated Cdk sites

Median of the normalised intensity profiles of **A.** SP and TP Cdk phosphosites, **B.** Phosphosites containing full or minimum Cdk motifs, **C.** Phosphosites grouped based on their phosphorylation timing were plotted. The number of phosphosites in each category was depicted in brackets. Colour coded lines in (C) indicate phosphorylation mid points for 40 to 70 min categories which correspond to 35, 44, 54 and 65 min in the control and 34, 38, 52 and 52 min in Clns Clb2(S-M) cells, respectively.


Control
Clns Clb2(S-M)
10min (11)
20min (87)
30min (202)
40min (226)
50min (127)
0
30
60
90
In Clns Clb2(S-M) cells, phosphosites are
strongly affected
moderately affected
hyper-P

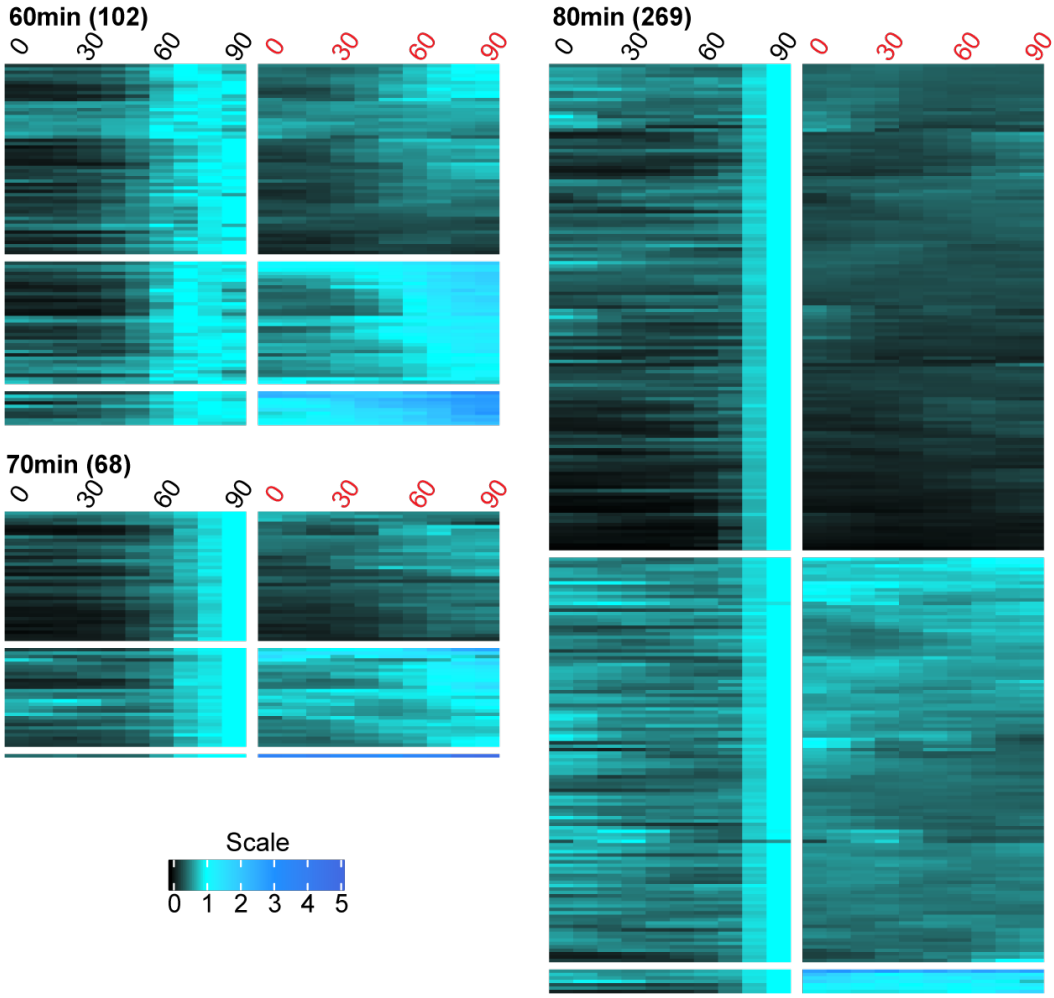


Figure 3.5. Global phosphorylation changes in Cdk phosphosites

Hierarchical clustering analysis of all phosphorylated Cdk phosphosites based on their phosphorylation timing. The dendrograms were generated with the help of Probir Chakravarty from the Crick Bioinformatics & Biostatistics STP. In each time point, phosphosites were divided into three groups to represent, from top to bottom, phosphosites that were strongly affected, moderately affected and hyperphosphorylated (hyper-P) in Clns Clb2(S-M) cells. The category containing phosphosites phosphorylated at 30 min was labelled as an example. This labelling applies to all time points. Each row represented a phosphorylated phosphosite. The total number of sites per time point was shown. Scale was set based on the normalised intensity values (see 2.4.2. Phosphoproteome data analysis).

3.2.3 Consequences of losing S phase cyclin specificity

The S phase cyclin Clb5-Cdk complexes exhibit specificity towards substrates implicated in replication (Loog and Morgan, 2005; Wilmes et al., 2004). Because the Clns Clb2(S-M) strain showed a delay in S phase entry (Figure 3.2.A), I addressed if this delay stemmed from the absence of Clb5-Cdk driven substrate phosphorylation. *In vitro* screening of Cdk targets revealed 14 Clb5-Cdk specific substrates (Loog and Morgan,

2005). 37 Cdk phosphosites that belong to these Clb5-Cdk substrates were phosphorylated at 22 min in the control strain which were delayed by 7 minutes in Clns Clb2(S-M) cells (Figure 3.6.A). On the contrary, 63 phosphosites on proteins that were phosphorylated with similar early timing in the control strain, but were not found to be specific for Clb5-Cdk (Loog and Morgan, 2005), were phosphorylated with similar timing in both control and Clns Clb2(S-M) cells.

A replication initiation factor Sld2 is an *in vitro* and *in vivo* Clb5-Cdk target whose timely phosphorylation relies on Clb5-Cdk kinase activity (Hu and Aparicio, 2005; Loog and Morgan, 2005; Zegerman and Diffley, 2007). Sld2 T84 phosphorylation completely vanished in Clns Clb2(S-M) cells compared to the control. This result is of great importance because the phosphorylation of T84 was proposed to be critical for DNA replication initiation and cell viability (Tak et al., 2006). Moreover, the phosphorylation of Sld2 Cdk phosphosites S188 and S100 were slightly affected and delayed, demonstrating that the phosphorylation of individual phosphosites are differentially regulated (Figure 3.6.B).

Next, I examined phosphorylation of the Clb5-Cdk targets Orc2 and Orc6, components of the origin recognition complex, whose phosphorylation is required to inhibit re-replication (Loog and Morgan, 2005; Nguyen et al., 2001; Weinreich et al., 2001). In the control strain, many early Orc2 and Orc6 phosphosites were simultaneously phosphorylated, whereas in Clns Clb2(S-M) cells phosphorylation of these sites was differentially affected (Figure 3.6.C). T24 and S206, the two most affected sites in Orc2, are located at 10 and 19 amino acid upstream of RxL and KxL motifs, respectively. This indicates the importance of Clb5-Cdk specific docking interactions to facilitate efficient phosphorylation of nearby sites. I also monitored the electrophoretic mobility shift of Orc2 and Orc6 by western blotting as a readout for their phosphorylation. Confirming my observations from the phosphoproteome analysis, both Orc2 and Orc6 showed slightly retarded mobility shifts and a low amount of phosphorylation in the Clns Clb2(S-M) strain compared to the control (Figure 3.6.D). Altogether, these findings demonstrate that S-Clb2-Cdk is not able to phosphorylate substrates implicated in DNA replication as

efficiently as Clb5-Cdk complexes and that cyclin substrate docking interactions are required for controlling phosphorylation of individual phosphosites.

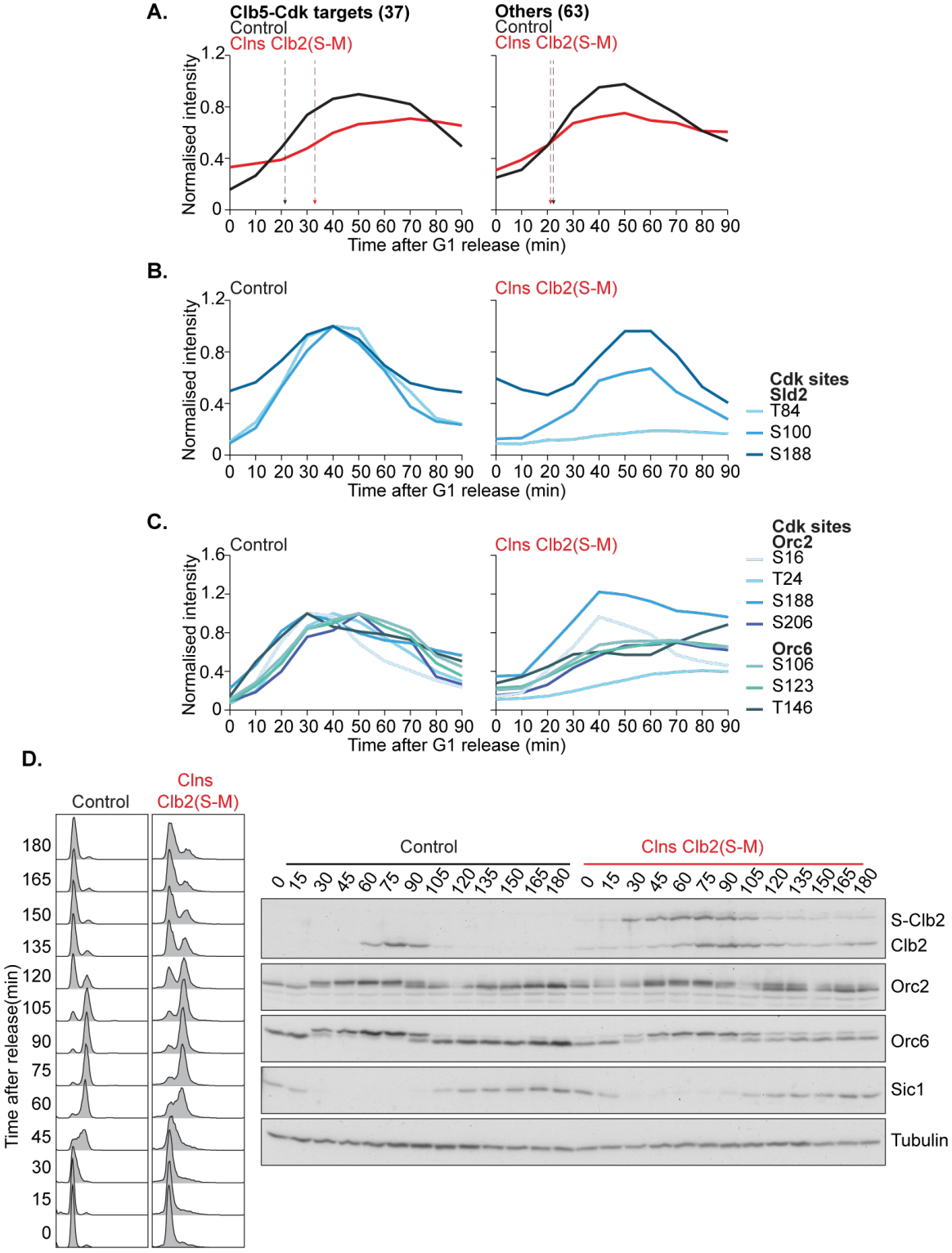


Figure 3.6. S-Clb2 fails at timely phosphorylation of Clb5-Cdk targets

(legend in the next page)

A. Median of the normalised intensity profiles of phosphosites that belong to *in vitro* Clb5-Cdk specific targets or other substrates which were not found to be specifically phosphorylated by Clb5-Cdk. Colour coded arrows indicated phosphorylation mid points 21 and 34 min for the Clb5-Cdk targets, 24 min and 21 min for the other substrates in the control and Clns Clb2(S-M) strains, respectively. Individual phosphorylated Cdk phosphosites found in **B.** Sld2, **C.** Orc2 and Orc6 were plotted. Note that T24 and S206 are nearby Clb5-Cdk docking motifs (K/R)xL. **D.** Cells were arrested by α-factor in G1 and released to a synchronous cell cycle progression through one cell cycle before re-arrest in the following G1. Flow cytometry analysis of DNA content was performed after staining cells with propidium iodide. Clb5, Clb2, Orc2, Orc6 and Sic1 antibodies were used for western blotting. S-Clb2 was fused to 6xHA epitope tag, hence it migrates slower than endogenous Clb2. Sic1 and Tubulin served as an internal cell cycle control and a loading control, respectively. Note the delay and reduced amount of electrophoretic mobility shift in Orc2 and Orc6 blots in Clns Clb2(S-M) cells compared to the shifts in control cells. A representative of three biological replicates was shown.

3.2.4 Impact of G2/M cyclin-Cdk specificity on substrate phosphorylation

The G2 cyclins Clb3/Clb4 were shown to specifically phosphorylate a set of cyclin-Cdk targets (Liakopoulos et al., 2003; Ord et al., 2020). Because the Clns Clb2(S-M) cells lacked Clb3/4, I asked if the G2 cyclin-Cdk specificity can be compensated by S-Clb2-Cdk activity. The spindle positioning factor Kar9 was proposed as a target of Clb3/4-Cdk complexes (Liakopoulos et al., 2003). I therefore monitored Kar9 electrophoretic mobility shift throughout the cell cycle in a time course experiment as before. In Clns Clb2(S-M) cells, the G2 specific phosphorylation of Kar9 was largely affected and the amount of phosphorylation judged by the slow migrating forms of Kar9 was lower compared to the control cells (Figure 3.7.A). In line with these observations, the phosphorylation of Kar9 Cdk sites detected in the phosphoproteome analysis were also impaired (Figure 3.7.B).

The HP dependent docking interactions between Clb3-Cdk complexes and a PxF motif bearing substrates were demonstrated *in vitro* (Ord et al., 2020). I next examined polar growth protein Boi1 and Cdc5 spindle pole body anchoring protein Csa1, both of which contain PxF motifs (Ord et al., 2020) (Figure 3.7.C). In Clns Clb2(S-M) cells, phosphorylation of Boi1 S393 that is positioned at 16 amino acids upstream of a PxF motif was drastically reduced. Similarly, phosphorylation of Csa1 S158 and S170 Cdk sites which reside within 40 amino acids upstream of a PxF motif were also affected in Clns Clb2(S-M) cells. Interestingly, the level of Csa1 S175 phosphorylation was not affected even though the onset of its phosphorylation showed a slight delay in Clns

Clb2(S-M) compared to the control strain. Together, these data indicate that G2 cyclin-Cdk specificity and Clb3-Cdk mediated docking interactions are required for timely and efficient substrate phosphorylation and cannot be substituted by S-Clb2-Cdk activity.

Since I demonstrated how the S cyclin-Cdk and G2 cyclin-Cdk docking site interactions shape the complex Cdk phosphorylation landscape during cell cycle progression, the next question then became whether phosphorylation timing of Clb2-Cdk targets change when Clb2 is expressed earlier in the cell cycle. To address this question, a late substrate Ndd1, whose phosphorylation is cell cycle regulated and mediated by Clb2-Cdk, was studied (Darieva et al., 2003; Reynolds et al., 2003). In a time course experiment, expression and degradation of Ndd1 showed similar timing in both control and Clns Clb2(S-M) cells. However, in the Clns Clb2(S-M) strain, Ndd1 phosphorylation was notably advanced and slow migrating forms of Ndd1 were present when Ndd1 was just starting to accumulate (Figure 3.8.A). In support of the western blotting analysis, phosphoproteome analysis revealed that the phosphorylation of most Ndd1 Cdk phosphosites in Clns Clb2(S-M) was advanced compared to the control (Figure 3.8.B). Increased phosphorylation of S168 and T236 correlated with the presence of nearby LxF motifs which were positioned 17 and 9 and amino acids downstream of the phosphosites, respectively. Ndd1 T319 was the only site whose phosphorylation was affected in Clns Clb2(S-M) cells. This site was located 7 amino acids upstream of a Clb5 specific KxL motif. Because T319 phosphorylation recruits Ndd1 to the Fkh2 transcription factor at the CLB cluster gene promoters to allow a complete activation of the CLB cluster gene transcription, impaired T319 phosphorylation could also indicate defective activation of the CLB cluster gene transcription in Clns Clb2(S-M) cells (Reynolds et al., 2003).

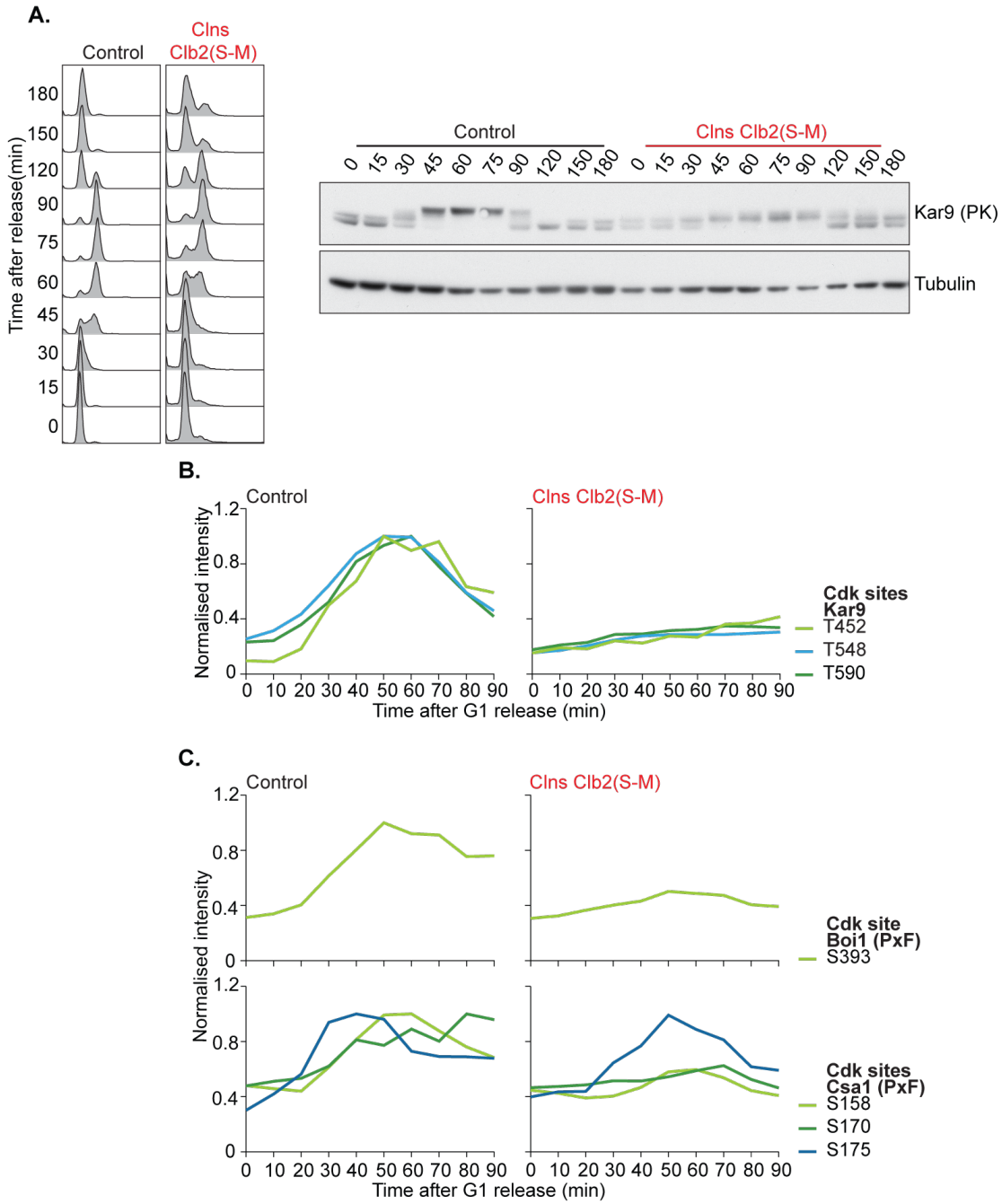


Figure 3.7. S-Clb2-Cdk is not able to compensate for the G2 cyclin-Cdk specificity

A. Cells were arrested by α-factor in G1 and released to a synchronous cell cycle progression through one cell cycle before re-arrest in the following G1. Flow cytometry analysis of DNA content was performed after staining cells with propidium iodide. Kar9 was fused to 3xPK epitope tag and detected using an antibody against PK. Tubulin served as a loading control. Note the delay and reduced amount of electrophoretic mobility shift in Kar9 in Clns Clb2(S-M) cells compared to the control cells. A representative of two biological replicates was shown. Individual phosphorylated Cdk phosphosites found in **B.** Kar9, **C.** Boi1 and Csa1 were plotted. For simplicity, four other Boi1 phosphosites which were not located in the vicinity of a docking motif were not plotted.

The two LxF motif containing proteins, Cdc6 and Bni1, were proposed to be Clb2-Cdk specific substrates (Ord et al., 2019b). Both Cdc6 T23 and Bni1 T1918 phosphosites are located at 22 and 20 amino acids upstream of an LxF motif, respectively. In Clns Clb2 (S-M) cells, phosphorylation of these sites was advanced compared to the control, confirming that S-Clb2 can employ LxF docking mechanisms to advance phosphorylation of the sites in the vicinity (Figure 3.8.C). The other Bni1 Cdk phosphosites detected in the phosphoproteome analysis, S261 and S993, were also phosphorylated on time in Clns Clb2 (S-M) cells. This result was unanticipated because both S261 and S993 are at an optimal distance to a Clb5-Cdk specific docking motif KxL (Figure 3.8.C), suggesting that in some cases cyclin-Cdk complexes can interchangeably phosphorylate sites that are optimal targets of a specific cyclin-Cdk complex. The underlying mechanisms behind this need to be further studied. To conclude, early expression of Clb2 is able to bring forward phosphorylation of late Clb2-Cdk targets, potentially by using its LxF substrate docking properties.

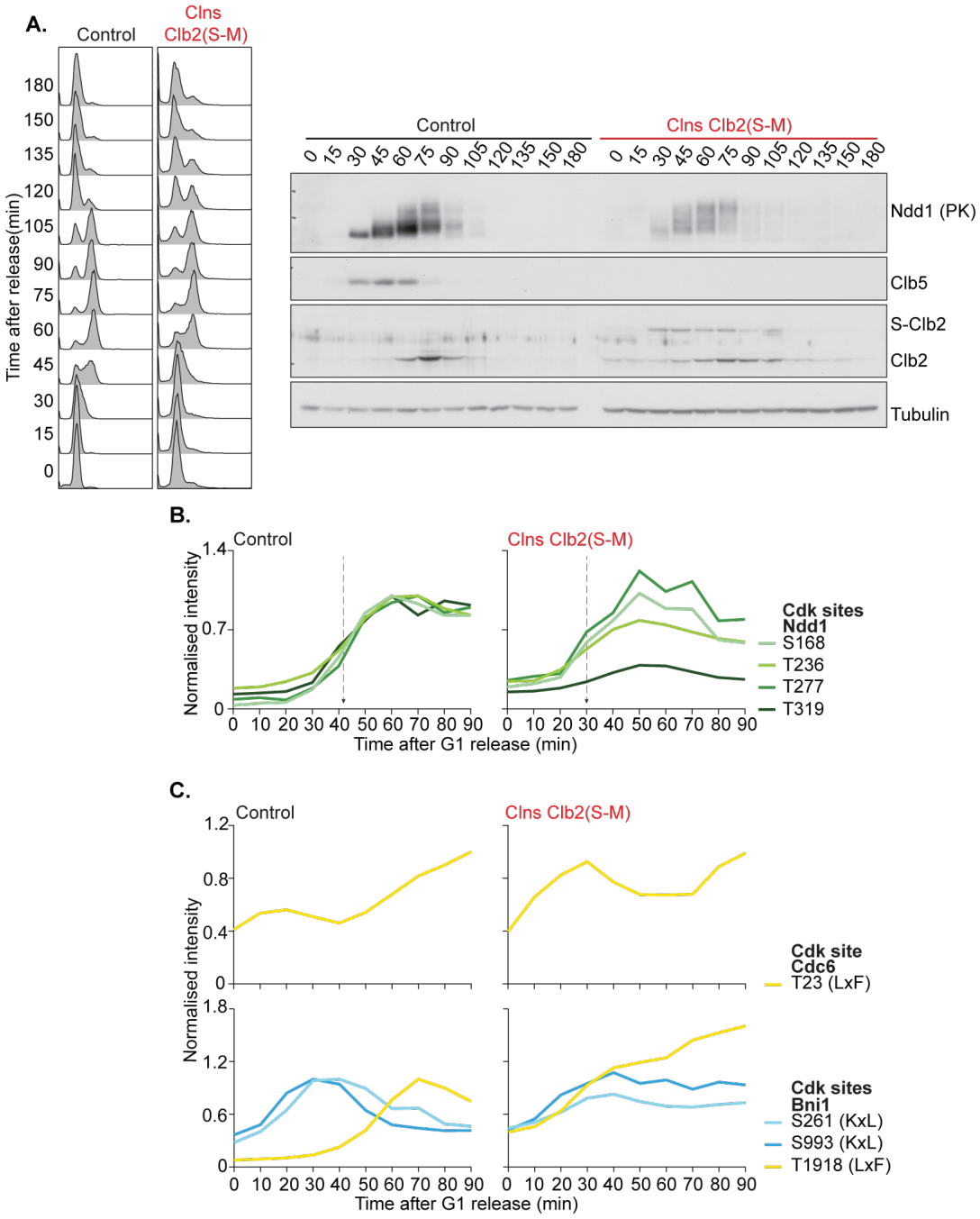


Figure 3.8. S-Clb2-Cdk can advance phosphorylation of Clb2-Cdk targets

A. Cells were arrested by α-factor in G1 and released before re-arrest in the following G1. Flow cytometry analysis of DNA content was performed after staining cells with propidium iodide. Ndd1 was fused to 3xPK epitope tag and detected using an antibody against PK. Clb5 and Clb2 antibodies were used for western blotting. S-Clb2 was fused to 6xHA epitope tag, hence it migrates slower than endogenous Clb2. Tubulin served as a loading control. Note the advanced and enhanced electrophoretic mobility shift in Ndd1 starting at 30 min after G1 release in Clns Clb2(S-M) cells. A representative of three biological replicates was shown. Individual phosphorylated Cdk phosphosites found in **B.** Ndd1, **C.** Cdc6 and Bni1 were plotted. In (B) arrows indicated phosphorylation mid points 42 and 30 min for Ndd1 in the control and Clns Clb2(S-M) strains, respectively. Ndd1 time course experiment in panel (A) was performed by my summer student, Florine Chretien.

3.3 Mitotic exit is compromised in Clns Clb2(S-M) cells

3.3.1 S phase and mitotic checkpoints are not activated in Clns Clb2(S-M)

In Clns Clb2(S-M) cells, Clb2 degradation was slow and incomplete for the duration of my time course experiments (Figure 3.2.A). Moreover, the extent and onset of dephosphorylation of Cdk targets were affected in Clns Clb2(S-M) cells compared to the control (Figure 3.6.D and 3.7.A). These observations could imply compromised mitotic exit as a result of checkpoint activation and subsequent inhibition of the APC/C. To address if there is a checkpoint activation in Clns Clb2(S-M) cells, I first assessed replication checkpoint activation by monitoring Rad53 phosphorylation in a time course experiment (Sanchez et al., 1996). However, I could not detect slower migrating phosphorylated forms of Rad53 in the Clns Clb2(S-M) strain (Figure 3.9.A). Next, I examined if the mitotic checkpoint was engaged in Clns Clb2(S-M) cells by monitoring Mad1 phosphorylation (Hardwick and Murray, 1995). However, I could not observe Mad1 electrophoretic mobility shift in Clns Clb2(S-M) cells. Together, these data suggest that the replication checkpoint and the mitotic checkpoint are not active in Clns Clb2(S-M) cells.

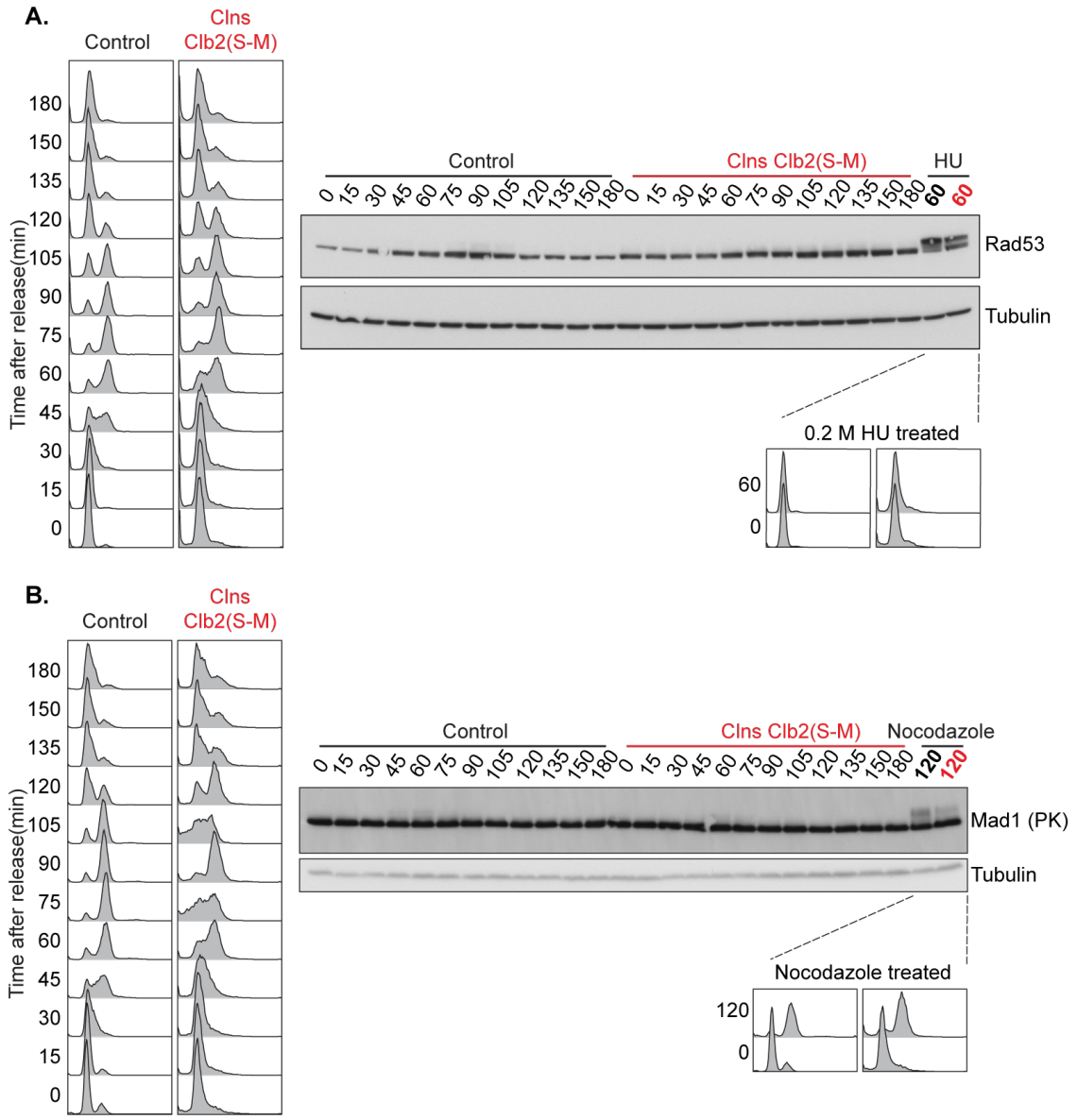


Figure 3.9. Replication and mitotic checkpoints are not activated in the Clns Clb2(S-M) strain

Cells were arrested by α-factor in G1 and released to a synchronous cell cycle progression through one cell cycle before re-arrest in the following G1. Flow cytometry analysis of DNA content was performed after staining cells with propidium iodide. Tubulin served as a loading control. **A.** Rad53 antibody was used for western blotting. **B.** Mad1 was fused to 3xPK epitope tag and detected using an antibody against PK. Positive controls for both the control and Clns Clb2(S-M) strains were also arrested in G1, but released into a medium containing **A.** 0.2 M hydroxyurea or **B.** 8 µg/ml nocodazole and collected at indicated times after G1 release. Strains used in these experiments were generated by my summer student, Florine Chretien. Representatives of two biological replicates were shown.

3.3.2 DNA damage signalling can delay mitotic exit

Securin also acts as an effector downstream of the budding yeast cell cycle checkpoints and is stabilised in response to checkpoint activation (Cohen-Fix et al., 1996; Yamamoto et al., 1996). I therefore surveyed the securin degradation pattern in a time course experiment. Examination of securin levels revealed a delay in securin degradation in Clns Clb2(S-M) cells. Moreover, I observed hyperphosphorylated forms of securin in the Clns Clb2(S-M) strain (Figure 3.10.A). In response to DNA damage, hyperphosphorylation of securin by Chk1 kinase makes it resistant to APC/C^{Cdc20} mediated degradation and results in a pre-anaphase arrest (Wang et al., 2001), suggesting that Clns Clb2(S-M) cells might be experiencing DNA damage. In support of this hypothesis, the three previously identified Chk1 phosphosites which get phosphorylated in response to DNA damage, S121, S132, S158, (Wang et al., 2001) were hyperphosphorylated in the Clns Clb2(S-M) strain compared to the control (Figure 3.10.B). Together, these findings suggest presence of an active DNA damage signalling pathway, albeit inactive replication and mitotic checkpoints.

Securin inhibits separase to prevent sister chromatid separation until anaphase onset and is also needed for separase nuclear accumulation (Jensen et al., 2001). The latter relies on securin phosphorylation at three Cdk sites, S277, S292, T304 which allows securin interaction with separase (Agarwal and Cohen-Fix, 2002). S292, one of the Cdk phosphosites that contributes to securin association with separase was detected in my phosphoproteome analysis. In Clns Clb2(S-M) cells, S292 phosphorylation was slightly delayed compared to control cells (Figure 3.10.B), pointing out a potential delay in separase nuclear accumulation, hence suspension of chromosome segregation and subsequent mitotic exit.

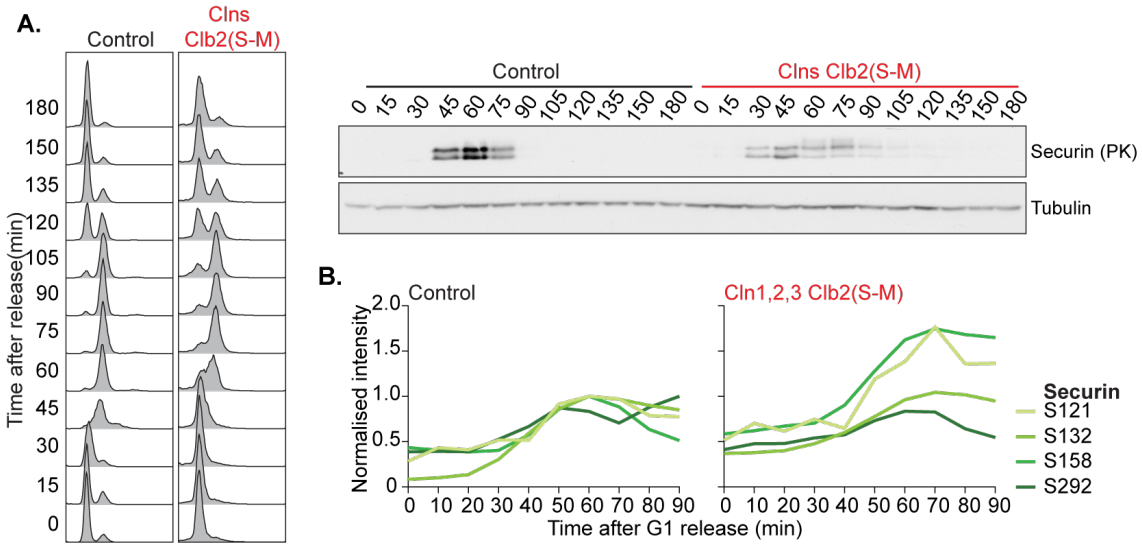


Figure 3.10. Securin is stabilised and hyperphosphorylated in Clns Clb2(S-M) cells

A. Cells were arrested by α-factor in G1 and released to a synchronous cell cycle progression through one cell cycle before re-arrest in the following G1. Flow cytometry analysis of DNA content was performed after staining cells with propidium iodide. Securin was fused to 3xPK epitope tag and detected using an antibody against PK. Tubulin served as a loading control. Note the delay in securin degradation and slow migrating hyperphosphorylated forms of securin in Clns Clb2(S-M) cells compared to the control cells. A representative of two biological replicates was shown. **B.** Individual phosphorylated Chk1 (S121, S132, S158) and Cdk (S292) phosphosites found in securin were plotted. Note that S292 was not hyperphosphorylated and delayed in the Clns Clb2(S-M) strain.

When securin degradation is delayed, it blocks mitotic exit by preventing separase from facilitating the Cdc14 release from nucleolus where it is kept inactive (Cohen-Fix and Koshland, 1999; Queralt et al., 2006; Queralt and Uhlmann, 2008b). Therefore, I next examined the timing of Cdc14 release from the nucleolus in a time course experiment. I used strains carrying an epitope tag at the C terminus of endogenous Cdc14 which allowed Cdc14 detection by indirect immunofluorescence. In Clns Clb2(S-M) cells, Cdc14 was retained in the nucleolus approximately 15 min longer compared to the control cells (Figure 3.11.A). Cdc14 is sequestered in nucleolus by its inhibitor protein Net1 and separase also has a role in promoting cyclin-Cdk mediated phosphorylation and dissociation of Net1 from Cdc14 (Azzam et al., 2004; Queralt et al., 2006). Consequently, I monitored the electrophoretic mobility shift of Net1 in a time course experiment which revealed a delay and a reduction in Net1 phosphorylation (Figure 3.11.B). Lastly, the analysis of 4 of 6 Cdk phosphosites in Net1 that are implicated in Cdc14 release (Azzam et al., 2004) showed that Net1 phosphorylation was indeed retarded and incomplete in the

Clns Clb2(S-M) strain (Figure 3.11.C). Overall, these results suggest that DNA damage signalling postpones execution of mitotic exit in Clns Clb2(S-M) cells.

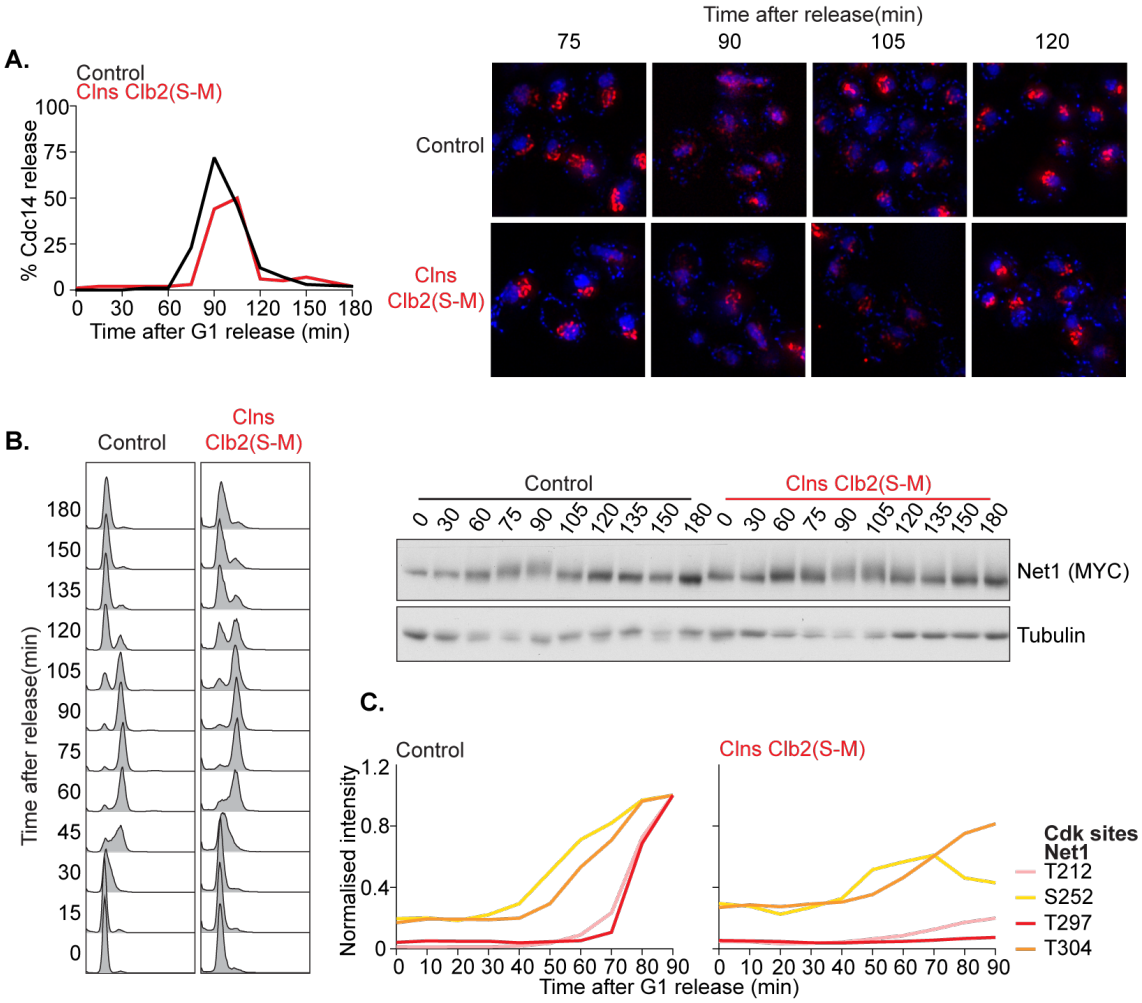


Figure 3.11. Mitotic exit is impaired in the Clns Clb2(S-M) strain

A. Cells were arrested by α-factor in G1 and released to a synchronous cell cycle progression through one cell cycle before re-arrest in the following G1. Cdc14 was fused to 9xPK epitope tag and detected using a primary antibody against PK and a secondary antibody conjugated to Cy3. A hundred cells per time point were quantified. Percentage of cells which lost the bright nucleolar signal were plotted. Representative images from indicated time points were shown. **B.** Cells were arrested by α-factor in G1 and released to a synchronous cell cycle progression through one cell cycle before re-arrest in the following G1. Flow cytometry analysis of DNA content was performed after staining cells with propidium iodide. Net1 was fused to 9xMYC epitope tag and detected using an antibody against MYC. Tubulin served as a loading control. A representative of two technical replicates was shown. **C.** Individual phosphorylated Cdk phosphosites implicated in Cdc14 release found in Net1 were plotted.

3.3.3 Absence of Clb5 affects Clb2 degradation and reduces cell fitness

To gain further insights into where the Clb2 degradation problem stemmed from, I investigated Clb2 degradation patterns in various strain backgrounds in a time course experiment. In summary, loss of Clb5 alone affected Clb2 degradation (Figure 3.12.A) and expression of S-Clb2 instead of Clb5 in the presence of all other CLNs and CLBs worsened the Clb2 degradation profile (Figure 3.12.B). Interestingly, absence of Clb1/3/4/6 did not affect Clb2 degradation timing in a strain harbouring wild type Clb5 and Clb2 in addition to CLNs (Figure 3.12.C). These observations imply an intriguing role for Clb5 in Clb2 degradation, potentially indirectly by preventing DNA damage during replication.

In an unperturbed cell cycle, a small pool of Clb2 is targeted for degradation by the APC/C^{Cdc20} in early anaphase. The Clb2 degradation is completed by the APC/C^{Cdh1} in late anaphase (Baumer et al., 2000). I therefore suspected that the Clb2 degradation problem could also stem from the incomplete activation of the APC/C^{Cdh1}. I then asked if the degradation problem was specific to Clb2 or could be a general problem observed in the other APC/C^{Cdh1} targets. To address this question I monitored the degradation pattern of another APC/C^{Cdh1} target Cdc5 in a time course experiment (Charles et al., 1998; Visintin et al., 2008). In fact, the Cdc5 degradation was also slow and incomplete for the duration of my time course experiment in both Clns Clb2(S-M) cells and in the strain lacking Clb5 (Figure 3.13.), suggesting a link between Clb5 and the APC/C^{Cdh1} activation.

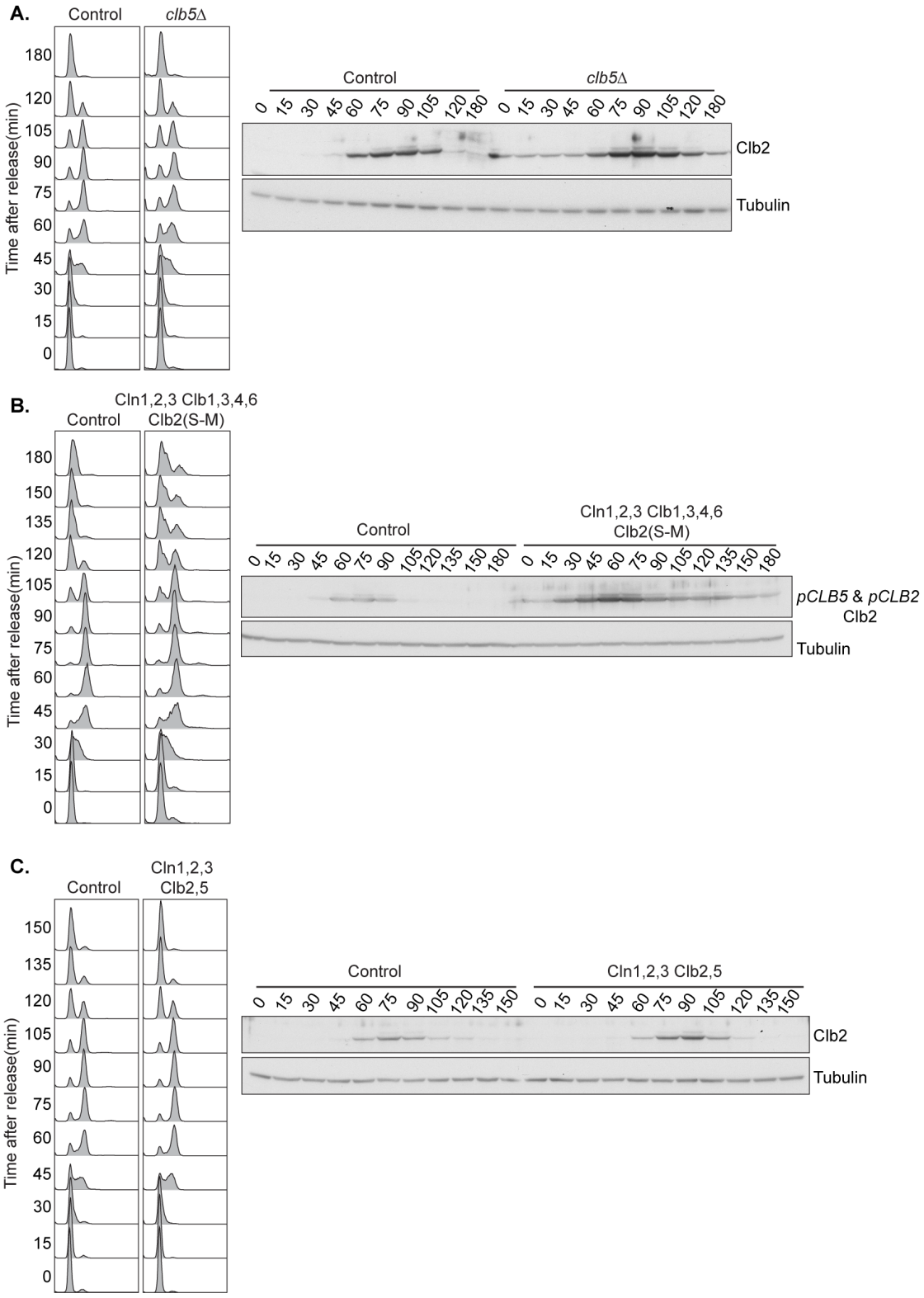


Figure 3.12. Loss of Clb5 results in Clb2 stabilisation

Cells were arrested by α-factor in G1 and released to a synchronous cell cycle progression through one cell cycle before re-arrest in the following G1. Clb2 antibody was used for western blotting. Tubulin served as a loading control. All strains were *swe1*Δ, additional genotypes as indicated. Representatives of three biological replicates were shown.

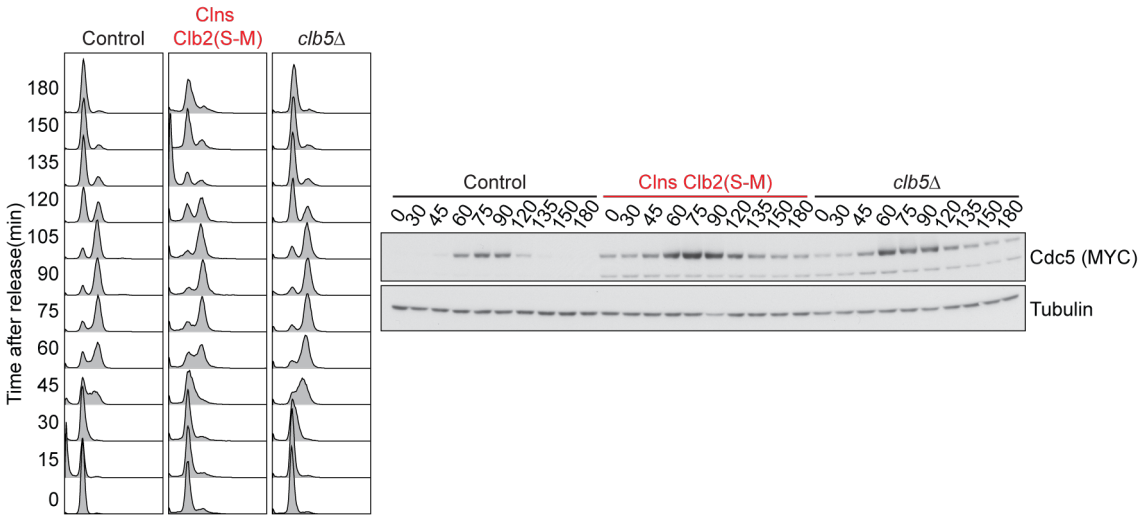


Figure 3.13. Cdc5 degradation is not complete in Clns Clb2(S-M) cells and in the strain lacking Clb5

Cells were arrested by α-factor in G1 and released to a synchronous cell cycle progression through one cell cycle before re-arrest in the following G1. Flow cytometry analysis of DNA content was performed after staining cells with propidium iodide. Tubulin served as a loading control. Cdc5 was fused to 9xMYC epitope tag and detected using an antibody against MYC. All strains were *swe1*Δ, additional genotypes as indicated. A representative of two biological replicates was shown.

Finally, I tested the importance of cyclin specificity under stress conditions by exposing the abovementioned cells as well as the Clns Clb2(S-M) strain to increased temperature and replication stress induced by HU in a spot assay (Figure 3.14.). All tested strains formed similar sized spots when grown in optimal conditions at 25°C on rich media; however, the Clns Clb2(S-M) strain showed a slight growth defect. Moreover, I found that stress sensitivity was mainly stemmed from the loss of Clb5 and was aggravated when Clb5 was replaced with Clb2 to express Clb2 earlier in than its usual timing. Strikingly, the cells lacking Clb1/3/4/6 behaved similar to the control cells when challenged under stress conditions. Together, these results demonstrate the importance of cyclin specificity, and especially the presence of Clb5, for fine tuning cell cycle progression and promoting cellular fitness to stress conditions in budding yeast.

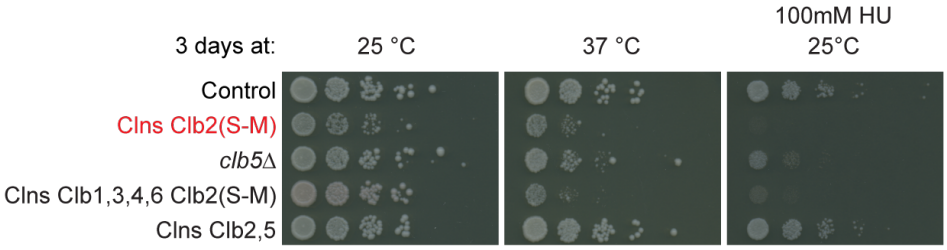


Figure 3.14. Absence of Clb5 results in sensitivity to cellular stress

Tenfold serial dilutions of cells with indicated genotypes were grown on rich media, YPD plates, for 3 days at the temperatures and treatments as indicated. A representative of two biological replicates was shown.

Chapter 4. G1 cyclin-Cdk specificity is required for cell proliferation

In Chapter 3, I addressed whether the mitotic cyclin-Cdk complex, Clb2-Cdk, is sufficient to promote S and M phases in the absence of CLBs in *S. cerevisiae*. I now expand this question to G1 and investigate the possibility of establishing a budding yeast strain which relies on Clb2-Cdk activity as a sole source of cyclin-Cdk activity throughout the cell division cycle.

In this chapter, I first illustrate that the strain relying on a G1 cyclin Cln2-Cdk and Clb2-Cdk in S and M phases results in a delay at the G1/S transition. I demonstrate that this delay can be rescued by Clb2 when expressed under control of a G1 cyclin *CLN2* promoter. Next, I remove the remaining G1 cyclin Cln2 from these cells and show that Clb2-Cdk is capable of supporting cell cycle transitions from G1 through S phase and into mitosis. This indicates that the Clb2-Cdk driven cell cycle in budding yeast meets the key expectations of the quantitative model in the absence of all other CLBs and CLNs. However, my findings indicate that the cells expressing Clb2-Cdk as a sole source of cyclin-Cdk activity is not able to support polarisation, bud formation and cell proliferation. This suggests that the G1 cyclin Cln2-Cdk complex is crucial for budding and cell viability and cannot be compensated by Clb2-Cdk. Therefore, I propose that the Cln2-Cdk complex is an essential qualitative component of the cell division cycle that is needed to complement the quantitative cell cycle control. Altogether, my findings provide insights into functional differences between G1 and mitotic cyclins and reveal that the budding yeast needs at least two distinct cyclin-Cdk complexes to couple cell division to morphogenetic developments.

4.1 Loss of CLNs delays the G1/S transition

Loss of G1 cyclins results in a lethal phenotype which can be rescued by the removal of the Clb-Cdk stoichiometric inhibitor Sic1(Tyers, 1996), indicating that it is enabling Cdk activation that drives the G1/S transition rather than G1 cyclin specificity. I therefore attempted to eliminate all G1 cyclins and Sic1 from Clns Clb2(S-M) in order to address if Clb2-Cdk alone can drive the cell cycle from G1 to mitosis. I successfully removed Cln1, Cln3 and Sic1 from Clns Clb2(S-M) cells, leaving Cln2 as an only G1 cyclin in these cells, hereafter referred to as Cln2 Clb2(S-M) (Figure 4.1.). However, unexpectedly, all my attempts to delete Cln2 in this strain failed. I also failed to establish a strain harbouring only Cln1 or Cln3 in G1 in cells driving the rest of the cell cycle with Clb2-Cdk.

I next asked if Cln2 can be made redundant in Cln2 Clb2(S-M) cells by expressing Clb2 in G1. Using a similar approach to the one I employed to drive S phase with an early expressed Clb2, I integrated a third copy of Clb2 expressed under control of the *CLN2* promoter, referred to as G1-Clb2, in Cln2 Clb2(S-M) cells, hereafter Cln2 Clb2(G1-S-M) (Figure 4.1.). I then attempted to delete Cln2 in this background. However, all my efforts were unsuccessful. This suggests that, likewise Cln2 Clb2(S-M) cells, Cln2 Clb2(G1-S-M) cells are also dependent on Cln2 for viability.

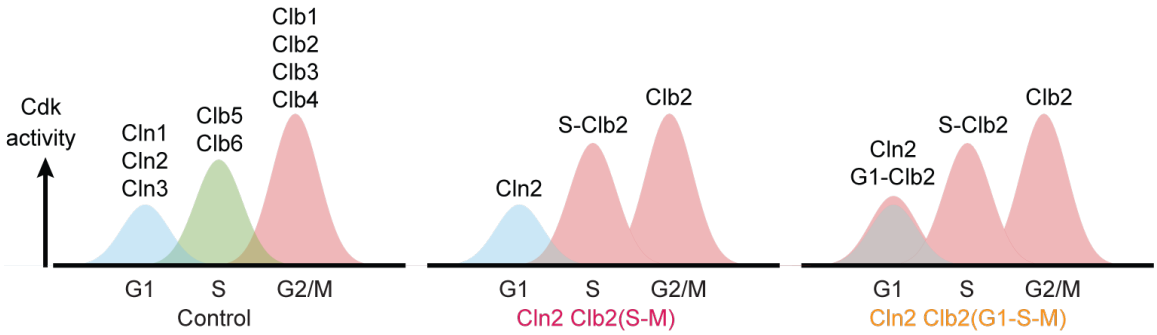


Figure 4.1. Schematics of the control, Cln2 Clb2(S-M) and Cln2 Clb2(G1-S-M) cells

Control cells harbour all nine budding yeast cyclins in different stages of the cell cycle. In Cln2 Clb2(S-M) cells Cln2 was the only G1 cyclin and two copies of Clb2, under the *CLB5* promoter (S-Clb2) and an endogenous *CLB2* promoter (Clb2) driving the S and M phases in the absence of remaining CLBs. The Cln2 Clb2(G1-S-M) strain was derived from Cln2 Clb2(S-M) cells by integrating a third copy of Clb2 expressed under control of the *CLN2* promoter. All strains lack the Cdk inhibitors Swe1 and Sic1.

To characterise the Cln2 Clb2(S-M) and Cln2 Clb2(G1-S-M) cells, I then performed a time course experiment by releasing cells to a synchronous cell cycle progression from the mating pheromone α-factor induced G1 arrest. The absence of Cln1 and Cln3 caused approximately a 30 min delay at the G1/S transition as assessed by DNA content analysis in Cln2 Clb2(S-M) cells compared to the control strain lacking Cdk inhibitors Sic1 and Swe1. In Cln2 Clb2(S-M) cells, Cln2 expression was also delayed by 15 min compared to the control (Figure 4.2.). This was consistent with the role of G1 cyclins in promoting each other's expression (Koch et al., 1996; Skotheim et al., 2008). In later stages of the cell cycle, Clb2 expression is believed to repress expression of G1 cyclins (Amon et al., 1993). Interestingly, in the Cln2 Clb2(G1-S-M) strain timing of G1-Clb2 expression coincided with Cln2 expression, indicating that presence of Clb2 did not affect Cln2 expression. In fact, G1-Clb2 expression partially rescued delayed replication and Cln2 expression (Figure 4.2.). Altogether, Clb2-Cdk was able to accelerate G1/S transition when expressed in G1 from *CLN2* promoter.

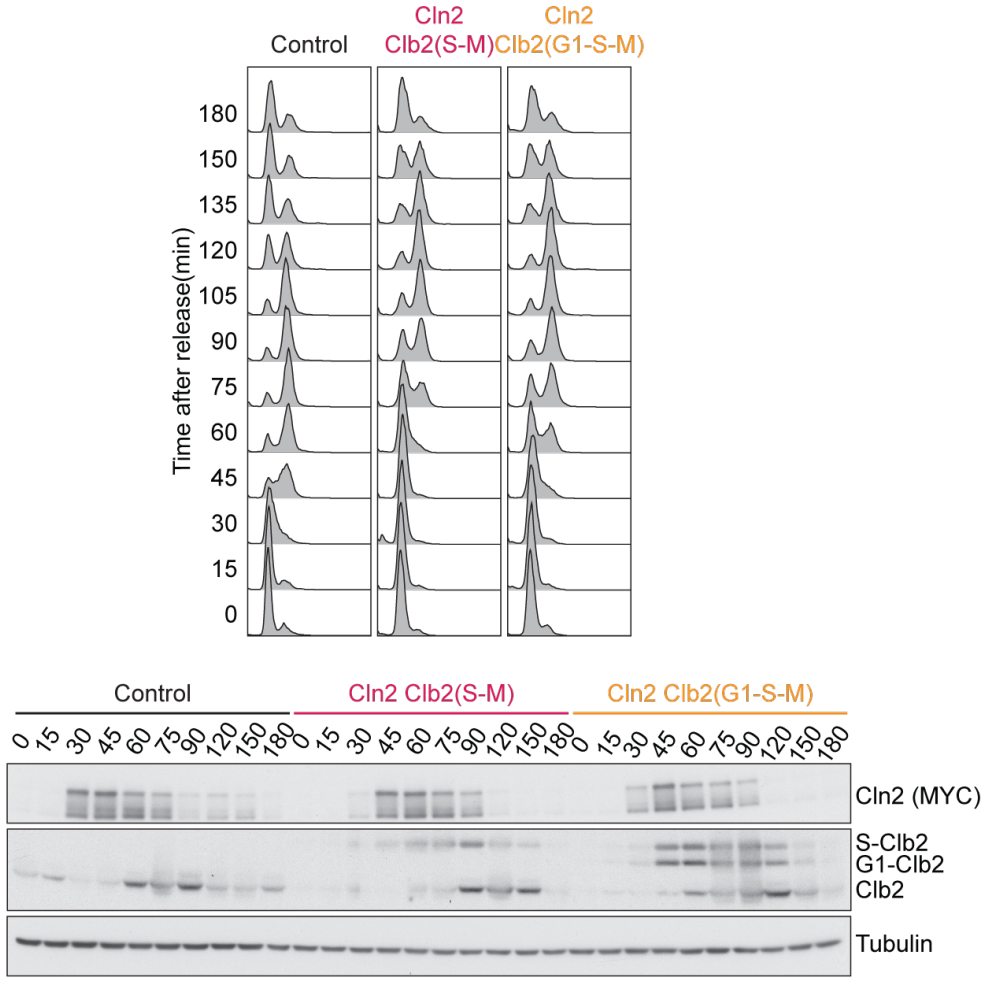


Figure 4.2. G1-Clb2 rescues G1/S transition delay

Cells were synchronised using α-factor and released to progress through the cell cycle before re-arrest in the following G1. Cell cycle progression was monitored by flow cytometry analysis of DNA content and western blotting. Clb2 expressed from the *CLN2* promoter was fused to a 3xHA epitope tag, leading to migration between *CLB5* promoter-expressed 6xHA epitope tagged Clb2 and endogenous Clb2. Clb2 was fused to 9xMYC epitope tag. Clb2 and MYC antibodies were used for western blotting. Tubulin served as a loading control. A representative of two biological replicates was shown.

4.2 Driving the cell cycle with a single cyclin-Cdk in budding yeast

4.2.1 Clb2-Cdk can promote replication and segregation in the absence of G1 cyclins

Because I failed to remove Cln2 from both Cln2 Clb2(S-M) and Cln2 Clb2(G1-S-M) cells, to study the role of Cln2 in these strain backgrounds I substituted endogenous *CLN2* promoter with a repressible *MET3* promoter, hereafter referred to as *MET3pr*Cln2

Clb2(S-M) and *MET3pr*Cln2 Clb2(G1-S-M), respectively. Presence of methionine in growth medium represses the *MET3* promoter and turns off Cln2 expression in these strains (Figure 4.3.)

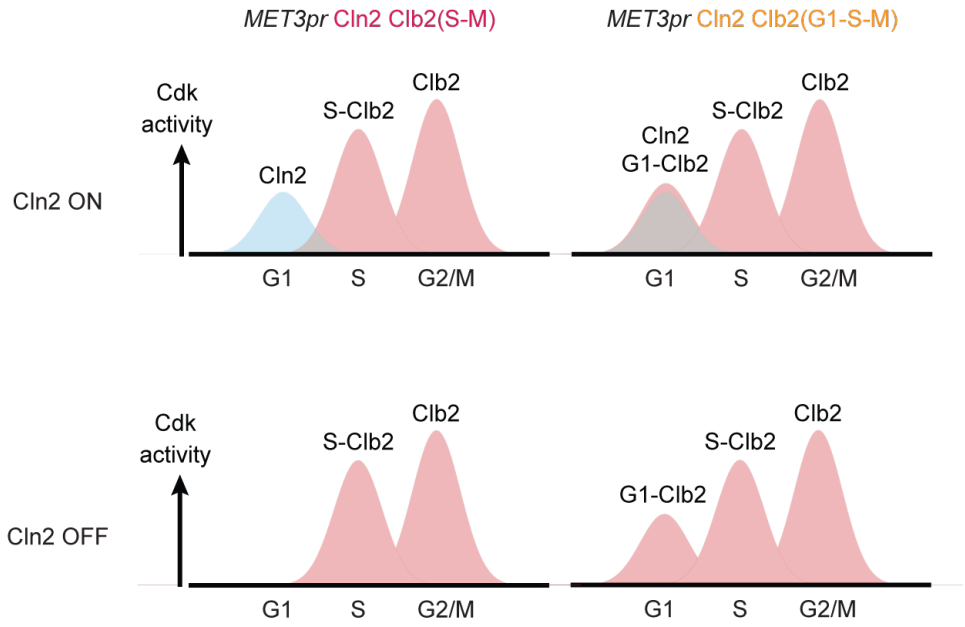


Figure 4.3. Schematics of the Cln2 Clb2(S-M) and Cln2 Clb2(G1-S-M) strains when Cln2 is expressed or repressed

In Cln2 Clb2(S-M) and Cln2 Clb2(G1-S-M) cells the endogenous *CLN2* promoter was replaced with a repressible *MET3* promoter. Cln2 expression was repressed (Cln2 OFF) when growth medium contained methionine. In the Cln2 OFF condition, the only source of cyclin-Cdk activity in *MET3pr*Cln2 Clb2(S-M) cells was Clb2(S-M). Likewise, in *MET3pr*Cln2 Clb2(G1-S-M) cells, Clb2(G1-S-M) was the sole source of cyclin-Cdk activity when Cln2 expression was silenced.

Establishment of the abovementioned Cln2 shut-off strains allowed me to address the role of Cln2 in these backgrounds in a time course experiment. I first arrested *MET3pr*Cln2 Clb2(S-M) and *MET3pr*Cln2 Clb2(G1-S-M) cells in G1 using mating pheromone α-factor and then released them into the CLN2 OFF condition. A separate culture of *MET3pr*Cln2 Clb2(G1-S-M) cells was also released into methionine free media, the Cln2 ON condition, to serve as a control. When Cln2 expression was ON, *MET3pr*Cln2 Clb2(G1-S-M) cells started to express G1, S and M-Clb2. This triggered DNA replication and phosphorylation of the cyclin-Cdk targets Orc2 and Orc6. Remarkably, when Cln2 expression was OFF, *MET3pr*Cln2 Clb2(G1-S-M) cells also accumulated G1, S and M-Clb2 starting at 150 min after from G1 release, which was followed by DNA replication

and phosphorylation of Orc2 and Orc6. In contrast, the *MET3pr*Cln2 Clb2(S-M) strain released into the Cln2 OFF condition remained arrested in G1. In these cells, I did not observe Clb2 expression, DNA replication or phosphorylation of Orc2 and Orc6 (Figure 4.4.A). This implies that G1-Clb2 can replace Cln2 and stimulate G1/S transition, albeit slower compared to the Cln2 ON condition.

I then analysed how Clb2-Cdk as a sole source of cyclin-Cdk drives cells from S phase into mitosis by performing immunostaining of the mitotic spindle and DNA (Figure 4.4.B). Interestingly, *MET3pr*Cln2 Clb2(G1-S-M) cells under the Cln2 OFF condition could not produce buds. Nevertheless, they could form a spindle and segregate their DNA into two within the same mother cell. Flow cytometry analysis of DNA content of these cells indicated that after completing the first round of replication the cells continued to accumulate more DNA. As expected, none of these observations were made in *MET3pr*Cln2 Clb2(S-M) cells which stayed as large unbudded cells for the duration of my experiment.

Repression of Cln2 in *MET3pr*Cln2 Clb2(S-M) and *MET3pr*Cln2 Clb2(G1-S-M) cells resulted in loss of viability in both strain backgrounds (Figure 4.5.). This underpins why my previous attempts to delete Cln2 in these cells were unsuccessful. Even though *MET3pr*Cln2 Clb2(G1-S-M) cells could duplicate their DNA and separate it into two in the absence of Cln2, they could not proliferate because they failed to form buds (Figure 4.4.B). Overall, my findings indicate that G1-Clb2 cannot compensate for the role of Cln2 in budding, but Clb2-Cdk alone can drive G1/S and G2/M transitions by promoting DNA replication and chromosome segregation.

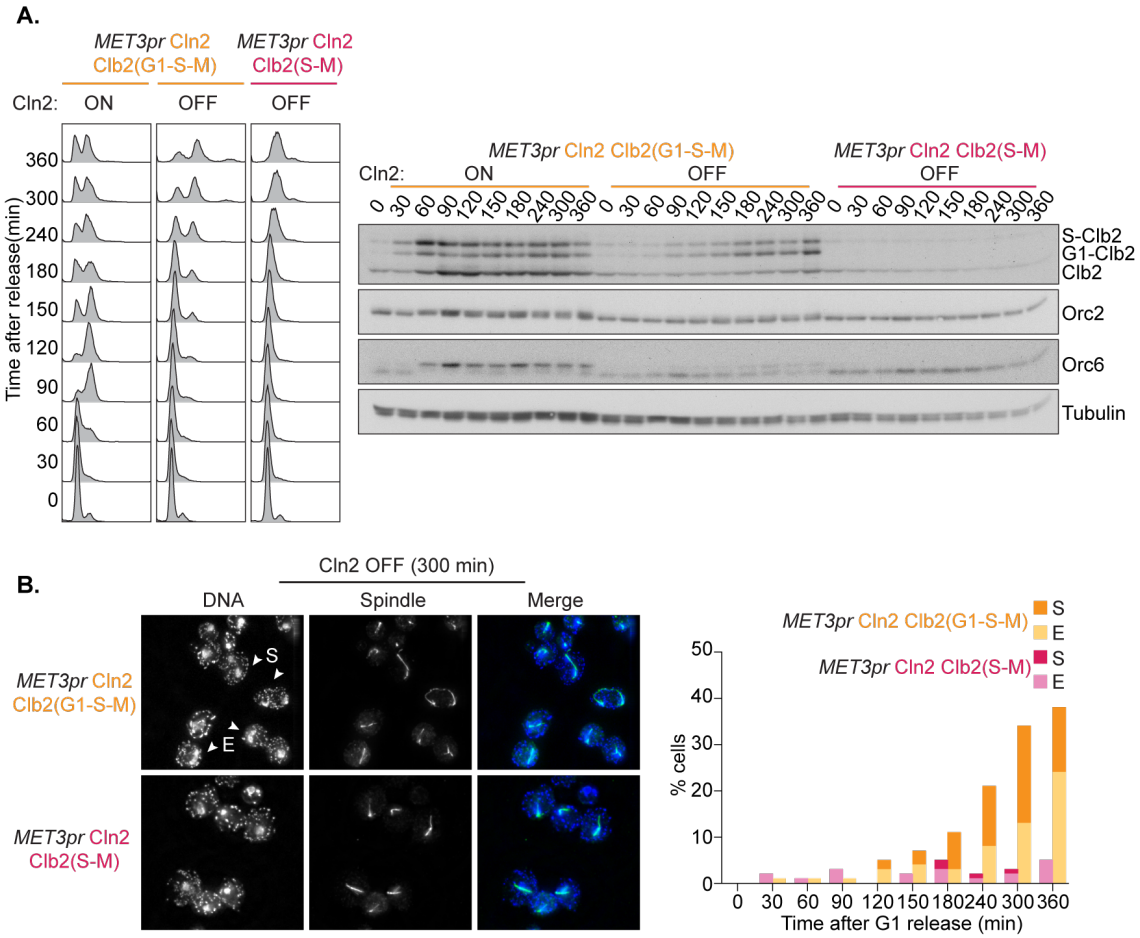


Figure 4.4. Clb2-Cdk driven cell division cycle in the absence of CLNs

Cells were arrested by α-factor in G1. *MET3pr*Cln2 Clb2(S-M) and *MET3pr*Cln2 Clb2(G1-S-M) cells were then released into medium with methionine (Cln2 OFF). A separate culture of *MET3pr*Cln2 Clb2(G1-S-M) cells were also released into medium lacking methionine (Cln2 ON) and served as a control. A representative of three biological replicates was shown. **A.** Flow cytometry analysis of DNA content was shown together with western blots of cyclin expression and cyclin-Cdk substrates Orc2 and Orc6. Clb2 expressed from the *CLN2* promoter was fused to a 3xHA epitope tag, leading to its migration between *CLB5* promoter expressed 6xHA epitope tagged Clb2 and endogenous untagged Clb2. Tubulin served as a loading control. Clb2, Orc2 and Orc6 antibodies were used for western blotting. **B.** Fields of *MET3pr*Cln2 Clb2(G1-S-M) and *MET3pr*Cln2 Clb2(S-M) cells in Cln2 OFF condition from the 300 min time point stained for the mitotic spindle and DNA were shown. 100 cells at each time point were quantified for elongated (E) or segregated (S) nuclei.

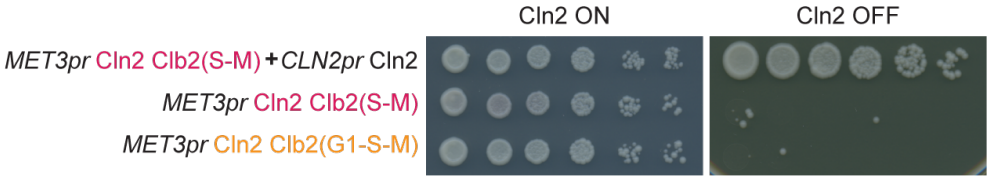


Figure 4.5. Cln2 is crucial for cell proliferation and viability

Fourfold serial dilutions of cells with indicated genotypes were grown on methionine lacking medium for Cln2 ON, and rich medium containing methionine for Cln2 OFF, for 3 days at 25°C. *MET3pr*Cln2 Clb2(S-M) cells harbouring a *CLN2pr*Cln2 plasmid integration served as a control. A representative of two biological replicates was shown.

4.2.2 Inhibition of G1/S transcriptional programme delays S phase entry

To gain insights into why Clb2 was not able to fully compensate for the loss of Cln2, I first focused on the G1/S transcriptional programme, activation of which relies on phosphorylation of the transcriptional inhibitors Whi5 and Stb1 by G1 cyclin-Cdk (Costanzo et al., 2004; de Bruin et al., 2008; de Bruin et al., 2004). Turning off Cln2 expression in *MET3pr*Cln2 Clb2(G1-S-M) indicated no G1 cyclin-Cdk kinase activity left in this strain (Figure 4.3.). I therefore hypothesised that these cells were incapable of overcoming Whi5 and Stb1 inhibition, which could in turn cause problems in producing a bud and an S phase entry delay as I previously observed (Figure 4.4.). To overcome transcriptional inhibition, I removed Whi5 or Stb1 from the *MET3pr*Cln2 Clb2(G1-S-M) cells. I then performed a time course experiment with three strains, *MET3pr*Cln2 Clb2(G1-S-M), *MET3pr*Cln2 Clb2(G1-S-M) with *whi5*Δ or *stb1*Δ. After releasing the cells from mating pheromone induced α-factor block in G1 into Cln2 ON and OFF conditions, I monitored their cell cycle progression by flow cytometry analysis of DNA content. No noticeable change in the timing of G1 progression was observed when Cln2 expression was ON (Figure 4.6.A). Strikingly, in the Cln2 OFF condition, loss of Whi5 accelerated replication initiation which was even more advanced in cells lacking Stb1 (Figure 4.6.B). However, neither deletion of *WHI5* nor *STB1* allowed bud formation and supported proliferation in the absence of Cln2. Moreover, deletion of Mbp1, a component of MBF transcriptional machinery which normally restrains transcription of MBF targets to G1 phase (de Bruin et al., 2006), in *MET3pr*Cln2 Clb2(G1-S-M) cells was also unsuccessful in promoting viability under Cln2 OFF conditions (Figure 4.6.C). These

findings suggest that the lethal phenotype of *MET3pr*Cln2 Clb2(G1-S-M) cells lacking Cln2 is not due to the inhibition of the G1/S transition, but the delay in S phase entry is.

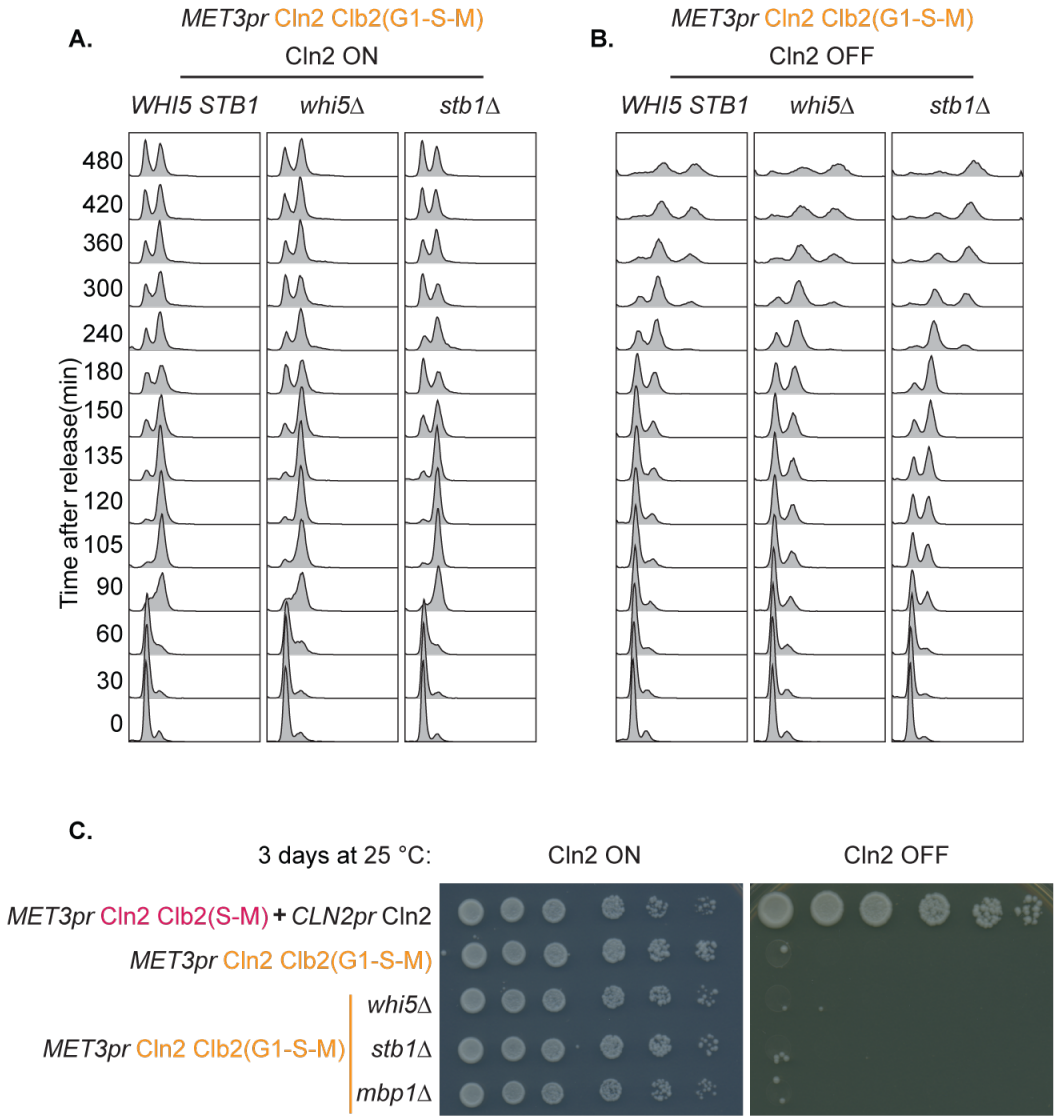


Figure 4.6. Inhibition of G1/S transcription delays S phase entry

Cells with indicated genotypes were synchronised in G1 by α-factor and released into **A.** Cln2 ON and **B.** Cln2 OFF conditions. Flow cytometry analysis of DNA content was performed after staining cells with propidium iodide. **C.** Fourfold serial dilutions of cells with indicated genotypes were grown on methionine lacking medium for Cln2 ON, and methionine containing rich medium for Cln2 OFF, for 3 days at 25°C. *MET3pr*Cln2 Clb2(S-M) cells harbouring a *CLN2pr* Cln2 plasmid integration served as a control. A representative of two biological replicates was shown.

4.2.3 Cln2 is vital for cell viability and proliferation

Cln2 is not only different than Clb2 in its role in triggering G1/S transcription, but also it possesses distinct structural properties and cellular localisation. To address why Clb2 could not replace Cln2, I next used the *MET3pr*Cln2 Clb2(S-M) strain which was introduced previously (Figure 4.3.) as a tool to study these differences. I aimed to establish various G1-Clb2 constructs and to express them in *MET3pr*Cln2 Clb2(S-M) cells, thereby testing their capacity to substitute Cln2.

In an attempt to narrow down the region of Cln2 that is required to promote budding and sustain cell proliferation, I first studied the structural differences between Cln2 and Clb2. A structure based sequence alignment of these two cyclins revealed two regions in the Cln2 cyclin core region, amino acids [134-156] and [218-265], which were not present in Clb2 (Figure 4.7.). Structural prediction analysis further revealed that these regions are expected to form loops and interact with potential binding partners (Figure 4.8.). In particular, the first loop covering the region [134-156] was predicted to create an additional interaction surface with the PSTAIRE helix of Cdk. The second loop [218-265], on the other hand, was larger and predicted to be more flexible. A potential function of this loop could be facilitating cyclin-Cdk substrate interactions. Interestingly, the second loop partially overlapped with a region previously shown to contain Cln2 NES [224-299] which could confer Cln2 specific functions to Cln1 (Quilis and Igual, 2012). I was therefore curious to study the function of these two additional loops in Cln2 further, and to address if they could also confer Cln2 specific functions to Clb2. Consequently, I created five different chimeras by swapping the cyclin core and N and C terminal regions of Cln2 and Clb2. Although the chimeras could be expressed as stable proteins under control of the *CLN2* promoter (Figure 4.9A), none of them was able to support cell growth in the absence of Cln2 (Figure 4.9.B).

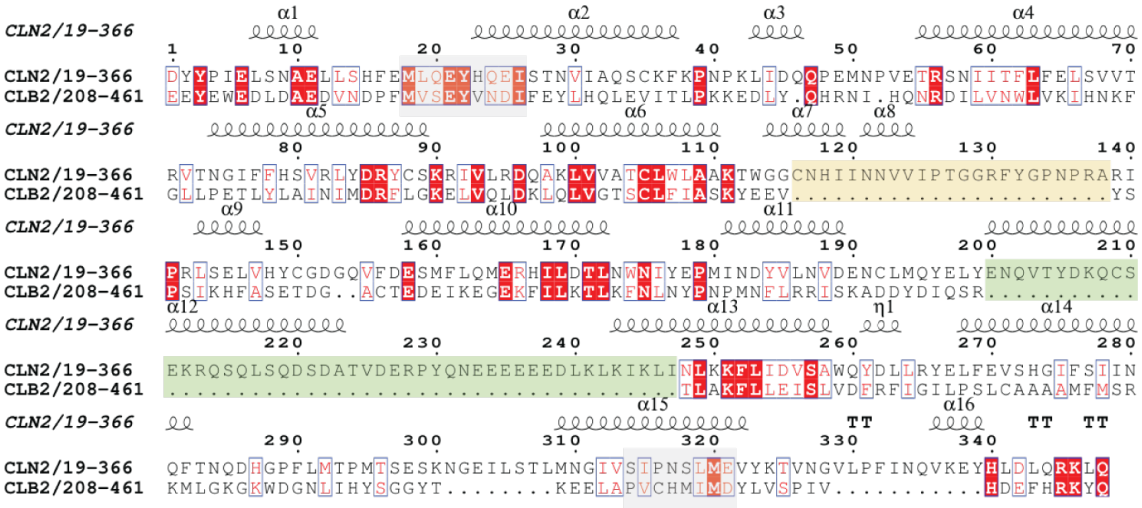


Figure 4.7. Structure based sequence alignment of Cln2 and Clb2 cyclin core

The conserved residues in Cln2 and Clb2 were highlighted with red boxes. Residues showing similar properties (e.g. polar, non-polar, acidic…) were framed and scripted in red. The regions highlighted in yellow [134-156] and green [218-265] were Cln2 specific and did not exist in Clb2. Homology regions used to create chimeras were shaded in grey. Structure based alignment of Cln2 and Clb2 cyclin cores was performed with the help of Stephane Mouilleron from the Crick Structural Biology STP.

I next investigated the localisation differences between Cln2 and Clb2. While cytoplasmic Cln2 is important for efficient budding (Skotheim et al., 2008), Clb2 is localised mainly to the nucleus (Bailly et al., 2003; Ord et al., 2019a). I therefore hypothesised that strong nuclear localisation of Clb2 could be a limiting factor in replacing Cln2. To increase cytoplasmic Clb2 levels, I deleted Clb2 nuclear localisation signal, Clb2$^{\Delta NLS}$, amino acids [176-213] (Bailly et al., 2003). When Clb2$^{\Delta NLS}$ construct placed under control of the *CLN2* promoter was integrated into *MET3pr*Cln2 Clb2(S-M) cells, it could not support viability upon loss of Cln2 (Figure 4.9.B).

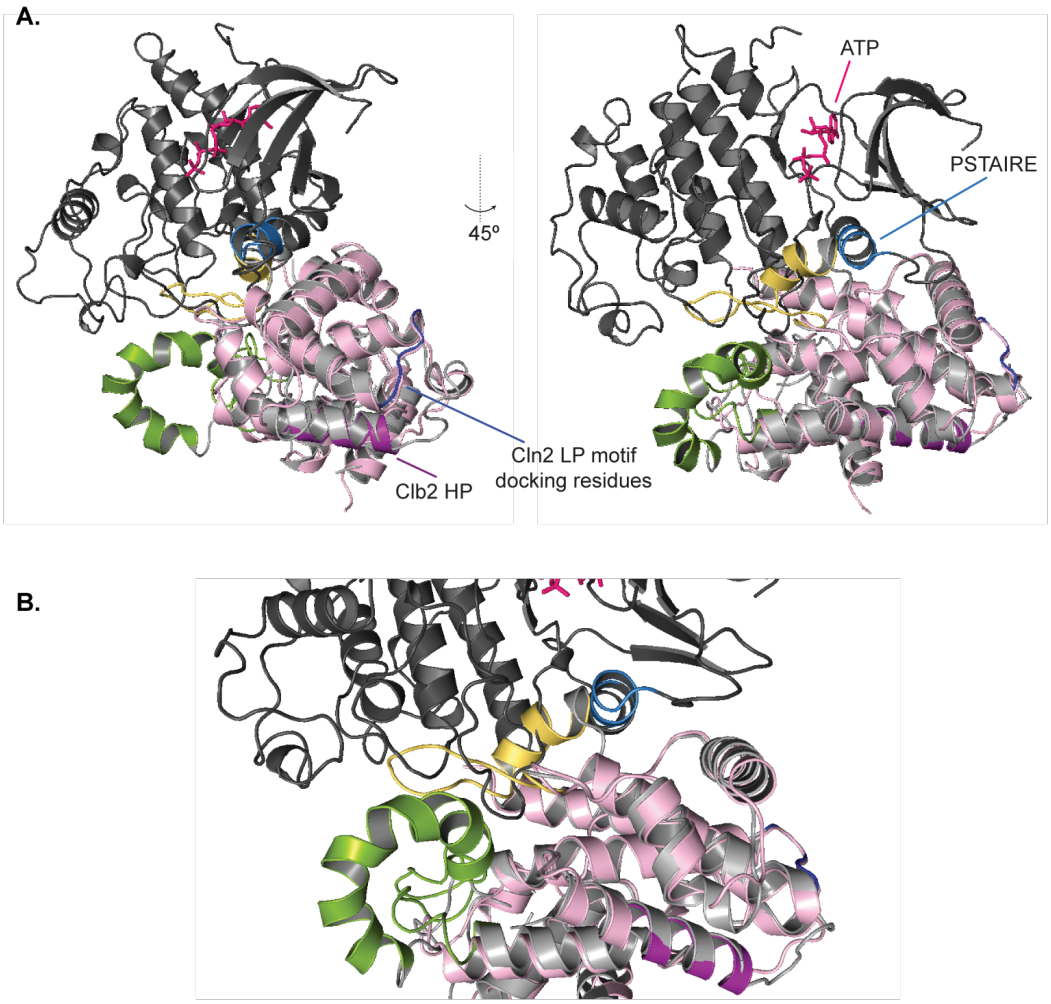


Figure 4.8. *In silico* modelling of Cln2 and Clb2 cyclin core

Conserved cyclin core of Cln2 (pink) and Clb2 (grey) were defined based on structure prediction and overlaid. Cdk2 (dark grey) (PDB ID: 1FIN) structure was then aligned to superimposed cyclins to show predicted binding differences. The Clb2 HP (purple), Cln2 LP docking residues (navy), ATP (magenta), conserved PSTAIRE helix for Cdk2 (blue) were shown. The regions highlighted in yellow [134-156] and green [218-265] were Cln2 specific and did not exist in Clb2. These regions were predicted to have loop structures and expected to be flexible in confirmation. **A.** Cln2-Clb2-Cdk2 model was shown in two different orientation to provide better visualisation of the interactions between cyclin-Cdk and the newly identified Cln2 loops. The model was rotated by 45° as indicated. **B.** Same as (A), focusing on the interface between the cyclins and Cdk2. Yellow loop was found to create an additional interaction surface with the PSTAIRE helix. Modelling of Cln2 and Clb2 cyclin cores was performed with the help of Stephane Mouilleron from the Crick Structural Biology STP.

Overexpression of Clb5 can compensate for the absence of G1 cyclin-Cdk complexes (Epstein and Cross, 1992; Schwob and Nasmyth, 1993) and Clb5 is highly dependent on its HP interactions to facilitate phosphorylation of Cdk substrates (Koivomagi et al.,

2011b; Loog and Morgan, 2005). To address if loss of G1-Cdk rescue by Clb5 relies on its docking mediated specificity for Cdk targets, I took advantage of the overall similar architecture of Clb5 and Clb2 and replaced the Clb2 HP ([259-270] QNRDILVNWLVK) with of Clb5 ([196-207] SMRTILVDWLVE), Clb2^{CLB5HP}. The expression of Clb2^{CLB5HP} was also insufficient to restore viability of *MET3pr*Cln2 Clb2(S-M) cells in the presence of methionine (Figure 4.9.B).

I then covalently fused Clb2 to the Cdk kinase subunit Cdc28 to address if a fusion protein approach similar to the Cdc13-Cdc2 fusion that allowed to establish the single cyclin-Cdk strain in fission yeast (Coudreuse and Nurse, 2010) would restore viability of *MET3pr*Cln2 Clb2(S-M) cells in the absence of Cln2. However, this attempt failed too (Figure 4.9.B).

To address the importance of Cln2 specific substrate targeting in an alternative way, I mutated the residues responsible for the LP motif docking interactions in Cln2, Cln2^{lpd}, that decreases Cln2-Cdk specific substrate phosphorylation (Bhaduri et al., 2015). Surprisingly, only the Cln2^{lpd} construct could compensate for the loss of all G1-Cdk in the *MET3pr*Cln2 Clb2(S-M) strain background. The rescue by Cln2^{lpd} was to a similar extent as the control construct which continued to express exogenous Cln2 when endogenous Cln2 was silenced (Figure 4.9.B). Altogether, my findings suggest that the fundamental role of Cln2 in supporting viability of *MET3pr*Cln2 Clb2(S-M) cells do not rely on Cln2 LP docking interactions. The intrinsic features that make Cln2 indispensable for budding and cell proliferation remain to be further studied.

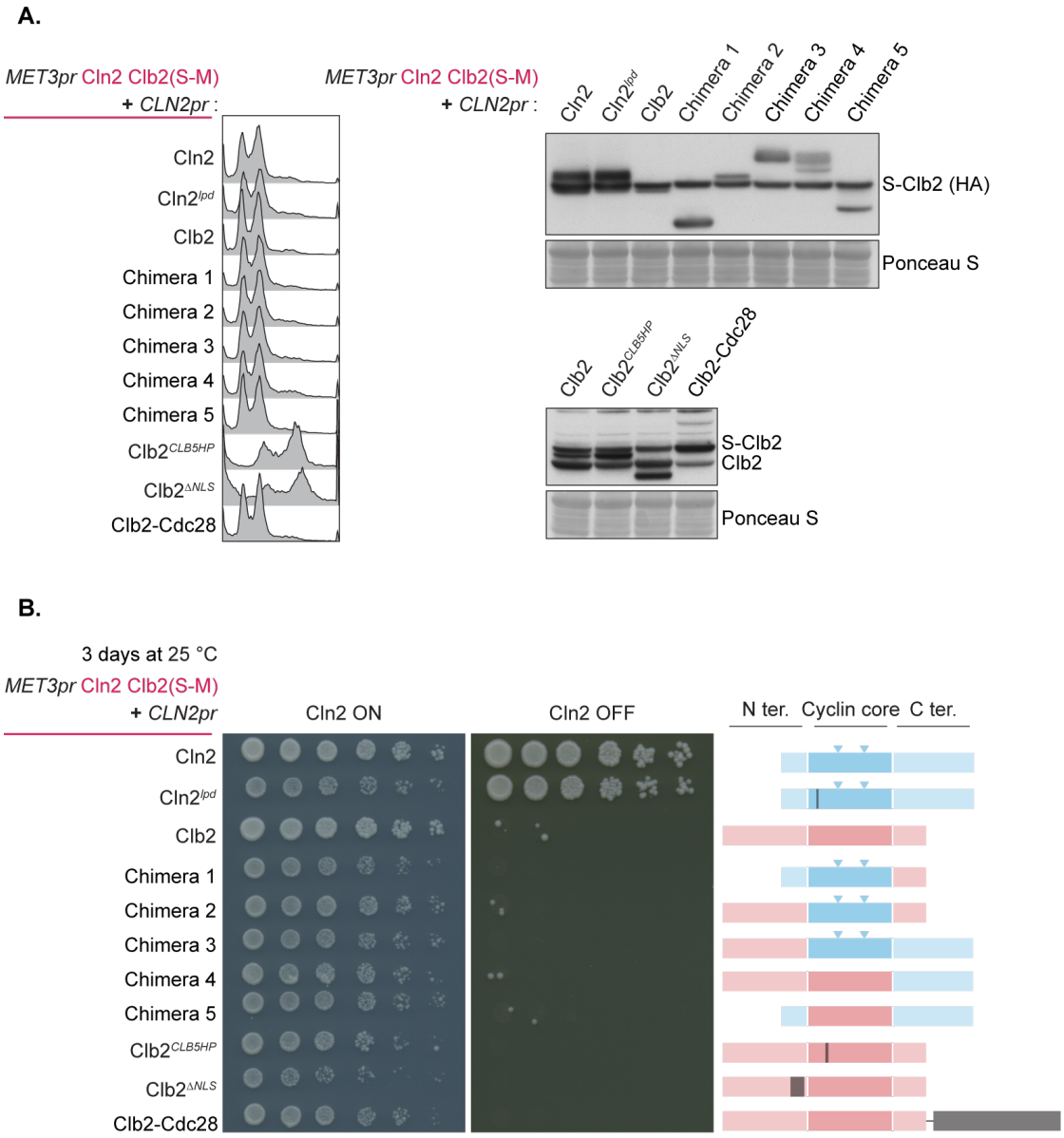


Figure 4.9. Cln2 is important for cell viability

A. Samples were harvested from exponentially growing asynchronous cultures. Constructs were fused to 3xHA epitope tag and expressed from the *CLN2* promoter except Clb2$^{\Delta NLS}$ and Clb2-Cdc28. Early expressed Clb2 from the *CLB5* promoter was fused to 3xHA. Clb2 and HA antibodies were used for western blotting. Ponceau S served as a loading control. Flow cytometry analysis of DNA content was performed after staining cells with propidium iodide. I observed that the cells expressing Clb2^{CLB5HP} and Clb2$^{\Delta NLS}$ constructs diploidised for yet an unknown reason. **B.** Fourfold serial dilutions of cells with indicated genotypes were grown on methionine lacking, Cln2 ON, and methionine containing, Cln2 OFF, media for 3 days at 25°C. Schematics represent the cyclin variants expressed under control of the *CLN2* promoter. Two Cln2 specific loop insertions are highlighted by arrowheads. Locations of engineered gene alterations are highlighted in dark grey. A representative of three biological replicates was shown.

4.2.4 Cln2 promotes polarisation and phosphorylation of budding substrates

Because the *MET3pr*Cln2 Clb2(G1-S-M) *whi5*Δ cells were unable to produce buds and could not support viability in the absence of Cln2 (Figure 4.6.), I asked if Cln2 was specifically needed to phosphorylate substrates implicated in the budding pathway. Amongst these substrates are the three G1 cyclin targets, Cdc24, Rga2 and Boi1, which are associated with the Cdc42 GTPase that is essential for polarisation and bud formation. They show electrophoretic mobility shifts due to phosphorylation by Cln2-Cdk complexes at bud emergence (McCusker et al., 2007) (see 1.3.2.1. G1 cyclin-Cdk regulates polarisation and budding). I therefore monitored changes in phosphorylation of Cdc24, Rga2 and Boi1 in a time course experiment where I synchronised *MET3pr*Cln2 Clb2(G1-S-M) *whi5*Δ cells in G1 using pheromone α-factor induced block and released into Cln2 ON or OFF condition. Cdc24, Rga2 and Boi1 showed mobility shifts at the time of budding, 60 min after G1 release, when cyclins accumulated in Cln2 expressing cells. However, I could not detect slower migrating forms of these proteins in cells lacking Cln2 expression even when G1- and S-Clb2 accumulated and initiated DNA replication (Figure 4.9.A). This suggests that Cln2-Cdk activity is needed to specifically phosphorylate targets facilitating bud formation.

Next, I investigated whether *MET3pr*Cln2 Clb2(G1-S-M) *whi5*Δ cells lacking Cln2 expression could promote polarisation which is an initial step of bud formation. I used phalloidin to stain actin filaments in the *MET3pr*Cln2 Clb2(G1-S-M) *whi5*Δ strain both in Cln2 ON and OFF conditions. Cells arrested with pheromone α-factor in G1 showed polarisation at shmoo tips. Following release from the G1 arrest, the cells expressing Cln2 retained polarity at the bud sites which was then directed to the bud neck prior to division. In contrast, polarised actin filaments at the shmoo tip became depolarised actin patches which were dispersed in the cells when Cln2 was OFF. I did not observe polarisation at any time for the duration of this experiment. Taken together, these data demonstrate that Cln2 plays a unique and crucial role in facilitating polarisation and budding by phosphorylating cyclin-Cdk substrates involved in budding pathway; this special feature of Cln2 cannot be substituted by Clb2-Cdk complexes.

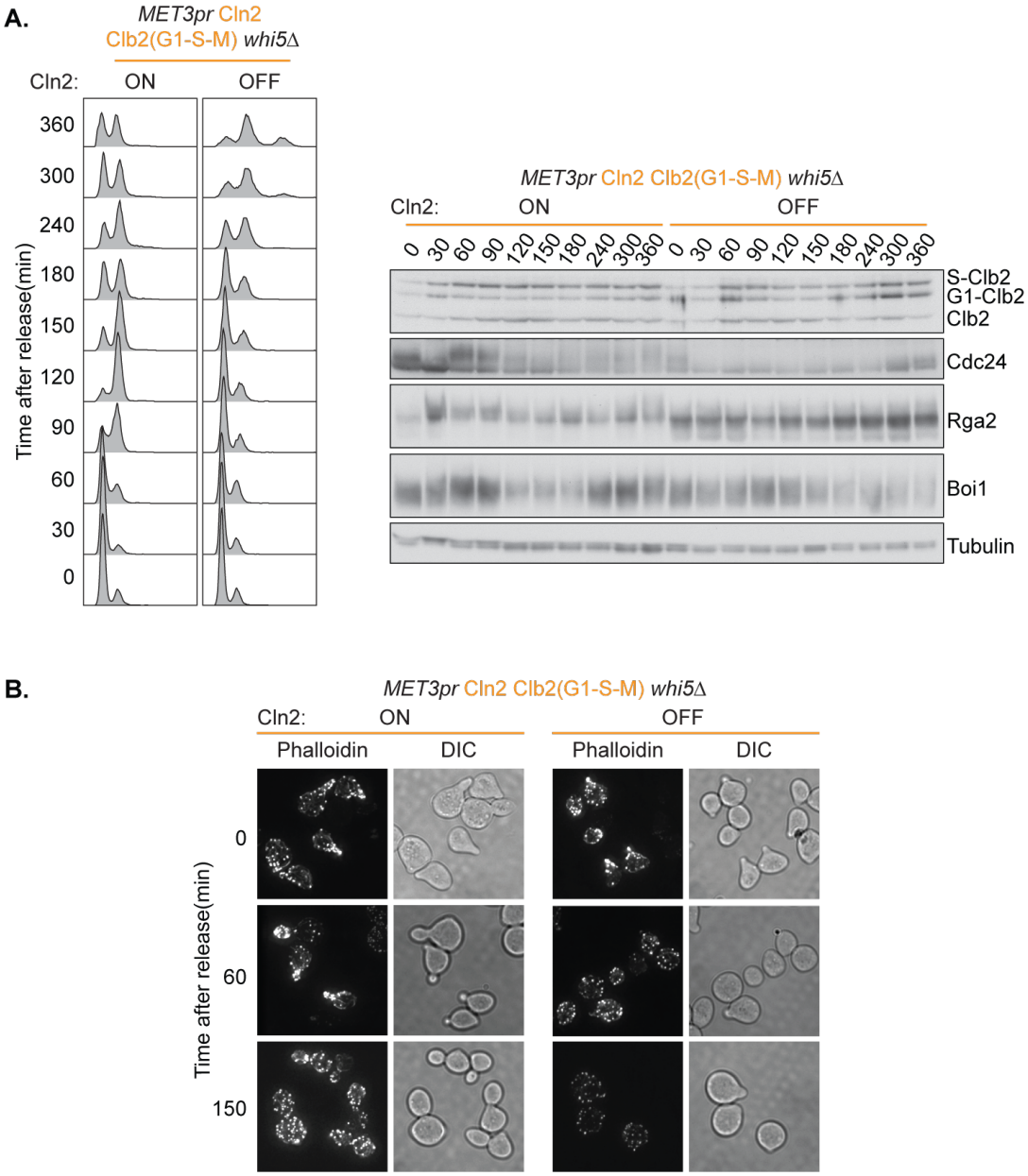


Figure 4.10. Cln2 allows polarisation and phosphorylation of budding substrates

Cells were arrested by α-factor in G1. *MET3pr*Cln2 Clb2(G1-S-M) *whi5*Δ were released into medium without (Cln2 ON) or with methionine (Cln2 OFF). A representative of two biological replicates was shown. **A.** Flow cytometry analysis of DNA content was shown together with western blots of cyclin expression and Cln2-Cdk substrates Cdc24, Rga2 and Boi1. Clb2 expressed from the *CLN2* promoter was fused to a 3xHA epitope tag, leading to its migration between *CLB5* promoter expressed 6xHA epitope tagged Clb2 and endogenous untagged Clb2. Tubulin served as a loading control. Clb2, Cdc24, Rga2 and Boi1 antibodies were used for western blotting. **B.** Fields of *MET3pr*Cln2 Clb2(G1-S-M) *whi5*Δ cells in Cln2 ON and OFF conditions from the indicated time point were stained with phalloidin to visualise actin filaments. Fluorescence was shown next to differential interference contrast (DIC) images.

Chapter 5. Discussion & Future directions

During my PhD studies, I addressed the relative contributions of qualitative cyclin specificity and quantitative Cdk kinase activity to timely and ordered cell division cycle in the budding yeast *S. cerevisiae*. My approach was trying to establish a budding yeast strain expressing a single cyclin-Cdk strain using a mitotic cyclin Clb2. Using this strain as a tool, my goal was then to investigate the impact of lacking remaining G1, S and G2/M cyclins to the cell cycle progression.

In this thesis, I propose that the cell division cycle relies on both qualitative and quantitative mechanisms governed by cyclin-Cdk complexes in budding yeast. My findings indicate that the nuclear division cycle can be driven by Clb2-Cdk complexes from G1 through S phase and into mitosis in the absence of the G1, S and G2/M cyclins. However, replacing G1 cyclins, in particular Cln2, with a mitotic cyclin Clb2 was not sufficient in promoting cell polarisation and budding in a strain relying solely on Clb2-Cdk activity. This implies a unique role of Cln2 to couple cell cycle progression to essential morphogenetic events.

In this chapter, I discuss my findings in the context of our current understanding of cell cycle regulation.

5.1 Docking interactions between CLBs and their substrates

Cyclins use hydrophobic surface residues to interact with SLiMs on their substrates. Budding yeast cyclins are excellent examples demonstrating specificity for distinct motifs. These motifs involve LP, NLxxxL or K/RxL, PxF and LxF which can be specifically recognised by Cln1/2, Clb5/6, Clb3 and Clb2, respectively. Cln2 interacts with the LP motif using the residues nearby to its hydrophobic patch (HP). However, all CLBs employ their HP to interact with the docking motifs (Bhaduri and Pryciak, 2011; Bhaduri et al., 2015; Faustova et al., 2020; Koivomagi et al., 2011b; Ord et al., 2020; Ord et al., 2019b).

How can CLBs use a conserved patch to recognise different SLiMs? There are two potential answers to this question. Firstly, the HP sequence is divergent among CLBs even though the HP itself is a conserved feature of cyclins (Figure 5.1.A). Therefore, it is likely that the differences in HP sequences allow specialised docking interactions between cyclin-Cdk complexes and their substrates. For instance, N terminal methionine residue of the HP is highly conserved in cyclins recognising RxL motifs such as Cyclin A2 and Clb5. This residue directly contributes to the interaction between the HP and RxL motif (Brown et al., 1999), but it is not conserved in Clb3 or Clb2 (Figure 5.1). This could explain why Clb3-Cdk and Clb2-Cdk are not able to phosphorylate RxL containing model substrates as efficiently as Clb5-Cdk (Koivomagi et al., 2011b; Ord et al., 2020). Secondly, because the motifs are short sequences, residues in the vicinity of these motifs could be critical to facilitate substrate docking. This idea suggests that SLiMs act as a minimal recognition sequence needed for cyclin-Cdk and substrate docking interactions, while the nearby residues fine tune the specificity and the timing of substrate phosphorylation. If true, this notion could also explain why the cyclin-Cdk complexes do not interact with all the (K/R)xL motif containing substrates in *S. cerevisiae* proteome, but instead selectively dock the motifs on the true cyclin-Cdk targets by recognising the flanking residues. In support of this idea, a recent study revealed that the residues surrounding the core RxL motif also play a role in determining cyclin specificity (Ord et al., 2020).

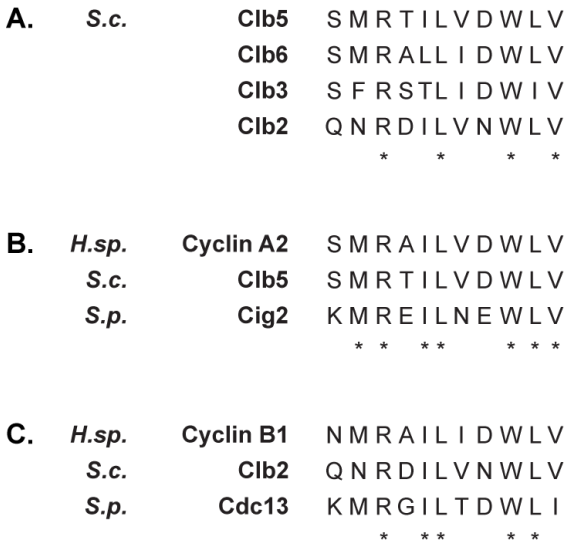


Figure 5.1. Cyclin HP alignment

Using Clustal Omega online tool developed by EMBL-EBI, hydrophobic patches of **A.** CLBs from *S. cerevisiae*, **B.** S phase cyclins and **C.** mitotic cyclins from indicated species were aligned. *S.c.*: *S. cerevisiae*, *S.p.*: *S. pombe*, *H.sp.*: *Homo sapiens*

5.1.1 Differences between Clb5 and Clb2

Analysis of the Clb2-Cdk driven S phase revealed that S-Clb2 is capable of firing early and late replication origins in the absence of CLB directed Cdk inhibitor Swe1 (Hu and Aparicio, 2005). However, an *in vitro* study conducted with purified proteins led to the identification of Clb5-Cdk specific S phase substrates which could not be efficiently phosphorylated by Clb2-Cdk (Loog and Morgan, 2005). This opens a question how efficiently can S-Clb2 trigger S phase and phosphorylate Clb5-Cdk specific substrates *in vivo*?

Because Clb2-Cdk complexes are intrinsically more potent than Clb5-Cdk in phosphorylating a generic substrate (Koivomagi et al., 2011b), one can expect S-Clb2 to override docking interaction requirements for the phosphorylation of S phase substrates. However, although I observed higher kinase activity earlier in the cell cycle in Clns Clb2(S-M) cells compared to the control, S-Clb2-Cdk could not prevent the delay in S phase entry. Moreover, the phosphoproteome analysis of Clns Clb2(S-M) cells revealed that the phosphorylation of Clb5-Cdk specific substrates by S-Clb2-Cdk was also affected. These findings suggest that the delay in S phase entry resulted from the inefficient

phosphorylation of Clb5-Cdk specific S phase targets by S-Clb2-Cdk. Because Clb5 and Clb2 are mainly nuclear and do not show major localisation differences (Bailly et al., 2003; Huisman et al., 2007; Ord et al., 2019a), it is likely that the differences between inefficient phosphorylation of Clb5-Cdk targets by Clb2-Cdk stemmed from differences in their HP (Figure 5.1.B and C). In fact, not only the conserved methionine residue implicated in the RxL motif interaction (Brown et al., 1999) is different in the Clb2 HP, but also the amino acids found at -1, +2 and +6 positions from the methionine residues vary between the Clb5 and Clb2 HP.

In fission yeast, a mitotic cyclin-Cdk fusion protein Cdc13-Cdc2 alone can trigger S and M phases without any cell cycle delays (Coudreuse and Nurse, 2010). Although cyclin-Cdk substrate docking interactions are not extensively studied in fission yeast, a recent study revealed that the Cdc13 HP is essential for global mitotic phosphorylation in the single cyclin-Cdk system (Basu et al., 2020). In this study, Cdc13 HP was found to be important for directing Cdc13-Cdc2 fusion to the SPB. When Cdc13 HP was mutated, the fusion protein could not localise to the SPB and phosphorylation of a set of specific cytoplasmic and SPB localised targets were affected. This suggests that cyclin substrate docking interactions are employed for phosphorylation of a set of specific substrates in fission yeast. Interestingly, the strain carrying Cdc13 HP mutant could still drive the S phase. It is therefore intriguing to ask how Cdc13-Cdk can efficiently target S phase cyclin Cig2-Cdk substrates.

In a strain background different than W303 which was the one used in this project, the S-Clb2 HP mutant can partially rescue *clb3,4,5,6*Δ lethality while the wild type S-Clb2 cannot (Cross and Jacobson, 2000). This suggests that either Clb2 HP targets some substrates which might be interfering with growth, or mutation of the HP in S-Clb2 allows sloppy fit of substrates, increasing the Clb2-Cdk substrate repertoire, and promoting growth. Therefore, studying the cell cycle progression and substrate phosphorylation patterns in Clns Clb2(S-M) cells expressing the S-Clb2 HP mutant will be interesting. Moreover, it will be also interesting to replace S-Clb2 HP with the Clb5 HP in Clns Clb2(S-M) cells. One can then ask if S-Clb2 harbouring Clb5 HP can now rescue the S phase entry delay and efficiently phosphorylate Clb5-Cdk substrates. Lastly, since

Cdc13-Cdc2 fusion is capable of driving S phase even when the Cdc13 HP is mutated, it will be intriguing to ask if Cdc13-Cdc2 could efficiently replace Clb5. It will be also curious to address how Cdc13-Cdc2 driven S phase would compare to the S phase driven by S-Clb2.

5.2 Can S-Clb2 rewire the multisite phosphorylation code of Cdk?

A Cdk multisite phosphorylation code has been proposed, in which a general set of rules determines the timing of cell cycle events and the cyclin-Cdk activity thresholds throughout the cell cycle. According to these rules, phosphorylation of a site depends on three determinants, namely, phosphosite identity (serine vs. threonine), distance between phosphosites and presence of a nearby cyclin docking motif. In addition, it is also believed that cyclins add a layer of complexity to these rules because successive cyclin waves can activate Cdk to different extent (Örd M., 2018).

5.2.1 S-Clb2-Cdk advanced phosphorylation of Ndd1

To identify the multisite phosphorylation code of Cdk, Örd et. al. utilised a biosensor containing phosphodegrons and monitored degradation timing of it as a read out for the phosphorylation by cyclin-Cdk complexes. They could then investigate, for example, how cyclin-Cdk docking interactions affect phosphorylation of their sensor, hence its degradation timing (Ord et al., 2019a). Early expression of Clb2 in S phase advanced degradation of the sensor containing a Clb2 docking motif LxF. This suggests that the changes in cyclin expression timing can affect phosphorylation of Clb2-Cdk specific targets. In line with this, my western blotting and phosphoproteome analyses showed advanced phosphorylation of a late substrate Ndd1 by S-Clb2 in Clns Clb2(S-M) cells, even though the cells were lacking the remaining CLBs. In fact, advanced phosphorylation of the Ndd1 was not surprising because Ndd1 contains three Clb2 specific LxF motifs. On the other hand, an unanticipated observation was the impaired phosphorylation of Ndd1 T319 phosphosite. T319 was the only affected site amongst the detected Cdk phosphosites in Clns Clb2(S-M) cells. Although T319 is located at 7 amino acids upstream of a KxL motif, it is not possible to attribute its impaired phosphorylation to being a Clb5-Cdk specific site for two reasons. Firstly, Ndd1 is not a good *in vitro*

Clb5-Cdk target (Koivomagi et al., 2011b; Ord et al., 2020). Secondly, the distance between the KxL motif and phosphosite is not optimal for docking interactions as the ideal distance is expected to range from 20 to 40 amino acids (Koivomagi et al., 2013). Therefore, it is yet unknown why the T319 site is not efficiently phosphorylated in Clns Clb2(S-M) cells. A motif which was not detected in my SLiM analysis as a true docking motif could be responsible for the regulation of T319 phosphorylation. This would not be surprising because predicting a docking motif has its own limitations due to the plasticity of conserved residues in SLiMs and the coincidental existence of SLiM-like peptide sequences in the proteome (Davey et al., 2012). In any case, it will be interesting to address the consequences of impaired T319 phosphorylation in Clns Clb2(S-M) cells further. This is because T319 phosphorylation is specifically needed for Ndd1 recruitment to the promoters of the CLB cluster genes and their transcription (Reynolds et al., 2003).

On another interesting note, in addition to Clb2-Cdk, Clb3-Cdk can also specifically phosphorylate Ndd1 *in vitro* (Koivomagi et al., 2011b; Ord et al., 2020). However, Ndd1 does not contain a PxF motif. This suggests a possibility that Clb3-Cdk might recognise an additional motif in Ndd1. Therefore, a mutational analysis of Ndd1 can lead to the identification of a novel docking residue in Ndd1 specific for Clb3-Cdk.

5.2.2 Every phosphosite is differentially affected by S-Clb2-Cdk activity

Early expressed Clb2 could also advance degradation of the sensor without a docking motif, or promote degradation of the sensor containing an RxL motif with a similar timing to wild type cells (Ord et al., 2019a). This is possibly because Clb2-Cdk complexes are intrinsically more active than S phase cyclin-Cdk complexes (Koivomagi et al., 2011b), allowing them to compensate for the loss of cyclin specificity in some cases.

In my phosphoproteome analysis, phosphorylation patterns of individual Cdk phosphosites greatly varied in Clns Clb2(S-M) cells as shown in heat maps. Division of each heat map into three subgroups revealed sites which were less phosphorylated, phosphorylated approximately at the same level and to a greater extent in Clns Clb2(S-M) cells compared to the control. With a closer examination of these heat maps, sites that were phosphorylated more and with advanced timing could be observed in Clns Clb2(S-

M) cells. In addition, in some other cases, phosphorylation of the sites by Clb2-Cdk alone was as efficient as the phosphorylation levels observed in control cells containing a full set of cyclins. These observations are pointing in the direction of observations made by Örd et. al.. However, to draw similar conclusions to theirs, I will need to take a bioinformatics approach to re-analyse my phosphoproteome data, this time by considering the presence of nearby SLiMs. However, because my heat maps clearly show differential phosphorylation landscape in Clns Clb2(S-M) cells compared to the control strain, based on my current analysis it is still plausible to propose that S-Clb2 can rewire the multisite phosphorylation code of Cdk.

Currently, it is possible to address how multisite phosphorylation codes help to refine the Cdk phosphorylation landscape using simple synthetic biology tools. In the future, it will be also interesting to develop, for example, biosensors which can integrate multiple inputs (e.g. change in subcellular localisation, interactions with other proteins…) at the same time. These biosensors can better reflect the biological complexity and can help us to gain more insights into the systems level control of the cell division cycle.

5.3 Impact of Clb5 deletion on Clb2 degradation

In an unperturbed cell cycle, Cdc14 release from the nucleolus allows mitotic exit, during which numerous substrates, including Cdh1, are dephosphorylated (Jaspersen et al., 1999; Visintin et al., 1998). Dephosphorylation of Cdh1 by Cdc14 is activatory so that the active APC/C^{Cdh1} promotes completion of cell cycle, targets many proteins such as Clb2 and Cdc5 for degradation and prevents mitotic cyclin accumulation until its inactivation at the end of G1. Clb3/4/5 are responsible for phosphorylation of Cdh1, hence inactivation of APC/C^{Cdh1}, to allow accumulation of Clb2 prior to mitosis (Simpson-Lavy et al., 2015). Although Clb2 was shown to be unstable in cells lacking Clb5 alone or Clb3/4/5 (Simpson-Lavy et al., 2015), I did not observe a reduction in Clb2 levels in neither *clb5*Δ nor Clns Clb2(S-M) cells. Instead, Clb2 was persistent throughout the cell cycle in both strain backgrounds.

In Chapter 3 (see 3.3. Mitotic exit is compromised in Clns Clb2(S-M) cells), I proposed that incomplete Clb2 degradation in Clns Clb2(S-M) could be an indication of the DNA damage checkpoint response which is activated by Chk1 mediated phosphorylation and stabilisation of securin. I also observed a delay in Cdc14 release from the nucleolus, indicating a mitotic delay in Clns Clb2(S-M) cells which could stem from persisting securin as it impedes mitotic exit by preventing separase from activating the Cdc14 phosphatase (Cohen-Fix and Koshland, 1999; Queralt et al., 2006). However, these observations do not fully explain why Clb2 is not completely degraded in Clns Clb2(S-M) cells and how Clb2 can be detected even in G1 when APC/C^{Cdh1} must be actively degrading Clb2. Because Clb2 degradation was also incomplete in *clb5*Δ cells, but not in the cells lacking all CLBs except Clb5 and Clb2, I propose that Clb5 must be playing a role in Clb2 degradation.

Cdh1 is known as an *in vitro* Clb5-Cdk specific substrate (Loog and Morgan, 2005) although it can also be phosphorylated by Clb2-Cdk and Clb3-Cdk complexes (Zachariae et al., 1998). Moreover, Clb2 degradation together with Cdc5 phosphorylation were shown to be prerequisites for APC/C^{Cdh1} mediated Cdc5 degradation (Simpson-Lavy et al., 2015). When I monitored Cdc5 degradation profile in a time course experiment in Clns Clb2(S-M) and *clb5*Δ cells, I indeed observed incomplete Cdc5 degradation. Although this experiment does not provide an answer as to why Clb2 and Cdc5 are not completely degraded, it suggests that APC/C^{Cdh1} is not able to promote complete degradation of these proteins. This could be because APC/C^{Cdh1} could not be fully activated in the absence of Clb5. I suspect that this regulation is indirect, and a phosphatase might be involved because Cdh1 needs to be dephosphorylated for APC/C^{Cdh1} activation. In future studies, it will be interesting to study the link between Clb5 and APC/C^{Cdh1} to fully understand how cyclins regulate APC/C to promote timely and accurate cell cycle progression.

5.4 Why Clb2 cannot compensate for Cln2?

Cln2 LP docking motif interactions facilitate cell polarisation and phosphorylation of Cln2 substrates (Bhaduri et al., 2015). However, the lethal phenotype of *MET3pr*Cln2

Clb2(S-M) cells in the absence of Cln2 was not dependent on LP docking interactions, suggesting that Cln2 LP docking site was not required to convey its critical role in this strain background. Could it therefore be that not the loss of Cln2-Cdk specificity, but early Clb2 expression interfered with cell polarisation in the cells relying solely only on Clb2-Cdk activity? This hypothesis is plausible because Clb2-Cdk promotes the apical to isotropic switch in budded cells and depolarises cortical actin cytoskeleton (Lew and Reed, 1993). Moreover, Clb2-Cdk was shown to prevent bud formation and to repress the SBF driven G1/S gene expression (Amon et al., 1994). Nevertheless, I consider an inhibitory role of Clb2 unlikely because G1-Clb2 did not prevent budding in the presence of Cln2. Instead, it accelerated the G1/S transition. In addition, Cln2 targets implicated in polarisation and budding pathways could not be phosphorylated by Clb2-Cdk, suggesting a crucial role for Cln2 in promoting substrate phosphorylation that Clb2 cannot achieve. Cln2 appears to do so using a novel substrate interaction that extends beyond the LP motif docking interactions. The structure based sequence alignment of Cln2 and Clb2 revealed two predicted loops in the Cln2 cyclin core. However, the Cln2-Clb2 chimeras I generated which involved these patches could not promote viability in *MET3pr*Cln2 Clb2(S-M) cells under Cln2 shut off condition. Therefore, further studies are needed to address what features make Cln2 indispensable and if these features are conserved in the other G1 cyclins.

5.4.1 Cln2 specialisation from an evolutionary perspective

Cyclins as cell cycle regulators were proposed to emerge after divergence of eukaryotes from archaea and bacteria (Gibson et al., 1994). A study on classification of cyclins based on phylogenetic analysis suggests that CLBs and CLNs are related to cyclin B, and CLNs diverged faster and more compared to CLBs (Cao et al., 2014). It is likely that during this fast evolution and further branching of the CLNs, *CLN2* specialised in functions such as triggering polarisation, promoting bud formation and recognising specific docking motifs on its targets that are different from CLB recognition motifs. Indeed, LP motif docking interactions were recently shown to be conserved among fungal G1 cyclins (Bandyopadhyay et al., 2020). This is consistent with the idea that ancestral fungi had at least a CLN cyclin and a CLB cyclin (Medina et al., 2016). This assumption would also align with the idea that throughout evolution Cln2 gained special functions which cannot

be substituted by Clb2. In the future, it will be interesting to address what triggered the need for harbouring more than one cyclin in ancestral fungi. Similarly, one can argue that the development of morphogenetic changes such as bud formation created such a need in budding yeast given that its counterpart fission yeast can live with a single cyclin-Cdk and display a simple morphogenetic life cycle of cell elongation and fission (Coudreuse and Nurse, 2010; Fisher and Nurse, 1996). Lastly, the cyclin B subfamily which also comprises CLNs and CLBs is conserved in fungi and animals, but the cyclin D subfamily is considered absent in fungi (Cao et al., 2014). Although the cyclin D subfamily does not seem to be evolutionarily conserved between fungi and animals, the common property of fungal G1 cyclins and cyclin D in higher eukaryotes could be that they are needed for special functions. The crucial role of Cln2 in polarisation and budding as suggested in this thesis, and the importance of cyclin D1 for the development of mammary glands and retina (Fantl et al., 1995; Sicinski et al., 1995) can be given as examples to these specialised functions. Therefore, it is likely that G1 cyclins evolved to provide specific inputs that cannot be provided by quantitative Cdk control to fine tune the cell cycle progression. Overall, understanding the evolution of cyclins will give insights into how the cell cycle machinery works across different organisms.

5.5 Can any other cyclin-Cdk complex drive the cell cycle in the absence of the remaining budding yeast cyclins?

Could I use another budding yeast cyclin to establish a single cyclin-Cdk system to drive the cell division cycle? Given its crucial role in cell polarisation and budding, would it be possible to drive the cell cycle with Cln2-Cdk, for example? Cln2-Cdk complexes show lower kinase activity towards a generic substrate (Koivomagi et al., 2011b) and localise mainly to the cytoplasm (Miller and Cross, 2000; Quilis and Igual, 2012). Because higher kinase activity levels and nuclear cyclin-Cdk localisation are required in later stages of the cell cycle, Cln2-Cdk alone is unlikely to be sufficient. A study addressing this question revealed that the overexpression of stabilised Cln2 fused to an NLS is sufficient to trigger DNA replication, but not chromosome segregation in cells lacking CLB kinase activity (Palou et al., 2015). Although the molecular mechanism behind this observation

is yet to be discovered, it suggests that earlier cyclins might not be fully capable of promoting later cell cycle events.

Although Cln2 cannot drive later cell cycle events, there are occasions where later expressed cyclins can take over G1 cyclin function. For instance, overexpressed Clb5 or an extra copy of Clb5 expressed from its own promoter can compensate for the absence of G1 cyclins (Epstein and Cross, 1992; Schwob and Nasmyth, 1993). However, expression of wild type Clb5 or its stabilised version from the *CLB5* promoter do not rescue the *clb1,2*Δ lethal phenotype (Cross et al., 1999; Epstein and Cross, 1992). This indicates that neither Clb5 expression levels nor its degradation limit Clb5's ability to compensate for Clb1/2. In future studies, it would be intriguing to characterise the *clb1,2*Δ strain overexpressing Clb5 in a time course experiment to determine which Clb1/2 specific tasks Clb5 fails to perform.

A stabilised version of the G2 cyclin Clb3 can compensate for the absence of both G1 and S phase cyclins, but cannot replace mitotic cyclins Clb1 and Clb2 (Pecani and Cross, 2016). However, Clb3 is the least concentrated cyclin amongst S and G2/M cyclins in the nucleus (Ord et al., 2019a). Being in the right place at the right time is important for the function of cyclins (Hegarat et al., 2020; Moore et al., 2003; Santos et al., 2012). Therefore, it will be interesting to test if increasing nuclear accumulation of stabilised Clb3 might allow establishment of a single cyclin-Cdk system in budding yeast.

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