Testing the cell cycle phase specificity of cyclins: can an earlier cyclin trigger a later event?

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Dedicated to

Abuji, Didi, Tathi, Khan bhai, Enkay, Noor,

And my beautiful wife

Insha

CONTENTS

ABSTRACT

Progression through the cell cycle is directed by cyclin dependent kinases (CDKs), which are essential for normal and cancer cell proliferation. CDKs are activated by association with cyclins specific to each cell cycle phase (G1, S, G2, and M). Thus, CDK associated with G1 phase cyclins promote the expression of proteins necessary for DNA replication, but is unable to activate it. CDK associated with S phase cyclins, in turn, triggers the activation of chromosomal origins of replication, but is unable to promote chromosome condensation and segregation of sister chromatids, which is carried out by CDK associated with mitotic cyclins.

Despite the essential role of CDKs in cell cycle progression, how the different cyclins promote specifically the various processes of the cell cycle was still an open question by the time this thesis was initiated. Since the discovery of cyclins by Nobel laureate Tim Hunt [Evans et al. (1983) Cell 33:389] it was assumed that cyclins confer substrate specificity. However, later on, fellow Nobel laureate Paul Nurse proposed an alternative *quantitative* model, [Stern & Nurse (1996) Trends Genet 12:345], based on the observation that successive waves of cyclins result in increased levels of CDK activity. While low levels of activity (CDK associated with G1 cyclins) are sufficient promote progression through the G1 phase and start the pro-S phase transcription program, would be unable to trigger replication. Moderate levels of activity (CDK associated with S phase cyclins) would be able to activate replication but not mitosic events, which would require the high CDK activity associated with M phase cyclins.

If correct, the quantitative model should fulfill two predictions, but only one was demonstrated by the Nurse lab. Eukaryotic unicellular yeast cells are able to survive with a single mitotic cyclin, which is notwithstanding able to orderly drive the cell through the different cell cycle phases [Fisher & Nurse (1996) EMBO J 15:850]. Therefore M-CDK activity is able to promote the previous phases of the cycle in the fission yeast *Schizosaccharomyces pombe* However, if a purely quantitative mechanism applies, the

complementary prediction should be true as well: an early cyclin to be able to trigger later events if expressed at levels high enough.

To test such prediction we generated a budding yeast *Saccharomyces cerevisiae* strain carrying a G1 cyclin G1 resistant to degradation, under a strong inducible promoter, and fused to a nuclear localization signal. Our results show that one such cyclin is capable of firing chromosome replication conditions in which the S phase cyclins, G2 and M are suppressed. Therefore, our results support the quantitative model against the requirement of substrate specificity. How eukaryotic cells prevent premature activation of the critical cell cycle processes that lead to genomic instability, seems therefore trust in the regulation of activity levels and limiting the presence of cyclin at specific time and space.

INTRODUCTION

1 Eukaryotic Cell Cycle

Every organism, however simple or complex needs to self sustain and the only way to make new cell is to duplicate already existing cell. In order to survive, these basic building blocks of the organism, cells, require to proliferate through a series of coordinated events were each individual cells duplicate there genomic data and divide themselves to complete a single cycle of life commonly referred as a cell cycle (Norbury and Nurse 1992).

The concept of cell division has been studied for over a century, however, the real hallmark was achieved in early 1950´s when Howard and Pelc's work on, *Vicia faba*, revealed that the cell goes through many discrete phases before and after cell division. It was shown that cell carries out DNA replication in specific phase of cell cycle and this phase being clearly separated from mitosis. It was later in 1970´s when Leland Hartwell, Paul Nurse and Timothy Hunt laid the ground breaking discoveries in cell cycle. Discovery of Cdc proteins by Hartwell using temperature sensitive techniques, discovery of cyclins by Tim Hunt and regulation of cell cycle by Paul Nurse opened the doorways for the most important findings in the field of cell cycle (explained in detail in latter segments).

Eukaryotic cell cycle basically consists of four distinct phases: a single round of chromosome duplication where entire genome is replicated taking place during the synthesis phase thus called as S-phase, followed by the segregation of replicated chromosomes into two daughter cells during mitosis phase also known as M-phase (Pardee 1989, Collins, Jacks et al. 1997, Johnson and Walker 1999). Both pivotal phases are preceded by gap phases known as Gap1 (G1) and Gap2 (G2) respectively (Figure 1). G1 occurs between M-phase and S-phase while as G2 occurs between S-phase and M-phase. During G1 phase, cells grow in size and ensure the presence of all proteins required for the process of replication before deciding for the entry into the synthesis phase (S-phase). During G2 phase, cells continue to grow while also

making sure of presence of all proteins required for mitosis and ultimate division of cell (Pardee 1989, Johnson and Walker 1999). The final M-phase is itself coupled into two processes, a major part consists of the dividing of two identical chromosomes into two daughter nuclei and cytokinesis, a process were cells divide their cytoplasm into two identical daughter cells.

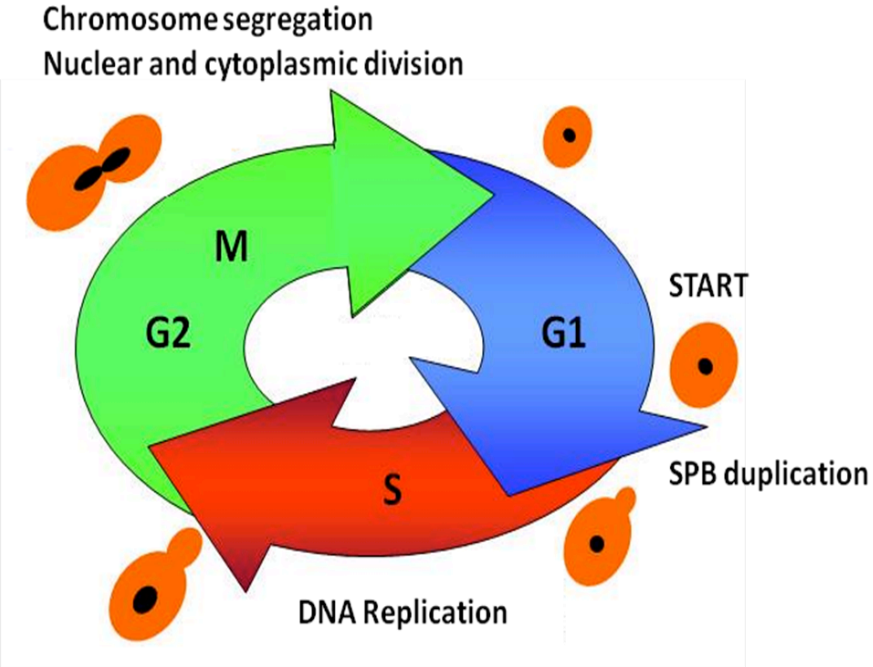


Figure 1. Schematic mitotic cell cycle of budding yeast *Saccharomyces cerevisiae*. After passing the START, cells are irreversibly committed to a new round of cell division. DNA replication takes place in the S-phase, while as cells segregate the replicated DNA into two sister chromatids followed by the nuclear and cytoplasmic division.

2. Why *Saccharomyces cerevisiae*

The details of cell cycle vary from one organism to other, including the time required to complete certain events, even in the same organism. However, organization of the cell cycle and its control system are essentially the same in all eukaryotic cells. The proteins required for the control system first appeared over a billion years ago. Remarkably, these proteins are so well conserved over the course of evolution that many of them function perfectly when transferred from a human cell to a yeast cell. Therefore, cell cycle and its regulation in a variety of organisms can be studied and findings pooled from all of them to assemble a unified picture of how eukaryotic cells proliferate.

The budding yeast *Saccharomyces cerevisiae* is the simplest and most powerful model systems for studying the genetics of cell cycle control among several other organisms like, *Xenopus*, sea urchins, *Drosophila melanogaster* and cultured mammalian cells. It is considered as a better model organism due to its numerous advantages over other model organisms, for example, *S. cerevisiae* is a small single celled eukaryotic organism with a short generation time (doubling time 1.25–2 hours at 30 °C) and can be easily cultured. These are all positive characteristics in that they allow for the swift production and maintenance of multiple specimen lines at low cost.

Also, in *S. cerevisiae,* upon transformation, new genes can either be added or deleted through homologous recombination. Furthermore, its ability to grow as a haploid has simplified the creation of gene knockout strains, which apparently is more intricate in other model organisms. And most importantly, as a eukaryote, *S. cerevisiae* shares the complex internal cell structure of plants and animals without the high percentage of non-

coding DNA that can confound research in higher eukaryotes. Therefore making *S. cerevisiae* a perfect tool for better understanding of cell cycle and its intricate mechanisms.

3. Cyclin Dependent Kinase (CDK)

As mentioned above *S. cerevisiae* has a same cell cycle as other eukaryotic organisms, gap1 phase followed by DNA replication, followed by gap2 phase and finally mitosis and division of cell. Cell cycle is a self sustaining process, however, in its essence; cell cannot drive itself without the help of well coordinated machinery. The core protein responsible for driving the cell cycle is a protein kinase known as Cyclin-dependent kinase (CDK).

Cyclin-dependent kinases are serine/threonine kinases that specifically phosphorylate proteins on their serine and threonine amino acid residues thus rendering the target protein either active or inactive. In budding yeast *S. cerevisiae*, this highly conserved protein is encoded by gene CDC28 (Cdk1 being the equivalent of cyclin dependent kinase in humans and Cdc2 in *S. pombe*) which regulates the progression of cell cycle in different phases (Hartwell 1976).

As the name of this protein suggests, this kinase protein is dependent on another set of proteins called cyclins. Cyclin dependent kinase proteins are the catalytic subunit of activated hetrodimer, where as the cyclins are regulatory subunits. The kinase activity of cyclin dependent kinase protein remains inactive unless they bind to its specific regulatory subunit, cyclins. While as cyclins alone confer no kinase activity (Morgan 1995, Pines 1995). Once activated by binding of cyclin, oscillating level of cyclin-cyclin dependent kinase complex orchestrates the regulation and progression of cell cycle through a series of biochemical reaction called phosphorylation that activates or inactivates target proteins in order to

coordinate the entry into the next phase of the cell cycle. Figure 2 (Pardee 1989, Norbury and Nurse 1991).

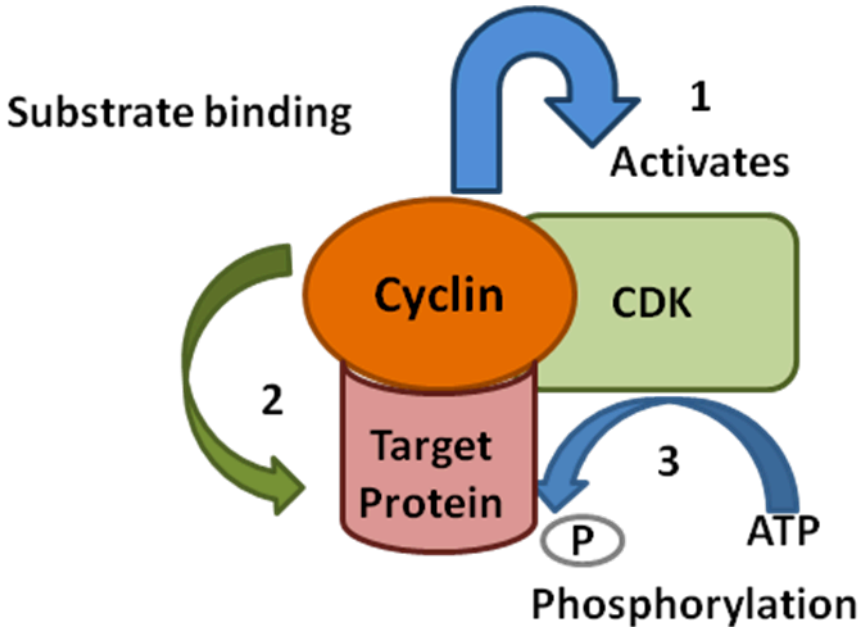


Figure 2. Shows the canonical representation of cyclin-CDK activity. 1. Cyclins bind to the CDK, thus, activating the complex. 2. The complex binds to the target protein and finally, 3. CDK transfers a $_{\gamma}$ phosphaste to the target protein.

Even though there are multiple CDK´s present in budding yeast *S. cerevisiae* like Cdc28, Kin28, Ctk1, Pho85 and Ssn3. Cdc28 however is alone sufficient and essential for the progression of cell cycle (Hartwell, Mortimer et al. 1973, Jallepalli and Kelly 1997). Moreover, early study of cdc mutants by Lee Hartwell showed that gene CDC28, encoding for Cdc28 protein is essential and temperature sensitive mutations of Cdc28 block the progression of cells at the Start (Hartwell 1974, Reed 1980, Mendenhall, Richardson et al. 1988).

The levels of cyclin dependent kinase (CDK) proteins relatively remains constant throughout the cycle, however, the cellular levels of cyclins increase and decrease as cell progresses

through the cell cycle, majorly due to the need of different cyclin-CDK combinations required during the different phases of the cell cycle. In case of *S. cerevisiae,* cyclin dependent kinase Cdc28 is activated by association with different cyclins, where each cyclin confers its specificity towards Cdc28 at different phases of the cell cycle, i.e., CDK confers specificity towards Cln1 and cln2 in G1 phase, Clb5 and Clb6 in S-phase and finally Clb1, Clb2, Clb3 and Clb4 in G2-M phase (Hartwell, Mortimer et al. 1973, Evans, Rosenthal et al. 1983, Schwob and Nasmyth 1993, Koch and Nasmyth 1994).

4. Cyclins

The part of the reason cyclins were discovered a decade after the discovery of cdc28 was due to their constant variability in cellular levels in a course of a cell cycle. They were rather named as cyclins due to their behaviour in appearing and disappearing in a cyclic manner (Evans, Rosenthal et al. 1983). Cyclins not only confer to the activation of CDK but also target the complex to specific sub cellular localisations to initiate processes like polarized growth at the site of bud emergence, promoting spindle pole body (SPB) (Lew and Reed 1993, Lew and Reed 1995).

The protein levels of cyclins are tightly regulated at the transcriptional level as well as their paths of degradations in order to control their presence at given specific cell cycle phase (Nash, Tokiwa et al. 1988, Hadwiger, Wittenberg et al. 1989, Richardson, Wittenberg et al. 1989, Ghiara, Richardson et al. 1991, Surana, Robitsch et al. 1991, Fitch, Dahmann et al. 1992, Richardson, Lew et al. 1992, Schwob and Nasmyth 1993, Donaldson, Raghuraman et al. 1998).

Cyclins are expressed and regulated in phase specific manner in different stages of cell cycle (Figure 3).

4.1 G1 cyclins (Cln1, Cln2 and Cln3): These cyclins associated with cyclin dependent kinase drive cell cycle from G1 to S-phase and also promote activities like spindle pole body duplication and budding (Lew and Reed 1995). Cyclin dependent kinase activity associated with Cln1 and Cln2 (which are closely related proteins with overlapping functions) are expressed in late G1 phase which upon association with Cdc28 activates its kinase activity (Wittenberg, Sugimoto et al. 1990, Nasmyth 1996). There mRNA accumulation in late G1 is dependent on two transcription factor complexes, MBF (Swi6p-Mbp1p) and SBF (Swi6p-Swi4p), which bind to MCB and SCB promoter elements, respectively (Cross, Hoek et al. 1994, Stuart and Wittenberg 1994). However, Cln3 on the other hand is not regulated under cell cycle transcription but instead as post-transcriptional modification (Tyers, Tokiwa et al. 1992, Cross and Blake 1993). Albeit, it rather plays a role in regulating the transcriptional activation of other G1 cyclins, Cln1 and Cln2 (Dirick, Bohm et al. 1995, Stuart and Wittenberg 1995).

4.2 S-phase cyclins (Clb5 and Clb6): Cyclin dependent kinase activity associated with S-phase cyclins brings about DNA replication. The levels of S-phase cyclins not only remain high in S-phase but remain such through G2 and early M phase, thus helping in promoting early events of mitosis and finally falling at onset of anaphase. Clb5 and Clb6 are expressed periodically throughout the cell cycle and are most abundant during late G1 (Schwob and Nasmyth 1993, Spellman, Sherlock et al. 1998). Promoters of CLB5 and CLB6 contain MCB (MluI cell cycle box) motifs, which are elements found in several DNA synthesis genes. The transcriptional activator MBF (MCB-binding factor), which is comprised of the Mbp1 and Swi6 proteins, bind to the MCB elements to activate transcription of CLB5 and CLB6 (Lew D.J et al 1997).

4.3 G2 cyclins (Clb3 and Clb4): These cyclins accumulate during S-phase and G2 phase. Cyclin dependent kinase activity associated with G2 cyclins promotes the transition of G2/M phase, and are also presumed to be essential of spindle assembly (Surana, Robitsch et al. 1991, Fitch, Dahmann et al. 1992).

4.4 M-phase cyclins (Clb1 and Clb2): As cells begin to enter mitosis, the levels of M-phase cyclins start to increase and reach to its peak when cells are in metaphase. Cyclin dependent kinase activity associated with M-phase cyclins promotes transition from G2 to M phase and through the process of mitosis, M-phase cyclins are degraded before the exit of mitosis (Schwob and Nasmyth 1993, Donaldson, Raghuraman et al. 1998).

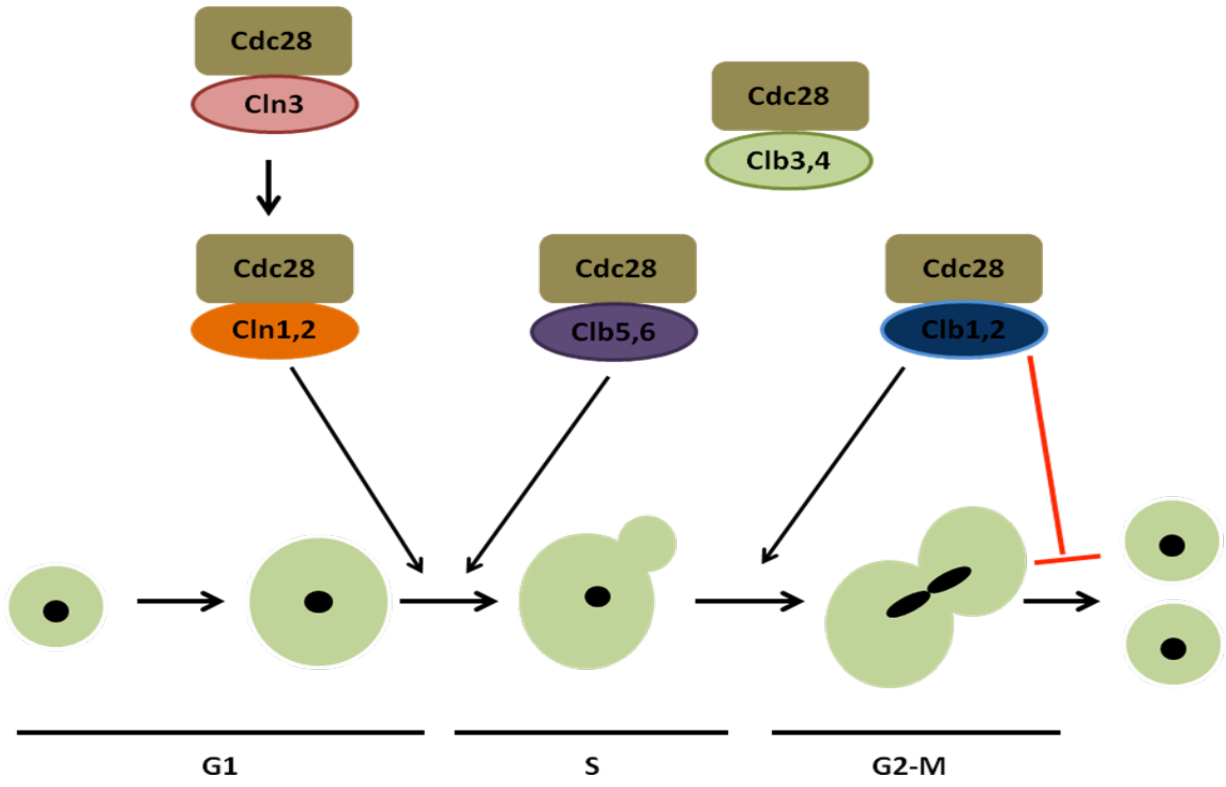


Figure 3. This diagram shows the orderly expression of different cyclins in *S. cerevisiae* in their respective phases. In budding yeast cell cycle, a single catalytic subunit (cdc28) drives the whole cell cycle by associating with specific regulatory subunits, cyclins, from different cell cycle phases.

5. Regulation of Cyclin Dependent Kinase activity (CDK-activity)

Since the progression of the cell cycle is coordinated and driven by the cyclin-CDK activities, it is more apparent to have the regulation of CDK activities rather tightly regulated in order to ensure the execution of cell cycle division with precision (Morgan 1997). Cell regulates the levels of cyclin-CDK activity by four critical mechanisms, CAK (CDK activation kinase) phosphorylation, binding of cyclins and there destruction, binding of cyclin dependent kinase inhibitor subunits (CKIs) and by regulatory inhibitory phosphorylation (Morgan 1995, Morgan 1997).

5.1 CDK activation kinases (CAKs): In budding yeast *S. cerevisiae*, cyclin dependent kinase proteins are phosphorylated by CDK activation kinase proteins at threonine 169 adjacent to the active site before cyclin-CDK complex is formed. Timing of this phosphorylation varies in different model organisms and moreover, CAK kinases are not regulated in cell cycle pathway manner, however, studies have shown that phosphorylation of CDK by CAK is pivotal for viability of cells (Ross, Kaldis et al. 2000). Figure 4.

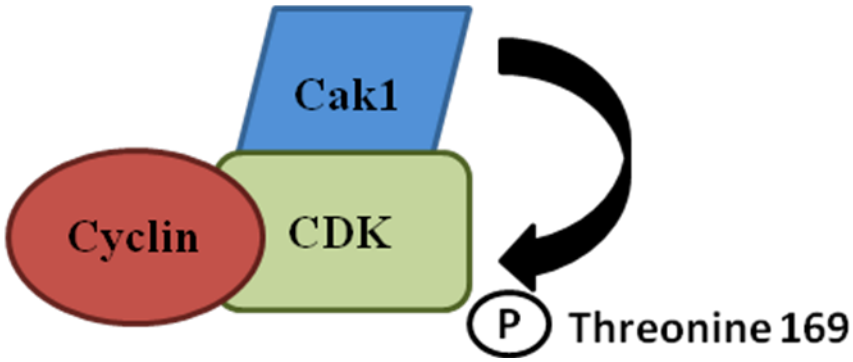
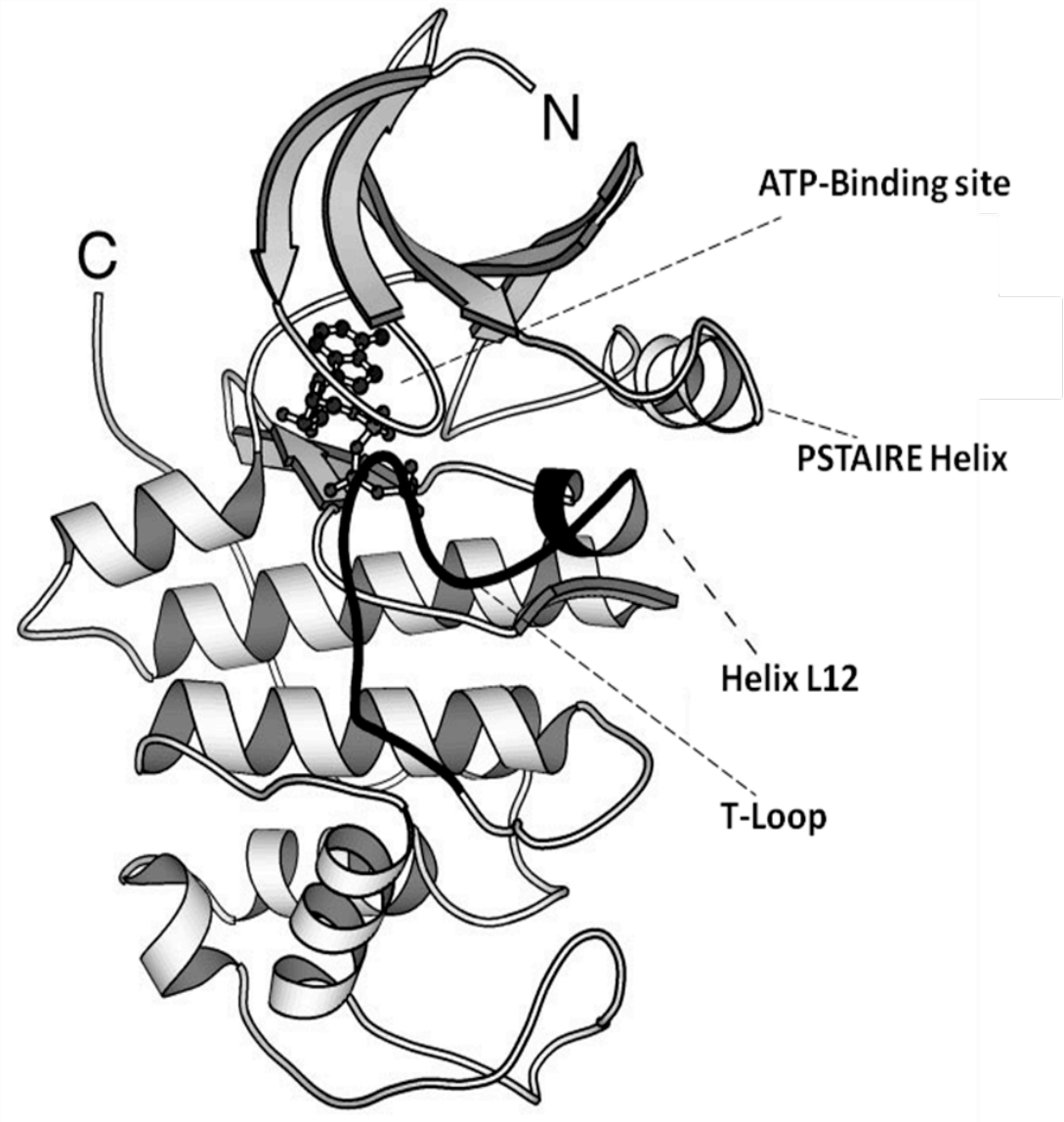


Figure 4. This diagram shows phosphorylation of threonine 169 by Cak1 which is followed by binding of cyclins.

5.2 Cyclin binding and degradation: Cyclin binding is a process where cyclins bind to an inactive CDK in order to expose the active site, or ATP-binding site, which is a cleft between a small amino-terminal lobe and a larger carboxy-terminal lobe. In its inactive form, Cyclin dependent kinase proteins have a flexible loop called the activation loop or T-loop which blocks the cleft. Due to the barrier of T loop and in absence of cyclin binding, the position of several key amino acid residues is not optimal for ATP-binding, thus rendering CDK inactive (Figure 5). CAKs phophorylate T loop at Thr 169 to increase the complex activity towards the cyclin binding (Ross, Kaldis et al. 2000).

With cyclin binding, the conformational change in two alpha helices permit the appropriate ATP binding. L12 helix that comes just before the T-loop in the primary sequence becomes a beta strand and helps rearrange the T-loop in a manner to prevent its blocking of active site. While as the other alpha helix called the PSTAIRE helix rearranges and helps change the position of the key amino acid residues in the active site, eventually leading to the activation of cyclin-cyclin dependent kinase complex activity (De Bondt, Rosenblatt et al. 1993, Morgan

1997).



Firgure 5. Shows the structure of cyclin dependent kinase. The smallerN-terminal lobe, containing a beta sheet and the PSTAIRE helix. Below N terminal lobe is larger C-terminal lobe. The active site cleft between the lobes contains ATP-binding site (ball and stick representation). The T-loop (residues 146–170), which blocks the cleft (dark black). And, the small L12 helix disrupts active site residues involved in ATP orientation (Morgan 1997).

Furthermore, the rising levels of cyclin-CDK activities are regulated by degrading the cyclins at the specific stages of cell cycle once the role of their functional properties are met. Cells have evolved with complex mechanisms and machineries to help degrade the cyclins via proteasome regulation to avoid the repetition of processes as well as eliminating the proteins to allow passage from one phase to the next. The two major proteasome complexes are Skp, Cullin, F-box containing complex (or SCF complex) and Anaphase Promoting Complex (APC).

SCF (*Skp1, Cullin, F-box*): G1 cyclins are highly unstable and are self regulated, which upon being phosphorylated are subjected to degradation under Skp, Cullin, F-box containing complex (or SCF complex) (Schneider, Patton et al. 1998). SCF complex is a multi-protein E3 ubiquitin ligase complex which helps in catalyzing the ubiquitination of proteins destined for proteasomal degradation.SCF is active throughout the cell cycle, and the stability of individual substrates is regulated by their phosphorylation and depending on different phosphate binding proteins (F box proteins) guiding different sets of substrates to destruction.e.g., Cdc4, Grr1 (Figure 6) (Murray 2004).

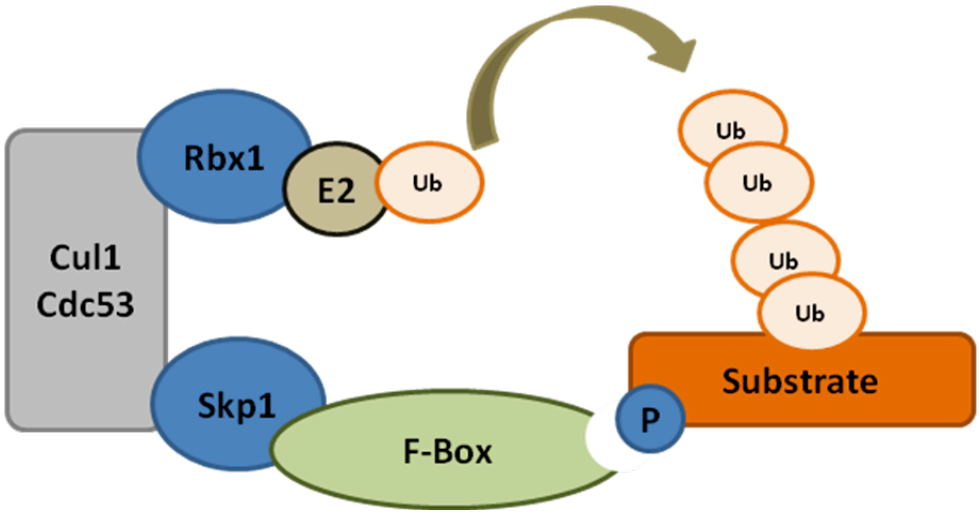


Figure 6. Schematic representation of SCF (Skp/cullin/F-box) complex. Composed of a core organization, the association of a cullin-like protein Cdc53, a protein containing a particular zinc finger domain Rbx1 which recruits E2-ubiquitin conjugate and phosphate binding F box proteins which guides different sets of substrates to destruction (Teixeira and Reed 2013).

Apart from degrading G1 cyclins, SCF complex has important roles in the ubiquitination of other proteins involved in the cell cycle. For example, the essential step to enter into the S-phase is achieved by the degradation of S and M-CDK inhibitor (also known as all CLB inhibitor) Sic1 by SCF complex coupled with the F-box protein Cdc4 (SCF^{cdc4}) (Feldman, Correll et al. 1997, Skowyra, Craig et al. 1997, Verma, Annan et al. 1997, Visintin, Craig et al. 1998, Yoshida, Asakawa et al. 2002, Queralt and Igual 2003, D'Amours, Stegmeier et al. 2004, Mayor, Lipford et al. 2005). While as Clns on the other hand are degraded by SCF coupled with F-box Grr1 (Barral, Jentsch et al. 1995, Skowyra, Craig et al. 1997, Patton, Willems et al. 1998, Schneider, Patton et al. 1998).

Cdc6, an essential protein required for Pre-RC assembely at the origin of replication is also degraded by SCF^{cdc4} mediated ubiquitination after first round of replication in order to prevent re-replication (Donovan, Harwood et al. 1997)

Among all Clbs (S phase and M phase cyclins), only Clb6, the paralog of Clb5 is degraded by SCF^{cdc4} because Clb6 lacks the destruction box motif responsible for the anaphase promoting complex-mediated destruction (which will be explained momentarily) however, contains putative Cdc4 degron motifs in the N terminus, therefore targeted to the SCF(Cdc4) ubiquitin ligase complex (Jackson, Reed et al. 2006).

Anaphase promoting complex (APC): All mitotic cyclins included S phase cyclin Clb5 are degraded by Anaphase promoting complex (APC), causing the inactivation of M-phase cyclin dependent kinase activity which is essential for exit from mitosis (Jaspersen, Charles et al. 1998, Visintin, Craig et al. 1998, Wasch and Cross 2002).

APC/C is a multi-protein E3 ubiquitin ligase complex like SCF complex which helps in catalyzing the ubiquitination of proteins destined for proteasomal degradation. APC core consists of 14 different proteins, including Apc2 cullin-like subunit that serves as a scaffold.

Apc11 RING-finger protein interacts with E2 enzyme responsible for elongating ubiquitin chains.

Unlike SCF complex, APC/C is not active throughout the cell cycle, albeit, its activation is achieved via further association with one of the two coactivator subunits: Cdc20 and Cdh1. Both the adapters recognise destruction motifs also known as degrons on C-terminal domains of target protein. The motifs present on target protein recognised by APC/C are the consensus sequence of RXXLXXXXN also known as D-box and KEN-box with consensus sequence of KENXXXN (Glotzer, Murray et al. 1991, Peters 2006, Barford 2011) (Figure 7).

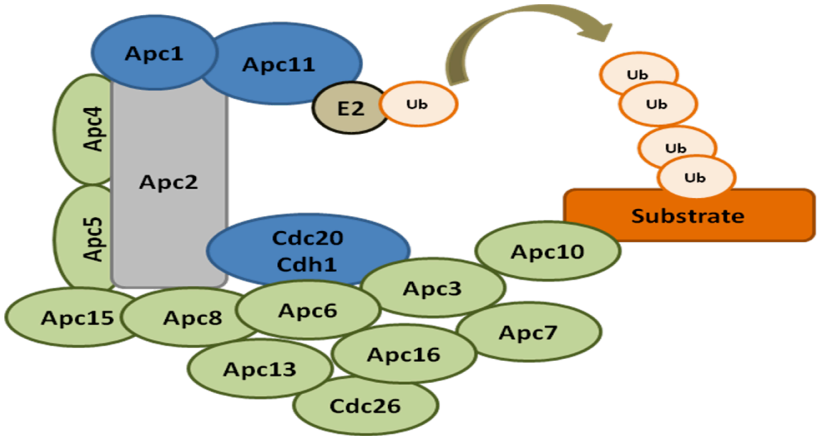


Figure 7. Schematic representation of APC (Anaphase promoting complex). Composed of a core organization, the association of a cullin-like protein Apc2, a protein containing a particular zinc finger domain Apc11 which recruits E2-ubiquitin conjugate and adapters Cdc20 and Cdh1, which recognise destruction motifs. Cdc26 is important for assembly of the complex (Teixeira and Reed 2013).

Anaphase promoting complex (APC) regulates the mitosis at two different steps, first at metaphase-anaphase transition followed by at the exit of the mitosis. At the metaphase-anaphase transition, APC associates with the co-activator Cdc20 (APC^{Cdc20}) and is responsible for the Securin/Pds1 proteasome pathway (Cohen-Fix, Peters et al. 1996, Visintin, Prinz et al. 1997). The protein Securin inhibits the separation of sister chromatids preventing the action of Separase/Esp1, which is to cleave protein Cohesin. Cohesin/Scc1 is a

protein that keeps two sister chromatids attached together, and upon activation of Separase, Cohesion is cleaved, thus, allowing chromosome segregation (Shirayama, Toth et al. 1999, Salah and Nasmyth 2000, Wang, Liu et al. 2001, Agarwal, Tang et al. 2003, Rahal and Amon 2008).

The second association of APC with Cdh1 (APC^{Cdh1}) is involved in the exit of mitosis, where all Clb cyclins are completely eliminated. Unlike APC^{cdc20}, APC^{cdh1} is inactive at the beginning of mitosis (Harper, Burton et al. 2002) due to phosphorylation of Cdh1 by cyclin dependent kinase activity associated with Clbs (Schwab, Lutum et al. 1997, Visintin, Prinz et al. 1997, Zachariae, Schwab et al. 1998, Kramer, Scheuringer et al. 2000). However, release of phosphatase Cdc14 from nucleus via FEAR and MEN (Mitotic Exit Network) pathways at the end of anaphase dephosphorylates Cdh1, causing the subsequent activation of APC^{Cdh1} (Jaspersen, Charles et al. 1998, Yoshida, Asakawa et al. 2002, D'Amours, Stegmeier et al. 2004). Upon activation of APC^{Cdh1}, APC^{Cdh1} promotes degradation of mitotic cyclins, leading to the inactivation of M-phase CDK activity and eventual exit from mitosis (Schwab, Lutum et al. 1997, Jaspersen, Charles et al. 1998, Visintin, Craig et al. 1998, Wasch and Cross 2002).

5.3 Cyclin dependent kinase inhibitors (CKIs): In order to prevent cell cycle events to run out of order, cyclin-dependent kinase inhibitor proteins (CKIs) interact with the cyclin-CDK complex and block its activity till its right time for cells to enter next stage. In budding yeast, CKIs are strong inhibitors of S-phase and M-phase CDK activities. CKIs inhibit the activity of S-phase-CDK activity at G1 to prevent untimely initiating of DNA replication. Once the levels of G1/S cyclin-CDK levels increase, the CKIs are phosphorylated and degraded by ubiquitin-mediated proteolysis pathway (Schwob, Bohm et al. 1994, Sheaff and Roberts 1996, Verma, Annan et al. 1997). In budding yeast *S. cerevisiae* , cyclin-dependent kinase inhibitor (CKI) Sic1 regulates the cell cycle at the G1 to S transition by inhibiting the activity

of the cyclin-dependent kinase (CDK) Cdc28. $p27^{KIP1}$ is the structural homologue of inhibitory domain of Sic1 in mammals, however, they lack the sequence homology (Lew 2003).

Sic1 is expressed at M/G1 transition and is dependent on the transcription factor Swi5 (Knapp, Bhoite et al. 1996, Toyn, Johnson et al. 1997, Aerne, Johnson et al. 1998). Sic1 is an inhibitor of the cyclin-CDK complexes containing S phase and M phase cyclins (Clbs) but not G1 (Cln) cyclin (Mendenhall 1993, Mendenhall, al-Jumaily et al. 1995). However, cells overcome the inhibition of Sic1 protein and enter S phase by phosphorylating and targeting it for degradation via ubiquitin-mediated proteolysis pathway (Sheaff and Roberts 1996). Cyclin dependent kinase Cdc28 associated with Clns phosphorylates Sic1 (Mendenhall, Jones et al. 1987, Wittenberg and Reed 1988, Schneider, Yang et al. 1996). Cyclin dependent kinase inhibitor protein Sic1 needs to be phosphorylated on a minimum of six of its nine potential phosphorylation sites and it becomes targeted for degradation (Verma, Annan et al. 1997, Nash, Tang et al. 2001, Mittag, Marsh et al. 2010). Phosphorylated Sic1 is bound by Cdc4, which is the substrate recognition subunit of the E3 ligase SCF-Cdc4 (Skowyra, Craig et al. 1997, Deshaies and Ferrell 2001, Nash, Tang et al. 2001). In conjunction with the E2 enzyme Cdc34, SCF^{Cdc4} polyubiquitinates Sic1 on N-terminal residues and once ubiquitinated, the polyubiquitin-binding protein Rpn10p targets Sic1 to proteasome for degradation (Feldman, Correll et al. 1997, Skowyra, Craig et al. 1997, Verma, McDonald et al. 2001, Petroski and Deshaies 2003, Kus, Caldon et al. 2004, Mayor, Lipford et al. 2005, Petroski and Deshaies 2005, Sadowski, Suryadinata et al. 2010).

The other cyclin dependent kinase inhibitor present in budding yeast is, Far1, Far1 inhibits Cln-Cdk1 complexes at Start, especially in presence of pheromone. Unlike Sic1, only one site on Far1 requires to be phosphorylated rather than six to target it for degradation (Peter and Herskowitz 1994, Gartner, Jovanovic et al. 1998).

5.4 Cyclin dependent kinase inhibition phosphorylation: Apart from activating phosphorylation by CAKs, CDK activities are also regulated by inhibitory phosphorylations by certain protein kinases. In budding yeast S. cerevisia, a protein kinase Swe1 inhibits the kinase activity of the main cell-cycle cyclin-dependent kinase Cdc28 through phosphorylation of a conserved tyrosine residue, tyrosine 19 (Y19) (Booher, Deshaies et al. 1993). Figure 8.

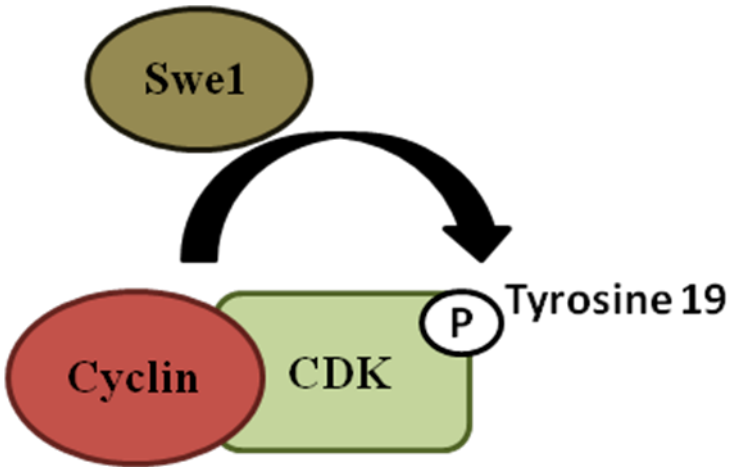


Figure 8. Schematic representation of blocking of M-CDK activity by inhibitory phosphorylation of CDK tyrosine 19 by kinase protein Swe1.

6. Transcription factors specific to the different phases of the cell cycle

While cell cycle progression is controlled by regulating levels of cyclin dependent kinase activity as explained in previous segments, other set of cell cycle control is carried out by transcriptional factors activated sequentially which regulates the transcription program periodically (Orlando, Lin et al. 2008).

These transcription factors essentially encode the expression of different specific cyclins of each phase of the cell cycle among other proteins. Cyclins themselves being under the category of periodically expressed genes and while as cyclin dependent kinase playing a role in the regulation of cell cycle transcription, however, are not exclusively responsible for establishing the periodic transcription program. This suggests that transcription program periodically works as an oscillator, independent of CDK activity (Orlando, Lin et al. 2008).

Nonetheless, the timely scheduled expression of genes required for cell cycle regulated processes such as DNA replication and mitosis is not alone sufficient for triggering these events, but the accurate execution of cell cycle events is more likely to require both properly timed transcription and post-transcriptional modifications mediated by cyclin dependent kinase (Orlando, Lin et al. 2008).

G1 transcription factors present in budding yeast *S. cerevisiae*, are known as SBF (Swi4 / 6 Cell-Cycle Box Binding Factor) and MBF (MLU And Cell-cycle Box Binding Factor) whose functional analogs to human is E2F (Cooper 2006). Upon activation of SBF and MBF, these transcription factors promote the entry into the S-phase from G1 phase, since cells cross the limiting step of cell cycle known as Start and thus committing the cell for the round of DNA replication and mitosis.

SBF (Swi4 / 6 Cell-Cycle Box Binding Factor) and MBF (MLU And Cell-cycle Box Binding Factor) bind to the promoters of 235 genes (Iyer, Horak et al. 2001, Simon, Barnett et al. 2001). SBF (Swi4 / 6 Cell-Cycle Box Binding Factor) binds the promoters of several other transcription factors, including HCM1, PLM2, POG1, TOS4, TOS8, TYE7, YAP5, YHP1, and YOX1 and ChIp-ChIp analysis has shown that the promoters of these genes with consistent roles in G1/S events including DNA replication, bud growth, and spindle pole complex formation (Horak, Luscombe et al. 2002).

6.1 Transcription factor SBF (Swi4 / 6 Cell-Cycle Box Binding Factor): This transcription factor consists of two proteins: a protein with transactivator activity, known as Swi6 and a protein with DNA binding activity, Swi4 (Nasmyth and Dirick 1991). Unlike Swi6, the expression of Swi4 varies throughout the cell cycle, peaking in G1 (MacKay, Mai et al. 2001).

Along with gene expression of critical G1 cyclins CLN1, CLN2, budding and biosynthesis of membranes (Breeden and Nasmyth 1987, Andrews and Herskowitz 1989, Taba, Muroff et al. 1991, Iyer, Horak et al. 2001, Simon, Barnett et al. 2001), SBF also controls the expression of OH which is a site-specific endonuclease required for gene conversion at the MAT locus (homothallic switching) at Start. Deletions in the promoter helped in the identification of a consensus sequence (CACGAAAA) that confers to Start-specific transcription (Nasmyth 1985). This specific sequence is recognized by Swi4 protein and is called as SCB (Swi4 / 6 Cell-Cycle Box) (Ogas, Andrews et al. 1991).

G1 transcription repressor, Whi5 binds to the SCB binding factor (SBF) at SCB target promoters in early G1. The phosphorylation of Whi5 by the cyclin dependent kinase activity associated with Cln3 relieves its repression and promoter binding (Costanzo, Nishikawa et al. 2004). pRB is the functional analog of Whi5 in human´s (de Bruin, McDonald et al. 2004).

6.2 Transcription factor MBF (MLU and Cell-cycle Box Binding Factor): MBF also consists of two proteins, one with transactivator activity same as in SBF, Swi6 and a DNA binging protein Mbp1 (Wijnen, Landman et al. 2002). MCB box (MLU and Cell-Cycle Box) which is recognized by Mbp1 has a consensus sequence (ACGCGT) of specific promoters that confers to Start-specific transcription (Verma, Patapoutian et al. 1991). MBF is responsible for the transcription of genes involved in DNA replication such as S phase cyclin CLB5 and CLB6. It is also involved in transcription of several other essential proteins required for DNA replication and DNA repair such as Pol1, Pol12, Rfa1, Cdc45, subunits of ribonucleotide reductase (RNR), and the cyclin dependent kinase activity inhibitor in M phase, Swe1 (Feldman, Correll et al. 1997, Iyer, Horak et al. 2001, Simon, Barnett et al. 2001)

6.3 Transcription Factor SFF: Timely transcription of cell cycle-regulated genes is organized into clusters, exhibiting similar patterns of regulation. In previous sections, we understood that in most cases periodic transcription is achieved by both repressive and activating mechanisms. Studies have shown the group of at least 35 yeast genes that are transcribed roughly from the end of S phase until nuclear division (Cho, Campbell et al. 1998, Spellman, Sherlock et al. 1998). Based on the CLB2 mitotic cyclin gene, this set of genes has been termed as the Clb2 cluster (Ghiara, Richardson et al. 1991, Surana, Robitsch et al. 1991). Other members of this family include CLB1, CDC5, CDC20, SWI5 and ACE2. Through the analysis of the SWI5 promoter, the insight into regulation of this gene cluster was first obtained. A protein complex was shown to be capable of binding to specific elements in the SWI5 promoter, thus coining the term Swi5 factor (SFF) (Taba, Muroff et al. 1991). Recent studies on SWI5 and CLB2, has revealed that SFF sites are binding sites for members of the forkhead family of transcription factors, especially two forkhead protein members, Fkh1 and Fkh2. (Kumar, Reynolds et al. 2000, Hollenhorst, Pietz et al. 2001) .

Although, activating pathway of SFF transcription factors is not fully understood, but proteins expressed under it are pivotal for the processes of M-phase and M/G1 phase.

6.4 Inactivation of Transcription Factors: In order to have limited and controlled transcription of MBF and SBF promoters, they are inactivated in different ways. MBF becomes inactive when repressor protein, Nrm1 binds to the MBF, with help of protein Mbp1, whose presence for recruitment is necessary (Koch, Moll et al. 1993, de Bruin, Kalashnikova et al. 2006). While as SBF inactivation is achieved by nuclear expulsion of transactivator protein Swi6 by cyclin dependent kinase activity associated with Clb6 and not Clb5 (Taba, Muroff et al. 1991, Sidorova, Mikesell et al. 1995, Queralt and Igual 2003, Geymonat, Spanos et al. 2004) (Figure 9 and Figure 10).

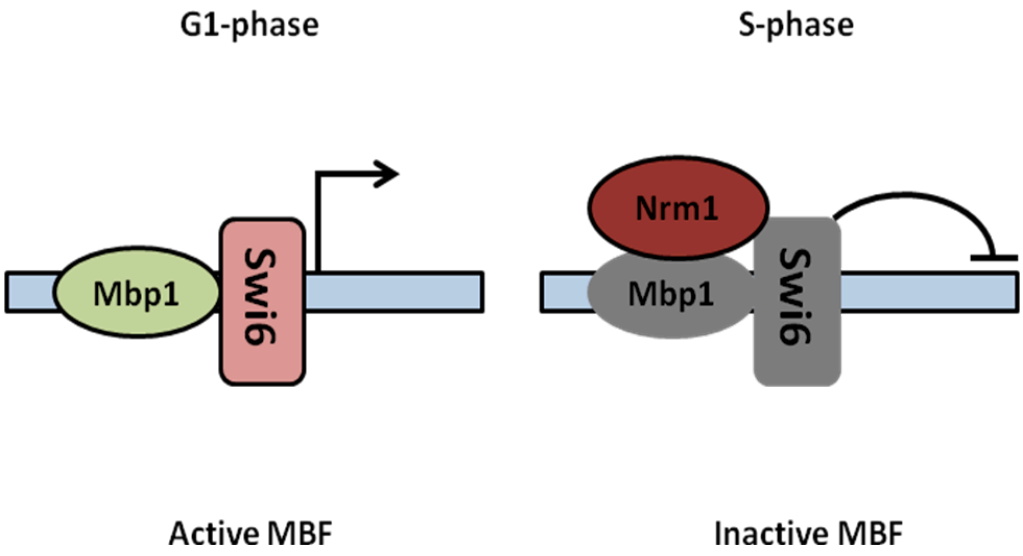


Figure 9. Diagram shows the inhibition of MBF-dependent transcription in S phase. Inactivation caused by binding of MBF repressor Nrm1.

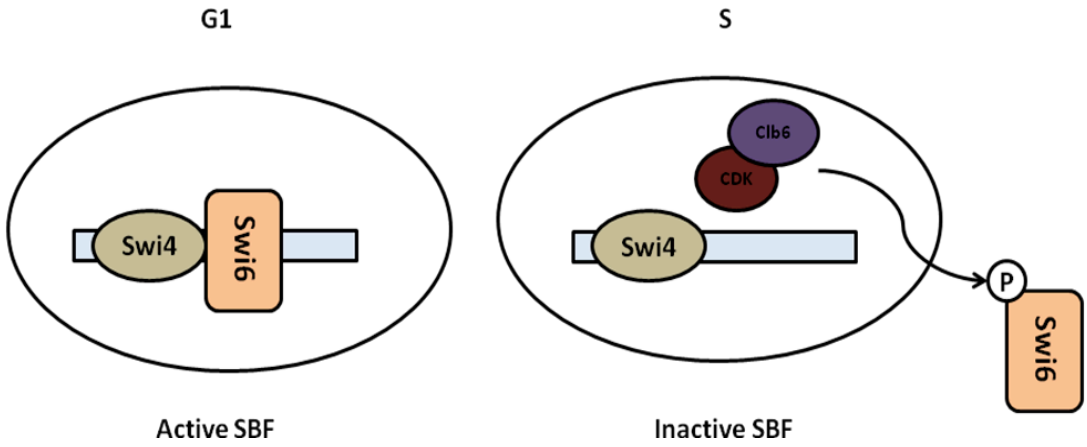


Figure 10. Diagram shows the inhibition of SBF-dependent transcription in S phase. Inactivation caused by nuclear exclusion of Swi6, triggered by phosphorylation by Clb6-cdc28.

7. Cell cycle progression

As previously explained, cell cycle is driven by properly timed transcription and post-transcriptional modifications mediated by cyclin-cyclin dependent kinase activities (Loog and Morgan 2005, Orlando, Lin et al. 2008). In this section, we will have detailed understanding of how cell cycle progresses from G1 till the end of the mitosis.

7.1 G1 Phase: In eukaryotic cell cycle, there is a critical point at which cells decide whether to enter the cell cycle (to complete a round of DNA replication, mitosis and cell division), remain in quiescence (also known as G0) or go for mating (in the presence of homothallic complementary type). This critical point in budding yeast *S. cerevisia* is called START; while as in human cells it is known as Restriction point. Cells cross this limiting junction only after

it has reached to an appropriate size and environmental conditions are feasible (Johnston, Pringle et al. 1977) (Hicks and Herskowitz 1976, Nasmyth 1983, Fitch, Dahmann et al. 1992).

Crossing Start is highly regulated till cells acquire the right size and proteins required for the process is properly accumulated. The process involves the activation of previously explained transcription factors (SBF, MBF), and G1/S phase promoters. The cyclin dependent kinase, Cdc28, activity associated with G1 cyclin Cln3 is the responsible for crossing the Start, Cln3-Cdc28 activity is tightly regulated in G1 phase with corresponding to cell cycle progression and cell growth (Hartwell and Unger 1977) (Johnston, Pringle et al. 1977).

As mentioned previously, transcription of Cln3 is not regulated by the cell cycle, rather by post-translational modifications, (Tyers, Tokiwa et al. 1992) (Cross and Blake 1993) moreover, prior to its function, Cln3 is inhibited at two levels. At first, a RNA binding protein, Whi3, involved in cell size, binds to the mRNA of Cln3 which leads to its translation in endoplasmic reticulum (Gari, Volpe et al. 2001, Wang, Gari et al. 2004), and in order to retain Cln3 in endoplasmic reticulum, N-terminal Cdc28 binding domain of Whi3 interacts with cyclin dependent kinase Cdc28 and Cln3-Cdc28 complex including members of HSP70 family, Ssa1 and Ssa2, thus restricting the nuclear accumulation of Cln3-Cdc28 complexes (Wang, Gari et al. 2004, Verges, Colomina et al. 2007). As G1 progresses and cell reaches to the optimal size, a J chaperone, Ydj1, interacts with the C-terminal regions of Cln3 and facilitates its phosphorylation by Cdc28 as a signal for degradation also plays a positive role in releasing the Cln3-Cdc28 complex from endoplasmic reticulum and allowing its nuclear accumulation (Verges, Colomina et al. 2007).

The accumulation of Cln3-Cdc28 complex in nucleus raises its kinase activity, which leads to the phosphorylation of Whi5. Whi5 is the inhibitor of SBF (Swi4 / 6 Cell-Cycle Box Binding

Factor) complex and upon being phosphorylated is excluded from the nucleus (Costanzo, Nishikawa et al. 2004, de Bruin, McDonald et al. 2004). Once SBF is activated, it is responsible for transcription of proteins involved in budding, biosynthesis of membranes and cell wall as well as G1 cyclin Cln1 and Cln2. On the other hand, pathway responsible for the activation of MBF is still unknown, however, it is responsible for transcribing pivotal proteins involved in DNA replication especially polymerase alpha subunits Pol1 and Pol12 as well as S-phase cyclins Clb5 and Clb6 (Figure 11) (Johnston, Morgan et al. 1996, Simon, Barnett et al. 2001).

With this sequential transcription of proteins and cyclin dependent kinase, cdc28 kinase activity associated with Cln3 in G1 progresses cell cycle past Start, once cells past Start, they enter and are committed into the cell cycle for a single round of DNA replication followed by mitosis and cell division with no possible point of return.

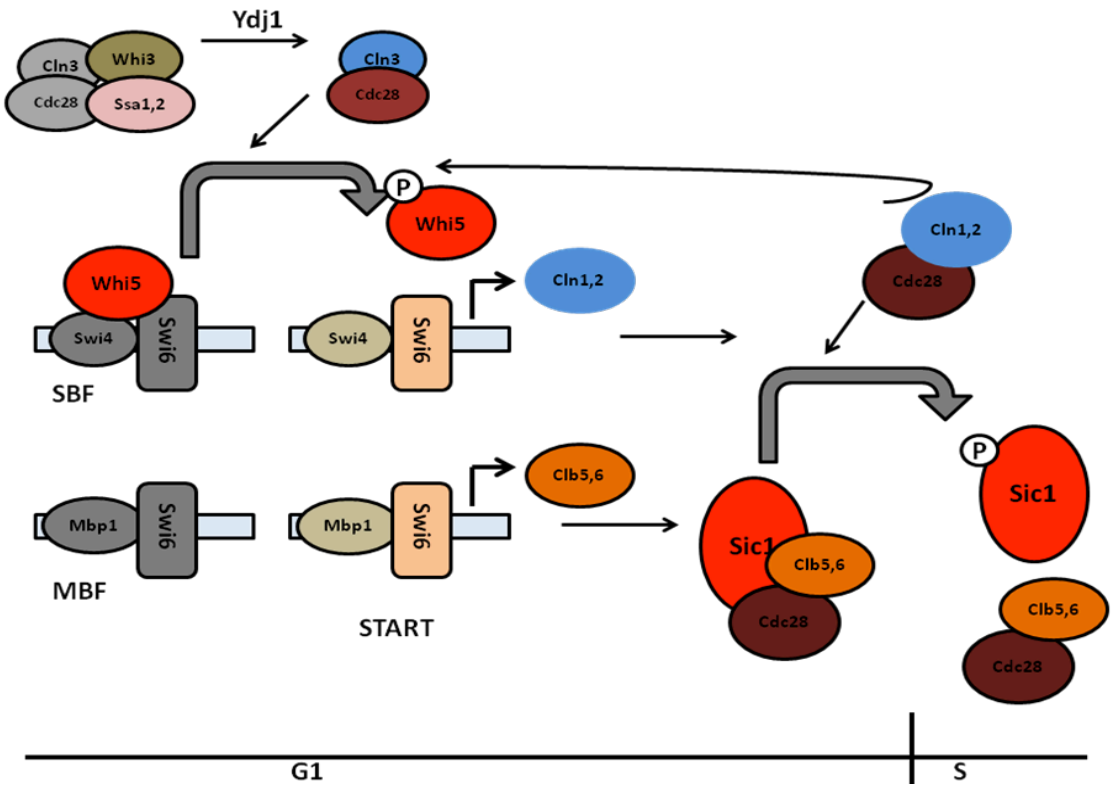


Figure 11. Shows the schematic diagram of G1 phase and G1-S transition. Cln3-Cdc28 is activated once cells reach the sufficient size, Whi5 involved in inactivation of transcriptional promoter of S phase SBF (Swi4-Swi6). The activation of SBF and MBF (Swi6-Mbp1) marks the crossing of Start. SBF transcribes, the G1 cyclins Cln1, 2, while MBF transcribes, the S phase cyclin Clb5, 6. The S-CDK activity, however, is inhibited by Sic1. The accumulation of CDK activity associated Cln1, 2 phosphorylates Sic1, marking it for destruction with subsequent activation of S-CDK and S phase.

7.2 S-phase: Once cells cross the Start, proteins required for replication are accumulated and recruited at specific positions. Before the beginning of S-phase, a protein known as Origins of Complex Recognition (ORC), recognizes and binds to a DNA sequence called the Autonomous Replication Sequences (ARS) which is considered as the origin of replication,

(Stinchcomb, Struhl et al. 1979, Bell and Stillman 1992). In budding yeast, ORC remains attached to origins throughout the cell cycle (Diffley and Cocker 1992). The protein ORC on the ARS acts as the platform for the other proteins that constitute the pre-RC (pre-Replication Complex).

The beginning of replication is carried out in two step mechanism, first step, licensing of origins, the recruitment of proteins in pre-RC are achieved by the sequential binding of Cdc6, which is required for the binding of another DNA replication licensing factor, Cdt1 (Devault, Vallen et al. 2002) followed by the recruitment of helicase MCM complex (Cocker, Piatti et al. 1996, Santocanale and Diffley 1996, Aparicio, Weinstein et al. 1997, Detweiler and Li 1997, Donovan, Harwood et al. 1997, Tanaka and Diffley 2002, Tanaka, Umemori et al. 2007) This Mcm2-7, cdc6, cdt1 containing complex bound to origin are called as pre-replicative complex. Pre-replication complex can be formed only during G1 phase due to low CDK activities (Zegerman and Diffley 2007).

In the meantime, MBF has transcribed the S-phase cyclins (as explained previously) Clb5 and Clb6. Clb5 and Clb6 are associated with cyclin dependent kinase, Cdc28, however, its kinetic activity is being inhibited by an all Clb inhibitor protein, Sic1 (explained in section 5.3). Sic1 inhibits Clb-Cdc28 kinase activity by blocking the active site of Cdc28 (Donovan, Toyn et al. 1994, Schwob, Bohm et al. 1994, Schneider, Yang et al. 1996). The accumulation Cdc28 kinase activity associated with Cln1 and 2 promotes the phosphorylation of Sic1, marking it for degradation by the ubiquitin-ligase complex SCFCdc4 (Schneider, Yang et al. 1996, Tyers 1996, Skowyra, Craig et al. 1997, Nash, Tang et al. 2001). The destruction of Sic1 causes the release of S phase cyclin dependent kinase activity (Clb5-Cdc28 and Clb6-Cdc28), thus marking second step in the beginning of DNA replication (S phase).

Rise in cyclin dependent kinase activity associated with Clb5, Clb6, commonly known as S-phase CDK activity and S-phase kinase Cdc7-Dbf4 (DDK) activity, both are essential for triggering the replication. Sld2 and Sld3 are the two essential constituents of the pre-RC and key targets of S-CDK (Tanaka, Umemori et al. 2007, Zegerman and Diffley 2007). While as DDK (Cdc7-Dbf4) phosphorylates Mcm4 subunit of the replicative helicase MCM, which promotes the formation of a stable complex between MCM and Cdc45 (Sheu and Stillman 2006).

After phosphorylation of two essential proteins, Sld2 and Sld3 by S-CDK activity, the pre-RC complex is converted into pre-IC (pre- Initiation Complex) upon binding of several other additional proteins at origins, including, Dbp11 (DNA replication initiation protein which helps in loading of DNA pol epsilon onto the pre-replication complexes at origins), DNA replication initiation factors, Cdc45 (recruits elongation machinery) and GINS (Sld5p, Psf1p, Psf2p, Psf3p) (Araki, Leem et al. 1995, Masumoto, Sugino et al. 2000, Kamimura, Tak et al. 2001, Takayama, Kamimura et al. 2003, Tanaka, Umemori et al. 2007).

Loading of Dbp11 and GINS, result in the activation of helicase and the opening of double-stranded DNA with the recruitment of DNA polymerases, processes finally commencing into DNA replication (Figure.12) (Aparicio, Weinstein et al. 1997, Aparicio, Stout et al. 1999, Labib, Tercero et al. 2000, Gambus, Jones et al. 2006, Kanemaki and Labib 2006, Moyer, Lewis et al. 2006). At the beginning of replication, not all origins fire at once, instead origins fire throughout the S-phase (Zegerman and Diffley 2009). While whole genome is being replicated throughout the S-phase, levels of M-phase cyclins start to increase towards the end of S-phase. Since levels of cyclin dependent kinase activity associated with Clb5 and Clb6 are not enough to trigger the mitosis, the timely scheduled transcription of proteins required for mitosis start to accumulate.

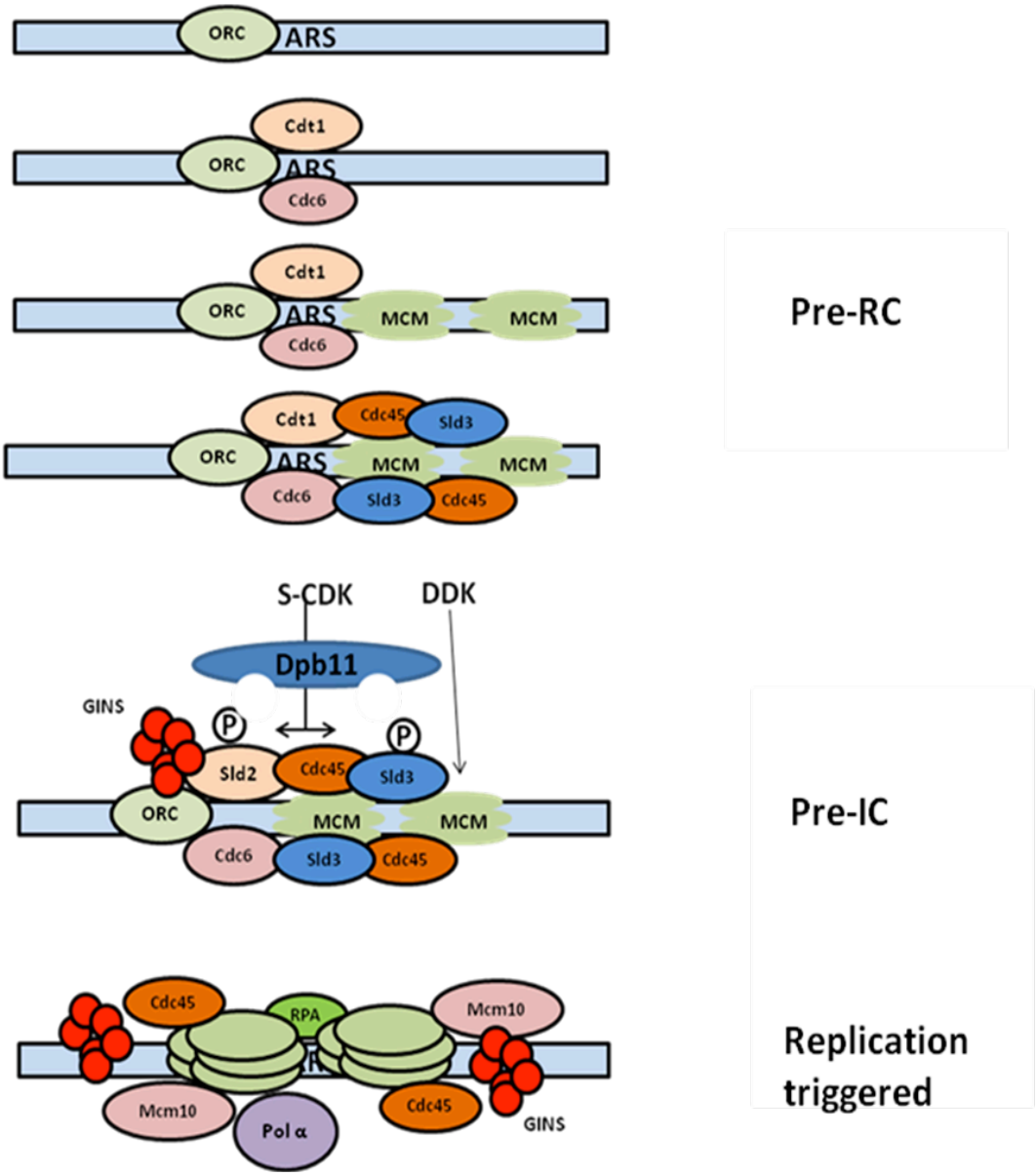


Figure 12. Depicts the schematic diagram of initial steps in the recruitment of proteins at the origin of replication in early S-phase. Followed by the triggering of replication by S-CDK and DDK activities in order to replicate the whole genome in S-phase.

7.3 M-phase: M-phase is brought about by cyclin dependent kinase activity associated with mitotic cyclins (Clb 1, 2, 3, 4). Though not much is known about the mitosis compared to S-phase, however, studies have shown that mitotic Clbs play roles involved in entry into mitosis, chromosome segregation and mitotic exit (Fitch, Dahmann et al. 1992, Richardson, Lew et al. 1992, Amon, Tyers et al. 1993, Miele 2004, Rahal and Amon 2008). High levels of M-CDK activity promotes nuclear envelope breakdown, spindle assembly and organization, chromosome condensation, and Golgi fragmentation, and also contributes towards the APC/C regulation (Nigg 2001, Miele 2004).

Chromosome segregation takes place during anaphase and is triggered by the disintegration of the protein linkages that hold sister chromatids together (Nasmyth 2002). After the completion of replication, chromosomes condense and align on the metaphase plate where spindle pole bodies attach to the kinetechores of each sister chromatid. However, chromatids are linked together, which is mediated by cohesin complexes (Scc1/Mcd1, Scc3, Smc1, and Smc3) (Nasmyth 2002). At the metaphase–anaphase transition, the Anaphase-Promoting Complex/Cyclosome (APC/C) associated with the co-activator Cdc20 (APC^{Cdc20}) targets Securin/Pds1 for proteosomal degradation. Securin/Pds1 is a protein that inhibits anaphase by binding separin/Esp1p, thence, blocking cyclin destruction and mitotic exit. Clb1, 2–CDK activity is required for the timely activation of Anaphase-Promoting Complex/Cyclosome (APC^{Cdc20}) (Rahal and Amon 2008). After degradation of Pds1, separin/Esp1p (a caspase-like cysteine protease) is free and cleaves the cohesin subunit Scc1/Mcd1 is, leading to the loss of cohesion between sister chromatids and subsequent chromosome segregation (Cohen-Fix, Peters et al. 1996) (Yamamoto, Guacci et al. 1996, Ciosk, Zachariae et al. 1998, Shirayama, Toth et al. 1999, Salah and Nasmyth 2000, Uhlmann, Wernic et al. 2000, Wang, Liu et al. 2001, Nasmyth 2002) (Agarwal, Tang et al. 2003, Rahal and Amon 2008).

The second part, exit from mitosis requires complete elimination of the Clbs and levels of cyclin dependent kinase activity. This is achieved by APC/C associated with Cdh1, since APC^{cdh1} is inactive at the beginning of mitosis (Harper, Burton et al. 2002) due to phosphorylation of Cdh1 by Clb1, 2 CDK activity (Schwab, Lutum et al. 1997, Visintin, Prinz et al. 1997, Zachariae, Schwab et al. 1998, Kramer, Scheuringer et al. 2000), its activation involves the release of phosphatase Cdc14 from nucleus via FEAR and MEN (Mitotic Exit Network) pathways at the end of anaphase, which dephosphorylates Cdh1, causing the subsequent activation of APC^{Cdh1} (Jaspersen, Charles et al. 1998, Yoshida, Asakawa et al. 2002, D'Amours, Stegmeier et al. 2004). Upon activation of APC^{Cdh1}, APC^{Cdh1} promotes degradation of mitotic cyclins, leading to the inactivation of M- CDK activity and eventual exit from mitosis (Schwab, Lutum et al. 1997, Jaspersen, Charles et al. 1998, Visintin, Craig et al. 1998, Wasch and Cross 2002).

8. Cyclin Dependent kinase control of cell cycle

So far we understood that cell cycle encompasses the ordered series of events for the purpose of survival of cell, where there chromosomal DNA gets duplicated and then distributed among two daughter cells. We also established that cells maintain to carry out the stages of cell cycle in an orderly fashion by regulating levels of cyclin dependent kinase activity, the oscillating activity of cyclin dependent kinase acts as a major regulator for cell cycle progression (Orlando, Lin et al. 2008).

However, since the discovery of cylins and cyclin dependent kinases over four decades ago, scientist are still not very clear about how these oscillating levels of cyclin dependent kinases are translated into ordered series of cellular events. The question how cyclin dependent kinase regulates cell cycle progression was raised for several reasons; 1) both pivotal phases, S-phase and M-phase are promoted by same catalytic subunit Cdk1 (Cdc2 in case of fission yeast and Cdc28 in case of budding yeast) (Nurse 1981). 2) cyclin dependent kinase activity associated with specific set of cyclins of a given phase can perform the functions of phase preceding it, while as cannot do the functions of phase following it. For example, Cdc28 activity associated with mitotic cyclins can trigger replication but cannot do the function of G1 cyclins (Fangfang Hu and Oscar M. Aparicio 2004). Similarly, Cdc28 activity associated with S-phase cyclins cannot trigger the events of mitosis but can rescue the all Cln null strain (Epstein and Cross 1992, Schwob, Bohm et al. 1994, Lopez-Girona, Mondesert et al. 1998).

In order to address this question, that, how one protein can fulfil two different roles in a cell cycle at two different times, several explanations were put forward by scientists. Two of them are of major importance. One of them, known as substrate specificity, put forward by Nobel laureate Timothy Hunt.

8.1 Substrate Specificity

Substrate specificity may refer to the specificity of cyclin dependent kinase activity towards a certain specific set of substrates when recruited by a cyclin in a given cell cycle phase. This concept originated due to the fact that three distinct cyclins, Cln1, Cln2, and Cln3 are required for transition of G1/S and entry into the S-phase (Nash, Tokiwa et al. 1988, Hadwiger, Wittenberg et al. 1989, Richardson, Wittenberg et al. 1989, Tyers, Tokiwa et al. 1992). While as different set of cyclins, Clb1, Clb2, Clb3 and Clb4 are required for the process of mitosis (Minshull, Blow et al. 1989, Ghiara, Richardson et al. 1991, Surana, Robitsch et al. 1991). Moreover, third set of cyclins (Clb5 and Clb6) are required for the timely onset of DNA replication (Epstein and Cross 1992, Schwob and Nasmyth 1993).

In addition to that, crystal structure study of S-phase cyclins showed the presence of Cdk consensus S/T-P recognition sites, and also identified an RxL peptide motif on CDK substrates which are readily recognized by the hydrophobic patch present on the S-phase cyclins (Adams, Sellers et al. 1996). The Similar patch is identified in G1 cyclins , however, not in mitotic cyclins (Petri, Errico et al. 2007, Day, Cleasby et al. 2009). The substrate recognition function of S-phase CDK provided by hydrophobic path of cyclin was confirmed in several Cdk substrates, suggesting RxL motif as a means by which S-phase cyclin recognizes certain specific substrates (Loog and Morgan 2005).

These findings, the timely transcriptional control of cyclins, and how each cyclin of each phase promotes the particular function of that phase might suggest that different cyclins act at different times with respect to their specificity towards target proteins to promote ordered cell cycle events. That is, G1 cyclins associated with cyclin dependent kinase might only phosphorylate targets required for G1/S transition, while as S-phase cyclins associated CDK

activity would only phosphorylate proteins responsible for DNA replication and finally, M-phase CDK activity provide specificity towards the entry and exit of mitosis.

Although, S-phase cyclins with cyclin-specific substrate recognition ability are required to trigger the replication by phosphorylating the two essential proteins, Sld2 and Sld3 (Zegerman and Diffley 2007) (Tanaka, Umemori et al. 2007), albeit, the rigorous test to check the requirement of S-phase cyclins showed that, cells can trigger replication on time and are viable in the absence of both S-phase cyclins (Clb5 and Clb6) (Fangfang Hu and Oscar M. Aparicio 2004). This suggests that the ability of triggering the replication is not limited to the S-phase cyclins alone. Similarly, cells with triple mutant of cln1, 2, and 3 are not viable since, it is important to have at least one G1 cyclin, which is required for proliferation and to trigger the Start (Richardson, Wittenberg et al. 1989), however, ectopic expression of S-phase cyclin Clb5 rescues the non-viability of triple mutant cells (Epstein and Cross 1992, Schwob, Bohm et al. 1994, Lopez-Girona, Mondesert et al. 1998). These findings suggest that G1 and S-phase cyclins are dispensable and are not pivotal for the sequential cell cycle progression; while as mitotic cyclins are essential.

8.2 Quantitative Model

As explained earlier, the question raised by observation, that, how can a single protein (Cdk1) be responsible for two apparently different process (DNA replication and Mitosis) and maintain the ordered cell cycle progression, Nobel Laureate Paul Nurse proposed a model known as, Quantitative model. According to this model, different levels of cyclin dependent kinase activity regulates the cell cycle progression. In simple terms, the low levels of cyclin dependent kinase activity associated with G1 cyclins trigger the events of G1-phase, however are not sufficient to trigger the DNA replication. While as, intermediate levels of cyclin dependent kinase activity associated with S-phase cyclins (Clb5 and Clb6) are enough to trigger the events of DNA replication but this amount of kinase activity is not enough to trigger the events of mitosis. Finally the higher level of cyclin dependent kinase activity associated with mitotic cyclins (Clb 1 and Clb2) bring about entry and exit from mitosis, where the cyclin dependent kinase activity falls and cells start with another cycle. Figure 13.

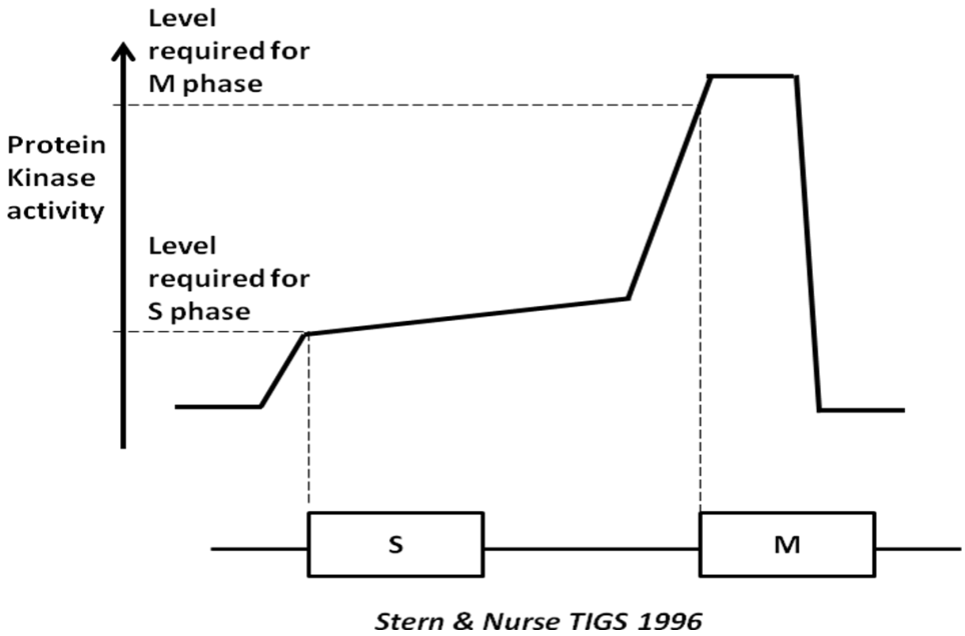


Figure 13. A quantitative model suggesting a single source of Cdk activity is sufficient for ordering sequential S phase and mitosis. S phase is triggered by an intermediate level of Cdk activity, while as mitosis depends on a higher kinase activity level. 1996)

The levels of CDK activity, one at the stage of triggering the DNA replication, the intermediate one and the other, high level of CDK activity at the time of mitosis are kept at two levels by inhibitory Cdk tyrosine phosphorylation by Swe1 (wee1 in case of fission yeast). Once CKI is inhibited, the CDK activity is boosted and rises to high levels to trigger the mitosis. However, recent study has shown the function of cyclin dependent kinase activity works in both the stages of replication as well as mitosis, without two distinct levels of cyclin dependent kinase activation (Coudreuse and Nurse 2010). Also, the regulation of single mitotic cyclin associated with Cdk activity can be achieved at the constant level of a fusion protein, where low, intermediate and high levels of CDK activity by chemical inhibition, and the concentrations are sufficient to drive orderly S-phase and M-phase (Coudreuse and Nurse 2010). These findings rationalize the existence of quantitative model, even though there is presence of other cyclins in cell, which could be just due to improvement in intricacy and delicacy of cell system over the course of evolution.

Although, quantitative model answers several impeding questions about regulation of cell cycle, it also raises some. Quantitative model to be utterly the reason for cell cycle regulation is far from being solved yet, some of the questions and challenges raised towards quantitative model are about understanding the timings of entry into the mitosis upon the completion of DNA replication and or exit from the mitosis after faithful segregation of replicated chromosomes, since it is basically dependent on synthesis of cylins and there timely destruction, because, if cyclin dependent kinase activity raises quickly before due time, the ordering of S-phase and M-phase will be hampered (Moore, Kirk et al. 2003, Coudreuse and Nurse 2010). Also, if basic requirement for cell cycle progression is not the substrate specificity, rather rising levels of cyclin dependent kinase activity, which dictates the progression of cell cycle, then cyclin dependent kinase activity associated with early cyclins

should be able to trigger latter events if expressed in higher level, at the right place and the right time. Addressing this question will be the major focus of this thesis.

OBJECTIVES

Objectives

The objective of this thesis is to validate the Quantitative model to explain how Cyclin Dependent Kinases regulate the orderly progression of cell cycle.

- We will explore the prediction required to validate a purely quantitative control: Whether an early cyclin bears the power to trigger a later cell cycle event if accumulated at high enough levels, at the right time, at the right place.
- In case a purely quantitative model applies, we will investigate the molecular barriers that are in place to avoid premature, deleterious events to take place.

MATERIALS AND METHODS

Materials and Methods:

9. Model organism:

In our study, we used budding yeast, *S. cerevisiae* as the model organism for the purpose of experiments. As previously mentioned in section 2, budding yeast is the perfect model organism for several reasons including small generation time (doubling time 1.25–2 hours at 30 °C), synchrony of the population, visual surveillance on progression of cell cycle according to the presence and size of the bud. Moreover, *S. cerevisiae* can be genetically modified to relative ease due to its prioritized repair of DNA double strand breaks by homologous recombination. With that context, if linear DNA is introduced into a cell of yeast with unprotected ends with homology to the genome sequence, integration would be of high frequency, thus replacing the corresponding genomic material. With the help of this technique we could mutant genes, or integrate them into the area of the genome that are of interest, by a simple process of transformation.

10. Yeast Genetic Background:

In order to have controlled access and technical advantages over the model organism, strains with modified genetic backgrounds were used, which made experimental procedures and results more accountable and simplified. Budding yeast *Saccharomyces cerevisiae* with the genetic background of W303 was used in this study (Thomas and Rothstein 1989) W303 has been modified with respect to the strains found in nature to stay in a haploid, the HO endonuclease mutant, which is the site-specific endonuclease required for gene conversion at the MAT locus (homothallic switching) (Schwob and Nasmyth 1993

Moreover, W303 is a mutant gene for each of the synthetic pathways of five essential nutrients: ADE2 (adenine, ade2-1), HIS3 (histidine, his3-11, 15), LEU2 (leucine, leu2-3, 112), TRP1 (synthesis of amino acid marker auxòtrofic trp1-1), and URA3 (synthesis of the nucleobase uracil, ura3-1). With the help of these auxotrophic markers available in strain, the selection of transformants was made highly probable, since the introduction of the wild type gene restores the ability of "auxotrophic marker gene" missing strain to live in a selective medium in the absence of the corresponding amino acid or nucleobase. Our work was based upon haploid strains, W303-1a, which allows synchronization of cells in G1-phase with pheromone α-factor. The α-factor pheromone is a natural peptide released by strains of type homothallic Mat α. The pheromone α-factor blocks Mat a type of haploid cells in G1 phase, in expectation of entering into the reproduction phase. The peptide Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr was used to synchronize cultures of Mat a in G1-phase in the absence of Mat α cells. Which acted as a pivotal tool, since, it makes it possible to release population of cells into S-phase synchronously.

The strains used in this study were bar1Δ. The BAR1 gene encodes a protease that removes the α-factor. bar1Δ mutant cells are sensitive towards the low concentrations of the peptide, being sufficient to synchronize cells at the concentrations of 100 times lower than in cells BAR1 which is 50 mg / ml.

10.1 Culture media:

YPD (1% (w / v) yeast extract, 2% (w / v) peptone, 2% (w / v) glucose) was used as rich medium while as other carbon source (sucrose, raffinose or galactose.) were used according to the needs of the experiment.

The minimal medium also known as SD media (Synthetic minimal medium Dextrose) containing 0.67% (w / v) yeast nitrogen base (YNB), 2% (w / v) glucose, and supplemented amino acids and/or nuclobase according to the selection required for the experiment, the following are the components required for desired supplementation: 40 mg / ml adenine, uracil, leucine, tryptophan, histidine.

The sporulation medium, RSM (Rich Sporulation Medium), consisting of 0.5% (w / v) yeast extract, 3% (w / v) potassium acetate, 0.001% glucose (w / v), supplemented with 0.16 mg / ml adenine and uracil, 0.08 mg / ml histidine, leucine, lysine, tryptophan, methionine and arginine 32 mg / ml tyrosine and 0.4 mg / ml phenylalanine.

Stocks of concentrated sugars, YNB, and amino nitrogen bases were sterilized by microfiltration (pore size 0.20 microns). Most of the sugars where sterilised this way rather than autoclaving, in order to prevent caramelization.

The medium used for counter selection of URA3 consists of 1 mg / ml 5-fluoroorotic (5-FOA) medium in SC (Synthetic Complete): 2 mg / ml Drop-out mix without Ura (Sigma), 0.67% (w / v) basis yeast nitrogen (YNB), 2% (w / v) glucose, 50 mg / ml uracil.

The medium LB (Lysogeny broth) consists of 1% (w / v) Bacto-Tryptone, 0.5% (w / v) yeast extract and 0.5% (w / v) NaCl.

The medium to prepare cells E. coli DH5α competent Psi-broth, contains 2% (w / v) Tryptone, 0.5% (w / v) yeast extract, 0.5% (w / v), MgSO4 · 7H2O, pH 7.6.

The solid media have the same composition as that described for liquid media plus 2% (w / v) agar.

10.2 Plasmids used in this study:

pFa6a family:

Plasmids family pFA6-KanMX6 (Bahler, Wu et al. 1998) were used to generate strains expressing proteins fused to a C-terminal tag. The pFA6a cassette used as a template for PCR amplification consists of the sequence of tag of interest and six copies of the gene KanMX geneticin antibiotic resistance (G418). The tags used in our case were 13myc (pFA6a-KanMX6-13myc) and 3HA (pFA6a-KanMX6-3HA).

pRS family:

Family of pRS series of plasmids (Sikorski and Hieter 1989) were used as the PCR template to amplify the genes of wild type auxotrophic markers corresponding to W303, in the process of generating strains of *S. cerevisiae* deletion mutants and as well as integrative plasmids.

PCM family:

The plasmid from PCM family were used in several experiments, these plasmids have activator/repressor expression system for budding yeast in which tetracyclines control in opposite ways the ability of tetR-based activator and repressor molecules to bind tetO promoters (Gari, Piedrafita et al. 1997).

10.3 Generating *Saccharomyces cerevisiae* strains:

10.3.1 Transformation of yeast

This method is used to transform the yeast cells upon weakening of the cell wall and transformation of genetic material of interest by heat shock. To perform the transformation, 12 ml culture of the parent strain in exponential growth (1. 10^7 cells / ml) were used. The pellets of cells were acquired by centrifuging at the speed of 3000 RPM for 3 minutes (Eppindorf centrifuge 5418, Fisher Bioblock Scientific 1-159, Sigma, centrifuge). The cells were washed twice with sterile water, and then washed once, later re-suspended in 0.1 M lithium acetate (pH 7.5), 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, at a concentration of 2. 10^9 cells / ml. To 50 µL of this cell suspension, 1-2 mg of DNA to be transformed was added, followed by 5 µL of ssDNA (10 mg / ml) (single-stranded DNA from salmon sperm, which helps transporter function by increasing the probability of passing the DNA into the cell).

This mixture is allowed to sit on ice for some time (from 5 minutes to 10 minutes) followed by addition of 300 µL of 50% (v / v) polyethylene glycol 3350, 0.1 M acetate lithium (pH 7.5), 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. The role of high weight polyethylene molecules is to occupy the space in the mixture, making the encounter between DNA and cells more probable. The mixture was vortexted at low speed to ensure the uniform solubilisation and then incubated at 30 ° C for 30 minutes, however, at 24 ° C in case of temperature sensitive mutants. After 30 minutes of incubation, DMSO was added to a final concentration of 10% (v / v) and cells were subjected to a heat shock of 15 minutes at 42 ° C, followed by 60 seconds on ice.

Cells were centrifuged (Eppindorf centrifuge 5418, Fisher Bioblock Scientific 1-159, Sigma, centrifuge) for 2 minutes, and pellets were re-suspended in 200 µL of TE pH 7.5 (10 mM Tris-HCl (pH 7.5), 1 mM EDTA) and seeded onto appropriate selective medium plates

(Schiestl and Gietz 1989, Gietz, St Jean et al. 1992, Bartel, Chien et al. 1993). The colonies were allowed to grow at 30 ° C (24 ° C in case of temperature sensitive mutants) for three to four days followed by second selection on fresh selective medium plates to ensure the purity of clones. Finally, modifications made in the strain were checked by either genomic PCR, functional assay or by Immunoblot of total cellular extracts (TCA extraction).

10.3.2 Deletion of genes in strains

Deletions of certain specific genes were achieved by transforming the cassette obtained from PCR amplification of the wild type auxotrophic markers available in vectors using the PRS and or pFA6a as a template. For the PCR amplification, primers were designed in a way that there 5´ tails had 60 nucleotide over hangs which were present in the sequences flanking the gene of interest to be deleted (Figure 14).

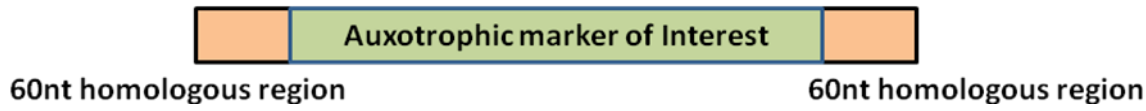


Figure 14. Cassette of PCR product used for the deletion of the gene.

Upon transformation of deletion PCR product, gene deletion occurred through homologous recombination, which happens by repair system activated by fragment of double strand DNA unprotected ends with a homologous region of the genome (Figure 15). Cell transformants were selected on minimal medium plates without supplemented amino acids and/or nuclobase according to the selection required for the deletion. For example, (SD-Ura, SD-Trp, SD-Leu,

SD-Ade, SD-His). The colonies were allowed to grow at 30 ° C (24 ° C in case of temperature sensitive mutants) for three to four days followed by second selection on fresh selective medium plates to ensure the purity of clones.

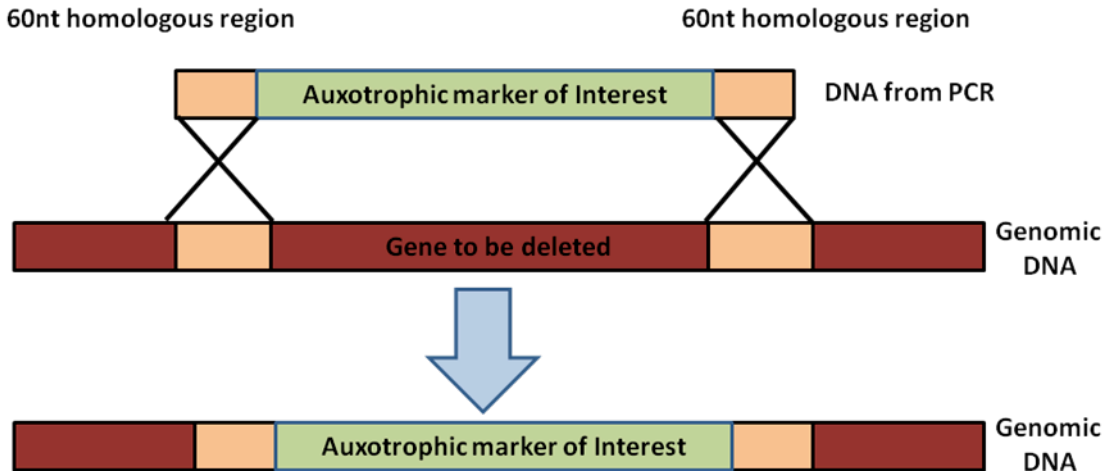


Figure 15. Shows the schematic diagram of the process of gene deletion by process of integration of auxotrophic marker by homologous recombination.

Finally, deletion of the genes was checked by either genomic PCR or by Immunoblot of total cellular extracts (TCA extraction). In case of PCR, it can be confirmed if the auxotrophic marker was integrated correctly into the locus of the gene of interest and not at nonspecific part in genome using transformed strain`s DNA as a template, and using primers external to the region replaced. The difference in length between the gene of interest and the marker can distinguish the clones in which the DNA was integrated in a specific gene of interest.

10.3.3 Tagging of genes

Open reading frames of genes of budding yeast can be tagged such that the corresponding protein expressed from its locus under its own promoter is fused with tag of interest. DNA to transform the protein of interest with tag comes from the PCR amplification of tag of

plasmid-family pFA6 KanMX6. The tags used in this case were 13myc (pFA6a-KanMX6-13myc) and 3HA (pFA6a-KanMX6-3HA) (Figure 16).

In order to tag the C terminal of the protein, two specific oligonucleotide primers were designed:

• Forward: at 5' 60 nucleotides corresponding features in the final sequence of the gene, excluding the STOP codon.

• Reverse: in the 5' 60 nucleotides present in a sequence corresponding to the region of genome downstream of the gene.

Using these primers, PCR generates a fragment that contains the tag and six copies of the gene geneticin /G418 KanMX antibiotic resistance, flanked by sequences homologous to the chromosomal region where integration takes place.

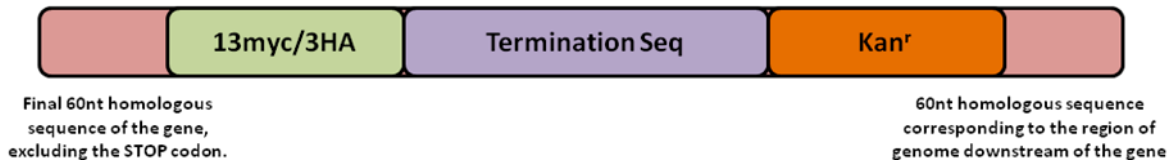


Figure 16. Showing the cassette of PCR product used to tag C-terminal of the protein.

Yeast cells were transformed with the resulting PCR product of interest for integration following the process as explained in (2.3.1**)**. The selection of transformants was carried out by resistance towards G418. Cells transformed with the G418 resistant gene requires time to express the resistance, thus cells were plated on rich medium (YPD) plates without selection. After 24 hours of incubation, resistant clones were selected by replica plating on rich medium

with 200 µg / ml G418. Positive colonies for correct tagging in the strain at the desired gene locus were checked by Immunoblot of total cellular extracts (TCA extraction) monoclonal antibodies against the tag (9E10 anti-myc or anti-HA 12CA5) while as using the corresponding parent strain as a negative control.

10.3.4 Sectors for second selection and functional assays

Upon transformation of a PCR product or integrative plasmid, colonies were passed through second selection by streaking the colony onto corresponding plates in form of sectors. Each colony was streaked on a sector and then re-spread from the end of the line in a zigzag form, such that individual colonies were produced.

Functional assay was also carried out in a same way as second selection. In functional assay, cells are grown on plates with specific carbon source or a drug, where specific gene of interest weather deleted or over-expressed renders cells unviable. For the sake of robustness, each plate was accompanied by streaking of a positive and negative control colonies.

10.3.5 Over-expression of Proteins

In the course of this thesis, several proteins were over-expressed to achieve desired results towards the cell cycle progression. Over-expression of proteins was obtained by cloning (cloning will be explained in next segment) gene of interest under the galactose-inducible, GAL1, 10. promoter in pRS family plasmids and transformed into the yeast strain. Transcription of gene was induced by adding the carbon source galactose to the culture media at will for the time period of one hour. The induction of this promoter was also turned off by adding rich source of carbon, dextrose, which yeast cells prefer over other sources of carbons, thus helping in the shut off of the Gal promoter.

11. Molecular Biology Techniques

11.1 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was used to amplify the sequences of genes of interest; which included, cloning of genes into desired plasmids with different family backgrounds (pRS, pCM, pFA6a), cloning of gens into generating deletion cassettes, integration of sequences leading to proteins fused to tags, replacement of promoters, inserting mutations in a wild type gene sequence.

In order to carry out amplification of genes, each specific gene required specific primers, primers were designed according to the need of an experiment (Shown in Table 3). In case of cloning plasmids, the oligonucleotides incorporating the restriction enzyme sequences at the 5 'end of target sequences were used to clone the PCR product into the appropriate vector. Furthermore oligonucleotides, incorporating the 60 nucleotides sequence flanking the region to be replaced at 5 'end where used in case of integration, deletion and tagging. Amplified products were checked by running a gel electrophoreses, stained with ethidium bromide.

The PCR reactions in the thermal cycler GeneAmp PCR System 2400 (Applied Biosystems) consisting of:

Step	<500bp	500-1000bp	1000-3000bp	>3000bp
1.Initial denature	95° C for 2 min	95° C for 2 min	95° C for 2 min	95° C for 2 min
2.Denature	95° C for 20 sec	95° C for 20 sec	95° C for 20 sec	95° C for 20 sec
3.Annealing	55° C for 10 sec	55° C for 10 sec	55° C for 10 sec	55° C for 10 sec
4.Extension	70° C for 10	70° C for 15	70° C for 20	70° C for 25

	s/kb	s/kb	s/kb	s/kb
Repeat Step 2-4	25 to 32 cycles, according to the need of the product.			

11.2 Electrophoresis of DNA in agarose gel

The agarose gel electrophoresis is a technique used to separate DNA molecules according to their linear size. This technique was used to verify all steps of cloning, as well as deletion and integration of genes in a yeast genome from PCR.

Gels using 1% (w / v) agarose (agarose D-1 Low EEO-TQM, Pronadisa) buffer TAE (50 mM Tris-acetic acid pH 8.5, 2 mM EDTA) with 0.5 µg / ml ethidium bromide. The ethidium bromide is a fluorescent agent which intercalates into the DNA that was retained during electrophoresis allowing the display to be illuminated with UV light (365 nm).

11.3 Cloning

The open reading frames to be cloned were amplified by using PCR with pure genomic DNA as a template. PCR products were purified using commercially available kits (QIAquick PCR purification kit, Qiagen) as well as homemade purification kits (Epoch). The quality and the concentration of DNA purified was analyzed by running gel electrophoresis stained with ethidium bromide.

100 ηg of purified PCR product was digested with restriction enzyme or enzymes (New England Biolabs) chosen to perform cloning, buffers and conditions required for the optimal function of enzymes were carried out as specified by the manufacturer. Like PCR products, the vectors where DNA was to be cloned were also digested with the same enzymes and conditions.

In order to make cloning more efficient, the vectors alone were dephosphorylated to prevent recirculation favoured ligation of the vector with itself. To achieve dephosphorylation, 5 units of Antarctic phosphatase (New England Biolabs) were added and incubated at 37 ° C for 15 minutes. Phosphatase was inactivated after incubation at 65 ° C for 5 minutes. The digested PCR product and vector both were purified (QIAquick PCR purification kit Qiagen) and ligated.

11.4 Ligation

The ligations were usually carried out at the ratio of 7:1 (vector : insert), which facilitated the meeting of the insert with the vector at close proximity. Ligation was performed using 1 unit of T4 ligase (Roche) in a final volume of 10 µL in the 10 x buffer supplied by the manufacturer. The mixture was incubated at 16 ° C overnight or at 25 ° C for two hours (quick ligation). The ligation mixture was transformed in *E. coli,* for the purpose of amplification of the plasmid.

11.5 *Escherichia coli* Transformation

E. coli DH5α competent cells (preparation of competent cells will be explained shortly) were used as the strain for the transformation process. 50 µL of competent cells and 10 µL ligation mixtures were mixed and incubated on ice for 30 minutes followed by a heat shock for 90 seconds at 42 ° C. After heat shock, cells were immediately cooled in ice-water for 60 seconds to reduce mortality and closing of cell membranes. 1 ml of LB media was added to the cells and incubated at 37 ° C for 1 hour in order to express the gene for resistance towards ampicillin before plating cells onto selective medium plates.

Finally, the cells were centrifuged at maximum speed (14000 RPM) (Eppindorf centrifuge 5418, Fisher Bioblock Scientific 1-159, Sigma, centrifuge) and pellets were re-suspended in

fresh 200 µl of LB and seeded on LB plates supplemented with 100 µg / ml ampicillin (LBA). Only those transformed cells containing vector with an ampicillin resistant gene (in principle only possible by ligation of the insert) resulted in colonies. Since the clones included in our thesis did not allow distinguishing of false positives for blue-white selection (Liu, Cashion et al. 1997), control plate was included for the efficiency of the ligation reaction with vector but without insert. The number of colonies obtained in the control would indicate the rate of false positives. An adequate number of individual colonies were inoculated into 5 ml of LBA at 37 ° C for overnight incubation. On the following day plasmid DNA was extracted and the presence of insert was checked by either digestion with restriction enzymes and/or by PCR. Results were checked by running gel electrophoresis of digested/amplified DNA stained with ethidium bromide.

11.6 Extraction of Plasmid

To extract plasmid DNA, 1.5 ml of saturated culture was taken from the overnight culture. Cells were centrifuged at maximum speed (14000 RPM) (Eppindorf centrifuge 5418, Fisher Bioblock Scientific 1-159, Sigma, centrifuge) for 90 seconds and supernatant was discarded. Pellets were resuspened in 200 µl of MX1 (50 mM TrisHCl, 10 mM EDTA pH 8.0, 100 µg/ml RNase A) suspenstion buffer by either vortexing or by pipette tip to avoid clumps and uniform solubilisation. 250 µl of buffer MX2 (0.2 M NaOH, 1 % SDS) (lysis solution) was added to the solution and mixed gently by inverting tubes 8-10 times. This lysis step was crucial and vortexing was avoided to prevent plasmid contamination with genomic DNA. Mixture was allowed to incubate at room temperature for 1-5 minutes followed by addition of 350 µl of neutralising buffer MX3 (4 M Guanidine hydrochloride, 0.5 M Potassium acetate, pH 4.2). Upon adding of neutralising buffer, solution was mixed quickly by inverting tubes 8-10 times to avoid partial neutralisation of lysate. Adding neutralising buffer turns solution into white precipitation form, this precipitate was centrifuged for 10 minutes at the speed of

13000 RPM (Eppindorf centrifuge 5418, Fisher Bioblock Scientific 1-159, Sigma, centrifuge). The supernatant was carefully placed in GenCatch^{TM} plus columns onto a collection tube and centrifuged at 5000 RPM for 60 seconds. Flow through was discarded and the column was washed once with 500 µl of WN (5 M Guanidine hydrochloride, 20 mM Tris-HCl, pH 6.6) wash buffer at 9000 RPM for 60 seconds. Flow through was again discarded and column was washed once with 700 µl WS (10 mM Tris-HCl pH 7.5, 80% ethanol) wash buffer at 9000 RPM for 60 seconds. Residual ethanol from WS buffer was removed by centrifuging column at 13000 RPM for two minutes. Finally, 50 µl of elution buffer (10 mM Tris-HCl, pH 8.5) was added to the column and incubated at room temperature for 5 minutes and centrifuged at 13000 RPM for 2 minutes collecting the eluted plasmid in a 1.5 ml eppindorf. The quality and quantification of plasmid was checked by running gel electrophoresis. Purified plasmid was either stored at -20 °C or carried on with the experiment.

11.7 Preparation *E. coli* DH5α competent cells using rubidium chloride

The competent cells for the purposes of *E. coli* transformation were made by chemical method using rubidium chloride which weakens the cell wall of bacteria in order to allow the transfer of genetic material into the cell at the time of heat shock.

A fresh single colony of *E. coli* DH5α was inoculated in 20 ml of LB (12 and incubated overnight at 37 ° C in an incubator with orbital agitation at 200 RPM. On the next day, 1 ml of this culture was used to inoculate 100 ml of Psi-broth medium (2% (w / v) Tryptone, 0.5% (w / v) yeast extract, 0.5% (w / v), MgSO4 · 7H2O, pH 7.6). The cells were allowed to grow at 37 ° C till they reached an optical density of range 0.48 to 0.6 at 550 nm absorbance; moreover, optical density not to exceed 0.7 was critical point in making competent cells. It was imperative after this point to perform rest of the steps at 4 ° C in cold room or on ice.

After obtaining desired OD, cells were allowed to cool down in ice-cold water for 15 minutes followed by harvesting cells by centrifuging for 5 minutes at 4 ° C at 3000 g (Beckman Coulter™, Rotor ID JLA-10,500). After removing the supernatant cells were gently resuspended in cold 40 ml of buffer TfbI (30 mM potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM manganese chloride, 15% (v / v) glycerol). Upon resuspension in cold room, cells were centrifuged again following the same conditions as above. Supernatant was discarded and pellets were resuspended in 4 ml of cold buffer TbfII (10 mM MOPS, 75 mM calcium chloride, 10 mM rubidium chloride, 15% (v / v) glycerol). After cells are resuspended in TbfII, they were left on ice for 15 minutes. At this point competent cells were either directly used for the transformation processes or aliquoted into 1.5 ml cold eppindorfs and stored at -80 ° C by rapid freezing in liquid nitrogen.

11.8 Yeast Genomic DNA Extraction

For genomic DNA extraction, single colony was inoculated into 5 ml of media, followed by centrifugation of saturated culture next day. The pellet was resuspended into lysis buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1% (v / v) SDS) using Gilson pipette. After resuspention, 400 µl by volume glass beads were added to the solution and 400 µl of phenol / chloroform / isoamyl alcohol (25:24:1). The cells were vortexed at high speed for 90 seconds to lyse the cells mechanically. Mixture was then set for centrifugation in order to separate the organic phase and the aqueous phase for 3 minutes at 14000 RPM, at room temperature. Second extraction was done by adding same volume of phenol / chloroform / isoamyl alcohol (25:24:1) to the aqueous solution, followed by vortexing and centrifuge. DNA precipitates were present in the aqueous phase due to alcohol precipitation by adding a volume of isopropanol equal to aqueous phase. After mixing the mixture quickly, samples were incubated on ice for at least 15 minutes to allow precipitation of DNA. The DNA was recovered by centrifugation in an Eppendorf 5418 centrifuge at 14,000 RPM, for 10 minutes

at room temperature. The DNA pellet was washed twice, each with 1 ml of ethanol 70% (v / v). Finally dissolved in 50 µL of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA + 0.01 mg / ml RNase. The samples were incubated at 37 ° C for 3 hours in order to solubilise DNA before using it as a template for PCR.

12. Cell Biology Techniques

12.1 Cell density of culture

The cell density of the cultures was determined by counting individual cells in a Neubauer chamber under an optical microscope. Culture samples were diluted by 1:10, 1:20, and 1:50 in water according to the density of parent culture. In order to have individual cells for counting, samples were sonicated for 9 seconds at 40W (sonicator Sonic Dismembrator (Dynatech)) prior to counting.

12.2 Experiments in unperturbed cell cycle

To study the behaviour of cell cycle progression in mutant strains under the unperturbed cell cycle conditions, we conducted experiments in rich medium (YPD) at 30 ° C as shown in (Figure 17).

A culture was inoculated from a fresh single colony in an incubator with orbital shaking at 200 RPM for overnight. The next morning density of culture was determined by counting the cells and diluted in rich medium (YPD) to 5.10^6 cells / ml in a volume appropriate to the size and number of aliquots required for the experiment.

The cells were allowed to grow until the cell density was doubled (one generation time), about 1.10^7 cells / ml. At this junction, first time point aliquot of growing asynchronous cells was taken and the rest of the cell culture was synchronized in G1 phase by adding 50 ng / ml α-factor (for bar1Δ strains) to the medium. After allowing cells to grow in presence of α-factor for 2 hours, cells were checked for proper synchronization in G1 phase. This was achieved by making 1:10 dilution of some of the culture with water and counted using Neubauer chamber, determining the budding index (BI). The budding index is number of budded cells divided by total number of cells multiplied by 100 which indicates the level of

synchronization in G1 phase of the culture. In a perfectly synchronized G1 phase culture, the budding index should be 0%, as cells in G1 should be. However, in practice, it is considered that a culture is well synchronized if budding index is less or equal to 5%. Once culture reached the optimal synchronization, one more time point for the purposes of experiment was taken followed by washing of cells three times with rich medium without α-factor for the release of the arrested cells in G1 phase, and then resuspended in rich medium without α-factor, allowing synchronous entry of cells in S phase. After release from α-factor and resuspension of cells in rich medium, time course was taken according to the need of the experiment.

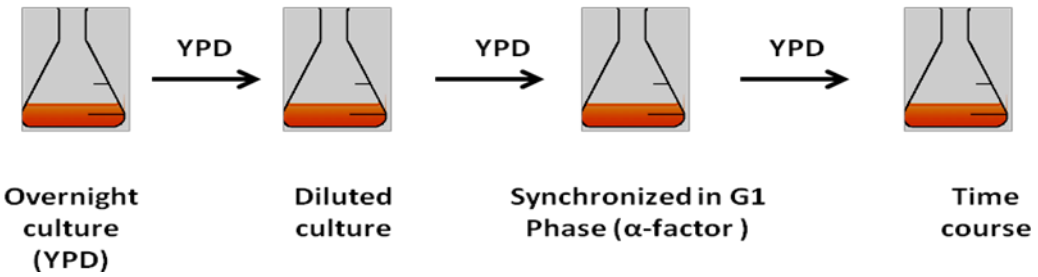


Figure 17. Shows the diagram of an experiment in an unperturbed cell cycle. Cells in exponential growth stages are synchronized in G1 phase by adding α-factor and are released for a synchronous S-phase and M-phase (YPD).

12.3 Experiments involved with over-expression of proteins

To study the behaviour of cell cycle progression when specific proteins were over-expressed during the cell cycle, we carried out experiments in rich medium, however poor source of carbon was used (Raffinose) such that, induction by better source of carbon would induce the protein of interest under galactose-inducible, GAL1, 10. promoter, as shown in (Figure 18).

A culture was inoculated from a fresh single colony in an incubator with orbital shaking at 200 RPM for overnight. The next morning density of culture was determined by counting the cells and diluted in rich medium (YPRaf) to 5.10^6 cells / ml in a volume appropriate to the size and number of aliquots required for the experiment. However, instead of YPD whose main source of carbon is dextrose, YPRaf was used in these experiments, having raffinose as the main source of carbon.

The cells were allowed to grow until the cell density was doubled (one generation time), about 1.10^7 cells / ml. At this junction, first time point aliquot of growing asynchronous cells was taken and the rest of the cell culture was synchronized in G1 phase by adding 50 ng / ml α-factor (for bar1Δ strains) to the medium. After allowing cells to grow in presence of α-factor for 2.5 hours, cells were checked for proper synchronization in G1 phase. This was achieved by making 1:10 dilution of some of the culture with water and counted using Neubauer chamber, determining the budding index (BI). The budding index is number of budded cells divided by total number of cells multiplied by 100 which indicates the level of synchronization in G1 phase of the culture. In a perfectly synchronized G1 phase culture, the budding index should be 0%, as cells in G1 should be. However, in practice, it is considered that a culture is well synchronized if budding index is less or equal to 5%. Once culture reached the optimal synchronization, one more time point for the purposes of experiment was taken. In order to induce the gene of interest at will, such that the protein of interest was over-

expressed, powdered Galactose was added to the culture to the final 2% sugar concentration. This step counted for induction and cells were allowed to grow in galactose media for 1 hour. After finishing the induction of cells for 1 hour, one more time point was taken for experimental purposes. Finally cells were washed three times with rich media without α-factor containing galactose for the release of the arrested cells in G1 phase. Once cells were released, they were resuspended in YPGal and time course was taken.

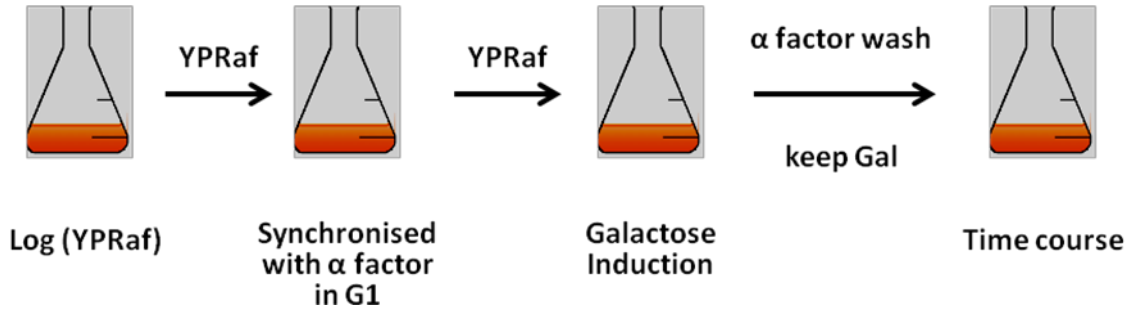


Figure 18. Shows the diagram of an experiment with galactose induction of genes in cell cycle. Cells in exponential growth stages are synchronized in G1 phase by adding α-factor, followed by induction by adding galactose and then released for a synchronous S-phase and M-phase (YPGal).

12.4 Experiments involved with temperature sensitive mutant proteins

To study the behaviour of cell cycle progression when specific proteins with temperature sensitive mutations were used during the cell cycle, we carried out experiments in rich medium YPRaf at 24 ° C (permissive temperature) as shown in (Figure 19).

A culture was inoculated from a fresh single colony in an incubator with orbital shaking at 200 RPM for overnight at 24 ° C. The next morning density of culture was determined by counting the cells and diluted in rich medium (YPRaf) to 5.10^6 cells / ml in a volume appropriate to the size and number of aliquots required for the experiment.

The cells were allowed to grow until the cell density was doubled (one generation time), about 1.10^7 cells / ml. At this junction, first time point aliquot of growing asynchronous cells was taken and the rest of the cell culture was synchronized in G1 phase by adding 50 ng / ml α-factor (for bar1Δ strains) to the medium. After allowing cells to grow in presence of α-factor for 3 hours, cells were checked for proper synchronization in G1 phase. This was achieved by making 1:10 dilution of some of the culture with water and counted using Neubauer chamber, determining the budding index (BI). In a perfectly synchronized G1 phase culture, the budding index should be 0%, as cells in G1 should be. However, in practice, it is considered that a culture is well synchronized if budding index is less or equal to 5%. Once culture reached the optimal synchronization, one more time point for the purposes of experiment was taken. Before induction, cell cultures were shifted from permissive temperature of 24 ° C to non permissive temperature of 38 ° C. Cell cultures were kept in non permissive temperature for 1 hour, enough time to bring about conformational change into the thermo-sensitive proteins that renders them non-functional. In order to induce the gene of interest at will, such that the protein of interest was over-expressed, powdered Galactose was added to the culture to the final 2% sugar concentration while keeping cells at

non-permissive temperature. This step counted for induction and cells were allowed to grow in galactose media for 1 hour. After finishing the induction of cells for 1 hour, one more time point was taken for experimental purposes. Finally cells were washed three times with pre-warmed rich media without α-factor containing galactose for the release of the arrested cells in G1 phase. Once cells were released, they were resuspended in pre-warmed YPGal and time course was taken while keeping cells in non-permissive temperature.

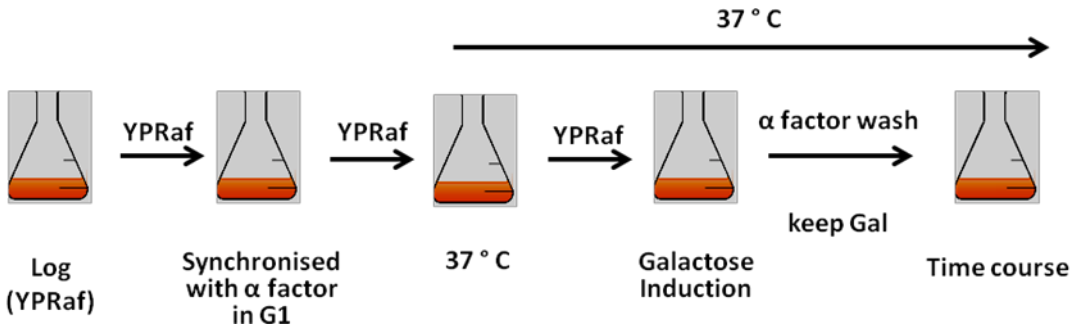


Figure 19. Shows the diagram of an experiment with temperature sensitive proteins and galactose induction of genes in cell cycle. Cells in exponential growth stages are synchronized in G1 phase by adding α-factor, followed by heat inactivation of thermo-sensitive proteins and induction by adding galactose and then released for a synchronous S-phase and M-phase (YPGal).

13. Biochemical Techniques

13.1 Total protein extraction from yeast cells using trichloroacetic acid (TCA)

Total protein extraction is an excellent method of extracting proteins from yeast cells effectively, while inactivating proteases, kinases and phosphotases immediately in the process.

The aliquots taken from experiments, either fresh or ones taken from -80 °C (thawed at first) were resuspended in 200 µl of 20% (w / v) TCA. After resuspension, 200 µl of sand glass by volume were added to cells which helped in breaking of cell wall by mechanical agitation

with sand glass. Cells were vortexed for 1 minute at maximum speed. Lysates were recovered and washed twice with sand glass vortexing and fresh 200 µl of 5% (w / v) trichloroacetic acid. Each volume of lysate added to the wash was recovered in order to maximize the amount of protein present in the lysate and in between dead spaces of the sand glass.

The denatured proteins which were insoluble in trichloroacetic acid were easily recovered by centrifuging the lysates at low speed of 3,000 RPM (Eppendorf centrifuge 5418) for 10 minutes at room temperature. Supernatant was discarded and pellets were dissolved in an appropriate volume of buffer implementation for electrophoretic samples (50 mM Tris-HCl pH 6.8, 8% (v / v) glycerol, 4% (v / v) 2 - mercaptoethanol, 1.6% (w / v) SDS, 0.008% (w / v) bromophenol blue). The residual remains of TCA exceeding the buffering capacity of the volumes of buffer commonly used was neutralized by balancing the pH of the samples by adding 0.2 volumes of 1 M Tris-base (This step helps in preventing protiolysis of samples in following step). Finally the samples were boiled for 5 minutes and then centrifuged at 3,000 RPM (Eppendorf 5418, centrifuge) for 5 minutes at room temperature, which helped in pelleting the insoluble material. The supernatants were either loaded immediately into a PAGE gel electrophoresis or stored at -20 °C for future use.

13.2 Polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate -Polyacrylamide gel electrophoresis (SDS-PAGE), is a technique widely used to separate the proteins according to their electrophoretic mobility (where mobility is a function of the length, conformation and charge of the molecule) and there molecular mass (Laemmli 1970).

Sodium dodecyl sulfate (SDS), an anionic detergent was applied to protein samples in order to linearize and impart a negative charge to linearized proteins. Binding of SDS to the

proteins imparted an even distribution of charge per unit mass, thereby resulted in a fractionation by approximate size during electrophoresis.

Gels of 1.5 mm thickness were used in this thesis while as different concentrations of acrylamide 7.5%, 10%, 12%, and 15% were used depending upon the molecular weight of the protein of interest. The ratio between acrylamide-bisacrylamide was always 37,5:1 (BioRad). Samples from TCA (explained in 5.1) were used to run in these gels. The proteins were scored according to their position on a gel with respect to the subsequent analysis of molecular weight ladders, Broad Range Prestained Starndar (Fermentas) and Broad Range Standard (BioRad).

Gels were run in electrophoresis buffer (25 mM Tris-glycine pH 8.3, 0.1% (w / v) SDS) using a generator-Power Pac Basic (BioRad). Following are the conditions of voltage and time used for different proteins:

- For the analysis of Sic1ΔN, 10% acrylamide, 15 minutes of stacking at 90 V and 60 minutes of 200 V.
- For the analysis of Cln2, 10% acrylamide, 15 minutes of stacking at 90 V and 75 minutes of 220 V.
- For the analysis of Clb5, 10% acrylamide, 15 minutes of stacking at 90 V and 65 minutes of 200 V.
- For the analysis of Clb2, 10% acrylamide, 15 minutes of stacking at 90 V and 60 minutes of 200 V.
- For the analysis of Swe1-13myc, 7.5 % acrylamide, 35 minutes of stacking at 120 V and 65 minutes of 195 V.
- For the analysis of the two forms of phosphorylated Pol12 (86 and 91 kDa), 7.5% acrylamide, 45 minutes at 120 V followed by 65 minutes 190 V.

- For the analysis of Cdc28 (34 kDa) 10% acrylamide, 15 minutes of stacking at 90 V and 50 minutes at 200V.

13.3 Western Blotting

The proteins separated by SDS-PAGE were transferred onto nitrocellulose membranes (Protran BA85) by semi-dry transfer (Panther Semidry Electrobotter, Owl Scientific). The transfer machine used constant amperage, adjusting according to the percentage of gel and the molecular mass of the protein to be transferred (2 to 3.2 mA/cm2) for 60 minutes. The transfer buffer was modified, containing SDS to increase the efficiency of transfer and alkaline pH, which minimized the number of cargo proteins that had zero to coincide with its isoelectric point (50 mM Tris-glycine pH 9.1, 0.0373% (w / v) SDS, 20% (v / v) methanol).

Once proteins were transferred onto a membrane, the membrane was stained with Ponceau S (0.2% (w / v) Ponceau S, 3% (w / v) TCA). Staining with Ponceau allowed the visualization of the most abundant proteins transferred to the membrane suggesting the control for correct transfer proteins, moreover, scanned images were used as a loading control. Removal of staining was imperative for the efficient immunobloting and was achieved by washing the membrane with TBST (100 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% (v / v) Tween-20) till membrane was clean for Ponceau staining.

The membranes were blocked by incubating them in TBST + 5% (w / v) skimmed milk powder, for 20 minutes on a shaker. Blocking of membrane by milk prevented the binding of an antibody to nonspecific parts of the membrane. Blocking agent (milk) was decanted and membranes were incubated in a dilution of primary antibody against specific protein or against the tag fused to the specific protein to be detected for a 120 minutes with gentle agitation on a shaker to promote uniform interaction of antibody with proteins on membrane

The primary antibodies and dilutions used in this study:

- Anti-Cln2 (Santacruz y-115, # sc-33624), 1:1000 dilution in TBST + 5% milk.
- Anti-Sic1 (Santacruz FL-284, # sc-50441), 1:1,000 dilution in TBST+5% milk.
- Anti-Clb5 (Santacruz yN-19, # sc-6704), 1:1,000 dilution in TBST+5% milk.
- Anti-Clb2 (Santacruz y-180, # sc-9071), 1:2,000 dilution in TBST+1% milk.
- 9E10 α-myc (mouse monoclonal, Roche), 1:2,000 dilution in TBST + 1% milk.
- 9E10 α-myc (monoclonal mouse hybridoma supernatant) diluted 1:500 in TBST.
- 12CA5 α-HA (mouse monoclonal, Roche) diluted 1:1000 in TBST + 1% milk.
- 12CA5 α-HA (monoclonal mouse hybridoma supernatant) diluted 1:100 in TBST.
- 6D2 Pol12-α (mouse monoclonal, given by Dr. Foiana Marco), dilution 1:2000 in TBST + 1% milk.
- Anti-pY15-Cdk1 (Cell Signaling #9111, anti human pY15-Cdk1, cross-reacts nicely with budding yeast pY19-Cdc28), 1:1,000 dilution in TBST + BSA.

After incubation of membrane in primary antibody for 2 hours, primary antibody was recovered and reused several times in the course of this study. Residual primary antibody was cleaned by washing the membrane three times with TBST. Membranes were then incubated in secondary antibody for 35 minutes. Secondary antibodies used in this study were as:

- For mouse primary antibodies, anti-mouse IgG conjugated to peroxidise Radish Rustica (HRP) (Dako P0161) 1:5.000 dilution in TBST + 5% milk.
- For Goat primary antibodies, anti-goat IgG conjugated to HRP (Santa Cruz Biotechnology sc-2020), diluted 1:2500 in TBST + 5% milk.
- For Rabbit primary antibodies, anti-Rabbit IgG-HRP (goat polyclonal, purified IgG, Dako P0448), dilution in 1:2,000 in TBST + 1% milk

13.4 Flow cytometry (FACS, Fluorescent Activated Cell Sorting)

Flow cytometry or simply known as FACS is a technique which allowed us to quantify the amount of DNA present in each cells in a sample. The results were achieved by staining the DNA with a fluorescent dye propidium iodide which intercalates in between the DNA molecules. With the help of this technique we determined the stage of the cell cycle cultures were in as well as the degree of synchrony and the rate of progression of the replication and cell division.

Before running the samples for cell sorting, cells were fixed, treated and stained. The sample aliquots taken from the experiments at around 1.10^7 cells per sample were centrifuged at the speed of 10000 RPM in an Eppendorf centrifuge 5418 and pellets were fixed with 1 ml of 70% (v / v) ethanol and incubated overnight at 4 ° C. On the next day, cells were rehydrated by washing them twice with 1 ml of 50 mM Tris-HCl pH 7.8, while giving a pause for two minutes between the washes to allow proper dissolving of pellet. Cells were incubated in the same buffer in the presence of 200 mg / ml RNase A, overnight at 24 ° C. In order to make propidium iodide seep into the cells, cell wall was partially made permeable by incubating them in 5 mg/ml pepsin dissolved in 50 mN HCl for 30 minutes at 37 ° C. Pepsin solution was activated prior to use by incubating at 37 ° C for 30 minutes. And same was inactivated by a single wash in 1 ml of FACS buffer (200 mM Tris-HCl pH 7.8, 211 mM NaCl, 78 mM MgCl2) to bring back to pH 7.8, in order to prevent the further degradation. All spins were carried out at 10000 RPM in an Eppendorf centrifuge 5418, and the pellets with 1.10^7 cells/ml were dissolved in 250 µl of FACS buffer with 16 µg/ml propidum iodide. This cell suspension was either used directly or stored at 4 ° C away from light.

For the analysis of flow cytometry, 50 µl of the cell suspension were added to the 1 ml of 50 mM Tris-HCl pH 7.8. Samples were sonicated for 9 seconds at 40W (sonicator Sonic

Dismembrator, Dynatech) to make sure that each reading corresponded to a single cell and prevented for example two cells containing 1C DNA to be counted as a single particle with a 2C DNA content. Samples were analyzed on a FACScan apparatus (Becton-Dickinson), reading the fluorescence of propidium iodide (FL2-H). The data represented graphically as histograms of the number of events (Y) front-FL2 fluorescence H (X) for 20,000 events. Graphs were plotted to see the progression of cell cycle were each peak represented the corresponding time point in the experiment and content of DNA present, as shown in (Figure 20).

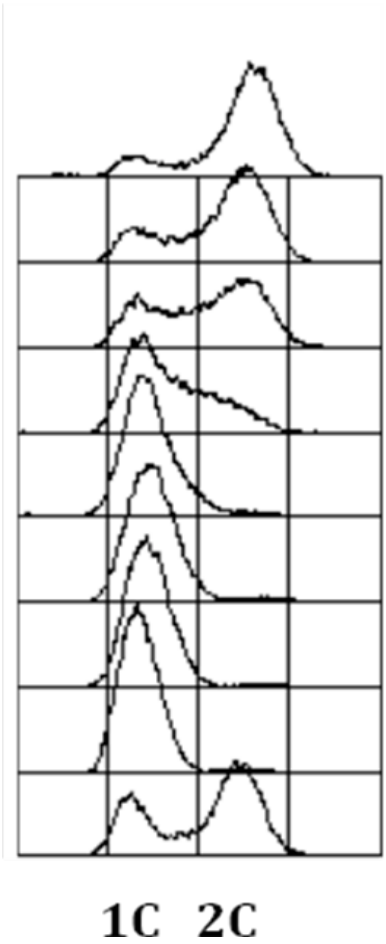


Figure 20. Shows the schematic diagram of a plot constructed on an experiment. 1C peaks represent the one content of DNA prior to replication, as cells pass through the cell cycle, upon replication DNA content is doubled and represented as two contents, 2C.

13.5 Microscopy

Microscopy was used to check the phenotype of cells as well as the behaviour of chromosomes upon the course of experiments. Stained samples from flow cytometry were checked under the microscope Nikkon eclipse 90i (CFI60 infinity optical system, i-Series Support Tools consisting of EZSetup, iSetup, and iControl; Nikon NIS-Elements Software.) which gave bright red flourescence of DNA material.

13.6 Immunoprecipitation (IP)

Immunoprecipitation is the method which helped in studying of interaction between two or more proteins in a complex *in-vivo* of the cell. Instead of using harsh acid, TCA, mild lysis buffer were used to extract the whole protein extract in its native condition.

Cell cultures with the number of cells at around 2.10^9 were centrifuged at 3000 RPM for 5 minutes in an (). Cell pellets were washed once with the lysis buffer (50 mM Tris-HCl pH 7.7, 75 mM NaCl, 1 mM EDTA, and 10 % (v / v) glycerol) and snap frozen using liquid nitrogen and stored at -80 °C. Prior to the extraction of the proteins, cells were thawed on ice cold water, and resuspended in 2 ml of cold lysis buffer , supplemented with protease inhibitors (1 mM AEBSF, 0.15 mM aprotinin, 1 mM leupeptin, 1 mM pepstatin), phosphatase inhibitors (0.5 mM sodium pyrophosphate, 2 mM NaF, 2 mM β-glycerophosphate), 1 mM DTT and 0.1% (v / v) nonidet-P40.

1.5 ml of glass beads by volume were added to the solution and vortexed 12 times for 60 seconds each with a gap of 60 seconds incubation on ice, in cold room (4 °C). The 60 seconds of gap in between the agitation allowed cells to cool down and prevent denaturation

of proteins due to heat produced during agitation. The lysates were allowed to sit on ice in the cold room for 60 minutes in order to maximize the extraction process. After incubation on ice, in order to extract the proteins and get rid of insoluble debris, cells were transferred to cold eppindorfs, and centrifuged at 14000 RPM for 30 minutes. The supernatant containing the total cell extract in its native conditions were transferred to a fresh cold eppindorf.

The immunoprecipitated proteins were incubated with primary antibody 3F10 anti-3HA Affinity Matrix (Roche). 3F10 is a monoclonal rat, with high affinity towards the HA epitope, which gives better performance in immunoprecipitacions compared to 12CA5 that were used in Immunoblotting. 20 µl of beads were incubated with the extract, where 10 µl were the sephearose beads with antibody attached and 10 µl was the storing solution of the antibody. The cell lysates were incubated with 3F10 for 1 h at 4 ° C on the rotation block. After this time, the mixture was centrifuged at 500 g, and extracts were removed, the immunoprecipitates were washed five times each with 1 ml of cold lysis buffer to remove excess proteins which did not get attached to the antibody. Finally, the proteins were released by boiling the samples in presence of Lammeli buffer. Protein-protein interactions were checked by running SDS- PAGE followed by immunoblotting.

13.7 *In-vitro* kinase assay

In-vitro kinase assay was used to measure the kinase activity of given proteins. In order to carry out *In-vitro* kinase assay, samples were collected, processed and immunoprecipitated as explained in previous segment.

Before carrying out kinase assay, last wash of cell samples with protein of interest bound to the beads is washed once with 25 mM MOPS buffer. Moreover, instead of incubating into 20 µl of antibody 3F10 anti-3HA Affinity Matrix (Roche), cell lysates were incubated with 40 µl of antibody. Such that, half of the samples were kept for western blotting while as other half

were used for kinase assay. 6 µl of HBII buffer (5 mM EGTA, 15 mM $MgCl_2$, 15 mM MOPS, 1 mM Na_2VO_3, 1 mM AEBSF, 0.15 mM aprotinin, 1 mM leupeptin, , 7 mM β-glycerophosphate, 1 mM DTT,) was added to the samples and incubated at room temperature for 15 minutes, followed by addition of 10 µl of reaction buffer. Reaction buffer is the 1:1 ration of two solutions, S1 and S2. **S1**: 100 mM ATP and 5 µCi of γ-[32P] - ATP (Amersham, GE Healthcare), **S2**: 4 mg/ml Histone H1 in 50 mM mops as a substrate. Samples were incubated at 30 °C for 15 minutes. Finally adding 5 µl of sample buffer, samples were boiled for 3 minutes. Kinase activity was checked by running SDS-PAGE followed by staining/destaining of the gel. At last, autoradiograph gel was exposed to a photographic film sensitive towards β radiation emitted by 32P incorporated into protein phosphorylation.

Table 1. Strains used in this study

Name	Genotype
W303-1a	MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100
YAM18	W303-1a MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100 bar1::URA3 GAL-Cln2-(7A)-NlsMycNls (TRP1)
YAM19	W303-1a MATa ade2-1 ura3-1 leu2-3,112 can1-100 bar1::URA3 GAL-Sic1C70 (HIS3)
YAM20	W303-1a MATa ade2-1 ura3-1 leu2-3,112 can1-100 bar1::URA3 Cln2(7A)-NlsMycNls (TRP1) GAL-Sic1C70 (HIS3)
YAM22	W303-1a MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 Clb6-del (KANMX6) bar1-del GAL-Cln2-(7A)-NlsMycNls (TRP1)
YAM24	W303-1a MATa ade2-1 ura3-1 trp1-1 leu2-3,112 can1-100 bar1-del his3::clb5::HIS3
YAM27	W303-1a MATa ade2-1 ura3-1 trp1-1 leu2-3,112 can1-100 bar1-del his3::clb5::HIS3 GAL-Sic1DelN (URA3)
YAM29	W303-1a MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 Clb6-del (KANMX6) bar1-del GAL-Sic1DelN (URA3)
YAM33	W303-1a MATa ade2-1 ura3-1 trp1-1 leu2-3,112 can1-100 bar1-del clb5::HIS3 GAL-Sic1DelN (URA3) GAL-Cln2-(7A)-NlsMycNls (TRP1)
YAM35	W303-1a MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 Clb6-del (KANMX6) bar1-del GAL-Sic1DelN (URA3) GAL-Cln2-(7A)-NlsMycNls (TRP1)
YAM36	W303-1a MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 bar1-del

	GAL-Sic1delN (URA3)
YAM42	W303-1a MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100 bar1-del GAL-Cln2-(7A)-NlsMycNls (TRP1)
YAM43	W303-1a MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100 bar1-del. GAL-Cln2-(7A)-NlsMycNls (TRP1) Gal-Sic1delN (URA3)
YAM44	W303-1a MATa ade2-1 ura3-1 trp1-1 leu2-3,112 can1-100 bar1-del his3::clb5::HIS3 clb6-del (kanmx) GAL-Sic1DelN (URA3)
YAM46	15Dau Mata ura3Dns clb1::URA3 clb2::LEU1 clb3::KanMx6 clb4::HIS2 clb5::ARG4 clb6::ADE1 GAL-CLB1:LEU2
YAM50	W303-1a MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100 bar1-del GAL-Cln2-(7A)-NlsMycNls (TRP1) GAL-Sic1delN (URA3)
YAM52	W303-1a MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100 bar1-del. GAL-Sic1delN (URA3), Swe1-13Myc (KanMX)
YAM53	W303-1a MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100 bar1-del. GAL-Cln2-(7A)-NlsMycNls (TRP1) GAL-Sic1delN (URA3) Swe1-13Myc (KanMX)
YAM54	W303-1a MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100 bar1-del. GAL-Sic1delN (URA3) Cdc28-3HA (KanMX)
YAM55	W303-1a MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100 bar1-del GAL-Cln2-(7A)-NlsMycNls (TRP1) GAL-Sic1delN (URA3) Cdc28-3HA (KanMX)
YAM56	W303-1a MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100 bar1-del. GAL-Sic1delN (URA3) Clb2-del::Leu2
YAM57	W303-1a MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100 bar1-del. GAL-Cln2-(7A)-NlsMycNls (TRP1) Sic1delN (URA3) Clb2-del::Leu2
YAM58	W303-1a MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100 bar1-del GAL-Sic1delN (URA3) Clb6-13Myc (KanMX)

YAM59	W303-1a MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100 bar1-del. GAL-Cln2- (7A)-NlsMycNls (TRP1) Gal-Sic1delN (URA3) Clb6-13Myc (KanMX)
YAM60	15Dau Mata ura3Dns clb1::URA3 clb2::LEU1 clb3::KanMx6 clb4::HIS2 clb5::ARG4 clb6::ADE1 GAL-CLB1:LEU2
YAM61	W303-1a MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100 bar1-del. Gal Sic1delN (URA3) Swe1-13Myc (KanMX) Hsl1-del::Leu2
YAM62	W303-1a MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100 bar1-del. . GAL-Cln2-(7A)-NlsMycNls (TRP1) Gal-Sic1delN (URA3), Swe1-13Myc (KanMX), Hsl1-del::Leu2
YAM64	W303-1a MATa ade2-1 ura3-1 trp1-1 leu2-3,112 can1-100 bar1-del his3::clb5::HIS3 . GAL-Cln2-(7A)NlsMycNls (TRP1) GAL-Sic1DelN (URA3)
YAM65	W303-1a MATa ade2-1 ura3-1 trp1-1 leu2-3,112 can1-100 bar1-del his3::clb5::HIS3 GAL-Cln2-(7A)-NlsMycNls (TRP1) clb6-del (kanmx) GAL-Sic1DelN (URA3)
YAM66	W303-1a MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100 bar1-del. GAL-Sic1delN (URA3) Clb2-3HA (KanMX)
YAM67	W303-1a MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100 bar1-del. GAL-Sic1delN (URA3) Clb5-3HA (KanMX)
YAM68	W303-1a MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100 bar1-del. GAL-Cln2-(7A)-NlsMycNls (TRP1) GAL-Sic1delN (URA3) Clb2-3HA (KanMX)
YAM69	W303-1a MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100 bar1-del. GAL-Cln2-(7A)-NlsMycNls (TRP1) GAL-Sic1delN (URA3) Clb5-3HA (KanMX)
YAM70	15Dau Mata ura3Dns clb1::URA3 clb2::LEU1 clb3::KanMx6 clb4::HIS2 clb5::ARG4 clb6::ADE1 GAL-CLB1:LEU2, tetO7-Cln2-(7A)-NlsMycNls (TRP1)

YAM71	W303-1a MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 clb1-del (without marker) clb2ts (allele VI) CDC14-3HA ura3::bar1::URA3 (Counter selected)
YAM72	W303-1a MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100 bar1-del. tetO7-Cln2-(7A)-NlsMycNls (TRP1) Gal-Sic1delN (URA3)
YAM73	W303-1a MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100 bar1-del. GAL-Cln2-(7A)-NlsMycNls (TRP1) GAL-Sic1 (9A)
YAM74	W303-1a MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 clb1-del (without marker) clb2ts (allele VI) CDC14-3HA ura3::bar1::URA3 (Counter selected), GAL-Cln2-(7A)-NlsMycNls (TRP1)
YAM75	W303-1a MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100 bar1-del GAL-Cln2-(7A)(WITHOUT, NLS) (TRP1) GAL-Sic1delN (URA3)
YAM76	W303-1a MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 bar1-del, GAL-Sic1(9A) (URA3)
YAM77	W303-1a MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 clb1-del (without marker) clb2ts (allele VI) CDC14-3HA ura3::bar1::URA3 (Counter selected), GAL-Sic1delN (URA3)
YAM78	W303-1a MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 clb1-del (without marker) clb2ts (allele VI) CDC14-3HA ura3::bar1::URA3 (Counter selected) GAL-Cln2-(7A)-NlsMycNls (TRP1)

Table 2. Constructs used in this study

Name	Construct description
pAM1	pRS306-GAL-MCS-13Myc Vector to clone ORFs under Gal promoter (to be expressed as C-ter 13 myc tag fusion).
pAM2	pRS306-GAL-Sic1delN
pAM3	pRS304-GAL-CLN2-NLS-1xMyc-NLS
pAM9	pRS304GAL-CLN2 (7A)-NLSmycNLS
pAM10	pCM252- CLN2 (7A)-NLSmycNLS
pAM11	pRS306- GAL Sic1 (9A)
pAM12	pRS304-GAL Clb2
pAM13	pRS304-GAL-CLN2(7A) without NLS

Table 3. Oligonucleotides used as primers in this study

Name	Sequence	Strain/Plasmid
Sic1ΔN-F	CGGATCCATGTCTTTTAAAAATGCGCCATT	pRS306-GAL-Sic1ΔN
Sic1ΔN-R	TCTCGCGGCCGCTCACAAGTCTTCCTCGGAGATTAGCTTTTGTTCATGC TCTTGATCCCTAGATTG	
Cln2-NlsmycNls- F	CGGGATCC ATGGCTAGTGCTGAACCAAG	pRS304-GAL-Cln2NLSmycNLS
Cln2-NlsmycNls- R1	CTCGGAGATTAGCTTTTGTTCGCCTTCCACCTTGCGCTTTTTCTTAGGT GGACCACTGCGTATTACTTGGGTATTGCCCATACC	
Cln2-NlsmycNls- R2	TCTCGCGGCCGCTCACCTCGATCCACCCTCAACTTTTCTTTTCTTTTTGGG GGACCCAAGTCTTCC	
Cln2-NlsmycNls- F	CGGGATCCATGGCTAGTGCTGAACCAAG	pRS304-GAL-Cln2-(7A) NLSmycNLS
Cln2-NlsmycNls- R1	CTCGGAGATTAGCTTTTGTTCGCCTTCCACCTTGCGCTTTTTCTTAGGT GGACCACTGCGTATTACTTGGGTATTGCCCATACC	
Cln2-NlsmycNls- R2	TCTCGCGGCCGCTCACCTCGATCCACCCTCAACTTTTCTTTTCTTTTTGGG GGACCCAAGTCTTCCTCGGAGATTAGCTTTTGTTC	
Sic1C70- F	CGGATCCATGGGTAAAAATCCCTTTGCATC	pRS303-GAL-Sic1C70
Sic1C70-R	TCTCGCGGCCGCTCAATGCTCTTGATCCCTAGATTG	
Clb3 Del-F (KNMX)	GCAACTGGCTCACCTACCCAC	YAM46
Clb3 Del-R (KNMX)	CCTCCAGAAAAGTCAATACAC	
Hsl1 del-F	CCAACTATATTCACTCCTTTATTATTTGTTATTAATATTTTTTATTTTTA CCACACGACGCGGTATTTTCTCCTTACGC	YAM61/62
Hsl1 del-R	CTCGTACGTTAAATTTTTCAAATTATTGTTGTATAATTATATAACATCT ATATAGAATAGATTGTACTGAGAGTGCAC	
Hsl1 CHK- F	GACAAGGTAAAAAGAACGAC	
Hsl1 CHK- R	CTTCGTGTCTCATGTCTCTCC	
Cln2- R	TCTCGCGGCCGCCTATATTACTTGGGTATTGC	YAM 75
Clb2-F	CGGATCCATGTCCAACCCAATAGAAAACAC	pRS304-GAL-Clb2
Clb2-R	TCTCGCGGCCGCTCATTCATGCAAGGTCATTAT	
Clb2p-Fa6a-F (3HA)	TCCGTCCAATGGGCTTTAAAGGTTAGAAAAAACGGCTATGATATAATG ACCTTGCATGAACGGATCCCCGGGTTAATTAA	YAM 66/68
Clb2-pFa6a-R (3HA)	GGTGTGAAATTTGTAAGAATAAATGGAAATGTGAAAAGAAATGATTA AAAAGCAGCCATAGAATTCGAGCTCGTTTAAAC	
Clb2-del-F	TATTTCCTTCCAAGAAGCCTTTTATTGATTACCCCCTCTCTCTCTTCATT GATCTTATAGGCGGTATTTTCTCCTTACGC	

Clb2-del-R	CATTTTATATGGACATTTATCGATTATCGTTTTAGATATTTTAAGCATC TGCCCCTCTTCAGATTGTACTGAGAGTGCAC	YAM56/57
chk-clb2-del-F	AACCTACTGCGAGCTATTCA	
chk-clb2-del-R	CGGAAACGGTTGAACTGAAA	
CLB5pfa6aF (3HA)	ACTTTCAAGTGGTGTACATCCGAAATGCATAGCAACTTTCAAAATCTA TTTAATCTTAAGCGGATCCCCGGGTTAATTAA	YAM67/69
CLB5pfa6aR (3HA)	ACATATATATAGATAGATAGATAGATGCAAAAAATTTTTTCCTTTTAG TTCAGCAAAAAGGAATTCGAGCTCGTTTAAAC	
chk-clb5-del-F)	CTTCAAGAGACTCAATCACC	YAM27
(chk-clb5-del-R)	GAAGTGAACACGCATATCAC	
Clb5-del-F	GCTTAATAATTAGCAGTAACGCGCTTTTCCCTGTATTTAAAGCCGCTGA ACACCTTTACGCGGTATTTTCTCCTTACGC	
Clb5-del-R	GATGATAATAGTAGTAATACTGGTGGTATTATATATGTTGAAGTGAAC ACGCATATCACAGATTGTACTGAGAGTGCAC	
Cdc28-pfa6a-F (3HA)	ATTAGCGCCAGAAGAGCAGCCATCCACCCCTACTTCCAAGAATCACGG ATCCCCGGGTTAATTAA	YAM54/55
Cdc28-pfa6a-R (3HA)	GCAGTAGCATTTGTAATATAATAGCGAAATAGATTATAATGCTTAGAA TTCGAGCTCGTTTAAAC	
Clb3 Del-F (NAT1)	CATGAAGCGCGCCATCGACATATACAACAGCGAAGGCCAGCAACTGG CTCACCTACCCACCGTACGCTGCAGGTCGAC	YAM82
Clb3 Del-R (NAT1)	AGAACACCCGAGTTTTCCTCCAGAAAAGTCAATACACATTACAATCTC CTCGTCAAAATCATCGATGAATTCGAGCTCG	
Swe1-13Myc-F	CGCAATGCAGGTGCTATTATCCAGGAAGACGACTTTGGACCTAAGCCA AAATTTTTTATA	YAM52/53
Swe1-13Myc-R	TAGAGCGGCCGCCACCGCGGTGGAGCTCCAATTCGCCCTATAGTGAGT CGTATTACAATTATGCTTGAAGCGGCTGTACT	
Clb6-del-F	GCTTCACTAATCTAGCGTTC	YAM 45/65
Clb6-del-R	AAGATGCAGGGGGTTAGCTGG	
Clb6-Chk-F	GCTAGGGTTAAGTTTTTGCC	
Clb6-Chk-R	CACGGTATCAGTTTTGTAGG	

RESULTS

Results:

14. Triggering of later events by early cylins

Each cyclin associates with its catalytic subunit cyclin dependent kinase to perform its function in phase specific manner, however, it is still uncertain, why early cyclins cannot trigger later events in cell cycle progression. Theory that suggests that increasing levels of cyclin dependent kinase activity is responsible for driving the cell cycle, then, prediction, that over-expressing early cyclin at the right time, to the right sub cellular location should bring about later event. To test this theory, we explored if G1 cyclin would be able to trigger replication which is a latter event with respect to the G1 phase when over-expressed and localised into the nucleus.

In order to study if G1 cyclin might trigger the replication, experiments were carried out in strains where G1 cyclin Cln2 tagged with two nuclear localization signal (NLS) sequences at the C-terminal and hyperstable form of all Clbs inhibitor Sic1ΔN, whose 50 amino acid long N-terminal sequence was deleted (explained in detail in section 5), were expressed under galactose-inducible promoter, GAL1, 10. Since Sic1ΔN is the strong inhibitor of S-CDK and M-CDK activity, strain consisting of only Sic1ΔN under galactose-inducible promoter blocked the cell cycle progression at the G1-S transition and thus acted as the control strain. While as strains consisting of both Sic1ΔN and Cln2- NlsmycNls under the galactose-inducible promoter acted as the experimental strain. Strains were checked in unperturbed conditions for proper cell cycle progression, and for integrated Sic1ΔN by functional assay prior to experiments (Figure 21).

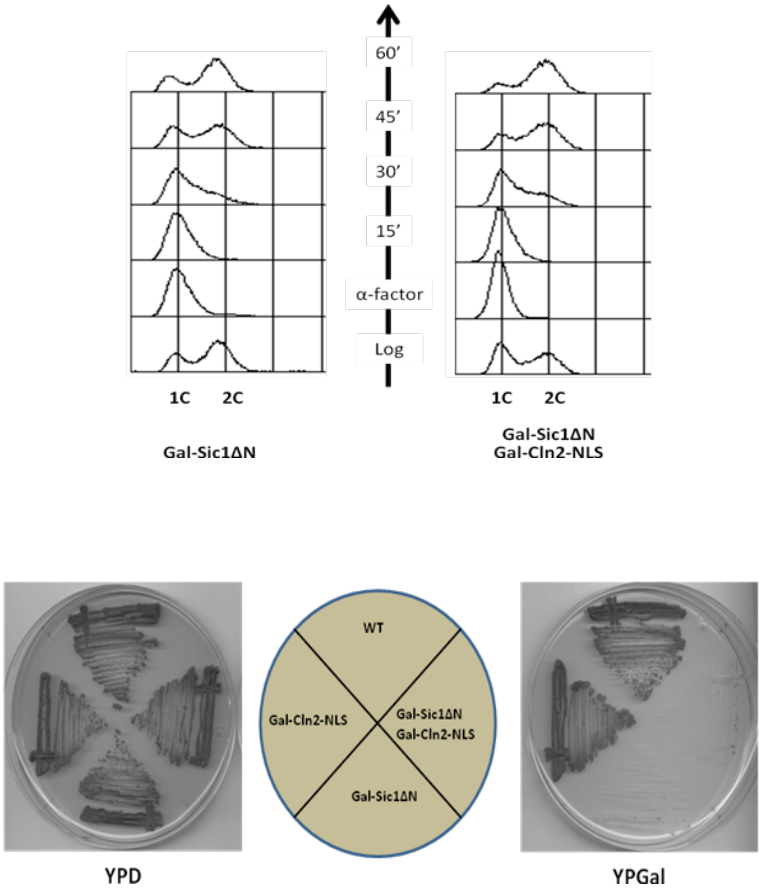


Figure 21. **A.** Flow cytometry shows the proper cell cycle progression in unperturbed conditions. **B.** Over-expresion of Sic1ΔN blocks cell cycle progression in both control and experimental strains.

However, flow cytometry results showed that over-expressing nuclear Cln2 in experimental strain could not trigger the replication in presence of an all Clb inhibitor. The reason behind the failure of Cln2 to trigger replication was lack of effective accumulation of protein due to its quick degradation by self regulating properties (Figure 22).

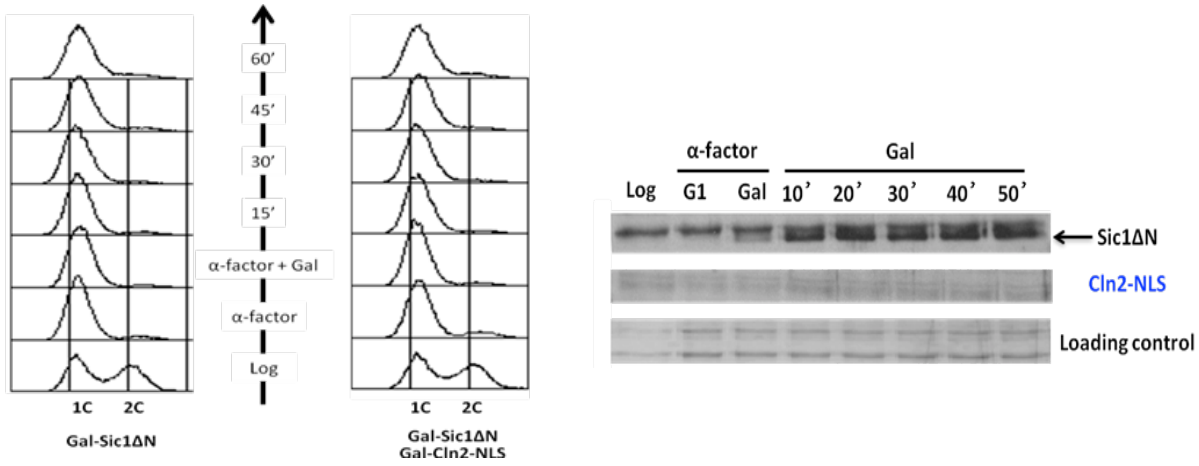


Figure 22. **A.** Flow cytometry results show, Cln2 was unable to trigger replication when over-expressed and localised to nucleus. **B.** Western blots shows accumulation of Sic1ΔN, however, no sizeable accumulation of nuclear Cln2 protein. Ponceau staining was used as loading control.

15. Hyperstable Cln2 triggers replication in absence of S and M-CDK activity

Since Cln2 was being self regulated at faster speed compared to its expression, the desired accumulation required for the purpose of increasing cyclin dependent kinase activity was not achieved. Hence, hyperstable form of Cln2 was used whose all seven S-P/T (Serine threonine) consensus sites for CDK phosphorylation were mutated to non-phosphorylatable form, alanine. With help of these mutations, protein was still functional, however, could not be identified and degraded by SCF ubiquitin ligase proteasome due to absence of phosphorylation of given sites required for identification of PEST site on Cln2 by F-box Grr1, thus rendering protein hyperstable.

Experiment was carried out in strains where hyperstable Cln2 (henceforth known as Cln2(7A)) tagged with two nuclear localization signal (NLS) sequences at the C-terminal and hyperstable form of all Clbs inhibitor Sic1ΔN, whose N-terminal degron sequence was deleted, were expressed under galactose-inducible promoter, GAL1, 10. Since Sic1ΔN blocks the S-CDK and M-CDK activity, strain consisting of only Sic1ΔN under galactose-inducible

promoter blocked the cell cycle progression at the G1-S transition and thus acted as the control strain. While as strains consisting of both Sic1ΔN and Cln2(7A)-NlsmycNls under the galactose-inducible promoter acted as the experimental strain. Strains were checked in unperturbed conditions for proper cell cycle progression, and for integrated Sic1ΔN by functional assay prior to experiments. Functional assay suggested that both strains were not viable upon over-expression of strong Clb inhibitor, while cells progressed in wild type form under unperturbed conditions (Figure 23).

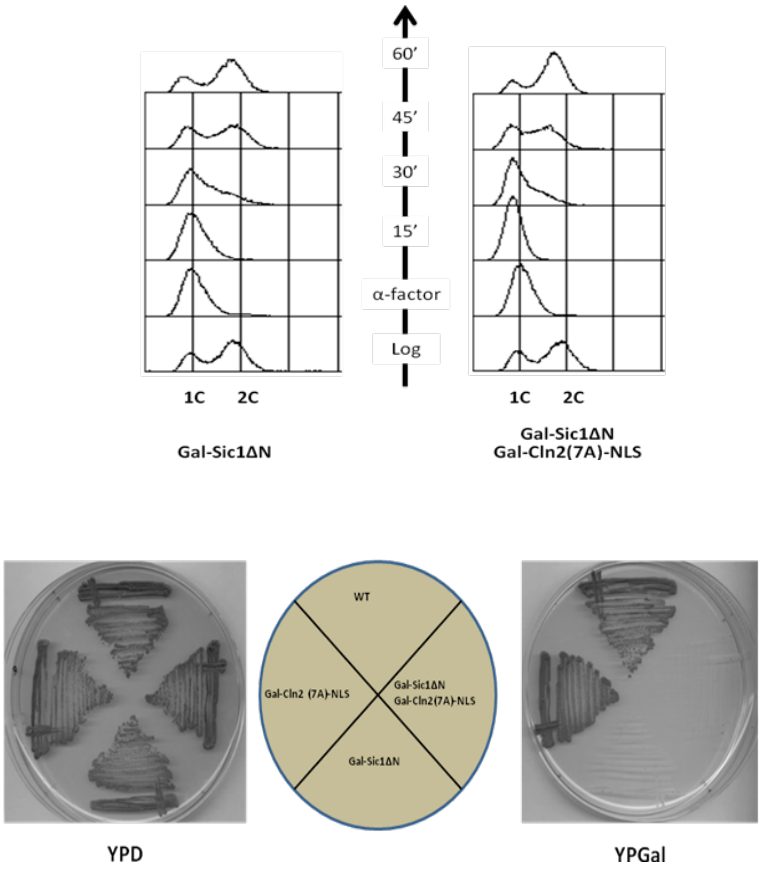


Figure 23. **A.** Flow cytometry shows the proper cell cycle progression in unperturbed conditions. **B.** Over-expresion of Sic1ΔN blocks cell cycle progression in both control and experimental strains.

Results showed the proper accumulation of hyperstable all Clbs inhibitor Sic1ΔN as well as hyperstable form of nuclear Cln2(7A) upon the induction of galactose-inducible promoter. Flow cytometry results showed that nuclear, hyperstable Cln2(7A) triggered the replication in absence of S and M-CDK activity as showen in (Figure 24).

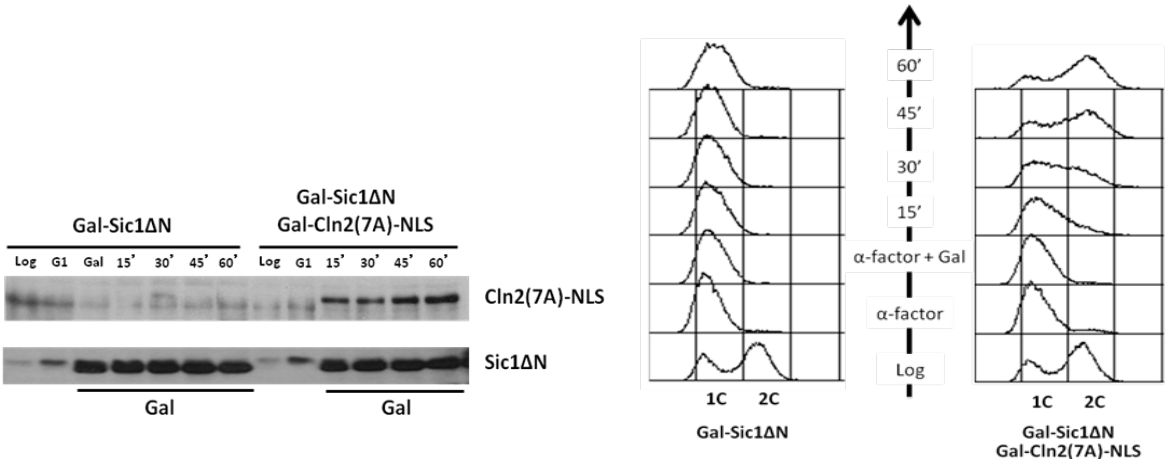


Figure 24 **A**. Western blots shows accumulation of Sic1ΔN, however, no sizeable accumulation of nuclear Cln2 protein. Ponceau staining was used as loading control. **B.**. Flow cytometry results show, Cln2 (7A) triggered replication when over-expressed and localised to nucleus in presence of strong Clb inhibitor Sic1ΔN.

Microscopic results showed the phenotype of cells compatible with effects of over-expression of strong Clb inhibitor Sic1ΔN. Cells released from α- factor arrest in G1 phase and upon induction into the medium containing galactose showed the phenotype with elongated buds due to their polar growth and failure to switch to isotropic growth because of absence of S and M-CDK activity. On the other hand, the phenotype of replicating cells was not as drastic as shown by control cells (Figure 25).

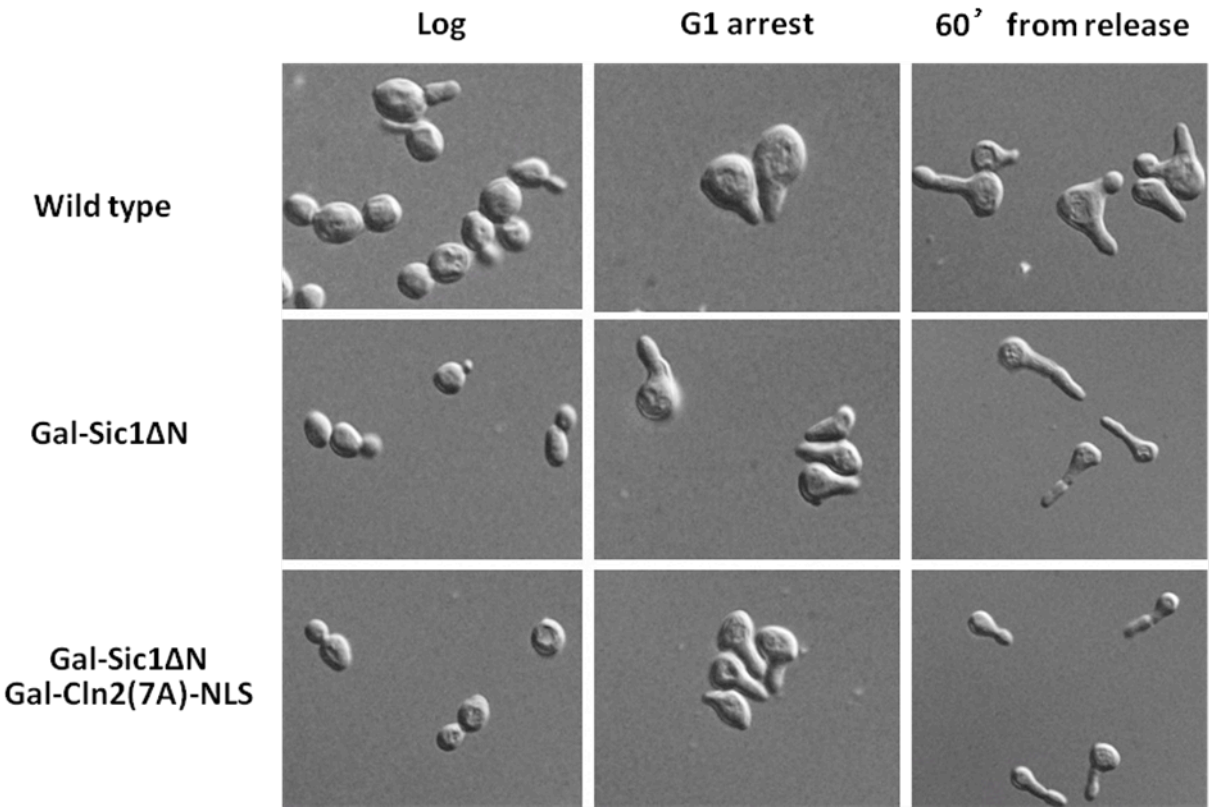


Figure 25. Shows the phenotype of cells upon release from G1 arrest. Cells in α-factor arrest show a specific phenotype called shmoo. Cells over-expressing Sic1ΔN show elongated buds, while as cells over-expressing nuclear Cln2(7A) also shows the elongated buds, however, not as dramatic as in former.

Possible reason for difference in phenotype among two strains was probably the leaky mitotic cyclins, which was not the case, since M-CDK inhibitor, Swe1 was expressed as early as start of replication in experimental strain. Also, Y-19 of Cdc28 was seen phosphorylated as early as 15 minutes after release from α-factor block. Both the results suggested that M-CDK activity was tightly inhibited including with the help of strong all Clb inhibitor Sic1ΔN (Figure 26).

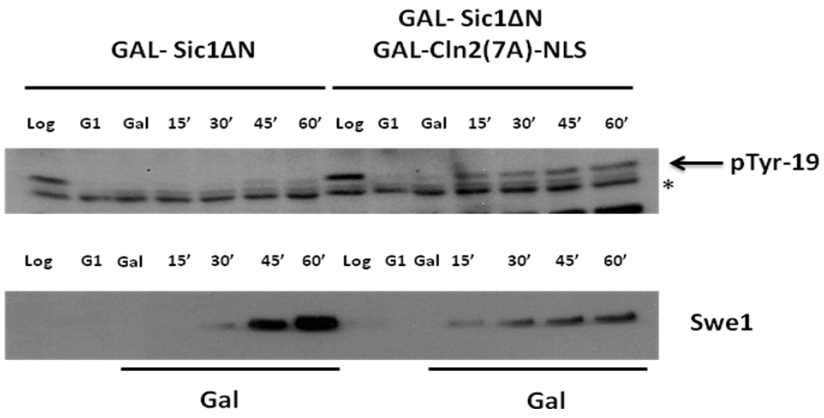


Figure 26. Shows that tyrosine 19 of Cdc28 in replicating cells is phosphorylated early compared to non-replicating cells. Also Swe1, that phosphorylates the tyrosine19 was expressed in accord to phosphorylation seen in both replicating and non replicating cells, confirming, block of M-CDK activity.

Due to the result of elongated buds by Sic1ΔN over-expression, it apparently was impossible to count buds and give representation of budding index in these experiments. Moreover, DNA staining by propidium iodide showed the early entrance into anaphase as well as disintegration of chromosomes in control cells after the release from α- factor arrest, while as replicating cells which over-express nuclear Cln2(7A) showed delayed or no anaphases with intact DNA material. However, the reason behind this behaviour is not well understood yet, as shown in (Figure 27).

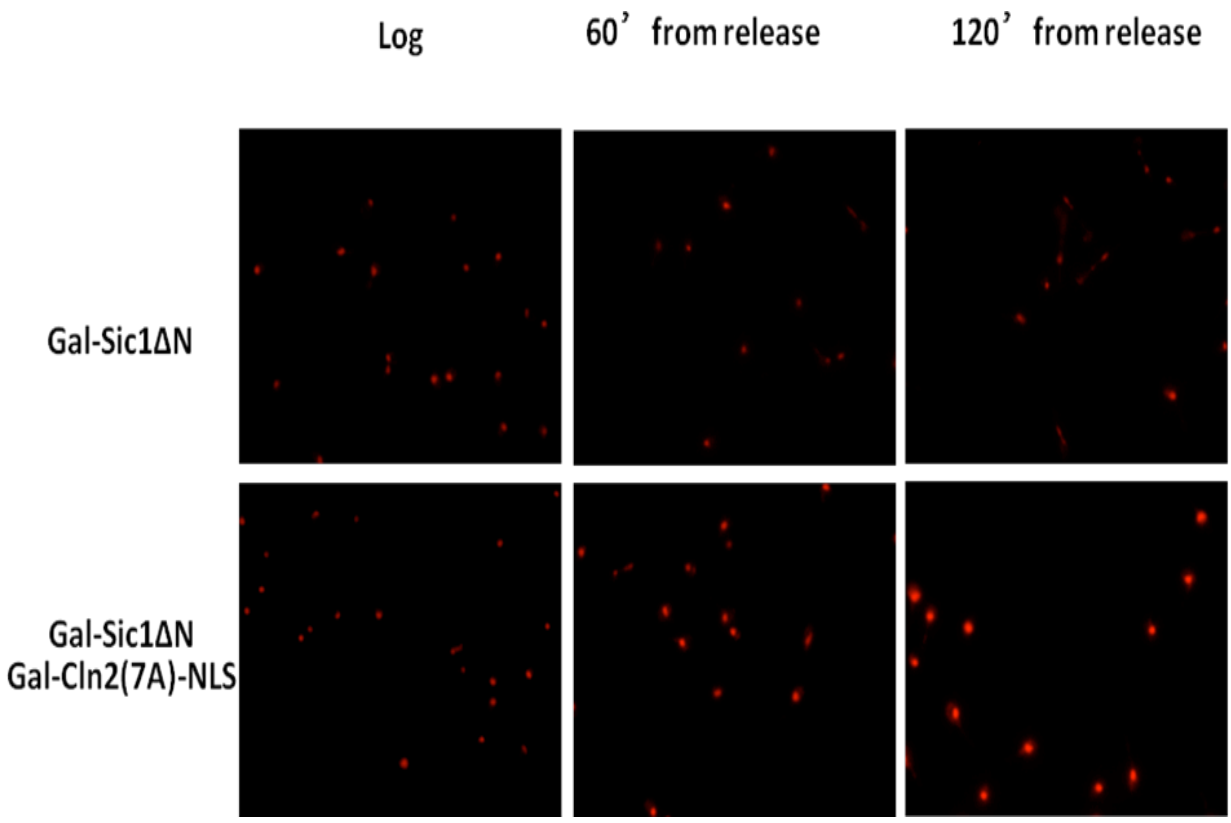


Figure 27. Shows the stained DNA of control and experimental strain upon release from G1 arrest. Cells over-expressing Sic1ΔN show disintegrated chromosomes and early anaphases, while as cells over-expressing both, Sic1ΔN and nuclear Cln2(7A) show delayed anaphase and compact DNA material.

16. Nuclear localization is essential for Cln2(7A) function in triggering replication

As shown in previous results, nuclear Cln2(7A) was properly accumulated compared to wildtype Cln2 protein and culminated into triggering replication in absence of S and M-CDK activity, however, it was imperative to explore if Cln2(7A) was required to be localized into the nucleus.

In order to check that, strains with background of Sic1ΔN were transformed with a copy of CLN2(7A) gene under the galactose inducible promoter lacking nuclear localization signal (NLS). Like previous experiments, strain with Sic1ΔN alone under the galactose inducible promoter acted as control strain, while as, strain with Sic1ΔN and Cln2(7A) without NLS under the galactose inducible promoter acted as experimental strain. Results showed that, indeed, nuclear localization signal was pivotal for the Cln2(7A) to trigger replication. Even though hyperstable protein Cln2(7A) was accumulated, at the phase level, almost equally as in previous experiment, however, flow cytometry results showed no progression of replication from one content DNA to two content. These results suggested that for Cln2(7A) to do its function, it must be localized into the nucleus rather than cytoplasm (Figure 28).

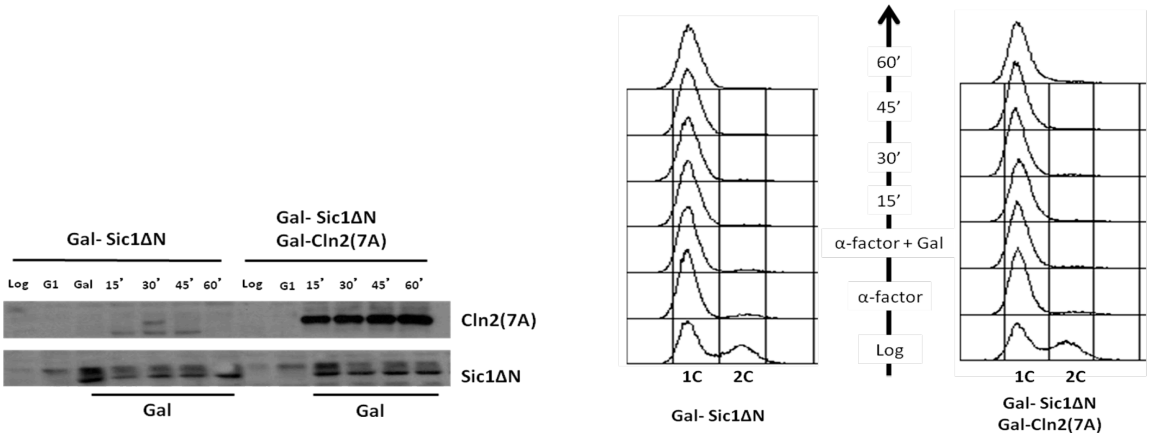


Figure 28. A. Accumulation of hyperstable Cln2 without nuclear localization signal and Clb inhibitor Sic1ΔN. B. Flow cytometry shows no replication in strains over-expressing hyperstable Cln2 lacking nuclear localization signal.

17. Sic1ΔN remains associated with CDK complex

Previous result showed that over-expressing nuclear Cln2(7A) in presence of strong Clb inhibitor Sic1ΔN resulted in triggering of replication. However, it also raised the question of possible override of Sic1ΔN by Cln2(7A), since Cln2 associated with Cdc28 in normal cell cycle phosphorylates Sic1 and leads it for destruction by SCF^{Cdc4} ubiquitin-mediated proteolysis pathway.

In order to explore whether Sic1ΔN in experimental strain was associated with Cdc28 complex rather than been disassociated due to over-expression of nuclear hyperstable Cln2, the CDK complex, Cdc28 was immunoprecipitated after one hour induction by galactose and release from α-factor block in both control and experimental strains. Upon pooling Cdc28 and complex associated with it from the cells, IP western was carried out against S-phase cyclin Clb5 and S and M- CDK inhibitor Sic1ΔN. Results showed the equal amounts of Clb5 and Sic1ΔN associated with Cdc28 in both control as well as experimental cells.

These results suggested that the replication was triggered by nuclear Cln2(7A) rather than leaky S-phase cyclins which might have created an artifactual result due to disassociation of Sic1ΔN from the complex (Figure 29).

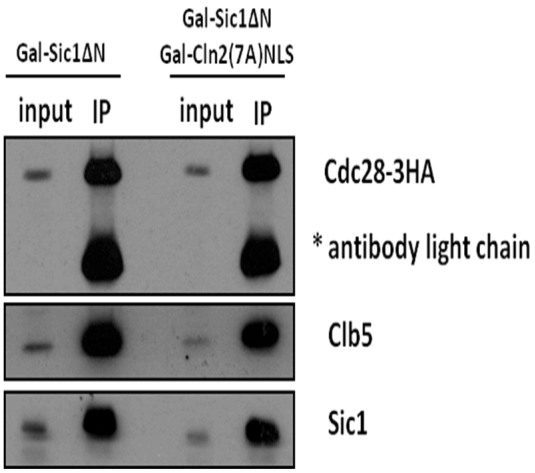


Figure 29. Shows the equal amounts of Clb5 and Sic1ΔN associated with Cdc28 in both strains containing Gal- Sic1ΔN (control strain) as well as strains containing Gal-Sic1ΔN and Gal- Cln2(7A)-NLS (experimental cells)

18. Nuclear Cln2(7A) mediated replication in Sic1(9A) and Sic1C70 background

Sic1ΔN has profoundly been used as S and M-CDK inhibitor in several studies, however, in our study, in order to reproduce our results, we used two other forms of hyperstable all Clb inhibitor Sic1 to achieve same results as in Sic1ΔN.

Sic1 has nine potential phosphorylation sites, out of which minimum of six require to be phophorylated in order to be targeted for degradation. In case of Sic1ΔN, first 50 amino acids were truncated, such that first four T/P sites were lost, thus left with only 5 possible sites to be phosphorylated which were not enough to target it for degradation. Therefore making Sic1ΔN hyperstable. In case of Sic1(9A), all nine S-T/P sites were mutated into alanine, thus rendering the protein hyperstable, since it could not be phosphorylated and targeted for degradation by SCF^{Cdc4} ubiquitin-mediated proteolysis pathway. Similarly, in case of Sic1C70, only final C-terminal 70 amino acids were used as all Clb inhibitor while as rest of the 216 amino acids were deleted (Figure 30).

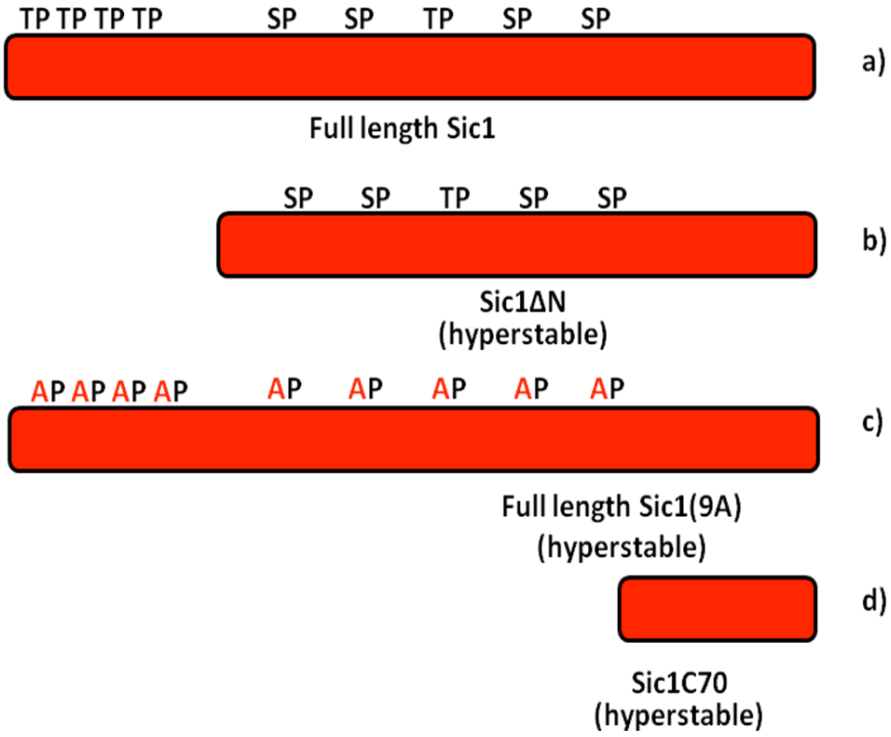


Figure 30. Shows the schematic diagram of different forms of all Clbs's inhibitor Sic1. a) full length, wild type Sic1. b) Sic1ΔN, hyperstable form of Sic1 made by truncating first 50 aminoacids, losing 4 T/P sites. c) Sic1(9A), hyperstable form of Sic1 having all S-T/P consensus sequence for CDK phosphorylation mutated to non-phosphorylating form. d) Sic1C70, hyperstable form of Sic1 having first 216 amino acids deleted losing all 9 consensus sequence for CDK phosphorylation.

Functional assay showed that both the inhibitors worked as efficiently as Sic1ΔN by blocking cell cycle progression once over-expressed under galactose-inducible promoter on rich media with galactose as a sugar source (Figure 31).

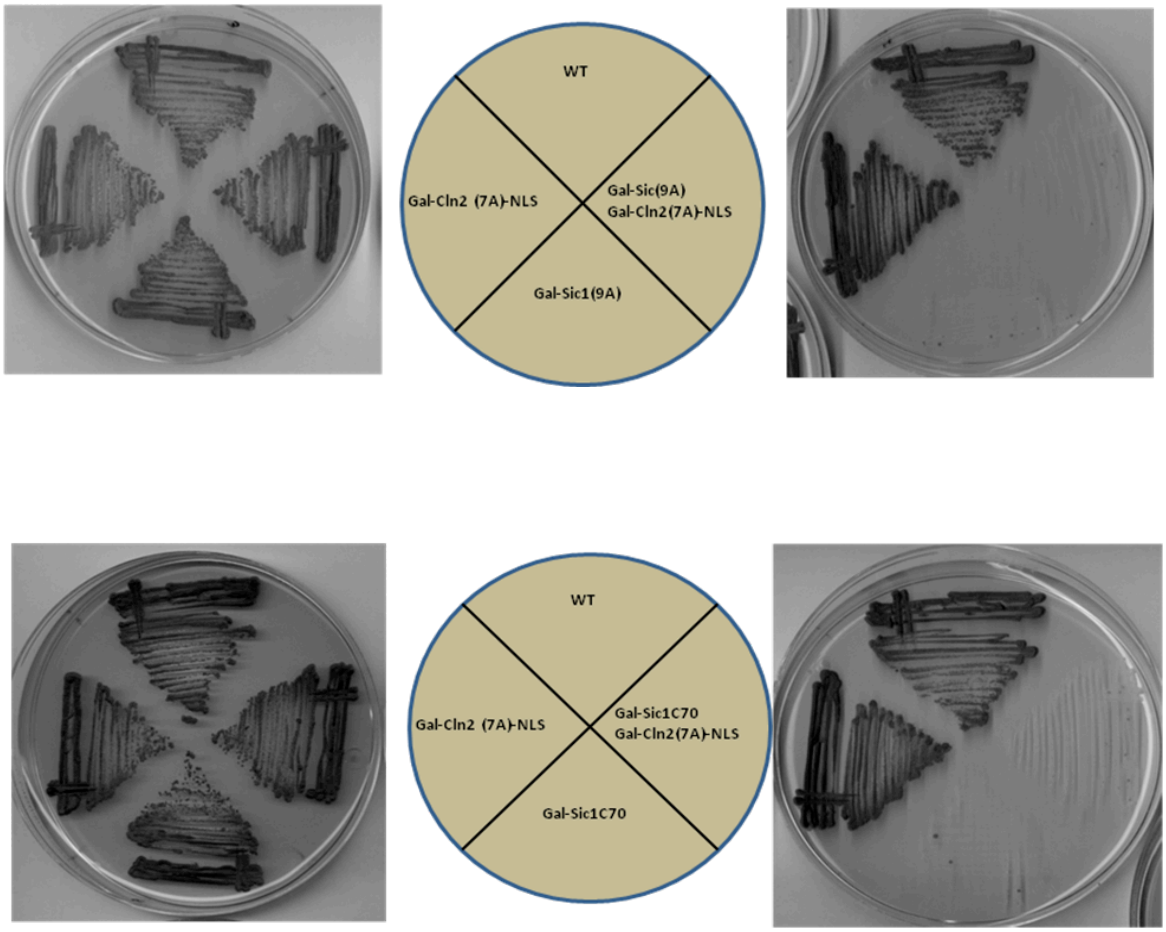


Figure 31. A. shows the functional assay of strains with Sic1(9A) on YPGAL plates. B. Shows the functional assay of strains with Sic1C70 on YPGAL plates.

Upon carrying experiments in strains containing Sic1(9A) and Sic1C70 alone as the control strains and Sic1(9A) and Sic1C70 including nuclear Cln2(7A) as experimental strains, results showed that, hyperstable nuclear Cln2 was able to trigger the replication in both the backgrounds. Thus making our previous result more concrete and robust. (Figure 32 and 33).

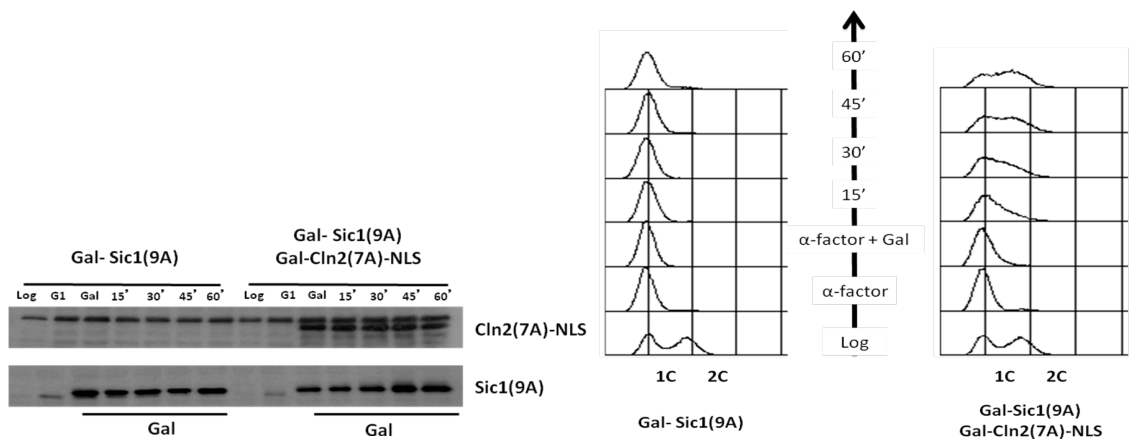


Figure 32. A. Shows the accumulation of nuclear hyperstable Cln2 B. Cytometry results showed that nuclear Cln2(7A) triggered replication in presence of all clb inhibitor Sic1(9A).

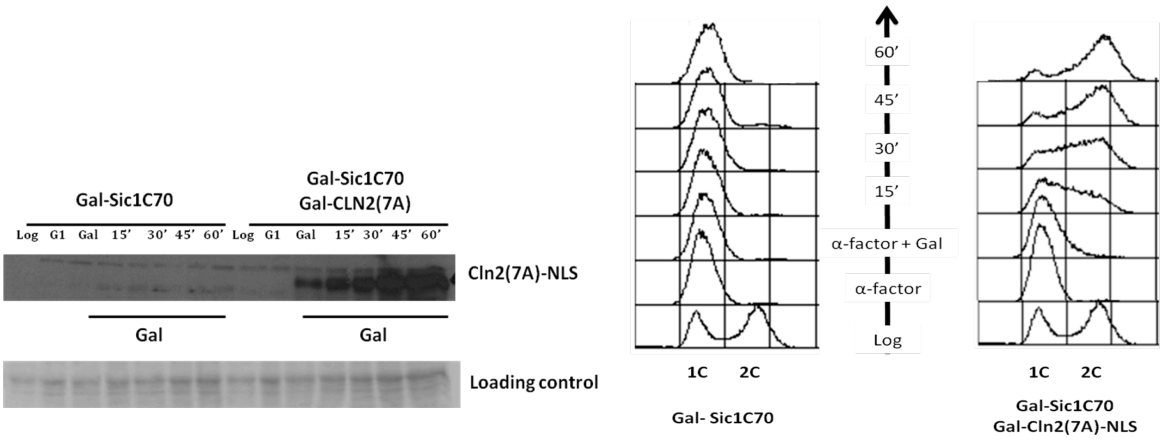


Figure 33. A. Shows the accumulation of nuclear hyperstable Cln2 B. Cytometry results showed that nuclear Cln2(7A) triggered replication in presence of all clb inhibitor Sic1C70.

19. S-phase cyclins play no role in replication mediated by nuclear Cln2(7A)

Although it is not very clear yet how MBF transcription factors are activated, however, several studies have shown positive loop of Cln2-Cdc28, which helps in further transcription of S-phase cyclins. In our study, due to presence of nuclear, hyperstable Cln2, it was imperative to explore the probability of high expression of major S-phase cyclin Clb5, which inadvertently, might override Sic1ΔN and be reason for replication. In order to investigate such possibility, levels of Clb5 protein were checked in both control and experimental strains. Immunoblotting results suggested that levels of S-phase cyclin, Clb5, were less if not more in experimental strain compared to control strain (Figure 34 A). Moreover, to make such observation rather robust, Clb5 associated with Cdc28 was immunoprecipitated and checked for its kinase activity towards histone H1 as its substrate. *In-vitro* results showed that Clb5-CDK complex had no kinase activity whatsoever in both replicating and non-replicating cells (Figure 34 B).

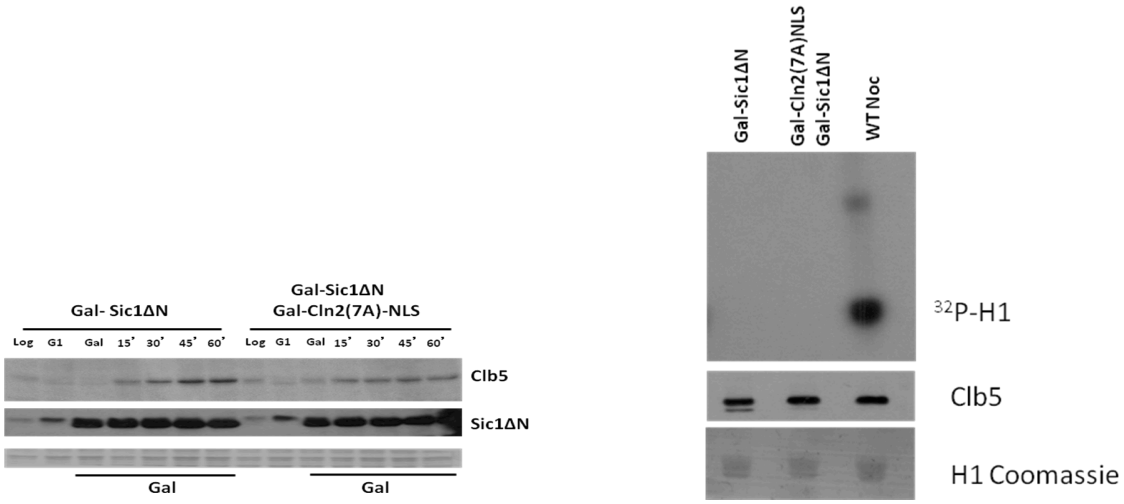


Figure 34. A. Shows that expression of Clb5 in replicating cells is less compared to amount of Clb5 present in non-replicating cells. B. *In vitro* kinase assay showing no kinase activity by Clb5-CDK complex. absence of S-phase cylins.

Furthermore, since Clb5 was only one of the two S-phase cyclins, Clb6 was still available, which is shown to have capability of triggering the replication in absence of Clb5 function. In order to address this question, both, CLB5 and CLB6 genes were deleted from the strains with back ground of Sic1ΔN (control strain) and Sic1ΔN; Cln2(7A)-NlsMycNls (experimental strain). Functional assay and experiment in unperturbed conditions showed block of cell cycle progression in cells overexpressing Sic1ΔN and late triggering of replication due to S-phase null cyclins respectively (Figure 35). Experiments were carried on both the strains and cytometry results showed that nuclear Cln2(7A) was still able to trigger replication in experimental strain (Figure 36).

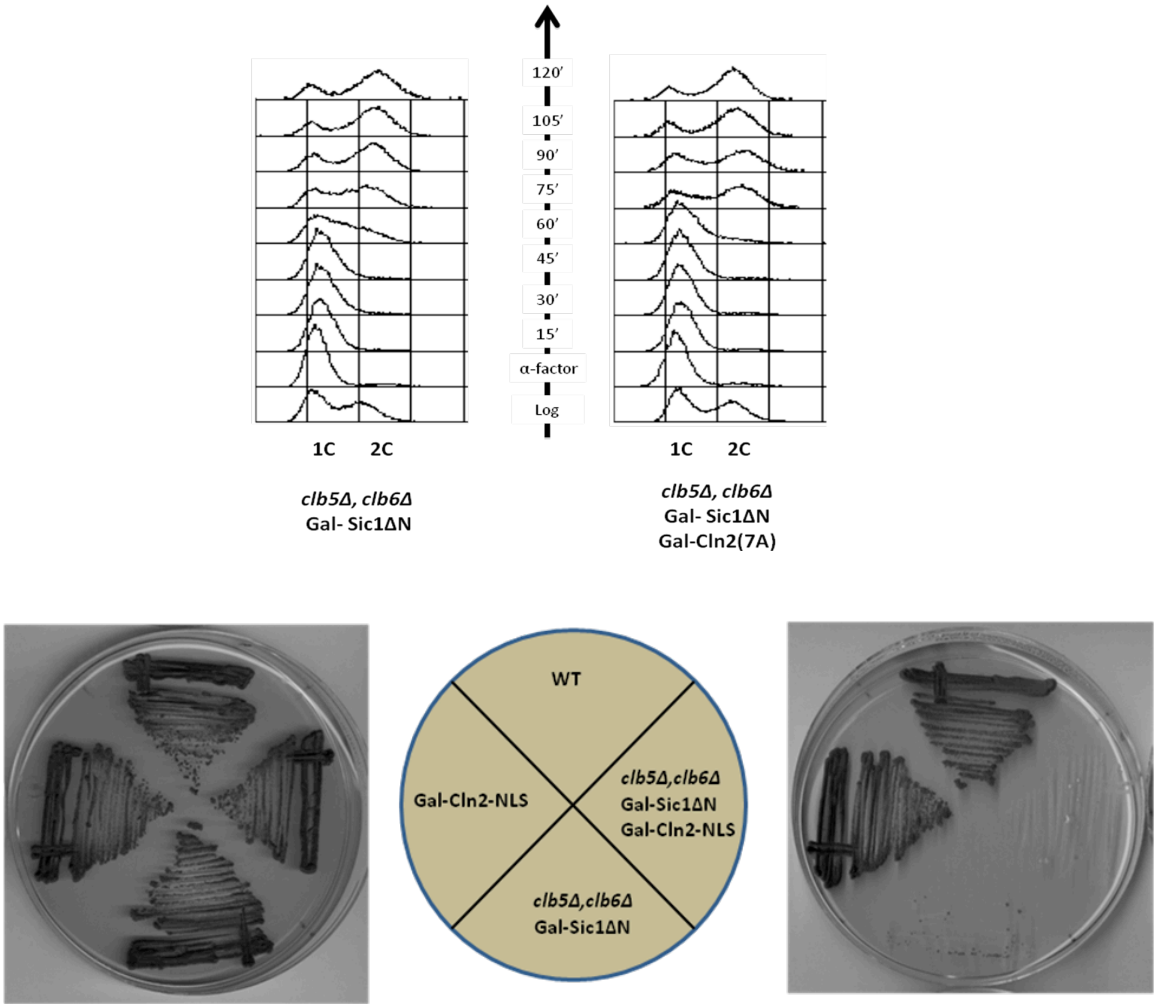


Figure 35. A. Flow cytometry shows cells trigger replication late in absence of S-phase cylins.

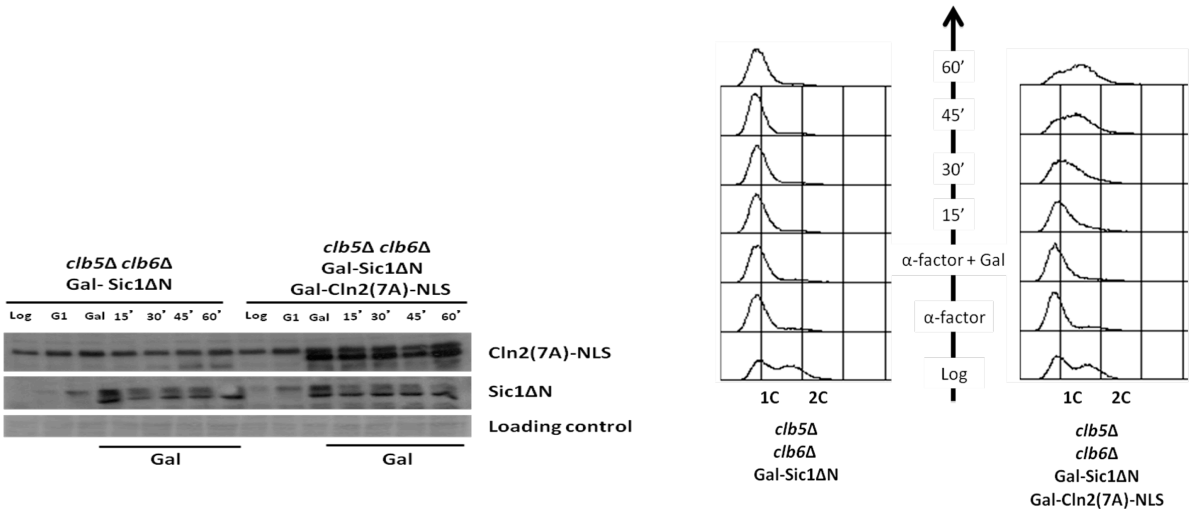


Figure 36. A. Accumulation of hyperstable Cln2(7A) and Sic1ΔN in cells with back ground of S-phase null cyclins. B. Flow cytometry shows cells overexpressing Cln2(7A)-NLS trigger replication in presence of Sic1ΔN and absence of S-phase cylins.

20. G1 cyclin Cln2 triggers later event of SFF activation

Like MBF, very less is known about the process of activation of SFF transcription factors. However, several researchers have speculated the role of S-phase associated cyclin dependent kinase activity play some role in it. According to our theory of quantity model, we presumed, if nuclear Cln2(7A) triggered replication which is a later event, it must also trigger the transcription of certain proteins which are expressed in later stages from G1-phase.

In order to explore this, major mitotic cyclin, Clb2, which is expressed from SFF transcription factor was checked by immunoblotting in both control and experimental strains. Results showed that indeed, Clb2 was expressed in replicating cells as early as at 15 minutes from release from α-factor block compared to non replicating cells, which was at around 60 minutes from α-factor block (Figure 37 A). This result showed that early phase cyclin could

trigger event of later phase, however, this result, as much it was supporting our theory, was also playing against our original observation of replication in cells that over-express Cln2(7A). The question raised here was, what if the observed replication in cells over-expressing nuclear Cln2(7A) was due to early appearance of mitotic cyclin Clb2 rather than nuclear Cln2(7A). To address this, Clb2 associated with Cdc28 was immunoprecipitated and checked for its kinase activity towards histone H1 as its substrate. *In-vitro* results showed that Clb2-CDK complex had very minute kinase activity compared to the kinase activity required for the purpose of mitosis as shown in positive control (Figure 37 B).

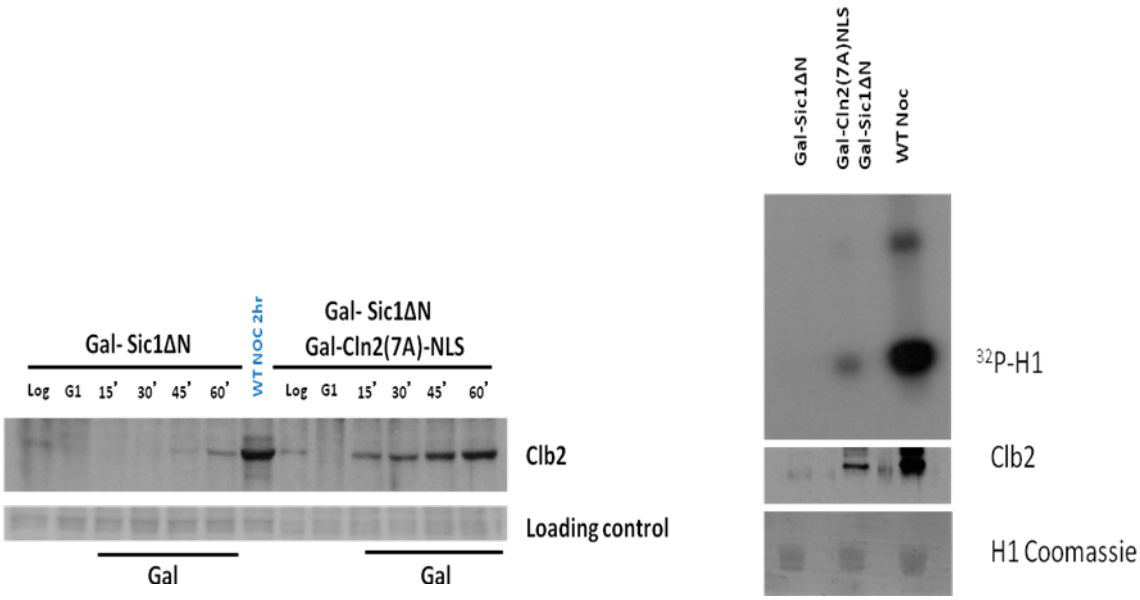


Figure 37 A. Shows the early expression of a mitotic cyclin under SFF transcription factor in cells over-expressing nuclear hyperstable Cln2. B. *In vitro* kinase assay shows very minute kinase activity associated with Clb2-CDK.

Nevertheless, *in-vitro* results showed miniature amount of kinase activity by Clb2-CDK, thus, it was more imperative to check if this amount of kinase activity was capable of carrying out M-CDK functions of Clb2. In order to check that, we used tracking of

phosphorylation of Pol12. Pol12 is a *bona fide* mitotic cyclin dependent kinase activity substrate as tested in our lab (Gloria Palou, Roger Palou, and David G. Quintana, submitted). The phosphorylation was checked by running western blot against Pol12 in several experimental and control strains. All the cells, replicating and non-replicating cells showed no phosphorylation of M-CDK substare whatsoever, suggesting, whatever residual or basal mitotic activity, which was seen in kinase assay was not enough to bring about replication (Figure 38).

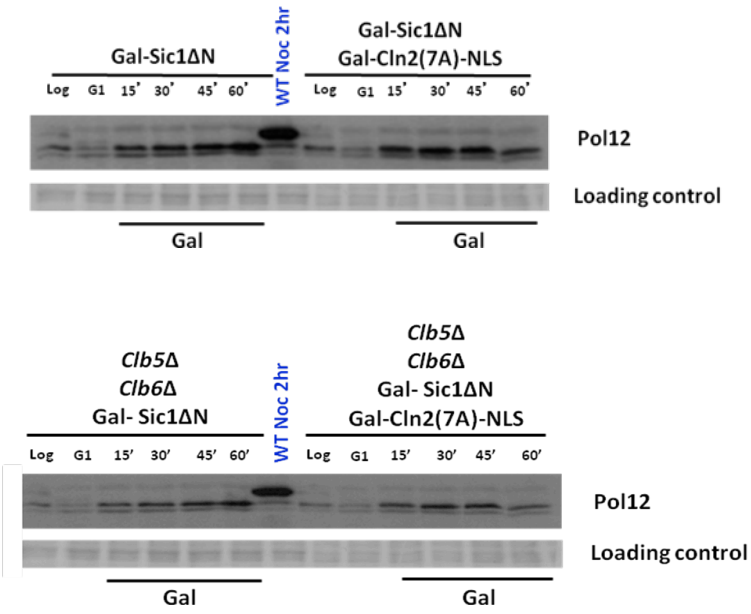


Figure 38. No phosphorylation of M-CDK substrate Pol12 in cells over expressing Cln2(7A)NLS in back ground of Sic1ΔN alone as well as Sic1ΔN and *clb5,clb6* deletion.

21. **M-CDK activity plays no role in nuclear Cln2(7A) mediated replication**

Previous results have shown that M-CDK activity (Clb2-Cdc28) was very low, and by checking M-CDK target phosphorylation (Pol12), it was apparent that this low amount of kinase activity wasn't enough to trigger the replication. However, to make results rather

convincing and more robust we carried out same experiment in two different ways to achieve convincing results, one in absence of major mitotic cyclin Clb2 expecting to see replication and second more importantly in presence of over-expressed Clb2 in back ground of Sic1ΔN. In first case, Clb2 was deleted from both the strains, control (Sic1ΔN alone) and experimental strain (Sic1ΔN and Cln2(7A)NlS). Cytometry results showed that, cells over-expressing nuclear Cln2(7A) triggered replication in presence of all Clb inhibitor Sic1N as well as absence of Clb2 cyclin (Figure 39).

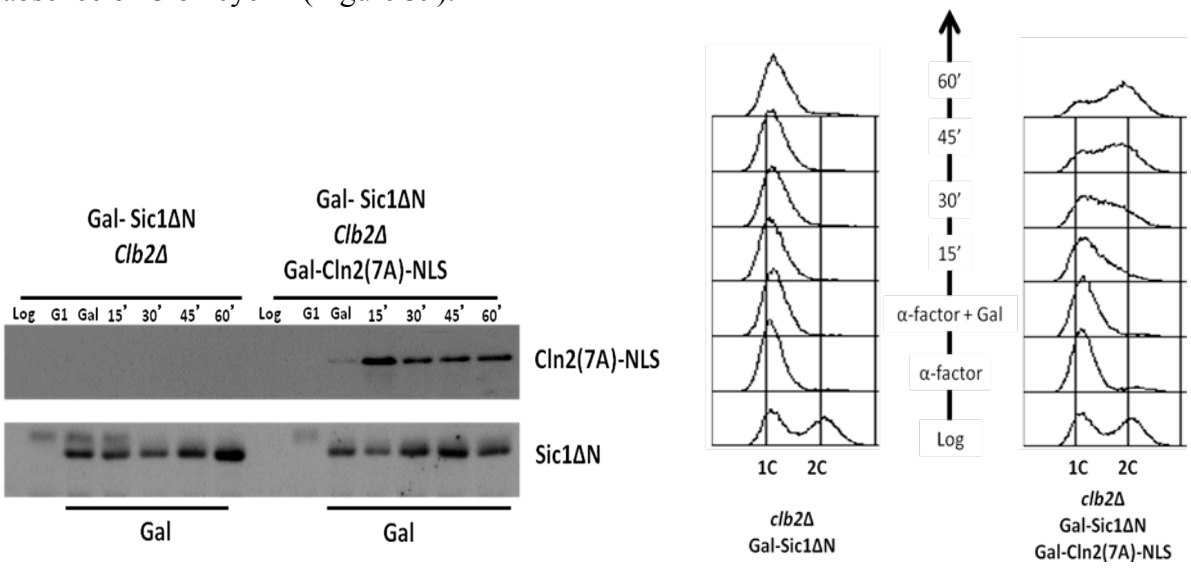


Figure 39. . Shows cells replicate when hyperstable nuclear Cln2 is over-expressed in back ground of Sic1ΔN and absence of major mitotic cyclin Clb2.

While in case of over-expressing Clb2 under the galactose-inducible promoter, over-expressed Clb2, which was higher than levels of Cln2(7A)Nls at the phase value couldn over-ride the Sic1ΔN and failed to trigger replication. These results reinforced the observation

made in previous section that Clb2-Cdc28 activity was not responsible for the triggering of replication in cells over expressing nuclear Cln2(7A) (Figure 40).

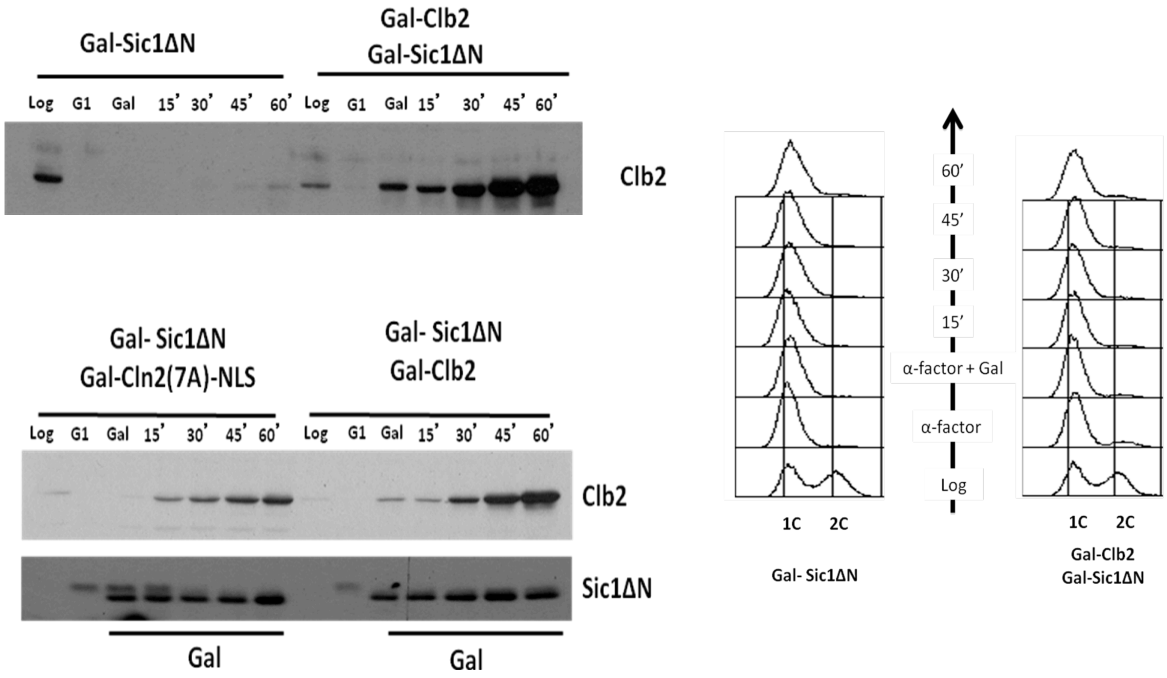


Figure 40. A. Accumulation of Clb2 under the galactose inducible promoter. B. Clb2 is unable to override the Sic1ΔN block of cell cycle progression.

Needless to say, Clb2 is not the only mitotic cyclin, Clb1 could also play role in triggering replication in absence of S-phase cyclins. However, we could not delete both the mitotic cyclins to check our strains for replication as cells are not viable in absence of both mitotic cyclins. To explore that possibility, we worked with the strain where Clb1 was deleted and Clb2 was temperature sensitive and can be rendered non-functional at will when required in the course of experiment. Cytometry results showed that cells still carried out replication in presence of all Clb inhibitor Sic1ΔN, absence of Clb1 and non-functional temperature sensitive Clb2 (Figure 41).

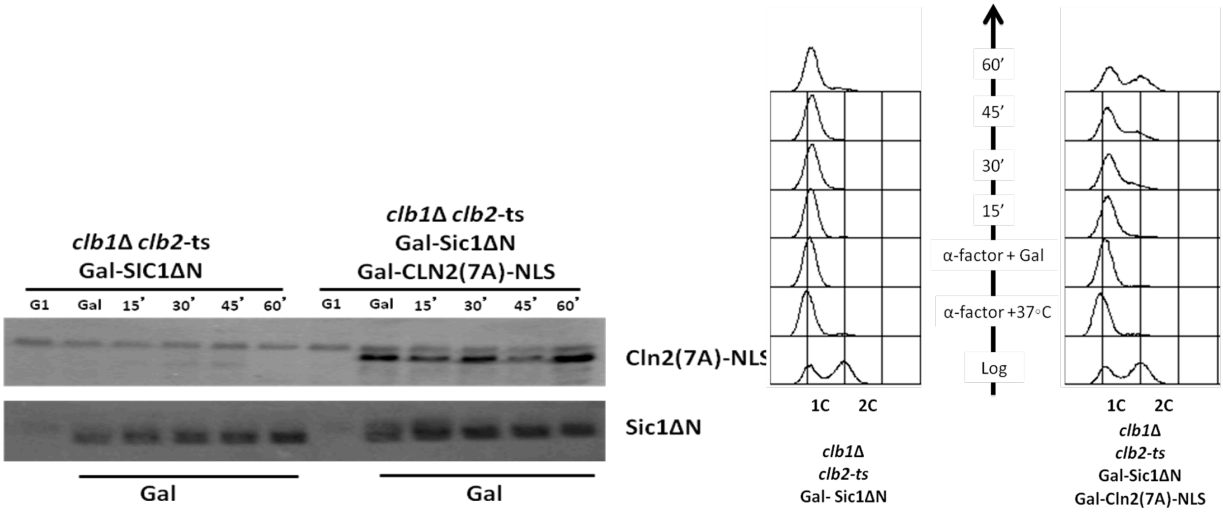


Figure 41. A. Accumulation of Cln2(7A)-NLS and Sic1ΔN in back ground of *clb1Δ clb2-ts*. B. Replication triggered by Cln2(7A)-NLS in presence of Sic1ΔN and absence of mitotic cyclins.

Finally, because these experiments were carried out separately in different strains, we attempted to construct a strain lacking S phase cyclins Clb5 and Clb6, and M phase cyclin Clb2, together with Sic1ΔN under the galactose inducible promoter. Unfortunately, it was not possible to work with this strain, since cells were barely viable.

In any case, the results shown in this section indicate that replication occurs when Cln2(7A)-NLS is induced in the presence of a strong Clb-CDK inhibitor and the absence of S-phase cyclins Clb5 and Clb6 or M-phase cyclins Clb1 and Clb2, supporting that replication can be triggered by a G1 cyclin when made hyperstable and accumulated in the nucleus.

DISCUSSION

It has been well understood that during the cell growth, cellular components double in number before they get divided in between two daughter cells. In eukaryotic cells, due to increased genome size, the S-phase which usually takes up about 1/3 of the cell cycle duration is separated from mitosis unlike prokaryotes (where two phases are thought to be inherently linked). This increased level of intricacy in cells has evolved the accurate cell cycle control which guards the duplication of chromosomes and there faithful distribution among daughter cells. As abundantly mentioned before, this replication of genetic material done in S-phase and condensing into chromosomes for the purposes of segregation into two newly forming daughter cells in mitosis is mediated by the oscillating levels of cyclin dependent kinase activities. However, it is not very clear yet, how these oscillating CDK activities bring about ordered series of cellular events.

Nevertheless, at present, there are several theories put forward by esteemed researchers, which mend to the enigma behind the cell cycle ordering. First among all is the surveillance mechanism of cell cycle also known by a term check point system, a system which coherently prevents later events to happen until earlier events are not faithfully completed yet. Since this surveillance mechanism prevents cyclin dependent kinase activities to trigger mitosis until replication of whole genome is completed without any insult to its genomic integrity, which by default is the ordering of S-phase and M-phase, it is considered to be a valid candidate for cause of cell cycle progression (Hartwell 1978, Hartwell and Weinert 1989). Studies with evidential results have shown that, even though surveillance mechanism do recognize the DNA damage and double strand breaks which apparently makes checkpoints to block the later events (Lisby, Rothstein et al. 2001, Bartek, Lukas et al. 2004) but on the other hand, ongoing replication tends to be invisible to surveillance mechanism. One of the studies has shown that in cells where DNA replication in S-phase is at very slow pace due to inefficient replication origin licensing has early onset of mitosis which results

into the mis-segregation, as well as DNA breaks at the time chromosomal segregation. The other checkpoint system, which decides and controls the transition into anaphase from metaphase, is known as mitotic checkpoint. However, progression into mitosis is largely independent of mitotic checkpoint, because when cells enter the mitosis, checkpoint components are only assembled at that time and once cells enter the anaphase, there is no longer monitoring of kinetochores attachment tension and checkpoint has already passed (Palframan, Meehl et al. 2006, Mirchenko and Uhlmann 2010). In spite of importance of un-adulterated DNA replication, and faithful segregation of chromosomes, the ordering of cell cycle progression seems to be checkpoint independent.

Since the discovery of cyclins and their unique cyclic behavior of appearing and disappearing at the specific phases of cell cycle made a compelling argument about their role in regulating the cell cycle progression. One of the relating concept originated due to the fact that three distinctive cyclins, Cln1, Cln2, and Cln3 are required for transition of G1/S and entry into the S-phase (Nash, Tokiwa et al. 1988, Hadwiger, Wittenberg et al. 1989, Richardson, Wittenberg et al. 1989, Tyers, Tokiwa et al. 1992). On the other hand, another set of cyclins (Clb5 and Clb6) are required for the timely onset of DNA replication. (Epstein and Cross 1992, Schwob and Nasmyth 1993), and last but not the least, third set of cyclins, Clb1, Clb2, Clb3 and Clb4 are required for the process of mitosis.(Minshull, Blow et al. 1989, Ghiara, Richardson et al. 1991, Surana, Robitsch et al. 1991). This discovery of cyclins and there phase specific function gave rise to the concept known as cyclin conferred substrate specificity model.

Further studies into the crystal structure of S-phase cyclins revealed the presence of Cdk consensus S/T-P recognition sites, and in addition to that also identified an RxL peptide motif on CDK substrates which are readily recognized by the hydrophobic patch present on the S-phase cyclins (Adams, Sellers et al. 1996). However mitotic cyclins lack this patch but is

identified in G1 cyclins. (Petri, Errico et al. 2007, Day, Cleasby et al. 2009). The substrate recognition function of S-phase CDK provided by hydrophobic path of cyclin was confirmed in several Cdk substrates, suggesting RxL motif as a means by which S-phase cyclin recognizes certain specific substrates (Loog and Morgan 2005). This observation, how each cyclin of each phase promotes the particular function of that phase might suggest that different cyclins act at different times with respect to their specificity towards target proteins to promote ordered cell cycle events and sequential cell cycle progression. However, on a closer look, studies have shown that, cells can trigger replication on time and are viable in the absence of both S-phase cyclins (Clb5 and Clb6). (Fangfang Hu and Oscar M. Aparicio 2004). Also triple mutant cells, *cln*1, 2, and 3 are not viable (Richardson, Wittenberg et al. 1989), however, ectopic expression of S-phase cyclin Clb5 rescues the non-viability of triple mutant cells. (Epstein and Cross 1992, Schwob, Bohm et al. 1994, Lopez-Girona, Mondesert et al. 1998). These findings suggest the ability of triggering the replication is not limited to the S-phase cyclins alone or a later cyclin can trigger the events and perform the function of early phase cyclin. The inference taken from these studies suggest that substrate specificity, as important as it is for the smooth functioning of intricate physiology of cell cycle, it is not the ultimate cause or reason for sequential and orderly cell cycle progression.

Recent studies have shown that minimal control network based on a single monomolecular CDK module drives the cell cycle. CDK oscillator associated with single cyclin, provides the timing and directionality to a circuit of two activity thresholds which defines two different phases of cell cycle prevailing over surveillance mechanism of S-phase. (Coudreuse and Nurse 2010). The oscillating activities are based upon the concept of quantitative model, where, different levels of cyclin dependent kinase activity regulate the cell cycle progression. These results not only suggest that, attainment of specific ratios of CDK to cyclins, substrate specificity mediated by cylins upon binding CDK and check point presence are not essential

for sequential cell cycle progression, but also advocates the quantitative model as the core control of cell cycle progression. Even though there is strong evidence towards the quantitative model to be the sole essential concept for orderly cell cycle progression, the question is yet far from being solved. For quantitative model to be the ultimate basal processes followed by cells to promote cell cycle progression, a prediction that, a cyclin from an early phase, if expressed in higher enough levels and located to the specific sub cellular localization at the right time, should bring about the events of latter phase, was the sole purpose of this study. Results in this study showed that early cyclins cannot do the function of latter cyclins even if localized to the right place, if not accumulated in higher amount. However, further experiments revealed that, early cyclin, a G1 cyclin, Cln2 can indeed trigger replication which is a latter event with respect to G1 phase. Replication was triggered by Cln2 when accumulated in higher levels using non degradable mutant protein as well as located into the nucleus in absence of S-phase and M-phase activity. Not only the replication, early cyclin also caused the early expression of later transcription factor SFF. Results also suggested that it was necessary for Cln2 to be localized to the nucleus to perform this function. These results highly complemented the already existing concept of quantitative model, where high levels of cyclin dependent kinase activity associated with a single cyclin could bring about the events of other phases of cell cycle when merely located at the right place on the right time. Now the question arises about the integrity of cell and it physiology, a cell is a clever creature on its own, and has evolved over a billion of years for its sustainability and survival. To address that, as we always spoke in terms of right place at the right time, perhaps cell takes it as wrong place at the wrong time. For instance, in a normal cell cycle, cyclin D1 (Which corresponds to Clns in yeast) gets relocalized from nucleus to cytoplasm for the purposes of degradation at the beginning of S-phase, however, studies have shown that some tumors contain several cells that have retained cyclin D1 in nucleus

throughout the cell cycle. In prostate cancer, 14% of primary tumors and all metastatic tumors had constant presence of cyclin D1 in there nucleus. Likewise, in esophageal cancer and mantle cell lymphomas showed 55% and 40% of high proportions of cells with nuclear cyclin D1 respectively. Such observation was not only found in the localization of cyclin but also in its levels as well (Lu, Gladden et al. 2003, Benzeno, Lu et al. 2006, Barbash and Diehl 2008) Studies have shown, 14% of esophageal cancer had mutations in F-box protein 4 (FBXO4), which renders it to loss of function and cannot code for SCF ubiquitin ligase component required for degradation of cyclin D1. These mutations not only rendered the cyclin hyperstable, but also lead to constitutive nuclear localization of cyclin D1(Moore 2013). Our results are in accord with the observation made from other studies in mammalian cells. In yeast, like any other cell, cells control their environment at a very intricate level, even though our results support the quantitative model and also boosts its accuracy few notches, we were able to fathom how quantitative model works and why an early cyclin should not be allowed to perform functions of latter event. As observed in our results, when cyclin Cln2 is not accumulated in higher amount, it was not able to trigger the replication, but could only trigger replication when accumulated in high amount in its hyperstable form, which is not in the benefit of cell since at the time of Cln2 expression, cells have not grown enough to enter S-phase neither are all proteins and complements fully expressed required for healthy and unadulterated genomic replication. To prevent such catastrophes as explained earlier by having constant Cyclin D1 in nucleus, cells self regulate the Cln2 such that it is not accumulated to high enough levels to cause pre-mature replication. Apart from the levels of cyclin, sub cellular localization also plays essential role in meditating the fate of cell. In our study, we proved that, even hyperstable form of Cln2 was not able to trigger the replication when devoid of nuclear localization signal. This also play in accord with the healthy cell cycle, since in normal cell cycle, cln2 in majorly cytosolic, thus preventing its early

devastating triggering of replication before due time, however, as mentioned previously, mutation in F-box 4, not only makes it hyperstable but also lead to constitutive nuclear localization of cyclin D1(Moore 2013).

Results shown in this study could have evolutionary implications, where a single cyclin associated with cyclin dependent kinase could drive cell cycle, while as other cyclins were introduced with course of time just in case for making intricate cell cycle processes run glib. However, in our study, we did not see triggering of mitosis by over-expressing early G1 cyclin. Partly it is presumed due to use of hyperstable Cln2 to trigger the replication, which cannot be degraded, where as we already know, for mitosis to occur, CDK activity should reduce to zero. To adjust and figure out if same could happen with the events of mitosis, whether G1 cyclin and or S-phase cylins can trigger events of mitosis would be the future focus of this study.

Conclusions

1- Cells regulate levels of cyclins by avoiding the accumulation at the wrong time.

2- Cell prevent deleterious effects by localising of cyclins and cyclin dependent kinase complex to sub- cellular localizations where there function imparts no risk to the integrity of cell.

3- G1 cyclin has potential of triggering the replication.

4- G1cyclin has potential of triggering latter events, e.g., SFF transcription.

5- In summary, early cyclins can trigger later events, if expressed in higher levels, at the right time, and at the right location.

6- Raising levels of G1cyclin dependent kinase activity triggered events of later phases, thus supporting the Quantitative model of cell cycle progression.

ABBREVIATIONS

Abbreviations	Full form
1C	1 content of DNA
2C	2 contents of DNA
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride HCL
BSA	Bovine serum albumin
CDK	Cyclin dependent kinase
M-CDK	M phase-Cyclin dependent kinase
S/CDK	S phase-Cyclin dependent kinase
DDK	Dbf4 dependent kinase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DSB	Double strand break
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
FACS	Fluorescence Activated Cell Sorting
Gal	Galactose

IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kb	Kilobase
LB	*Lysogeny Broth*
LBA	*Lysogeny Broth* with Ampicillin
MCM	*minichromosome maintenance*
NP-40	Nonidet P-40
ORC	origin recognition complex
PAGE	Polyacrylamide gel electrophoresis
PCR	*Polymerase Chain Reaction*
Raf	Rafinose
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
ssDNA	Single stranded DNA
TAE	Tris base, acetic acid and EDTA.
TBS	Tris base, Salt.
TCA	Trichloroacetic acid
TE	Tris/EDTA buffer
TEMED	N, N, N', N'- Tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane

WT	Wild type
YP	*Yeast extract/Peptone*
YPD	Rich media with Glucose (*Dextrose*)
YPGal	Rich media with galactose (*Galactose*)
YPRaff	Rich media with rafinose (*Raffinose*)
YPSucr	Rich media with sucrose (*Sucrose*)

CHEMICALS USED

Chemical used	Company- Reference
Acrylamide:Bisacrylamide37,5:1)	BioRad (ref. 161-0148)
Agarose	Laboratoris Conda (D-1 low EEQ-GQT, ref. 8017)
Agar	Laboratoris Conda (ref. 1800.00)
AEBSF	Melford (ref. MB2003)
Ampicillin	Melford (ref- A0104)
Anti- c-myc (clone 9E10)	Roche (ref. 11667149001)
Anti-3HA (clone 12CA5)	Roche (ref. 11583816001)
Anti-IgG-HRP (anti goat)	Santacruz (ref. SC-2020)
Anti-IgG-HRP (anti rat)	Dako (ref. P0161)
Anti-HA High affinity Matrix (clone 3F10)	Roche (ref. 11815016001)
Aprotinina	Roche (ref. 1583794)
APS	Melford (ref. A1512-100G)
Coomassie blue	Sigma (Brilliant Blue 250 R)
Ethidium bromide	Bio-Rad (ref. 161-0433)
BSA certified free of immunoglobulins	Sigma (ref. A7030)
Magnesium Chloride	Sigma (ref. M-2670)

Manganese Chloride	Merck (ref. 105927)
Methanol	SCHARLAB ref (ME03022500)
DMSO	Sigma (ref. D2650-100ML)
DNA polymerase (PCR)	KOD hot polymerase Novagen ref- 71086-3 Taq Polymerase ref- NEB- M0273S
dNTPs (A,T,G,C)	Roche
Restriction Enzymes	New England Biolabs
Yeast extract	BD (BactoTM Yeast Extract, ref. 212750) Laboratoris Conda (ref. 1702.00)
EDTA	Sigma (ref. E-6758)
EGTA	Sigma (ref. E-8145)
Phenol-Chloroform:Isoamyl Alcohol	Sigma (ref. P3803-250ML)
Antarctic phosphatase	New England Biolabs (Ref. M02895)
Glycine	Melford (ref. G0709)
Galactose	Sigma (ref. G0750-100G)
Glucose	Merck (ref. 36922074)
G418	Melford (ref. G0175)
Immunoblot detection- Amersham ECL	GE ref- (RPN2209)
Propidium iodide	Sigma (ref. P4170-1G)
IPTG	Melford (ref. MB1008)
Leupeptin	Roche (ref. 253286)

Lysozyme	Sigma (ref. L6876-1G)
Skim milk powder	Nestle
Marker DNA	New England Biolabs (1 Kb ladder, ref. N3232S)
Marker protein	Fermentas (ref. SM0441)
Pepsin	Sigma (ref. P7012-1G)
Pepstatin	Roche (ref. 11524488001)
Peptone	BD (BactoTM Peptone, ref. 211677) OXOID (Bacteriological Peptone, ref. 736279)
Ponceau-S	Sigma (ref. P-3504)
Raffinose	Sigma (ref. R0250-100G)
Ribonuclease A (RNase A)	Sigma (ref. P5125-100MG)
SDS	Melford (ref. S1030)
Sucrose	Sigma (ref.S7903-250MG)
T4 DNA ligase–	Roche (ref. 12759622)
TCA	Sigma (ref. T0699-250ML)
TEMED	BioRad (ref. 101-0800)
Triptone	Laboratoris Conda (ref. 1612.00)
Tris base	Sigma (ref. T-1503)
Tween-20	Bio-Rad (ref. 170-6531)
Uracil	Sigma (ref. U-0750)

Yeast nitrogen base	Difco (ref. 291940)
2-Propanol	Fluka (ref. 59304-100ML)
5-FOA	Melford (ref. 703-95-7)

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