Novel Modes of Regulation of Cyclin Dependent Kinase Cdk1

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March, 2014

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A thesis submitted in conformity with the requirements for the degree of

Doctor in Biochemistry, Molecular Biology and Biomedicine

Universitat Autònoma de Barcelona

March, 2014

Thesis Director **PhD Candidate**

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Dedicated to

My family, my wife Xinjie Zhang

And my beautiful daughter Wenxi Zeng

ABSTRACT

Cyclin dependent kinases are drive cell division cycle progression in eukaryotic cells. In the model eukaryotic organism *Saccharomyces cerevisiae* (budding yeast) a single Cyclin Dependent Kinase, Cdk1, is essential and sufficient to drive the cell cycle. Alternately bound to G1, S and G2/M phase cyclins, Cdk1 regulates cell cycle transcriptional programs, chromosome replication and segregation, spindle dynamics, polarized cell growth, morphogenesis, etc. Misregulated CDK activity induces unscheduled proliferation as well as genomic instability.

Given its essential function in cell cycle progression, Cdk1 is tightly regulated by binding partners (cyclins, Cks1 and Cyclin dependent Kinase Inhibitors -CKIs) and post-translational modifications. However, many details on Cdk1 regulation remain unknown, such as how G1 or mitotic CDK activities are inhibited in response to challenging conditions.

When the cell cycle progression is challenged by genotoxic stress such as DNA replication stress or DNA damage, a surveillance mechanism, the S phase checkpoint is activated to protect the integrity of the genome. In the budding yeast the S phase checkpoint is mediated by the Mec1 kinase (ATR/ATM in humans) and its downstream effector kinase Rad53 (Chk2 in humans).

To explore whether the effector kinase Rad53 regulates Cdk1 in response to genotoxic stress, we have been exploring two main avenues: (1) Cdk1 phosphorylation by the S phase checkpoint effector kinase Rad53 and (2) Rad53 dependent regulation of Cdk1 associated factors.

With respect to the first question, taking advantage of a Rad53 *in vitro* kinase assay, we show that recombinant Cdk1 is directly phosphorylated by Rad53. We also proteomically identified two sites of Cdk1 (Ser46, Ser258) phosphorylated by Rad53 *in vitro*. Cells carrying the non-phosphorylatable Cdk1 allele (Cdk1-2A) display a *wee* phenotype, compatible with increased/unrestrained CDK activity. Cells carrying the phosphomimetic Cdk1 allele (Cdk1-2E) are elongated and larger in size than wild type cells. Moreover, we also assign and quantify the different phosphorylation forms of Cdk1 *in vivo* using Phos-tag electrophoresis technology.

With respect to the second question, we have proteomically identified proteins associated with Cdk1 in the presence of replication stress in a Rad53 dependent manner. The product of the unknown function gene YPL014W, which we name Cip1 (for Cdk1 Interacting Protein 1), with the highest score, is further studied. Our data shows that Cip1 is a cell cycle regulated protein. In addition, the abundance of Cip1 increases in a Rad53 dependent manner upon DNA replication stress. Overexpression of Cip1 blocks cells in G1 and stabilizes the S-phase-Cdk1 inhibitor Sic1 *in vivo*. Moreover, Cip1 specifically interacts with G1 phase Cln2-Cdk1 but not with S phase Clb5-Cdk1 or M phase Clb2-Cdk1. Cip1 inhibits Cln2-CDK activity both *in vivo* and *in vitro*. Our finding suggests that Cip1 may be a novel CKI of G1 phase CDK activity.

CONTENTS

1 INTRODUCTION

1.1 THE CELL CYCLE OF BUDDING YEAST

Eukaryotes, ether yeasts with one single cell, or multi-cellular plants and mammals, renew their life through a similar cell division. The decision to start a cell division is critical for cell viability. A eukaryotic cell will not divide into two unless the genetic material is faithfully duplicated and equally distributed. The ability to transfer the identical copy of genetic material to the daughter cells with profound precision decides the survival of an organism, no matter how few or how many cells it contains. It is of great importance for cells to initiate cell division only in the appropriate environmental condition and correct genetic background. In single-celled organisms, premature entry into cell division leads to abnormal cells without the proper raw materials and structures necessary to complete the cell division. In mammals, unregulated cell proliferation can lead to cancer (more details in section 1.2.2).

The molecular architecture of cell division is broadly conserved between unicellular and multi-cellular eukaryotes. The budding yeast *Saccharomyces cerevisiae* in many respects is an ideal organism and star model in eukaryotic cell cycle research.

First of all, yeasts are the *E.coli* of eukaryotes. They share many of the technical advantages which lead to rapid progress in eukaryote molecular genetics, like cheap media, rapid growth, a well-defined genetics, and most important, a highly versatile DNA transformation system. Budding yeast represents a simple eukaryote whose genome can be easily manipulated. It has only a slightly greater genetic complexity than bacteria. Secondly, budding yeast *Saccharomyces cerevisiae* was the first eukaryote whose genome was completely sequenced. So we have complete information of all the genes and protein sequences (SGD, *Saccharomyces* Genome Database http://www.yeastgenome.org/). Thirdly, many of the genes and pathway networks present in budding yeast are well conserved in higher eukaryotes. Therefore knowledge gained about the cell cycle control in budding yeast can be used to build regulatory networks of more sophisticated higher eukaryotes.

1.1.1 Cell cycle phases

Working with *Saccharomyces cerevisiae*, Leland H. Hartwell and coworkers identified genes specifically involved in cell cycle control, namely CDC-genes (cell division cycle genes) (Culotti & Hartwell, 1971; L H Hartwell, 1971a, 1971b; L. H. Hartwell, Culotti, & Reid, 1970). Genetic analysis of the collection of cdc mutants exhibited a picture of budding yeast cell cycle from the easily visualized uniform morphological changes while it progresses through the cell cycle. The cell cycle is viewed as a cyclical progression through four phases in the following order (Mitchison & Creanor, 1971): G1 (gap 1), S (synthesis), G2 (gap 2), and M (mitosis) (Figure 1.1).

From Leland Hartwell's observation of morphological and nuclear positioning of cdc mutants, specific stages of the cells affected were characterized (Culotti & Hartwell, 1971; L H Hartwell, 1971a, 1971b; L. H. Hartwell et al., 1970). Cells in G1 phase which is also called growth phase 1 were figured by being unbudded. Once budded with a small bud, cell is identified as starting DNA replication in S phase. At the meantime, cells replicate its spindle pole bodies. Hartwell named this point as START where the cell commits to division rather than to mating (L H Hartwell, 1974; S I Reed, 1980). After S phase cells in G2 phase (growth phase 2) or M phase have larger buds.

In general, the first three phases G1, S and G2 phases are collectively known as interphase and M phase is composed of two tightly coupled processes, mitosis and cytokinesis. Each phase can not be triggered unless the previous one progresses properly and completes. In S-phase (DNA synthesis) DNA replication occurs and sister chromatid separation and nuclear division take place during M phase (mitosis). During the late mitosis, cytokinesis initiates so that a mitotic cell splits into two which ensures that the identical copy of genetic material is maintained from one generation to the next. S and M phases in general are separated by two gaps, so called as G1 phase and G2 phase, which are critical control

points for the subsequent phases. From the end of M phase in the previous cell cycle until the start of DNA synthesis is called G1. During this phase, the cell growth in size is increasing and the biosynthetic activities remain a high rate to produce all the material required in S phase, mainly for DNA replication. During G2, the gap between DNA synthesis and mitosis, the cell continues to grow and prepare material for following mitosis. (Figure 1.1)

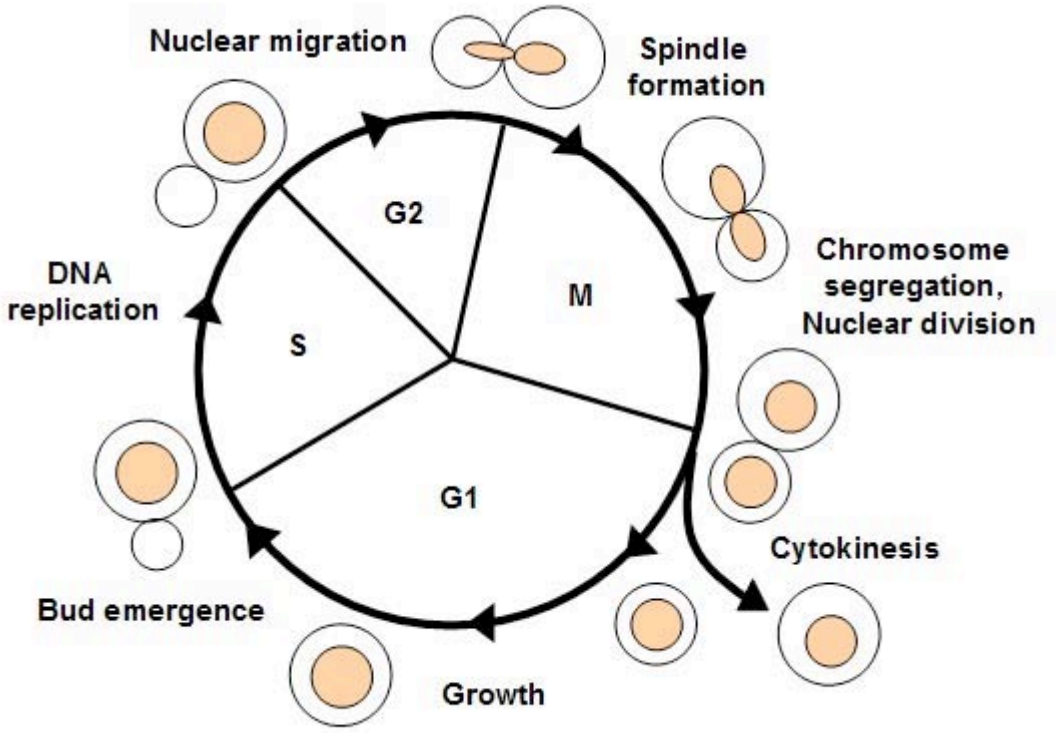


Figure 1.1 Cell cycle of S. cerevisiae. The budding yeast cell cycle is characterized by visualized uniform morphological changes and nuclear position that are associated with different phases of the cell division cycle. The cell cycle is viewed as a cyclical progression through four phases in the following order: G1 (gap 1), S (synthesis), G2 (gap 2), and M (mitosis). In G1, cells are irreversibly committed to a new round of cell division. In S phase, DNA is replicated. The replicated DNA migrates into the buds in G2 and two sister chromatids segregate and nuclear division takes place in M phase followed by cytoplasmic division (cytokinesis).

1.1.2 Cdks and cyclins

The Nobel Prize in Physiology or Medicine 2001 was awarded jointly to Leland H. Hartwell, Tim Hunt and Paul M. Nurse for their discoveries of key regulators of the cell cycle. As mentioned in section1.1.1, Leland Hartwell isolated temperature sensitive mutants wherein genes controlling the cell cycle were dysfunctional (mutated). One of these genes, he named CDC28 gene which was observed to control the progression of the G1 phase (L. H. Hartwell et al., 1970). Later, Paul Nurse discovered CDC2 gene as the rate limiting step controlling the onset of M phase and later identified also as a rate limiting factor for the onset of S phase and required for G1/S and G2/M transition (Paul Nurse, Thuriaux, & Nasmyth, 1976). In 1987, he used complementation strategy and cloned the human homologue gene CDK1 (M. G. Lee & Nurse, 1987). CDC28 identified by Leland Hartwell in budding yeast as well as homologue genes identified by Paul Nurse in both fission yeast and humans is the highly conserved gene which encodes cyclin dependent kinase 1 (Cdk1). Cdk1 as a serine/threonine kinase is a key player in cell cycle regulation.

In eukaryotic cells, the cell cycle progression is tightly controlled by cyclin dependent kinases (CDKs). There are six conserved CDKs in the budding yeast *S. cerevisiae* (J. M. Lee & Greenleaf, 1991; Liao et al., 1995; Simon, Seraphin, & Faye, 1986; Toh-e, Tanaka, Uesono, & Wickner, 1988; Yao, Neiman, & Prelich, 2000): Cdk1 (also known as Cdc28), Pho85, Kin28, Srb10 (also known as Cdk8 and Ssn3Ccn3), Ctk1 and Bur1(also known as Sgv1). Unlike in higher organisms, in budding yeast only Cdk1; a single CDK is essential and sufficient to drive all phases of the cell division cycle.

1.1.2.1 Expression and function of cyclins

As the name implies, the cyclin dependent kinases (CDKs) are activated by their cyclin partners (Norbury & Nurse, 1991) which are periodically synthesized and degraded (Evans, Rosenthal, Youngblom, Distel, & Hunt, 1983). As important as Leland Hartwell and Paul

Nurse found CDKs, working in sea urchin eggs, Tim Hunt shared the Nobel Prize for discovering cyclins which bind to CDKs and regulate their activity (Evans et al., 1983).

In budding yeast there are totally nine cyclins (Cln and Clb subgroups) that associate with Cdk1 in different phases throughout the cell cycle (Figure 1.2). During the cell cycle, different cyclins binding to Cdks in different phases form cyclin-Cdk complexes. These complexes are responsible for specific events during each phase, such as DNA replication in S phase, microtubule formation and chromatin remodelling in M phase. Cyclins are grouped into different classes according to the function in different cell cycle phases (Dahmann, Diffley, & Nasmyth, 1995; Daniel J. Lew, Marini, & Reed, 1992; G. D. Moore, Ayabe, Kopf, & Schultz, 1996): G1 cyclins (Cln3), G1/S cyclins (Cln1/2), S cyclins (Cln5/6) and M cyclins (Cln1/2/3/4). In general, each cyclin level is increasing to a peak just before the cell is ready to progress to the next stage of the cell cycle, and degraded until it is needed in the next cycle again.

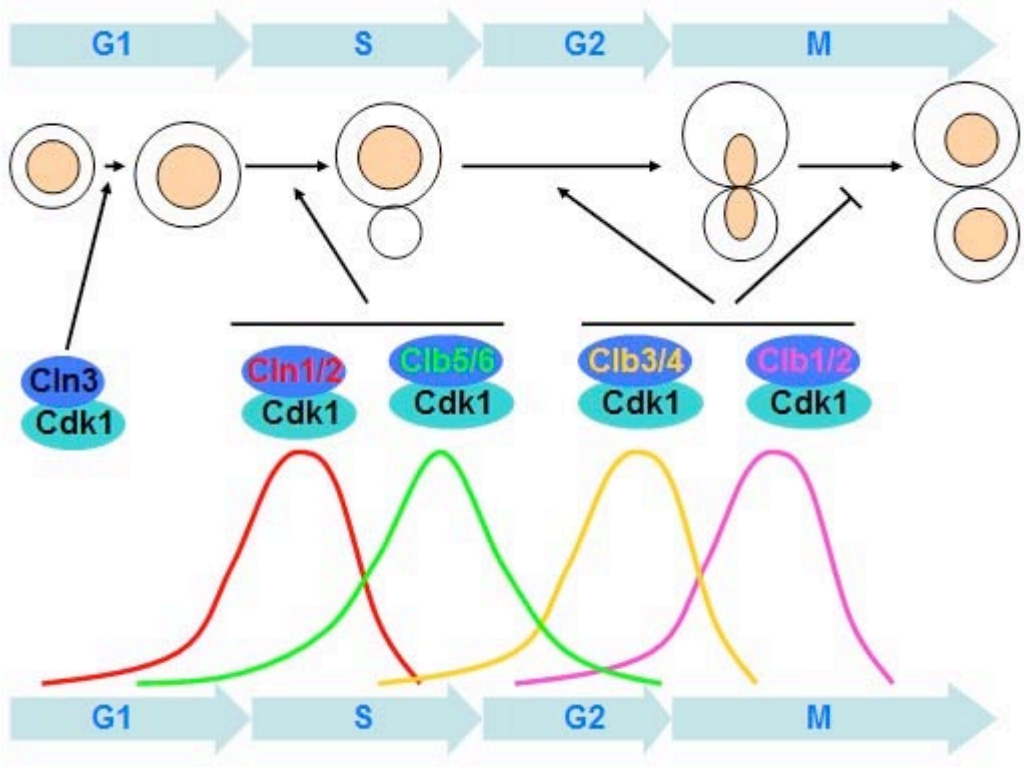


Figure 1.2 The orderly expression and function of cyclins in S. cerevisiae. The molecular events underlying cell cycle phase transitions are controlled by cyclin-Cdk1 complexes. In G1 phase, Cln3-Cdk1 activates the expression of a cluster of genes (CLN1/2). Cln1/2-Cdk1 activity is important for the G1 /S transition. Clb3/4-Cdk1 promotes later processes in S and G2phases, whereas Clb1/2-Cdk1 promotes processes at the G2 /M transition. Inactive of Mitosis CDK (Clb1/2/3/4-Cdk1) activity is required for cytokinesis. The colored curves represent the expression of cyclins through the cell cycle.

Cln1, Cln2 and Cln3 express in G1 phase which are involved in entry into S phase. The three cyclins have different functions by binding and activating CDK1. G1 cyclin Cln3 is increasing gradually throughout the cell cycle in response to cell growth, peaking in early G1 phase (McInerny, Partridge, Mikesell, Creemer, & Breeden, 1997). It is the main regulator linking cell growth to the cell cycle and also the most upstream regulator of START, because it induces the mRNA transcription of CLN1 and CLN2 genes (Dirick, Bohm, & Nasmyth, 1995; Stuart & Wittenberg, 1995; Tyers, Tokiwa, & Futcher, 1993). Upon transcriptional stimulated by Cln3 in middle G1 phase, G1/S cyclins Cln1 and Cln2, bind Cdk1 to trigger progression through START and initiate the processes leading to DNA replication. The increase of G1/S cyclins is accompanied by S phase CDK activity, which principally is suppressed by various braking systems in G1 phase until the G1/S cyclins are shuttled down (See section 1.2.3.2).

Other six cyclins Clb1-6 function after the G1/S cyclins in the cell cycle. These six Clbs are all B-type cyclins in budding yeast. Among them Clb5 and Clb6 are the two S cyclins function mainly on DNA replication. In general, their levels rise during late G1 and accumulate throughout S till M phase (Schwob & Nasmyth, 1993; Spellman et al., 1998). However their expression levels are different. Expression of both Clb5 and Clb6 is induced

during G1 phase, while Clb5 keeps quite stable until mitosis, but Clb6 is degraded soon at the G1/S border (Jackson, Reed, & Haase, 2006).

Mitosis cyclins Clb3 and Clb4 are present from S phase until late M phase (Fitch et al., 1992) which are involved in DNA replication, spindle assembly, and the G2/M-phase transition (Fitch et al., 1992; Surana et al., 1991). The other two M cyclins Clb1 and Clb2 are expressed during the G2-M phase and degraded at the end of M phase (Ghiara et al., 1991; Surana et al., 1991). Clb2-Cdk1 is essential for entry into mitosis, the activity of which is high in middle M phase (Richardson, Lew, Henze, Sugimoto, & Reed, 1992).

1.1.2.2 Cdk1 dependent cell polarized growth and isotropic growth

In budding yeast, cells divide into two through budding with bud formation and growth which therefore need a polarized growth along the horizontal axis to the bud. At commitment to a new cell cycle at START, cell decides to select a bud site to start the cell division. Once the bud emerges, cortical actin patches initially accumulate at the frontier of the bud, and cables (another form of actin cytoskeleton) extend into the bud which triggers the bud polarized growth (Pruyne & Bretscher, 2000).

It has been shown that cyclin dependent kinase Cdk1 can trigger polarization of the cortical actin cytoskeleton to the pre-bud site and promote the polarized growth in G1-S, and causes depolarization of the cortical actin cytoskeleton and secretory apparatus in G2 (D J Lew & Reed, 1993) (the red dots shows the distribution of actin, Figure 1.3). In detail, when Cdk1 is bound to G1/S cyclins (Cln1, Cln2), the Cln-CDK restricts cortical actin cytoskeleton to an polarized distribution and hence promotes the transition from isotropic growth to polarized growth at START, while when bound to mitotic cyclins (Clb1, Clb2), Cdk1 activity can promote the re-distribution of actin throughout the bud which therefore switches the

polarized growth back to isotropic growth during M phase (D J Lew & Reed, 1993, 1995) (Figure 1.3).

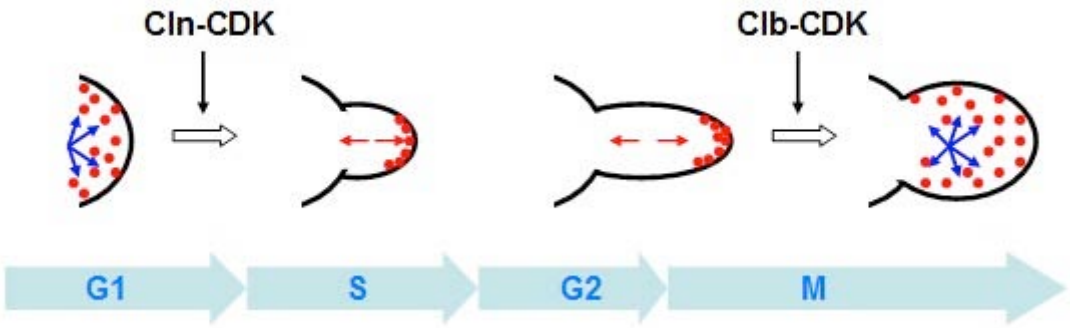


Figure 1.3 Scheme of Cln-CDK regulated polarized growth and Clb-CDK regulated isotropic growth in the cell cycle of budding yeast. The two main polarized and isotropic growth events in cell cycle: the polarization of cell bud in late G1, triggered by Cln-CDK activity (Cln1/2-Cdk1); and the polarized-isotropic switch in early G2, triggered by Clb-CDK activity (Clb1/2-Cdk1). Actin (red) distribution is indicated.

It has been shown that overexpression the ectopic Cln1 or Cln2 under Gal promoter causes hyperpolarization of the cortical actin patches and hence elongated buds (D J Lew & Reed, 1993) as illustrated in Figure 1.4. Cell lacking Clb2 exhibit dramatically enhanced filamentous growth (Ahn et al., 2001). Moreover, overexpression of the Clb-CDK inhibitor Sic1 (see section 1.2.3.2) which blocks Clb-CDK activity, therefore produces cells with an elongated buds (Nugroho & Mendenhall, 1994).

The Cln-CDK activity triggers the establishment of cell polarity at the G1/S transition, and the polarized growth of bud must be sustained through S, G2 phases. However, the live time of G1/S cyclins (Cln1, Cln2) is quite short only until S phase (see section 1.1.2.1). Therefore, there is an interesting paradox that cell bud polarized growth is continuous through the whole period but the Cln-CDK activity is short lived. There is an explanation by

system hysteresis, where the control of cell polarized growth is “bistable” (Ferrell, 2002) and the polarized growth could sustain after Cln-CDK activity diminishes (Rancati & Li, 2007). Or there exist an unknown factor which sustains the state of polarization after the establishment by Cln-Cdk1.

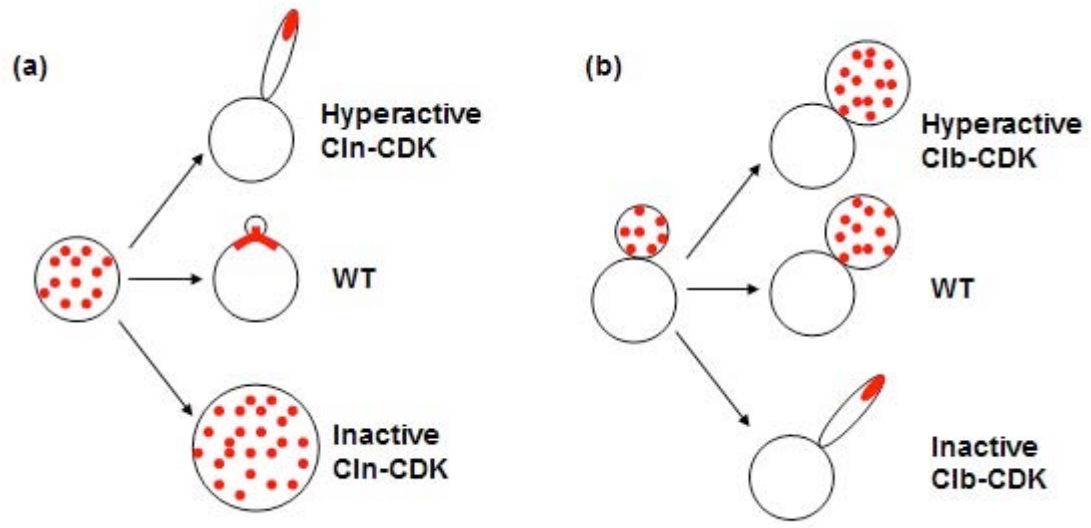


Figure 1.4 Scheme of actin distribution regulated by CDK activity. (a) Wildtype G1 cells polarize the actin (red dots) to bud necks. Inactivation of Cln-CDK activity results in a dispersed distribution of actin, which leads to a cell size much larger than normal G1 cells. Hyperactivation of Cln-CDK results in premature actin polarization and the filamentous growth of the buds. (b) Wildtype budded cells depolarize the actin distribution and switch the isotropic growth in G2 phase. Inactivation of Clb-CDK activity causes these cells to hyperpolarize their actin to the bud tip. And hyperactivation of Clb-CDK depolarizes the actin distribution from dispersed pattern, therefore generates a much more round and bigger bud compared to wildtype cell.

1.1.3 Transcriptional regulation of the cell cycle

It is a common strategy for cells that gene expression starts just when or a little bit early before the gene products are needed to avoid unnecessary synthesis. For example, the

expression of histone genes is induced during S phase and periodically induced only in S phase, to produce sufficient histones for the assembly of chromatins during DNA synthesis (L. Hereford, Bromley, & Osley, 1982; L. M. Hereford, Osley, Ludwig, & McLaughlin, 1981; Osley & Hereford, 1981). And also cyclins as mentioned in section 1.1.2, are periodically synthesized and peak in different phases. Many other genes also exhibit clear oscillations in transcript levels and/or protein level at different stages in the cell cycle. High throughput microarray data and other experiments have allowed discovering the clusters of the timely transcription of cell cycle-regulated genes (approximately 20% of protein-encoding genes) (Fraser, 2013; Spellman et al., 1998; Tavazoie, Hughes, Campbell, Cho, & Church, 1999). The same cluster of genes exhibit similar patterns of regulation in cell cycle. Cell cycle transcriptional control occurs through a tightly connected network which is required for cell cycle regulated processes. The CDK activity oscillation and also cluster genes expression oscillation promote cell cycle events progression.

1.1.3.1 Cln-CDK and SBF, MBF during G1/S transition

The accurate G1/S transition of the cell cycle is crucial for the regulation of eukaryotic cell proliferation. Lack of control on G1/S transition promotes cancer.

In budding yeast, at late G1 phase, when the cells decide whether to commit to enter into S phase at this point called START (L H Hartwell, 1974) which is analogous to the restriction point in mammalian cells (Zetterberg, Larsson, & Wiman, 1995). More than 200 genes are transcriptional activated and initiates G1/S phase transition (Cho et al., 1998). The transcription of these G1/S genes is primarily determinated by two alternative heterodimeric transcription factors, Swi4-Swi6 (SBF, Swi4/6 cell cycle box binding factor) and Mbp1-Swi6 (MBF, Mlu I binding factor) (Moll, Dirick, Auer, Bonkovsky, & Nasmyth, 1992), which are the functional analog of human E2F (Cooper, 2006). The SBF and MBF complexes are

comprised of different DNA-binding components, Swi4 in SBF and Mbp1 in MBF, and a common component, Swi6, the trans-activating component (Breeden, 1996). Mbp1 recognizes the *MluI* cell cycle box (MCB) ACGCG (Koch, Moll, Neuberg, Ahorn, & Nasmyth, 1993), due to the presence of the *MluI* restriction site in the consensus sequence, and SBF recognizes the Swi4/6 cell cycle box (SCB) CRCGAAA (Andrews & Herskowitz, 1989; Breeden & Nasmyth, 1987). SBF and MBF recognize and bind to these *cis*-acting regulators (SCB and MCB) in the promoters of G1-specific gene cluster.

MBF targets include those genes encoding proteins involved in DNA replication and DNA repair (CLB5, CLB6, RNR1, POL12, RFA1, etc) (Iyer et al., 2001; Spellman et al., 1998), whereas SBF targets include those involved in events such as bud emergence and growth, and spindle pole body (SPB) duplication (CLN1, CLN2, GIN4, HCM1, etc) (Horak et al., 2002; Iyer et al., 2001; Spellman et al., 1998).

In budding yeast, Cln3-CDK is the key regulator that promotes G1-specific transcriptional activation of MBF and SBF-dependent genes. As shown in Figure 1.5, in early G1, the transcription repressor Whi5 binds and inhibits SBF (Costanzo et al., 2004) until cells reach a certain mass (Cross, 1988, 1989), and then Whi5 gets phosphorylated by Cln3-Cdk1 hence releases from SBF which causes the depressession of the SBF-dependent transcription (Costanzo et al., 2004). This regulation is analogous to the regulation of E2F (like SBF) by the tumour suppressor Rb (homologue of Whi5) in humans (R. A. de Bruin, McDonald, Kalashnikova, Yates 3rd, & Wittenberg, 2004). CLN1/2 as the SBF regulated genes express and lead to the accumulation of Cln1/2-Cdk1 which provides additional positive feedback regulation of Whi5 and SBF (Skotheim, Di Talia, Siggia, & Cross, 2008) (Figure 1.5). SBF inactivation is achieved in a negative feedback regulation. MBF-dependent transcriptional cyclin Clb6 accumulates and targets the SBF transactivator Swi6 for nuclear exclusion in S phase (Taba, Muroff, Lydall, Tebb, & Nasmyth, 1991).

One of the major players in promoting G1/S transition is the MBF-dependent transcriptional corepressor Nrm1 which accumulates and as a negative feedback binds to MBF at target promoters, hence inhibits the transcription (R. A. de Bruin et al., 2006; Koch et al., 1993) (Figure 1.5). Transcriptional activation of MBF still remains largely unknown and recently it was shown that Cln3-Cdk1 may activate MBF by regulating the activity of the MBF-associated protein Stb1 (Robertus A M de Bruin, Kalashnikova, & Wittenberg, 2008).

In addition to the regulation in unperturbed cell cycle, the G1-specific transcriptional machinery is as well tightly regulated by checkpoints in response to DNA replication stress or DNA damage (see section 1.3.3).

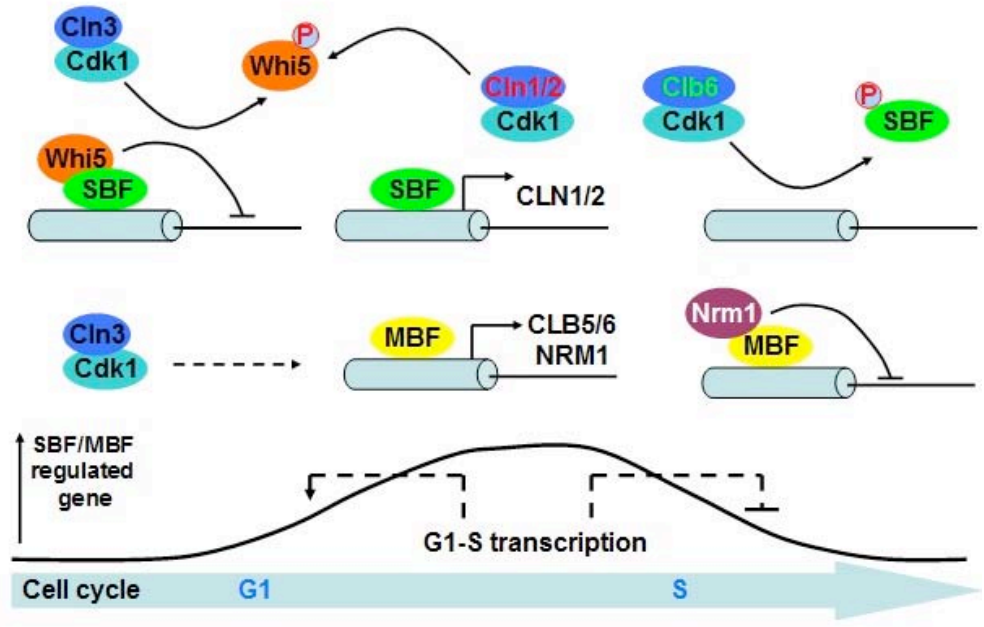


Figure 1.5 Schematic models of SBF/MBF regulatory circuits that drive gene expression through G1/S transition. During G1, SBF (Swi4-Swi6) is bound to the promoters target genes (Cln1/2 etc) in complex with the transcriptional inhibitor Whi5, which represses transcription. Phosphorylated Whi5 by Cln3-Cdk1 releases from SBF at the promoters, which in turn activates the transcription. SBF-dependent accumulation of Cln1/2 resulting from initial transcriptional activation bound to CDK is involved in a positive feedback loop to further phosphorylate Whi5, which leads to

stronger activation of G1/S transcription. MBF bound to the promoters target genes (CLB5/6, NRM1, etc) activates the transcription. In G1/S transition, MBF-dependent Clb6 accumulation leads to the phosphorylation of SBF and hence release from the promoter which stops the transcription. As an MBF target gene, the product Nrm1 is bound to MBF at target promoters, which represses expression as cells enter S phase. This is the additional negative feedback loop involved to repress the MBF transcription.

1.1.3.2 Transcriptional circuitry of the cell cycle

Periodic transcription of cell cycle-regulated genes is crucial to cell cycle progression. These genes encode critical cell cycle regulators as well as proteins related to cell cycle cycle functions. This transcription regulation networks during cell cycle are integrated mainly through both repressive and activating mechanisms, as the example of regulation of SBF/MBF dependent transcription. These cell cycle-regulated genes can be grouped into clusters that share similar patterns of regulation, like CLN2 cluster in G1/S transition (see the previous section 1.1.3.1). Apart from SBF/MBF regulated G1/S cluster, there are clusters expressed in other different phases. Such as histone gene cluster is periodically expressed in S phase which includes genes essential for DNA synthesis like HTA2, HTB2 (L. Hereford et al., 1982). Also there are the S/G2 gene cluster characterized by CLB3 and CLB4 transcriptional expression and G2/M gene cluster including CLB1, CLB2 and WHI5 etc.

Although key regulators of many waves of cell cycle transcription have been identified, the regulation of the complex networks is still exclusive. In recent years, a lot of work have provided significant insight and led to the visualization of the complex transcriptional networks based on bioinformatics strategy and genome-wide analysis (Horak et al., 2002) . Although the networks are complex and highly integrated, the general mechanisms of transcriptional regulation are seems widely applied during cell cycle

progression. First, most of the transcription regulators (activators or repressors) themselves are directly regulated in the networks. Such as the MBF regulated Nrm1 is the repressor of MBF dependent regulation system (similar positive and negative feedback loops discussed in previous section 1.1.3.1). Another example is the restriction of Mcm1-mediated gene expression in M/G1 is controlled by the transcriptional repressor Yox1 which is regulated by SBF. The second potential regulation model in the networks is mediated through cell cycle dependent expression of cyclin genes. The best example is Cln3 mediated activation of SBF. Mcm1-dependent expression of CLN3 gene during M/G1, leads to activation of Cln3-Cdk1 which phosphorylates and inactivates Whi5, thereby activates SBF regulation. There is more such well defined regulation which cooperates to control the sequential waves of transcription.

1.2 REGULATION OF CYCLIN DEPENDENT KINASE

Cdks are the master regulators that control the cell cycle progression. Their regulatory function in the cell cycle is evolutionarily conserved in all known eukaryotes. In humans, more than nine Cdks were found and at least four of them (Cdk1, Cdk2, Cdk3, Cdk4) are directly involved in cell cycle control (E A Nigg, 1995). Each CDK transient associates with a specific subset of regulatory cyclins subunits. Their role is to regulate the activity of hundreds of proteins through preferential phosphorylation at Ser (S) or Thr (T) residues in the minimal consensus sequence S/T-P and optimal S/T-P-x-K/R (where x is any amino acid) (Erich A. Nigg, 1993).

Since the 1980´s CDK activity has been found to be regulated through the cell cycle and upon cyclin binding in both budding and fission yeasts (Beach, Durkacz, & Nurse, 1982). There exist many additional CDK regulatory mechanisms under complex control. In this section, mainly three different regulation mechanisms of CDK activity are discussed as

following: the binding of cyclins, modifications by other protein kinases and cyclin-dependent kinase inhibitors (CKIs) (Figure 1.6). All of these regulators modified CDK activity, kinase conformation, substrate specificity, and/or change the subcellular localization and thereby generate an interlinked series of Cdk oscillators that drive the cell cycle progression at right time and right place (D O Morgan, 1997).

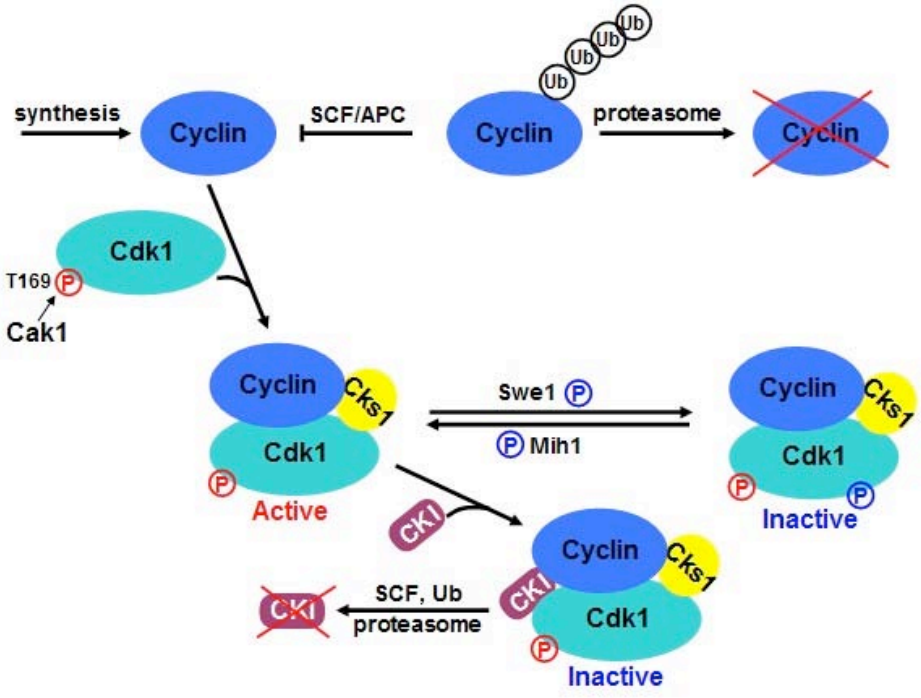


Figure 1.6 Scheme illustrating general aspects of CDK regulation. Monomeric Cdk1 has little kinase activity until it is phosphorylated by CDK-activating kinase Cak1 at Thr169 on the T-loop and interacts with a cyclin. The wave of cyclins available is balanced by the rates of their synthesis and degradation. Cyclins are targeted for ubiquitin-dependent degradation in the 26S proteasome by Skp1-Cullin-Fbox ubiquitin ligase complex (SCF) and Anaphase Promoting Complex (APC). The cyclin-Cdk complexes can be inactivated by cyclin-dependent kinase inhibitors (CKIs) or the inhibitory phosphorylation at Tyr19 by Swe1. CKIs can be targeted for ubiquitin-dependent degradation in the 26S proteasome by SCF. The inhibitory phosphorylation at Tyr19 can be removed by the phosphotase Mih1. Cks1 is the Cyclin-dependent kinase regulatory subunit and adaptor. P in the circle represents phosphorylated residue (red-activating, blue-inhibitory). Ub represents ubiquitin.

1.2.1 Cyclin and Cks1 binding

1.2.1.1 Cyclin binding

Cyclins were first discovered by Tim Hunt as periodic proteins that appeared and destroyed in synchrony with early embryonic cleavage divisions in sea urchins (Evans et al., 1983). The structure of Cdks is evolutional highly conserved in species. As the structure of human Cdk2 shown in Figure 1.7, Cdks have the same two-lobed structure as other protein kinases. The amino-terminal lobe (N) contains primarily of beta sheet (red arrows) and the PSTAIRE helix (the α1 helix), and the large carboxyl-terminal lobe (C) is primarily composed alpha helices (blue). Two alpha helices in carboxyl-terminal lobe make a critical contribution to the control of Cdk activity.

CDKs are completely inactive in the absence of cyclins. Upon cyclin binding, the PSTAIRE helix which interacts directly with cyclin, rearranges the key residues better position for ATP binding. Meanwhile, the helix L12, adjacent to the T-loop modifies its conformation to a beta strand which leads to the reconfiguration of T-loop and therefore exposure the active site for the kinase reaction (De Bondt et al., 1993; Jeffrey et al., 1995).

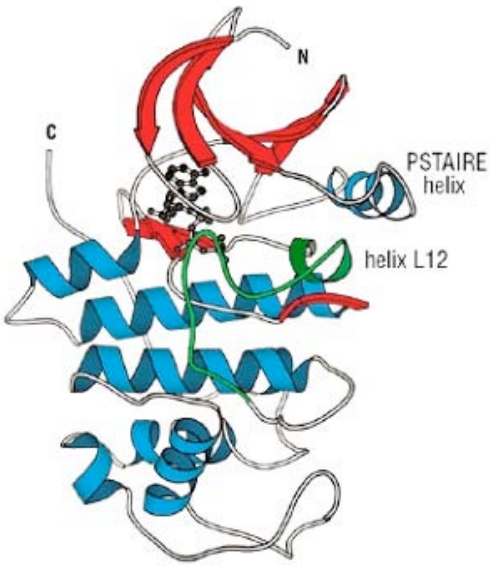


Figure 1.7 Structure of human Cdk2, determined by X-ray crystallography. The amino-terminal lobe (N) contains primarily of beta sheet (red arrows) and the PSTAIRE helix, and the large carboxyl-terminal lobe (C) is primarily composed alpha helices (blue). The ATP substrate is represented as a ball-and-stick model. The T-loop is highlighted in green. A small alpha helix helix L12 (green) adjacent to the T-loop modifies the conformation when cyclin binding. (David O. Morgan, 2007).

1.2.1.2 Cyclin degradation

Cell cycle regulated abundance of the subunits of CDK is an important factor for forming the complex at a right time through cell cycle progression. The expression levels of CDK1 gene and also CKS1 are kept constant during the cell cycle. Cdk1 and Cks1 therefore are always available for being in complex with cyclins. The crucial regulation is dependent on the regulatory subunit, cyclins as they are synthesized and degraded in a cell cycle regulated manner.

One control for cyclin synthesis is through well controlled transcriptional system which supplies the mRNA template for its translation (see section 1.1.3). Cyclin abundance is controlled not only through transcriptional regulation but also through regulation of their degradation. Different cyclins are tightly control through different ubiquitin mediated proteolysis mechanisms (Deshaies, 1997). The two major ubiquitin ligase complexes involved are SCF (Skp1, Cdc53/Cullin, F-box containing complex) and the APC (Anaphase Promoting Complex). The SCF and APC complexes are E3 ubiquitin ligases that target substrates with polyubiquitin on lysine residues for degradation by the 26S proteasome (Steven I Reed, 2003) (Figure 1.7).

G1/S cyclins Cln1 and Cln2 are unstable in G1, and their degradation is mediated by SCF complexes (Lanker, Valdivieso, & Wittenberg, 1996; Schneider et al., 1998). SCF is active throughout the cell cycle, and the destruction signal of individual substrates for SCF

system is the phosphorylation state on degradation sites (degrons). Different F box proteins (Cdc4, Grr1) bound to the phosphate of the substrate and confer substrate specificity (Rana et al., 2004). In particular for Cln2, after the self regulatory autophosphorylation of degradation sites, a PEST motif (Proline, Glutamic, Serine, Threonine rich sequence) is exposed and recognized by F-box protein Grr1 (Lanker et al., 1996). The adaptor protein Skp1 together with Cullin like protein Cdc53 recruits the phosphate binding Grr1, which then guide the substrate Cln2 to poly-ubiquitilation and finally 26S protease mediated degradation. Ubiquitilation of Cln3 is mediated by both SCF-Cdc4 and SCF-Grr1 in a similar mechanism as degradation of Cln2 (Landry, Doyle, Toczyski, & Benanti, 2012; Li et al., 2006).

In addition to Clns, B-type cyclin Clb6 but not Clb5, is also targeted by SCF-Cdc4 complexes (Jackson et al., 2006). The other B-type cyclins (mitosis cyclins and Clb5) are degraded by the Anaphase-Promoting Complex (APC, also known as Cyclosome). Unlike the SCF ubiquitin ligase complexes, APC is not active throughout the cell cycle, and it is not active until associates with one of the two coactivator subunits (Cdc20 and Cdh1) which are recruited at different times of mitosis and then confer the substrates specificity (Visintin, Prinz, & Amon, 1997). During the early mitosis, APC is activated in complex with Cdc20 and targets Clb5 and the mitotic cyclins for degradation (Shirayama, Toth, Galova, & Nasmyth, 1999; Wäsch & Cross, 2002). In late mitosis, the APC exchanges the adaptor protein Cdc20 for Cdh1 to change the set of substrates which allowed the complete degradation of the mitotic cyclins and cells enter cytokinesis.

1.2.1.3 Cdk1kinase subunit Cks1

In addition to the regulatory subunit cyclin, a evolutionarily conserved CDK small subunit Cks1 in budding yeast has been identified directly bound to CDK with high affinity (Hadwiger, Wittenberg, Mendenhall, & Reed, 1989; S I Reed, Hadwiger, Richardson, &

Wittenberg, 1989). Cdk kinase subunit (Cks) proteins are highly conserved in all eukaryotes and play essential roles cell cycle control (Pines, 1996). However, their regulation on CDK activity and the biological function mechanism are still largely unknown.

Cks1 (molecular weight 18 KD) in budding yeast is the largest Cks protein discovered so far. The essential functions of Cks proteins in cell cycle progression have been shown through genetic and biochemical experiments, including the function involving in G1/S phase and G2/M phase transitions (Tang & Reed, 1993) as well as transcriptional regulation (Yu, Baskerville, Grünenfelder, & Reed, 2005). Recent research has proposed a clear role of Cks1 as a phosphoadaptor for regulating CDK activity. Cks recognizes and interacts with specific phosphorylated sequences in CDK substrates, which is required for efficient multisite phosphorylation of a large subset of CDK substrates and also for budding-yeast viability (Kõivomägi et al., 2013; McGrath et al., 2013).

1.2.2 CDK regulation by phosphorylation

Transcriptional and translational regulation of Cdk1 has not been considered important through the cell cycle. However the posttranslational regulation of Cdk1 is crucial to govern CDK activity in addition to regulation by cyclins. Cyclin dependent kinase have two different phosphorylation sites: one activating phosphorylation site on the T loop, and one (or two) inhibitory phosphorylation site(s) near the ATP binding site (more details will be discuss in the following sections). Apart from the phosphorylatary modification, Cdk1 was recently found to be acetylated on lysine 40 (K40) close to the kinase domain (PSTAIRE helix) (Choudhary et al., 2009), but the function of Cdk1 acetylation is still unknown.

1.2.2.1 Thr169 activating phosphorylation by Cak1

It is well known that most kinases trigger their activation by autophosphorylating a site in their T-loop. However this is not the case for cyclin dependent kinase activation. Instead, the T-loop phosphorylation is carried out through another kinase, cyclin dependent kinase activating kinases (CAKs), Cak1 in budding yeast (Kaldis, Sutton, & Solomon, 1996; Solomon, Lee, & Kirschner, 1992) (Figure 1.6). For full activation, CDKs require not only cyclin binding, but also the phosphorylation on T-loop by CAKs. In budding yeast, Cak1 phosphorylates Thr169 equivalent to Thr161 in fission yeast and human Cdk2-Thr160. Phosphorylation in this site help to rearrange T-loop position to release the space for substrates binding and also change the residues position for ATP binding (Jeffrey et al., 1995; A. A. Russo, Jeffrey, & Pavletich, 1996).

Surprisingly, CAK activity is not cell cycle regulated (Kaldis et al., 1998), however it is essential for CDKs activation as deletion of Cak1 in budding yeast causes lethality (Kaldis et al., 1996). The cell cycle independent regulation of Cak1 indicates that binding of cyclins is the main determinant of Cdk1 activity. Moreover, unlike in higher eukaryotes, the activating phosphorylation of Cdk1 precedes cyclin binding in yeast, which is known from the fact that a non-phosphorylatable Cdk1 (Cdk1-T169A) associates with cyclin less efficiently compared to wild type Cdk1 in immunoprecipitation (Ross, Kaldis, & Solomon, 2000).

1.2.2.2 Tyr19 inhibitory phosphorylation by Swe1

In addition to the activating phosphorylation on T-loop, CDK is also regulated by inhibitory phosphorylation. In budding yeast, inhibitory phoshorylation takes place at a single conserved Tyr19 (Y19) residue which is regulated by kinase Swe1 and phosphotase Mih1. Swe1 (Wee1 in S. pombe) blocks cells entry into mitosis through Cdk1 inhibitory phosphorylation on Y19 (Y15 in S. pombe) (Booher, Deshaies, & Kirschner, 1993; Sorger &

Murray, 1992) and this phosphorylation is reversed by the protein phosphatase Mih1 (Cdc25 in S. pombe) (Russell, Moreno, & Reed, 1989) (Figure 1.6). The inhibitory phosphorylation is highly conserved. In fission yeast S. pombe, this inhibitory phosphorylation on Y15 is catalyzed by two kinases, Wee1 and the redundant Mik1 (Lundgren et al., 1991). In mammalian cells, there are two Cdk1 inhibitory phosphorylation sites, Y15 and the adjacent T14. The phosphorylations are catalyzed by Wee1 on Tyr15 and Myt 1 on both Thr14 and Tyr15 (Booher, Holman, & Fattaey, 1997; F. Liu, Stanton, Wu, & Piwnica-Worms, 1997; McGowan & Russell, 1993; Parker & Piwnica-Worms, 1992). At the G2/M transition, the Cdk1 inhibitory phosphorylation is reversed by the phosphatase Mih1 (Cdc25 in S. pombe and human), which results in an abrupt activation of Cdk1 and cell cycle progression into mitosis (Gautier, Solomon, Booher, Bazan, & Kirschner, 1991; Lundgren et al., 1991).

Apart from the essential role of Cdk1 inhibitory phosphorylation in unperturbed cell cycle progression, it has a important function in response to genotoxic stress. It's well known in fission yeast that in response to genotoxic stress the S phase checkpoint blocks M-CDK activity by regulating the balance of the wee1/cdc25 activites on Tyr15 phosphorylation (Furnari, Rhind, & Russell, 1997; Rhind, Furnari, & Russell, 1997). This tyrosine phosphorylation regulation of Cdk1 is considered as a universal mechanism for mitosis entry which is conserved in S. pombe, Xenopus, chicken and human cells (Draetta et al., 1988; Gould & Nurse, 1989; Krek & Nigg, 1991; Solomon, Glotzer, Lee, Philippe, & Kirschner, 1990). But in budding yeast the role of M-CDK control in normal cell cycle, or in response to genotoxic stress, seems independent of Cdk1 tyrosine phosphorylation from the fact that mutations preventing Cdk1 phosphorylation by Swe1 do not lead to premature mitosis (Amon, Surana, Muroff, & Nasmyth, 1992; Sorger & Murray, 1992). Despite Cdk1-Y19 nonphosphorylatable mutants do not show premature mitosis, it can not exclude that tyrosine phosphorylation of Cdk1 regulation is important for M-CDK control. Because Swe1 kinase

which is normally eliminated at the G2/M transition, is stabilized in the presence of genotoxic stress (H. Liu & Wang, 2006). This fact suggests that the tyrosine phosphorylation is indeed required in response to genotoxic stress in S. cerevisiae. It rather indicates that an additional, redundant control operates in budding yeast together with Cdk1-Y19 phosphorylation regulation.

1.2.3 CDK inhibitors (CKIs)

The third way to regulate CDK activity is mediated by CDK inhibitors (CKIs) which bind and inactivate cyclin-Cdk complexes (Figure 1.6).

In human cells, based on their structure homology and affinity on different CDKs and cyclins, CDK inhibitors (CKIs) can be divided into two families: INK4 (INhibitors of Cdk4) and Cip/Kip (CDK interacting protein/Kinase inhibitory protein) (Sherr & Roberts, 1999). The INK4 family members p16 (INKa), p15 (INK4b), p18 (INK4c) and p19 (INK4d) exclusively affect the activity of D-type cyclin-dependent kinases (CDK4 and CDK6) (Guan et al., 1994; Hannon & Beach, 1994; Hirai, Roussel, Kato, Ashmun, & Sherr, 1995; Serrano, Hannon, & Beach, 1993; Sherr & Roberts, 1995). The INK4 binds to Cdk4/6 and inhibit CDK activity by forcing the CDK subunit rearange into a conformation with less affinity to cyclin (Brotherton et al., 1998).

The CIP/KIP family members p21 (CIP1/WAF1), p27 (KIP1) and p57 (KIP2) are able to inhibit the activity of a broader spectrum of cyclin-CDK complexes with higher specificity to the G1 and S phase CDKs (Harper, Adami, Wei, Keyomarsi, & Elledge, 1993; M. H. Lee, Reynisdóttir, & Massagué, 1995; Polyak, Kato, et al., 1994; Xiong, Hannon, et al., 1993). Unlike INK4 family, CIP/KIP can bind both cyclin and Cdk, determinded by their cyclin binding motif and CDK binding motif (Chen, Jackson, Kirschner, & Dutta, 1995; Luo, Hurwitz, & Massagué, 1995). CKIs are important regulators in cell cycle control. Lack of

CKIs will lead to uncontrolled cell division and therefore promots tumorigenesis (Marcos Malumbres & Barbacid, 2009) (see section 1.2.3.3).

In budding yeast, there are mainly two known inhibitors for cyclin-Cdk1 complexes Far1 and Sic1. Far1 is an important regulator in the mating pathway, where haploid cells of opposite mating type synchronize their cell cycles by arresting at START in response to mating pheromone (Peter & Herskowitz, 1994). Far1 is able to inactivate Cln-CDK activity, and thus arrest the cell cycle at START in G1 (Peter & Herskowitz, 1994; Tyers & Futcher, 1993). Sic1 is important in regulating the cell cycle at G1/S transition and mitotic exit.

1.2.3.1 Far1and Sic1

FAR1 (Factor ARrest) was originally identified as a gene necessary for cell cycle arrest in response to mating pheromone alpha factor (Chang & Herskowitz, 1990). Far1 acts at the final step in the alpha-factor response pathway through binding to Cln-Cdk1 complex and thus directly inhibiting CDK activity (Peter & Herskowitz, 1994).

The other inhibitor in budding yeast, Sic1, is a specific Clb-Cdk1 inhibitor (Mendenhall, 1993) that plays a critical role in G1/S transition. In late G1 phase, Clb5/6-Cdk1 (S-CDK) complexes are tightly bound by Sic1 hence in an inhibited state. It was shown that the C terminal 70 amino acid fragment of Sic1 (residues 215 to 284, figure 1.8) is enough to function as a CDK inhibitory domain(Hodge & Mendenhall, 1999). While as G1 cyclin Cln1/2-Cdk1 complexes accumulate and phosphorylate Sic1 at multiple sites leading to its degradation via SCF-Cdc4 mediated ubiquitin-proteasome proteolysis thus releasing S-CDK activity (Figure 1.8) (Nash et al., 2001; Skowyra, Craig, Tyers, Elledge, & Harper, 1997; Verma, Annan, et al., 1997). Sic1 needs to be phosphorylated at least any six of the nine CDK consensus S/TP sites for binding to Cdc4 and triggering degradation (Figure 1.8) (Nash et al., 2001; Verma, Annan, et al., 1997). in vitro (Verma, Feldman et al. 1997).

In fission yeast, Rum1 is proposed to be a functional homolog of Sic1 and is the only CKI described to date (Sánchez-Díaz, González, Arellano, & Moreno, 1998). In mammalian cells, CDK inhibitor p27/Kip1 is structurally and functionally related to Sic1(Barberis et al., 2005). Similarly, p27/Kip1 plays a critical role preventing the premature onset of DNA replication by inhibiting cyclin A-Cdk2 complexes as cyclin A is expressed in G1(Barberis, 2012; Barberis et al., 2005).

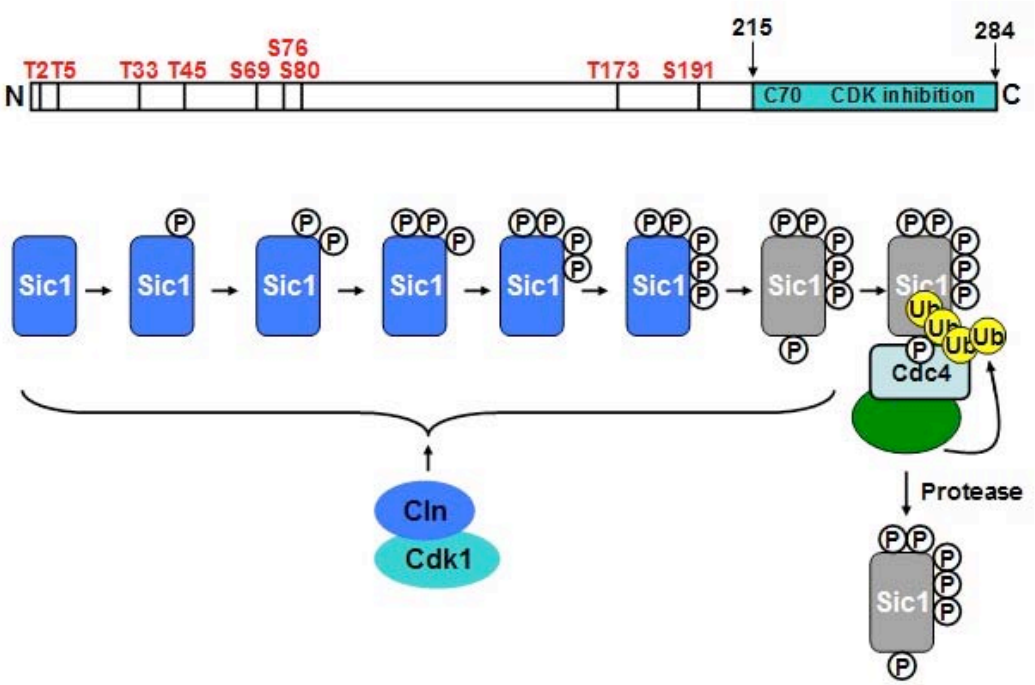


Figure 1.8 Schematic model of the cyclin-dependent kinase inhibitor Sic1 degradation. The upper diagram represents the CDK phosphorylation sites and the CDK interaction motif. Sic1 has nine CDK phophorylation sites: T2, T5, T33, T45, T48, S69, S76, S80, T173, and S191, all shown in red. The truncated, inhibitory version of Sic1, Sic1C70 is shown in light blue. Sic1 needs at least six sites phosphorylation by Cln-Cdk1 followed by the degradation through SCF.

Because cyclins lack sequence similarity of between species, it is difficult to identify the CDK inhibitors by sequence homology search. Sic1 only shares 33% identity with Rum1 in fission yeast and 21% with p27/Kip1 in humans. In budding yeast Sic1 is the only CKI

identified so far involving in cell cycle progression regulation. It prevents the premature onset of DNA replication by inhibiting S-CDK complexes as S phase cyclins are expressed in G1. However, no specific cell cycle inhibitor of G1-CDK activity has been identified in budding yeast. And yet, cells should be able to respond to changes that challenge the upcoming round of chromosome replication, such as a sudden change in nutrient availability, once START has been crossed and G1 cyclins are already being expressed and accumulated.

1.2.3.2 CKIs and cancer

Unlike single CDK regulation in budding yeast, the human cell cycle is controlled by a subfamily of cyclin-dependent kinases (CDKs), the regulation of which is more complex through cyclin binding, phosphorylation as well as inhibitors. The famous CDK inhibitors (CKIs) in human cells are Ink4 family (INK4A, INK4B, INK4C and INK4D), and Cip/Kip family (p21, p27 and p57) which were identified as tumor suppressors in 1990s (Guan et al., 1994; Matsuoka et al., 1995; Polyak, Kato, et al., 1994; Polyak, Lee, et al., 1994; Xiong, Zhang, & Beach, 1993; Xiong, Hannon, et al., 1993). The function of Cip/Kip family is far beyond cyclin dependent kinase inhibitors. One important role is the regulation of transcription as a regulator apart from the inhibitor of CDKs in cell cycle control.

Deregulation of cell cycle is considered as one and the most crucial one of the first steps for tumor cells transformation. The activity of CDKs is deregulated in cancer cells owing to changes on the pathways that control the CDK activity, such as modifications on CDK inhibitors (CKIs). In many cancers, Inactivation of CKIs, which leads to deregulated CDK activity and impede cell cycle progression, results in uncontrolled cell proliferation (M Malumbres & Barbacid, 2001). Usually it is the proteins which control G1/S transition that are commonly deregulated, such as the tumor suppressor protein Rb (Whi5 in budding yeast) (more detail in section 1.1.3.1). Hypophosphorylated Rb in complex with E2F acts as a

growth suppressor and blocks the transcription of G1/S clusters thus prevents S phase entry (a similar mechanism in budding yeast discussed in section 1.1.3.1) (Hiebert, Chellappan, Horowitz, & Nevins, 1992). When it is time for cells to enter S phase, cyclin D-Cdk4/6 and cyclin E-Cdk2 phosphorylate Rb, which results in disassociation of Rb from E2F and hence activation of the E2F dependent transcription.

There are multiple mechanisms of Rb inactivation in several major cancers (Murphree & Benedict, 1984). Apart from direct mutations of Rb gene, in a large set of cancers Rb protein is indirectly down regulated through hyperactivation of CDK activity, such as loss function of CKI.

1.3 CELL CYCLE CHECKPOINTS

As discussed in above chapter, the eukaryote cell cycle is an ordered set of events where late events tightly depend on the completion of early events. For maintaining the defined sequence of events and keeping genomic stability, eukaryote cells have developed a surveillance mechanism known as checkpoints that was first scientifically proved by Leland Hartwell: Checkpoints, controls that ensure the order of cell cycle events (L H Hartwell & Weinert, 1989). Checkpoints follow the transduction cascades signal formed by sensor elements, transducers and central kinase effectors. These kinases transmit and amplify the signal, act on different target proteins and block the progression of the cycle (Weinert & Hartwell, 1988). Multiple checkpoints in eukaryotes have been identified at various stages of the cell cycle which ensure that later cellular events are not triggered until previous ones complete. For instance, when DNA damage is present, to ensure the integrity of chromosomes replication in S phase before segregation initiation in M phase, one cell cycle checkpoint blocks entry into mitosis until the problems are solved. The first checkpoint gene RAD9, was discovered in *Saccharomyces cerevisiae* by Leland Hartwell in search of

radiation sensitive mutants (Weinert & Hartwell, 1988). Radiation sensitive mutant rad9 was shown to arrest in the G2 phase in response to ionizing radiation as well as DNA damage resulting from incomplete replication (L H Hartwell & Weinert, 1989; Weinert & Hartwell, 1988).

Actually the environment of cell growth and division is rarely ideal and precise duplication of eukaryotic genome is full of challenge. Eukaryotic cells have developed multiple checkpoints to preserve both viability and genome integrity. Lack of checkpoint genes can lead to mutations accumulation and genomic instability which disrupt normal growth control and eventually promote carcinogenesis (L H Hartwell & Kastan, 1994; Kolodner, Putnam, & Myung, 2002).

1.3.1 S phase checkpoint and Rad53 dependent response

Cells are constantly under the pressure of endogenous or exogenous agents that cause DNA damage or interfere with DNA replication. When cells have DNA damage or DNA replication stress in S phase, cells activate the crucial surveillance mechanism, the S phase checkpoint that arrests cell cycle progression and facilitate DNA repair pathways until the problems are solved. Although DNA damage happen at any stage of the cell cycle, the S phase is the most vulnerable period to the genotoxic stress because the replicating chromosome and double-stranded DNA are more accessible to the genotoxic agents (B.-B. S. Zhou & Bartek, 2004). Moreover, during the process of DNA synthesis unrepairable damage could turn into a permanent mutation.

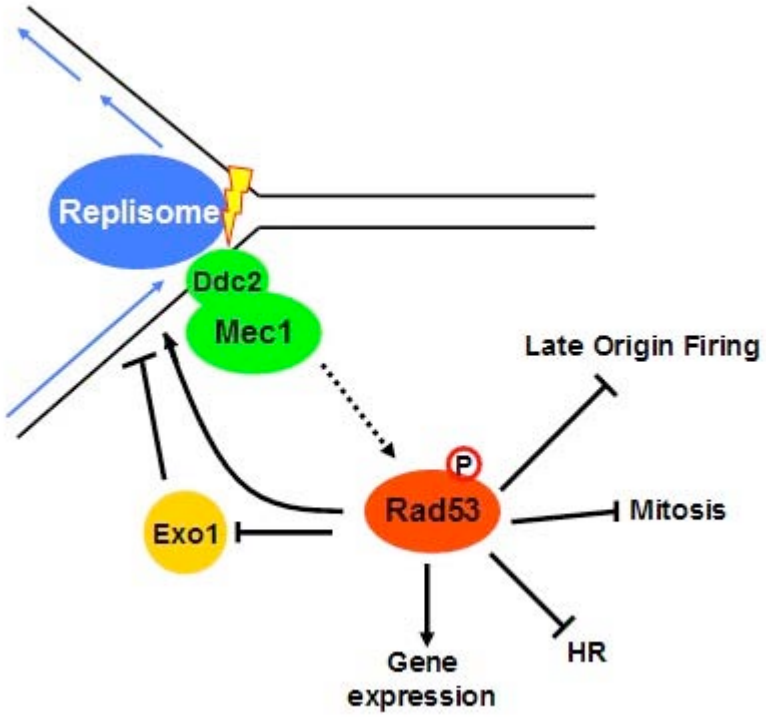


Figure 1.9 Schematic model of Rad53 dependent S phase checkpoint response. When cells are under conditions that threaten DNA replication, such as DNA replication stress or DNA damage, Mec1-Rad53 mediated S phase checkpoint pathway gets activated. Ddc2-Mec1 dependent activation of downstream effector kinase Rad53 stabilizes the DNA replication forks through Exo1 dependent or independent manner, inhibits the late origin firing, activates gene expression and prevents cells entry into mitosis and unscheduled recombination (HR) until the problems are solved.

The backbone elements and pathways that constitute the S phase checkpoint in eukaryotic cells are fairly understood. In the budding yeast *Saccharomyces cerevisiae* replication forks stalled due to damaged DNA or upon replication stress, lead to the activation of the central transducer protein kinases Mec1 and Tel1 (Sanchez et al., 1996), the orthologs of human ATM and ATR. Mec1, in turn, activates the effector protein kinases Chk1 and Rad53 (Sanchez et al., 1999), the orthologs of human Chk1 and Chk2, respectively. The checkpoint response includes the stabilization of stalled replisomes, suppression of

recombination at arrested replication forks, block of yet unfired chromosomal origins of replication (Santocanale & Diffley, 1998; Tercero & Diffley, 2001), preventing cells entry into mitosis and segregation of sister chromatids (Cohen-Fix & Koshland, 1997) and also activating a transcriptional response characterized by the induction of ribonucleotide reductase genes to counteract the stress (Z. Zhou & Elledge, 1993) (Figure 1.9), the more details will be discussed in the following section (section 1.3.2).

The checkpoint effector kinases Chk1 and Rad53 in S phase are members of the family of serine threonine kinases CHK which are evolutionarily conserved (Melo & Toczyski, 2002). The center kinase effector of S phase checkpoint in budding yeast S. cerevisiae is Rad53. Rad53 gets activated through direct phosphorylation by upstream kinase Mec1 and also autophosphorylation (Ma, Lee, Duong, & Stern, 2006; Pellicioli et al., 1999). In response to genotoxic stress activated Rad53 is recruited to the damaged site, where through interaction with the corresponding adapter Rad9 and Mrc1(Pellicioli et al., 1999; Sanchez et al., 1999).

S. cerevisiae mec1 and rad53 mutants show a strong sensitivity to all type of DNA damage or DNA replication stress (Allen, Zhou, Siede, Friedberg, & Elledge, 1994). Cells lacking Rad53 cause multiple defects, including impaired checkpoint activation, problems of replication fork stall recovery, and also excess histone stress (Allen et al., 1994; Gunjan & Verreault, 2003; Sidorova & Breeden, 1997).

In humans, mutations in S phase checkpoint pathway kinases like ATM/ATR lead to familial cancer syndromes, such as Ataxia telangiectasia (AT, also referred to as Louis–Bar syndrome) (Boder, 1985). Mutations in the human tumor suppressor CHK2 (ortholog of Rad53) cause familial breast cancer and Li-Fraumeni syndrome (Bell et al., 1999; Shaag et al., 2005). It is well accepted that the S phase checkpoint is an anti-cancer barrier in the

beginning of the process of tumorigenesis (Bartkova, Bakkenist, et al., 2005; Bartkova, Horejsí, et al., 2005).

1.3.2 Rad53 dependent gene expression

The S phase checkpoint response is mediated through a complex protein networks that tranduce the detected problems and regulate multiple cellular processes (see section 1.3.1). Among these proteins, the checkpoint kinases Mec1/Tel1, Rad53 play essential roles in the networks. Mec1 and Tel1 are involved in the upstream of the pathway, sensing the damaged DNA or stalled replication stress, while Rad53 functions as a centrol effector kinase to regulate the downstream response processes including gene expression.

1.3.2.1 The Rad53 protein kinase

Rad53 is an essential protein kinase to the S phase checkpoint response. It was named as the mutant is extremely sensitive to X-rays and bleomycin in the screen (C. W. Moore, 1978). Rad53 protein contains a central serine/threonine kinase domain which is flanked by a ForkHead Associated 1 (FHA1) domain in N-terminal and a C-terminal FHA2 domain (Durocher, Henckel, Fersht, & Jackson, 1999). The FHA domain is a phospho-specific protein-protein interaction motif which has been found in many signaling proteins (Hofmann & Bucher, 1995). The FHA domains of Rad53 are shown to mediate the interaction of Rad53 with both upstream and downstream of the DNA checkpoint signaling pathways (Schwartz, Lee, Duong, Eminaga, & Stern, n.d.; Smolka et al., 2006). For example, DNA damage triggered Rad9-dependent activation of Rad53 is mediated via binding to the upstream Rad9 through the FHA domains of Rad53. The phosphorylation-dependent association of Rad53 with the chromatin assembly factor Asf1 is also mediated through FHA1 of Rad53 (Emili, Schieltz, Yates, & Hartwell, 2001).

Apart from the roles in S phase checkpoint response, Rad53 has an essential role in DNA replication of unperturbed cell cycle, since deletion of Rad53 leads to cell lethality. However this lethality can be avoided by increasing the level of deoxyribonucleotides (dNTP) which is simply achieved by either overexpression of the large catalytic subunit of the ribonucleotide reductase (Rnr1) or by deletion of SML1, a gene encoding an inhibitor of Rnr1 (Huang, Zhou, & Elledge, 1998; Sanchez et al., 1996; X Zhao, Muller, & Rothstein, 1998). Additionally, Rad53 is also involved in the regulation of polarized cell growth in response to DNA replication stress with the fact that *rad53*Δ cells shows a elongated buds but this phenotype is not readily observed in WT cells nor in unstressed *rad53*Δ cells (Smolka et al., 2006).

1.3.2.2 Rad53 regulated transcription

To ensure the genomic stability in the face of genotoxic stress including DNA replication stress and DNA damage, cells activate checkpoint pathway where the central effector kinase Rad53 triggers several downstream events (discussed in section 1.3.1). Among these response, Rad53 reprogrammes the gene expression including the induction of genes involved in DNA replication and repair, thereby promotes to solve the problems (Bastos de Oliveira, Harris, Brazauskas, de Bruin, & Smolka, 2012; Jaehnig, Kuo, Hombauer, Ideker, & Kolodner, 2013; Travesa et al., 2012; Z. Zhou & Elledge, 1993). The reprogramme of gene expression is crucial for genomic stability; however the mechanism is still largely unknown.

One of the response transcription pathways is Rad53 mediated Dun1 regulation. In response to genotoxic stress, Rad53 activates Dun1 kinase, which subsequently phosphorylates and inactivates the transcriptional repressor Crt1, thereby upregulates expression of genes including those encoding ribonucleotide reductase (Rnr2, Rnr3, Rnr4) (Huang et al., 1998; Z. Zhou & Elledge, 1993). But the large Rnr1 subunit is not controlled

by Crt1. It is inactive until it is phosphorylated by Dun1 and released from the ribonucleotide reductase inhibitor Sml1(X Zhao et al., 1998). These RNR enzymes catalyze the ribonucleotides (NTPs) to deoxyribonucleotides (dNTPs) as the materials for DNA synthesis and repair (Elledge, Zhou, & Allen, 1992; Xiaolan Zhao & Rothstein, 2002). It therefore suggests that the essential role of Rad53 in unperturbed S phase is to promote and monitors to synthesis enough dNTP for DNA synthesis. Apart from the dNTP for chromatin duplication, Rad53 is also found to regulate the level of histone which is another crucial material for chromatin assembly (Gunjan & Verreault, 2003). Rad53 monitors the excess histones by direct binding to them and mediated via its kinase activity (Gunjan & Verreault, 2003).

The most important role of RAD53 is the S phase checkpoint effector in the response pathway. In recent years, the phosphorylation state of Rad53 in response to different genotoxic insults has been studied (Albuquerque et al., 2008; Smolka et al., 2005).

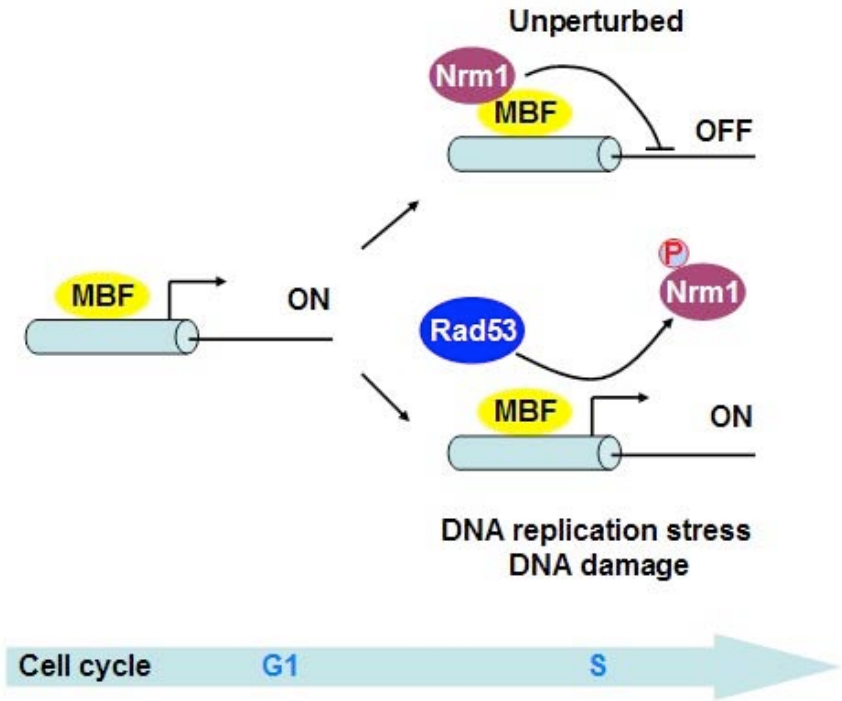


Figure 1.10 DNA replication stress regulates MBF targets via Rad53-dependent inactivation of Nrm1. The MBF-dependent transcription is inactivated (OFF) as cells enter S phase by the corepressor Nrm1 in complex with MBF. In response to DNA replication or DNA damage, Rad53 is activated and phosphorylates Nrm1 which leads to the release of Nrm1 from the promoter and hence activates the MBF-dependent transcription.

Apart from Rad53-Dun1-Crt1 pathway response to replication stress, another parallel Rad53-Nrm1-MBF de-repression pathway represents a larger part of the checkpoint transcriptional response (R A M de Bruin et al., 2008; Travesa et al., 2012).

Genome-wide expression analysis of cells treated with different methyl methane sulfonate (MMS), hydroxyurea (HU) or camptothecin (CPT) shows that S phase checkpoint specifically induces MBF-regulated genes but not SBF-regulated genes (Bastos de Oliveira et al., 2012; Travesa et al., 2012). Most of the MBF targets strong upregulation is mediated via Rad53-dependent inactivation of the MBF co-repressor Nrm1(R A M de Bruin et al., 2008; Travesa et al., 2012) (Figure 1.10). As we discussed in section 1.1.3.1, in unperturbed cell cycle progression, MBF is a main transcription factor to regulate the gene transcription in G1/S transition. And Nrm1 behaves as a corepressor in complex with MBF to inactivate the transcription in S phase (Figure 1.5 and Figure 1.10). Unlike Rad53-Dun1-Crt1 pathway, the MBF reactivation is also conserved in fission yeast where Yox1 is also required in addition to Nrm1(Caetano, Klier, & de Bruin, 2011; R A M de Bruin et al., 2008).

More than these well defined mechanisms, Rad53 mediated transcription regulation in response to genotoxic stress still largely unknown. A recent genome-wide expression analysis have mapped a global transcriptional regulatory between checkpoint kinases and downstream transcription factors (TFs) (Jaehnig et al., 2013). This network includes nine TFs (Swi6, Gcn4 Ndd1, Msn4, Mcm1, Fkh2, Mbp1, Swi4 and Swi6), many of which have Rad53-dependent phosphorylation sites, as regulators in response to genotoxic stress. Still, Rad53

dependent regulatory circuit represents only a small part of the checkpoint transcriptional response. There are many other checkpoint kinases and kinase-independent transcription factors play roles in the complex networks.

2 OBJECTIVES

Cyclin dependent kinase Cdk1 drives cell cycle progression in the model eukaryotic organism *Saccharomyces cerevisiae* (budding yeast). Cdk1 is tightly regulated by the binding of activatory partners (cyclins), regulatory subunits (Cks1) and Cyclin dependent Kinase Inhibitors (CKIs), and also by activatory and inhibitory phosphorylations. However, many details on Cdk1 regulation, such as how G1 or mitotic CDK activities are inhibited in response to conditions that threaten genomic integrity, remain unknown.

The main objective of the PhD work presented herein is **to identify novel regulation modes of the cell cycle master kinase Cdk1, in budding yeast**.

More specifically, our work is aimed at adressing the following specific objectives:

- To study Cdk1 regulation by phosphorylation, with a specific interest in changes mediated by the S phase checkpoint effector kinase Rad53 in response to genotoxic stress.

- To try and identify novel Cdk1 regulatory associated factors, in particular as part of the S phase checkpoint response to genotoxic stress.

- To provide insight on how the budding yeast *Saccharomyces cerevisiae* regulates the window of time comprised from Start to the onset of S phase, and progression into M phase, in response to genotoxic insults.

3 MATERIALS AND METHODS

3.1 YEAST GROWTH AND MANIPULATION

3.1.1 *Saccharomyces cerevisiae* genetic background

In this study, budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) was used as a model organism for the purpose of experiments. Among many normal standard strains, budding yeast *Saccharomyces cerevisiae* with the genetic background of W303 was used (Thomas & Rothstein, 1989).

W303 has been modified in different genes from the true wile type strain in nature for purpose of easier genetic manipulation. Firstly, to make the strain stay in MATa or MATalpha haploid, the HO gene which encodes an endonuclease responsible for initiating mating-type switching (Kostriken, Strathern, Klar, Hicks, & Heffron, 1983) was mutated to avoid the spontaneous generation of diploid cells (Nasmyth, 1983). Secondly, W303 was modified with point mutation in five essential genes, each involved in essential nutrients synthetic pathways as shown below, *ade2-1* (defect for adenine synthesis), *his3-11, 15* (defect for histidine synthesis), *leu2-3, 112* (defect for leucine synthesis), *trp1-1* (defect for trptophan synthesis), and *ura3-1* (defect for synthesis of the nucleobase uracil,). Since the defect in essential nutrients, the strain will not grow in a selective medium in the absence of the corresponding amino acid or nucleobase unless the wild type gene was introduced which restores the ability of mutated auxotrophic marker gene in the strain. This profile made the selection of transformants highly probable.

Moreover, our strains were made from Mat a haploid strains, W303-1a, which allows the synchronization of cells in G1 phase by adding pheromone alpha factor. The alpha factor is a kind of peptide pheromone which is secreted by Mat α strains to arrest Mat a type of haploid cells at pre-START. The extraneous peptide Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr was used to synchronize Mat a cells in G1-phase in the absence of Mat α cells. There is a protease gene Bar1 which helps cells find mating partners, cleaves and

inactivates alpha factor allowing cells to recover from α-factor induced cell cycle arrest (Barkai, Rose, & Wingreen, 1998; Chan & Otte, 1982; Ciejek & Thorner, 1979). In order to use alpha factor efficiently, we deleted Bar1 gene in W303-1a strain, which made cells sensitive to even one hundred times lower alpha factor concentrations.

3.1.2 Yeast culture media

Yeast rich medium YP (1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) sugar as carbon source) was used in the experiments. The sugar was either glucose (YPD), raffinose (YPRaf) or galactose (YPGal) accordingly. For solid medium, agar was added to 2% (p/v).

Synthetic Dextrose (SD) Minimal Medium: 0.67% (p/v) Yeast Nitrogen Base (YNB), 2% (p/v) Glucose, 40 μg/ml Adenine (Ade), Uracil (Ura), Leucine (Leu), Tryptophan (Trp) and Histidine (His). For solid medium, agar was added to 2% (p/v).

5-FOA medium used for counter-selection of URA3 cassette: 1 mg/ml 5-fluoroorotic (5-FOA) in SC (Synthetic Complete) medium, 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, 2% (p/v) agar.

3.1.3 Yeast transformation

Our yeast transformation in this study is modified from Lithium method (Ito, Fukuda, Murata, & Kimura, 1983). It is used to transform the competent yeast cells with genetic material of interest by heat shock after weakening of the cell wall.

For each transformation, $1 \cdot 10^8$ cells in exponential growth were pelleted 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 with sterile water to get rid of all the medium, and then washed once with sterile 0.1 M lithium acetate/TE prepared fresh from sterile 10X stocks (stock of 1 M lithium acetate pH 7.5; 10 x TE pH 7.5), later

resuspended in the same buffer at a concentration of $2 \cdot 10^9$ cells/ml. Around 1 mg of DNA to be transformed was added to 50 µL of this cell suspension, mixed with 5 µL of 10 mg/ml ssDNA (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 at most few hours or directly followed by addition 300 µl of Li/PEG. (Prepare Li/PEG freshly from 1 volume of 1 M LiAc pH 7.5, 1 volume of 10 x TE, and 8 volumes of 50% PEG 4000). High weight polyethylene molecules here are used as crowding agent to occupy the space in the mixture, increasing the encounter between DNA and cells. The mixture was vortexted at low speed and then incubated at 30°C for 30 minutes mixing occasionally if unshaken (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 the mixture was vortexed gently but thoroughly at medium power. The cells were then put for heat shock at 42°C for15 minutes, followed by 60 seconds on ice. Cells were centrifuged for 2 minutes, and pellets were resuspended in a convenient volume of sterile TE pH 7.5 and plated on appropriate selective medium plates.

The transformants need to be checked by genomic PCR, functional assay or western blotting accordingly.

3.1.4 Yeast Genomic DNA Extraction

In this study, genomic DNA from yeast was extracted using phenol/chloroform/isoamyl alcohol (25:24:1). Briefly, 1 ml of overnight culture was pelleted and resuspended into lysis buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.0, and 1 mM EDTA, 0.1% (v/v) SDS). After resuspention, 400 µl glass beads and 400 µl of phenol/chloroform/isoamyl alcohol (25:24:1) were added. The mixture was vortexed at high speed for 90 seconds to extract the cells.

Mixture was then centrifuged for 3 minutes at 14000 RPM to separate the organic phase and the aqueous phase at room temperature. The aqueous solution was recovered and extracted again by adding 400 µl of phenol/chloroform/isoamyl alcohol (25:24:1) and vortexing and centrifuge as before. The aqueous phase was mixed with the same volume of isopropanol to precipitate DNA on ice for at least 15 minutes. Subsequently, the DNA was recovered by centrifuge at 14,000 RPM, for 10 minutes at room temperature. The DNA pellet was washed twice with 1 ml of ethanol 70% (v/v) and finally dissolved in 50 µL of TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 0.01 mg/ml RNase) at 37 ° C for 3 hours.

3.2 MOLECULAR BIOLOGY TECHNIQUES

3.2.1 Plasmids and constructs

3.2.1.1 pRS plasmids

The pRS series plasmids (Sikorski & Hieter, 1989) were used as the PCR template to amplify the genes of wild type auxotrophic markers (URA, HIS, TRP and LUE), in the process of generating W303 deletion mutants.

3.2.1.2 pFa6a plasmids

The pFA6-KanMX6 family of plasmids (Bahler et al., 1998) were used to generate strains with the target protein fused with a C-terminal tag. The pFA6a plasmids were used as the PCR template to amplify the product with the sequence of favorite tag and six copies of KanMX gene genetic in antibiotic resistance to geneticin G418. The tags used in this study were 13MYC (pFA6a-KanMX6-13MYC) and 3HA (pFA6a-KanMX6-3HA). pFA6-KanMX6 family was also used as the PCR template to amplify the KanMX gene in the process of generating W303 deletion mutants.

3.2.1.3 pGEX plasmid

pGEX are glutathione S-transferase (GST) gene fusion vectors which offer a Tac promoter for chemically inducible, high-level protein expression in *Ecoli*. In this study, we used pGEX-6P family containing a Prescission protease digestion site between GST and the target protein which was allowed to generate a recombinant protein without GST tagged.

3.2.1.4 Constructs used in this study

All constructs created and used in this study are listed in Table 3.1.

Table 3.1 List of constructs

Name	Description
pFL1	pGEX-6P-3-CDK1
pFL2	pGEX-6P-3-CKS1
pGQ10	pGEX-6P-1-RAD53
pAT28	pGEX-6P-1-Rad53(K227A)-kd
pFL15	pRS306-CDK1
pFL21	pRS306-CDK1-S46E,S258E
pFL24	pRS306-CDK1-S46A,S258A
pFL25	pRS306-CDK1-Y19F
pFL26	pRS306-CDK1-T18V
pFL27	pRS306-CDK1-T169V
pFL32	pRS306-CDK1-T18V,Y19F
pFL33	pRS306-CDK1-Y19F,T169V
pFL44	pGEX-6P-3-Cip1
pFL45	pRS306-Gal-Cip1-13MYC

3.2.2 Polymerase chain reaction (PCR) and DNA Electrophoresis

3.2.2.1 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) (Mullis & Faloona, 1987) was used to amplify DNA in vitro by using specific couple primers that define the sequence of interest required. In this study, we used KOD hot start DNA polymerase (Novagen) in PCR to delete genes, tag genes, clone sequences, and Taq polymerase in PCR to check yeast transformants.

For PCR using KOD kit, program of 50 µl reaction (shown in Table 3.2) was performed by GeneAmp PCR system 2400 (Applied Biosystem) as described below, following the initial polymerase activation and denature at 95ºC for 2 min, the reaction repeated for 30 cycles with each cycle the same program as denature at 95ºC for 20 secs, primer annealing at 55ºC for 10 sec and extension 30 sec/Kb DNA at 70ºC. Followed 30 cycles, there is 7 min whole extension at 70ºC to finish DNA synthesis.

Table 3.2 KOD PCR reaction components

Component	Volume
Template (around 200 ng/µl)	1 µl
Forward primer (25 µM)	1.5 µl
Reverse primer (25 µM)	1.5 µl
25 mM MgCl2	3 µl
dNTPs (2 mM each)	5 µl
10X PCR Buffer for KOD	5 µl
PCR Grade Water	32 µl
KOD polymerase (1 U/µl)	1 µl

For PCR using Taq polymerase, program of 50 µl reaction (shown in Table 3.3) was performed by PCR system as described below, following DNA denature at 94ºC for 2 min, the reaction repeated for 30 cycles with each cycle the same program as denature at 94ºC for 30 secs, primer annealing at 55ºC for 30 sec and extension 1 min/Kb of DNA at 72ºC. Followed 30 cycles, there is 7 min whole extension at 72ºC to finish DNA synthesis.

Table 3.3 Taq PCR reaction components

Component	Volume
Template (around 200 ng/µl)	1 µl
Forward primer (25 µM)	1 µl
Reverse primer (25 µM)	1 µl
dNTPs (2.5 mM each)	4 µl
10X Taq Buffer	5 µl
PCR Grade Water	37.8 µl
Taq polymerase (1 U/µl)	0.2 µl

Amplified PCR products were checked by agarose gel electrophoresis.

3.2.2.2 DNA electrophoresis in Agarose gel

Agarose gel electrophoresis was used to separate DNA fragments according to their linear size (Thorne, 1966). Gels were cast at 1% (w/v) agarose (agarose D-1 Low EEO-TQM, Pronadisa) in TAE buffer (50 mM Tris-acetic acid pH 8.5, 2 mM EDTA) with 0.5 µg/ml ethidium bromide (EtBr). Ethidium bromide is a fluorescent dye that intercalates into the nucleic acids during the electrophoresis, allowing visualization of DNA bands upon

illumination with UV light (365 nm) (Waring, 1965). Gels were run in TAE buffer at 8 V/cm of inter-electrode distance.

3.2.3 Site-directed DNA mutagenesis

The site-directed DNA mutagenesis of plasmid was modified based on Single-Primer Reactions IN Parallel (SPRINP) method (Edelheit, Hanukoglu, & Hanukoglu, 2009) using KOD hot start DNA polymerase (Novagen). Two parallel polymerase chain reactions (PCRs) were performed as shown in Table 3.4, with each reaction using only one primer (forward or reverse primer), designed to contain a expect mutation in the center of oligo. In this manner the annoying formation of tandem repeats of the region around the primers, due to strand displacement by the thermo polymerase, is avoided.

Table 3.4 Reaction setup for site-directed DNA mutagenesis of plasmid

	Reaction 1		Reaction 2	
	Volume	Final concentration	Volume	Final concentration
Construct template		20 ng/µl		20 ng/µl
Forward primer (25 µM)	1.6 µl	1.6 µM	-	-
Reverse primer (25 µM)	-	-	1.6 µl	1.6 µM
25 mM MgCl2	1.5 µl	0.2 mM	1.5 µl	0.2 mM
dNTPs (2 mM each)	2.5 µl	0.2 mM	2.5 µl	0.2 mM
10X PCR Buffer for KOD	2.5 µl	1X	2.5 µl	1X

PCR Grade Water	X µl		X µl	
KOD polymerase (1 U/µl)	0.5 µl	0.02 U/µl	0.5 µl	0.02 U/µl
Total reaction volume	25 µl		25 µl	

The 25 µl PCR program was performed by GeneAmp PCR system 2400 (Applied Biosystem) as described below, following the initial polymerase activation and denature at 95ºC for 5 min, the reaction repeated for 30 cycles with each cycle the same program as denature at 95ºC for 50 s, primer annealing at 55ºC for 50 s and extension 1min/Kb of construct DNA at 68ºC. Followed 30 cycles, there is 7 min whole extension at 68ºC to finish DNA synthesis.

- Polymerase activation and initial DNA denature: 5min, 95^{o}C

- 30 cycles:

- Denature (95^{o}C, 50 sec)

- Annealing (55^{o}C, 50 sec)

- Extension (68^{o}C, 1min/Kb construct DNA)

-7 min, 68^{o}C

The parallel PCR products were combined to a total volume of 50 µl and kept at 95ºC for 5 min to denature the construct DNA (both template and PCR product), then followed by gradually cooling to 37ºC for random annealing of denatured parental construct template and PCR products strands. The annealing program was also carried out by GeneAmp PCR system 2400 described as: 95 ^{o}C, 5 min; 90 ^{o}C, 1 min; 80 ^{o}C, 1min; 70 ^{o}C, 30 sec; 60 ^{o}C, 30 sec; 40 ^{o}C, 30 sec; 37 ^{o}C 1 min.

To digest the methylated parental plasmid strands, the annealed product was incubated at 37ºC O/N in 50 µl reaction in which, 45 µl annealed product, 5 µl NEB1 buffer

and 1.5 µl DPNI (20U/µl New England Biolabs) were mixed. The O/N product was transformed to DH5α competent cells. The plasmids from three random colonies were extracted using E poch Miniprep Kit. Mutations were confirmed by DNA sequencing.

3.2.4 Primers used in this study

All primers used in this study are listed in Table 3.5

Table 3.5 List of primers

Name	Sequence	Construct / Strain
oGQ83(CDC28clonBamF)	CGGATCCATGAGCGGTGAATTAGCAAATTAC	
oGQ84(CDC28clonSalR)	CCGGTCGACTTATGATTCTTGGAAGTAGG	pFL1
oFL1(CKS1 pGEX-6P BamHI F)	CGCGGATCCATGTACCATCACTATCACGCCTTCC	
oFL2(CKS1 pGEX-6P XhoI R)	CCGCTCGAGCTAGGAGATTTGGGGTGGAAC	pFL1
oFL59(Cdc28clon-BamHI-F)	CGCGGATCCATTATCGTTCTCGAGATAG	pFL15
oFL60(Cdc28clon-XhoI-R)	CGGCTCGAGTTATGATTCTTGGAAGTAG	YFL41 YFL46 YFL64
oFL61(Cdc28-S46A-F)	GAAAATAAGACTAGAGGCTGAAGACGAGGGTGTTC	
oFL62(Cdc28-S46A-R)	GAACACCCTCGTCTTCAGCCTCTAGTCTTATTTTC	pFL24 YFL20
oFL65(Cdc28-S258A-F)	GCGCAGAAAAGACCTAGCACAAGTGGTACCAAGTC	YFL24
oFL66(Cdc28-S258A-R)	GACTTGGTACCACTTGTGCTAGGTCTTTTCTGCGC	
oFL63(Cdc28-S46E-F)	GAAAATAAGACTAGAGGAGGAAGACGAGGGTGTTC	
oFL64(Cdc28-S46E-R)	GAACACCCTCGTCTTCCTCCTCTAGTCTTATTTTC	pFL21 YFL23
oFL67(Cdc28-S258E-F)	GCGCAGAAAAGACCTAGAACAAGTGGTACCAAGTC	YFL30
oFL68(Cdc28-S258E-R)	GACTTGGTACCACTTGTTCTAGGTCTTTT	

	CTGCGC	
oFL89(Cdc28-del-F)	ATCTATCAGAATTAGAGCCTTAAAAAAAG	
	CCGCAAGACAGGTTAAAAAGGAATAGAAT	
	TAGCGGTATTTTCTCCTTACGC	
oFL90(Cdc28-del-R)	CTAGGCTATAATGACAGTGCAGTAGCATT	YFL43
	TGTAATATAATAGCGAAATAGATTATAATG	YFL44
	CAGATTGTACTGAGAGTGCAC	YFL45
oFL91(Cdc28-del-chk-F)	GTAATTACAGAGACCTTTG	YFL39
oFL92(Cdc28-del-chk-R)	TGTTCCGTATATGACGATG	YFL34
		YFL36
		YFL37
		YFL38
		YFL20
		YFL23
		YFL24
		YFL30
oFL95(cdc28-T18V-F)	GAAAGTCGGTGAAGGTGTATACGGTGTT	
	GTTTAT	pFL26
oFL96(cdc28-T18V-R)	ATAAACAACACCGTATACACCTTCACCGA	YFL37
	CTTTC	
oFL97(cdc28-Y19F-F)	AGTCGGTGAAGGTACATTCGGTGTTGTTT	
	ATAAAG	pFL25
oFL98(cdc28-Y19F-R)	CTTTATAAACAACACCGAATGTACCTTCA	YFL38
	CCGACT	
oFL99(cdc28-TY18, 19VF-F)	GAAAGTCGGTGAAGGTGTATTCGGTGTT	
	GTTTATAAAG	pFL32
oFL100(cdc28-TY 18, 19VF-R)	CTTTATAAACAACACCGAATACACCTTCA	YFL39
	CCGACTTTC	
oFL101(cdc28-T169V-F)	CCGTTGAGAGCTTACGTACATGAAATTGT	pFL27
	TAC	YFL41
oFL102(cdc28-T169V-R)	GTAACAATTTCATGTACGTAAGCTCTCAA	
	CGG	
oFL127(cdc28-Cter-seq-F)	TGGTACCAAGTCTAGATCCA	
		YFL63
oFL128(cdc28-3GFP-pRS-R)	ACAGGAAACAGCTATGACCATGATTACGC	YFL64
	CAAGCTCGGAATTAACCCTCACTAAAGGG	
	AAGACAGTGCAGTAGCATTTGTA	
CDC28-pFA6a-F	ATTAGCGCCAGAAGAGCAGCCATCCACC	YFL34

	CCTACTTCCAAGAATCA	YFL36
	CGGATCCCCGGGTTAATTAA	YFL43
oFL30 (pRS30X-3HA-R)	ACAGGAAACAGCTATGACCATGATTACGC	YFL44
	CAAGCTCGGAATTAACCCTCACTAAAGGG	YFL45
	AAGAATTCGAGCTCGTTTAAAC	YFL46
oFL105(Cip1-del-F)	CTTCAACCTGTAATTCCCTTCTTAGTAAAG	
	CGAACTAGAACCAGTTTAATAGGATATAG	
	AGCGGTATTTTCTCCTTACGC	
oFL106(Cip1-del-R)	TTCATTAAGATAGTGTCATTATAGGTCATG	YFL53
	TATTGTGTGCTCCTGACTGTTCGGCACTA	YFL110
	TAGATTGTACTGAGAGTGCAC	
oFL109(srl3-del-F)	TATTTTCAAGAGGATGGAAGAAGAACCTG	
	AGCACAAACGGATAAATAATAGACGAATA	
	TAGCGGTATTTTCTCCTTACGC	
oFL110(srl3-del-R)	AAGCCATGTATATATGCGAAACCCGAATC	YFL54
	ATTAGTTCAGTCCGATGAGGACATCTTCA	YFL68
	ATAGATTGTACTGAGAGTGCAC	
oFL137(Cip1-pGEX-6P-BamHI-F)	CGCGGATCCATGCTGCTGGAAAGATTGCA	pFL44
oFL138(Cip1-pGEX-6P-XhoI-R)	CCGCTCGAGTTACCTTTTAAGACGCAATAAATCTC	pFL45
oFL140(pro + Cip1-BamHI-R)	CGCGGATCCTTTTAAGACGCAATAAATCTC	
oFL141(Cip1-F)	ATGCTGCTGGAAAGATTGCA	YFL114
oFL142(Cip1-R-URA3-F)	GAATGTGAAGAAAAGAGAAAGTGTAATAG	YFL110
	AATTTCATTAAGATAGTGTCATTATAGGTC	YFL109
	AAGATTGTACTGAGAGTGC AC	YFL108
		YFL107
		YFL106
		YFL105
		YFL100
		YFL101
		YFL102
oFL143(Cln2-3HA-F)	GGTAAATCTAGCAGTGCCTCATCTTTAAT	
	TTCTTTTGGTATGGGCAATACCCAAGTAA	
	TACGGATCCCCGGGTTAATTAA	YFL114
oFL144(Cln2-3HA-R)	CTCTTCATATTATATGTCGTCGCTTCTTAT	YFL115
	CCCTTTAAGTTATGAATTGTTGAGCTTAG	
	GGAATTCGAGCTCGTTTAAAC	

3.2.5 General manipulation of *Escherichia coli*

3.2.5.1 Escherichia coli Transformation

Transformation of competent E. coli DH5α, BL21 (DE3) RIL and Rosetta 2 plys E with different DNA resource were identical. Briefly, a few micro liters of DNA resource and 150 µl of competent E.coli incubate for 30 min on ice followed by 60 sec heat shock at 42ºC, and then the cells were immediately kept on ice for 1 min.

1 ml of LB media (1% (p/v) bactotryptone, 0.5% (p/v) yeast extract and 0.5% (p/v) NaCl) (Bertani 2004) was added to the cells and incubated at 37°C for 1 hour for the expression of resistance to ampicillin before plating cells onto selective medium plates.

After incubation, the cells were centrifuged at maximum speed (14000 RPM) (Eppindorf centrifuge 5418) and pellets were resuspended in fresh 200 µl of LB media and seeded on LB plates supplemented with 100 µg/ml ampicillin (LBA plates). The colonies were checked by plasmid digestion, PCR and/or sequencing.

3.2.5.2 E. coli competent cells preparation

A large amount of DH5α competent cells were prepared using rubidium chloride method in this study. 2 ml of saturated O/N culture was diluted into 200 ml of PSI-broth medium (2% (p/v) tryptone, 0.5% (p/v) yeast extract and 0.5% (p/v) MgSO4·7H2O, pH 7.6 with KOH.). And the culture was incubate at 37ºC to reach OD600 ≈ 0.48 (never let them pass 0.6, late exponential). After 15 min chilling in ice-water, the cells were harvested (4000g for 5 min, 4ºC) in a chilled, sterile 250 ml centrifuge bottle and resuspended gently with chilled TfbI (30 mM potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM manganese chloride, 15% glycerol). The cells were spinned down (4000g for 5 min, 4ºC) and resuspended gently with chilled TfbII (10 mM MOPS, 75 mM calcium chloride, 10 mM

rubidium chloride, 15% glycerol). After 15 min on ice, every 150 µl aliquot competent cells were put into pre-chilled, sterile eppendorf tubes, quickly chilled in liquid nitrogen and kept in -80ºC.

In this study, BL21 (DE3) RIL and Rosetta 2 plys E. coli were used for recombinant protein expression. For this purpose, competent cells preparation by using cold $CaCl_2$ is a simple and convenient way for a small amount E. coli ready for plasmid transformation. Briefly, a single colony was picked up and incubated O/N in LB medium. Next morning, 150 µl of saturated culture was diluted into 5 ml of fresh medium and cells were grown for 1h 30min. The exponential growing cells were then harvested (3000 rpm, 4ºC, 5 min) and resuspended gently on ice with 500 µl of 50 mM $CaCl_2$.

3.2.5.3 Plasmid Miniprep

Ecoli plasmid was extracted by using GenCatch (TM) Plasmid DNA Mini-Prep Kit (Epoch) following the instruction as bellow. The pellet of 1-5 ml overnight culture was collected by centrifuge at maximum speed (14000 RPM) for 1 min and then resuspended in 200 µl of MX1 buffer (50 mM Tris HCl, 10 mM EDTA pH 8.0, 100 µg/ml RNase A). 250 µl of lysis solution MX2 (0.2 M NaOH, 1 % SDS) was then added and mixed gently by inverting upside down the tubes for 5-10 times. The lysate mixture need to incubate at room temperature for 1-5 minutes followed by addition of 350 µl of neutralizing buffer MX3 (4 M Guanidine hydrochloride, 0.5 M Potassium acetate, pH 4.2). Once adding of neutralizing buffer, solution was mixed immediately by inverting upside down the tubes 5-10 times to make the lysate fully neutralization. White precipitation of the neutralization was centrifuged for 10 minutes at the maximal speed. The supernatant was carefully placed into GenCatch^{TM} columns and centrifuged at 5000 RPM for 1 min.

Flow through was discarded and the column was washed with 500 µl of WN buffer (5 M Guanidine hydrochloride, 20 mM Tris-HCl, pH 6.6) and repeated once with 700 µl WS buffer (10 mM Tris-HCl pH 7.5, 80% ethanol) at 9000 RPM for 1 min. Additional 13000 RPM for two minutes is needed to removed all the residual ethanol in previous wash buffer. Finally, 50 µl of elution buffer (10 mM Tris-HCl, pH 8.5) was added to the center of column and incubated for 5 minutes and then centrifuged at 13000 RPM for 1 minute eluting the plasmid in a 1.5 ml eppendorf tube. The quality and quantification of plasmid was checked by running gel electrophoresis.

3.2.6 Generation of genetically modified S. cerevisiae strains

3.2.6.1 Gene Deletion

The method used in this study was a PCR-based strategy to generate a deletion of the gene in the yeast genome. Deletion of a certain specific gene was achieved by transforming the PCR product containing the wild type auxotrophic marker. And the wild type auxotrophic marker was franked with 60 nucleotides over hangs in 5′ and 3′ terminals, which were the sequences flanking the gene of interest to be deleted. The long primers (80nt) include two functional regions. In order to delete the complete coding sequence, the 60 nucleosides complementary to the gene of interest are upstream of the ATG in F primer and downstream of the stop codon in R primer. To amplify the auxotrophic marker in plasmids, the 3′ terminal additional 20 nucleosides of both F primer and R primer should be overlapping the marker (shown in Figure 3.1).

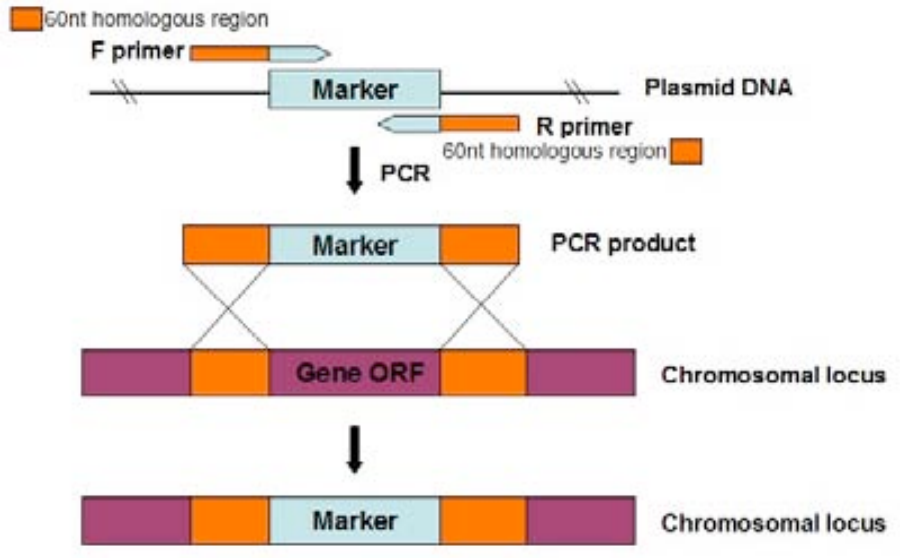


Figure 3.1 Schematic diagram of the process of gene deletion by integration the PCR cassette by homologous recombination.

The cassette then was transformed into yeast cells where, by homologous recombination, will replace the target gene ORF. The transformants were selected on minimal medium plates lacking the corresponding amino acid or nucleobase according to auxotrophic marker used for deletion. The colonies will grow in around three days followed by second selection on fresh selective medium plates to ensure the purity of clones.

Deletion of the genes was checked by either genomic PCR or western blot against the encoding protein. If the deleted gene has a sensibly different size than the marker used, a simple genomic PCR will be enough for checking. A couple of 21 nucleoside oligos, a bit upstream and downstream of the deletion will be used as the primers in PCR. If the deletion product has the same size as the original gene, then the couple primer should be one annealing to a bit upstream or downstream of the deletion, and the other one annealing to the marker, which will give band in positive colony.

3.2.6.2 Protein Tagging

Tagging a certain specific gene in the C terminal was achieved by transforming the cassette obtained from PCR amplification of the tag and KanMX marker fragment available in vectors using pFA6a as a template, which is similar as gene deletion (Shown in Figure 3.2). Important details were different from gene deletion strategy. First, the fragment to be replaced was a more than one hundred nucleotides sequence downstream of the target gene ORF. The 60 homologous nucleotides in the forward primer are the final sequence of the gene ORF, excluding the STOP codon. And the 60 homologous nucleotides in the reverse primer are corresponding to the more than 100nt downstream genome of the gene ORF. Using these primers, PCR product contains the tag 13MYC/3HA and six copies of KanMX, flanked by sequences homologous to the chromosomal region downstream of the gene ORF.

Yeast cells were transformed with the PCR product of interest for integration as described above. Cells transformed with the KanMX gene requires longer time to express the G418 antibiotic resistance, thus cells were first plated on rich medium (YPD) plates without selection and followed by replica plating on rich medium with 200 μg/ml G418 after 24 hours. Colonies were checked by western blot using monoclonal antibodies against the tag (9E10 anti-myc or anti-HA 12CA5) with the corresponding parent strain as a negative control.

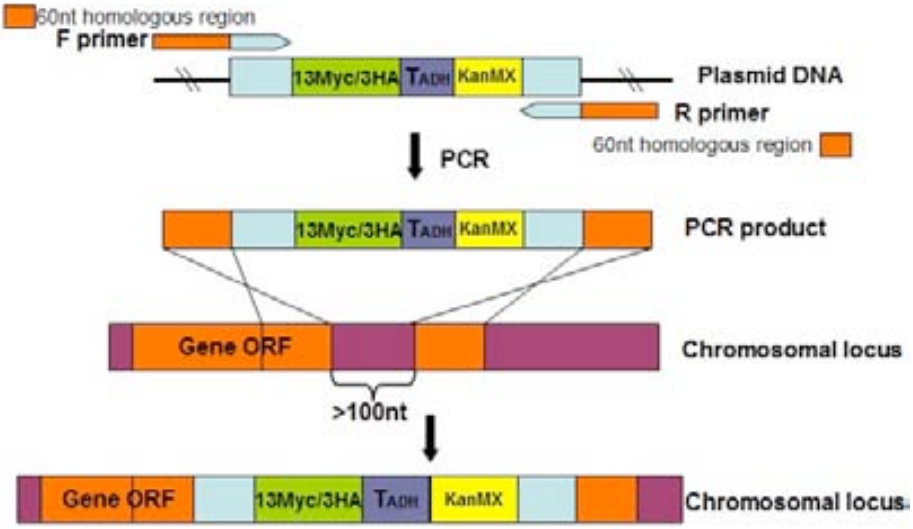


Figure 3.2 Schematic diagram of the process for C-terminal tagging of proteins by integration the PCR cassette by homologous recombination.

3.2.6.3 Integration of ectopic genes for protein over-expression

The GAL1 promoter has been incorporated upstream of the target ORF for over-expression in yeast in the presence of galactose. In this study, pRS family with GAL1, 10 promoter before multiple cloning sites was used to insert the ORF of interest for over-expression. Gal promoter is extremely sensitive to the carbon source galactose.

3.2.7 Strains used in this study

All the strains created and used in this study are listed in Table 3.6.

Table 3.6 List of strains

Name	Genotype
W303-1a	MATa *ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100*
YFL3	W303-1a *bar1*Δ
YFL4	W303-1a CDK1-3HA:KanMX6 *bar1*Δ

YFL12	W303-1a CDK1-3HA:KanMX6 *swe1*Δ::TRP1 *bar1*Δ::URA3
YFL13	W303-1a CDK1-3HA:KanMX6 *rad53*Δ::LUE2 *swe1*Δ::TRP1 *sml1Δ* *bar1*Δ::URA3
YFL14	W303-1a CDK1-3HA:KanMX6 *sml1Δ bar1Δ*
YFL20	W303-1a CDK1(S46A,S258A):URA3 *cdk1*Δ::TPR1 *bar1Δ*
YFL23	W303-1a CDK1(S46E,S258E):URA3 *cdk1*Δ::TPR1 *bar1Δ*
YFL24	W303-1a CDK1(S46A,S258A):URA3 *cdk1*Δ::TPR1 *swe1*Δ::LUE2 *bar1Δ*
YFL30	W303-1a CDK1(S46E,S258E):URA3 *cdk1*Δ::TPR1 *swe1*Δ::LUE2 *bar1Δ*
YFL31	W303-1a *rad53*Δ::LUE2 *sml1*Δ CDK1-3HA:KanMX6 *bar1*Δ::URA3
YFL32	W303-1a *sad1-1* CDK1-3HA:KanMX6 *bar1*Δ::URA3
YFL34	W303-1a CDK1(S46A,S258A)-3HA:KanMX6 *cdk1*Δ::TPR1 *bar1*Δ
YFL36	W303-1a CDK1(S46E,S258E)-3HA:KanMX6 *cdk1*Δ::TPR1 *bar1*Δ
YFL37	W303-1a CDK1(T18V):URA3 *cdk1*Δ::TPR1 *bar1*Δ
YFL38	W303-1a CDK1(Y19F):URA3 *cdk1*Δ::TPR1 *bar1*Δ
YFL39	W303-1a CDK1(TY18,19VF):URA3 *cdk1*Δ::TPR1 *bar1*Δ
YFL41	W303-1a CDK1(T169V):URA3 *bar1*Δ
YFL43	W303-1a CDK1(T18V)-3HA:KanMX6 *cdk1*Δ::TPR1 *bar1*Δ
YFL44	W303-1a CDK1(Y19F)-3HA:KanMX6 *cdk1*Δ:TPR1 *bar1*Δ
YFL45	W303-1a CDK1(TY18,19VF)-3HA:KanMX6 *cdk1*Δ::TPR1 *bar1*Δ
YFL46	W303-1a CDK1(T169V)-3HA:KanMX6 *bar1*Δ
YFL53	W303-1a *swe1*Δ::TRP1 *cip1*Δ::LUE2 *bar1*Δ
YFL54	W303-1a *swe1*Δ::TRP1 *srl3*Δ::LUE2 *bar1*Δ
YFL60	A364A MATa ura1 ade1 ade2 tyr1 his7 lys2 gal1-1 *cdc13-ts* CDK1-

	3HA:KanMX6
YFL61	W303-1a CDK1-3HA:KanMX6 CDK1:URA3 *bar1*Δ
YFL62	W303-1a CDK1-3HA:KanMX6 CDK1(Y19F):URA3 bar1Δ
YFL63	W303-1a CDK1(Y19F)-3GFP:KanMX6 *cdk1*Δ::TRP1, *bar1Δ*
YFL64	W303-1a CDK1(T169V)-3GFP:KanMX6 *bar1Δ*
YFL67	W303-1a CDK1-3HA:KanMX6 CDK1(T169V):URA3 *bar1Δ*
YFL68	W303-1a *srl3*Δ *bar1*Δ
SHM187	W303-1a CDK1-3xGFP:KanMX6
YFL63	W303-1a CDK1(Y19F)-3GFP:KanMX6, *cdk1*Δ::TRP1, bar1Δ
YFL64	W303-1a CDK1(T169V)-3GFP:KanMX6, bar1Δ
YMM1546	W303-1a TUB-3GFP *ycg1-2*:kan
YFL86	W303-1a CLB2-3HA:KanMX6 *bar1*Δ
YFL87	W303-1a CLB2-3HA:KanMX6 *swe1*Δ::LUE2 *bar1*Δ
YFL95	W303-1a GAL-HistoneH3:URA3 *bar1*Δ
YFL97	W303-1a GAL-CIP1-13MYC:URA3 *bar1*Δ
YFL98	W303-1a *sml1*Δ GAL-CIP1-13MYC:URA3 *bar1*Δ
YFL99	W303-1a *rad53*Δ::LUE2 *sml1*Δ GAL-CIP1-13MYC:URA3 *bar1*Δ
YFL100	W303-1a CIP1-13MYC:URA3 *bar1*Δ
YFL101	W303-1a *sml1*Δ CIP1-13MYC:URA3 *bar1*Δ
YFL102	W303-1a rad53Δ::LEU2 sml1Δ CIP1-13MYC:URA3 *bar1*Δ
YFL103	W303-1a CLB5-3HA:KanMX6 *bar1*Δ
YFL104	W303-1a CLB2-3HA:KanMX6 *bar1*Δ
YFL105	W303-1a CLB5-3HA: KanMX6 CIP1-13MYC:URA3 *bar1*Δ
YFL106	W303-1a CLB2-3HA:KanMX6 CIP1-13MYC:URA3 *bar1*Δ
YFL108	W303-1a CDK1-as CIP1-13MYC:URA3

YFL109	W303-1a CIP1-13MYC:URA3 GAL-SIC1(9A):LUE2 *bar1*Δ
YFL110	W303-1a *cip1*Δ::LUE2 *bar1*Δ
YFL111	W303-1a GAL-CIP1-13MYC:URA3 GAL-SIC1(9A):LUE2 *bar1*Δ
YFL112	W303-1a GAL-CLN2(7A) -2xNLS:TRP1 GAL- SIC1C70:HIS3 *bar1*Δ
YFL113	W303-1a GAL-CLN2(7A) -2xNLS:TRP1 GAL- SIC1C70:HIS3 GAL-CIP1-13MYC:URA3 *bar1*Δ
YFL114	W303-1a GAL-CIP1-13MYC:URA3 Cln2-3HA: KanMX6 *bar1*Δ
YFL115	W303-1a GAL-CIP1-13MYC:URA3 GAL-SIC1(9A):LUE2 Cln2-3HA: KanMX6 *bar1*Δ
YFL116	W303-1a GAL-CLN2(7A) -2xNLS-13MYC:KanMX6 GAL-SIC1C70:HIS3 *bar1*Δ
YFL117	W303-1a CIP1-13MYC:URA3 Cln2-3HA: KanMX6 *bar1*Δ
YFL118	W303-1a rad53Δ::LEU2 sml1Δ CIP1-13MYC:URA3 Cln2-3HA: KanMX6 *bar1*Δ
YFL119	W303-1a CIP1-13MYC:URA3 GAL-SIC1(9A):LUE2 Cln2-3HA: KanMX6 *bar1*Δ

3.3 CELL BIOLOGY AND MICROSCOPY

3.3.1 Basic cell cycle time course experiment module

In order to characterize a protein of interest throughout the unperturbed cell cycle, like protein abundance, phosphorylation, or trace the cell cycle progression in target mutants, or observe the phenotype of a particular mutant throughout the cell cycle, we carried out experiments in rich medium (YPD) at 24°C or 30°C as shown in Figure 3.3.

Usually if not mentioned elsewhere, overnight culture from a fresh single colony growing in an incubator with orbital shaking at 220 RPM was diluted in rich medium (YPD) to 5.10^6 cells / ml in a required volume according to the downstream experiment.

The culture was then growing until the cell density was doubled corresponding to one generation time in the certain condition (1.5 h for WT cells at 30°C), to 1.10^7 cells/ml. A aliquot of the culture at this point considered as early exponential phase was taken and the rest was synchronized in G1 phase (START) by adding α factor (50 ng/ml for bar1Δ strains). After growing for one doubling time plus 20 min in YPD with α factor, cells were checked for proper synchronization in G1 phase, determined by the budding index (BI). The budding index is budded cells number divided by total cells number multiplied by 100% which indicates the extent of synchronization in G1 phase of cell population. Once the culture reached the optimal synchronization (BI<5%), a aliquot of the culture at this point was taken and the rest α factor synchronized cells were washed three times with pre-warmed rich medium to release the arrested cells from G1 phase to the rich medium, allowing synchronous entry of cells into S phase. After release from G1 into rich medium, time course with required intervals (10, 15 or 30 min) was collected accordingly.

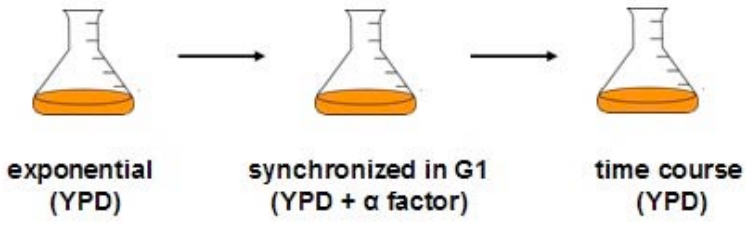


Figure 3.3 Diagram of the basic cell cycle time course experiment module.

3.3.2 Experiments involving cell cycle to genotoxic stress

To study the behavior of a target protein in response to genotoxic stress (HU, MMS or CPT) or the cell cycle progression in the presence of genotoxic stress in a given mutant. We conducted experiments in rich medium (YPD) at 24°C or 30°C as shown in Figure 3.4. We proceed the same way described in 3.3.1 to synchronize cells in G1 phase with the pheromone α factor. Once we see the proper G1 synchronization, the cells are washed three times in rich medium without α factor to release of the arrested cells, and resuspended in rich medium without α-factor but with a genotoxic agent, allowing synchronous entry into S phase compromised replication stress or DNA damage. Replication stress was generated in the presence of 200 mM hydroxyurea (HU); DNA damage was generated in the presence of 0.033% (v/v) Methyl methane sulfonate (MMS).

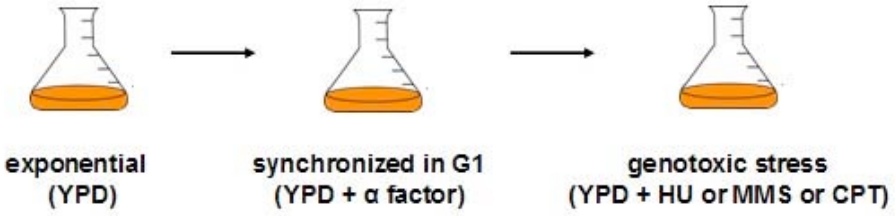


Figure 3.4 Diagram of a cell cycle time course experiment involving genotoxic stress.

3.3.3 Experiments involving the protein over-expression

In order to explore the effect or cellular phenotype caused by over-expression of a target protein throughout cell cycle progression, or to study the behavior of cell cycle progression when a particular protein was over-expressed during the cell cycle, we performed

experiments in rich medium YPRaffinose and induced the over expression of the protein under GAL1, 10 promoter by adding galactose as shown in Figure 3.5.

Overnight culture from a fresh single colony growing in an incubator with orbital shaking at 220 RPM was diluted in rich medium (YPRaff) to 5.10^6 cells / ml in a required volume according to the downstream experiment. The culture was then growing until the cell density was doubled corresponding to one generation time in the certain condition (1.5 h for WT cells at 30°C), to 1.10^7 cells/ml. A aliquot of the culture at this point considered as early exponential phase was taken and the rest was synchronized in G1 phase (START) by adding α factor (50 ng/ml for bar1Δ strains). Once the culture reached the optimal synchronization (BI<5%), a aliquot of the culture at this point was taken and the rest culture were added with galactose to final 2% (w/v) to induce the protein expression for 30 min or 1 h. After induction of the protein, one more time point aliquot was taken for experimental purpose and the rest cells were washed three times with rich media YPGal without α-factor to release the G1 arrested cells. Finally the cells were resuspended in YPGal and time course with required intervals (15 or 30 min) was taken.

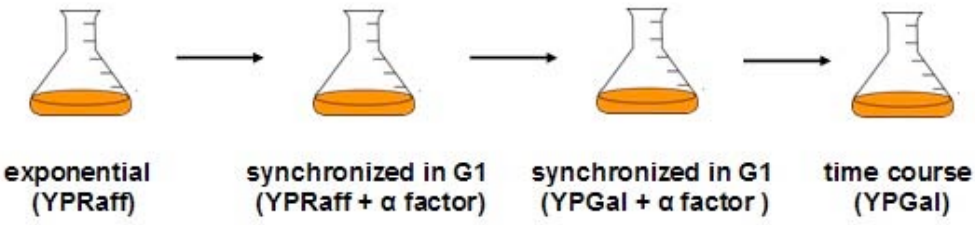


Figure 3.5 Diagram of a cell cycle time course experiment involving the induction of a protein.

3.3.4 Flow cytometry (FACS, Fluorescent Activated Cell Sorting)

Fluorescent Activated Cell Sorting (FACS) was used in this study for analysis of cellular DNA content following cell staining with propidium iodide (PI). This approach reveals distribution of cells in three different stages of the cycle: G1 (1C content), G2/M (2C content) and S (intermediate content).

For FACS sample preparation, around $1 \cdot 10^7$ cells taken from the experiments were collected at 10000 RPM for 2 minutes at room temperature. The pellet was resuspended and fixed in 1 ml of 70% ethanol and incubated overnight at 4°C. The fixed cells were washed twice with 50 mM Tris HCl pH 7.8 with centrifuge at 10,000 RPM, the supernatant discarded. Cells were then resuspended in 500 µl of 50 mM Tris pH 7.8 containing 200 µg of DNase-free RNase A and incubated overnight at 24°C. After removal of RNA, cells were resuspened and incubated with 0.5 ml of pepsin solution at 37°C for 30 min to make DNA accessible to propidium iodide by limited protease digestion. 30 min before use, pepsin was dissolved at 5 mg/ml in 50 mN HCl and activated at 37°C for 30 min. After pepsin treatment, cells were washed with 1 ml of FACS buffer (200 mM Tris-HCl pH 7.8, 211 mM NaCl, 78 mM $MgCl_2$) to inactivate and get rid of most of the pepsin. All spins were carried out at 10000 RPM for 2 min. The cells were finally dissolved and get stained in 250 µl of FACS buffer with 16 µg/ml propidum iodide for 30 min.

For flow cytometry analysis, 50 µl of stained cells in FACS buffer were transferred into the FACS tubes (Falcon 2054) containing 1 ml of 50 mM Tris HCl pH 7.8. Samples were sonicated at 40W for 10 seconds (sonicator Sonic Dismembrator, Dynatech) to separate cells group, otherwise couples of G1 cells will show up as apparent 2C DNA content. Around 20,000 cells were taken and analyzed on a FACScan apparatus (Becton-Dickinson), reading the fluorescence of propidium iodide at channel FL2.

DNA content was normalized using wild-type cells in log phase. Relative DNA content indicates the proportion of cells in G1 (1C content), G2/M (2C content) and S (intermediate content).

3.3.5 Microscopy

Fluorescence microscopy Nikkon eclipse 90i (CFI60 infinity optical system) was used in this study to examine the cellular morphologic phenotype, nuclear behavior and also protein localization. 95% methanol fixed cells were taken for checking the cellular morphologic phenotype. Propidium iodide stained samples as prepared for FACS were checked for nucleus observation. Cells expressing wild type or mutant alleles of Cdk1-3GFP were used to examine the localization in certain conditions.

3.4 BIOCHEMISTRY TECHNIQUES

3.4.1 Total protein extraction using trichloroacetic acid (TCA)

The total proteins extraction using trichloroacetic acid (TCA) in his study is one of the most efficient methods, based on simultaneous precipitation and denaturation with TCA. The samples were resuspended in 200 µl of 20% (v/v) TCA and added with 200 µl of glass beads. Cells were vortexed harshly for 1 minute at maximum speed to fully break the cell wall. Lysates were recovered to fresh Eppendorf tubes and the glass beads were washed twice with 200 µl of 5% (w/v) TCA by vortexing. All the TCA lysate was recovered together to maximize the amount of proteins in the lysate.

The insoluble precipitated and denatured total proteins in trichloroacetic acid were pelleted by centrifuging at speed of 3,000 RPM for 10 minutes at room temperature, supernatant discarded. And the pellets were dissolved in Lammeli buffer. The residual

remains of TCA exceeding the buffering capacity were neutralized by adding 0.2 volumes of 1 M Tris-base to balance the PH. The samples were boiled for 5 minutes and then centrifuged at 3,000 RPM for 5 minutes at room temperature. After this, the soluble supernatants were loaded into a SDS-PAGE gel for electrophoresis.

3.4.2 Polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), is a classical technique used to separate the proteins according to their mobility in the gel (Laemmli, 1970). SDS is applied as an anionic detergent to linearize proteins and also to impart a negative charge to the proteins. Then the protein electrophoretic mobility is only determined by their molecular mass.

Separation gels with different concentrations of acrylamide (7.5%, 10%, and 12%) were used according to the molecular weight of the target proteins. And 4% of stacking gel was always used above the resolution gel (The recipe shown in Table 3.7). Molecular weight ladder, Broad Range Prestained Standard (Fermentas) or Broad Range Standard (BioRad) was used for scoring the position of the target proteins.

Table 3.7 Poly-acrylamide gel components

	7.5%	10%	12%	4% STACK
1M Tris pH 8.8 (pH 6.8 for stacking)	3.7 ml	3.7 ml	3.7 ml	375 µl
40% acrylamide mix	1.9 ml	2.5 ml	3.0 ml	300 µl
20% SDS	50 µl	50 µl	50 µl	15 µl
10% APS	100 µl	100 µl	100 µl	30 µl
TEMED	10 µl	10 µl	10 µl	5 µl
H_2O	4.2 ml	3.6 ml	3.1 ml	2.3 ml

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).

3.4.3 Phostag SDS-PAGE electrophoresis

Phos-tag regent was purchased from the Phos-Tag Consortium (http://www.phos-tag.com). Phostag™ SDS-PAGE was used to separate and identify the phosphorylation modification from unphosphorylated proteins (Kinoshita, Kinoshita-Kikuta, & Koike, 2009). Phos-tag regent specifically retarded phosphorylated proteins in the SDS-PAGE because of phosphate affinity to phos-tag.

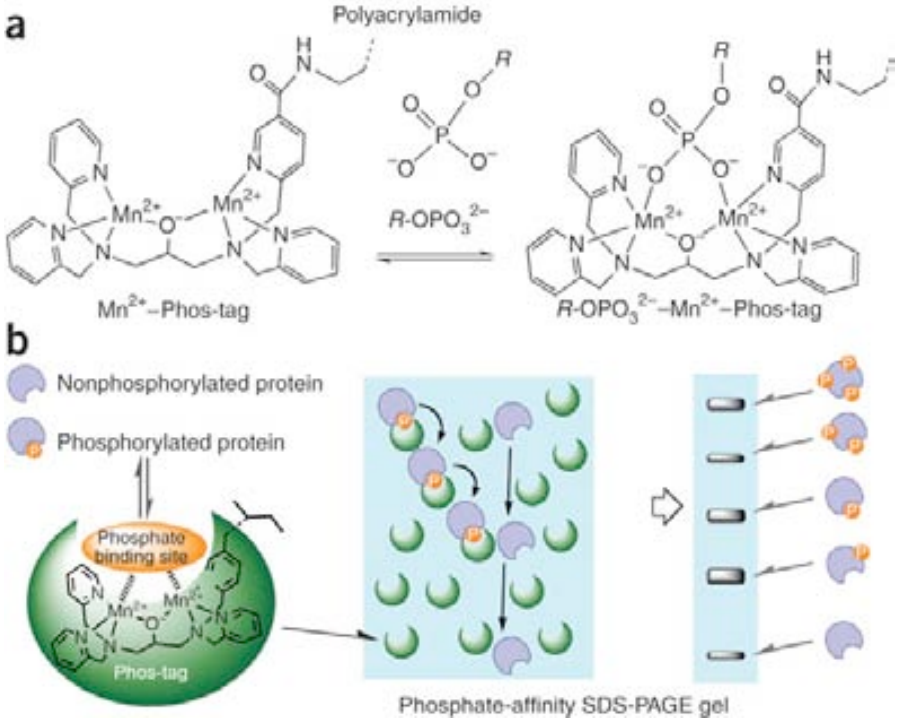


Figure 3.6 Phosphate-affinity Mn^{2+}-Phos-tag SDS-PAGE for the mobility-shift detection of phosphor-proteins (Kinoshita et al., 2009)**.** (a) Structure of the polyacrylamide-bound Mn^{2+}-Phos-tag and scheme of the reversible capturing of a phosphomonoester dianion ($R\text{-}OPO_3^{2-}$) by Mn^{2+}-Phos-tag and (b) schematic representation for the principle of Mn^{2+}-Phos-tag SDS-PAGE.

As shown in Figure 3.6, a, polyacrylamide-bound Mn^{2+}-Phos-tag has the capability to capture reversiblely a phosphomonoester dianion (*R*-OPO_3^{2-}), And the polyacrylamide-bound Mn^{2+}-Phos-tag shows preferential trapping of the phosphorylated proteins, which means the more phosphorylation sites the protein has, it gets more retarded in the polyacrylamide-bound Mn^{2+}-Phos-tag gel compared to its nonphosphorylated counterparts (shown in Figure 3.6, b).

The stacking gel preparation was the same as regular SDS-PAGE and phostag and $MnCl_2$ were only added to cast acrylamide gels. In this study, only 7.5% (v/v) SDS PAGE with 50 μM Phostag and 12% (v/v) SDS PAGE with 25 μM Phostag were used. Briefly, 50 or 100 μl of 5 mM Phostag and 50 or 100 μl of 10 mM were added to mix solution before TEMED and APS, as shown in Table 3.8.

Table 3.8 Phosphate-affinity Mn^{2+}-Phos-tag Poly-acrylamide gel components.

	7.5% SDS PAGE with 50 μM Phostag	12% SDS PAGE with 25 μM Phostag
H2O	4.04 ml	3.0 ml
1M Tris pH 8.8	3.7 ml	3.7 ml
40% acrylamide mix	1.9 ml	3.0 ml
20% SDS	50 μl	50 μl
5 mM Phostag	100 μl	50 μl
10 mM $MnCl_2$	100 μl	50 μl
10% APS	100 μl	100 μl
TEMED	10 μl	10 μl

The gel was electrophoresed at 90V for 20 min and subsequently at 160 mV for 60 min. Before transferring to a nitrocellulose membrane, the phostag SDS-PAGE was soaked in transfer buffer with 1 mM EDTA for 10 min and in transfer buffer without EDTA for another 10 min. Finally, the proteins in the gel were transferred to a nitrocellulose membrane using the Bio-Rad wet transfer apparatus (Invitrogen) at 100V for 70 min.

3.4.4 Western Blotting

The proteins separated by SDS-PAGE were then transferred onto nitrocellulose membrane (Protran BA85) using semi-dry transfer system (Panther Semidry Electrobotter, Owl Scientific) with modified transfer buffer (50 mM Tris-glycine pH 9.1, 0.0373% (w/v) SDS, 20% (v/v) methanol). Following the transfer, nitrocellulose membranes were stained with Ponceau (0.2% (w/v) Ponceau S, 3% (w/v) TCA) to visualize the most abundant proteins used as a loading control and subsequently destained with TBST (100 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween-20).

The nitrocellulose membrane was incubated in 5% skimmed milk in TBST for 30 minutes to block the non-specific binding space of the membrane, agitating at room temperature on a rocking platform.

Subsequently, the membrane was washed briefly with TBST and incubated with the diluted primary antibody for 2 hours on the rocking platform at room temperature. The primary antibodies and dilutions used in this study were:

α-Cdc28 (Goat IgG, Santacruz yc-20), diluted 1:500 in TBST+5% milk

α-RAD53 (Goat IgG, Santacruz yC-19), diluted 1:500 in TBST+5% milk

α-Cln2 (Rabbit IgG, Santacruz y-115), diluted 1:1000 in TBST+5% milk.

α-Sic1 (Rabbit IgG, Santacruz FL-284), diluted 1:1,000 in TBST+5% milk.

α-Clb5 (Goat IgG, Santacruz yN-19), diluted 1:1,000 in TBST+5% milk.

α-Clb2 (Rabbit IgG, Santacruz y-180), diluted 1:2,000 in TBST+1% milk.

α-myc (9E10, monoclonal mouse hybridoma supernatant) diluted 1:500 in TBST.

α-HA (12CA5, monoclonal mouse hybridoma supernatant) diluted 1:100 in TBST.

α-Pol12 (mouse monoclonal 6D2, given by Dr. Foiana Marco), diluted 1:2000 in TBST + 1% milk.

α-pY15-CDK1 (anti pY15-CDK1 also pY19-Cdc28, Cell Signaling), diluted 1:1,000 in TBST + BSA.

Following incubation with primary antibodies, nitrocellulose membranes were washed 5 minutes in TBST for 3 times. Nitrocellulose membranes were then incubated with horse-radish peroxidase (HRP) conjugated secondary antibodies for 35 min at room temperature. The secondary antibodies and dilutions used in this study were:

α-mouse IgG-HRP (rabbit polyclonal, Dako P0161) 1:5.000 dilution in TBST + 5% milk.

α-goat IgG-HRP (donkey polyclonal, Santa Cruz Biotechnology sc-2020), diluted 1:2500 in TBST + 5% milk.

α-Rabbit IgG-HRP (goat polyclonal, purified IgG, Dako P0448), dilution in 1:2,000 in TBST + 1% milk

After incubation with the secondary antibody, the solution was removed, and the membranes were then washed 3 times with TBST, 5 minutes each. Finally, proteins were then visualised by incubating the nitrocellulose membrane in enhanced chemi-luminescence (ECL) reagent (GE) for 5 minute, and exposed to sensitive photographic film (Super RX Film, Fujifilm) for the required amount of time.

3.4.5 Immunoprecipitation (IP)

Immunoprecipitation is used to enrich the protein of interest and also to identify the protein-protein interaction in a complex *in vivo*.

Cell pellets from cultures with the number of cells at $1 \cdot 10^8$ - $1 \cdot 10^9$ were collected and washed once with the lysis buffer (50 mM Tris-HCl pH 7.8, 125 mM NaCl, 1 mM EDTA, and 10 % (v/v) glycerol, 0.1% (v/v) nonidet-P40) and snap frozen using liquid nitrogen and kept in -80 °C freezer. Before extraction, 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) and phosphatase inhibitors (0.5 mM sodium pyrophosphate, 2 mM NaF, 2 mM β-glycerophosphate).

All the following procedures were done in the 4°C cold room. 1 ml of pre-chilled cold glass beads (0.5 mm diameter) were added to the cells in chilled Duran glass tubes aand vortexed 1 min for 10 times, in between with 1 min rest on ice. The mixture was incubated on ice for 1 hour to be fully extracted. After incubation on ice, extraction was transferred to a new cold eppendorf tube, and centrifuged at 14000 RPM for 20 minutes to clean the extract. The supernatant lysate were transferred to a fresh cold 1.5 ml tube.

Before Co-Immunoprecipitation, the total protein concentration in the cell lysate was determined by Bio-Rad assay against a standard curve produced using known concentrations of bovine serum albumin (BSA) (Sigma) in 1 ml of 1:5 diluted Bio-Rad reagent (Bio-Rad Laboratories). 2µl of cell lysate was also added to 1 ml of Bio-Rad reagent diluted 1:5 with sterile water. Protein concentration in cell lysate was read at 595nm wavelength. Cell lysates were diluted to the same concertration (1 mg/ml used for Co-IP followed by western blotting, 10 mg/ml for Cln2-CDK kinase assay and GST-pull down assay).

The cleaned lysate containing 3HA or 13MYC tagged protein of interest was incubated accordingly with 20 µl of Anti-HA HA-7 or Anti-myc HA-7 agarose matrix (Sigma A2095, mouse monoclonal purified IgG1) and rotated for 1 h at 4°C. After this, the samples were then centrifuged at 500g for 1 minute at 4°C and supernatant discarded. The beads were then washed four times with 1ml of cold lysis buffer. Finally, the proteins were

released by boiling the samples in presence of Lammeli buffer. Enriched proteins were resolved by SDS-PAGE and checked by western blotting.

3.4.6 Large scale immunoprecipitation for mass spectroscopy

For identification of CDK1 phosphorylation sites and also its interaction proteins by mass spectroscopy proteomic analysis, a large amount of cells expressing CDK1-3HA was collected and extracted. Around 20g of cell pellet was collected and washed with 150 ml ice cold lysis buffer (50 mM Tris-HCl pH 7.8, 125 mM NaCl, 1 mM EDTA, and 10 % (v/v) glycerol, 0.1% (v/v) nonidet-P40) and finally resuspended in 0.25 volumes (v/w) of lysis buffer with 4 times inhibitors (4 mM AEBSF, 0.6 mM aprotinin, 4 mM leupeptin, 4 mM pepstatin, 2 mM sodium pyrophosphate, 8 mM NaF, 8 mM β-glycerophosphate). And the cell juice was then sucked and extrude into 50 ml Falcon tube containing liquid nitrogen by 1 ml syringe to make the mixture to be solid popcorns. After liquid nitrogen fizzle off, the cell popcorns were stored at -80°C.

The solid cell popcorns were extracted by coffee mill with dry ice mixed throughout the process. All the popcorns were grinded with dry ice for 20 min with 10 sec on and 10 sec off in between. After this, the yeast powder was transferred into a fresh pre-chilled Falcon tube and mixed well with 10 ml lysis buffer and 0.05% NP-40 on ice for I hour to allow extraction take place.

After 1 hour extraction on ice, the mixture was clarified for 1 hour at 100,000g using ultracentrifuge (Beckman). The lysate was then incubate with 100 µl of pre balanced Anti-HA 3F10 affinity matrix (Roche 11 815 016 001, rat monoclonal, purified IgG1) and rotated for 1 h at 4°C, followed by 5 times washes with lysis buffer. The sample was divided in to two tubes. In one tube the proteins were released from the beads by mixing with 50 µl of room temperature 8M urea in 100 mM Tris pH 8.0 for 5 min. After releasing, the supernatant

containing IPed proteins was transferred into a new tube for mass spectroscopy analysis of CDK1 interaction proteins. In the other tube, the purified CDK1-3HA were released by boiling in 50 µl of Laemmli buffer for 5 min and then separated by SDS-PAGE. The gel was then stained with Coomassie solution, and followed by destaining with destainer (50% methanol 10% acetic acid) for as little as sufficient to distinguish the protein bands. The CDK1-3HA band corresponding to 40 KD position was cut and washed 10 min for 3 times in Milli-Q water. The gel purified CDK1 will be analyzed for phosphorylation sites by mass spectroscopy.

3.4.7 Recombinant proteins expression and purification

For induced expression of recombinant proteins, BL21 (DE3) RIL *E. Coli* competent bacteria were transformed with pEGX-6P-3 constructs expressing GST-Rad53, GST-Rad53-KD, GST-Cks1, GST-Cip1 and Rosetta 2 plys *E. Coli* competent bacteria was transformed with pEGX-6P-3 constructs expressing GST-CDK1. O/N culture was diluted to 250 ml LB+ampicillin (additional chloramphenicol for Rosetta 2 plys) medium and grown at 37^{o}C to reach an OD600 of 0.5-0.6 (no less, no more). For better soluble recombinant proteins quality induction, the temperature, concentration of Isopropyl β -D-thiogalactopyranoside (IPTG) and induction time were modified according to different proteins. For GST-Rad53 and GST-Rad53-KD induction, the cultures were shifted to 24^{o}C and grown for 4 h with 200 rpm shaking, induced by adding 0.5 mM TPTG. For GST-CDK1 expression, the culture was shift to grow at 24^{o}C for 3 h or 16^{o}C for 5 h with 150 rpm shaking, induced by adding 0.1 mM TPTG. For GST-Cks1, the culture was grown at 37^{o}C for 2 h with 1 mM TPTG and 200 rpm shaking. For GST-Cip1 the culture was grown at 24^{o}C for 3 h with 1 mM TPTG and 200 rpm shaking.

The culture was spined down in centrifuge bottles at 8,000 g, 10 min, 4°C and the pellet was resuspended with 50 ml of ice-cold lysis buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 2 mM DTT, 1.5 mM EDTA, 1 mM PefaBloc). All the same culture was pooled and spined in a single bottle again and then resuspended in 20 ml of ice-cold lysis buffer. The cell suspension sample was then frozen in liquid nitrogen and kept in -80ºC freezer.

The sample was thaw in ice water. Final 0.1 mg/ml lysozyme (Sigma) was added and incubated on ice for 30 min followed by sonication in ice with 5 pulses (50 w, 20 kHz, 30 sec) and 1 min pause in between. To help solubilize the over-expressed protein, triton X-100 was added to final 1% and the lysate was rocked in the cold room for 1 h and then clarified at 12,000 g, 10 min, 4 °C. The extract was transferred into 50 ml falcon tube containing 200 µl of glutathione beads bed volume (previously transferred as 4 volumes of pre-equilibrated 25% slurry in lysis buffer) and incubated in cold room for 1 h. The glutathione beads were collected by 500g, 1 min centrifugation at 4°C and washed 5 times for 10 min each wash with ice-cold wash buffer (50 mM Tris-HCl pH 8.0, 1.25 M NaCl, 1.5 mM EDTA, 1 mM DTT, 1 mM PefaBloc). For GST-CDK1 sample, the glutathione beads were washed with modified ice-cold wash buffer (50 mM Tris-HCl pH 8.0, 1.25 M NaCl, 1.5 mM EDTA, 1 mM DTT, 1 mM PefaBloc, 10% glycerol) and additional Triton X-100 for first two washes (0.5% for first time wash, 0.1% for second wash).

For in vitro kinase assay, the proteins were released by cleaving the GST moiety with Pre-Scission protease according to the manufacturer (GE). After equilibration by washing 3 times with PreScission buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT), the beads was incubated O/N with the PreScission protease rotating in the cold room (ratio: 200 µl of drained beads+200 µl of PreScission buffer+5 µl PreScission protease). The PreScission buffer for CDK1 was modified as 50 mM Tris-HCl pH 7.5, 25 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.4% NP-40 and 10% Glycerol.

3.4.8 GST pull down assay

GST pull-down assay is used to identify physical interactions between a probe protein and unknown targets and also to confirm suspected interactions between proteins. In this study, recombinant GST-YPL014 fusion protein was expressed and purified as the probe protein to confirm the interaction with CDK1.

The native extraction of the input cells was done as the same mentioned in IP extraction. And then the GST pull down assay was performed using GST-Cip1 as the probe protein and GST alone as negative control. Briefly, clean the yeast cell lysate by incubation with 25 µl glutathione-agarose beads with GST for 1 hour in cold room with rotate mixing. Centrifuge the mixture at 500g for 2 min at 4 °C. The pre-cleaned cell lysate supernatant was then divided into two tubes, each containing 50 µl of glutathione-agarose beads. Around 10 µg the GST-Cip1 fusion protein and more than 10 µg of GST were added into these two different tubes and incubate for 2 hours in cold room with rotate mixing. Wash the beads four times with 1 ml of ice-cold GST wash buffer (50 mM Tris-HCl pH 7.8, 125 mM NaCl, 1 mM EDTA, and 0.1 % (v / v) NP-40, 1 mM AEBSF, 0.15 mM aprotinin, 1 mM leupeptin, 1 mM pepstatin, 0.5 mM sodium pyrophosphate, 2 mM NaF, 2 mM β-glycerophosphate).

Finally, the proteins associated were released by boiling the samples in presence of Lammeli buffer. Protein-protein interactions were checked by running SDS- PAGE followed by Western blotting.

3.4.9 Rad53 *in vitro* kinase assay

GST-Rad53 and GST fused candidate substrate proteins were separately expressed in 250 ml cultures of Escherichia coli BL21 (DE3) RIL (GST-CDK1 in Rosetta 2 plys), and purified by

affinity chromatography using glutathione-Sepharose beads. The proteins were all cleavage released from the GST moiety by Pre-Scission protease.

The reaction was performed in screwed cap 1.8 ml plastic vials with rubber o-ring containing Rad53, candidate substrate and the kinase buffer as bellow:

- 1 µg of Rad53 or the same volume of the same buffer in the corresponding kinase-less control reaction.
- 200 ng-1 µg of substrate protein (1 µg with CKS1 or 200 ng with CDK1) or the same volume of the same buffer in the corresponding substrate-less control reaction.
- kinase buffer to 30 µl total volume (50 mM Tris HCl pH 8, 10 mM MgCl2, 10 mM MnCl2, 1mM DTT, 1mM AEBSF, 2 mM NaF, 100 µM ATP, and 5 µCi of [γ-32P] ATP).

The reaction was then incubated at 30 °C for 35 min in thermomixer at minimal shaking. The reaction was stopped by addition of 5×Laemmli sample buffer and boiled for 5 min. The full sample was loaded on 12% SDS-PAGE, and the gel was stained with Coomassie blue and scanned. Autoradiography was performed O/N.

Under the same conditions, except modification of the reaction time (35min, 50min, 65min and 80min) for ^{32}P incorporation to the substrate, CDK1 reached a plateau after 65 min incubation. The in vitro kinase assay without ^{32}P (only add cold ATP) was then performed with 65 min reaction time. Control reactions were carried out under identical conditions except for the presence of Rad53. Samples were resolved by SDS-PAGE and Coomassie Blue-stained. The gel slices containing the CDK1 were excised from the gel and sent to Dr. James Wohlschlegel (UCLA) for identifying the phosphorylated sites.

3.4.10 Cdk1 *in vitro* kinase assay

In vitro kinase assays were carried out with G1-CDK complexes purified from whole cell extracts by immunoprecipitation. To increase the amount of Cln2, hyperstable Cln2-7A-13MYC under GAL1, 10 promoter were induced and purified as the G1-CDK kinase.

3 µl of 2×HBII buffer (30 mM MOPS pH 7.4, 10 mM EGTA, 30 mM $MgCl_2$, 2 mM Na_2VO_3, 2 mM AEBSF, 0.3 mM aprotinin, 3 mM leupeptin, , 14 mM β-glycerophosphate, 2 mM DTT,) were added to 10 µl of immunoprecipitate beads, and incubated at room temperature for 15 minutes.

In order to study the effect of Cip1 to G1-CDK activity, the mixture was then added with rCip1 or the PreScission buffer used to release rCip1. Samples were then added with 2 µl 100 mM ATP and incubated at 30°C for 15 minutes.

The sample were then added with the reaction buffer: 3 µl 100 mM ATP and 2 µl 5 µCi of γ-[^{32}P]-ATP (GE), 5 µl 4 mg/ml Histone H1 (the substrate in the kinase reaction) in 50 mM MOPS pH7.4. Samples were incubated at 30°C for 15 minutes in thermomixer at minimal shaking. The reaction was stopped by addition of 5×Laemmli sample buffer and boiled for 5 min. The full sample was loaded on 12% SDS-PAGE, and the gel was stained with Coomassie blue and scanned. The kinase activity was measured by autoradiography with sensitive film (Super RX Film, Fujifilm).

4 RESULTS AND DISCUSSION

4.1 CDK1 REGULATION BY PHOSPHORYLATION

4.1.1 Multiple in vivo phosphorylation forms of Cdk1

As we know that not all the phosphorylated proteins show electrophoresis mobility shifts in regular SDS PAGE gel. Cdk1 is one case among them. In order to study the regulation of Cdk1 phosphorylation, we established a simple biochemical method to analyze the phosphorylation forms of Cdk1 by recently developed phospho-affinity gel electrophoresis (Kinoshita et al., 2009).

4.1.1.1 Quantification of different phosphorylation forms of Cdk1 in vivo

To investigate the phosphorylation pattern of total Cdk1, we first examined how many phosphorylation forms of Cdk1 can be resolved in Phos-tag SDS-PAGE. We observed reproducibly three Cdk1 bands in WT cells resolved by phostag in the gel (Figure 4.1). We next assigned the phosphorylation state of each band using Cdk1 mutants with corresponding non-phosphorytable Val or Phe substitutions at known phosphorylation sites (as shown in Figure 4.1), Thr18 (mutant T18V), Tyr19 (mutant Y19F), and Thr169 (mutant T169V) and the double mutant T18V, Y19F. The mutated Cdk1 was tagged with 3HA under its own promoter and the endogenous copy was deleted, and for T169V mutant, the original Cdk1 was kept for its viability since Thr169 is essential for Cdk1 activation. To maximally visualize all the different phosphorylated forms of CDK1, cells in S phase in the presence of hydroxyurea were collected for analysis.

For evaluating the phosphorylation forms, we compared those mutants with WT Cdk1 to see which bands were lost so that we know which band contains the corresponding amino acid phosphorylation. As we can see in Figure 4.1, compared with WT Cdk1, the upper band was lost in Y19F mutant, and the upper two bands disappeared in T169V mutant. However,

all three Cdk1 bands were shown in T18V mutant as well as in WT. This assign unveil that multiple phosphorylation forms of Cdk1 exist in vivo, and that Thr169 phosphorylation is required for Tyr19 phosphorylation. But we did not detect Thr18 phosphorylation which could be because phostag affinity gel is not sensitive enough to resolve all the phosphorylation forms or there is no Thr18 phosphorylation existing in budding yeast.

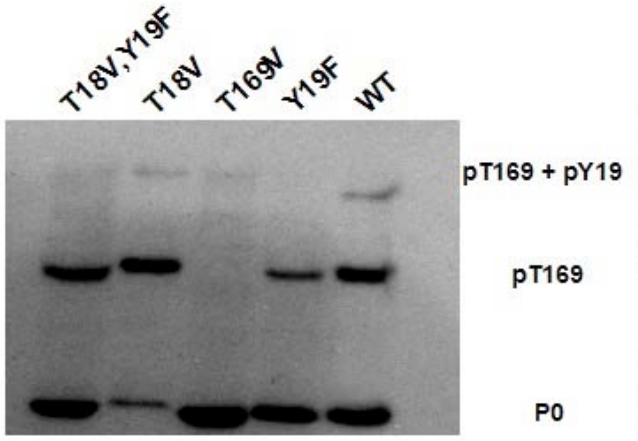


Figure 4.1 Phosphorylation forms of Cdk1 *in vivo* aligned using Phostag electrophoresis. Exponentially growing WT cells (YFL4) and the indicated mutants (T18V: YFL43; Y19F: YFL44; T169V: YFL46; T18V,Y19F: YFL45) were synchronized with α-factor (G1) and allowed to progress into the S phase in the presence of hydroxyurea (1h 0.2M HU). The proteins in whole cell extraction were seperated using phostag gel and analyzed by immunoblot with anti-HA (Cdk1) antibodies.

The stoichiometry of Cdk1 phosphorylation significantly impacts cell cycle progression. We next used the phospho-affinity gel electrophoresis to systematically monitor the phosphorylation levels during cell cycle and different treatment. As we know, it is unlikely to compare the absolute quantification of different phosphorylation forms with either phosphorylation-independent antibody or antibody against specific phosphorylation site in

regular SDS PAGE followed by immunoblotting. Here, we used normal antibody which was sufficient for detection and quantification of different phosphorylation states of Cdk1 by immunoblotting after Phos-tag SDS-PAGE.

The phosphorylation pattern of Cdk1 was investigated during synchronous cell cycle progression and different treatments. As shown in Figure 4.1 and 4.2, phos-tag gel resolved highly reproducible three Cdk1 bands, and regular SDS PAGE gel showed one band without any mobility shift. Coincident with cell cycle regulated Tyr19 phosphorylation wave shown by anti-pTyr19 immunoblotting, the upper bands in different time course or treatments were well evaluated to phosphorylation form of pThr169 pTyr19. However, the bands in the middle and lower evaluated as Thr169 phosphorylation form and non-phosphorylated form respectively are constantly stable throughout cell cycle and upon genotoxic stress (HU, MMS).

Interestingly, the level of the inhibitory phosphorylation of Cdk1 in Try19 is quite low. As we can see in Figure 4.2, the population of Try19 phosphorylated Cdk1 stands only a small fraction of total Cdk1, despite Tyr19 phosphorylation is very important to regulate Cdk1 activity in cell cycle progression.

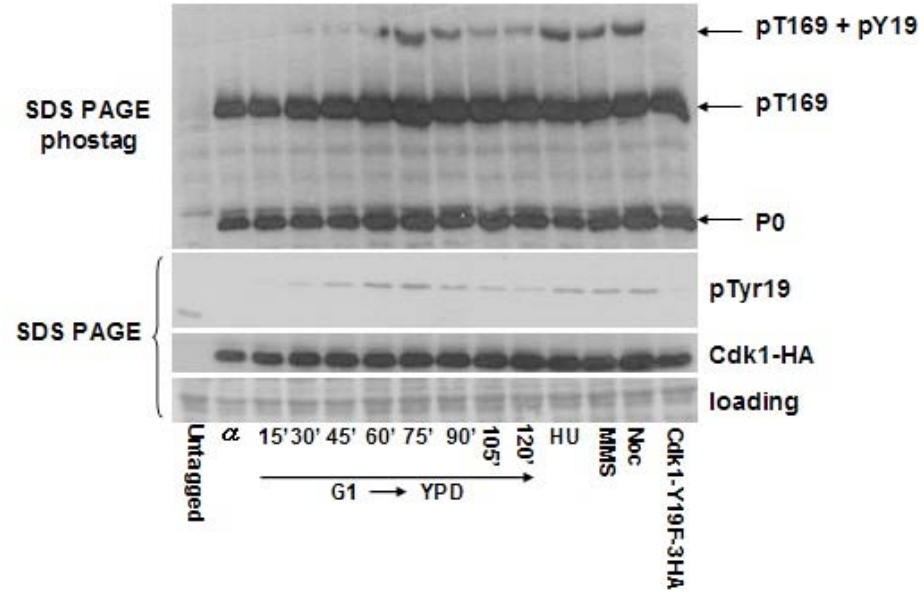


Figure 4.2 Quantification of different phosphorylation forms of Cdk1 throughout cell cycle and upon genotoxic stress. Exponentially growing cultures (YFL4, Cdk1-3HA; YFL44, Cdk1-Y19F-3HA) were synchronized with α-factor (G1). The cells were then released from the G1 arrest by several washes with medium lacking α-factor and either allowed to progress into the S phase without stress (YPD), or allowed to progress into S phase in present of genotoxic stress (1h HU) or synchronized in G2/M (2h Noc) as indicated. For Cdk1-Y19F-3HA, cells were taken after 1h HU treatment. Cells with untagged Cdk1 were used as control for the crossreative band of HA antibodies. Proteins in whole cell extraction were separated by phostag gel and analyzed by immunoblot with anti-HA (Cdk1) antibodies. A Ponceau S-stained region of the same membrane used for immunoblot is shown as a loading control.

4.1.1.2 Cdk1 pThr169 levels require constant de novo synthesis of Cdk1

The levels of Cdk1 Thr169 phosphorylation and nonphosphorylation form of Cdk1 are constantly stable throughout the cell cycle. Thr169 of Cdk1 in budding yeast is phosphorylated by CDK-activating kinase Cak1 (Thuret, Valay, Faye, & Mann, 1996) which is not a cell cycle dependent kinase. Moreover, in our proteomic study, we found Cak1 physically bound to Cdk1. This information suggested that Cak1 kinase bound to Cdk1 could

constantly phosphorylate Thr169 of *de novo* Cdk1. To check if the stability of Thr169 phosphorylation form and also the nonphosphorylation form of Cdk1 is due to the constant *de novo* expression of Cdk1, we carried out experiments using cycloheximide (CHX) to block proteins synthesis. Cells in different phases of cell cycle arrested by either α-factor (G1), hydroxyurea (S), Noc (G2/M) were treated with cycloheximide to block the proteins synthesis. As shown in Fig, the bands corresponding to the unphosphorylated Cdk1 (P0) were gone due to block of the proteins synthesis which confirmed that the P0 Cdk1 form is the *de novo* expressed Cdk1. Moreover, P0 bands are still invisible even when the total proteins loading were increased (Figure 4.3 (b)) which suggested that Cdk1 was expressed and phosphorylated at Thr169 constantly compared with the general total proteins level. In addition, the level of Thr169 phosphorylated Cdk1 (P1) significant decreased upon block of the proteins synthesis which suggested Cdk1 pThr169 levels require constant de novo synthesis of Cdk1. However, the level of the inhibitory phosphorylation form of Cdk1 (P2) did not change much in the presence of cycloheximide, which might due to longer half life time compared with unmodified Cdk1.

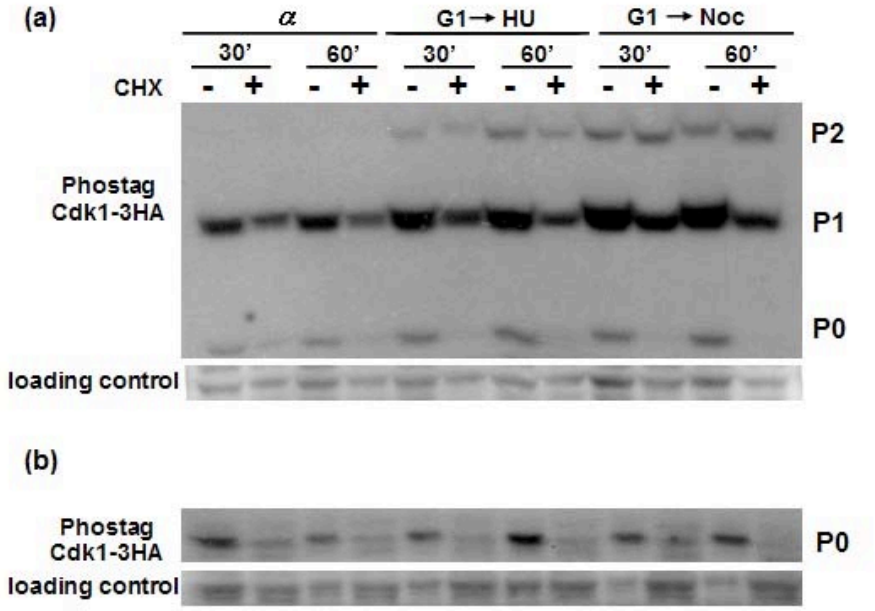


Figure 4.3 Cdk1 P1 levels require constant P0 *de novo* synthesis. A log phase culture (YFL4, Cdk1-3HA) was synchronized with α-factor (G1). The G1 arrest culture was then divided into three for different processes. One culture was directly treated with/without cycloheximide (CHX), keeping the presence of α-factor (α). And one culture was then released from the G1 arrest by several washes with medium lacking α-factor and allowed to progress into the S phase in the presence of hydroxyurea. After 1 h of incubation cycloheximide was added, keeping the presence of hydroxyurea (G→HU). And another culture was allowed to progress into the G2/M phase in the presence of Noc. After 2 h of incubation, cycloheximide was added, keeping the presence of Noc (G→Noc). The samples were taken at 30 min and 60 min after adding cycloheximide, and the proteins in whole cell extracts were seperated by Phos-tag electrophoresis and analyzed by immunoblot with anti-HA (Cdk1) antibodies. In (a), cell extrats were loaded according to the same cells number. In (b), total proteins with the same concentration were loaded. A Ponceau S-stained region of the same membrane used for immunoblot is shown as a loading control.

4.1.1.3 Swe1 is the only kinase phosphorylating Cdk1 at Tyr19

As discussed in section 1.2.2.2, the inhibitory phosphorylation of Cdk1 on Tyr15 (Y15) in fission yeast S. pombe is catalyzed by two kinases, Wee1 and the redundant Mik1. In mammalian cells, there are two Cdk1 inhibitory phosphorylation sites, Tyr15 (Y15) and the adjacent Thr14 (T14). The phosphorylations are catalyzed by Wee1 on Tyr15 and Myt 1 on both Thr14 and Tyr15. No evidence shows the exist of phosphorylation on the homologous T18 of Cdk1 in budding yeast. Useing the phospho-affinity gel electrophoresis, we did not detect any band corresponding to T18 phsophorylation (Figure 4.1, 4.4).

We next want to know whether there is any paralog kinase apart from Swe1 phosphorylation Cdk1 on Y19. We first did the homologue search (BLAST) using the Wee1 paralog kinase either Mik1 (fission yeast) or Myt1 (human) in Saccharomyces Genome

Database (SGD http://www.yeastgenome.org/). However, we did not get any hit through BLAST, which suggests there is no such homology kinase as Mik1 in fission yeast and Myt1 in humans. To confirm this, we seperated total Cdk1 from cells lacking Swe1 by phospho-affinity gel electrophoresis. It is well known that to prevent premature entry into mitosis, Y19 Cdk1 is phosphorylated in S phase without stress or in present of DNA replication stress. As we see in Figure 4.4, compared with the identified Cdk1 phosphorylation forms (the left panel), Cdk1 Y19 phosphorylation form was not detected in ether unperturbed S phase or stressed S phase (HU) as long as SWE1 gene is deleted (the left panel). This result confirmed that unlike in fission yeast and humans, there is no homology kinase that can substitute for Swe1 phosphorylation Cdk1 on Y19.

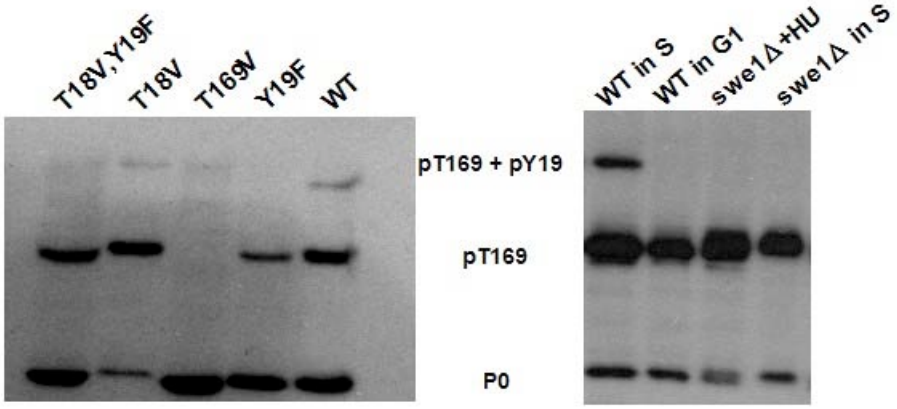


Figure 4.4 Swe1 is the only kinase phosphorylating Tyr19 in budding yeast. The left panel is from Fig for comparison. In the right panel, WT cells were released into S phase from G1 arrest (45 min in YPD) or kept in α-factor (G1). *swe1*Δ cells were released from G1 arrest and allowed to progress into the S phase (45 min YPD) without stress or in the presence of hydroxyurea (1h HU). Proteins in whole cell extracts were separated by phostag gel and analyzed by immunoblot with anti-HA (Cdk1) antibodies.

4.1.1.4 Only a population of Clb2-Cdk1 is inhibited by Y19 phosphorylation in response to replication stress

Our results reproduciblely showed that Y19 phosphorylation of Cdk1 represented only a small population of total Cdk1 in both normal cell cycle and in response to genotoxic stresses (Figure 4.2). Then we ask whether M-Cdk1 complexes are totally included in this small Cdk1 population, which indicates that all the M-Cdk1 is inhibited through Cdk1-Y19 phosphorylation.

To check this question, we synchronized the WT cells and *swe1*Δ cells in pre-G1 phase using α-factor, and released the cells into S phase in in the presence of hydroxyurea to block M-CDK activity. From this condition, the major mitosis CDK, Clb2-Cdk1 complexes were immunoprecipitated and separated by phospho-affinity gel electrophoresis. As shown in Figure 4.5, the input cell lysate of WT cells (the first lane) reproduciblely presented three different Cdk1 forms, p0, pT169 and pT169pY19 as usual, and pT169pY19 form was gone in *swe1*Δ cells (the second lane). In addition, Cdk1 Y19 phosphorylation is still just a small fraction of taltal Cdk1. Intrestingly, two different Clb2-Cdk1 populations were separated (Lane 3) including Y19 phosphorylation inhibitory Clb2-Cdk1 (the upper band in Lane 3) and non-pY19 Clb2-Cdk1 (the lower band in Lane 3). These two forms of Clb2-Cdk1 have more or less identical amount. In the control, as expected pY19 was not presented and non-pY19 Clb2-Cdk1 was the only form of Clb2-Cdk1 (Lane 3). Moreover, the nonphosphorylated Cdk1 was not associated with Clb2 (p0 position in Lane 3 and 4).

From this result, we concluded that at least two different mechanisms exsited to regulate M-CDK activity inresponse to DNA replication stress. Apart from the conserved Cdk1 tyrosine inhibitory phostorylation, another half of Clb2-Cdk1 was blocked through unidentified mechanisms. Argue with the opinion that the dispensability of the Swe1 control

in S. cerevisiae, we propose at there are other unidentified controls, redundant with Cdk1-Y19 phosphorylation, regulating M-CDK activity.

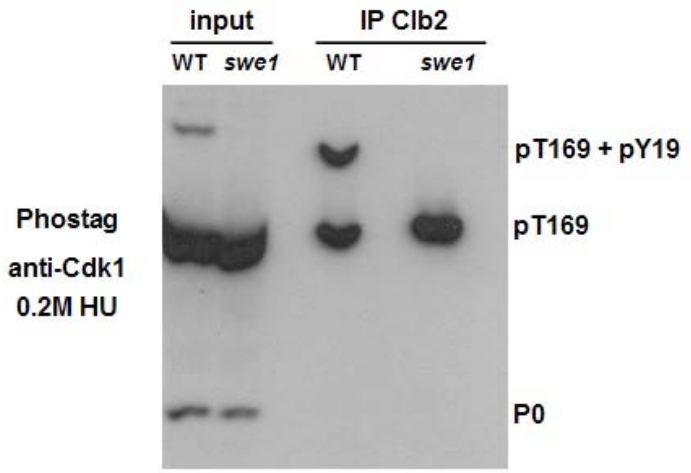


Figure 4.5 A population of Clb2-Cdk1, but not all, is inhibited by Tyr19 phosphorylation in response to replication stress. Exponetial growing cultures (YFL86 (Clb2-3HA), YFL87(Clb2-3HA, *swe1*Δ)) were synchronized with α-factor (G1) and allowed to progress into the S phase in the presence of hydroxyurea (1h 0.2M HU). Cells were then extracted followed by immuno-precipitation using agrose beads cross-linked with antibody against HA (Clb2). The precipitated proteins were then seperated by phostag gel followed by immunoblot against Cdk1. The cell lysates were loaded as input control.

In our lab Pol12 (the B subunit of the DNA polymerase alpha-primase complex) was well defined as the *bona fide* specific substrate of Clb1/2-Cdk1 to monitor M-CDK activity *in vivo* (unpublished data in our lab). It has been well demonstrated that in a *clb1*Δ *clb2*-ts strain, Pol12 is not phosphorylated at the restrictive temperature, despite the presence of S phase cyclins Clb5 and Clb6, and G2 cyclins Clb3 and Clb4. The M-CDK phosphorylation of Pol12 is abolished in the presence of HU as the checkpoint blocks the M-CDK activity in response to DNA replication stress. Compatible with the result in Figure 4.5, *swe1* null

mutants or Cdk1-Y19F non-phosphorytable mutants remain competent to inhibit the M-CDK activity on Pol12 in response to DNA replication stress (unpublished data in our lab).

The two different approaches suggest that there is a redundant mechanism together with Tyr19 inhibitory phosphorylation on Cdk1 to block M-CDK in response to replication stress. To check if the novel phosphorylation of Cdk1 by Rad53 as well as the novel Cdk1 associated factor Cip1 identified in this study (shown in the following sections) contribute to block the M-CDK activity, we constructed Cdk1(2A) *swe1*Δ double mutant strain and *Cip1*Δ *swe1*Δ double mutant strain and did the same time course experiment as described in Figure 4.6. However, the same as wiletype cells, both Cdk1(2A) *swe1*Δ double mutants and *Cip1*Δ *swe1*Δ remain competent to inhibit the M-CDK activity on Pol12 in response to DNA replication stress.

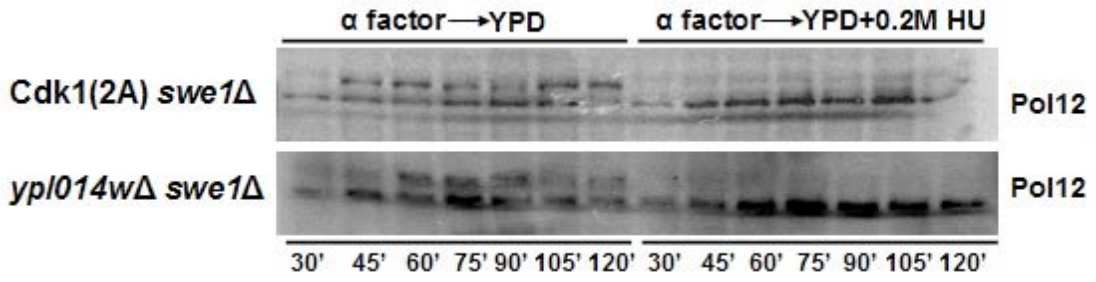


Figure 4.6 Cdk1(2A) *swe1*Δ and Cdk1(2A) *swe1*Δ remain competent to inhibit the M-CDK activity on Pol12 in response to DNA replication stress. Cdk1(2A) *swe1*Δ (YFL24) and Cdk1(2A) *swe1*Δ (YFL53) cells were synchronized in G1 phase and released into either YPD or medium containing hydroxyurea (YPD+HU) to generate replication stress. Samples were taken at the indicated points. Whole cell extracts were analyzed by immunoblot with anti-Pol12 antibodies.

4.1.1.5 pThr169 is required for Clb2 binding to Cdk1

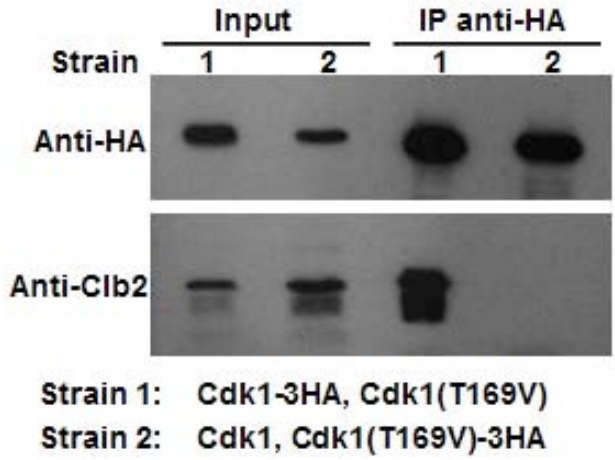


Figure 4.7 Cdk1(T169V) is defective to bind Clb2 in vivo. Exponetial growing cultures (strain 1, YFL46; Strain 2, YFL67) were extracted followed by immuno-precipitation using agrose beads cross-linked with antibody against HA (Cdk1-3HA or Cdk1(T169V)-3HA). The precipitated proteins were then seperated and immunoblotted against Clb2. The cell lysates were loaded as input control.

4.1.2 Checkpoint Rad53 kinase phosphorylates Cdk1 in vitro

In eukaryotic cells a surveillance mechanism, the S phase checkpoint, responds to genotoxic insults to protect the integrity of the genome. In the eukaryotic model organism *Saccharomyces cerevisiae* (budding yeast) the S phase checkpoint is mediated by the Mec1 kinase and its downstream effector kinase Rad53. As part of its response, the checkpoint inhibits Mitotic Cyclin Dependent Kinase (M-CDK) activity to block cell cycle progression until the stress is overcome. Whereas the control mechanism has long been well defined in the fission yeast *Schizosaccharomyces pombe* (Rhind et al., 1997) (Furnari et al., 1997), it remains elusive in budding yeast.

Our lab has dissected such control in two redundant branches (unpublished data). One of the pathways coincides with the control in fission yeast, as Mec1 stabilizes the Swe1

kinase, which in turn phosphorylates the Tyr19 residue in the Cdc28 catalytic subunit, resulting in the specific inhibition of CDK activity associated to mitotic cyclins, while leaving S-CDK activity unaffected. The second pathway is under Rad53 control, and might either target a mediator protein or directly target one or more of the M-CDK subunits.

In this study, we explore whether the checkpoint directly regulates the Cyclin Dependent Kinase subunit (Cdk1 or Cks1) that drives cell cycle progression.

4.1.2.1 Cdk1 and Cks1 are phosphorylated by Rad53 in vitro

To determine whether Cdk1 and Cks1 are Rad53 substrates, we studied whether these two recombinant proteins are phosphorylated by Rad53 using *in vitro* kinase assay. Overexpression of Rad53 in E. coli results in hyperphosphorylated active form (Palou et al., 2010; Pellicioli et al., 1999). This is a more powerful approach to determine phosphorylation than relying on electrophoretic mobility shifts, which are often undetectable and never demonstrate directness. In addition, this in vitro approach set up in our lab has so far proved to be completely specific (Duch et al., 2011; Palou et al., 2010).

The recombinant proteins Rad53 as kinase and Cdk1 as well as Cks1 as candidate substrates were expressed and purified in *E.coli* cells (see Materials and Methods 3.4.7). Before in vitro kinase assay, the GST moieties were removed by incubation with PreScission protease. We carried out in vitro kinase assay with 1μg of Rad53 recombinant, 1 μg CKS1 and 200 ng with Cdk1 (see Materials and Methods 3.4.9). As controls for the specificity of the in vitro kinase reaction, we included a single reaction without kinase substrate, and another single kinase reaction without substrate. Hyperphosphorylated, active Rad53 was incubated with the substrates in the presence of 100 μM ATP and 5 μCi of [γ-32P] ATP. The samples were resolved by SDS-PAGE and Coomassie Blue-stained and the phosphorylated proteins were detected by autoradiography (^{32}P).

As shown in Figure 4.1, Lane 2, 3, 7, Cdk1 alone, Cks1 alone as well as both Cdk1, Cks1 without Rad53 show no band autorad at the position of Cdk1 or Cks1 size, so the phosphorylation of Cdk1 and Cks1 (Lane 4, 5 and 6) are not due to any contaminated kinase co-purified with Cdk1 or Cks1. Furthermore, the first lane in the presence of Rad53 and without substrates, only detected is the corresponding Rad53 autophosphorylation, so the phosphorylation by Rad53 (Lane 4, 5 and 6) is not due to any protein co-purified with recombinant Rad53. Significantly, none of the contaminant proteins (marked with stars) is phosphorylated by Rad53 in the assay. This result shows that Cdk1 and Cks1 are phosphorylated by Rad53 in vitro.

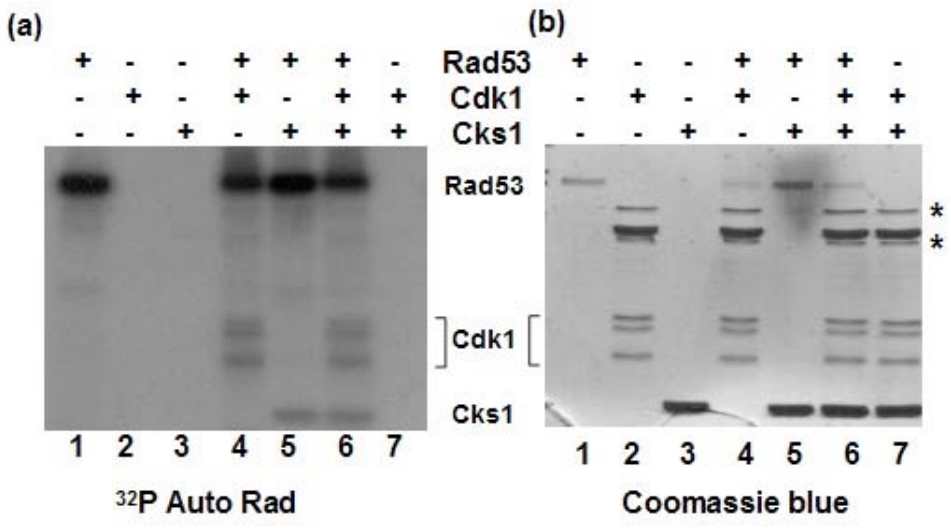


Figure 4.8 Cdk1 and Cks1 are directly phosphorylated by the checkpoint effector kinase Rad53 *in vitro*. (a) The samples were resolved by SDS-PAGE and Coomassie Blue-stained. (b) The phosphorylated proteins were detected by autoradiography (^{32}P). (1) reconbinant Rad53, (2) reconbinant Cdk1 (3) reconbinant Cks1, (4) reconbinant Rad53 and Cdk1, (5) reconbinant Rad53 and Cks1, (6) reconbinant Rad53, Cdk1, and Cks1, (7) reconbinant Cdk1 and Cks1. The additional bands visible in the Coomassie stain (marked with stars) correspond to E. coli proteins that coelute with GST-Cdk1

4.1.2.2 Rad53 phosphorylates Cdk1 at S46 and S258 in vitro

Using Rad53 *in vitro* kinase assay, we found the S phase checkpoint kinase Rad53 phosphorylated Cdk1 and Cks1 in vitro. We next prepare samples to identify the phosphorylation sites on Cdk1. Before that we carried out the same in vitro kinase assay to find out the plateau of Cdk1 phosphorylation by Rad53. Under the same conditions, except modification of the reaction time (35min, 50min, 65min and 80min) for ^{32}P incorporation to Cdk1, as seen in Figure 4.2, Rad53 and Cdk1 got more phosphorylated according to increased reaction time. And Cdk1 reached a plateau after 65 min incubation with Rad53. The *in vitro* kinase assay without ^{32}P (only add cold ATP) was then performed with 65 min reaction time. Control reactions were carried out under identical conditions but lack Rad53. Samples were resolved by SDS-PAGE and Coomassie Blue-stained. The gel slices containing the Cdk1 were excised for identifying the phosphorylated sites.

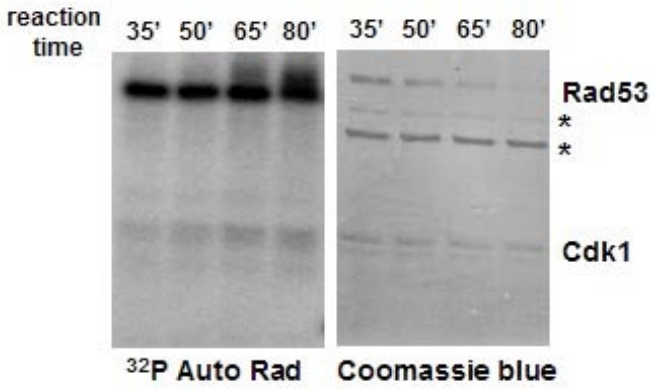
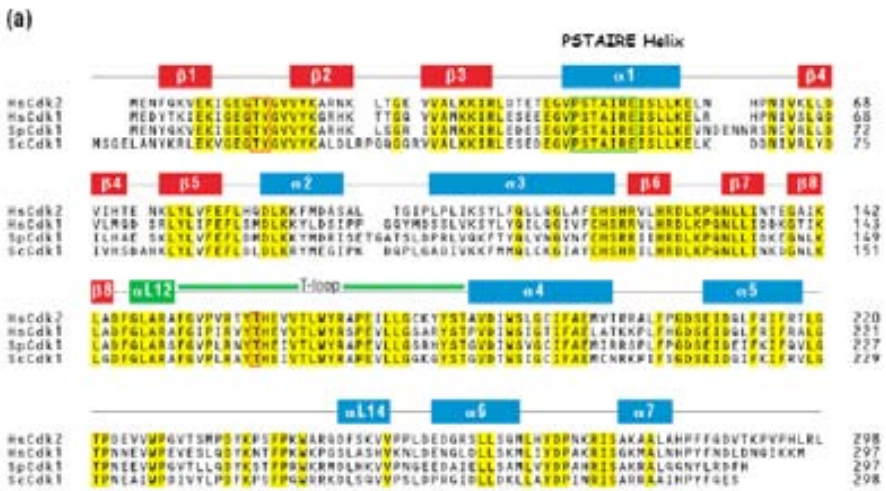


Figure 4.9 Rad53 kinase assay plateau

Two phosphorylated sites Ser46 and Ser258 were specifically identified by mass spectrometry on Cdk1 that had been incubated with Rad53, but not on mock incubated Cdk1.

From the alignment of homologue Cdk1 in different species, the Ser46 amino acid is conserved in human Cdk1 and Cdk2. And the homlogous sites of Ser46 in human Cdk1 and Cdk2 are located in human Chk2 (homolog of Rad53) target motif RxxS/T (O'Neill et al., 2002). In S pombe, the homologous site in Cdk1 is a negative charge aspartate (Figure 4.3). As seen in the aligned linear sequence compared with tertiary structure of human Cdk2 (Figure 4.3), Ser 46 is located in the N-terminus of Cdk1 and it precedes the PSTAIRE helix (enclosed in green box), which is a crucial docking motif for cyclins binding (De Bondt et al., 1993; Radzio-Andzelm, Lew, & Taylor, 1995). Interestingly, Ser46 had been reported as a phosphorylation site by CK2 (G. L. Russo et al., 2000; G. L. Russo, van den Bos, & Marshak, 2001). The homolog T39 in human Cdk2 has been suggested to be phosphorylated by AKT (DaSilva, 2008). And the other site Ser258 is located in the linker α helix 14, which is an important docking motif for Cks1 binding (Bourne et al., 1996).



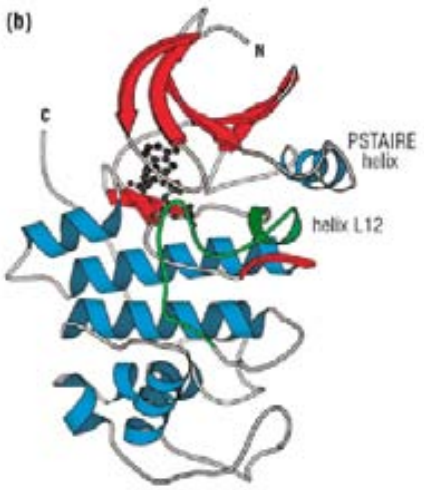


Figure 4.10 S46 and S258 localize to the pockets for Cyclins and CKs1 binding. (a) Amino-acid sequences of S. cerevisiae ScCdk1 alignment with major Cdks in humans HsCdk1, HsCdk2, S. pombe SpCdk1. Yellow residues are identical in all four kinases. Above the alignment, secondary motifs of human Cdk2 are shown with the tertiary structure in panel (b). (b) Tertiary structure of human Cdk2, determined by X-ray crystallography (David O. Morgan, 2007).

4.1.2.3 Mutation of S46 and S258 of Cdk1 alters cell size

To examine the role of Rad53 mediated Cdk1 phosphorylation in vivo, we generated the corresponding non-phosphorytable allele Cdk1-S46A, S258A (Cdk1-2A) and the phosphomimetic allele Cdk1-S46E, S258E (Cdk1-2E) and generated strains carrying such allele as single copy of the CDK1 gene. To determine the functional relevance of Ser46 and Ser258 phosphorylation of Cdk1, we were looking for phenotype change between wile type cells and the corresponding mutants. As seen in Figure 4.4 (a), cells carrying the non-phosphorytable Cdk1 allele (Cdk1-2A) show a *wee* phenotype, compatible with increased/unrestrained CDK activity (P Nurse & Thuriaux, 1980) and cells carrying the phosphomimetic Cdk1 allele (Cdk1-2E) are larger in size than wild type cells, elongated and longer in M phase, compatible with reduced/retarded M-CDK activity.

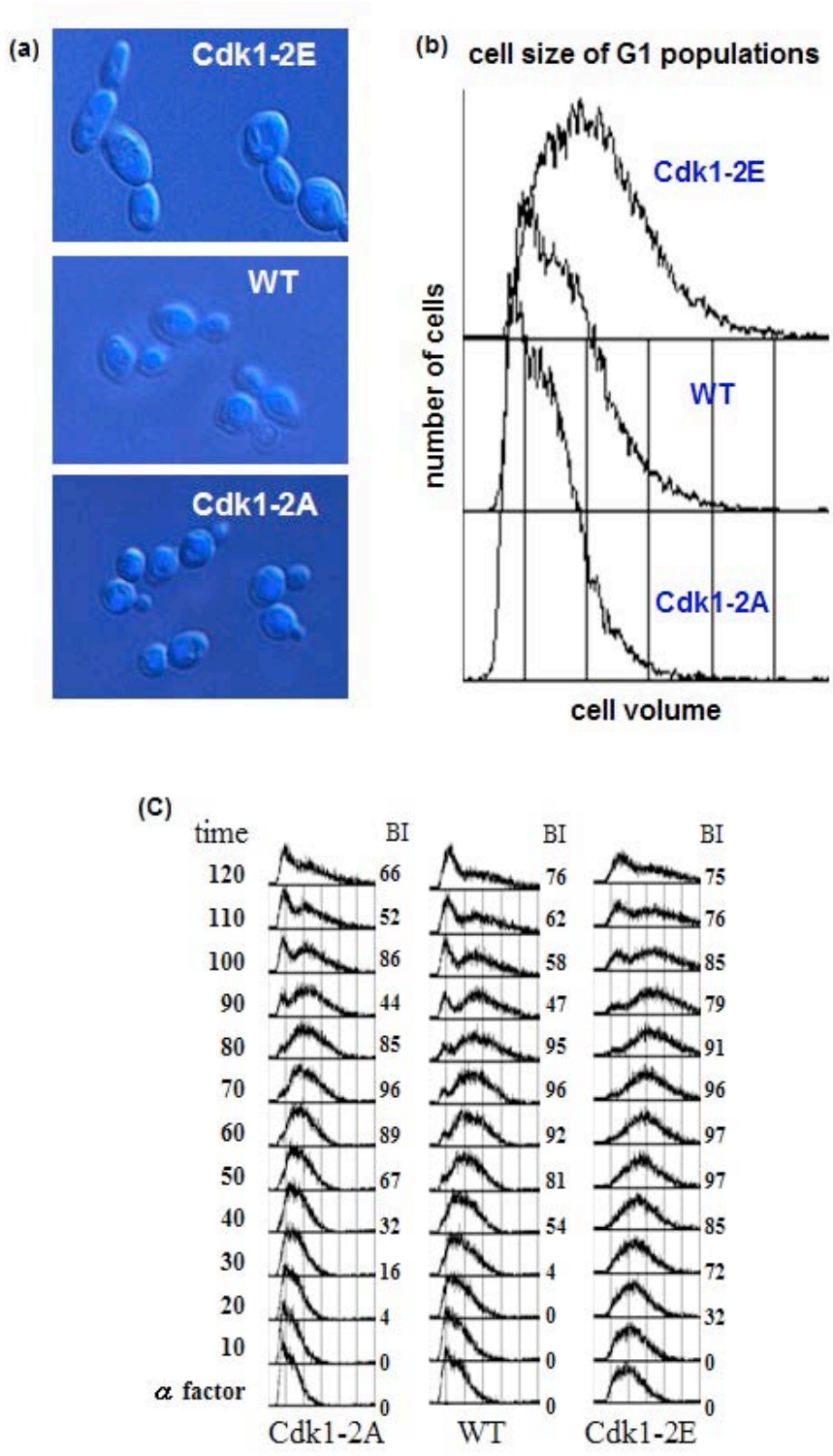


Figure 4.11 Morphologies altered in Cdk1 S46 and S258 mutants. (a) DAPI stained log phase non-phosphorylatable mutant Cdk1-2A (YFL20) and phosphomimetic mutant Cdk1-2E (YFL23) compared with WT cells under fluorescence microscopy. Photos were shown in a same scale. (b) Cell size analysis of G1 arrested Cdk1-2A and Cdk1-2E mutants by flowcytometry. (c) Cell size

analysis of Cdk1 S46 and S258 mutants throughout cell cycle released from G1 arrest. Cells were taken at the indicated time points after release. Budding indexes (BI) are shown as measures of synchronicity and cell cycle progression.

To quantify the difference of cell size, we sychronized the cells in G1 phase by adding α factor and more than 2000 cells in each sample were collected by a FACScan apparatus. As shown in Figure 4.4 (b), the population of cells with smaller size in Cdk1-2A strain is much more than in wile type cells and the larger size population of Cdk1-2E strain is significant higher compared with wile type cells. With cells released from G1 phase through the cell cycle, cells are growing bigger and bigger from G1, S, G2 till M phase. Cells carrying the non-phosphorytable Cdk1 allele (Cdk1-2A) show a constant smaller size and cells carrying the phosphomimetic Cdk1 allele (Cdk1-2E) are constantly larger in size compared with wile type cells (see Figure 4.4 (c)).

4.1.2.4 Phosphomimetic Cdk1 mutant shortens G1/S transition

To determine whether non-phosphorytable allele Cdk1-2A and the phosphomimetic allele Cdk1-2E would have an effect on cell cycle progression, we performed a FACS assay to show the cell cycle progression after G1 arrest release. Wile type cells, Cdk1-2A cells and also Cdk1-2E cells were growing in rich media until early exponential phase and then were synchronized with α factor. Cells were then released and aliquots were taken every 15 min intervals for cell cycle analysis. As seen in Figure 4.5 the different distributions of DNA content in exponential phase (exp) cells suggest that Cdk1-2A and Cdk1-2E have opposite defect compared with wile type cells which compatible with problems in G1-S transition or defect in G2/M phase. The same as suggested in exponential phase, we observed a significant and highly reproducible decrease of the time from alpha factor release to S phase in the cell expressing Cdk1-2E compared with wile type cells (shadowed with yellow box in Figure 4.5).

The wile type and Cdk-2A cells had similar S phase entry times. This shortened G1 phase was not due to different doubling times as shown in 90 min these three strains start to divide identically.

Although phosphomimetic mutation of S46 and S258 amino acid of Cdk1 have no significant effect on cellular proliferation, they progress faster through G1-S transition the G1 phase compared with wile type cells due to the phosphomimetic alteration suggest a novel CDK regulation.

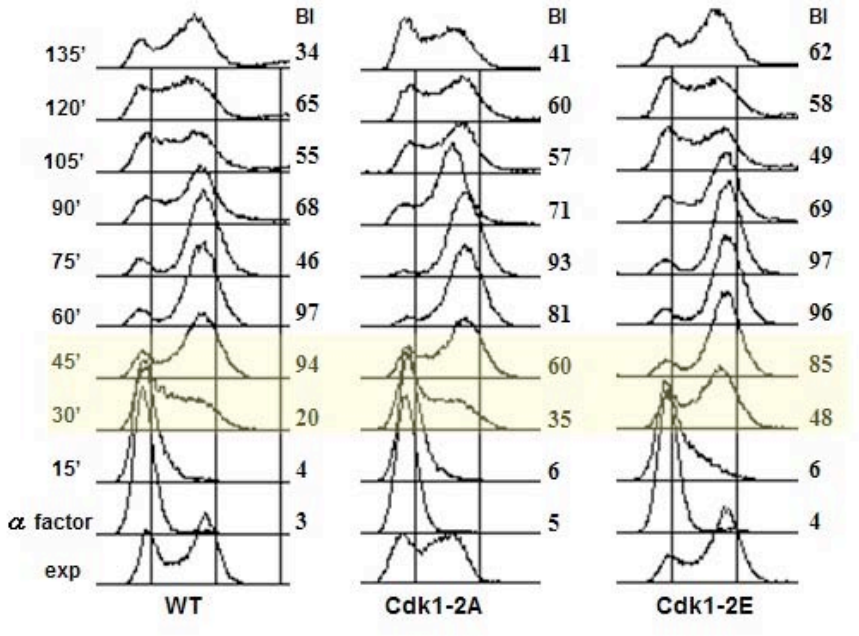


Figure 4.12 Cdk1-2E progresses faster through S phase, Cdk1-2A more slowly compared with WT cells using FACS. Exponetial growing cultures (WT strain YGP30, Cdk1-2A strain YFL20, Cdk1-2E strain YFL23) were synchronized with α-factor (G1). The cells were then released from the G1 arrest by several washes with medium lacking α-factor and allowed to progress into the S phase. The samples were taken at the indicated time points. Fluorescence-activated cell sorting analysis of DNA content. Budding indexes (BI) are shown as measures of synchronicity and cell cycle progression. The progresion difference were shown in a yellow shadow.

We next checked whether non-phosphorytable allele Cdk1-2A cells have any defect in response to genotoxic stress where the checkpoint Rad53 is activated. First, five-fold serial dilutions of exponential cells were spotted onto YPD plates with or without genotoxic agents including HU, MMS or CPT and incubated at 30 ^{o}C for four days**.** However, we did not see any significant sensitivity of the S46 and S258 mutants to those genotoxic stress shown in Figure 4.6 (a). And then we carried out experiment to test if the mutants loss the ability to recover from DNA replication stress. Cells released from α-factor arrest in G1 phase to HU. After 1 hour HU treatment, cells were washed and released to YPD without HU. Samples in every 15 min intervals were analyzed by flow cytometry. As shown in Figure 4.6 (b), neither Cdk1-2A nor Cdk1-2E mutant has defect on recovering from HU.

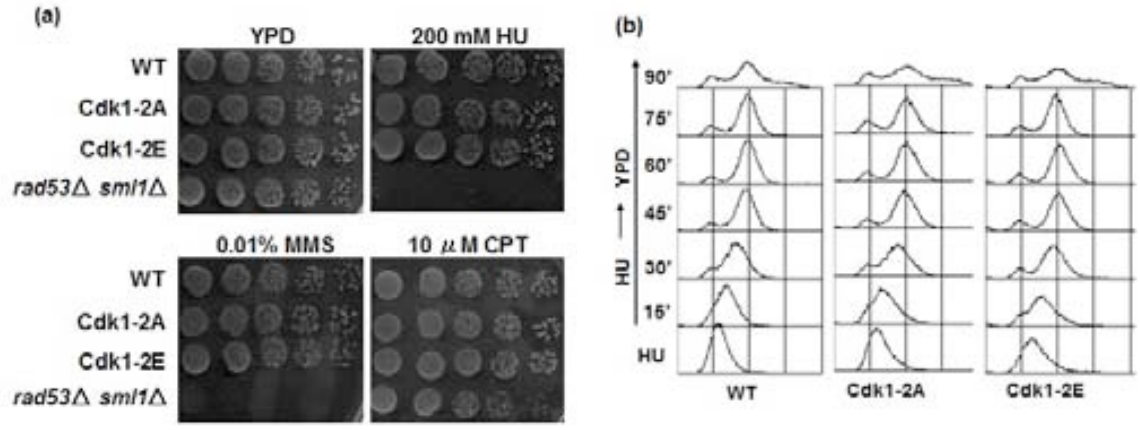


Figure 4.13 Cdk1-2A cells are not sensitive to genotoxic stress. (a) Cdk1-2A cells are not sensitive to different genotoxic stress regents. 5-fold serial dilutions of the indicated cell cultures, growing exponentially in YPD, were spotted onto plates containing YPD, YPD supplemented with 200 mM hydroxyurea, YPD supplemented with 0.01% MMS or YPD supplemented with 10 μM CPT. The wild type strain (WT) and the checkpoint mutant rad53-del were included as controls for sensitivity to genotoxic stress. (b) Cdk1-2A cells have no defect on recovering from HU. Exponential growing cells were synchronized in G1 and released into S phase in the presence of hydroxyurea (1h HU). After 1 h of incubation, HU was washed away,

and the cells were allowed to recover from replication stress in rich medium (YPD). The samples were taken at the indicated time points.

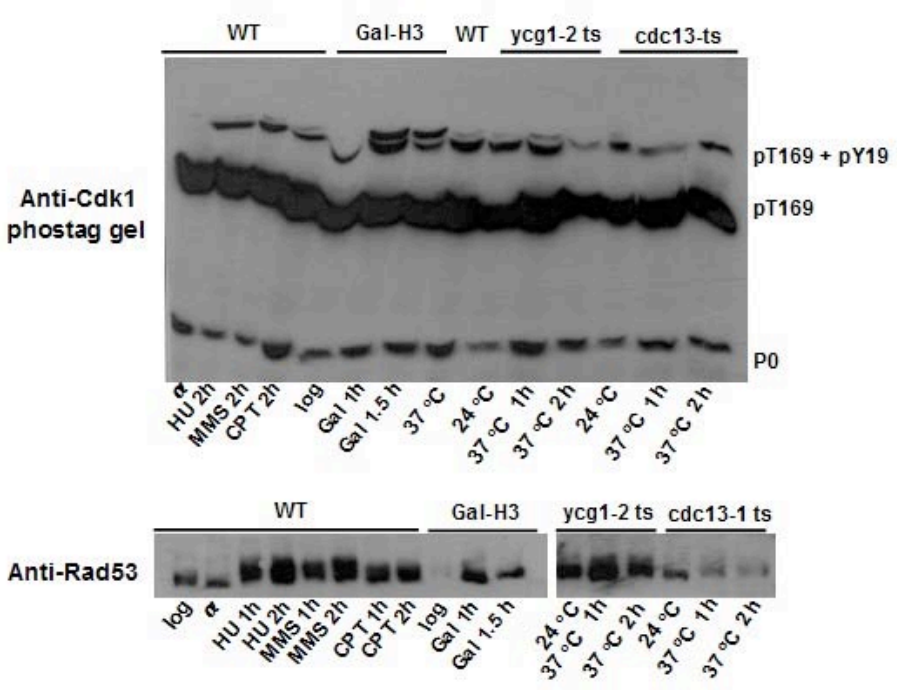


Figure 4.14 Cdk1 phosphorylation state in different Rad53 dependent/required genotoxic stress conditions.

4.2 A NOVEL CDK1 ASSOCIATED FACTOR, YPL014W (CIP1)

4.2.1 Identification of YPL014W as a novel Cdk1 associated protein

4.2.1.1 Proteomic analysis of Cdk1 associated factors

The cell division cycle of the yeast *S. cerevisiae* is driven by a unique Cdk (cyclin-dependent kinase) cdk1, which bound to one of nine cyclin subunits, different substrates and other associated proteins in different cell cycle phases as well as genotoxic conditions. The S phase checkpoint center kinase Rad53 attenuates and protects DNA replication, triggers an *ad hoc* transcriptional response, and blocks cell cycle progression until the problem is solved. In this study,

we carried out a targeted proteomics study using immuno-purification coupled to mass spectrometry.

In order to identify proteins that associate with Cdk1 in a Rad53 dependent manner upon DNA replication stress, we performed large-scale immuno-precipitation of Cdk1 on cell lysates from hydroxyurea stressed rad53-del cells as well as stressed WT cells as control (Figure 4.15). In order to get more specific associated proteins, the large scale immuno-precipitation was washed 5 times with strong wash buffer containing 125 mM NaCl.

In this proteomic study, we identified totally 1078 candidate proteins bound to Cdk1 in stressed cells, in which 452 proteins interacted with Cdk1 in both rad53-del and WT strains, 536 proteins associated with Cdk1 only in rad53-del strain and of our interest there were 90 proteins binding to Cdk1 only in stressed WT cells not with Cdk1 when Rad53 was knocked out (see Figure 4.15).

This proteomic study is quite specific and reliable. First, we immuno-purified microgram grade of in vivo Cdk1 proteins which is even visual as strong as the input IgG chains in coomassie stained gel (shown in Figure 4.15). Second, we found many well known Cdk1 binding proteins in the list, including the cyclins, CDK regulatory subunit and adaptor Cks1, CDK inhibitor Sic1, Cdk1-activating kinase Cak1 among others (data not shown). As seen in Figure 4.15, the group of associated proteins only in one of the two strains (more than half of the identified proteins) is dependent on Rad53. Our aim and interest is the group of 90 proteins shown in Figure 4.15 and Table 4.1, which are the Cdk1 associated proteins bound to Cdk1 only when Rad53 is present. In the Table 4.1, those identified 90 proteins were ranked according to the frequency of peptide occurrence.

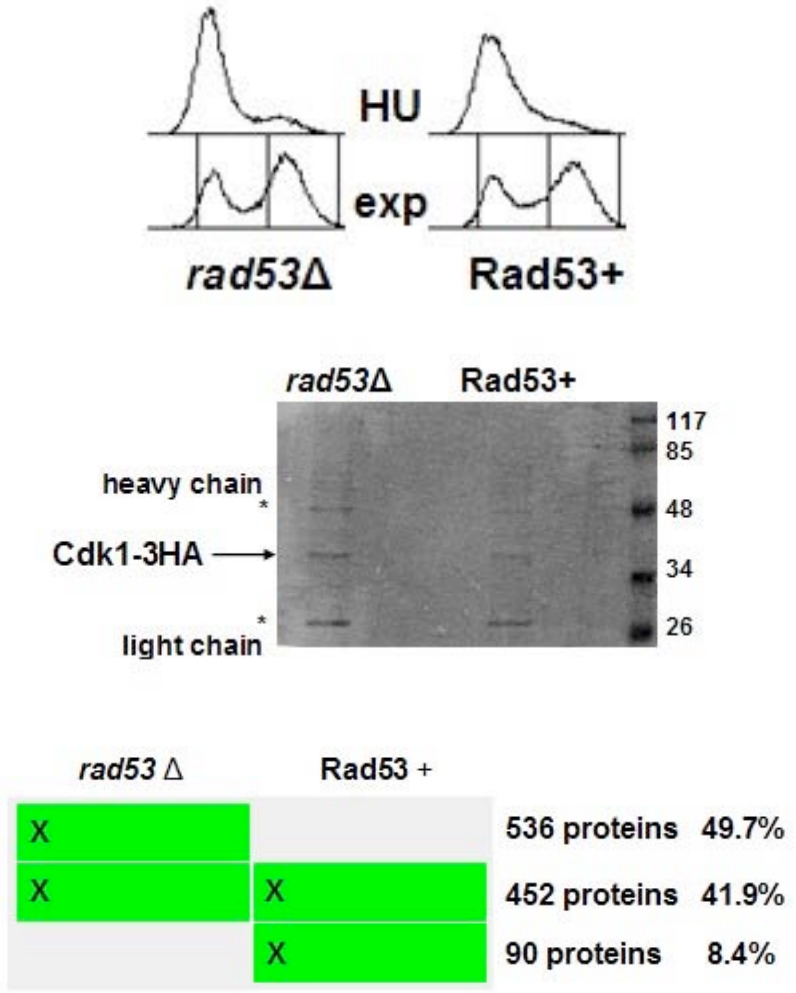


Figure 4.15 Cdk1 interactors identified in *rad53*Δ vs Rad53+ cells by mass spectroscopy.

There are a large amount of well known proteins (39 out of 90, shown in blue code in Table 4.1) directly connected to Cdk1 and/or DNA replication stress response. In the list we found plenty of interactors whose abundance increase in response to DNA replication stress, like Hch1, Mbf1, Rbl2, et al and also some interactors like Pbp4, Scd6 and YLR114C et al, forms nuclear or cytoplasmic foci upon DNA replication stress. There are also some proteins which are well known Cdk1 proteins connected to Rad53, like Srl3, the second one in the list is potential Cdk1 substrate who suppresses the lethality of a rad53 null mutation when

overexpressed. Still there are also 9 proteins with unknown function which were identified as potential Rad53 dependent Cdk1 interactors. Among these highly potential Rad53 dependent Cdk1 interactors found in this study, we are exploring the unknown function protein YPL014W ranked No.1 in the list and leave the rest candidate for future work.

Table 4.1 Proteins associated with Cdk1 in stressed WT cells but not in stressed rad53 null cells

Cdk1 interactors	description
YPL014W	unknown function (Cdk1 associated protein proved in this study)
SRL3	when overexpressed, suppresses the lethality of a rad53 null mutation; potential Cdk1 substrate; the paralog of WHI5
HCH1	protein abundance increases in response to DNA replication stress
RPC19	RNA polymerase subunit
ADH4	Alcohol dehydrogenase isoenzyme type IV
MBF1	Transcriptional coactivator; protein abundance increases in response to DNA replication stress
CUP1-2	Metallothionein; binds copper and mediates resistance to high concentrations of copper and cadmium
CUP1-1	Paralog of CUP1-2
TIF11	Translation initiation factor eIF1A
YAL049C	Cytoplasmic protein involved in mitochondrial function; null mutant displays reduced frequency of mitochondrial genome loss
PRE1	Beta 4 subunit of the 20S proteasome; localizes to the nucleus throughout the cell cycle, Cdk1 physical interaction
RBL2	required for protection from excess free beta-tubulin; protein abundance increases in response to DNA replication stress
REC104	forms a complex with Rec102p and Spo11p necessary during the initiation of recombination
DUT1	preventing incorporation of uracil into DNA during replication; critical for the maintenance of genetic stability
RPO26	RNA polymerase subunit
YNL010W	Putative protein of unknown function with similarity to

	phosphoserine phosphatases
CAF20	Phosphoprotein of the mRNA cap-binding complex involved in translational control
YKU70	Subunit of the telomeric Ku complex (Yku70p-Yku80p); involved in telomere length maintenance; relocates to sites of double-strand cleavage to promote nonhomologous end joining during DSB repair
POA1	Phosphatase that is highly specific for ADP-ribose 1"-phosphate; a tRNA splicing metabolite
TMA20	Protein of unknown function that associates with ribosomes; null mutant exhibits translation defects; has homology to human oncogene MCT-1; protein abundance increases in response to DNA replication stress
HRT3	Putative SCF-ubiquitin ligase F-box protein; identified in association with Cdc53p, Skp1p and Ubi4
CDC37	Essential Hsp90p co-chaperone; necessary for passage through the START phase of the cell cycle, Cdk1 physical interactions
PHO3	Constitutively expressed acid phosphatase similar to Pho5p
FSH1	Putative serine hydrolase; sequence is similar to the human candidate tumor suppressor OVCA2
PBP4	Pbp1p binding protein; relative distribution to the nucleus increases upon DNA replication stress
SKP1	Evolutionarily conserved kinetochore protein; part of the SCF ubiquitin ligase complex, the CBF3 complex that binds centromeric DNA, and the RAVE complex that regulates assembly of the V-ATPase; protein abundance increases in response to DNA replication stress
YER156C	Putative protein of unknown function
CLN1	G1/S cyclin involved in regulation of the cell cycle
SCD6	Repressor of translation initiation; forms cytoplasmic foci upon DNA replication stress
SPS2	Protein expressed during sporulation
TUB3	Alpha-tubulin; associates with beta-tubulin (Tub2p) to form

	tubulin dimer, which polymerizes to form microtubules, Cdk1 genetic interaction
YCR087C-A	Putative protein of unknown function
YER030W	Histone chaperone required for the stabilization of the Chz1p-Htz1-H2B complex; null mutant displays weak sensitivity to MMS and benomyl; protein abundance increases in response to DNA replication stress
RAD23	Damaged DNA binding
MRPL49	Mitochondrial ribosomal protein of the large subunit
SAM4	S-adenosylmethionine-homocysteine methyltransferase
ENT4	Protein of unknown function
TWF1	Twinfilin
ASI3	Putative integral membrane E3 ubiquitin ligase
YGL242C	Putative protein of unknown function
LHP1	RNA binding protein required for maturation of tRNA and U6 snRNA precursors
CLB4	B-type cyclin involved in cell cycle progression
ECM4	Omega class glutathione transferase
IDI1	Isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IPP isomerase)
ADE4	Phosphoribosylpyrophosphate amidotransferase, Cdk1 genetic interaction
PIN3	Negative regulator of actin nucleation-promoting factor activity; interacts with Las17p, a homolog of human Wiskott-Aldrich Syndrome protein (WASP)
ARG4	Argininosuccinate lyase
POL32	Third subunit of DNA polymerase delta; involved in chromosomal DNA replication; required for error-prone DNA synthesis in the presence of DNA damage and processivity
EAP1	eIF4E-associated protein, competes with eIF4G for binding to eIF4E; functions independently of eIF4E to maintain genetic stability
RKI1	Ribose-5-phosphate ketol-isomerase

PYC1	Pyruvate carboxylase isoform
ADE17	Enzyme of 'de novo' purine biosynthesis
DBP1	Putative ATP-dependent RNA helicase; protein abundance increases in response to DNA replication stress
SER33	3-phosphoglycerate dehydrogenase
QRI1	UDP-N-acetylglucosamine pyrophosphorylase; protein abundance increases in response to DNA replication stress
CDC10	Component of the septin ring at the mother-bud neck act as scaffolds for recruiting cell division factors; protein abundance increases under DNA damage stress
YRB2	Protein of unknown function, phosphorylated by Cdk1
RPN3	proteasome role in cell cycle control, Cdk1 physical interaction
TUP1	involved in the establishment of repressive chromatin structure through interactions with histones H3 and H4, Cdk1 genetic interaction
MET22	Bisphosphate-3'-nucleotidase
PGU1	Endo-polygalacturonase
CLN2	G1/S cyclin involved in regulation of the cell cycle
YLR114C	Conserved protein involved in exocytic transport from the Golgi; relocalizes from bud neck to cytoplasm upon DNA replication stress
CUE5	Ubiquitin-binding protein, phosphorylated by Cdk1
LST4	Protein possibly involved in a post-Golgi secretory pathway
MAP2	Methionine aminopeptidase
RPC82	RNA polymerase III subunit
TIF3	Translation initiation factor eIF-4B
TFA1	TFIIE large subunit; involved in recruitment of RNA polymerase II to the promoter, activation of TFIIH, and promoter opening
LAS1	Protein required for pre-rRNA processing at both ends of ITS2; required for the G1/S cell cycle transition; human ortholog Las1L
RCK1	kinase involved in the response to oxidative stress; identified as suppressor of S. pombe cell cycle checkpoint mutations
REF2	RNA-binding protein, phosphorylated by Cdk1

DSN1	Essential component of MIND kinetochore complex (Mtw1p Including Nnf1p-Nsl1p-Dsn1p); important for chromosome segregation, phosphorylated by Cdk1
GGA2	Protein that regulates Arf1p, Arf2p to facilitate Golgi trafficking
GAD1	Glutamate decarboxylase; involved in response to oxidative stress
APP1	Phosphatidate phosphatase
MDM32	Mitochondrial inner membrane protein
CRN1	Coronin
VPS5	Nexin-1 homolog
YIL067C	Uncharacterized protein of unknown function
PTK2	Putative serine/threonine protein kinase; involved in regulation of ion transport across plasma membrane, Cdk1 physical interaction
XRS2	Protein required for DNA repair; component of the Mre11 complex, which is involved in double strand breaks, meiotic recombination, telomere maintenance, and checkpoint signaling
AAP1	Arginine/alanine amino peptidase
CDC13	Single stranded DNA-binding protein found at TG1-3 telomere G-tails; key roles in regulation of telomerase, telomere end protection, and conventional telomere replication, Cdk1 physical interaction
SEC24	Component of the Sec23p-Sec24p heterodimer of the COPII vesicle coat
PMA2	Plasma membrane H+-ATPase
ECM17	Sulfite reductase beta subunit
NMA111	Serine protease and general molecular chaperone; involved in response to heat stress and promotion of apoptosis
HFA1	Mitochondrial acetyl-coenzyme A carboxylase; relocalizes from mitochondrion to cytoplasm upon DNA replication stress
SPT6	Nucleosome remodeling protein
TOR2	PIK-related protein kinase and rapamycin target

4.2.1.2 Identification of YPL014W (Cip1) as a novel Cdk1 interactor

We identified an unknown function protein YPL014W as a highly potential Cdk1 interactor with the highest frequency of peptide occurrence in the proteomic study. We next explored the expression and function in cell cycle in this part. Hereafter we use Cyclin dependent kinase Interacting Protein 1 (Cip1) instead of the systematic name YPL014W since it was identified as a novel Cdk1 associated protein.

To confirm the association between Cip1 and Cdk1, we immuno-precipitated Cip1 with the C terminus 13 repeat myc tag in the exponential growing cells followed by immunoblotting against Cdk1. As shown in Figure 4.16 (a), Cdk1 was reproducible fished with Cip1-13myc using agarose beads cross linked with antibody against myc.

As Cdk1 alternately associates with different cyclins as a complex in different cell phase, we next want to check if Cip1 could be a candidate regulator alternately bound to Cdk1-cyclin. For this, we chose one cyclin in each type (G1/S cyclin Cln2, S cyclin Clb5 and M cyclin Clb2) as the target cyclins to analyze the association with Cip1. In order to make the all the co-immunoprecipitation identical, the cyclins were tagged with the same C terminus 3 HA, and Cip1 with 13myc. All the corresponding strains were collected in mid exponential growing state. As shown in Figure 4.16 (b), with G1/S cyclin Cln2-3HA co-immunoprecipitation, we detected strong signal of Cip1 corresponding to the correct size as input control showed by immunoblotting. However, Cip1 was not detected in the S cyclin Clb5 or M cyclin Clb2 co-immunoprecipitation (Figure 4.16 (b)). So through co-immunoprecipitation experiment, we confirmed the association of Cdk1 and Cip1 and in addition Cip1 alternately interacted with G1/S cyclin Cln2 but not S cyclin Clb5 or M cyclin Clb2, which suggests that Cip1 may do the function in G1 phase related to Cln2-Cdk1 complex.

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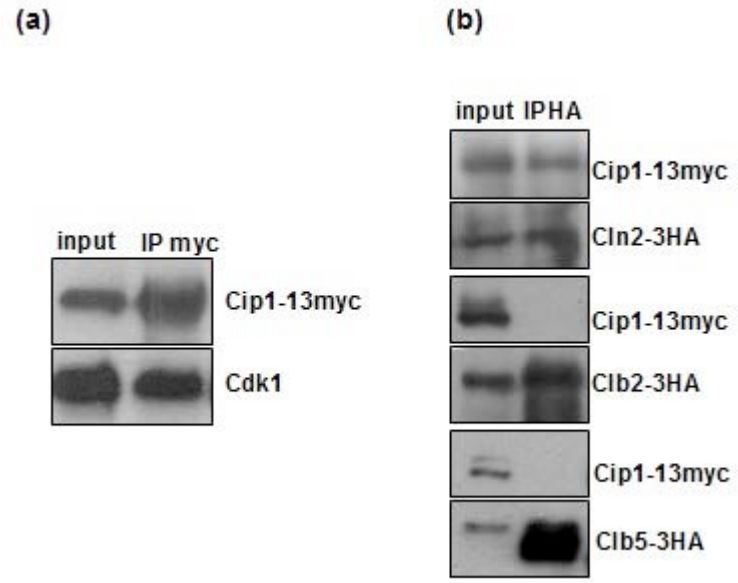


Figure 4.16 Cip1 specifically interacts with Cln2-Cdk1 but with not Clb2 or Clb5 by Co-Immunoprecipitation. (a) Exponential growing cells YFL100 (Cip1-13myc) were extracted followed by immuno-precipitation using agrose beads cross-linked with antibody against myc. The IPed proteins were analysed by immonoblot with anti-Cdk1 antibodies. (b) Exponential growing cells YFL117 (Cln2-3HA, Cip1-13myc), YFL106 (Clb5-3HA, Cip1-13myc) and YFL105 (Clb2-3HA, Cip1-13myc) were extracted followed by immuno-precipitation using agrose beads cross-linked with antibody against HA (Cln2, Clb5 or Clb2) as indicated. The IPed proteins were seperated analysed by immonoblot with anti-myc (Cip1) antibodies.

4.2.1.3 Cip1 is a putative Cdk1 inhibitor

No obvious sequence homologue of Cip1 was not found in the databases, as indicated by low scores from BLAST (Altschul, Gish, Miller, Myers, & Lipman, 1990). However, the alignment of Cip1 reveals a side-by-side conserved match to the CDK binding motif present in human Cip/Kip CKI p21 (Figure 4.17). Similarly, budding yeast Sic1 which is the functional homologue of human Cip/Kip CKI p27, has very low sequence homology but the inhibitory domain of Sic1 is functionally and structurally related to the inhibitory domain of

p27 (Barberis et al., 2005). The loss of sequence homology of CKI between species mostly is due to the evolutional diversity of cyclins which hence leads to the structurally diversity of both cyclins and their binding factors.

Figure 4.17 Alignment of Cip1 (YPL014W) with human Cdk inhibitor p21 (Cip1). The protein sequences of Cip1 (Gene ID: 856093) and p21 (Gene ID: 1026) from NCBI genebank were aligned by ClustalW http://www.ebi.ac.uk/Tools/clustalw2/ with default settings. The extent of conservation is labelled below according to ClustalW definitions: identical residues (*); conservative substitutions (:); semi-conservative substitutions (.). The Cdk binding motif of p21 is enclosed with a red box.

To investigate whether Cip1 is a putative inhibitor, first we asked whether Cip1 could directly bind to Cdk1 *in vitro*. To know the information, we carried out GST pull down assay considered to analyze the direct binding between two proteins. The lysate extracted from exponential growing WT cells was cleaned by recombinant GST protein and then was incubated with recombinant GST-Cip1 bound glutathione-agarose beads or GST bound ones

as control. As seen in Figure 4.18 (a), Cdk1 is physically bound to recombinant GST-Cip1 but not with GST alone. The coomassie stained gel shows that GST-Cip1 used as probe is even much lesser than the control GST; still we detect Cdk1 with GST-Cip1 not with GST alone. This GST pull down assay well proved the physical interaction of Cip1 and Cdk1.

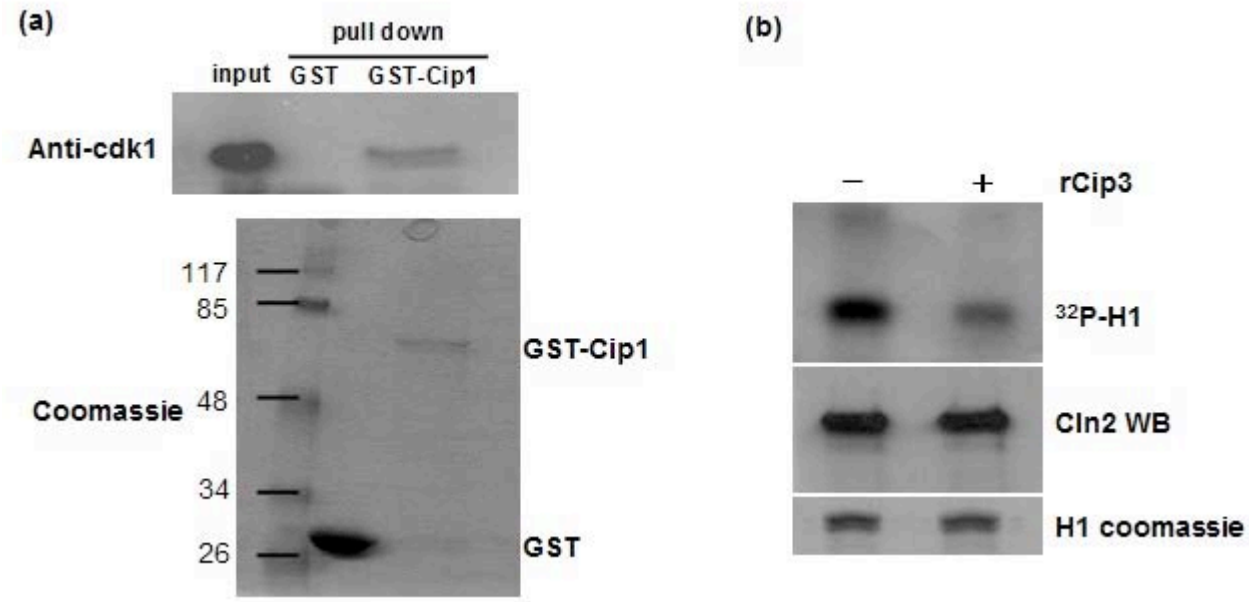


Figure 4.18 Cip1 physically interacts with Cdk1 and inhibits Cln2-CDK activity. (a) GST pull down assay. Glutathione-agarose beads affinity bound with GST-Cip1 (GST as control) as probe were incubated with pre-cleaned WT cell lysate for 2 h and followed by 5 washes, the pull-downed proteins were analyzed by immunoblot with anti-Cdk1 antibody. The upper panel showed the immunoblot against Cdk1, the lysate was loaded as input control. The lower panel showed the recombinant GST-Cip1 and recombinant GST proteins as the probes. (b) CDK kinase assay. Cln2-Cdk1 complexes were co-immunoprecipitated from lysate of cells overexpressing hyperstable Cln2 (YFL116). Recombinant Cip1 or the same amount of elution buffer was incubated with Cln2-Cdk1 and the substrate histone H1 in the presence of ATP (^{32}P). The samples were resolved by SDS-PAGE and Coomassie Blue-stained. Activity of histone H1 was detected by autoradiography (^{32}P).

As shown in 4.2.1.2, Cip1 interacts with Cln2, we next explored whether the recombinant Cip1 (rCip1) could directly inhibit Cln2-CDK activity. We purified the recombinant GST-Cip1 and released rCip1 by cleaving the GST moiety with Pre-Scission protease. Cln2-Cdk1 was co-purified and assayed by its ability to transfer ATP (^{32}P) to histone H1. In order to get enough Cln2, we overexpressed and coimmunoprecipetated hyperstable form of Cln2 (Cln2-7A) from cells arrested in G1 by overexpression of Clb-Cdk1 inhibitory Sic1 (Sic1-70C). Because it is possible that Cln2-Cdk1 could phosphorylate recombinant Cip1 in the assay, we first incubated IPed Cln2-Cdk1 with rCip1 in the presence of ATP before adding the substrate histone H1 and ATP (^{32}P) to avoid the competetion of ATP (^{32}P) between histone H1 and rYL014W.

As we can see in Figure 4.18 (b), addition of rCip1 resulted in a prominent decrease of CDK kinase activity on histone H1 compared with the control where only the same volume of elution buffer was added. Coomassie-blue staining confirmed that equal amounts of histone H1 were added and immunoblot against Cln2 showed the same amounts of Cln2 were added in both reactions. This *in vitro* kinase assay argues strongly that Cip1 directly inhibit the ability of Cln2-Cdk1.

The unpublished results in our lab suggest that high activity of Cln2-CDK triggers DNA replication. In the experiment, last C terminus 70 amino acids peptide Sic1C70 under Gal1, 10. promoter was used as Clb-CDK inhibitor. The C-terminal 70 amino acids peptide is the CDK inhibitory motif of Sic1 (See Figure 1.8) which is considered as the strongest S, G2, M-CDK inhibitor when overexpressed (John Diffley, personal communication). In this strain, cells were blocked at G1/S transition due to lack of Clb-CDK activity (see strain 1 in Figure 4.18). In the second strain cells are still lack Clb-CDK activity, but a nuclear located hyperstable Cln2 under Gal1, 10 promoter (Gal-Cln2 (7A) NLS) accumulated which can trigger DNA replication as shown in Figure 4.18. If Cip1 is a G1-CDK inhibitor, this DNA

replication triggered by high level of Cln2 could be inhibited by Cip1 accumulation. The same as what we expected, overexpression of Cip1 in deed blocked the DNA replication induced by high activity of Cln2. Figure 4.18 (b) shows the same hyperstable Cln2 accumulated in both strains. Results from this experiment indicate that Cip1 could inhibit Cln2-CDK activity *in vivo*.

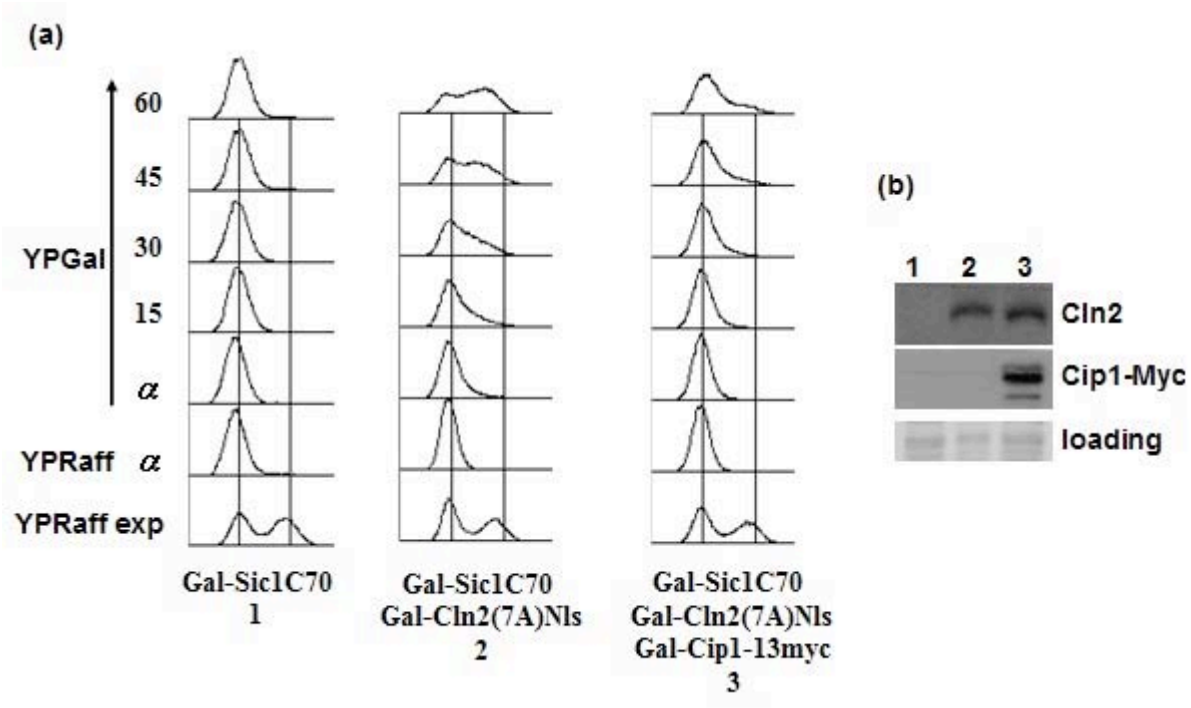


Figure 4.19 Over-expression of Cip1 inhibits the DNA replication triggered by hyper-activated Cln2-Cdk1. Log phase cultures of indicated strains 1, 2 and 3 (YAM19, YFL112, YFL113) grown in YP raffinose were synchronized with α-factor (G1). Galactose was added to a final concentration of 2% while keeping the presence of α-factor for 30 min. After 30 min induction, the cells were then released to YPGal with induction of Cip1. Samples were taken at the indicated points. (a) fluorescence-activated cell sorting analysis of DNA content. Budding indexes (BI) are shown as measures of synchronicity and cell cycle progression. (b) whole cell extracts were analyzed by immunoblot with anti-Cln2 and anti-Myc (Cip1) antibodies shown as a loading control. A Ponceau S-stained region of the same membrane used for immunoblot is shown as a loading control.

4.2.2 Overexpression of Cip1 blocks G1/S transition

4.2.2.1 Overexpression of Cip1 blocks cell entry into S phase

Evidences show that Cip1 interacts with and inhibits Cln2-Cdk1 both *in vitro* and *in vivo*. We then checked if there was any phenotype of Cip1-null mutant as well as Cip1 overexpression cells compared with WT. As we see in Figure 4.20 (a), FACS result shows that over-expression of Cip1 significantly inhibits cells entry into S phase. Even till 120 min upon alpha factor release into YPGal, cells overexpressing Cip1 under Gal1,10 promoter is tightly arrested in G1 phase, while as most of WT cells have already entered S phase at 60 min upon release. And cells overexpressing Cip1 induced by galactose show a significant larger size compared with un-induced cells (Figure 4.20 (b)). Moreover, the expression of S phase cyclin Clb5 is inhibited by overexpression of Cip1 as seen in Figure 4.20 (c). As control, the western blotting against Cip1 showed a proper overexpression induced by galactose compared with un-induced one.

Over-expression of Cip1 blocks cells in G1 with larger cell size, opposite to this *cip1*Δ cells are expected to be faster entry into S phase with smaller size. As shown in Figure 4.21 (a), at 18^{o}C where cells progress slowly enough to see obvious difference, while as *Cip1*Δ cells progress G1-S transition identically to WT cells. However, the cell size of *Cip1*Δ strain is obvious smaller than WT. To quantify the difference of cell size, cells were arrested in G1 by adding α factor, and more than 2000 cells in each sample were collected by a FACScan apparatus. As shown in Figure 4.21 (b), most of *Cip1*Δ cells have smaller size than WT in G1 phase.

*cip1*Δ cells show slightly smaller size but no difference in G1/S progression which could be due to exist of another redundant control. Above all, overexpression of Cip1 blocks cells in G1 phase with larger cell size and deletion of Cip1 leads to smaller size, which

suggests that Cip1 as a Cln2-Cdk1 associated protein may act as a Cdk1 negtive regulator blocking S phase entry when over-expressed.

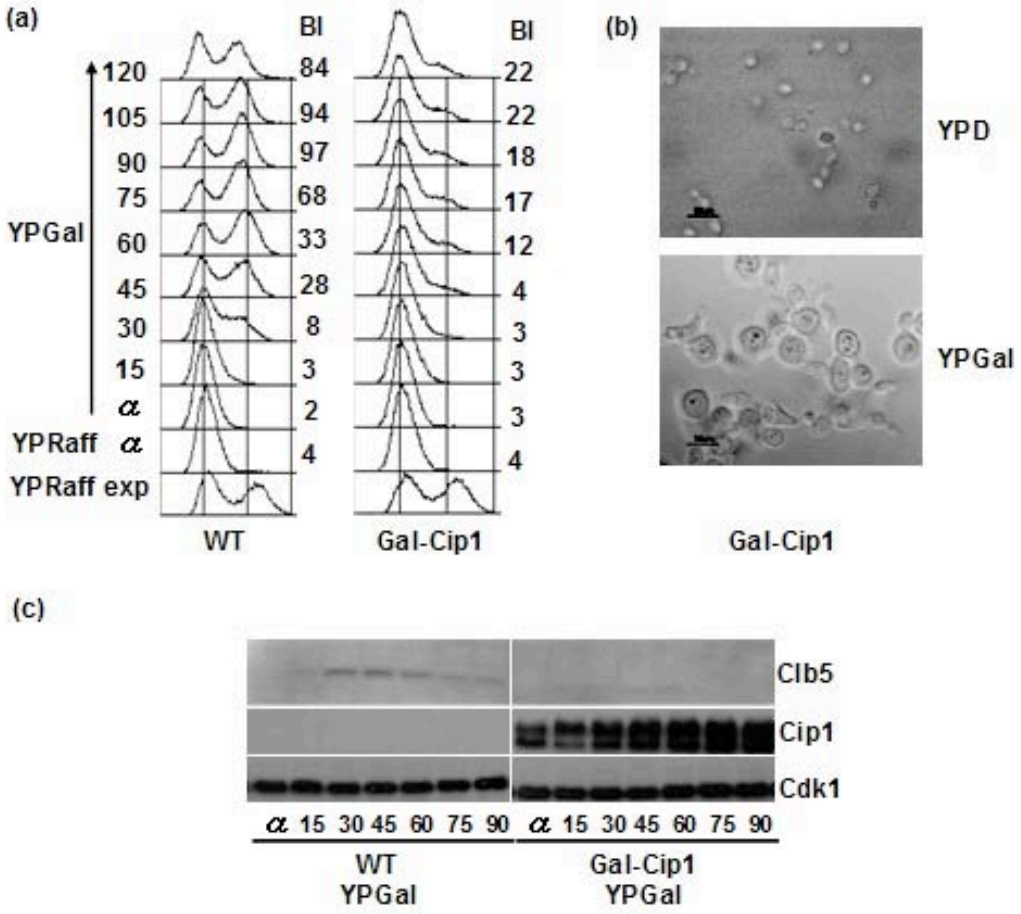


Figure 4.20 Over-expression of Cip1 leads to cells G1 arrest. Log phase cultures of GAL-Cip1-13myc (strain YFL97) or control cells (strain YGP30) grown in YP raffinose were synchronized with α-factor (G1). Galactose was added to a final concentration of 2% while keeping the presence of α-factor for 30 min. After 30 min induction, the cells were then released to YPGal with induction of Cip1. Samples were taken at the indicated points. (a) fluorescence-activated cell sorting analysis of DNA content. Budding indexes (BI) are shown as measures of synchronicity and cell cycle progression. (b) A Log phase culture of GAL-Cip1-13myc (strain YFL97) were growing in YPD medium or in rich medium containing 2% galactose (YPGal) for overnight induction of Cip1. (c) whole cell extracts were analyzed by immunoblot with anti-Myc (Cip1), anti-Clb5 and with anti-Cdk1 antibodies shown as a loading control.

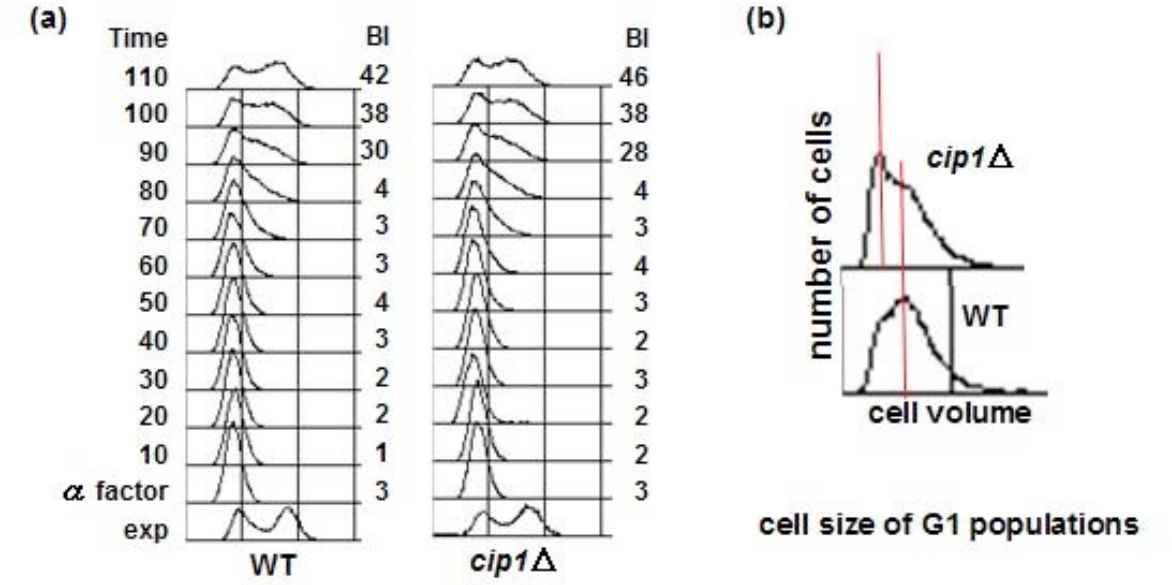


Figure 4.21 *Cip1*Δ cells show slightly smaller size but the same G1/S progression time compared with WT cells. (a) Fluorescence-activated cell sorting analysis of DNA content in WT and *Cip1*Δ cells. Exponetial growing cultures (WT strain YGP30, *Cip1*Δ strain YFL110) were synchronized with α-factor (G1). The cells were then released from the G1 arrest by several washes with medium lacking α-factor and allowed to progress into the S phase. The samples were taken at the indicated time points. Budding indexes (BI) are shown as measures of synchronicity and cell cycle progression. (b) FL2 channel of cell sorting shows the cell distribution upon cell volume.

4.2.2.2 Sic1 is stable in cells over expressing Cip1

Sic1 is a good specific G1-CDK target *in vivo*. As shown in Figure 4.22 (a) once Sic1 is phosphorylated on at least six of its nine potential phosphorylation sites of Cln-CDK1, it gets targeted for SCF mediated degradation (Sheaff & Roberts, 1996; Verma, Feldman, & Deshaies, 1997). Since Cip1 might be a Cln2-CDK inhibitor, we next want to check if the activity is inhibited *in vivo* when Cip1 is over-expressed by detecting Sic1 protein level after G1 release. As we see in Figure 4.22 (b), as control Sic1 protein gets degraded soon after G1 release in WT cells, however, in cells over-expressing Cip1 Sic1 gets very stable for a long

time after G1 release. A Ponceau S-stained region of the same membrane used for immunoblot is shown as a loading control. It again suggests that Cip1 is a putative Cln-Cdk1 negtive regulator.

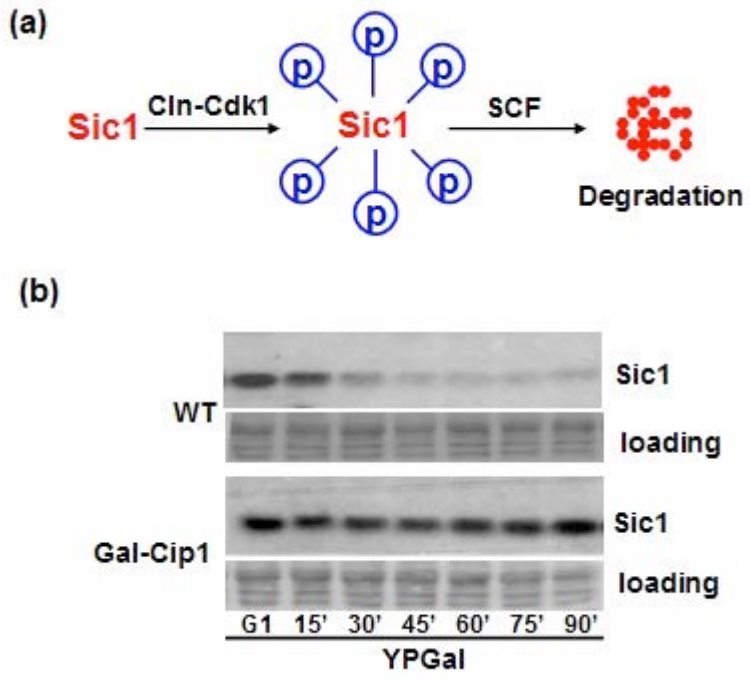


Figure 4.22 Sic1 is stable in cells over-expressing Cip1. (a) Cln-Cdk1 mediated degradation of Sic1 (b) Log phase cultures of GAL-Cip1-13myc (strain YFL97) or control cells (strain YGP30) grown in YP raffinose were synchronized with α-factor (G1). Galactose was added to a final concentration of 2% while keeping the presence of α-factor for 30 min. After 30 min induction, the cells were then released to YPGal with induction of Cip1. Samples were taken at the indicated points. Whole cell extracts were analyzed by immunoblot with anti-Myc (Cip1). A Ponceau S-stained region of the same membrane used for immunoblot is shown as a loading control.

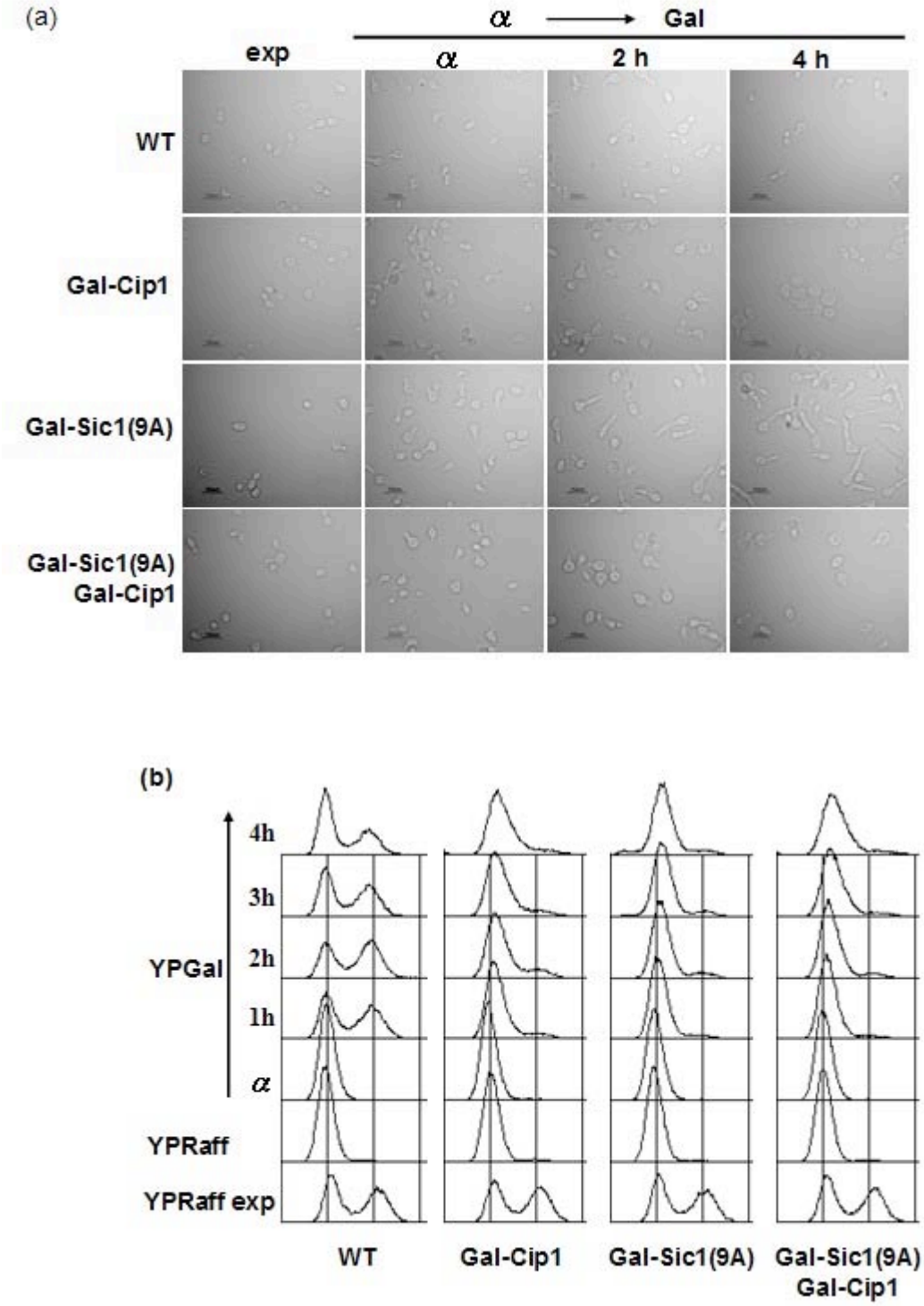
4.2.2.3 Overexpression of Cip1 inhibits Cln2 expression

To further explore whther the target of overexpressed Cip1 is G1 cyclin, we generated strains where Clb-CDK is completely inhibited and hence show polarized growth phenotype. The G1 cyclins Cln1 and Cln2 activate Cdk1 to direct polarized growth while as Clb cyclins

promote isotropic growth and they are the balance to trigger cell morphology alternation through G1-S-G2/M phases. Over-expression of Clb inhibitor Sic1 results in cells with elongated buds (Nugroho & Mendenhall, 1994) due to their polarized growth promoted by Cln-CDK activity, and failure to switch to isotropic growth lacking Clb-CDK activity (discussed in section 1.1.2.2).

If Cip1 is a negtive regulator of Cln-Cdk1, overexpression of Cip1 should block the polarized growth and inhibit the elongated buds of cells over-expressing Sic1. To prove this hypothesis, we over-expressed a hyperstable form of Sic1 (Sic1-9A), in which all nine potential Cdk1 phosphorylation S-T/P sites were substituted with non-phosphorytable Ala, hence it could not be phosphorylated and targeted for degradation. And at the same background we also over-expressed Cip1. As shown in Figure 4.23, log phase cultures were synchronized with α -factor in pre-G1 and then proteins under Gal1, 10 promoter were induced and released from G1 arrest. As shown in microscopy images Figure 4.23 (a) and (b) fluorescence-activated cell sorting analysis of DNA content, cells have larger size and were blocked tightly in G1 even till 4 hours after release from α-factor arrest when over-expressing Cip1 while as cells have elongated buds when over-expressing stable Sic1(9A), and what proves our hypothesis is when over-expressing both proteins, cells do not bud and show a larger size and round morphology.

This inhibition is not due to different accumulation of Sic1 in different strains, as shown in Figure 4.23 (c), in double mutants with Cip1 and Sic1 (9A) both under Gal1, 10 promoter, Cip1 and Sic1(9A) have the same accumulation proteins compared with the single mutants. A Ponceau S-stained region of the same membrane used for immunoblot is shown as a loading control. Coincident to the morphology phenotype, cells containing Gal-Sic1 (9A) can not survive in YPGal plate due to lack of Clb-CDK activity. And cells over-expressing Cip1 in YPGal plate can not grow well as WT cells (Figure 4.23 (d)).


(a)
α → Gal
exp
α
2 h
4 h
WT
Gal-Cip1
Gal-Sic1(9A)
Gal-Sic1(9A)
Gal-Cip1
(b)
4h
3h
2h
1h
α
YPGal
YPRaff
YPRaff exp
WT
Gal-Cip1
Gal-Sic1(9A)
Gal-Sic1(9A)
Gal-Cip1

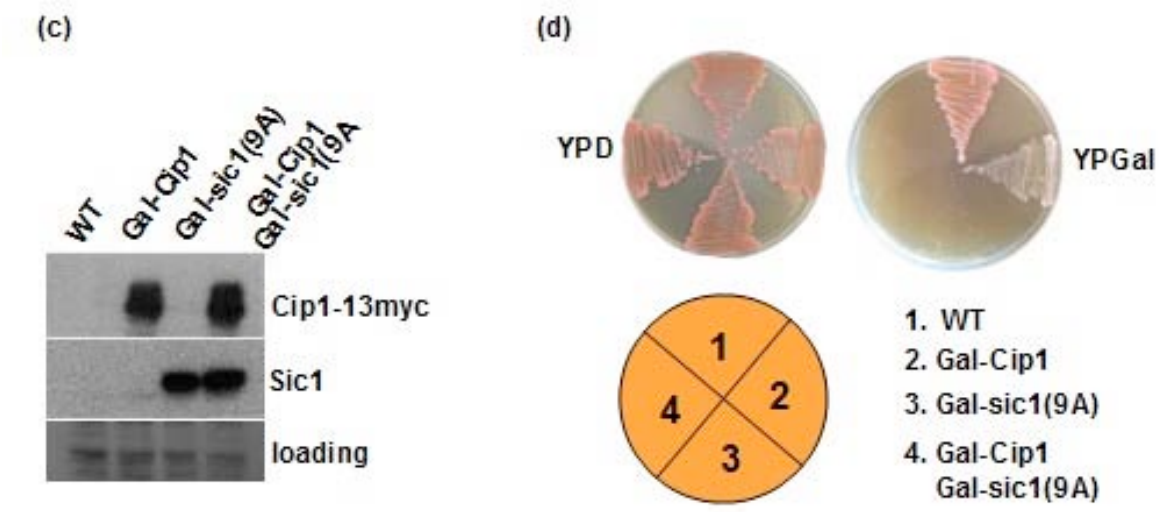


Figure 4.23 Overexpression of Cip1 rescues the polarized phenotype of cells expressing high stable Sic1. Log phase cultures of YFL119 (GAL-Cip1-13myc, Cln2-3HA), YFL114 (GAL-Sic1(9A), Cln2-3HA), YFL115 (GAL-Cip1-13myc, GAL-Sic1(9A), Cln2-3HA) or control WT cells YFL117 (Cln2-3HA) grown in YP raffinose were synchronized with α-factor (G1). Galactose was added to a final concentration of 2% while keeping the presence of α-factor for 30 min. After 30 min induction, the cells were then released to YPGal. Samples were taken at the indicated points. (a) the microscopy pictures of the cell morphorlogy in normasik phase with 50 μm scale (b) fluorescence-activated cell sorting analysis of DNA content. (c) whole cell extracts were analyzed by immunoblot with anti-Myc (Cip1) or anti-Sic1 antibodies shown as a loading control. A Ponceau S-stained region of the same membrane used for immunoblot is shown as a loading control. (d) function assay showing the viability of the mutants indicated.

Cip1 overexpression makes Sic1 stable (Section 4.2.2.2) and also inhibits Cln-CDK directed cell polarized growth. To distinguish wether it is due to direct inhibition of Cln-CDK

activity or negative inhibition of Cln expression, we probed the expression of Cln2 by quantitative western blot using a Cln2-3HA strain that harbors a C-terminally HA tagged allele of CLN2. As seen in Figure 4.24, in WT cells Cln2-3HA protein steadily accumulated and reach peak within 45 min after release, whereafter it declined sharply as cells exited G1. By contrast, level of Cln2 protein got stable in Sic1(9A) overexpressed cells. However, once Cip1 is overexpressed, Cln2 expression is blocked completely. Therefore, stabilise of Sic1 and rescue of cell polarized growth directed by Cln-CDK when Cip1 are overexpressed are due to negative inhibition of Cln expression instead of Cln2-CDK activity.

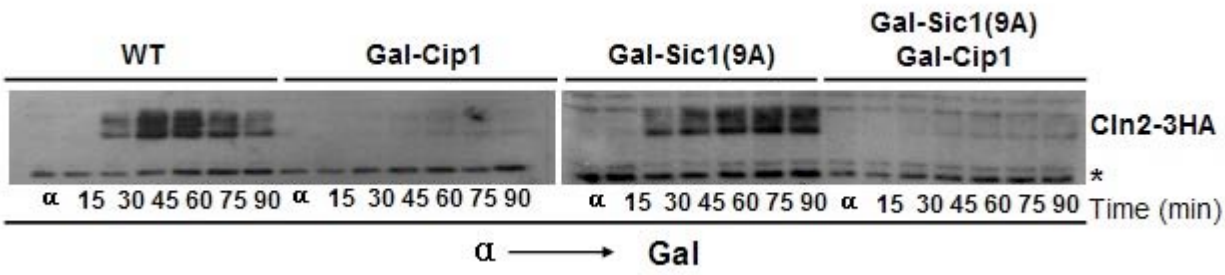


Figure 4.24 Overexpression of Cip1 inhibits Cln2 expression. Log phase cultures of YFL119 (GAL-Cip1-13myc, Cln2-3HA), YFL114 (GAL-Sic1(9A), Cln2-3HA), YFL115 (GAL-Cip1-13myc, GAL-Sic1(9A), Cln2-3HA) or control WT cells YFL117 (Cln2-3HA) grown in YP raffinose were synchronized with α-factor (G1). Galactose was added to a final concentration of 2% while keeping the presence of α-factor for 30 min. After 30 min induction, the cells were then released to YPGal. Samples were taken at the indicated points. Whole cell extracts were analyzed by immunoblot with anti-HA (Cln2). The crossreactive bands (star) are shown as a loading control.

4.2.3 Regulation of Cip1 upon DNA replication stress

4.2.3.1 Cip1 is a cell cycle regulated protein

Cip1 is a Cdk1 associated protein and only interacts with G1 cyclin. We next examined whether the Cip1 protein is modified in cell cycle-dependent manner. To monitor modifications of Cip1 during cell cycle, we synchronized cells in pre-G1 with α-factor and washed the pheromone away, enabling synchronous progression through cell cycle. Protein samples were taken every 10 intervals for 3 hours, and Cip1-13myc was monitored by western blots. We observed the Cip1-13myc as smeared bands, with one or more fractions showing delayed migration in a cell cycle-dependent manner (Figure 4.25). By comparison with FACS analysis of samples taken in parallel, we found that the Cip1 shift started from S phase of the cell cycle, but was specifically lost in G1 phase.

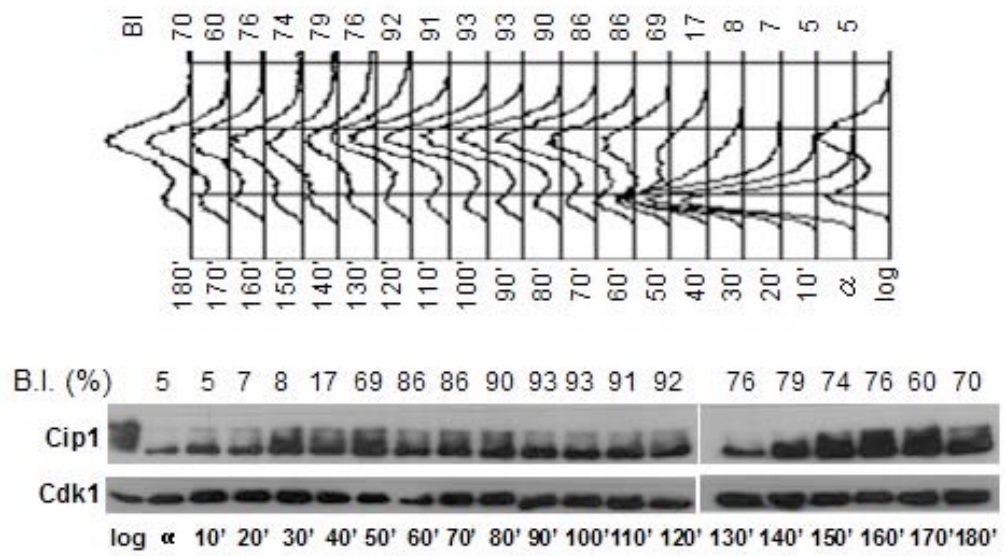


Figure 4.25 Cip1 is cell cycle regulated. A log phase culture of WT cells (YFL110: Cip1-13myc) was synchronized with α-factor (G1). The cells were then released from the G1 arrest by several washes with medium lacking α-factor. The samples were taken at the indicated time points. The up panel, fluorescence-activated cell sorting

analysis of DNA content. The lower panel, whole cell extracts were analyzed by immunoblot with anti-Myc (Cip1) and anti-Cdk1 antibodies as a loading control.

4.2.3.2 Rad53 dependent increase of Cip1 abundance upon DNA replication stress

Since Cip1 was identified as a Cdk1 associated protein in DNA replicatin stress from our proteomic screen, we next explored whether it is regulated in response to DNA replication stress. The cells synchronized in G1 with α-factor were synchronously released into S phase, either in the presence or in the absence of 0.2 M HU, a reagent that generates replication stress by depleting the pool of dNTPs. As shown in Figure 4.26 (a), Cip1 protein level change a little through cell cycle as discussed in 4.2.2.2. However, when cells enter the S phase in the presence of HU, Cip1 abundance increased dramatically. As Cip1 is associated with Cln2 in normal cell cycle, we then wanted to know whether Cln2 is stable in HU and therefore Cip1 may function as a Cln2-Cdk1 binding factor. However, as seen in Figure 4.26 (a), Cln2 disapeared the same time as Cln2 is eliminated in an unperturbed S phase.

In addition, as seen in Figure 4.26 (b), similarly as HU treatment, Cip1 equally accumulated when cells enter the S phase in the presence of methylmethane sulfonate, a reagent that generates DNA methylation damage as well as when cells enter G2/M in the presence of camptothecin which inhibits the DNA enzyme topoisomerase I. These three different types of genotoxic stress are known to activate checkpoint kinase Rad53. It suggests that increase of Cip1 abundance could be dependent on Rad53.

To determine whether increase of Cip1 abundance depends on Rad53 checkpoint response, we repeated the experiment described in the legend to Figure 4.26, now with a *rad53* null mutant. As shown in Figure 4.27, the *rad53* null mutant is unable to stabilize Cip1, despite the fact that DNA replication is effectively blocked (HU).

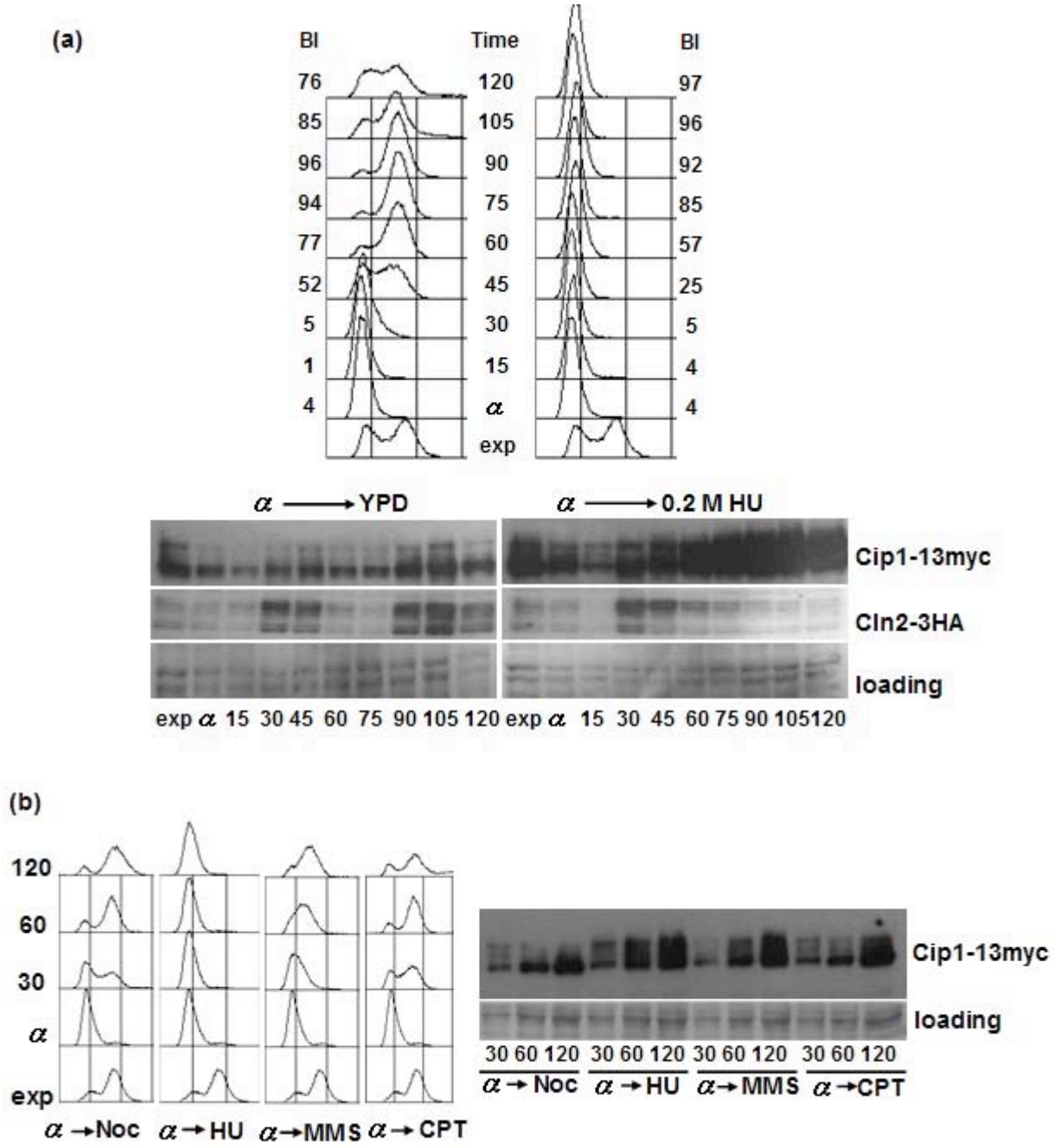


Figure 4.26 Cip1 stablizes upon DNA replication and DNA damage. (a) A log phase culture of WT cells (YFL117: Cip1-13myc, Cln2-3HA) was synchronized with α-factor (G1). The cells were then released from the G1 arrest by several washes with medium lacking α -factor and allowed to progress into the S phase with out stress (YPD) or in the presence of HU. The samples were taken at the indicated time points. Upper panel, fluorescence-activated cell sorting analysis of DNA content.

Lower panel, whole cell extracts were analyzed by immunoblot with anti-Myc (Cip1) or anti-HA (Cln2) antibodies. A Ponceau S-stained region of the same membrane used for immunoblot is shown as a loading control. (b) The WT cells (YFL110) were released from the G1 arrest allowed to progress into G2 in the presence of Noc, or in the presence of HU, MMS, CPT respectively. The samples were taken at the indicated time points. Left panel, fluorescence-activated cell sorting analysis of DNA content. Right panel, whole cell extracts were analyzed by immunoblot with anti-Myc (Cip1). A Ponceau S-stained region of the same membrane used for immunoblot is shown as a loading control.

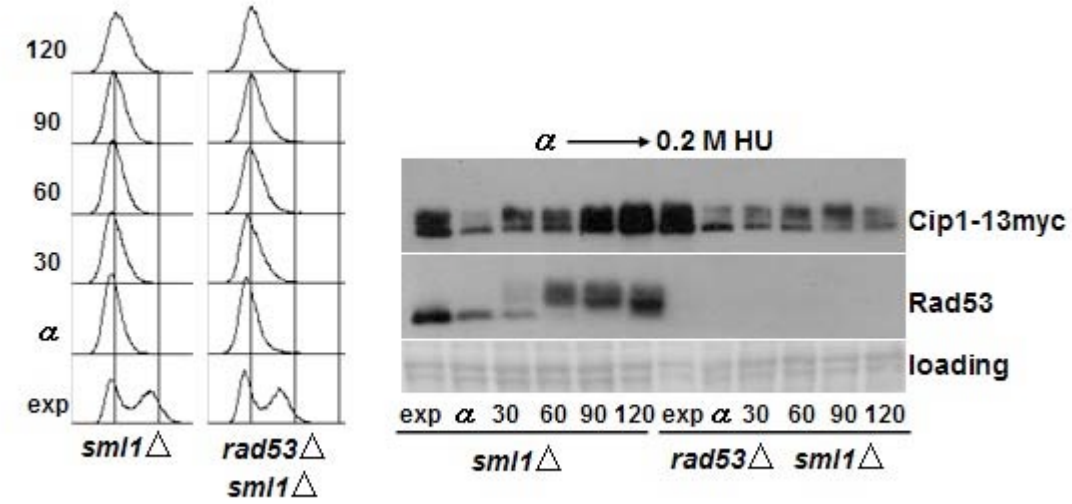


Figure 4.27 Rad53 dependent increase of Cip1 abundance upon DNA replication stress. log phase cultures of Rad53 positive strain (*sml1*Δ, YFL101) and *rad53*Δ mutants (*rad53*Δ *sml1*Δ, YFL102) were synchronized with α-factor (G1). The cells were then released from the G1 arrest by several washes with medium lacking α-factor and allowed to progress into the S phase in the presence of HU. The samples were taken at the indicated time points. left panel, fluorescence-activated cell sorting analysis of DNA content. Right panels, whole cell extracts were analyzed by immunoblot with anti-Myc (Cip1) or anti-Rad53 (Rad53) antibodies. A Ponceau S-stained region of the same membrane used for immunoblot is shown as a loading control.

CONCLUSIONS

Conclusions as a result of this thesis work:

Block 1

1- We resolved, assigned and and quantified the different phosphorylation forms of Cdk1 during the cell cycle.

2- Analysis of the M-CDK population (Clb2-Cdk1) suggests that additional mechanisms to inhibit M-CDK activity are in place in response to DNA replication stress in budding yeast, apart from Cdk1-Tyr19 inhibitory phosphorylation in response to DNA replication stress in budding yeast. This observation may explain the dispensability of Tyr19 phosphorylation in budding yeast.

3- We identified a novel, direct phosphorylation of Cdk1 by the S phase checkpoint Rad53 *in vitro*: Ser46 and Ser258. Ser46 had been suggested to be regulated by CKII in budding yeast and the homologous site by AKT in human cells.

4- We generated the corresponding non-phosphorylatable Cdk1 allele (Cdk1-2A) and phosphomimetic allele (Cdk1-2E). Cells driven by the non-phosphorylatable Cdk1-2A allele display a *wee* phenotype, compatible with increased/unrestrained CDK activity. Cells driven by the phosphomimetic Cdk1 allele (Cdk1-2E) are larger in size than wild type cells, compatible with reduced/retarded M-CDK activity.

Block 2

5- Through a biochemical-proteomic strategy, we identified a number of Rad53 dependent Cdk1 associated/lost factors in the presence of replication stress.

6- We have characterized one of the proteins specifically associated to Cdk1 in a Rad53 dependent manner in the presence of replication stress, the product of the previously unknown function gene YPL014W. We name the gene/protein *CIP1*, for Cdk1 Interacting Protein 1.

7- The expression of Cip1 is regulated in a Rad53 dependent manner in response to DNA replication stress.

8- Cip1 is bound to G1-specific Cln2-Cdk1 complexes, but not to S phase Clb5-Cdk1 or M phase Clb2-Cdk1 complexes.

9- Our results are compatible with Cip1 being a specific G1 CKI, whose role might be to halt entry into S phase once cells have crossed Start and accumulation of Cln2-Cdk1 would otherwise target Sic1 for destruction.

ABBREVIATIONS

Abbreviations	Full form
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride HCL
DTT	Dithiothreitol
SDS	Sodium dodecyl sulfate
NP-40	Nonidet P-40
PAGE	Polyacrylamide gel electrophoresis
TEMED	N, N, N', N'- Tetramethylethylenediamine
TAE	Tris base, acetic acid and EDTA buffer
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TE	Tris and EDTA buffer
Tris	Tris(hydroxymethyl)aminomethane
BSA	Bovine serum albumin
DMSO	Dimethyl sulfoxide
ssDNA	Single stranded DNA
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Phos-tag	Phosphate-binding tag
LBA	Lysogeny Broth with Ampicillin
YPD	Rich medium with dextrose (glucose)
YPGal	Rich medium with galactose
YPRaff	Rich medium with raffinose
FACS	Fluorescence Activated Cell Sorting
GST	Glutathione S-transferase
HU	Hydroxyurea
MMS	methyl methanesulfonate
CPT	Camptothecin

REFERENCES

Ahn, S. H., Tobe, B. T., Fitz Gerald, J. N., Anderson, S. L., Acurio, A., & Kron, S. J. (2001). Enhanced cell polarity in mutants of the budding yeast cyclin-dependent kinase Cdc28p. *Molecular biology of the cell*, *12*(11), 3589–600. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=60278&tool=pmcentrez&rendertype=abstract

Albuquerque, C. P., Smolka, M. B., Payne, S. H., Bafna, V., Eng, J., & Zhou, H. (2008). A multidimensional chromatography technology for in-depth phosphoproteome analysis. *Molecular & cellular proteomics : MCP*, *7*(7), 1389–96. doi:10.1074/mcp.M700468-MCP200

Allen, J. B., Zhou, Z., Siede, W., Friedberg, E. C., & Elledge, S. J. (1994). The SAD1/RAD53 protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. *Genes & development*, *8*(20), 2401–15. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7958905

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of molecular biology*, *215*(3), 403–10. doi:10.1016/S0022-2836(05)80360-2

Amon, A., Surana, U., Muroff, I., & Nasmyth, K. (1992). Regulation of p34CDC28 tyrosine phosphorylation is not required for entry into mitosis in S. cerevisiae. *Nature*, *355*(6358), 368–71. doi:10.1038/355368a0

Andrews, B. J., & Herskowitz, I. (1989). The yeast SWI4 protein contains a motif present in developmental regulators and is part of a complex involved in cell-cycle-dependent transcription. *Nature*, *342*(6251), 830–833. doi:10.1038/342830a0

Bahler, J., Wu, J. Q., Longtine, M. S., Shah, N. G., McKenzie 3rd, A., Steever, A. B., … Pringle, J. R. (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. *Yeast*, *14*(10), 943–951. doi:10.1002/(SICI)1097-0061(199807)14:10<943::AID-YEA292>3.0.CO;2-Y

Barberis, M. (2012). Molecular systems biology of Sic1 in yeast cell cycle regulation through multiscale modeling. *Advances in experimental medicine and biology*, *736*, 135–67. doi:10.1007/978-1-4419-7210-1_7

Barberis, M., De Gioia, L., Ruzzene, M., Sarno, S., Coccetti, P., Fantucci, P., … Alberghina, L. (2005). The yeast cyclin-dependent kinase inhibitor Sic1 and mammalian p27Kip1 are functional homologues with a structurally conserved inhibitory domain. *The Biochemical journal*, *387*(Pt 3), 639–47. doi:10.1042/BJ20041299

Barkai, N., Rose, M. D., & Wingreen, N. S. (1998). Protease helps yeast find mating partners. *Nature*, *396*(6710), 422–3. doi:10.1038/24760

Bartkova, J., Bakkenist, C. J., Rajpert-De Meyts, E., Skakkebaek, N. E., Sehested, M., Lukas, J., … Bartek, J. (2005). ATM activation in normal human tissues and testicular cancer. *Cell cycle (Georgetown, Tex.)*, *4*(6), 838–45. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15846060

Bartkova, J., Horejsí, Z., Koed, K., Krämer, A., Tort, F., Zieger, K., … Bartek, J. (2005). DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature*, *434*(7035), 864–70. doi:10.1038/nature03482

Bastos de Oliveira, F. M., Harris, M. R., Brazauskas, P., de Bruin, R. A. M., & Smolka, M. B. (2012). Linking DNA replication checkpoint to MBF cell-cycle transcription reveals a distinct class of G1/S genes. *The EMBO journal*, *31*(7), 1798–810. doi:10.1038/emboj.2012.27

Beach, D., Durkacz, B., & Nurse, P. (1982). Functionally homologous cell cycle control genes in budding and fission yeast. *Nature*, *300*(5894), 706–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6757758

Bell, D. W., Varley, J. M., Szydlo, T. E., Kang, D. H., Wahrer, D. C., Shannon, K. E., … Haber, D. A. (1999). Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. *Science (New York, N.Y.)*, *286*(5449), 2528–31. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10617473

Boder, E. (1985). Ataxia-telangiectasia: an overview. *Kroc Foundation series*, *19*, 1–63. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2415689

Booher, R. N., Deshaies, R. J., & Kirschner, M. W. (1993). Properties of Saccharomyces cerevisiae wee1 and its differential regulation of p34CDC28 in response to G1 and G2 cyclins. *The EMBO journal*, *12*(9), 3417–26. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=413617&tool=pmcentrez&rendertype=abstract

Booher, R. N., Holman, P. S., & Fattaey, A. (1997). Human Myt1 is a cell cycle-regulated kinase that inhibits Cdc2 but not Cdk2 activity. *The Journal of biological chemistry*, *272*(35), 22300–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9268380

Bourne, Y., Watson, M. H., Hickey, M. J., Holmes, W., Rocque, W., Reed, S. I., & Tainer, J. A. (1996). Crystal structure and mutational analysis of the human CDK2 kinase complex with cell cycle-regulatory protein CksHs1. *Cell*, *84*(6), 863–74. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8601310

Breeden, L. (1996). Start-specific transcription in yeast. *Current topics in microbiology and immunology*, *208*, 95–127. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8575215

Breeden, L., & Nasmyth, K. (1987). Cell cycle control of the yeast HO gene: cis- and trans-acting regulators. *Cell*, *48*(3), 389–397. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3542227

Brotherton, D. H., Dhanaraj, V., Wick, S., Brizuela, L., Domaille, P. J., Volyanik, E., … Laue, E. D. (1998). Crystal structure of the complex of the cyclin D-dependent kinase Cdk6 bound to the cell-cycle inhibitor p19INK4d. *Nature*, *395*(6699), 244–50. doi:10.1038/26164

Caetano, C., Klier, S., & de Bruin, R. A. M. (2011). Phosphorylation of the MBF repressor Yox1p by the DNA replication checkpoint keeps the G1/S cell-cycle transcriptional program active. *PloS one*, *6*(2), e17211. doi:10.1371/journal.pone.0017211

Chan, R. K., & Otte, C. A. (1982). Physiological characterization of Saccharomyces cerevisiae mutants supersensitive to G1 arrest by a factor and alpha factor pheromones. *Molecular and cellular biology*, *2*(1), 21–9. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=369749&tool=pmcentrez&rendertype=abstract

Chang, F., & Herskowitz, I. (1990). Identification of a gene necessary for cell cycle arrest by a negative growth factor of yeast: FAR1 is an inhibitor of a G1 cyclin, CLN2. *Cell*, *63*(5), 999–1011. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2147873

Chen, J., Jackson, P. K., Kirschner, M. W., & Dutta, A. (1995). Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. *Nature*, *374*(6520), 386–8. doi:10.1038/374386a0

Cho, R. J., Campbell, M. J., Winzeler, E. A., Steinmetz, L., Conway, A., Wodicka, L., … Davis, R. W. (1998). A genome-wide transcriptional analysis of the mitotic cell cycle. *Mol Cell*, *2*(1), 65–73. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9702192

Choudhary, C., Kumar, C., Gnad, F., Nielsen, M. L., Rehman, M., Walther, T. C., … Mann, M. (2009). Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science (New York, N.Y.)*, *325*(5942), 834–40. doi:10.1126/science.1175371

Ciejek, E., & Thorner, J. (1979). Recovery of S. cerevisiae a cells from G1 arrest by α factor pheromone requires endopeptidase action. *Cell*, *18*(3), 623–635. doi:10.1016/0092-8674(79)90117-X

Cohen-Fix, O., & Koshland, D. (1997). The anaphase inhibitor of Saccharomyces cerevisiae Pds1p is a target of the DNA damage checkpoint pathway. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(26), 14361–6. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=24978&tool=pmcentrez&rendertype=abstract

Cooper, K. (2006). Rb, whi it's not just for metazoans anymore. *Oncogene*, *25*(38), 5228–5232. doi:10.1038/sj.onc.1209630

Costanzo, M., Nishikawa, J. L., Tang, X., Millman, J. S., Schub, O., Breitkreuz, K., … Tyers, M. (2004). CDK activity antagonizes Whi5, an inhibitor of G1/S transcription in yeast. *Cell*, *117*(7), 899–913. doi:10.1016/j.cell.2004.05.024

Cross, F. R. (1988). DAF1, a mutant gene affecting size control, pheromone arrest, and cell cycle kinetics of Saccharomyces cerevisiae. *Mol Cell Biol*, *8*(11), 4675–4684. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3062366

Cross, F. R. (1989). Further characterization of a size control gene in Saccharomyces cerevisiae. *J Cell Sci Suppl*, *12*, 117–127. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2561422

Culotti, J., & Hartwell, L. H. (1971). Genetic control of the cell division cycle in yeast. 3. Seven genes controlling nuclear division. *Experimental cell research*, *67*(2), 389–401. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/5097524

Dahmann, C., Diffley, J. F., & Nasmyth, K. A. (1995). S-phase-promoting cyclin-dependent kinases prevent re-replication by inhibiting the transition of replication origins to a pre-replicative state. *Curr Biol*, *5*(11), 1257–1269. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8574583

DaSilva, T. (2008). *Cdk2 Phosphorylation on Threonine39 by AKT and Its Implication on Cyclin Binding, Cellular Localization, and Cell Cycle Progression. University of Miami.*

De Bondt, H. L., Rosenblatt, J., Jancarik, J., Jones, H. D., Morgan, D. O., & Kim, S. H. (1993). Crystal structure of cyclin-dependent kinase 2. *Nature*, *363*(6430), 595–602. doi:10.1038/363595a0

De Bruin, R A M, Kalashnikova, T. I., Aslanian, A., Wohlschlegel, J., Chahwan, C., Yates, J. R., … Wittenberg, C. (2008). DNA replication checkpoint promotes G1-S transcription by inactivating the MBF repressor Nrm1. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(32), 11230–5. doi:10.1073/pnas.0801106105

De Bruin, R. A., Kalashnikova, T. I., Chahwan, C., McDonald, W. H., Wohlschlegel, J., Yates 3rd, J., … Wittenberg, C. (2006). Constraining G1-specific transcription to late G1 phase: the MBF-associated corepressor Nrm1 acts via negative feedback. *Mol Cell*, *23*(4), 483–496. doi:10.1016/j.molcel.2006.06.025

De Bruin, R. A., McDonald, W. H., Kalashnikova, T. I., Yates 3rd, J., & Wittenberg, C. (2004). Cln3 activates G1-specific transcription via phosphorylation of the SBF bound repressor Whi5. *Cell*, *117*(7), 887–898. doi:10.1016/j.cell.2004.05.025

De Bruin, Robertus A M, Kalashnikova, T. I., & Wittenberg, C. (2008). Stb1 collaborates with other regulators to modulate the G1-specific transcriptional circuit. *Molecular and cellular biology*, *28*(22), 6919–28. doi:10.1128/MCB.00211-08

Deshaies, R. J. (1997). Phosphorylation and proteolysis: partners in the regulation of cell division in budding yeast. *Current opinion in genetics & development*, *7*(1), 7–16. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9024629

Dirick, L., Bohm, T., & Nasmyth, K. (1995). Roles and regulation of Cln-Cdc28 kinases at the start of the cell cycle of Saccharomyces cerevisiae. *EMBO J*, *14*(19), 4803–4813. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7588610

Draetta, G., Piwnica-Worms, H., Morrison, D., Druker, B., Roberts, T., & Beach, D. (1988). Human cdc2 protein kinase is a major cell-cycle regulated tyrosine kinase substrate. *Nature*, *336*(6201), 738–44. doi:10.1038/336738a0

Duch, A., Palou, G., Jonsson, Z. O., Palou, R., Calvo, E., Wohlschlegel, J., & Quintana, D. G. (2011). A Dbf4 mutant contributes to bypassing the Rad53-mediated block of origins

of replication in response to genotoxic stress. *The Journal of biological chemistry*, *286*(4), 2486–91. doi:10.1074/jbc.M110.190843

Durocher, D., Henckel, J., Fersht, A. R., & Jackson, S. P. (1999). The FHA domain is a modular phosphopeptide recognition motif. *Molecular cell*, *4*(3), 387–94. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10518219

Edelheit, O., Hanukoglu, A., & Hanukoglu, I. (2009). Simple and efficient site-directed mutagenesis using two single-primer reactions in parallel to generate mutants for protein structure-function studies. *BMC biotechnology*, *9*, 61. doi:10.1186/1472-6750-9-61

Elledge, S. J., Zhou, Z., & Allen, J. B. (1992). Ribonucleotide reductase: regulation, regulation, regulation. *Trends in biochemical sciences*, *17*(3), 119–23. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1412696

Emili, A., Schieltz, D. M., Yates, J. R., & Hartwell, L. H. (2001). Dynamic interaction of DNA damage checkpoint protein Rad53 with chromatin assembly factor Asf1. *Molecular cell*, *7*(1), 13–20. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11172707

Evans, T., Rosenthal, E. T., Youngblom, J., Distel, D., & Hunt, T. (1983). Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell*, *33*(2), 389–396. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6134587

Ferrell, J. E. (2002). Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability. *Current opinion in cell biology*, *14*(2), 140–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11891111

Fitch, I., Dahmann, C., Surana, U., Amon, A., Nasmyth, K., Goetsch, L., … Futcher, B. (1992). Characterization of four B-type cyclin genes of the budding yeast Saccharomyces cerevisiae. *Mol Biol Cell*, *3*(7), 805–818. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1387566

Fraser, H. B. (2013). Cell-cycle regulated transcription associates with DNA replication timing in yeast and human. *Genome biology*, *14*(10), R111. doi:10.1186/gb-2013-14-10-r111

Furnari, B., Rhind, N., & Russell, P. (1997). Cdc25 mitotic inducer targeted by chk1 DNA damage checkpoint kinase. *Science (New York, N.Y.)*, *277*(5331), 1495–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9278510

Gautier, J., Solomon, M. J., Booher, R. N., Bazan, J. F., & Kirschner, M. W. (1991). cdc25 is a specific tyrosine phosphatase that directly activates p34cdc2. *Cell*, *67*(1), 197–211. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1913817

Ghiara, J. B., Richardson, H. E., Sugimoto, K., Henze, M., Lew, D. J., Wittenberg, C., & Reed, S. I. (1991). A cyclin B homolog in S. cerevisiae: chronic activation of the Cdc28 protein kinase by cyclin prevents exit from mitosis. *Cell*, *65*(1), 163–174. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1849458

Gould, K. L., & Nurse, P. (1989). Tyrosine phosphorylation of the fission yeast cdc2+ protein kinase regulates entry into mitosis. *Nature*, *342*(6245), 39–45. doi:10.1038/342039a0

Guan, K. L., Jenkins, C. W., Li, Y., Nichols, M. A., Wu, X., O'Keefe, C. L., … Xiong, Y. (1994). Growth suppression by p18, a p16INK4/MTS1- and p14INK4B/MTS2-related CDK6 inhibitor, correlates with wild-type pRb function. *Genes & development*, *8*(24), 2939–52. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8001816

Gunjan, A., & Verreault, A. (2003). A Rad53 kinase-dependent surveillance mechanism that regulates histone protein levels in S. cerevisiae. *Cell*, *115*(5), 537–49. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14651846

Hadwiger, J. A., Wittenberg, C., Mendenhall, M. D., & Reed, S. I. (1989). The Saccharomyces cerevisiae CKS1 gene, a homolog of the Schizosaccharomyces pombe suc1+ gene, encodes a subunit of the Cdc28 protein kinase complex. *Molecular and cellular biology*, *9*(5), 2034–41. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=362996&tool=pmcentrez&rendertype=abstract

Hannon, G. J., & Beach, D. (1994). p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature*, *371*(6494), 257–61. doi:10.1038/371257a0

Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., & Elledge, S. J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell*, *75*(4), 805–16. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8242751

Hartwell, L H. (1971a). Genetic control of the cell division cycle in yeast. IV. Genes controlling bud emergence and cytokinesis. *Experimental cell research*, *69*(2), 265–76. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4950437

Hartwell, L H. (1971b). Genetic control of the cell division cycle in yeast. II. Genes controlling DNA replication and its initiation. *Journal of molecular biology*, *59*(1), 183–94. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/5283752

Hartwell, L H. (1974). Saccharomyces cerevisiae cell cycle. *Bacteriological reviews*, *38*(2), 164–98. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=413849&tool=pmcentrez&rendertype=abstract

Hartwell, L H, & Kastan, M. B. (1994). Cell cycle control and cancer. *Science (New York, N.Y.)*, *266*(5192), 1821–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7997877

Hartwell, L H, & Weinert, T. A. (1989). Checkpoints: controls that ensure the order of cell cycle events. *Science*, *246*(4930), 629–634. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2683079

Hartwell, L. H., Culotti, J., & Reid, B. (1970). Genetic Control of the Cell-Division Cycle in Yeast, I. Detection of Mutants. *Proceedings of the National Academy of Sciences*, *66*(2), 352–359. doi:10.1073/pnas.66.2.352

Hereford, L., Bromley, S., & Osley, M. A. (1982). Periodic transcription of yeast histone genes. *Cell*, *30*(1), 305–10. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6751560

Hereford, L. M., Osley, M. A., Ludwig, T. R., & McLaughlin, C. S. (1981). Cell-cycle regulation of yeast histone mRNA. *Cell*, *24*(2), 367–75. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7016339

Hiebert, S. W., Chellappan, S. P., Horowitz, J. M., & Nevins, J. R. (1992). The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. *Genes & development*, *6*(2), 177–85. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1531329

Hirai, H., Roussel, M. F., Kato, J. Y., Ashmun, R. A., & Sherr, C. J. (1995). Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. *Molecular and cellular biology*, *15*(5), 2672–81. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=230497&tool=pmcentrez&rendertype=abstract

Hodge, A., & Mendenhall, M. (1999). The cyclin-dependent kinase inhibitory domain of the yeast Sic1 protein is contained within the C-terminal 70 amino acids. *Molecular & general genetics : MGG*, *262*(1), 55–64. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10503536

Hofmann, K., & Bucher, P. (1995). The FHA domain: a putative nuclear signalling domain found in protein kinases and transcription factors. *Trends in biochemical sciences*, *20*(9), 347–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7482699

Horak, C. E., Luscombe, N. M., Qian, J., Bertone, P., Piccirrillo, S., Gerstein, M., & Snyder, M. (2002). Complex transcriptional circuitry at the G1/S transition in Saccharomyces cerevisiae. *Genes Dev*, *16*(23), 3017–3033. doi:10.1101/gad.1039602

Huang, M., Zhou, Z., & Elledge, S. J. (1998). The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor. *Cell*, *94*(5), 595–605. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9741624

Ito, H., Fukuda, Y., Murata, K., & Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. *Journal of bacteriology*, *153*(1), 163–8. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=217353&tool=pmcentrez&rendertype=abstract

Iyer, V. R., Horak, C. E., Scafe, C. S., Botstein, D., Snyder, M., & Brown, P. O. (2001). Genomic binding sites of the yeast cell-cycle transcription factors SBF and MBF. *Nature*, *409*(6819), 533–538. doi:10.1038/35054095

Jackson, L. P., Reed, S. I., & Haase, S. B. (2006). Distinct mechanisms control the stability of the related S-phase cyclins Clb5 and Clb6. *Mol Cell Biol*, *26*(6), 2456–2466. doi:10.1128/MCB.26.6.2456-2466.2006

Jaehnig, E. J., Kuo, D., Hombauer, H., Ideker, T. G., & Kolodner, R. D. (2013). Checkpoint Kinases Regulate a Global Network of Transcription Factors in Response to DNA Damage. *Cell Reports*, *4*(1), 174–188. Retrieved from http://www.sciencedirect.com/science/article/pii/S221112471300274X

Jeffrey, P. D., Russo, A. A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J., & Pavletich, N. P. (1995). Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex. *Nature*, *376*(6538), 313–320. doi:10.1038/376313a0

Kaldis, P., Pitluk, Z. W., Bany, I. A., Enke, D. A., Wagner, M., Winter, E., & Solomon, M. J. (1998). Localization and regulation of the cdk-activating kinase (Cak1p) from budding yeast. *J Cell Sci*, *111 (Pt 2*, 3585–3596. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9819350

Kaldis, P., Sutton, A., & Solomon, M. J. (1996). The Cdk-activating kinase (CAK) from budding yeast. *Cell*, *86*(4), 553–564. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8752210

Kinoshita, E., Kinoshita-Kikuta, E., & Koike, T. (2009). Separation and detection of large phosphoproteins using Phos-tag SDS-PAGE. *Nature protocols*, *4*(10), 1513–21. doi:10.1038/nprot.2009.154

Koch, C., Moll, T., Neuberg, M., Ahorn, H., & Nasmyth, K. (1993). A role for the transcription factors Mbp1 and Swi4 in progression from G1 to S phase. *Science*, *261*(5128), 1551–1557. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8372350

Kõivomägi, M., Ord, M., Iofik, A., Valk, E., Venta, R., Faustova, I., … Loog, M. (2013). Multisite phosphorylation networks as signal processors for Cdk1. *Nature structural & molecular biology*. doi:10.1038/nsmb.2706

Kolodner, R. D., Putnam, C. D., & Myung, K. (2002). Maintenance of genome stability in Saccharomyces cerevisiae. *Science (New York, N.Y.)*, *297*(5581), 552–7. doi:10.1126/science.1075277

Kostriken, R., Strathern, J. N., Klar, A. J. S., Hicks, J. B., & Heffron, F. (1983). A site-specific endonuclease essential for mating-type switching in Saccharomyces cerevisiae. *Cell*, *35*(1), 167–174. doi:10.1016/0092-8674(83)90219-2

Krek, W., & Nigg, E. A. (1991). Differential phosphorylation of vertebrate p34cdc2 kinase at the G1/S and G2/M transitions of the cell cycle: identification of major phosphorylation sites. *The EMBO journal*, *10*(2), 305–16. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=452647&tool=pmcentrez&rendertype=abstract

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*(5259), 680–685. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/5432063

Landry, B. D., Doyle, J. P., Toczyski, D. P., & Benanti, J. A. (2012). F-box protein specificity for g1 cyclins is dictated by subcellular localization. *PLoS genetics*, *8*(7), e1002851. doi:10.1371/journal.pgen.1002851

Lanker, S., Valdivieso, M. H., & Wittenberg, C. (1996). Rapid degradation of the G1 cyclin Cln2 induced by CDK-dependent phosphorylation. *Science*, *271*(5255), 1597–1601. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8599119

Lee, J. M., & Greenleaf, A. L. (1991). CTD kinase large subunit is encoded by CTK1, a gene required for normal growth of Saccharomyces cerevisiae. *Gene expression*, *1*(2), 149–67. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1820212

Lee, M. G., & Nurse, P. (1987). Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2. *Nature*, *327*(6117), 31–5. doi:10.1038/327031a0

Lee, M. H., Reynisdóttir, I., & Massagué, J. (1995). Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes & development*, *9*(6), 639–49. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7729683

Lew, D J, & Reed, S. I. (1993). Morphogenesis in the yeast cell cycle: regulation by Cdc28 and cyclins. *The Journal of cell biology*, *120*(6), 1305–20. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2119756&tool=pmcentrez&rendertype=abstract

Lew, D J, & Reed, S. I. (1995). Cell cycle control of morphogenesis in budding yeast. *Current opinion in genetics & development*, *5*(1), 17–23. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7749320

Lew, Daniel J., Marini, N. J., & Reed, S. I. (1992). Different G1 cyclins control the timing of cell cycle commitment in mother and daughter cells of the budding yeast S. cerevisiae. *Cell*, *69*(2), 317–327. Retrieved from http://www.sciencedirect.com/science/article/pii/0092867492904126

Li, W. J., Wang, Y. M., Zheng, X. De, Shi, Q. M., Zhang, T. T., Bai, C., … Wang, Y. (2006). The F-box protein Grr1 regulates the stability of Ccn1, Cln3 and Hof1 and cell morphogenesis in Candida albicans. *Molecular microbiology*, *62*(1), 212–26. doi:10.1111/j.1365-2958.2006.05361.x

Liao, S. M., Zhang, J., Jeffery, D. A., Koleske, A. J., Thompson, C. M., Chao, D. M., … Young, R. A. (1995). A kinase-cyclin pair in the RNA polymerase II holoenzyme. *Nature*, *374*(6518), 193–6. doi:10.1038/374193a0

Liu, F., Stanton, J. J., Wu, Z., & Piwnica-Worms, H. (1997). The human Myt1 kinase preferentially phosphorylates Cdc2 on threonine 14 and localizes to the endoplasmic

reticulum and Golgi complex. *Molecular and cellular biology*, *17*(2), 571–83. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=231782&tool=pmcentrez&rendertype=abstract

Liu, H., & Wang, Y. (2006). The function and regulation of budding yeast Swe1 in response to interrupted DNA synthesis. *Molecular biology of the cell*, *17*(6), 2746–56. doi:10.1091/mbc.E05-11-1093

Lundgren, K., Walworth, N., Booher, R., Dembski, M., Kirschner, M., & Beach, D. (1991). mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. *Cell*, *64*(6), 1111–22. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1706223

Luo, Y., Hurwitz, J., & Massagué, J. (1995). Cell-cycle inhibition by independent CDK and PCNA binding domains in p21Cip1. *Nature*, *375*(6527), 159–61. doi:10.1038/375159a0

Ma, J.-L., Lee, S.-J., Duong, J. K., & Stern, D. F. (2006). Activation of the checkpoint kinase Rad53 by the phosphatidyl inositol kinase-like kinase Mec1. *The Journal of biological chemistry*, *281*(7), 3954–63. doi:10.1074/jbc.M507508200

Malumbres, M, & Barbacid, M. (2001). To cycle or not to cycle: a critical decision in cancer. *Nature reviews. Cancer*, *1*(3), 222–31. doi:10.1038/35106065

Malumbres, Marcos, & Barbacid, M. (2009). Cell cycle, CDKs and cancer: a changing paradigm. *Nature reviews. Cancer*, *9*(3), 153–66. doi:10.1038/nrc2602

Matsuoka, S., Edwards, M. C., Bai, C., Parker, S., Zhang, P., Baldini, A., … Elledge, S. J. (1995). p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene. *Genes & development*, *9*(6), 650–62. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7729684

McGowan, C. H., & Russell, P. (1993). Human Wee1 kinase inhibits cell division by phosphorylating p34cdc2 exclusively on Tyr15. *The EMBO journal*, *12*(1), 75–85. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=413177&tool=pmcentrez&rendertype=abstract

McGrath, D. A., Balog, E. R. M., Kõivomägi, M., Lucena, R., Mai, M. V, Hirschi, A., … Rubin, S. M. (2013). Cks confers specificity to phosphorylation-dependent CDK signaling pathways. *Nature structural & molecular biology*. doi:10.1038/nsmb.2707

McInerny, C. J., Partridge, J. F., Mikesell, G. E., Creemer, D. P., & Breeden, L. L. (1997). A novel Mcm1-dependent element in the SWI4, CLN3, CDC6, and CDC47 promoters activates M/G1-specific transcription. *Genes & development*, *11*(10), 1277–88. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9171372

Melo, J., & Toczyski, D. (2002). A unified view of the DNA-damage checkpoint. *Current opinion in cell biology*, *14*(2), 237–45. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11891124

Mendenhall, M. D. (1993). An inhibitor of p34CDC28 protein kinase activity from Saccharomyces cerevisiae. *Science (New York, N.Y.)*, *259*(5092), 216–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8421781

Mitchison, J. M., & Creanor, J. (1971). Further measurements of DNA synthesis and enzyme potential during cell cycle of fission yeast Schizosaccharomyces pombe. *Exp Cell Res*, *69*(1), 244–247. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/5124486

Moll, T., Dirick, L., Auer, H., Bonkovsky, J., & Nasmyth, K. (1992). SWI6 is a regulatory subunit of two different cell cycle START-dependent transcription factors in Saccharomyces cerevisiae. *Journal of cell science. Supplement*, *16*, 87–96. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1297653

Moore, C. W. (1978). Responses of radiation-sensitive mutants of Saccharomyces cerevisiae to lethal effects of bleomycin. *Mutation research*, *51*(2), 165–80. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/80746

Moore, G. D., Ayabe, T., Kopf, G. S., & Schultz, R. M. (1996). Temporal patterns of gene expression of G1-S cyclins and cdks during the first and second mitotic cell cycles in mouse embryos. *Molecular reproduction and development*, *45*(3), 264–75. doi:10.1002/(SICI)1098-2795(199611)45:3<264::AID-MRD2>3.0.CO;2-Q

Morgan, D O. (1997). Cyclin-dependent kinases: engines, clocks, and microprocessors. *Annual review of cell and developmental biology*, *13*, 261–91. doi:10.1146/annurev.cellbio.13.1.261

Morgan, David O. (2007). *The Cell Cycle, Principles of Control*. OUP Oxford.

Mullis, K. B., & Faloona, F. A. (1987). Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol*, *155*, 335–350. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3431465

Murphree, A. L., & Benedict, W. F. (1984). Retinoblastoma: clues to human oncogenesis. *Science (New York, N.Y.)*, *223*(4640), 1028–33. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6320372

Nash, P., Tang, X., Orlicky, S., Chen, Q., Gertler, F. B., Mendenhall, M. D., … Tyers, M. (2001). Multisite phosphorylation of a CDK inhibitor sets a threshold for the onset of DNA replication. *Nature*, *414*(6863), 514–21. doi:10.1038/35107009

Nasmyth, K. (1983). Molecular analysis of a cell lineage. *Nature*, *302*(5910), 670–676. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6339953

Nigg, E A. (1995). Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle. *BioEssays : news and reviews in molecular, cellular and developmental biology*, *17*(6), 471–80. doi:10.1002/bies.950170603

Nigg, Erich A. (1993). Cellular substrates of p34cdc2 and its companion cyclin-dependent kinases. *Trends in Cell Biology*, *3*(9), 296–301. Retrieved from http://www.sciencedirect.com/science/article/pii/096289249390011O

Norbury, C., & Nurse, P. (1991). Cyclins and cell cycle control. *Current biology : CB*, *1*(1), 23–4. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15336197

Nugroho, T. T., & Mendenhall, M. D. (1994). An inhibitor of yeast cyclin-dependent protein kinase plays an important role in ensuring the genomic integrity of daughter cells. *Molecular and cellular biology*, *14*(5), 3320–8. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=358698&tool=pmcentrez&rendertype=abstract

Nurse, P, & Thuriaux, P. (1980). Regulatory genes controlling mitosis in the fission yeast Schizosaccharomyces pombe. *Genetics*, *96*(3), 627–37. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1214365&tool=pmcentrez&rendertype=abstract

Nurse, Paul, Thuriaux, P., & Nasmyth, K. (1976). Genetic control of the cell division cycle in the fission yeast Schizosaccharomyces pombe. *MGG Molecular & General Genetics*, *146*(2), 167–178. doi:10.1007/BF00268085

O'Neill, T., Giarratani, L., Chen, P., Iyer, L., Lee, C.-H., Bobiak, M., … Rathbun, G. A. (2002). Determination of substrate motifs for human Chk1 and hCds1/Chk2 by the oriented peptide library approach. *The Journal of biological chemistry*, *277*(18), 16102–15. doi:10.1074/jbc.M111705200

Osley, M. A., & Hereford, L. M. (1981). Yeast histone genes show dosage compensation. *Cell*, *24*(2), 377–84. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7016340

Palou, G., Palou, R., Guerra-Moreno, A., Duch, A., Travesa, A., & Quintana, D. G. (2010). Cyclin regulation by the s phase checkpoint. *J Biol Chem*, *285*(34), 26431–26440. doi:10.1074/jbc.M110.138669

Parker, L. L., & Piwnica-Worms, H. (1992). Inactivation of the p34cdc2-cyclin B complex by the human WEE1 tyrosine kinase. *Science (New York, N.Y.)*, *257*(5078), 1955–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1384126

Pellicioli, A., Lucca, C., Liberi, G., Marini, F., Lopes, M., Plevani, P., … Foiani, M. (1999). Activation of Rad53 kinase in response to DNA damage and its effect in modulating phosphorylation of the lagging strand DNA polymerase. *The EMBO journal*, *18*(22), 6561–72. doi:10.1093/emboj/18.22.6561

Peter, M., & Herskowitz, I. (1994). Direct inhibition of the yeast cyclin-dependent kinase Cdc28-Cln by Far1. *Science (New York, N.Y.)*, *265*(5176), 1228–31. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8066461

Pines, J. (1996). Cell cycle: reaching for a role for the Cks proteins. *Current biology : CB*, *6*(11), 1399–402. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8939596

Polyak, K., Kato, J. Y., Solomon, M. J., Sherr, C. J., Massague, J., Roberts, J. M., & Koff, A. (1994). p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes & development*, *8*(1), 9–22. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8288131

Polyak, K., Lee, M. H., Erdjument-Bromage, H., Koff, A., Roberts, J. M., Tempst, P., & Massagué, J. (1994). Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell*, *78*(1), 59–66. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8033212

Pruyne, D., & Bretscher, A. (2000). Polarization of cell growth in yeast. I. Establishment and maintenance of polarity states. *Journal of cell science*, *113 (Pt 3*, 365–75. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10639324

Radzio-Andzelm, E., Lew, J., & Taylor, S. (1995). Bound to activate: conformational consequences of cyclin binding to CDK2. *Structure (London, England : 1993)*, *3*(11), 1135–41. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8591024

Rana, J. S., Mittleman, M. A., Sheikh, J., Hu, F. B., Manson, J. E., Colditz, G. A., … Camargo Jr., C. A. (2004). Chronic obstructive pulmonary disease, asthma, and risk of type 2 diabetes in women. *Diabetes Care*, *27*(10), 2478–2484. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15451919

Rancati, G., & Li, R. (2007). Polarized cell growth: double grip by CDK1. *Current biology : CB*, *17*(15), R600–3. doi:10.1016/j.cub.2007.05.065

Reed, S I. (1980). The selection of amber mutations in genes required for completion of start, the controlling event of the cell division cycle of S. cerevisiae. *Genetics*, *95*(3), 579–88. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1214248&tool=pmcentrez&rendertype=abstract

Reed, S I, Hadwiger, J. A., Richardson, H. E., & Wittenberg, C. (1989). Analysis of the Cdc28 protein kinase complex by dosage suppression. *Journal of cell science. Supplement*, *12*, 29–37. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2699737

Reed, Steven I. (2003). Ratchets and clocks: the cell cycle, ubiquitylation and protein turnover. *Nature reviews. Molecular cell biology*, *4*(11), 855–64. doi:10.1038/nrm1246

Rhind, N., Furnari, B., & Russell, P. (1997). Cdc2 tyrosine phosphorylation is required for the DNA damage checkpoint in fission yeast. *Genes & development*, *11*(4), 504–11. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9042863

Richardson, H., Lew, D. J., Henze, M., Sugimoto, K., & Reed, S. I. (1992). Cyclin-B homologs in Saccharomyces cerevisiae function in S phase and in G2. *Genes Dev*, *6*(11), 2021–2034. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1427070

Ross, K. E., Kaldis, P., & Solomon, M. J. (2000). Activating phosphorylation of the Saccharomyces cerevisiae cyclin-dependent kinase, cdc28p, precedes cyclin binding. *Molecular biology of the cell*, *11*(5), 1597–609. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=14870&tool=pmcentrez&rendertype=abstract

Russell, P., Moreno, S., & Reed, S. I. (1989). Conservation of mitotic controls in fission and budding yeasts. *Cell*, *57*(2), 295–303. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2649252

Russo, A. A., Jeffrey, P. D., & Pavletich, N. P. (1996). Structural basis of cyclin-dependent kinase activation by phosphorylation. *Nature structural biology*, *3*(8), 696–700. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8756328

Russo, G. L., van den Bos, C., & Marshak, D. R. (2001). Mutation at the CK2 phosphorylation site on Cdc28 affects kinase activity and cell size in Saccharomyces cerevisiae. *Molecular and cellular biochemistry*, *227*(1-2), 113–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11827161

Russo, G. L., van den Bos, C., Sutton, A., Coccetti, P., Baroni, M. D., Alberghina, L., & Marshak, D. R. (2000). Phosphorylation of Cdc28 and regulation of cell size by the protein kinase CKII in Saccharomyces cerevisiae. *The Biochemical journal*, *351*(Pt 1), 143–50. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1221344&tool=pmcentrez&rendertype=abstract

Sanchez, Y., Bachant, J., Wang, H., Hu, F., Liu, D., Tetzlaff, M., & Elledge, S. J. (1999). Control of the DNA damage checkpoint by chk1 and rad53 protein kinases through distinct mechanisms. *Science (New York, N.Y.)*, *286*(5442), 1166–71. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10550056

Sanchez, Y., Desany, B. A., Jones, W. J., Liu, Q., Wang, B., & Elledge, S. J. (1996). Regulation of RAD53 by the ATM-like kinases MEC1 and TEL1 in yeast cell cycle checkpoint pathways. *Science (New York, N.Y.)*, *271*(5247), 357–60. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8553072

Sánchez-Díaz, A., González, I., Arellano, M., & Moreno, S. (1998). The Cdk inhibitors p25rum1 and p40SIC1 are functional homologues that play similar roles in the regulation of the cell cycle in fission and budding yeast. *Journal of cell science*, *111 (Pt 6*, 843–51. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9472012

Santocanale, C., & Diffley, J. F. (1998). A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication. *Nature*, *395*(6702), 615–8. doi:10.1038/27001

Schneider, B. L., Patton, E. E., Lanker, S., Mendenhall, M. D., Wittenberg, C., Futcher, B., & Tyers, M. (1998). Yeast G1 cyclins are unstable in G1 phase. *Nature*, *395*(6697), 86–89. doi:10.1038/25774

Schwartz, M. F., Lee, S.-J., Duong, J. K., Eminaga, S., & Stern, D. F. (n.d.). FHA domain-mediated DNA checkpoint regulation of Rad53. *Cell cycle (Georgetown, Tex.)*, *2*(4), 384–96. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12851493

Schwob, E., & Nasmyth, K. (1993). CLB5 and CLB6, a new pair of B cyclins involved in DNA replication in Saccharomyces cerevisiae. *Genes Dev*, *7*(7A), 1160–1175. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8319908

Serrano, M., Hannon, G. J., & Beach, D. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature*, *366*(6456), 704–7. doi:10.1038/366704a0

Shaag, A., Walsh, T., Renbaum, P., Kirchhoff, T., Nafa, K., Shiovitz, S., … King, M.-C. (2005). Functional and genomic approaches reveal an ancient CHEK2 allele associated with breast cancer in the Ashkenazi Jewish population. *Human molecular genetics*, *14*(4), 555–63. doi:10.1093/hmg/ddi052

Sheaff, R. J., & Roberts, J. M. (1996). End of the line: proteolytic degradation of cyclin-dependent kinase inhibitors. *Chemistry & biology*, *3*(11), 869–73. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8939714

Sherr, C. J., & Roberts, J. M. (1995). Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes & development*, *9*(10), 1149–63. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7758941

Sherr, C. J., & Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes & development*, *13*(12), 1501–12. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10385618

Shirayama, M., Toth, A., Galova, M., & Nasmyth, K. (1999). APC(Cdc20) promotes exit from mitosis by destroying the anaphase inhibitor Pds1 and cyclin Clb5. *Nature*, *402*(6758), 203–207. doi:10.1038/46080

Sidorova, J. M., & Breeden, L. L. (1997). Rad53-dependent phosphorylation of Swi6 and down-regulation of CLN1 and CLN2 transcription occur in response to DNA damage in Saccharomyces cerevisiae. *Genes & development*, *11*(22), 3032–45. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=316703&tool=pmcentrez&rendertype=abstract

Sikorski, R. S., & Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics*, *122*(1), 19–27. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2659436

Simon, M., Seraphin, B., & Faye, G. (1986). KIN28, a yeast split gene coding for a putative protein kinase homologous to CDC28. *The EMBO journal*, *5*(10), 2697–701. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1167171&tool=pmcentrez&rendertype=abstract

Skotheim, J. M., Di Talia, S., Siggia, E. D., & Cross, F. R. (2008). Positive feedback of G1 cyclins ensures coherent cell cycle entry. *Nature*, *454*(7202), 291–6. doi:10.1038/nature07118

Skowyra, D., Craig, K. L., Tyers, M., Elledge, S. J., & Harper, J. W. (1997). F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell*, *91*(2), 209–219. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9346238

Smolka, M. B., Albuquerque, C. P., Chen, S., Schmidt, K. H., Wei, X. X., Kolodner, R. D., & Zhou, H. (2005). Dynamic changes in protein-protein interaction and protein phosphorylation probed with amine-reactive isotope tag. *Molecular & cellular proteomics : MCP*, *4*(9), 1358–69. doi:10.1074/mcp.M500115-MCP200

Smolka, M. B., Chen, S., Maddox, P. S., Enserink, J. M., Albuquerque, C. P., Wei, X. X., … Zhou, H. (2006). An FHA domain-mediated protein interaction network of Rad53 reveals its role in polarized cell growth. *The Journal of cell biology*, *175*(5), 743–53. doi:10.1083/jcb.200605081

Solomon, M. J., Glotzer, M., Lee, T. H., Philippe, M., & Kirschner, M. W. (1990). Cyclin activation of p34cdc2. *Cell*, *63*(5), 1013–24. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2147872

Solomon, M. J., Lee, T., & Kirschner, M. W. (1992). Role of phosphorylation in p34cdc2 activation: identification of an activating kinase. *Molecular biology of the cell*, *3*(1), 13–27. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=275499&tool=pmcentrez&rendertype=abstract

Sorger, P. K., & Murray, A. W. (1992). S-phase feedback control in budding yeast independent of tyrosine phosphorylation of p34cdc28. *Nature*, *355*(6358), 365–8. doi:10.1038/355365a0

Spellman, P. T., Sherlock, G., Zhang, M. Q., Iyer, V. R., Anders, K., Eisen, M. B., … Futcher, B. (1998). Comprehensive identification of cell cycle-regulated genes of the yeast Saccharomyces cerevisiae by microarray hybridization. *Mol Biol Cell*, *9*(12), 3273–3297. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9843569

Stuart, D., & Wittenberg, C. (1995). CLN3, not positive feedback, determines the timing of CLN2 transcription in cycling cells. *Genes Dev*, *9*(22), 2780–2794. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7590253

Surana, U., Robitsch, H., Price, C., Schuster, T., Fitch, I., Futcher, A. B., & Nasmyth, K. (1991). The role of CDC28 and cyclins during mitosis in the budding yeast S. cerevisiae. *Cell*, *65*(1), 145–161. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1849457

Taba, M. R., Muroff, I., Lydall, D., Tebb, G., & Nasmyth, K. (1991). Changes in a SWI4,6-DNA-binding complex occur at the time of HO gene activation in yeast. *Genes Dev*, *5*(11), 2000–2013. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1936990

Tang, Y., & Reed, S. I. (1993). The Cdk-associated protein Cks1 functions both in G1 and G2 in Saccharomyces cerevisiae. *Genes & development*, *7*(5), 822–32. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8491379

Tavazoie, S., Hughes, J. D., Campbell, M. J., Cho, R. J., & Church, G. M. (1999). Systematic determination of genetic network architecture. *Nature genetics*, *22*(3), 281–5. doi:10.1038/10343

Tercero, J. A., & Diffley, J. F. (2001). Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint. *Nature*, *412*(6846), 553–7. doi:10.1038/35087607

Thomas, B. J., & Rothstein, R. (1989). Elevated recombination rates in transcriptionally active DNA. *Cell*, *56*(4), 619–630. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2645056

Thorne, H. V. (1966). Electrophoretic separation of polyoma virus DNA from host cell DNA. *Virology*, *29*(2), 234–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4287545

Thuret, J. Y., Valay, J. G., Faye, G., & Mann, C. (1996). Civ1 (CAK in vivo), a novel Cdk-activating kinase. *Cell*, *86*(4), 565–76. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8752211

Toh-e, A., Tanaka, K., Uesono, Y., & Wickner, R. B. (1988). PHO85, a negative regulator of the PHO system, is a homolog of the protein kinase gene, CDC28, of Saccharomyces cerevisiae. *Molecular & general genetics : MGG*, *214*(1), 162–4. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3067079

Travesa, A., Kuo, D., de Bruin, R. A. M., Kalashnikova, T. I., Guaderrama, M., Thai, K., … Wittenberg, C. (2012). DNA replication stress differentially regulates G1/S genes via Rad53-dependent inactivation of Nrm1. *The EMBO journal*, *31*(7), 1811–22. doi:10.1038/emboj.2012.28

Tyers, M., & Futcher, B. (1993). Far1 and Fus3 link the mating pheromone signal transduction pathway to three G1-phase Cdc28 kinase complexes. *Molecular and cellular biology*, *13*(9), 5659–69. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=360296&tool=pmcentrez&rendertype=abstract

Tyers, M., Tokiwa, G., & Futcher, B. (1993). Comparison of the Saccharomyces cerevisiae G1 cyclins: Cln3 may be an upstream activator of Cln1, Cln2 and other cyclins. *EMBO J*, *12*(5), 1955–1968. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8387915

Verma, R., Annan, R. S., Huddleston, M. J., Carr, S. A., Reynard, G., & Deshaies, R. J. (1997). Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase. *Science (New York, N.Y.)*, *278*(5337), 455–60. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9334303

Verma, R., Feldman, R. M., & Deshaies, R. J. (1997). SIC1 is ubiquitinated in vitro by a pathway that requires CDC4, CDC34, and cyclin/CDK activities. *Molecular biology of the cell*, *8*(8), 1427–37. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=276167&tool=pmcentrez&rendertype=abstract

Visintin, R., Prinz, S., & Amon, A. (1997). CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis. *Science (New York, N.Y.)*, *278*(5337), 460–3. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9334304

Waring, M. J. (1965). Complex formation between ethidium bromide and nucleic acids. *J Mol Biol*, *13*(1), 269–282. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/5859041

Wäsch, R., & Cross, F. R. (2002). APC-dependent proteolysis of the mitotic cyclin Clb2 is essential for mitotic exit. *Nature*, *418*(6897), 556–62. doi:10.1038/nature00856

Weinert, T. A., & Hartwell, L. H. (1988). The RAD9 gene controls the cell cycle response to DNA damage in Saccharomyces cerevisiae. *Science (New York, N.Y.)*, *241*(4863), 317–22. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3291120

Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., & Beach, D. (1993). p21 is a universal inhibitor of cyclin kinases. *Nature*, *366*(6456), 701–4. doi:10.1038/366701a0

Xiong, Y., Zhang, H., & Beach, D. (1993). Subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation. *Genes & development*, *7*(8), 1572–83. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8101826

Yao, S., Neiman, A., & Prelich, G. (2000). BUR1 and BUR2 encode a divergent cyclin-dependent kinase-cyclin complex important for transcription in vivo. *Molecular and cellular biology*, *20*(19), 7080–7. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=86248&tool=pmcentrez&rendertype=abstract

Yu, V. P. C. C., Baskerville, C., Grünenfelder, B., & Reed, S. I. (2005). A kinase-independent function of Cks1 and Cdk1 in regulation of transcription. *Molecular cell*, *17*(1), 145–51. doi:10.1016/j.molcel.2004.11.020

Zetterberg, A., Larsson, O., & Wiman, K. G. (1995). What is the restriction point? *Current Opinion in Cell Biology*, *7*(6), 835–842. Retrieved from http://www.sciencedirect.com/science/article/pii/0955067495800670

Zhao, X, Muller, E. G., & Rothstein, R. (1998). A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools. *Molecular cell*, *2*(3), 329–40. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9774971

Zhao, Xiaolan, & Rothstein, R. (2002). The Dun1 checkpoint kinase phosphorylates and regulates the ribonucleotide reductase inhibitor Sml1. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(6), 3746–51. doi:10.1073/pnas.062502299

Zhou, B.-B. S., & Bartek, J. (2004). Targeting the checkpoint kinases: chemosensitization versus chemoprotection. *Nature reviews. Cancer*, *4*(3), 216–25. doi:10.1038/nrc1296

Zhou, Z., & Elledge, S. J. (1993). DUN1 encodes a protein kinase that controls the DNA damage response in yeast. *Cell*, *75*(6), 1119–27. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8261511

ACKNOWLEDGEMENTS

First and foremost, my biggest thanks go to my supervisor Dr. David Garcia Quintana, for his best training to me; for the knowledge and scientific thinking he transferred to me; for his dedication and guide on my project; also for his trust on me and leaving me freedom to follow my own interest. He promotes me realize the beauty of science!

I would like to thank my lab mates, Gloria Palou, Roger Palou, Asrar Ahmad Malik, Ping Ren and David Montpeyó for their support on my lab work. I would like to thank Asrar Ahmad Malik, for his friendship and endless help. I am thankful to Gloria Palou and Roger Palou, for their scientific advice and experience shared to me. I would also like to thank Ping Ren who helped me with my bench work.

I am also thankful to members of other groups in our department, for discussions, suggestions, and also happy atmosphere in the lab. Bea, Neus, Txell, Josu, Albert, Yibin, Arash, Pablo, Maribel, Guillem, Liying, Vidhya, Ateh, Adrian, Marco, Elodia, Mateu, and other nice bosses of the unit, Prof. Esteve Padrós, Prof. Mireia Duñach, Dr. Toni Morros, Dr. Joan Manyosa, Dr. Manel Sabés, Dr. Josep Cladera, Dr. Alex and Dr. Tzvetana Lazarova.

I want to sincerely thank James Wohlschlegel from UCLA for his support on mass spectrometry analysis. Thanks to Chinese Scholarship Council (CSC) for the grant.

I would like to thank my family members, my parents, and Guixiang, Fanxiang, Jiasheng, Pingzhong for their endless support and love.

Finally, my love and thanks go to my dearest wife Xinjie, for her dedication, love and support in these years. I thank my little baby Wenxin for giving me so much happiness and strength. I dedicate this thesis to them.

Fanli Zeng

Barcelona, Spring, 2014