Control of Cytokinesis by the Mitotic Cyclin Dependent Kinase M-Cdk1

Ping Ren

PhD Thesis

Control of Cytokinesis by the Mitotic Cyclin Dependent Kinase M-Cdk1

PhD Program in
Biochemistry, Molecular Biology and Biomedicine

Department of Biochemistry and Molecular Biology

June 1, 2017

PhD candidate

Ping Ren

Thesis Supervisor

Dr. David G. Quintana

Dedicated to my family, my wife Deyin Kong,
my son Weiyao Ren
without whom none of this would have been possible

ABSTRACT

Abstract

Cytokinesis is the final regulated process in the eukaryotic mitotic cell division cycle. Cells enter cytokinesis once that chromosome segregation is satisfactorily completed. During cytokinesis cells physically separate, giving place to two daughter cells. Defects in the control of cytokinesis result in aneuploidies and genomic instability. A key controller of cytokinesis is the mitotic Cdk1 (M-Cdk1) activity. Our project derives from the observed correlation between the onset of cytokinesis and the termination of M-Cdk1 activity. Complementary to such observation, expression of a hyperstable allele of the mitotic cyclin Clb2, blocks cytokinesis. Thus, the very same activity that pushes cells into mitosis and promotes mitotic entry and anaphase, blocks the occurrence of premature cytokinesis. These observations suggest that one or more proteins, essential to trigger cytokinesis, are inhibited by M-Cdk1 phosphorylation. The identity of such critical M-Cdk1 substrate/s is unknown. Therefore, the goal of this thesis is to gain insight on how M-Cdk1 prevents premature cytokinesis during anaphase in the model eukaryotic organism *Saccharomyces cerevisiae*. The thesis work reveals that the Mitotic Exit Network is unexpectedly partially activated in the presence of prevailing high M-Cdk1 activity, and drives the release of the Cdc14 phosphatase to the cytoplasm under such conditions.

CONTENTS

CONTENTS

CONTENTS

1. INTRODUCTION

Control of Cytokinesis by the Mitotic Cyclin Dependent Kinase M-Cdk1

1.1. The Eukaryotic Cell Cycle

The mitotic cell cycle is the biological process through which one cell duplicates its genetic material and divides to give place to two daughter cells.

The archetypical mitotic cell cycle of eukaryotic cells is subdivided into four discrete phases (Figure 1) (Mitchison & Creanor 1971). Each phase was defined on the basis of chromosomal events and designated as G1 phase (for **G**ap **1**), S phase (for DNA **S**ynthesis), G2 phase (for **G**ap **2**) and M phase (for **M**itosis), respectively. During S phase chromosome duplication (replication) takes place. During M phase takes place the balanced segregation of the replicated chromosomes. As cells exit mitosis they divide into two daughter cells.

The object of interest of this thesis is the M phase. M phase sub-divides in turn in 5 distinct mitotic phases, based on the changes in the nucleus and cytoskeleton morphology (mainly the mitotic spindle): Prophase, prometaphase, metaphase, anaphase and telophase, which also (must) occur in a strict sequential order. Chromosome condensation, the first conspicuous signal, represents mitotic entry and the onset of prophase. Also, in prophase kinetochores assemble, the

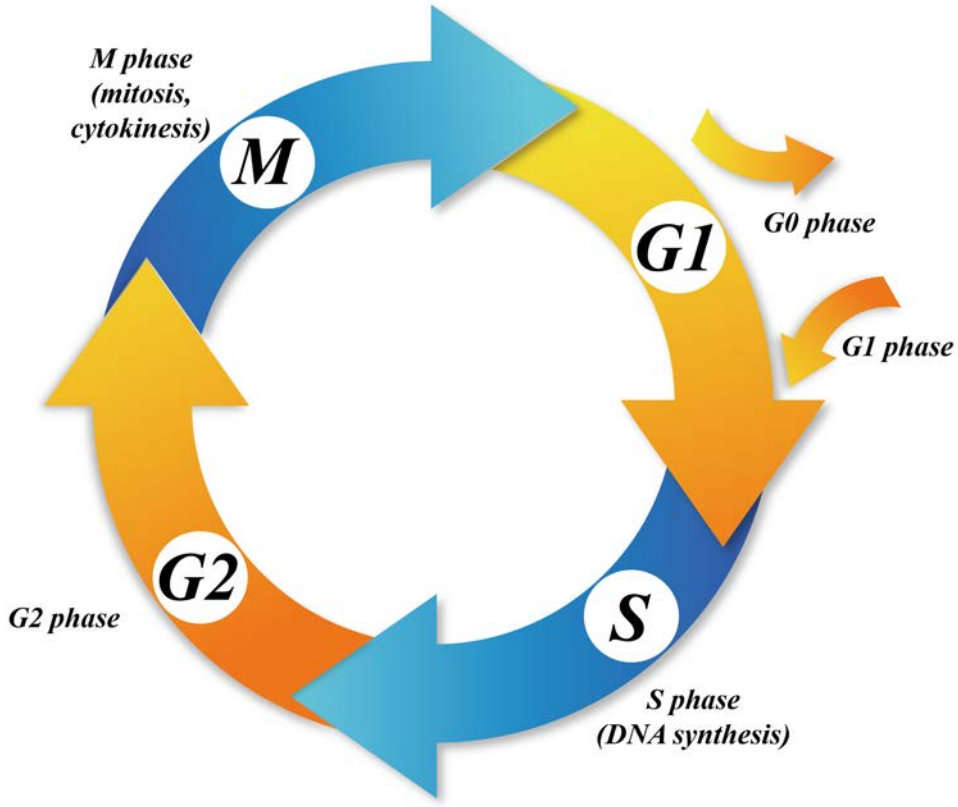


Figure 1. The eukaryotic mitotic cell cycle. Representation of the main phases of the cell cycle: G1, S, G2 and M phase. Normally, G1 phase and G2 phase are responsible for cell growth, transcription and translation of specific cell cycle proteins. In S phase and M phase, the two critical events for genetic inheritance take place: Chromosome replication and chromosome segregation (Mitchison & Creanor 1971). G_o a resting state in which cells do not cycle. Cells may enter the mitotic cell cycle at G1, and also may quit the cell cycle anytime between division and the so-called Restriction point in G1 (see 1.4.2).

protein structure found at the centromere of a chromatid to which spindle microtubules later attach for chromosome segregation. In higher eukaryotes centromeres are now duplicated and the mitotic spindle is launched to form. During the following mitotic phase, prometaphase, chromosomes continue condensing and migrating to the center of the cell, and the mitotic spindle commences to capture

chromosomes at the kinetochore. When the mitotic spindle captures all chromosomes and perfectly aligns them at the metaphase that marks metaphase. During metaphase, each sister chromatid is attached to microtubules and oriented to opposite poles of the cell and under bipolar tension. However, cohesion between sister chromatids blocks segregation. This is a critical control point and cells only enter the next mitotic phase in the absence of DNA damage and if all the chromosomes are attached to the spindle and under bipolar tension. When both conditions are satisfied cells may enter anaphase, marked by loss of cohesion between sister chromatids, chromosome segregation and spindle elongation. Finally, when chromosome segregation is completed. cell may enter telophase. In telophase, the mitotic spindle starts disassembling, chromosome decondensation begins, and nuclear envelope re-appears in higher eukaryotes. After mitosis is completed (karyokinesis, separation of nuclei) the cell must still split in two (cytokinesis, separation if two cells). During cytokinesis plasma ingression takes place, dividing the cytoplasm into two daughter cells (see 1.5.5).

1.2. Cell Cycle Control

Cell cycle progression is highly regulated to ensure that the genetic information is inherited unaltered (genomic integrity). Loss of genomic integrity results in loss of viability and generation of genomic instability. Genomic instability is the leading force that in higher eukaryotes drives malignant transformation.

Current knowledge of eukaryotic cell cycle regulation reveals three major *modes* of control. First, the sequence of critical cell cycle events is unidirectional and irreversible. Thus, the first cell cycle event cells commit to a round of mitotic division cycle once the pro-proliferative signaling prevails and the cell has reached a critical mass to support a round of division. Such point is called Restriction point in mammalian cells and START in budding yeast. After crossing the R point, cells must trigger a transcriptional wave that produces the proteins required for one round of chromosome replication which in turn must precede mitotic chromosome segregation and that must precede cytokinesis. Either premature entry into replication, or segregation before chromosome replication is complete, or cytokinesis before chromosomes segregation is finished, results in the loss of genomic integrity. Cells base such directionality on the regulated expression and destruction of the critical activities and substrates required at each given time of the cell cycle. As an example, chromosome segregation will only start if (1) mitotic cyclins are present to activate the mitotic Cyclin Dependent Kinase (M-Cdk1) catalytic subunit, (2) the right substrates have been expressed, and (3) Pds1/Securin is eliminated via the Ubiquitin ligase APC^{Cdc20} to release the activity of Esp1/Separase (see 1.5.1).

A second control mode ensures that each critical event occurs once and only once per cycle. Such control is based on two major, connected strategies. One, li-

censing factors that are essential to trigger an event are lost upon activation. The other, kinase activities that are essential to trigger a particular cell cycle event also inhibit licensing factor reload. An illustrative example is the way how eukaryotic cells avoid re-replication. The Cyclin Dependent Kinase activity that triggers DNA replication (S-Cdk1) requires a number of licensing factors to fire origins of replication (Cdc6, Cdt1, MCM). Upon firing, those factors are lost from individual origins. In addition, the very same S-Cdk1 prevents that licensing factors may re-load to a fired origin.

The third major control mode is constituted by *feed-forward* surveillance mechanisms, better known as checkpoints, that whenever problems that threaten genomic integrity are detected, temporarily halt the cell. Thus, the so-called Spindle Assembly Checkpoint (SAC) blocks anaphase until all chromosomes are attached at the spindle and under bipolar tension (see 1.4.2.1). Later in the cell cycle, the Spindle Position Checkpoint (SPOC) blocks mitotic exit until the two divided nuclei are fully segregated into the bodies of the future daughter cells (see 1.4.2.2)

1.3. The Model Eukaryotic Organism *Saccharomyces cerevisiae* (Budding Yeast)

1.3.1. *S. cerevisiae* as model organism in cell cycle research

The budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) is one of the most widely used model organisms to study fundamental aspects of eukaryotic cell biology in many laboratories, throughout the world, for many decades.

Budding yeast *S. cerevisiae* was the first completely sequenced eukaryotic organism (Goffeau et al. 1996). The entire genome information and databases are available in the *Saccharomyces* Genome Database (SGD, www.yeastgenome.org). Most genes, proteins and mechanisms first identified in budding yeast *S. cerevisiae* are found conserved in humans and in other higher eukaryotes (Guarente 1988).

In addition, several attributes make budding yeast extremely valuable for the study of cell cycle control. First, *S. cerevisiae* has a short doubling time compared to other eukaryotic cells (90 minutes at 30°C). Second, it is easily grown in quite economic media. Third, budding yeast is easy to transform, and several nutrient selectable markers introduced in laboratory strains make it very easy to select transformed strains. Fourth, budding yeast is extremely amendable for genetic manipulation, as it readily integrates DNA fragments by homologous recombina-

tion. Such integrations are targeted to the site of interest by sequence homology with an extremely high efficiency. Finally, because laboratory strains can be kept in a stable haploid mitotic cycle, a single genetic modification has a direct phenotypic effect in the cell.

In all, budding yeast is an ideal model to dissect the intricacies of eukaryotic cell cycle regulation. Based on a long sequence of discoveries, most of the findings are likely to be conserved in human cells and other higher eukaryotes.

1.3.2. Specifics of the *S. cerevisiae* cell cycle

Budding yeast was chosen in the milestone work by Leland Hartwell in the early 1970's to track cell cycle progression in the pre-molecular biology era (Hartwell et al. 1970) During G1 phase, *S. cerevisiae* cells are oval-shaped. The beginning of the cell cycle (START) is marked by the emergence of a bud that will eventually give place to *the* daughter cells. Because bud growth continues during S, G2 and M phases, microscopic inspection of the bud size allows to determine the cell cycle phase. During mitosis the bud size is nearly as large as the *mother* body. Cells with such morphology are called dumbbells. At the time of cell division, the bud size remains slightly smaller than the mother size. Because of that, in the budding yeast cell cycle a *daughter* cell and a *mother* cell result upon asymmetric division instead of two daughter cells. For that reason, *daughter* cells take longer to reach the critical mass to cross START at the next cell cycle.

Additional singularities of the budding yeast cell cycle, compared with the cell cycle of mammalian cells, are the duplication and segregation of the centrosomes (called Spindle Pole Bodies, SPB) in S phase; the assembly of a cross-nuclear spindle in S phase; and the lack of dissolution of the nuclear envelope during mitosis.

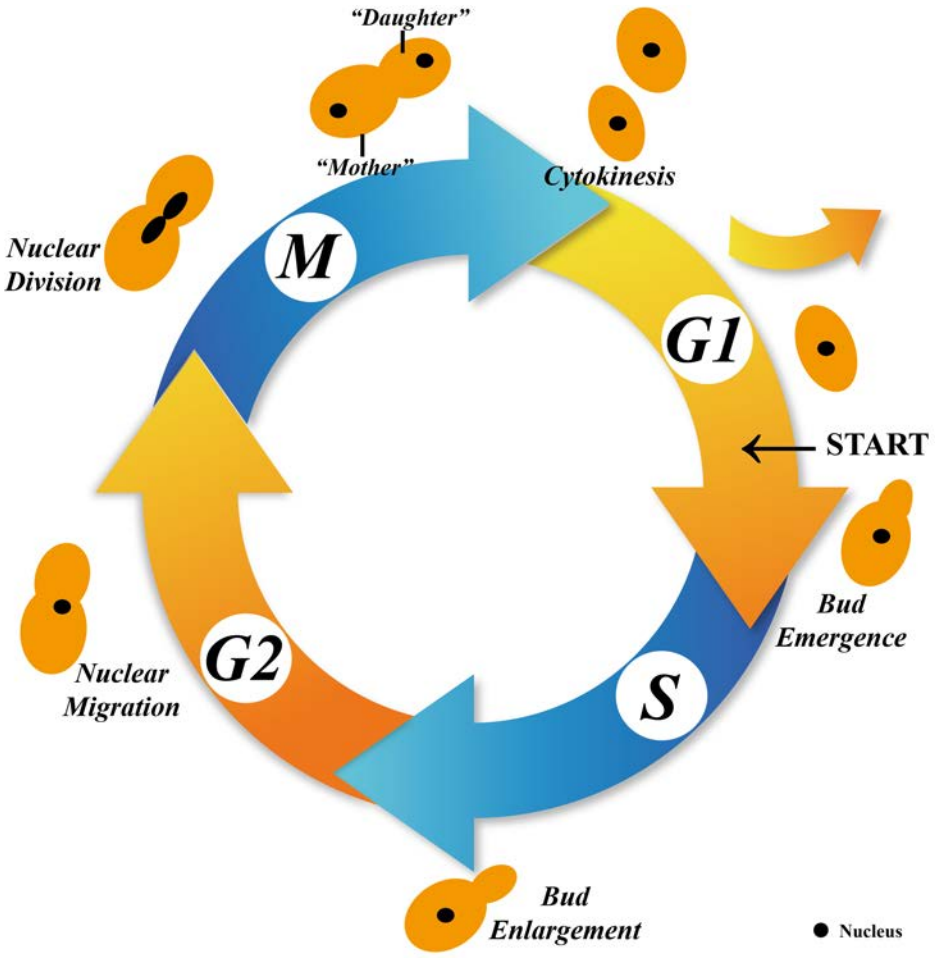


Figure 2. The cell cycle in *S. cerevisiae*. START marks the point of no return where cells commit to a complete round of chromosome replication, segregation and division. Bud size allows to easily identify the cell cycle phase by light microscopy. Nuclear envelope does not dissolve in M phase. Division is asymmetric as the bud/daughter never gets to reach the size of the mother.

1.4. Cell Cycle Control in *S. cerevisiae*

1.4.1. Cyclin Dependent Kinase Cdk1 activity

Cyclin-dependent kinases (CDKs) are the engine that drives cell cycle progression. CDKs are evolutionarily conserved in all known eukaryotes. CDKs are activated by binding to phase specific activatory subunits termed cyclins.

In *S. cerevisiae* only one CDK is essential for cell cycle progression, Cdk1 (encoded by the *CDC28* gene). The levels of Cdk1 remain constant and in excess throughout the cell cycle. The function of Cdc28 was first described in a conditional genetic screen for essential genes in cell cycle progression carried out by later Nobel laureate Lee Hartwell (Hartwell 1974; Hartwell et al. 1970). Such milestone works demonstrated for the first time ever the existence of a control on cell cycle. Inactivation of the original conditional Cdc28 alleles resulted in large, unbudded cells arrested at START with unreplicated DNA.

CDKs are proline-driven kinases, with a minimal phosphorylation consensus sequence S/T-P, and a preferential phosphorylation sequence S/T-P-X-K/R, where X is any amino acid and K/R are the basic amino acids lysine or arginine.

Several modes of Cdk1 regulation are in place in *S. cerevisiae.*

1.4.1.1. Cyclins

Cyclins are a group of activating proteins, originally discovered in sea urchin embryos by Tim Hunt (Evans et al. 1983), who got the Nobel prize for the discovery. The expression and degradation of cyclins are tightly controlled, and therefore cyclin levels oscillate during the cell cycle in all known eukaryotes (Amon et al. 1993).

Nine different phase-specific cyclins are present in budding yeast. Three G1-specific cyclins (Cln1-3) and six B-type cyclins (Clb1-6). Release of Cln3 is responsible to unleash the transcriptional oscillator at START. G1 cyclins Cln1 and Cln2 are expressed upon START and drive cells to the G1-S transition. S phase cyclins Clb5 and Clb6 are trigger DNA replication. The role of G2/M cyclins Clb3 and Clb4 is dispensable, whereas M phase cyclins Clb1 are responsible to trigger mitosis and also to block premature cytokinesis difference (Bloom & Cross 2007) (Figure 3).

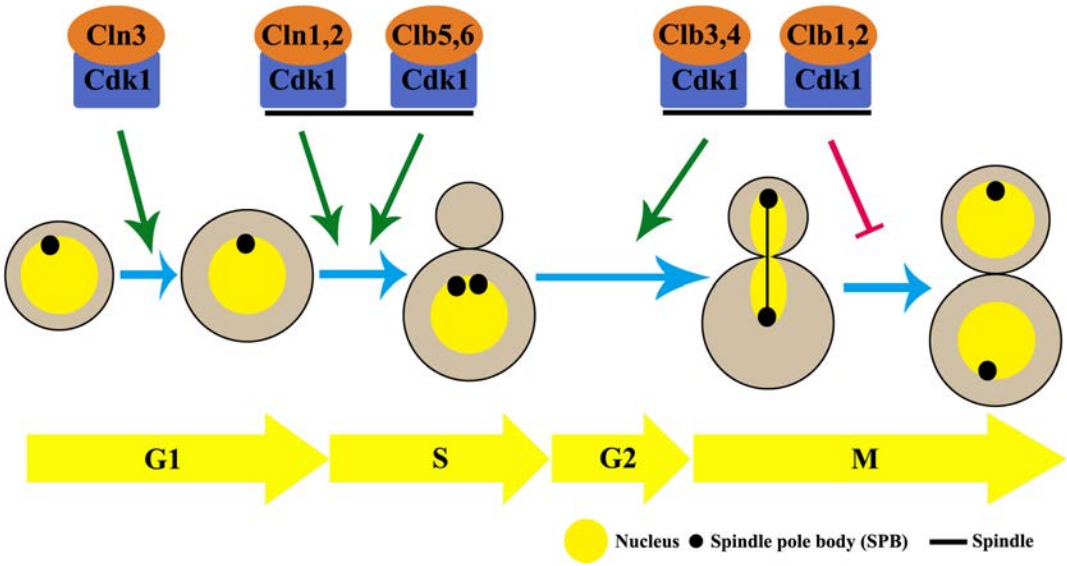


Figure 3. The cyclin-Cdk1 complexes in *S. cerevisiae*. Representation of the main cyclin-Cdk1 complexes of the cell cycle. The only CDK in budding yeast *S. cerevisiae*, Cdk1, associated with different phase specific cyclins at different phase points, forming a specific cyclin-Cdk1 complex that tightly regulates the cell cycle transitions during the cell cycle. The morphology of budding yeast during the cell cycle is presented by the schematic.

Mitosis, the focus of interest of this thesis, involves four different cyclins. Clb3 and Clb4, expressed in late S phase and present until anaphase (Fitch et al. 1992). The catalytic activity of Clb3,4-Cdk1 complexes plays an accessory role in early mitotic events (Mendenhall & Hodge 1998; Richardson et al. 1992; Lew & Reed 1993). M-specific cyclins Clb1 and Clb2 are transcribed at G2 and accumulate until they peak at metaphase (Fitch et al. 1992). The double inactivation arrests cells with a single undivided nucleus and a short spindle (Fitch et al. 1992; Surana et al. 1991). Clb1 and Clb2 are required to activate the APC^{Cdc20} (see 1.5.1) that triggers anaphase, and also to promote spindle elongation during anaphase (Raha & Amon

2008).

In turn, mitotic cyclins are destroyed upon mitotic exit by APC^{Cdh1} (see 1.5.4), a necessary condition for cytokinesis to occur (see 1.5.5).

1.4.1.2. Phosphorylation control of Cdk1

Cdk1 activity is also regulated by phosphorylation.

The CDK-Activating Kinase Cak1 phosphorylates Cdk1 at a highly conserved threonine-169 residue located at the kinase activatory T-loop (Kaldis et al. 1996). T-loop phosphorylation triggers a conformational change that facilitates ATP binding and enhances the affinity of Cdk1 for cyclin binding (Ross et al. 2000). Cak1 phosphorylation precedes cyclin binding.

On the other hand, the Swe1 kinase plays a negative control on Cdk1 by inhibitory phosphorylation on a highly conserved tyrosine-19 residue. Swe1 differently inhibits the activities of mitotic cyclin-Cdk1 complexes, affecting essentially Clb1 and Clb2 associated activity only (Booher et al. 1993). Swe1 mediates the cell cycle arrest elicited by the DNA damage checkpoint, that blocks the segregation of damaged or incompletely replicated chromosomes (Palou et al. 2015).

1.4.2. Checkpoints

The checkpoint concept was put forward by Hartwell and Weinert in 1989, when they demonstrated that a surveillance mechanism is responsible for blocking cells entry into mitosis upon DNA damage (Hartwell & Weinert 1989; Weinert & Hartwell 1988). As expectable, in humans the DNA damage checkpoint acts as an anti-cancer barrier in early tumorigenesis(Bartek et al. 2007; Bartkova et al. 2005; Bartkova et al. 2006; Karakaidos et al. 2005).

In general, checkpoints characteristically ensure that critical cell cycle events are successfully completed before progression to a subsequent phase is allowed. Loss of checkpoint function results in genomic instability (Hartwell et al. 1994), which is the driving force that fuels cancer transformation (Cahill et al. 1999; Gatenby & Gillies 2008). Mechanistically, checkpoints are signal transduction pathways triggered by intracellular signals, and are constituted by sensor complexes, central transducer kinases, and downstream effector kinases. Two different checkpoints operate in mitosis (Figure 4).

1.4.2.1. The Spindle Assembly Checkpoint (SAC)

The SAC blocks progression to anaphase until each and every chromosome is attached to the spindle and under bipolar tension (Rieder et al. 1995; Vanoosthuyse et al. 2009), thus preventing the occurrence of unbalanced chromosome segregation and aneuploidy. The SAC prevents anaphase by blocking the activation of the Anaphase

Promoting Complex by Cdc20 (see 1.5.1). Unattached kinetochores promote the formation of a Mitotic Checkpoint Complex (MCC), where SAC proteins Mad2, Bub3 and Bub3R1/Mad3 repress Cdc20 activation of the APC.

1.4.2.2. The Spindle Position Checkpoint (SPOC)

Once cells are in anaphase, the SPOC keeps mitotic exit inhibited until the segregated nuclei reach opposite poles of the cell. Because spindles occasionally misalign and anaphase occurs in the mother cell body, absence of a functional SPOC results in binucleate and anucleate cells (Kops et al. 2005).

Active SPOC inhibits the Mitotic Exit Network (MEN, see 1.5.3). Most of MEN components are assembled at the Spindle Pole Bodies (SPB). Kin4 coats the mother cell cortex, inhibiting MEN upstream protein Tem1 at the SPBs. If the spindle is misaligned, Tem1 at both SPBs is thus inhibited. Upon proper spindle orientation, only Tem1 at the mother SPB (mSPB) is inhibited(D'Aquino et al. 2005; Pereira & Schiebel 2005). On the other hand, Lte1 an activator of the MEN, localizes selectively at the bud compartment. Thus, upon correct alignment, when the daughter SPB (dSPB) reaches the bud cortex at the end of anaphase, MEN activation takes place (Bertazzi et al. 2011; Falk et al. 2011).

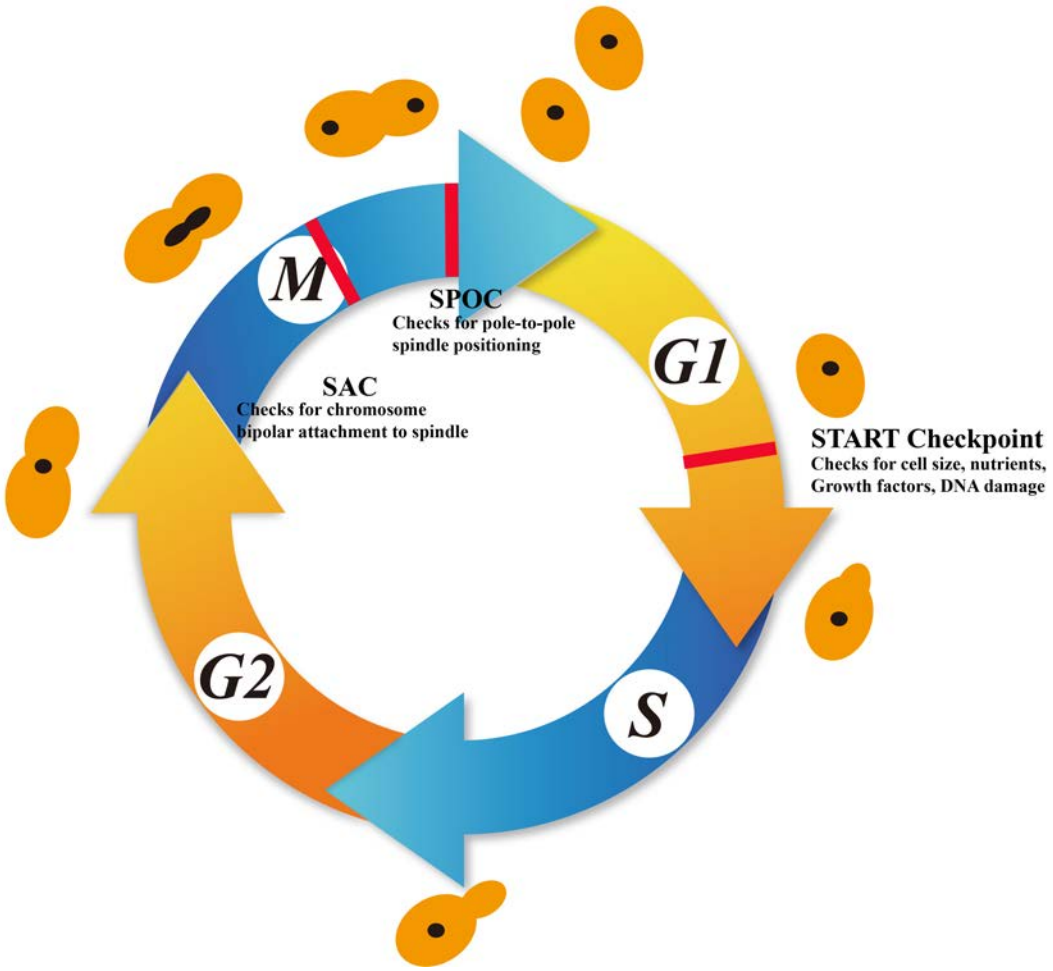


Figure 4. The Mitotic Checkpoints in *S. cerevisiae*. In metaphase, the Spindle Assembly Checkpoint (SAC) prevents anaphase until all chromosomes are under bipolar attachment at the spindle, thus preventing aneuploidy. In anaphase, the Spindle Position Checkpoint prevents mitotic exit until one of the SPBs enters the bud, thus ensuring a balances karyokinesis.

1.5. Control of Mitosis

Mitotic Clb1,2-Cdk1 activity is required to trigger mitosis (Fitch et al. 1992; Surana et al. 1991). Clb1,2-Cdk1 activity activates the APC^{Cdc20} that triggers anaphase, and also to promote spindle elongation during anaphase (Rudner & Murray 2000; Raha & Amon 2008).

1.5.1. Activation of the Anaphase Promoting Complex APC^{Cdc20}

Unlike other ubiquitin ligases with constitutive activity, the APC only activates upon the regulated association with one of two sub-stoichiometric activators: Cdc20 and Cdh1. Inactivation of the Spindle Assembly Checkpoint (see 1.4.2.1) allows APC activation by Cdc20 (APC^{Cdc20}), which also requires M-Cdk1 activity (Rudner & Murray 2000; Raha & Amon 2008). On the other hand, M-Cdk1 activity keeps Cdh1 inactive until mitotic exit (see 1.5.4).

The essential function of the APC^{Cdc20} is to eliminate Pds1/Securin (Hardwick and Murray 1995). Pds1/securin is a chaperone that inhibits the protease Esp1/separase. In turn, Esp1/separase is responsible for the cleavage of cohesin required for sister chromatid segregation (Uhlmann et al. 2000; Yamamoto et al. 1996; Ciosk et al. 1998; Uhlmann et al. 1999). Esp1/Separase also plays a role as part of the FEAR

network.

1.5.2. The FEAR network

The FEAR (cdc Fourteen Early Anaphase Release) network is triggered by the APC^{Cdc20} dependent activation of Esp1/Separase. Esp1/Separase downregulates the $PP2A^{Cdc55}$ phosphatase that keeps Net1 hypophosphorylated. Hypophosphorylated Net1 retains the mitotic exit phosphatase Cdc14 sequestered at the nucleolus (Shou et al. 1999) in an inactive state (Traverso et al. 2001) (Figures 5 and 6). Since M-Cdk1 activity is present from prophase, $PP2A^{Cdc55}$ prevents the premature release of Cdc14 (Queralt et al. 2006). In anaphase, decreased $PP2A^{Cdc55}$ activity allows that two mitotic kinases, M-Cdk1 and FEAR kinase Cdc5 phosphorylate Net1, promoting the transient release of active Cdc14 to the nucleoplasm release (Azzam et al. 2004).

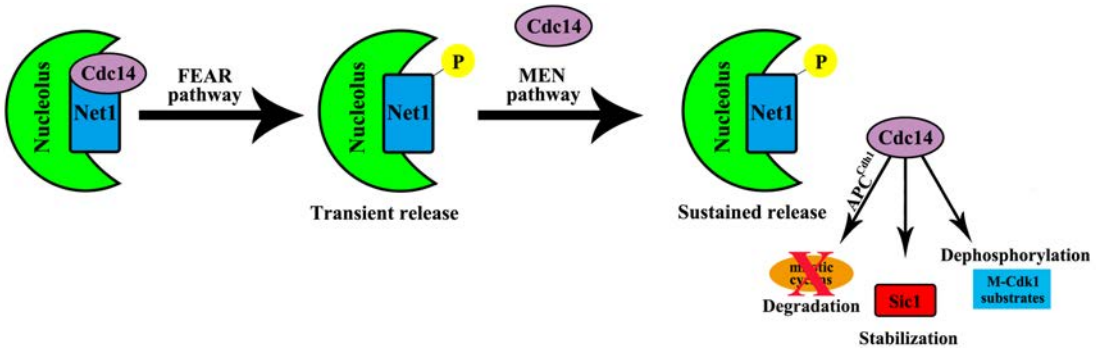


Figure 5. Regulation of the Cdc14 phosphatase and mitotic exit. Before anaphase, Cdc14 is sequestered in the nucleolus by Net1. At the onset of anaphase, upon SAC inactivation, FEAR activation results in a transient release of Cdc14 to the nucleoplasm. Upon completion of anaphase and SPOC inactivation, MEN activation provides a sustained release of Cdc14 into the

cytoplasm. Released Cdc14 activates the ubiquitin ligase APC^{Cdh1}, which degrades the mitotic cyclins. Lack of M-Cdk1 activity and active, fully released Cdc14, reverses the phosphorylation of a number of dual substrates, which triggers cytokinesis, the last stage of the mitotic cell cycle.

The Cdc14 phosphatase is essential to trigger mitotic exit. For cells to exit mitosis and undergo cytokinesis, Cdk1 must be inactivated and Cdk1 phosphorylations must be reverted by Cdc14 (Visintin et al. 1998). However, the full release of Cdc14 requires activation of the Mitotic Exit Network (MEN).

1.5.3. The Mitotic Exit Network (MEN)

MEN is kept inactive during anaphase by the Bfa1-Bub2 complex, under the SPOC (see 1.4.2.2 and Figure 6). Upon SPOC inactivation, the most upstream component of the MEN, Tem1 activates the MEN signaling cascade. Tem1 activates the Cdc15 kinase, which in turn activates the downstream effector kinase Dbf2-Mob1 (cells have a Dbf2 paralog, Dbf20, the double deletion is lethal). One of the essential roles of the Dbf2-Mob1 kinase is to phosphorylate Cdc14 to mask a nuclear localization signal (NLS), which allows Cdc14 to diffuse to the cytoplasm (Figure 6). Eventually, Dbf2-Mob1 kinase migrates from the SPBs, the place of activation, to the bud-neck, where it promotes Actomyosin Ring (AMR) contraction (Meitinger et al. 2011; Meitinger et al. 2013).

1.5.4. Activation of the Anaphase Promoting Complex APC^{Cdh1}

The full Cdc14 release overrides M-Cdk1 phosphorylation of Cdh1 which then can bind and activate the ubiquitin ligase APC (Jaspersen et al. 1999). Cdh1 is the second activator subunit of the ubiquitin ligase APC. APC^{Cdh1} avidly targets Clb cyclins for destruction (Wäsch & Cross 2002), as well as the cytokinesis essential protein Iqg1 (see 1.5.5.1). The termination of M-Cdk1 activity and the full activation of Cdc14 reverts Cdk1 phosphorylations, an event essential to trigger cytokinesis (Visintin et al. 1998).

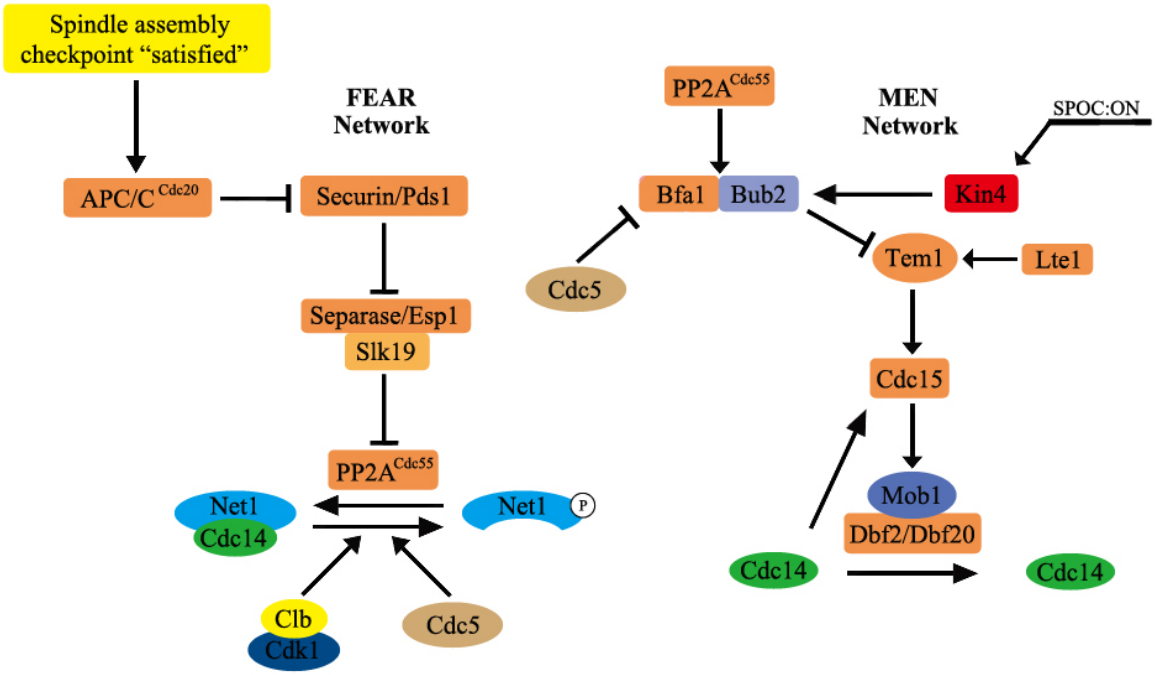


Figure 6. Control of Cdc14 release by the FEAR and the MEN networks. At the onset of

anaphase, the ubiquitin ligase APC^{Cdc20} is activated (M-Cdk1 activity + SAC inactivation) and targets Pds1/Securin for destruction, which releases Esp1/Separase activity. Esp1/Separase downregulates the $PP2A^{Cdc55}$ phosphatase, that can now be counteracted by M-Cdk1 and Cdc5 kinases on Net1. Phosphorylated Net1 releases Cdc14 to the nucleoplasm. At the end of anaphase, the dSPB reaches the Lte1-rich bud (daughter body), which counteracts the inhibitory action of SPOC Kin4 on MEN upstream element Tem1. Tem1 activation unleashes the MEN cascade, mediated by Cdc15. Downstream MEN kinase Dbf2-Mob1 phosphorylates Cdc14 that can now occupy the cytoplasm, counteracting preexisting M-Cdk1 phosphorylation, a necessary condition for cytokinesis to start.

1.5.5. Cytokinesis

Cytokinesis is the process that leads to the physical separation of the mother and daughter cells. Defects in cytokinesis result in regression of the cleavage furrow, leading to bi-nucleate cells, which are viable, albeit genomically unstable (Fujiwara et al. 2005; Ganem et al. 2007; Steigemann et al. 2009). On the other hand, premature cytokinesis causes chromosome breakage and aneuploidy, due to abscission before chromosome segregation is complete (Mendoza et al. 2009), which is likely to have direct implications in tumorigenesis (Daniel P. Cahill et al. 1999; Gatenby & Gillies 2008)

In eukaryotic cells cytokinesis is driven by contraction of an Actomyosin Ring, that guides plasma membrane ingression. In cell-walled *S. cerevisiae* coordinated septation occurs too.

1.5.5.1. Actomyosin Ring (AMR) contraction

AMR contraction provides the driving force for cytokinesis. In budding yeast AMR contraction must be coordinated with the ingression of the plasma membrane and guided primary septum formation. Actomyosin ring is assembled by a sequential order throughout the cell cycle (Lippincott & Li 1998), it begins before bud emergence and stabilizes by the accumulation of septins at the presumptive bud site. Next, the type-II myosin heavy chain protein Myo1 migrates to the nascent bud neck and is immobilized thereby interacting with the septin-binding protein Bni5 (Lee et al. 2002). By the beginning of mitosis, the type-II myosin light chain protein Mlc1 assembles to the bud neck (Boyne et al. 2000) and is responsible for the recruitment of the essential cytokinesis protein Iqg1 to the AMR (Shannon & Li 2000). Iqg1 plays a central role to assemble the AMR (Naylor & Morgan 2014). When M-Cdk1 drops Iqg1 is targeted for destruction by the APC/C^{Cdh1}, which defines the signal for AMR disassembly (Tully et al. 2009), which presumably promotes primary septum formation.

1.5.5.2. Primary Septum Formation

The primary septum is a thin chitin-rich layer of the cell wall that assembles centripetally at the mother-bud neck during AMR contraction. Primary septum formation mirrors AMR assembly, which is then followed by the sequential localization of several organizing proteins at the bud neck: The septum promoting proteins (Hof1, Inn1, and Cyk3) and the chitin synthase Chs2.

Chs2 is a chitin-synthesizing enzyme chiefly responsible for assembly of the primary septum at the mother-daughter neck during contraction of the AMR in cytokinesis. Chs2 is expressed in metaphase and sequestered at the endoplasmic reticulum (ER) in a M-Cdk1 dependent manner (Chuang & Schekman 1996). Upon mitotic exit Chs2 is de-phosphorylated by the activation of Cdc14. Chs2 dephosphorylation triggers Chs2 re-localization to the bud-neck (Zhang, Kashimshetty, Ng, et al. 2006). Chs2 primary septum deposition activity requires a complex with Inn1, and Cyk3.

Coupling AMR contraction and primary septum formation requires that Chs2 associates at the bud neck with an Ingression Progression Complex (IPC) composed of Inn1, Hof1 and Cyk3 (Foltman et al. 2016). Inn1 localizes to the IPC only when M-Cdk1 phosphorylation is reversed by Cdc14 activity (Palani et al. 2012). Therefore, Chs2 and Inn1 may constitute two substrates through which high M-Cdk1 activity precludes the onset of cytokinesis.

1.6. A role for M-Cdk1 in the control of cytokinesis

A key controller of cytokinesis is the mitotic Cdk1 (M-Cdk1) activity. Our project derives from the observed correlation between AMR contraction and the decrease in mitotic Cdk1 activity upon APC^{Cdh1} activation in late mitosis (Visintin et al. 1998; Wäsch & Cross 2002). Complementary to such observation, expression of hyperstable forms of the mitotic cyclins prevents cytokinesis, and cells arrest in telophase with separated nuclei (karyokinesis) but unable to divide (Ghiara et al. 1991). The very same activity that pushes cells into mitosis and promotes anaphase and karyokinesis, elegantly blocks the occurrence of premature cytokinesis. A similar control operates in S phase, where S-Cdk1 triggers DNA replication but also prevents re-replication by inhibiting the re-assembly of chromosomal pre-replication complexes (Dahmann et al. 1995).

A M-Cdk1 control makes sense, as cytokinesis must be temporally and spatially coordinated with genome segregation, and continued M-Cdk1 activity is required during anaphase (Raha & Amon 2008). Only when a number of indicators of a successful anaphase are met, cytokinesis should be safely allowed. Indeed, cytokinesis is triggered by the so-called Mitotic Exit Network (MEN), that activates only when it receives the *OK* from signals that anaphase is successfully completed (Rock & Amon 2009).

MEN activation results in the full release of Cdc14, that reverts M-Cdk1 phosphorylation and also terminates M-Cdk1 activity by activating the ubiquitin ligase APC^{Cdh1} (Jaspersen et al. 1999). APC^{Cdh1} targets mitotic cyclins for destruction via proteasome.

The above described observations suggest that one or more proteins, essential to trigger cytokinesis, are kept inactive by M-Cdk1 phosphorylation. The nature of such critical M-Cdk1 substrate/s is unknown and the goal of our work was to provide information on the essential M-Cdk1 targets that prevent premature cytokinesis.

2. OBJECTIVES

OBJECTIVES

The general objective of this thesis work was to investigate how the Mitotic Cyclin Dependent Kinase (M-Cdk1) restrains cytokinesis.

The specific objectives were:

- To set up an experimental system to test the bypass of the control that M-Cdk1 exerts on cytokinesis.
- To study how M-Cdk1 blocks mitotic exit and cytokinesis.
- To attempt the identification of the essential M-Cdk1 substrates that block premature cytokinesis.

3. MATERIALS AND METHODS

3.1. Yeast Techniques

3.1.1. Genetic background

All experiments were carried out on *Saccharomices cerevisiae* (budding yeast) W303 genetic background(Thomas & Rothstein 1989). Haploid cells mating type Mat a strains were used (W303-1a). To avoid the spontaneous generation of MAT α (W303-1b) and diploid cells, the heterothallic HO gene is mutated in this background.

The W303 genetic background also carries point mutations in five genes in different metabolic synthesis pathways, which interrupt the *de novo* synthesis of the corresponding amino acid or nucleobase. The genes are: *ade2-1,* which rends cells unable to synthesize nucleobase adenine; *trp1-1*, unable to synthesize the amino acid tryptophan; *his3-11,15*, unable to synthesize the amino acid histidine; *leu2-3,112*, unable to synthesize amino acid leucine; and *ura3-1*, unable to synthesize nucleobase uracil. These mutations may be used as auxotrophic selection markers for the isolation of autotrophic restored transformants when the transforming DNA carries a wild type copy of the corresponding gene.

In addition, W303-1a haploid cells can be naturally arrested in pre-START G1 phase in presence of the mating pheromone alpha-factor. Alpha-factor is 13 amino acid peptide (Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr) naturally

secreted by W303-1b MAT α haploid cells. To further improve the degree of synchronization with the pheromone, the *BAR1* gene was deleted. *BAR1* expresses an extracellular protease that cleaves α-factor and inactivates it (MacKay et al. 1988), allowing Mat a haploid cells to resume the cell cycle and escape the G1 arrest (Ciejek & Thorner 1979). The *bar1*Δ strains arrest tightly for several hours, and yet recover fast and synchronously when the pheromone is washed away.

For pre-START G1 synchronization alpha-factor was added to 50 ng/ml in the medium, and cells were incubated for a doubling time. Budding indexes <5% were a condition of quality of synchronization.

3.1.2. Yeast growth conditions

The cells of budding yeast *S. cerevisiae* were usually grown in standard liquid yeast extract-peptone-dextrose (YPD), which normally consisted of 1% Bacto yeast extract (Conda, #1702), 2% Bacto peptone (Oxoid, #LP0037) and 2% glucose (Merck, #14431-43-7). Nevertheless, based on different needs of experiments, glucose could be shifted to other carbon sources and complemented with liquid yeast extract-peptone (YP). Raffinose (Melford, # R0812), sucrose (Sigma-Aldrich, #S7903) and galactose (Duchefa Biochemie, #G0810) were widely applied in this study. For liquid yeast extract-peptone-dextrose-adenine (YPDA), additional 0.1mg/ml of adenine hemisulfate salt (Sigma-Aldrich, #A3159) was

supplemented as required. For solid YPDA plates, 2% bacto-agar (Conda, #1800) was supplemented with normal YPDA components and autoclaved together.

Supplemented minimal medium (SMM) was composed of 0.67% yeast nitrogen base without amino acids (BD, # 291940), 2% carbon sources (depend on the purposes of applications, glucose was normally used) and five auxotrophic requirements that included 5mg/ml adenine hemisulfate salt, 10mg/ml histidine (Sigma-Aldrich, #H8000), 10mg/ml leucine (Sigma-Aldrich, #L8912), 2mg/ml tryptophan (Sigma-Aldrich, #T8941) and 2mg/ml uracil (Sigma-Aldrich, #U0750). For solid synthetic minimal dextrose (SD) plates, 2% Bacto-agar was initially autoclaved with demineralized water. Later, other purified essential components (five auxotrophic requirements and carbon source) were added, respectively. For solid minimal auxotrophic selection plates, all above components supplemented but only omitted the auxotrophic requirement of interest, which was depended on the choice of the selection marker.

In additional, for solid antibiotic-contained selection plates, one of following antibiotics (200mg/L of Geneticin (Melford, #G0175), 100mg/L of Nourseothricin (Werner Bioagents, #51000) or 300mg/L of Hygromycin B (Invitrogen, #ant-hm-5)) was supplemented in hand touchable autoclaved YPD-agar medium, which was depended on the choice of antibiotic resistance gene marker.

5-fluoro-orotic acid (5-FOA) was a fluorinated derivative of the pyrimidine precursor orotic acid that was used to counter-select *ura3* auxotrophic restored transformants, in order to get rid of functional *ura3* gene and isolated for *ura3* auxotrophic mutants. For solid 5-FOA plates, 2% Bacto-agar was initially autoclaved with demineralized water, then following compounds were added, respectively, that included 1mg/ml 5-FOA (Melford, #F5001), 2mg/ml drop-out mix without uracil (Sigma-Aldrich, #Y1501), 0.67% yeast nitrogen base, 2% glucose and 50mg/ml uracil.

3.1.3. Cell synchronization

All strains in this thesis work were all deleted *BAR1* gene, thus G1 arrest was achieved by adding 50ng/ml final concentration of the mating pheromone alpha-factor. Cells were periodically checked under the light microscope and determined both by the appearance of a pear-shaped “schmoo” and by FACS analysis of DNA content. To release the synchronized cells, cells were washed and transferred into media with no pheromone.

For arrest the cells in metaphase, Nocodazole (LKT) was added at 5 μg/ml, subsequently, every 90 minutes half volume of Nocodazole was required to supply, in order to block the cells tightly and avoid the cells escape. Cells were periodically checked under the light microscope and determined both by the appearance of a dumbbell-shaped and by FACS analysis of DNA content.

In addition, arrest cells in metaphase also can be achieved by Cdc20 depletion, which required Cdc20 under the control of the *Met3* promoter. When using *MET-Cdc20* strains, the cells were grown in media without methionine and 2% of glucose. To arrest cells, 2mM final concentration of methionine was added, and the metaphase arrest was checked both by morphology of the cells and by FACS analysis of DNA content. For release from the metaphase arrest, cells were washed and transferred to media without methionine.

3.1.4. DNA Vectors

The DNA vectors in this study for molecular cloning are used yeast integrative plasmids that could propagate in both *E.coli* and *S.cerevisiae.* Furthermore, Applying with these integrative plasmids that allow either replication of molecules or by an introduction of exogenous DNA into the yeast genome via a mechanism that called homologous recombination and genetic change persisted after cell division.

3.1.4.1 Prototrophic gene-carrier vectors

In this study, the prototrophic gene-carrier vectors (pRS402 (*ADE2*), pRS303 (*HIS3*), pRS304 (*TRP1*), pRS305 (*LEU2*) and pRS306 (*URA3*))(Brachmann et al. 1998; Christianson et al. 1992; Chee & Haase 2012) were wildly employed for genetic manipulations, including PCR-mediated gene knockout and gene integration, according to following advantages:

- Appropriate length for genetic manipulation,
- High copy number for *E.coli*,
- Contained multiple unique restriction sites, facilitate molecular cloning,
- Suitable for white-blue color selection,
- Covered all W303-1a MAT a prototrophic genes.

For PCR-mediated gene knockout, the gene of interest was deleted or swapped by using the method of PCR-mediated gene disruption (Wach et al. 1994). The prototrophic gene deletion cassettes were generated by PCR amplification with two specific primers that tailed 60 nucleotides homologous sequences to swap the chromosomal sequences of interest. For gene integration, the prototrophic gene-carried vectors were constructed on a hybrid vector backbone that included sequences of multiple cloning sites (MCS). Thus, these vectors were widely used for molecular cloning that allowed introducing heterogeneous genes and/or mutagenized genes, along with the result of homologous recombination integrated into corresponding auxotrophic genetic loci in the host genome.

3.1.4.2 Antibiotic resistance gene-carrier vectors

The antibiotic resistance gene carried vectors were used for two priorities, one was PCR-mediated gene deletion (Wach et al. 1994) and the other was PCR-mediated epitope tagging (Bähler et al. 1998). In this study, pFa6a-*kan*MX6 serial vectors,

pFa6a-*nat*MX6 serial vectors, and pFa6a-*hph*MX6 serial vectors were all wildly employed (Hentges et al. 2005).

Due to each genetic manipulation was required to accompany with a selection marker gene, but the number of available selectable prototrophic marker genes were restricted that limited for sequential mutation. Thus, the antibiotic resistance genes were widely used as alternatives to prototrophic markers for PCR-mediated gene deletion, the antibiotic resistance genes were amplified by PCR with two specific primers that tailed with 60 nucleotides homologous sequence to swap the chromosomal sequences of interest. Furthermore, these antibiotic resistance genes were derived from either bacteria or fungi and lacking homology to yeast genome, thus only integrated transformants could neutralize the toxic of antibiotic and grow on antibiotic contained plates, whereas non-integrated cells were not. For PCR-mediated epitope tagging, because of lack of homology to yeast genome, thus the pFa6a-MX6 vectors were commonly used as the backbone to generate different epitope-tagging vectors. Moreover, in order to facilitate for protein detection, tandem copies of epitopes or fluorescent proteins were integrated in the pFa6a-MX6 vectors, such as six copies of the influenza virus hemagglutinin (6xHA), 13 copies of the human c-myc (13xMyc), green fluorescent protein (GFP) and its variant (mCherry) (Bähler et al. 1998). Therefore, relied on above basic tool kits that were widely provided us a starting point to dissect the functions of any gene of interest.

3.1.5. Yeast transformation

This high-efficiency yeast transformation protocol was adapted from alkali cations method (Ito et al. 1983) that was treated intact yeast cells with lithium acetate (LiAc), in order to weaken the cell walls and induce competence of yeast cells, subsequence, the divalent cations were displaced by LiAc/polyethylene glycol (PEG) and additional organic solvent DMSO before heat shocking. The purposes of these approaches were to facilitate introducing exogenous DNA into yeast cells by heat shocking upon transient pores generating and enhance the efficiency of yeast transformation (Gietz et al. 1995; Gietz et al. 1992; Schiestl & Gietz 1989).

The parental strains of choice were initially inoculated into 10ml of liquid YPDA and incubated at 30°C for overnight. The density of overnight culture was counted by using hemacytometer and the overnight culture was diluted into 10 ml of liquid YPDA with a cell density of $5x10^6$/ml. Upon cell density reached $2x10^7$/ml, the exponential cells were pelleted by centrifugation at 3000 RPM for 5 minutes at room temperature in a 15ml falcon.

Pelleted cells were washed twice with 10ml of sterilizing water and washed once with 1ml sterile TE/LiAc buffer (100mM LiAc, 10mM Tris, 1mM EDTA, pH 7.5), resuspended in 72µl sterile TE/LiAc buffer. Then 8µl of a 10mg/ml de-

natured single-stranded carrier DNA (boiled at 95 °C for 10 minutes and chilled on ice before use) and either 9µl of PCR products or 5µl of linearized DNA plasmids were supplemented into the cell suspension, respectively. Thereafter, 500µl of LiAc/TE/PEG buffer (100mM LiAc, 10mM Tris, 1mM EDTA, 40%(w/v) PEG (Sigma- Aldrich, #95904)) was supplemented and resuspended the cell pellet by briskly vortex mixing. Then, incubated the cells at 30 °C for 30 minutes.

Next, 60µl of DMSO (Sigma-Aldrich, #D2650) was added and tubes were mixed vigorously. The cells were followed by heat-shocked in a 42°C water bath for 15 minutes and chilled down in ice for another 3 minutes for the sake of terminate the shock.

Finally, the cells were pelleted by centrifugation at 3,000 RPM for 3 minutes and resuspended in either 200µl sterile water or liquid YPDA before plating. For integration of prototrophic marker, the cells were directly spread on solid auxotrophic minimal selection plates by lacking the auxotrophic requirement of interest and transformants can be isolated after incubation at 30°C for 3-4 days. For integration of antibiotic-resistance marker, the cells were initially spread on solid YPDA plates for overnight at 30°C, followed by replicated on solid antibiotic-contained selection plates and transformants can be isolated after incubation at 30°C for 2-3 days.

The positive transformants were selected by several molecular biological techniques. The most common instrumentations were phenotype observation, growth test, PCR amplification (described at 3.2.2), and western blot (described at 3.4.3).

3.1.6. Ectopic protein over-expression from the *GAL1* promoter

The endogenous Clb2 (without N-terminal) was subcloned into an integrative *pRS30X* vector (described at 3.1.4.1) and downstream of the inducible promoter. In this study, the inducible promoter *GAL1,10* was selected to conditionally over-express Clb2 by an addition of galactose into the cell culture.

Upon yeast transformation (described at 3.1.5), the linearized plasmid was integrated into yeast genome due to homologous recombination. In this study, over-expression of Clb2 is known to block the cell cycle progression. Thus, the transformants were picked and streaked on minimal auxotrophic selection plates and YPGal plates, respectively. The positive mutants only grow on minimal auxotrophic selection plates but failed to grow on YPGal plates.

Furthermore, there is an alternative to rapidly activate *GAL* promoter transcription by integration of additional chimerical transcriptional activator into the yeast genome. This chimerical transcriptional activator was abbreviated as ADGEV, which contained a DNA binding domain of Gal4 with a hormone-binding domain

of human estrogen receptor and a transactivation domain of the herpex virus protein VP16 (Louvion et al. 1993). For complete activation of *GAL* promoter, only required adding small volumes of beta-estradiol and no longer rely on galactose induction. The advantage of this approach is induction of *GAL* promoter could execute even in presence of glucose.

3.2. Molecular Biology Techniques

3.2.1. Yeast genomic DNA preparation

This protocol was based on glass-bead preparation protocol that dissolved genomic DNA in phenol/chloroform, precipitated DNA by low temperature and accumulated genomic DNA by high-speed centrifugation.

Transformants were initially inoculated in 3ml liquid YPDA at 30°C for overnight. Then 1ml harvest culture was transferred into a screw-cap vial, cells were spun down by centrifugation at 14000 RPM for 1 minute and then cell pellet was resuspended in 400µl lysis buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1% SDS). 200µl of acid-washed glass beads (0.5 mm diameter) was supplemented into cell suspension and then whole screwed vial was vortexed on highest setting for 90 seconds in order to break cell wall. Thereafter, 400µl of phenol/chloroform/isoamyl alcohol (25:24:1) mixture was supplemented into lysate and also vortexed on highest setting for another 90 seconds to de-chromatinize chromosomal DNA and dissolve chromosomal DNA into extract aqueous layer. Subsequence, extract aqueous layer (top) was isolated from the lysate by centrifugation at 14000 RPM for 5 minutes at room temperature and transferred to a new eppendorf. In order to increase the purity of extract aqueous layer, phenol/chloroform/isoamyl alcohol (25:24:1) mixture was supplemented again, vortexed, centrifuged and extracted as above.

Thereafter, two volumes of 96% ice-cold ethanol were mixed with extract aqueous layer and kept eppendorf at -20°C for 15 minutes to precipitate chromosomal DNA. Then, chromosomal DNA was accumulated by centrifugation at 14000 RPM for 20 minutes at room temperature. DNA pellet was washed twice with 70% ethanol and reconstituted in 50µl TE buffer containing RNase A (10 mM Tris·HCl pH 8.0, 1 mM EDTA, 10µg/ml RNase A) after 3 hours at 37 °C. Finally, all genomic DNA samples were preserved at -20°C. For each polymerase chain reaction (PCR), 1µl of genomic DNA sample was used as template.

3.2.2. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (Mullis & Faloona 1987) is one of the most powerful and important techniques to examine gene replacement, which included gene disruption (described in 3.2.2.1), C-terminal tagging (described in 3.2.2.2) and molecular cloning (described in 3.2.3). PCR was used to amplify a piece of DNA fragment *in vitro* by applying specific oligonucleotide primers to generate thousands or millions of copies of targeted DNA sequence. These PCR-based products were provided different possibilities to yeast genetic research.

In this study, all gene-specific primers were designed based on the paper of versatile PCR-based toolbox(Janke et al. 2004). For gene disruption-deletion in budding yeast *S. cerevisiae* that required homologous recombination, thus two

gene-specific primers were tailed with approximately 60 nucleotides homologous sequence to allow PCR products replacing the chromosomal sequences of a target and each prime was ended with a universal sequence (21 nucleotides) to amplify various selectable markers from plasmid templates. The flanking sequences were specially described in 3.2.2.1. For transformants checking, two gene-specific primers were only contained 21 nucleotides homologous sequences to amplify the upstream and downstream of the gene of interest at least 100 nucleotides apart. For molecular cloning, two gene-specific primers were tailed with 21 nucleotides homologous sequences to the gene of interest and ended with specific restriction endonuclease sequence.

Additionally, PCR was a thermal cycling-based method. A reliable PCR machine was allowed using time increment program to maximize complete DNA synthesis and high-fidelity DNA polymerases were crucial for all PCR-related works by its high efficiency and simple protocol. In this study, all PCR reactions were completed in DNA Engine Dyad thermal cycler (Bio-rad) and various DNA polymerases were applied for different purposes.

The PCR reaction mix (shown in Table 1) and cycle profile (shown in Table 2) were as follow:

- PCR reaction mix (Table 1)

1µl of template DNA (Plasmid or genomic DNA)

5µl of 10x DNA Polymerase Buffer

8.75µl of 2mM dNTPs

3.2µl of 10µM forward primer

3.2µl of 10µM reverse primer

0.6µl of DNA polymerase mixture

H_2O fill up to 50µl

Note: Additional of 1µl DMSO was supplemented into PCR reaction mix, if the NAT1 cassette was needed to amplify.

- PCR cycle profile (Table 2)

95ºC for 4 minutes

95ºC for 30 seconds

54ºC for 30seconds

68ºC for 1 minute per kb

→ 30 cycles

68ºC for 7 minutes

4ºC for 24 hours

Eventually, 5µl of PCR products were loaded and analyzed on an Agarose-TAE gel by DNA electrophoresis.

3.2.2.1. Gene disruption

Gene disruption is one of most efficient molecular-genetic analyses approaches that allowed generating a null mutation by deleting a putative open reading frame of a particular gene, investigated the phenotypes and deduced the functional identity of the gene. Finally attempted to elucidate the physiological role in vivo of the gene in budding yeast *S.cerevisiae.* Thus, one-step gene replacement method (Rothstein 1983) was wildly developed. Furthermore, in order to complete delete or swap the gene of interest on yeast chromosome that required a single yeast transformation by inserting an extraneous DNA fragment into yeast cells, thereby the gene of interest was completely deleted or swapped by homologous recombination.

Traditionally, the entire endogenous open reading frame (ORF) of the gene of interest was precisely deleted by using the method of PCR-mediated gene disruption (Wach et al. 1994) and the typical gene deletion flanking homologous sequences should overlap at upstream of the ATG and downstream of the stop codon. In this study, the entire gene deletion cassette (referred above as extraneous DNA fragment) was generated by PCR amplification that contained an entire selection marker gene plus gene-specific flanking homologous sequences. Along with the homologous sequences, the entire gene deletion cassette would sufficient for homologous recombination and the target gene would be completely swapped in its chromosomal locus. The basic scheme for gene deletion was depicted in Figure 7.

In addition, the entire selection marker gene was amplified from one of the prototrophic-carried vectors or antibiotic resistance gene carried vectors by using two gene-specific oligonucleotide primers that were tailed with approximately 60 nucleotides flanking homologous sequences to the target gene at 5' end and hybridized with 21 nucleotides universal sequences at 3' end that was homologous to the sequence of selection marker gene.

Additional, due to PCR-mediated gene deletion cassettes were recombinogenic and contained free DNA ends. Thus, upon introduction of this cassettes into yeast cells, which were greatly stimulated the homologous recombination repair mechanism and resulting in efficient uptake of selection marker cassette and simultaneous recombination into the locus of the target gene. After yeast transformation, the cells were plated on the corresponding solid selection plates that according to the selection marker gene of choice. Transformants can be selected after 3-4 days incubation at 30°C and second round of selection was to eliminate the false positives and enhance the purity of positive transformants.

Finally, transformants (defined here as the cells who carried the correctly integrated deletion cassette) were verified by PCR of genomic DNA employing with two check oligonucleotide primers (21 nucleotide-long) that specifically annealed at outside (upstream or downstream) the region of the gene of interest. The expected size of PCR product was obtained only if the deletion cassette was correct-

ly inserted into the genome by homologous recombination repair. However, in some cases, upon the size of deletion cassette was the same as the gene of interest, thus, the PCR verification should employ with two different annealed check primers, one primer called as external primer that annealed adjacent area of genomic DNA and the other one called as internal primer that amplifies part of integrated selection marker, only positive transformants provided PCR products.

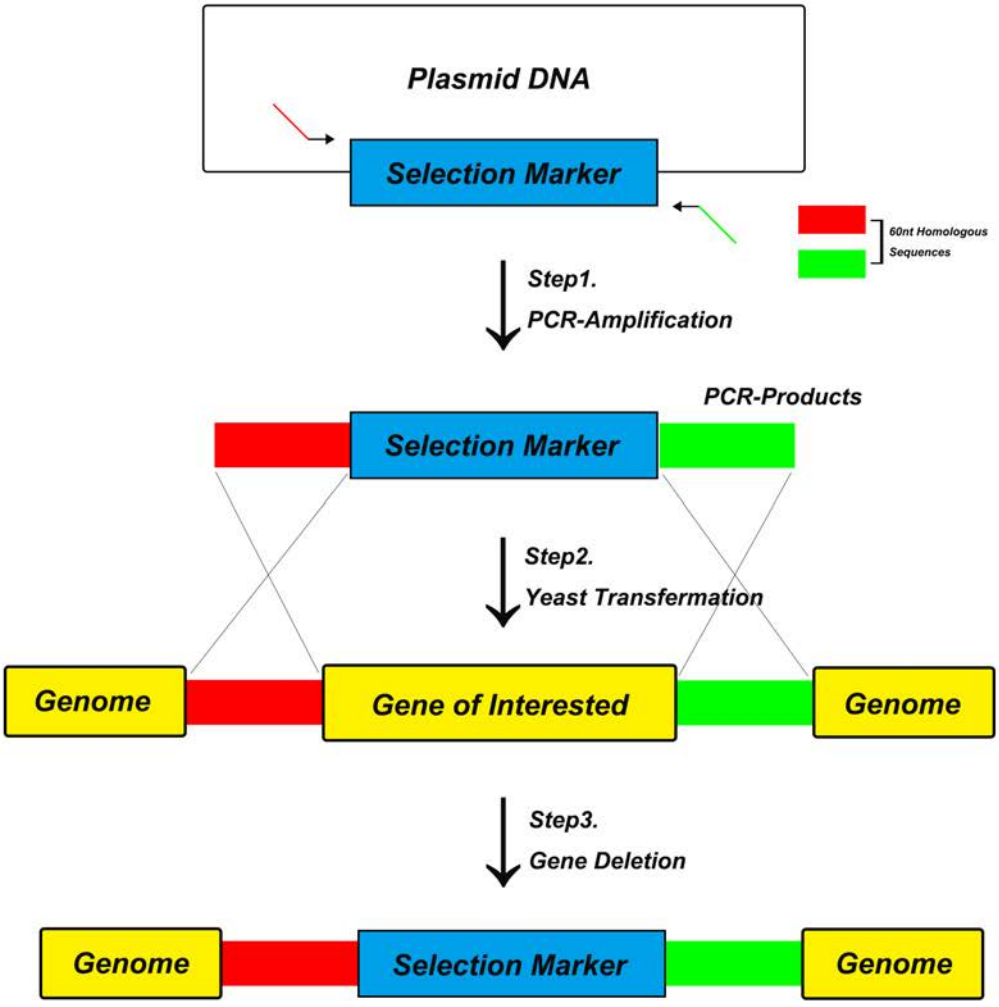


Figure 7. The principle of gene disruption. The plasmid contained a cassette, which consisted of a selection marker and additional sequences. The primers contained 21bp universal sequences that allowed amplification of cassettes (Step 1)

and 60bp gene-specific homologous sequences that help PCR product to recognize the desired genomic location (Step 2). Upon yeast transformation, an integrative cassette for gene disruption is integrated into yeast genome due to homologous recombination (Step 3).

3.2.2.2. C-Terminal tagging

Epitope tagging is a widespread approach for analysis the intricate functions of a particular protein in budding yeast *S.cerevisiae* and it is also achieved by a simple PCR-mediated strategy (Schneider et al. 1995) that allowed inserting a short stretch of amino acids into chromosomal gene and constituting an epitope into the protein of interest. The most typical approach for epitope tagging is inserted various epitopes sequence into the C-terminus of the chromosomal gene, because the tag is engineered into the genomic locus. As a consequence, the tagged protein is expressed and regulated similarly to its authentic protein. Furthermore, the protein was tagged with small peptide sequences such as HA, MYC that allowed for protein detection by western blot or fused with fluorescent markers such as GFP, mCherry that allowed visualizing the localization of the protein of interest by fluorescent microscope.

Normally, the PCR-mediated tag DNA fragment was contained an epitope tag DNA sequence plus a selection marker, then introduced into yeast cells by a simple yeast transformation and tagged to the gene of interest by homologous recombination repair. The strategy was similar to the one described above at gene dis-

ruption and the basic scheme for epitope tagging was depicted in Figure 8. In this study, the tag DNA fragment was amplified from one of desired *pFa6a-MX6* tool-kits with two gene-specific oligonucleotide primers. But in this part, one of gene-specific primer was tailed 50 nucleotides of flanking homologous sequences that must overlap before the STOP-codon (excluding STOP) to the gene of interest at 5' end and followed by 21 nucleotides universal sequences that homologous the sequence of desired tag (HA or MYC) at 3' end and the other one was tailed 50 nucleotides of reserved complement flanking homologous sequences that must overlap downstream of the STOP-codon (including STOP) to the gene of interest at 5' end and followed by 21 nucleotides universal sequences that homologous the sequence of the entire selection marker of choice at 3' end (Janke et al. 2004).

After transformation, the cells were initially plated on the solid YPDA plates for overnight growth then replicated to corresponding solid antibiotic-contained plates. Transformants can be selected after 2-3 days incubation at 30°C and the second round of selection was to eliminate the false positives and enhance the purity of correct transformants.

The correct transformants can be verified by PCR of genomic DNA or by checking for expression of the tagged protein under the florescent microscope or using western blotting.

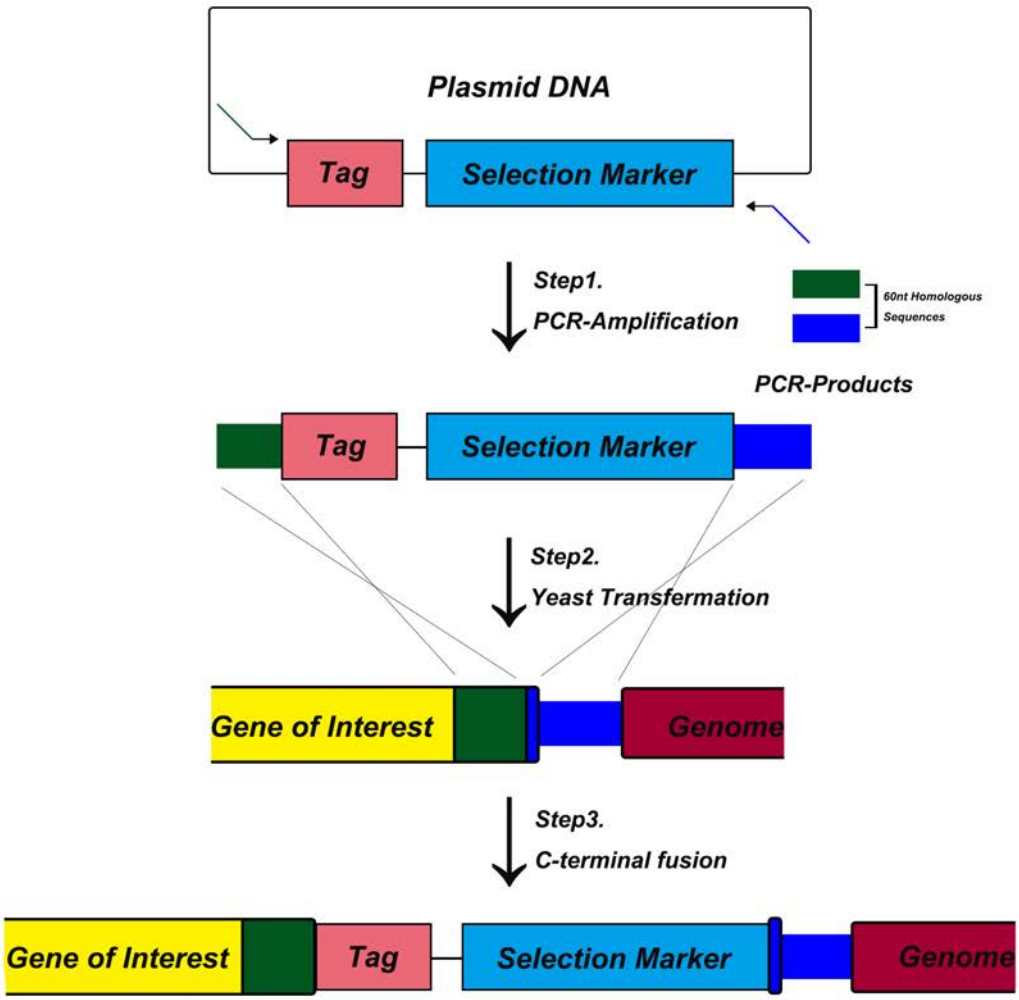


Figure 8. The principle of C-terminal tagging. The plasmid contained a cassette, which consisted of a selection marker and additional sequences. The primers contained 21bp universal sequences that allowed amplification of cassettes (Step 1) and 60bp gene-specific homologous sequences that help PCR product to recognize the desired genomic location (Step 2). Upon yeast transformation, an integrative cassette for C-terminal gene fusion was integrated into yeast genome due to homologous recombination (Step 3).

3.2.2.3 DNA directed point mutagenesis

Single primer site-directed DNA mutagenesis(Edelheit et al. 2009)was optimized from traditional site-directed mutagenesis methods by employing single PCR oligonucleotide primer that contained the desired mutation to generate cloned DNAs with modified sequence, in comparison with other double-primer mutagenesis methods (Kumar & Rajagopal 2008; Braman et al. 1996), using single PCR oligonucleotide primer that could avoid primer-primer annealing caused sequence mismatching and formation of tandem repeats of primers.

The specific-PCR reactions were performed by using a single primer that carried a mutation was exhibited in Table 3 and PCR cycle profile was shown in Table 4.

- PCR reaction components (Table 3)

500ng of template DNA plasmid

2.5µl of 10xDNA Polymerase Buffer

2.5µl of 2mM dNTPs

4µl of 10µM forward primer/ reverse primer

0.5µl of DNA polymerase

H_2O fill up to 25µl

- PCR cycle profile (Table 4)

95ºC for 5 minutes

95ºC for 50 seconds

55ºC for 50seconds

68ºC for 1 minute per kb

→ 30 cycles

68ºC for 7 minutes

4ºC for 24 hours

After PCR amplification, two PCR reactions were mixed together in one PCR vial and put PRC vial in PCR machine to re-anneal. Initially, the vial was incubated at 95ºC for 5 minutes to allow denaturing double strands of plasmid templates and releasing PCR products that carrying the mutation and parental plasmid strand. Thereafter, stepwise decreased incubation temperature until 37ºC by following procedures, 90ºC for 1 minute, 80ºC for 1 minute, 70ºC for 30 seconds, 60ºC for 30 seconds, 40ºC for 30 seconds, 37ºC for 1 minute, in order to randomly re-anneal PCR products and parental plasmid strands.

After the re-annealing step, the mixture was purified by QIAquick PCR purification kit and eluted in 45µl elution buffer (EB). 1.5µl of restriction endonuclease DpnI (NEB, # R0176S) and 5µl of NEB buffer1 were separately supplemented and the whole mixture was incubated at 37ºC for overnight. The parental plasmid strands were digested by DpnI digestion (DpnI is a frequent cutter and only recognized the sites where were methylated), because of they were extracted

from DH5α competent *E.coli* cells and methylated by cellular DNA methylases, whereas only newly amplified PCR produces were remained and intact.

In the end, all digested products were transformed into DH5α competent *E.coli* cells and DNA mini-preps were performed by QIAprep spin miniprep kit as described in 3.2.4.3 and positive mutations were identified by DNA sequencing.

3.2.2.4 Agarose gel electrophoresis

Agarose gel electrophoresis was an analytical technique widely applied to measure and separate DNA molecules based on the size of DNA cassettes (Waring 1965).

Agarose gel was cast by suspending 0.5g of agarose powder (agarose D-1 Low EEO-TQM, Pronadisa) in 50ml of 1X TAE buffer (50 mM Tris-acetic acid pH 8.5, 2 mM EDTA). After boiling and cooling to below 60ºC, 5µl of SYBR safe DNA gel stain was added (Invitrogen, #S33102). SYBR safe DNA gel stain was a highly sensitive stain for visualization of DNA bands in agarose gels upon illumination with UV light (365 nm) and with less hazardous than ethidium bromide (EtBr) by its specific formulation.

According to DNA fragment size, the electrophoresis was carried out in TAE at 80-120V for the time necessary to achieve a good fragment resolution in elec-

trophoresis tank. The samples were run in parallel and the size of the DNA was monitored by comparison with 1kb DNA ladder (NEB, #N3232S). The resolved DNA was visualized under a UV transilluminator.

3.2.3. Molecular cloning

3.2.3.1. *Classical* cloning: Restriction enzyme digestion

Restriction digests were performed using NEB enzymes and buffers according to the manufacturer's instructions. Molecular cloning followed by a robust "cut and paste" system where a defined DNA fragment could digest from one plasmid and paste to another. The desired DNA fragments were amplified by PCR reaction with gene-specific primers as described in 3.2.2.1 or digested from other vector plasmids by correspondence restriction endonucleases. Then desire DNA fragments were either purified by QIAquick PCR purification kit (QIAGEN, #28104) or cut and recovered by QIAquick gel extraction kit (QIAGEN, #28704). Thereafter, the sizes of DNA fragments were checked in agarose gel by DNA electrophoresis as described in 3.2.2.4. The yields of purified DNA fragments were measured by nanodrop (Thermo).

The recipient plasmid and insert PCR products were both digested with identical restriction endonucleases and digestion procedures were strictly followed by the instructions of the manufacturer. Moreover, in order to increase the efficiency of cloning and also prevent recipient plasmid self-ligation, Antarctic phosphatase

(NEB, # M0289S) and its reaction buffer (NEB, # B0289S) were recommended to supplement with linearized recipient plasmid to further catalyze and remove 5' phosphate under 37 °C for 30 min and inactivated by using 70 °C for 5 min. Finally, digested PCR products and phosphatase-treated recipient plasmids were both purified by PCR purification kit and yields were measured by nanodrop again.

3.2.3.2. *Classical* cloning: Ligation

10µl of the reaction system was used to recommend facilitating ligation reaction. The best yield ratios between insert DNA fragment and linearized recipient plasmid was 7, then 1µl of T4 DNA ligase (Roche, #10481220001) and 1µl of its ligation buffer were supplemented, respectively. Meanwhile, a negative sample was prepared by adding identical components but used water to replace insert DNA fragments, in order to measure the background of vector self-ligation. In the end, two reaction vials were incubated at 16°C for overnight in a thermal cycler and entire ligation mixture were mixed into DH5α competent *E.coli* cells.

3.2.3.3. Gibson assembly cloning

According to this new method (Gibson et al. 2009), molecular cloning was no longer needs for idiographic restriction sites and meanwhile available for multiple overlapping DNA fragments joining in a single isothermal reaction. The recipient plasmid was either digested by restriction endonucleases or amplified by PCR

with two specific primers that only included 21 nucleotides homologous sequence. Besides, the insert DNA fragments were obtained by PCR amplification with two specific primers. Each prime contained 51 nucleotides, which consisted of 21 nucleotides homologous sequence that allowed amplifying the sequence of interest and 30 nucleotides complementary sequence that allows overlapping recipient plasmid and inserts DNA. The yields were both measured again by nanodrop after related purification.

In Gibson assembling system, it required 300ng of insert DNA fragment and 100ng of recipient plasmid and mixed with 10µl of 2x Gibson cloning master mix that consisted of T5 exonuclease, Phusion DNA polymerase, and Taq DNA ligase, finally water filled up to 20µl. Followed by the reaction mixture was incubated at 50°C for 30 minutes and chilled down on ice for 10 minutes. In the end, 2µl of ligation mixture was transformed into DH5α competent *E.coli* cells (Figure 9).

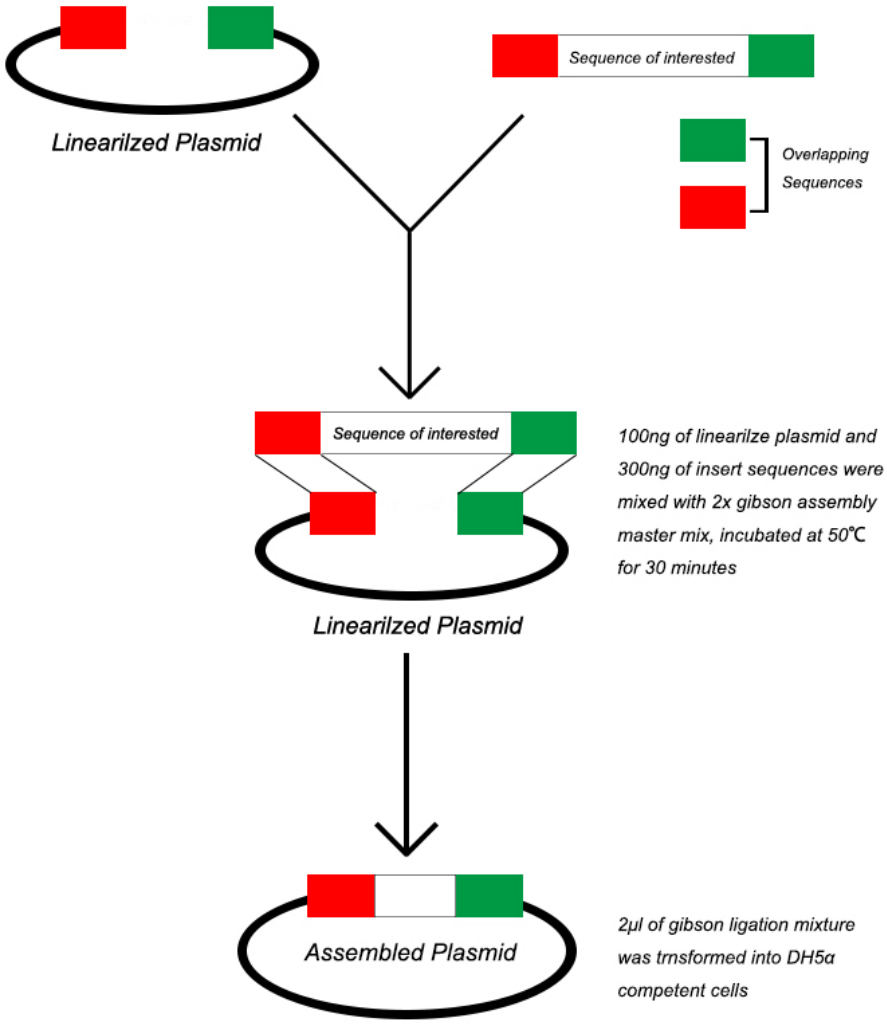


Figure 9. The principle of Gibson assembly cloning. Two linearized DNA fragments (PCR-amplified DNA inserts and linear vectors) were sharing the same overlapping ends (green and red) and the ratio between these two linearized DNA fragments is 3. Thereafter, the inserts and vectors were gently mixed with Gibson assembly master mix and then incubated at 50°C for 30 minutes. After that, 2µl of ligation mixture was transformed into DH5α competent *E. coli* cells.

3.2.4. Bacterial techniques

3.2.4.1 Bacterial culture

Escherichia coli (*E. coli*) cells were normally grown on lysogeny broth (LB) at 37 °C. For liquid LB, it composed of 1% bactotryptone (Conda, #1612), 0.5% yeast extract and 0.5% sodium chloride (NaCl, Melford, #S0520)(Bertani 2004). For solid LB plates, 2% bacto-agar was supplemented with above LB components and autoclaved together. For solid LB-ampicillin plates (LBA), 50 mg/ml of ampicillin (Melford, #A0104) was supplemented after LB-agar medium autoclaving.

3.2.4.2 Transformation of Competent Cells with Plasmid DNA

DH5α cell was one of the most frequently chemically competent *E.coli* strain for routine cloning applications and blue/white selection. In this study, DH5α competent *E.coli* cells were prepared by a chemical method that using calcium chloride to weaken the cells and induce competence of the cells.

For *E.coli* transformation, all traditional ligation mixture or 2µl of Gibson ligation mixture was supplemented in a freshly thawed aliquot of DH5α competent *E.coli* cells and incubated on ice for 30 minutes. Then, cells were heat-shocked for 40 seconds at 42°C and chilled on ice for 3 minutes to stop the shock. Followed by 1ml pre-warmed LB was added and incubated for 1 hour at 37°C to allow cells integrating the plasmid and to express ampicillin resistance

gene before plating. Thereafter, the cells were pelleted by centrifugation at 14000 RPM for 1 min, removed all but approximately 200µl of LB. Gently resuspended cell pellet and plated on solid LBA plates. In the end, the plates were incubated for overnight at 37°C, only transformed cells grown on LBA plates and generated colonies.

In this study, the vector plasmids were using *pRS* family that derived from pBluescripe and retained the *LacZ* gene. Thus, allowed us to utilize blue-white screening method upon the LBA plates were supplemented into X-Gal and IPTG. Once the insert DNA fragment was introduced into multiple cloning sites sequence of recipient plasmid, which caused alpha complementation coding sequence disrupt and inactivated the beta-galactosidase enzyme, resulting in white color colonies. Otherwise, non-inserted colonies appeared blue color.

3.2.4.3 Isolation of Plasmid DNA From *E. coli*

In this study, all plasmid DNA was purified using the QIAprep spin miniprep kit according to the manufacturer's instructions (QIAGEN, #27104).

A single colony was picked and inoculated into 4ml of LB which supplemented with 100µg/ml of ampicillin for overnight at 37°C. The day after, 1ml of the overnight culture was transferred into an eppendorf and cell pelleted by centrifugation at 13000 RPM for 1 minute and the supernatant was removed by aspiration. Then,

200µl buffer P1 was added and fully suspended the pellet by gently pipetting. Followed by 250µl buffer P2 was added into lysis, mixed gently by inverting the eppendorf 4-6 times and incubated for 5 minutes at room temperature. Thereafter, 350µl buffer N3 was added and mixed immediately and thoroughly.

A compact white pellet was accumulated by centrifugation at 13000 RPM for 10 minutes. The supernatant was carefully poured into the QIAprep 2.0 spin column and centrifuged at 13000 RPM for 1 minute. Then, spin column was washed once by 500µl buffer PB and 700µl buffer PE, respectively. Additional 3 minutes full speed centrifugation was required for the sake of removing residual completely. All flow-throughs were discarded during above processes.

Later placed the spin column in a clean and well-labeled eppendorf. 40µl of water was dropped in the middle of the column and incubated for 3minutes at room temperature. Finally, DNA plasmid was eluted by centrifugation at 13000 RPM for 3 minutes. The primary screening was checked by agarose gel electrophoresis, and further analysis was based on restriction endonuclease digestion or PCR amplification. The yields of DNA mini-preps were measured by nanodrop.

3.2.5. Frozen strain stocks

For frozen stocks of W303-1a genetic manipulated strains, the strains were initially inoculated with 5ml YPDA medium at 30°C for overnight. Then, a

1ml overnight culture was resuspended in 8ml YPDA medium to allow saturated strains recovering. Finally, 600µl of exponentially growing culture was mixed with 600µl of 60% glycerol (30% v/v) in an appropriate labeled cryogenic tube (Nunc, # 377267) and preserved aseptically at -80 °C.

For frozen stocks of plasmid-transformed *E. coli* strains, the strains were initially inoculated with a 5ml LB-Ampicillin medium at 37°C for overnight. Then, 600µl of the overnight culture was mixed with 600µl of 60% glycerol (30% v/v) in an appropriate labeled cryogenic tube and preserved aseptically at -80 °C.

3.3. Cell Biology and Microscopy

3.3.1. Basic Cell Cycle Experiment

A fresh single colony was inoculated in a 15ml of complete synthetic media (absence of methionine) supplemented with glucose (GLU-CSM), and incubated with orbital shaking for overnight at 25°C. The following morning, cell density was counted by using a Neubauer chamber through the light microscope. An early log phase culture ($5x10^6$ cells/ml) was required for the downstream time course experiment (Figure 10).

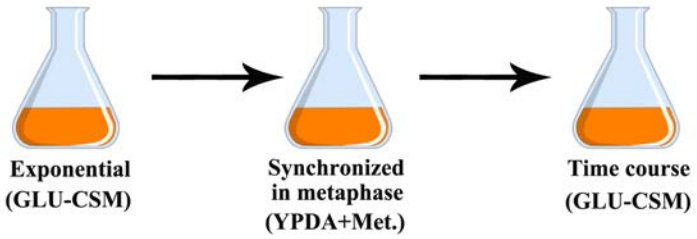


Figure 10. The Steps of Regular Time Course Experiment.

In order to synchronize cells in early metaphase, 2mM of methionine was added. After one doubling time, cells were checked and determined by a dumbbell- shaped index lower than 5%. If necessary, additional time was given. Once cells reached the optimal synchronization, 1ml of aliquot cell culture was taken for further analysis. The rest of culture was washed three times with methionine-free media to release the cells from the arrest. Aliquots were taken at the desired intervals during the desired extension of time.

3.3.2. Time course experiments with inducible over-expression

In order to conditionally over-express the gene of interest, the mutants were generated by carrying an ectopic, integrated copy of the gene of interest under the *Gal1,10* promoter (described at 3.1.6).

Cells were processed the same steps as described at 3.1.3 to synchronize cells in early metaphase. As described at 3.1.6, additional chimerical transcriptional activator was integrated into yeast genome, thus for full induction of *Gal1,10* promoter that required to add beta-estradiol in presence of glucose. After 30 minutes induction, cells were spun down and resuspended in methionine-free plus beta-estradiol media. Thereafter, cells were re-entered into cell cycle progression in presence of over-expressed protein of interest (Figure 11).

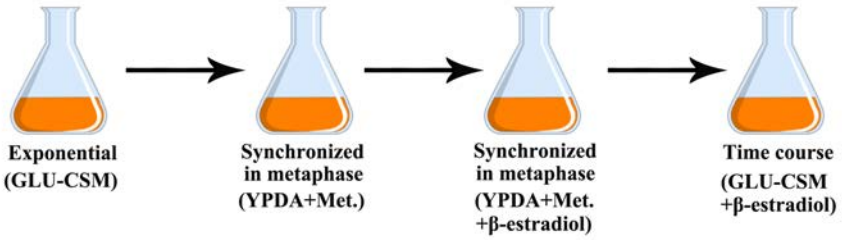


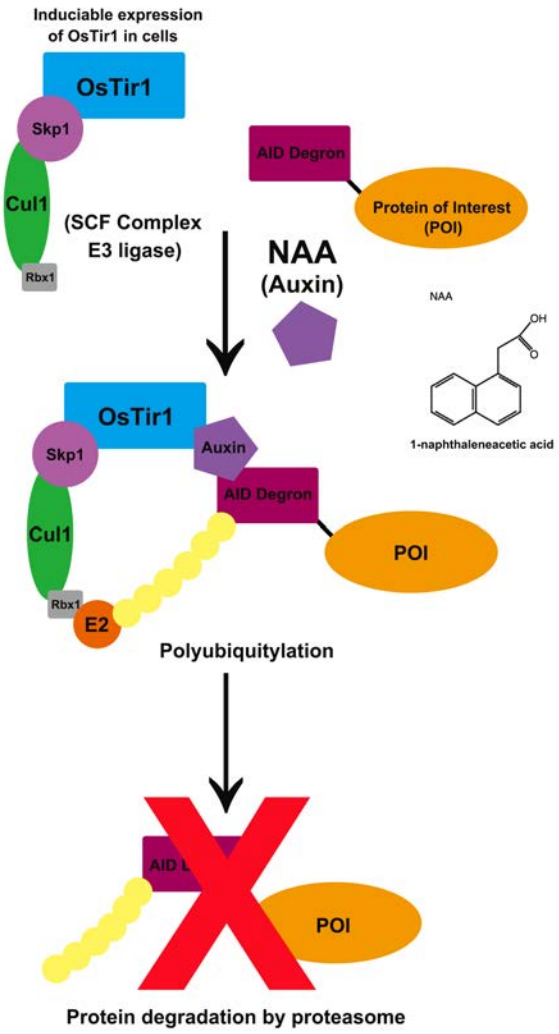
Figure 11. Steps of Time Course Experiment for Protein Overexpression.

3.3.3. Time course experiments with inducible protein degradation

In order to precisely dissect the cytokinesis process and characterize the role of each participating essential protein plays in the controlled progression of cell division. The auxin-induced degron (AID) technology allowed us to conditionally de-

plete the protein of interest at the exact point of cytokinesis where the protein was required. The mutants were generated by tagging PCR-based degron at the C-terminus of a protein of interest (described at 3.2.2.2). Moreover, a unique TIR1 F-box protein was required to integrate into the genome (Nishimura & Kanemaki 2014). Subsequently, TIR1 formed an E3 ubiquitin ligase SCFTIR1 complex to recognize degron fusion proteins in the presence of auxin, resulting in rapid polyubiquitylation and degradation of the protein of interest (Figure 12). In order to conditionally over-express the gene of interest, the mutants were generated by carrying an ectopic, integrated copy of the gene of interest under the *Gal1,10* promoter (described at 3.1.6).

Cells were processed the same steps as described at 3.1.3 to synchronize cells in early metaphase. As described at 3.1.6, additional chimerical transcriptional activator was integrated into yeast genome, thus for full induction of *Gal1,10* promoter that required to add beta-estradiol in presence of glucose. After 30 minutes induction, cells were spun down and re-suspended in methionine-free plus beta-estradiol media. Thereafter, cells were re-entered into cell cycle progression in presence of over-expressed protein of interest (Figure 13).



F

igure 12. The Principle of The AID System. OsTIR1 was amplified from *Oryza sativa* (rice) and subcloned under the control of a galactose inducible *Gal1,10* promoter for conditional expression. OsTIR1 associated with endogenous SCF components to form an E3 ubiquitin ligase SCF^{TIR1} complex. In the presence of auxin, SCF^{TIR1} complex poly-ubiquitinated the AID-tagged proteins and rapidly depleted proteins by the proteasome.

Cells were processed the same steps as described at 3.1.3 to synchronize cells in early metaphase. 3mM of auxin was supplemented and incubated with 60 minutes

to completely deplete the protein of interest. Thereafter, cells were spun down and resuspended in methionine-free plus auxin media, cells were blocked at the exact point of cytokinesis where the protein was required (Figure 13).

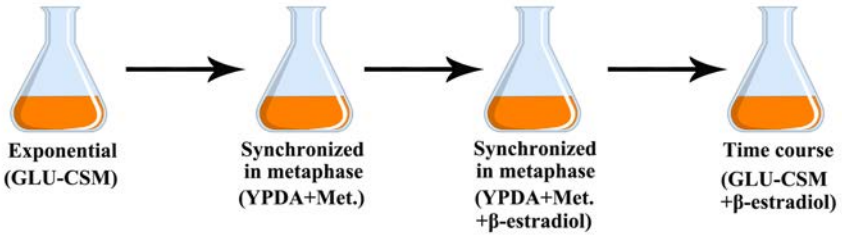


Figure 13. The Steps of Time Course Experiment for Protein Degradation.

3.3.4. Fluorescence microscopy and image analysis

In order to well understanding the subcellular localization of the fluorescently labeled proteins in living cells. The image acquisition, in this study, was performed in a wide-field microscope (Leica AF 600) with an Andor DU-885K-CSO-#VP camera. Image acquisition settings were depended on the protein being imaged and image of 4.5um stacks spaced 0.3um apart were acquired.

For the time course experiments, synchronous cells were released in complete synthetic media and 5ul of cell culture was placed on a microscope slide at each time point and avoiding cells drying, image acquisition was completed in 20 minutes and at least 100 individual cells were imaged. For image visualization, the open-source image analysis software Fiji was employed.

3.4. Biochemical Techniques

3.4.1. Preparation of yeast protein extracts

Yeast protein extracts were prepared using the TCA (Trichloroacetic acid) method. Generally, the TCA was one of the most common protein precipitation agents that were widely used for extracts of total proteins in budding yeast studies. Furthermore, TCA was as an inorganic salt denaturant that usually used to completely inactive proteins and precipitates them from the lysates. In this study, the TCA method was combined with glass beads beating, which could tolerate thoroughly cracking cell walls and highly enhance the performance for protein extracts.

1ml cell culture was collected by centrifugation at 14000 RPM for 1 minute and washed once by ice-cold water in order to completely remove the media. Cell pellets were re-suspended in 200µl 20% (v/v) TCA solution and vortexed harshly with 200µl of glass beads (0.5 mm diameter) for 90 seconds under maximum speed to thoroughly crack the cell walls and precipitate all proteins. Subsequently, lysates were transferred into a new eppendorf. The remains were continued to re-suspend in 200µl of 5% (v/v) TCA solution twice and lysates were gathered in the same eppendorf.

The lysates were spun down by centrifugation at 14000 RPM for 10 minutes. The cleared supernatant was carefully removed and the white protein pellet was re-suspended in 1x Laemmli sample buffer (50 mM Tris-HCl pH 6.8, 8% (v/v) glycerol, 4% (v/v) 2-mercaptoethanol, 1.6% (w/v) SDS and 0.008% (w/v) bromo-phenol blue). Thereafter, 0.2 volumes of 1 M Tris-base were added in order to neutralize the pH value of the TCA residual remains. Before loading the soluble supernatants onto the regular SDS-PAGE gel electrophoresis, the samples were required boiling at 95°C for 5 minutes to denature proteins and then centrifuged at 3000RPM for 10 minutes at room temperature to isolate insoluble material.

3.4.2 SDS-Polyacrylamide gel electrophoresis

The denaturing polyacrylamide gel electrophoresis (PAGE) is a classic technique that could provide different types of information about the proteins and allow separating proteins based on their electrophoretic mobility. However, adding additional anionic detergent sodium dodecyl sulfate (SDS) in protein samples, which denatures and binds to the protein to make them evenly negatively charged. Thus, SDS-PAGE separates proteins according to their molecular mass, all SDS-bound proteins migrate to the positive charged when a current is applied, and the proteins with less mass move faster than those with larger mass.

In this study, we therefore selected 1.5mm thick gel and casted 7.5%, 10% or 12% (v/v) of resolving gels, but ratio between acrylamide and bis-acrylamide was al-

ways constant (37.5:1, BioRad) in 370mM Tris pH8.8, 0.1% (w/v) SDS. Moreover, in order to obtain optimal resolution of proteins, a stacking gel was cast on top of resolving gel with 4% of acrylamide in 125mM Tris pH6.8, 0.1% (w/v) SDS.

SDS-bound protein samples were allowed migrating in cast SDS-PAGE with 1x running buffer (25 mM Tris-Glycine pH 8.3, 0.1% (w/v) SDS) in electrophoresis tanks from BioRad. To monitor the position of the proteins in the gel and subsequently on the membrane, a broad range pre-stained protein marker (Fermentas) was used. Furthermore, for analyzing different proteins of interest, the conditions of % acrylamide, voltage and running time are the difference.

- For analysis of two forms of phosphorylated Pol12 (86 and 91 kDa), 7.5% acrylamide, 50 minutes at 120 V followed by 62 minutes 195 V.
- For the analysis of Clb2, 10% acrylamide, 15 minutes of stacking at 90 V and 60 minutes of 200 V.
- For the analysis of Sic1, 10% acrylamide, 15 minutes of stacking at 90 V and 60 minutes of 200 V.
- For the analysis of Gal-Clb2ΔN-13myc, 7.5 % acrylamide, 25 minutes of stacking at 90 V and 60 minutes of 200 V.
- For the analysis of Ask1-6HA, 7.5 % acrylamide, 25 minutes of stacking at 90 V and 60 minutes of 200 V.
- For the analysis of Iqg1-6HA, 7.5 % acrylamide, 25 minutes of stacking at 90 V and 60 minutes of 200 V.

3.4.3. Western blotting

Separated proteins were transferred onto pre-equilibrated nitrocellulose membrane (Protean BA85) by applying semi-dry electrotransfer apparatus (Panther Semi-dry Electroboltter, Owl Scientific). Transfer buffer contained 50mM Tris-Glycine pH9.1, 0.0373% (w/v) SDS and 20% (v/v) methanol and entire semi-dry transfer was carried out at 0.11mA/cm^2 for 1 hour. The efficiency of transfer was confirmed by Ponceau S stain, which contained 0.2%(w/v) Ponceau S and 3%(w/v) TCA. Subsequently, scanned stained membrane to be used as loading control and erased the Ponceau S stain by three times wash with TBST (100 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween-20).

The membrane was incubated with a 5% skimmed milk solution in TBST for 30 minutes at room temperature. The membrane was then incubated with the diluted primary antibody for 1 hour at room temperature. The primary antibodies concentrations were as following:

- Anti-Pol12 (Mouse monoclonal 6D2) diluted 1:2000 in TBST+ 1% milk.
- Anti-HA (Mouse monoclonal supernatant 12CA5) diluted 1:100 in TBST without milk.
- Anti-Sic1 (Rabbit polyclonal, Santacruz FL-284) diluted 1:1000 in TBST+5% milk.
- Anti-Clb2 (Rabbit IgG, Santacruz y-180), diluted 1:2000 in TBST+1% milk.
- Anti-Myc (Mouse monoclonal, Roche 11 667 149 001) diluted 1:1000 in TBST +5% milk.

After incubation with the primary antibody, the membrane was washed in an excess of TBST three times for 15 minutes. Afterward, horseradish peroxidase (HRP) coupled secondary antibody was incubated with the membrane in TBST containing 1-5% milk for additional 35 minutes at room temperature. The secondary antibodies concentrations were as following:

- α-mouse IgG-HRP (rabbit polyclonal, Dako P0161) diluted 1:3,000 in TBST + 1% milk
- α-rabbit IgG-HRP (goat polyclonal, Dako P0448) diluted 1:2,000 in TBST without milk.

After incubation with the secondary antibody, the membrane was washed a further three times and incubated with enhanced chemiluminescence (ECL) reagent (GE) for 5 minutes according to the manufacturer's instructions. After that, the membrane was exposed to sensitive photographic film (Super RX Film, Fujifilm).

4. RESULTS

Control of Cytokinesis by the Mitotic Cyclin Dependent Kinase M-Cdk1

As stated in the introduction, mitotic Cdk1 activity blocks premature cytokinesis. To identify the essential M-Cdk1 substrates in the control of cytokinesis, we hypothesized that a strain carrying non-phosphorylatable alleles of the critical M-Cdk1 substrates should override the M-Cdk1 control, and therefore cells should undergo cytokinesis in the presence of high M-Cdk1.

4.1. Setting up the system: Blocked cytokinesis by inducible, stable, high M-Cdk1 activity

We first set up the background strain, to confirm that high M-Cdk1 activity did indeed block cytokinesis (Surana et al. 1993; Ghiara et al. 1991) in our hands.

Because cells eventually override the over-expression of wild type mitotic cyclins, we created a strain that integrated a galactose-inducible, ectopic copy of the hyper-stable mitotic cyclin CLB2ΔN allele. We then checked whether over-expression of CLB2ΔN affects cell proliferation. As shown in Figure 14, whereas it grew normally in YPD medium, the GAL-CLB2ΔN strain failed to form colonies in YPGal medium. Compatible with a block in cytokinesis.

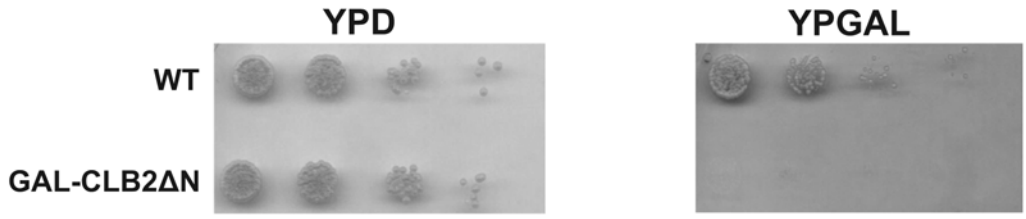


Figure 14. GAL-CLB2ΔN cells fail to proliferate in Galactose medium. Ten-fold serial dilutions of control parental cells and GAL-CLB2ΔN cells on a YPD and YPGal plates. Plates were incubated at 25ºC for 2 days.

We therefore next asked whether the arrest indeed occurs in cytokinesis and after successful karyokinesis. For that, we monitored karyokinesis (nuclei visualized with mCherry-HTB2), plasma membrane abscission (plasma membrane visualized with GFP-SPO20), presence / contraction / disassembly of the actomyosin ring (AMR visualized with mCherry-MYO1), and DNA content (propidium iodide flow cytometry).

MET3-CDC20 ADGEV GAL-CLB2ΔN HTB2-mCherry cells, and isogenic control cells lacking the GAL-CLB2ΔN gene, were grown to mid-exponential phase. Methionine was then added to synchronize cells at metaphase by depletion of Cdc20. β-estradiol was subsequently added to induce the over-expression of CLB2ΔN. Finally, methionine was washed out to allow for Cdc20 expression and the activation of the APC^{Cdc20}, allowing cells to progress through mitosis.

As shown in Figure 15, both control and cells over-expressing Clb2ΔN were successfully arrested in metaphase, as evidenced by the dumbbell morphology, single nucleus by the bud-neck, and 2N DNA content. Upon induction, Clb2ΔN effectively accumulates in the experimental strain, as evidenced by western blot against Clb2. The accumulation of Clb2ΔN does not affect karyokinesis, as control cells divide with a normal nucleus and experimental cells show two separated nuclei. However, whereas the control cells divide (single unbudded cells, drop in budding index, DNA content from 2N to 1N), accumulation of Clb2ΔN in the experimental cells block cytokinesis, as cells remain with a dumbbell morphology and a 2N DNA content.

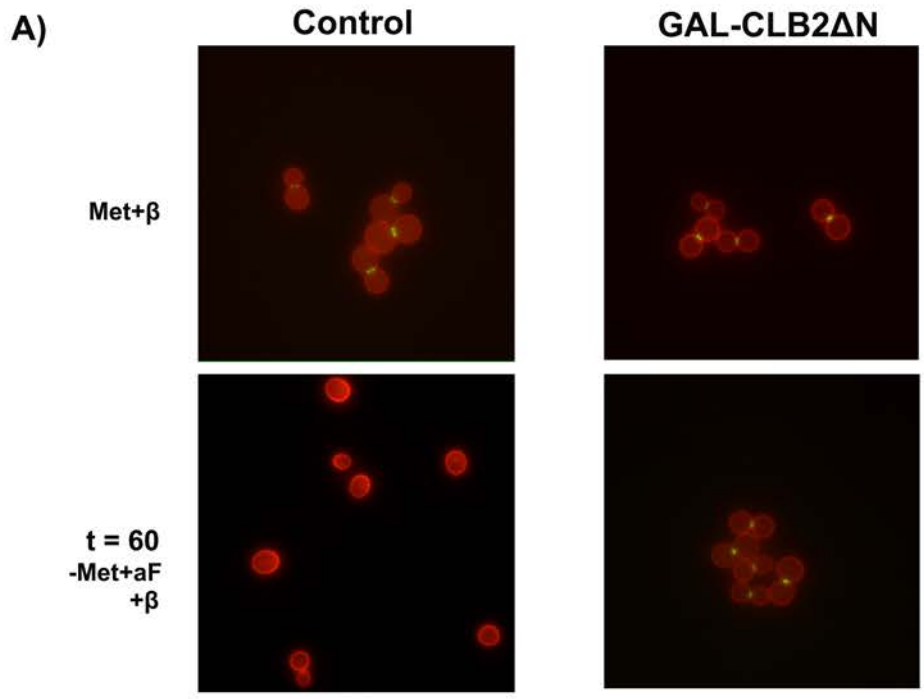


Figure 15. Cells expressing GAL-CLB2ΔN arrest in telophase. Cells were synchronized in metaphase, β-estradiol was then added to allow the expression of GAL-CLB2ΔN, and then cells were allowed to resume cell cycle progression (t=0). Alpha-factor was added to trap the divided cells in G1. (A) Live cell fluorescence microscopy imaging. (B-D) See next page.

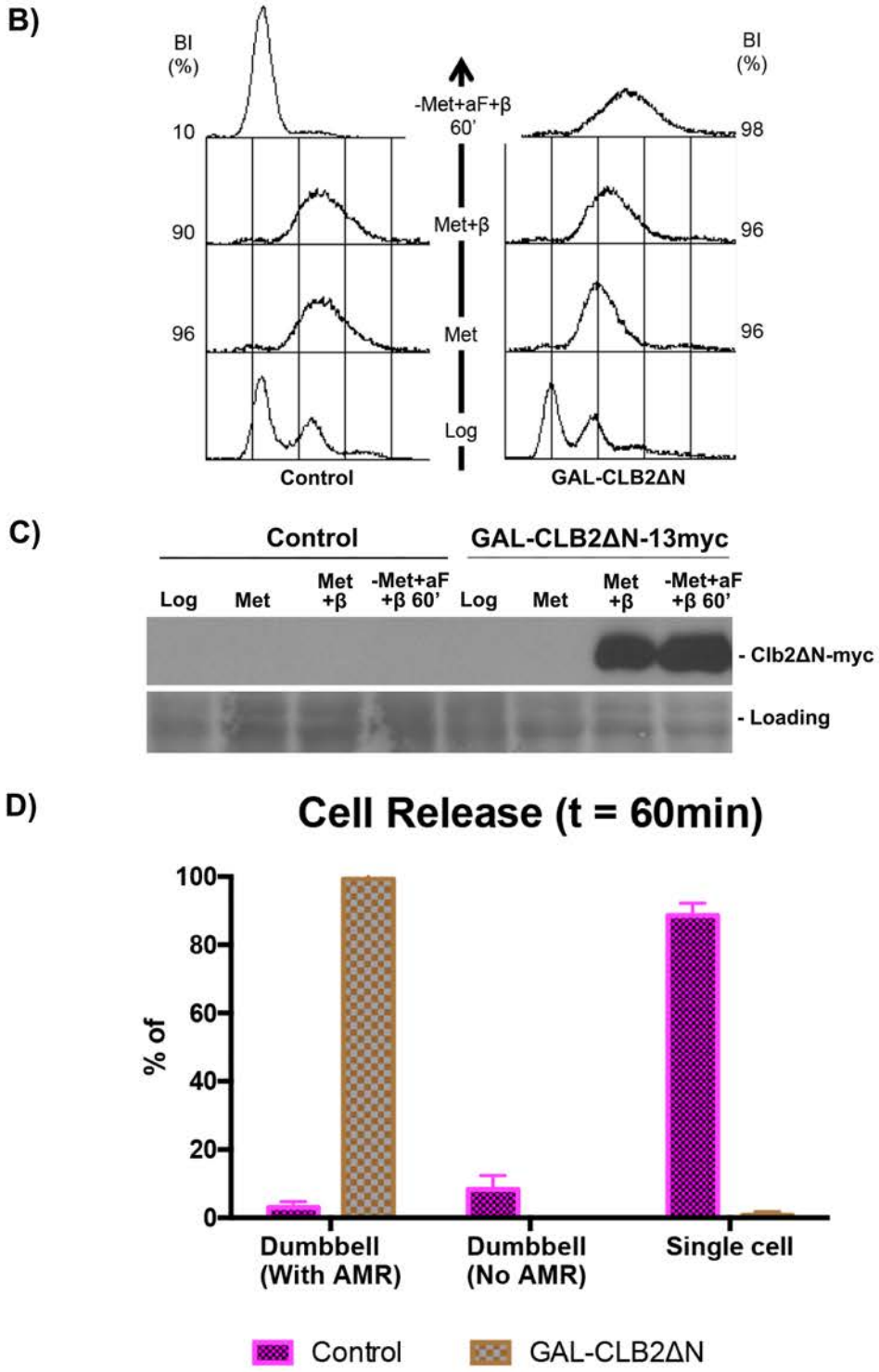
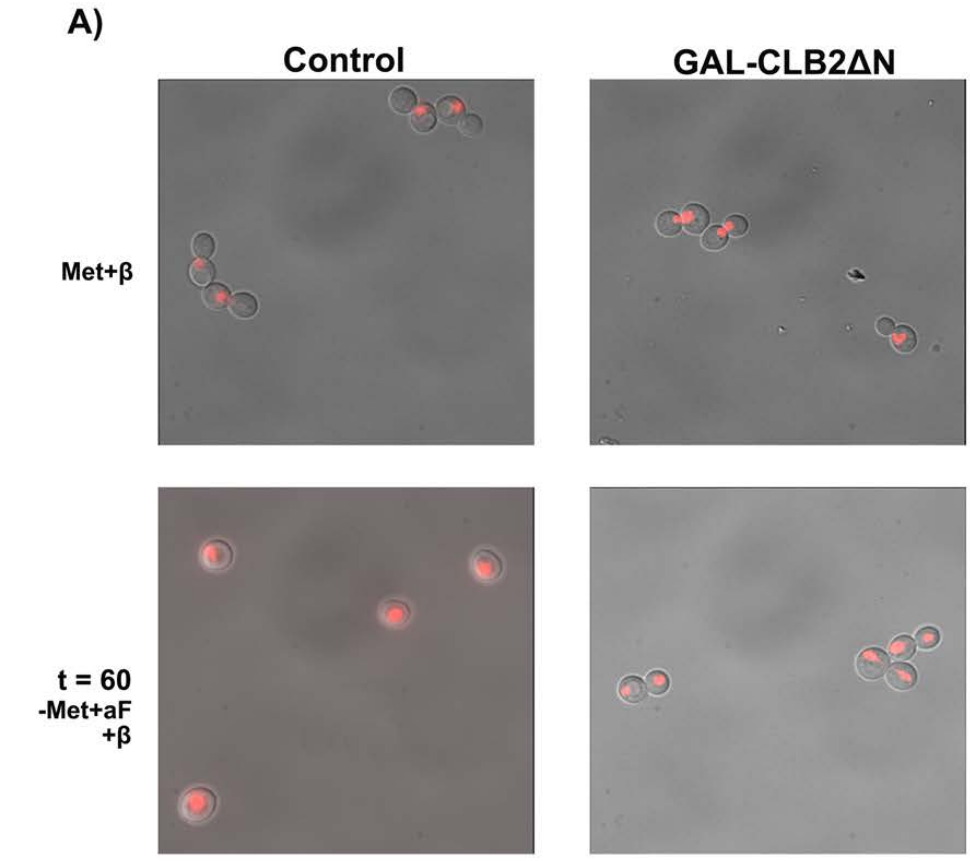


Figure 15 (continued). (B) DNA content analysis as monitored by flow cytometry of propidium iodide stained cells. Budding indexes are included for the shown time-points. (C) Western blot on whole cell extracts. A strip of the membrane, stained with Ponceau S, is shown as loading control. (D) Statistics of morphology and nuclear status. Bars are mean ± standard deviation (n = 3).

The same experiment was next carried out to monitor cytokinesis based on plasma membrane and AMR images. MET3-CDC20 ADGEV GAL-CLB2ΔN MYO1-mCherry GFP-SPO20 cells, and isogenic control cells lacking the GAL-CLB2ΔN gene, were grown to mid-exponential phase. Methionine was then added to synchronize cells at metaphase by depletion of Cdc20. β-estradiol was subsequently added to induce the over-expression of CLB2ΔN. Finally, methionine was washed out to allow for Cdc20 expression and the activation of the APC^{Cdc20}, allowing cells to progress through mitosis. For all selected time-points budding indexes were counted and DNA content was analyzed by means of propidium iodide flow cytometry. AMR and plasma membranes were visualized by means of fluorescence microscopy on live cells, and the number of dumbbell cells with or without an AMR, and unbudded cells, were counted.

As shown in Figure 16, control cells go through cytokinesis normally, and 60 minutes after release from the metaphase arrest 60% of the cells becoming unbudded (trapped in G1 with alpha-factor). Cell division is further confirmed by DNA cytometry going from 2N to a mixture of 2N and 1N. However, cells where CLB2ΔN accumulation was induced (1) maintain a dumbbell morphology, (2) with a single cytoplasm, (3) presence of AMR, and (4) remain with a 2N DNA content.



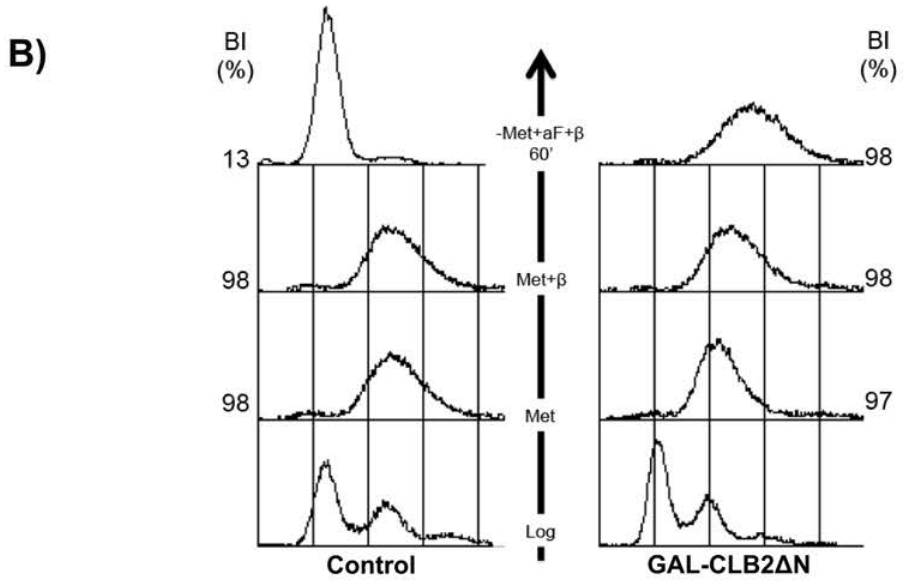


Figure 16. Cells expressing GAL-CLB2ΔN fail to undergo cytokinesis. Cells were treated as described in Figure 15. (A) Live cell fluorescence microscopy imaging. (B) DNA content analysis as monitored by flow cytometry of propidium iodide stained cells. Budding indexes are included for the indicated time-points. (C) See next page.

C)

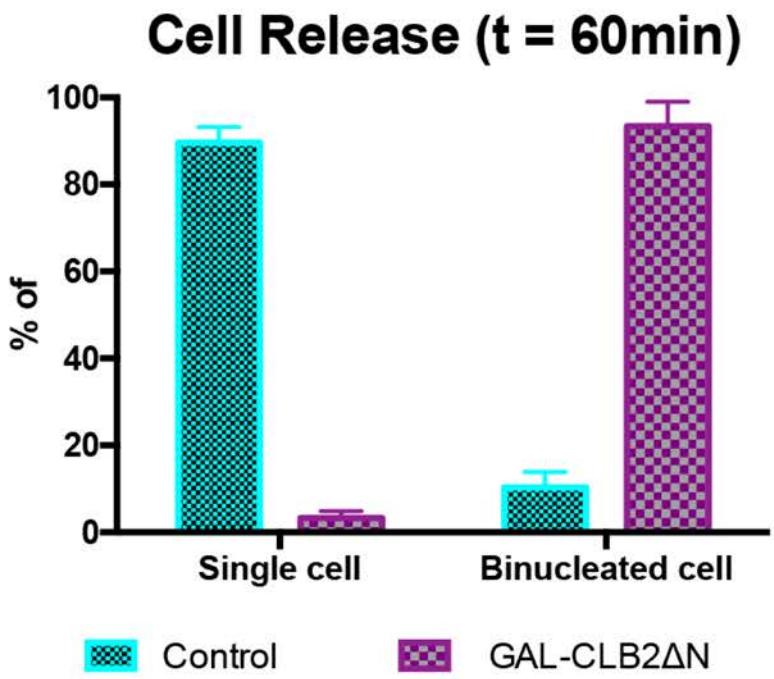


Figure 16. (continued). (C) Statistics of morphology and nuclear status. Bars are mean ± standard deviation (n = 3).

In all, our experimental approach works to block cytokinesis and therefore can be used to investigate a bypass of the M-Cdk1 regulation of cytokinesis.

4.2. A Mitotic Exit Network (MEN) M-Cdk1-bypass

Different transducers of the MEN pathway are inhibited by M-Cdk1 (see introduction). In brief, the MEN kinase Cdc15 is inhibited by M-Cdk1 phosphorylation. Cdc15 activation is required for Mob1-Dbf2 activation. In addition, both Mob1 and Dbf2 are also inhibited by M-Cdk1 phosphorylation. Mob1-Dbf2 activity is required to phosphorylate Cdc14 at its nuclear localization signal, thereby releasing Cdc14 to the cytoplasm. The cytoplasmic localization of Cdc14 correlates with the onset, and is likely to be the trigger, of cytokinesis.

We hypothesized that bypassing M-Cdk1 control on Cdc15, Mob1 and Dbf2 might result in the cytoplasmic release of Cdc14 in the presence of high M-Cdk1 activity, and that such release alone might prevail over M-Cdk1 and trigger cytokinesis.

We therefore generated Cdc15, Mob1 and Dbf2 alleles that cannot be phosphorylated by Cdk1, by replacing Serine or Threonine amino acid residues in SP/TP consensus Cdk1 phosphorylation sites with the non-phosphorylatable amino acid Alanine. Thus, the seven SP/TP sites in Mob1 (Ser10, Ser36, Thr41, Ser80, Thr85, Thr105, Ser180) and the 7 SP/TP sites in Dbf2 (Thr26, Ser51, Ser53, Ser83, Ser378, Thr413, Thr493) were mutated. The alleles are named Mob1-7A and Dbf2-7A. For Cdc15 we used a previously published Cdc15-7A allele (Jaspersen

& Morgan 2000). All the alleles were confirmed by DNA sequencing.

We next generated strains carrying the non-phosphorylatable alleles as sole version of the respective genes. Of note, the mutations were viable and morphologically identical to the wild-type strain (see Figures in this section).

4.2.1. Mob1-Dbf2 M-Cdk1-bypass

We first examined whether the MEN downstream kinase Mob1-Dbf2, responsible for the cytoplasmic release of Cdc14, constitutes the critical M-Cdk1 control point to block cytokinesis. MET3-CDC20 ADGEV GAL-CLB2ΔN MOB1-7A DBF2-7A GFP-SPO20 MYO1-mCherry cells, and isogenic control cells carrying the wild-type versions of the two MEN genes, were grown to mid-exponential phase. Methionine was then added to synchronize cells at metaphase by depletion of Cdc20. β-estradiol was subsequently added to induce the over-expression of CLB2ΔN. Finally, methionine was washed out to allow for Cdc20 expression and the activation of the APC^{Cdc20}, allowing cells to progress through mitosis. For all selected time-points cell density and budding indexes were counted. DNA content was analyzed by means of propidium iodide flow cytometry. AMR and plasma membranes were visualized by means of fluorescence microscopy on live cells.

As shown in Figure 17, the Mob1-Dbf2 double M-Cdk1-bypass strain remains

blocked prior to cytokinesis identically as the wild type control overexpressing Clb2ΔN. (1) Cell density does not increase, (2) the population remains in a dumbbell morphology, (3) with a 2N DNA content, (4) a visible AMR, and (5) no plasma membrane ingression.

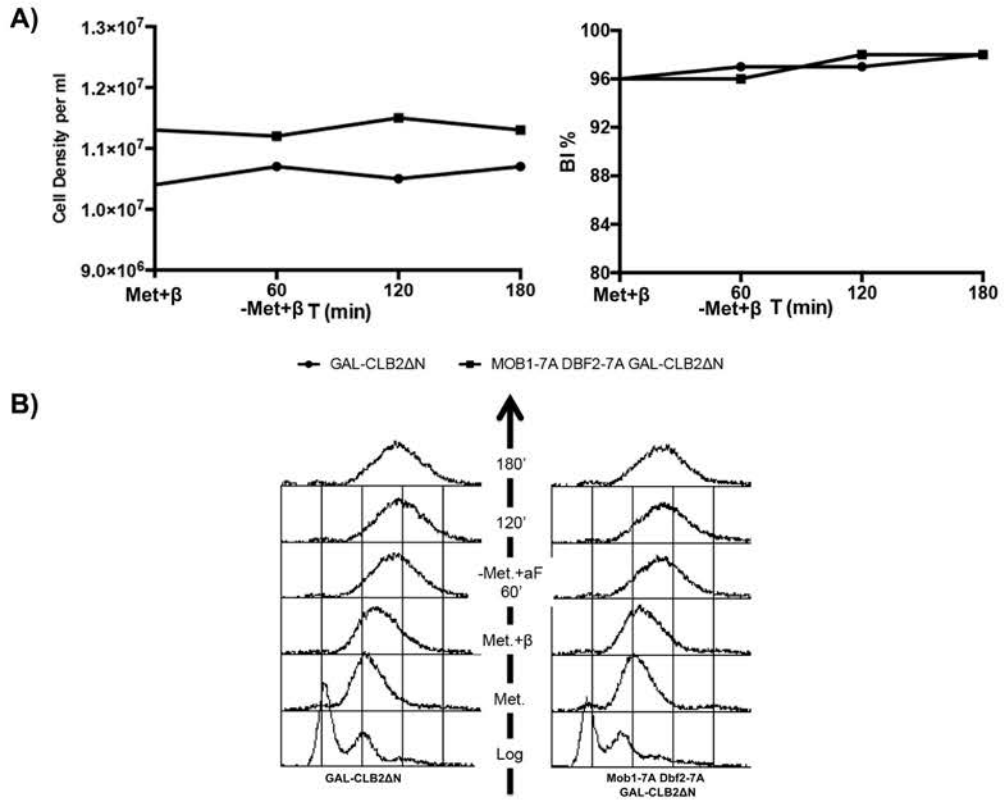


Figure 17. The MOB1-7A DBF2-7A M-Cdk1-bypass mutant does not undergo cytokinesis in the presence of high, stable levels of M-Cdk1 activity. Cells were synchronized in metaphase (MET3-CDC20), then expression of GAL-CLB2ΔN was induced with β-estradiol, and finally cells were allowed to resume cell cycle progression by addition of Methionine (t=0). (A) Cell densities and budding indexes. (B) DNA content analysis as monitored by flow cytometry of propidium iodide stained cells. (C) See next page.

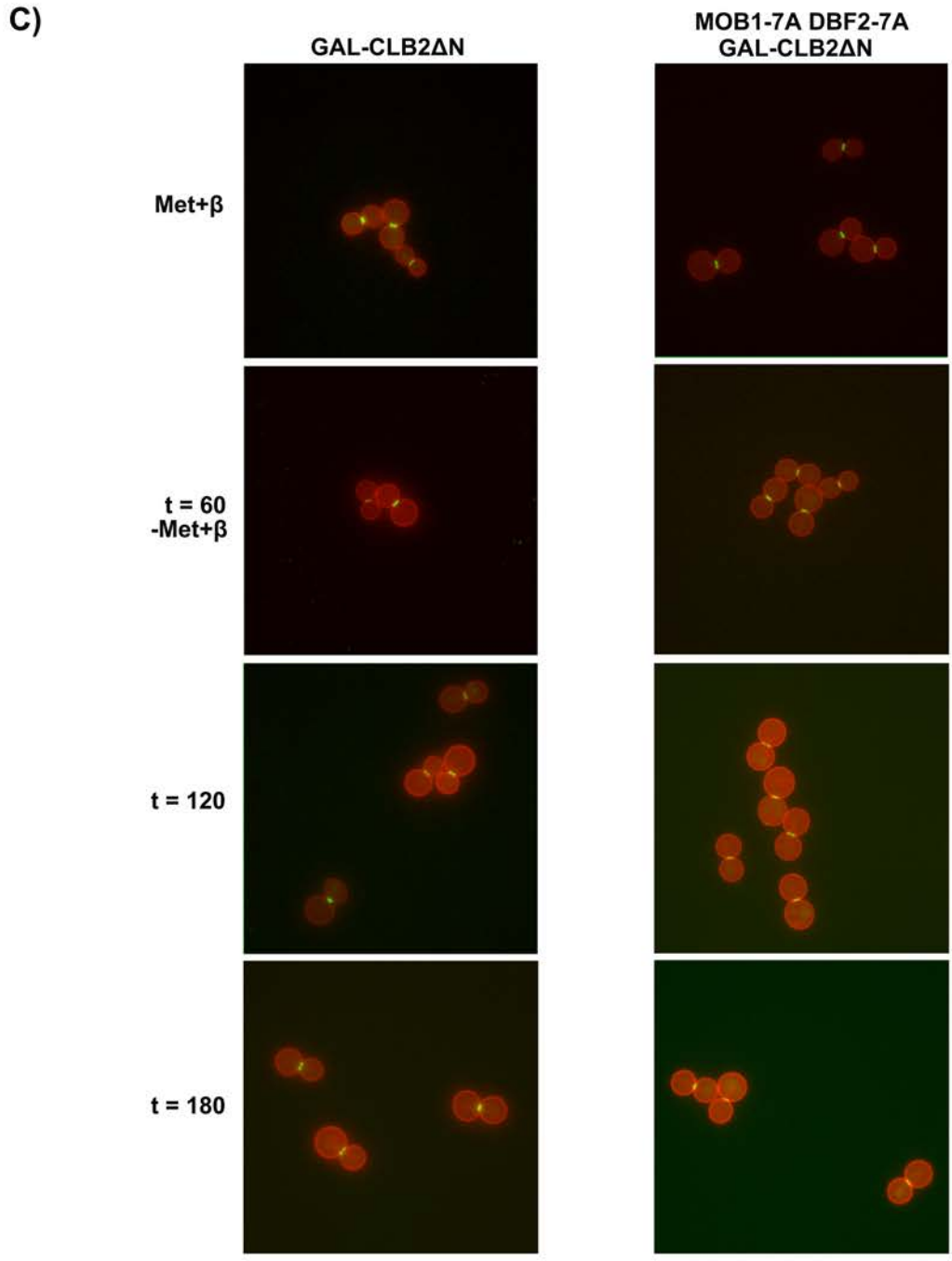


Figure 17 (continued). (C) Live cell fluorescence microscopy imaging.

4.2.2. Cdc15 Mob1-Dbf2 Cdk1-bypass

Because Mob1-Dbf2 activation requires upstream Cdc15 activation, and given that Cdc15 is also under the negative control of M-Cdk1, we next explored whether a

triple Cdc15, Mob1, Dbf2, M-Cdk1-bypass strain would be sufficient to allow cytokinesis despite the presence of high, stable levels of M-Cdk1 activity. MET3-CDC20 ADGEV GAL-CLB2ΔN CDC15-7A MOB1-7A DBF2-7A GFP-SPO20 MYO1-mCherry cells, and isogenic control cells carrying the wild-type versions of the three MEN genes, were grown to mid-exponential phase. Methionine was then added to synchronize cells at metaphase by depletion of Cdc20. β-estradiol was subsequently added to induce the over-expression of CLB2ΔN. Finally, methionine was washed out to allow for Cdc20 expression and the activation of the APC^{Cdc20}, allowing cells to progress through mitosis. For all selected time-points cell density and budding indexes were counted. DNA content was analyzed by means of propidium iodide flow cytometry. AMR and plasma membranes were visualized by means of fluorescence microscopy on live cells.

Again, the triple mutant arrests prior to cytokinesis identically as the wild type control overexpressing Clb2ΔN. As shown in Figure 18, (1) cell density does not increase, (2) the population remains in a dumbbell morphology, (3) with a 2N DNA content, (4) a visible AMR, and (5) no plasma membrane ingression.

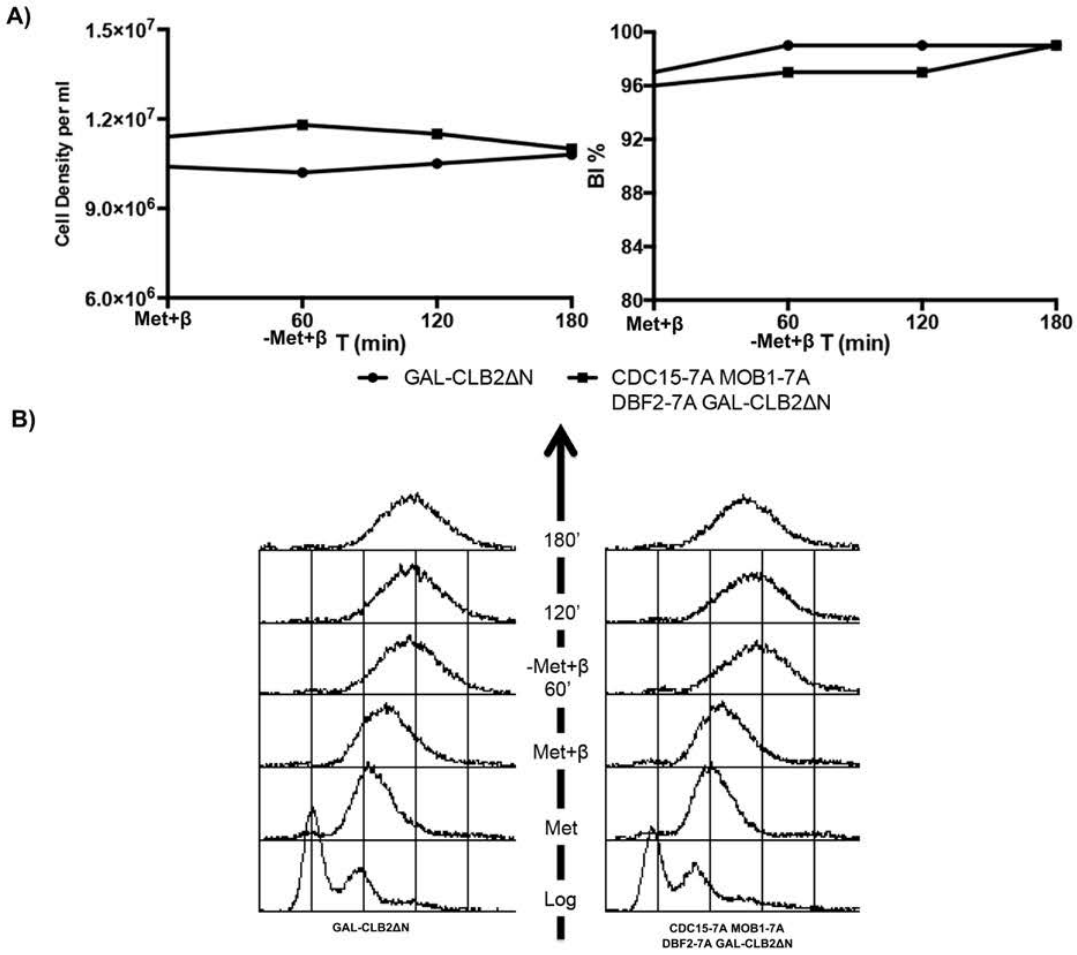


Figure 18. The CDC15-7A MOB1-7A DBF2-7A M-Cdk1-bypass mutant does not undergo cytokinesis in the presence of high, stable levels of M-Cdk1 activity. Cells were synchronized in metaphase (MET3-CDC20), then expression of GAL-CLB2ΔN was induced with -estradiol, and finally cells were allowed to resume cell cycle progression by addition of Methionine (t=0). (A) Cell densities and budding indexes. (B) DNA content analysis as monitored by flow cytometry of propidium iodide stained cells. (C) See next page.

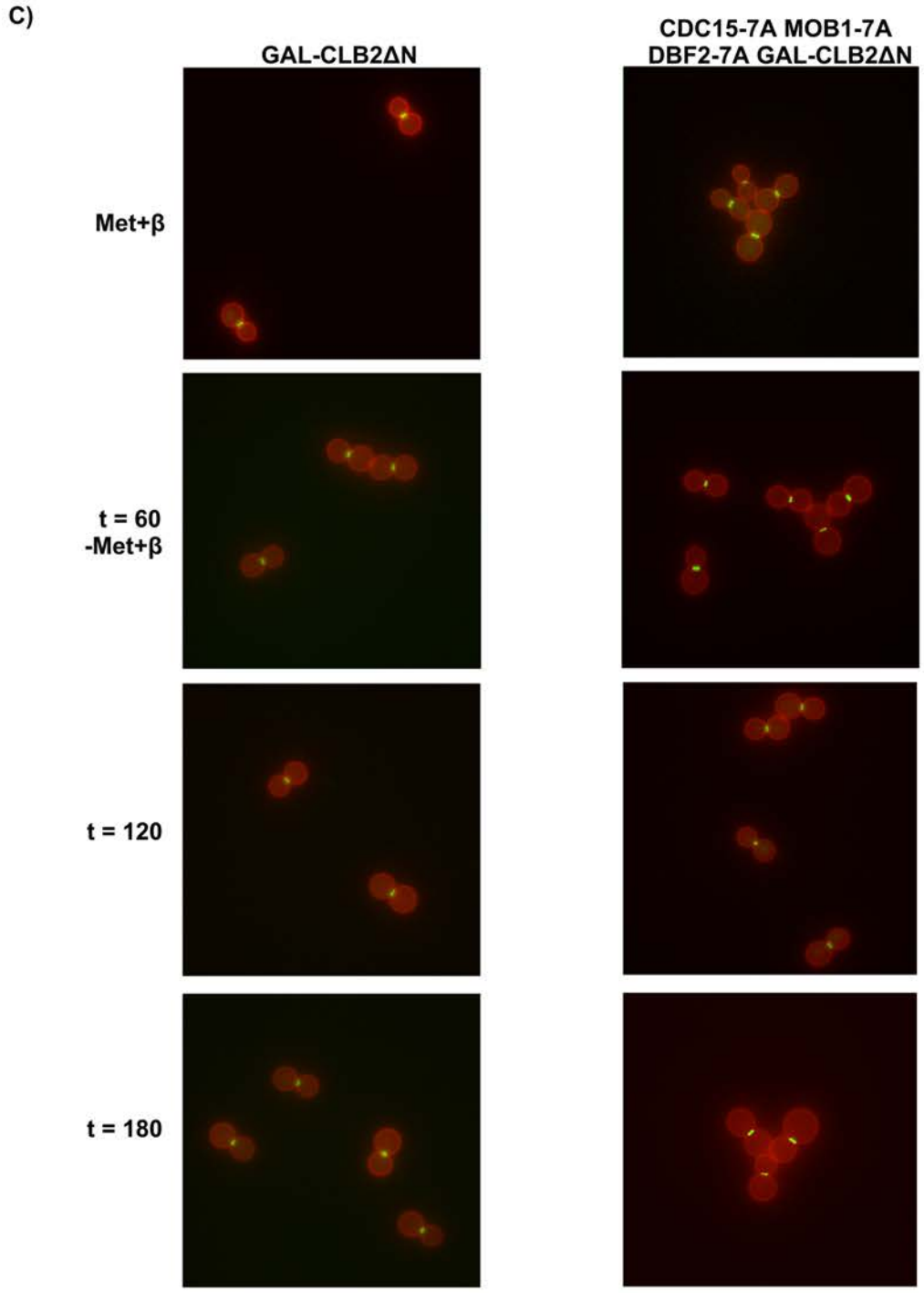


Figure 18 (continued). (C) Live cell fluorescence microscopy imaging.

4.2.3. Cdc15 Mob1-Dbf2 Dbf20 Cdk1-bypass

We next hypothesized that the presence of wild type DBF20 might work as a

dominant negative lock in the presence of M-Cdk1 activity. As explained in the introduction, DBF20 and DBF2 are paralog genes. They have overlapping functions based on two observations: (1) Despite each is individually dispensable for viability, the single mutants have a dumbbell phenotype (delay in mitotic exit); (2) the double mutant is lethal. Therefore, it cannot be ruled out that, depending on their relative affinities for Mob1, Dbf20 phosphorylated by Cdk1 might take over the non-phosphorylatable Dbf2 allele, thus keeping Mob1 kinase activity off.

To test such hypothesis, a MET3-CDC20 ADGEV GAL-CLB2ΔN CDC15-7A MOB1-7A DBF2-7A DBF20-1A GFP-SPO20 MYO1-mCherry strain was generated and tested for entry into cytokinesis in the presence of stable M-Cdk1 levels. The experimental strain and a control strain carrying the wild-type versions of the four MEN genes were grown to mid-exponential phase. Methionine was then added to synchronize cells at metaphase by depletion of Cdc20. β-estradiol was subsequently added to induce the over-expression of CLB2ΔN. Finally, methionine was washed out to allow for Cdc20 expression and the activation of the APC^{Cdc20}, allowing cells to progress through mitosis. For all selected time-points cell density and budding indexes were counted. DNA content was analyzed by means of propidium iodide flow cytometry. AMR and plasma membranes were visualized by means of fluorescence microscopy on live cells.

Yet again, the MEN M-Cdk1-bypass strain arrests prior to cytokinesis identi-

cally as the wild type control overexpressing Clb2ΔN. As shown in Figure 19, (1) cell density does not increase, (2) the population remains in a dumbbell morphology, (3) with a 2N DNA content, (4) a visible AMR, and (5) no plasma membrane ingression.

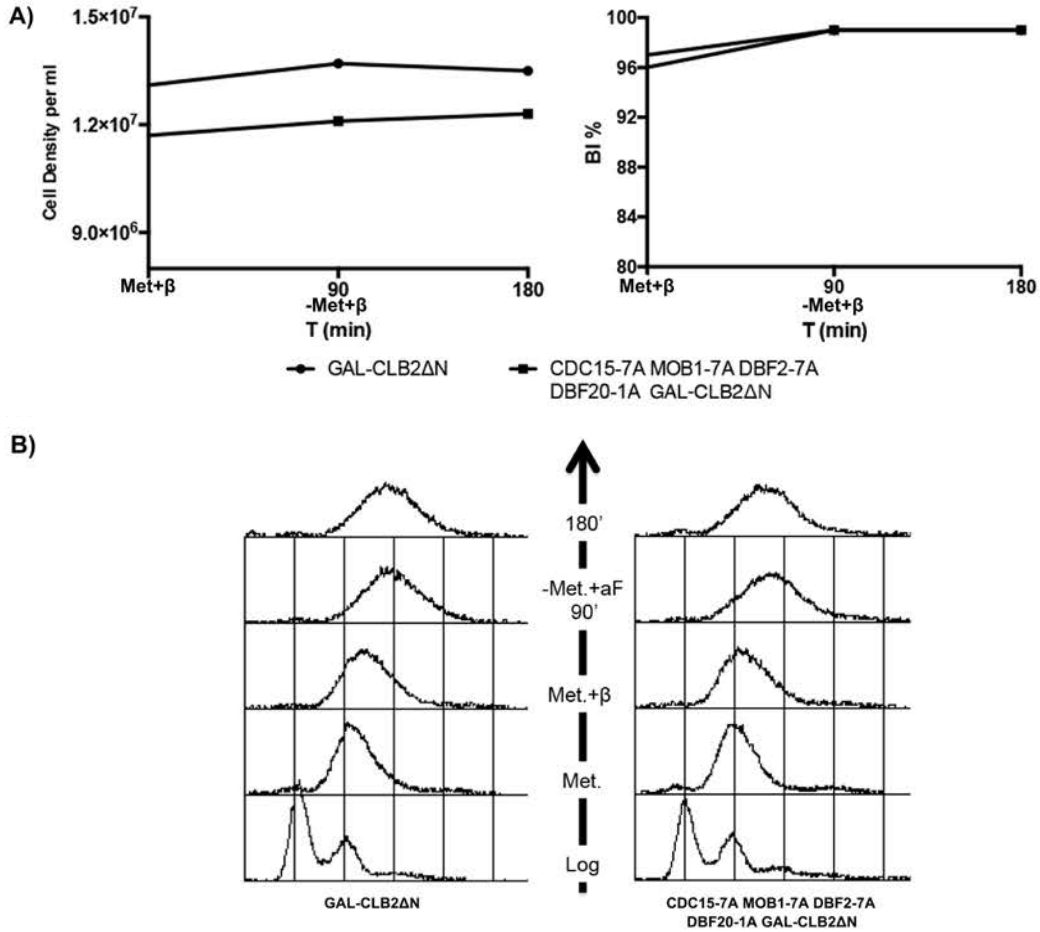


Figure 19. The CDC15-7A MOB1-7A DBF2-7A Dbf20-1A M-Cdk1-bypass mutant does not undergo cytokinesis in the presence of high, stable levels of M-Cdk1 activity. Cells were synchronized in metaphase (MET3-CDC20), then expression of GAL-CLB2ΔN was induced with β-estradiol, and finally cells were allowed to resume cell cycle progression by addition of Methionine (t=0). (A) Cell densities and budding indexes. (B) DNA content analysis as monitored by flow cytometry of propidium iodide stained cells (C) See next page.

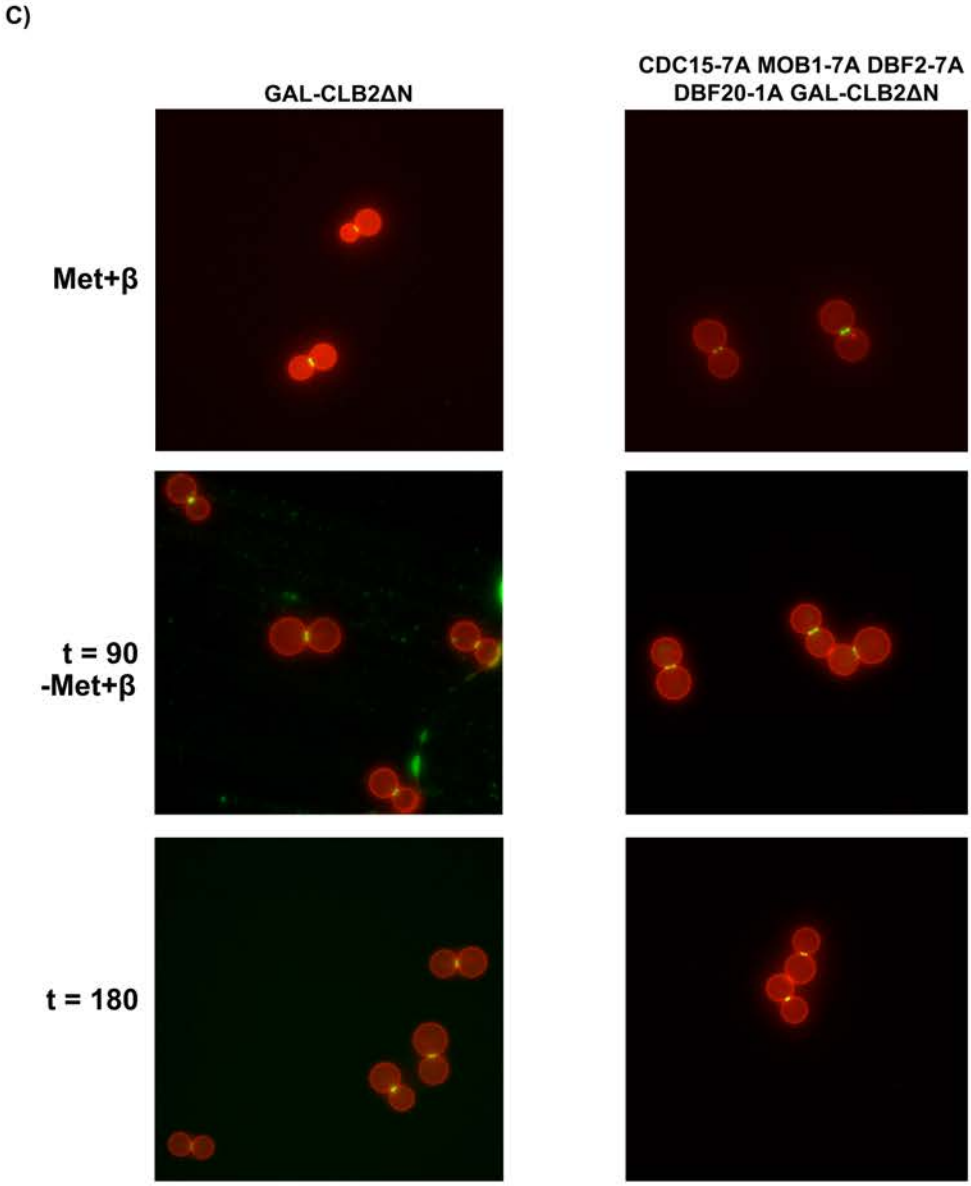


Figure 19 (continued). (C) Live cell fluorescence microscopy imaging.

In summary, a Mitotic Exit Network (MEN) Cdk1-bypass, involving non-phosphorylatable alleles of Cdc15, Dbf2, Dbf20 and Mob1, fails to enter cytokinesis in the presence of stable M-Cdk1 levels.

4.3. Cytoplasmic Cdc14 under stable, high M-Cdk1

Different explanations are possible for the lack of cytokinesis in the MEN Cdk1-bypass. For instance, Cdc14 might not be released to the cytoplasm ("active Cdc14") despite the MEN alleles cannot be inhibited by Cdk1. Alternatively, Cdc14 might be released but unable to prevail over Cdk1 on common substrates whose dephosphorylation is essential for cytokinesis, such as, perhaps, components of the Ingression Progression Complex.

To distinguish between the two options, the subcellular localization of Cdc14 was investigated. We first compared experimental strain MET3-CDC20 ADGEV GAL-CLB2ΔN CDC15-7A MOB1-7A DBF2-7A with a control strain carrying the wild-type version of the three MEN genes. The strains were doubly labelled to visualize Cdc14 (Cdc14-GFP) and nuclei (HTB2-mCherry) by means of fluorescence microscopy on live cells. Cells were grown to mid-exponential phase and then synchronized in metaphase by depletion of Cdc20 (MET3-CDC20). Clb2ΔN over-expression was then induced by the addition of β-estradiol. At this point, Cdc14 was moonlighting in the nucleolus (Figure 20). Subsequently, methionine was washed out to allow for the re-expression of Cdc20, activation of the APC^{Cdc20}, and progression through mitosis. As seen in Figure 20C, in agreement with the previous experiments shown above, in both cases cells arrest in telophase, dumbbell shaped, bi-nucleated, and with a 2N DNA content. Cdc14 localization is found all

over the cell, indicating cytosolic release. However, such release does not depend on the MEN M-Cdk1-bypass but on the presence of high M-Cdk1 activity, as unexpectedly it occurs in the control strain as well.

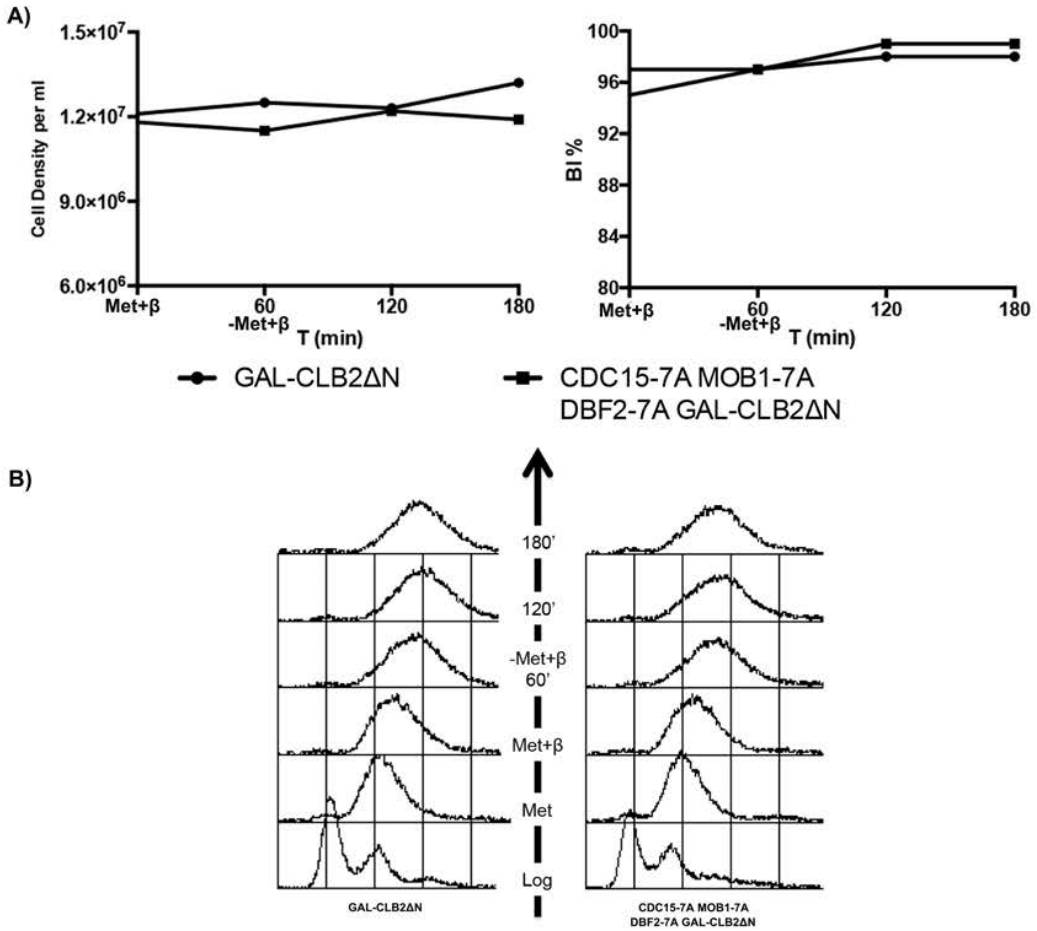


Figure 20. CDC14 subcellular localization in a MEN M-Cdk1-bypass strain under stable, high M-Cdk1. Cells were synchronized in metaphase (MET3-CDC20), then expression of GAL-CLB2ΔN was induced with β-estradiol, and finally cells were allowed to resume cell cycle progression by addition of Methionine (t=0). (A) Cell densities and budding indexes. (B) DNA content analysis as monitored by flow cytometry of propidium iodide stained cells. (C) See next page.

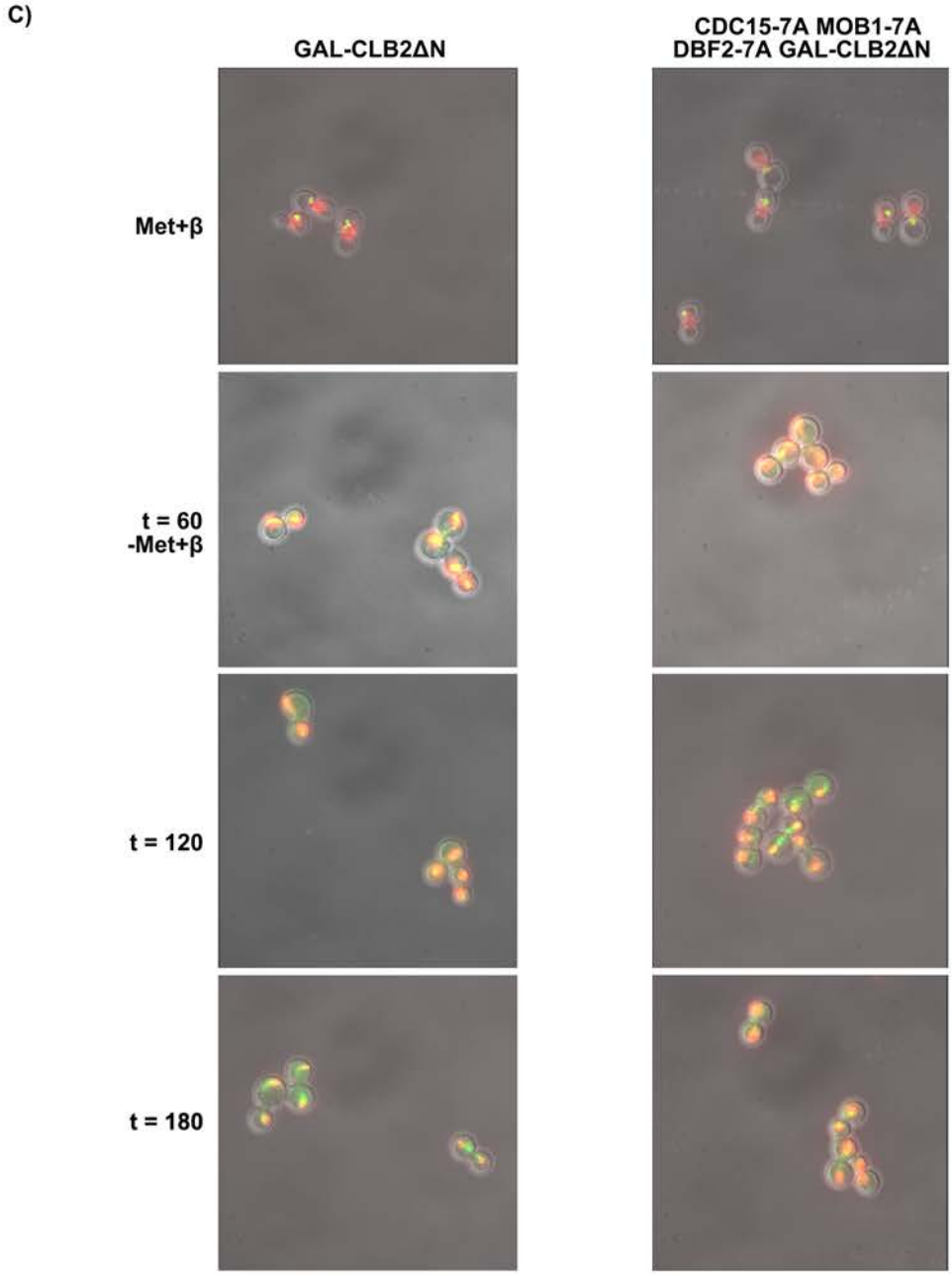


Figure 20 (continued). (C) Live cell fluorescence microscopy imaging.

One such observation is, in principle, in contradiction with the reported inhibition of the MEN pathway by M-Cdk1 (König et al. 2010), which keeps Cdc14 restricted to the nucleus. To further check our observation, we next studied the

Cdc14 subcellular localization in the ADGEV MET3-CDC20 CDC14-GFP backbone strain, comparing two isogenic strains with GAL-CLB2ΔN as the only variable. Thus, cells under exponential growth were synchronized in metaphase by depletion of Cdc20 (MET3-CDC20). Clb2ΔN over-expression was then induced by the addition of β-estradiol. At this point, Cdc14 localized to the nucleolus in both strains (Figure 21A). Subsequently, methionine was washed out to allow for the re-expression of Cdc20, the activation of the APC^{Cdc20}, and progression through mitosis. Alpha-factor was added to trap the divided cells in G1. For all selected time-points cell density and budding indexes were counted. DNA content was analyzed by means of propidium iodide flow cytometry. Nuclei and Cdc14 localization were visualized by means of fluorescence microscopy on live cells.

As shown in Figures 21B and C, control cells eventually divided and Cdc14 returned to a nucleolar localization. However, cells over-expressing Clb2ΔN arrested in telophase with a dumbbell morphology, two nuclei, a 2N DNA content, and cytosolic Cdc14. Therefore, the cytosolic release of Cdc14 occurs as a result of high M-Cdk1 activity, either as a cause or only because it blocks cells differently than MEN mutants. In any case, the Cdc14 localization suggests that the MEN kinase Dbf2-Mob1, which is responsible for the cytoplasmic localization of Cdc14, may be unexpectedly active during the high M-Cdk1 arrest.

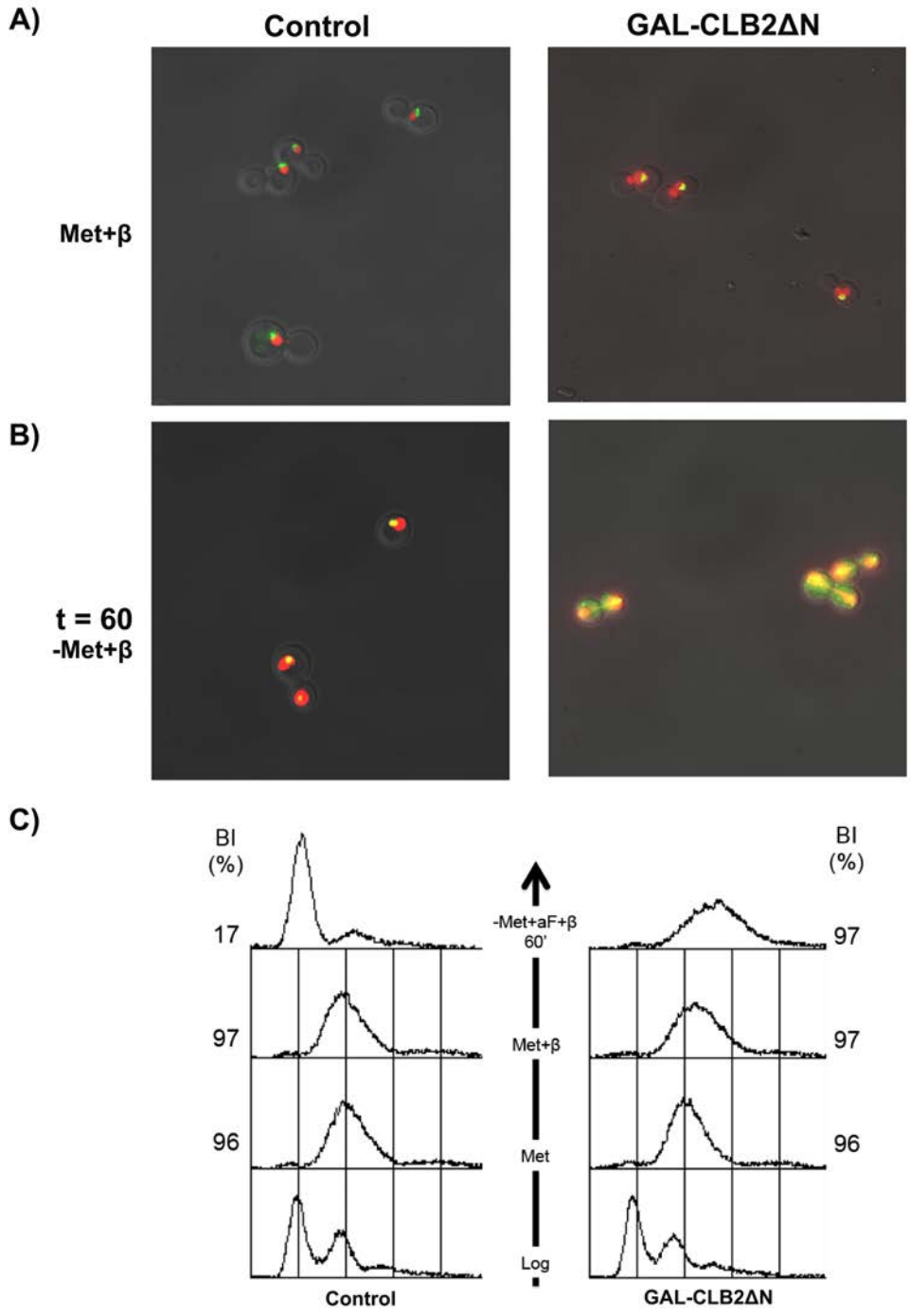


Figure 21. CDC14 subcellular localization under stable, high M-Cdk1. Cells were synchronized in metaphase (MET3-CDC20), then expression of GAL-CLB2ΔN was induced with β-estradiol, and finally cells were allowed to resume cell cycle progression by addition of Methionine (t=0). Alpha-factor was added to trap the divided cells in G1. (A) Live cell fluorescence microscopy imaging of cells at t = 0. (B) Live cell fluorescence microscopy imaging of cells at t = 60 min from the metaphase release. (C) DNA content analysis as monitored by flow cytometry of propidium iodide stained cells. Budding indexes are included for the shown time-points.

4.4. M-Cdk1 activity prevails over released Cdc14 activity

The release of Cdc14 under high M-Cdk1 described in 4.1.3 above contradicts previous reports indicating that M-Cdk1 activity keeps MEN inactive and therefore Cdc14 in the nucleus (König et al. 2010). In addition, released Cdc14 is unable to trigger cytokinesis under these experimental conditions. We next wished to explore which was the balance between M-Cdk1 and Cdc14 activities by examining different common substrates.

4.4.1. Stability of endogenous Clb2 levels

Mitotic cyclins serve as markers to monitor the balance between M-Cdk1 and Cdc14 activities. When the later prevails over the former, APC^{Cdh1} activates and completely eliminates Clb1 and Clb2. We therefore examined the levels of endogenous Clb2 in our experimental conditions. The prediction is, only if Cdc14 prevails over M-Cdk1, Clb2 should be eliminated in cells under stable, high M-Cdk1.

MET3-CDC20 ADGEV GAL-CLB2ΔN and control cells lacking the GAL-CLB2ΔN gene were grown to mid-exponential phase. Cells were then synchronized in metaphase by depletion of Cdc20 (MET3-CDC20). β-estradiol was

then added to both cultures, to induce Clb2ΔN over-expression in the experimental strain. Subsequently, methionine was washed out to allow for the re-expression of Cdc20, the activation of the APC^{Cdc20}, and progression through mitosis. Alpha-factor was added to trap the divided cells in G1. As shown in Figure 22C, Clb2 accumulates at the metaphase arrest in both strains. However, Clb2 levels drops by 60 minutes upon the metaphase release in the control strain. By then cells are dividing, as assessed by the increase in cell density, the decrease in the budding index (Figure 22A), and also the 1N DNA content (Figure 22B). In contrast, the strain that over-expresses Clb2ΔN shows stable levels of endogenous Clb2, suggesting that APC^{Cdh1} remains inactive, which is suggestive of M-Cdk1 activity prevailing over the released Cdc14.

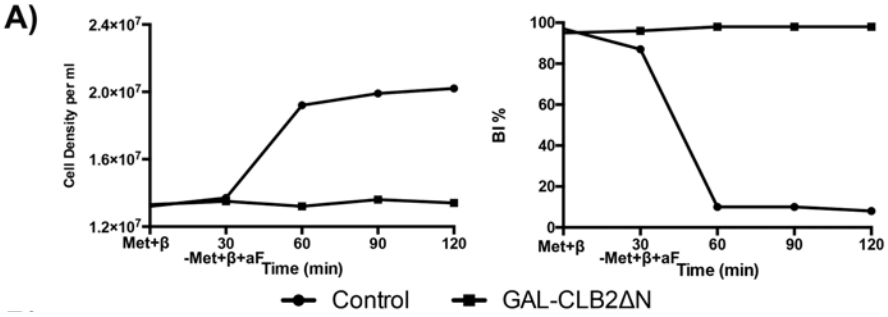


Figure 25. Stable levels of endogenous Clb2 under stable, high M-Cdk1. Cells were synchronized in metaphase (MET3-CDC20), then expression of GAL-CLB2ΔN was induced with β-estradiol, and finally cells were allowed to resume cell cycle progression by addition of Methionine (t=0). Alpha-factor was added to trap the divided cells in G1. (A) Cell densities and budding indexes. (B and C) See next page.

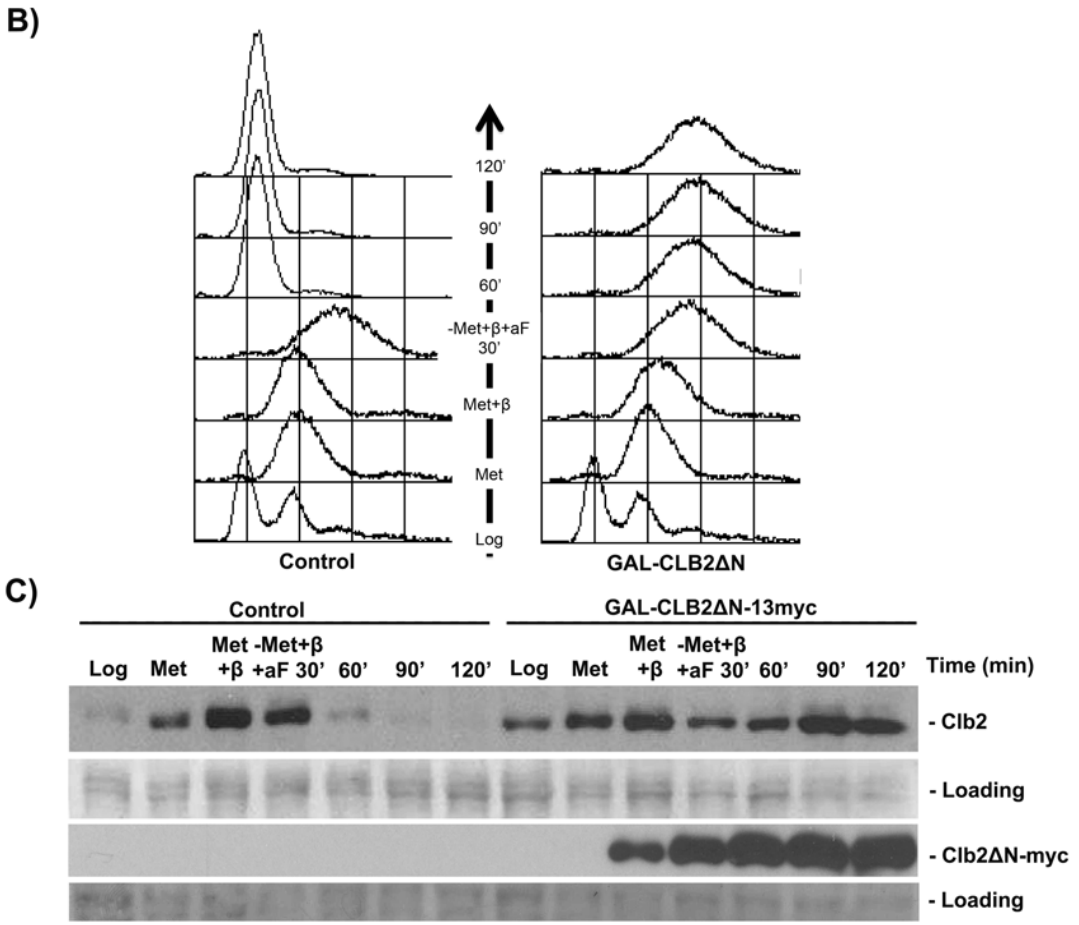


Figure 22 (continued). (B) DNA content analysis as monitored by flow cytometry of propidium iodide stained cells. Budding indexes are included for the shown time-points. (C) Western blot on whole cell extracts. The anti-Clb2 antibody recognizes the 19 N-terminal amino acids and therefore detects only the endogenous cyclin but not Clb2ΔN. The hyperstable form is fused at the C-terminus with a 13myc tag and is detected with anti-myc antibodies. A Ponceau S stained strip of the respective membranes is shown as loading control.

4.4.2. Sic1 abundance

Sic1 accumulates upon mitotic exit as a result of dephosphorylation of the transcription factor Swi5 by Cdc14 (Visintin et al. 1998). In addition, Cdk1 activity phosphorylates Sic1 targeting it for destruction via the SCF^{Cdc4} ubiquitin ligase. To further test the balance between M-Cdk1 and Cdc14 we analyzed the levels of Sic1 in

our experimental system. The prediction is, only if Cdc14 prevails over M-Cdk1, Sic1 should accumulate in cells under stable, high M-Cdk1.

MET3-CDC20 ADGEV GAL-CLB2ΔN and control cells lacking the GAL-CLB2ΔN gene were grown to mid-exponential phase. Cells were then synchronized in metaphase by depletion of Cdc20 (MET3-CDC20). β-estradiol was then added to both cultures, to induce Clb2ΔN over-expression in the experimental strain. Subsequently, methionine was washed out to allow for the re-expression of Cdc20, the activation of the APC^{Cdc20}, and progression through mitosis. Alpha-factor was added to trap the divided cells in G1. As shown in Figure 23, Sic1 accumulates in the control cells 60 minutes upon release, the time where cell division occurs. The presence of alpha-factor, that blocks divided cells in G1, keeps Sic1 stable for the rest of the experiment. However, Sic1 fails to accumulate in cells that over-express Clb2ΔN, supporting that under these experimental conditions M-Cdk1 prevails over Cdc14.

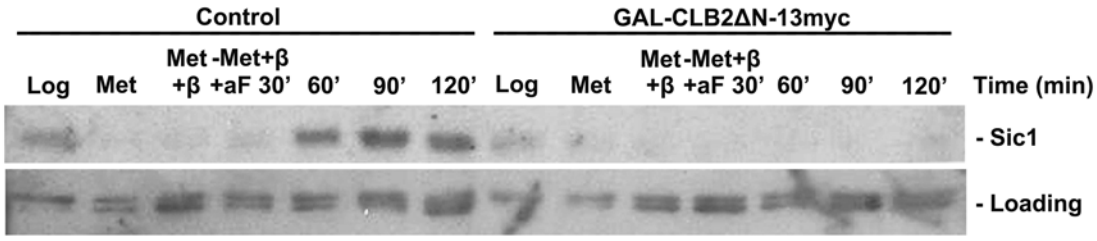


Figure 23. Sic1 fails to accumulate under stable, high M-Cdk1. Cells were synchronized in metaphase (MET3-CDC20), then expression of GAL-CLB2ΔN was induced with β-estradiol, and finally cells were allowed to resume cell cycle progression by addition of Methionine (t=0). Alpha-factor was added to trap the divided cells in G1. Whole cell extracts were im-

munoblotted with specific anti-Sic1 antibodies. The bottom panel shows an immunoblot cross-reactive band as control of equal loading.

4.4.3. M-Cdk1-dependent phosphorylation of Pol12

The B subunit of DNA polymerase alpha – primase (Pol12) is a *bona fide* substrate for M-Cdk1 and Cdc14 an excellent marker to monitor the activity balance between the kinase and the phosphatase. The prediction is, only if Cdc14 prevails over M-Cdk1, Pol12 should revert to the hypo-phosphorylated form in cells under stable, high M-Cdk1.

MET3-CDC20 ADGEV GAL-CLB2ΔN and control cells lacking the GAL-CLB2ΔN gene were grown to mid-exponential phase. Cells were then synchronized in metaphase by depletion of Cdc20 (MET3-CDC20). β-estradiol was then added to both cultures, to induce Clb2ΔN over-expression in the experimental strain. Subsequently, methionine was washed out to allow for the re-expression of Cdc20, the activation of the APC^{Cdc20}, and progression through mitosis. Alpha-factor was added to trap the divided cells in G1. As shown in Figure 24, Pol12 dephosphorylates by 30-60 minutes upon release in the control cells, in good correlation with the timing of Cdc14 release. The presence of alpha-factor, that blocks divided cells in G1, keeps Pol12 in the low phosphorylation form for the rest of the experiment. However, Pol12 remains phosphorylated throughout the experiment in cells that over-express Clb2ΔN, supporting that under these experimental condi-

tions M-Cdk1 prevails over Cdc14.

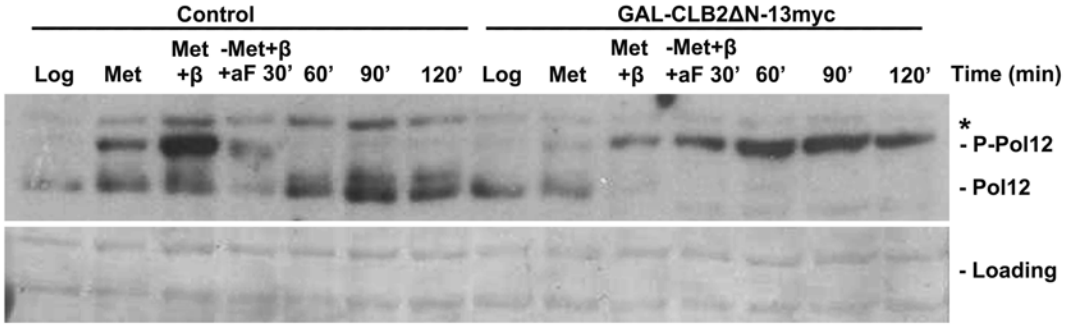


Figure 24. Pol12 remains phosphorylated under stable, high M-Cdk1. Cells were synchronized in metaphase (MET3-CDC20), then expression of GAL-CLB2ΔN was induced with β-estradiol, and finally cells were allowed to resume cell cycle progression by addition of Methionine (t=0). Alpha-factor was added to trap the divided cells in G1. Whole cell extracts were immunoblotted with specific anti-Pol12 antibodies. A Ponceau S stained strip of the membrane is shown as loading control.

4.4.4. Ask1 phosphorylation and Cdc20 levels

Ask1 is another *bona fide* substrate for M-Cdk1 and Cdc14. We chose to monitor Ask1 phosphorylation because it is expectably more sensitive to changes in the M-Cdk1/Cdc14 balance than Pol12. Ask1 phosphorylation is reversed by the FEAR-released Cdc14 at early anaphase, in the presence of relatively high Cdk1 activity (Chin et al. 2012). Therefore, the prediction is that if Cdc14 prevails over M-Cdk1, Ask1 should revert to the hypo-phosphorylated form in cells under stable, high M-Cdk1.

In the same experiment, we also monitored the levels of Cdc20. Cdc20 is de-

stroyed by APC^{Cdh1} by the times cells undergo mitotic exit. In turn, full APC^{Cdh1} activity depends on Cdc14. Therefore, the prediction is that only if Cdc14 prevails over M-Cdk1, Cdc20 should be eliminated in cells under stable, high M-Cdk1.

MET3-CDC20 ADGEV GAL-CLB2ΔN ASK1-HA and control cells lacking the GAL-CLB2ΔN gene were grown to mid-exponential phase. Cells were then synchronized in metaphase by depletion of Cdc20 (MET3-CDC20). β-estradiol was then added to both cultures, to induce Clb2ΔN over-expression in the experimental strain. Subsequently, methionine was washed out to allow for the re-expression of Cdc20, the activation of the APC^{Cdc20}, and progression through mitosis. Alpha-factor was added to trap the divided cells in G1. As shown in Figure 25, in control cells Ask1 electrophoretical mobility increases 30 minutes upon release from the metaphase arrest. At the same time, Cdc20, expressed from the MET3 promoter, accumulates. As cells progress through the cell cycle, Ask1 remains in its underphosphorylated form and Cdc20 is eliminated, indicating activation of APC^{Cdh1}, both observations in agreement with Cdc14 prevailing over M-Cdk1. In sharp contrast, in cells over-expressing Clb2ΔN, Cdc20 levels remain stable throughout the time of the experiment, suggesting that M-Cdk1 activity prevails over Cdc14 and keeps Cdh1 hyperphosphorylated and APC^{Cdh1} inactive. The case is more complex for Ask1. Despite presumably high M-Cdk1 activity, the electrophoretical mobility of Ask1 increases by 30 min upon metaphase release, a time

compatible with FEAR dependent release of Cdc14. However, the mobility decreases to an intermediate degree at later time points, indicating that under these experimental conditions M-Cdk1 prevails over Cdc14.

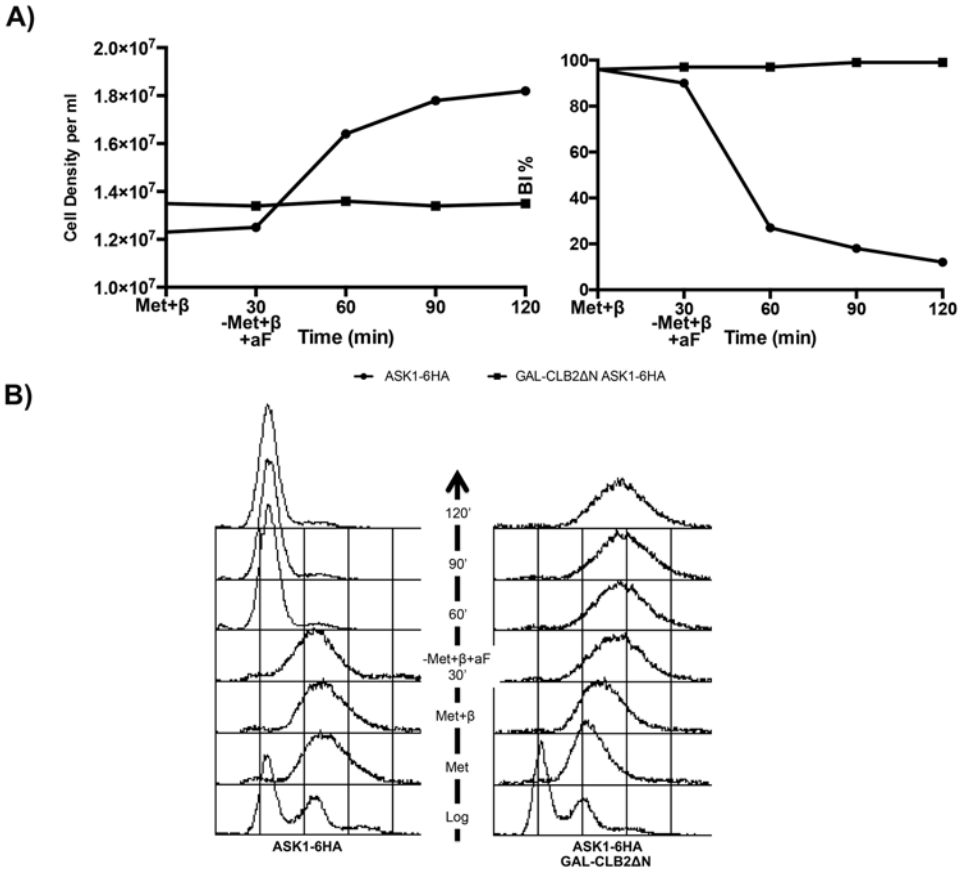


Figure 25. Electrophoretical mobility of Ask1 and abundance of Cdc20 under stable, high M-Cdk1. Cells were synchronized in metaphase, β-estradiol was then added to allow the expression of GAL-CLB2Δ, and then cells were allowed to resume cell cycle progression (t=0). Alpha-factor was added to trap the divided cells in G1. (A) Cell densities and budding indexes. (B) DNA content analysis as monitored by flow cytometry of propidium iodide stained cells. (C) See next page.

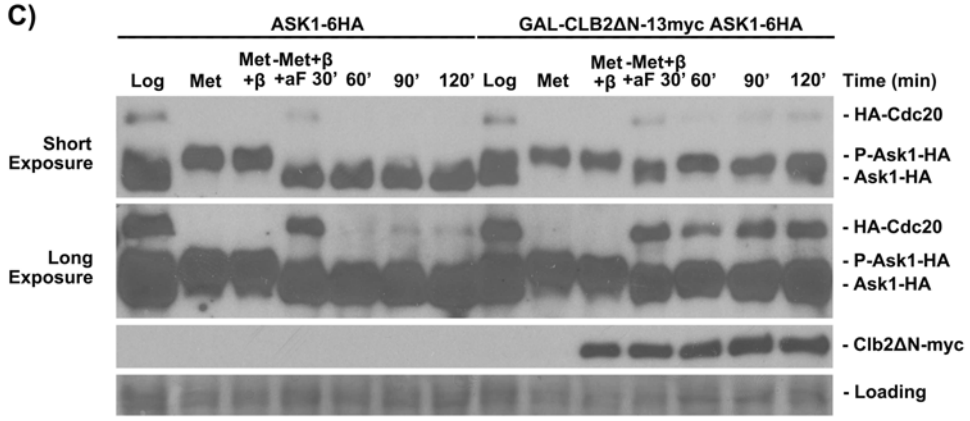


Figure 25 (continued). (C) Whole cell extracts were immunoblotted with the indicated specific antibodies. A Ponceau S stained strip of the membrane is shown as loading control.

4.4.5. Stability of Iqg1 abundance

As additional control of the M-Cdk1/Cdc14 balance, we monitored the abundance of Iqg1, a member of the Ingression Progression Complexes (Foltman et al. 2016). Iqg1 is targeted for destruction by the APC^{Cdh1}, whose activation depends on Cdc14 reversing the M-Cdk1 dependent phosphorylation on Cdh1 (Jaspersen et al. 1999).

MET3-CDC20 ADGEV GAL-CLB2ΔN IQG1-HA and control cells lacking the GAL-CLB2ΔN gene were grown to mid-exponential phase. Cells were then synchronized in metaphase by depletion of Cdc20 (MET3-CDC20). β-estradiol was then added to both cultures, to induce Clb2ΔN over-expression in the experimental strain. Subsequently, methionine was washed out to allow for the re-expression of

Cdc20, the activation of the APC^{Cdc20}, and progression through mitosis. Alpha-factor was added to trap the divided cells in G1. As shown in Figure 26, in control cells endogenous levels of Iqg1 drop by 30 minutes upon release from the metaphase arrest. In contrast, Iqg1 abundance remains stable throughout the experiment, further supporting that Cdh1 remained inactive due to M-Cdk1 phosphorylation.

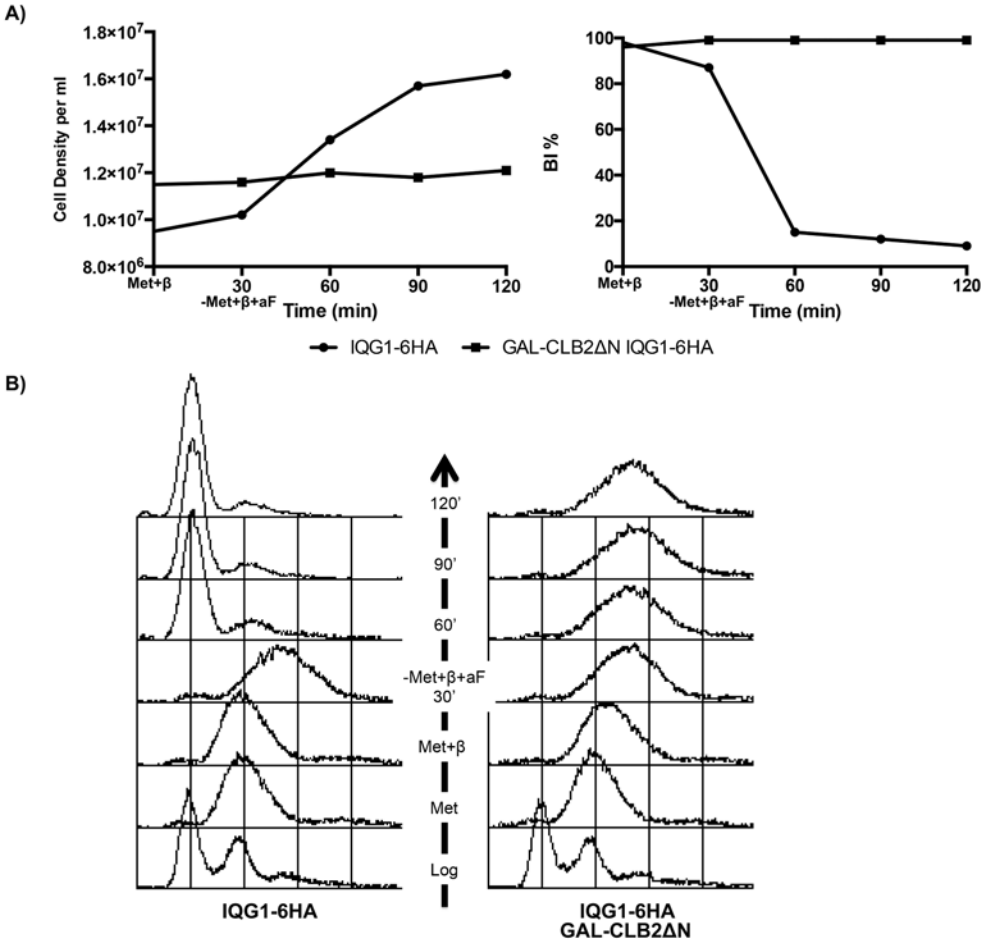


Figure 26. Abundance of Iqg1 under stable, high M-Cdk1. Cells were synchronized in metaphase, β-estradiol was then added to allow the expression of GAL-CLB2ΔN, and then cells were allowed to resume cell cycle progression (t=0). Alpha-factor was added to trap the divided cells in G1. (A) Cell densities and budding indexes. (B) DNA content analysis as

monitored by flow cytometry of propidium iodide stained cells. (C) See next page.

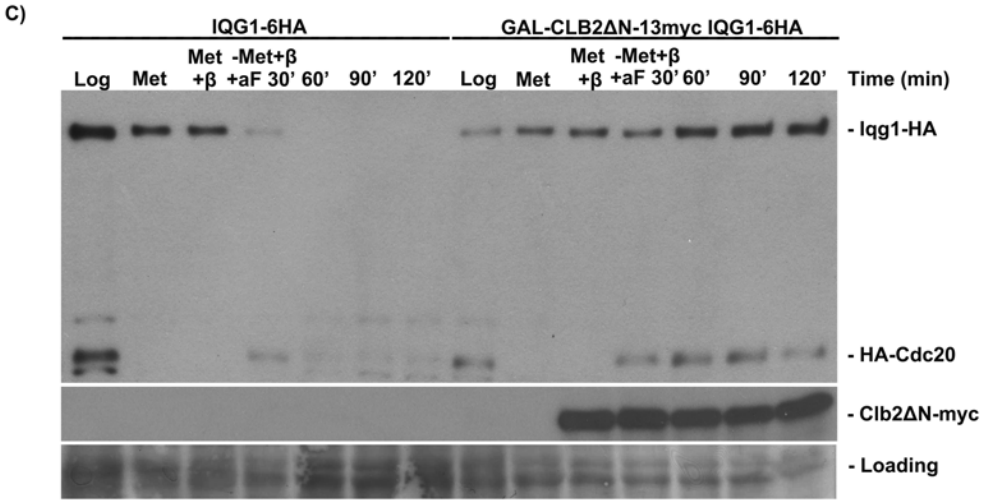


Figure 26 (continued). (C) Whole cell extracts were immunoblotted with the indicated specific antibodies. A Ponceau S stained strip of the membrane is shown as loading control.

4.5. The Mitotic Exit Network (MEN) is active under stable, high M-Cdk1 activity

According to the results shown above, under our experimental conditions M-Cdk1 activity prevails over Cdc14 activity (4.1.4). And yet, Cdc14 is released to the cytoplasm (4.1.3), which is expected to require MEN activation. As will be discussed in detail later, the two observations together indicate the existence of one or more M-Cdk1 and Cdc14 targets, downstream of the MEN dependent release of Cdc14, whose dephosphorylation is essential for cytokinesis. From this point, our work focused on two different objectives. The first objective was to reconcile our observed cytoplasmic release of Cdc14 under high M-Cdk1 activity, in apparent contradiction with previous reports (König et al. 2010) (section 4.1.5). The second objective was to try to abrogate the M-Cdk1 control of cytokinesis at a downstream level downstream of the MEN release of Cdc14 (section 4.1.6).

To address the first objective, we asked whether MEN was active or inactive under high M-Cdk1 activity. Active MEN would explain the cytoplasmic release of Cdc14, which requires phosphorylation by Dbf2-Mob1 to mask the Cdc14 nuclear localization signal (Mohl et al. 2009). On the other hand, inactive MEN would reconcile our observed release and the previous reports that M-Cdk1 keeps MEN inactive, pointing to an alternative release mechanism under our experi-

mental conditions.

4.5.1. The cytoplasmic release of Cdc14 under high M-Cdk1 requires the MEN kinase Cdc15

We wished to study whether the observed cytoplasmic release of Cdc14 under high M-Cdk1 activity was dependent or independent on MEN activation. Because CDC15 is an essential gene, we took advantage of a conditional, thermosensitive mutant strain, *cdc15-2* (Shou & Deshaies 2002).

MET3-CDC20 ADGEV GAL-CLB2ΔN CDC14-GFP mCherry-HTB2 cells carrying the *cdc15-2* allele as sole copy of Cdc15, were compared to an isogenic strain carrying the wild type CDC15. Cells were grown at the permissive temperature (25ºC). At mid-exponential phase growth methionine was added to synchronize cells at metaphase by depletion of Cdc20. The cultures were then shifted to the restrictive temperature of 37ºC. β-estradiol was then added to induce the over-expression of CLB2ΔN. Finally, cells were allowed to progress into mitosis by removal of methionine. The subcellular localization of Cdc14 was then investigated by means of live cell fluorescence microscopy imaging.

As shown in Figure 27, at t = 0 cells in the two cultures display a dumbbell morphology, a single nucleus by the bud-neck, and a nucleolar localization of

Cdc14. Upon release, the wild type CDC15 cells over-expressing Clb2ΔN behave as expected from previous experiments, remaining undivided with two nuclei, and displaying the expected diffuse Cdc14 distribution all over the cell. However, under identical conditions, the *cdc15-2* cells fail to release Cdc14 from the nucleolus. Our results indicate that the MEN kinase Cdc15 is required for the observed cytosolic release of Cdc14, and therefore, that MEN may be active under high M-Cdk1. The nucleolar (re-)localization is unlikely to be due to SPOC signaling an incomplete anaphase, as nuclei look completely segregated.

4.5.2. MEN kinase Mob1 localizes to the Spindle Pole Bodies (SPB) under high M-Cdk1

To further check whether MEN is active under our high M-Cdk1 experimental conditions, we investigated the SPB localization of the MEN kinase Mob1. The recruitment of Mob1 at the SPBs depends on the previous recruitment of MEN upstream components (Cdc15, Tem1) and Dbf2, and marks the "ON" status of the MEN pathway (Visintin & Amon 2001).

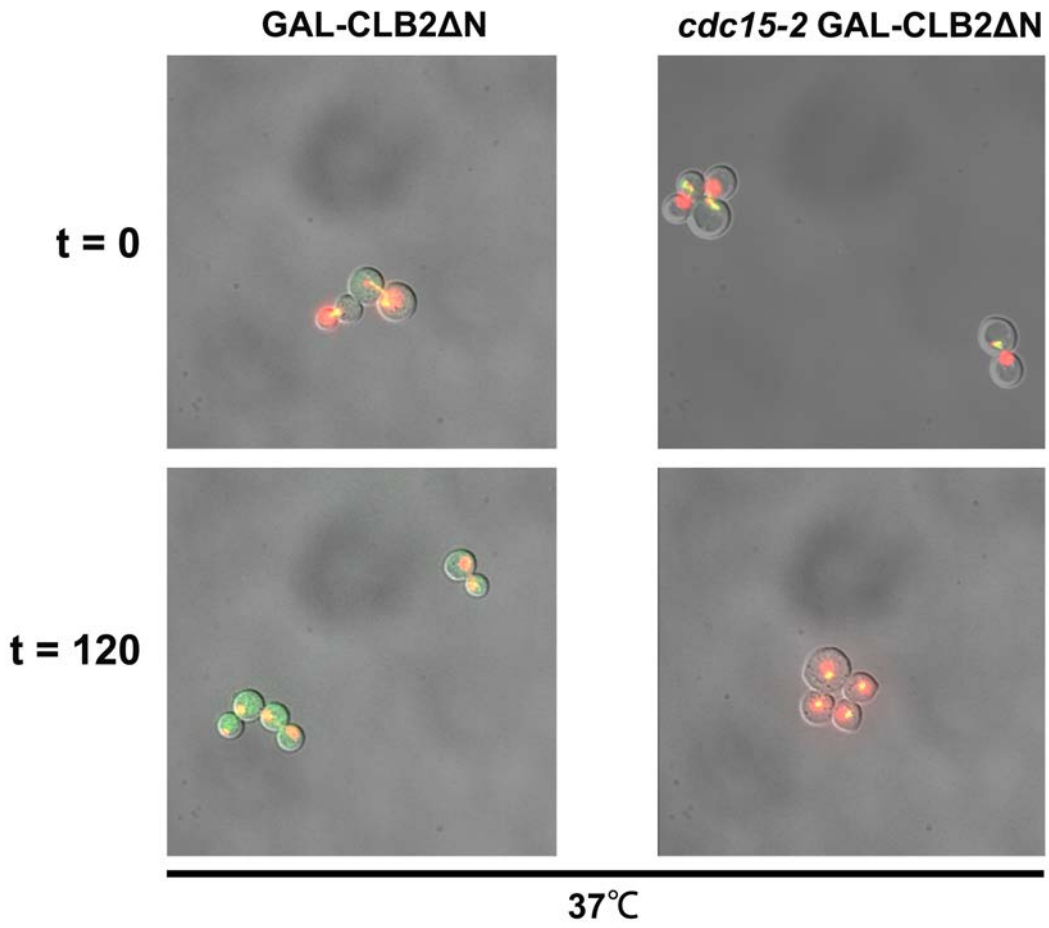


Figure 27. The cytoplasmic release of Cdc14 under high M-Cdk1 requires the MEN kinase Cdc15. Live cell fluorescence microscopy imaging. See text for experimental details. Comparison of CDC15 and *cdc15-2* cells, both at 37ºC and over-expressing Clb2ΔN.

MET3-CDC20 ADGEV GAL-CLB2ΔN MOB1-GFP SPC42-mCherry cells were grown to mid-exponential phase. Methionine was then added to synchronize cells at metaphase by depletion of Cdc20. β-estradiol was subsequently added to induce the over-expression of CLB2ΔN. Finally, cells were allowed to progress into mitosis by removal of methionine. The subcellular localization of Mob1 was then investigated by means of live cell fluorescence microscopy imaging, using the

SPB protein Spc42 as reference.

As shown in Figure 28, Mob1 shows a SPB localization in the presence of high M-Cdk1, suggesting that the MEN pathway is active under these conditions.

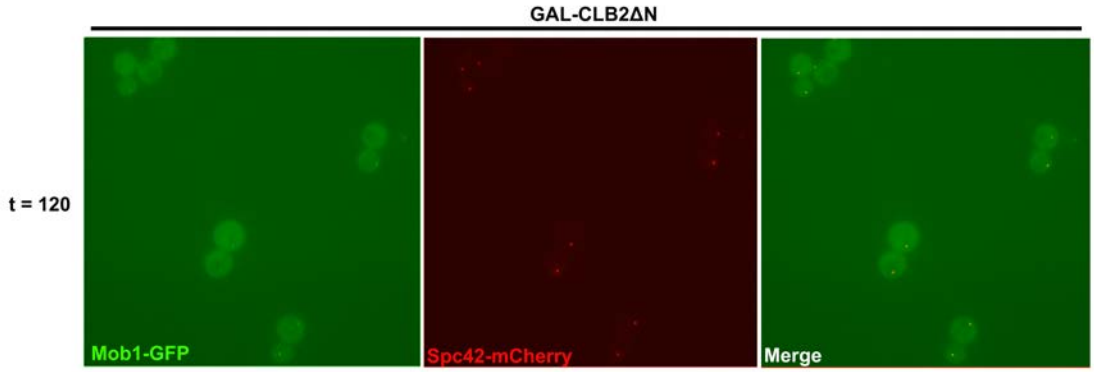


Figure 28. Mob1 localizes to the SPBs in the presence of high M-Cdk1 activity. Live cell fluorescence microscopy imaging. See text for experimental details.

4.6. An Ingression Progression Complex (IPC) M-Cdk1-bypass

In the preceding sections, experimental evidence has been presented showing that under high M-Cdk1 activity Cdc14 is released to the cytoplasm. However, released Cdc14 is unable to overcome the high M-Cdk1 activity under our experimental conditions, as cells fail to undergo cytokinesis. Therefore, our next objective was to identify the essential M-Cdk1/Cdc14 substrates downstream of MEN that block cytokinesis while phosphorylated by M-Cdk1.

As first choice, we shortlisted members of the Ingression Progression Complexes (IPCs) that bridge actomyosin ring contraction and primary septum formation. Among the IPC components Myo1, Iqg1, Hof1, Inn1, Cyk3 and Chs2, two of them, Inn1 and Chs2 are (1) essential for cytokinesis and (2) their recruitment at the IPC depends on M-Cdk1/Cdc14 (Foltman et al. 2016). A third component, Iqg1, must be degraded by the APC^{Cdh1} for cytokinesis to occur (Tully et al. 2009). Given that APC^{Cdh1} activity is under the control of M-Cdk1/Cdc14 (Jaspersen et al. 1999), we hypothesized that conditional elimination of Iqg1 might contribute to abrogate the M-Cdk1 control on cytokinesis.

4.6.1. Inn1 Chs2 M-Cdk1-bypass

Sequence analysis shows that Inn1 has five putative Cdk1 phosphorylation sites (Thr182, Thr349, Ser364, Ser437, Ser398) and Chs2 has four of them (Ser14, Ser60, Ser69, Ser100). We therefore mutated all the putative Cdk1 phosphorylation sites in Inn1 and Chs2 to the non-phosphorylatable amino acid alanine, generating the INN1-5A allele and CHS2-4A alleles. We then used them to generate strains with the non-phosphorylatable alleles as sole copy of the respective gene. The strains carrying these two alleles are viable and morphologically identical to the isogenic wild-type strain.

MET3-CDC20 ADGEV GAL-CLB2ΔN INN1-5A CHS2-4A GFP-SPO20 MYO1-mCherry cells, and isogenic control cells carrying the wild-type versions of the two IPC genes, were grown to mid-exponential phase. Methionine was then added to synchronize cells at metaphase by depletion of Cdc20. β-estradiol was subsequently added to induce the over-expression of CLB2ΔN. Finally, methionine was washed out to allow for Cdc20 expression and the activation of the APC^{Cdc20}, allowing cells to progress through mitosis. For all selected time-points cell density and budding indexes were counted. DNA content was analyzed by means of propidium iodide flow cytometry. AMR and plasma membranes were visualized by means of fluorescence microscopy on live cells.

As shown in Figure 29, cells carrying the Inn1-5A Chs2-4A double

M-Cdk1-bypass alleles remain blocked prior to cytokinesis identically as the wild type control overexpressing Clb2ΔN. (1) Cell density does not increase, (2) the population remains in a dumbbell morphology, (3) with a 2N DNA content, (4) a visible AMR, and (5) no plasma membrane ingression. Therefore, additional M-Cdk1/Cdc14 substrates whose dephosphorylation is essential to allow cytokinesis are missing in the bypass.

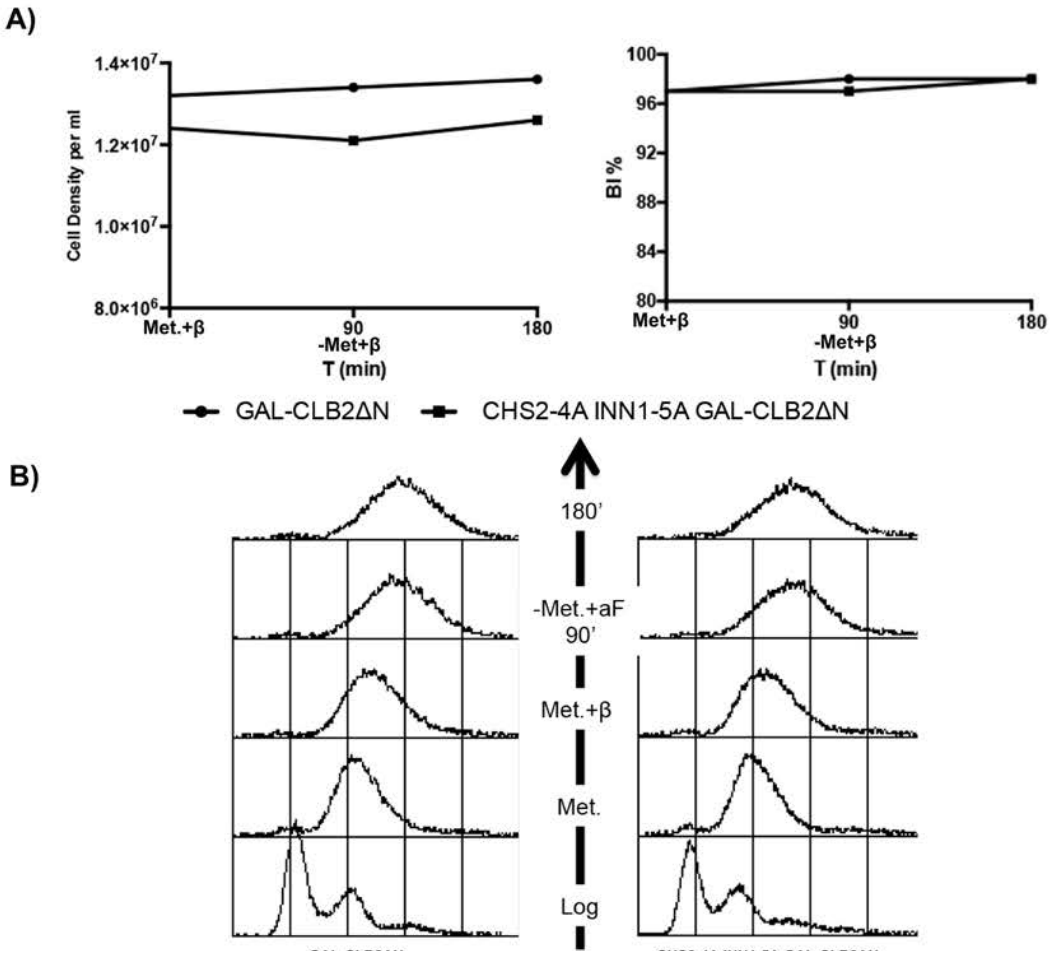
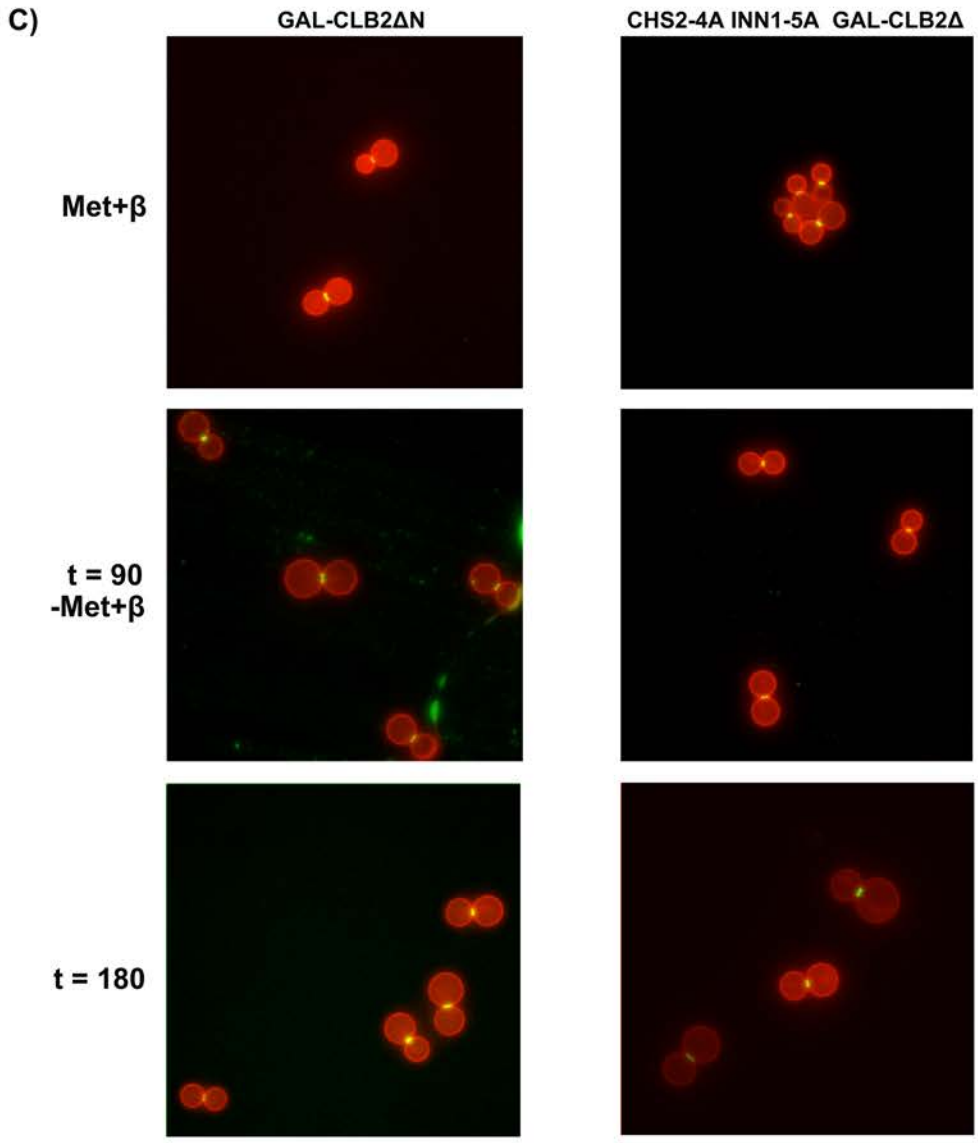


Figure 29. The INN1-5A CHS2-4A M-Cdk1-bypass mutant does not undergo cytokinesis in the presence of high, stable levels of M-Cdk1 activity. Cells were synchronized in metaphase (MET3-CDC20), then expression of GAL-CLB2ΔN was induced with β-estradiol, and finally cells were allowed to resume cell cycle progression by addition of Methionine (t=0). (A) Cell densities and budding indexes. (B) DNA content analysis as monitored by flow cytometry of propidium iodide stained cells. Budding indexes are included for the shown

time-points. (C) Live cell fluorescence microscopy imaging.



4.6.2. Inn1 Chs2 Iqg1 M-Cdk1-bypass

A clear control level under M-Cdk1 is the APC^{Cdh1} activity Cdc14 (Jaspersen et al. 1999). Aside from the mitotic cyclins, Iqg1 seems to be a critical APC^{Cdh1} target to exit mitosis (Tully et al. 2009). We therefore chose to generate a conditional IQG1 AID (Auxin Induced Degron) mutant that could be destroyed at will. It is not possible

to work with a null *iqg1* mutant because it is an essential gene, required for proper mitosis earlier on. The strains carrying the AID-IQG1 allele combined with the INN1-5A CHS2-4A are viable (Figure 30) and morphologically identical to the isogenic wild-type strain. In addition, the AID-IGQ1 fusion was tested functionally: Upon addition of auxin, the experimental strain fails to grow whereas the control strain remains viable (Figure 30).

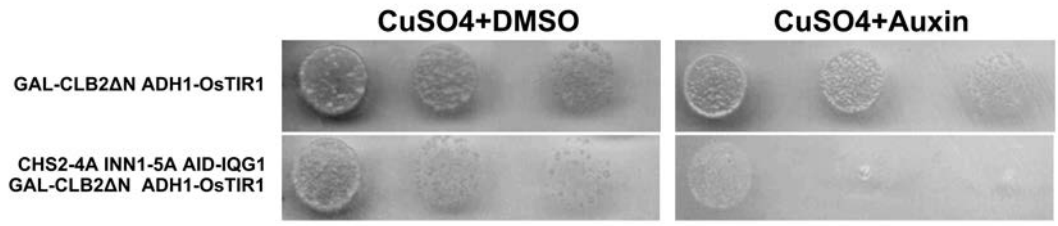


Figure 30. A test of the normal viability of the AID-IQG1 allele and the loss of Iqg1 function in the presence of auxin. Ten-fold serial dilutions of control parental cells and parental control cells on plates containing or not auxin. Plates were incubated at 25ºC for 2 days.

MET3-CDC20 ADGEV GAL-CLB2ΔN INN1-5A CHS2-4A ADH-OsTIR1 CUP1-AID-IQG1 GFP-SPO20 MYO1-mCherry cells, and isogenic control cells carrying wild-type versions of the three IPC genes, were grown to mid-exponential phase. Methionine was then added to synchronize cells at metaphase by depletion of Cdc20. β-estradiol was subsequently added to induce the over-expression of CLB2ΔN. Methionine was washed out to allow for Cdc20 expression and the activation of the APC^{Cdc20}, allowing cells to progress through mitosis and arrest at

telophase. 60 minutes later auxin was added to trigger the destruction of Iqg1 and incubated for 120 more minutes. For all the selected time-points, cell density and budding indexes were counted and DNA content was analyzed by means of propidium iodide flow cytometry. AMR and plasma membranes were visualized by means of fluorescence microscopy on live cells.

As shown in Figure 31, cells carrying the Inn1-5A Chs2-4A AID-Iqg1 triple M-Cdk1-bypass remain blocked prior to cytokinesis identically as the control cells. (1) Cell density did not increase, (2) the population remained in a dumbbell morphology, (3) with a 2N DNA content, (4) a visible AMR, and (5) no plasma membrane ingression.

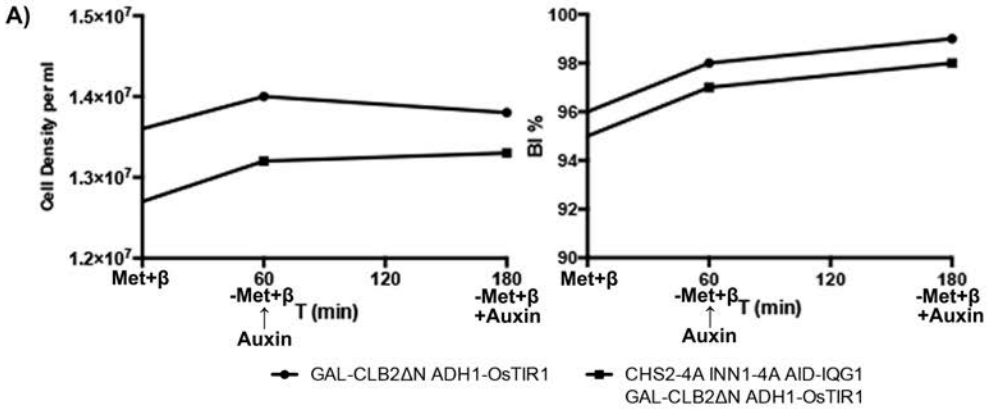


Figure 31. The INN1-5A CHS2-4A AID-IQG1 M-Cdk1-bypass mutant does not undergo cytokinesis in the presence of high, stable levels of M-Cdk1 activity. (A and B) Cells were synchronized in metaphase (MET3-CDC20), then expression of GAL-CLB2ΔN was induced with β-estradiol, cells were allowed to resume cell cycle progression by addition of methionine (t=0). 60 minutes after the metaphase release auxin was added to target Iqg1 for destruction via proteasome. Cells were incubated for 120 more minutes. (A) Cell densities and budding indexes. Budding indexes are included for the shown time-points. (B) See next page.

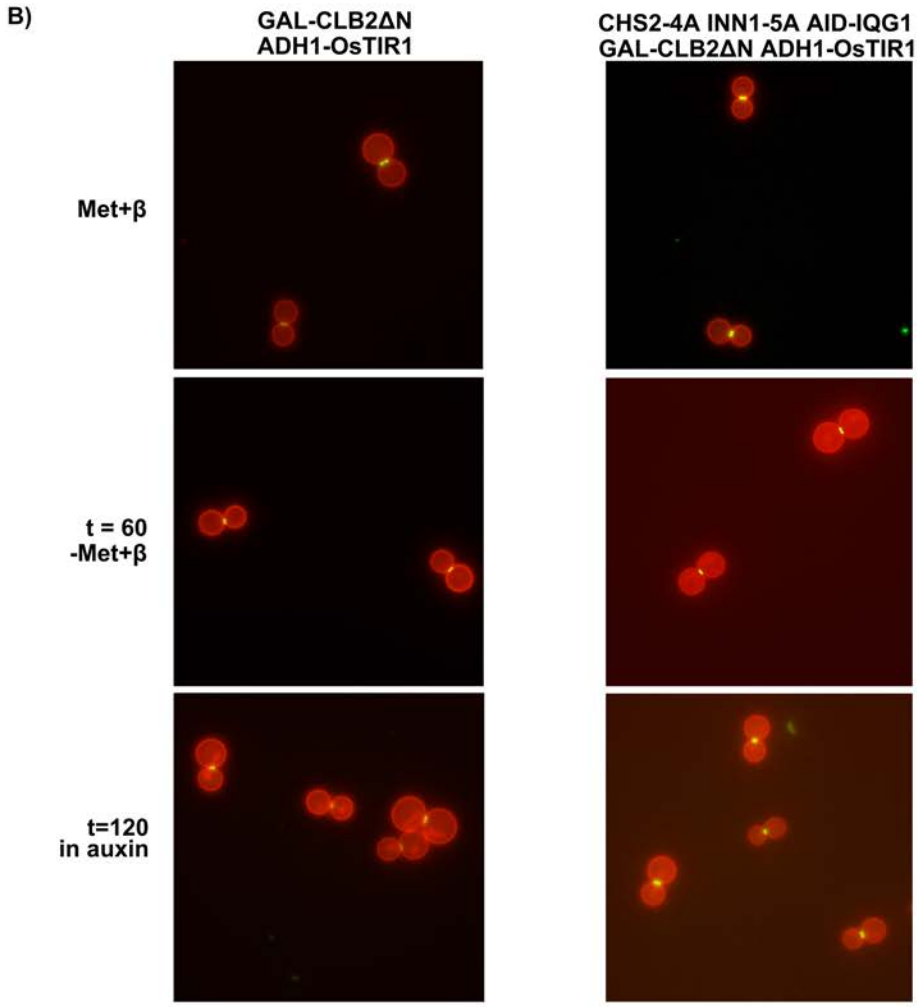


Figure 31 (continued). (B) Live cell fluorescence microscopy imaging.

Therefore, additional M-Cdk1/Cdc14 substrates whose dephosphorylation is essential to allow cytokinesis remain unidentified

5. DISCUSSION

5.1. Control of cytokinesis by M-Cdk1

Cytokinesis is the final step of cell division, by which cells physically separate after chromosomes segregation is satisfactorily completed, giving place to two daughter cells.

In eukaryotic cells an actomyosin ring (AMR) assembles during the mitotic cell cycle, defining a cleavage furrow. At the onset of cytokinesis, activation of the Mitotic Exit Network (MEN) switches the balance of activities between the mitotic Cdk1 kinase and the Cdc14 phosphatase. As a result, AMR contracts, leading to furrow ingression. An Ingression Progression Complex (IPC), fully assembled at the AMR upon cytokinesis, couples contraction to primary septum formation and cell wall deposition (Meitinger & Palani 2016; Foltman et al. 2016).

Defects in cytokinesis result in regression of the cleavage furrow, leading to bi-nucleate cells, which are viable, albeit genomically unstable (Fujiwara et al. 2005; Ganem et al. 2007; Steigemann et al. 2009). On the other hand, premature cytokinesis causes chromosome breakage and aneuploidy, due to abscission before chromosome segregation is complete (Mendoza et al. 2009), which is likely to have direct implications in tumorigenesis (Daniel P. Cahill et al. 1999; Gatenby & Gillies 2008).

DISCUSSION

A key controller of cytokinesis is the mitotic Cdk1 (M-Cdk1) activity. Our project derives from the observed correlation between AMR contraction and the decrease in mitotic Cdk1 activity upon APC^{Cdh1} activation in late mitosis (Wäsch & Cross 2002). Complementary to such observation, expression of hyperstable forms of the mitotic cyclins prevents cytokinesis, and cells arrest in telophase with separated nuclei (karyokinesis) but unable to divide (Ghiara et al. 1991). The very same activity that pushes cells into mitosis and promotes anaphase and karyokinesis, elegantly blocks the occurrence of premature cytokinesis. A similar control operates in S phase, where S-Cdk1 triggers DNA replication but also prevents re-replication by inhibiting the re-assembly of chromosomal pre-replication complexes (Dahmann et al. 1995).

A M-Cdk1 control makes sense, as cytokinesis must be temporally and spatially coordinated with genome segregation, and continued M-Cdk1 activity is required during anaphase (Raha & Amon 2008). Only when a number of indicators of a successful anaphase are met, cytokinesis should be safely allowed. Indeed, cytokinesis is triggered by the so-called Mitotic Exit Network (MEN), that activates only when it receives the *OK* from signals that anaphase is successfully completed (Rock & Amon 2009).

DISCUSSION

MEN activation results in the full release of Cdc14, that reverts M-Cdk1 phosphorylation and also terminates M-Cdk1 activity by activating the ubiquitin ligase APC^{Cdh1} (Jaspersen et al. 1999). APC^{Cdh1} targets mitotic cyclins for destruction via proteasome.

The above described observations suggest that one or more proteins, essential to trigger cytokinesis, are kept inactive by M-Cdk1 phosphorylation. The nature of such critical M-Cdk1 substrate/s is unknown and **the goal of our work was to provide information on the essential M-Cdk1 targets that prevent premature cytokinesis.**

Our experimental approach took advantage of a GAL-CLB2ΔN strain, where Clb2 is rendered hyperstable by deletion of the N-terminus that contain the destruction box and KEN box sequences recognized by the APC ubiquitin ligases. CLB2ΔN was put under the strong, inducible GAL promoter. Cells that accumulate Clb2ΔN are unable to divide and arrest in telophase with two properly segregated nuclei cytokinesis (Surana et al. 1993; Wäsch & Cross 2002). We hypothesized that a strain carrying non-phosphorylatable alleles of the M-Cdk1 essential targets through which M-Cdk1 blocks cytokinesis, should be able to divide in the presence of continuous M-Cdk1 activity generated by the over-expression of a hyperstable form of mitotic cyclin Clb2.

We first shortlisted candidate substrates based on (1) being *bona fide* M-Cdk1 and Cdc14 substrates, and (2) playing an essential role in cytokinesis. These criteria resulted in a 5-strong shortlist: Cdc15, Dbf2, Mob1, Inn1 and Chs2. The first three proteins are indeed central elements of the MEN pathway, responsible to release Cdc14. The other two are part of the more downstream control of cytokinesis, now known as Ingression Progression Complex (Foltman et al. 2016).

Another M-Cdk1/Cdc14 controlled substrate essential for cytokinesis is Cdh1 (Jaspersen et al. 1999). Because Cdh1 has 11 putative Cdk1 phosphorylation sites, we chose to manipulate its critical substrate Iqg1 instead. In addition to mitotic cyclins Clb1 and Clb2, Iqg1 must be degraded by the APC^{Cdh1} for cytokinesis to occur (Tully et al. 2009). The presence of mitotic cyclins associated activity is taken care in our approach by the non-phosphorylatable alleles. We therefore hypothesized that the conditional elimination of Iqg1 might contribute to bypass the M-Cdk1 control on APC^{Cdh1}.

5.2. MEN activation and Cdc14 subcellular localization under high M-Cdk1

We initially selected Cdc15, Dbf2, Mob1 as essential M-Cdk1 targets to block premature cytokinesis. The Cdc15 kinase is essential for cytokinesis. Cdc15 is

kept inactive by M-Cdk1 (König et al. 2010) and reversion of such phosphorylation quires Cdc14 (Jaspersen & Morgan 2000). In turn, Cdc15 activity is required for Dbf2-Mob1 activation (Mah et al. 2001). The Dbf2-Mob1 kinase is also essential for cytokinesis, migrates to the bud-neck just at the time of AMR contraction, and such localization requires the downregulation of M-Cdk1 (Frenz et al. 2000) Also, Dbf2-Mob1 has been proposed to be kept inactive by M-Cdk1 (König et al. 2010). Mob1-Dbf2 activity is required to phosphorylate Cdc14 at its nuclear localization signal, thereby releasing Cdc14 to the cytoplasm (Mohl et al. 2009).

Therefore, as it turns out, the three candidates indeed constitute a module of M-Cdk1 control. It makes sense that for as long as M-Cdk1 activity is required in anaphase, MEN remains (at least partially) inactive. As three of the shortlisted candidates were MEN transducer kinases, at the onset of the project we visualized that a MEN M-Cdk1-bypass alone could result in MEN activation in the presence of high M-Cdk1 activity, likely resulting in full Cdc14 release. From there, two possible outcomes were possible: (1) released Cdc14 would prevail over high M-Cdk1, dephosphorylating downstream cytokinesis substrates, and allowing cell division; (2) released Cdc14 would not prevail over high M-Cdk1, and cell division would require the identification of additional essential downstream cytokinesis M-Cdk1 substrates.

DISCUSSION

MEN activity and Cdc14 cytosolic release under high M-Cdk1

Our results show cytosolic Cdc14 release under our experimental conditions (4.1.3). However, out of expectation, the release is not the result of the MEN M-Cdk1-bypass, as it also happens in control cells under high M-Cdk1 activity. The released Cdc14 fails to override the high M-Cdk1, as cells do not undergo cytokinesis. Therefore, additional substrates must be identified (section 5.3 below).

The simplest explanation for the cytosolic release of Cdc14 does not require M-Cdk1 participation and M-Cdk1 merely *freezes* cells in that situation by block in cell cycle progression. We propose that the observed Cdc14 release is the result of MEN activation at the dSPB upon completion of karyokinesis and SPOC inactivation (Caydasi et al. 2017). FEAR-released nuclear Cdc14 dephosphorylates Cdc15 upon SPOC inactivation, which promotes Cdc15 binding to SPBs, which in turn recruits Dbf2-Mob1 (König et al. 2010; Visintin & Amon 2001; Rock & Amon 2009; Caydasi et al. 2017; Menssen et al. 2001). Therefore, the SPB localization of Mob1 is a consequence of MEN activity (Caydasi et al. 2017).

Our results indeed do provide support for MEN activity. (1) The cytosolic release of Cdc14 to the cytosol (4.1.3) is known to require Mob1-Dbf2 phosphorylation to mask a NLS (Mohl et al. 2009). (2) The observed Cdc14 cytosolic release does require Cdc15 (activity). When a conditional mutant allele of Cdc15 is rendered

inactive, Cdc14 is detected in the nucleolus (4.1.5). Such nucleolar re-localization is unlikely to be due to SPOC signaling an incomplete anaphase, as nuclei look completely segregated.

In any case, there is not full MEN activation under our experimental conditions. Mob1(-Dbf2) fail(s) to localize to the bud-neck. Under normal conditions Mob1 is recruited to the SPB by active Cdc15. Upon activation, Mob1-Dbf2 migrates from the SPBs to the bud-neck, which precedes AMR contraction (Frenz et al. 2000). Because Mob1-Dbf2 is a kinase on its own, and is essential to trigger cytokinesis, it is likely that one of its essential roles is to phosphorylate proteins at the bud-neck required for cytokinesis, such as, perhaps Myo1 to contract. However, under our experimental high M-Cdk1 conditions, Mob1 stays at the SPB and no migration is seen to the bud-neck. Whereas there seems to be sufficient Mob1-Dbf2 for the cytosolic release of Cdc14, continued SPB localization is likely to preclude other essential Dbf2-Mob1 functions that require re-localization. In future work it will be of interest to explore the subcellular localization of the non-phosphorylatable alleles Mob1-7A Dbf2-7A under high M-Cdk1, and see whether they localize to the bud-neck under such conditions. Our results suggest that the phosphorylation of Cdc14 by Dbf2-Mob1 likely occurs at the SPBs, and precedes the re-localization of the kinase at the bud-neck.

If MEN is at least sufficiently active to target Cdc14 to the cytosol in the presence of high M-Cdk1 activity. This observation is in apparent contradiction with the previously published inhibition of Cdc15, Mob1 and Dbf2 by M-Cdk1 activity (König et al. 2010). However, the lack of Mob1 re-localization to the bud-neck indicates that MEN is not fully active in our high M-Cdk1 experimental settings, which reconciles our observations with the proposed inhibitory role of M-Cdk1 on MEN.

Does MEN activation at the dSPB signal to the mSPB?

A conundrum to address in future work is the fact that Mob1 is found associated not only at the dSPB but also at the mSPB (Figure 31). Specific dSPB association would be expected from our activation model. Also, the asymmetric localization of Cdc15 to the mother SPB has been reported by other authors (Menssen et al. 2001). The recruitment of Mob1 at the SPBs depends on the previous recruitment of MEN upstream components (Cdc15, Tem1) and Dbf2, and marks the "ON" status of the MEN pathway (Visintin & Amon 2001). We do not know at this time whether activation of MEN at the dSPB may have an effect *in trans* at the mSPB and whether the mediator is Cdc14 itself or a different element.

Despite Mob1 signal is found at the two SPBs, differences are still visible between mother and daughter. Thus, in all our experiments with wild type cells

over-expressing Clb2ΔN, the Cdc14 signal shows a specific accumulation at the dSPB but not at the mSPB (see for instance Figure 24). Interestingly, such asymmetric localization is however lost in cells that carry the M-Cdk1-bypass for the MEN module (CDC15-7A DBF2-7A MOB1-7A) over-expressing Clb2ΔN (see for instance Figure 23), suggesting that the lack of Cdc14 accumulation at the mSPB may result from control of one or more of the MEN kinases by Cdk1 specifically at the mother moiety.

A novel regulation for nuclear-nucleolar Cdc14 localization?

An interesting additional information can be inferred from our results. It had been previously reported that Cdc14 returns to the nucleolus in the absence of MEN signaling (Yoshida et al. 2002). We now show that MEN signaling is essential to block Cdc14 from returning to the nucleolus even when high M-Cdk1 activity is present. Therefore, high M-Cdk1 does not work as a dominant *super-FEAR* mechanism that keeps the nucleolar sequestering protein Net1 blocked by phosphorylation (Azzam et al. 2004). It therefore must be an advantage for the cell to abort mitotic exit by restoring nuclear Cdc14 to the nucleolus whenever problems during anaphase delay MEN activation. After the initial FEAR release of Cdc14 to the nucleus, M-Cdk1 seems to be unable to target Net1 anymore. Such experimental evidence is suggestive of a licensing mechanism where a factor recruiting M-Cdk1 to Net1 is lost upon the FEAR release. It will be of interest to investigate

whether Spo12, a nucleolar protein bound to Net1, itself released from the nucleolus by M-Cdk1 phosphorylation, plays one such role.

5.3. M-Cdk1 activity prevails over Cdc14 activity under our experimental conditions

We initially hypothesized that bypassing M-Cdk1 control on Cdc15, Mob1 and Dbf2 might result in the cytosolic release of Cdc14 in the presence of high M-Cdk1 activity, and that such release alone might perhaps prevail over M-Cdk1 and trigger cytokinesis.

However, cytokinesis does not occur in cells carrying the MEN M-Cdk1-bypass, despite Cdc14 release to the cytosol, and despite it is expected that the lack of phosphorylation allows Mob1-Dbf2 localize to the AMR to trigger contraction. Lack of cytokinesis indicates suggests that additional targets M-Cdk1 essential for cytokinesis remain inhibited.

Indeed, several biochemical markers for M-Cdk1/Cdc14 activity balance all indicate that M-Cdk1 prevails over Cdc14 in our experimental settings (4.1.4). (1) Sic1 protein fails to accumulate. The presence of Sic1 depends on Swi5 transcription and Sic1 protein stabilization. Both events require that Cdc14 activity prevails

over M-Cdk1 activity. (2) Mitotic cyclin Clb2, Cdc20 and Iqg1 remain stably present, indicating lack of APC^{Cdh1} activity. (3) The B subunit of the DNA alpha polymerase / primase (Pol12) remains phosphorylated. Pol12 is a bona fide specific M-Cdk1 substrate (Palou et al. 2015). (4) Nuclear Cdc14 substrate (Bouchoux & Uhlmann 2011) Ask1 is only transiently dephosphorylated, suggesting effective FEAR Cdc14 release, but prevailing M-Cdk1 activity afterwards.

Because M-Cdk1 prevails over released Cdc14, the system continues to be valid to attempt the full identification of the essential M-Cdk1 targets to keep cytokinesis blocked, which we further attempted as discussed next.

5.4. Towards a full bypass of M-Cdk1 control of cytokinesis

Inn1 and Iqg1, two members of the so-called Ingression Progression Complex (IPC) (Foltman et al. 2016), were included in our original shortlist for candidate substrates for M-Cdk1 control of cytokinesis (see 5.1). The IPC couples actomyosin ring contraction (AMR) and primary septum formation (Foltman et al. 2016). Inn1 was shortlisted because it is essential for cytokinesis and localization at the bud neck is regulated by M-Cdk1/Cdc14 (Foltman et al. 2016). Iqg1 plays an essential scaffold role in the IPC, and was shortlisted because, aside of the mitotic

cyclins, it is the major essential target of the APC^{Cdh1} to allow cells progress through cytokinesis (Tully et al. 2009). Despite Iqg1 also contains multiple Cdk1 phosphorylation sites and requires Cdc14 dephosphorylation (Naylor & Morgan 2014), we first attempted to bypass the control on Iqg1 by regulating its presence. Because IQG1 is essential, we generated a conditional Auxin Induced Degron (AID) mutant that could be destroyed at will upon addition of auxin to the medium.

We also identified Chs2 as a likely critical M-Cdk1 target. Chs2 activity is required for primary septum formation, which is in turn required for full AMR contraction and complete membrane ingression (Meitinger & Palani 2016). M-Cdk1 retains Chs2 at the endoplasmic reticulum by direct phosphorylation (Teh et al. 2009). The release of Cdc14 triggers Chs2 localization to the mother-daughter neck, where it associates with the IPC (Zhang, Kashimshetty, Kwee, et al. 2006).

We therefore generated non-phosphorylatable alleles for Inn1 and Chs2, and a conditional degron allele for Iqg1. The rationale being that lack of M-Cdk1 phosphorylation would allow the recruitment of Inn1 and Chs2 at the IPC despite high M-Cdk1 activity. And later on, Iqg1 could be eliminated at will, thus mimicking the essential elimination of the protein in the absence of APC^{Cdh1} activity. However, the triple Cdk1-bypass INN-5A CHS2-4A AID-IQG1 fails to undergo cytoki-

nesis in the presence of high M-Cdk1 regardless of the presence or the controlled absence of Iqg1.

At the time to conclude the experimental work for this thesis, we are working to distinguish whether other essential targets remain inhibited or in fact, phosphorylation of AID-Iqg1 precludes proper IPC assembly of the IPC. To this respect, we are currently working to generate strains carrying a non-phosphorylatable Iqg1 allele (Naylor & Morgan 2014), both under its own promoter and as an AID conditional mutant.

6. CONCLUSIONS

CONCLUSIONS

1. We have set up a system to test the bypass of the control that Mitotic Cyclin Dependent Kinase (M-Cdk1) exerts on cytokinesis. Cells over-expressing hyper-stable mitotic cyclin Clb2 arrest with fully segregated nuclei and an assembled, uncontracted Actomyosin Ring (AMR).

2. Under these conditions the arrested cells display cytosolic release of Cdc14, suggestive of activation of the Mitotic Exit Network (MEN).

3. Indeed, the cytosolic localization of Cdc14 depends on the presence of the MEN kinase Cdc15, which further supports MEN activation under high M-Cdk1 activity.

4. Further experimental evidence for MEN activation under high M-Cdk1 activity is the localization of the MEN kinase Mob1, and also Cdc14, at the Spindle Pole Bodies (SPB).

5. Our results indicate that the Dbf2-Mob1 phosphorylation that grants Cdc14 access to the cytoplasm, occurs at the SPBs, and precedes Dbf2-Mob1 re-localization to the bud-neck.

6. MEN activation occurs despite high M-Cdk1 activity prevails over Cdc14 activity, as measured by the status of several biochemical markers: absence of Sic1,

stable Clb2, Cdc20 and Iqg1 levels, and the phosphorylated forms of Pol12 and Ask1.

7. Therefore M-Cdk1 is not a universal inhibitor of MEN activation. We propose that upon successful completion of anaphase, inactivation Spindle Position Checkpoint (SPOC) likely allows MEN activation at the daughter SPB (dSPB).

8. However, MEN activation is not complete under our experimental settings, as Mob1 does not co-localize with the AMR at the bud-neck.

9. In addition, there is no AMR contraction or membrane ingression, indicating that Cdc14 does not prevail over high M-Cdk1 activity.

10. Therefore, the system remains suitable for the original purpose of testing the essential M-Cdk1 targets that block cytokinesis.

11. The nucleolar localization of Cdc14 in cdc15 mutants indicates that M-Cdk1, which is the activity responsible for the FEAR promoted nuclear localization of Cdc14, is unable to long-term keep the phosphatase in the nucleoplasm even at high kinase levels.

12. Such loss of power is suggestive of a licensing mechanism where a factor re-cruiting M-Cdk1 to Net1 is lost upon the FEAR release, thus allowing cells abort

mitotic exit by restoring Cdc14 to the nucleolus whenever problems during anaphase delay MEN activation.

13. Two M-Cdk1-bypass strains tested so far, creating non-phosphorylatable MEN and Ingression Promoting Complex (IPC) modules, fail to allow cytokinesis in the presence of stable, high levels of M-Cdk1 and fully released Cdc14. Such result indicates that additional essential targets remain inhibited. Future work will be addressed a generating a viable combined bypass strain.

14. The collection of non-phosphorylatable alleles generated during this thesis provides a useful toolbox for the future dissection of the hierarchy between the different essential mediators of cytokinesis.

7. BIBLIOGRAPHY

Amon, A. et al., 1993. Mechanisms that help the yeast cell cycle clock tick: G2 cyclins transcriptionally activate G2 cyclins and repress G1 cyclins. *Cell*, 74(6), pp.993–1007.

Azzam, R. et al., 2004. Phosphorylation by Cyclin B – Cdk Underlies Release of Mitotic Exit Activator Cdc14 from the Nucleolus. *SCIENCE*, 305, pp.516–519.

Bähler, J. et al., 1998. Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. *Yeast*, 14(10), pp.943–951.

Bartek, J., Bartkova, J. & Lukas, J., 2007. DNA damage signalling guards against activated oncogenes and tumour progression. *Oncogene*, 26(56), pp.7773–7779. Available at: http://www.nature.com/doifinder/10.1038/sj.onc.1210881.

Bartkova, J. et al., 2005. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature*, 434(7035), pp.864–870. Available at: http://www.nature.com/doifinder/10.1038/nature03482.

Bartkova, J. et al., 2006. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature*, 444(7119), pp.633–637. Available at: http://www.nature.com/doifinder/10.1038/nature05268.

Bertani, G., 2004. Lysogeny at mid-twentieth century:P1, P2, and other experimental systems. *J Bacteriol*, 186(3), pp.595–600.

Bertazzi, D.T., Kurtulmus, B. & Pereira, G., 2011. The cortical protein Lte1 promotes mitotic exit by inhibiting the spindle position checkpoint kinase Kin4. *Journal of Cell Biology*, 193(6), pp.1033–1048.

Bloom, J. & Cross, F.R., 2007. Multiple levels of cyclin specificity in cell-cycle control. *Nature reviews. Molecular cell biology*, 8(2), pp.149–160.

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), pp.3417–3426.

Bouchoux, C. & Uhlmann, F., 2011. A Quantitative Model for Ordered Cdk Substrate Dephosphorylation during Mitotic Exit. *Cell*, 147(4), pp.803–814.

Boyne, J.R. et al., 2000. Yeast myosin light chain , Mlc1p , interacts with both IQGAP and Class II myosin to effect cytokinesis. *Journal of cell science*, 4543, pp.4533–4543.

Brachmann, C.B. et al., 1998. Designer deletion strains derived from Saccharomyces

cerevisiae S288C: A useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast*, 14(2), pp.115–132.

Braman, J., Papworth, C. & Greener, A., 1996. Site-directed mutagenesis using double-stranded plasmid DNA templates. *Methods in molecular biology*, 57, pp.31–44.

Cahill, D.P. et al., 1999. Genetic instability and darwinian selection in tumours. *Trends in Biochemical Sciences*, 24(12), pp.57–60.

Cahill, D.P. et al., 1999. Genetic instability and darwinian selection in tumours (Reprinted from Trends in Biochemical Sciences, vol 12, Dec., 1999). *Trends in Genetics*, 15(12), pp.M57–M60.

Caydasi, A.K. et al., 2017. Temporal and compartment-specific signals coordinate mitotic exit with spindle position. *Nature Communications*, 8, p.14129.

Chee, M.K. & Haase, S.B., 2012. New and Redesigned pRS Plasmid Shuttle Vectors for Genetic Manipulation of Saccharomyces cerevisiae. *Genes|Genomes|Genetics*, 2(5), pp.515–526.

Chin, C.F. et al., 2012. Dependence of Chs2 ER export on dephosphorylation by cytoplasmic Cdc14 ensures that septum formation follows mitosis. *Molecular Biology of the Cell*, 23(1), pp.45–58.

Christianson, T.W. et al., 1992. Multifunctional yeast high-copy-number shuttle vectors. *Gene*, 110(1), pp.119–122.

Chuang, J.S. & Schekman, R.W., 1996. Differential Trafficking and Timed Localization of Two Chitin Synthase Proteins, Chs2p and Chs3p. *Journal of Cell Biology*, 135(3), pp.597–610.

Ciejek, E. & Thorner, J., 1979. Recovery of S. cerevisiae a cells from G1 arrest by alpha-factor pheromone requires endopeptidase action. *Cell*, 18(3), pp.623–635.

Ciosk, R. et al., 1998. An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. *Cell*, 93(6), pp.1067–1076.

D'Aquino, K.E. et al., 2005. The protein kinase Kin4 inhibits exit from mitosis in response to spindle position defects. *Molecular Cell*, 19(2), pp.223–234.

Dahmann, C., Diffley, J.F.X. & 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. *Current Biology*, 5(11), pp.1257–1269.

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, p.61.

Evans, T. et al., 1983. Cyclin: A protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell*, 33(2), pp.389–396.

Falk, J.E., Chan, L.Y. & Amon, A., 2011. Lte1 promotes mitotic exit by controlling the localization of the spindle position checkpoint kinase Kin4. *Proceedings of the National Academy of Sciences of the United States of America*, 108(31), pp.12584–90. Available at: http://www.pnas.org/content/108/31/12584.long.

Fitch, I. et al., 1992. Characterization of four B-type cyclin genes of the budding yeast Saccharomyces cerevisiae. *Molecular biology of the cell*, 3(7), pp.805–818.

Foltman, M. et al., 2016. Ingression Progression Complexes Control Extracellular Matrix Remodelling during Cytokinesis in Budding Yeast. *PLOS Genetics*, 12(2), pp.1–33.

Frenz, L.M. et al., 2000. The budding yeast Dbf2 protein kinase localises to the centrosome and moves to the bud neck in late mitosis. *J Cell Sci*, 113 Pt 19, pp.3399–3408.

Fujiwara, T. et al., 2005. Cytokinesis failure generating tetraploids promotes tumorigenesis in p53-null cells. *Nature*, 437(7061), pp.1043–1047. Available at: http://www.nature.com/doifinder/10.1038/nature04217.

Ganem, N.J., Storchova, Z. & Pellman, D., 2007. Tetraploidy, aneuploidy and cancer. *Current Opinion in Genetics and Development*, 17(2), pp.157–162.

Gatenby, R.A. & Gillies, R.J., 2008. A microenvironmental model of carcinogenesis. *Nature Reviews Cancer*, 8(1), pp.56–61.

Ghiara, J.B. et al., 1991. A cyclin B homolog in S. cerevisiae: Chronic activation of the Cdc28 protein kinase by cyclin prevents exit from mitosis. *Cell*, 65(1), pp.163–174.

Gibson, D.G. et al., 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods*, 6(5), pp.343–345.

Gietz, D. et al., 1992. Improved method for high efficiency transformation of intact

yeast cells. *Nucl Acid Res*, 20(6), p.1425.

Gietz, R.D. et al., 1995. Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. *Yeast*, 11(4), pp.355–60.

Goffeau, A. et al., 1996. Life with 6000 Genes. *Science*, 274(October), pp.546–567.

Guarente, L., 1988. UASs and Enhancers : Common Mechanism of Transcriptional Activation in Yeast and Mammals Minireview. *Cell*, 52, pp.303–305.

Hartwell, L.H., 1974. Saccharomyces cerevisiae cell cycle. *Bacteriological reviews*, 38(2), pp.164–198.

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 of the United States of America*, 66(2), pp.352–359.

Hartwell, L.H. & Weinert, T.A., 1989. Checkpoints : control that ensure the order of cell cycles events. *Science*, 246, pp.629–633.

Hentges, P. et al., 2005. Three novel antibiotic marker cassettes for gene disruption and marker switching in Schizosaccharomyces pombe. *Yeasr*, 22(13), pp.1013–1019.

Ito, H. et al., 1983. Transformation of intact yeast cells treated with alkali cations. *Journal of Bacteriology*, 153(1), pp.163–168.

Janke, C. et al., 2004. A versatile toolbox for PCR-based tagging of yeast genes: New fluorescent proteins, more markers and promoter substitution cassettes. *Yeast*, 21(11), pp.947–962.

Jaspersen, S.L., Charles, J.F. & Morgan, D.O., 1999. Inhibitory phosphorylation of the APC regulator Hct1 is controlled by the kinase Cdc28 and the phosphatase Cdc14. *Current Biology*, 9(5), pp.227–236.

Jaspersen, S.L. & Morgan, D.O., 2000. Cdc14 activates Cdc15 to promote mitotic exit in budding yeast. *Current Biology*, 10, pp.615–618.

Kaldis, P., Sutton, A. & Solomon, M.J., 1996. The Cdk-Activating Kinase (CAK) from Budding Yeast. *Cell*, 86, pp.553–564.

Karakaidos, P. et al., 2005. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature*, pp.907–913.

König, C., Maekawa, H. & Schiebel, E., 2010. Mutual regulation of cyclin-dependent kinase and the mitotic exit network. *Journal of Cell Biology*, 188(3), pp.351–368.

Kops, G.J.P.L., Weaver, B. a a & Cleveland, D.W., 2005. On the road to cancer:

aneuploidy and the mitotic checkpoint. *Nature reviews. Cancer*, 5(10), pp.773–85.

Kumar, R. & Rajagopal, K., 2008. Single-step overlap-primer-walk polymerase chain reaction for multiple mutagenesis without overlap extension. *Analytical Biochemistry*, 377(1), pp.105–107.

Lee, P.R. et al., 2002. Bni5p , a Septin-Interacting Protein , Is Required for Normal Septin Function and Cytokinesis in Saccharomyces cerevisiae. *Molecular and Cellular Biology*, 22(19), pp.6906–6920.

Lew, D.J. & Reed, S.I., 1993. Morphogenesis in the yeast cell cycle: Regulation by Cdc28 and cyclins. *Journal of Cell Biology*, 120(6), pp.1305–1320.

Lippincott, J. & Li, R., 1998. Dual Function of Cyk2, a cdc15/PSTPIP Family Protein, in Regulating Actomyosin Ring Dynamics and Septin Distribution. *Journal of Cell Biology*, 143(7), pp.1947–1960.

Louvion, J.F., Havaux-Copf, B. & Picard, D., 1993. Fusion of GAL4-VP16 to a steroid-binding domain provides a tool for gratuitous induction of galactose-responsive genes in yeast. *Gene*, 131(1), pp.129–134.

MacKay, V.L. et al., 1988. The Saccharomyces cerevisiae BAR1 gene encodes an exported protein with homology to pepsin. *Proceedings of the National Academy of Sciences of the United States of America*, 85(1), pp.55–9.

Mah, A.S., Jang, J. & Deshaies, R.J., 2001. Protein kinase Cdc15 activates the Dbf2-Mob1 kinase complex. *Proceedings of the National Academy of Sciences*, 98(13), pp.7325–7330. Available at: http://www.pnas.org/cgi/doi/10.1073/pnas.141098998.

Meitinger, F. et al., 2013. Dual function of the NDR-kinase Dbf2 in the regulation of the F-BAR protein Hof1 during cytokinesis. *Molecular Biology of the Cell*, 24(9), pp.1290–1304.

Meitinger, F. et al., 2011. Phosphorylation-dependent regulation of the F-BAR protein Hof1 during cytokinesis. *Genes and Development*, 25(8), pp.875–888.

Meitinger, F. & Palani, S., 2016. Actomyosin ring driven cytokinesis in budding yeast. *Seminars in Cell and Developmental Biology*, 53, pp.19–27. Available at: http://dx.doi.org/10.1016/j.semcdb.2016.01.043.

Mendenhall, M.D. & Hodge, A.E., 1998. Regulation of Cdc28 cyclin-dependent protein kinase activity during the cell cycle of the yeast Saccharomyces cerevisiae.

Microbiology and molecular biology reviews : MMBR, 62(4), pp.1191–1243.

Mendoza, M. et al., 2009. A mechanism for chromosome segregation sensing by the NoCut checkpoint. *Nature Cell Biology*, 11(4), pp.477–483. Available at: http://www.nature.com/doifinder/10.1038/ncb1855.

Menssen, R., Neutzner, A. & Seufert, W., 2001. Asymmetric spindle pole localization of yeast Cdc15 kinase links mitotic exit and cytokinesis. *Current Biology*, 11(5), pp.345–350.

Mitchison, J.M. & Creanor, J., 1971. Further measurements of DNA synthesis and enzyme potential during cell cycle of fission yeast Schizosaccharomyces pombe. *Experimental Cell Research*, 69(1), pp.244–247.

Mohl, D.A. et al., 2009. Dbf2-Mob1 drives relocalization of protein phosphatase Cdc14 to the cytoplasm during exit from mitosis. *The Journal of cell biology*, 184(4), pp.527–539.

Mullis, K.B. & Faloona, F.A., 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods in Enzymology*, 155(C), pp.335–350.

Naylor, S.G. & Morgan, D.O., 2014. Cdk1-dependent phosphorylation of Iqg1 governs actomyosin ring assembly prior to cytokinesis. *Journal of Cell Science*, 127, pp.1128–1137.

Nishimura, K. & Kanemaki, M.T., 2014. Rapid Depletion of Budding Yeast Proteins via the Fusion of an Auxin-Inducible Degron (AID). *Curr Protoc Cell Biol*, pp.1–16.

Palani, S. et al., 2012. Cdc14-dependent dephosphorylation of Inn1 contributes to Inn1-Cyk3 complex formation. *Journal of Cell Science*, 125(13), pp.3091–3096.

Palou, G. et al., 2015. Three Different Pathways Prevent Chromosome Segregation in the Presence of DNA Damage or Replication Stress in Budding Yeast. *PLoS Genetics*, 11(9), pp.1–22.

Pereira, G. & Schiebel, E., 2005. Kin4 kinase delays mitotic exit in response to spindle alignment defects. *Molecular Cell*, 19(2), pp.209–221.

Raha, R. & Amon, A., 2008. Mitotic CDKs control the metaphase-anaphase transition and trigger spindle elongation. *Genes and Development*, 22(11), pp.1534–1548.

Richardson, H. et al., 1992. Cyclin-B homologs in Saccharomyces cerevisiae function

in S phase and in G2. *Genes and Development*, pp.2021–2034.

Rieder, C.L. et al., 1995. The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores. *Journal of Cell Biology*, 130(4), pp.941–948.

Rock, J.M. & Amon, A., 2009. The FEAR network. *Current Biology*, 19(23), pp.1–10.

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), pp.1597–1609.

Rothstein, R.J., 1983. One-step gene disruption in yeast. *Methods in enzymology*, 101(1981), pp.202–211.

Rudner, A.D. & Murray, A.W., 2000. Phosphorylation by Cdc28 activates the Cdc20-dependent activity of the anaphase-promoting complex. *Journal of Cell Biology*, 149(7), pp.1377–1390.

Schiestl, R.H. & Gietz, R.D., 1989. High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Current Genetics*, 16(5–6), pp.339–346.

Schneider, B.L. et al., 1995. Use of polymerase chain reaction epitope tagging for protein tagging in Saccharomyces cerevisiae. *Yeast*, 11(13), pp.1265–1274.

Shannon, K.B. & Li, R., 2000. A myosin light chain mediates the localization of the budding yeast IQGAP-like protein during contractile ring formation. *Current Biology*, 10, pp.727–730.

Shou, W. et al., 1999. Exit from Mitosis Is Triggered by Tem1-Dependent Release of the Protein Phosphatase Cdc14 from Nucleolar RENT Complex. *Cell*, 97, pp.233–244.

Shou, W. & Deshaies, R.J., 2002. essential requirement for Cdc15 in exit from mitosis in S . cerevisiae. *BMC Genetics*, 12, pp.1–12.

Steigemann, P. et al., 2009. Aurora B-Mediated Abscission Checkpoint Protects against Tetraploidization. *Cell*, 136(3), pp.473–484. Available at: http://dx.doi.org/10.1016/j.cell.2008.12.020.

Surana, U. et al., 1993. Destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. *The EMBO journal*, 12(5), pp.1969–1978.

Surana, U. et al., 1991. The role of CDC28 and cyclins during mitosis in the budding yeast S. cerevisiae. *Cell*, 65(1), pp.145–161.

Teh, E.M., Chai, C.C. & Yeong, F.M., 2009. Retention of Chs2p in the ER requires N-terminal CDK1-phosphorylation sites. *Cell cycle*, 8(18), pp.2964–74.

Thomas, B.J. & Rothstein, R., 1989. Elevated recombination rates in transcriptionally active DNA. *Cell*, 56(4), pp.619–630.

Traverso, E.E. et al., 2001. Characterization of the Net1 Cell Cycle-dependent Regulator of the Cdc14 Phosphatase from Budding Yeast *. *Journal of Biological Chemistry*, 276(24), pp.21924–21931.

Tully, G. et al., 2009. The Anaphase-promoting Complex Promotes Actomyosin- Ring Disassembly during Cytokinesis in Yeast. *Molecular biology of the cell*, 20, pp.1201–1212.

Uhlmann, F. et al., 2000. Cleavage of Cohesin by the CD Clan Protease Separin Triggers Anaphase in Yeast. *Cell*, 103, pp.375–386.

Uhlmann, F., Lottspeich, F. & Nasmyth, K., 1999. Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. *Nature*, 400(6739), pp.37–42.

Vanoosthuyse, V. et al., 2009. Overcoming inhibition in the spindle checkpoint Overcoming inhibition in the spindle checkpoint. *Genes & Development*, (1997), pp.2799–2805.

Visintin, R. et al., 1998. The Phosphatase Cdc14 Triggers Mitotic Exit by Reversal of Cdk-Dependent Phosphorylation. *Molecular cell*, 2, pp.709–718.

Visintin, R. & Amon, A., 2001. Regulation of the mitotic exit protein kinases Cdc15 and Dbf2. *Molecular biology of the cell*, 12(10), pp.2961–2974.

Wach, A. et al., 1994. New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. *Yeast*, 10(13), pp.1793–1808.

Waring, M.J., 1965. Complex formation between ethidium bromide and nucleic acids. *Journal of Molecular Biology*, 13(1), pp.269–282.

Wäsch, R. & Cross, F.R., 2002. APC-dependent proteolysis of the mitotic cyclin Clb2 is essential for mitotic exit. *Nature*, 418(6897), pp.556–562.

Weinert, T.A. & Hartwell, L.H., 1988. The RAD9 Gene Controls the Cell Cycle Response to DNA Damage in Saccharomyces cerevisiae. *Science*, 241, pp.317–

22.

Yamamoto, A., Guacci, V. & Koshland, D., 1996. Pds1p, an inhibitor of anaphase in budding yeast, plays a critical role in the APC and checkpoint pathway(s). *Journal of Cell Biology*, 133(1), pp.99–110.

Yoshida, S., Asakawa, K. & Toh-e, A., 2002. Mitotic Exit Network controls the localization of Cdc14 to the Spindle Pole Body in Saccharomyces cerevisiae. *Current Biology*, 12(11), pp.944–950.

Zhang, G., Kashimshetty, R., Kwee, E.N., et al., 2006. Exit from mitosis triggers Chs2p transport from the endoplasmic reticulum to mother-daughter neck via the secretory pathway in budding yeast. *Journal of Cell Biology*, 174(2), pp.207–220.

Zhang, G., Kashimshetty, R., Ng, K.E., et al., 2006. Exit from mitosis triggers Chs2p transport from the endoplasmic reticulum to mother–daughter neck via the secretory pathway in budding yeast. *Journal of Cell Biology*, 174(2), pp.207–220.

ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS

I would like to express my special appreciation to my mentor Dr. David G. Quintana for the continuous support of my PhD study and related research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my PhD study.

Besides my mentor, I would like to thank my thesis committee members: Prof. Enric Herrero Perpiñán, Prof. Jordi Torres-Rosell, and Dr. Alba Duch Larrégola, for their insightful comments and encouragement, but also for the tough questions which encouraged me to widen my research from various perspectives.

My sincere thanks also goes to Dr. Manuel Mendoza and his group members: Trini, Andrea, Micheal, Arun, Nicola, Francesca, Petra, Tsveti, for all the helps when the time I was in group and bringing me the most happiest moment during my entire PhD life.

I also would like to thank former and present lab members Roger Palou, Asrar Ahmad Malik, Fanli Zeng and Alberto Zurita Carpio for giving me help and supports. I am thankful to Roger Palou and Fanli Zeng, for their scientific advice and experience shared to me, I would also like to thank Alberto Zurita Carpio who helped me with my bench work.

ACKNOWLEDGEMENTS

Finally, I would like to thank the colleagues in my department: Bea, Neus, Josu, Aida, Albert, Pablo, Maribel, Guillem, Vidhya, Elena and Alex, for discussions, suggestions, and also for providing the happy atmosphere in the lab.

ANNEXES

Primers used in this study

All primers used in this study are listed in Table 5

Table 5. List of primers

NAME	SEQUENCE
oPing16 (MOB1-del-F)	CGGTTTTTTTGTTAGTATCTTCAACAAAATTTTCCTAATTCAA-TAAGGACTTCAATTTCCGCGGTATTTTCTCCTTACGC
oPing17 (MOB1-del-R)	TTTTGTGTCCCATGCATGGAAGAATACAACCTACAA-GCAGACTTATATAAATATACAATAAGATTGTACTGAGAGTG-CAC
oFL71 (MOB1-chk-F)	CTTATGGCACAAAAGTACAC
oFL72 (MOB1-chk-R)	GATTCTGAATTACAAGATCA
oFL55 (DBF2-del-F)	GCACAAGAAACAGCATACTAACCCAGAAGAAGAGAC-CAATTGGTTTTCCGGTCATGGTTAGCGGTATTTTCTCCTTACGC
oFL56 (DBF2-del-R):	AAGCGTTCATAGGTGCTAGTTGAAAAGTTTGCATGAA-GCTCGTTAAAGCTAATTATATCGAGATTGTACTGAGAGTGCAC
oFL17 (DBF2clon-BamHI-F)	CGCGGATCCGGGCTCTTCTACATACTTG
oFL18 (DBF2clon-XhoI-R)	CGGCTCGAGCGAATGCAAGACAAGAATT
oFL57 (DBF2-del-chk-F)	TCGCCAGAGATGGTTCAAATG
oFL58 (DBF2-del-chk-R)	ATGGGATGGACCGTTACCGTG
oPing13 (CDC15-del-R)	CAGATGCGTTTTCAGTATTGGAAGGTTCACAATTCTATA-TATAGTGTTAATGTAATGCTGATCGATGAATTCGAGCTCG
oPing14 (CDC15-del-F)	GTATACGGGAGATCTGCCTCACAAAAGGTATATCTTCTACTT-GGAGGCTCGAGGAAGGACCGTACGCTGCAGGTCGAC
oPing7 (CDC15-del-chk-F)	ATCGGCATGGGCATGAGCAG
oPing8 (CDC15-del-chk-R)	GCTGCTGCTGAGGACTACGG
oPing145 (OSTIR1-CHK-F)	ATGACGTACTTCCCGGAGGAG
oPing146 (OSTIR1-CHK-R)	CTAGCAGCAGAACCGGAGTCG
oPing78 (CDC14-AID-F)	CTACAAGCGCCGCCGGTGGTATAAGAAAAA-TAAGTGGCTCCATCAAGAAACGTACGCTGCAGGTCGAC
oPing79 (CDC14-AID-R)	TAAGTTTTTTTATTATATGATATATATATATATAAAAATGAAA-TAAATTAATCGATGAATTCGAGCTCG
oPing130 (CDC14-AID-CHK-F)	GAGCTCAGCCGTCCCACAGAC
oPing131 (CDC14-AID-CHK-R)	GGTGAAGTCCGAGCCATGACG
oPing80 (MOB1-AID-F)	CGGCTGATTTTGGTCCGCTGTTAGAATTAGTGATGGAGTT-GAGGGATAGGCGTACGCTGCAGGTCGAC
oPing81(MOB1-AID-R)	GCATGGAAGAATACAACCTACAAGCAGACTTATATAAA-TATACAATACTAATCGATGAATTCGAGCTCG
oPing132 (MOB1-AID-CHK-F)	GTGGAATGTTTGATGAGGTGG
oPing133 (MOB1-AID-CHK-R)	GCTAACCAATAACCATACCAG
oPing82 (CDC15-AID-F)	CCAAAGATAAAAGTGAC-GGCTTTTCCGTCCCCATTACAACATTTCAAACACGTACGCTG-CAGGTCGAC
oPing83 (CDC15-AID-R)	CTGTATTATTTCTCTATATATGTATGTATGCACATGCAATTCC-TACATTAATCGATGAATTCGAGCTCG
oPing134 (CDC15-AID-CHK-F)	GACAGCCGGATCCTATAAAGC
oPing135 (CDC15-AID-CHK-R)	GTATTCTGACCTTGGTGGGTG

oPing100 (CHS2-AID-F)	CATGGGAAGTCTCTAAATTAGACTTAC-CAAATGTTTTCCACAAAAAGGGCCGTACGCTGCAGGTCGAC
oPing101 (CHS2-AID-R)	TGAGAAAAAAGAGGGAATGACGAGAAATTAGCTGAAAAA-TACTGGCATTAATCGATGAATTCGAGCTCG
oPing136 (CHS2-AID-CHK-F)	GGAAACAGATTGTGGAAACAG
oPing137 (CHS2-AID-CHK-R)	CTAAAACAGTGCGCTCTCTAC
oPing102 (CYK3-AID-F)	TCGCTGACTCTGGTATTGGGTGGTCCGTTTTTGCTGAATGGTT-GTGCGTACGTACGCTGCAGGTCGAC
oPing103 (CYK3-AID-R)	TTGAATGATACAGATTATAGCGCTGTAAAAAAATTT-GTGAAAAACGTTTAATCGATGAATTCGAGCTCG
oPing104 (CYK3-AID-CHK-F)	TCATTCGAAGTTATTCAAAG
oPing105 (CYK3-AID-CHK-R)	CATTTTTGGGTCTTTCCATC
oPing127 (HOF1-AID-F)	GGATAGGATTAATTCCCTATAATTTCATTCAGCTACTG-CATCAAGGTCTTCGTACGCTGCAGGTCGAC
oPing128 (HOF1-AID-R)	CTTTTTTTCTTTTATCAGAAAACTAGTAAAATTGA-TATACATCGAGATCAATCGATGAATTCGAGCTCG
oPing138 (HOF1-AID-CHK-F)	CCTGTGGCAGTAAGAGGAACG
oPing139 (HOF1-AID-CHK-R)	CGTACCAAAATACTGTCCTGC
oPing36 (INN1-replace-R)	CATTTTTCTTTATGGTTTAGATCGGCGGTGAGGGCG-CAACAGATTTATGTATACATATTGGCGGTATTTTCTCCTTACGC
oPing47 (INN1-del-CHK-F)	CCTCTGCTGATCATGAATGAC
oPing52 (Chs2-del-R)	CTACATTATATTTGGTATTGAAACATGTATATTATA-TATAAAAAGTCTAAGAGAGAAATGGCGG-TATTTTCTCCTTACGC
oPing53 (CHS2-del-CHK-F)	ATTACTGTTGATTTCGTGAC
oPing188 (CHS2-420bp-F)	CTGTTTCCCTTGCCTTATACG
oPing35 (DBF20-replace-R)	CTTGGTTTCATAATTTTGG-TAATCATTCAAAATGGAAAATGTTAGCACATCTTGTT-GCTGGCGGTATTTTCTCCTTACGC
oPing90 (DBF20-del-chk-F)	CTGTAGTCGGCTAGATCACAG
oPing163 (GAL4-BD-F)	CCAAGTGTCTGAAGAACAACTGGGAG
oPing164 (VP16-R)	CTACCCACCGTACTCGTCAATTCCAAGGGC
oPing177 (DBF2-6HA-S3-F)	GCATCTTATTCAACGGACTGGAACAC-TCAGACCCCTTTTCAACCTTTTACCGTACGCTGCAGGTCGAC
oPing178 (DBF2-6HA-S2-R)	AAGCTAATTATATCGCGGCGAATGCAAGACAA-GAATTCATTTTTACGCTAATCGATGAATTCGAGCTCG
oPing185 (KAN-replace-LEU2-F)	CTACCCTATGAACATATTCCATTTTGTAATTTCGTGTCGTTTC-TATTATGGGATCCCCGGGTTAATTAAG
oPing186 (KAN-replace-LEU2-R)	TGGCCGAGCGGTCTAAGGCGCCTGATTCAAGAAATATCTT-GACCGCAGTTGAATTCGAGCTCGTTTAAAC
oPing37 (CHS2-replace-R)	CTACATTATATTTGGTATTGAAACATGTATATTATA-TATAAAAAGTCTAAGAGAGAAATGGCGGTATTTTCTCCTTACGC
oPing153 (CBF1-S3)	CAAGAGAACGAAAGAAAAAGCACTAGGAGCGA-TAATCCACATGAGGCTCGTACGCTGCAGGTCGAC
oPing154 (CBF1-S2)	GATACATAGGGAGACTCGAAATACATTTAGC-TATCTATTTTTAACTCTCAATCGATGAATTCGAGCTCG
oPing190 (ASK1-S3)	CGAAAGAAGTACCAAAGCCTGGGACCATCATTCATTTTTC-TACGAATAGACGTACGCTGCAGGTCGAC
oPing191 (ASK1-S2)	CGTTCTGATATTCATCACTAGTAAAAATT-GTATGTACTTATTTATTCTAATCGATGAATTCGAGCTCG
oPing192 (CHS2-S3)	CATGGGAAGTCTCTAAATTAGACTTAC-CAAATGTTTTCCACAAAAAGGGCCGTACGCTGCAGGTCGAC

oPing193 (CHS2-S2)	GAGAAAAAAGAGGGAATGACGAGAAATTAGCTGAAAAA-TACTGGCATTAATCGATGAATTCGAGCTCG
oMM256 (URA3-start-R)	CAACAGTACCCTTAGTATATTC
oMM873 (HTB2-mCherry-F)	CATGCCGTCTCCGAAGGTACTAGGGCTGTTACCAAA-TACTCCTCCTCTACTCAAGCCGACGGTGCTGGTTTAATTAAC
oMM874 (HTB2-mCherry-R)	GCCACTAATAAAAAGAAAACATGACTAAATCACAATACCTAG-TGAGTGACGAATTCGAGCTCGTTTAAAC
oMM890 (KAN-check)	CGCGATTAAATTCCAACATGG
oMM1235 (MYO1-S3-F)	CGAAAAATATTGATAGTAACAATGCACAGAGTAAAATTTTCAG-TCGTACGCTGCAGGTCGAC
oMM1236 (MYO1-S2-R)	ATAAAGGATA-TAAAGTCTTCCAAATTTTTAAAAAAAAGTTCGTTAATCGATGAA TTCGAGCTCG
oMM2453 (TRP1-chk-R)	GGCTCTCTTGCCTTCCAACCC

Plasmids used in this study

All plasmids used in this study are listed in Table 6

Table 6. List of plasmids

PLASMID ID	DESCRIPTION
BASIC VECTORS FOR INTEGRATION IN YEAST	
266	pRS306-Mob1p-Mob1(7A)
275	pRS306-Dbf2p-Dbf2(7A)
279	pRS304-PGal-Clb2△N
293	pRS306- PGal-Clb2△N-13myc
303	pRS306-Chs2p-Chs2(WT)
304	pRS306-Inn1p-Inn1(WT)
309	pRS306-Chs2p-Chs2(4A)
312	pRS306-Dbf20p-Dbf20(WT)
318	pRS306-Dbf20p-Dbf20(1A)
320	pRS306-Inn1p-Inn1(5A)
321	pRS304-PGal-Clb2△N-13myc
322	pRS306-Chs2p-Chs2(6A)
265	pRS305-Cdc15p-Cdc15(7A)-3HA
pMM428	pRS303-GFP-SPO20$^{(51-91)}$
pMM180	pRS305-Met3-3HA-Cdc20

pMM195	ADEGV (URA+) estradiol induction
pMM185	ADH1-OsTIR1 (URA)
pMM186	PGAL-OsTIR1 (URA)
VECTOR FOR EPITOPE TAGGING IN YEAST	
pMM68	Template to C-ter tag protein with yeGFP, HIS3MX6
pMM287	Template to C-ter tag protein with mCherry, hphNT1
pMM111	Template to C-ter tag protein with yeGFP, hphNT1
pMM42	Template to C-ter tag protein with mCherry, HIS3MX6
pMM102	Template to C-ter tag protein with 6xHA, hphNT1
pMM189	Template to C-ter tag protein with IAA17, KANMX6
313	Template to C-ter tag protein with IAA17, hphNT1

Strains used in this study

All strains are W303 background. Strains construction is using standard method and listed in Table 7

Table 7. List of strains

STRAIN	GENOTYPE
W303-1a	MATa *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*
YPR42	W303-1a *bar1*△ GAL-CLB2△N-13myc:URA3
YPR43	W303-1a *bar1*△ Mob1-7A *mob1*△ Dbf2-7A *dbf2*△ Cdc15-7A:LEU2 *cdc15*△::NAT1 GAL-CLB2△N-13myc:URA3
YPR142	W303-1a *bar1*△ Gal-OsTIR1:URA3 *cdc14*-AID-6HA:HYG
YPR144	W303-1a *bar1*△ Gal-OsTIR1:URA3 *mob1*-AID-6HA: HYG
YPR151	W303-1a *bar1*△ Gal-OsTIR1:URA3 *cdc15*-AID-6HA: HYG
YPR152	W303-1a *bar1*△ Gal-OsTIR1:URA3 *chs2*-AID-6HA: HYG
YPR156	W303-1a *bar1*△ Gal-OsTIR1:URA3 *hof1*-AID-6HA: HYG *cyk3*-AID-9myc:KANMx6
YPR170	W303-1a *bar1*△ Gal-OsTIR1:URA3 *cdc14*-AID-6HA:HYG PMET3-3HA-CDC20:LEU2 GFP-SPO20$^{(51-91)}$:HIS3
YPR171	W303-1a *bar1*△ Gal-OsTIR1:URA3 *mob1*-AID:HYG PMET3-3HA-CDC20:LEU2 GFP-SPO20$^{(51-91)}$:HIS3
YPR172	W303-1a *bar1*△ Gal-OsTIR1:URA3 *cdc15*-AID:HYG PMET3-3HA-CDC20:LEU2 GFP-SPO20$^{(51-91)}$:HIS3
YPR173	W303-1a *bar1*△ Gal-OsTIR1:URA3 *chs2*-AID:HYG PMET3-3HA-CDC20:LEU2 GFP-SPO20$^{(51-91)}$:HIS3
YPR174	W303-1a *bar1*△ Gal-OsTIR1:URA3 *hof1*-AID-6HA:HYG

	cyk3-AID-9myc:KANMx6 PMET3-3HA-CDC20:LEU2 GFP-SPO20$^{(51-91)}$:HIS3
YPR175	W303-1a *bar1*△ Gal-OsTIR1:URA3 PMET3-3HA-CDC20:LEU2 GFP-SPO20$^{(51-91)}$:HIS3
YPR177	W303-1a *bar1*△ CDC14-yeGFP:HYG HTB2-mCherry:HIS3 PMET3-3HA-CDC20:LEU2 ADGEV:URA3
YPR178	W303-1a *bar1*△ MYO1-mCherry:HYG GFP-SPO20$^{(51-91)}$:HIS3 PMET3-3HA-CDC20:LEU2 ADGEV:URA3
YPR205	W303-1a *bar1*△ MYO1-mCherry:HYG GFP-SPO20$^{(51-91)}$:HIS3 PMET3-3HA-CDC20:LEU2 ADGEV:URA3 GAL-CLB2△N-13myc:TRP1
YPR206	W303-1a *bar1*△ CDC14-yeGFP:HYG HTB2-mCherry:HIS3 PMET3-3HA-CDC20:LEU2 ADGEV:URA3 GAL-CLB2△N-13myc:TRP1
YPR209	W303-1a *bar1*△ Mob1-7A *mob1*△ Dbf2-7A *dbf2*△ Cdc15-7A:ADE2 *cdc15*△::NAT1 CDC14-yeGFP:HYG HTB2-mCherry:HIS3 PMET3-3HA-CDC20:LEU2 ADGEV:URA3 GAL-CLB2△N-13myc:TRP1
YPR210	W303-1a *bar1*△ Mob1-7A *mob1*△ Dbf2-7A *dbf*2△ Cdc15-7A:ADE2 *cdc15*△::NAT1 MYO1-mCherry:HYG GFP-SPO20$^{(51-91)}$:HIS3 PMET3-3HA-CDC20:LEU2 GAL-CLB2△N-13myc:TRP1 ADGEV:URA3
YPR214	W303-1a *bar1*△ Mob1-7A *mob1*△ Dbf2-7A *dbf*2△ MYO1-mCherry:HYG GFP-SPO20$^{(51-91)}$:HIS3 PMET3-3HA-CDC20:LEU2 GAL-CLB2△N-13myc:TRP1 ADGEV:URA3
YPR231	W303-1a *bar1*△ PMET3-3HA-CDC20:LEU2 ADGEV:URA3 ASK1-6HA:HIS3
YPR240	W303-1a *bar1*△ PMET3-3HA-CDC20:LEU2 ADGEV:URA3 GAL-CLB2△N-13myc:TRP1 ASK1-6HA:HIS3
YPR241	W303-1a *bar1*△ PMET3-3HA-CDC20:LEU2 ADGEV:URA3 IGQ1-6HA:HIS3
YPR291	W303-1a *bar1*△Mob1-7A *mob1*△ Dbf2-7A *dbf*2△ Cdc15-7A:KANMx6 *cdc15*△::NAT1 GAL-CLB2△lN-13myc:TRP1 Dbf20-1A ADGEV:URA3 MYO1-mCherry:HYG PMET3-3HA-CDC20:LEU2 GFP-SPO20$^{(51-91)}$:HIS3
YPR302	W303-1a *bar1*△ PMET3-3HA-CDC20:LEU2 ADGEV:URA3 GAL-CLB2△N-13myc:TRP1 IQG1-6HA:HIS3
YPR311	W303-1a *bar1*△ Chs2-4A *chs2*△::KANMx6 Gal-CLB2△N-13myc:TRP1 Inn1-5A ADGEV:URA3 MYO1-mCherry:HYG PMET3-3HA-CDC20:LEU2 GFP-SPO20$^{(51-91)}$:HIS3
YPR319	W303-1a *bar1*△ Gal-CLB2△N-13myc:TRP1 ADGEV:URA3 MYO1-mCherry:HYG GFP-SPO20$^{(51-91)}$:HIS3 PADH1-OSTIR1-9myc:ADE2
YPR326	W303-1a *bar1*△ Gal-CLB2△N-13myc:TRP1 ADGEV:URA3 MYO1-mCherry:HYG GFP-SPO20$^{(51-91)}$:HIS3 PADH1-OsTIR1-9myc:ADE2 PMET3-3HA-CDC20:LEU2
YPR329	W303-1a *bar1*△ Chs2-4A *chs2*△::NAT1 Gal-CLB2△N-13myc:TRP1 Inn1-5A ADGEV:URA3 MYO1-mCherry:HYG GFP-SPO20$^{(51-91)}$:HIS3 PCUP1-AID-*iqg1*:KANMx6 PADH1-OsTIR1-9myc:ADE2
YPR330	W303-1a *bar1*△ Chs2-4A *chs2*△::NAT1 Gal-CLB2△N-13myc:TRP1 Inn1-5A ADGEV:URA3 MYO1-mCherry:HYG GFP-SPO20$^{(51-91)}$:HIS3

	PCUP1-AID-*iqg1*:KANMx6 PADH1-OsTIR1-9myc:ADE2 PMET3-3HA-CDC20:LEU2
YPR331	W303-1a *bar1*△ ADGEV:URA3 Gal-CLB2△N-13myc:TRP1 SPC42-mCherry:HYG PMET3-3HA-CDC20:LEU2 MOB1-GFP:HIS3

The W303 background is from Thomas et al. (1989). The YPR strains were all created on purpose for this work.