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Molecular mechanisms underlying Cdc14 activation during mitotic exit in *Saccharomyces cerevisiae*

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Table of Contents

Figure Index **8**

Tables Index **10**

Abstract **11**

1. Introduction **13**
The eukaryotic cell cycle: an overview 17
Cyclin-dependent kinases (Cdks): the workhorses of the cell cycle 20
Checkpoint 24
Walking through mitosis: chromosome segregation and mitotic exit 26
The Yeast mitosis 31
Mitotic entry 32
Exit from Mitosis 34
The metaphase-anaphase transition 34
Telophase-G1 35
Regulation of Cdc14 during mitosis in *S. cerevisiae* 38
Regulation of Cdc14/Cfi1 interaction 41
The Cdc Fourteen Early Anaphase Release (FEAR) network 42
The Mitotic Exit Network (MEN) 46
Inactivation of FEAR network and MEN 52
Signals controlling FEAR network and MEN activity 53
The multiple roles of Cdc14 during late stages of mitosis 57
Functions of Cdc14 released by the FEAR network 58
Functions of Cdc14 released by the MEN 61
Cdc14 function and regulation in other eukaryotes 62

2. Materials and Methods **67**
Plasmids, primers and strains 67
Plasmids and Primers 67
Bacterial strains 67
Yeast strains 67
Media and growth conditions 69
Media for *E.coli.* 69
Media for *S. cerevisiae* 69
Procedures 70
DNA manipulation techniques 70
Enzymatic restriction of DNA 70
Purification of DNA inserts from agarose gel 71
Ligation 71
DNA amplification 71
Transformation 72
Escherichia coli transformation 72
Yeast transformation 73
DNA extraction 74
Plasmid DNA isolation from E. coli (mini prep) 74
Plasmid DNA isolation from E. coli (maxi prep) 74
Smash and Grab yeast genomic DNA isolation 74
Teeny genomic yeast DNA isolation 75

Site-directed mutagenesis of *CDC14* 76
Agarose gel electrophoresis 77
Protein techniques 77
Protein extraction 77
Protein extraction from TCA treated yeast cells 78
Immunoprecipitation and phosphatase assay 80
SDS polyacrylamide gel electrophoresis 81
Western blot hybridization 82
Kinase Assays 83
Cdc5 kinase assay 83
Dbf2 kinase assay 86
Clb2 kinase assay 88
Yeast procedures 88
Tetrad dissection and analysis 88
BAR-test/alpha factor sensitivity 89
Synchronization experiments 90
In situ indirect immunofluorescence 92
Percentage of budded cells (budding index) 94
Fluorescence-activating cell sorting (FACS) 95
Nuclei staining (DAPI staining) 95

3. Results 102
An overview: What do we know about Cdc14 release? 102
Cdc5 and one between Clb-Cdks and MEN are required for Cdc14 release 104
Mitotic exit requires the power of two kinases: Cdc5 and either Clb-Cdks or MEN 108
Cdc14 re-sequestration occurs concomitantly with Clb2 degradation in MEN mutants 111
Clb2 stabilization is not sufficient to sustain Cdc14 release in the absence of MEN 113
Clb-Cdks stabilization maintains Cdc14 released in the absence of MEN 117
Clb-Cdks stabilization maintains Cdc14 released in the absence of MEN 121
Clb5 is a *bona fide* FEAR network component 121
Oscillations are impaired in the absence of both MEN and Clb2-Cdk-mediated phosphorylation of Cfi1 124
Oscillations are impaired in Cdc5 mutants 127
A negative feedback loop including Cdc5, Cdc14 and Cdh1 generates a Cdc14 oscillatory release 129
APC/C^{Cdh1} is not the only form of the APC/C capable of degrading Cdc5 136
Cdc5 undergoes cycles of synthesis and degradation in the presence of high levels of stable Clb2 140
Cdc14 promotes its own sequestration in multiple ways 142
The fraction of Cdc14 involved in the oscillatory phenotype is the one that specifically binds Cfi1 145
What is the molecular significance of the two-hit model? 147
Cdc14 and Cfi1 phosphorylation are important for Cdc14 release 148
Do Clb2 and Dbf2 modulate Cdc5 activity? 153
High levels of Cdc5 induce a post-translational modification on Clb2 156
High levels of Cdc5 induce the ectopic phosphorylation of Clb2 157
Clb2 is phosphorylated during early anaphase in wild-type cells 159
In a *cdc5* FEAR-defective mutant Clb2 is not hyperphosphorylated during early anaphase 160
How is the post-translational modification affecting the Clb2-Cdk complex? 163
Cdc5 is not required to modulate Dbf2 activity 166
4. Discussion and future plans 169

Cdc14 release from its inhibitor requires the power of two kinases....170
Molecular interpretation of the “two-hit” model....170
Clb2 is phosphorylated in a Cdc5-dependent manner....173
The “two-hit” model provides a nice explanation for cell cycle progression....177
A model for Cdc14 dynamics at the exit from mitosis....177
Final remarks....179
Why oscillations?....179

References....181

List of Abbreviations

APC/C	Anaphase-Promoting Complex/Cyclosome
APS	Ammonium Persulphate
BFB	Bromophenol Blue
BSA	Bovine Serum Albumine
CAK	Cdk-Activating Kinase
Cdk	Cyclin-Dependent Kinase
CIP	Calf Intestinal Phosphatase
CKI	Cdk-Inhibitor
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetracetate
EtBr	Ethidium Bromide
HU	Hydroxyurea
FACS	Fluorescence-Activated Cell Sorting
FEAR	Cdc Fourteen Early Anaphase Release
GFP	Green Fluorescent Protein
IF	Immunofluorescence
kb	Kilobase
kDa	Kilodalton
MEN	Mitotic Exit Network
MTOC	Microtubule Organizing Center
NOC	Nocodazole
OD	Optical Density
ON	Overnight
PBD	Polo-Box Domain

PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
Plk	Polo-like kinase
PP1	Protein Phosphatase 1
PP2A	Protein Phosphatase 2A
rpm	Rounds per minute
RT	Room temperature
SAC	Spindle Assembly Checkpoint
SPB	Spindle Pole Body
SDS	Sodium Dodecyl Sulphate
SPOC	Spindle Position Checkpoint
Tm	Melting temperature
TCA	Trichloroacetic acid
TRIS	Tris-(hydroximethyl)-aminomethane
YNB	Yeast Nitrogen Base

Figure Index

Introduction

Figure 1.1 Evolution of a tumor……...14

Figure 1.2 Drawings of mitosis by Walther Flemming…….…...16

Figure 1.3 Chromosomal abnormalities in cancer cells…….…..…17

Figure 1.4 Overview of the eukaryotic cell cycle. ……..……19

Figure 1.5 Mechanisms regulating cyclin-dependent kinase (Cdk) activity…….21

Figure 1.6 Regulation of cell cycle progression by cyclin-Cdk complexes……..…22

Figure 1.7 Stages of M phase (mitosis and cytokinesis) in a vertebrate cell…….……..27

Figure 1.8 Type of microtubules-kinetochore attachments…….…….30

Figure 1.9 APC/C triggers the metaphase-anaphase transition…….…..…..35

Figure 1.10 APC/C promotes exit from mitosis……...36

Figure 1.11 The protein phosphatase Cdc14 triggers exit from mitosis in *S. cerevisiae*….37

Figure 1.12 Regulation of Cdc14 during the cell cycle…….....…..39

Figure 1.13 The Cdc Fourteen Early Anaphase Release (FEAR) network…….…..…43

Figure 1.14 The Mitotic Exit Network (MEN)……..…..….47

Figure 1.15 Localization of MEN components during the cell cycle……..……50

Figure 1.16 Functions of Cdc14 released by the FEAR network and the MEN…….……58

Figure 1.17 Overview of Cdc14 orthologs…….…….66

Results

Figure 3.1. Cdc5 requires a partner kinase for promoting Cdc14 release……107

Figure 3.2. A “two-hit” mechanism underlies the release of Cdc14……110

Figure 3.3. Cdc5 is partially degraded in MEN mutant cells……...112

Figure 3.4. Identification of the minimal galactose concentration necessary for an homogeneous telophase arrest……..115

Figure 3.5. Non-degradable Clb2 promotes periodic cycles of Cdc14 release and sequestration……..…116

Figure 3.6. The metaphase to anaphase transition: Rationale for the experiment described in Figure 3.7……...119

Figure 3.7. Stabilization of S- and M-phase cyclins maintain Cdc14 released in MEN mutants……...120

Figure 3.8. Clb5 is a FEAR network component……...123

Figure 3.9. Cdc14 oscillations fulfill the kinase requirement of the two-hit model……..126

Figure 3.10. Modulating Cdc5 activity interferes with the oscillations…….....128

Figure 3.11. Negative feedback loop generate oscillators…….....133

Figure 3.12. A Cdc14 loss-of-function mutant interferes with the oscillatory phenotype134

Figure 3.13. Cdc5 stabilization and Cdh1 depletion interfere with the oscillatory phenotype……...135

Figure 3.14. APC/C dependent degradation and *de novo* protein synthesis are required for Cdc14 endocycles…….....138

Figure 3.15. A model for Cdc14 endocycles……..139

Figure 3.16. Cdc14 re-sequestration requires both Cdc5 inactivation and Cdc14 phosphatase activity……...144

Figure 3.17. The pool of Cdc14 involved in the oscillations is the one bound to Cfi1….146

Figure 3.18. Cdc15 overexpression results in Cfi1 phosphorylation in S phase arrested cells……150

Figure 3.19. Overexpression of *CDC5dBΔ* does not induce the ectopic phosphorylation of Cdc14 or Cfi1 in cells arrested in G1…….151

Figure 3.20. The concomitant overexpression of Cdc5 and Cdc15 is sufficient to promote the ectopic phosphorylation of both Cdc14 and Cfi1 in G1-arrested cells……152

Figure 3.21. Cdc5 overexpression resulted in an increase of its activity also in G1 arrested cells……155

Figure 3.22. Overexpression of Cdc5 induces the phosphorylation of Clb2……158

Figure 3.23. Clb2 is not phosphorylated during early anaphase in *cdc5-ad1* cells……..162

Figure 3.24. Cdc5 does not modulate Clb2 activity…….164

Figure 3.25. Clb2 activity does not require active Cdc5……..165

Figure 3.26. Cdc5 does not affect Dbf2 activity……..167

Figure 3.27. *GAL-CDC15(1-750)* does not require Cdc5 activity to mediate Dbf2 activation……..168

Tables Index

Materials and Methods

Table 2.1. Plasmids used in this study.....96

Table 2.2. Primers used in this study.....97

Table 2.3. Bacterial strains used in this study.....99

Table 2.4. Yeast strains used in this study.....100

Abstract

In budding yeast, progression through anaphase and exit from mitosis are controlled by the conserved protein phosphatase Cdc14. The activity of Cdc14 is regulated in space and time by changes in its subcellular localization. For most of the cell cycle up to metaphase, the phosphatase is sequestered in the nucleolus, by binding to a competitive inhibitor called Cfi1 (also known as Net1) (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999). During anaphase, Cdc14 is released from its inhibitor by the sequential activation of two signaling cascades, the Cdc Fourteen Early Anaphase Release (FEAR) network and the Mitotic Exit Network (MEN). Once released Cdc14 spreads throughout the nucleus and the cytoplasm, where it reaches its targets and promotes progression through and exit from mitosis (Pereira et al., 2002; Shou et al., 1999; Stegmeier et al., 2002; Visintin et al., 1999; Yoshida et al., 2002). Several *in vivo* and *in vitro* observations suggest that phosphorylation of Cdc14 and/or Cfi1 is responsible for the dissociation of Cdc14 from its inhibitor. Three kinases have been implicated in the process: the polo-like kinase Cdc5, the Clb2-Cdk complex and the MEN kinase Dbf2 (Azzam et al., 2004; Geymonat et al., 2003; Hu and Elledge, 2002; Hu et al., 2001; Mohl et al., 2009; Pereira et al., 2002; Queralt et al., 2006; Stegmeier et al., 2002; Visintin et al., 2003; Yoshida and Toh-e, 2002).

The aim of my project was to assess the contribution of the above-mentioned kinases and to identify the molecular mechanisms by which these kinases mediate the release of Cdc14 from its inhibitor. By modulating the kinases of interest alone or in mutual combination we found that Cdc14 is released from the nucleolus by the combined activity of two kinases, Cdc5 always and either Clb-Cdks or Dbf2. Once active, Cdc14 triggers a negative feedback loop that, in the presence of stable levels of mitotic cyclins, generates periodic cycles of Cdc14 release and sequestration. Similar phenotypes have been described for yeast bud formation and centrosome duplication. A common theme emerges

where events that must happen only once per cycle, although intrinsically capable of oscillations, are limited to one occurrence by their coupling with the cyclin-Cdk cell cycle engine.

1. Introduction

In the early 1800 it became clear that all living organisms are composed of individual units called cells. As growth, development and survival of all living organisms rely on the reproduction of those cells, understanding the cell reproduction process is a fundamental problem in biology. Understanding cell reproduction is also important for understanding human diseases such as cancer that can be defined as a disease of cell reproduction. A cell within a tissue acquires a mutation that allows it to grow more rapidly than its neighboring cells. Through the acquisition of additional mutations the progeny of that cell can form into a tumor that can eventually escape that tissue and result in malignant cancer that spreads through the body (Morgan, 2007), Fig.1.1.

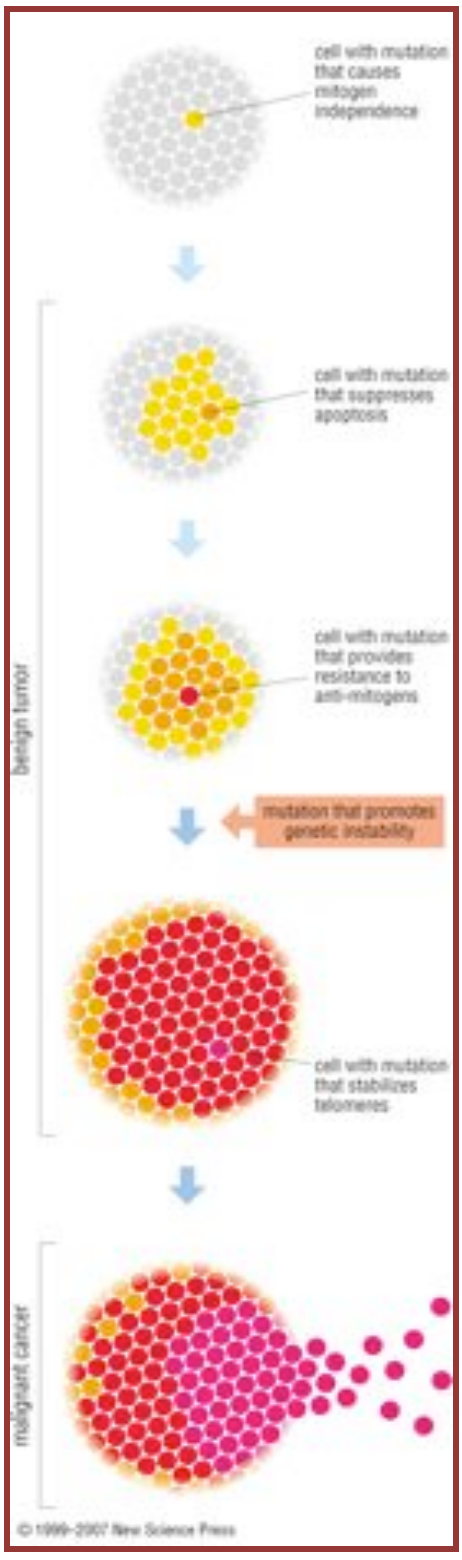

Fig. 1.1 Evolution of a tumor
Tumor evolution begins with a mutation that provides a cell with a competitive advantage, by enabling the cell to proliferate more rapidly than its neighbors. Over a period of many years, the descendants of that mutant cell acquire additional mutations that allow them to overcome the various regulatory barriers that restain cell proliferation. From: The Cell Cycle: Principle of Control by David O Morgan.

How do cells reproduce? This fundamental question has occupied scientists for more than a century. Nearly a century ago, Walter Sutton, observed that during meiotic cell division each sperm or egg receives only one chromosome of each type. Sutton had been observing grasshopper cells, where chromosomes have quite distinct shapes. He published his findings in 1902 and a year later, made an even stronger argument to connect Mendel's laws of heredity and the behavior of chromosomes in his paper *The Chromosomes in Heredity*. In many ways, Sutton reiterated the work of Walther Fleming and, later, of Theodor Boveri, a German scientist who in the late 1880s and early 1890s observed that chromosome numbers are cut in half as fertilized sea urchin eggs mature, and concluded that sperm and egg nuclei have half sets of chromosomes. Boveri further described the detrimental effect of unequal segregation of chromosomes on these cells and their progeny (Boveri, 1902), Fig. 1.2, and, later, he postulated that such misdistribution of chromosomes might be a cause for tumor development and birth defects (Boveri, 1902).

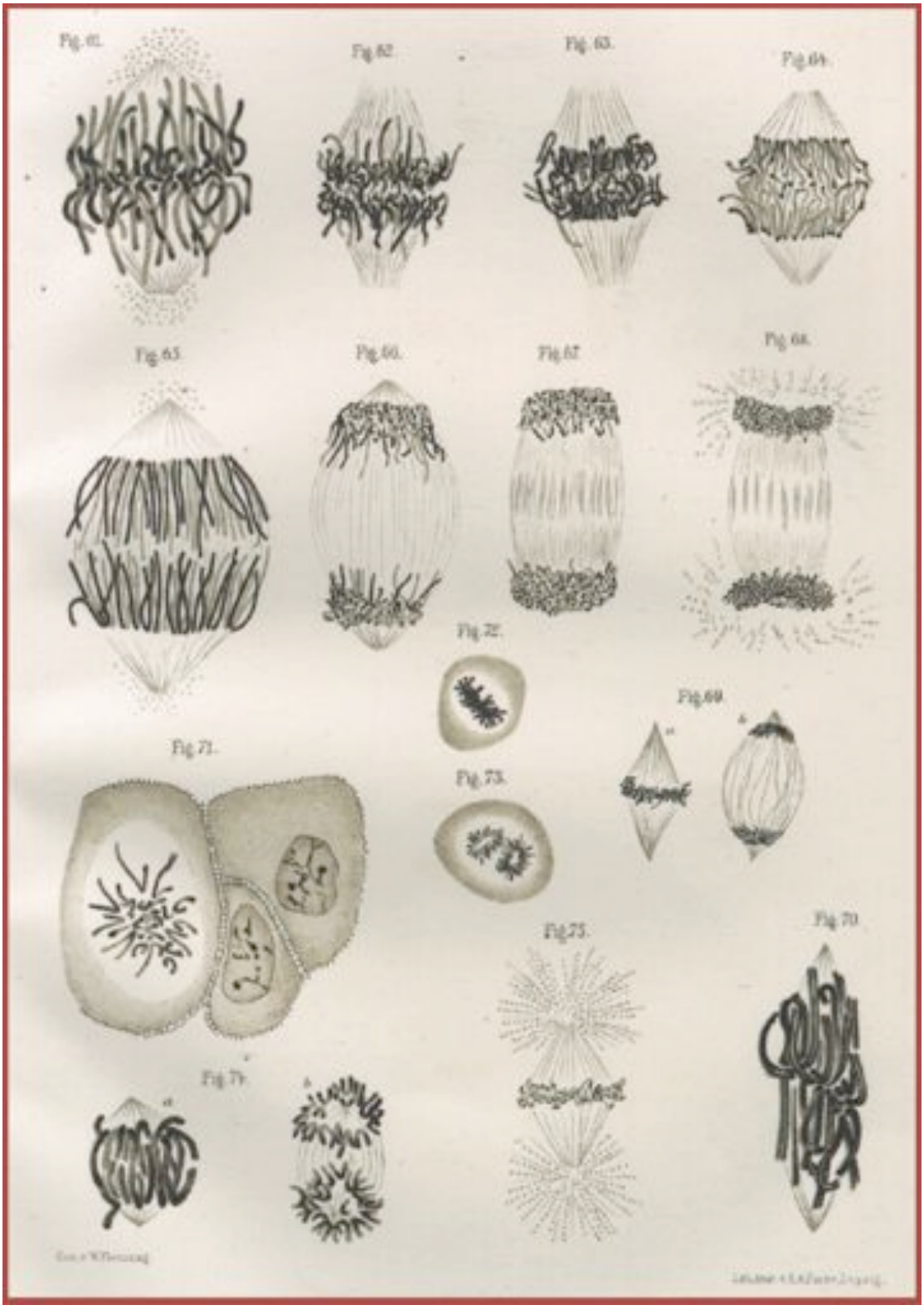

Figure 1.2 Drawings of mitosis by Walther Flemming

Since then, errors in chromosome segregation have been shown to have dire consequences, Fig 1.3. Missegregation during meiosis in gamentogenesis can lead to chromosomal abnormalities such as Down, Klinefelter, Prader-Willi and Angelman syndromes, and are a leading cause of infertility (Bittel et al., 2005; Jiang et al., 2004; Lowe et al., 2001; Tempest et al., 2004). Errors during mitosis can cause chromosome instability and cancer (for reviews see (Draviam et al., 2004; Storchova et al., 2004)). Therefore, a detailed knowledge of the mechanism driving chromosome segregation is vital for understanding and preventing these problems.

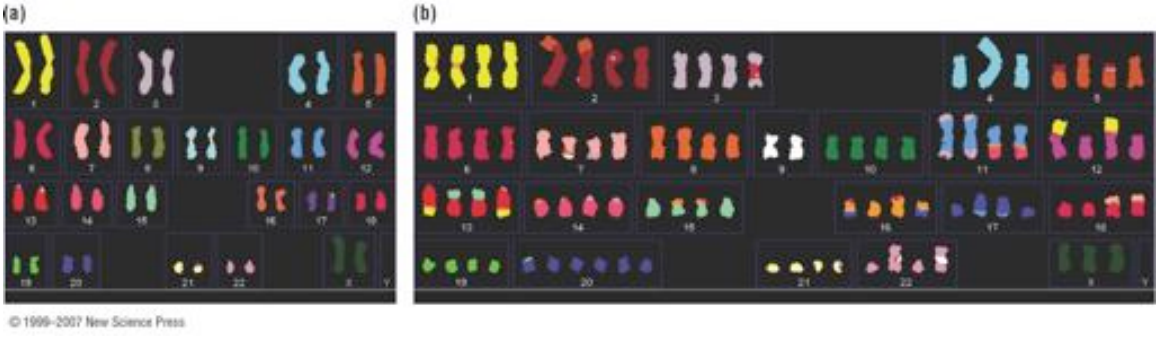

Fig. 1.3 Chromosomal abnormalities in cancer cells
Karyotype of a normal (**a**) and a cancer (**b**) cell. In the chromosome complement of a cancer cell are present extra copies of several chromosomes and translocations between chromosomes, as revealed by the presence of chromosomes with multiple colors.

The eukaryotic cell cycle: an overview

The mitotic cell cycle is a strictly ordered sequence of events that leads to the production of two daughter cells identical to the mother cell, Figure 1.4. A central regulatory system dictates the ordering of the steps by initiating each event at the appropriate time. Although in part predetermined, the schedule can be adjusted based on extemporary needs and incoming problems.

The goal of the mitotic cell cycle is to replicate the genome and pass identical copies to two daughter cells, so as to maintain genomic ploidy (number of chromosomes) from one generation to the next.

Biologists have long been fascinated by the complexity of cell division and mystified

by its high fidelity. The most intriguing part of cell division is how cells manage to scrupulously make so that all events occur in a tightly regulated manner, at the right time and in the appropriate order. Our current understanding of the cell cycle is a synthesis, based on work from numerous investigators who studied the cell cycle in flies, frog extracts, clam embryos, human cancer cells and yeast cells. Studies in budding yeast cell division, in particular, have contributed significantly to our understanding of genes that control cell cycle progression. Although the complexity is increased throughout the evolution, the molecular mechanisms involved in the key events of the cell cycle and their regulation are highly conserved throughout the eukaryotic organisms (Morgan, 2007). The universal architecture of the cell division program consists of four fundamental phases: G1 ("first gap"-unreplicated chromosomes) where the commitment to cell division occurs, S ("synthetic") phase during which the DNA is replicated, G2 ("second gap"-replicated chromosomes) where the cell further grows and prepares to divide, and the mitotic phase (M phase) during which nuclear division, where duplicated chromosomes are equally segregated into the daughter nuclei (mitosis), and cytoplasmic division, when cells physically split (cytokinesis), occur, Fig. 1.4.

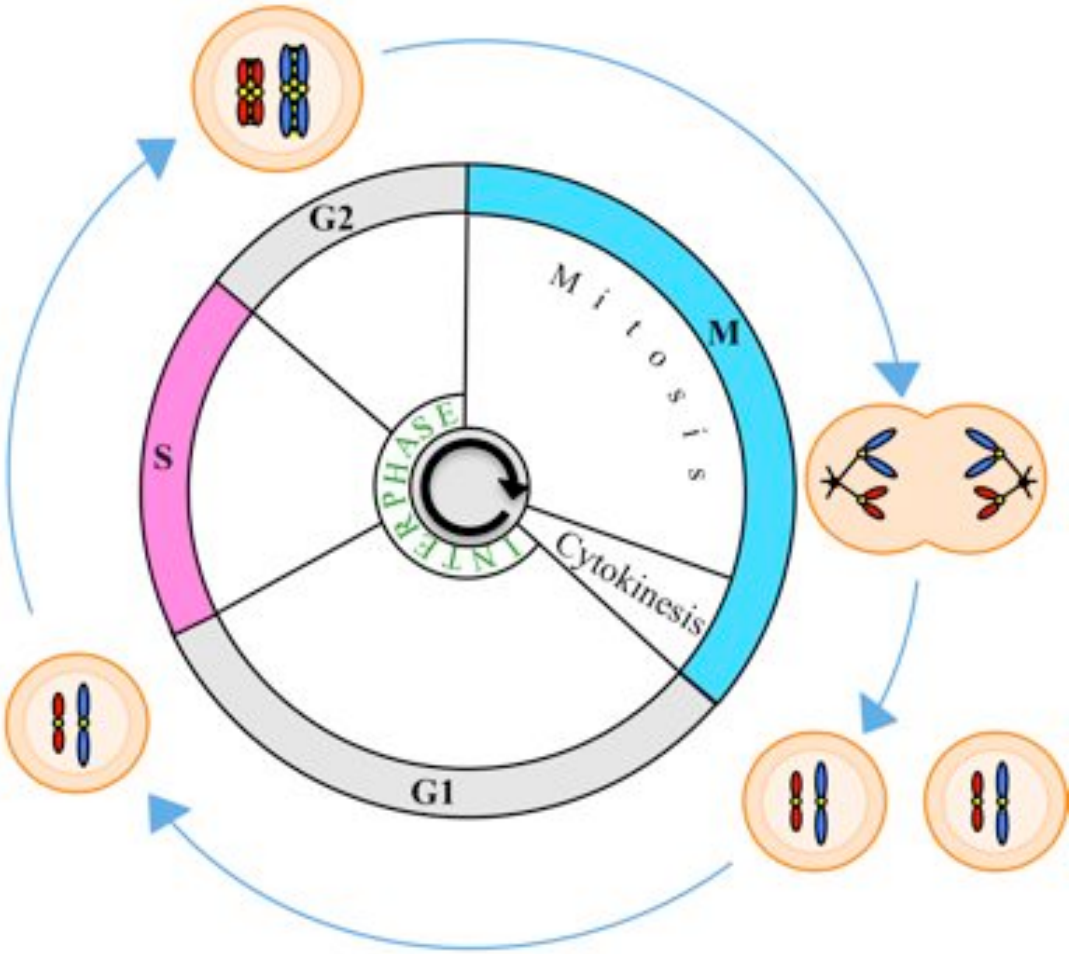

Figure 1.4 Overview of the eukaryotic cell cycle
The diagram illustrates the division of a hypothetical eukaryotic cell with two chromosomes. The major events of the cell cycle are chromosome duplication and chromosome segregation. Chromosome duplication occurs during S phase and results in each chromosome consisting of two tightly-associated identical DNA molecules (sister-chromatids). Sister-chromatids are next segregated into daughter nuclei during mitosis and finally packaged into individual daughter cells during cytokinesis. Mitosis and cytokinesis are collectively called M phase. G1 and G2 are gap phases, G1 preceding and G2 following S phase, respectively. G1, S and G2 phases are collectively called interphase. Cell growth occurs throughout the cell cycle.

Importantly, after completing M phase cells return in G1, hence the division program is cyclical. Between the two major stages (S phase and M phase), the "gap phases" provide time for the mother cell to grow and to control whether it is ready to enter into the next phase. It is intuitive that the alternation of DNA duplication and chromosomes segregation as much as directionality for this process (G1→S→G2→M→G1) is instrumental to maintain ploidy (Morgan, 2007). Indeed, to prevent cells from backsliding, cell-cycle transitions are coordinated by irreversible events that are finely integrated in reaction networks (Csikasz-Nagy et al., 2007).

Cyclin-dependent kinases (Cdks): the workhorses of the cell cycle

At the heart of this system are the cyclin-dependent kinases (Cdks) that drive progression through the cell cycle (Nigg, 2001). The activity of the Cdk oscillates during the cell cycle in response to changes in their association with regulatory cyclin subunits, by binding to stoichiometric Cdk inhibitors (CKIs) and by inhibitory tyrosine phosphorylation (Keaton et al., 2007; Murray et al., 2004) Fig. 1.5.

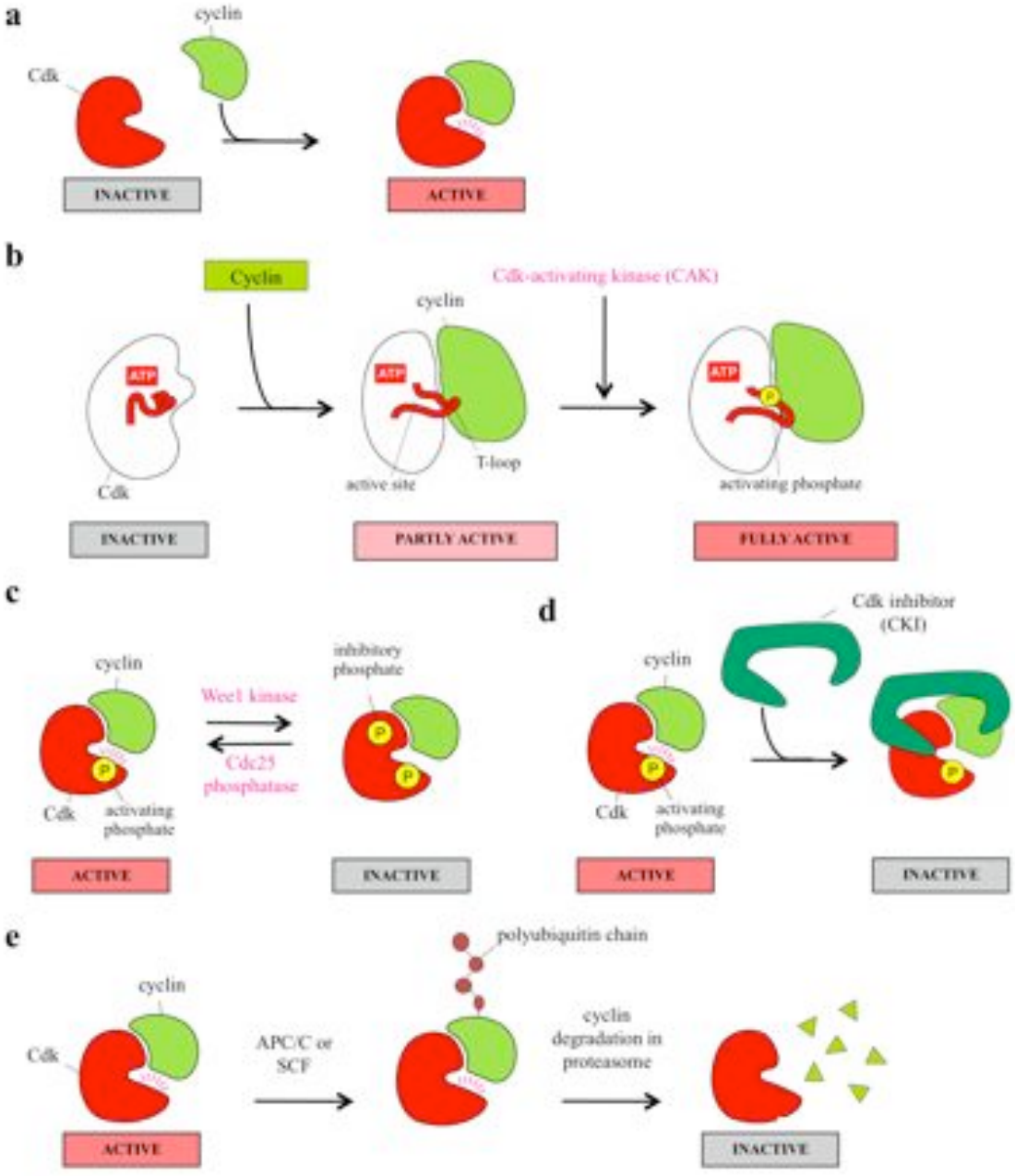

Figure 1.5 Mechanisms regulating cyclin-dependent kinase (Cdk) activity
Cdk enzymatic activity is primarly controlled by binding to an activating subunit called cyclin (**a**). Full activation of a Cdk also requires the phosphorylation of a threonin residue in the T-loop of the kinase domain by Cdk-activating kinases (CAKs; **b**). The fully active cyclin-Cdk complex can be inhibited by phosphorylation on a tyrosine residue close to the ATP-binding site of the enzyme by Wee1-like kinases, while dephosphorylation of this residue by Cdc25-like phosphatases leads to reactivation of the cyclin-Cdk complex (**c**). The activity of cyclin-Cdk complexes is also negatively regulated by binding to Cdk inhibitor proteins (CKIs; **d**). Cyclin ubiquitylation by APC/C or SCF promotes cyclin degradation and Cdk inactivation (**e**). Modified from Alberts, Molecular Biology of the Cell, 2002.

Distinct cyclin-Cdk complexes form at specific cell-cycle stages and initiate the events of S and M phase and, at each of the irreversible cell-cycle transitions (G1-S, G2-M, M-G1), one of these Cdk regulators is ubiquitinated and subsequently degraded by the proteosome (Reed et al., 2003). Hence there are cyclins associated with G1 (cyclin D in vertebrates and Cln1, Cln2 and Cln3 in budding yeast), S phase (Cyclin E and A, Clb5 and Clb6 in budding yeast) and mitosis (cyclin B and A and Clb1, Clb2, Clb3 and Clb4 in budding yeast), Fig. 1.6.

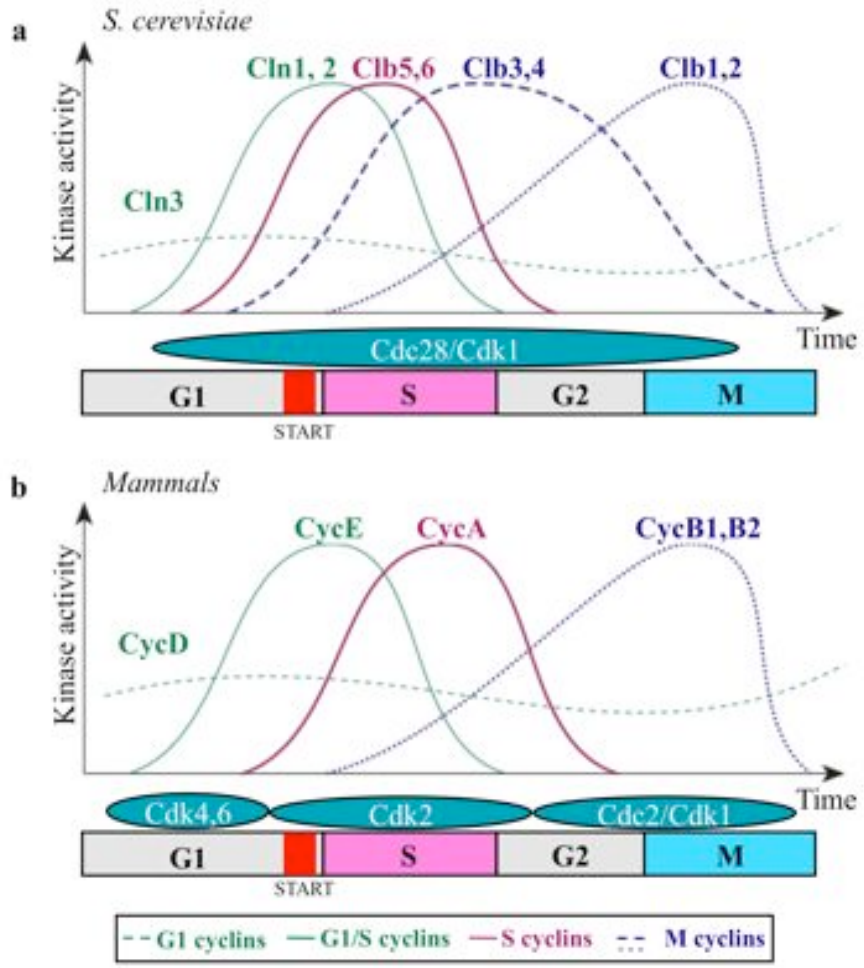

Figure 1.6 Regulation of cell cycle progression by cyclin-Cdk complexes
Activation of distinct cyclin-Cdk complexes at different stages of the cell cycle triggers the various cell cycle events in simple eukaryotic organisms as budding yeast (**a**) as well as in higher eukaryotes as mammals (**b**). Unlike budding yeast, higher eukaryotes possess several Cdks. The levels of the three major cyclin types oscillate during the cell cycle while the concentrations of Cdks (not shown) do not change and exceed the amount of cyclins, thus the activity of cyclin-Cdk complexes raise and fall in parallel with cyclin levels. Expression of G1 cyclins is stimulated in response to external factors (nutrient levels in *S. cerevisiae*, growth factors in mammals). G1-Cdk complexes induce the expression of G1/S cyclins thereby promoting the formation of active G1/S-Cdk complexes which trigger progression through the Start in late G1. S-Cdk complexes form at Start and initiate DNA replication at the beginning of S phase. M-Cdk complexes form during G2 and promote progression through the G2/M transition and the early events of mitosis. Exit from mitosis requires the inactivation of M-Cdks (both S- and M-Cdks in *S. cerevisiae*).

Rather than differences on substrate specificity, the multiplicity of cyclins account for duplication and divergence that enable different family members to perform biological functions that are distinct in space and time (Murray et al., 2004). First discovered in early embryonic cell cycle (Evans et al., 1983), complexes of Cdk2 with either cyclin E or cyclin A coordinate the G1/S transition, DNA replication and possibly initiate nuclear envelope breakdown (NBD) (Strausfeld et al., 1996). Once this is complete, Cdk1/cyclin B complexes orchestrate mitotic events such as NBD, Golgi fragmentation, centrosome separation, chromosome condensation and spindle assembly (Murray et al., 2004). Although mainly overlapping the function of their metazoan functional homologues, a series of distinct cyclins governs mitosis in budding yeast. The major S-phase cyclin, Clb5, exhibits features that are reminiscent of vertebrate cyclin A: its expression increases in late G1, it helps to stimulate chromosome duplication and it appears to have some functions during mitosis. Unlike cyclin A, Clb5 is destroyed just before anaphase, together with securin (a negative regulator of chromosome segregation). The major mitotic cyclin, Clb2, is crucial for spindle assembly and progression to metaphase. Some Clb2 protein is destroyed at the same time as Clb5 and securin, but most remains stable until after anaphase (Bloom and Cross, 2007).

Cdk activity drives cell-cycle progression as far as metaphase, but progression into anaphase and beyond depends on another major regulatory component, the APC/C, an E3 ubiquitin ligase which ubiquitinates several regulatory proteins and thereby targets them to the proteosome for destruction (Peters, 2006; Thornton et al., 2006). As cells proceed through mitosis, the APC/C is activated in two ways: its subunits are phosphorylated and its interaction with an activating protein, named Cdc20, increases (Peters, 2002). Remarkably, both changes are stimulated by the activation of Cdk1. Leaving mitosis, the APC/C remains active but switches from being dependent on Cdc20 to being dependent on a related protein known as Cdh1 for its activity (Visintin et al., 1997). Unlike Cdc20, Cdh1 is inhibited by Cdk1 dependent phosphorylation (Jaspersen et al., 1999; Zachariae et al.,

1998) and this is one of the reasons why late mitosis and mitotic exit require the timely and complete inactivation of Cdk1 and destruction of cyclin B, which is itself ubiquitinated by Cdc20-directed APC/C. In this way, Cdks and ubiquitin machinery mutually regulate each other, allowing the coupled, oscillating waves of kinase activity and proteolysis to order the events of mitosis (Peters, 2006).

Checkpoint

While phase-specific cyclin activity and regulated proteolysis confer an order to cell division events, cell cycle checkpoints render the transition between phases sensitive to the completion of key cellular tasks and the presence of errors. The original paradigm for cell cycle checkpoint was provided by Weinart and Hartwell in a genetic screen for *S. cerevisiae* mutants with an impaired response to DNA damage (Weinert and Hartwell, 1988). Whereas wild type yeast cells exposed to low doses of X-ray irradiation arrest in G2 and do not enter mitosis until genome damage has been resolved, *rad9* cells fail to arrest, enter mitosis, and show decreased viability. However, *rad9* mutants survive irradiation if mitosis is prolonged by treatment with the MT depolymerizing agent nocodazole. Notably, loss of Rad9 protein does not impact cell cycle progression or cell viability in the absence of DNA damage (Hartwell and Weinert, 1989; Weinert and Hartwell, 1989). These results suggested that Rad9 is not directly involved in repairing the DNA but that it becomes critical under circumstances of severe DNA damage. Thus, early models defined checkpoints as non-essential backup systems that monitor but do not participate in the underlying cell division process. Formally, the minimal checkpoint requirements are thought to consist of a sensor, which detects underlying errors; a transducer, which relays and perhaps amplifies the sensed signal and an effector, which halts the cell cycle until damage has been repaired. In reality, these tasks may rely on shared components, and crosstalk or feedback between them may exist. Additionally, checkpoints may possess a shutoff step to ensure that prolonged cell cycle arrest following repair does not impair the

health of a cell.

Since the *RAD* screen, cell cycle checkpoints that monitor multiple DNA structural lesions, replication fork progress, unreplicated DNA, spindle position, and chromosome segregation in mitosis and meiosis have been identified (Bartek et al., 2004; Zhou et al., 2000).

While many elements of cell cycle checkpoints are conserved in evolution, further characterization of yeast and metazoan checkpoints has modified the classical model in two broad ways. First, it has become clear that many checkpoints not only alter the cell cycle but also actively regulate the appropriate response, i.e. repair or apoptosis. For example, budding yeast Rad24 participates in both DNA double strand break (DSB) repair and cell cycle arrest (Aylon and Kupiec, 2003). Second, while canonical checkpoint genes are non-essential in yeast because they do not contribute to core processes, genetic deletion of homologous genes in higher eukaryotes has proven lethal (Basu et al., 1999; Brown et al., 2003; Dobles et al., 2000; Kitagawa et al., 1999). At least three non-exclusive models can explain these findings. First, the lesions monitored by such checkpoints occur in nearly every cell cycle in higher organism and checkpoint activity is made necessary by the frequency of its use; unlike with Rad9, a basal level of activity exists in the absence of an exogenous insult. Second, any given checkpoint activity is not active in every cell cycle, but the survival of a multicellular organism can be compromised by checkpoint failure in a few cells of a specific stages of development. Third, a checkpoint protein in higher organism may participate in the underlying repair process or contribute directly to cell cycle progression. Metazoan checkpoint proteins often have more complex multidomain structures than their yeast homologues and serve to integrate arrest and repair functions.

The requirement for cell cycle checkpoints in the fitness of multicellular organisms is illustrated by their frequent disruption in hereditary and acquired cancers (Zhivotovsky et al., 2004; Lobrich et al., 2007).

Walking through mitosis: chromosome segregation and mitotic exit

Although mitosis is a continuous process, chromosomal movements and other cytological changes allow it to be arbitrarily divided into a series of sub-phases, known as prophase, prometaphase, metaphase, anaphase and telophase, Fig. 1.7.

During **prophase** the duplicated genomes condense into compacted chromosomes, each consisting of a pair of sister chromatids that are held together by cohesin proteins. Chromosome compaction is essential for guaranteeing the ease of movements necessary to separate the sister chromatids along the mitotic spindle. Next to chromosome condensation, other events that characterize this phase include the beginning of mitotic spindle formation and nucleolar break down, Fig. 1.7a. **Prometaphase** starts with the break down of the nuclear envelope. This event allows the spindle to invade the nuclear region and capture the condensing chromosomes. Spindle fibers or microtubules (MT) capture the chromosomes by their kinetochores, large proteinaceous structures that assemble on centromeres, Fig. 1.7b. In **metaphase**, microtubules pull chromosomes to the equator of the cell: the so-called metaphase plate. Aligned chromosomes are held in position by kinetochore-microtubule interactions, Fig. 1.7c. During **anaphase**, sister chromatids are separated and can now be considered individual chromosomes. Meanwhile, spindle fibers shorten, pulling the individual chromosomes to the opposite poles of the cell, Fig. 1.7d.

Finally, in **telophase** separating chromosomes reach the two opposite poles, Fig. 1.7e. The new daughter nuclei and nuclear envelopes start to reform and chromosomes decondense. The mitotic spindle breaks down and the cytoplasm begins to divide, a process that is completed during cytokinesis and results in the formation of the two daughter cells (Morgan, 2007).

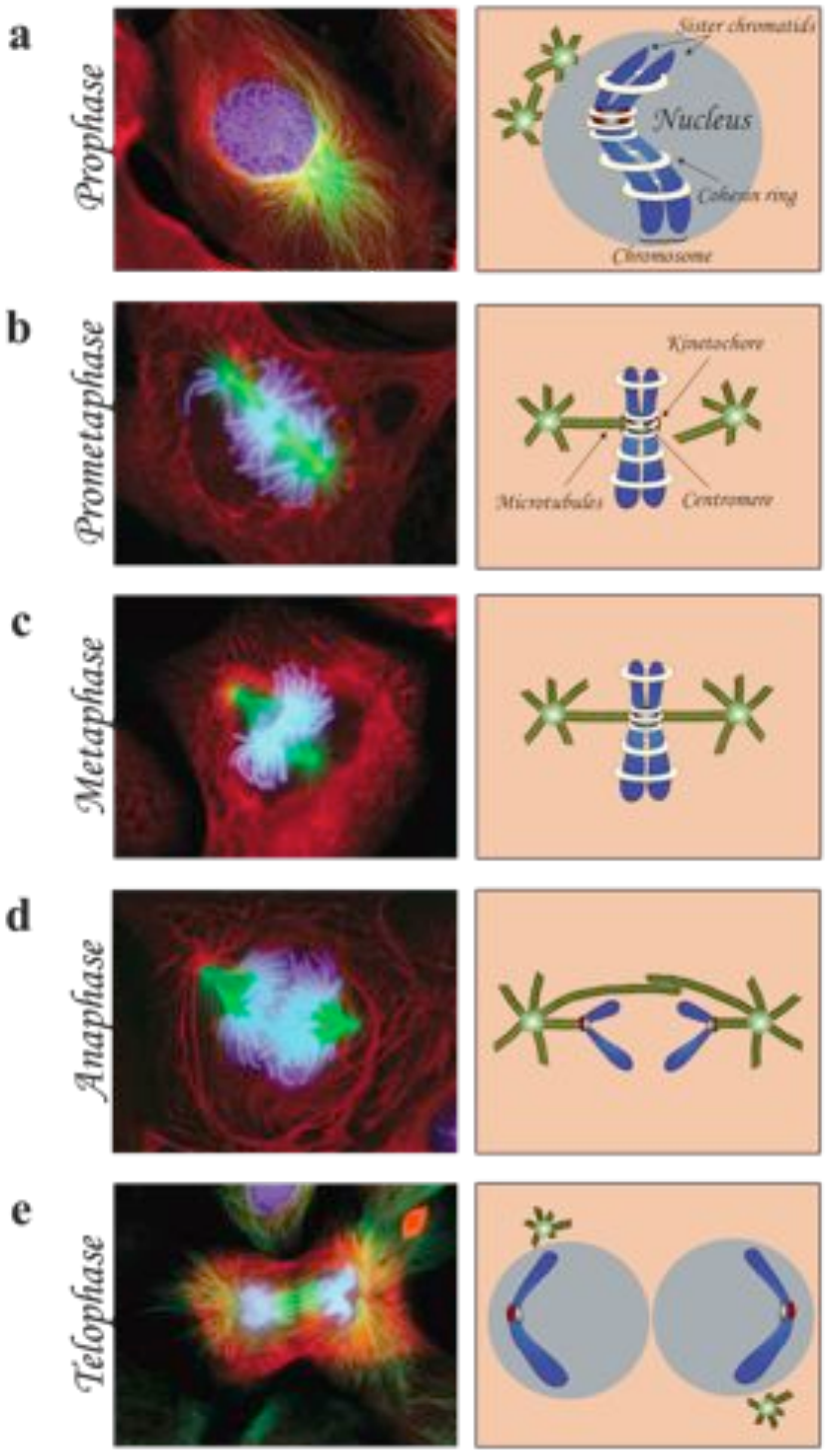

Figure 1.7 Stages of M phase (mitosis and cytokinesis) in a vertebrate cell
Schematic representation of the events occurring during mitosis and cytokinesis in vertebrates. Adapted from Visintin and Ciliberto, Encyclopedia of System Biology. Images are a courtesy of Dr. Conly Rieder.

Proper chromosome segregation requires that duplicated chromosomes (sister chromatids) are bi-polarly attached to microtubules (MTs). Bi-polar attachment is achieved when sister chromatids bind microtubules emanated by opposite poles. Microtubules are hollow filaments approximately 25 nm diameter (Amos and Klug, 1974). Each tubule forms from the parallel association of 13 linear tubulin protofilaments, which in turn form from GTP-dependent polymerization of αβ-tubulin heterodimers (Weisenberg and Deery, 1976). The asymmetry of the tubulin heterodimer imposes directionality on the MTs. α-tubulin subunits are exposed at the more stable minus end of the MT and tend to be buried in MTOCs. β-tubulin subunits are exposed at the less stable, dynamically growing plus end of the MTs. The behavior of individual MTs is characterized by "dynamic instability" (Mitchison et al., 1984), meaning that the polymer switches stochastically between phases of growth, pauses, and sudden disassembly by a GTP dependent mechanism. Microtubules dynamicity directs spindle assembly and contributes to the forces necessary for chromosome segregation. When a microtubule encounters (by chance) a specialized structure in the chromosome's primary constriction –the kinetochore– it is captured and stabilized. As spindle assembly progresses, kinetochore fibers, which are bundles of microtubules that link each kinetochore to opposite spindle poles, form. Concurrently, as a result of the stabilization of microtubules by the kinetochores, the initially symmetric array of astral microtubules is transformed into a typical spindle-like shape.

Despite the appealing simplicity of the search-and-capture model, this mechanism could not explain mitosis in cells that lack centrosomes, for example plant cells and oocytes. A variety of experiments have shown that chromatin plays a key role in spindle assembly in acentrosomal cells (Heald et al., 1996). Addition of DNA to *Xenopus* eggs and extracts lacking centrosomes results in microtubule formation near chromatin; kinetochores are not required for microtubule assembly under these conditions (Karsenti et al., 1984). Further experiments show that the small GTPase Ran, in its active GTP-bound form, can stimulate microtubule formation in the absence of centrosomes (Carazo-Salas et

al., 1999). GTP–Ran is generated near chromosomes because its exchange factor, Rcc1, localizes to chromatin (Carazo-Salas, 1999). Ran promotes spindle assembly by releasing spindle-assembly factors and molecular motors from inhibition by importin α and β (Ems-McClung et al., 2004; Gruss et al., 2001). A unifying model proposed by Wadsworth and Khodjakov suggests that spindle assembly might proceed by a generally conserved acentrosomal mechanism in all higher eukaryotes (Wadsworth and Khodjakov, 2004). They argued that, where present, the role of centrosome is to integrate preassembled spindle components such as kinetochore fibers and microtubule bundles into a common spindle, and to position the spindle in the cell. Recent observations in Jan Ellenberg laboratory by quantitative high-resolution confocal live-imaging further enrich this model. Looking at chromosome segregation driven by the acentrosomal spindle of maturing mouse oocytes, Ellenberg and colleagues showed that a microtubule network in prophase promotes the formation of multiple microtubules organizing centers (MTOCs) that in turn self organize in a fully functional barrel-shaped spindle in a kinesin-5 dependent manner (Schuh et al., 2007).

The terminology that describes the microtubules-kinetochore attachments accounts for both kinetochore orientation and the pole of origin of interacting microtubules, Fig. 1.8. Monotelic attachment is a normal stage of microtubules-kinetochore attachment and chromosome biorientation processes. In this condition only one kinetochore in a sister chromatid pair is attached to kinetochore microtubules, while the other is not. In contrast, in syntelic attachment, both sister kinetochores face and become attached to the same pole. Finally, merotelic attachments occur when either one or both sister kinetochores bind microtubules that arise from both poles even though they orient toward opposite poles. However, the only kinetochore-microtubule interaction resulting in proper chromosome segregation is the amphitelic or bipolar attachment. In the latter case, the sister kinetochores face opposite poles and each kinetochore binds only spindle microtubules irradiating from the pole it is facing.

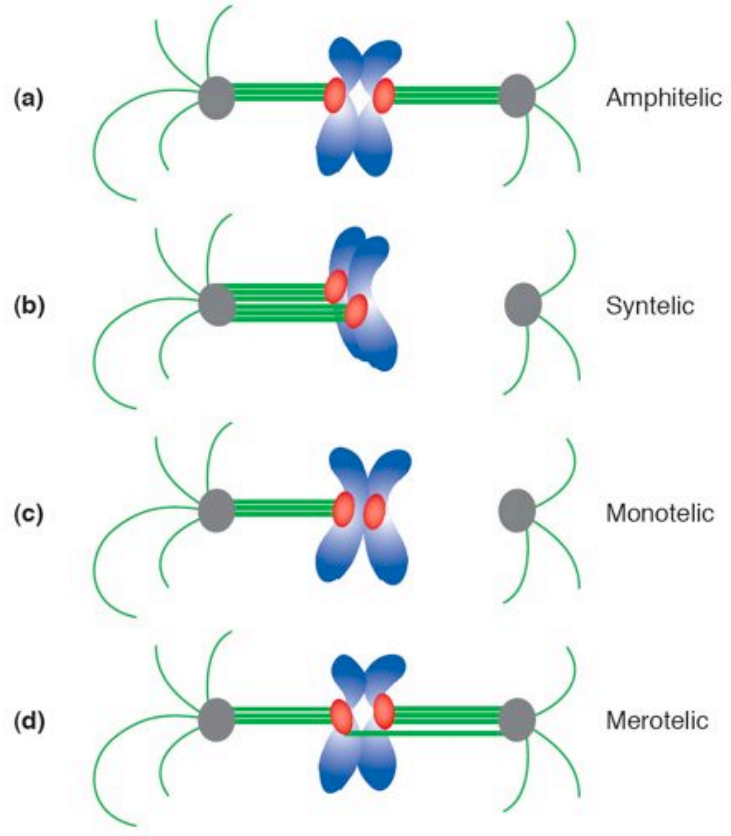

Figure 1.8 Type of microtubules-kinetochore attachments
(adapted from (Pinsky and Biggins, 2005)).

Sister chromatids are held together from S-phase through metaphase by cohesin. The cohesin complex – composed of two SMC proteins, Smc1 and Smc3, and two non-SMC components Scc1 and Scc3 – loads onto DNA during replication (Guacci et al., 1997; Nasmyth, 2002; Uhlmann, 2004). The discovery that cohesin's cleavable α-kleisin subunit Scc1 bridges the two heads of V-shaped Smc1/Smc3 heterodimers suggests that cohesin's stable association with chromatin arise from the trapping of double helical DNA molecules inside the tripartite ring created by Scc1, Smc1, and Smc3 (Haering et al., 2002; Ivanov et al., 2005).

Once all kinetochores have matured bipolar attachment to the mitotic spindle, the cell is defined as being in metaphase. Now, chromosome congress toward the center of the spindle to form the metaphase plate and tension is generated at kinetochores. APC/C then targets securin Pds1 for degradation (Yamamoto et al., 1996a; Yamamoto et al., 1996b), which in turn activates Separase, a protease that cleaves the Scc1 subunit of cohesin,

allowing the sisters to separate (Ciosk et al., 1998).

Metaphase ends with the rapid and almost synchronous separation of all the sister chromatids, which begin to segregate to opposite poles of the spindle (anaphase A), followed by elongation of the spindle itself. Once each set of sister chromatids has reached opposite spindle poles, the chromatids begin to decondense, the nuclear envelope re-forms and the mitotic spindle disassembles (telophase).

The Yeast mitosis

In most organisms, the accumulation of mitotic Cdk activity is prevented by inhibitory phosphorylation on Tyr15 mediated by Wee1 family of kinases. This event delays mitotic entry providing time to the cells to continue their growth and preparation for cell division (Morgan, 2007). This "Gap time" is called G2 phase. Only when Cdks are dephosphorylated by Cdc25 family phosphatase, mitotic Cdks can be activated and cells can progress into metaphase. Expression of a mutant version of Cdk, that can no longer be tyrosine phosphorylated causes premature mitotic entry in *S. pombe* (Gould and Nurse, 1989) or human cells (Krek and Nigg, 1991), but not in budding yeast (Amon et al., 1992). The phosphorylation of this specific tyrosine occurs also in budding yeast but its primary function is to delay the cell cycle in response to morphogenesis checkpoint activated by perturbation of the actin cytosckeleton (Keaton and Lew, 2006). Therefore, budding yeast lacks a canonical G2 phase and does not undergo a typical G2/M transition. However, in G2/M M-cyclins (*CLB1, 2, 3* and *4*) expression is switched on. This process depends, at least in part, on M-Cdk activity itself. Indeed, the rising M-Cdk activity promotes the activation of the gene regulatory factor Mcm1-Fkh1/2-Ndd1 which trigger the expression of the *CLB2* cluster genes, comprising genes encoding for mitotic regulators. This cluster includes genes encoding for M-cyclin Clb1 and Clb2, the APC/C activator Cdc20 and the polo-like kinase Cdc5.

Mitotic entry

In contrast to higher eukaryotes, *Saccharomyces cerevisiae* undergoes a closed mitosis and possess a relatively simple spindle in which the main MTOCs spindle pole bodies (SPBs) remain embedded in the nuclear envelope and chromosomes are bound to microtubules throughout the cell cycle with the possible exception of a brief period during the replication of centromeres (Adams and Kilmartin, 2000). This results in close association between chromosomes and SPBs from telophase through prometaphase in budding yeast (Guacci et al., 1997).

During entry into mitosis sister-chromatids condense into a rod-like structure in order to be easily moved and separated. Two sets of microtubles nucleated by the duplicated, opposing spindle pole bodies (SPBs) interdigitate in the spindle midzone to form a bipolar array of microtubules called mitotic spindle. Next, duplicated spindle pole bodies (SPBs) migrate towards the opposite site of the nucleus. Faithful chromosome segregation during mitosis requires sister-chromatids to be properly aligned on the mitotic spindle prior to cohesion loss. Sister chromatids are aligned in metaphase by the microtubule based mitotic spindle, which attaches to individual chromatids via the kinetocore, a large protein complex that assembles on centromeric DNA. Unlike metazoans, however, budding yeast chromosomes do not undergo congression *per se*. *S. cerevisiae* kinetochores do adopt a highly characteristic bilobed metaphase configuration analogous to the metaphase plate (Goshima et al., 2000; He et al., 2000). This bilobed localization pattern is the result of both transient sister separation caused by tension across centromeres and chromosome oscillation and it changes subtly on a timescale of seconds as the extent of overlap among microtubules varies (He et al., 2000). Similarly to Metazoans, microtubules attachment to kinetocores proceeds through a random "search and capture" mechanism, which does not guarantees proper sister-chromatids orientation (McIntosh et al., 2002; Walczak and Heald, 2008). Of course, to guarantee the accurate

segregation of the duplicated chromosomes, the attachment of all the sister-chromatids to the mitotic spindle has to occur in a bipolar manner, with the two chromatids of a pair bound to microtubules originating from opposite poles. Dedicated surveillance mechanisms exist to monitor and correct improper sister-chromatids orientation. Cells determine if sister-chromatids are properly attached and oriented on the spindle by monitoring the status of tension at sister-kinetocores. Tension is generated across sister-kinetocores when cohesin complexes linking sister-chromatids resist pulling forces exerted by the microtubules emanating from opposite spindle poles (Dewar et al., 2004; Tanaka et al., 2002; Tanaka et al., 2005). Co-oriented sister-chromatids, or those pairs in which only one sister is attached to a microtubule, are unable to generate tension. Kinetocore-microtubule attachments that fail to generate tension are detached by the Aurora family kinases Ipl1 (Biggins et al., 2001; Dewar et al., 2004; Tanaka et al., 2002), that activates the spindle assembly checkpoint (SAC). The SAC is a surveillance mechanism that monitors the segregation of the genetic material during mitosis, and delays the onset of anaphase until all the requirements are satisfied. Even if a single kinetocore is not properly attached, a "wait" anaphase signal is generated that prevents sister-cromatids separation. Once SAC is active it delays the metaphase-anaphase transition until all sister-chromatids are properly bi-oriented on the spindle. The uninterrupted destabilization of improper kinetocore-microtuble attachment by Ipl1 ensures that all sister-chromatids re-establish a correct microtubule attachment now able to generate tension. Only when tension is detected across all sister-chromatids, will the SAC be satisfied and metaphase proceed into anaphase.

The kinases regulating the entry into mitosis are the Clb1-4 mitotic cyclin-Cdks whose transcription has already initiated in G2 phase and whose activation is permitted by the degradation of their inhibitors Sic1 and Swe1. Sic1 is targeted to proteasomal degradation by phosphorylation mediated by the Cln1/Cln2 G1/S-phase cyclins. While responsible for Swe1 degradation is the bud neck-localized fraction of the Polo-like kinase

Cdc5 that phosphorylates and targets Swe1 for proteosomal degradation via APC/C^{Cdh1}.

Exit from Mitosis

Exit from mitosis is the process by which sister chromatids are abruptly separated through the cleavage of the cohesin complex and then pulled towards the opposite poles of the spindle, culminating in the splitting of two identical cells. It is initiated by the activation of the Anaphase-Promoting Complex or Cyclosome (APC/C) and by the down-regulation of Cdk1 complexes. Next, the phosphate groups that Cdk1 added to its targets and allowed cells to enter mitosis must be removed so that the cells can exit from mitosis, a process taken care of by specific phosphatases (De Wulf et al., 2009; Queralt and Uhlmann, 2008).

Within mitotic exit, two major transitions are identified: i) the metaphase-anaphase transition and ii) the telophase-G1.

The metaphase-anaphase transition

Passage from metaphase to anaphase is triggered by the dissolution of cohesion between the sister-chromatids. At anaphase onset, the Scc1/Mcd1 subunit of the cohesin complex is cleaved by a CD clan family protease called Separase (Esp1 in *S. cerevisiae*, reviewed in (Nasmyth, 2001)), which unlinks the sister chromatids and allow them to be pulled to the opposite poles of the cell (Ciosk et al., 1998; Uhlmann et al., 1999). Prior to anaphase, Separase is kept inactive by the binding of Securin (Pds1 in budding yeast) (Ciosk et al., 1998; Yamamoto et al., 1996). Pds1 plays two opposing roles in Esp1 regulation: an activating one promoting the nuclear import of Esp1 (Jensen et al., 2001; Agarwal and Cohen-Fix, 2002), and an inhibitory one by keeping Separase inactive through the binding (Ciosk et al., 1998). The crucial event that make Separase free is the activation at the metaphase-anaphase transition of the anaphase promoting complex APC/C in association with its specificity factor Cdc20, that once active targets Psd1 for

proteosomal degradation (Ciosk et al., 1998; Cohen-Fix et al., 1996; Peters, 2006), Fig. 1.9. At this time APC/C^{Cdc20} targets for degradation also S- and M-phase cyclins a process that culminates in cyclins full and partial elimination, respectively.

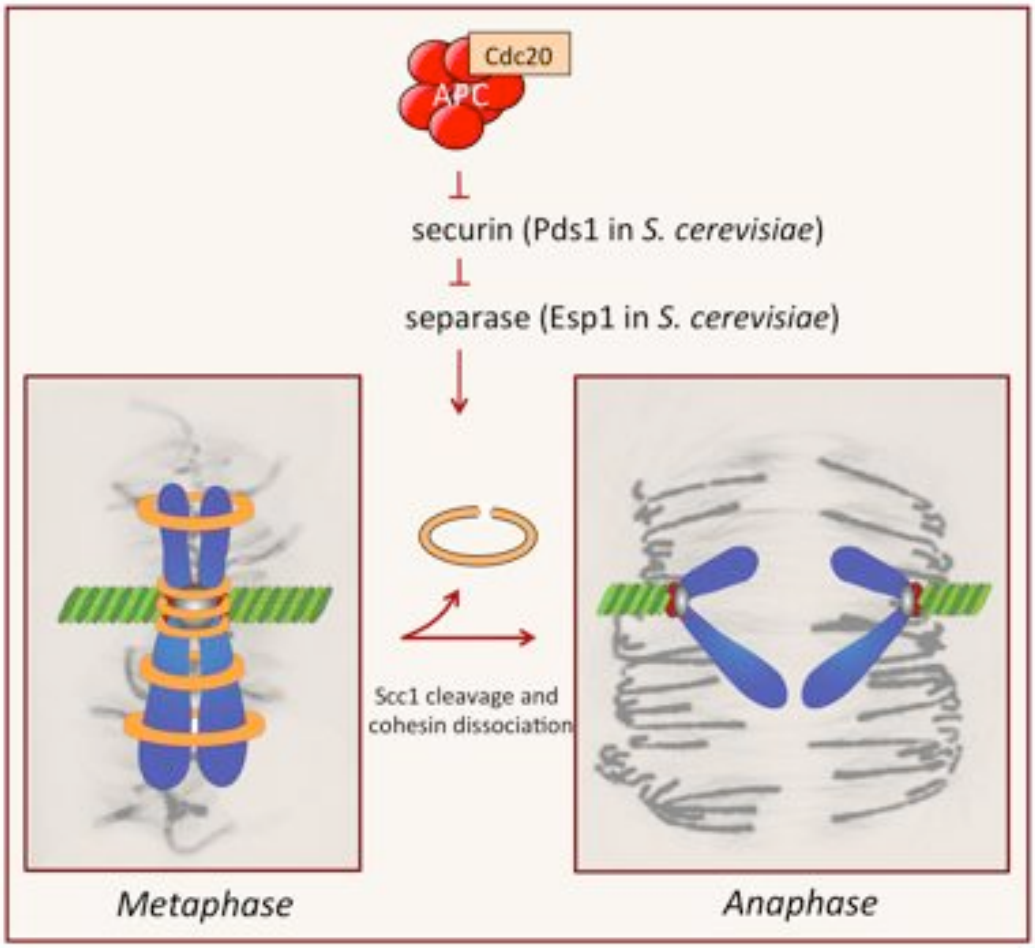

Figure 1.9 APC/C triggers the metaphase-anaphase transition
In all the eukaryotic organisms APC/C^{Cdc20} ubiquitylates securin (Pds1 in *S. cerevisiae*) and thereby targets it for proteolysis by the proteasome. This process liberates separase (Esp1 in *S. cerevisiae*) and allows it to mediated the proteolytic cleavage of the cohesin subunit Scc1. This event triggers the initiation of sister-chromatid separation.

Telophase-G1

The telophase-G1 transition requires the complete inactivation of the Clb-Cdk complexes and removal of the phosphate groups that Cdk added to its targets.

In contrast to most other eukaryotes in which M-Cdks inactivation is completed at the meta-to-anaphase transition, in budding yeast a pool of mitotic Cdk activity persists until anaphase spindle elongation is complete (telophase) (Jaspersen et al., 1998;

Shirayama et al., 1994; Surana et al., 1993). In this organism, this pool of mitotic Cdk activity is primarily inactivated during later stages of anaphase by the APC/C now associated with its specificity factor Cdh1, which targets mitotic cyclins for ubiquitin-mediated proteolysis, Fig. 1.10 (Peters, 2002; Visintin et al., 1997; Zachariae et al., 1998).

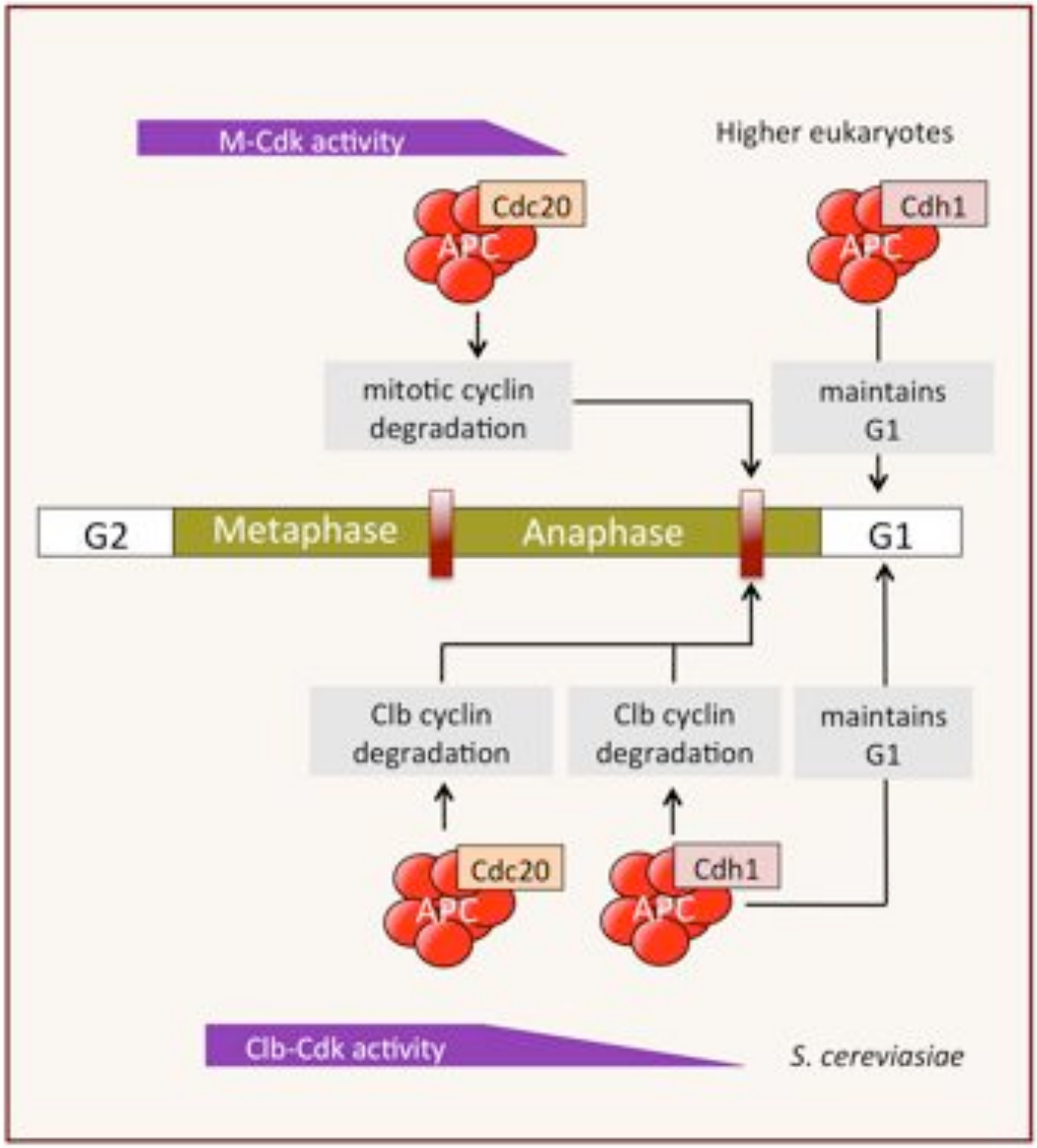

Figure 1.10 APC/C promotes exit from mitosis
In eukaryotic organisms, exit from mitosis requires the inactivation of M-Cdks, which is mainly obtained by the degradation of mitotic cyclins (Clb cyclins in *S. cerevisiae*). In most eukaryotes, mitotic cyclin degradation is initiated and completed at the metaphase-anaphase transition by the APC/C^{Cdc20}. In contrast, in budding yeast a pool of Clb-Cdk activity persists until late anaphase. In this organism, Clb cyclin degradation is initiated by APC/C^{Cdc20} at the metaphase-anaphase transition and completed by APC/C^{Cdh1} in late anaphase. In all eukaryotes, the APC/C^{Cdh1} maintains mitotic Cdks (Clb-Cdks in *S. cerevisiae*) inactive in G1.

Accumulation of the Cdks inhibitor Sic1, which directly binds to, thereby inhibiting, the cyclin-Cdk complexes, further ensures the precipitous inactivation of the M-Cdk at the end of mitosis (Mendenhall, 1993; Schwob et al., 1994). Responsible for both the activation of the APC/C^{Cdh1} and the accumulation of Sic1, is the highly conserved protein phosphatase Cdc14 (Visintin et al., 1998), Fig. 1.11.

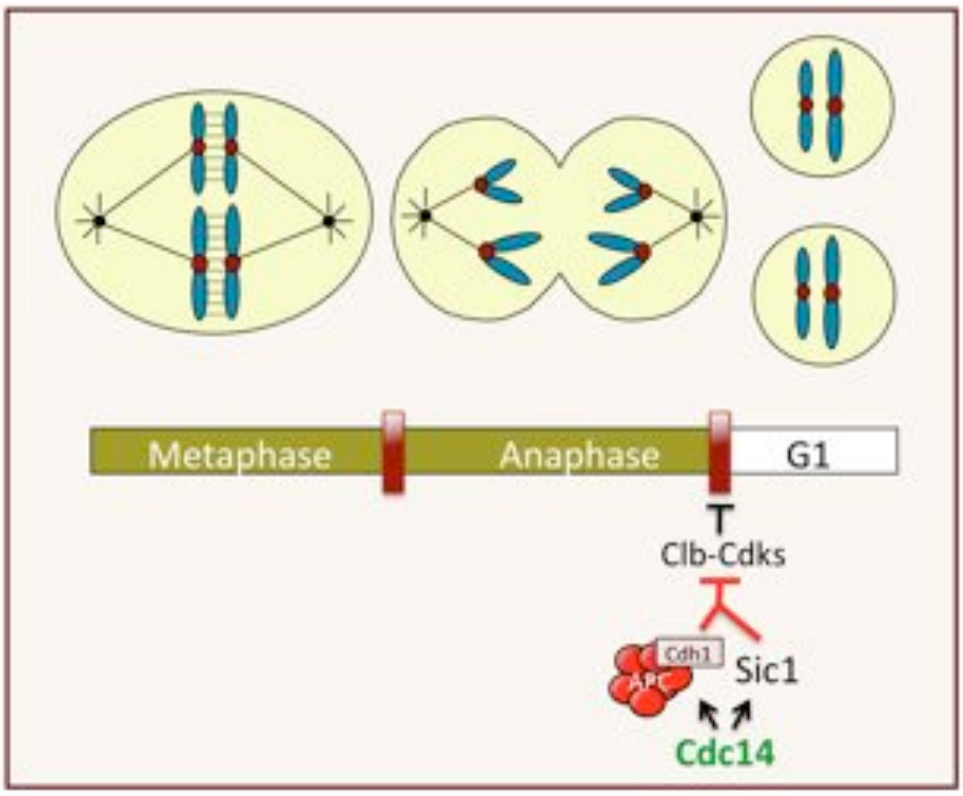

Figure 1.11 The protein phosphatase Cdc14 triggers exit from mitosis in *S. cerevisiae*
In the budding yeast *S. cerevisiae* the protein phosphatase Cdc14 is responsible for triggering Clb-Cdk inactivation in late mitosis by promoting both the activation of the APC/C^{Cdh1} and the accumulation of the Clb-Cdk inhibitor Sic1.

Beside cyclin-Cdks inactivation, the fate of the substrates that had been phosphorylated by cyclin-Cdks is of equal importance. When the cells have to exit mitosis cyclin-Cdks phosphorylation have to be reverted. This further contributes to the resetting of the cell cycle to G1. The timing of Cdks substrates dephosphorylation is not dictated simply by the time of cyclin-Cdk kinase inactivation (via cyclin degradation) but also by the dynamics of specialized phosphatases able to catalyze the reverse reaction. As for the Cdk dependent phosphorylation, also the dephopshorylation of Cdks targets occurs in a

specific order to guarantee the correct sequence of cell cycle events (De Wulf et al., 2009; Sullivan and Morgan, 2007).

In budding yeast responsible for this task is the protein phosphatase Cdc14. Indeed cell lacking Cdc14 function arrest in late anaphase with high mitotic Cdks activity (Visintin et al., 1998; Wan et al., 1992). Conversely, overexpression of *CDC14* results in inappropriate mitotic Cdks inactivation (Visintin et al., 1998). Cdc14 promotes mitotic Cdks inactivation by reversing Cdks phosphorylation events. Cdc14 dephosphorylates Cdh1 making it able to bind APC/C, thereby activating the complex (Visintin et al., 1998; Zachariae et al., 1998; Jaspersen et al., 1998). This APC/C activation, as previously said, induces the degradation of M-cyclins. Furthermore Cdc14 phosphatase dephosphorylates the Cdks inhibitor Sic1 and its transcription factor Swi5. Swi5 dephosphorylation induces its translocation from the cytoplasm to the nucleus where it triggers *SIC1* transcription. While dephosphorylation of Sic1 prevents its degradation by the ubiquitin-protein ligase SCF^{Cdc4}, resulting in Sic1 stabilization (Moll et al., 1991; Knapp et al., 1996; Feldman et al., 1997; Skowyra et al., 1997; Visintin et al., 1998; Jaspersen et al., 1999). Thus, Cdc14 provides the accumulation of Sic1 and thus the inhibition of M-Cdks. Cdc14 has many other substrates in the cell, and it is likely that Cdc14 dephosphorylate many if not all Clb-Cdks substrates, contributing to the regulation of multiple processes during anaphase.

Regulation of Cdc14 during mitosis in *S. cerevisiae*

The activity of Cdc14 is controlled by changes in its subcellular localization. For most of the cell cycle up to metaphase Cdc14 is kept inactive in the nucleolus by the binding to its competitive inhibitor Cfi1 (also known as Net1) (Shou et al., 1999; Visintin et al., 1999). During anaphase, Cdc14 becomes released from its inhibitor, spreading in the nucleus and in the cytoplasm, where it dephosphorylates its substrates. The dissociation of Cdc14 from its inhibitor during anaphase is controlled by the sequential activation of two regulatory networks (Stegmeier and Amon, 2004). At the onset of anaphase, the Cdc

Fourteen Early Anaphase Release (FEAR) network initiates the release of Cdc14 from its inhibitor and the phosphatase spreads throughout the nucleus, while during the later stages of anaphase the Mitotic Exit Network (MEN) induces further release of Cdc14 and results in Cdc14 being released both in the nucleus and in the cytoplasm and maintains the phosphatase in its released state, Fig. 1.12.

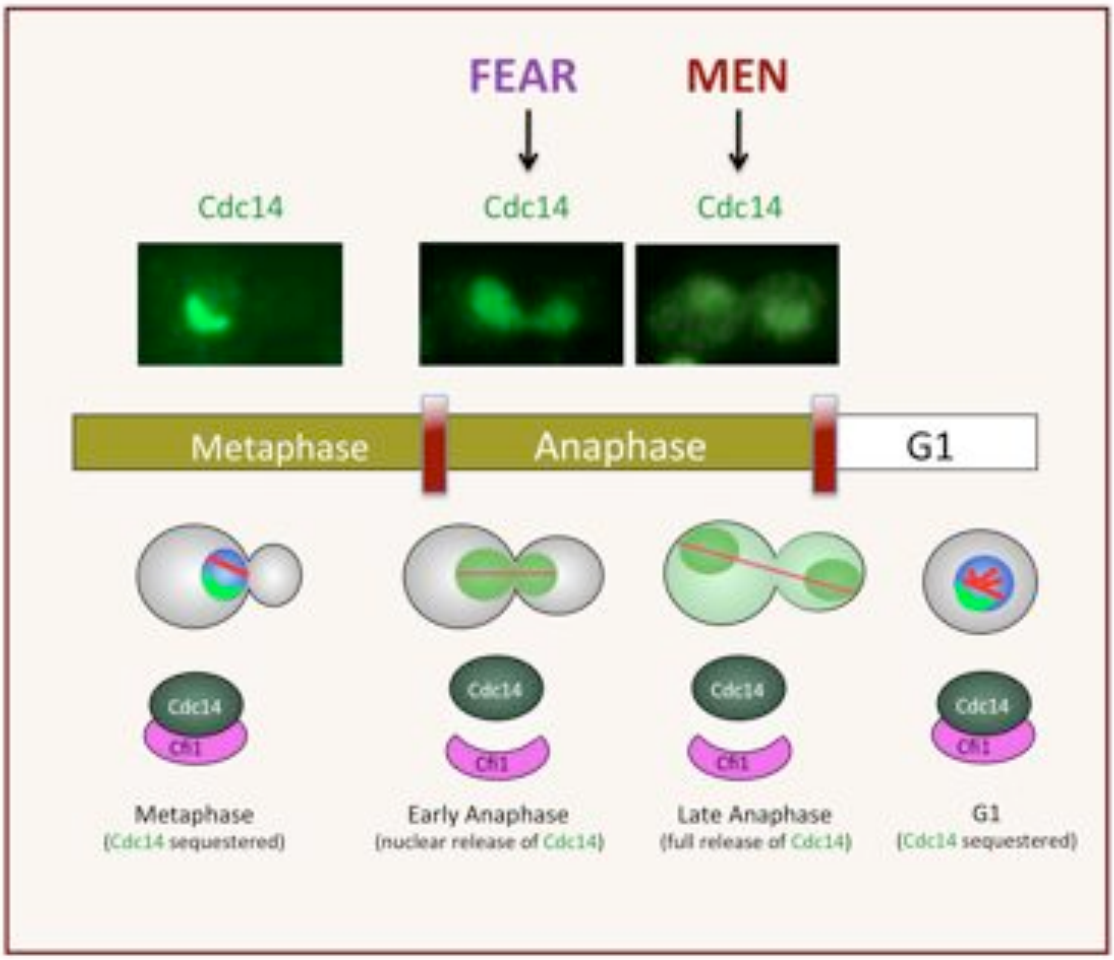

Figure 1.12 Regulation of Cdc14 during the cell cycle
The activity of Cdc14 is controlled by changes in its intracellular localization. From G1 up to metaphase, Cdc14 is sequestered in the nucleolus by binding to its inhibitor Cfi1. During anaphase Cdc14 is released from its inhibitor as a consequence of the sequential activation of two signaling networks. The FEAR network promotes a transient nuclear release of Cdc14 from the nucleolus during early anaphase. The Mitotic Exit Network (MEN) promotes a sustained release of the phosphatase from its inhibitor both in the nucleus and into the cytoplasm during later stages of anaphase. When exit from mitosis is completed Cdc14 is resequestered in the nucleolus. Concentrations of Cdc14 are shown in different shades of green, where dark green corresponds to high amount of Cdc14 and light green corresponds to low amount of Cdc14; nucleus, blue; microtubules, red lines.

The qualitative difference in Cdc14 release caused by the FEAR network (primarily nuclear) and by the MEN (nuclear and cytoplasmic) is at the basis of Cdc14 function regulation. Interestingly, two screens aimed to identify loss-of-function mutants able to bypass the mitotic exit defect of the MEN mutant identified proteins involved in the nuclear-cytoplasmic transport (Asakawa and Toh-e, 2002; Shou and Deshaies, 2002). In addition to give rise to a qualitative difference in Cdc14 release, there are quantitative differences in the extent that FEAR network and MEN promote exit from mitosis. Indeed, the release of Cdc14 induced by the MEN is essential for mitotic exit as MEN mutant arrest in late anaphase with high levels of mitotic Cdk activity (Jaspersen et al., 1998; Shirayama et al., 1994b; Yoshida et al., 2002). In contrast, the FEAR-dependent release of Cdc14 is required for a timely exit from mitosis but it is not essential for this process. This is demonstrated by the fact that mutation in FEAR network components delay, but do not prevent, exit from mitosis (Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002). Furthermore the Cdc14 release induced by the FEAR network is transient, as in absence of MEN signaling Cdc14 is not maintained in its released state but it is re-sequestered in the nucleolus (Pereira et al., 2002; Shou et al., 1999; Stegmeier et al., 2002; Visintin et al., 1999; Yoshida et al., 2002). These observations imply that Cdc14 activated by the FEAR network does not lower mitotic Cdks enough to trigger exit from mitosis. This inability may be explained by the fact that the FEAR network is not able to direct large amount of Cdc14 to the cytoplasm, where its main targets for mitotic exit Cdh1 and Swi5 are located, and that the FEAR network activity is restricted to a brief window during early anaphase (Nasmyth et al., 1990). Despite qualitative and quantitative differences in FEAR network and MEN-mediated Cdc14 activation, the question arises as to why budding yeast uses two pathways rather then one to regulate Cdc14 activity. Employing multiple pathways may allow for more elaborate regulation of exit from mitosis. Another possibility is that the Cdc14 fraction released by the FEAR network performs function during mitosis that are different from that of the Cdc14 fraction release by the MEN. A lot

of studies underscored this idea. Indeed, Cdc14 released by the FEAR network has been implicated in regulating different processes during anaphase, such as chromosomes segregation and localization of chromosomal passenger proteins (see below, Pereira et al., 2003; D'Amurs et al., 2004). A hypothesis is that the levels of Cdc14 activity needed to accomplish these tasks are lower than the one needed to promote exit from mitosis. Hence, a pathway that activates briefly Cdc14 would allow the phosphatase to accomplish these early anaphase events, while a pathway that leads to the complete release and a full activation of the Cdc14 would allow the phosphatase to perform its mitotic exit-promoting function. Furthermore, the fact that different anaphase events depend on different and increasing amounts of Cdc14 activity might help to establish the order by which they occur (Bouchoux and Uhlmann, 2011).

Regulation of Cdc14/Cfi1 interaction

Although it is clear that the FEAR network and the MEN promote the release of Cdc14 from its inhibitor during anaphase, the molecular mechanisms by which they break the complex apart are only partially understood. The observation that both Cdc14 and Cfi1 are phosphorylated during anaphase and that the timing of these phosphorylations correlate with the timing or Cdc14 release from the nucleolus, raised the possibility that the complex dissociation was regulated by this post-transcriptional modification (Shou et al., 2002; Visintin et al., 2003; Yoshida and Toh-e, 2002). Consistent with this notion, the phosphorylation of Cfi1 destabilises the complex *in vitro* (Shou et al., 2002a). Moreover it has been demonstrated that the phosphorylation of Cfi1 by the FEAR network is essential for the release of Cdc14 during the early stages of anaphase (Azzam et al., 2004).

Responsible for the phosphorylation events leading to the dissociation of the complex is a combination of specific kinases involved in both the FEAR network and the MEN that leads to the dissociation of Cdc14 from its inhibitor in early anaphase and then in late anaphase respectively (see next session).

The Cdc Fourteen Early Anaphase Release (FEAR) network

That a MEN-independent release of Cdc14 existed was first discovered in MEN mutants. Indeed, several groups observed Cdc14 is transiently released into the nucleus during early anaphase even in the absence of MEN activity (Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002). Several proteins, collectively referred to as FEAR network, are required for the release and thus activation of Cdc14 during the early stages of anaphase, Fig 1.13.

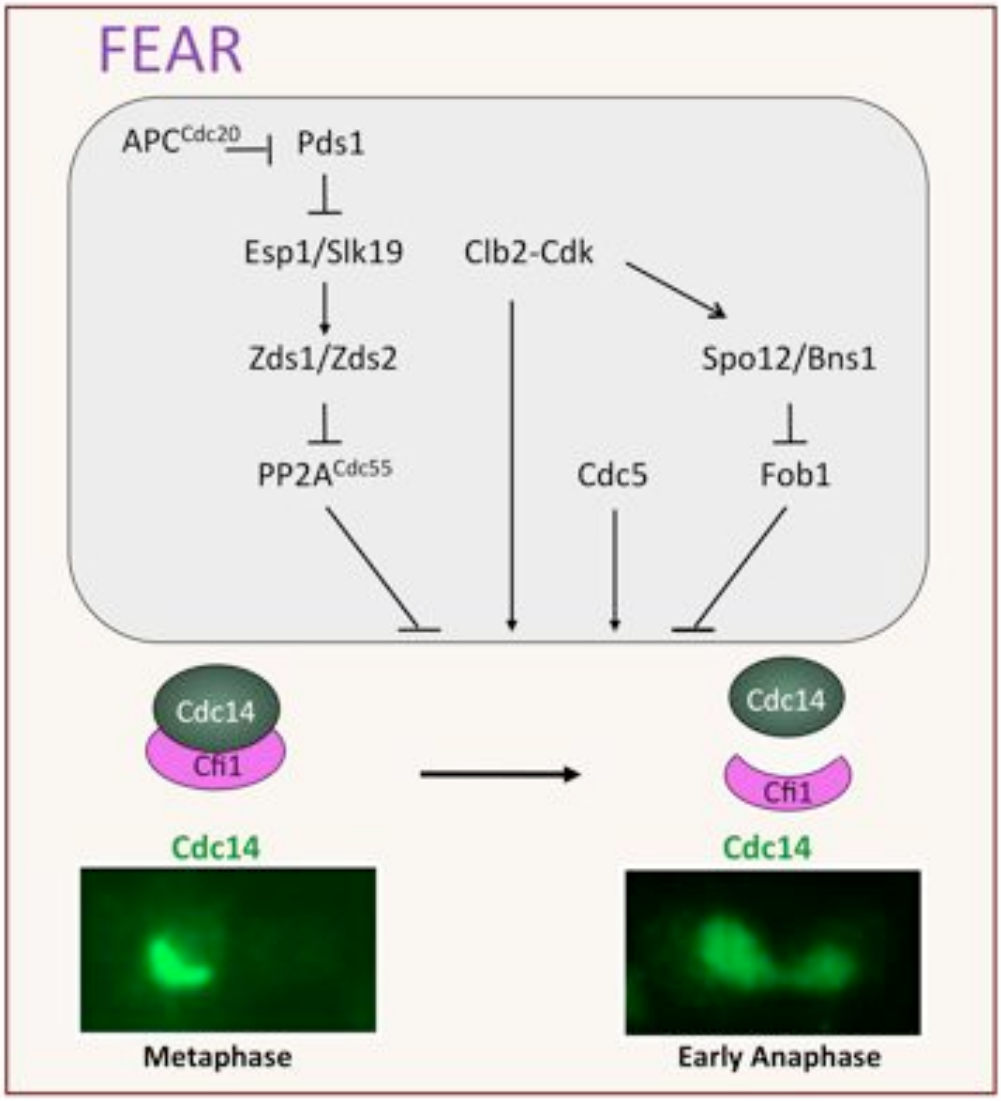

Figure 1.13 The Cdc Fourteen Early Anaphase Release (FEAR) network
At anaphase onset, the degradation of securin (Pds1) induced by the APC/C^{Cdc20} leads to the activation of separase (Esp1), an essential component of the FEAR network. Separase, together with Slk19, induces the downregulation of of the protein phosphatase $PP2A^{Cdc55}$, a process that requires the Cdc55-interacting proteins Zds1 and Zds2. This allows the Clb2-Cdk-dependent phosphorylation of Cfi1 and Spo12, which lead to the dissociation of Cdc14 from its inhibitor. Cfi1 phosphorylation by Clb2-Cdk directly promotes the dissociation of the Cdc14-Cfi1 complex. Spo12 phosphorylation by Clb2-Cdk induces the protein to inhibit Fob1, which inhibits the dissociation of the Cdc14-Cfi1 complex. The activity of the polo-like kinase Cdc5 is also required for the release of the phosphatase at this stage of the cell cycle.

The FEAR network is comprised of the separase Esp1, the separase-binding protein Slk19, the small nucleolar protein Spo12 and its homologue Bns1, the replication fork block protein Fob1, the PP2A posphatase bound to its regulatory subunit Cdc55

(PP2A^{Cdc55}), the Clb2-Cdk kinase complex and the Polo-like kinase Cdc5 (Azzam et al., 2004; Pereira et al., 2002; Queralt et al., 2006; Stegmeier et al., 2002; Sullivan and Uhlmann, 2003; Yoshida and Toh-e, 2002). Among all, positive regulators of the FEAR are Esp1, Slk19, Spo12, Bns1, Clb2-Cdk and Cdc5, while proteins that regulate the FEAR network activity in a negative manner are Fob1 and PP2A^{Cdc55}. Epistasis analysis suggests that the FEAR network consists of at least two parallel branches, with *SPO12*, *BNS1* and *FOB1* functioning in one branch of the pathway, and *ESP1* and *SLK19* in the other (Sullivan and Uhlmann, 2003; Visintin et al., 2003). The Polo-like kinase Cdc5 acts downstream of, or in parallel to, the *ESP1-SLK19* branch. The position of Cdc5 within the FEAR is difficult to determine because Cdc5 is also a regulator of the MEN pathway. It is however likely that Cdc5 is the ultimate effector of the FEAR network, as its overexpression suppresses the defects in Cdc14 release of cells impaired in both *SPO12* and *ESP1* branches and it is sufficient to promote ectopic release of Cdc14 in absence of MEN and Esp1 activity (Visintin et al., 2008; Visintin et al., 2003). Little is known about the relationship among FEAR network components. Slk19 is cleaved by Esp1 at the metaphase-anaphase transition (Sullivan et al., 2001) but Slk19 cleavage is not required for its mitotic exit function (Stegmeier et al., 2002; Sullivan et al., 2003). Remarkably, Esp1 promotes the release of Cdc14 independently of its protease function (Buonomo et al., 2003; Sullivan et al., 2003), but the nature of this protease-independent signalling mechanism remains elusive. Rather than Slk19 being regulated by Esp1, it seems that they function together to promote Cdc14 release from the nucleolus. Slk19 forms a complex with Esp1 targeting Esp1 to kinetocores and the spindle midzone (Sullivan et al., 2003). Thus it is possible that Slk19 promotes the protease-independent function of Esp1. Importantly, both the protease dependent and independent function of Esp1 are inhibited by the securin Pds1, thus enabling securin to restrain both the onset of sister-chromatids separation and FEAR-mediated activation of Cdc14 (Sullivan and Uhlman, 2003). A study by Azzam and colleagues provided insights into the mechanisms by which the FEAR

network promotes the release of Cdc14 from its inhibitor Cfi1. They showed that Cfi1 phosphorylation by Clb2-Cdk on six Cdk consensus sites is essential for the release of Cdc14 from the nucleolus during early anaphase. Indeed mutants carrying mutations of these 6 residues into alanine, an amino acid that can no longer be phosphorylated, exibit defects in Cdc14 release from the nucleolus during early anaphase (Azzam et al., 2004). The FEAR negative regulator $PP2A^{Cdc55}$ initially antagonizes the Clb2-dependent phosphorylation of Cfi1 keeping Cfi1 itself in an hypophosphorylated form during metaphase (Queralt et al., 2006). At the metaphase-anaphase transition, once securin is degraded, Esp1 in complex with Slk19 triggers the downregulation of $PP2A^{Cdc55}$. Although the mechanism by which Esp1 promotes $PP2A^{Cdc55}$ inactivation remains unknown, $PP2A^{Cdc55}$ inactivation results in an increase of Clb2-Cdk-dependent phosphorylation of Cfi1, that culminates in the dissociation of Cdc14 from its inhibitor (Queralt et al., 2006).

Cdc5 plays an essential role in Cdc14 release from the nucleolus (Stegmeier et al, 2002; Yoshida et al., 2002; Visintin et al., 2008). Indeed, *cdc5* mutant cells are impaired in the release of Cdc14 during both early and late anaphase. When overexpressed, Cdc5 can release Cdc14 from the nucleolus in the absence of MEN function (Visintin et al., 2003). Moreover inactivation of the kinase is sufficient to induce ectopic re-sequestration of Cdc14 in the nucleolus (Visintin et al., 2008). The way through which Cdc5 contributes to Cdc14 release from the nucleolus during early anaphase is still unknown. It has been observed that the overexpression of *CDC5* induces the ectopic release of Cdc14 with a concomitant appearance of phosphorylated forms both Cdc14 itself and Cfi1 (Visintin et al., 2003). Both Cdc14 (unpublished data) and Cfi1 (Shou et al., 2002) are phosphorylated by Cdc5 *in vitro* and Cfi1 phosphorylation by Cdc5 has been demonstrated to induce the dissociation of the complex Cdc14-Cfi1. Thus, Cdc5 could mediate Cdc14 release in early anaphase by directly promoting Cdc14 and/or further Cfi1 phosphorylation. Consistent with this hypothesis Cdc5 directly interact with Cdc14 *in vitro* (Rahal and Amon, 2008). Furthermore, Cdc5 promotes the release of Cdc14 during early anaphase also in an indirect

way. It induces Clb2-Cdk-dependent phosphorylation of Cfi1 through a $PP2A^{Cdc55}$ independent mechanism (Liang et al., 2009). At anaphase onset, Cdc5 phosphorylates, thus promoting its degradation, the M-Cdks inhibitor Swe1. Swe1 degradation results in Clb-Cdks activation thus leading to a Clb2-Cdk dependent phosphorylation of Cfi1 culminating in Cdc14 release from the nucleolus (Liang et al., 2009).

Several studies provided insights into the mechanism by which Spo12, together with the FEAR inhibitor Fob1, promotes the release of Cdc14 from Cfi1. The replication fork block Fob1 directly binds Cfi1, preventing the dissociation of Cdc14 from the complex (Stegmeier et al., 2004). Spo12 also binds this multicomplex, and once it is phosphorylated by Clb2-Cdk kinase in early anaphase (Tomson et al., 2009), it induces a conformational change in Fob1 which reduces its ability to inhibit the dissociation of Cdc14-Cfi1 complex.

Recently chromatin has also been implicated in regulating the release of Cdc14 from the nucleolus during early anaphase. More specifically, monoubiquitination of histone H2B and methylation of histone H3 seem to be required for Cdc14 release from the nucleolus at this cell cycle stage (Hwang et al., 2009). The modified chromatin structure would allow the access of the FEAR network components to the Cdc14-Cfi1 complex (Hwang et al., 2009).

The Mitotic Exit Network (MEN)

The Mitotic Exit Network (MEN) was the first signaling network identified to regulate the subcellular localization of Cdc14 (Shou et al., 1999; Visintin et al., 1999). The MEN resembles a Ras-like signaling cascade and is comprised of the GTPase Tem1, a positive regulator Lte1, the two-component GAP (GTPase-activating protein) Bub2-Bfa1/Byr4, the protein kinases Cdc5, Cdc15 and Dbf2, the Dbf2-associated factor Mob1, and the scaffold protein Nud1 (Bardin and Amon, 2001). Inactivation of this signaling cascade causes cells to arrest in late telophase with Cdc14 sequestered in the nucleolus

(Shou et al., 1999; Visintin et al., 1999). A combination of genetic and biochemical investigations suggested the model for MEN signalling outlined in Fig. 1.14.

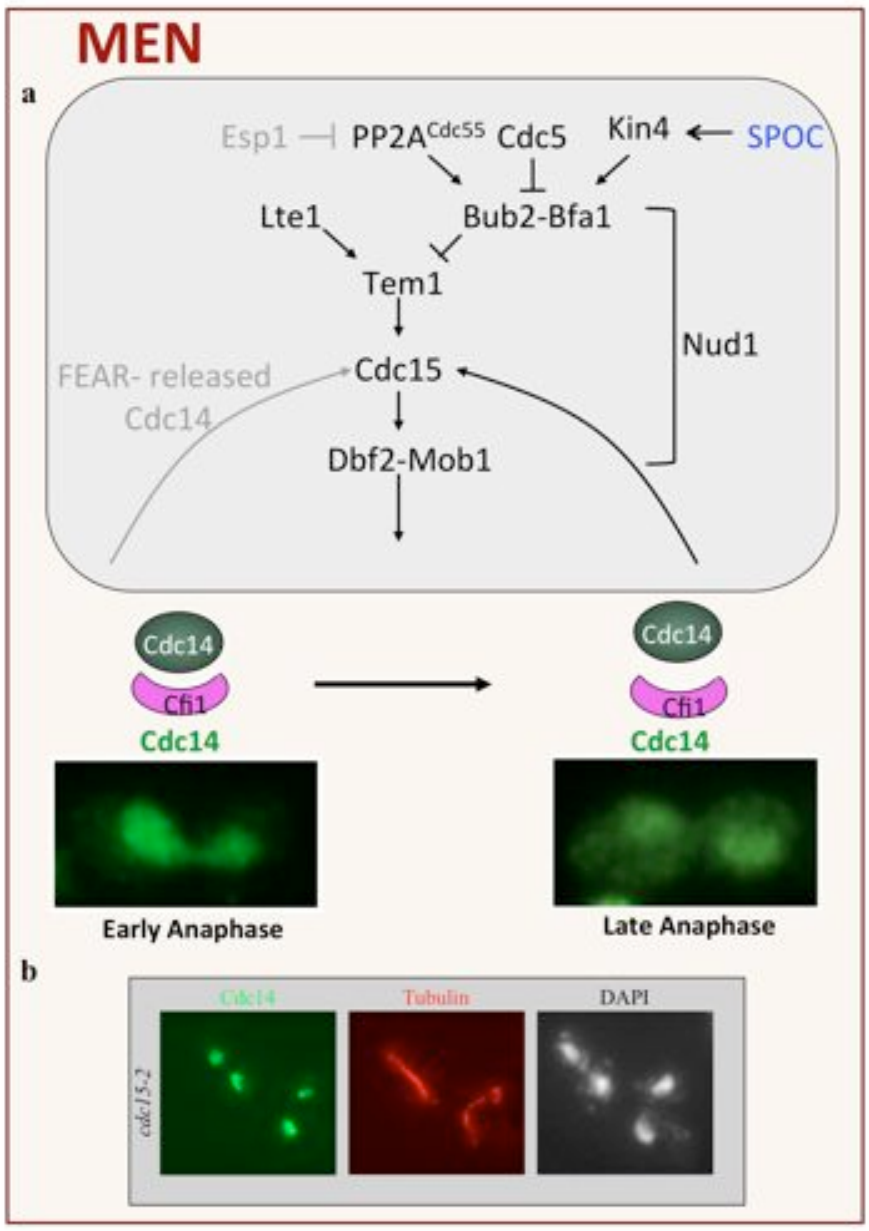

Figure 1.14 The Mitotic Exit Network (MEN)
(a) The MEN is a Ras-like signaling cascade which promotes the release of Cdc14 from its inhibitor Cfi1 during late anaphase. Although the MEN is sufficient to promote the release of Cdc14 from Cfi1, in an umperturbed cell cycle FEAR network activity contributes to MEN activation (shown in gray). The GTPase Tem1 acts at the top of the MEN and is positively regulated by Lte1 and negatively regulated by the Bub2-Bfa1 complex. Once activated, Tem1 propagates the signal to the protein kinase Cdc15, which in turn activates Dbf2-Mob1. Nud1 acts as a scaffold for Tem1, Cdc15, Dbf2, Bub2 and Bfa1 on the spindle pole body (SPB). MEN activation is achieved by: (1) inactivation of Bub2-Bfa1 promoted by Cdc5 activity and by separase (Esp1)-dependent downregulation of the protein phosphatase $PP2A^{Cdc55}$; (2) dephosphorylation of Cdc15 by the FEAR network-released Cdc14; (3) movement of the Tem1-bearing SPB into the bud, where Lte1 is located; (4) inactivation of Kin4, a key component of the spindle position checkpoint (SPOC; shown in blue), which prevents MEN activation until the spindle is correctly aligned along the mother-bud axis. Active MEN promotes a sustained release of Cdc14 from the nucleolus both in the nucleus and in the cytoplasm. Once released Cdc14 further stimulates MEN activity. (**b**) Terminal phenotype of the *cdc15-2* MEN mutant. *cdc15-2* cells arrest in telophase with Cdc14 sequestered in the nucleolus.

The activated form of Tem1, which is likely but not proven to be the GTP-bound form, is thought to propagate the signal to the downstream protein kinase Cdc15. Cdc15, in turn, stimulates Dbf2 kinase activity, a process that requires the Dbf2-associated factor Mob1 (Bardin and Amon, 2001). Tem1's GTPase activity is negatively regulated by the GAP complex Bub2-Bfa1 in a GAP dependent and independent manner (Bardin et al., 2000; Fraschini et al., 2006). The Bub2-Bfa1 complex inhibits Tem1 protein in two ways: on one hand by promoting the hydrolysis of GTP to GDP, switching off the protein activity, and on the other hand simply by binding. The GAP activity is given by Bub2 (Geymonat et al., 2003) which carries a GAP TBC (Tre-2, Bub2 and Cdc16; (Neuwald et al, 1997)) domain, whereas the Bfa1 subunit is responsible for the physical interaction of the GAP complex with Tem1 (Ro et al., 2002). Recent observations suggested that a mutant version of the Bub2-Bfa1 complex depleted for its GAP activity towards Tem1, is still able to inhibit mitotic exit in response to the activation of the spindle position checkpoint (SPOC; Fraschini et al., 2006), thereby suggesting that Bub2-Bfa1 may completely inhibit Tem1 through a GAP-independent mechanism.

Lte1 is a positive regulator of Tem1 which shares homology with the guanosine nucleotide exchange domain of the Ras-guanosine nucleotide exchange factor (GEF) Cdc25 (Shirayama et al., 1994). Since *TEM1* was isolated as a high copy number suppressor of the cold sensitivity *lte1* mutant, Lte1 was initially proposed to function as a GEF for Tem1 (Shirayama et al, 1994; Keng et al., 1994). However recent studies suggests that Lte1 does not rely on nucleotide-exchange activity for activating Tem1 and suggest that Lte1 regulates Tem1 by influencing the subcellular localization of Bfa1 (Geymonat et al., 2009); which appears to be an important factor in regulating MEN activation (Pereira et al., 2001; Fraschini et al., 2006; Pereira and Schiebel, 2005; Molk et al, 2004). The polo-like kinase Cdc5 has long been thought to be a component of the MEN signalling cascade, as cells lacking *CDC5* exhibit a phanotype similar to that of MEN mutants, arresting in late anaphase with Cdc14 sequestered in the nucleolus (Jaspersen et al., 1998; Kitada et al.,

1993; Lee et al., 2001a). Recent studies, however suggested that Cdc5 is not a core component of the MEN signalling cascade. Instead, Cdc5 is essential for exit from mitosis because it activates the MEN pathway in multiple ways. Cdc5 phosphorylates Bfa1, which results in the inhibition of the Bub2-Bfa1 GAP activity (Geymonat et al., 2003; Hu et al., 2001; Pereira et al., 2002). Cdc5 may also regulate Lte1 (Lee et al., 2001b). Lastly, Cdc5 activates Dbf2 in a *BUB2*-independent manner, at least in part by promoting the FEAR-dependent release of Cdc14, which results in Cdc15 dephosphorylation and thus activation (Jaspersen and Morgan, 2000; Lee et al., 2001a; Stegmeier et al., 2002). Together, these MEN activating functions of Cdc5 are likely to account for the complete loss of MEN signalling in *cdc5* mutants (Visintin et al., 2003). Activation of the MEN is regulated in many additional ways. Cdc15 itself has a potent autoinhibitory C-terminal domain (Bardin et al., 2003), but its regulation remains elusive. The spatial segregation of the MEN components Lte1 and Tem1 also contributes to the temporal coordination of MEN activation, Fig. 1.15.

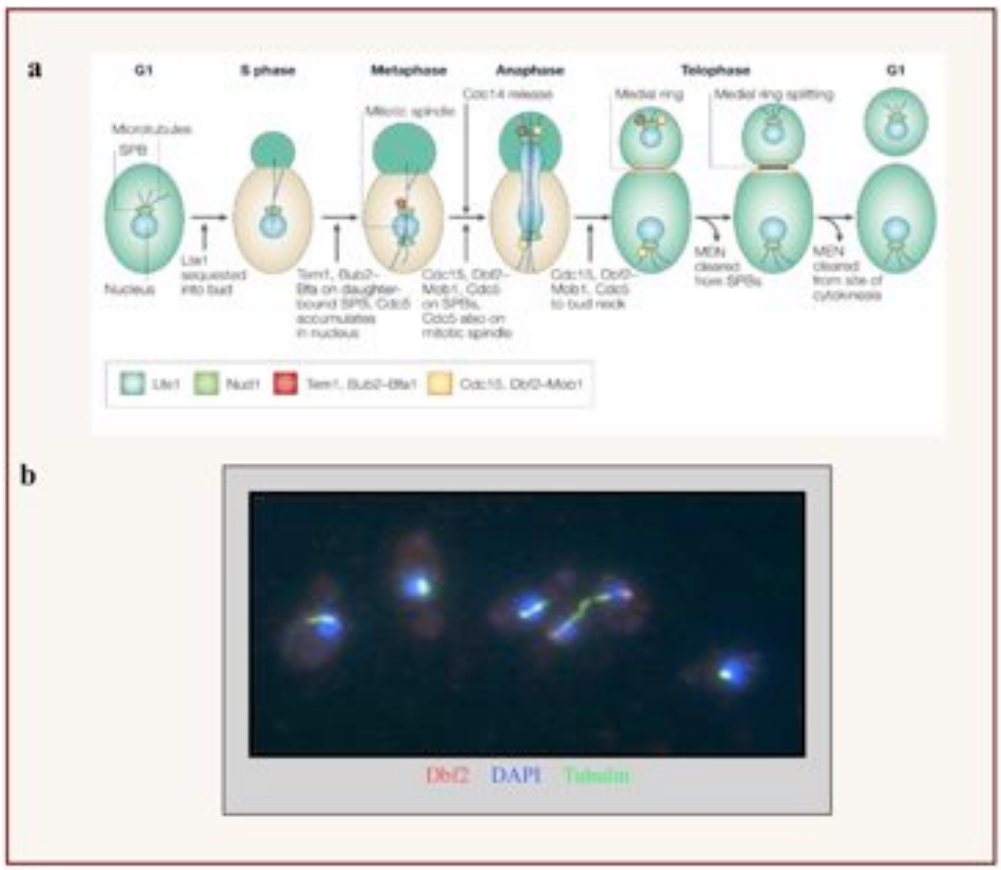

Figure 1.15 Localization of MEN components during the cell cycle
(a) Lte1 is present throughout the cell during G1, localizes to the bud (mainly to the bud cortex) during S phase and mitosis and spreads into the cytoplasm of both the mother and the daughter cells after the nucleus has migrated into the bud. Tem1 and Bub2-Bfa1 localize to both spindle pole bodies (SPBs) at the time of SPB duplication (not shown) and, as the mitotic spindle forms, are found more concentrated at the daughter-directed SPB. A small amount of Tem1 however is still present at the mother-bound SPB (not shown) while Bub2-Bfa1 completely disappears from the mother-bound SPB. During anaphase Cdc15 and Dbf2-Mob1 are recruited to SPBs. Nud1 functions as a scaffold for MEN components. Cdc5 localizes to the nucleus during S phase, metaphase and anaphase and is found also on SPBs and the site of cytokinesis during anaphase. Taken from Bardin and Amon, 2001. (**b**) Localization of Dbf2 during the cell cycle analyzed by indirect immunofluorescence (IF). Dbf2 localizes to SPBs during anaphase.

Tem1 resides on the single spindle pole body in G1, gets distributed with similar amounts on both SPBs when they are duplicated and later on, at metaphase to anaphase transition, it becames concentrated at the daughter-directed SPB (dSPB; Bardin et al., 2000; Molk et al., 2004; Pereira et al., 2000). However a small amount of Tem1 is still present on the mother cell SPB, also after anaphase (Molk et al., 2004; Pereira et al., 2000). Bub2-Bfa1 subcellular localization follows essentially the same pattern, with the exception that the complex completely disappears from the SPB remaining in the mother

cell at the onset of anaphase (Molk et al., 2004; Pereira et al., 2000). Bub2-Bfa1 are required for the localization of Tem1 on SPBs during interphase and early mitosis, but not during anaphase (Pereira et al., 2000). The MEN activator Lte1 is confined in the bud (mainly at the bud cortex) throughout most of the cell cycle and spreads into the cytoplasm of both the mother and the daughter cells after the nucleus has migrated into the bud (Molk et al., 2004). The subcellular localization of many other MEN components is also cell cycle regulated. Cdc5 localizes on SPBs throughout the cell cycle except in G1 when it is degraded by the APC/C^{Cdh1} (Shirayama et al., 1998; Song et al., 2000), while the MEN components Cdc15, Dbf2 and Mob1 localize on the cytoplasmic face of the SPBs during anaphase (Molk et al., 2004; Menssen et al., 2001; Cenamor et al., 1999; Bardin et al., 2000; Visintin et al., 2001). The SPB component Nud1 is thought to function as a scaffold for the Bub2-Bfa1-Tem1 complex and helps to recruit other MEN components onto the SPBs (Gruneberg et al., 2000). Cdc15 and Dbf2 localize to both SPBs, while, as previously said, Tem1 and the Bub2-Bfa1 complex associate preferentially with the daughter-bound SPB. Interestingly the future destination (daughter versus mother cell) rather then age of SPB (old versus newly synthesized) determines which SPB recruits the Tem1-Bub2-Bfa1 complex (Pereira et al., 2001). The spatial segregation of Lte1 and Tem1 led to an appealing model for explaining how nuclear division is coupled with mitotic exit. According to this model, Tem1 is kept inactive at SPBs by Bub2-Bfa1 until the Tem1-bearing SPB migrates into the bud at anaphase onset. Only in this case will Tem1 be exposed to its activator Lte1. Thanks to this spatial segregation, misaligned spindle would prevent MEN activation and delay mitotic exit until errors are corrected, thus ensuring the balanced chromosome partitioning between the mother and the daughter cells (Bardin et al., 2000; Pereira et al., 2000).

This model would imply that the pool of Tem1 localized on the d-SPB would be responsible for triggering MEN activation and mitotic exit. However recent studies have highlighted an important role also for the pool of Tem1 localized on the SPB remaining in

the mother cell. This pool would control the timing of mitotic exit as persistence of Bub2-Bfa1 at this SPB during anaphase prevents mitotic exit in cells where Tem1 activity is partially impaired (Fraschini et al., 2006). Consistent with this idea Bub2-Bfa1 is retained on both SPBs in anaphase cells with improperly positioned spindle (Pereira et al., 2001).

The molecular mechanism through which MEN induces the release of Cdc14 from its inhibitor Cfi1 in late anaphase is still unknown. Some observations lead to the conclusion that MEN contributes to the dissociation of the phosphatase from its inhibitor by promoting phosphorylation of Cfi1. Indeed, a correlation exists between the release of the phosphatase and the phosphorylation of both Cdc14 and Cfi1. Overexpression of a hyperactive form of Cdc15, known to ectopically activate the downstream kinase Dbf2, induces the extopic phosphorylation of Cfi1 (Visintin et al., 2003). Furthermore Cfi1 has been identified as a putative Dbf2-substrate (Mah et al., 2005). The MEN pathway other than promote the release of Cdc14 it further promotes its accumulation in the cytoplasm. Responsible for this Cdc14-cytoplasmic localizationis the protein kinase Dbf2 that by phosphorylating two residues of Cdc14 close to its nuclear localization signal (NLS) (Mohl et al., 2009) allows for cytoplasmic retention of the phosphatase.

Inactivation of FEAR network and MEN

The inactivation of Cdc14 after mitotic exit has been completed is as important for succesfull cell division as its activation during anaphase. This is illustrated by the severe growth defects exhibited by cells with unconstrained Cdc14 activity (Visintin et al., 1998). Therefore, it is important that FEAR network and MEN are inactivated once mitotic exit has been completed and cells have entered G1. FEAR network activity appears to be restricted to a very brief time during early anaphase, as Cdc14 becomes re-sequestered into the nucleolus during late anaphase in cells lacking a functional MEN (Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002). How the activation of the FEAR network is restricted to the duration of early anaphase is unknown. However the mechanisms that

regulate FEAR and MEN inactivation at the end of mitosis are well understood. Interestingly, Cdc14 itself plants the seeds for its own demise. The most important substrate of Cdc14, once fully activated by the MEN pathway, is the APC/C^{Cdh1}. By dephosphorylating Cdh1, and thus activating the APC/C^{Cdh1} complex, Cdc14 promotes its own re-sequestration. The most important APC/C^{Cdh1} target at this time is Cdc5, the key factor for Cdc14 release and maintainance. Thus, since Cdc5 is a component of the FEAR network and activator of the MEN its degradation leads to the silencing of both the two pathways (Visintin et al., 2008). Cdc5 degradation represents the major mechanism responsible for Cdc14 inactivation at the end of mitosis. Beside Cdc5, another APC/C^{Cdh1} substrate that once degraded contributes to FEAR network silencing is the small nuleolar protein Spo12. Indeed, the phosphorylation of two serine residues of Spo12 by Clb2-Cdk is required for FEAR network function. Given that these phosphorylation sites conform to Cdc14 consensus, it is possible that Cdc14 itself quenches FEAR network activation by dephosphorylating Spo12 (Shah et al., 2001; Stegmaier et al., 2004). Regarding MEN inactivation, Cdc14 dephosphorylates both Bfa1 and Lte1, thus presumably restoring the GAP-activity of Bub2-Bfa1 complex towards Tem1 (Geymonat et al., 2003; Pereira et al., 2002) and triggering the release of Lte1 from the bud cortex. The cortical release of Lte1 decreases its concentration in the bud, which is believed to be required for efficient Tem1 activation (Bardin et al., 2000; Jensen et al., 2002).

Signals controlling FEAR network and MEN activity

Many mitotic processes, such as the onset of sister-chromatid separation, mitotic spindle disassembly and cytokinesis are irreversible. Therefore, to ensure the successful completion of mitosis, it is essential that mitotic exit is tightly coordinated with other cell cycle events. Different works have identified some of the cellular signals that control the activation of the FEAR network and the MEN, shedding light onto the mechanisms

whereby Cdc14 activation and hence the coordination of late mitotic events is accomplished.

Coordination of chromosome segregation and exit from mitosis through the FEAR network

Faithful chromosome segregation requires that exit from mitosis is temporally coordinated with partitioning of the genetic material between the progeny. The fact that two important regulators of sister-chromatid separation, the separase Esp1 and the polo-kinase Cdc5, also promote the release of Cdc14 as part of the FEAR network, provides a molecular explanation as to how cells ensure that exit from mitosis does not occur prior to the onset of sister-chromatid separation (Stegmeier et al., 2002; Sullivan and Uhlman, 2003). Conversely, the fact that FEAR network-induced Cdc14 activation promotes the segregation of telomeres and rDNA regions (described in detail below) ensures that sister-chromatid separation is completed before cells exit from mitosis (D'Amours et al., 2004).

Preventing mitotic exit in response to microtubule and DNA damage through inhibition of MEN and FEAR network

In presence of mitotic spindle or DNA damage, cells have to delay sister-chromatid separation and mitotic exit in order to provide time for error correction. Two surveillance mechanisms, known as the spindle assembly chechpoint (SAC) and the DNA damage checkpoint monitor defects in the attachment of microtubles to kinetocores and DNA damage, respectively (Nyberg et al., 2002; Lew et al, 2003). The target of both checkpoints is the securin Pds1. By preventing degradation of Pds1 they prevent sister chromatid separation and the activation of the FEAR network and furthermore antagonize MEN function. The spindle assembly checkpoint components Mad1, Mad2, Mad3, Bub1 and Bub3 are the one responsible for the inhibition of Pds1 degradation via APC/C^{Cdc20} inhibition (reviewed in Lew et al., 2003), whereas Bub2 and Bfa1 are the components

involved in preventing MEN activation in response to spindle damage. It has been proposed that the activation of the spindle checkpoint prolongs the inhibitory function of the Bub2-Bfa1 complex on MEN cascade activation, possibly influencing Bub2-Bfa1 GAP-activity (Hu and Elledge, 2002). Furthermore it has been also proposed that the Bub2 and Bfa1 SAC components are needed to prevent MEN activation in response to any mitotic checkpoints (Lee et al., 2001b).

Regarding the DNA damage checkpoint, the DNA damage effector protein Chk1 directly phosphorylate Pds1, protecting it from APC/C^{Cdc20} ubiquitilation (Sanchez et al., 1999; Wang et al., 2001); Rad53 inhibits the interaction between Cdc20 and the securin Pds1 itself (Agarwal et al., 2003), and the c-AMP-dependent protein kinase PKA directly phosphorylate Cdc20 blocking its interaction with the substrates (Searle et al., 2004), thereby all inhibiting the metaphase anaphase transition and sister-chromatid separation in presence of DNA damage to preserve genomic stability.

Preventing mitotic exit in response to incorrect spindle position through regulating MEN activity

In order to ensure that each daughter cell receives exactly one DNA complement during cell division, the division site must bisect the mitotic spindle. In fission yeast and higher eukaryotes, the division site is determined by the position of the mitotic spindle and signalling between the cell membrane and the mitotic spindle apparatus is likely to be important to coordinate chromosomes segregation and cytokinesis in space. In *S. cerevisiae* the division site is pre-determined and therefore the proper orientation of the spindle apparatus through the mother-bud neck prior to mitotic spindle disassembly and cytokinesis is crucial for the fidelity of chromosomes segregation. It thus come as no surprise that mechanisms exist that prevent exit from mitosis until the bud, the future daughter cell, receives a complete DNA complement. The activity of a surveillance mechanism termed “the spindle orientation checkpoint”: (SPOC) blocks exit from mitosis

until the spindle is correctly oriented. One mechanism that helps prevent exit from mitosis in cells with a mis-positioned mitotic spindle is the spatial segregation of MEN components through which cell senses the position of the spindle. Indeed, the MEN activator Lte1 becomes sequestered at the bud cortex during bud formation while Tem1 resides specifically on the daughter-bound SPB (Bardin et al., 2000; Pereira et al., 2000). Thus, delivery of Tem1 bearing SPB into the bud, that occurs only if the mitotic spindle is correctly oriented, is likely to promote Tem1 and thus MEN activation during anaphase (Bardin et al., 2000; Pereira et al., 2000). When the spindle is not correctly aligned, Tem1 is not exposed to Lte1 and thus mitotic exit is prevented. This idea is consistent with the finding that overexpression of *LTE1*, which causes the protein to be present in the mother cell as well as the daughter cell, allows cells with mis-oriented spindles to exit from mitosis (Bardin et al., 2000). In the same way, when the maintenance of Lte1 in the bud is disrupted by the inactivation of septins, which form a diffusion barrier for membrane associated proteins (Barral et al., 2000; Hofken et al., 2002), cells with a mis-positioned nucleus exit from mitosis (Castillion et al., 2003).

Spatial restriction of Lte1 and Tem1 is not the only mechanism that prevents cells with misaligned spindles from exiting mitosis. Indeed, it has been shown that *BUB2* and *BFA1* deletion allows cells to exit from mitosis even if in presence of an improperly aligned spindle (Pereira et al., 2000). Interestingly, the phosphorylation of Bfa1 and its asymmetric localization to the daughter bound SPB are regulated in response to spindle orientation (Hu et al., 2001). Responsible for this regulating phosphorylation on Bub2-Bfa1 complex, in response to improper spindle positioning, is the protein kinase Kin4, a component of the SPOC (D'Aquino et al., 2005; Pereira and Schiebel, 2005). During an unperturbed cell cycle for most of the time Kin4 localizes through the mother cell cortex, while during anaphase it is concentrated in the bud neck. It associates with the mother-bound SPB mirroring the localization of Bub2-Bfa1 (D'Aquino et al., 2005; Pereira and Schiebel, 2005).

However, in cells with mis-positioned spindles both Kin4 and Bub2-Bfa1 associate with both SPBs during anaphase allowing Kin4 to phosphorylate Bfa1. This phosphorylation prevents Bfa1 from Cdc5 inhibitory phosphorylation, thus freezing the Bub2-Bfa1 complex in an active state and preventing the activation of the MEN (D'Aquino et al., 2005; Pereira and Schiebel, 2005; Maekawa et al., 2007). Recent studies highlighted that the protein phasphatase $PP2A^{Rts1}$ and the protein kinase Elm1 are essential for the function of Kin4 in the SPOC (Caydasi et al., 2010; Chan et al., 2009). Based on the restriction of Kin4 to the mother cell cortex and Lte1 to the bud cortex, it has been proposed that Kin4 establishes a domain of MEN inhibition in the mother cell and Lte1 creates a domain of MEN activation in the bud (Bardin et al., 2000; Chan and Amon, 2010; D'Aquino et al., 2005; Pereira et al., 2000). According to this "zone model", the Tem1-bearing SPB has to escape the zone of inhibition and enter in the zone of activation for MEN to become active and to promote exit from mitosis (Chan and Amon, 2010; Chan and Amon, 2009).

The multiple roles of Cdc14 during late stages of mitosis

In the last years it became clear that Cdc14 not only promotes mitotic Cdks inactivation and exit from mitosis, but also a variety of other cellular events, including rDNA and telomere segregation, mitotic spindle dynamics and cytokinesis. Remarkably, the execution of these diverse events relies on Cdc14 activated by different regulatory networks, Fig. 1.16.

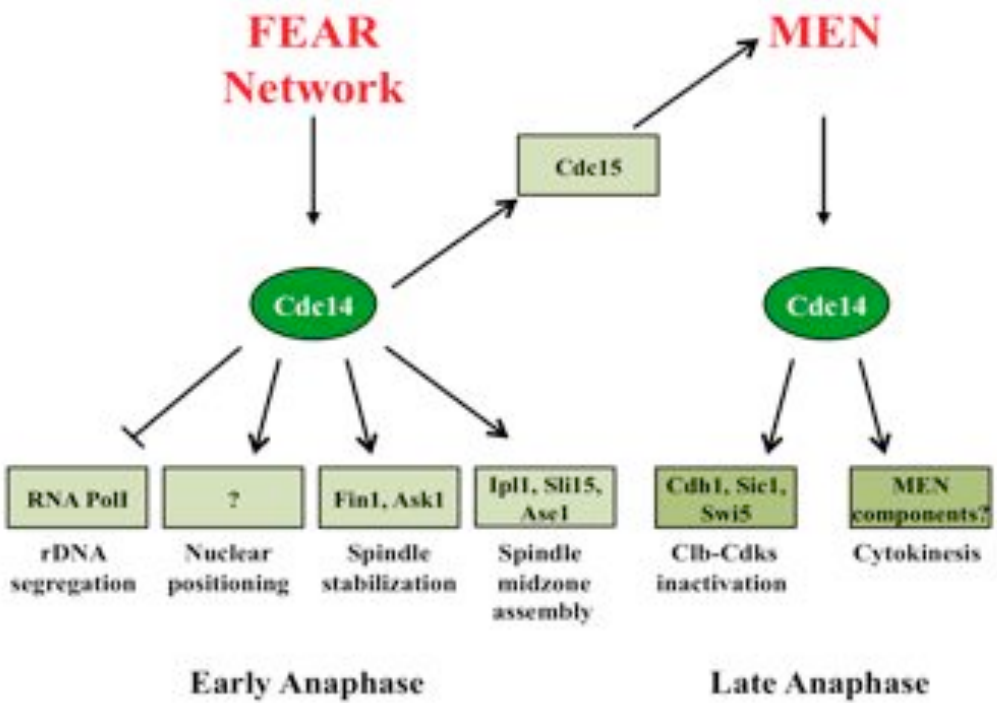

Figure 1.16 Functions of Cdc14 released by the FEAR network and the MEN
Cdc14 orchestrates the anaphase events. FEAR released Cdc14 regulates many aspects of chromosome movement. The phosphatase promotes rDNA segregation by inducing the silencing of rDNA transcription by RNA PolI, which results in the recruitment of condensins to rDNA. Cdc14 stabilizes the anaphase spindle by dephosphorylating several microtubule-binding proteins such as Ask1 and Fin1. Cdc14 promotes the localization of the microtubule-bundling protein Ase1 and the chromosomal passenger complex Ipl1-Sli15 to the spindle midzone and controls nuclear position. Cdc14 also stimulates the MEN, which is required to maintain Cdc14 in a released state during late anaphase. Once activated, the MEN further promotes Cdc14 activity. Cdc14 released by MEN bring about exit from mitosis by dephosphorylating Cdh1, Swi5 and Sic1, which results in mitotic Cdk inactivation.

Functions of Cdc14 released by the FEAR network

Although Cdc14 activated by the FEAR network is not essential for cell proliferation, it helps to coordinate several mitotic events, thereby contributing to the maintenance of genomic stability. The importance of the FEAR network is highlighted by the dramatic loss of viability exhibited by cells that progress through anaphase in its absence (D'Amours et al., 2004). Cdc14 released by the FEAR network promotes the completion of chromosomes segregation. Indeed, it has been observed that cells lacking FEAR network activity are impaired in the segregation of telomeres and rDNA array sequences. Given that cells lacking MEN activity do not exhibit such defects, it appears that the transient activation of Cdc14 by the FEAR network is sufficient for the separation

of these late segregating chromosomal regions. rDNA arrays and sister-chromatid's telomeres are linked by cohesin-independent linkages (D'Amours et al., 2004; Sullivan et al., 2004). Cdc14 released by the FEAR network is responsible for the dissolution of these cohesin-independent linkages. Several observations point to the possibility that Cdc14 mediates rDNA segregation by promoting the enrichment of condensins, which are protein complexes required for chromosomes condensation (Bhalla et al., 2002; Hirano, 2000), at the rDNA locus. First, cells carrying mutation in subunits of the condensin complex exhibit nucleolar segregation defects similar to that of a Cdc14 loss of function mutant (Bhalla et al., 2002; Freeman et al., 2000). Secondly, *CDC14* overexpression induces ectopic enrichment of condensins at the rDNA locus and ectopic rDNA segregation (D'Amours et al., 2004). Lastly, the inactivation of condensins prevents rDNA segregation in *CDC14* overexpressing cells (D'Amours et al., 2004). Furthermore, recently it has been shown that Cdc14 released by the FEAR network promotes the removal of RNA PolI subunit from the nucleolus, thereby inhibiting the transcription of rDNA in anaphase (Clemente-Blanco et al., 2009; Tomson et al., 2006). The silencing of rDNA genes during anaphase is thought to be necessary for allowing condensin's access to rDNA chromatin (Clemente-Blanco et al., 2009). The observation that Cdc14 induces the sumoylation of the condensin subunit Ycs4 during anaphase raises the interesting possibility that Cdc14 targets condensins to the rDNA by promoting Ycs4 sumoylation. How Cdc14-dependent condensation facilitates rDNA segregation remains unclear.

An additional function of FEAR-released Cdc14 is to control nuclear positioning. In budding yeast, the nucleus is positioned at the bud neck during metaphase. At anaphase onset microtubule motor proteins associated with the cytoplasmic microtubules emanating from the daughter-bound SPB pull the nucleus into the bud. Concomitantly, microtubule motor proteins associated with the mitotic spindle push the two SPBs apart. The combination of these microtubule-associated forces results in the equal partitioning of the genetic material between the mother and the daughter cells. Cdc14 released by the FEAR

network has been implicated in the generation of the mother-directed cytoplasmic-microtubule pulling forces which contribute to the equal distribution of the duplicated chromosomes between the mother and the daughter cells. In cells lacking separase function sister-chromatids are not segregated and the undivided nucleus is pulled into the bud (Ross et al., 2004). How Cdc14 promotes the generation of these forces remains unknown.

Cdc14 released by the FEAR network induces the stabilization of the anaphase mitotic spindle, which is essential for the correct segregation of sister-chromatids in a wide variety of processes. Cdc14 dephosphorylates several microtubule-interacting proteins such as Fin1 and Ask1, which allows them to interact with and thus stabilize the elongating spindle (Woodbury et al, 2007; Higuchi et al, 2005). Cdc14 contributes to the stabilization of the spindle midzone by regulating the subcellular localization of the chromosomal passenger proteins (Pereira et al., 2003). The spindle midzone is a very fragile site of the anaphase spindle, where interpolar microtubules overlap. FEAR-released Cdc14 promotes the translocation of the chromosomal passenger proteins Ipl1 and Sli15 (Aurora B kinase and INCENP in higher eukaryotes, respectively) to the mitotic spindle and spindle midzone, ensuring stabilization of the anaphase spindle. Other than dephosphorylating Sli15, Cdc14 also dephosphorylates the microtuble-bundling protein Ase1, translocating it to the spindle midzone. The Ipl1-Sli15 complex and Ase1 then recruit other factors which stabilize the elongating spindle, critical among them being Esp1-Slk19 complex (Khmelinskii et al., 2007).

Lastly, the activation of Cdc14 by the FEAR network contributes to the MEN activation (Stegmeier et al., 2002). First, Cdc14 released by the FEAR network promotes the dephosphorylation of Cdc15 kinase, thus enhancing Cdc15's mitotic exit function (Jaspersen et al., 2000; Stegmeier et al., 2002). The mechanism by which dephosphorylation of Cdc15 promotes its mitotic exit function remains unknown. Dephosphorylation may enhance its interaction with the upstream Tem1, its localization on SPBs or antagonize the inhibitory function of Cdc15's C-terminal domain. Secondly,

Cdc14 has been proposed to inactivate the Bub2-Bfa1 complex in a phosphatase-independent manner during early anaphase (Pereira et al., 2002). Shortly after its release from the nucleolus, Cdc14 binds Bub2 at the daughter SPB, inactivating Bub2-Bfa1 complex activity (Pereira et al., 2002; Yoshida et al., 2002a). While the binding of Cdc14 released by the FEAR network to the Bub2-Bfa1 complex would inactivate its GAP activity in early anaphase, dephosphorylation of Bub2 by Cdc14 during late mitosis would re-activate the GAP-complex (Geymonat et al., 2003; Pereira et al., 2002). However further investigations are needed to better understand the nature of this phosphatase-independent function of Cdc14 and the mechanism that prevents Cdc14 from dephosphorylating Bub2 in early anaphase. Notably, during early anaphase Cdc14 dephosphorylates its targets despite the presence of high mitotic Cdks activity throughout the cell. Therefore, regions within the cell that contain a high local concentration of Cdc14 during early anaphase, such as kinetochores, the mitotic spindle and SPBs are most likely to harbour yet unidentified targets of Cdc14 released by the FEAR network.

Functions of Cdc14 released by the MEN

Cdc14 released by the MEN is mainly responsible for promoting exit from mitosis, as this transition does not occur in absence of MEN function (Jaspersen et al., 1998; Surana et al., 1993). Several observation, however, indicate that Cdc14 and MEN also regulate cytokinesis, independently of their mitotic exit function. When the need for MEN function in mitotic exit is bypassed, either by weakening the Cdc14/Cfi1 interaction or by overexpression of the Cdk inhibitor Sic1, severe cytokinesis defects become apparent (Jimenez et al., 1998; Lippincott et al., 2001). The notion that MEN regulates cytokinesis is also consistent with the translocation of the MEN components Cdc15, Dbf2, Mob1 and Cdc5 to the bud neck (the site of actomyosin ring constriction in budding yeast) at the time of cytokinesis (Frenz et al., 2000; Xu et al., 2000). Furthermore, the cytokinesis function of at least some MEN components appears to require Cdc14 function, as is the case for the

accumulation of Dbf2 and Mob1 at the bud neck (Frenz et al., 2000; Yoshida et al., 2001). It is possible therefore that, after the MEN promoted Cdc14 activation, Cdc14 itself dephosphorylates Cdk consensus site in MEN components to promote their localization to the bud neck, where they will then regulate cytokinesis.

Cdc14 function and regulation in other eukaryotes

Homologues of Cdc14 exist in most if not all eukaryotes nevertheless their biochemical similarities, their function and regulation significantly differ among the species, Fig. 1.17.

Fission yeast Like its cousin in *S. cerevisiae*, the fission yeast ortholog Clp1 (also known as Flp1), antagonizes mitotic Cdk activity. Furthermore, the activity of Clp1 is at least in part regulated by a signal pathway (SIN-septation initiation network) homologous to the MEN. Despite these similarities, the mechanism whereby Clp1 and Cdc14 accomplish mitotic Cdk inactivation and the cellular processes regulated by these two phosphatases appear to be quite different. In contrast to Cdc14, Clp1 is not essential for cell cycle progression and mitotic exit, but rather controls mitotic entry and coordinates cytokinesis with entry in the next cell cycle (Cueille et al., 2001; Trautmann et al., 2001). The most important role of Clp1 seems to be to maintain low mitotic Cdk activity during G2 in response to a cytokinesis checkpoint. Indeed, activation of the cytokinesis checkpoint in fission yeast leads to a cell cycle arrest in G2. This cell cycle arrest requires the continuous inhibition of mitotic Cdks, which is mediated by Clp1 (Cueille et al., 2001; Trautmann et al., 2001). The observation that Clp1 localizes in the nucleolus during some cell cycle stages and it is released during others raises the possibility that some aspect of Cdc14 and Clp1 regulation are shared between the two yeasts. During G1 and S phase, Clp1 localizes predominantly to the nucleolus, but in contrast to its budding yeast counterpart a small portion resides at SPBs (Cueille et al., 2001; Trautmann et al., 2001). Furthermore, while

Cdc14 is released from the nucleolus during anaphase, Clp1 is released from the nucleolus as cells enter in mitosis localizing to the mitotic spindle and to kinetochores, and later it is found to the medial ring, site of cytokinesis (Cueille et al., 2001; Trautmann et al., 2001). Kinetocores-localized Clp1 has been proposed to function together with Aurora B kinase to repair mono-orientation of sister-chromatids, thus ensuring their correct segregation (Trautmann et al., 2004). Similarly to the budding yeast Cdc14, Clp1 regulates the spindle midzone stability by dephosphorylating several midzone-binding proteins such as Ase1 and the kinesin 6-motor protein Klp9 (Fu et al., 2009; Khmelinskii et al., 2009). Whether Clp1 is bound to an inhibitor when it resides in the nucleolus is at present unclear. However, a regulatory network homologous to the MEN termed the septation-initiation network (SIN) appears to control Clp1 localization in a manner similar to the MEN (Bardin et al., 2001; Cueille et al., 2001; Trautmann et al., 2001). Although SIN does not regulate Clp1 localization during an unperturbed cell cycle, this signalling network is required to maintain Clp1 in a released state when the cytokinesis checkpoint has been activated (Cueille et al., 2001; Trautmann et al., 2001). Thus, maintainance of Cdc14 and Clp1 in its released state relies on conserved pathways. How Clp1 is initially released from the nucleolus remains unclear but this release does not depend on homologues of the FEAR network (Chen et al., 2006).

C. elegans Depletion of Cdc14 by RNA interference (RNAi) in *C. elegans* has been reported to causes cytokinesis defects, most likely due to a failure to form an intact central spindle, leading to multinucleated cells and causing embryonic lethality (Gruneberg et al., 2002). A different study, however, showed that null *CeCDC14* worms were viable and lacked mitotic and cytokinetic defects (Saito et al., 2004). How CeCdc14 would promote the central spindle formation is unknown, but it is noteworthy that the phosphatase is required for the localization of Zen-4, a kinesin-related motor protein required for central spindle formation, to the mitotic spindle (Gruneberg et al., 2002). Consistent with a role in cytokinesis and central spindle function, CeCdc14 localizes to the central spindle (spindle

midzone) during anaphase and to the midbody during telophase, similar to *S. pombe* Clp1, but the phosphatase has neither been detected in the nucleolus nor on centrosomes (Grunberg et al., 2002). Whether CeCdc14 is regulated by signaling pathways homologous to the MEN and SIN is also unclear. RNA interference of several potential MEN and SIN homologues in *C. elegans* did not lead to embryonic lethality (Gruenberg et al., 2002). It is possible that the RNAi-mediated depletion was incomplete or that redundant factors exist in *C. elegans*. Alternatively, the MEN/SIN components may not be necessary during embryonic division or these pathways evolved to fulfill functions specific to yeast.

Human The human genome encodes two Cdc14 homologues, hCdc14A and hCdc14B. The roles of these two phosphatases are poorly understood, but an involvement in mitotic exit and cytokinesis is possible. *In vitro* studies have shown that hCdc14, just like its yeast counterpart, has a clear preference for substrates of proline-directed kinases (Visintin et al., 1998; Trautmann et al., 2001; Kaiser et al., 2002), which is further supported by the crystal structure of the core domain of hCdc14A (Gray et al., 2003). Furthermore hCdc14A can dephosphorylate APC/C^{Cdh1} *in vitro* (Bembenek et al., 2001). Although this finding indicates that hCdc14 can bring about mitotic Cdks inactivation, this Cdc14 isoform appears to primarily regulate centrosomes function *in vivo*. Indeed, overexpression of hCdc14A leads to premature centriole splitting in S-phase and formation of an excessive number of aberrant mitotic spindles (Kaiser et al., 2002; Mailand et al., 2002). Conversely, depletion of hCdc14A by RNAi leads to centrosomes duplication, mitotic and cytokinetic defects (Mailand et al., 2002). Consistent with its proposed role in regulating centrosome function, hCdc14A localizes to this organelle (Bembenek et al., 2001; Kaiser; Mailand). Much less is known about the function of hCdc14B. Intriguingly, however, hCdc14B localizes to the nucleolus during interphase and is dispersed at the onset of mitosis (Bembenek and Kaiser, 2002). Based on RNAi depletion experiments hCdc14B has been implicated in the regulation of mitotic spindle assembly, centriole duplication, mitotic exit and the G2 DNA damage checkpoint (Basserman et al., 2008; Cho et al., 2005; Wu et al.,

2008). Interestingly, it has been recently found that in response to DNA damage checkpoint activation hCdc14B becomes released from the nucleolus and it activates the APC/C^{Cdh1}, that in turn leads Plk1 to the proteosomal degradation (Bassermann et al., 2008). Interestingly this is the same circuit activated by the yeast Cdc14, during exit from mitosis. More recent observations showed that deletion of both the hCdc14 isoforms does not cause growth or mitotic defects (Berdougo et al., 2008; Mocciaro et al., 2010).

From the studies of Cdc14 functions in different eukaryotes emerged a picture where the mitotic function of the *S. cerevisiae* Cdc14 has not been conserved through the evolution. However, the finding that the introduction of both hCdc14A and hCdc14B into fission yeast and Cdc14B in budding yeast rescue respectively the *clp1* and *cdc14* loss of function phenotypes, suggests that at least some properties of these phosphatases have been conserved throughout the evolution (Vazquez-Novelle et al., 2005). Further studies are necessary to assess the regulation and physiological function of Cdc14 phosphatases in organisms other than yeasts.

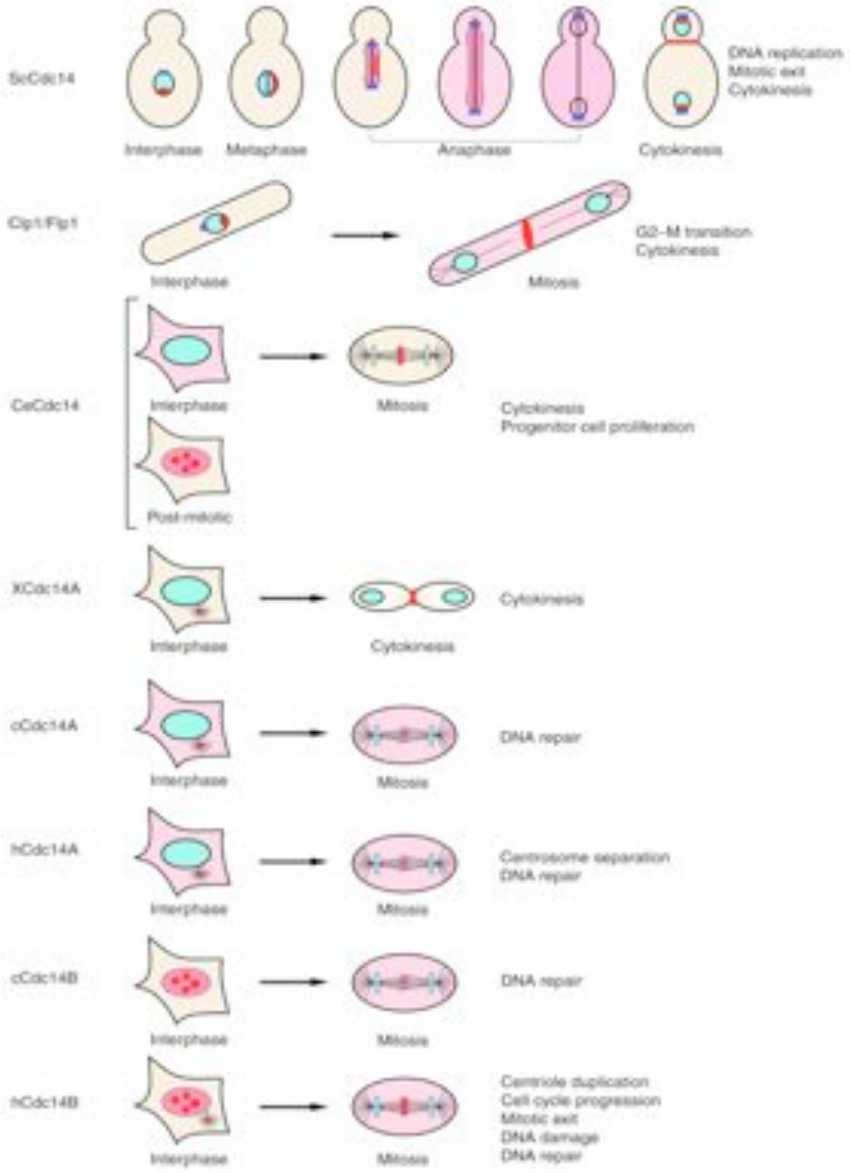

Figure 1.17 Overview of Cdc14 orthologs
Localization of Cdc14 orthologs during different stages of the cell cycle and possible functions of the phosphatase in each organism are shown. Concentrations of Cdc14 are shown in different shades of red, where dark red corresponds to high amount of Cdc14 and light red corresponds to low amount of Cdc14. DNA, light blue; *S.cervisiae* spindle pole bodies, dark blue rectangles; centrosomes, dark blue circles; microtubules, black lines. From Mocciaro & Schiebel, 2010.

2. Materials and Methods

Plasmids, primers and strains

Plasmids and Primers

Plasmids and primers used in this study are listed in Table 2.1 and Table 2.2, respectively.

Bacterial strains

The genotypes of all the bacterial strains used as hosts for plasmid amplification and construction are listed in Table 2.3. Cells were provided chemically competent for transformation.

Yeast strains

All the strains are derivatives of W303 (*ade2-1, can1-100, trp1-1 leu2-3, 112, his3-11, 15, ura3*). The relevant genotypes of all the yeast strains used in this study are listed in Table 2.4. The majority of the strains used in this study was generated by dissecting sporulated heterozygous diploid strains obtained by crossing haploid strains of opposite sexes (see below for procedure).

Construction of CDC14 mutants

Plasmids pFM1, pFM2, pFM3, pFM4, pFM5, pFM6, pFM7, pFM8, pFM9, pFM10, pFM11, pFM12, pFM13, pFM14, pFM15, pFM16, pFM17, pFM18, pFM20, pFM21 carrying *CDC14* alleles encoding for versions of the protein in which the T10, T140, S260, T391, S471 and T517 have been mutated, alone or in combination to alanine (A) or aspartate (D) were generated by PCR-based site-directed mutagenesis as described below. Integrative plasmid Rp49 (YIplac204) containing a HindIII *3HA-CDC14* fragment under

the control of its own promoter were used as template in the amplification reaction. The obtained plasmids were first sequenced to confirm the presence of the desired mutations with primers listed in Table 2.2 and then digested with Bsg1 so to direct the integration of these mutant alleles of *CDC14* at the *TRP1* locus of the yeast strain Ry1 (wild type), Ry1219 (a heterozygous diploid deleted in *CDC14- cdc14Δ*). Amounts of wild-type and mutated Cdc14 proteins were checked by Western Blot, in order to identify strains that have the same amount of integrants, and concomitantly the amount that ensures the physiological levels of Cdc14 as compared to wild type. Diploids obtained were selected, sporulated and dissected.

Media and growth conditions

Media for *E.coli.*

LB (DIFCO)	1% Bactotryptone
	0.5% Yeast extract
	1% NaCl
	pH 7.25
LB agar	LB + 2% agar (DIFCO)
LB amp	LB + 50 μg/ml ampicillin

Strains were grown at 37°C.

Media for *S. cerevisiae*

YEP	1% Yeast extract
	2% bactopeptone
	0.015% L-tryptophan
	pH 5.4
YEP agar	YEP + 2% agar (DIFCO)

Before using, YEP was supplemented with 2% glucose (YEPD) or 2% raffinose (YEPR) or 2% raffinose and 2% galactose (YEPRG) and 300 μM adenine.

Minimum media: SC	0.15% yeast nitrogen base (YNB, DIFCO) without amino acids and ammonium sulfate 0.5% ammonium sulfate 200 nM inositol
SC agar	SC + 2% agar

Before using SC was supplemented with 2% glucose or 2% raffinose or 2% raffinose and 2% galactose and amino acids as required. Strains were grown at room temperature (23°C).

Procedures

DNA manipulation techniques

Enzymatic restriction of DNA

DNA samples were digested with the appropriate restriction enzymes using conditions described in (Sambrook, 1989) or the own provider's instructions (NEB).
For diagnostic DNA restriction 0,5-2 μg of plasmid DNA was digested for 2 hrs at 37°C with 1-10 units of the appropriate restriction enzyme. The volume was made up depending on the DNA volume and concentration to 20-50 μl with the appropriate buffer and ddH_2O.

For preparative DNA restriction, 5-10 μg of plasmid DNA were incubated for 2 hrs at 37°C with 1-10 units of restriction enzyme. The enzymes sensitive to heat inactivation were inactivated at 65°C for 20'. The enzymes not sensitive to heat inactivation and the restrictions preparative for yeast transformation were inactivated at 65°C for 5' with 6 mM EDTA pH 8. The DNA was then precipitated adding 1/10 volume 3 M NaAc and 3 volumes 100% isopropanol and then pelleted at 13000 rpm for 15'. The pellet was washed

with 200 μl of 70% ethanol and finally resuspended in 10 μl ddH_2O in the case of integrative plasmids to be transformed into yeast.

Purification of DNA inserts from agarose gel

Cut DNA was loaded into an agarose gel and DNA fragments were separated by electrophoresis. The DNA fragment of interest was excised from the agarose gel with a sharp scalpel. DNA extraction was performed with QIAquick Gel Extraction Kit (Quiagen) following the manufacturer's instructions. DNA fragments were eluted in 30-50 μl of ddH2O.

Ligation

50 ng vector DNA was ligated with a 3- and 6-fold molar excess of insert DNA in the following conditions:

10X T4 DNA ligase buffer	1 μl
T4 DNA ligase	1 μl
ddH2O	up to 10 μl

Reactions were incubated ON at 16°C. After incubation the entire ligation reaction was used to transform *E.coli*.

DNA amplification

DNA was amplified using polymerase chain reaction (PCR). PCR was performed using genomic yeast DNA or plasmid DNA as template. Amplification of a DNA fragment requires two oligonucleotides flanking the region of interest, working as primers for the DNA polymerase. Phusion DNA polymerase (Finnzymes) and ExTaq (TaKaRa) DNA polymerase were used.

The reaction mix :

template DNA	1 μl
5X/10X Buffer	1X
dNTPs	0.2 mM
forward primer	1 μM
reverse primer	1 μM
DNA polymerase	1-2 units
ddH2O	to 20 μl

DNA amplification was performed with a Biometra T3000 Thermocycler with the following parameters:

1. heat shock step	5’	at 95°C
2. denaturation step	1’	at 95°C
3. annealing step	1’	at 50-55°C
4. extension step	1’/kb	at 72°C
5. repeat 25 times steps from 2 to 4		
6. extension step	10’	at 72°C
7. end	hold	at 4°C

Transformation

Escherichia coli transformation

50 μl of fresh chemically competent DH5alpha cells were thawed on ice for approximately 10 minutes prior to the addition of plasmid DNA or the ligation mixture. Cells were incubated with DNA on ice for 30 minutes and then subjected to a heat shock for 30-45’’ at 37° C. After the heat shock, cells were returned to ice for 2’. Finally 950 μl

of LB medium was added to the reaction tube. Cell suspension was incubated on a shaker at 37°C for 45' before plating onto LB plates containing the appropriate antibiotic. Plates were incubated overnight at 37°C.

Yeast transformation

Yeast cells were grown ON in 50 ml of YEPD or of the appropriate medium allowing them to reach the stationary phase. Next morning the cell culture was diluted to OD_{600} = 0.2 and allowed to grow several cycles until it had reached an OD_{600} of 0.5-0.8. Cells were then harvested at 3000 rpm for 3' and washed with 50 ml of sterile double-distilled (dd) H_2O. The pellet was then transferred to an eppendorf tube with 1 ml of ddH_2O and washed with 1 ml 1X TE/1X LiAc solution. Cells were then resuspended in 250 μl 1X TE/1X LiAc solution. Aliquots of 50 μl of competent cells were used for each transformation reaction.

Transformation mix:

cell suspension	50 μl
PEG/TE/LiAc solution (40% PEG, 1X TE, 1X LiAc)	300 μl
10 mg/ml single-stranded salmon sperm denatured DNA	5 μl
DNA	"x" μl (max up to 10 μl)

After gentle mixing, the transformation reaction was incubated on a rotating wheel for 30' at RT. Cells were heat-shocked at 42°C for 15 min and then centrifuged for 3' at 3000 rpm. The pellet was resuspended in 200 μl of 1X TE and the cell suspension was plated on appropriate selective medium.

Solutions:

10 mg/ml single-stranded salmon sperm denatured DNA

10X LiAc (1.0 M, pH 7.0)

50% PEG 4000

10X TE (0.1 M Tris-HCl pH 7.5, 10 mM EDTA)

DNA extraction

Plasmid DNA isolation from E. coli (mini prep)

Clones picked from individual colonies were used to inoculate 2 ml LB supplemented with 50 μg/ml ampicillin and grown ON at 37°C. Bacterial cells were transferred to micro-centrifuge tubes and pelleted for 5' at 8000 rpm. Minipreps were performed with QIAprep Spin Miniprep Kit (Quiagen) following the manufacturer's instructions. Plasmids were eluted in 30 μl ddH2O.

Plasmid DNA isolation from E. coli (maxi prep)

Plasmid-containing cells were inoculated in 100 ml LB amp and they were grown at 37°C ON. Cells were then recovered by centrifuging 10 min at 5000 rpm. Maxipreps were performed with QIAprep Spin Maxiprep Kit (Quiagen) following the manufacturer's instructions. Plasmids were eluted in 500 μl ddH2O.

Smash and Grab yeast genomic DNA isolation

Cells picked from an individual colony were inoculated in 200 μl of Lysis buffer. 200 μl of phenol/chlorophorm/isoamyl alcohol and 1 volume of glass beads were added to the cell suspensions and the tube was shaked 10' on Vxr Ika-Vibrax shaker. The tube was centrifuged 4' at 13000 rpm and the upper aqueous layer was transferred to a new tube.

This step was repeated another time. 1 ml ice-cold 100% ethanol was added to precipitate DNA. After gently mixing, the tube was centrifuged 4' at 13000 rpm. Supernatant was removed, the pellet was air-dried and DNA resuspended in 50 µl of 1X TE.

Solutions

Lysis buffer: 2% Triton X-100
1% SDS, 100 mM NaCl
10 mM Tris-HCl pH 8.0
1 mM EDTA pH 8.0

phenol/clorophorm/isoamyl alcohol 25:24:1 (SIGMA)

10X TE: 0.1 M Tris-HCl pH 7.5
10 mM EDTA

Teeny genomic yeast DNA isolation

Yeast cells of the desired strain were grown in 10 ml YEP containing the appropriate sugar to stationary phase. Cells were collected by centrifuging and then washed with 1 ml of a solution containing 0.9 M sorbitol, 0.1 M EDTA pH 7.5. Pellet was then transferred into 0.4 ml of a solution containing 0.9 M sorbitol, 0.1 M EDTA pH 7.5 and 14 mM β-mercaptoethanol. After mixing, 0.1 ml of a 2 mg/ml solution of Zymoliase 100T were added and the tube was incubated at 37°C up to spheroplasts formation (20'-30'), checked by optical microscopy. After 30'' of centrifugation, the pellet was carefully resuspended in 0.4 ml TE 1X (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8). After addition of 90 µl of a solution containing 1.5 ml of EDTA pH 8.5, 0.6 ml of Tris base and 0.6 ml 10% SDS, the tube was mixed and incubated 30' at 65°C. 80 µl of 5M potassium acetate (KAc) were

added and then the tube was incubated on ice for at least 1 hour. The tube was then centrifuged 15', the supernatant was transferred in a new tube and DNA was then precipitated and washed with 100% ethanol. Dried pellet was carefully resuspended in 0.5 ml of TE 1X. 25 µl of 1 mg/ml RNAse was added to the tube and the solution was incubated 20' at 37°C. DNA was then precipitated by addition of 0.5 ml of isopropanol and then centrifuged. Pellet was washed with 70% cold ethanol, air-dried and finally resuspended in 50 µl of TE 1X.

Site-directed mutagenesis of *CDC14*

CDC14 was mutagenized according to the QuickChange Site-Directed mutagenesis kit (Stratagene) instructions. Plasmid Rp49 containing *CDC14* open reading frame (ORF) under the control of its endogenous promoter was used as template in the amplification reactions. Primers listed in Table 2.2 were used to introduce the specific mutations. The mutagenesis mix contained 5 µl buffer, 3 µl quick solution, 10 ng DNA template 125 ng for each primer, 1 µl dNTP mix, 1 µl *Pfu Turbo* DNA polymerase and water up to 50 µl. The template DNA was next eliminated with DpnI digestion, a restriction enzyme that cleaves only methylated DNA. 2µl of the mixture was used to transform XL10-Gold bacterial cells in order to recover and amplify the mutated plasmids. The plasmids were next sequenced to ascertain that the only mutation they carried was the one we intended to insert. The primers used in the sequencing reaction are listed in Table 2.2. Plasmids pFM3, pFM4, pFM9, pFM10, pFM13, pFM14 were used as templates for another round of mutagenesis to generate plasmids containing multiple mutations to A or to D in *CDC14*.
Amplification of mutated *CDC14* was performed with the following PCR parameters:

1. heat shock step	30''	at 95°C
2. denaturation step	30''	at 95°C
3. annealing step	1'	at 55°C

4. extension step (2'/Kb)	17'	at 68°C
5. repeat 18 times steps from 2 to 4		
6. extension step	7'	at 68°C
7. end	hold	at 4°C

Agarose gel electrophoresis

Following the addition of 1/5 volume of bromophenol blue (BPB) solution, DNA samples were loaded on 0.8% -1% agarose gels along with DNA markers. Gels were made in 1X Tris-Acetate-EDTA (TAE) buffer containing 10 μg/ml ethidium bromide and run at 80-120 volts (V) until desired separation was achieved. DNA bands were visualized under a UV lamp (radiation wavelength 260 nm).

Solution:

BPB solution: 0.2% BFB in 50% glycerol

10X TAE buffer: 0.4 M Tris acetate
0.01 M EDTA

Protein techniques

Protein extraction

10 ml of a cell culture at OD_{600} = 0.2-1 were collected and centrifuged for 2' at maximum speed. The resulting pellet was washed with 1 ml of cold 10 mM Tris-HCl pH 7.5, transferred to 2 ml Sarstedt tubes and frozen in liquid nitrogen in order to better preserve protein integrity. The pellet was then resuspended in 100 μl of lysis buffer supplemented with complete protease inhibitor cocktail (Roche) and phosphatases inhibitors (60 mM β-glycerol phosphate, 0.1 mM Na orthovanadate, 5 mM NaF, 15 mM p-

Nitrophenylphosphate). An equal volume of acid-washed glass beads (Sigma) was added (leaving a layer of supernatant over the beads) and the tubes were subjected to 3 rounds of Fast Prep (speed 6.5 for 45'') at 4°C in order to break the cells. Cell breakage was checked under the optical microscope. Lysed cells were transferred to a fresh tube. In order to quantify the protein content, 10 µl of the lysate were diluted 1:3 with cold 50 mM Tris-HCl pH 7,5/ 0,3 M NaCl and 3 µl were used in the Biorad protein quantification assay. The absorbance was read at λ= 595 nm. 50 µl of 3x SDS blue loading buffer was then added to each sample. The samples were boiled at 95ºC for 5', centrifuged at 13000 rpm for 3' and the supernatant, containing the final protein extract, was collected in a new microcentrifuge tube. Extracts were stored at -20°C.

Solutions:

Lysis buffer: 50 mM Tris-HCl pH 7,5
1 mM EDTA pH 8
50 mM DTT

3X SDS blue loading buffer: 9% SDS
30% glycerol
0.05% Bromophenol blue
6% β-mercaptoethanol
0.1875 M Tris-HCl pH 6.8

Protein extraction from TCA treated yeast cells

10 ml of a cell culture at OD_{600} = 0.2-1 were collected and centrifuged for 2' at maximum speed. The resulting pellet was resuspended in an equal volume of ice-cold 5% trichloroacetic acid (TCA) and incubated 10' on ice. After centrifuging 2' at maximum speed at 4°C, the pellet was transferred with 1 ml 5% TCA to a 2 ml Sarstedt tube. The

tube was centrifuged at 4°C and the supernatant discarded. The pellet was frozen in liquid nitrogen in order to better preserve protein integrity. Pellet was then washed with 1 ml room temperature absolute acetone and air-dried. The pellet was next resuspended in 100 µl of lysis buffer supplemented with complete protease inhibitor cocktail (Roche) and phosphatases inhibitors (60 mM β-glycerol phosphate, 0.1 mM Na orthovanadate, 5 mM NaF, 15 mM p-Nitrophenylphosphate). An equal volume of acid-washed glass beads (Sigma) was added (leaving a layer of supernatant over the beads) and the tubes were subjected to 3 rounds of Fast Prep (speed 6.5 for 45'') at 4°C in order to break the cells. Cell breakage was checked under the optical microscope. 50 µl of 3X SDS blue loading buffer was then added to each sample. The samples were boiled at 95ºC for 5', centrifuged at 13000 rpm for 3' and the supernatant, containing the final protein extract, was collected in a new microcentrifuge tube. Extracts were stored at -20°C.

Solutions:

Lysis buffer:	50 mM Tris-HCl pH 7,5
	1 mM EDTA pH 8
	50 mM DTT
3X SDS blue loading buffer:	9% SDS
	30% glycerol
	0.05% Bromophenol blue
	6% β-mercaptoethanol
	0.1875 M Tris-HCl pH 6.8

Immunoprecipitation and phosphatase assay

50 ml of a cell culture were treated as described in Material and Methods-TCA yeast protein extraction. Cell breakage was checked under the optical microscope. SDS to a final concentration of 1% was added and the sample was boiled 5' at 95°C. 9 volumes of NP40 buffer containing 2 mg/ml BSA were added, the tube was gently mixed and then centrifuged 10' at 13000 rpm at 4°C. After centrifugation, the supernatant was transferred, avoiding the debris on the bottom of the tube, into a new eppendorf tube. This step was repeated another time. Rabbit anti-Clb2 antibodies (Santa Cruz) were added at a dilution of 1:50 to 500 μl of protein extract and samples were incubated on a rotating wheel for 1 hour at 4°C. 50 μl of Protein G-conjugated agarose beads (PIERCE) suspension pre-incubated (at least 30' on the rotating wheel at 4°C) with 5 mg/ml BSA, were next added to each sample. The samples were then incubated on the rotating wheel at 4°C for 2 hours. After incubation with the beads, the beads bound to the protein of interest were washed:

2x	1 ml of NP40 buffer
1x	1 ml of NP40 buffer + 1% β-mercaptoethanol
2x	1 ml of NP40 buffer 1% β-mercaptoethanol + 2 M Urea
1x	1 ml of 10 mM Tris-HCl pH 7.5

During each wash:

- samples were centrifuged for 1' at 3000 rpm at 4°C
- supernatant was removed paying attention at not disturbing beads

After the last wash, 1 ml 1X calf intestinal phosphatase (CIP) buffer (NEB) was added to the beads, the tube was gently agitated and the suspension was centrifuged at 3000 rpm. After discarding the supernatant 45 μl of 1X CIP buffer were added to the sample together with 5 μl of CIP. 50 μl of 1X CIP buffer were added to a control sample. Samples were incubated at 37°C for 30'. Samples were then washed twice with 1 ml NP40 buffer. After

the last wash, samples were centrifuged at 3000 rpm and supernatants were removed. 30 μl of 3X SDS blue loading buffer were added, samples were boiled 5' at 95°C and then loaded on SDS-PAGE.

SDS polyacrylamide gel electrophoresis

Proteins were separated based on their molecular weight on 6%, 8% or 10% polyacrylamide gels. Gels were prepared from 30%, 30:0.8 acrylamide:bisacrylamide mix (Sigma) and 4X Separating buffer or 4X Stacking buffer and an appropriate amount of ddH2O. As polymerization catalysts ammonium persulphate (APS) and TEMED (BDH) were used. Gels were run in SDS-PAGE Running buffer at 50-150 V for 2-3.5 hrs. For Phos-tag gels, 40 μM of Phos-tag (NARD Institute, Ltd) and 80 μM $MnCl_2$ were added to the separating gel. Before transfer to nitrocellulose the gels were incubated in 1X Western Transfer Buffer supplemented with 1 mM EDTA for 10', in order to chelate the Mn ions, and then washed in 1X Western Transfer Buffer for 10'.

Solutions:

30%, 30:0.8 acrylamide:bisacrylamide mix (Sigma).

4X Separating buffer:	1.5 M Tris base pH 8.8 with glacial acetic acid
	0.4% SDS
4X Stacking buffer:	0.25 M Tris base pH 6.8 with glacial acetic acid
	0.2% SDS
10X SDS-PAGE Running buffer:	2 M glycine
	0.25 M Tris-HCl
	0.02 M SDS, pH 8.3

Western blot hybridization

Proteins were transferred in western transfer tanks to nitrocellulose (Protran, Whatman) in 1X Western Transfer buffer at 30 V ON or 100 V for 1.5-2 hrs. Ponceau S staining was used to roughly reveal the amount of protein transferred onto the filters.

Membranes were blocked with:

- 3% milk in PBS-T for Western blots using anti-HA, anti-Myc, anti-GFP, anti-Clb2 and anti-Pgk1 antibodies, 1 hour at RT
- 5% ovalbumin in PBS-T for Western blots using anti-Cdc14 and anti-Cdc5 antibodies, 5 hours at RT
- 5% milk in TBS-T for Western blots using anti-Clb5 antibodies, 3 hours at RT

After blocking, membranes were incubated with the primary antibody:

- 1:1000 mouse anti-Myc (Covance, 9E10), 1:1000 mouse anti-HA (Covance), 1:5000 mouse anti-Pgk1 (Invitrogen), 1:1000 rabbit anti-Clb2 (Santa Cruz), 1:1000 mouse anti-GFP (Chemicon), diluted in 1% milk/ 1% BSA/ PBS-T for 2 hrs at RT or ON at 4˚C
- 1:1000 goat anti-Cdc14 (Santa Cruz), 1:1000 goat anti-Cdc5 (Santa Cruz) diluted in 1% ovalbumin/ PBS-T, ON at 4°C
- 1:1000 goat anti-Clb5 (Santa Crutz) diluted in 5% milk/TBS-T, 3 hrs at RT or ON at 4°C

Membranes were then washed 3 x 15’ in PBS-T or TBS-T, after which they were incubated with the horseradish-peroxidase-conjugated secondary antibody:

- 1:10000 anti-mouse, 1:10000 anti-rabbit in 1% milk/ 1%BSA/ PBS-T for 1 hour
- 1:5000 anti-goat in 1% ovalbumin/ PBS-T or TBS-T for 1 hours

After incubation with the secondary antibody, the membrane was washed 3 x 15' in PBS-T or TBS-T and the bound secondary antibody was revealed using ECL (Enhanced Chemiluminescence, Amersham).

Solutions:

1X Western Transfer buffer:	0.2 M glycine
	0.025 M Tris base
	20% methanol
10X PBS buffer:	1.37 M NaCl
	27 mM KCl
	14.7 mM KH_2PO_4
	80 mM Na_2HPO_4

PBS-T buffer: 0.1% Tween, 1X PBS

Kinase Assays

Cdc5 kinase assay

25 ml of a cell culture at OD_{600}: 0.5 were treated as described in (Charles et al., 1998).

Cells were centrifuged for 2' at maximum speed. The resulting pellet was transferred with 1 ml Tris-HCl pH 7.4 to a 2 ml Sarstedt tube. The tube was centrifuged at 4°C and the supernatant discarded. The pellet was frozen in liquid nitrogen in order to better preserve protein status. Pellet was then resuspended in 100-150 µl of LLB breakage buffer supplemented with complete protease inhibitor cocktail (Roche) and phosphatases inhibitors (60 mM β-glycerol phosphate, 0.1 mM Na orthovanadate, 5 mM NaF, 15 mM p-

Nitrophenylphosphate). An equal volume of acid-washed glass beads (Sigma) was added (leaving a layer of supernatant over the beads) and the tubes were subjected to 3 rounds of Fast Prep (speed 6.5 for 45'') at 4°C in order to break the cells. Cell breakage was checked under the optical microscope. The samples were centrifuged twice at maximum speed at 4°C for 10' to clean up the extract. Supernatant was transferred to a new tube avoiding cellular debris at the bottom and lipid layer at the top. Protein concentration was determined by Bradford Assay (see TRIS protein extraction for details). 500 ug of extract were used for the immunoprecipitation. An appropriate volume of LLB breakage buffer was added to each tube so that all tubes are at the same volume. Mouse anti-HA antibodies (Covance) were added at a dilution of 1:50 and samples were incubated on a rotating wheel for 30' at 4°C. 20 μl of slurry Protein A-conjugated agarose beads (PIERCE) were next added to each sample. The samples were then incubated on the rotating wheel at 4°C for 1 hour. After incubation with the beads, the beads bound to the protein of interest were washed:

2x	1 ml of LLB
1x	1 ml of High-Salt QA
1x	1 ml of 5KB
2x	1 ml of KB

During each wash:

- samples were centrifuged for 1' at 2000 rpm at 4°C
- supernatant was removed paying attention at not disturbing beads

Beads bound to the protein of interest were resuspended with 30 ul of Reaction mix and incubated for 15' at RT. Then 15 ul of 3X sample buffer was added to stop the reaction

and samples were boiled for 3' at 95°C. Samples were spun down at maximum speed for 5' and then loaded on a 12.5% gel. The gel was fixed in 10% Ac. Acetic, 10% MeOH for 20-30', dryed for 1-3 hrs depending on the size and then exposed.

Solutions:

LLB buffer:	50 mM HEPES pH 7.4
	0.1% NP40
	5 mM NaF
	5 mM β-glycerol phosphate
	1 mM $MgCl_2$
	1 mM DTT
	75 mM KCl
LLB Breakage Buffer:	LLB
	1X protease inhibitor cocktail (PIC, Roche)
	100 mM PMSF
	0,1 M Na orthovanadate
High-Salt QA buffer:	20 mM Tris-HCl pH 7.6
	250 mM KCl
	1 mM $MgCl_2$
	1 mM DTT
5KB buffer:	50 mM HEPES pH 7.4
	200 mM KAc
	10 mM $MgCl_2$
	5 mM $MnCl_2$

	1 mM DTT
KB buffer:	50 mM HEPES pH 7.4
	60 mM KAc, 10 mM $MgCl_2$, 5 mM $MnCl_2$

Kinase reaction mix:

1 mg/ml Casein (Sigma) in KB buffer	29.6 μl
10 mM ATP	0.15 μl
6000 Ci/mmol gamm-ATP	0.25 μl

Dbf2 kinase assay

15 ml of a cell culture at OD_{600}: 0.5 were collected and centrifuged for 2' at maximum speed. The resulting pellet was transferred with 1 ml Tris-HCl pH 7.4 to a 2 ml Sarstedt tube. The tube was centrifuged at 4°C and the supernatant discarded. The pellet was frozen in liquid nitrogen in order to better preserve protein status. Pellet was then resuspended in 100-150 μl of NP40 buffer supplemented with complete protease inhibitor cocktail (Roche) and phosphatases inhibitors (60 mM β-glycerol phosphate, 0.1 mM Na orthovanadate, 5 mM NaF, 15 mM p-Nitrophenylphosphato). An equal volume of acid-washed glass beads (Sigma) was added (leaving a layer of supernatant over the beads) and the tubes were subjected to 3 rounds of Fast Prep (speed 6.5 for 45'') at 4°C in order to break the cells. Cell breakage was checked under the optical microscope. The samples were centrifuged twice at maximum speed at 4°C for 10' to clean up the extract. The supernatant was transferred to a new tube avoiding cellular debris at the bottom and lipid layer at the top. Protein concentration was determined by Bradford Assay (see TRIS protein extraction for details). 300 ug of extract were used for the immunoprecipitation. An appropriate volume of LLB breakage buffer was added to each tube so that all tubes had

the same volume. Mouse anti-Myc antibodies (Covance) were added at a dilution of 1:50 and samples were incubated on a rotating wheel for 1 hour at 4°C. 20 μl of slurry Protein A-conjugated agarose beads (PIERCE) were next added to each sample. The samples were then incubated on the rotating wheel at 4°C for 2 hours. After incubation with the beads, the beads bound to the protein of interest were washed:

4x 1 ml of NP40 buffer

2x 1 ml of 25 mM mops

During each wash:

- samples were centrifuged for 1' at 2000 rpm at 4°C
- supernatant was removed paying attention at not disturbing beads

Beads bound to the protein of interest were resuspended with 6 ul of HBII buffer and incubated for 15' at RT. 10 ul of kinase reaction mix was added to the samples. Samples were incubated for 15' at RT. Then 10 ul of 3X sample buffer was added to stop the reaction. Samples were spun down at maximum speed for 5' and then loaded on a 12.5% gel. The gel was fixed in 10% Ac. Acetic, 10% MeOH for 20-30', dryed for 1-3 hrs depending on the size and then exposed.

Solutions:

NP40 buffer: 150 mM NaCl
50 mM Tris-HCl pH 7.5
1% NP40

HBII buffer: 60 mM β-glycerol phosphate
15 mM Mops
15 mM $MgCl_2$

5 mM EGTA

1 mM DTT

1X protease inhibitor chocktail (PIC- Roche)

0.1 mM Na orthovanadate

15 mM p-Nitrophenylphosphate

Kinase reaction mix:

equal volume of SOL1 and SOL2

SOL1:

100 mM cold ATP	1.1 μl
6000 Ci/mmol gamm-ATP	3 μl
H2O	275 μl

SOL2:

4 mg/ml Histon H1 in 50 mM Mops

Clb2 kinase assay

Same as for Dbf2. Rabbit anti-Clb2 (SantaCrutz) were addedat a dilution of 1:50.

Yeast procedures

Tetrad dissection and analysis

MATa and *MATα* strains were mixed on the appropriate solid medium and incubated overnight at permissive conditions. The next day, cells from the cross mixture were streaked to single colonies on selective medium and incubated at the appropriate temperature, allowing for selection of diploid cells. Single colonies grown under selective conditions were next amplified on rich media for 1 day. This step greatly increases the efficiency of sporulation. The next day diploids were patched onto sporulation plates (1%

KAcetate, all amino acids at 1/4 of the normal concentration) to induce meiosis and sporulation by starvation. After ~ three days diploids have efficiently sporulated and maturated and tetrads can now be dissected.

In order to separate individual spores the wall of the ascus or tetrad is removed by enzymatic digestion.

Digestion conditions: a toothpick full of tedrads is resuspended into:

200 μl ddH_2O

2 μl 10 mg/ml zymolase 100T (Seikagaka, Biobusiness)

The digestion mixture was then incubated at 30°C for 5' in order to enzymatically digest the asci sac. Then, 1 ml ddH_2O was added to dilute the mix and 20 μl were dripped in a line onto the appropriate agar plate. Individual tetrads were dissected using the Nikon dissection microscope. Spores were left to grow at RT for 3-5 days. Colonies were replica plated onto selective media to define their genotype.

BAR-test/alpha factor sensitivity

The *BAR1* gene (BARrier to the α-factor response) encodes for a protease that is secreted into the periplasmic space of *MATa* cells (Sprague, 1981). This protease cleaves and inactivates the α-mating factor pheromone allowing cells to recover from α-factor-induced cell cycle arrest. Transcription of *BAR1* in *MATα* haploids and *MATa*/*MATα* diploids is repressed. *BAR1* is a *MATa* specific gene, whose transcription is stimulated by the presence of α-factor (Kronstad et al., 1987; Manney et al., 1983). *MATa* cells that lack the Bar1 protein are supersensitive to an α-factor-induced G1 arrest (Sprague, 1981). When assessing the genotype of yeast strains, the presence or absence of the Bar1 protein is assessed with what we call the "BAR-test".

MATa cells (one streak through a patch) were resuspeded in 50 μl of the appropriate medium. An appropriate volume of the appropriate medium containing 0.8% agarose was boiled. When cooled down, 3 ml of agarose/medium were mixed with the cell suspension mixture and poured into a small Petri dish. The Petri dish was put on ice until the mixture was solid. A small dot of *MATα* tester strain was put in the center of the dish. The dish was incubated at RT for 1-2 days. *bar1* mutant strains, being highly sensitive to the presence of α-factor, do not grow in proximity of the *MATα* cells and produce a halo around the tester strain.

Synchronization experiments

Cells were grown ON in the appropriate volume of YEPD or YEPR at 23°C in a water shaking bath. The day after, cells were diluted to OD_{600} = 0.2 in fresh medium and left to grow for 2 hrs.

G1 arrest and release:

In order to synchronize cells in G1-phase, cell cultures were then diluted again to OD_{600} = 0.2 and added with 5 μg/ml α-mating factor synthetic peptide (Primm). After 90' incubation, 2.5 μg/ml α-factor was re-added to the culture. The G1 arrest was considered complete when more than 90% of the cells have shmoo. For G1 arrest experiments, to maintain cells in G1, 2.5 μg/ml α-factor was added every 90' until the end of the experiment. For synchronization experiments, after the arrest was complete, cells were released from the G1 block. α-factor was washed out by filtration, using between 5 to 10 volumes of medium without the pheromone. Cells were next released into the appropriate fresh medium in the absence of the pheromone.

S phase and Metaphase arrests and releases:

For S phase or metaphase arrest experiments: cells were first pre-synchronized in G1 by addition of α-factor and next released in a medium containing 10 mg/ml hydroxyurea (HU, Sigma) or 15 μg/ml nocodazole (NOC, Sigma) dissolved in DMSO, respectively. In the case of a nocodazole arrest, 7.5 μg/ml nocodazole was re-added to the culture after 90' incubation.

For synchronization experiments, after the arrest was complete, cells were released from blocks. Drugs were washed out by filtration, using between 5 to 10 volumes of medium without the without them, in the case of nocodazole releases 1% of DMSO was added to the washing media. Cells were next released into the appropriate fresh medium.

Note: when releasing from nocodazole it is advisable to add 1% DMSO also in the flask of release.

Special treatments:

- Expression of genes under the control of the *GAL1-10* promoter, was achieved by adding 2% galactose to the medium.
- Repression of genes under the control of the *MET3* promoter, was achieved by adding 8mM of fresh methionine to the medium (re-add 4mM methionine every hours).
- ATP analogue-sensitive alleles (*cdc15-as1* and *cdc28-as1*) were inactivated by adding 5μM 1NM-PP1 to the cell culture (Bishop et al., 2001).
- The ATP analogue-sensitive allele *cdc5-as1* (Zhang et al., 2005), was inactivated by adding 5 μM CMK to the medium.
- Temperature sensitive alleles were inactivated by incubating the colture to the restrictive temperature.

Reagents:

Purified α-factor synthetic peptide (Primm), 5 mg/ml stock dissolved in ddH2O

Hydroxyurea (Sigma), powder

Nocodazole (Sigma), 15 mg/ml stock dissolved in DMSO

1NM-PP1 (Toronto Research Chemicals), 20 mM stock in DMSO

CMK (Accenda Tech), 10 mM stock in DMSO

***In situ* indirect immunofluorescence**

1.5 ml of a cell culture at OD_{600} = 0.2-0.4 were collected by centrifugation and incubated 10' at RT or ON at + 4°C in 1 ml fixative solution (3.7% formaldehyde in 0.1 M KPi pH 6.4). Cells were pelleted and washed 3 times with 1 ml of 0.1 M KPi pH 6.4 followed by a wash with 1 ml of sorbitol-citrate solution. Cells were then resuspended in 200 µl of digestion solution and incubated at 30°C in order to enzymatically digest the cell wall, creating spheroplasts. Spheroplasts are osmotically fragile and lyse in a hypotonic solution; 1.2 M sorbitol maintains an isotonic environment in order to avoid cell lysis. The low pH helps in slowing down the endogenous cell proteolytic activity. Digestion was checked by looking for burst spheroplasts when mixed with an equal volume of 1% SDS under an optical microscope. When the digestion was complete, the obtained spheroplasts were pelleted at 2000 rpm for 2' and washed with 1 ml of sorbitol-citrate solution. The pellet was then resuspended in an appropriate volume of sorbitol-citrate solution (from 10 to 50 µl, depending on the pellet size). 5 µl of the resuspended spheroplasts were then loaded on a 30-well slide previously coated with 0.1 % polylysine (Sigma). To further fix cells, the slide was then put in cold methanol for 3', followed by 10'' in cold acetone. Cells were then incubated, in a humid dark incubation chamber, with primary antibody/ies at the following conditions:

- 1:100 rat anti-tubulin (Oxford-Biotechonology) in PBS-BSA for 90'

- 1:150 mouse anti-HA (Covance) in PBS-BSA for 90'
- 1:300 goat anti-Cdc14 (Santa Cruz) in PBS-ovalbumin for 2 hours
- 1:500 mouse anti-Nop1 (EnCor Biotechnology) in PBS-BSA for 90'

After incubation, cells were washed 5x with PBS-BSA or PBS-ovalbumin and incubated with secondary antibody at the following conditions:

- 1:100 FITC-conjugated anti-rat in PBS-BSA for 90'
- 1:1000 Cy3-coniugated anti-mouse in PBS-BSA for 90'
- 1:500 Cy3-coniugated anti anti-goat in PBS-ovalbumin for 2 hours
- 1:500 Cy3-coniugated anti-mouse in PBS-BSA for 90'

Cells were then washed 5x with PBS-BSA or PBS-ovalbumin and then treated with a DAPI mount solution. The slide was covered with a coverslip and sealed with nail polish. Immunofluorescence was analysed with a Leica DMR HC BIOMED fluorescence microscope using a 100X immersion-oil objective.

Solutions:

0.1 M KPi buffer pH 6.4:	27.8 ml 1 M K_2HPO_4
	72.2 ml 1 M KH_2PO_4
	900 ml ddH2O
Fixative solution:	3.7% formaldehyde
	0.1 M KPi pH 6.4
Sorbitol-citrate solution:	1.2 M Sorbitol
	0.1 M phospho-citrate pH 5.9

Digestion solution:	1.2 M sorbitol-citrate
	10% glusulase
	0.1 mg/ml zymolase
PBS-BSA:	1% crude BSA (Sigma)
	0.04 M K_2HPO_4
	0.01 M KH_2PO_4
	0.15 M NaCl
	0.1% NaN_3
PBS-ovalbumin:	1% crude ovalbumin
	0.04 M K_2HPO_4
	0.01 M KH_2PO_4
	0.15 M NaCl
	0.1% NaN_3
DAPI mount solution:	0.04 M K_2HPO_4
	0.01 M KH_2PO_4
	0.15 M NaCl
	0.1% NaN_3
	0.05 μg/ml DAPI
	0.1% p-phenylenediamine
	90% glycerol

Percentage of budded cells (budding index)

1 ml of culture was collected and incubated 10' at RT in 1 ml fixative solution (3.7% formaldehyde in 0.1 M KPi pH 6.4). Cells were next washed 1x with 0.1 M KPi pH

6.4. Fixed cells were sonicated and a 5 μl aliquot was scored under the light microscope for the presence or absence of the bud. 200 cells were scored for each sample.

Fluorescence-activating cell sorting (FACS)

1.5 ml of a cell culture at OD_{600} = 0,2 - 0,4 were collected by centrifugation and suspended in a Tris-HCl/ethanol solution for 15'. Cells were then washed with 1 ml of 50 mM Tris-HCl pH 7.4 and suspended in the same buffer containing 1 mg/ml of RNase. Samples were incubated ON at 37°C. The day after the cells were collected by centrifugation and washed with 1 ml FACS buffer and stained in the same buffer containing 55 μg/ml propidium iodide (Fluka). Samples were then diluted 10-fold in 50 mM Tris-HCl pH 7.4 and analyzed using a Becton Dickinson FACScan.

Solutions:

Tris-HCl/Ethanol solution:	250 mM Tris-HCl pH 7.4
	70% ethanol
	50 mM Tris-HCl pH 7.4
FACS buffer:	200 mM Tris-HCl pH 7.4
	200 mM NaCl
	78 mM $MgCl_2$

Nuclei staining (DAPI staining)

1 ml of a cell culture at OD_{600} = 0.2-0.4 were collected by centrifugation and incubated 10' at RT in 1 ml of 70% ETOH. Cells were pelleted and then resuspended in 20 μl of DAPI 0.001 mg/ml.

Plasmid	Template	Primers	Mutated AA
pFM1	YIplac204-3HA-CDC14	FM56-FM57	T10A
pFM2	YIplac204-3HA-CDC14	FM58-FM59	T10D
pFM3	YIplac204-3HA-CDC14	FM9-FM10	T140A
pFM4	YIplac204-3HA-CDC14	FM11-FM12	T140D
pFM5	YIplac204-3HA-CDC14	FM1-FM2	S260A
pFM6	YIplac204-3HA-CDC14	FM3-FM4	S260D
pFM7	YIplac204-3HA-CDC14	FM13-FM14	S391A
pFM8	YIplac204-3HA-CDC14	FM15-FM16	S391D
pFM9	YIplac204-3HA-CDC14	FM5-FM6	S471A
pFM10	YIplac204-3HA-CDC14	FM7-FM8	S471D
pFM11	YIplac204-3HA-CDC14	FM-60-FM61	T517A
pFM12	YIplac204-3HA-CDC14	FM62-FM63	T517D
pFM13	pFM3	FM56-FM57	T10A-T140A
pFM14	pFM4	FM58-FM59	T10D-T140D
pFM15	pFM13	FM1-FM2	T10A-T140A-S260A
pFM16	pFM14	FM3-FM4	T10D-T140D-S260D
pFM17	pFM9	FM13-FM14 FM60-FM61	T391A-S471A-T517A
pFM18	pFM10	FM15-FM16 FM62-FM63	T391D-S471D-T517D
pFM19	pFM13 + pFM17	N/A	T10A-T140A- S391A- S471A-T517A
pFM20	pFM14 + pFM18	N/A	T10D-T140D-S391D- S471D-T517D
pFM21	pFM19	FM1-FM2	T10A-T140A-S260- S391A-S471A-T517A
pFM21	pFM20	FM3-FM4	T10D-T140D-S260- S391D-S471D-T517D

Table 2.1: Plasmids used in this study.
N/A applies to plasmid obtained by cloning rather then via PCR directed-mutagenesis.

Name	Sequence (5'-3')	Purpose
FM1	ggtacatgtcctgatcttgccattgtaaaaaactttgttgg	Cdc14 mutagenesis
FM2	ccaacaaagtttttttacaatggcaagatcaggacatgtacc	Cdc14 mutagenesis
FM3	ggtacatgtcctgatcttgacattgtaaaaaactttgttggtgc	Cdc14 mutagenesis
FM4	gcaccaacaaagtttttttacaatgtcaagatcaggacatgtacc	Cdc14 mutagenesis
FM5	gcaacaactcagatgacgaagccatgcatgataccaacg	Cdc14 mutagenesis
FM6	cgttggtatcctgcatggcttcgtcatctgagttgttgc	Cdc14 mutagenesis
FM7	gcaacaactcagatgacgaagacatgcaggataccaacg	Cdc14 mutagenesis
FM8	cgttggtatcctgcatgtcttcgtcatctgagttgttgc	Cdc14 mutagenesis
FM9	gcggatttcgaaatcgcaattcaagatgtggtttatgg	Cdc14 mutagenesis
FM10	ccataaaccacatcttgaattgcgatttcgaaatccgc	Cdc14 mutagenesis
FM11	gcggatttcgaaatcgatattcaagatgtggtttatggcg	Cdc14 mutagenesis
FM12	cgccataaaccacatcttgaatatcgatttcgaaatccgc	Cdc14 mutagenesis
FM13	ggtgaattaagagatttagccatgacgccgc	Cdc14 mutagenesis
FM14	gcggcgtcatggctaaatctcttaattcacc	Cdc14 mutagenesis
FM15	ggtgaattaagagatttagacatgacgccgccatcc	Cdc14 mutagenesis
FM16	ggatggcggcgtcatgtctaaatctcttaattcacc	Cdc14 mutagenesis
FM56	ggagtgtatacctcgacaacgcgatcgagttcc	Cdc14 mutagenesis
FM57	ggaactcgatcgcgttgtcgaggtatacactcc	Cdc14 mutagenesis
FM58	ggagtgtatacctcgacaacgacatcgagttcctgc	Cdc14 mutagenesis
FM59	gcaggaactcgatgtcgttgtcgaggtatacactcc	Cdc14 mutagenesis
FM60	ccgcagatgatgccatactaagacaactattgcc	Cdc14 mutagenesis
FM61	ggcaatagttgtcttagtatggcatcatctgcg	Cdc14 mutagenesis
FM62	ccgcagatgatgacatactaagacaactattgcc	Cdc14 mutagenesis
FM63	ggcaatagttgtcttagtatgtcatcatctgcgg	Cdc14 mutagenesis
FM45	ggatttcggtcccatgaacattgg	Cdc14 sequencing
FM46	gccggttattctaatgcgg	Cdc14 sequencing
FM47	ccgctaacgagtgtattggc	Cdc14 sequencing
FM48	gacgacaagagggttgcgc	Cdc14 sequencing
FM49	catctgcatcgaccagacaacg	Cdc14 sequencing
FM50	gatgtccaatgttcatgggac	Cdc14 sequencing

FM51	gagaattcaaccttaccac	Cdc14 sequencing
FM52	cgtaatccaacttcccatgc	Cdc14 sequencing
FM53	ggaagcttttattcgaaatccgcattaga	Cdc14 sequencing
FM54	gtggtataagaaaaataagtggctc	Cdc14 sequencing
FM55	cggggtaccaattatttcttgatggagccac	Cdc14 sequencing

Table 2.2: Primers used in this study.

Name	Genotype
***E. coli* DH5α™**	*F Φ80 dlacZ ΔM15 Δ(lacZYA-argF) U169 deoR recA1 endA1 hsdR17 (r_K-, m_K+) supE44 λ- thi-1 qyrA96 relA1*
***E. coli* XL10-Gold®(Stratagene)**	*TetR Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F′ proAB lacIqZΔM15 Tn10 (TetR) Amy CamR]*

Table 2.3: Bacterial strains used in this study.

Strain	Relevant Genotype
Ry1	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+*
Ry278	*MATa, CDC14-3HA*
Ry394	*MATa, CFI1-3MYC, CDC14-3HA*
Ry430	*MATa, GAL-CLB2dBdel::URA3, CDC14-3HA*
Ry446	*MATa, clb5::URA3, CDC14-3HA*
Ry448	*MATa, GAL-CLB2dBdel::URA3, CFI1-3MYC, CDC14-3HA*
Ry798	*MATa, 1xGAL-CDC5(dN70aa)-HA::URA3*
Ry995	*MATa, cdc15::TRP1-GAL-GFP-CDC15(1-750)-HIS3, leu2::CDC15-HA3-LEU2, CFI1-3MYC, CDC14-3HA*
Ry1132	*MATa, cdc15::CDC15-as1(L99G).::URA3, CDC14-3HA*
Ry1324	*MATa, ura::GAL-3Myc-CDC5::URA3, cdc15::CDC15-as1(L99G)::URA3, CDC14-3HA, CFI1-3MYC*
Ry1325	*MATa, ura::GAL-3Myc-CDC5::URA3, CDC14-3HA, CFI1-3MYC*
Ry1345	*MATa, cdc15::TRP1-GAL-GFP-CDC15(1-750 –HIS3, leu2::CDC15-HA3-LEU2, 1xGAL-CDC5(dN70aa)-HA::URA3, CFI1-3MYC, CDC14-3HA*
Ry1353	*MATa, cdc15::TRP1-GAL-GFP-CDC15(1-750)-HIS3, leu2::CDC15-HA3-LEU2, 1xGAL-CDC5(dN70aa)-HA::URA3, cdc28d::cdc28-as1, CFI1-3MYC, CDC14-3HA*
Ry1356	*MATa, 1xGAL-CDC5(dN70aa)-HA::URA3, cdc28d::cdc28-as1, CFI1-3MYC, CDC14-3HA*
Ry1358	*MATa, 1xGAL-CDC5(dN70aa)-HA::URA3, CFI1-3MYC, CDC14-3HA*
Ry1387	*MATa, GAL-CLB2dBdel::URA3*
Ry1388	*MATa, GAL-CLB2dBdel::URA3, sic1::TRP1*
Ry1392	*MATa, GAL-CLB2dBdel::URA3, cdc5::KanMX6, ura3::CDC5dBdel::URA3, CDC14-3HA*
Ry1393	*MATa, GAL-CLB2dBdel::URA3, cdc5-1, CDC14-3HA*
Ry1394	*MATa, GAL-CLB2dBdel::URA3, cdc15::CDC15-as1(L99G)::URA3, CDC14-3HA*
Ry1428	*MATa, GAL-CLB2dBdel::URA3, cdc14::CDC14-yEGFP::KanMX*
Ry1452	*MATa, GAL-CLB2dBdel::URA3, cdc23-1, CDC14-3HA*
Ry1457	*MATa, GAL-CLB2dBdel::URA3, cdc20-3, CDC14-3HA*
Ry1466	*MATa, GAL-CLB2dBdel::URA3, cdh1::HIS3, CDC14-3HA*
Ry1510	*MATa, cdc5-ad1(L251W)*
Ry1538	*MATa, MET3-CDC20::URA3, pds1::URA3, cdc15::CDC15-as1(L99G)::URA3, clb5::URA3, CDC14-3HA*
Ry1545	*MATa, cdc15::TRP1-GAL-GFP-CDC15(1-750)-HIS3, leu2::CDC15-HA3-LEU2, 1xGAL-CDC5(dN70aa)-HA::URA3, CFI1-3MYC, CDC14-3HA*
Ry1547	*MATa, cdc15::TRP1-GAL-GFP-CDC15(1-750)-HIS3, leu2::CDC15-HA3-LEU2, GAL-CLB2dBdel::URA3, CDC14-HA*
Ry1558	*MATa, MET3-CDC20::URA3, pds1::URA3, cdc15::CDC15-as1(L99G)::URA3, CDC14-3HA*

Ry1573	*MATa, cdc14-1*
Ry1575	*MATa, GAL-CLB2dBdel::URA3, cdc14-1*
Ry1588	*MATa, clb5::URA3*, *cdc15::CDC15-as1(L99G)::URA3, CDC14-3HA*
Ry1606	*MATa, GAL-CLB2dBdel::URA3, cdc14-1, cdc5L158G*
Ry1607	*MATa, GAL-CLB2dBdel::URA3, cdc5L158G*
Ry1687	*MATa, GAL-CLB2dBdel::URA3, CDC5::Venus*
Ry1695	*MATa/MATalpha, CDC14/cdc14-1-6HA::kanMX6*
Ry1708	*MATa, GAL-CLB2dBdel::URA3, tof2::kanMX6, CDC14-3HA*
Ry1710	*MATa, GAL-CLB2dBdel::URA3, cfi1::URA3, CDC14-3HA*
Ry1715	*MATa, GAL-CLB2dBdel::URA3, net1::his5, net1-6Cdk-TEV-myc9::TRP1, cdc15::CDC15-as1(L99G)::URA3*
Ry2016	*MATa, ura::GAL-3Myc-CDC5::URA3, CLB2-3HA*
Ry2020	*MATa, CLB2-3HA*
Ry2248	*MATa, cdc15::TRP1-GAL-GFP-CDC15(1-750)-HIS3, leu2::CDC15-HA3-LEU2, 1xGAL-CDC5(dN70aa)-HA::URA3, DBF2-3MYC*
Ry2254	*MATa, cdc15::TRP1-GAL-GFP-CDC15(1-750)-HIS3, leu2::CDC15-HA3-LEU2, DBF2-3MYC*
Ry2257	*MATa, 1xGAL-CDC5(dN70aa)-HA::URA3, DBF2-3MYC*
Ry2446	*MATa, cdc5L158G*
Ry3536	*MATa, cdc15::TRP1-GAL-GFP-CDC15(1-750)-HIS3, leu2::CDC15-HA3-LEU2, dbf2-2*
Ry3540	*MATa, cdc15::TRP1-GAL-GFP-CDC15(1-750)-HIS3, leu2::CDC15-HA3-LEU2, cdc5L158G*

Table 2.4: Yeast strains. The table includes strains made and use in this work.

3. Results

An overview: What do we know about Cdc14 release?

Several observations suggest that the release of Cdc14 from its inhibitor Cfi1 (also known as Net1) is mediated by phosphorylation. *In vitro* studies suggest that phosphorylation triggers the dissociation of Cdc14 from its inhibitor Cfi1 (Shou and Deshaies, 2002; Shou et al., 1999). Furthermore, *in vivo* the release of Cdc14 correlates with an increased phosphorylation of Cdc14 and Cfi1 (Visintin et al., 2003). In agreement with the idea that phosphorylation controls the dissociation of the Cdc14-Cfi1 complex is the observation that the most downstream components of the networks that control Cdc14 release (FEAR and MEN) are kinases. Indeed the Polo-like kinase Cdc5, Clb2-Cdk and the most-downstream MEN kinase Dbf2 have all been implicated in this process (Azzam et al., 2004; Geymonat et al., 2003; Hu and Elledge, 2002; Hu et al., 2001; Mohl et al., 2009; Pereira et al., 2002; Queralt et al., 2006; Stegmeier et al., 2002; Visintin et al., 2003; Yoshida and Toh-e, 2002). More specifically, work from Azzam and colleagues (Azzam et al., 2004) supports the idea that the Clb2-Cdk kinase is responsible for Cfi1 phosphorylation. Phoshorylation that lead to the dissociation of Cdc14 from its inhibitor during early anaphase (Azzam et al., 2004; Sullivan et al., 2004). The authors showed that recombinant Clb2-Cdk could disrupt the Cdc14-Cfi1 complex *in vitro* (Azzam et al., 2004). Moreover, they identified several residues in Cfi1 that appears to be phosphorylated by Cdk1 *in vivo*. Interestingly, yeast strains carrying an allele of Cfi1 where these sites were replaced by alanine (an amino acid that cannot be phosphorylated) exhibited a kinetic of Cdc14 release reminiscent of the one characteristic of FEAR network mutants. This finding suggests that Clb2 is a FEAR network component and that Clb2-Cdk-mediated phosphorylation of Cfi1 is the mechanism via which the FEAR network controls the Cdc14-Cfi1 interaction (Azzam et al., 2004).

Of all the mitotic exit network components identified to date, the kinase Dbf2 is the most downstream acting and as such the most likely candidate for controlling the phosphorylation status of the Cdc14-Cfi1 complex in the later stages of anaphase. Although data in support of this hypothesis are still missing, Mohl and colleagues (Mohl et al., 2009) recently identified sites in Cdc14 whose phosphorylation by Dbf2 results in a cytoplasmic accumulation of the phosphatase. Whether Dbf2 is also responsible for phosphorylation events that trigger the dissociation of Cdc14 from Cfi1 is still unknown.

Of the three kinases mentioned above, the Polo-like kinase Cdc5 seems to take the lion's share. Cdc5 is part of the FEAR network and acts as an activator of the MEN (Stegmeier et al., 2002). It is unique among FEAR and MEN components in that it is required throughout anaphase for maintaining Cdc14 in its released state (Visintin et al., 2008). When overexpressed, Cdc5 is capable of inducing a release of Cdc14 in stages of the cell cycle during which the phosphatase is normally sequestered (Visintin et al., 2003). Similarly, recombinant Cdc5 dissociates the Cdc14-Cfi1 complex *in vitro* (Shou et al., 2002). Both *in vitro* and *in vivo*, the ability of Cdc5 to disrupt the interaction between Cdc14 and Cfi1 is associated with the ability of the protein to induce directly or indirectly phosphorylation of both Cdc14 and Cfi1 (Shou et al., 2002; Visintin et al., 2003). Although we have established a framework of how mitotic exit is controlled in budding yeast, important questions remain to be addressed. For instance, the molecular mechanism that regulates the Cdc14-Cfi1 interaction remains unclear and how the three kinases interact to induce a timely and complete release of Cdc14 is still unknown.

My PhD aimed to provide answers to those questions. More specifically I wished to reveal the mechanism(s) underlying the phospho-driven (dis)association of Cdc14 with its inhibitor Cfi1.

Cdc5 and one between Clb-Cdks and MEN are required for Cdc14 release

To evaluate the minimal requirement for mitotic kinases Clb2-Cdk, Cdc5 and Dbf2 to promote the release of Cdc14, we analyzed the consequences of modulating the activity of these kinases either alone or in mutual combination in the G1 and S phases of the cell cycle. In these phases, both endogenous Clbs and Cdc5 are absent (G1-phase) or just start to accumulate (S-phase), MEN is inactive and there is no contribution of other mitotic regulators; thus Cdc14 is maintained sequestered in the nucleolus.

Ectopic expression of the kinases Cdc5 and Clb2 was obtained by placing their coding sequence under the control of the galactose-inducible and glucose-repressible promoter *GAL1-10*. As for Dbf2, its ectopic activation was achieved by modulating the expression of an allelic variant of MEN kinase Cdc15 (*CDC15*(1-750)), truncated of its auto-inhibitory loop. Overexpression of this hyperactive allele of Cdc15 was shown to result in the ectopic activation of Dbf2 (Bardin et al., 2003; Visintin et al., 2003). As for Clb2 and Cdc5, the coding sequence of this allele of *CDC15* was placed under the control of the *GAL1-10* promoter.

We started to assess the ability of Cdc5 and Cdc15 to release Cdc14 from the nucleolus in G1 arrested cells. As Cdc5 is a target of the APC/C^{Cdh1} and the APC/C^{Cdh1} is active in G1 (Shirayama et al., 1998), we analyzed the contribution of Cdc5 in this phase of the cell cycle, by overexpressing an allele of *CDC5* that is resistant to degradation by the APC/C^{Cdh1} (Shirayama et al., 1998). APC/C substrates carry recognition motifs known as destruction boxes and KEN boxes. The core sequence of destruction-box motifs is RxxLxxxxN, while the KEN box comprises four essential ammino acids, KENxxxN (Harper et al., 2002). These sequences contain sufficient information to confer APC/C-dependent ubiquitination on a protein. Cdc5 contains two destruction boxes at its N-terminus, which are essential for the protein's degradation during exit from mitosis

(Charles et al., 1998; Shirayama et al., 1998). To determine whether overexpression of *CDC5* was sufficient to trigger the release of Cdc14 in G1, we examined the consequences of expressing an allele of Cdc5 that lacks the first 70 amino acids of the protein. This fragment contains both Cdc5's destruction boxes (*CDC5ΔN70*, henceforth *CDC5dBΔ*) (Shirayama et al., 1998).

Cells carrying one integrant of the *GAL-CDC5dBΔ* construct, the *GAL-CDC15(1-750)* and both *GAL-CDC5dBΔ* and *GAL-CDC15(1-750)* were arrested in G1. When the arrest was complete, as judged by more than 90 percent of cells having shmoos, the pheromone was re-added to the medium to maintain the cells blocked in G1 together with galactose to drive ectopic overexpression of *CDC5* and *CDC15*. Cdc14 localization was analyzed by indirect immunofluorescence (IF) using anti-Cdc14 antibodies. We found that in G1 arrested cells, neither the overexpression of a non-degradable form of Cdc5 (*GAL-CDC5dBΔ*), nor of a hyperactive version of Cdc15 (*GAL-CDC15(1-750)*) was able to promote the release of Cdc14 from the nucleolus. In contrast, the concomitant *GAL1-10* driven expression of non-degradable Cdc5 and hyperactive Cdc15 induced a Cdc14 release, Fig. 3.1a. We conclude that Cdc5 and MEN interact, directly or indirectly, to induce the ectopic release of Cdc14 in G1 arrested cells.

As overexpression of Clb-Cdk complexes quickly push yeast cells into S-phase, the contribution of Clb2-Cdk on Cdc14 release was analyzed in S-phase arrested cells. To obtain an S-phase arrest, we treated cells with hydroxyurea (HU), a drug that inhibits the ribonucleoside reductase and prevents DNA synthesis. *GAL-CLB2dBΔ, GAL-CDC15(1-750),* and *GAL-CLB2dBΔ GAL-CDC15(1-750)* cells were pre-synchronized in G1 and then released into growth medium containing HU. Once cells where homogeneously arrested in S-phase, as judged by more than 90 percent of cells having small buds, the media was supplemented with galactose to induce expression from the GAL-promoter. Here, neither the overexpression of an allele of Clb2 resistant to degradation (*GAL-CLB2dBΔ*) nor of the hyperactive form of Cdc15 (*GAL-CDC15(1-750)*) or the combined overexpression of Clb2

(*GAL-CLB2dBΔ*) and Cdc15 (*GAL-CDC15(1-750)*) induced a release of Cdc14, Fig. 3.1b. However, differently from the G1 phase, overexpression of Cdc5 succeeded in releasing Cdc14 in S-phase arrested cells, Fig. 3.1b.

As our G1 data suggest that Cdc5 is not sufficient to induce Cdc14 release on its own we searched for the kinase that cooperates with Cdc5 to release Cdc14 in S phase arrested cells. Previous work has shown that the ability of Cdc5 to ectopically release Cdc14 in this cell cycle phase does not require MEN activity (Visintin et al., 2008). As endogenous Clb-Cdk complexes start to accumulate in this phase of the cell cycle we hypothesized that these complexes could cooperate with Cdc5. We tested our hypothesis by asking whether *CDC5dBΔ* could release Cdc14 in S phase-arrested in the absence of Clb-Cdks activity. To inactivate Clb-Cdk complexes we took advantage of an allele of *CDC28* (the sole Cdk in budding yeast) that is sensitive to the ATP analogue NM-PP1 (*cdc28-as1*; (Bishop et al., 2001)). Of note, we refer to Clb-Cdk complexes rather then Clb2-Cdk because by inactivating directly *CDC28*, we inhibited all the complexes that the kinase forms at this specific phase (Clb5-Cdk, the S-phase predominant species; Clb2-Cdk, the mitotic predominant species). Interestingly we observed that Cdc5dBΔ is no more able to induce Cdc14 when Clb-Cdk complexes are inhibited. Thus, in agreement with our hypothesis we found that the release of Cdc14 by high levels of Cdc5 in S phase-arrested cells requires Clb-Cdks activity, Fig. 3.1c. As for the cells arrested in G1, the concomitant expression of Cdc5 (*GAL-CDC5dBΔ*) and MEN (*GAL-CDC15(1-750)*) rendered the presence of the Clb-Cdks activity dispensable, Fig. 3.1c.

Taken together, our results show that the ectopic release of Cdc14 from the nucleolus requires the combined activity of two kinases, Cdc5 and either Clb-Cdks or the MEN kinase Dbf2. We refer to this double kinase requirement as the “two-hit” model (Fig. 3.2a, (Manzoni et al., 2010)).

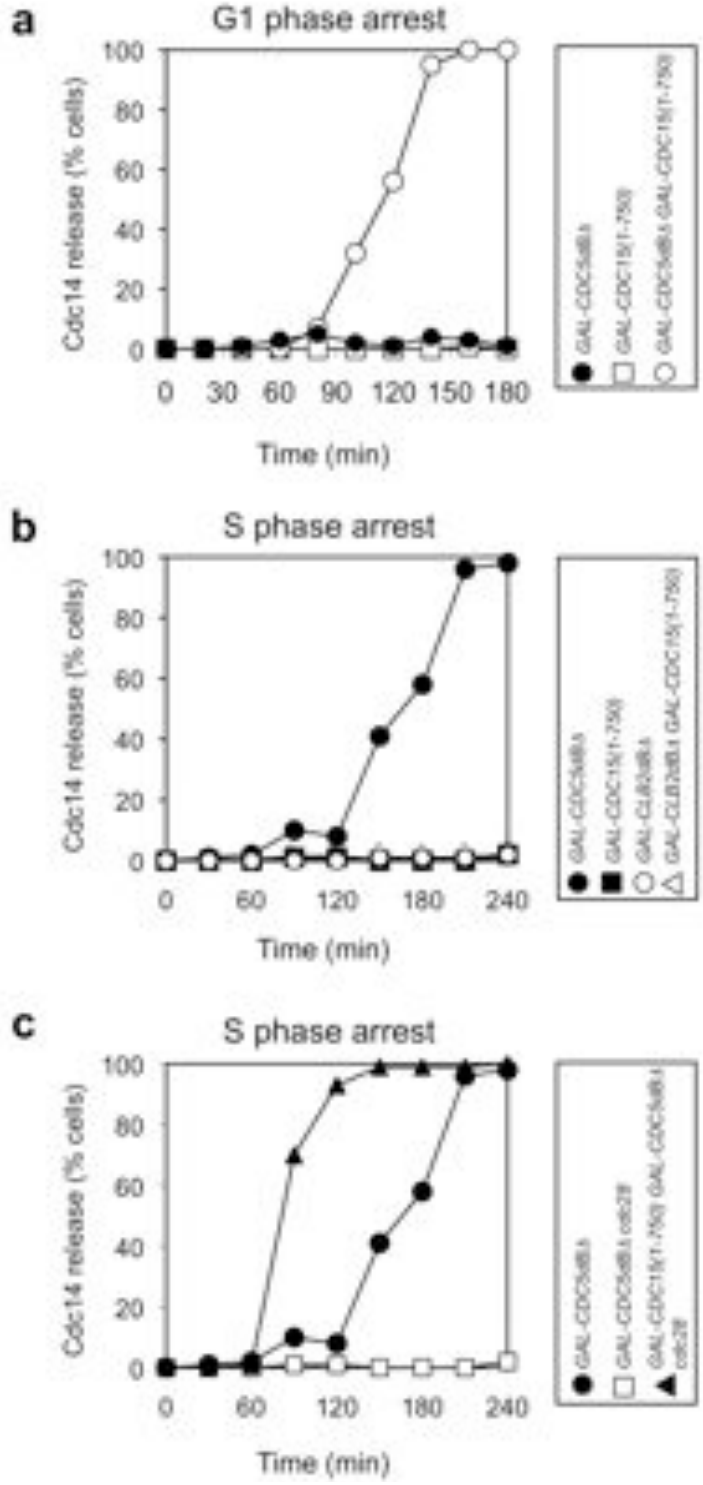

Figure 3.1 Cdc5 requires a partner kinase for promoting Cdc14 release
(**a**) *GAL-CDC5dBΔ* (Ry1358, *GAL-CDC5DN70*), *GAL-CDC15(1-750)* (Ry995) and *GAL-CDC5dBΔ GAL-CDC15(1-750)* (Ry1345) cells were arrested in G1 by α-factor in YEPR. When arrest was complete the media was supplemented with 2% galactose (YEPR+G) to induce the expression of *CDC5dBΔ* and *CDC15(1-750).* The percentage of cells with Cdc14 released from the nucleolus was determined at the indicated times. (**b**) *GAL-CDC5dBΔ* (Ry1358, *GAL-CDC5DN70*), *GAL-CDC15(1-750)* (Ry995)*, GAL-CLB2dBΔ (*Ry448) and *GAL-CDC15(1-750) GAL-CLB2dBΔ* (Ry1547) cells were arrested in G1 with α-factor in YEPR and released in YEPR fresh media supplemented with HU to induce a synchronous arrest in S phase. Once cells were arrested, the media was supplemented with 2% galactose (YEPR+G) to induce the expression of *CDC5dBΔ, CLB2dBΔ* and CDC15. The percentage of cells with Cdc14 released from the nucleolus was determined at the indicated times. (**c**) *GAL-CDC5dBΔ* ((Ry1358, *GAL-CDC5DN70*), *GAL-CDC5dBΔ cdc28-as1* (Ry1356)*, GAL-CDC5dBΔ GAL-CDC15(1-750) cdc28-as1* (Ry1353) cells were treated and analyzed as described in (**b**), with the difference that concomitantly with galactose addition, cells were supplemented with 5μM of 1NM-PP1 analog 9 (Bishop et al., 2001) to inhibit Cdc28 activity.

Mitotic exit requires the power of two kinases: Cdc5 and either Clb-Cdks or MEN

The observation that Cdc5 requires Clb-Cdks or MEN activity to release Cdc14 in phases other than anaphase suggests an appealing interpretation to explain how the correct timing of Cdc14 activation is achieved during a normal cell cycle. Moreover, it provides a nice explanation for a wide variety of mutants. At anaphase onset, the activation of the APC/C^{Cdc20} results in the degradation of securin (Pds1 in budding yeast) and in the activation of separase (Esp1 in budding yeast). Active separase has been proposed to downregulate $PP2A^{Cdc55}$ phosphatase activity (Queralt et al., 2006) resulting in a first wave of Cdk-dependent Cfi1 phosphorylation (Queralt et al., 2006). As in this cell cycle stage Cdc5 and Clb-Cdks have reached their peak concentration it is conceivable to think that at anaphase onset Cdc5 and Clb2-Cdk would be responsible to initiate Cdc14 release, Fig. 3.2a. At this stage, while Cdc5 and Clb2-Cdk are active, MEN is not (Stegmeier and Amon, 2004), as most of its components are inhibited by Clb-Cdks phosphorylation (Konig et al., 2010) and the GTPase Tem1 still resides in the mother cells, a space conceived as "MEN inhibitory zone" (Bardin et al., 2000; Chan and Amon, 2010). However, MEN is activated shortly thereafter (Stegmeier and Amon, 2004), when Clb2 is partially degraded by the APC/C^{Cdc20} (Morgan, 2007). Hence it can maintain Cdc14 in its released state by taking over the role of Clb2-Cdk when the activity of the latter goes below a critical threshold. These events change the ratio of kinase/phosphatase activity in favor of the phosphatase thereby diminishing the rate of inhibitory phosphorylation on MEN components and ultimately leading to complete activation of the MEN, Fig. 3.2a (Bouchoux and Uhlmann, 2010; Konig et al., 2010).

The "two-hit" model would also explain Cdc14 dynamics in mutants lacking MEN, in agreement with (Queralt et al., 2006). FEAR and MEN are not redundant. In MEN mutants, the release of Cdc14 brought about by the FEAR network is transient and limited

to the nucleus and hence is insufficient to complete exit from mitosis, Fig. 3.2b, and (Pereira et al., 2002; Stegmeier et al., 2002; Yoshida and Toh-e, 2002). Only by the additional contribution of MEN can the release of Cdc14 be prolonged in time resulting in Cdc14 spreading into the cytoplasm as well, Fig. 3.2c and (Mohl et al., 2009; Stegmeier and Amon, 2004).

According to our model, the decrease of Clb2-Cdk activity is not compensated for in MEN mutants, and might be the reason why the FEAR-mediated Cdc14 release is transient (Manzoni et al. 2010) Fig. 3.2b. If this interpretation is correct then Cdc14 re-sequestration in MEN mutants should mirror the pattern of Clb2 degradation and it should be possible to make Cdc14 release permanent, even in the absence of MEN by stabilizing Clb2.

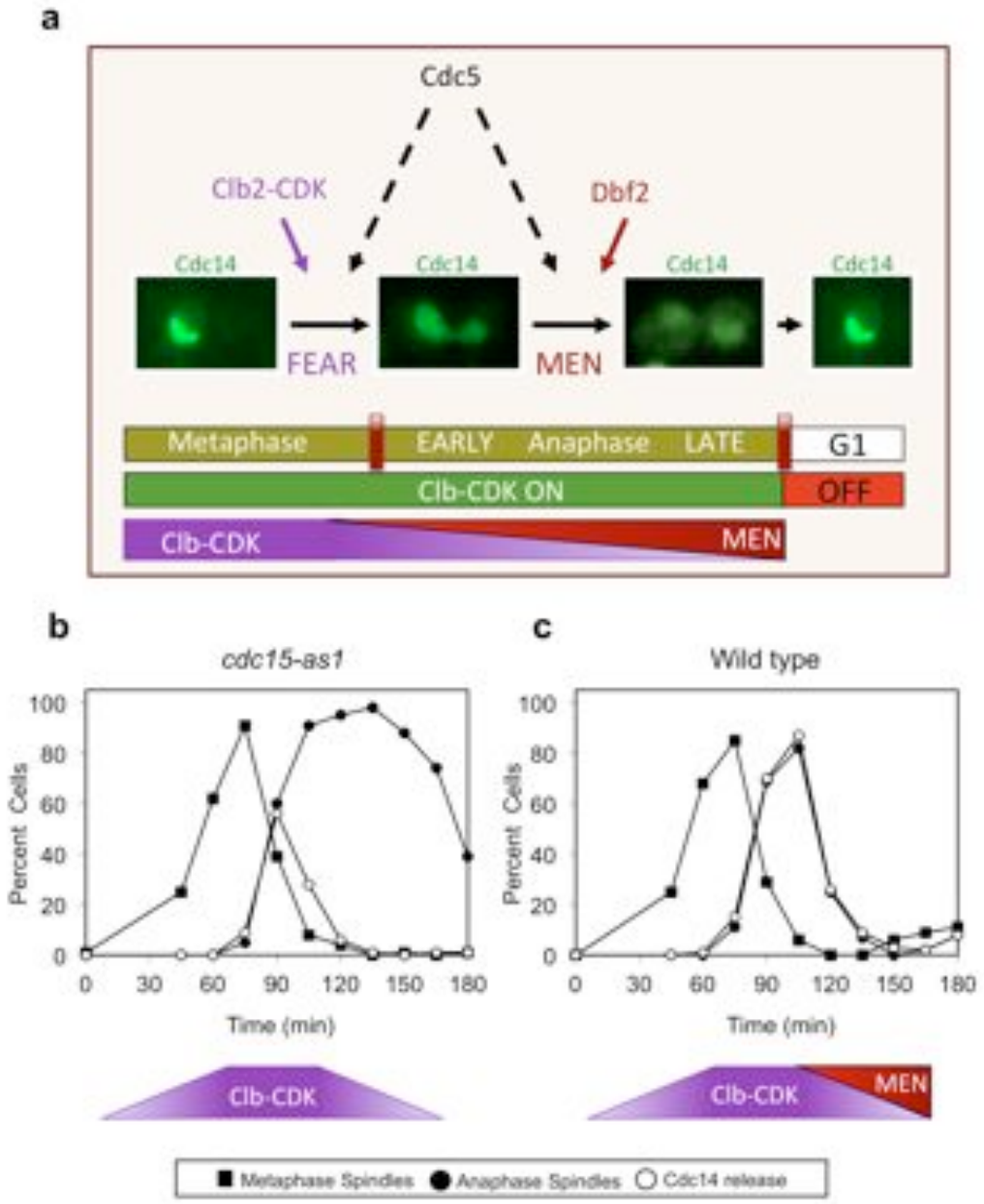

Figure 3.2 A "two-hit" mechanism underlies the release of Cdc14
(**a**) Release of Cdc14 requires the activity of two kinases: Cdc5 always and one between Clb2-Cdk and MEN kinase Dbf2. This model provides a nice explanation for what happens in an unperturbed cell cycle. Here Clb2-Cdk and Cdc5 initiate the release of Cdc14 by the FEAR network. At the metaphase to anaphase transition Clb2 is partially degraded by the APC/C^{Cdc20}, while the MEN start to be activated. Thus the role of Clb2 is taken over by Dbf2 when its activity goes below a critical threshold, sustaining the release of Cdc14 in the later stages of anaphase. (**b-c**) *cdc15-as1* (**b**) and wild type (**c**) cells were arrested in G1 with α-factor in YEPD at 23°C and released in fresh YEPD media supplemented with the Cdc15-as1 inhibitor (1NM-PP1 analog 9 (Bishop et al., 2001)). At the indicated time-points samples were taken to determine the percentage of metaphase spindles (closed squares), anaphase spindles (closed circles) as well as the percentage of cells with Cdc14 released from the nucleolus (open circles).

Cdc14 re-sequestration occurs concomitantly with Clb2 degradation in MEN mutants

To test whether Clb2 degradation could be the reason why the release of Cdc14 mediated by the FEAR network is transient, we compared the kinetics of Cdc14 release and Clb2 degradation in wild type and MEN mutant cells. To inactivate the MEN we used the ATP analog-sensitive allele of *CDC15* (*cdc15-as1*), (Bishop et al., 2001). Wild type and *cdc15-as1* cells were arrested in G1 and synchronously released in media lacking the pheromone, but containing the *cdc15-as1* inhibitor. Cell cycle progression was assessed by scoring mitotic spindle formation. The dynamics of Cdc14 release and sequestration were followed by indirect *in situ* immunofluorescence. The levels and mobility of Clb2 were investigated by western-blot hybridization. In agreement with our hypothesis wild-type cells maintained Cdc14 released throughout anaphase, Fig. 3.3a; whereas MEN mutant *cdc15-as1* (Bishop et al., 2001) cells re-sequestered Cdc14 into the nucleolus. They did so concomitantly with the reduction of Clb2 levels triggered by the APC/C^{Cdc20}, Fig. 3.3b (Mohl et al., 2009; Stegmeier and Amon, 2004).

From this series of experiments we concluded that reduction in the levels of Clb2 maybe the reason why the release of Cdc14 mediated by the FEAR network is transient.

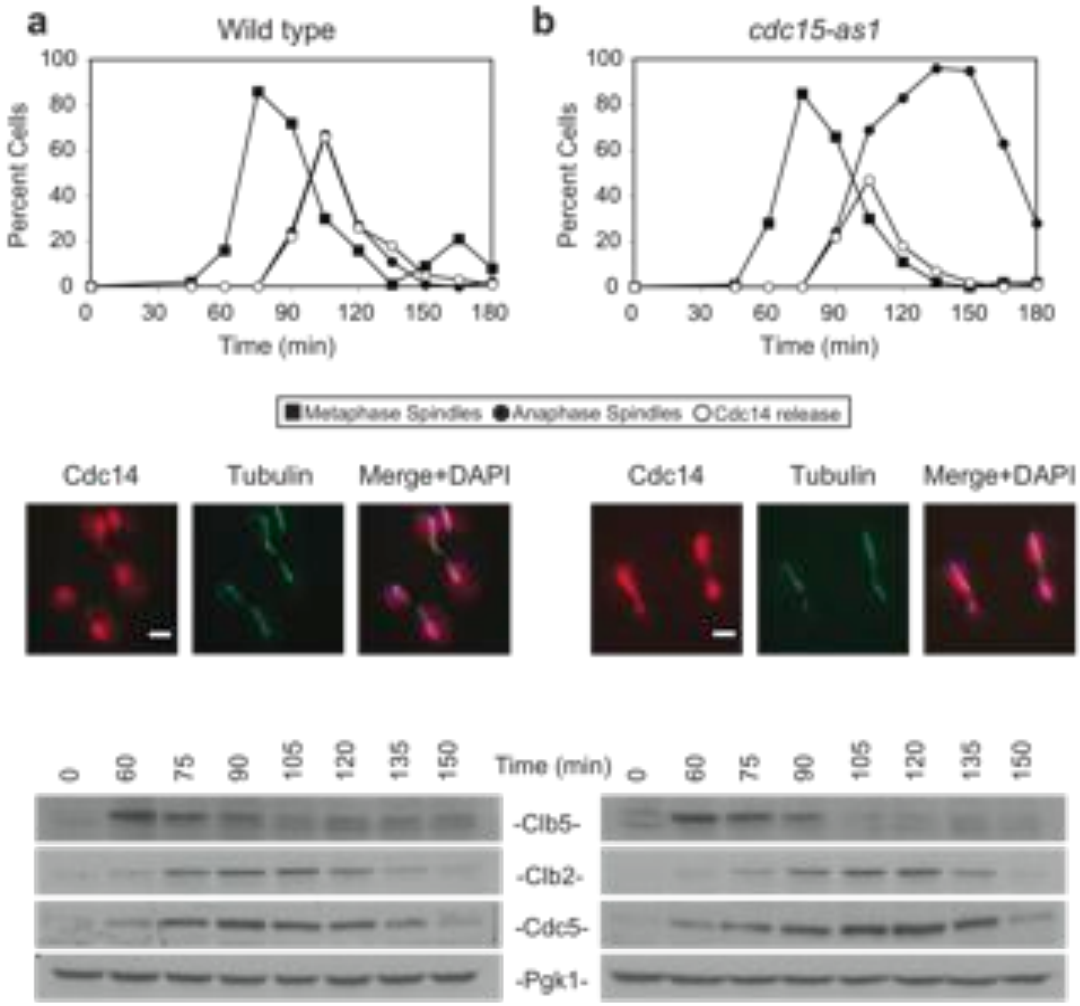

Figure 3.3 Cdc5 is partially degraded in MEN mutant cells
Wild type (Ry278, **a**) and *cdc15-as1* (Ry1132, **b**) cells were arrested in G1 with α-factor in YEPD at 23°C and released in fresh YEPD media supplemented with the Cdc15-as1 inhibitor (1NM-PP1 analog 9 (Bishop et al., 2001)). At the indicated time-points samples were taken to determine the percentage of metaphase spindles (closed squares), anaphase spindles (closed circles) as well as the percentage of cells with Cdc14 released from the nucleolus (open circles). Examples of Cdc14 localization for the 105 min. time-point are shown. Cdc14 is shown in red, tubulin in green and DAPI in blue. Bar, 3μm. Clb5, Clb2, Cdc5 and Pgk1 protein levels were analysed by western blot hybridization. Pgk1 protein was used as an internal loading control in immunoblots.

Clb2 stabilization is not sufficient to sustain Cdc14 release in the absence of MEN

If this hypothesis is correct, it should be possible to release Cdc14 permanently, even in the absence of MEN, by stabilizing Clb2.

To test this hypothesis, we tracked Cdc14 localization in a synchronous population of cells overexpressing a non-degradable form of Clb2 (*GAL-CLB2dBΔ*) that are impaired in MEN activity due to the ATP analog-sensitive allele of *CDC15* (*cdc15-as1*) (Bishop et al., 2001). We expressed Clb2dBΔ at low and high doses to cover a wide range of Clb2 concentrations. As for the low doses, we first determined the minimal galactose concentration necessary to arrest the cells in mitosis. *GAL-CLB2dBΔ* cells exit from mitosis when grown in media with 0.025% galactose, Fig. 3.4a, but are blocked in the presence of 0.05% galactose, Fig. 3.4b. We thus grew *GAL-CLB2dBΔ cdc15-as1* cells in 0.05% galactose to express what we call 'low doses' of non-degradable Clb2. For the 'high doses', we used 1% galactose. Nucleolar integrity was assessed by imaging the nucleolar protein Nop1 (Tollervey et al., 1991), Fig. 3.5 a, b, c.

In the presence of non-degradable Clb2 expressed at high doses, more *cdc15-as1* mutant cells released Cdc14 as compared to the *cdc15-as1* single mutant (compare the release at 135 minutes in Fig. 3.5a with the release at 105 minutes in Fig. 3.3b). Interestingly, we noticed that the spatial pattern of Cdc14 release changed from a nuclear FEAR-type to a nuclear and cytoplasmic release, resembling the one mediated by MEN (compare Fig. 3.5e (135 minutes) and Fig. 3.3b). However, this shift towards a MEN-type release was incomplete, as assessed by the fraction of cells that underwent release and by localization of Cdc14, because Cdc14 was not completely released in the cytoplasm in the entire anaphase population. Indeed, if the Cdc15 inhibitor was added 120 minutes after the release, Cdc14 release appeared more similar to that in wild-type cells, Fig. 3.5b. This result suggests that Clb2-Cdk and MEN have overlapping but not identical roles. This

notion is consistent with recent results obtained by Mohl and colleagues showing the need for Cdc14 phosphorylation by Dbf2 on its NLS for its cytoplasmatic retention (Mohl et al., 2009).

When non-degradable Clb2 was expressed at low doses, Cdc14 localization dynamics remained similar to those in *cdc15-as1* cells for both the small fraction of anaphase cells with Cdc14 released, Fig. 3.5d, and the nuclear localization.

Regardless of the presence of non-degradable Clb2, Cdc14 eventually re-entered the nucleolus in both cases. While with low doses it remained there, with high doses, Cdc14, after some time was released again. Noticeably, the alternation of sequestration and release of Cdc14 was not an isolated event, but occurred 3 to 4 times during the 5 hours of observation, Fig. 3.5a, and during each cycle Cdc14 was always fully released into the cytoplasm, Fig. 3.5e.

In conclusion, our data suggest that the stabilization of Clb2 is not sufficient to maintain Cdc14 released in MEN mutants.

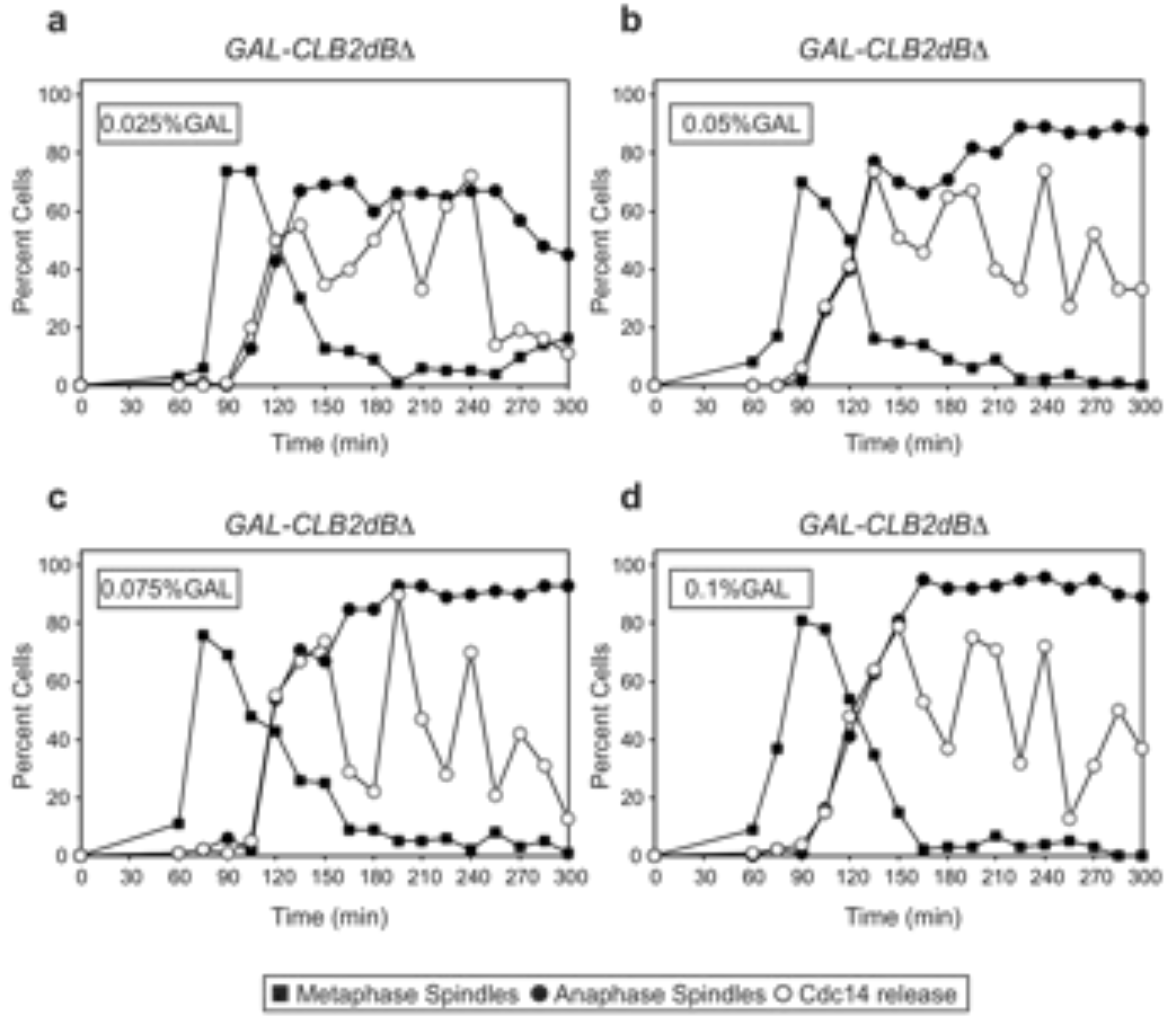

Figure 3.4 Identification of the minimal galactose concentration necessary for an homogeneous telophase arrest

(**a-d**) *GAL-CLB2dBΔ* (Ry430) cells were arrested with α-factor in YEPR and released into medium supplemented with different concentrations of galactose (0,025%, **a**; 0,05%, **b**; 0,075%, **c**; 0,1%, **d**) to induce the expression of Clb2. The percentage of cells with metaphase spindles (closed squares), anaphase spindles (closed circles) as well as the percentage of cells with Cdc14 released from the nucleolus (open circles) was determined at the indicated times.

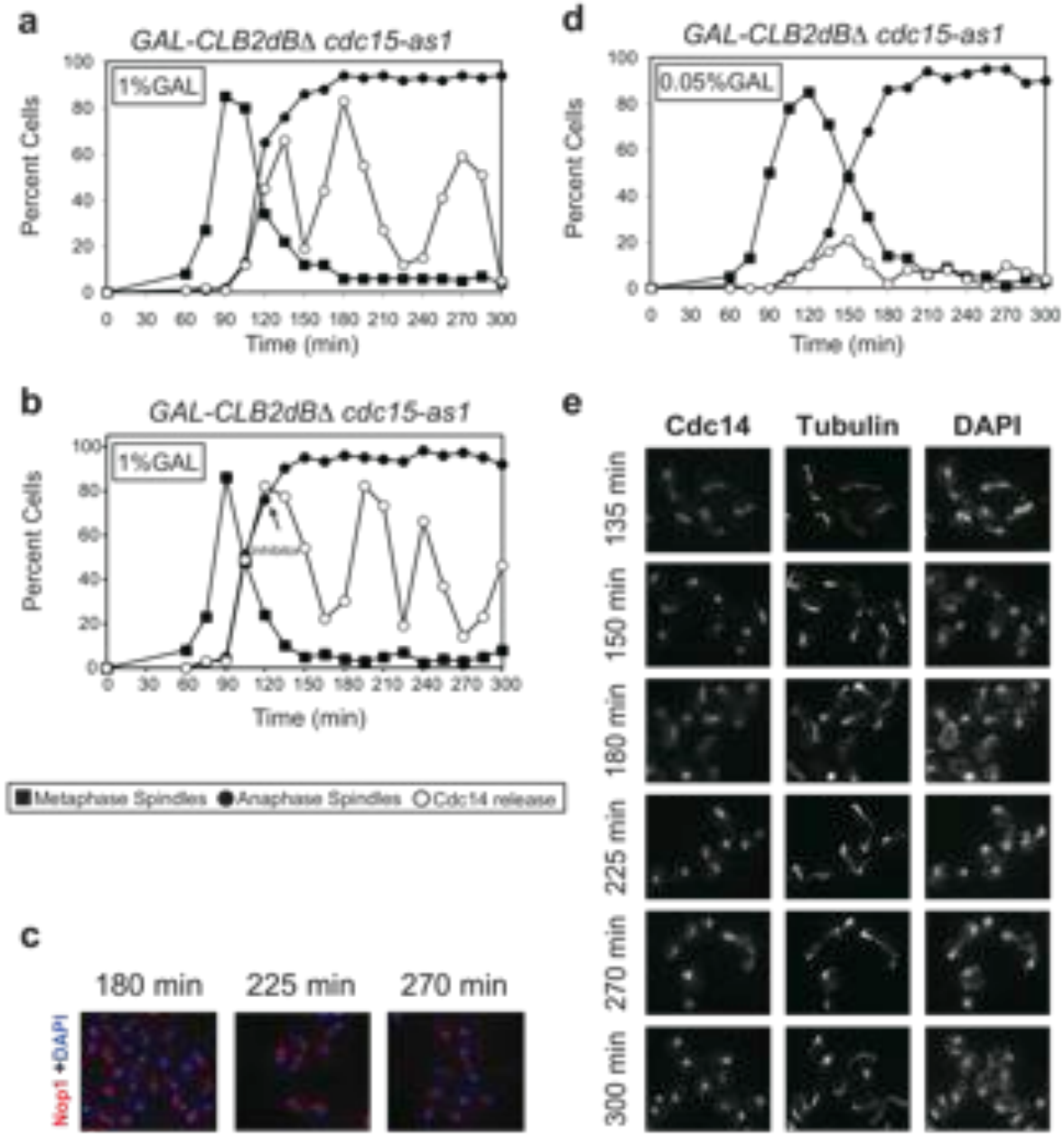

Figure 3.5 Non-degradable Clb2 promotes periodic cycles of Cdc14 release and sequestration
(**a,b,d**) *GAL-CLB2dBΔ cdc15-as1* (Ry1394) cells were arrested with α-factor in YEPR and released into medium supplemented with 1% (**a,b**) and 0,05% (**d**) galactose to induce the expression of Clb2. 5 μM 1NM-PP1 analog 9 (Bishop et al., 2001) was added at the time of (**a,c**) or two hours after the release to inactivate the *cdc15-as1* allele. The percentage of cells with metaphase spindles (closed squares), anaphase spindles (closed circles) as well as the percentage of cells with Cdc14 released from the nucleolus (open circles) was determined at the indicated times. (**c**) At the indicated time-points the nucleolar protein Nop1 was stained to assess the integrity of the nucleolus during the course of the experiment. Nop1 is shown in red and DAPI in blue. (**e**) The localization of Cdc14 and the morphology of the mitotic spindle and nuclei for representative fields of cells from (**a**) are shown for various time-points.

Clb-Cdks stabilization maintains Cdc14 released in the absence of MEN

If in MEN mutants Cdc14 is sequestered even in the presence of stable Clb2, what other mechanisms contribute to Cdc14 sequestration? We wished to verify whether other Clbs besides Clb2 could play a role in this process. This hypothesis is consistent with the results of our experiments in cells arrested in S-phase, where Cdc5 requires Clb-Cdks activity to release Cdc14, Fig. 3.1c, as by inactivating Cdc28 directly we inhibited all Clb-Cdk complexes at once. To test whether stabilization of Clbs other than Clb2 maintains Cdc14 released in MEN mutants, we stabilized all Clbs at physiological levels by depleting Cdc20 in a strain lacking *PDS1*. Depletion of Cdc20 allows for cyclin stabilization, whilst Pds1 deletion allows for cohesin cleavage and passage into anaphase Fig. 3.6.

More specifically, the coding sequence of Cdc20 was placed under the control of the methionine repressible *MET3* promoter. To deplete Cdc20, *MET-CDC20 pds1Δ* cells were first arrested in G1, in a synthetic complete medium lacking methionine (SC-Met). In G1, the APC/C^{Cdh1} targets Cdc20 for degradation. Cells depleted for Cdc20 were next synchronously released into the cell cycle into YEPD media added with methionine to repress the transcription of Cdc20, thereby preventing *de novo* Cdc20 synthesis. As Cdc20 activity besides triggering Clbs degradation is also important for the degradation of securin, a process essential for sister chromatid separation, deletion of *PDS1* in our mutant is necessary to allow cells depleted for Cdc20 to transit into anaphase, Fig. 3.6a, b. *MET-CDC20 pds1Δ* cells, however, arrest in telophase, due to their inability to destroy cyclins, Fig. 3.6b. Interestingly, these cells arrest with Cdc14 released from the nucleolus, Fig. 3.6b and Fig. 3.7a and (Shirayama et al., 1999) and as such represent the perfect experimental set up to determine whether and which flavor of Clb has to be stabilized to render MEN activity dispensable for Cdc14 release. To address this question, we inactivated MEN via the *cdc15-as1* allele in a *MET-CDC20 pds1Δ* background. Strikingly, we found that inactivation of MEN did not alter the kinetics of Cdc14 release observed in *MET-CDC20*

pds1Δ cells Fig.3.7b (compare with Fig. 3.7a), Fig. 3.7c. These results indicate that degradation of other Clbs besides Clb2 is the reason why Cdc14 is re-sequestered in MEN mutants.

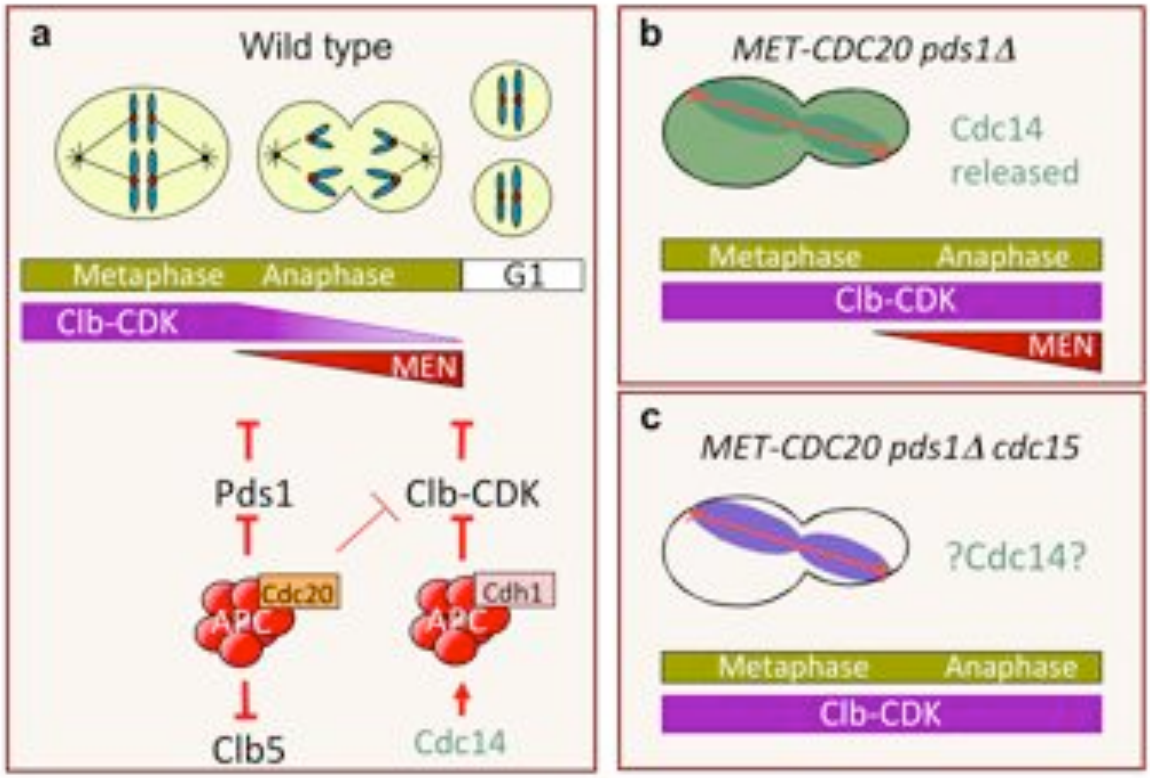

Figure 3.6 The metaphase to anaphase transition: Rationale for the experiment described in
(**a**) The metaphase to anaphase transition is triggered by the APC/C^{Cdc20}. In budding yeast the APC/C^{Cdc20} triggers the degradation of securin (Pds1), thereby allowing for sister chromatid separation; the degradation of S phase cyclin Clb5, a potent antagonist of phosphatase Cdc14, thereby allowing for activation of the phosphatase and progression through anaphase; and partial degradation of mitotic cyclin Clb2. (**b**) Cells depleted for Cdc20 that lack Pds1 deletion arrests in telophase with Cdc14 fully released. Cdc14 activity in this setting is opposed by kinase Clb5-Cdk. (**c**) The question we wish to address is whether endogenous stabilization of Clbs is sufficient to maintain Cdc14 release also in the absence of MEN.

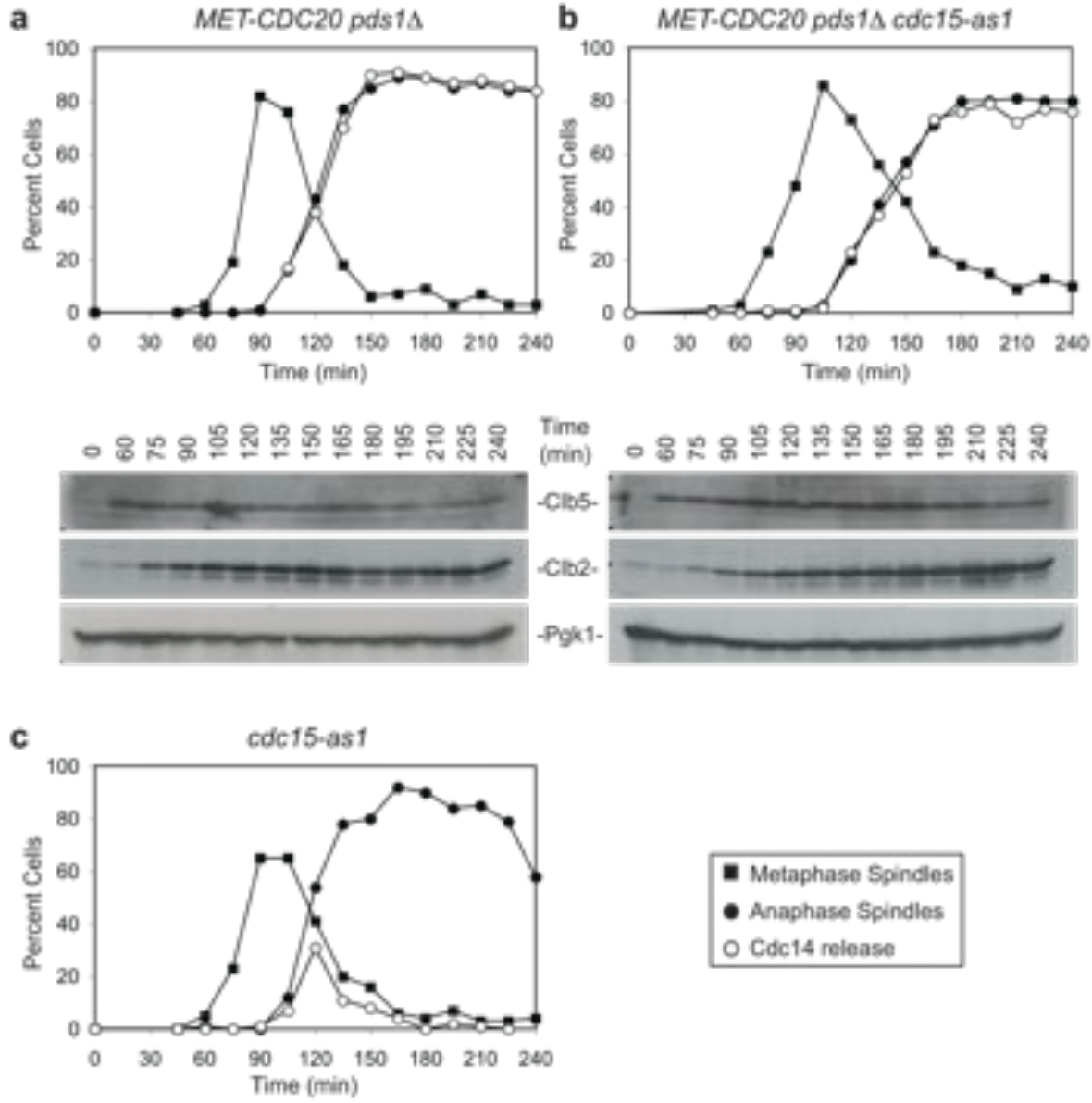

Figure 3.7 Stabilization of S- and M-phase cyclins maintain Cdc14 released in MEN mutants
MET-CDC20 pds1Δ cdc15-as1 (Ry1558, **a**-**b**) and *cdc15-as1* (Ry1132, **c**) cells were arrested in G1 with α-factor in a synthetic complete medium lacking methionine (SC-Met). When arrest was complete cells were released into YEPD media lacking the pheromone, but added with 8 mM methionine to repress the expression of Cdc20. 5 μM 1NM-PP1 analog 9 (Bishop et al., 2001) to inactivate the *cdc15-as1* allele was added to (**b**) and (**c**) . The percentages of cells with metaphase spindles (closed squares), anaphase spindles (closed circles) as well as the percentage of cells with Cdc14 released from the nucleolus (open circles) were determined at the indicated times. Clb5, Clb2 and Pgk1 protein levels were assessed by western blot hybridization (**a**, **b**). Pgk1 protein was used as an internal loading control in immunoblots.

Clb-Cdks stabilization maintains Cdc14 released in the absence of MEN

As Clb5 is the predominant species among S-phase cyclins, and as it is degraded at the metaphase to anaphase transition, Fig. 3.3a, b, we set out to verify its contribution to Cdc14 release in the absence of MEN. To this aim, we analyzed the consequences of deleting *CLB5* in a *MET-CDC20 pds1Δ cdc15-as1* mutant. To our surprise we found that, in this mutant, Cdc14 was never released from the nucleolus, Fig. 3.8a. This result points to Clb5 being a novel member of the FEAR pathway. Indeed, the pattern of Cdc14 release in the *MET-CDC20 pds1Δ cdc15-as1 clb5Δ* mutant in the absence of the Cdc15 inhibitor, and as such with an active MEN, closely resembles the typical phenotype of FEAR mutants, Fig. 3.8b, where Cdc14 release is delayed during early anaphase.

We conclude that the stabilization of both S phase and mitotic cyclins is sufficient to render full and stable the release of Cdc14. This result is consistent with the hypothesis that S-phase cyclin degradation contributes to Cdc14 sequestration.

Clb5 is a *bona fide* FEAR network component

The observation that deletion of *CLB5* gene (*clb5Δ*) leads to: i) a delay in the release of Cdc14 in MEN competent cells (*MET-CDC20 pds1Δ clb5Δ*) Fig. 3.8b, and ii) a complete absence of Cdc14 release in cells lacking MEN activity (*MET-CDC20 pds1Δ clb5Δ cdc15-as1*), Fig.3.8a, point to Clb5 as a novel FEAR network component. Indeed, FEAR network mutants are impaired in the release of Cdc14 during early anaphase, when the mitotic spindle is between 3 and 7 μm, and exhibit a delay of about 10 minutes in mitotic exit (Stegmeier et al., 2002). To better assess if Clb5 plays a role in the FEAR network or if the phenotype we observe is caused by the genetic background in which we performed the experiment, we analyzed progression through the cell cycle of a synchronous population of cells carrying only a deletion of *CLB5* (*clb5Δ*). To this aim,

wild-type, *clb5Δ* and *clb5Δ cdc15-as1* cells first arrested in G1 and then synchronously released into the next cell cycle. We monitored cell cycle progression by following the kinetics of mitotic spindle formation. The contribution of Clb5 in promoting Cdc14 release was assessed by imaging Cdc14 by indirect IF. In wild-type cells Cdc14 was released from the nucleolus in the entire anaphase population, Fig. 3.8c, while *clb5Δ* cells showed a delay in Cdc14 release from the nucleolus during early anaphase, Fig. 3.8d. Moreover, similarly to all FEAR network mutants analysed to date, we found that a deletion of *CLB5* in combination with MEN inactivation (*cdc15-as1*) resulted in cells being impaired in releasing Cdc14, Fig. 3.8e. Taken together, we conclude that Clb5 is a *bona fide* member of the FEAR network. As Clb5 is degraded at the metaphase-anaphase transition, a role for the kinase in promoting Cdc14 release is difficult to envision. We believe that elucidating this mechanism will provide important information about cell cycle logic.

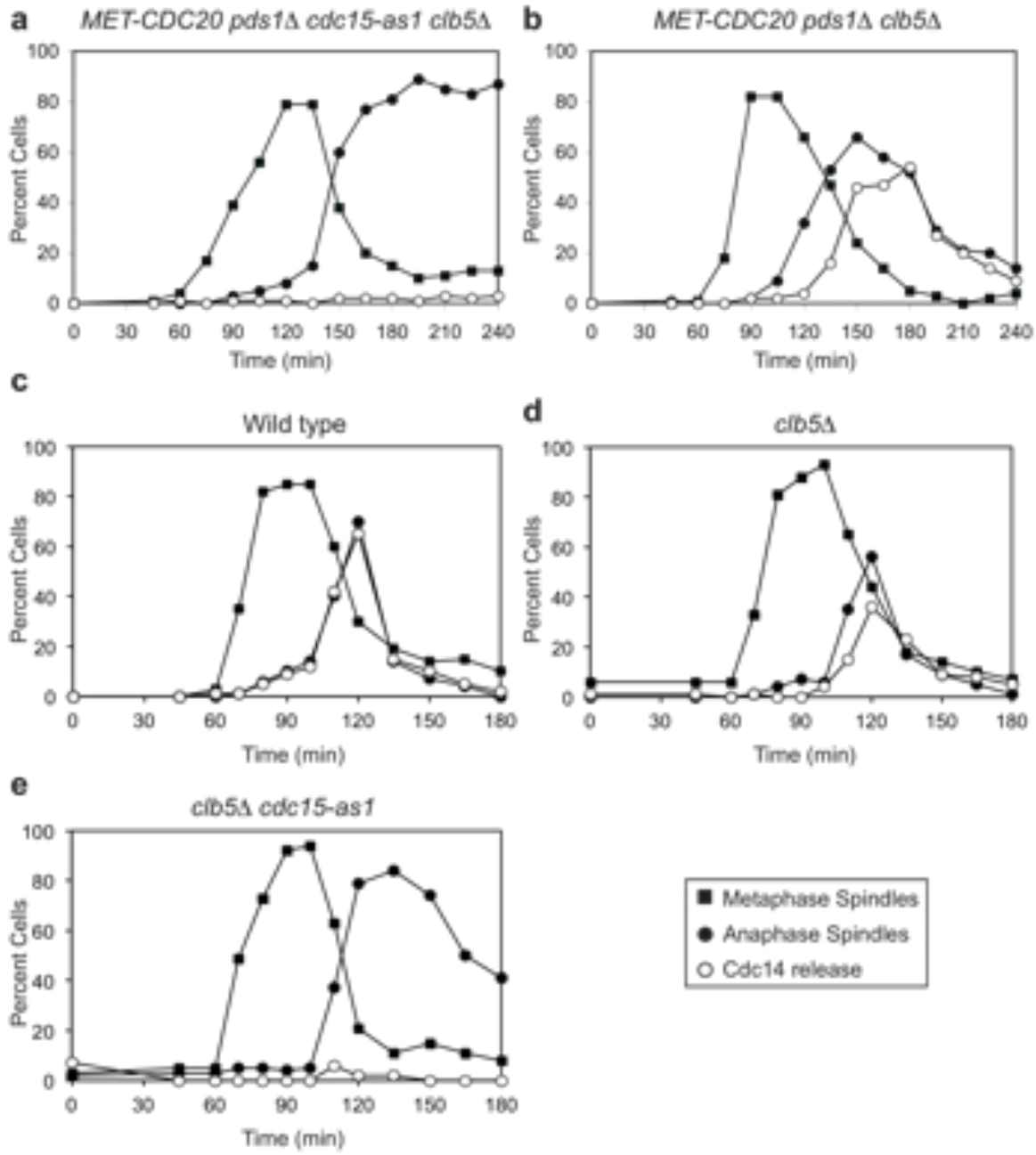

Figure 3.8 Clb5 is a FEAR network component
(**a, b**) *MET-CDC20 pds1Δ clb5Δ cdc15-as1* (Ry1538) cells were arrested in G1 with α-factor in a synthetic complete medium lacking methionine (SC-Met). When arrest was complete cells were released into YEPD media lacking the pheromone, but added with 8 mM methionine to repress the expression of Cdc20 in the presence (**a**) or absence (**b**) of 5 μM Cdc15-as1 inhibitor (1NM-PP1 analog 9 (Bishop et al., 2001)).. The percentages of cells with metaphase spindles (closed squares), anaphase spindles (closed circles) as well as the percentage of cells with Cdc14 released from the nucleolus (open circles) were determined at the indicated times. (**c-e**) Wild type (Ry1, **c**), *clb5Δ* (Ry446, **d**) and *clb5Δ cdc15as1* (Ry1588, **e**) cells were arrested with α-factor in YEPR and released into medium supplemented with 5 μM 1NM-PP1 analog 9 (Bishop et al., 2001) to inactivate the *cdc15-as1* allele. Cell samples were analyzed as in (**a, b**).

Oscillations are impaired in the absence of both MEN and Clb2-Cdk-mediated phosphorylation of Cfi1

Although Cdc14 was re-sequestered in the presence of both high and low doses of non-degradable Clb2, oscillations occurred only in cells blocked in anaphase by high doses. It is possible that also in this setting Clb5 is required for Cdc14 release and accordingly that the first sequestration event is related with its degradation. However, at the metaphase-anaphase transition, the APC/C^{Cdc20} is activated. As such, it is unlikely that Clb5 is present during the ensuing cycles of Cdc14 release and sequestration. Here, we can hypothesize that in MEN mutants high doses of non-degradable Clb2 can compensate for the lack of Clb5, and trigger an additional mechanism that leads to multiple cycles of Cdc14 release and sequestration. What is the molecular circuit underlying this oscillatory behavior? First, we asked whether the kinase requirement for Cdc14 release that we have identified above (i.e., the two-hit hypothesis whereby Cdc5 and either MEN or Clb-Cdks are necessary for Cdc14 release) was satisfied during the oscillations. According to our model, clear predictions can be made: 1) oscillations should persist in the presence of at least one between MEN and Clb2-Cdk, 2) should be abolished by the simultaneous removal of both MEN and Clb2-Cdk, 3) should occur when both are present, and 4) should be abolished by the inactivation of Cdc5.

We thus moved to test the predictions. We have already shown that high doses of Clb2-Cdk can sustain oscillations in the absence of MEN, Fig. 3.5a, suggesting that Clb2-Cdk, together with endogenous Cdc5 are sufficient to sustain the oscillations. We next wished to determine whether MEN and Cdc5, without the contribution of Clb2-Cdk, could sustain the oscillations. To test whether MEN alone can drive the oscillations, we interfered with the ability of Clb2-Cdk to release Cdc14 by using an allele of Cfi1 (*cfi1-6Cdk*) lacking six Clb2-Cdk phosphorylation sites. This mutant is no longer phosphorylated by Clb2-Cdk. It shows a delay in releasing Cdc14 typical for FEAR

mutants (Azzam et al., 2004) and allows us to selectively eliminate the contribution of Clb2-Cdk to the release of Cdc14. In agreement with our prediction we found that in the presence of high doses of stable Clb2, in the *cfi1-6Cdk* mutant cells, although Cdc14 was released with a delay, oscillations arose nevertheless, Fig. 3.9a.

The simultaneous removal of MEN and Clb2-Cdk can arrest the oscillatory behavior, as observed when *cdc15-as1* was inhibited with the analog sensitive inhibitor in combination with the *cfi1-6Cdk*, both when the inhibitor was added before or after the initial release, Fig. 3.9b, c. Finally, we confirmed that oscillations arise when both MEN and Clb2-Cdk are present, Fig. 3.9d, e. So far, we have observed oscillations only in the presence of high levels of stabilized Clb2. Given that both Clb2-Cdk and MEN alone can sustain the oscillations, we asked whether the presence of MEN would allow low levels of stable Clb2-Cdk to undergo periodical cycles of Cdc14 release and re-sequestration. We found this to be the case, Fig. 3.9e. Because the release of Cdc14 in the simultaneous presence of Clb2-Cdk and MEN resembles that observed in wild-type cells, Fig. 3.10b compare to 3.10a, we decided to investigate the molecular nature of the oscillations in this setting, using both high and low doses of non-degradable Clb2.

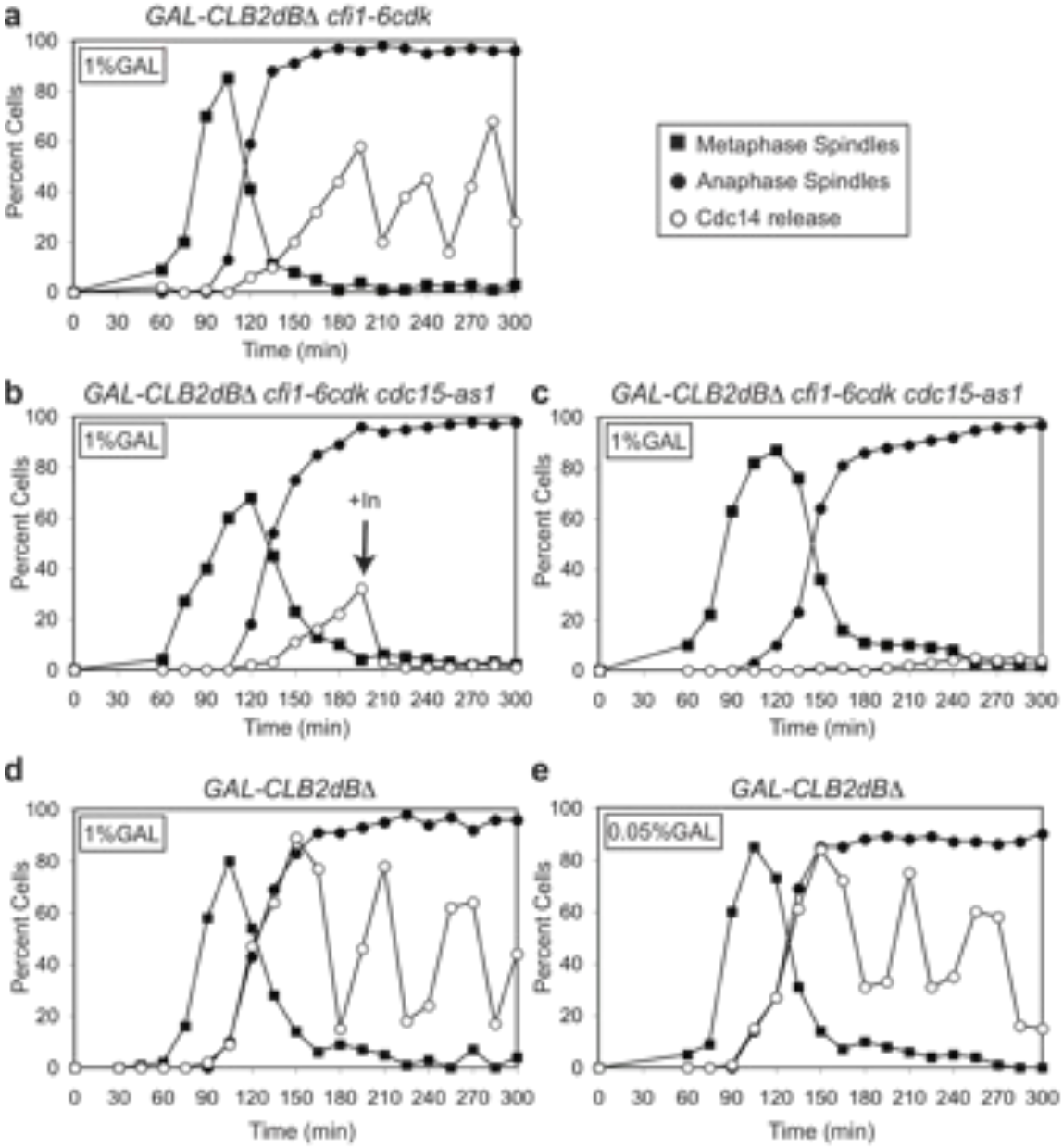

Figure 3.9 Cdc14 oscillations fulfill the kinase requirement of the two-hit model
(**a-c**) *GAL-CLB2dBΔ cfi1-6Cdk* (Ry1545) and*GAL-CLB2dBΔ cfi1-6Cdk cdc15-as1* (Ry1715) cells were arrested with α-factor in YEPR and released into medium supplemented with 1% galactose to induce the expression of Clb2. 195 minutes into the release (**a, b**) or at the moment of the release (**c**) 5 μM of the 1NM-PP1 analog 9 inhibitor (Bishop et al., 2001) was added to inactivate the *cdc15-as1* allele. The percentage of cells with metaphase spindles (closed squares), anaphase spindles (closed circles) as well as the percentage of cells with Cdc14 released from the nucleolus (open circles) was determined at the indicated times. The arrow indicates the time of inhibitor addition (+In; **b**) (**d, e**) *GAL-CLB2dBΔ* (Ry430) cells were arrested in G1 with α-factor in YEPR and released into fresh medium supplemented with 1% (**d**) or 0.05% (**e**) galactose. Cell samples were analyzed as in (**a**).

Oscillations are impaired in Cdc5 mutants

The final prediction of the "two-hit" model is that Cdc5 is continuously required to generate oscillations. If this is the case, then a loss-of-function allele of *CDC5* should sequester Cdc14 without further release. To address this point we looked at the consequences of inhibiting Cdc5 on Cdc14 endocycles. *GAL-CLB2dBΔ cdc5-1* cells where arrested in G1 and synchronously released in the next cell cycle in the presence of galactose. Since Cdc5 is required for the release of Cdc14, the allele was inactivated after a large fraction of the population had reached anaphase (as assessed by DAPI staining, 165 minutes after release). The prediction was confirmed experimentally by using the temperature sensitive mutant *cdc5-1*, with no distinction between high and low doses, Fig. 3.10c, d. The re-sequestration of Cdc14 was not a mere consequence of the temperature shift because the *GAL-CLB2dBΔ* single mutant strain showed oscillations at 37^{o}C as well, 3.10e.

In conclusion, our data suggest that the periodical release and sequestration of Cdc14 is in agreement with the kinase requirement as proposed by the "two-hit" model.

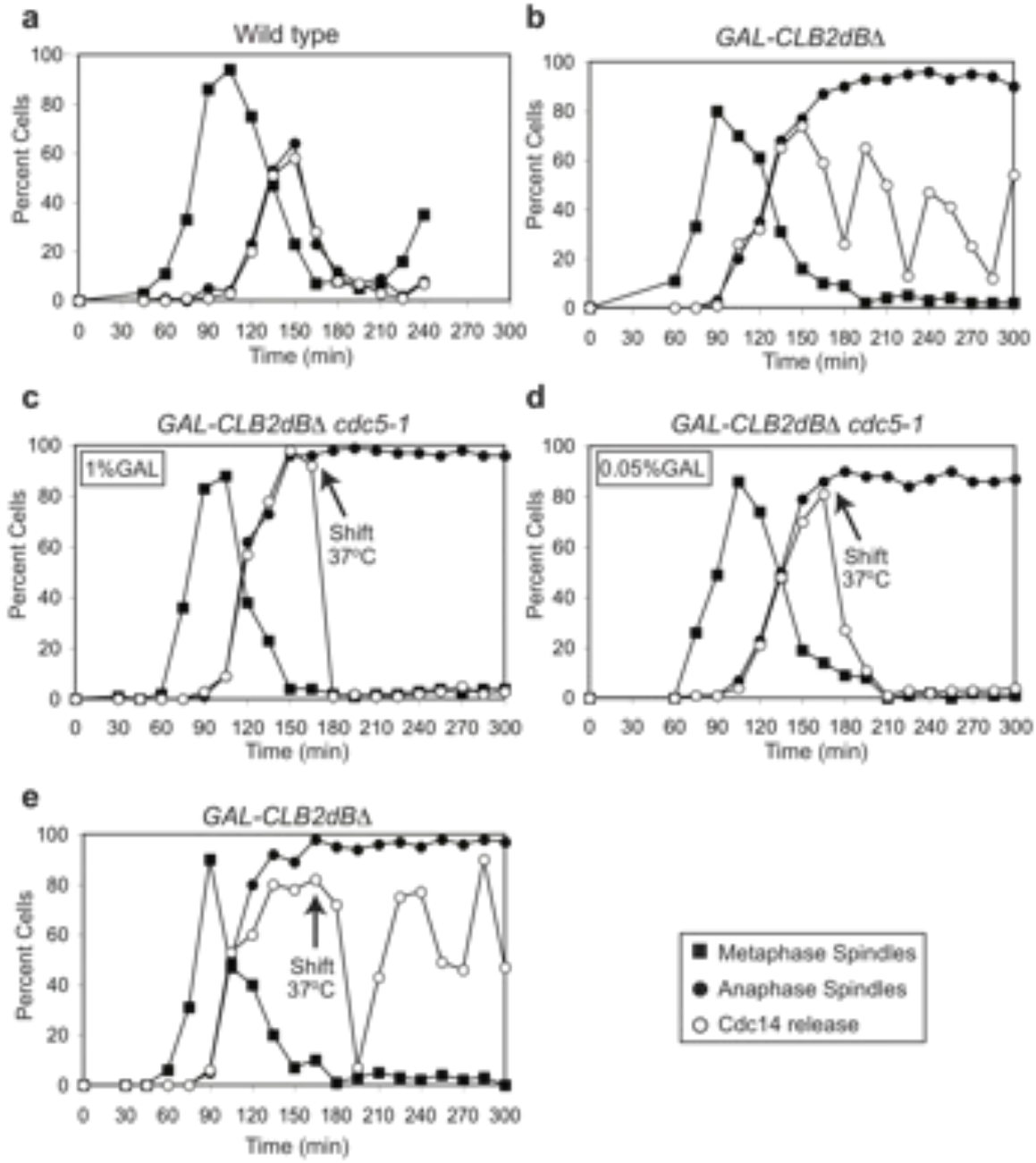

Figure 3.10 Modulating Cdc5 activity interferes with the oscillations
(**a-b)** Wild type (Ry278, **a**) and *GAL-CLB2dBΔ* (Ry430, **b**) cells were arrested with α-factor in YEPR and released into fresh media. The percentage of cells with metaphase spindles (closed squares), anaphase spindles (closed circles) as well as the percentage of cells with Cdc14 released from the nucleolus (open circles) was determined at the indicated times. (**c, d**) *GAL-CLB2dBΔ cdc5-1* (Ry1393) cells were arrested in G1 with α-factor in YEPR and released into fresh medium supplemented with 1% (**c**) or 0.05% (**d**) galactose. Cells were shifted at the restrictive temperature 165 minutes after the release. Cell samples were analyzed as in (**a**). (**e**) *GAL-CLB2dBΔ* (Ry430) cells were treated as in (**a**). Cells were shifted at the restrictive temperature 165 minutes after the release. Cell samples were analyzed as in (**a**). The arrow indicates the time of the shift to the restrictive temperature (**c-e**).

A negative feedback loop including Cdc5, Cdc14 and Cdh1 generates a Cdc14 oscillatory release

Having established that the oscillatory phenotype satisfies the kinase requirement that controls Cdc14 release in an unperturbed cell cycle, we moved to investigate the molecular nature of these oscillations in the *GAL-CLB2dBΔ* strain, both at high and low doses of non-degradable Clb2. We observed that either the simultaneous inactivation of MEN and Clb2-Cdk or Cdc5, Fig.3.9b, c and Fig 3.10c, d, interfered with the oscillatory phenotype. We set to understand which of these two activities is periodically regulated during the oscillations. A simultaneous and coordinated activation and inactivation of Clb2-Cdk and MEN is difficult to envision. A much easier explanation is that oscillations are driven by regulation of the Polo-like kinase Cdc5. Based on theoretical studies, oscillations are generated by a negative feedback loop. A negative feedback loop capable to give rise to oscillations is composed by at least three elements (Novak and Tyson, 2008). At the end of mitosis, Cdc14 initiates a negative feedback loop composed of Cdc5-Cdc14-APC/C^{Cdh1}-Cdc5 (Visintin et al., 2008). During exit from mitosis Cdc5 triggers the Cdc14 release; active Cdc14 dephosphorylates the APC/C cofactor Cdh1 leading to the formation (activation) of the APC/C^{Cdh1} complex. APC/C^{Cdh1} activation results in Cdc5 degradation, and hence in Cdc14 re-sequestration, Fig 3.11. We asked whether this negative feedback loop could be responsible for the oscillatory behavior observed in the presence of high levels of stable Clb2. To answer this question we analyzed the involvement of these three components in the generation of the oscillations by interfering with their activity in the *GAL-CLB2dBΔ* setup.

We already assessed the contribution of Cdc5 to the oscillatory phenotype. We reasoned that if Cdc5 takes part in the loop, and its function is to activate Cdc14, Cdc14 itself should be part of the loop, giving rise to the oscillations. If Cdc14 belongs to the loop and is required for its own re-sequestration (Tomson et al., 2009; Visintin et al., 2008), we

predict that in a Cdc14 loss-of-function mutant the loop cannot be closed and Cdc14 should always be released. We tested this hypothesis using the temperature-sensitive *cdc14-1* mutant. Indeed, in this mutant Cdh1 should be inactive, Cdc5 stable and Cdc14 constitutively released. Experimentally *GAL-CLB2dBΔ* cells carrying the *cdc14-1* allele were pre-synchronized in G1 and then released in fresh media containing galactose. 180 minutes after the release, when the cells had reached anaphase and had Cdc14 released, the double mutant was shifted to the restrictive temperature (37^{o}C). We followed this strategy for every temperature sensitive mutant. In agreement with our hypothesis, we found that upon inhibition of Cdc14, the oscillations were lost and the phosphatase remained released from the nucleolus in the presence of both low and high levels of stable Clb2, Fig. 3.12a, b. As previously shown, this is not a mere consequence of the temperature shift because the *GAL-CLB2dBΔ* single mutant oscillated at 37^{o}C as well, Fig. 3.10e.

A possible caveat to this experiment is that the *cdc14-1* mutant protein at the restrictive temperature could be misfolded and thus unable to bind to its inhibitor. In this case, we should observe Cdc14 constantly released, independently of Cdc5 stabilization. We tackled this problem in two manners. First, we assessed whether the *cdc14-1* arrest was reversible once cells were shifted back to the permissive temperature (23^{o}C) with the *de novo* protein synthesis inhibited. To this aim *cdc14-1* mutant cells were arrested in G1 by α-factor pheromone and then released into fresh media lacking pheromone at the restrictive temperature (37^{o}C). When the majority of cells had reached the telophase arrest as determined by nuclear morphology (DAPI staining, 3 hr into the release), the culture was shifted back to the permissive temperature in the presence of 1 mg/ml cycloheximide (inhibits *de novo* protein synthesis). We found that the *cdc14-1* arrest was reversible, Fig. 3.12c, because the already synthesized *cdc14-1* mutant protein that suffered the thermal shock was able to be re-sequestered from its inhibitor. Second, we examined whether in presence of a wild type catalytically active copy of *CDC14*, the *cdc14-1-6HA* mutant protein could bind Cfi1 at the restrictive temperature. To address this question we

synchronized a heterozygous *CDC14/cdc14-1-6HA* diploid strain in S-phase with 10 mg/ml hydroxyurea. When the arrest was complete, the cells were released in fresh media at the restrictive temperature (37^{o}C). We observed that the mutant *cdc14-1-6HA* protein in this strain underwent release and sequestration at the restrictive temperature with wild-type kinetics Fig. 3.12d, e. We conclude that in our experimental set up Cdc14-1 stays released because it is enzymatically inactive.

In the negative feedback loop, Cdc14 induces its own sequestration by indirectly destabilizing Cdc5. If the loop works as expected, we predict that stabilizing Cdc5 should abolish the oscillations. We tested this hypothesis using a non-degradable mutant of Cdc5 *(CDC5dBΔ)* (Visintin et al., 2008), and indeed in this setting Cdc14 was always in a released state, for both high and low doses, Fig. 3.13a, b. Although it is possible that other mechanisms such as opposing phosphatases might also contribute to modulate Cdc5 activity, the result is in agreement with the notion that the negative regulation occurs via Cdc5 degradation. This is not obvious, as in the presence of high levels of Clb2, APC/C^{Cdh1} should be inhibited (Peters, 2006). We can explain this discrepancy supposing that the bulk of Cdh1 is phosphorylated and thus inhibited, but the spatial localization of the component of the circuit allows to a minor population of Cdh1 to be active. This possibility, however, allows a clear prediction: in a *cdh1Δ* strain, oscillations should be lost and Cdc14 fully released. We tested this prediction and confirmed that oscillations were lost, a result confirmed for both high and low doses, Fig. 3.13c, d. However the prediction was only partially confirmed since Cdc14 localization in anaphase cells was intermediate in nature. Indeed, in cells with high doses of non-degradable Clb2 (1% galactose), approximately half of the cells had Cdc14 released while the other half had Cdc14 sequestered, Fig.3.13c. Cells with low doses of stable Clb2 (0.05% galactose) also had an intermediate phenotype, but more than 50% of anaphase cells had Cdc14 released, Fig.3.13d. Because *cdh1Δ* cells are difficult to arrest in G1, we reasoned that such an intermediate phenotype could be a consequence of poor synchronization and thus due to an

averaging out of the oscillations observed at the population levels. This possibility, however, is unlikely since wild-type cells released from a metaphase arrest induced by nocodazole, although poorly synchronous, show oscillations, while the *cdh1Δ* mutant confirmed the intermediate phenotype, Fig 3.13e, f.

In detail, *GAL-CLB2dBΔ cdh1Δ* cells were first synchronized in G1 by 5 mg/ml of α-factor pheromone for 3 hr and then released into medium lacking pheromone supplemented with 15 mg/ml of the microtubule-depolimerizing drug nocodazole. Nocodazole treatment activates the spindle assembly checkpoint and results in a metaphase arrest. When the arrest was complete cells were released in growth medium lacking nocodazole supplemented with galactose to induce the expression of Clb2. *GAL-CLB2dBΔ* cells, released from a metaphase block, showed oscillations in Cdc14 release and sequestration, Fig 3.13e whereas *GAL-CLB2dBΔ cdh1Δ* cells exhibited the same intermediate phenotype observed in the G1 synchronization experiment, Fig. 3.13f compare to 3.13c.

Taken together, our data suggest that the oscillations in Cdc14 localization are driven by the same negative feedback loop that controls mitotic exit. This loop is composed of Cdc14, APC/C^{Cdh1} and Cdc5.

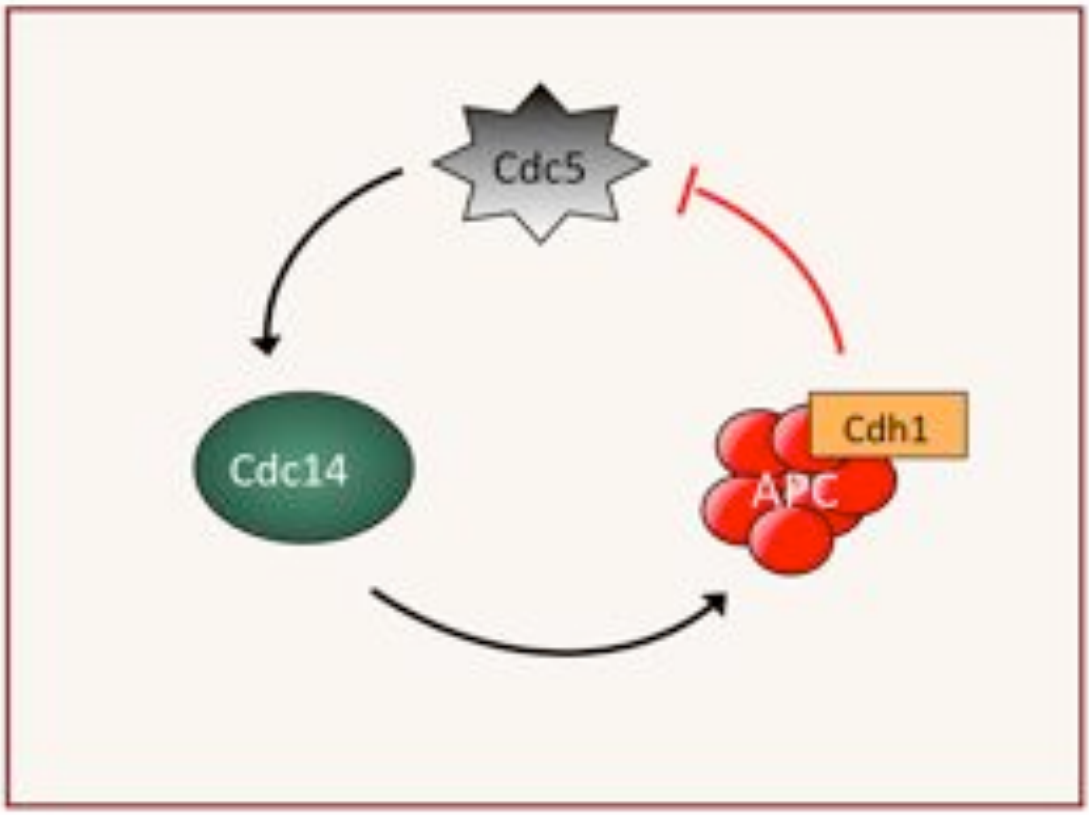

Figure 3.11 Negative feedback loop generate oscillators
Theoretical studies suggest that oscillators are generated by negative feedback loops. Cdc14 activity at the end of mitosis is controlled by a negative feedback loop composed of Cdc5, Cdc14 and the APC/C^{Cdh1}. Here, Cdc5 triggers Cdc14 release and activation. Once active Cdc14 dephosphorylates Cdh1 promoting the formation of the APC/C^{Cdh1} complex. APC/C^{Cdh1} in turns ubiquitinates Cdc5 leading to the its proteosomal degradation. Cdc5 degradation in turns results in Cdc14 re-sequestration and closes the loop. We wondered whether this negative feedback loop is the one responsible for Cdc14 "endocycles".

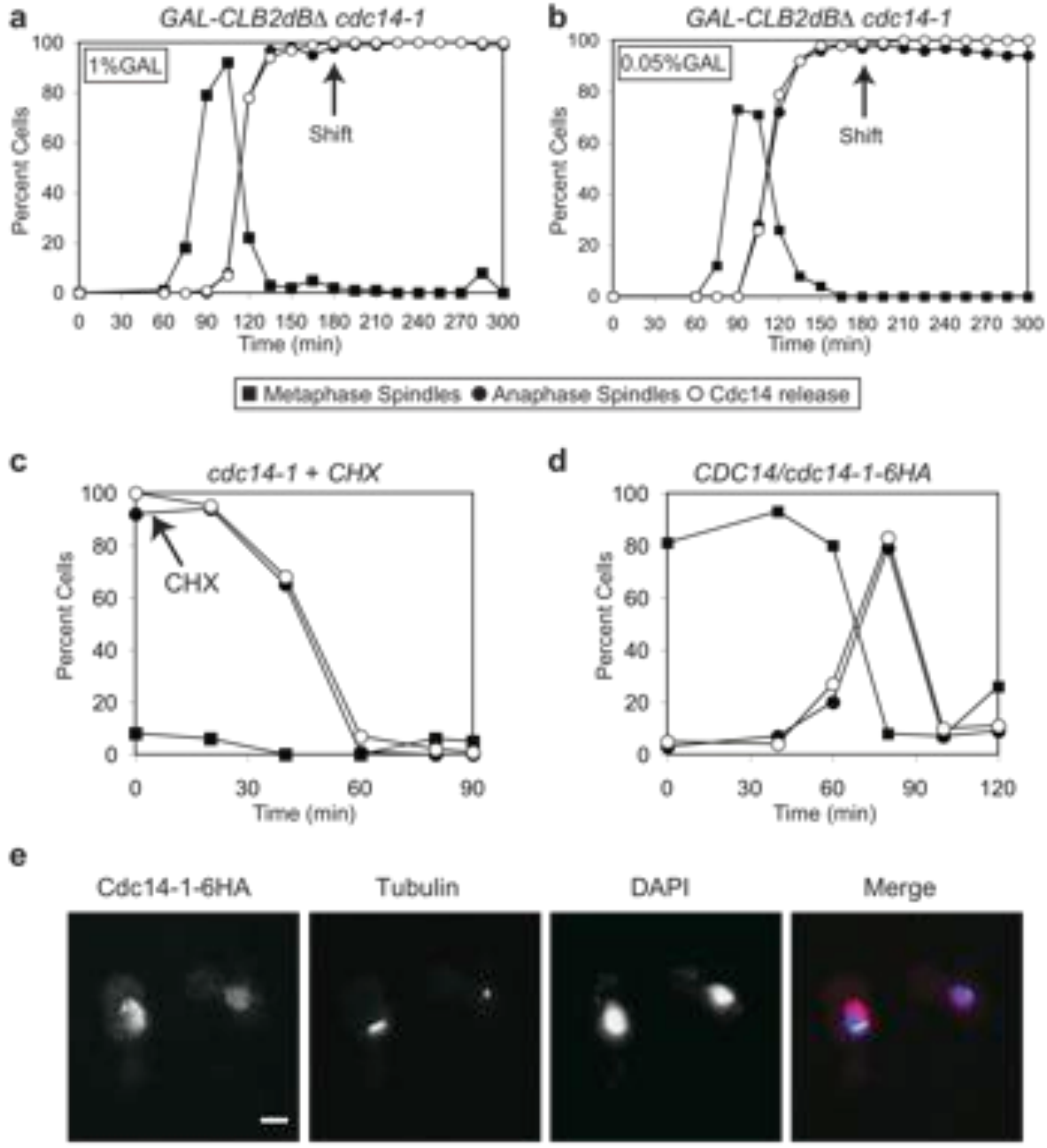

Figure 3.12 A Cdc14 loss-of-function mutant interferes with the oscillatory phenotype
(**a, b**) *GAL-CLB2dBΔ cdc14-1* (Ry1575) cells were arrested with α-factor in YEPR and released into medium supplemented with 1% (**a**) or 0.05% (**b**) galactose to induce the expression of Clb2. The percentage of cells with metaphase spindles (closed squares), anaphase spindles (closed circles) as well as the percentage of cells with Cdc14 released from the nucleolus (open circles) was determined at the indicated times. The arrow indicates the time of the temperature shift (180 minutes). (**c**) *cdc14-1* (Ry1573) cells were arrested in G1 with α-factor (5μg/ml) in YEPD and then released into medium lacking pheromone at the restrictive temperature of 37°C. When the majority of cells reached the telophase arrest (3 hr into the release), the colture was shifted back to the permissive temperature in the presence of 1mg/ml cycloheximide to inhibit *de novo* protein synthesis. Cells were analysed as in (**a**-**b**). (**d**) Heterozygous diploid *CDC14/cdc14-1-6HA* (Ry1695) cells were arrested in S phase with 10 mg/ml hydroxyurea in YEPD. When arrest was complete cells were released in a fresh YEPD medium at the restrictive temperature of 37°C. Samples were taken at the indicated time points to analyze the percentage of metaphase spindles (closed squares), anaphase spindles (closed circles) as well as the percentage of cells with Cdc14-1-6HA released from the nucleolus (open circles). Representative cells of the 120 minute timepoint are shown in (**e**). Cdc14-1-6HA is shown in red, tubulin in green and DAPI in blue.

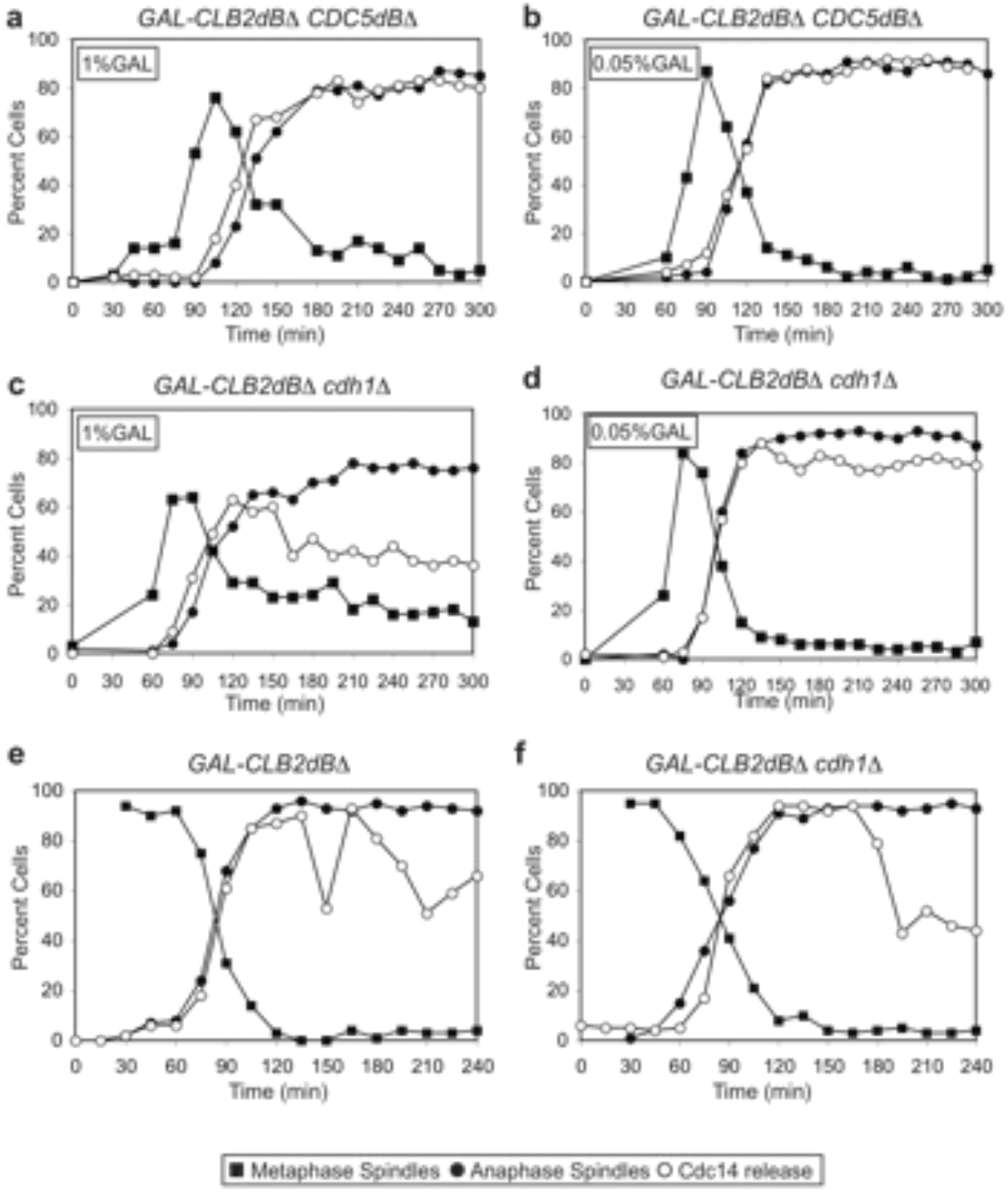

Figure 3.13 Cdc5 stabilization and Cdh1 depletion interfere with the oscillatory phenotype
(**a**-**d**) *GAL-CLB2dBΔ CDC5dBΔ* (Ry1392, **a**, **b**) and *GAL-CLB2dBD cdhΔ* (Ry1466, **c**, **d**) cells were arrested with α-factor in YEPR and released into medium supplemented with 1% (**a**, **c**) or 0.05% (**b**, **d**) galactose to induce the expression of Clb2. (**e**, **f**) *GAL-CLB2dBΔ* (Ry430, **e**) and *GAL-CLB2dB*Δ *cdh1*Δ (Ry1466, **f**) cells were pre-synchronized in G1 with α-factor (5μg/ml) and then released into medium lacking pheromone supplemented with 15μg/ml nocodazole to arrest cells in metaphase. When the arrest was complete cells were released into a medium lacking nocodazole supplemented with 1% galactose to induce the expression of Clb2 and 1% DMSO. The percentage of cells with metaphase spindles (closed squares), anaphase spindles (closed circles) as well as the percentage of cells with Cdc14 released from the nucleolus (open circles) was determined at the indicated times.

APC/C^{Cdh1} is not the only form of the APC/C capable of degrading Cdc5

Puzzled by the observation that *GAL-CLB2dBΔ cdh1Δ*, and *GAL-CLB2dBΔ CDC5dBΔ* mutants behave differently, and having established that the intermediate phenotype of the *GAL-CLB2dBΔ cdh1Δ* strain was not due to an averaging out of the oscillations resulting from poor synchronization, we further examined the contribution of Cdc5 degradation to Cdc14 "endocycles", by analyzing the pattern of Cdc14 localization in cells carrying a temperature sensitive allele of *CDC23* (*cdc23-1)*, a core component of the APC/C (reviewed in (Peters, 2006)). Since the APC/C is required for the metaphase-anaphase transition, being responsible, together with its cofactor Cdc20, for separase (Esp1) activation, the *cdc23-1* allele was inactivated after a large fraction of the population had reached anaphase (180 minutes after the release from the G1 block). In agreement with our interpretation, oscillations were lost in *GAL-CLB2dBΔ cdc23-1* mutant. In these cells, both at high and low doses of Clb2dBΔ, Cdc14 did not oscillate but, contrarily to *cdh1Δ*, it was released in the entire anaphase population, Fig. 3.14a, b. This finding is in agreement with the possibility that APC/C-dependent degradation of Cdc5 promoted by Cdc14 is required for the oscillatory behavior. We confirmed the role of protein synthesis and degradation in the generation of oscillations by blocking protein synthesis with cycloheximide 180 minutes after having overexpressed non-degradable Clb2. In these conditions Cdc14 was sequestered and was never released, Fig.3.14e.

This observation led us to hypothesize that other forms of the APC/C could be responsible for Cdc5 degradation. Thus, we verified whether the periodic release of Cdc14 was affected by Cdc20, the other co-factor of the APC/C (Peters, 2006). When *CLB2dBΔ* was overexpressed in a *cdc20-3* mutant, we observed periodic sequestration and release of Cdc14 regardless of the expression levels of non-degradable Clb2, Fig.3.14c, d, thus showing that Cdc20 is not implicated in generating the oscillatory loop. This result together with what we observed in the *cdc23-1* and *cdh1Δ* mutants strongly points to a role

for Cdh1 in the generation of oscillations. This finding raises the possibility that the spatial localization of the components of this circuit allows Cdc14 to activate a subset of Cdh1, even in the presence of high levels of Clb2-Cdk.

As for the distribution of Cdc14 in *cdh1Δ* cells, we hypothesized that although in this strain Cdc5 is degraded in an APC-dependent manner, the negative feedback loop was destroyed and thus oscillations were lost. As for the APC-dependent and Cdh1-independent mechanism that drives Cdc5 degradation, our results hinted to a possible role for Cdc20. In *cdc15-as1* cells, APC/C^{Cdh1} is not activated because Cdc14 is only transiently released from the nucleolus early in anaphase, and thus Cdc20, a substrate of APC/C^{Cdh1}, is stabilized. Here, Cdc5 levels decreased with the same kinetics as Clb2, Fig. 3.3b, suggesting that similarly to Clb2, Cdc5 could also be partially degraded in an APC/C^{Cdc20}-dependent manner. This possibility is plausible as *GAL-Clb2dBΔ cdh1Δ* cells accumulate Cdc20. Indeed, on one hand Cdc20 is continuously synthesized, being part of a cluster of genes whose expression depends on Clb2-Cdk activity (the Clb2 cluster), on the other hand it is stabilized by the lack of Cdh1, which is responsible for its degradation, Fig. 3.15a. As such we can envision that in this context, Cdc5 synthesis (Clb2-CDK-dependent (Darieva et al., 2003)) and Cdc5 degradation (APC/C-dependent, reviewed in (Peters, 2002)) find an equilibrium, Fig 3.15a. This intermediate value of Cdc5 could result in some cells having Cdc14 sequestered and others released.

The observation that Cdc20 does not affect the oscillations if Cdh1 is present is consistent with the fact that in wild-type cells the synthesis of Cdc20 is counterbalanced by its APC^{Cdh1}-dependent degradation, Fig 3.15a. In conclusion, our results support the hypothesis that APC/C^{Cdh1}-dependent degradation of Cdc5 provides the main contribution to the generation of our oscillations and that oscillations in Cdc14 localization are driven by the negative feedback loop which includes Cdc14, Cdh1 and Cdc5 and controls Cdc14 re-localization at the end of mitosis (Visintin et al., 2008).

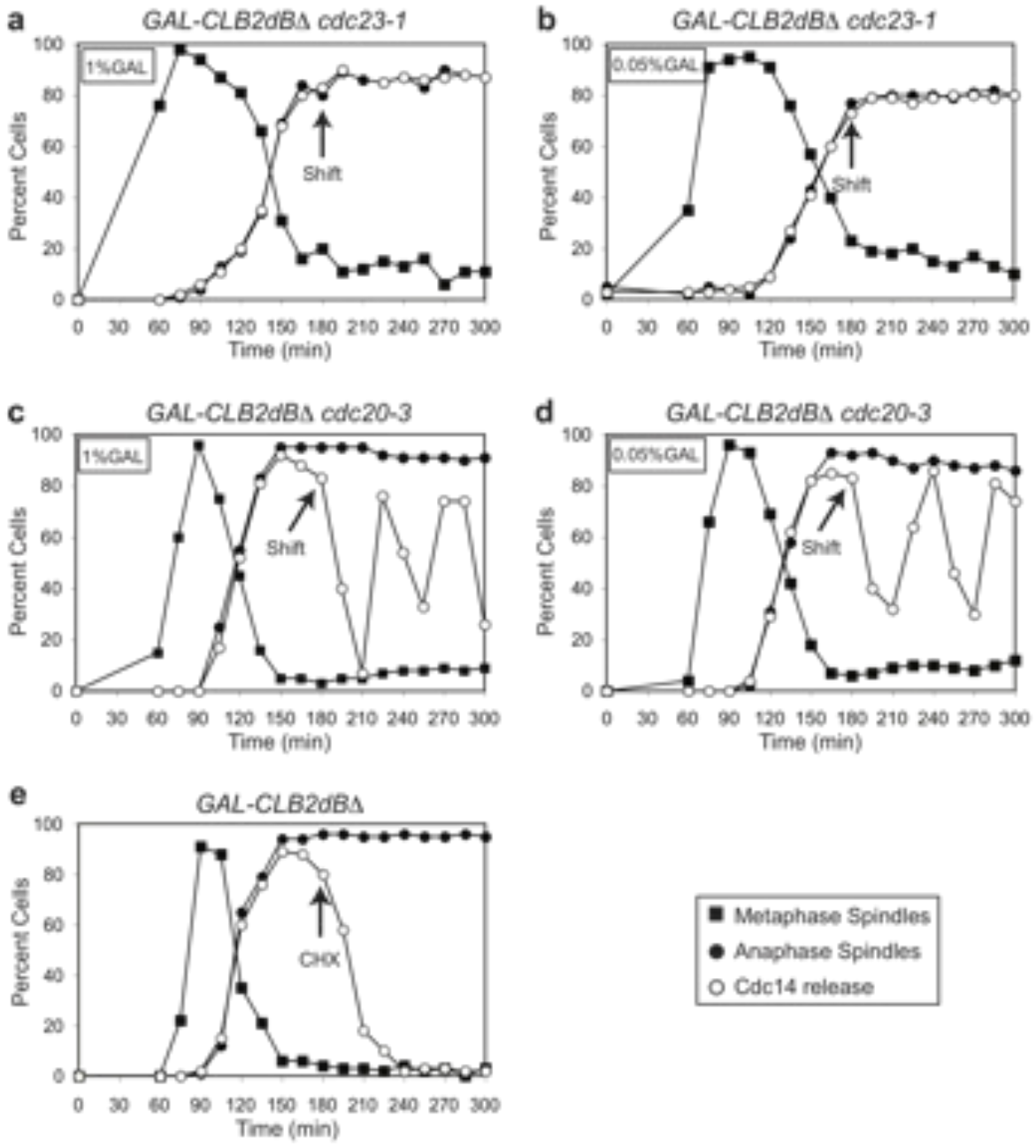

Figure 3.14 APC/C dependent degradation and *de novo* protein synthesis are required for Cdc14 endocycles

(**a**-**d**) *GAL-CLB2dBΔ cdc23-1* (Ry1452, **a**,**b**) and *GAL-CLB2dBΔ cdc20-3* (Ry1457, **c**, **d**) cells were arrested with α-factor in YEPR and released into fresh medium supplemented with 1% (**a**, **c**) or 0.05% (**b**, **d**) galactose to induce the expression of Clb2. Cells were shifted at the restrictive temperature 180 minutes after the release (arrow). The percentages of cells with metaphase spindles (closed squares), anaphase spindles (closed circles) as well as the percentage of cells with Cdc14 released from the nucleolus (open circles) were determined at the indicated times. (**e**) *GAL-CLB2dBΔ* (Ry430) cells were arrested and released as in (**a**). 180 minutes after the release the cultures were added with 1 mg/ml of cycloheximide (arrow) to stop protein synthesis. Samples were analyzed as in (**a**).

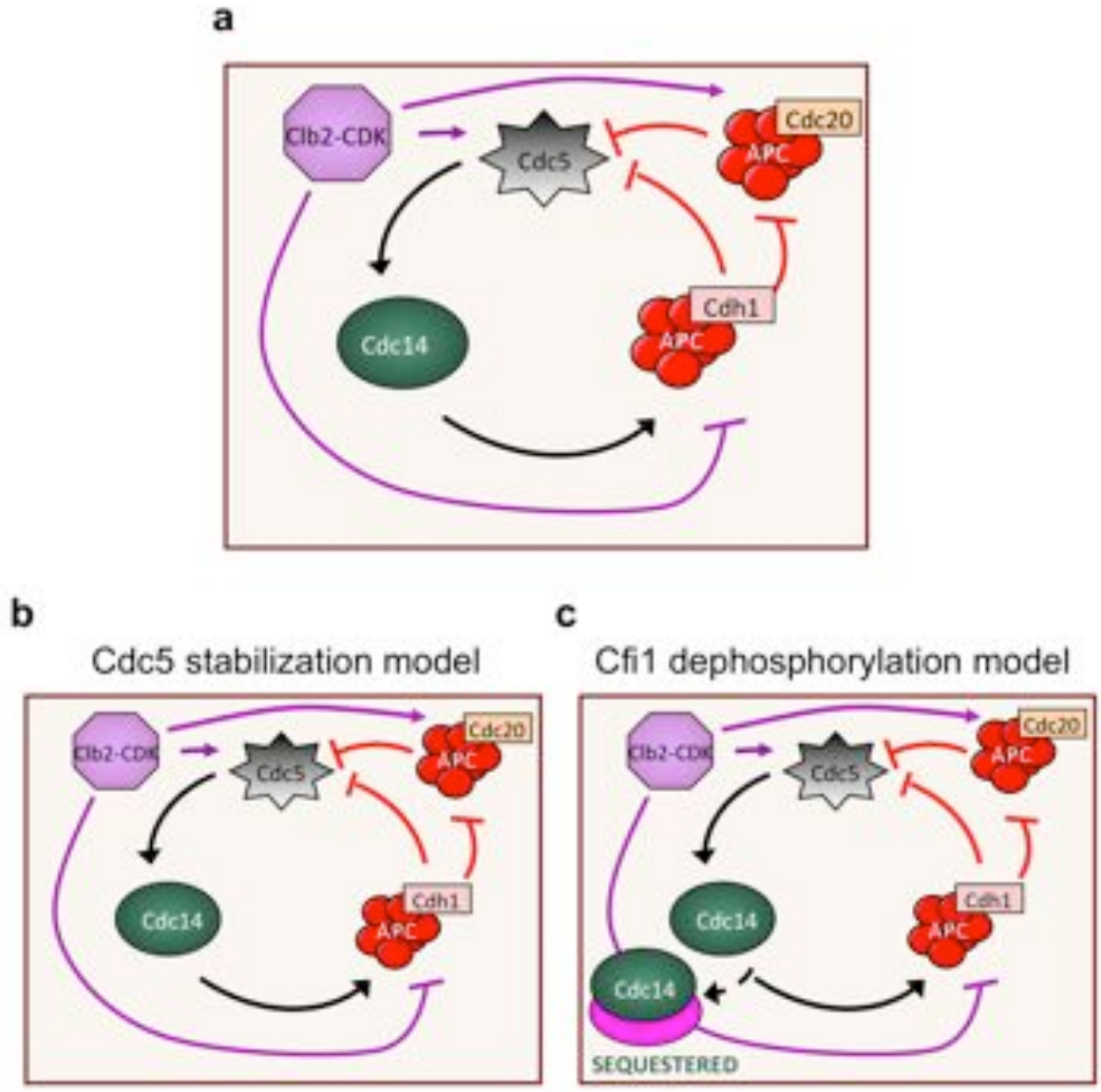

Figure 3.15 A model for Cdc14 endocycles
(**a**) How do Cdc14 endocycles arise? In our experimental set up Cdc5 is continuously synthesized being part of the Clb2 cluster genes. In *GAL-CLB2dBΔ cdh1*Δ cells oscillations are lost due to the involvement of Cdh1 in the loop, and the intermediate phenotype can be explained by an equilibrium between Cdc5 Clb2-dependent synthesis and Cdc5 APC/CCdc20-dependent degradation, the latter being stabilized by the lack of Cdh1. In *GAL-CLB2dBΔ*, where Cdh1 is active Cdc20 is degraded. Thus oscillations can occur. (**b**) Hypothesis 1: "Cdc5 stabilization model": Cdc14 does not induces Cdc5 degradation indirectly activating APC/CCdh1 complex, but also directly dephosphorylating Cdc5 making the kinase a good substrate for the degradation. (**c**) Hypothesis 2: "Cfi1 dephosphorylation model": besides triggering Cdc5 degradation Cdc14 dephosphorylates other substrate, of which Cfi1 is a good candidate, that once dephosphorylated contributes to the re-sequestration of Cdc14 itself.

Cdc5 undergoes cycles of synthesis and degradation in the presence of high levels of stable Clb2

Cycles of Cdc14 release and sequestration should be mirrored by cycles of phosphorylation and de-phosphorylation of Cdh1, and by cycles of synthesis and degradation of Cdc5. As changes in the phosphorylation status can be difficult to appreciate we focused our attention on Cdc5. As mentioned, Cdc5 is essential for Cdc14 release. Hence, its degradation mediated by the APC/C^{Cdh1} should be opposed by its re-synthesis for a new cycle of Cdc14 release to occur. We already showed, Fig. 3.14e, that *de-novo* protein synthesis is required for the oscillation. That this synthesis concerns Cdc5 is a likely possibility as Cdc5 belongs to the Clb2-cluster gene (Peters, 2006). We can hypothesize that in our setting the presence of Clb2 at high levels sustains Cdc5 re-synthesis after the kinase is degraded. To visualize changes in the levels of Cdc5 in our experimental set up we employed different strategies. Population analysis of Cdc5 levels by western blot hybridization gave only partial answers, likely because of the heterogeneity of the cells in the population. Of note, the sensitivity of this technique is not able to assess differences in the 2-fold range of magnitude.

To avoid complications due to population analysis we addressed the problem of Cdc5 degradation with a real-time single cell analysis. Before analyzing Cdc5 kinetics at single cell level, we established the right imaging conditions to identify oscillations in Cdc14 release and re-sequestration. For this purpose, a *GAL-CLB2dBΔ* strain carrying a GFP-tagged version of Cdc14 in combination with Cherry-tagged tubulin was grown in Sc-medium with 2% Sucrose and arrested in G1 by adding α-factor pheromone. When arrest was complete cells were loaded in a microfluidic chamber, and released in Sc-medium with 2% Sucrose and 2% Galactose to induce the expression of *GAL-CLB2dBΔ*. Time-lapse imaging was performed at 30°C and frames were taken every 15 minutes. In

agreement with what we found during our population studies, Cdc14 endocycles where visible also at single cell level (data not shown).

Having established the right conditions for oscillations to be reproduced, we moved to visualize over time an allele of Cdc5 tagged with Venus characterized in the laboratory of Dr. Yves Barral. This allele was shown to have wild-type kinetics of synthesis and degradation. *GAL-CLB2dBΔ CDC5*-Venus cells were grown in Sc-medium with 2% Sucrose and imaged in Sc-medium with 2% Sucrose and 2% Galactose to induce the expression of *GAL-CLB2dBΔ*. Cells were then loaded into a microfluidic chamber and the time-lapse imaging was performed at 30°C taking frames every 20 minutes. These experiments were performed in collaboration with Fabrice Coudron, a Post-Doc in the laboratory of Yves Barral. In agreement with our hypothesis in the presence of high levels of non-degradable Clb2 we observed fluctuations in the Cdc5 levels, as judged by the disappearance and re-appearance of the Cdc5 signal (data not shown).

Taken together our experiments support the notion that cycles of Cdc5 synthesis and degradation underlie the Cdc14 endocycles. We conclude that once Cdc14 is fully released, due to a combination of FEAR and MEN, the negative feedback loop is operational: Cdc14 activates Cdh1, APC/C^{Cdh1} degrades Cdc5 and Cdc5 degradation induces Cdc14 re-sequestration. In the presence of physiological levels of degradable Clb2, this event can occur only once, because upon APC/C^{Cdh1} activation, Clb2 is also degraded and cells exit from mitosis. Instead, in our *GAL-CLB2dBΔ* system Clb2dBΔ is not degraded. Furthermore it behaves as a transcription factor for the Clb2 cluster gene, of which Cdc5 is part, thereby continuously stimulating Cdc5 transcription. Thus, Cdc5 is continuously synthesized, initiating new loops of release and re-sequestration.

Cdc14 promotes its own sequestration in multiple ways

Having established the role of Cdc5 degradation in setting the oscillations we wished to understand another inconsistency found during our analyses. Our results showed that *GAL-CLB2dBΔ cdc23-1,* Fig. 3.14a, b, and *GAL-CLB2dBΔ cdc14-1,* Fig. 3.12a, b, mutants share the same phenotype, having Cdc14 permanently released, contrarily to *GAL-CLB2dBΔ cdh1Δ* cells*,* Fig. 3.13c, d, where Cdc14 is only partially released. The observation that *GAL-CLB2dBΔ cdc14-1,* Fig. 3.12a, b, and *GAL-CLB2dBΔ cdh1Δ* cells Fig. 3.13c, d, behave differently cannot be explained by the minimal circuit we proposed so far (Cdc14 → Cdh1 —| Cdc5 → Cdc14). In this setting the only role played by Cdc14 is to activate Cdh1. This discrepancy suggests that Cdc14 could perform additional roles to induce its own sequestration. Given that both the inactivation of Cdc14 and of the APC/C (i.e., *cdc23-1*) cause a complete release of Cdc14, one possibility is that the *cdc14-1* mutant stabilizes Cdc5 as efficiently as the *cdc23-1* mutant. We can envision one scenario that we called "the Cdc5 stabilization hypothesis" whereby Cdc5 is stabilized by phosphorylation (possibly by Clb2-Cdk) while its dephosphorylation, catalyzed by Cdc14, makes the kinase a good substrate for both the APC/C^{Cdc20} and APC/C^{Cdh1}. Thus Cdc14 not only induces Cdc5 degradation indirectly activating APC/C^{Cdh1} complex, but also directly dephosphoryates Cdc5 making the kinase a good substrate for degradation, Fig. 3.15b.

Alternatively, the different phenotypes of *cdh1Δ* and *cdc14-1* could be explained if Cdc14 would induce its own sequestration, besides triggering Cdc5 degradation, via dephosphorylating other substrates, of which Cfi1 is a good candidate. Once dephosphorylated Cfi1 contributes to the re-sequestration of Cdc14 itself (as previously proposed by Tomson and colleagues (Tomson et al., 2009)), Fig. 3.15c. We refer to it as "Cfi1 dephosphorylation model". To discriminate between these two models we analyzed the consequences of inactivating Cdc14 and Cdc5 simultaneously. According to the Cdc5 stabilization model, the *GAL-CLB2dBΔ cdc14-1 cdc5-1* double mutant should phenocopy

a *GAL-CLB2dBΔ cdc5-1* single mutant, Fig. 3.10c, d, re-sequester Cdc14 and never release it again because the inactivation of Cdc5 itself should overcome its own stabilization due to the lack of Cdc14 activity. In contrast, the Cfi1 phosphorylation model predicts that the double mutant should phenocopy the *GAL-CLB2dBΔ cdc14-1* mutant with Cdc14 permanently released because Cfi1 phosphorylation could not be increased but also not decreased, Fig. 3.12a, b. Thus, *GAL-CLB2dBΔ, GAL-CLB2dBΔ cdc14-1, GAL-CLB2dBΔ cdc5-1* and *GAL-CLB2dBΔ cdc14-1 cdc5-1* cells were arrested in G1 by α-factor pheromone and then synchronously released in the presence of galactose to induce Clb2 expression. When the majority of the cells reached anaphase, 180 minutes after the release, the culture was shifted to the restrictive temperature (37^{o}C). In agreement with the Cfi1 dephosphorylation hypothesis Cdc14 was always released in the double mutant Fig. 3.16d. We conclude that Cdc14 induces its own re-sequestration in additional ways besides contributing to the destabilization of Cdc5 mediated by the APC/C^{Cdh1}.

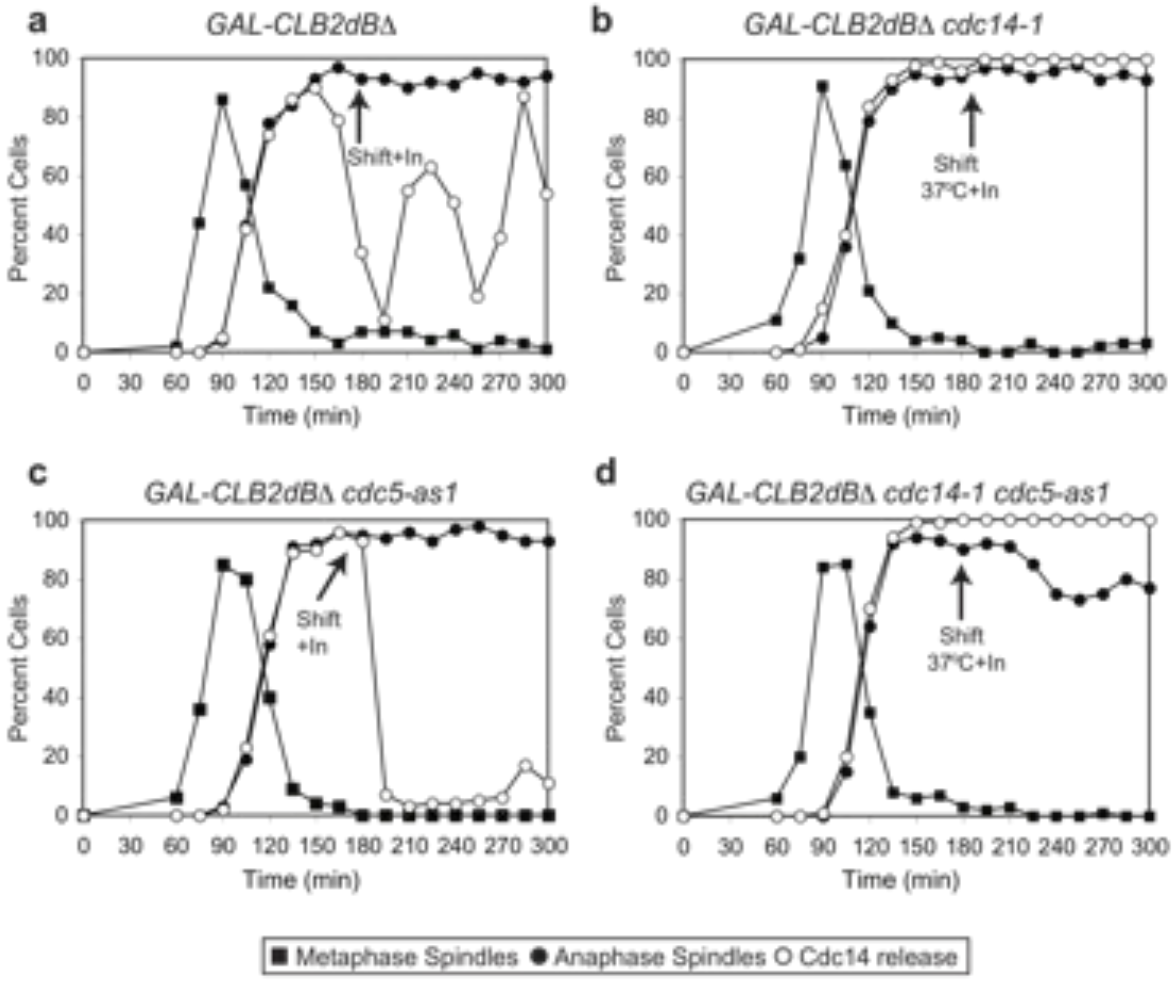

Figure 3.16 Cdc14 re-sequestration requires both Cdc5 inactivation and Cdc14 phosphatase activity
(**a-d**) *GAL-CLB2dBΔ* (Ry430, **a**) *GAL-CLB2dBΔ cdc14-1* (Ry1575, **b**), *GAL-CLB2dBΔ cdc5-as1* (*cdc5L158G,* Ry1607, **c** (Snead et al., 2007; Zhang et al., 2005)) and *GAL-CLB2dBD cdc14-1 cdc5-as1* (*cdc5L158G*, Ry1606, **d**) cells were arrested with α-factor (5 μg/ml). After 3 hr cells were released into medium lacking pheromone supplemented with 1% galactose to induce the expression of Clb2. 180 minutes after the release cells were shifted at 37°C and supplemented with 10 μM of the *cdc5-as1* inhibitor (CMK) (Snead et al., 2007) (arrow). The percentages of cells with metaphase spindles (closed squares), anaphase spindles (closed circles) as well as the percentage of cells with Cdc14 released from the nucleolus (open circles) were determined at the indicated times.

The fraction of Cdc14 involved in the oscillatory phenotype is the one that specifically binds Cfi1

Having identified the molecular circuit responsible for generating Cdc14 endocycles, we decided to elucidate the pool of Cdc14 that is responsible for this phenotype. Cdc14 phosphatase is sequestered in the nucleolus in a complex termed RENT (REgulator of Nucleolar silencing and Telophase exit) consisting of Cdc14 itself, Cfi1 and the silencing factor Sir2 (Huang and Moazed, 2003; Shou et al., 1999; Visintin et al., 1999). A recent protein interaction analysis identified Tof2 as physically interacting with RENT complex components (Huang and Moazed, 2006). Furthermore it directly binds to Cdc14 *in vitro* and genetically interacts with the phosphatase *in vivo* (Geil et al., 2008; Waples et al., 2009). Interestingly, Cfi1 and Tof2 shear a significant homology at their N-terminus, and it has been shown that this Tof2 N-terminal domain, as for Cfi1, is the one responsible for the Tof2/Cdc14 interaction (Waples et al., 2009). Similar to Cfi1, Tof2 localizes to the nucleolus and remains nucleolar during anaphase. It was recently proposed that also Tof2, next to Cfi1, affects Cdc14 localization (Waples et al., 2009). As such, we decided to investigate which fraction of Cdc14 is involved in the periodic release, the one sequestered by Cfi1 or the one sequestered by Tof2. To this aim we looked at the consequences of deleting Tof2 and Cfi1 in our oscillatory system. *GAL-CLB2dBΔ tof2Δ* and *GAL-CLB2dBΔ cfi1Δ* cells were arrested in G1 by α-factor pheromone and synchronously released into the next cell cycle into fresh media containing galactose to induce Clb2 expression. Although it is possible that the interaction between Tof2 and Cdc14 is affected during the periodical cycles of Cdc14 release/re-sequestration, we found that deleting Tof2 did not affect the oscillations, Fig. 3.17a. On the contrary, the interaction with Cfi1 is key for the oscillations, as *GAL-CLB2dBΔ cfi1Δ* strain in the presence of high levels of stable Clb2 did not oscillate, but released constantly Cdc14, Fig. 3.17b. Thus, we demonstrated that the pool of Cdc14 involved in the oscillations is the one regulated by Cfi1.

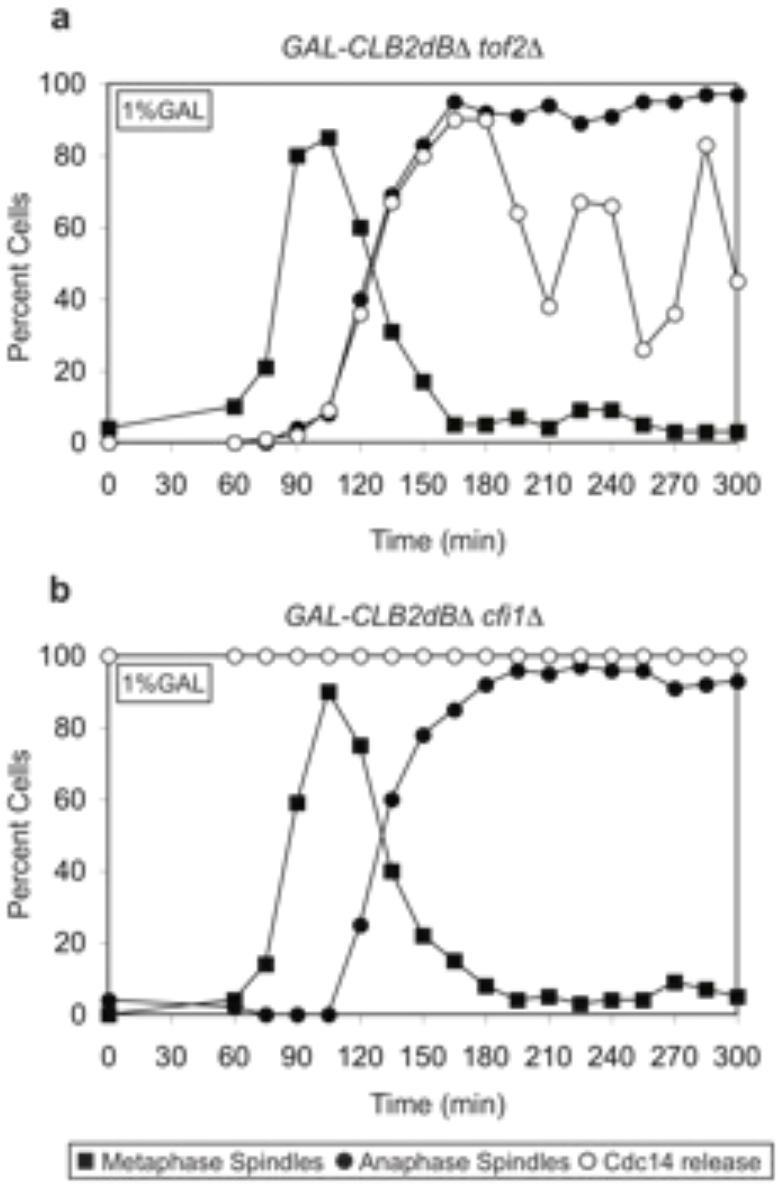

Figure 3.17 The pool of Cdc14 involved in the oscillations is the one bound to Cfi1
(**a**, **b**) *GAL-CLB2dBΔ tof2Δ* (Ry1708, **a**) and *GAL-CLB2dBΔ cfi1Δ* (Ry1710, **b**) cells were arrested with α-factor in YEPR and released into medium supplemented with 1% galactose to induce the expression of Clb2. The percentage of cells with metaphase spindles (closed squares), anaphase spindles (closed circles) as well as the percentage of cells with Cdc14 released from the nucleolus (open circles) was determined at the indicated times.

What is the molecular significance of the two-hit model?

Having identified the circuit responsible for the oscillation we wished to better understand the molecular significance of this double-kinase requirement. Different scenarios can be envisioned. The observation that the activity of Cdc5 is always required for Cdc14 release suggests an attractive hypothesis. Polo-like kinases (Plks), of which Cdc5 is the unique member in budding yeast, possess a conserved C-terminal polo-box domain (PBD; (Elia et al., 2003a; Lee and Erikson, 1997; Lee et al., 1998). The PBD interacts with Plk substrates or docking proteins after undergoing phosphopriming by another kinase (Elia et al., 2003b). The binding of Plk to these proteins results in Plk localization in the vicinity of its substrates and thus allows Plk-dependent phosphorylation events. It is therefore possible that priming by Clb-Cdks or Dbf2 is required to build up Cdc5 activity. Phosphorylation of Cdc14 and/or Cfi1 by Cdc5 could then promote the dissociation of Cdc14 from its inhibitor. Reinforcing this hypothesis are six Clb2-Cdk-dependent phospho-residues that were identified in Cfi1 (Azzam et al., 2004). Cells carrying an allele of *CFI1* encoding a version of the protein in which these residues have been mutated to alanine (*cfi1-6Cdk*), a non-phosphorylatable amino acid, display defects in releasing Cdc14 during early anaphase. Remarkably, one of these residues (Thr 212) is part of an optimal phospho-binding motif recognized by PBD (Elia et al., 2003b). Alternatively Cdc5 and Clb-Cdk or Dbf2 could have parallel functions in phosphorylating Cfi1 and/or Cdc14 thereby promoting the release of Cdc14 from its inhibitor (Azzam et al., 2004; Mohl et al., 2009; Visintin et al., 2003; Yoshida and Toh-e, 2002). Lastly, the MEN kinase Dbf2 and Clb-Cdk complexes could be required for Cdc5 activation and/or *vice versa*. Supporting this idea is that fact that the activation of Cdc5 requires the phosphorylation of a conserved threonine residue (Thr 242), located in its T-loop, by Cdc28 (Mortensen et al., 2005), the only Cdk in budding yeast.

The fact that Cdc5, Clb-Cdks and MEN kinases are interdependent, as Cdc5 kinase activity requires Clb-Cdk, MEN activation requires Cdc5, and finally MEN activity (e.g. Cdc15 kinase) is antagonized by Clb-Cdks, renders the elucidation of the molecular mechanism underlying the "two-hit" model rather challenging. It is in fact likely that various regulatory mechanisms co-exist to ensure a tight control over the activity of these powerful multitasking mitotic regulators. To shed light onto this process I investigated each of these possibilities.

Cdc14 and Cfi1 phosphorylation are important for Cdc14 release

Data present in the literature (Azzam et al., 2004; Visintin et al., 2003) suggest that one important function of the kinase that co-operates with Cdc5 to promote Cdc14 release from the nucleolus (Clb-Cdks or the MEN) is to phosphorylate Cfi1. Supporting this conclusion, Azzam and colleagues showed that Clb2-Cdk is required for Cfi1 phosphorylation during early anaphase while we found that overexpression of *CDC15(1-750)* in both G1- and S phase-arrested cells resulted in the ectopic phosphorylation of Cfi1, Fig. 3.18b and Fig. 3.20b. Our data also support the idea that Cfi1 phosphorylation serves as a priming step to build up Cdc5 activity. Indeed, when expressed in G1, where neither Clb-Cdks nor MEN kinases are active, differently than in all other stages of the cell cycle, Cdc5 not only was not capable of triggering the ectopic release of Cdc14, but also did not induce the phosphorylation of Cdc14 and/or of Cfi1, Fig 3.19b. However, the concomitant overexpression of *CDC5* and *CDC15(1-750)* re-established both the ectopic release of Cdc14, in agreement with (Manzoni et al., 2010), and changes in the electrophoretic mobility of both Cdc14 and its inhibitor, Fig. 3.20a. The observation that the pattern of Cfi1 phosphorylation in cells expressing only *CDC15(1-750)* or both *CDC5* and *CDC15(1-750)* is similar, Fig. 3.20b and Fig. 3.20a, as evidenced by our Western blots, allows us to further speculate that the priming events serve to mediate Cdc14

phosphorylation by Cdc5. In support to this "priming" model we identified (unpublished data) by mass spectrometry a residue in Cfi1 that lies in a putative Dbf2 recognition sequence (Mah et al., 2005), which is specifically phosphorylated in anaphase and is part of a minimal PBD-phosphobinding motif (Elia et al., 2003b). To test our model we will assess the consequences, on the phosphorylation and release of Cdc14, of mutating this residue into alanine, a non-phosphorylatable amino acid, and to aspartic acid, which mimics constitutive phosphorylation. Similarly, we will mutate other putative Dbf2-dependent phosphorylation sites within Cfi1 and putative Cdc5-dependent phosphorylation sites within Cdc14. The analysis of the phenotypes of cells conditionally expressing these different mutant proteins will provide further insight about the relevance of the priming model. Clear predictions as to the expected phenotypes can be made. For instance, if Cfi1 phosphorylation functions exclusively as a mediator of Cdc5-dependent phosphorylation, we expect cells expressing a mutant variant of Cdc14, carrying all putative sites phosphorylated by Cdc5 mutated into aspartic acid (D), to have Cdc14 released from the nucleolus during the entire cell cycle. Interestingly, cells carrying a mutant allele of *CDC14* encoding a form of the protein where six putative Cdc5-dependent phosphorylation sites have been mutated into aspartic acid exhibit an altered kinetic of Cdc14 release with the phosphatase released for most of the cell cycle (unpublished data). *Vice versa*, a mutant allele of Cdc14 where the same sites have been mutated into alanine is unviable (unpublished observation). We are currently testing the localization of these mutant Cdc14 proteins in a strain carrying the *cdc14-1* temperature sensitive mutation.

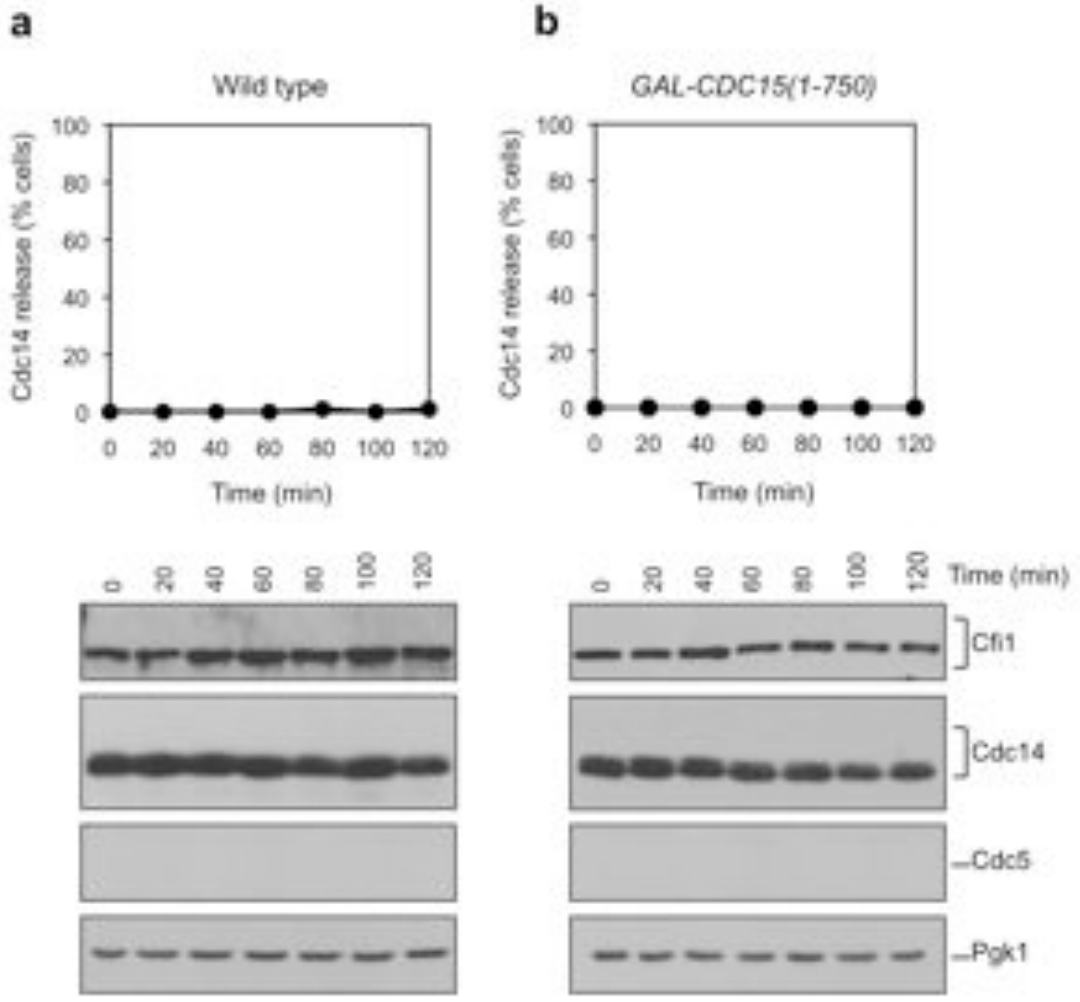

Figure 3.18 Cdc15 overexpression results in Cfi1 phosphorylation in S phase arrested cells
(**a**, **b**) Wild-type (Ry394, **a**) and *GAL-CDC15(1-750)* (Ry995, **b**) cells carrying a *CDC14-3HA* and a *CFI1-3MYC* fusions were arrested in G1 with α-factor in YEPR (5 μg/mL) at 23°C. When the arrest was complete, cells were released into YEPR lacking pheromone but containing HU (10 mg/mL). When cells had arrested in S phase, 2% galactose was added to induce the expression of Cdc15. Samples were taken at the indicated times to determine the percentage of cells with Cdc14 released from the nucleolus and proteins levels and mobility in SDS-PAGE of Cdc14-3HA, Cfi1-3Myc and 3Myc-Cdc5. Pgk1 was used as an internal loading control in Western blot analysis.

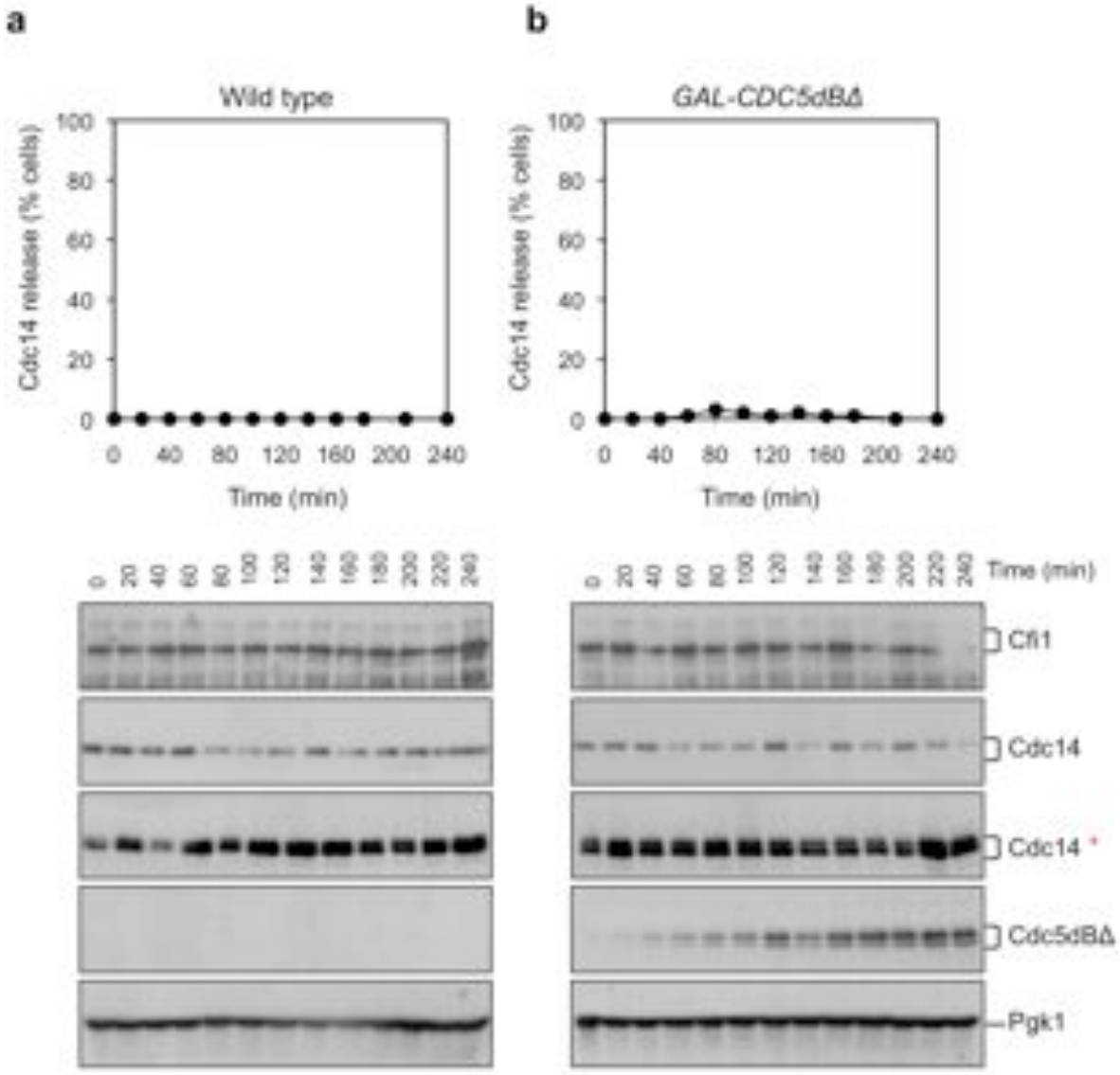

Figure 3.19 Overexpression of *CDC5dBΔ* does not induce the ectopic phosphorylation of Cdc14 or Cfi1 in cells arrested in G1

(**a, b**) Wild-type (Ry394, **a**) and *GAL-CDC5dBΔ-3HA* (Ry1358, **b**) cells carrying a *CDC14-3HA* and a *CFI1-3MYC* fusions were arrested in G1with α-factor (5 μg/ml) in YEPR at 23°C. When more than 90% of cells were unbudded, 2% galactose was added to induce the expression of Cdc5. To maintain the G1 block, α-factor pheromone (2.5 μg/ml) was re-added to the media every 90 min after galactose addition. Samples were taken at the indicated times to determine the percentage of cells with Cdc14 released from the nucleolus and proteins levels and mobility in SDS-PAGE or in Phos-tag SDS-PAGE (*) of Cdc14, Cfi1 and Cdc5dBΔ. Pgk1 was used as an internal loading control in Western blot analysis.

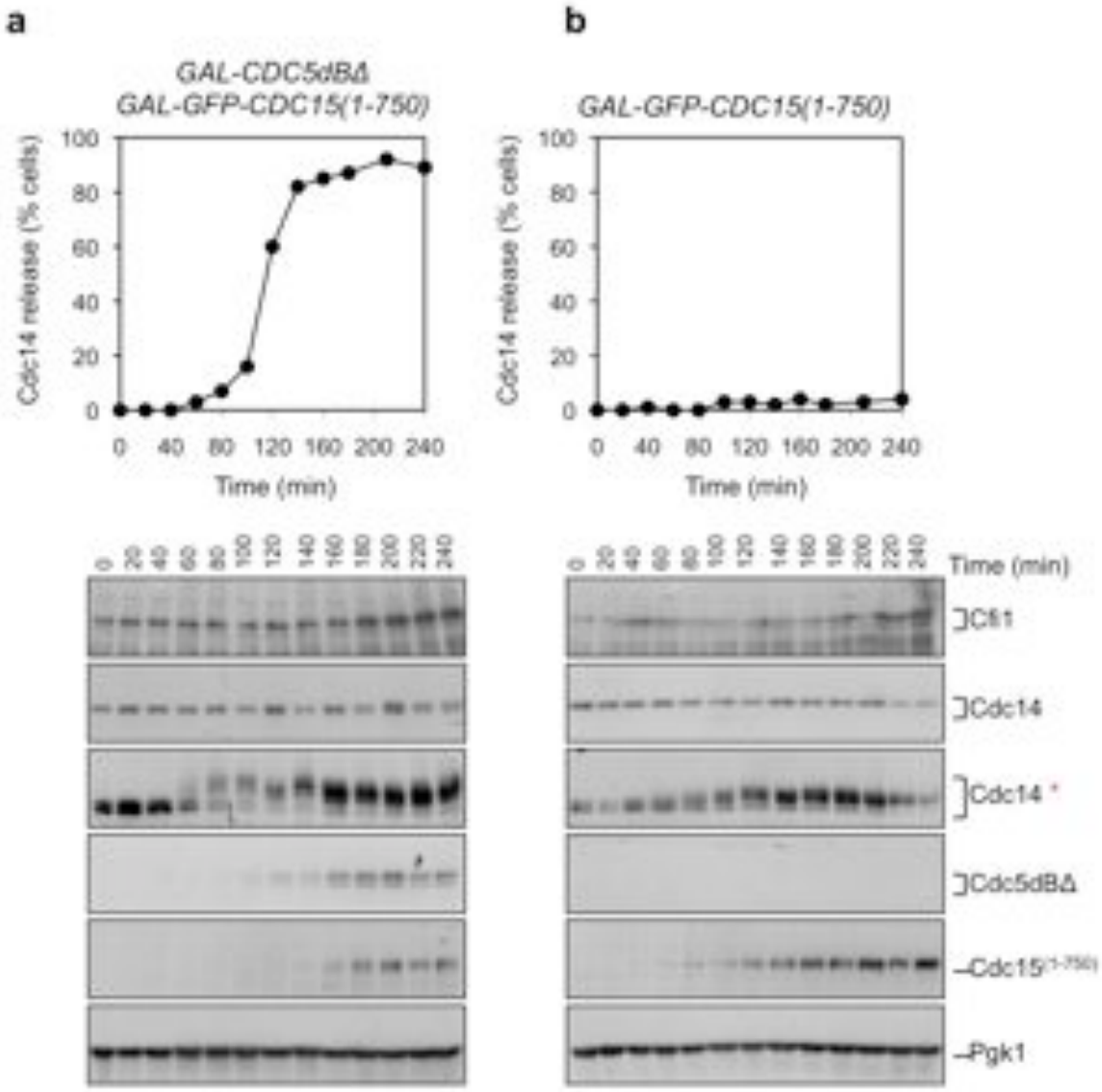

Figure 3.20 The concomitant overexpression of Cdc5 and Cdc15 is sufficient to promote the ectopic phosphorylation of both Cdc14 and Cfi1 in G1-arrested cells
(**a**, **b**) *GAL-GFP-CDC15(1-750)* (Ry995, **a**) and *GAL-CDC5dBΔ-HA GAL-GFP-CDC15(1-750)* (Ry1345, **b**) cells carrying a *CDC14-3HA* and a *CFI1-3MYC* fusions were arrested in G1 in YEPR with α-factor pheromone (5 μg/ml) at 23°C. When the arrest was complete, 2% galactose was added to induce the expression of Cdc5 and Cdc15. To maintain the G1 block, α-factor pheromone (2.5 μg/ml) was re-added to the media every 90 min after galactose addition. Samples were taken at the indicated times to determine the percentage of cells with Cdc14 released from the nucleolus and proteins levels and mobility in SDS-PAGE or in Phos-tag SDS-PAGE (*) of Cdc14, Cfi, Cdc15 and Cdc5dBΔ. Pgk1 served as an internal loading control in Western blot analysis.

Do Clb2 and Dbf2 modulate Cdc5 activity?

Our data are also consistent with Clb-Cdks and the MEN kinase Dbf2 being required to directly activate Cdc5. Supporting this hypothesis is the observation that Cdc5 activation requires the phosphorylation of a conserved threonine residue by Cdc28 (Mortensen et al., 2005). We found a putative site for Dbf2 kinase phophorylation nearby in Cdc5 T-loop. We mutated this site into alanine and will analyse the consequences of this mutation on Cdc5 activity. Although we cannot exclude that this extra layer of regulation is involved in the process, the observation that cells expressing a mutant form of Cfi1 lacking the 6 Cdk-dependent phosphorylation sites are defective in releasing Cdc14 from the nucleolus during early anaphase, when both Clb2-Cdk and Cdc5 are present, argues that Cfi1 phosphorylation by Clb-Cdks is essential to mediate Cdc14 release. It will be interesting to assess if in this mutant the phosphorylation of Cdc14 is delayed as well.

Moreover if it possible that the "partner" kinases contribute to full Cdc5 activation under physiological conditions, we do not think that this is the major contribution of Clb2 and Dbf2 to Cdc14 release. Indeed, when we compared Cdc5 kinase activity at different cell cycle stages we found that, albeit with a delay and to a lesser extent than in S- and M-phase, overexpression of Cdc5 resulted in an increase of its kinase activity also in G1 arrested cells, but in this phase of the cell cycle Cdc5 is not capable of inducing ectopic release of Cdc14, Fig 3.21a compare to 3.21b, c.

Nevertheless for completion we will investigate the contribution of Cdc5 "partner" kinases to Cdc5 activation itself by comparing the activity of Cdc5 in G1-arrested cells, when the kinase is expressed alone or in combination with the other kinases involved in the process. We will also test, in the different mutant backgrounds, the consequences of overexpressing a mutant allele of *CDC5* encoding a Cdc5 protein that can no longer be activated by Clb-Cdks (Mortensen et al., 2005). This way we will assess not only the relevance of this activating phosphorylation in the process but also, in case the partner

kinases serve to activate Cdc5, if Clb-Cdks and MEN contribute to Cdc5 activation via the same molecular mechanism.

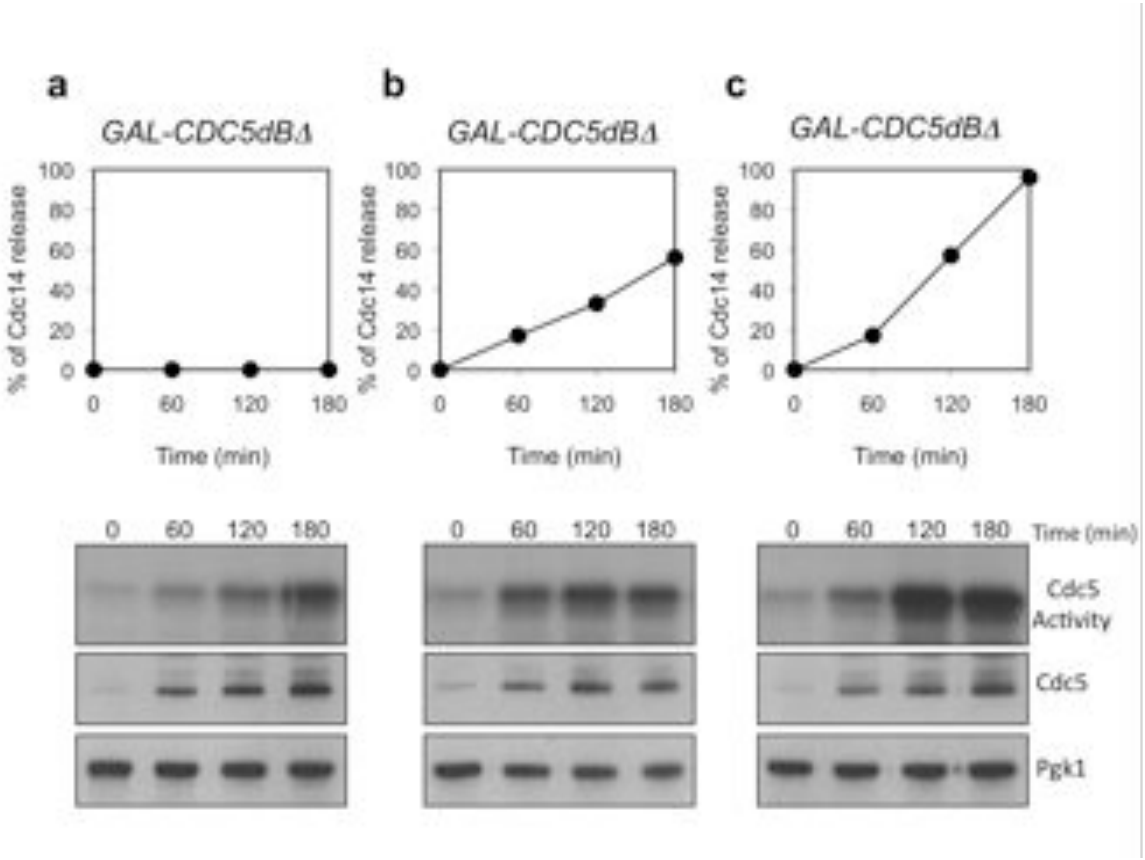

Figure 3.21 Cdc5 overexpression resulted in an increase of its activity also in G1 arrested cells
(**a**) *GAL-CDC5dBΔ* (Ry798) cells were arrested in G1 in YEPR with α-factor pheromone (5μg/ml) at 23°C. When more than 90% of cells were unbudded, 2% galactose was added to induce the expression of Cdc5. To maintain the G1 block, α-factor pheromone (2.5 μg/ml) was re-added to the media every 90 min after galactose addition. Samples were taken at the indicated times to determine the percentage of cells with Cdc14 released from the nucleolus. (**b-c**) *GAL-CDC5dBΔ* (Ry798) were pre-synchronised in G1 as described in (a). When arrest was complete cells were released into 10mg/ml HU (b) and 15μM nocodazole, to achieve an S- and M-phase arrest respectively. Cdc5 kinase activity on Casein and western blot analysis of Cdc5 protein level and mobility in SDS-PAGE are shown. Pgk1 was used as an internal loading control in Western blot analysis.

High levels of Cdc5 induce a post-translational modification on Clb2

During the course of our experiments in which *CDC5* was overexpressed we noticed a slower migrating form of Clb2 appearing concomitantly with ectopic release of Cdc14. This observation suggested the possibility that this Cdc5-dependent modification of Clb2 rendered Clb2 competent to phosphorylate Cfi1 in the FEAR network context. To test this possibility we analyzed Clb2 modification in response to *CDC5* overexpression in wild-type and *cdc15-as1* mutant cells, the latter allowing to alleviate the contribution of MEN, if any, in the process. Wild-type, *GAL-CDC5* and *GAL-CDC5 cdc15-as1* cells, were arrested in G1 by α-factor and synchronously released from the block in a medium containing HU and 1NM-PP1, to arrest cells in S phase and to inactivate the MEN, respectively. When the arrest was complete, as judged by more than 95 percent of the cells having a bud, *CDC5* overexpression was induced by adding galactose to the medium. To assess post-translational modifications of Clb2 we analyzed Clb2 electrophoretic mobility on SDS-PAGE. Clb2 was visualized by Western blot analysis with anti-Clb2 antibodies. Our data showed that in wild-type cells Cdc14 was not released from the nucleolus and that the electrophoretic mobility of Clb2 did not change through the course of the experiment, excluding that this modification of Clb2 was simply accumulating as a result of a prolonged arrest in S phase, Fig. 3.22a. On the contrary, overexpression of *CDC5* in wild-type cells induced a modification on Clb2 concomitantly with the release of Cdc14 from the nucleolus both in the presence or absence of MEN activity, 3.22b-c. These observations suggest that Cdc5 induces in a MEN-independent manner, directly or indirectly, a post-translational modification on Clb2, which might be relevant for the role that Clb2 plays in Cdc14 release.

High levels of Cdc5 induce the ectopic phosphorylation of Clb2

Having established that Cdc5 induces a post-translational modification of Clb2, we characterized the nature of this modification. Because Cdc5 is a kinase, the most obvious hypothesis was the modification of Clb2 to be phosphorylation. *GAL-CDC5* cells were arrested in S phase by treatment with HU. When the arrest was complete, *CDC5* overexpression was induced. Clb2 was immunoprecipitated, using anti-Clb2 antibodies, from a protein extract obtained from *GAL-CDC5* cells after three hours of *CDC5* induction. The immunoprecipitated Clb2 protein was incubated in the presence or absence of the Calf Intestinal Phosphatase (CIP). Clb2 mobility in SDS-PAGE was analyzed by Western blot using anti-Clb2 antibodies. While two differently migrating forms of Clb2 were detectable when the protein was not treated with the CIP, only the faster migrating form was detectable when the protein was incubated with the phosphatase Fig. 3.22d. This finding indicated that high levels of Cdc5 are capable of inducing, directly or indirectly, Clb2 phosphorylation.

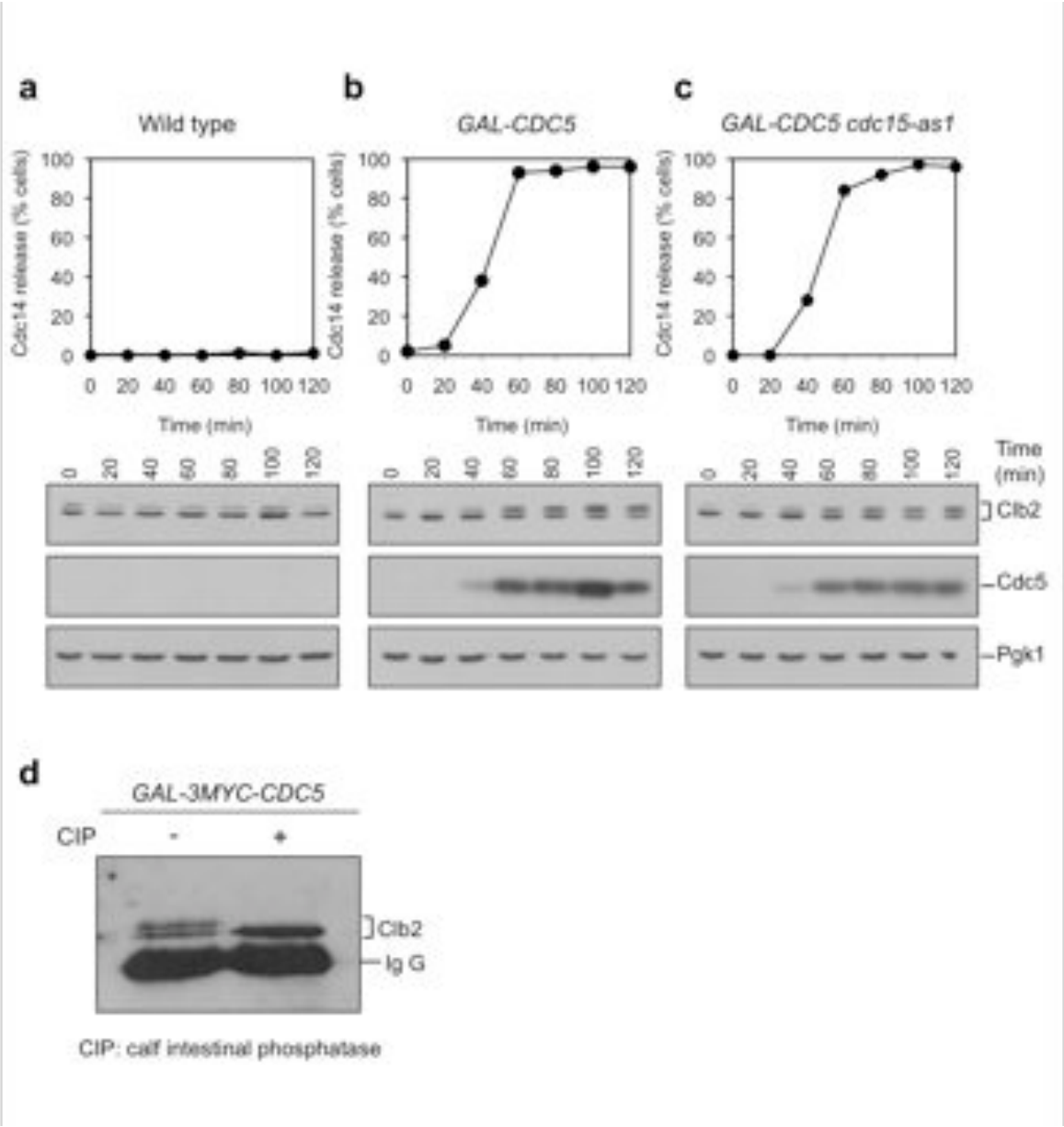

Figure 3.22 Overexpression of Cdc5 induces the phosphorylation of Clb2
(**a**-**c**) Wild-type (Ry394, **a**), *GAL-3MYC-CDC5* (Ry1325, **b**) and *GAL-3MYC-CDC5 cdc15-as1* (Ry1324, **c**) cells carrying a *CDC14-3HA* and a *CFI1-3MYC* fusions were arrested in G1 in YEPR with α-factor pheromone (5μg/mL) at 23°C. When the arrest was complete, cells were released into YEPR lacking pheromone but containing HU (10mg/mL) and 1NM-PP1 (5μM) to arrest them in S phase and to inhibit Cdc15-as1, respectively. When the arrest was complete, galactose was added to induce the expression of Cdc5. Samples were taken at the indicated times to determine the percentage of cells with Cdc14 released from the nucleolus (top) and Clb2 and Cdc5 protein levels and mobility in SDS-PAGE (bottom). Pgk1 was used as an internal loading control in Western blot analysis. (**d**) *GAL-3MYC-CDC5* (Ry1325) cells were arrested in S phase as described in (**a**). Two and a half hours after galactose addition, a sample was taken to perform Clb2 immunoprecipitation. A phosphatase assay with the calf intestinal phosphatase (CIP) was performed on the immunoprecipitated Clb2 protein. Western blot analysis of Clb2 protein level and mobility in SDS-PAGE is shown.

Clb2 is phosphorylated during early anaphase in wild-type cells

To examine whether the phosphorylation of Clb2 by Cdc5 is relevant for Clb2 function rather than being an artefact due to overexpression of *CDC5*, we analyzed the levels and pattern of Clb2 during an unperturbed cell cycle. If phosphorylated Clb2 is the active form of the protein within the FEAR network, a clear prediction can be made. Indeed we predict that the phosphorylated Clb2 will appear concomitantly with the release of Cdc14 during early anaphase and will disappear as cells progress through anaphase. Wild-type cells were arrested in G1 by treatment with α-factor and then synchronously released into the next cycle. The synchrony of the cell population was assessed by monitoring the kinetics of mitotic spindle formation. Cdc14 release from the nucleolus was analyzed by indirect IF. To examine Clb2 phosphorylation we performed conventional SDS-PAGE and Phos-tag SDS-PAGE, followed by Western blot analysis using anti-Clb2 antibodies. We found that, as cells entered in anaphase, Cdc14 was released from the nucleolus and remained in the released state until the mitotic spindle was disassembled. Clb2 levels increased as cells progressed through the cell cycle, reached a peak during metaphase and the protein was degraded as cells exited from mitosis, Fig. 3.23a. Of note, a hyperphosphorylated form of Clb2 accumulated at anaphase onset (70 minute time point) concomitant with the early release of Cdc14 from the nucleolus and disappeared as cells progressed through anaphase (90 minute time point), Fig. 3.23a. This result shows that a correlation exists between Clb2 phosphorylation and the early release of Cdc14 mediated by the FEAR network, supporting the possibility that Clb2 phosphorylation is mechanistically involved in promoting Cdc14 release during early anaphase.

In a *cdc5* FEAR-defective mutant Clb2 is not hyperphosphorylated during early anaphase

To assess if this phosphorylation of Clb2 is in any way important for Clb2 function within the FEAR network, we analyzed progression trough the cell cycle and the Clb2 phosphorylation state in a specific mutant of *CDC5*, *cdc5-ad1* (adaptation defective). Besides their most defect in escaping from a checkpoint-mediated cell cycle arrest in the presence of irreparable double-strand breaks (a process called "adaptation"; (Toczyski et al., 1997), *cdc5-ad1* cells have recently also display defects in the release of Cdc14 during early anaphase under unperturbed conditions (Jin and Wang, 2006; Toczyski et al., 1997). If, as we hypothesize, Cdc5 is required for the phosphorylation of Clb2 and if phosphorylated Clb2 is the FEAR network effector, we expect a lack of Clb2 hyperphosphorylation during early anaphase in the FEAR-defective *cdc5-ad1* mutant. To assess this hypothesis, wild-type and *cdc5-ad1* cells were arrested in G1 with α-factor and then synchronously released into the next cell cycle. We monitored cell cycle progression by analyzing via FACS the kinetics of DNA replication and via indirect IF the kinetics of mitotic spindle formation. The pattern of Clb2 mobility was analyzed by performing conventional SDS-PAGE and Phos-tag SDS-PAGE followed by Western blot analysis with anti-Clb2 antibodies. We found that wild-type and *cdc5-ad1* cells entered and progressed through the cell cycle up to the first stages of mitosis with similar kinetics but, in agreement with (Jin and Wang, 2006), *cdc5-ad1* cells did not release Cdc14 from the nucleolus during early anaphase as is typical for FEAR network mutants. Fig.3.23b. Consistent with their defect in releasing Cdc14 during early anaphase, *cdc5-ad1* cells displayed a 10 minute delay in exiting from mitosis compared to wild-type cells. Both in wild-type and *cdc5-ad1* cells, Clb2 levels increased during progression through S phase up to metaphase whereas Clb2 was degraded as cells entered into the next cell cycle Fig. 3.23b. In agreement with our hypothesis we found that, while in wild-type cells a

hyperphosphorylated form of Clb2 accumulated at anaphase onset concomitant with the release of Cdc14 during early anaphase (100 minute time point), in *cdc5-ad1* cells this hyperphosphorylated form of Clb2 did not accumulate Fig.3.23b. This observation suggests that the Cdc5-dependent phosphorylation of Clb2 may be relevant for the function of Clb2 in promoting the release of Cdc14 from the nucleolus during early anaphase. This set of experiments were performed in collaboration with Guendalina Mimun another graduate student in the lab.

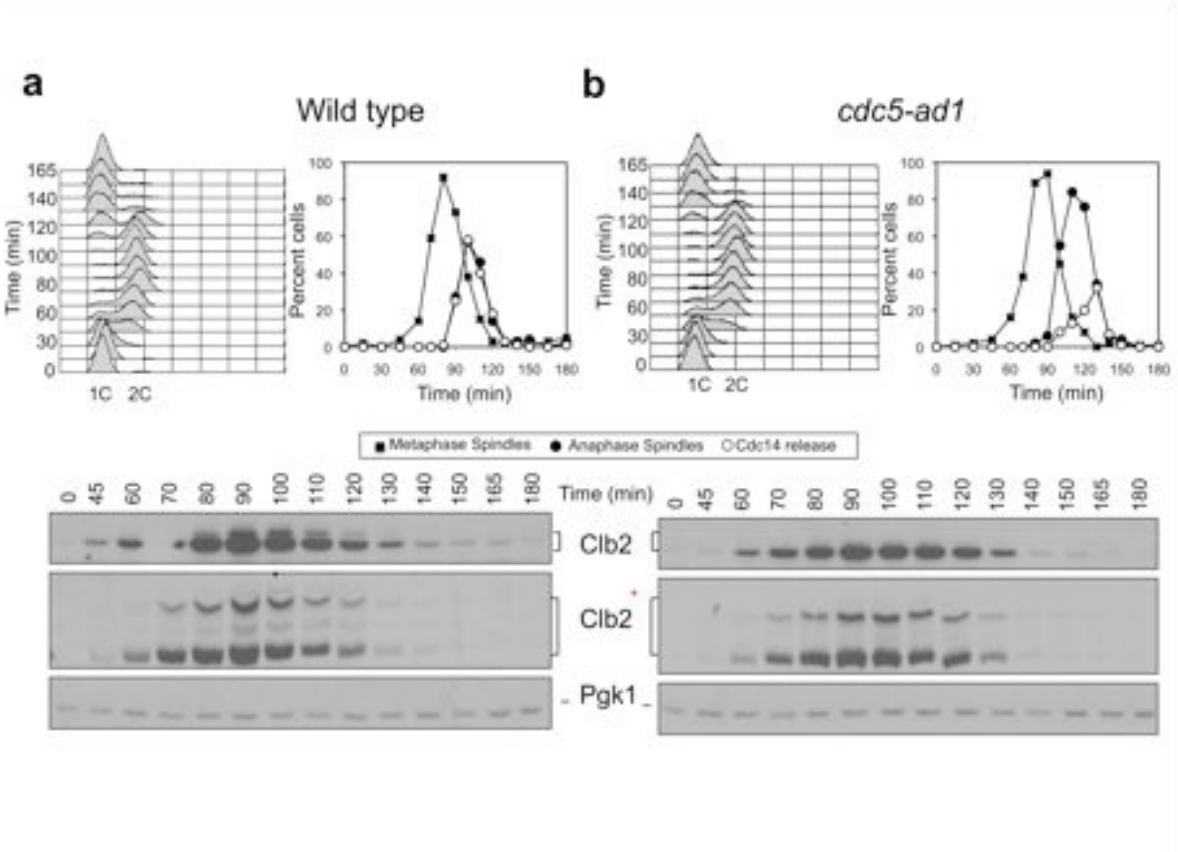

Figure 3.23 Clb2 is not phosphorylated during early anaphase in *cdc5-ad1* cells

(**a**, **b**) Wild-type (Ry1, **a**) and *cdc5-ad1* (Ry1510, **b**) cells were arrested in G1 in YEPD with α-factor pheromone (5 μg/mL) and next released in fresh YEPD lacking pheromone. α-factor pheromone was readded 90 min after release to prevent entry into a subsequent cell cycle. In each panel (a and b): the graph on the left shows the DNA content of the cell population measured by FACS analysis; the graph on the right shows the percentages of cells with metaphase spindles (closed squares), anaphase spindles (closed circles) and Cdc14 released from the nucleolus (open circles); Western blot analysis shows Clb2 protein levels and mobility in SDS-PAGE and in Phos-tag SDS-PAGE (*) at the indicated times. Pgk1 served as an internal loading control in Western blot analysis. * indicates hyperphosphorylated Clb2.

How is the post-translational modification affecting the Clb2-Cdk complex?

To tackle the latter question we analyzed the consequences of these phosphorylation events on Clb2-Cdk activity. Clb2 activity was assessed using histone H1 as a substrate. We found that high levels of Cdc5 result in a decrease in Clb2-Cdk activity, Fig. 3.24a compare to Fig. 3.24b. This result is in agreement with our observation that Cdc5 overexpression results in a general decrease of total levels of Clb2. Although overexpression of Cdc5 induces Clb-Cdk inactivation, we cannot exclude that in physiological conditions the Cdc5 kinase activity may contribute to Clb2-Cdk activity. To test this possibility we compared the kinetics of Clb2 activity between a wild type and a loss of function allele of *CDC5*. Wild type and *cdc5-as1* cells were synchronously released from a G1 arrest into media containing the Cdc5 inhibitor. Clb2 kinase activity was assessed over histone H1. Our data indicate that Cdc5 activity is not required to modulate Clb2 kinase activity, Fig 3.25b compare to 3.25a.

The hypothesis that we favor to explain these apparently conflicting observations is that Cdc5-mediated phosphorylation of Clb2, although necessary for Clb2 role within the FEAR network, likely by causing a relocation of the kinase, ultimately leads to its inactivation. We speculate that this may be the Clb2 pool degraded at the metaphase to anaphase transition. It will bw interesting to see if Cdc5 induces re-localization of Clb2, possibly in the nucleolus where it can reach its substrates Cfi1.

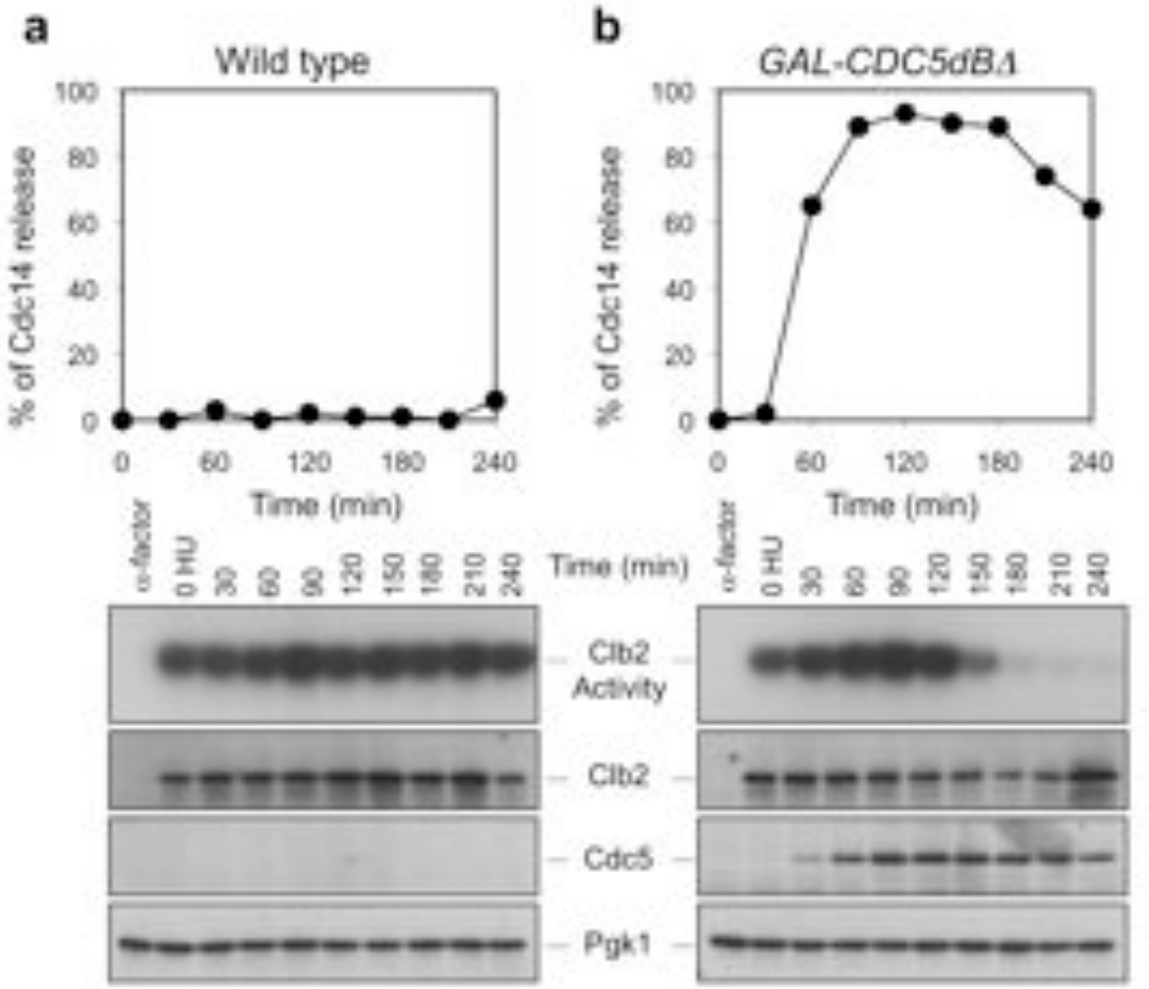

Figure 3.24 Cdc5 does not modulate Clb2 activity
(**a**, **b**) Wild type (Ry2020, **a**) and *GAL-CDC5dBΔ* (Ry 2016, **b**) cells carrying a HA tagged version of Clb2, were arrested in G1 with α-factor in YEPR (5 μg/mL) at 23°C. When the arrest was complete, cells were released into YEPR lacking pheromone but containing HU (10 mg/mL). When cells had arrested in S phase, 2% galactose was added to induce the expression of Cdc5. Samples were taken at the indicated times to determine the percentage of cells with Cdc14 released from the nucleolus (top) and to perform Clb2 immunoprecipitation. A kinase assay was performed on the immunoprecipitated Clb2 protein. Western blot analysis of Clb2 and Cdc5 protein level and mobility in SDS-PAGE is shown. Pgk1 was used as an internal loading control in Western blot analysis.

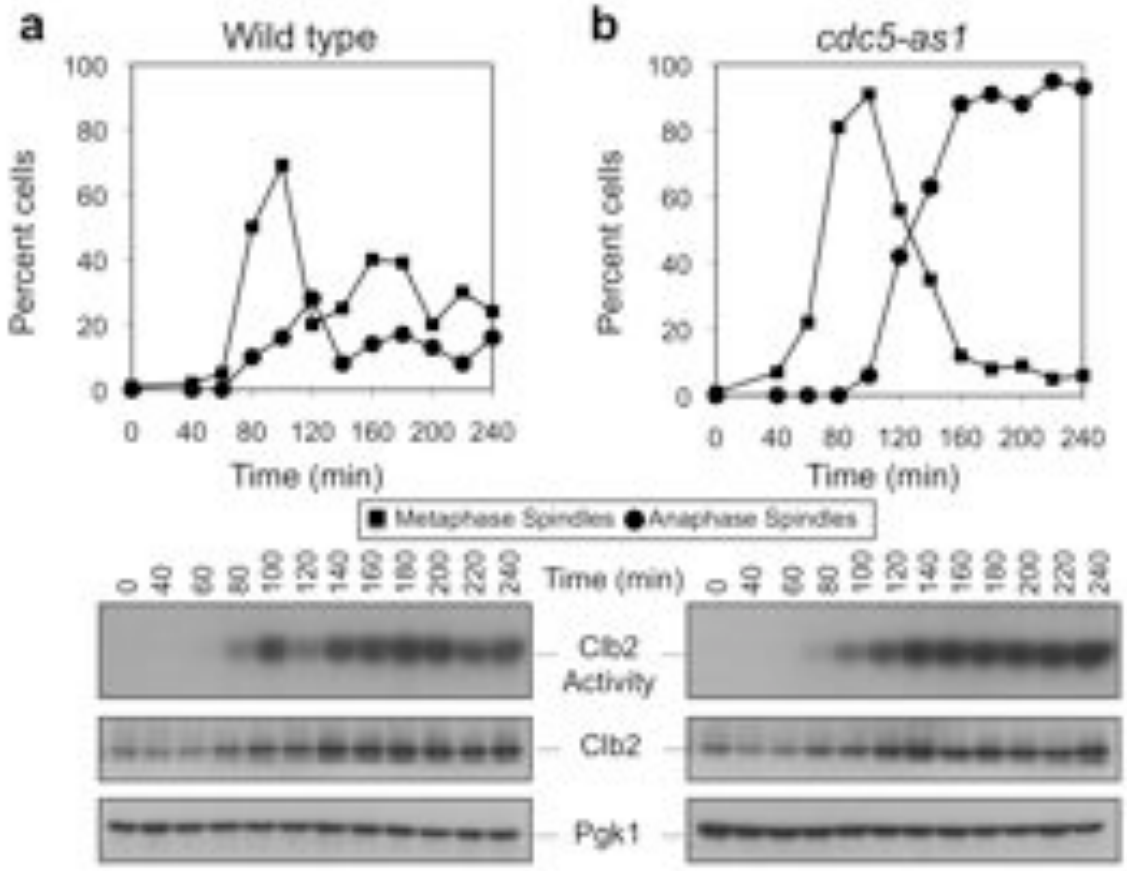

Figure 3.25 Clb2 activity does not require active Cdc5
(**a**, **b**) Wild type (Ry1, **a**) and *cdc5-as1* (Ry 2446, **b**) cells were arrested in G1 with α-factor in YEPR (5 μg/mL) at 23°C and released into fresh media lacking the pheromone but supplemented with 10 μM of the *cdc5-as1* inhibitor (CMK) (Snead et al., 2007). The percentage of cells with metaphase spindles (closed squares) and anaphase spindles (closed circles) was determined at the indicated times. Immunoprecipitation and kinase assay was performed on Clb2 protein. Western blot analysis of Clb2 and Cdc5 protein level and mobility in SDS-PAGE is shown. Pgk1 was used as an internal loading control in Western blot analysis.

Cdc5 is not required to modulate Dbf2 activity

Overexpression of the *GAL-CDC15(1-750*) allele in S phase was reported (Bardin et al., 2003) to induce ectopic activation of kinase Dbf2. We wished to determine whether this was true also in cells arrested in G1. To this aim *GAL-CDC15(1-750*) cells carrying a *DBF2-3Myc* fusion were arrested in G1 and Dbf2 activity was assessed on histone H1 as substrate. Our data show that also in G1 arrested cells overexpression of hyperactive Cdc15 resulted in the ectopic activation of Dbf2, Fig 3.26a. To further investigate the contribution of Cdc5 alone or in combination with Cdc15 to this process *GAL-CDC5dBΔ-3HA* and *GAL-CDC5dBΔ-3HA GAL-CDC15(1-750*) cells both carrying a *DBF2-3Myc* fusion were tested for Dbf2 activity, Fig. 3.26b, c. Our results suggest that Cdc5 does not contribute to Dbf2 activity, nor alone nor in combination with Cdc15 Fig. 3.26b, c. Similarly in S-phase arrested cells the ability of the *GAL-CDC15(1-750)* does not require Cdc5, Fig. 3.27a, b. However the ability of Cdc5 and Cdc15 to ectopically release Cdc14 requires active Dbf2 as indicated by the observation that adding a loss of function allele of *DBF2* to the *GAL-CDC5dBΔ-3HA GAL-CDC15(1-750*) cells, results in the inability of these constructs to induce the ectopic release of the phosphatase, Fig 3.27c, d.

Taken together, our results exclude a role for Cdc5 in modulating its partner kinases. It will be interesting to see if Cdc5 induces a re-localization of Dbf2.

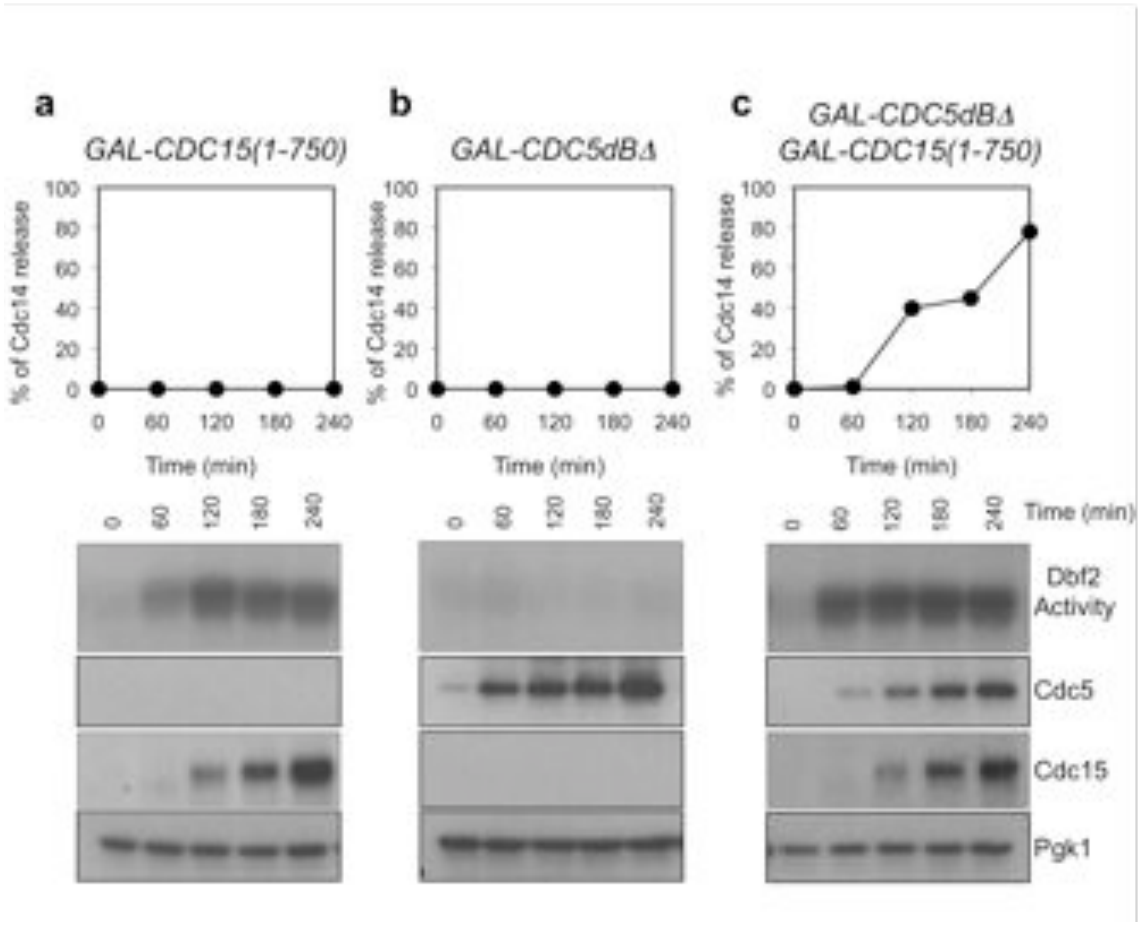

Figure 3.26 Cdc5 does not affect Dbf2 activity
(**a**-**c**) *GAL-CDC15(1-750)* (Ry2254, **a**), *GAL-CDC5dBΔ* (Ry2257, **b**) and *GAL-CDC5dBΔ GAL-CDC15(1-750)* (Ry 2248, **c**) with a Myc-tagged version of Dbf2 cells were arrested in G1 with α-factor (5 μg/ml) in YEPR at 23°C. When more than 90% of cells were unbudded, 2% galactose was added to induce the expression of Cdc5. To maintain the G1 block, α-factor pheromone (2.5 μg/ml) was re-added to the media every 90 min after galactose addition. Samples were taken at the indicated times to determine the percentage of cells with Cdc14 released. Immunoprecipitation and kinase assay was performed on the Dbf2-3Myc protein. Western blot analysis of Cdc5 and Cdc15 protein level and mobility in SDS-PAGE is shown. Pgk1 was used as an internal loading control in Western blot analysis.

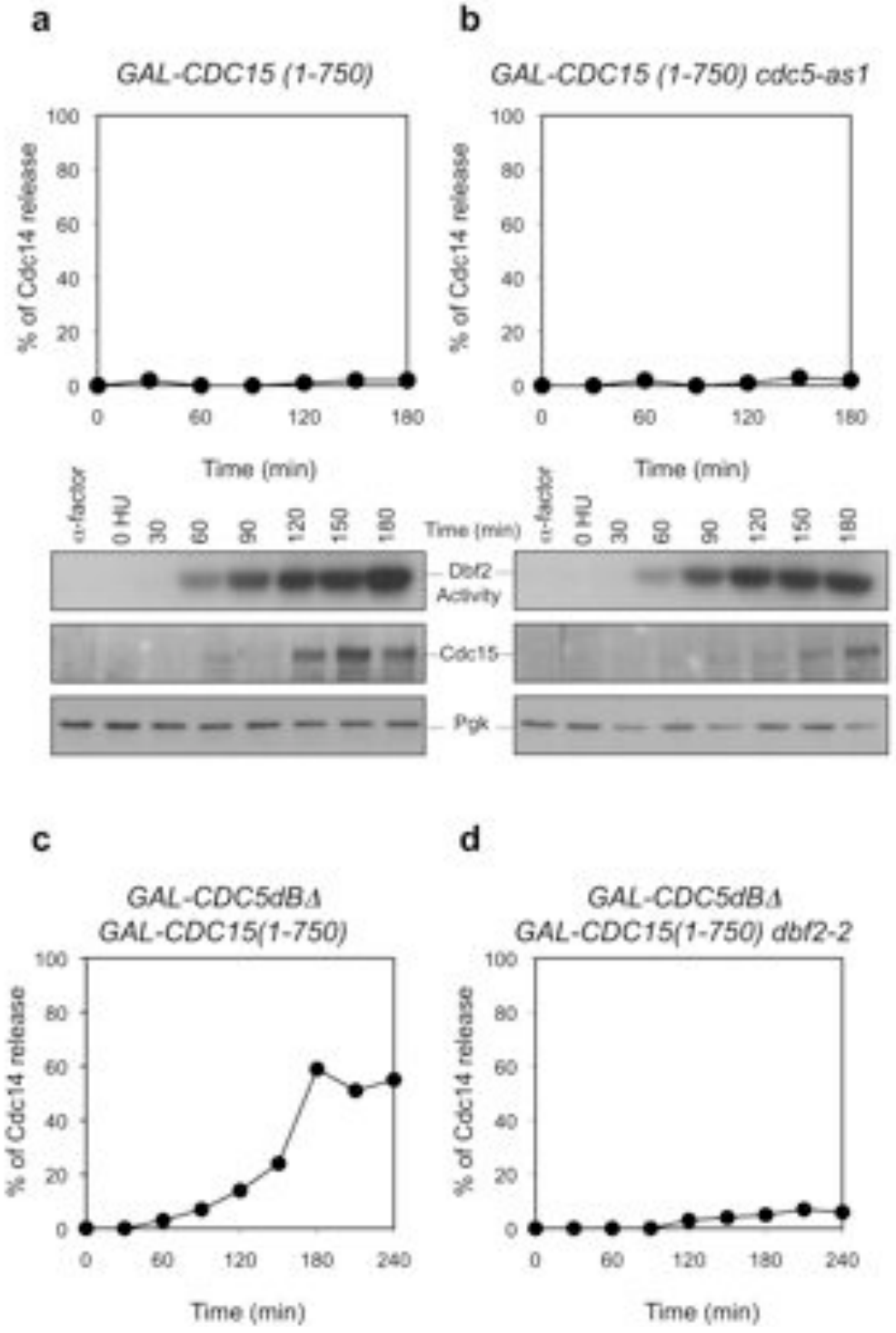

Figure 3.27 *GAL-CDC15(1-750)* does not require Cdc5 activity to mediate Dbf2 activation
(**a**, **b**) *GAL-CDC15(1-750)* (Ry2254, **a**) and *GAL-CDC15(1-750) cdc5-as1* (Ry3539, **b**) cells were arrested in G1 in YEPR with α-factor pheromone (5μg/ml) at 23°C. When the arrest was complete, cells were released into YEPR lacking pheromone but containing HU (10mg/mL) to arrest them in S phase. Once cells were arrested 2% galactose was added to induce Cdc15 overexpression and 10 μM of the *cdc5-as1* inhibitor (CMK) (Snead et al., 2007) was supplemented to inhibit Cdc5. Samples were taken at the indicated times to determine the percentage of cells with Cdc14 released. Immunoprecipitation and kinase assay was performed on the Dbf2-3Myc protein. Western blot analysis of Cdc15 protein level and mobility in SDS-PAGE is shown. Pgk1 was used as an internal loading control in Western blot analysis. (**c**, **d**) *GAL-CDC5dBΔ GAL-CDC15(1-750)* (Ry2248, c) and *GAL-CDC5dBΔ GAL-CDC15(1-750) dbf2-2* (Ry3536, **d**) cells were pre-synchronized in G1 in YEPR with α-factor pheromone (5μg/mL) at 23°C. and then released into YEPR lacking pheromone but containing HU (10mg/mL) to arrest them in S phase. When the arrest was complete, cells were shifted at the restrictive temperature to inactivate Dbf2. Samples were analyzed as in (**a**).

4. Discussion and future plans

The studies presented in this thesis aim to characterize the molecular mechanisms that regulate the interaction between Cdc14, a key regulator of mitotic exit, and its inhibitor Cfi1. The activity of Cdc14 is controlled by changes in its cellular localization (Shou et al., 1999; Traverso et al., 2001; Visintin et al., 1999). From G1 up to metaphase, Cfi1 sequesters Cdc14 in the nucleolus. During nuclear division Cdc14 is released from Cfi1 and first spreads into the nucleus, subsequently into the cytoplasm (Jin et al., 2008; Shou et al., 1999; Visintin et al., 1999). This sequential release (and concomitant activation) of Cdc14 is mediated by the consecutive activation of two signalling cascades: the Cdc-Fourteen Early Anaphase Release (FEAR) network and the Mitotic Exit Network (Pereira et al., 2002; Shou et al., 1999; Stegmeier et al., 2002; Visintin et al., 1999; Yoshida and Toh-e, 2002). FEAR and MEN ensure a timely correct execution of mitotic events (Shou et al., 1999). Components of both cascades include various kinases suggesting that cell cycle-regulated phosphorylation may mediate the interaction between Cdc14 and Cfi1. A number of observations support this hypothesis. *In vitro* studies suggest that phosphorylation is responsible for the dissociation of Cdc14 from its inhibitor Cfi1 (Shou et al., 2002; Shou et al., 1999). In addition, *in vivo* phosphorylation of Cfi1 and possibly also of Cdc14 correlates with the release of the latter from the nucleolus (Shou et al., 2002; Shou et al., 1999; Visintin et al., 2003; Yoshida and Toh-e, 2002).

In this study we aimed to identify the minimal kinase requirement for induction of the ectopic release of Cdc14 from the nucleolus with the goal of elucidating the molecular mechanisms controlling the Cdc14/Cfi1 interaction.

Cdc14 release from its inhibitor requires the power of two kinases

A role for Clb-Cdks, Cdc5 and the MEN effector kinase Dbf2 in the release of Cdc14 has already been proposed (Azzam et al., 2004; Geymonat et al., 2003; Hu and Elledge, 2002; Hu et al., 2001; Mohl et al., 2009; Pereira et al., 2002; Queralt et al., 2006; Stegmeier et al., 2002; Visintin et al., 2003; Yoshida and Toh-e, 2002). However, the molecular mechanism via which Cdc14 is released remains unknown. To tackle this issue we analyzed the consequences of modulating the above-mentioned kinases alone or in mutual combination in the G1 and S phase of the cell cycle. In both phases, the kinases of interest are either absent or just start to accumulate. In addition, there is no contribution of other mitotic regulators, and Cdc14 is sequestered in the nucleolus. Our results showed that the release of Cdc14 requires the activity of two kinases, Cdc5 plus the Clb-Cdk complexes or the MEN effector kinase Dbf2. Interestingly, MEN and Clb2-Cdks appeared to have partially overlapping roles, up to the point that Clb2-Cdk can replace MEN activity if stably expressed at high levels. MEN, however, remained essential if Clb2-Cdk levels were low. Indeed, our data suggest that the S-phase cyclin Clb5, other than Clb2, is essential for the early anaphase release of Cdc14 in this setting and in physiological conditions. The involvement of Clb5 in Cdc14 release is at odds with the well-known role of Clb5 as an anaphase inhibitor. More studies will be required to elucidate the role of this cyclin during mitotic exit. We speculate that elucidating Clb5 function within the FEAR network will improve our understanding of how cells coordinate DNA replication with mitotic exit.

Molecular interpretation of the “two-hit” model

The molecular significance of this double-kinase requirement is still unknown, but different scenarios can be envisioned. The observation that the activity of Cdc5 is always

required for Cdc14 release suggests an attractive hypothesis. Polo-like kinases (Plks), of which Cdc5 is the unique member in budding yeast, possess a conserved C-terminal polo-box domain (PBD) (Elia et al., 2003a; Lee and Erikson, 1997; Lee et al., 1998). The PBD interacts with Plk substrates or docking proteins after they have undergone phosphopriming by another kinase (Elia et al., 2003b). The binding of Plk to these proteins allows Plk-dependent phosphorylation events to occur. It is therefore possible that priming by Clb-Cdk and Dbf2 is required to build up Cdc5 activity. Phosphorylation of Cdc14 and/or Cfi1 by Cdc5 could then promote the dissociation of Cdc14 from Cfi1. Supporting this hypothesis is the observation that cells carrying an allele of *CFI1* encoding for a version of the protein in which six Clb2-Cdk-dependent phospho-residues have been mutated to alanine (*cfi1-6Cdk*), a non-phosphorylatable amino acid, display defects in the release of Cdc14 during early anaphase (Azzam et al., 2004). Remarkably, one of these residues (Thr 212) is part of an optimal phospho-binding motif recognized by PBD (Elia et al., 2003b). An alternative model foresees that Cdc5 and Clb-Cdks or Dbf2 have parallel functions in phosphorylating Cfi1 and/or Cdc14, (Azzam et al., 2004; Mohl et al., 2009; Visintin et al., 2003; Yoshida and Toh-e, 2002). Finally, the MEN kinase Dbf2 and Clb-Cdk complexes could be required for Cdc5 activation and/or *vice versa*. Supporting the latter idea is the observation that activation of Cdc5 requires Cdc28-mediated phosphorylation of a conserved threonine residue within its T-loop, (Thr 242). Cdc28 (Mortensen et al., 2005) is the only Cdk in budding yeast. As we said the fact that Cdc5, Clb-Cdks and MEN kinases are known to be interdependent, renders likely the possibility that multiple mechanisms are involved.

Phosphorylation data from our laboratory together with reports in the literature (Azzam et al., 2004; Visintin et al., 2003) suggest that one important function of the kinases that co-operate with Cdc5 (Clb-Cdks or the MEN) is to phosphorylate Cfi1. Azzam and colleagues showed that Clb2-Cdk is required for Cfi1 phosphorylation during early anaphase while we found that overexpression of *CDC15(1-750)* in both G1 and S phase-

arrested cells resulted in the ectopic phosphorylation of Cfi1. Our data also support the idea that Cfi1 phosphorylation serves as a priming step to build up in Cdc5 activity. Indeed, when expressed in G1, where neither Clb-Cdks nor MEN kinases are active, Cdc5 not only was incapable of triggering the ectopic release of Cdc14, but also did not induce the phosphorylation of Cdc14 and/or Cfi1. However, the simultaneous expression of *CDC5* and *CDC15(1-750)* re-established the ectopic release of Cdc14 and phopshorylation of both Cdc14 and Cfi1. The observation that the pattern of Cfi1 phosphorylation in cells expressing only *CDC15(1-750)* or both *CDC5* and *CDC15(1-750)* is similar, allows us to speculate that priming events serve ultimately to mediate Cdc14 phosphorylation by Cdc5. In support of this "priming" model we identified (unpublished data) by mass spectrometry a residue in Cfi1 (S259) that lies in a putative Dbf2 recognition sequence (Mah et al., 2005), that is specifically phosphorylated in anaphase and is part of a minimal PBD-phosphobinding motif (Elia et al., 2003b). To test the "priming model" we will next examine the consequences on the phosphorylation and release of Cdc14, of mutating this residue into alanine, an amino acid that can no longer be phosphorylated, and into aspartic acid, which mimics phosphorylation. In addition, putative Dbf2-phosphorylation sites within Cfi1, and putative Cdc5-dependent phosphorylation sites within Cdc14, identified by sequence analysis (PHOSIDA) will be mutated. The analysis of the phenotype of cells conditionally expressing these mutant proteins will provide additional insights into the relevance of the priming model. Clear predictions as to the expected phenotypes can be made. For instance, if Cfi1 phosphorylation functions exclusively as a mediator of Cdc5-dependent phosphorylation, we expect that cells expressing a mutant variant of Cdc14 whose putative Cdc5 phosphorylation sites were mutated into aspartic acid, to have Cdc14 released from the nucleolus during the entire cell cycle. Interestingly, cells carrying a mutant allele of *CDC14* encoding for a form of the protein where six putative Cdc5-dependent phosphorylation sites have been mutated into aspartic acid, exhibit an altered kinetic of Cdc14 release. This phosphatase was released for most of the cell cycle. *Vice*

versa, a mutant allele of *CDC14* where the same sites were mutated into alanine is unviable (unpublished data). We are currently testing the localization of these mutant Cdc14 proteins in a yeast strain carrying the *cdc14-1* temperature-sensitive mutation. Our results, besides supporting the "priming model", are also consistent with Clb-Cdks and the MEN kinase Dbf2 being required to directly activate Cdc5. Supporting this hypothesis is the observation that Cdc5 activation requires the phosphorylation of a conserved threonine residue by Cdc28 (Mortensen et al., 2005). Although we cannot exclude that this extra layer of regulation is involved in the process, the observation that cells expressing a mutant form of Cfi1 lacking the 6 Cdk-dependent phosphorylation sites are defective in releasing Cdc14 from the nucleolus during early anaphase in presence of both Clb2-Cdk and Cdc5, argues that Cfi1 phosphorylation by Clb-Cdks is essential to mediate Cdc14 release. It will be interesting to see if in this mutant the phosphorylation of Cdc14 is delayed as well. The contribution of the Cdc5 "partner" kinases to Cdc5 activation will be taken into consideration as well. Although our preliminary data seem to exclude that the three kinases modulate each other's activities. Nevertheless, we expect that the finalization of our analysis will finally unveil the molecular mechanism underlying the dissociation of Cdc14 from its inhibitor.

Clb2 is phosphorylated in a Cdc5-dependent manner

The Clb2-Cdk mitotic complex has been proposed to phosphorylate Cfi1 during the early stages of anaphase, thereby triggering the initial release of Cdc14 from the nucleolus (Azzam et al., 2004). Our studies envision for the Clb2-Cdk complex within the FEAR network the same role played by the MEN kinase Dbf2 during the later stages of mitosis; that is to promote the phosphorylation of Cfi1. This result is in agreement with (Azzam et al., 2004). Indeed, the observation that (1) the ectopic release of Cdc14 in S phase-arrested cells brought about by high levels of Cdc5 was MEN-independent (Visintin et al., 2008)

but relied on the presence of Clb-Cdk complexes, (2) requirement for Clb-Cdk activity is dispensable if MEN is ectopically activated (Manzoni et al., 2010) and that (3) the ectopic activation of MEN triggers the ectopic phosphorylation of Cfi1 (Visintin et al., 2003), support the notion that the Clb2-Cdk complex in the FEAR network is required for phosphorylating Cfi1. If correct, one would expect that similarly to the hyperactivation of MEN, overexpression of *CLB2* would result in the ectopic phosphorylation of Cfi1. So far, our result did not confirm this prediction (data not shown). Although we can not exclude that we were not able to detect phosphorylated Cfi1 due to technical limitations of the procedure used to analyze the phospho-state of the protein, this observation raises alternative possibilities. First Clb-Cdk complexes other than Clb2-Cdk promote the phosphorylation of Cfi1 as members of the FEAR network; in agreement with this possibility, Clb5 has been hypothesized to be a novel FEAR network component (Manzoni et al., 2010). Second, Clb2 needs to be regulated, by post-translational modification/s and/or interactions with specific partners, to promote Cfi1 phophorylation and Cdc14 release from the nucleolus. The existence of an additional activation step required for Clb2-mediated function within the FEAR network is appealing because it could explain why, differently than all other FEAR network components, high levels of Clb2 are not able to induce Cdc14 release from the nucleolus when cells are arrested in metaphase using nocodazole (data not shown). This result is in conflict with what is described by Azzam and collaborators who showed that the ectopic expression of *CLB2* induces the ectopic release of the phosphatase from the nucleolus in metaphase (Azzam et al., 2004). The reasons for this discrepancy are currently unclear, but we suspect they are due to the different experimental procedures employed. While Clb2-Cdk-dependent phosphorylation of Cfi1 is essential for the release of Cdc14 in early anaphase (Azzam et al., 2004), our findings with *GAL-CLB2dBΔ* cells suggests that high levels of Clb2 are not sufficient to induce this event. During the course of our experiments we noticed that overexpressing *CDC5* induced an electrophoretic mobility shift of the Clb2 protein, which we later found

to be due to phosphorylation. This Cdc5-mediated phosphorylation of Clb2 did not require MEN activity. Interestingly, the appearance of phospho-Clb2 correlated with the kinetics of Cdc14 release from the nucleolus and the appearance of phospho-Cdc14 and phospho-Cfi1. These observations made us wonder whether this Cdc5-mediated phosphorylation of Clb2 was relevant for the role of the Clb2-Cdk complex within the FEAR network. Supporting this idea are the findings that: (1) hyperphosphorylated Clb2 appears concomitantly with the release of Cdc14, during the early stages of anaphase in an unperturbed cell cycle of wild-type cells, (2) Clb2 is not hyperphosphorylated during early anaphase in cells carrying an allele of *CDC5* encoding for a protein defective in its FEAR functions (*cdc5-ad1*), and (3) cells overexpressing a FEAR-defective allele of *CDC5* (*GAL-cdc5-ad1*) in S phase-arrested cells, are not only impaired in promoting the ectopic release of Cdc14 but also in triggering the ectopic phosphorylation of Clb2 (data not shown). Encouraged by these preliminary observations we decided to further investigate the significance of this cell cycle-regulated Clb2 phosphorylation. We searched for putative Cdc5-dependent phosphorylation sites in Clb2. We identified and mutated two Clb2 residues, both into alanine or aspartic acid. Next, we assessed the consequences on cell cycle progression and Cdc14 release of introducing these mutated alleles of *CLB2* as the sole source of Clb2 cyclin in the cell. Our preliminary data showed that a modified Clb2 that cannot be phosphorylated ($Clb2^{S181AS389A}$) is unable to rescue the FEAR defect associated with *clb2Δ* mutant cells. Regardless of the expression of the $Clb2^{S181AS389A}$ protein, cells deleted for *CLB2* remained defective in releasing Cdc14 from the nucleolus. In agreement with our hypothesis, this mutant $Clb2^{S181AS389A}$ is never hyperphosphorylated. These data are consistent with a direct or indirect requirement for Cdc5 activity to phosphorylate Clb2 in one or both of the sites we identified. In contrast, the expression of a phospho-mimicking version of Clb2 ($Clb2^{S181DS389D}$) restored the kinetics of Cdc14 release from the nucleolus and exit from mitosis of a *clb2Δ* mutant as observed in wild-type cells. The observation that this "hyperactive" Clb2 does not trigger

the release of Cdc14 in phases other than anaphase is consistent with the possibility that besides triggering Clb2-phospharylation, an additional role for Cdc5, such as phosphorylating Cdc14, exists. It will be important to determine whether this "hyperactive" Clb2 can ectopically phosphorylate Cfi1.

To further assess the relevance of the Cdc5-mediated phosphorylation of Clb2 on these sites we will: (1) analyze the phosphorylation pattern of the non-phosphorylatable $Clb2^{S181AS389A}$ following overexpression of *CDC5* in S phase-arrested cells. Given the phenotype exhibited by the mutant, we expect that overexpressing *CDC5* will not induce an ectopic phosphorylation of this mutant version of Clb2; (2) analyze by Phos-tag SDS-PAGE the mobility of Clb2 in various genetic combinations; (3) address how this post-translational modification is affecting the Clb2-Cdk complex. To tackle the latter question we will analyze the consequences of these phosphorylation events on the fate of the protein, in terms of localization and substrate specificity. In particular, we will consider the hypothesis that phospho-Clb2 may accumulate into a specific cellular compartment. Indeed, phosphorylation of cyclin B1 (the human ortholog of *CLB2*) by Plk1 (the human ortholog of *CDC5*) on S133 (corresponds to S389 in *S.c.*Clb2) has been reported to induce the translocation of the protein from the cytoplasm into the nucleus, thus promoting the G2-M transition of the cell cycle (Yuan et al., 2002). We speculate that, similarly, phospho-Clb2 would migrate into (a) particular cellular compartment(s), for example into the nucleolus, where its target Cfi1 is located. To investigate this possibility we will analyze Clb2 localization on a genome-wide scale by ChIP-on-chip and specifically at the nucleolus by performing ChIP assays and indirect immunofluorescence on spread nuclei, a technique that allows the detection of variations in the concentration of a protein in specific subnuclear locations, before and after *CDC5* overexpression. In parallel we are raising antibodies that recognize specifically phosphorylated Clb2. This will be a powerful tool to investigate the dynamics of a subpopulation of the Clb2-Cdk complex.

The "two-hit" model provides a nice explanation for cell cycle progression

The two-kinase requirement for Cdc14 release explains Cdc14 dynamics in wild-type and mutant cells. At anaphase onset, wild-type cells release Cdc14 by the concerted action of Cdc5 and Clb-Cdk complexes. Clb-Cdk includes both S- and M-phase cyclins of which Clb5 and Clb2 are the predominant species, respectively. Later in the cell cycle, after the APC/C^{Cdc20} triggers the degradation of Clb5 and partially that of Clb2, the decrease in Clb-Cdk activity is compensated by the activation of the MEN. This stepwise mechanism of Cdc14 release ensures a faithful execution of the anaphase program. It also makes sure that either Clb-Cdk or MEN is always active during mitosis (Clb-Cdks before and MEN after) to sustain the release of Cdc14. Therefore in agreement with previous work (Visintin et al., 2008), it must be the degradation of Cdc5 by the APC^{Cdh1} that causes the re-sequestration of Cdc14 at the end of mitosis. Indeed, both *CDC5dBΔ* and *cdh1Δ* strains have a very large fraction of cells with Cdc14 released.

A model for Cdc14 dynamics at the exit from mitosis

We challenged the "two-hit" model by analyzing Cdc14 localization in the presence of non-degradable Clb2, expressed at high and low levels. In the presence of high doses, Clb2-Cdk has a double role: it prevents cells from exiting mitosis and induces the release of Cdc14 (together with MEN, which is nevertheless dispensable in this setting). The latter of clb2-Cdk role is shared with Cdc5 as both are required for the release of Cdc14. When Cdc5 is degraded, as a consequence of Cdc14 release and APC/C^{Cdh1} activation, Clb2-Cdk cannot promote the release of Cdc14 alone. As a consequence, Cdc14 moves back into the nucleolus. Nevertheless, Clb2-Cdk manages to block cells in mitosis, allowing Cdc5 to be resynthesized and to induce a new round of Cdc14 release and sequestration. In this context, Clb2-Cdk likely plays an additional third role, namely the

promotion of *CDC5* transcription. *CDC5*, together with *CLB2* belongs to the "*CLB2* cluster genes", a set of about 30 genes whose expression depends upon Clb2 (Darieva et al., 2003).

When the doses of non-degradable Clb2 are reduced, the presence of MEN becomes essential for full Cdc14 release similarly to a normal cell cycle. As Cdh1 is activated, Cdc5 degradation causes the re-sequestration of Cdc14, followed by MEN-driven cycles of Cdc14 release and sequestration. In presence of both high and low doses of Clb2, the role of Clb5 is likely limited to the initial release of Cdc14, after which it becomes degraded. Its presence is dispensable at high levels of stable Clb2, whereas it becomes essential when Clb2 is stabilized at low doses in the absence of MEN.

We were surprised to observe that APC/C^{Cdh1} was required for the oscillations in the presence of non-degradable Clb2. This result is in contrast to the notion that Clb2-Cdk inhibits APC/C^{Cdh1} (Nasmyth, 1996). Similarly, Clb2-Cdk has been reported to inhibit MEN (reviewed in (Stegmeier and Amon, 2004) and (Konig et al., 2010)). In our system, however, MEN seems to be active even in the presence of high levels of stable Clb2. When we used the *cfi1-6Cdk* mutant, we found that oscillation increased in a MEN-dependent manner in the presence of active Clb2-Cdk arresting cells in mitosis. At present, we cannot give an explanation for these results. Possibly, they point to the fact that the subcellular localization of the kinases and phosphatases, whose delicate balance controls Cdc14 localization, cannot be neglected if we wish to understand the regulatory network that controls exit from mitosis.

Final remarks

The goal of my thesis research was to elucidate the molecular mechanisms that control the activity of Cdc14, a key regulator of mitotic exit in *Saccharomyces cerevisiae*. Although our picture is still incomplete, my work shed new light onto this process. We found that the combination of Cdc5 with either Clb-Cdks or Dbf2 is sufficient to promote Cdc14 release from the nucleolus. Our results provide an explanation of how the interplay of the above mentioned kinases results in the release of Cdc14 at the right time during the cell cycle. Moreover, we found that once fully released, Cdc14 triggers a negative feedback loop that, in the presence of stable levels of mitotic cyclins, triggers periodic cycles of Cdc14 release and sequestration. As similar oscillatory phenotypes have been described for yeast bud formation and centrosome duplication, our observations suggest a paradigm whereby events capable of repeating themselves multiple times are restrained to occur once per cycle by their coupling to the cyclin-Cdk engine.

Why oscillations?

Our results raise the question of the physiological significance of the oscillations in Cdc14 localization. We believe that what we report contributes to the understanding of the principles underlying the coordination between the cell cycle engine (i.e. cyclin-Cdk activities) and the events triggered by the engine itself. The oscillatory behavior reported here is not an isolated example, but resembles other cases of periodical phenotypes that emerge when the cell cycle engine is blocked. In budding yeast, the deletion of Clbs results in a periodical budding in G1 (Haase and Reed, 1999). In both budding yeast and *Drosophila melanogaster*, the deletion or knock down of mitotic cyclins arrests cells before mitosis, but centrosomes nevertheless undergo periodic rounds of divisions (Haase et al., 2001; McCleland and O'Farrell, 2008). Mutants for three major control mechanisms preventing DNA re-replication can be blocked in G2/M with high levels of Clb2-Cdk and

undergo multiple cycles of re-replication (Nguyen et al., 2001). These seemingly different systems share basic properties with themselves and with our system: they are all triggered by the cell-cycle engine and normally occur once per cell cycle. They show periodic behavior when the engine is blocked.

Do these systems share properties at the molecular level? The molecular details of the circuits we have mentioned are not as well known as the circuit we analyzed here. However, their capability to oscillate implies that they all include at least one negative feedback loop. This fits with the need for these circuits to reset to their initial state after one cycle, thereby preparing for the forthcoming cell cycle. However, their capability to oscillate implies much stronger requirements than the simple negative feedback loop: they must have enough non-linearity, sufficient time-delays and proper balancing of the rate constants, all conditions required for oscillations (Novak and Tyson, 2008). All these conditions are not obvious, and indeed the oscillatory behavior in the presence of constant high levels of mitotic kinase came to us as a surprise.

We thus believe that an important question to be asked is why events coupled to the cell cycle engine, that take place only once per cycle, have evolved to an oscillatory regime, normally hidden by their coupling to the cell cycle machinery. The answer to this question is unknown, but surely worth further investigations. Here we propose that an oscillatory dynamic guarantees a resetting of the molecular circuit to its initial conditions less dependent on the Cdk input than a simple negative feedback loop thus avoiding intermediate outcomes of the circuit. In the case of Cdc14, that would be a state with only a fraction of Cdc14 re-sequestered in the nucleolus at the end of mitosis, with the APC/C^{Cdh1} only partially active and with intermediate levels of Clb2. Such a condition would leave cells in a state of unclear physiological condition, partially mitotic and partially G1. The oscillatory dynamics, even if limited to one cycle, makes sure that this is avoided.

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