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Regulation of chromosome segregation by conserved phosphatase Cdc14 and kinase Cdc5

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List of abbreviations

APC/C	Anaphase promoting complex or cyclosome
ATP	Adenosine triphosphate
CAK	CDK-activating kinase
CDK	Cyclin-dependent kinase
CEN	Centromere
CPC	Chromosomal passenger protein
DDC	DNA damage checkpoint
DDR	DNA damage checkpoint response
DNA	Deoxyribonucleic acid
FEAR	Cdc14 early anaphase release (network)
GAP	GTPase-activating protein
GEF	Guanine nucleotide exchange factor
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
KT	Kinetochore
MAP	Microtubules-associated protein
MAPK	Mitogen-activated protein kinase
MCC	Mitotic checkpoint complex
MEN	Mitotic exit network
MT	Microtubule
MTOC	Microtubule-organizing centre
NLS	Nuclear localization signal
NTP	Nucleoside triphosphate
PBD	Polo-box domain
Plk	Polo-like kinase
PTPA	Phosphotyrosyl phosphatase activator
SAC	Spindle assembly checkpoint
SMC	Structural maintenance of chromosomes
SPB	Spindle pole body
SPOC	Spindle position checkpoint
Ub	Ubiquitin

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Materials and methods

Abstract

The faithful transmission of the replicated genome from the mother to the daughter cell requires the correct establishment of linkages between the duplicated chromosomes (sister chromatids) and their bi-orientation on the mitotic spindle. Chromosome segregation initiates only after each sisters pair is correctly aligned onto the microtubules emanating from the spindle poles. Next, Esp1-mediated cleavage of cohesin is required to trigger anaphase onset while the physical segregation of the separated sisters is next driven by spindle activity. However, this scenario appears to be more complicated involving additional factors driving the sister chromatid segregation process (i.e. the Top2-mediated resolution of replication catenates). In budding yeast, anaphase progression and exit from mitosis require the protein phosphatase Cdc14 whose activation relies on two consecutive protein pathways, the FEAR network and the MEN. As the polo-like kinase Cdc5 is a component of both pathways its activity is essential to Cdc14 release and in its absence Cdc14 is never released.

By combining loss-of-function alleles of Cdc5 and Cdc14 we obtained double mutant cells that had cohesin cleaved but still arrested with undivided nuclei and short bipolar spindles. Anaphase spindle elongation initiates quickly after cohesin removal (anaphase A) and then switches to a slower elongation rate (anaphase B) due to changes in spindle behaviour mediated by motor proteins and microtubule-associated enzymes. Although some residual cohesion between sister chromatids seems to contribute to the terminal phenotype of *cdc14 cdc5* cells, our data indicate that anaphase B is the main mitotic defect of these cells.

We conclude that Cdc5 and Cdc14 are redundantly involved in activating spindle activity following cohesion resolution, suggesting the existence of a regulatory network

that coordinates sister chromatid separation with spindle elongation after cohesin cleavage. Importantly, we identified the motor protein Cin8 as a (direct or indirect) target of Cdc5 in the regulation of spindle elongation.

1. Introduction

1.1 The eukaryotic cell cycle

The process by which a eukaryotic cell divides into two identical daughter cells is referred to as mitotic cell cycle. This division cycle involves two sequential events that ensure the correct transmission of the genome from one generation to the next: the duplication of the chromosomes during the S ("synthesis") phase of the cycle and their subsequent segregation into the daughter cells during the M ("mitotic") phase. The S and the M phase are connected by two gap phases allowing cells to prepare for both events: gap phase 1 (G1) between M and S phase, and gap phase 2 (G2) between S and M phase. Together, G1, S and G2 are referred to as interphase, representing the longest part of the cell cycle. G1 is characterized by high levels of protein synthesis resulting in a doubling of the cell size. Later in G1, in response to intra- and extra-cellular signals, the cell decides whether or not it will initiate the cell division process. This deciding moment is defined as the "restriction point" in mammals or START in yeast (Pardee 1974, Hartwell and Unger 1977, Pardee 1989, Blagosklonny and Pardee 2002). Only when the environment is favourable for division, the cell commits to a new round of replication. Otherwise, cell cycle progression will be paused and the cell enters a state of quiescence referred to as G0 phase. As soon as conditions become favourable, the cell will enter G1 and start a new division cycle.

G1 is followed by the S phase, characterized by DNA replication. The original and duplicated chromosomes are associated to each other and hence referred to as sister chromatids. The cohesion between sister chromatids relies on a protein ring complex

named cohesin complex that encircles both chromatids (Michaelis, Ciosk et al. 1997, Uhlmann and Nasmyth 1998, Tanaka, Fuchs et al. 2000, Gruber, Haering et al. 2003). However, in recent years it has become clear that factors like topological linkages and/or catenates may also contribute to the linkage between sisters (Lavoie, Hogan et al. 2004, Pereira and Schiebel 2004, Sullivan, Higuchi et al. 2004, Bermejo, Branzei et al. 2008, Diaz-Martinez, Gimenez-Abian et al. 2008).

Once DNA replication has been completed the cell enters the G2 phase. The pre-mitotic G2 phase represents a period of rapid cell growth and protein synthesis in anticipation of the segregation of the sister chromatids into the two daughter cells, as occurs in M phase. Importantly, multiple systems of surveillance of the cell cycle (referred to as checkpoints) can prevent entry into M phase and arrest cells in G2 in response to incomplete DNA replication, DNA damage, perturbations of cell polarity (in budding yeast) (McMillan, Sia et al. 1998), misaligned sister chromatids and/or spindle positioning problems.

The G2 phase ends with the onset of the M phase, collectively composed of mitosis and cytokinesis. Mitosis is a fast process that equally separates the genetic material giving rise to the two identical nuclei. Cytokinesis refers to the physical separation of the cells and involves the separation of the cytoplasm and cell content in two.

Although mitosis varies a lot between species it can be cytologically divided into serial phases: prophase, prometaphase (not in yeast), metaphase, anaphase and telophase. In prophase the genetic material condenses into discrete pairs of duplicated chromosomes. During prometaphase the nuclear envelope breaks down (only in animal cells) and the cell builds up the mitotic spindle via which the sister chromatids segregate. The spindle consists of tubulin polymers (microtubules, MTs) that extend from two opposite positioned cellular structures named centrosomes (or spindle pole bodies in yeast, SPBs). Concomitantly, the cell assembles on the centromeric region of each

chromosome a protein complex named kinetochore (KT) (>60 proteins in yeast and >150 in metazoans) that attaches the sister chromatids to the spindle MTs. Each sister chromatids pair must be bi-oriented onto the spindle, meaning that the two sisters must be bound from MTs emanating from the opposite centrosomes. At this point of the cell cycle the spindle assembly checkpoint monitors if all sister chromatids are bi-oriented. This checkpoint will arrest the cells in metaphase even if only one unattached chromosomes, or misbound (e.g. mono-oriented) sisters pair is sensed. This delay allows cells to detach the misaligned sister(s) and then bi-orient it (them) to the spindle. Only when each sisters pair is bi-oriented will the spindle checkpoint be satisfied and allow cells to proceed into anaphase. At anaphase onset, cohesin is cleaved resulting in a separation of the sister chromatids. Later in anaphase changes in the spindle dynamics underlie the segregation of the chromatids toward the opposite spindle poles. In telophase, once the sisters have reached the SPBs, the mitotic spindle becomes dismantled, two new nuclear membranes are generated to enclose each set of SPB-associated chromosomes into a separated nucleus (not in yeast) and chromosomes decondense.

Telophase represents the end of mitosis but cytokinesis is the last event required to finish cell division. Cytokinesis leads indeed to the physical separation of cytoplasm, cell content and cell membrane into the two daughter cells, each one containing one exact copy of the genetic information of the mother. This process is generally achieved through a contractile actomyosin ring that pinches and cuts the cell into two but of course additional specie-specific events are essential to complete it.

1.2 *Saccharomyces cerevisiae* as a model system

The cell cycle machinery and the basic mechanisms of cell cycle regulation are highly conserved in the eukaryotic kingdom. The actual knowledge about this complicated process has derived indeed from studying the cell cycle in different organisms. The unicellular budding yeast *Saccharomyces cerevisiae* is a simplified form of eukaryotic organism and its small genome, both haploid (*1n* DNA content) and diploid (*2n* DNA content) proliferation states and "easy" genetic and biochemical manipulations are few of the many advantages associated with using yeast as a model system.

Cell division in budding yeast is asymmetric because proteins, membrane compartments and organelles are asymmetrically distributed between the mother and daughter cell. Consistent with this, the daughter cell is smaller than the mother cell and thus in yeast it is highly important that the daughter cell increases in size before it starts its own cell division cycle (Chant 1999).

The budding yeast cell cycle initates with a single unbudded cell (typical G1 cell). G1 is for the budding yeast the longest and more controlled phase of the cell cycle. It represents the time when yeast integrates intra- and extra-cellular signals to decide its fate. Already in G1, a yeast cell determines the site from which the bud will emerge (Lew and Reed 1993) requiring a specific polarization of the cell before it initiates to divide. The yeast-specific morphogenetic checkpoint at the G1-S transition, delays entry into S phase in case of polarity defects (McMillan, Sia et al. 1998). Proper cell polarity as well as nutrient starvation are not the only limiting factors at the START in yeast. Mating pheromones in the surrounding environment are able to switch a haploid cell from a "proliferation mode" to a "mating mode". In this case, the cell arrests in G1 in preparation to fuse with a second haploid yeast cell of the opposite mating type, resulting in a diploid yeast cell (Kurjan 1993).

Between G1 and S the cell buds. This bud (the future daughter cell) will grow until its final size is obtained at the end of mitosis. The neck between the mother and bud (also named bud-neck) corresponds to the plane of separation of the daughter cell from the mother cell at the end of cell division. The forming mitotic spindle has therefore to be oriented along the mother-bud axis (with one pole in the mother cell and the other pole in the bud) to ensure an equal distribution of the replicated chromosomes between the two cells (Merlini and Piatti 2011).

Another peculiarity of yeast is that in contrast to animal cells, which perform an "open mitosis" (the nuclear envelope breaks down before mitosis and reforms after it), yeasts undergo a "closed mitosis". In yeast the nucleus remains intact until cytokinesis and the process of chromosome segregation and the proteins required for it are present within the intact nucleus.

1.3 Cell cycle regulation

1.3.1 Cyclin dependent kinases (CDKs)

1.3.1.1 CDKs regulation

Every step of the division cycle is the direct consequence of the previous one and is a precondition for the following one. Basic elements orchestrating cell cycle progression are a class of kinases named cyclin-dependent kinases (or CDKs) that drive the different cell cycle phases in a timely and consequential manner.

As indicated by the name, CDKs are regulated through the association of the kinase with a set of regulatory subunits called cyclins. Moreover, cyclin-CDK complexes need to be phosphorylated by specific CDK activating kinases (CAKs) on the Thr160 residue within the CDK T-loop (activating loop) to become active. In higher eukaryotes CAK activity occurs only once cyclin is bound to CDK. In budding yeast, the activity of the CAK named Cak1 does not require the binding between CDK and cyclin (Enke, Kaldis et al. 2000). Additional levels of regulation include the interaction of the cyclin-CDK complexes with specific CDK inhibitors and/or post-translational modifications of both cyclin and kinase subunit. Nevertheless, cyclin binding remains the limiting event in the regulation of CDKs.

The CDK active site is placed in a cleft between two lobes of the protein. Monomeric CDK is completely inactive because its T-loop occludes the cleft entrance where the kinase interacts with its substrates. The ATP-binding site, located depth in the cleft, is also mis-oriented in a monomeric CDK. Cyclin binding induces deep conformational changes in the CDK molecular structure: the repositioning of the T-loop, to remove the occlusion at the cleft entrance, and the remodelling of the ATP-binding site, to facilitate the binding of the ATP and the phospho-transfer reaction (De Bondt, Rosenblatt et al.

1993, Jeffrey, Russo et al. 1995). CAK phosphorylation of the T-loop stabilizes the cyclin-CDK interaction and positively acts on the substrate-binding site (Russo, Jeffrey et al. 1996, Brown, Noble et al. 1999).

Besides contributing to CDK activation, cyclins drive the kinase to its target thereby determining its substrate specificity (Peeper, Parker et al. 1993). On the substrate CDKs phosphorylate serine and threonine residues found within the consensus sequence S/T-P-X-K/R (Songyang Z. 1994, Zhang J. 1994, Srinivasan, Koszelak et al. 1995, Holmes and Solomon 1996). The sequential phosphorylations of proteins required for specific events of the cell cycle dictate the proper cell cycle progression.

Eukaryotic cells usually have several CDKs to regulate the cell cycle but Cdc28 is the sole CDK in budding yeast. Cdc28 determines the proper timing of the various cell cycle phases (DNA replication, entry into mitosis, spindle elongation, chromosome segregation and cytokinesis) by associating with one of the nine phase-specific yeast cyclins (Cln1-3 and Clb1-6). G1 phase cyclins Cln1-3 are required for bud formation and spindle pole body duplication. DNA replication during S phase requires the B-type cyclins Clb5-6. Lastly, B-type cyclins Clb1-4 regulate mitotic events (Bloom and Cross 2007). Conversely, exit from mitosis requires Clb-CDKs inactivation (Zachariae and Nasmyth 1999).

Cellular CDK level is constant through the cell cycle, while the levels of cyclins and other regulators of CDKs (like CDK inhibitors) vary according to the cell cycle phase. The oscillation of these regulatory elements requires a controlled synthesis and well-timed down-regulation of the protein. Indeed, degradation at specific times of key cell cycle regulators is required to allow cell cycle progression. In particular, degradation is usually mediated by two major ubiquitin ligase complexes: the F-box containing complex (SCF complex) and the anaphase promoting complex or cyclosome (APC/C), wich are associated with co-factors that confer substrate specificity.

1.3.1.2 Cyclin-CDK complexes and cell cycle progression

Cln3 is the unique cyclin present in all the cell cycle phases but nevertheless it is mostly detectable in late M/early G1 phase. Similarly, Cln1-2 and Clb5-6 cyclins synthesis peaks in G1 phase. For this reason they are defined as early-expressed cyclins when compared with Clb1-4 cyclins. This specific timing of expression reflects different regulatory mechanisms of transcription (Bloom and Cross 2007).

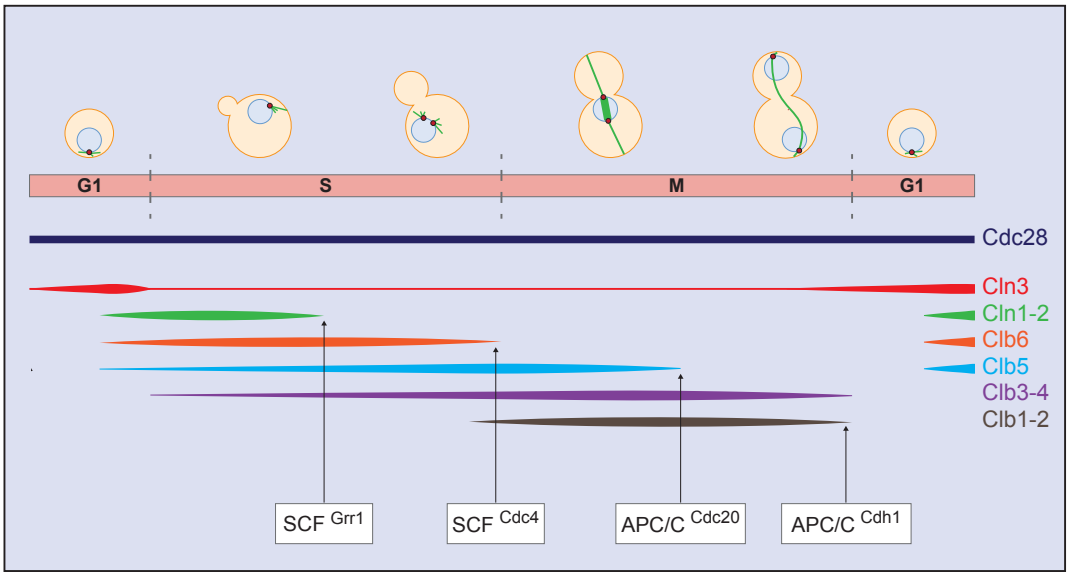

Figure 1.1. Cell cycle regulation of cyclins. CDK activation requires cyclin association. While CDK protein levels are constant, each cyclin exists only in a specific time window throughout the cell cycle. The figure shows how the cyclins encoded in yeast are expressed during the cell cycle as well as the ubiquitin ligase complex responsible for their time-regulated degradation.

The heterodimeric transcription factor SBF (composed of Swi4 and Swi6) and the related MBF transcription factor (composed of Mbp1 and Swi6) overlap in function for the activation of the early-expressed cyclins (Bean 2005).

Cln3-Cdc28 is required to allow the transcription of these cyclins because it is responsible for the inhibitory phosphorylation of Whi5, a transcriptional repressor of SBF (Costanzo, Nishikawa et al. 2004, de Bruin, McDonald et al. 2004). Cln1/2-Cdc28 and Clb5/6-Cdc28, which result from this transcription activity, contribute to Whi5

phosphorylation, thus providing a positive-feedback loop (Dirick and Nasmyth 1991). G1 cyclins Cln1-2 once bound to Cdc28 are phosphorylated by Cdc28 itself and are soon targeted for degradation through the SCF^{Grr1} complex (Barral, Jentsch et al. 1995, Willems, Lanker et al. 1996, Skowyra 1997). In this regard, a role of the protein phosphatase PP2A to their stabilization has been proposed (McCourt, Gallo-Ebert et al. 2013).

All three possible Cln-Cdc28 complexes can be inhibited via a specific pheromone-induced response, resulting in the activation of the specific inhibitor of Cln-associated CDKs, Far1 (Peter and Herskowitz 1994). The ubiquitin ligase SCF^{Cdc4} drives the degradation of Far1 in the absence of mating pheromones (Blondel, Galan et al. 2000). SCF^{Cdc4} is also required to target for degradation the Clb-CDK complexes inhibitor, Sic1. The degradation of this inhibitor is essential to enter S phase (Schwob, Bohm et al. 1994). Moreover, SCF^{Cdc4} recognises Sic1 only when previously phosphorylated by Clns-Cdc28 and Clbs-Cdc28 (Feldman, Correll et al. 1997, Skowyra 1997, Verma 1997).

S phase cyclins Clb5 and Clb6 are synthesised in late G1 (Jackson, Reed et al. 2006). These two cyclins show overlapping role in promoting the initiation of DNA replication but only Clb5 seems also to be required to block re-replication at later stage in S phase (Ikui, Archambault et al. 2007). This additional role of Clb5 reflects a different stability of the two proteins. Clb6 becomes indeed rapidly degraded at the G1-S transition by the SCF^{Cdc4} while Clb5 is degraded at the metaphase-to-anaphase transition by the APC/C^{Cdc20} (Visintin, Prinz et al. 1997, Jackson, Reed et al. 2006). High Clb5 levels until metaphase reflect the requirement of the cyclin to inhibit the initiation of chromosome segregation in addition to its main role in promoting DNA synthesis (Jin, Richmond et al. 2009, Liang, Richmond et al. 2013), as well.

Clb3-4 production starts in S phase suggesting possible roles in DNA replication and mitosis (Grandin and Reed 1993). The most important cyclins in mitotic progression, Clb1-2, are instead expressed at the G2-M transition, concomitantly with a group of genes known as *CLB2* gene cluster (Futcher 2000, Futcher 2002). This cluster includes Clb1-2 cyclins and other important mitotic genes (like Swi5, Cdc5 and Cdc20) and is regulated by the Mcm1-Fkh2 complex associated with the coactivator Ndd1 (Koranda, Schleiffer et al. 2000). Clb2-Cdc28 is required to phosphorylate Ndd1 and Fkh2, allowing Ndd1-dependent Mcm1-Fkh2 recruitment to the *CLB2* promoter (Reynolds 2003) (Darieva 2003) and serve to facilitate the Fkh2-Ndd1 interaction (Pic-Taylor 2004), respectively. Clb2-Cdc28 complexes are also involved in repressing the transcription of G1 cyclins via SBF phosphorylation. Upon completion of mitosis, mitotic cyclin Clb1-4 degradation is required for mitotic exit to occur. Their degradation depends on the APC/C^{Cdh1} (Zachariae, Schwab et al. 1998, Schwab, Neutzner et al. 2001). Actually, although the complete degradation of Clb2 rests upon the APC/C^{Cdh1} the cyclin is partially degraded already at the metaphase-to-anaphase transition by the APC/C^{Cdc20} (Baumer, Braus et al. 2000, Yeong, Lim et al. 2000).

An additional level of regulation is based on post-translational phosphorylations. For example, mitotic Clb-Cdc28 complexes are negatively regulated through the Swe1-dependent phosphorylation of the Tyr19 residue (Booher, Deshaies et al. 1993) and positively regulated through the dephosphorylation of the same residue by the Mih1 tyrosine phosphatase (Russell 1989). Swe1 is itself negatively regulated by Clb-Cdc28 complexes (and another important mitotic kinase, the polo-like kinase Cdc5) via phosphorylation, a signal that induces Swe1 degradation by the SCF^{Met30} prior to mitotic entry (Asano, Park et al. 2005) and allows the full activation of Clb-Cdc28 complexes. Degradation of Swe1 requires also its translocation to the bud-neck, event that is mediated by the protein phosphatase PP2A. The absence of PP2A results in a stabilized

Swe1 and delayed mitotic entry (Xu, Xing et al. 2006). Also Mhi1 is tightly regulated, in interphase Mhi1 is phosphorylated and kept inhibited by the Yck1-2 kinases while during mitotic entry PP2A-mediated dephosphorylation activates Mih1 (Pal, Paraz et al. 2008).

Of note, the morphogenetic pathway, that monitors cell polarity establishment between START and nuclear division, acts through the Swe1-mediated inhibition of Clb-CDK complexes (McMillan, Sia et al. 1998, Sia, Bardes et al. 1998).

1.3.2 Mitotic kinases

CDKs are considered the key regulators of the cell cycle. However other kinases play important roles for mitotic progression. Interestingly, CDK substrates include few of these kinases, such as the polo-like kinases and the Aurora family of kinases, which are directly involved in the regulation of specific mitotic events.

1.3.2.1 The Polo family

Polo-like kinases (Plks) are integral parts of the cell cycle engine. Plks are conserved serine/threonine kinases orthologues of the *Drosophila melanogaster* Polo kinase, originally identified during a screen for genes involved in SPBs behaviour (Sunkel and Glover 1988, Llamazares, Moreira et al. 1991). Metazoans have multiple Plks (Plk1-4) that are associated to diverse structures and functions whilst *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* carry only one Plk, the Cdc5 (Kitada, Johnson et al. 1993) and Plo1 kinase (Ohkura, Hagan et al. 1995) respectively.

All polo-like kinases share a closely related molecular structure with a N-terminal catalytic domain and a conserved polo-box domain (PBD) in the non-catalytic C-terminal region. Most Plks possess a PBD composed of two conserved sequences named polo-boxes (PB1-2) that function as a single unit in determining Plk localization

and activity (Lee, Grenfell et al. 1998, Nigg 1998). Moreover, the PBD determines the preference of Plks for substrates containing optimal phospho-sequences of Ser-pThr/pSer-Pro/X (Elia, Cantley et al. 2003, Elia, Rellos et al. 2003).

Plks localization is highly dynamic. Human Plk1 moves from the cytoplasm and nucleus to centromeres in early mitosis and, later in mitosis, a fraction of the protein localizes to the spindle midzone (Golsteyn, Schultz et al. 1994). Despite the structural similarity between Plk1 and Cdc5, the yeast Plk does not localize to the spindle midzone. Cdc5 moves from the nucleus and localizes to the SPBs and to the bud-neck ring structures, required for cytokinesis (Song, Grenfell et al. 2000).

At the level of regulation, Cdc5 resembles Plk1. As part of the *CLB2* gene cluster, Cdc5 levels increase from late S/G2 to early M phase and then slightly decrease during anaphase. Cdc5 activation requires at least a Cdc28-mediated phosphorylation in the conserved Thr242 residue of its T-loop (Mortensen, Haas et al. 2005). In late mitosis, Cdc5 must be degraded. Its degradation depends on the ubiquitin-ligase APC/C^{Cdh1} and continues throughout G1 (Cheng, Hunke et al. 1998, Shirayama, Zachariae et al. 1998).

Although *plk* mutants are defective is SPBs maturation and separation (Sunkel and Glover 1988, Lane and Nigg 1996), the yeast Cdc5 seems not to be involved in the regulation of these events so that *cdc5* mutant are not associated to SPBs defects. Yeast Cdc5 acts at the G2/M transition to promote the degradation of the Clb-CDK inhibitor Swe1 (Park, Song et al. 2003). The additional positive role of human Plk1 on the CDK-activator Cdc25 (orthologue of yeast Mhi1) (Nakajima, Toyoshima-Morimoto et al. 2003) has not been reported in budding yeast. Cdc5 is then required at the metaphase-to-anaphase transition to promote sister chromatid separation through the phosphorylation of cohesin, that maintains the cohesion between the sisters up to metaphase (Alexandru, Uhlmann et al. 2001). Plks are also required for exit from mitosis and cytokinesis. In budding yeast, Cdc5 is essential for mitotic exit. This topic

will be covered in more detail later in this thesis. The role of Cdc5 during cytokinesis is limited to a positive regulation of the Rho1 GTPase, an activator of the cytokinetic actomyosin ring (Yoshida, Kono et al. 2006).

1.3.2.2 Aurora B/Ipl1

Aurora B is a conserved serine/threonine kinase essential for chromosome condensation, sister chromatid segregation and cytokinesis. In mammals, Aurora B belongs to the Aurora kinase family that comprises also Aurora A, involved only in centrosome functions (Glover, Leibowitz et al. 1995), and Aurora C, which works in germ-line cells with less clear functions. In budding yeast, there is a single Aurora protein kinase named Ipl1 (orthologue of Aurora B), originally identified in a screen for mutants with increased ploidy (chromosome number) (Chan and Botstein 1993, Francisco, Wang et al. 1994).

Ipl1 is expressed only in mitosis and it is part of the conserved chromosome passenger complex (CPC) that further includes Sli15 (hINCENP), Bir1 (hSurvivin) and Nbl1 (hBorealin) (Kim, Kang et al. 1999, Tanaka, Rachidi et al. 2002). Early in mitosis Ipl1 localizes to chromosomes and later it associates with kinetochores (KTs). Here, through the phosphorylation of KT proteins (Ruchaud, Carmena et al. 2007), Ipl1 is required for the spindle assembly checkpoint to regulate microtubule-kinetochore attachments and establish a bipolar orientation of chromosomes on the mitotic spindle (Skoufias, Andreassen et al. 2001, Liu, Vader et al. 2009, Maresca and Salmon 2009, Uchida, Takagaki et al. 2009). As such, *ipl1* mutants fail to segregate sister chromosomes to opposite poles (Biggins, Severin et al. 1999, Biggins and Murray 2001). At anaphase Ipl1 transfers to the spindle to drives spindle disassembly tracking the plus ends of the spindle MTs back to the poles (Buvelot, Tatsutani et al. 2003).

1.3.3 Protein degradation

Protein degradation plays a crucial role in cell cycle progression. Cyclins and CDK inhibitors activities are timely restricted through cycles of synthesis and degradation. The phase-specific intracellular concentration of such proteins depends on the ubiquitin-proteasome proteolytic pathway. The ubiquitylation machinery rests upon two distinct events: the polyubiquitylation of target molecules and their subsequent degradation by the 26S proteasome system.

Polyubiquitylation can be considered as a "death-signal" for the targeted protein and consists in the covalent attachment of 8 kDa molecules called ubiquitin (Ub) onto a lysine residue (K48) of the growing Ub chain linked to the protein substrate, through a sequential mechanism (Barford 2011). Ub-attachment is mediated by the cooperation of three different enzymes: an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase (Martin 2010).

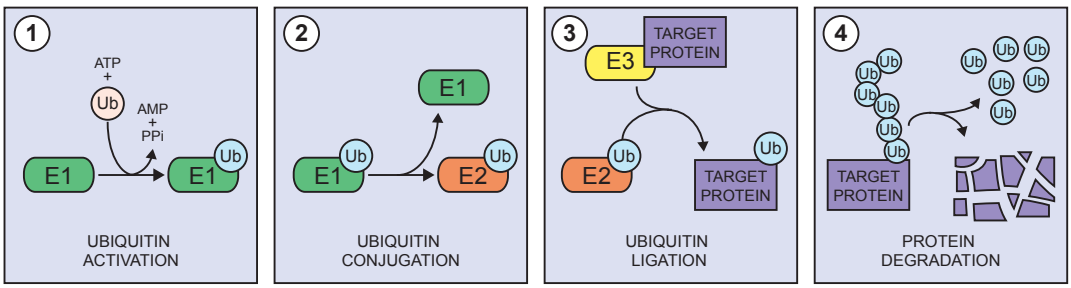

Figure 1.2. The ubiquitin-mediated degradation pathway. Schematic representation of the four major events driving protein degradation through the ubiquitylation system: (1) Ub activation by the E1 enzyme, (2) Ub conjugation to the E2 enzyme, (3) repeated E3-mediated transfer of Ub from E2 to the target protein to form a poly-Ub chain on the substrate and, finally, (4) degradation of the poly-Ub linked target protein.

Ub is first activated and attached via its C-terminal glycine residue to the catalytic cysteine of the E1 enzyme through a thioester bond and then is transferred to the catalytic cysteine of the E2 enzyme. Thereafter, the E3 enzyme catalyzes the transfer of the Ub from the Ub-charged E2 enzyme to a specific lysine residue of the substrate

protein to form an isopeptide bond. Moreover, the polyubiquitylation process requires specific combination of E2/E3 enzymes, able to use lysine residues on Ub already substrate-conjugated to catalyze the sequential addition of further Ubs to each other, leading to the assembly of poly-Ub chains (Pickart and Eddins 2004, Petroski and Deshaies 2005). Among these 3 classes of enzymes, the E3 seems to be the most implicated in recognizing specific substrates and in regulating cell cycle progression. It is possible to identify two major types of E3 enzymes, both belonging to the Ring-H2-finger-containing E3 Ub-ligase family: the F-box containing complex (SCF complex) and the anaphase promoting complex or cyclosome (APC/C).

1.3.3.1 The SCF complex

The SCF complex mainly controls the G1-S transition by targeting for degradation G1 cyclins and CDK inhibitors (Bai 1996, Feldman, Correll et al. 1997, Skowyra 1997). Additional roles in other cell cycle phases have been identified as well. In *S. cerevisiae* this complex consists of three essential elements (Skp1, the scaffold protein Cdc53/Cullin1 and the RING-finger protein Rbx1/Roc1/Hrt1) and a variable component responsible for substrate specificity. The latter is named F-box protein because it interacts with the Skp1 subunit via its F-box domain (Bai 1996). Budding yeast encodes for at least 15 F-box proteins including Cdc4, Grr1 and Met30 that have been characterized to be involved in cell cycle progression. Particularly, they all depend on the E2 ubiquitin-conjugating enzyme Cdc34. SCF complexes are active throughout all phases of the cell cycle and their activities depend on the state of phosphorylation of their substrates, which is often dependent on Cdc28 (Skowyra 1997). For example, at the G1-S transition the *S. cerevisiae* F-box protein Cdc4 recognizes sequences of the CDK inhibitor Sic1 only if previously phosphorylated by Cdc28 on at least 6 of the 9 CDK-phosphorylation sites (Nash, Tang et al. 2001, Orlicky, Tang et al. 2003). Cdc34-

SCF^{Cdc4} mediated degradation of Sic1 is essential for the activation of the S phase specific Clb5-Cdc28 complexes.

In G1 Cdc34-SCF^{Cdc4} drives also the degradation of Clns inhibitor Far1 in the absence of mating pheromones (Blondel, Galan et al. 2000) and, following the G1-S transition, of the essential component of the replication machinery Cdc6 to avoid re-replication (Drury, Perkins et al. 1997). Cdc34-SCF^{Grr1} is required for Cln1-2 degradation at the G1-S transition (Kishi and Yamao 1998) while Cdc34-SCF^{Met30} targets the CDK inhibitor Swe1 for degradation to allow entry into mitosis (Kaiser, Sia et al. 1998).

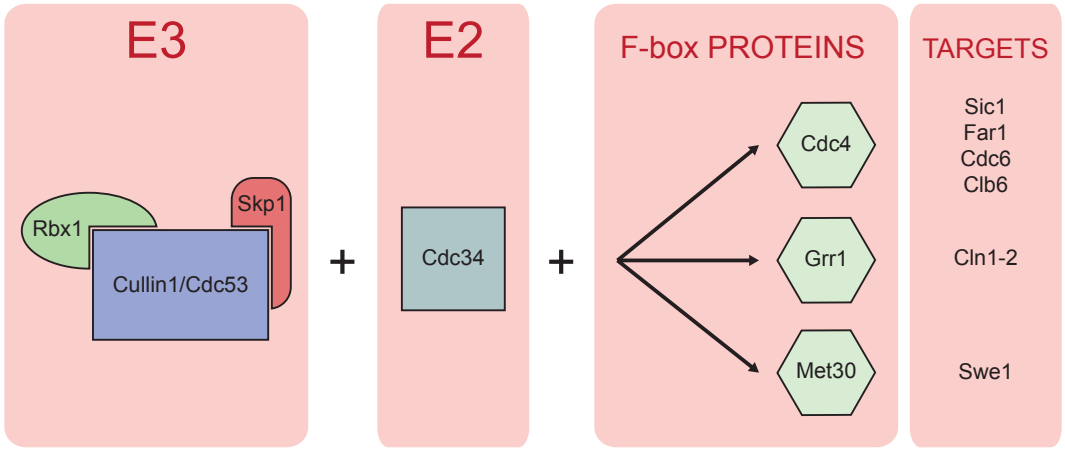

Figure 1.3. The SCF degradation pathway. SCF substrate specificity relies on the association of the core components of the complex (Cullin1/Cdc53, Skp1 and Rbx1) with a specific F-box protein. Cdc4, Grr1 and Met30 are the more important mitotic F-box proteins in budding yeast and they all act through the association with the E2 Ub-conjugating enzyme Cdc34.

1.3.3.2 The anaphase promoting complex or cyclosome (APC/C)

The APC/C is composed of two sub-complexes, a catalytic core including the Cullin domain Apc2 and the RING H2 finger subunit Apc11, and a second sub-complex consisting of three tetratricopeptide repeat (TPR) subunits (Cdc16, Cdc23 and Cdc27), each one present as homodimer to shape the so-called arc lamp of the APC/C (Zhang, Roe et al. 2010, Primorac 2013). In *Saccharomyces cerevisiae* the APC/C builds poly-Ub chains linked to the target proteins. Initiation and subsequent elongation of these

chains depend on two different E2 enzymes (Ubc4 and Ubc1, respectively) (Rodrigo-Brenni and Morgan 2007).

APC/C activation requires the interaction of the core APC/C complex with substrate specific activator proteins like Cdc20 and Cdh1/Htc1, typified as the mitotic co-activators of the APC/C (Ama1 is another APC/C co-activator, mainly involved in the meiotic cycle) (Cooper 2000, Okaz, Arguello-Miranda et al. 2012). Cdc20 activates the ubiquitin ligase at the metaphase-to-anaphase transition to induce (i) sister chromatid separation through the degradation of the anaphase inhibitor Pds1 and (ii) the down-regulation of mitotic CDKs. Later on Cdh1 directs the APC/C towards its late mitotic targets and is important for inducing (i) exit from mitosis and (ii) to maintain cells in a G1 state (Dawson 1995, Schwab 1997, Visintin, Prinz et al. 1997, Zachariae, Schwab et al. 1998). To ensure the proper temporal succession of these two events (sister chromatid separation and exit from mitosis) Cdc20 and Cdh1 activities are deeply coordinated. In metaphase high levels of CDKs assure the phosphorylation of the core subunits of the APC/C, a prerequisite for APC/C-Cdc20 interaction. APC/C^{Cdc20} induces a decreased CDK activity promoting its own inactivation and allowing the formation of an active APC/C^{Cdh1} complex. Indeed, Cdh1 binds the APC/C only when de-phosphorylated (Kramer, Scheuringer et al. 2000).

From a structural point of view these two co-activators are very similar and they both bind APC/C via specific motifs: the C-box and the KILR (lysine-isoleucine-leucine-arginine tetrapeptide) motifs, mapping at the N-terminal domain (Schwab, Neutzner et al. 2001, Zhang and Lees 2001, Izawa and Pines 2012), and the IR (isoleucine-arginine dipeptide) motif, mapping at the C-terminal domain of the protein (Vodermaier, Gieffers et al. 2003, Thornton, Ng et al. 2006).

Co-activators not only activate the APC/C enzyme but they are also required to present the substrates to its catalytic core. Substrates usually interact with the co-activators

through sequence motifs called degrons, like the D (destruction)-box (RXXLXXXXN) (Glotzer 1991) and the KEN (lysine-glutamate-asparagine)-box (KENXXXN) (Pfleger, Lee et al. 2001). The existence of two possible degron sequences highlights additional levels of specificity in substrates identification, with the APC/C^{Cdc20} having a strong dependence on the D-box and the APC/C^{Cdh1} able to interact with both D- and KEN-boxes. Binding affinities to these motifs is also conferred by the amino acid sequence surrounding the motifs (Pfleger and Kirschner 2000).

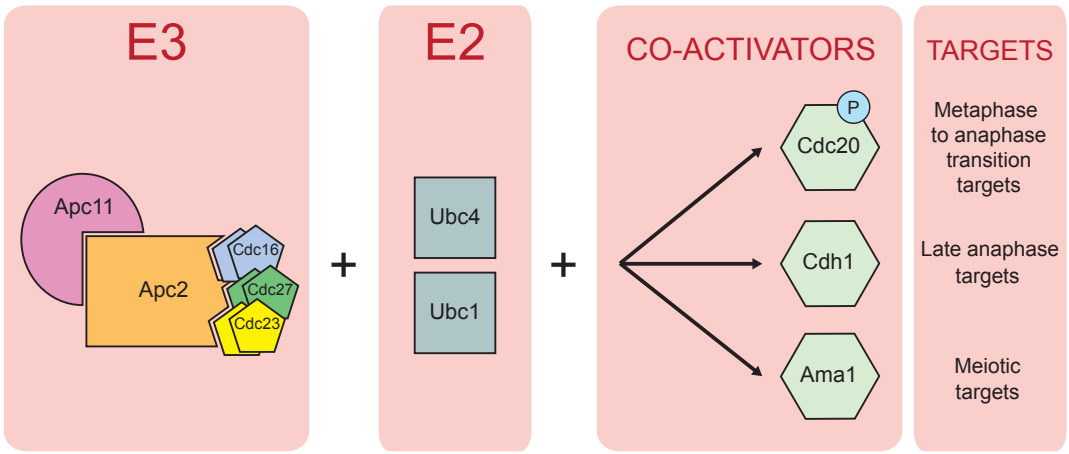

Figure 1.4. The APC/C degradation pathway. In budding yeast the APC/C complex collaborates with two E2 ubiquitin-conjugating enzymes: Ubc4, required for the mono-ubiquitylation of APC targets, and Ubc1, that promotes poly-Ub chains extension on the pre-attached Ubs. The interaction with specific co-activators is then required for substrate specificity.

1.3.4 Mitotic phosphatases

For decades phosphorylation and protein degradation have been considered the cellular mechanisms that drive the cell cycle. Accumulating evidence has focussed on phosphatases as active players in cell cycle engine. It is now clear that substrate dephosphorylation after kinase phosphorylation does not simply hinge on kinases down-regulation but also on the activity of specific phosphatases (reviewed in (De Wulf, Montani et al. 2009). Phosphatases activity seems to be especially important in the final

steps of mitosis to reverse the phosphorylation events mediated by the mitotic kinases that are required to drive the cell cycle up to metaphase. Dephosphorylation of mitotic phospho-substrates is a prerequisite to reset the cell cycle machinery in view of a new round of cell division. However protein phosphatases also have important roles before and during mitosis.

The importance of phosphatases goes in parallel with the good balance between phosphatases and opposing kinases. In this regard, the interplay between the Clb5-Cdc28 kinase complex and the phosphatase Cdc14 can be an example. The Clb5-Cdc28 complex has an important role in preventing anaphase entry through the inhibitory phosphorylation of factors required for spindle stabilization and elongation (Jin, Richmond et al. 2009, Liang, Richmond et al. 2013). These factors have to be dephosphorylated to become active in the spindle elongation process so that progression into anaphase requires Cdc14 activation but it is also strongly facilitated by the time-regulated Clb5 degradation at the metaphase-to-anaphase. In this way, Clb5 down-regulation helps Cdc14 in the dephosphorylation of that specific Clb5 targets, because they cannot be re-phosphorylated once dephosphorylated by Cdc14 (Jin, Richmond et al. 2009, Liang, Richmond et al. 2013).

1.3.4.1 Protein phosphatase type 2A (PP2A)

Protein phosphatases type 2A (PP2A) are conserved serine/threonine phosphatases. Budding yeast PP2A are holoenzymes composed of the Tpd3 scaffold protein, a catalytic subunit (Pph21 or Pph22) and one regulatory subunit between Rts1 or Cdc55 (Stark 1996). Sub-cellular localization and activity of PP2A are dictated by the regulatory subunit that also determines the substrate specificity by changing the catalytic pocket of the phosphatase and by binding to the substrate (Xu, Chen et al. 2008). A second level of regulation comes from post-translational modifications such as threonine as well as tyrosine phosphorylation and methylation of the PP2A catalytic

domain. PP2A are also regulated by specific phosphotyrosyl phosphatase activators (PTPAs). *S. cerevisiae* encodes two PTPAs: Ypa1, with specific role at the G1-S transition (Van Hoof, Janssens et al. 2000), and Ypa2, with functions in M phase (Van Hoof, Janssens et al. 2001).

PP2A associated with the regulatory subunit Cdc55 ($PP2A^{Cdc55}$) regulates the G1-S transition because it is required to maintain Cln1-2 levels in G1. Phosphorylated Clns are quickly degraded via the SFC; thus $PP2A^{Cdc55}$ dephosphorylation of these cyclins result in their stabilization (McCourt, Gallo-Ebert et al. 2013).

Additionally, $PP2A^{Cdc55}$ regulates both mitotic entry and mitotic exit. Upon entry into mitosis the phosphatase indirectly regulates the activity of Clb-CDK complexes by promoting the degradation of the CDK inhibitor kinase Swe1 and the activation of the CDK activator phosphatase Mih1 (Minshull, Straight et al. 1996). Moreover, *cdc55* mutants lack a functional spindle assembly checkpoint and allow Clb-CDKs inactivation by inhibitory phosphorylation in cells with defective spindles (Wang and Burke 1997). $PP2A^{Cdc55}$ is also required to prevent the activation of the phosphatase Cdc14 prior to anaphase. In budding yeast, Cdc14 plays a pivotal role in promoting mitotic exit. One important function of $PP2A^{Cdc55}$ is to prevent premature exit from mitosis by controlling the phosphatase Cdc14 (Queralt, Lehane et al. 2006, Wang and Ng 2006, Queralt and Uhlmann 2008).

Recently, two paralogues, Zds1 and Zds2, have been identified as regulators of the Cdc55 regulatory subunit of PP2A. Both Zds proteins interact with Cdc55 via their ZH4 C-terminal domain to form a tight stoichiometric complex with $PP2A^{Cdc55}$ (Yasutis, Vignali et al. 2010, Wicky, Tjandra et al. 2011). Of note, Zds1-2 positively act with $PP2A^{Cdc55}$ to promote mitotic entry (regulating only the $PP2A^{Cdc55}$-dependent dephosphorylation of Mih1 and not the $PP2A^{Cdc55}$-dependent dephosphorylation of Swe1) (Wicky, Tjandra et al. 2011) whilst they oppose $PP2A^{Cdc55}$ roles in mitotic exit

(Zds1-2 enhance Cfi1 phosphorylation and Cdc14 release) (Queralt and Uhlmann 2008). This apparently contrasting behaviour has been explained through a possible role of the Zds proteins in confining Cdc55 to the cytoplasm. Zds1-2 are required to exclude Cdc55 from the nucleus/nucleolus thereby allowing Cfi1 phosphorylation and Cdc14 release (Rossio and Yoshida 2011).

The regulatory subunit Rts1 seems to play a prominent role in the stress response yet additional roles in cell cycle regulation have been proposed (Shu, Yang et al. 1997). $PP2A^{Rts1}$ promotes the normal accumulation of G1 cyclins regulating their transcription in a Whi5-independent manner. $PP2A^{Rts1}$ seems to work in a distinct network that link nutrient availability, cell size, and G1 cyclins transcription (Artiles, Anastasia et al. 2009). Moreover, $PP2A^{Rts1}$ has been proposed as a member of the molecular pathway that controls the alignment of the mitotic spindle along the polarized mother-bud axis, the spindle position checkpoint (SPOC). Proper localization and function of the essential SPOC component kinase Kin4 depend indeed on $PP2A^{Rts1}$-dependent dephosphorylation of the kinase (Chan and Amon 2009). $PP2A^{Rts1}$ has however a prominent role in meiosis I to prevent cohesin cleavage at chromosome centromeric regions. Cdc5-mediated phosphorylation of cohesin rings enhanced cohesins dissolution but $PP2A^{Rts1}$ is recruited to the centromere by Sgo1 and once there it dephosphorylates the cohesin complexes preventing their cleavage (Xu, Cetin et al. 2009). The further permanence of $PP2A^{Rts1}$ at the centromeres up to meiosis II hinges on Ipl1 (Yu and Koshland 2007).

1.3.4.2 Protein phosphatase type 1 (PP1 or Glc7)

In addition to PP2A, also the serine/threonine protein phosphatase type 1 (PP1) is a cell cycle key regulator, with molecular structure and mechanisms of regulation similar to those of PP2A. PP1 regulates different aspects of cell cycle progression and interestingly both metazoans PP1 and the orhtologue in yeast, Glc7, are involved in

kinetochore stabilization. To date, Glc7 is the only phosphatase that localise to KTs counteracting Ipl1-mediated phosphorylations once bi-orientation of sister chromatids is achieved (Francisco, Wang et al. 1994). This phosphatase is regulated through the association with PP1-interacting proteins (PIPs), able to direct PP1 to the sub-cellular region of its substrates and also able to regulate its activity (Egloff, Johnson et al. 1997). PP1-interacting proteins bind to PP1 through one or more short and degenerate docking motifs, of which the more characteristic are the "RVxF", the "SILK" and the "MyPhoNE" motifs (Egloff, Johnson et al. 1997, Hendrickx, Beullens et al. 2009).

The nuclear protein Ypi1 is an essential PP1-interacting protein that associates with Sds22 to positively regulate Glc7 through the formation of a nuclear ternary complex (Bharucha, Larson et al. 2008) although it was initially described as a Glc7 inhibitor *in vitro* (Garcia-Gimeno, Munoz et al. 2003, Pedelini, Marquina et al. 2007). *ypi1* mutants arrest at the G2-M transition with short mitotic spindle, phenotype that depends on the activation of the G2/M checkpoint (Bharucha, Larson et al. 2008) or on Swe1 stabilization (Marquina, Queralt et al. 2012).

The kinetchore protein Fin1 has been proposed as a candidate to promote the localization of Glc7 to the KTs, although its contribute is not essential. With a positive feedback loop, Glc7 dephosphorylation of Fin1 localises Fin1 at the KTs, which in turn enhances Glc7 accumulation at the KTs, too (De Wulf, Montani et al. 2009, Hendrickx, Beullens et al. 2009).

Multiple evidences also indicate that Glc7 opposes Ipl1 kinase in regulating the microtubule-kinetochore attachments to the spindle (Francisco, Wang et al. 1994, Bloecher and Tatchell 1999). The Glc7-mediated dephosphorylation of KT proteins previously phosphorylated by Ipl1 contributes indeed to the spindle assembly checkpoint silencing once chromosome bi-orientation is achieved (Rosenberg, Cross et al. 2011).

1.3.4.3 Cdc14 phosphatase

Cdc14 is a conserved dual specificity protein phosphatase that drives mitotic exit in budding yeast (Visintin, Craig et al. 1998, Stegmeier and Amon 2004). Whether this role is conserved in higher eukaryotes is still unclear. More conserved roles for Cdc14 have been shown in early anaphase in order to stabilize and elongate the mitotic spindle as well as later in mitosis to induce cytokinesis (Mocciaro and Schiebel 2010). This phosphatase shows a spatial-temporal regulation and is active only in anaphase, the temporal window in which its function is required. From G1 to metaphase Cdc14 is kept inactive in the nucleolus as it is bound to its inhibitor Cfi1 (also called Net1) (Shou, Seol et al. 1999, Visintin, Hwang et al. 1999). Cdc14 activation at anaphase onset requires its dissociation from Cfi1, event that let the phosphatase to spread into the nucleus and subsequenetly into the cytoplasm to reach its targets. The binding of Cdc14 to Cfi1 is regulated via phosphorylation/dephosphorylation events so that the two proteins interact only when not phosphorylated and their phosphorylation is required for their dissociation. Up to metaphase, $PP2A^{Cdc55}$ keeps Cfi1 in a dephosphorylated state hence preventing the dissolution of Cdc14-Cfi1 interaction (Queralt et al., 2006; Y. Wang & Ng, 2006). At the metaphase-to-anaphase transition, the protein separase Esp1 becomes active to induce anaphase entry by removing the cohesion between sister chromatids. In parallel, Esp1 acts through Zds1 and Zds2 to downregulate $PP2A^{Cdc55}$ and therefore enables Cfi1 phosphorylation as well as Cdc14 release (Sullivan and Uhlmann 2003) (Queralt & Uhlmann, 2008). Two consecutive pathways, the Cdc14 early anaphase release (FEAR) network and the mitotic exit network (MEN), drive the dissociation of Cdc14 from Cfi1 through the phosphorylation of both proteins. In telophase, Cdc14 drives its own re-sequestration and inactivation (Pereira, Manson et al. 2002, Visintin, Tomson et al. 2008).

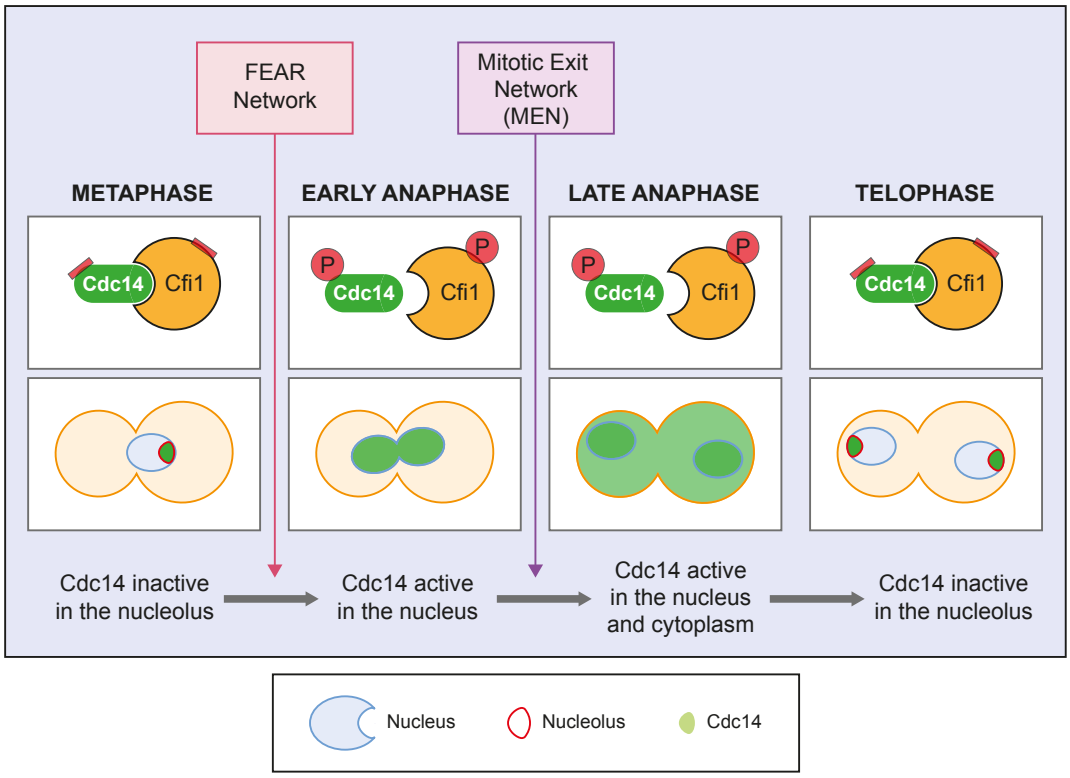

Figure 1.5. Cdc14 phosphatase regulation. Cdc14 activity is regulated through changes in its localization. At anaphase onset two consecutive pathways (the FEAR network and the MEN) mediate Cdc14 activation inducing the release of the phosphatase from its nucleolar inhibitor Cfi1. The interaction between Cdc14 and Cfi1 depens on the phosphorylation status of the two proteins.

This well characterized mechanism of Cdc14 regulation restrains the activity of Cdc14 to anaphase. Nevertheless roles of Cdc14 prior to anaphase are slowly emerging. For example it has been proposed that up to metaphase the nucleolar Cdc14 is required to maintain dephosphorylated and hence inactive Spo12, one of the proteins involved in its own activation at the metaphase-to-anaphase transition (Tomson, Rahal et al. 2009). This discrepancy could be explained by the coexistence of two nucleolar pools of Cdc14: one inactive and one already active. In addition to the major pool of nucleolar Cdc14 kept inactive through the association with Cfi1, a minor pool of Cdc14 exists that is bound to Tof2. As well as Cfi1, Tof2 localizes to the nucleolus and directly binds Cdc14. Unlike to the inhibitory role of Cfi1 on Cdc14, the effect of the binding with Tof2 remains controversial. In one study it is suggested that Tof2 supports the activity

of Cdc14 (Geil, Schwab et al. 2008), yet another proposes that similarly to Cfi1 also Tof2 acts as a Cdc14 inhibitor (Waples, Chahwan et al. 2009). It is possible to allocate these pre-anaphase specific functions of Cdc14 to this pool of Tof2-bound Cdc14 (Geil, Schwab et al. 2008). Nevertheless other data indicate that the dephosphorylation of the Dsn1 kinetochore protein in metaphase requires Cdc14, supporting also the possibility of a non-nucleolar pool of Cdc14 active prior to anaphase (Akiyoshi and Biggins 2010).

1.3.5 Cell cycle checkpoints

A successful cell division requires the coordination between the different cell cycle phases such that each one starts at the right moment: DNA replication should always come before chromosome segregation, followed by cell division. Hartwell and Weinert in 1989 first provided evidence for pathways by which the initiation of late events is dependent on the completion of early events (Hartwell 1989). They proved the existence of such control mechanisms studying budding yeast mutants surviving DNA damage following irradiation. They found that a wild type cell paused its division in the presence of damaged DNA, until reparation of the damage. Conversely, *rad9* mutants kept on dividing even when irradiated and subsequently died. They also demonstrated that the protein Rad9 is not essential for cell viability *per se* but becomes indispensable for arresting cell division in the presence of damaged DNA, as a member of a control system that they called "checkpoint" (Hartwell 1989).

Checkpoints are protein pathways that monitor the entrance in the different cell cycle phases: in the presence of an uncompleted cell cycle event they send inhibitory signals to delay cell cycle progression and solve the problem. Checkpoints activity is therefore essential to preserve genome integrity and cell viability under adverse circumstances. Even if with some differences between different species, these mechanisms of control are highly conserved.

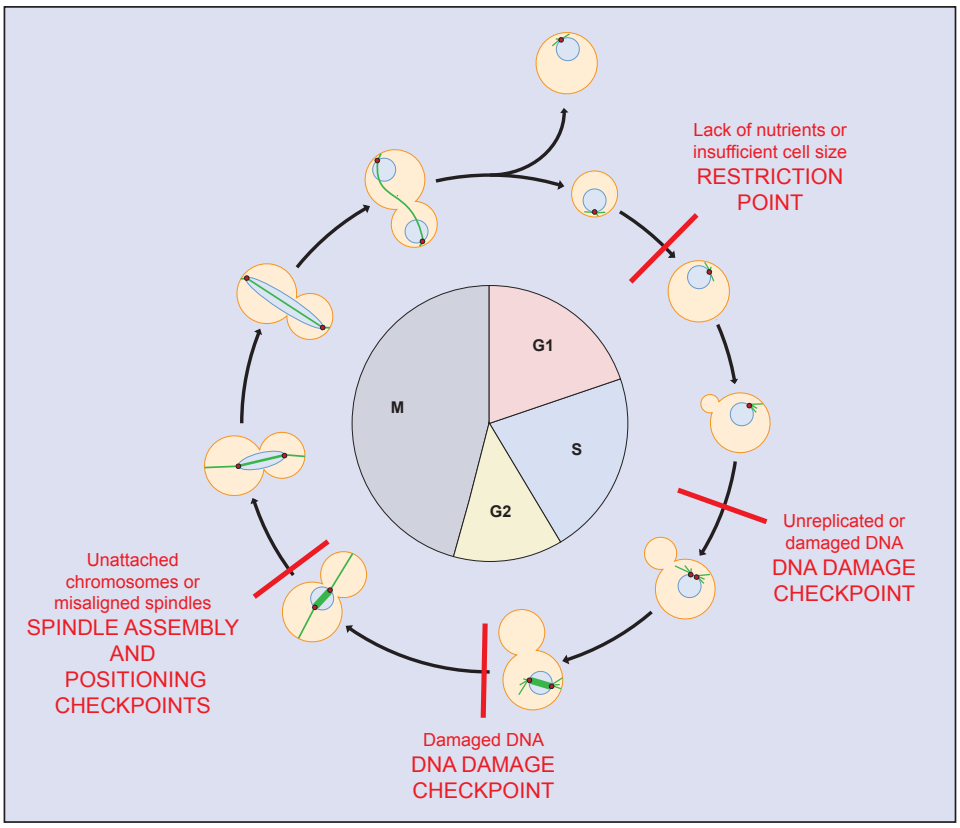

Figure 1.6. Yeast cell cycle major checkpoints.

1.3.5.1 The G1 (restriction) checkpoint

The essential G1 cyclin Cln3 has to be accumulated over a certain threshold to allow the transcription of Cln1-2 and Clb5-6. These cyclins are in turn required to drive post-mitotic G1 cells into S phase, restarting a new round of DNA replication and cell division. G1-S transition therefore corresponds to an irreversible commitment to the cell cycle. It is no surprise that the first cell cycle checkpoint, known as "restriction point" in mammals and START in yeast (Pardee 1974, Hartwell and Unger 1977, Pardee 1989, Blagosklonny and Pardee 2002) acts late in G1 to monitor this transition.

A relationship between cell size and cell commitment to division has been described from bacteria (Donachie 1968) to mammals (Killander 1965). Also in *S. cerevisiae* the START transition requires the cell to reach a critical cell size, referred to as the "setpoint" (Hartwell, Culotti et al. 1974, Johnston, Pringle et al. 1977). However yeast

cells enter the cell division cycle also depending on nutrient availability and the absence of factors like mating pheromones.

Nutrient starvation activates START by causing a decreased protein synthesis, that prevents the accumulation of Cln3. It also promotes Cln3 degradation (Gallego, Gari et al. 1997, Parviz, Hall et al. 1998). Differently, the presence of mating pheromones arrests cell in G1 inducing the inhibition of Cln-CDKs. The pheromone bound to the Ste2 receptor triggers a mitogen-activated protein kinase (MAPK) cascade that culminates with the expression of multiple mating genes. Among these gene products, the Far1 kinase, inhibits Cln-CDK complexes (not Clb-CDK complexes), thereby blocking the cells in G1 in response to pheromone (Peter and Herskowitz 1994).

1.3.5.2 The DNA damage checkpoint (DDC)

Damaged DNA or blocked DNA replication can determine cell cycle arrest at different cell cycle phases: at the G1-S transition, during the S phase and at the G2-M transition.

The cellular response to the DDC (DDR) is a kinases-dependent signal transduction pathway that involves sensing a DNA lesion and then transducing it to downstream elements directly involved in arresting the cell cycle in response to DDC activation.

The phosphoinositol-3-kinase-related Mec1 (homolog of human ATM/ATR) is an essential element of the budding yeast DDR acting both as “sensor” and “transducer”. Downstream transmission of the signal requires the activation of the adaptor protein Rad9. Rad9 is usually phosphorylated in the cell but needs Mec1-dependent hyper-phosphorylation to interact with one between two possible “effectors”: Chk1 or Rad53 (Navas, Sanchez et al. 1996, Sanchez, Bachant et al. 1999). Rad9-mediated interaction between Mec1 and the “effectors” allows them to be phosphorylated and hence activated by Mec1. Once activated, Rad53 and Chk1 mediate different kinds of DDR (Sanchez, Bachant et al. 1999). Chk1 acts mainly trough the phosphorylation of Pds1, an anaphase inhibitor that has to be degraded at the metaphase-to-anaphase transition.

Chk1-mediated phosphorylation of Pds1 prevents its degradation and hence anaphase entry (Sanchez, Bachant et al. 1999).

The Mec1-dependend phosphorylation of Rad53 serves as a priming for Rad53 auto-phosphorylation, an essential step for its full activation. When active, Rad53 phosphorylates Dun1 (Bashkirov, Bashkirova et al. 2003, Lee, Schwartz et al. 2003) thereby initiating the Rad53-Dun1 branch of the DDR. One of the consequences of this activation is the inhibition of exit from mitosis by phosphorylating (and activating) the MEN-inhibitor Bfa1-Bub2 (Hu, Wang et al. 2001) and through the inhibitory phosphorylation of Cdc5, a key element in promoting mitotic exit (Sanchez, Bachant et al. 1999). Rad53 is also the central "effector" of the DDC in S phase. Rad53-Dun1 can mediate the degradation of the ribonucleotide reductase inhibitor Sml1 (Zhao and Rothstein 2002). Rad53-dependent Sml1 reduction is required to initiate DNA replication after DNA damage as well as during normal cell growth (Zhao, Chabes et al. 2001). Rad53-Dun1 activation is also required to upregulate the RNR complex that catalyzes the rate-limiting step in the synthesis of dNTP from NTP, in response to DNA damage (Huang, Zhou et al. 1998). Finally, DDC can pause G1 through the inhibitory phosphorylation of Swi6 by Rad53, the transcription factor that is required for the expression of the G1 cyclin Cln1-2 (Sidorova and Breeden 1997).

1.3.5.3 The mitotic checkpoint

The mitotic checkpoint, also called spindle assembly checkpoint (SAC), arrests cells in metaphase until all the sister chromatids are bi-oriented on the mitotic spindle (sister KTs have to be attached to MTs emanating from opposite SPBs; amphitelic attachment) (Musacchio and Salmon 2007). Syntelic (sister KTs attached to the same SPB), merotelic (one of the two sister KTs attached to both the SPBs) or monotelic (between the two sister KTs one is attached to one pole whereas the other KT is not bound)

attachments are incompatible with an equal distribution of the chromosomes between the two daughter cells (Lampson, Renduchitala et al. 2004, Pinsky and Biggins 2005).

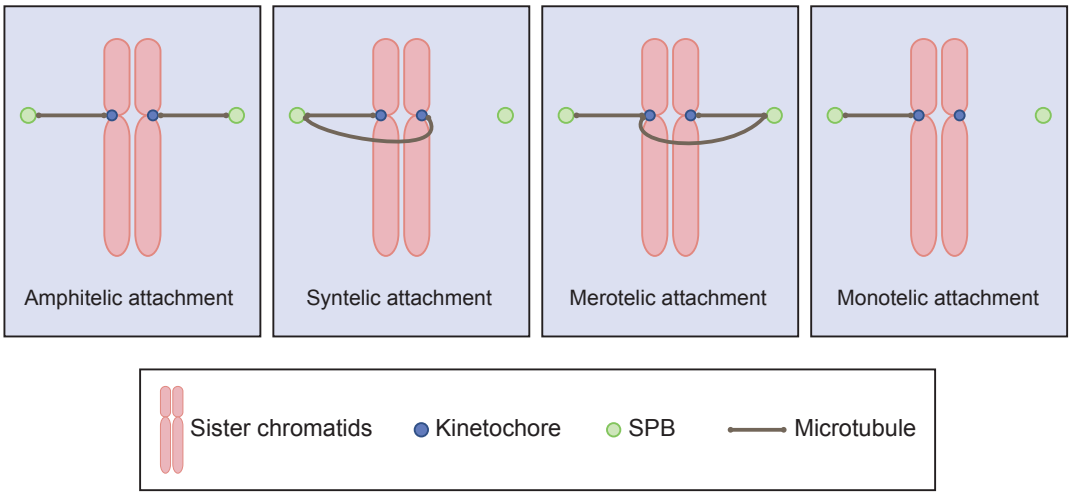

Figure 1.7. Types of kinetochore-microtubule attachments. MTs binding to KTs can occur in different ways. The requirement for SAC satisfaction and hence anaphase entry is that each sister chromatid becomes attached form MTs emanating from opposite SPBs (amphitelic attachment). Syntelic, merotelic and monotelic attachments do not ensure proper chromosome segregation and hence are sensed as problems to be solved by the SAC.

Un-attached or not bi-oriented chromosomes trigger the SAC, which in turn halts the cells in metaphase by inhibiting the APC/C^{Cdc20} (Musacchio and Salmon 2007). The key component of the SAC is the mitotic checkpoint complex (MCC), which is composed of Mad2, BubR1/Mad3 and Bub3. The MCC inhibits the APC/C through the direct association of Mad2 with APC/C cofactor Cdc20. In addition, Bub1, Mad1 and Mps1 are other members of the SAC that help the MCC-mediated inhibition of Cdc20 (Musacchio and Salmon 2007, Vleugel, Hoogendoorn et al. 2012). Only after the interaction between Mad2 and Mad1, Mad2 is able to bind Cdc20. Indeed, the interaction Mad2-Mad1 changes the Mad2 status from an open-conformation (O-Mad2) to a closed-conformation (C-Mad2), increasing its affinity for Cdc20 and ensuring efficient MCC formation (De Antoni 2005, Nezi, Rancati et al. 2006).

In addition to the “mis-attached signal” it has also been proposed that SAC responds to a “low-tension signal”, generated by the absence of centromeric chromatin stretching between sister KTs (Skoufias, Andreassen et al. 2001). This second kind of SAC response has been suggested to depend on the activity of the Ipl1 kinase (Biggins and Murray 2001). Ipl1 forms a complex with Sli15 that up to metaphase localizes at the interface between the centromeric regions of paired sister chromatids (Kim, Kang et al. 1999). Ipl1-mediated phosphorylation of different KT proteins decreases their affinity to MTs, destabilizing the KT-MT attachments. Bi-orientation generates tension that space KTs from inner sister centromeres preventing Ipl1 from phosphorylating its KTs substrates, resulting in stable KT-MT attachments. The Glc7 phosphatase enhances this stabilization by dephosphorylating the previously phosphorylated Ipl1 substrates (Francisco, Wang et al. 1994, Bloecher and Tatchell 1999, Rosenberg, Cross et al. 2011), once bi-orientation has been achieved. Upon a “low-tension signal” Ipl1 generates unattached KTs to activate the SAC (Cheeseman, Anderson et al. 2002, Tanaka, Rachidi et al. 2002, Pinsky, Kung et al. 2006). The kinase Mps1 seems to play the same role of Ipl1 in re-orienting erroneous KT-MT attachments that does not generate tension (Maure, Kitamura et al. 2007), although the relationship between the two kinases is still matter of debate (Maure, Kitamura et al. 2007, Bermejo, Branzei et al. 2008, Jelluma, Brenkman et al. 2008, Hewitt, Tighe et al. 2010, Maciejowski, George et al. 2010, Santaguida, Tighe et al. 2010).

1.3.5.4 The spindle position checkpoint (SPOC)

In budding yeast the site of cleavage between the mother cell and the bud (corresponding to the bud-neck) is determined in G1, long before the assembly of the mitotic spindle (Casamayor & Snyder, 2002; Pruyne & Bretscher, 2000). This peculiarity causes the requirement of mechanisms to capture and shorten the cytoplasmatic microtubules at the bud-neck to orient the spindle relative to the cleavage

plane (Kusch, Meyer et al. 2002). Proper positioning of the mitotic spindle along the mother-bud axis is indeed essential for accurate chromosome segregation. Hence, when yeast cells encounter problems in spindle orientation a surveillance mechanism known as the spindle position checkpoint (SPOC), delays the cell cycle until the misaligned spindles become correctly oriented. This checkpoint prevents exit from mitosis by inhibiting the MEN network involved in the release of Cdc14 (Pereira, Hofken et al. 2000, Caydasi, Ibrahim et al. 2010). The SPOC is mainly composed of the Bfa1-Bub2 complex, Cdc5 and Kin4. During a normal cell cycle, Bfa1 is inhibited by Cdc5-mediated phosphorylation to allow exit from mitosis (Hu, Wang et al. 2001). If the spindle is improperly oriented, Kin4 phosphorylates Bfa1, thereby preventing the Cdc5-mediated inhibitory phosphorylation and promoting its negative role in mitotic exit (D'Aquino, Monje-Casas et al. 2005, Pereira and Schiebel 2005).

This regulatory network is based on the sub-cellular localization of its components. Kin4 is localized on the mother cell cortex through most of the cell cycle (only in late anaphase it moves to the bud-neck) (D'Aquino, Monje-Casas et al. 2005, Pereira and Schiebel 2005) and its localization depends on the phosphatase $PP2A^{Rts1}$, suggesting that the phosphatase is an additional SPOC component (Chan and Amon 2009). The MEN inhibitor Bfa1-Bub2 complex initially localizes to both the SPBs but as soon as the spindle elongates in the bud it relocates exclusively on the SPB that enters the bud (Fraschini, D'Ambrosio et al. 2006). This transfer of the Bfa1-Bub2 complex impedes Kin4 kinase, confined into the mother cell, to counteract the inhibitory phosphorylation of Bfa1-Bub2 mediated by Cdc5. If the spindle fails to orientate properly through the bud-neck both SPBs remain in the mother cell associated with the Bfa1-Bub2 complex. Kin4 is now capable of counteracting Cdc5 and maintaining MEN inactive (Piatti, Venturetti et al. 2006).

1.4 Mitotic transitions

1.4.1 The metaphase-to-anaphase transition

Anaphase entry is defined by the separation of sister chromatids and requires the removal of cohesin from chromosomes through the proteolytic cleavage of the cohesin subunit Scc1 (Uhlmann, Lottspeich et al. 1999, Uhlmann, Wernic et al. 2000). Scc1 cleavage is mediated by the caspase-related protease (separase) Esp1 that prior to anaphase is held inactive by the securin Pds1. Only when all sister chromatids are bipolarly attached to the spindle and the SAC is satisfied will the APC/C^{Cdc20} be activated, thereby targeting Pds1 for degradation leading to Esp1 release and activation (Cohen-Fix, Peters et al. 1996, Ciosk, Zachariae et al. 1998, Shirayama, Toth et al. 1999, Uhlmann, Wernic et al. 2000).

The interaction between Pds1 and Esp1 also seems to be regulated by post-translational modifications. In particular Cdc28 directly phosphorylates Pds1 on multiple sites and these events seem to be crucial for the ability of Pds1 to interact efficiently with Esp1 (Agarwal and Cohen-Fix 2002). Importantly, the Pds1-Esp1 interaction not only inhibits Esp1 but it is also required to promote Esp1 nuclear localization. Pds1 is indeed a nuclear protein (Jensen, Segal et al. 2001).

Although Pds1 is a key regulator of the metaphase-to-anaphase transition at least one other mechanism may exist that control the timing of Scc1 cleavage, independently of Pds1. Indeed cells lacking Pds1 are viable and separate sister chromatids with kinetics similar to wild type cells, indicating a cell cycle regulation of Scc1 cleavage even if in the absence of Pds1. In regards of this, a role has been suggested for S phase cyclins in sister chromatids cohesion. Clb5 and Clb6 indeed seem to act in parallel with Pds1 to ensure the cohesion between sister chromatids so that *clb5Δ clb6Δ* cells show cohesin defects, not due to a premature activation of Esp1 but reflecting a less-efficient cohesins

loading onto chromosomes in early S phase (Hsu, Erickson et al. 2011). Moreover, the complete cleavage of Scc1 is strongly enhanced by Cdc5-mediated phosphorylation of Scc1 on serine residues adjacent to the Esp1-recognition sites. Cdc5-mediated phosphorylation of Scc1 also facilitates its removal from chromosomes after the cleavage (Alexandru, Uhlmann et al. 2001, Hornig and Uhlmann 2004).

Cohesion between sister chromatids opposes the pulling forces exerted by the mitotic spindle to separate them, preserving the identity of each pair of chromosomes until bi-orientation is achieved. At anaphase onset, the loss of cohesion between sister chromatids allows the spindle forces, no more counteracted, to move the separated sisters toward opposite poles (Shirayama, Toth et al. 1999, Tanaka, Fuchs et al. 2000, Uhlmann, Wernic et al. 2000). The Esp1-mediated cleavage of cohesin rings at the metaphase-to-anaphase transition is both indispensible and sufficient to segregate the bulk of the genome (Uhlmann, Wernic et al. 2000), although some specific DNA regions require additional tricks to be properly segregated.

For example telomeres and the rDNA repeat arrays maintain topological linkages until anaphase in spite of cohesin cleavage. The resolution of these topological entanglements is then a prerequisite to allow the complete segregation of these DNA loci and of the genome as a whole (D'Amours, Stegmeier et al. 2004, Pereira and Schiebel 2004, Sullivan, Higuchi et al. 2004, Wang, Yong-Gonzalez et al. 2004).

Cohesin-independent mechanisms of cohesion might be mediated also by catenates, arising with the replication process (Murray and Szostak 1985). The rotation of the DNA around its axis ahead of the replication fork leads to the formation of intermolecular intertwines between the two emerging sister chromatids. These catenates physically link together the two sisters and thus must be removed by the topoisomerase II (Top2) for chromosome segregation to occur (Peter, Ullsperger et al. 1998, Lucas, Germe et al. 2001).

1.4.2 Exit from mitosis

Progression through mitosis relies on the Cdc28 kinase associated with the mitotic cyclins Clb1-4 (Hartwell, Culotti et al. 1974). Exit from mitosis is therefore dependent on the inactivation of these Clb-Cdc28 complexes and on the reversal of all the Cdc28-mediated phosphorylation events previously required for mitotic progression. In budding yeast both events are driven by Cdc14, so that cells lacking its function arrest in anaphase with high levels of Clb-CDK activity (Visintin, Craig et al. 1998, Stegmeier and Amon 2004),

Cdc14 down-regulates Clb-CDK complexes via two parallel mechanisms. On one hand it dephosphorylates and activates the co-activator of the ubiquitin ligase APC/C, Cdh1 (Visintin, Craig et al. 1998, Jaspersen 1999). APC/C^{Cdh1} is then directly responsible for targeting to degradation amongs other the Clb cyclins (Schwab 1997, Visintin, Prinz et al. 1997, Zachariae, Schwab et al. 1998). On the other hand, Cdc14 promotes the accumulation of the CDK inhibitor Sic1, by both dephosphorylating and thus stabilizing Sic1 and by enhancing its transcription through the dephosphorylation of its transcription factor Swi5 (Visintin, Craig et al. 1998).

Cdc14 activity is regulated through changes in its localization. For most of the cell cycle (from G1 till metaphase) the phosphatase is kept inactive in the nucleolus through the association with its inhibitor Cfi1/Net1. Activation of Cdc14 requires its release from the nucleolus, allowing the phosphatase to reach and dephosphorylate its substrates (Shou, Seol et al. 1999, Visintin, Hwang et al. 1999, Traverso, Baskerville et al. 2001).

Cdc14 release and hence activation is regulated via two consecutive pathways. At anaphase onset the Cdc14 early anaphase release (FEAR) network regulates only a transient release of the protein from the nucleolus to the nucleoplasm. FEAR-mediated activation of Cdc14 is not essential for exit from mitosis. FEAR mutants only show 10-20 minutes delay in exit from mitosis but they have problems in anaphase events like

nuclear positioning (Ross and Cohen-Fix 2004), spindle assembly and stabilization, (Khmelinskii, Lawrence et al. 2007, Khmelinskii, Roostalu et al. 2009) and rDNA segregation (D'Amours, Stegmeier et al. 2004, Pereira and Schiebel 2004, Sullivan, Higuchi et al. 2004, Wang, Yong-Gonzalez et al. 2004). Later in anaphase the mitotic exit network (MEN), drives the full and complete release of Cdc14 that can now move also into the cytoplasm (Shou, Seol et al. 1999). Unlike FEAR, MEN mutants are no more able to proceed after anaphase indicating the MEN as an essential network for mitotic exit (Jaspersen, Charles et al. 1998). The polo like kinase Cdc5 is the only protein shared by the two pathways. Hence, its depletion results in a complete lack of Cdc14 release and activity during anaphase. Its overexpression is sufficient to promote the release of Cdc14 in cell cycle phases other than anaphase (Visintin, Stegmeier et al. 2003).

The interaction between Cdc14 and Cfi1 is strongly dependent on the phosphorylation status of the two proteins. While their association requires both the proteins to be un-phosphorylated, their dissociation requires specific phosphorylation events on both Cfi1 and Cdc14 (Visintin, Stegmeier et al. 2003). A “two-hit model” describes the release of Cdc14 through a time-regulated phosphorylation of both Cfi1 and Cdc14 (Manzoni et al., 2010). According to this model the polo like kinase Cdc5 is the kinase responsible for the phosphorylation of Cdc14 while Cfi1 phosphorylation is mediated by a “Cdc5-partner kinase” that changes accordingly to the cell cycle moment. In particular, Clb5-CDK and Clb2-CDK complexes phosphorylate Cfi1 contributing, togheter with Cdc5, to the first release of Cdc14 (Manzoni et al., 2010)(Azzam, Chen et al. 2004). Clb5 degradation at anaphase onset is followed by a progressive degradation of Clb2 so that MEN activation becomes essential to compensate for the inactivation of the two Clbs and to sustain the release of Cdc14 in late anaphase. In this model the MEN-kinase Dbf2 cooperates with Cdc5 for the second phase of Cdc14 release, phosphorylating

Cfi1 (Manzoni, Montani et al. 2010). Moreover, Cdc5 seems to preferentially phosphorylate substrates already phosphorylated by other kinases (Elia, Cantley et al. 2003, Elia, Rellos et al. 2003) ((Rahal and Amon 2008). Based on the "two-hit model", it is possible to speculate that priming by Clbs–CDK and Dbf2 serves to build up Cdc5 activity (Manzoni et al., 2010).

1.4.2.1 The MEN network

The MEN network is a Ras-like GTPase signal transduction pathway that regulates exit from mitosis (Jaspersen, Charles et al. 1998). It comprises the GTPase Tem1, the GTPase-activating protein (GAP) complex Bub2-Bfa1, the putative GTP-GDP exchange factor (GEF) Lte1, and the kinases Cdc5, Cdc15 and Dbf2, the latter in association with Mob1.

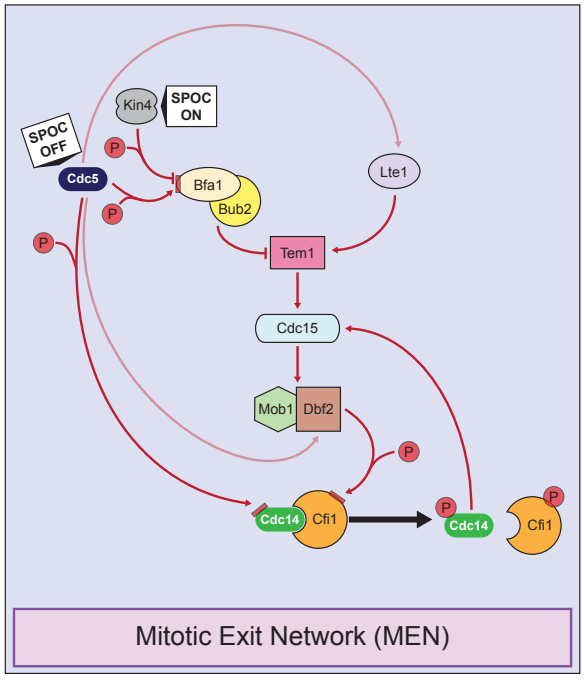

Figure 1.8. The mitotic exit network (MEN). The MEN is essential to maintain Cdc14 active (released from its nucleolar inhibitor Cfi1) in the later stages of anaphase. MEN major components and their interplay are shown in the scheme.

Interestingly, the specificity in the localization of the MEN components coordinates exit from mitosis with spindle position. The scaffold protein Nud1 anchors Tem1 on the SPB that will enter into the bud, while the Tem1-activator Lte1 moves to the bud cortex as the bud emerges (Bardin, Visintin et al. 2000). In this way the activation of Tem1 requires the migration of the Tem1-bearing SPB into the bud, allowing Tem1 to meet its activator. A second requirement for Tem1 activation is the Cdc5-mediated inhibition of Bfa1, resulting in the dissociation of the Bub2-Bfa1 complex (Hu, Wang et al. 2001). In the presence of a misaligned spindle, the SPOC inhibits mitotic exit by interfering with the inactivation of the Bub2-Bfa1 complex (Pereira, Hofken et al. 2000). Once Tem1 is active, Cdc15 phosphorylates Dbf2, thereby activating the Dbf2-Mob1 kinase complex (Mah, Jang et al. 2001). In particular, a "scaffold-assisted" process is also required for Dbf2-Mob1 activation. This process required a Cdc15-mediated phosphorylation of Nud1 as prerequisite for the following phosphorylation of Dbf2. Phosphorylated Nud1 acts as a phospho-docking site to which Dbf2 binds to be itself phosphorylated by Cdc15 (Rock, Lim et al. 2013). Dbf2 is indeed the MEN kinase suggested to be responsible for Cfi1 phosphorylation (Manzoni, Montani et al. 2010). Dbf2 activity is also required to allow the translocation of Cdc14 from the nucleus into the cytoplasm. This happens through a Dbf2-mediated phosphorylation of Cdc14 on serine and threonine residues adjacent to its nuclear localization signal (NLS), thereby abrogating its NLS activity (Mohl, Huddleston et al. 2009).

Cdc5 contribution to the MEN is not limited to the inactivation of the Bub2-Bfa1 complex (Hu, Wang et al. 2001) but Cdc5 directly regulates also Lte1 and Dbf2, activating the MEN in different ways. Significantly, *cdc5* mutants completely lack MEN signalling (Visintin, Stegmeier et al. 2003).

Cdc14 released by the FEAR network and MEN positively regulates Cdc15, thereby auto-enhancing its own MEN-mediated release through a positive feedback loop (Bardin, Boselli et al. 2003).

Later in anaphase, however, Cdc14 has to be re-sequestered into the nucleolus to avoid severe growth defects and let the cells proceed into the next cell cycle. Interestingly, Cdc14 drives its own inactivation through negative feedback loops. On one hand Cdc14 induces the inactivation of the MEN through the dephosphorylation and thus re-activation of the MEN-inhibitor Bfa1 (Pereira, Manson et al. 2002). More importantly, Cdc14-mediated down-regulation of Clbs-CDK activities triggers the activation of the APC/C^{Cdh1}, which in turn targets Cdc5 to degradation thereby silencing the MEN (Visintin, Tomson et al. 2008, Lu and Cross 2010, Manzoni, Montani et al. 2010).

1.4.2.2 The FEAR network

The FEAR network is known as the pathway responsibles for the transient release of Cdc14 that is detectable in MEN mutants in early anaphase (Stegmeier, Visintin et al. 2002). The existence of an additional pathway controlling the early-anaphase release of Cdc14 was originally suggested by data showing that MEN is active later in anaphase after Cdc14 had already been released from the nucleolus into the nucleus (Visintin and Amon 2001). Evidence indicating that the protease Esp1 has additional roles in promoting exit from mitosis (Cohen-Fix and Koshland 1999, Tinker-Kulberg and Morgan 1999, Sullivan and Uhlmann 2003) allowed the identification Esp1 as the first member of the FEAR network. Additional members of the FEAR network are the Cdc5 kinase, the Clb1/2-CDKs complexes, the protein phosphatase type 2A (PP2A) associated with Cdc55, the replication fork block protein Fob1, the kinetochore protein Slk19, and the nucleolar protein Spo12 (as well as its paralog Bns1) (Stegmeier, Visintin et al. 2002, Rock and Amon 2009).

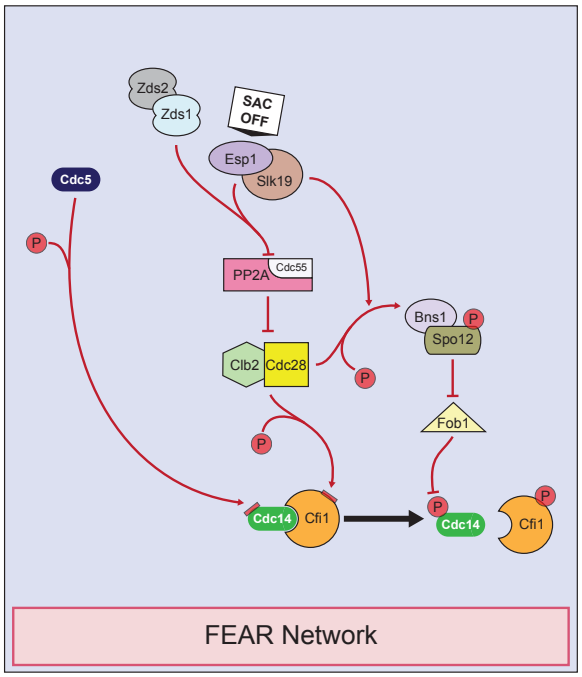

Figure 1.9. The FEAR network. The FEAR network mediates Cdc14 activation at anaphase onset inducing the dissociation of the phosphatase from its nucleolar inhibitor Cfi1. The major components of this network and their interplay are shown in the scheme.

At anaphase onset the Esp1/Slk19 complex seems to downregulate the phosphatase $PP2A^{Cdc55}$ (Queralt, Lehane et al. 2006, Wang and Ng 2006, Yellman and Burke 2006), allowing the Clb2-mediated phosphorylation of Cfi1 and the release of Cdc14 (Azzam, Chen et al. 2004). Moreover, two Cdc55-interacting proteins (Zds1-2) cooperate with Esp1 to downregulate $PP2A^{Cdc55}$ (Queralt and Uhlmann 2008). In parallel, the Esp1/Slk19 complex indirectly promotes the Clb2-mediated phosphorylation and activation of Spo12. Active Spo12 is then required to antagonize Fob1, the negative regulator of Cdc14 that prior to anaphase stabilizes its association with Cfi1 (Stegmeier, Huang et al. 2004, Tomson, Rahal et al. 2009). In this network of proteins Cdc5 can be placed downstream of and/or in parallel to the Esp1/Slk19 complex. Indeed it has been shown that Cdc5 is able to interact with Esp1 and Slk19 (Rahal and Amon 2008) and to phosphorylate Cdc14 *in vivo* and *in vitro* (Shou, Azzam et al. 2002, Yoshida, Asakawa et al. 2002, Visintin, Stegmeier et al. 2003).

Once active, the FEAR network mediates the release of Cdc14 through the Clb1/2-mediated phosphorylation of six CDK consensus sites on Cfi1 (Azzam, Chen et al. 2004).

1.5 Mitotic players

1.5.1 Condensins

A proper distribution of the genetic material between mother and daughter cell requires appropriate changes in the chromosome structure. Upon entry into mitosis single chromosomes must be compacted into individual entities to be further segregated by the mitotic spindle. This process is known as chromosome condensation and strongly relies on multi-protein complexes referred to as condensins (Koshland and Strunnikov 1996).

Condensins comprise two structural maintenance of chromosome (SMC) proteins, Smc2 and Smc4, and three regulatory non-SMC elements. SMCs have an ATP-binding cassette (ABC) ATPase "head" domain connected through a 50-nm-long anti-parallel coiled coil to a "hinge" domain. Smc2 and Smc4 dimerize via their "hinge" domains while their "head" domains are bridged by a kleisin subunit (Brn1 in budding yeast) forming a closed structure required to encircle chromosomes. The kleisin subunit has the additional role of recruiting to condesin other two components of the complex, two proteins containing multiple alpha-helical HEAT repeats (Ycs4 and Ycg1 in budding yeast) (Neuwald and Hirano 2000). Interestingly, a mutation of any of the five condensin subunits results in incomplete chromosome segregation in anaphase in all organisms studied so far.

Metazoans have two condensin complexes (condensin I and II) that bind to mitotic chromosomes with different spatio-temporal rules (Ono, Losada et al. 2003, Hirota, Gerlich et al. 2004, Ono, Fang et al. 2004), whereas *S. cerevisiae* has a unique condesin complex. Moreover, most eukaryotes show a multi-sites phospho-regulation of the condensin complex, involving CDKs as well as Polo and Aurora kinases (Bazile, St-Pierre et al. 2010), yet in yeast mechanisms that underlie condensin regulation are less clear.

In budding yeast, condensin seems to have the more important function in the segregation of rDNA loci and hence condensin has been found to concentrate to rDNA regions although it associates also with pericentromeric and peritelomeric chromosome regions (Freeman, Aragon-Alcaide et al. 2000). While the bulk of the genome is already condensed in metaphase, rDNA condenses later and maintains catenations until anaphase. rDNA condensation correlates with rDNA disentanglement and they both are dependent on Cdc14. rDNA condensation depends on the early-anaphase Cdc14 activation. FEAR-released Cdc14 regulates condensins enrichment at the rDNA inducing the sumoylation of the Ycs4 condensin component (D'Amours, Stegmeier et al. 2004). rDNA condensation during anaphase also requires the phosphorylation of condensin component Ycg1 by Ipl1, whose activity is up-regulated at the metaphase-to-anaphase transition (Lavoie, Hogan et al. 2004). Cdc14-mediated enrichment of condensins to rDNAs in anaphase is a prerequisite to further disentangle these loci. Top2 is the enzyme responsible of catenates resolution and its recruitment to rDNA depends on the anaphase Ycs4 accumulation mediated by Cdc14 (Bhalla, Biggins et al. 2002, D'Amours, Stegmeier et al. 2004, Sullivan, Higuchi et al. 2004).

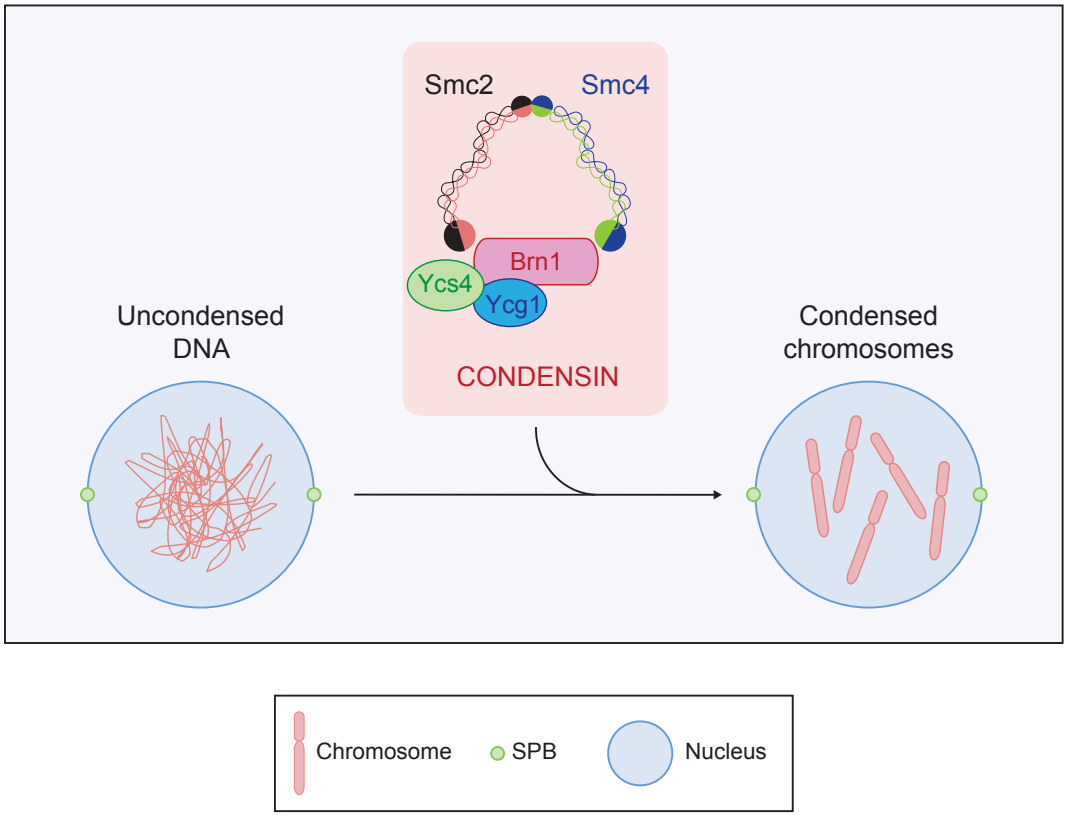

Figure 1.10. Condensins. Condensin subunits are shown as well as their role in chromosome condensation is schematically depicted.

1.5.2 Cohesins

Accurate cell division requires that each daughter cell receives one exact copy of every chromosome. Since the time of their synthesis in S phase until the moment of their separation in anaphase every pair of duplicated chromosomes must remain tethered together to maintain their couple-identity (Uhlmann and Nasmyth 1998).

Establish sister chromatid cohesion means pairing the two sisters together and this process mainly depends on the protein complex named cohesin. The cohesin complex is composed of four subunits: two members of the SMC family, Smc1 and Smc3, and two non-SMC proteins, the α-kleisin subunit Scc1/Mcd1 and Scc3 (Guacci, Koshland et al. 1997, Michaelis, Ciosk et al. 1997). Smc1 and Smc3 are rod-shaped proteins with an ABC ATPase domain connected via long coiled-coil to a dimer-forming hinge domain. Using their dimerization domains they create V-shaped Smc1/Smc3 heterodimers while

their ATPase ends are bridged by the Scc1 subunit of the complex. The result is a tripartite ring complex that topologically traps sister chromatids in close proximity within its structure (Haering, Lowe et al. 2002, Gruber, Haering et al. 2003). The Scc3 subunit of the complex binds the C-terminal region of Scc1 after its interaction with the two SMC proteins but its role is debatable. It has been proposed that Scc3 as well as Pds5 are cohesin maintenance factors that stabilize the ring and its association to the chromosomes (Panizza, Tanaka et al. 2000). Other data suggest a role of Scc3 and Pds5 mainly during cohesion establishment in S phase (Kulemzina, Schumacher et al. 2012).

Even if the cohesion between sister chromatids is established during DNA replication (Uhlmann and Nasmyth 1998) cohesin complexes already bind to the chromosome shortly before S phase (Michaelis, Ciosk et al. 1997).

Loading of cohesins onto chromosomes is a multi-step process initially mediated by the Scc2-Scc4 complex (Ciosk, Shirayama et al. 2000). Soon after, the establishment of the cohesion requires the S phase-specific acetyltransferase Ctf7/Eco1 (Skibbens, Corson et al. 1999, Toth, Ciosk et al. 1999). Ctf7/Eco1 acetylates two lysine residues in the ATPase domain of Smc3 to establish the cohesive structure between sisters (Rolef Ben-Shahar, Heeger et al. 2008, Zhang, Shi et al. 2008, Heidinger-Pauli, Onn et al. 2010). Ctf7/Eco1 acetylation of Smc3 seems to counteract the activity of the protein Wlp1/Rad61 that is instead involved in the destabilisation of chromatin-bound cohesin (Chatterjee, Zakian et al. 2013).

Centromere-bound cohesin is essential for the proper attachment of chromosomes to the mitotic spindle. Soon after it yields to the resulting forces generated by the mitotic spindle causing sisters' centromere and proximal chromatin to split before the onset of anaphase. The remaining cohesins sticked to the neighboring MTs regions become now primary to prevent the complete and premature split of the sisters (Tanaka, Fuchs et al. 2000). Cohesin contribution to sister cohesion varies upon the region of the

chromosomes considered, and it is much higher at telomeric regions than at chromosome arms or pericentromeric regions (Diaz-Martinez, Gimenez-Abian et al. 2008). This evidence opens to the possibility that the residual cohesion in these particular loci could be mediated by catenation (Bermejo, Branzei et al. 2008). Indeed, centromeric catenation seems to be actively preserved up to anaphase when Top2 is specifically sumoylated to become enriched at centromeres and to remove the persisting catenates (Bachant 2009).

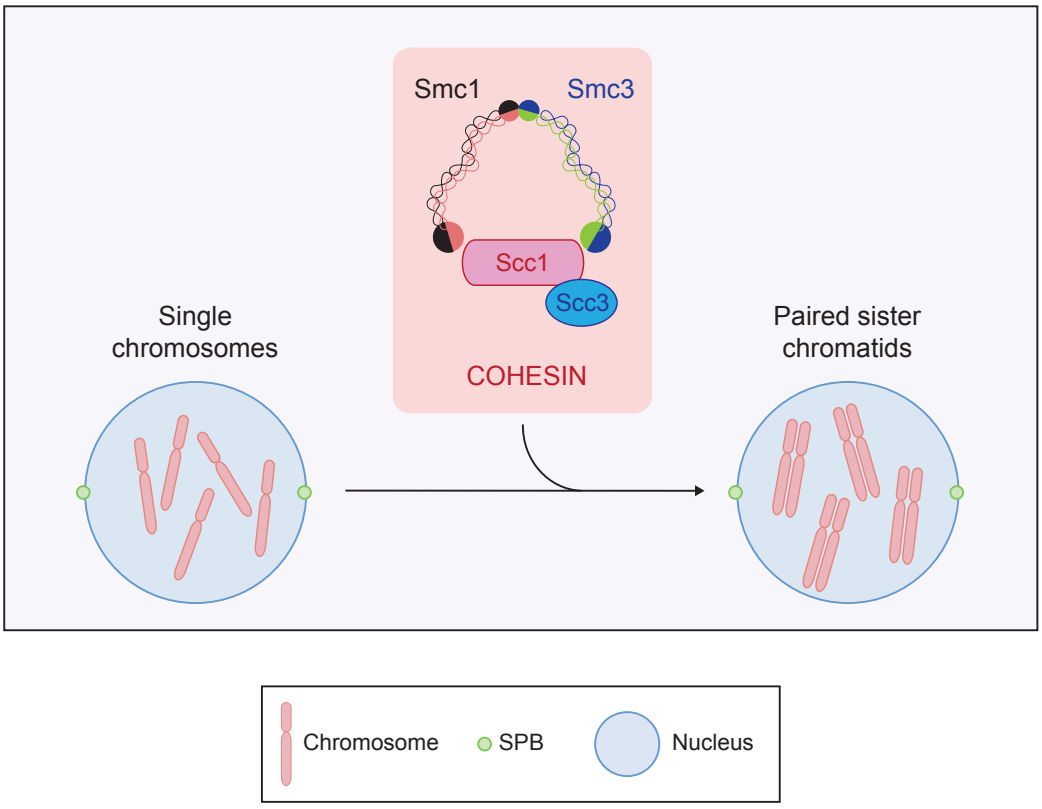

Figure 1.11. The cohesin ring complex. Cohesin subunits are shown as well as their role in sister chromatid cohesion is schematically depicted.

1.5.3 Kinetochores

The link between replicated chromosomes and spindle MTs is mediated by a protein structure called kinetochore (KT). Kinetochores establish the bipolar alignment of the sister chromatids onto the mitotic spindle but they are also involved in sensing erroneous attachments and triggering the spindle assembly checkpoint.

Kinetochores assemble on centromeres (CENs) containing (a) nucleosome(s) carrying the histone H3 variant CENP-A, named Cse4 in budding yeast (Mellone and Allshire 2003, Black and Bassett 2008). Higher eukaryotes have "regional CENs" that are extended regions of DNA assembling multiple kinetochores to bind multiple MTs. *S. cerevisiae* possesses instead "point CENs" corresponding to short DNA sequence of 125 bp on which one KT assembles that binds only one MT (Hegemann and Fleig 1993).

KT can be divided into inner KT and outer KT: the inner part directly binds chromosome CENs whereas the outer part interacts with spindle MTs. Moreover the inner KT seems to be directly involved in the recruitment of the outer KT.

The inner KT comprises the CBF3 complex (composed of a dimer of Cep3, a dimer of Ndc10, and a Skp1:Ctf13 heterodimer) (Lechner and Carbon 1991) and the proteins Cbf1 (Mellor, Jiang et al. 1990) and Mif2 (Meluh and Koshland 1995). CBF3 is required for the recruitment of all KT components so that cells lacking this complex fail to establish KT-MT attachments *in vivo* and *in vitro* (Goh and Kilmartin 1993, Sorger, Severin et al. 1994, He, Rines et al. 2001).

The KT-MT binding interface is composed of three sub-complexes referred to as "linker" complexes: the Spc105 complex, the MIND complex and the Ndc80 complex. All these complexes contribute to establish a proper KT-MT attachment site but the central element in the connection between MTs and the centromeric platform is the Ndc80 complex (Alushin and Nogales 2011, Tooley and Stukenberg 2011). There are

six to eight Ndc80 complexes per kinetochore in budding yeast (Joglekar, Bouck et al. 2006) and each Ndc80 complex is composed of two heterodimers: Ndc80:Nuf2 and Spc24:Spc25 (Wei, Sorger et al. 2005). The N-termini of Ndc80 and Nuf2 bind to the MT (Wei, Al-Bassam et al. 2007) while the Spc24:Spc25 heterodimer attaches to the MIND complex (composed of Mtw1, Nsl1, Nnf1, and Dsn1) (De Wulf, McAinsh et al. 2003). The heterotrimeric complex composed of Spc105 and Kre28 is the third complex responsible for bridging centromeres and MTs and also directly binds to MTs (Pagliuca, Draviam et al. 2009).

Likely, mechanisms exist to alter the KT structure upon MT binding. The DASH complex (Dam1, Duo1, Dad1-4, Ask1, Spc19, Spc34, and Hsk3,) is a heterodecamer complex bound to MTs that moves from MTs to KTs after the KT-MT attachment (Janke, Ortiz et al. 2002, Li, Bachant et al. 2002). The DASH complex migrates to KTs to adapt the KTs to the new status of bound-KTs. It contributes to the maintenance of bipolar KT-MT interaction auto-assembling closed rings around spindle MTs (Miranda, De Wulf et al. 2005, Westermann, Avila-Sakar et al. 2005).

DASH component (like Dam1) as well as inner (like Mif2 and Ndc10) and outer (like Ndc80 and Dsn1) KT components are regulated by Ipl1-mediated phosphorylation events, suggesting a multiple levels of regulation of this kinase on KTs.

Yeast KTs comprises also lot of motor proteins and MT-associated proteins (MAPs) that seem to be recruited via the three “linker” complexes at outer KTs (Pagliuca, Draviam et al. 2009).

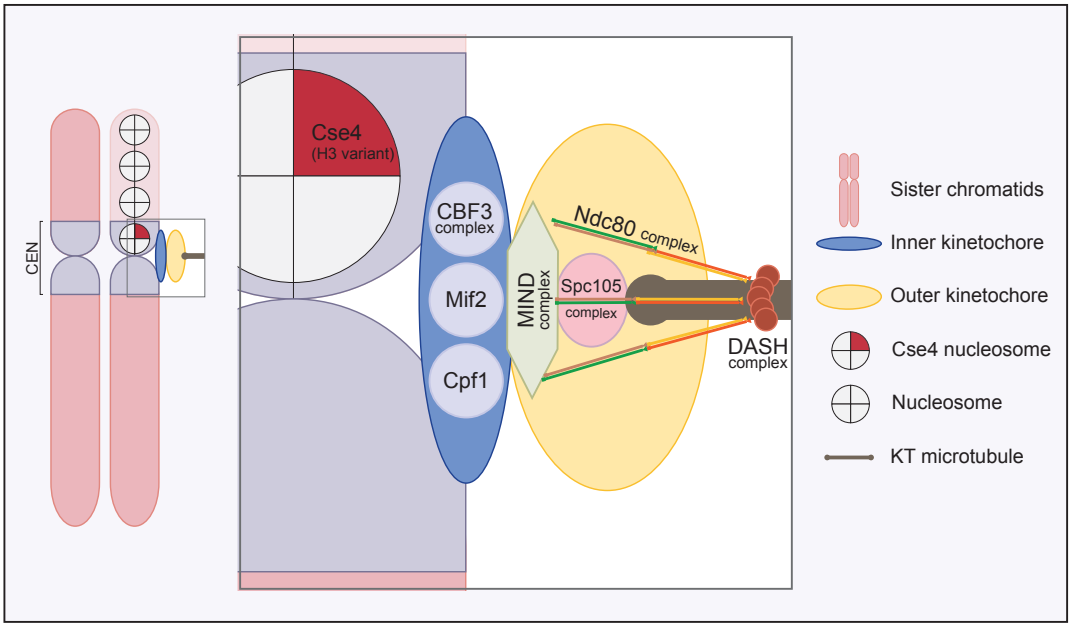

Figure 1.12. The *S. cerevisiae* kinetochore. Kinetochore assembles hierarchically from preformed subcomplexes on centromeric DNA (CEN). The main complexes of the inner and the outer part of yeast kinetochores are shown.

1.5.4 The mitotic spindle

The mitotic spindle is responsible for chromosome segregation during mitosis. Central elements of the mitotic spindle are microtubules (MTs), 25 nm tubular polymers composed of 13 protofilaments of αβ-tubulin heterodimers. Mitotic MTs are nucleated by a group of proteins, inserted within the nuclear membrane, that form the spindle pole bodies (SPBs). SBPs are considered the yeast microtubule-organizing centre (MTOC) with the γ-tubulin complex (composed of γ-tubulin or Tub4, Spc97 and Spc98) being the effective element specialized in MTs nucleation (Schiebel 2000, Vinh, Kern et al. 2002, Kollman, Polka et al. 2010). In particular, the minus-ends of the MTs are nucleated at the SPB while the plus-ends extend far from the SPB and bind the kinetochores.

Each SPB comprises 18 proteins (Huh, Falvo et al. 2003, Keck, Jones et al. 2011), 16 of which essential for cell viability. The MTOC can be divided in three regions: an inner plaque that faces the nucleoplasm and nucleates the nuclear MTs of the mitotic spindle,

a central plaque placed in the plane of the nuclear envelope and the outer plaque that faces the cytoplasm and nucleates the cytoplasmic (or astral) MTs, required for positioning the nucleus during the division cycle (Winey 2012).

Spc42 is one of the major components of the central plaque and is in contact with the outer plaque component Cnm67 and with the only protein connecting the central plaque to the inner plaque, Spc110 (Kilmartin 1993, Muller, Snydsman et al. 2005). The N-terminus of Spc110 binds to the γ-tubulin (Tub4) complex promoting its role in MTs nucleation (Knop 1997, Nguyen 1998, Vinh, Kern et al. 2002, Kollman, Polka et al. 2010). Cdc28- and Mps1-mediated phosphorylation of this Spc110 N-terminal domain of Tub4 controls its nucleation activity as well (Friedman, Kern et al. 2001, Huisman, Smeets et al. 2007).

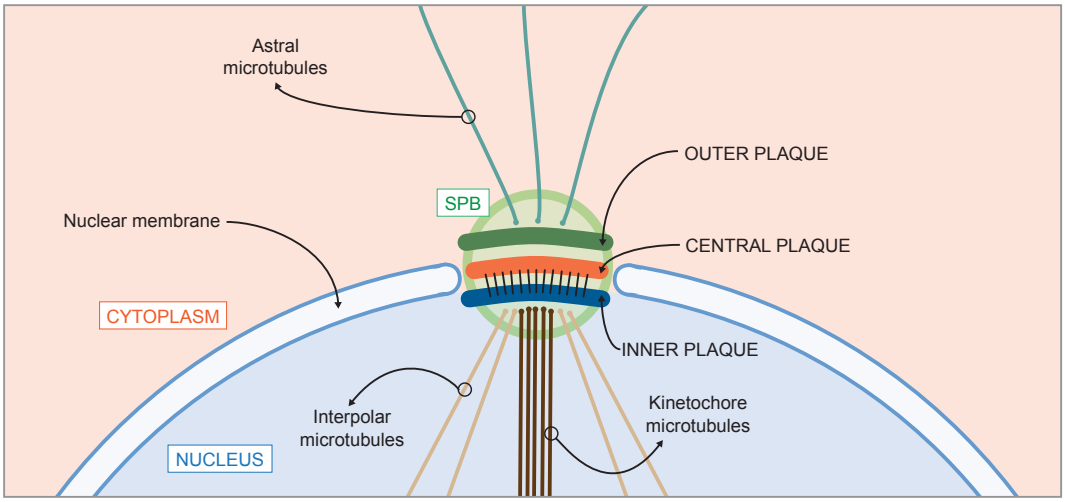

Figure 1.13. The yeast microtubule-organizing centre. Schematic representation (i) of the yeast MTOC structure (named SPB) and (ii) of the different types of MTs (astral, interpolar or kinetochore MTs) nucleate from the cytoplasmic or the nuclear side of the SPB.

At a certain point in G1 phase the SPB has to be duplicated as a prerequisite for the formation of a bipolar mitotic spindle. This event occurs in a step-wise manner starting with the duplication of the single SBP. Then the two side-by-side just duplicated SBPs

are separated and pushed apart by the activities of the SPBs component Sfi1 (Anderson, Prudden et al. 2007), Cdc28 (Fitch, Dahmann et al. 1992) and the motor proteins Cin8 and Kip1 (Roof, Meluh et al. 1992, Saunders and Hoyt 1992).

The organization of the mitotic spindle in *S. cerevisiae* has been analyzed by computer-assisted reconstruction from electron micrographs of serially cross-sectioned spindles (Winey, Mamay et al. 1995) and by high-voltage electron tomography of cell sections (O'Toole, Winey et al. 1999). These studies confirmed that the mitotic spindle is composed of two classes of nuclear MTs, both nucleated by the SPBs. In particular, sixteen kinetochore MTs directly contact chromosomes in a 1:1 ratio while about four interpolar MTs emanating from each SPB in the nucleoplasm, interdigitate at their plus-ends to stabilize the bipolar spindle (Winey, Mamay et al. 1995, O'Toole, Winey et al. 1999).

The extending plus-ends of MTs are highly dynamic and alternate phases of growth (rescue) and shortening (catastrophe). This phenomenon, defined as dynamic instability, allows kinetochore MTs to “explore” the space and to find and bind to sister chromatids resulting in a bipolar alignment of all sisters onto the mitotic spindle.

1.6 Mitotic spindle dynamics

1.6.1 Mitotic spindle orientation and positioning

Spindle positioning requires the interaction between astral MTs and the cell cortex (Shaw 1997, Yeh, Yang et al. 2000). Kar9 and dynein are the central members of two redundant pathways controlling this process (Miller 1998, Moore, Stuchell-Brereton et al. 2009), and the lack of both these pathways is lethal.

The Kar9 pathway facilitates nucleus migration to the bud-neck before anaphase entry. This pathway orientates astral MTs from one SPB into the bud using actin filaments of the cytockeleton as rails. The microtubule-binding protein Bim1 mediates the interaction between Kar9 and MTs, while the class-V myosin motor Myo2 is responsible for its transportation (Beach D.L.; Thibodeaux 2000, Gardner, Haase et al. 2008).

Dynein unique function is required after Kar9 to generate the force needed for nuclear positioning (Eshel, Urrestarazu et al. 1993, Li, Yeh et al. 1993). Dynein is a large cytoplasmic polypeptide member of the minus-end directed family of motor proteins that specifically localizes on astral MTs. In particular, dynein is targeted to the plus-end of astral MTs through the cooperation between the microtubule-binding protein Bik1 and the plus-end directed kinesin Kip2 (Carvalho, Gupta et al. 2004) and once there it is retained at their plus-ends by the Pac1-Ndl1 complex (Lee, Kaiser et al. 2005). Then dynein is “offloaded” from MTs to the cortex in a process that requires the multi-subunit complex dynactin (Moore, Li et al. 2008) and the cortical protein Num1, that acts also as dynein receptor on non-motile cell cortex foci (Heil-Chapdelaine 2000). On the cortex, dynein is activated and drives the sliding of the astral MTs along the cortex through a lateral contact between them, pulling the MTs and the attached SPB in the direction of the contact with the cortex (Moore, Stuchell-Brereton et al. 2009). The

timing of dynein movements and activity is cell cycle regulated and precisely some of the elements responsible for anaphase entry seem to be directly involved in this control. Examples are some proteins of the DNA damage checkpoint (Dotiwala, Haase et al. 2007) and FEAR network components (Dotiwala, Haase et al. 2007).

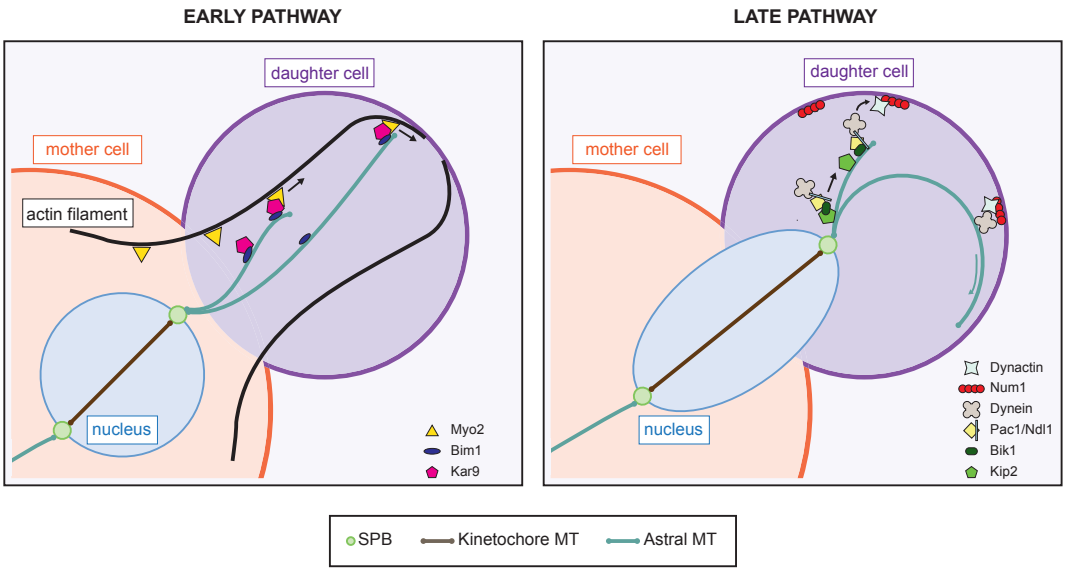

Figure 1.14. Mitotic spindle orientation in budding yeast. Two sequential pathways drive the positioning of the nucleus and with-it of the elongating mitotic spindle along the mother-bud axis. Before anaphase entry the Kar9 pathway mediates the movement of the nucleus close to the bud-neck while its following stretching between the mother and the daughter cell depends upon the dynein pathway.

1.6.2 Mitotic spindle elongation

Anaphase spindle elongation after the removal of the cohesion between sister chromatids is divided into an anaphase A and anaphase B step. Anaphase A is characterized by the movement of the separated chromosomes toward the SPB they are in contact with and this pole-ward movement is mediated by the shortening of the kinetochore MTs. During anaphase B the complete segregation of the two sets of SPBs-associated chromosomes is achieved through a progressive increase in the length of the interpolar MTs and hence in the pole-to-pole distance (Pearson, Maddox et al. 2001).

Moreover, the full elongation of the spindle in anaphase B happens in two steps. In a first phase the spindle elongates fast (0.54 μm/min) and from the typical length of a metaphase spindle (1,5-2 μm) it reaches a length of 4-6 μm. Then, MT elongation rate decreases (0.21 μm/min) allowing the spindle to reach the final length of 8-10 μm (Straight, Sedat et al. 1998, Movshovich, Fridman et al. 2008).

Anti-parallel interpolar MTs emanating from opposite SPBs overlap in the spindle midzone. This is important to maintain spindle bipolarity and to generate forces required for spindle elongation. In this domain MTs organize in highly ordered geometrical arrays and both organization and functions of this domain depend on motor proteins and microtubule-associated proteins (MAPs).

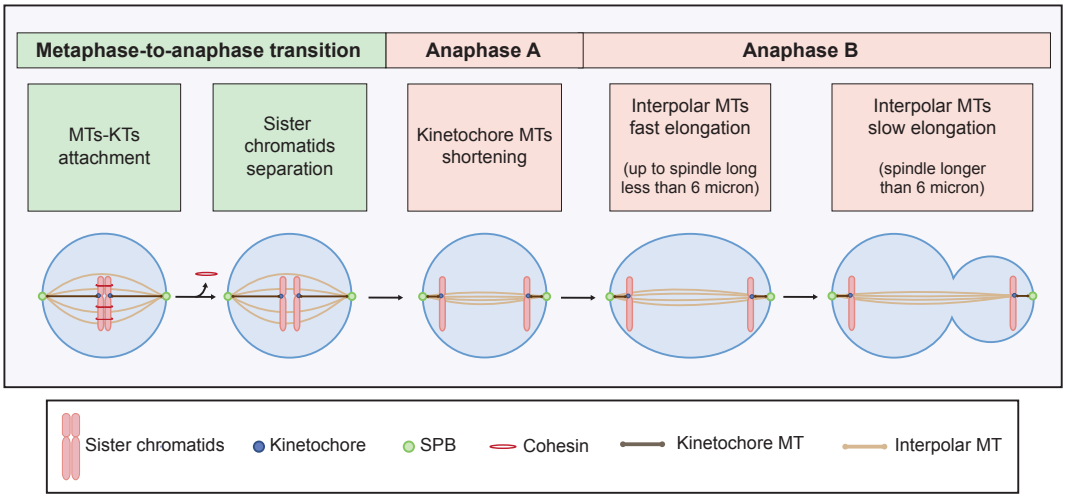

Figure 1.15. Spindle dynamics and chromosome segregation. Chromosome segregation starts after cohesin cleavage at the metaphase-to-anaphase transition. Soon after cohesin dissolution a shortening in the kinetochore MTs length mediates a pole-ward movement of the chromosomes (anaphase A) while later on a progressive elongation of the interpolar MTs drives the complete segregation of the sister chromatids (anaphase B). Anaphase B spindle elongation is characterized by a switch from fast to slow in the elongation rate.

1.6.2.1 Microtubule-associated proteins (MAPs)

Phospshorylation is the main mechanism of MAPs regulation and thus the balance between kinase activity and that of counteracting phosphatases plays an essential role in dictating spindle behaviour. Upon entry into mitosis mitotic-CDKs drive a dramatic increase in MT dynamics to allow the formation of the mitotic spindle and the attachment of the spindle MTs to sister chromatid KTs. Further on spindle elongation and chromosome segregation require the stabilization of the anaphase spindles and indeed the metaphase-to-anaphase transition coincides with a strong decrease in MT dynamics. Esp1 activation triggers anaphase entry resolving the cohesion between sister chromatids but at the same time induces Cdc14 activation as a member of the FEAR network. Once active the early anaphase-released phosphatase regulates through dephosphorylation lots of MAPs required to stabilize the elongating anaphase spindles (Higuchi and Uhlmann 2005).

In budding yeast, one of the major midzone MAPs is Ase1. Ase1 homodimers bundle anti-parallel MTs at the spindle midzone (Schuyler, Liu et al. 2003) constituting a landmark for the recruitment of all the other midzone organizers. Thus, spindle midzone assembly in anaphase strongly depends on the regulation of Ase1. Ase1 is expressed in mitosis, likely after bipolar spindle formation, and its APC/C^{Cdh1}-mediated degradation contributes to the disassembly of the spindle upon exit from mitosis (Juang, Huang et al. 1997). Before anaphase onset, Ase1 is phosphorylated by Clb5-Cdc28 but must be dephosphorylated by Cdc14 to guarantee spindle midzone assembly. The lack of its dephosphorylation results in the delocalization of the other midzone components and delays the switch between fast and slow elongation rate of the spindle (Khmelinskii, Lawrence et al. 2007).

Once bound, Ase1 recruits the Esp1-Slk19 complex, whose role is to center and limit the spindle midzone to the middle of the mitotic spindle (Jensen, Segal et al. 2001,

Sullivan and Uhlmann 2003). Esp1-Slk19 recruitment to the spindle also requires the chromosomal passenger complex (CPC) (Ruchaud, Carmena et al. 2007). This complex is associated with KTs up to metaphase. Then, the dephosphorylation of the CPC component Sli15 by Cdc14 re-localizes the complex to the spindle MTs. The CPC requires this repositioning to fulfil its role in spindle midzone assembly (Pereira and Schiebel 2003). Premature CPC binding to MTs is prevented through combinatory phosphorylation of Sli15 by CDKs and Ipl1 itself (Nakajima, Cormier et al. 2011).

It has become clear that the main role of Cdc14 in regulating the spindle behaviour seems to be in limiting the activity of different MAPs to anaphase. Furthermore, the role of the phosphatase is not limited to Ase1 and Sli15 but additional proteins associated to the spindle are Cdc14 substrates. The spindle-stabilizing protein Fin1 is kept detached from the spindle when phosphorylated by Clb5-Cdc28, requires Cdc14-mediated dephosphorylation to interact with SPBs and MTs of elongating anaphase spindles and upon mitotic exit is targeted to degradation by the activated APC/C^{Cdh1} ubiquitin ligase (Woodbury and Morgan 2007). The kinetochore DASH component Ask1 is another mitotic protein whose activity is regulated through CDK phosphorylation and Cdc14-mediated dephosphorylation (Li and Elledge 2003). Also the MAP Stu1 (Yin, You et al. 2002) needs to be dephosphorylated by Cdc14 to localize to the spindle. These Cdc14-dependent dephosphorylation events directly contribute to reduce MT dynamics at anaphase onset (Higuchi and Uhlmann 2005).

Of note, in addition to the role of Cdc14 in spindle stabilization also sumoylation has important functions. Lots of kinetochore-associated proteins need to be sumoylated to move from KTs to anaphase spindles contributing to their stability (Montpetit, Hazbun et al. 2006).

1.6.2.2 Kinesin-related motor proteins

Microtubule motor proteins use the energy derived from ATP hydrolysis to generate the mechanical energy required to move and segregate spindle-attached chromosomes. *S. cerevisiae* genome encodes for five kinesin-related motor proteins (Cin8, Kip1, Kar3, Kip3 and Kip2). The big overlap in functions of these proteins underlies the fact that none of them is individually essential for cell viability although their involvement in essential events of cell division (Hildebrandt and Hoyt 2000).

Cin8 and Kip1 are part of the kinesin-5 (BimC) subfamily of motor proteins. Although BimC family members are generally processive plus-end directed motor proteins it has been shown that Cin8 can switch its directionality and move in both the directions on spindle MTs even if the significance of this peculiarity is not yet fully understood (Gerson-Gurwitz, Thiede et al. 2011, Roostalu, Hentrich et al. 2011).

Cin8 and Kip1 share a high sequence similarity in the motor (force-producing) domain and act by sliding apart anti-parallel MTs to generate an outwardly-directed force important to fulfil their functions in spindle dynamics. Before anaphase onset Cin8 and Kip1 are required for SBPs separation and bipolar spindle formation (Hoyt, He et al. 1992, Roof, Meluh et al. 1992). During anaphase they stabilize the spindle midzone (Fridman, Gerson-Gurwitz et al. 2009) and regulate the switch from the fast to the slow phase of anaphase B spindle elongation. In particular Kip1 and Cin8 control anaphase B at different levels but the respective involvement of the two motors in the rapid initial elongation of the spindle rather than in the second and slower phase of spindle elongation is still confused (Straight, Sedat et al. 1998, Movshovich, Fridman et al. 2008, Gerson-Gurwitz, Movshovich et al. 2009).

In addition to the motor domain Cin8 possesses a self-association domain as well as a tetramerization domain but it is active only as homotetramer. This particular structure seems to be essential for its role in cross-linking and sliding apart anti-parallel MTs as

supported by the fact that homodimers of Cin8 can bind to MTs but are not functional (Hildebrandt, Gheber et al. 2006).

Kinesin-5 motor expression is cell cycle regulated and their expression pattern resembles the one of mitotic cyclins. They peak in mitosis and their stability relies on the APC/C ubiquitin-mediated pathway. In particular, Cin8 is degraded late in anaphase through the APC/C^{Cdh1} (Hildebrandt and Hoyt 2001) while Kip1 seems to be degraded earlier in anaphase by the APC/C^{Cdc20} (Gordon and Roof 2001). Their mechanisms of regulation are instead yet poorly understood. Their early functions (i.e. SPBs separation and bipolar spindle formation) seem to be regulated by Clbs-Cdc28 complexes in two parallel ways. On one hand CDKs phosphorylation of Cdh1 keep the APC/C^{Cdh1} inactive indirectly stabilizing the motors (Crasta, Huang et al. 2006, Crasta, Lim et al. 2008), on the other hand mitotic CDKs directly phosphorylate Cin8 and Kip1 promoting their activity in SPBs separation and bipolar spindle formation (Chee and Haase 2010). A different kind of phospho-regulation of the motors during anaphase spindle elongation has been proposed. According to this data Cdc14 released at the metaphase-to-anaphase transition seems to dephospharylate Cin8 promoting its interaction with the short anaphase spindle typical for this cell cycle phase and promoting its fast elongation. At late anaphase, once Cdc14 is no more active and the phosphorylated form of Cin8 prevails, Cin8 dissociates from the spindle thereby reducing its elongation rate (Avunie-Masala, Movshovich et al. 2011). Finally, a third level of regulation implicates CDKs and the midzone organizer Ase1. CDK-mediated phosphorylation of Ase1 seems to inhibit the Cin8 interaction with MTs up to metaphase. At anaphase onset the FEAR-released Cdc14 dephosphorylates Ase1 and induces Cin8 recruitment to spindle midzone to drive spindle elongation (Khmelinskii, Roostalu et al. 2009).

Kar3 is a kinesin-14 family member characterized by a non-processive powerstroke minus-end directed motility (deCastro, Fondecave et al. 2000). Kar3 produces inwardly-directed forces antagonistic to the outwardly-directed forces generated by the kinesin-5 motors (Saunders, Lengyel et al. 1997). Its activity depends on the heterodimerization with either of the non-motor proteins Cik1 or Vik1, two accessory subunits associated to different functionality (Page, Satterwhite et al. 1994, Manning, Barrett et al. 1999). Kar3/Vik1 localizes to SPBs while Kar3/Cik1 mainly concentrates on astral MTs (Manning, Barrett et al. 1999). According to their specific localization pattern Kar3/Vik1 cross-links and stabilizes parallel MTs at the SPBs while Kar3/Cik1 slides, stabilizes, and depolymerizes cytoplasmic MTs and seems to be the complex involved in spindle and nucleus orientation and positioning (Maddox, Stemple et al. 2003, Sproul, Anderson et al. 2005, Gardner, Haase et al. 2008, Chen, Rayment et al. 2011).

Kip3 belongs to the kinesin-8 family of motor proteins and possess a plus-end specific MTs depolymerase activity that correlates with its catastrophe-promoting effect in cells (Gupta, Carvalho et al. 2006, Varga, Helenius et al. 2006). Kip3 functionality depends on cooperative interactions with other Kip3 motors (Varga, Leduc et al. 2009) and varies accordingly to the cell compartments. This motor localizes predominantly onto cytoplasmic MTs and SPBs to regulate astral MTs length and nuclear migration (DeZwaan, Ellingson et al. 1997). Kinesins-8 are required to limit MTs length preferentially promoting catastrophe of longer MTs over shorter ones (Tischer, Brunner et al. 2009), and negatively regulating the MTs elongation rate (Du, English et al. 2010). Kip3 also contributes to metaphase KTs clustering in the nucleus (Wargacki, Tay et al. 2010).

Kip2 as well as Kip3 are prevalently cytoplasmic motor proteins, but Kip2 localizes exclusively on astral MTs and not at SPBs. Provided with MTs polymerizing activity

Kip2 antagonizes Kip3 in the process of nuclear positioning. Loss of Kip2 results in short astral MTs. In contrast, in *kip3* mutants the cytoplasmic MTs are significantly longer than normal (Cottingham and Hoyt 1997, Miller, Heller et al. 1998). Kip2 serves also to target cargo-proteins such as Bik1 and dynein to the plus-ends of astral MTs, required to orient the nucleus towards the bud-neck (Carvalho, Gupta et al. 2004).

1.6.3 Spindle disassembly

The breaking down of the spindle in telophase must be tightly coordinated with the end of chromosome segregation. During spindle elongation the number of interpolar MTs reduces from around four MTs emanating form each SPBs to one or two. Spindle disassembly requires the separation of the spindle halves and the depolymerization of these few interpolar MTs from their plus ends (Maddox, Bloom et al. 2000). The split of the spindle requires the disassembly of the spindle midzone and hence strongly depends on the activation of the ubiquitin ligase APC/C^{Cdh1} that targets to degradation lots of midzone stabilizing proteins, among which Ase1 (Juang, Huang et al. 1997), Fin1 (Woodbury and Morgan 2007) and Cin8 (Hildebrandt and Hoyt 2001). Great contribution to MTs depolymerization comes from Ipl1 (Buvelot, Tatsutani et al. 2003). This kinase destabilizes the spindle at least through the phosphorylation-mediated detachment of the MTs plus-ends binding protein Bim1. Loss of Bim1 from plus-ends of interpolar MTs makes the shortening MTs unable to switch to rescue (Zimniak, Stengl et al. 2009). Additionally, Ipl1 seems also to activate the disassembly factor She1, whose mechanisms of function is however not clear (Woodruff, Drubin et al. 2010). Finally, also the depolymerizating activity of Kip3 is necessary for the depolymerization of interpolar MTs (Straight, Sedat et al. 1998).

2. Materials and methods

2.1 Plasmids, primers and strains

2.1.1 Plasmids and primers

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

2.1.2 Bacterial strains

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

2.1.3 Yeast strains

All the *Saccharomyces cerevisiae* yeast strains are isogenic to the W303 (*ade2-1, can1-100, trp1-1 leu2-3,112, his3-11,15, ura3*) background except for the mating type tester strains Ry72 and Ry73. The majority of the strains used in this study were generated by dissecting sporulated heterozygous diploid strains obtained by crossing haploid strains of opposite mating type (see section 2.5.1 for procedure). The relevant genotypes of all the yeast strains used in this study are listed in **Table 2.4**.

2.2 Media and growth conditions

2.2.1 Media for *Escherichia coli*

Bacterial cells were grown in LB medium.

LB: 1% bactotryptone (DIFCO)
0.5% yeast extract (DIFCO)
1% NaCl
pH 7.25

LB were supplemented with 50 μg/ml ampicillin (LB + amp). For solid media 2% agar (DIFCO) was added to the medium. All strains were grown at 37°C.

2.2.2 Media for *Saccharomyces cerevisiae*

Yeast cells were grown in rich medium (YEP) or synthetic minimal medium (SC).

YEP: 1% yeast extract
2% bactopeptone
0.015% L-tryptophan
pH 5.4

YEP were supplemented with 300 μM adenine and either 2% glucose (YEPD), or 2% raffinose (YEPR) or 2% raffinose and 2% galactose (YEPRG) as carbon sources. For solid media 2% agar (DIFCO) was added to the medium.

SC: 0.15% yeast nitrogen base (YNB, DIFCO) without amino acids and ammonium sulfate.
0.5% ammonium sulfate
200 nM inositol

SC were supplemented with either 2% glucose (SCD), or 2% raffinose (SCR) or 2% raffinose and 2% galactose (SCRG) as carbon sources and amino acids as required. For solid media 2% agar (DIFCO) was added to the medium.

All strains were grown at 23°C unless otherwise stated. Growth conditions for individual experiments are described in the corresponding figure legend.

2.3 DNA-based procedures

2.3.1 *Escherichia coli* transformation

50 µl of fresh chemically competent Top10 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' 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 + amp plates. Plates were incubated overnight (ON) at 37°C.

2.3.2 Plasmid DNA isolation from *Escherichia coli* (mini prep)

Clones picked from individual colonies were used to inoculate 2 ml LB + amp 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 30µl of sterile double-distilled water (ddH_2O).

2.3.3 Plasmid DNA isolation from *Escherichia coli* (maxi prep)

Plasmid-containing cells were inoculated in 100 ml LB + amp and they were grown ON at 37°C. Cells were then recovery by centrifuging 10' at 5000 rpm. Maxipreps were performed with QIAprep Spin Maxiprep Kit (Quiagen) following the manufacturer's instructions. Plasmids were eluted in 500 µl of ddH_2O.

2.3.4 High efficiency LiAc-based 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 0.4-0.7. Cells were then harvested at 3000 rpm for 3' and washed with 50 ml of ddH_2O. The pellet was then transferred to an eppendorf tube with 1 ml of ddH_2O and washed with 1 ml of 1X TE/LiAc solution. Cells were then resuspended in 250 µl of 1X TE/LiAc solution. 50 µl aliquots of competent cells were used for each transformation reaction with 300 µl of 1X PEG/TE/LiAc solution, 5 µl of 10 mg/ml single-stranded salmon sperm denatured DNA and "x" µl (max up to 10 µl) of DNA. After gentle mixing, the transformation reaction was incubated on a rotating wheel for 30' at room temperature (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 auxotrophy selective medium. In case of selection for resistance to antibiotic G418 the pellet was resuspended in 200 µl of 1X TE and the cell suspension was plated on YEPD to allow cells to recover after heat-shock before exposure to antibiotic. After 2 days the resulting colonies were replica plated on a YEPD plate containing 220 µm/ml of G418.

<u>10X TE</u>:	0.1 mM Tris, bring to pH 8.0 with HCl
	10 mM EDTA pH 8
<u>10X LiAc</u>:	1 M LiAc, bring to pH 7.0 with acetic acid

1X TE/LiAc: 1X TE
1X LiAc

1X PEG/TE/LiAc: 1X TE
1X LiAc
40% PEG 4000

2.3.5 Smash and Grab yeast genomic DNA isolation

Cells picked from individual yeast colonies were inoculated in 200 µl of Lysis buffer. 200 µl of phenol/clorophorm/isoamyl alcohol 25:24:1 (SIGMA) and 1 volume of glass beads were added to the cell suspensions and the tubes were shaked 10' on Vxr Ika-Vibrax shaker. Tubes were then centrifuged 2x 4' at 13000 rpm and the upper aqueous layer was transferred to new tubes. 1 ml ice-cold 100% ethanol was added to precipitate DNA. After gently mixing, tubes were centrifuged 4' at 13000 rpm. Supernatants (SN) were removed, pellets were air-dried and DNA resuspended in 50 µl of 1X TE.

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

2.3.6 Teeny yeast genomic DNA extraction

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 solution I. Pellet was then transferred into 0.4 ml of solution I added with 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. After addition of 90 µl of solution II the tube was mixed and incubated 30' at 65°C. 80 µl of potassium acetate (KAc) 5 M were added and then the tube was incubated in 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 cold 70% ethanol, air-dried and finally resuspended in 50 µl of TE 1X.

Solution I:	0.9 M sorbitol
	0.1 M EDTA pH 7.5
Solution II:	1.5 ml of EDTA pH 8.5
	0.6 ml of Tris base
	0.6 ml 10% SDS

2.3.7 Enzymatic restriction of DNA

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 (New England Biolabs, NEB). 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.

2.3.8 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 interesting region, working as primers for the DNA polymerase. Phusion DNA polymerase (Finnzymes) and ExTaq (TaKaRa) DNA polymerase were used.

Reaction mix:	template DNA	1 μl
	reaction buffer	1X
	dNTPs	0.2 mM
	forward primer	1 μM
	reverse primer	1 μM
	DNA polymerase	1-2 units
	ddH_2O	to 20 μl

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

1. heat shock step	5’	at 95°C
2. denaturation step	1’	at 95°C
3. annealing step	1’	at 50-58°C
4. extension step	1’/kb	at 72°C
5. repeat 20-25 times steps from 2 to 4		
6. extension step	10’	at 72°C
7. end	hold	at 4°C

2.3.8.1 PCR-mediated gene deletion

Gene deletion has been performed as described in Longtine M. S. et al. 1998 (Longtine, McKenzie et al. 1998). To delete *SGO1* we transformed wild type (Ry1), *cdc14-1* (Ry1574), *cdc5-as1* (Ry2446) and *cdc14-1 cdc5-as1* (Ry1602) cells with the PCR fragment obtained using Rp89 plasmid as template and SgoD_F and SgoD_R as primers. *SGO1* deletion has been checked via PCR on the entire genome of the transformed strains (see section 2.3.5 for details) using F_SgoC2 and R_SgoC as primers.

2.3.9 Agarose gel eletrophoresis

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).

BPB solution:	0.2% BFB in 50% glycerol
10X TAE buffer:	0.4 M Tris acetate
	0.01 M EDTA

2.3.10 Purification of DNA from agarose gel

Cut DNA was first loaded into an agarose gel to separate the DNA fragments by electrophoresis. The DNA fragment of interest was then 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 ddH_2O.

2.3.11 DNA 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 (New England Biolabs, NEB) and ddH_2O up to 10 μl. Reactions were incubated ON at 16°C. After incubation the entire ligation reaction was used to transform *E. coli*.

2.4 Protein-based procedures

2.4.1 Yeast 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 SN over the beads) and the tubes were subjected to 3-5 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 95°C for 5', centrifuged at 13000 rpm for 3' and the SN, containing the final protein extract, was collected in a new microcentrifuge tube. Extracts were stored at -20°C.

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

2.4.2 Yeast 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' in 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 SN discarded. The pellet was frozen in liquid nitrogen in order to better preserve protein integrity. Pellet was then washed with 1 ml RT absolute acetone and air-dried. The pellet was next resuspended in 100 µl of lysis buffer (see section 2.4.1 of materials and methods for the receipt) 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 3-5 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 (see section 2.4.1 of materials and methods for the receipt) was then added to each sample. The samples were boiled at 95°C for 5', centrifuged at 13000 rpm for 3' and the SN, containing the final protein extract, was collected in a new microcentrifuge tube. Extracts were stored at -20°C.

2.4.3 Recombinant protein expression in yeast

The coding sequence of the *CIN8* gene wild type and mutated in 4 or 11 putative Cdc5-consensus sites (*cin8-4A* and *cin8-11A*, respectively) were ordered to GenScript. The company sent us these synthetic genes cloned BamHI-SalI into pUC57 plasmids (called Mp1, Mp2 and Mp3, respectively). *CIN8* wild type, *cin8-4A* and *cin8-11A* coding sequences were excise from Mp1, Mp2 or Mp3 plasmid to be cloned into the centromeric Rp173 plasmid, under the control of the endogenous *CIN8* promoter (841bp long). To this aim Mp1, Mp2 and Mp3 plasmids were cut with a double enzymatic digestion using both BamHI and SalI restriction enzymes to obtain the coding sequences of interest. The fragments corresponding to *CIN8* wild type, *cin8-4A* or *cin8-11A* coding sequences have been cloned into the Rp173 plasmid to obtain Mp4, Mp5 and Mp6 plasmid, respectively. 841bp of the endogenous *CIN8* promoter was amplified by PCR using as template the genome of a wild type W303 strain (Ry1), extracted (as indicated in section 2.3.6), and PcinN_F and PcinN_R as primers. The promoter was cloned KpnI-BamHI into Mp4, Mp5 or Mp6 plasmid to obtain Mp7, Mp8 and Mp9 plasmid, respectively. Mp7, Mp8 or Mp9 plasmid was then transformed into the strain of interest.

Here below are reported the mutated sequences (from the "start" codon ATG to the "stop" codon TAG) of *cin8-4A* and *cin8-11A*.

- *cin8-4A* coding sequence:

ATGCCAGCGGAAAACCAAAATACGGGTCAAGATAGAAGCTCCAACAGCATCA
GTAAAAATGGCAACTCTCAGGTTGGATGTCACACTGTTCCTAATGAGGAACTG
AACATCGCTGTAGCTGTGCGATGCAGAGGAAGGAATGAAAGGGAAATTGCTAT
GAAAAGCTCCGTTGTGGTAAATGTTCCAGATATTACAGGTTCTAAAGAAATTG
CCATTAACACGACGGGAGATACCGGTATAACTGCTCAAATGAATGCCAAGAGA
TACACAGTGGACAAAGTCTTCGGTCCCGGCGCTTCCCAGGATCTAATTTTTGAT
GAAGTGGCGGGCCCATTATTCCAGGATTTCATTAAAGGTTACAATTGCGCCGTA
CTGGTATATGGTATGACGTCAACAGGTAAAACATATACAATGACGGGCGACGA
AAAGTTATATAATGGTGAATTGAGCGATGCAGCAGGAATTATACCGAGGGTTC
TTTTGAAGTTGTTTGACACATTGGAACTACAACAGAACGATTACGTAGTAAAAT
GTTCGTTCATTGAACTCTACAACGAAGAATTGAAGGACCTCTTGGACAGCAATA
GCAACGGCTCTAGTAATACTGGCTTTGACGGCCAATTTATGAAAAAATTGAGG
ATTTTTGATTCAAGCACAGCAAATAATACCACTAGCAACAGTGCTAGTAGTTCC

AGGAGTAATTCTAGGAACAGTTCTCCGAGGTCATTAAATGATCTAACACCTAA
AGCTGCTCTATTAAGAAAAAGGTTAAGGACAAAATCACTGCCGAATACCATCA
AGCAACAGTATCAACAACAACAGGCAGTGAATTCCAGGAACAACTCTTCCTCT
AACTCTGGCTCTACCACTAATAATGCTTCTAGTAACACCAACACAAATAACGGT
CAAAGAAGTTCGATGGCTCCAAATGACCAAACTAATGGTATATACATCCAGAA
TTTGCAAGAATTTCACATAACAAATGCTATGGAGGGGCTAAACCTATTACAAA
AAGGCTTAAAGCATAGGCAAGTAGCGTCCACTAAAATGAACGATTTTTCCAGT
AGATCTCATACCATTTTTACAATCACTTTGTATAAGAAGCATCAGGATGAACTA
TTTAGAATTTCCAAAATGAATCTTGTGGATTTAGCTGGTTCAGAAAACATCAAC
AGATCCGGAGCATTAAATCAACGTGCCAAAGAAGCTGGTTCAATCAACCAAGC
TCTATTGACGCTGGGCAGGGTCATAAACGCACTCGTAGATAAAAGCGGCCATA
TACCTTTCCGTGAATCGAAATTGACCCGCCTGCTTCAAGATGCCCTGGGTGGTA
ATACGAAAACCGCACTAATTGCTACTATATCGCCTGCAAAGGTAACTTCTGAAG
AAACCTGCAGTACATTAGAGTATGCTTCGAAGGCTAAAAACATTAAGAACAAG
CCGCAACTGGGTTCATTTATAATGAAGGATATTTTGGTTAAAAATATAGCTATG
GAATTAGCAAAGATTAAATCCGATTTACTCTCTACAAAGTCCAAAGAAGGAAT
ATATATGAGCCAAGATCACTACAAAAATTTGAACAGTGATTTAGAAAGTTATA
AAAATGAAGTTCAAGAATGTAAAAGAGAAATTGAAAGTTTGACATCGAAAAAT
GCATTGCTAGTAAAAGATAAATTGAAGTCAAAAGAAACTATTCAATCTCAAAA
TTGCCAAATAGAATCATTGAAAACTACCATAGATCATTTAAGGGCACAACTAG
ATAAACAGCATAAAACTGAAATTGAAATATCCGATTTTAATAACAAACTACAG
AAGTTGACTGAGGTAATGCAAATGGCCCTACATGATTACAAAAAAAGAGAACT
TGACCTTAATCAAAAGTTTGAAATGCATATTACTAAAGAAATTAAAAAATTGA
AATCTACACTGTTTTTACAATTAAACACTATGCAACAGGAAGCTATTCTTCAAG
AGACTAATATCCAACCAAATCTTGATATGATCAAAAATGAAGTACTGACTCTTA
TGAGAACCATGCAAGAAAAAGCTGAACTAATGTACAAAGACTGTGTGAAGAA
AATTTTAAACGAATCTCCTAAATTCTTCAATGTTGTTATTGAGAAAATCGACAT
AATAAGAGTAGATTTCCAAAAATTTTATAAAAATATAGCCGAGAATCTTTCTGA
TATTAGCGAAGAAAATAACAACATGAAACAGTACTTAAAAAACCATTTTTTCA
AGAATAACCATCAAGAATTACTGAATCGTCATGTGGATTCTGCTTATGAAAAT
ATTGAGAAGAGAACAAACGAGTTTGTTGAGAACTTTAAAAAGGTCCTAAATGA
CCACCTTGACGAAAATAAAAAACTAATAATGCAGAATCTGACAACTGCAACCA
GCGCGGTTATTGATCAAGAAATGGATCTGTTTGAACCCAAGCGCGTTAAATGG
GAAAATGCATTTGATCTGATAAATGATTGTGACTCCATGAATAACGAATTCTAT
AATAGCATGGCAGCGACGCTATCGCAAATCAAGAGTACTGTTGATACATCATC
AAATTCGATGAATGAGGCTATTTCAGTCATGAAAGGACAAGTGGAAGAATCGG
AGAACGCTATATCCCTTTTGAAGAACAATACCAAATTTAATGATCAATTTGAGC
AGCTTATTAACAAGCATAACATGTTGAAAGATAACATTAAAAATTCGATAACA
TCAACACACTCTCATATAACTAATGTGGATGATATCTATAATACGATTGAAAAC
ATAATGAAAAACTATGGTAACAAGGAAAACGCTACCAAAGACGAAATGATCG
AGAACATATTGAAGGAAATACCAAATCTAAGTAAGAAAATGCCGTTAAGGTTA
TCAAACATAAATAGCAATTCAGTGCAAAGTGTAATATCGCCCAAAAAGCATGC
AATTGAAGATGAAAACAAATCCAGTGAAAATGTGGACAATGAGGGCTCGAGA
AAAATGTTAAAGATTGAATAG

- cin8-11A coding sequence:

ATGCCAGCGGAAAACCAAAATACGGGTCAAGATAGAAGCTCCAACAGCATCA
GTAAAAATGGCAACTCTCAGGTTGGATGTCACACTGTTCCTAATGAGGAACTG
AACATCGCTGTAGCTGTGCGATGCAGAGGAAGGAATGAAAGGGAAATTGCTA
TGAAAAGCTCCGTTGTGGTAAATGTTCCAGATATTACAGGTTCTAAAGAAATTG
CCATTAACACGACGGGAGATACCGGTATAACTGCTCAAATGAATGCCAAGAGA
TACACAGTGGACAAAGTCTTCGGTCCCGGCGCTTCCCAGGATCTAATTTTTGAT
GAAGTGGCGGGCCCATTATTCCAGGATTTCATTAAAGGTTACAATTGCGCCGT
ACTGGTATATGGTATGACGTCAACAGGTAAAACATATACAATGACGGGCGACG
AAAAGTTATATAATGGTGAATTGAGCGATGCAGCAGGAATTATACCGAGGGTT
CTTTTGAAGTTGTTTGACACATTGGAACTACAACAGAACGATTACGTAGTAAAA
TGTTCGTTCATTGAACTCTACAACGAAGAATTGAAGGACCTCTTGGACAGCAAT
AGCAACGGCTCTAGTAATACTGGCTTTGACGGCCAATTTATGAAAAAATTGAG
GATTTTTGATTCAAGCACAGCAAATAATACCACTAGCAACAGTGCTAGTAGTTC
CAGGAGTAATTCTAGGAACAGTTCTCCGAGGTCATTAAATGATCTAACACCTAA

AGCTGCTCTATTAAGAAAAAGGTTAAGGACAAAATCACTGCCGAATACCATCA
AGCAACAGTATCAACAACAACAGGCAGTGAATTCCAGGAACAACTCTTCCTCT
AACTCTGGCTCTACCACTAATAATGCTTCTAGTAACACCAACACAAATAACGGT
CAAAGAAGTTCGATGGCTCCAAATGACCAAACTAATGGTATATACATCCAGAA
TTTGCAAGAATTTCACATAACAAATGCTATGGAGGGGCTAAACCTATTACAAA
AAGGCTTAAAGCATAGGCAAGTAGCGTCCACTAAAATGAACGATTTTTCCAGT
AGATCTCATACCATTTTTACAATCACTTTGTATAAGAAGCATCAGGATGAACTA
TTTAGAATTTCCAAAATGAATCTTGTGGATTTAGCTGGTTCAGAAAACATCAAC
AGATCCGGAGCATTAAATCAACGTGCCAAAGAAGCTGGTTCAATCAACCAA**GC**
TCTATTGACGCTGGGCAGGGTCATAAACGCACTCGTAGATAAAAGCGGCCATA
TACCTTTCCGTGAATCGAAATTGACCCGCCTGCTTCAAGAT**G**C**C**CTGGGTGGTA
ATACGAAAACCGCACTAATTGCTACTATATCGCCTGCAAAGGTAACTTCTGAAG
AAACCTGCAGTACATTAGAGTATGCTTCGAAGGCTAAAAACATTAAGAACAAG
CCGCAACTGGGTTCATTTATAATGAAGGATATTTTGGTTAAAAATATA**G**CTATG
GAATTAGCAAAGATTAAATCCGATTTACTCTCTACAAAGTCCAAAGAAGGAAT
ATATATGAGCCAAGATCACTACAAAAATTTGAACAGTGATTTAGAAAGTTATA
AAAATGAAGTTCAAGAATGTAAAAGAGAAATTGAAAGTTTGACATCGAAAAAT
GCATTGCTAGTAAAAGATAAATTGAAGTCAAAAGAAACTATTCAATCTCAAAA
TTGCCAAATAGAATCATTGAAAACTACCATAGATCATTTAAGGGCACAACTAG
ATAAACAGCATAAAACTGAAATTGAAATATCCGATTTTAATAACAAACTACAG
AAGTTGACTGAGGTAATGCAAATGGCCCTACATGATTACAAAAAAAGAGAACT
TGACCTTAATCAAAAGTTTGAAATGCATATTACTAAAGAAATTAAAAAATTGA
AATCTACACTGTTTTTACAATTAAACACTATGCAACAGGAA**GC**TATTCTTCAAG
AGACTAATATCCAACCAAATCTTGATATGATCAAAAATGAAGTACTGACTCTTA
TGAGAACCATGCAAGAAAAAGCTGAACTAATGTACAAAGACTGTGTGAAGAA
AATTTTAAACGAATCTCCTAAATTCTTCAATGTTGTTATTGAGAAAATCGACAT
AATAAGAGTAGATTTCCAAAAATTTTATAAAAATATAGCCGAGAATCTTTCTGA
TATTAGCGAAGAAAATAACAACATGAAACAGTACTTAAAAAACCATTTTTTCA
AGAATAACCATCAAGAATTACTGAATCGTCATGTGGATTCT**GC**T**T**ATGAAAAT
ATTGAGAAGAGAACAAACGAGTTTGTTGAGAACTTTAAAAAGGTCCTAAATGA
CCACCTTGACGAAAATAAAAAACTAATAATGCAGAATCTGACAACTGCAACCA
GCGCGGTTATTGATCAAGAAATGGATCTGTTTGAACCCAAGCGCGTTAAATGG
GAAAAT**GCA**TTTGATCTGATAAATGATTGTGACTCCATGAATAACGAATTCTAT
AATAGCATGGCAGCGACGCTATCGCAAATCAAGAGTACTGTTGATACATCATC
AAATTCGATGAATGAG**GC**TATTTCAGTCATGAAAGGACAAGTGGAAGAATCGG
AGAACGCTATATCCCTTTTGAAGAACAATACCAAATTTAATGATCAATTTGAGC
AGCTTATTAACAAGCATAACATGTTGAAAGATAACATTAAAAATTCGATAACA
TCAACACACTCTCATATAACTAATGTGGATGATATCTATAATACGATTGAAAAC
ATAATGAAAAACTATGGTAACAAGGAAAACGCTACCAAAGACGAAATGATCG
AGAACATATTGAAGGAAATACCAAATCTAAGTAAGAAAATGCCGTTAAGGTTA
TCAAACATAAATAGCAATTCAGTGCAAAGTGTAATATCGCCCAAAAAGCATGC
AATTGAAGATGAAAACAAATCCAGTGAAAATGTGGACAATGAGGGCTCGAGA
AAAATGTTAAAGATTGAATAG

2.4.4 SDS polyacrylamide gel elettrophoresis

Appropriate amount of proteins (usually from 50 to 100 μg of total extract) 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), 4X Separating buffer, 2X Stacking buffer and an appropriate amount of ddH_2O. As polymerization catalysis ammonium persulphate (APS) and TEMED (BDH) were used. 1.5 mm thick polyacrylamide gels were run in 1X running buffer at 100-150 V for 2-3.5 hrs.

4X Separating buffer: 1.5 M Tris base, bring to pH 8.8 with glacial acetic acid
0.4% SDS

2X Stacking buffer: 0.25 M Tris base, bring to pH 6.8 with glacial acetic acid
0.2% SDS

10X Running buffer: 2 M glycine
0.25 M Tris-HCl
0.02 M SDS
pH 8.3

2.4.5 Western blot hybridization

Proteins were transferred in Western transfer tanks to nitrocellulose (Protran, Whatman) in 1X 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 proteins transferred onto the filters.

Membranes were blocked with:

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

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) or 1:1000 rabbit anti-Clb2 (Santa Cruz) diluted in 1% milk/1% BSA/1X PBS-T for 2 hours at RT or ON at 4°C.

- 1:1000 goat anti-Cdc5 (Santa Cruz) diluted in 1% ovalbumin / 1X PBS-T, ON at 4°C.
- 1:1000 goat anti-Clb5 (Santa Crutz) diluted in 5% milk / 1X TBS-T, 3 hours at RT or ON at 4°C.

Membranes were then washed 3 x 15' in 1X PBS-T or 1X 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 / 1X PBS-T for 1 hour.
- 1:5000 anti-goat in 1% ovalbumin / 1X PBS-T or 1X TBS-T for 1 hours.

After incubation with the secondary antibody, the membrane was washed 3 x 15' in 1X PBS-T or 1X TBS-T and the bound secondary antibody was revealed using ECL (Enhanced Chemiluminescence, Amersham).

1X 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

10X TBS buffer: 25mM Tris base
150mM NaCl
2 mM KCl

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

1X TBS-T buffer: 0.1% Tween
1X TBS

2.4.6 Clb2 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 SN 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 lysis buffer. An equal volume of acid-washed glass beads (Sigma) was added (leaving a layer of SN over the beads) and the tubes were subjected to 3-5 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. SN was transfer to new tube avoiding cellular debris on the bottom and lipid layer on the top. Protein concentration was determined by Bradford Assay (see materials and methods section 2.4.1 for details). 150 µg of extract were used for the immunoprecipitation. A right volume of NP40 buffer supplemented with complete protease inhibitor cocktail (Roche) and phosphatases inhibitors was added to each tube so that all tubes are at the same volume (50-60 µl was usually the desired amount). Rabbit anti-Clb2 (Santa Cruz) 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 and then SN was removed paying attention not disturbing beads.

Beads bound to the protein of interest were resuspended with 6 μl of HBII buffer and incubated for 15' at RT. 10 μl of kinase reaction mix was added to samples. Samples were incubated for 15' at RT. Then 10 μl of 3X sample buffer was added to stop the reaction. Samples were spin down at maximum speed for 5' and then loaded on a 12.5% gel. 1.5 mm thick polyacrylamide gel was run in 1X running buffer at 100 V for 1-2 hours. After run the gel was fixed in the destaining solution for 20-30', carefully washed with ddH_2O and then immersed for 10' in 50% glycerole. After these washes gel was dryed for 1-3 hrs depending on the size and then exposed.

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

NP40 lysis buffer:
NP40 buffer
60 mM β-glycerol phosphate
0.1 mM Na orthovanadate
15 mM p-Nitrophenylphosphate
5 mM NaF
1mM DTT
1X Protease inhibitor cocktail (Roche)

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
	ddH_2O	275 μl
SOL2:	4 mg/ml Histon H1 in 50 mM Mops	

Destaining solution: 75 ml Acetic acid
50 ml Methanol
875 ml ddH_2O

2.5 Yeast procedures

2.5.1 Tetrads dissection and analysis

MATa and *MATα* strains were mixed on solid medium, appropriate for the growth of both the haploids, and incubated ON 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 to induce meiosis and sporulation by starvation. After 3-5 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. A toothpick full of tedrads is resuspended into the digestion mixture. The digestion mixture is then incubated at 37°C for 3' in order to enzymatically digest the ascus wall. Then, 1 ml ddH_2O is 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 23°C for 3-5 days. Colonies were replica plated onto selective media to define their genotype.

Digestion mixture: 198 µl ddH_2O
2 µl 10 mg/ml zymolase 100T (Seikagaka, Biobusiness)

Sporulation plates: 30 g K-Acetate
60 g Agar (DIFCO)
all amino acids at 1/4 of the normal concentration
up to 3l with ddH_2O

2.5.2 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 and Herskowitz 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 (Manney 1983, Kronstad, Holly et al. 1987). *MATa* cells that lack the Bar1 protein are supersensitive to of α-factor-induced G1 arrest (Sprague and Herskowitz 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.

2.5.3 Synchronization experiments

2.5.3.1 G1 phase arrest and release

Cells were grown ON in the appropriate medium 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. Cells were then diluted again to OD_{600} = 0.2 and added with 5 μg/ml α-mating factor synthetic peptide dissolved in ddH_2O (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. 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.

2.5.3.2 S phase arrest and release

Cells were grown ON in the appropriate medium 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. Cells were then pre-synchronized in G1 by addition of α-factor and next released in a medium containing 10 mg/ml hydroxyurea (HU, Sigma) (powder added directly to the medium). For synchronization experiments, after the arrest was complete, cells were released from the block. Drug was washed out by filtration, using between 5 to 10 volumes of medium without it. Cells were next released into the appropriate fresh medium.

2.5.3.3 Nocodazole-mediated metaphase arrest and release

Cells were grown ON in the appropriate medium 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.

Cells were then pre-synchronized in G1 by addition of α-factor and next released in a medium containing 15 μg/ml nocodazole (NOC, Sigma) dissolved in DMSO. 7.5 μg/ml nocodazole was re-added to the culture after 90' incubation. Once the arrest was complete, cells were released from the block. Drug was washed out by filtration, using between 5 to 10 volumes of medium added with 1% of DMSO. Cells were next released into the appropriate fresh medium added with 1% of DMSO.

2.5.2 Regulation of gene expression

To regulate the expression of specific proteins we used yeast strains in which the encoding genes were cloned under the control of regulatable promoters, like the *pGAL1-10* promoter (West, Chen et al. 1987) or the *pMET3* promoter (Care, Trevethick et al. 1999) or under the control of the auxin-based degron (Freeman, Aragon-Alcaide et al.) system (Nishimura, Fukagawa et al. 2009).

The *pGAL1-10* promoter quickly induces the expression of a downstream fused-gene after the addition of galactose (usually at 2%) to cells growing in media containing a poor carbon source as raffinose. Conversely, *pGAL1-10* can be turned off by the addition of glucose to a galactose-containing medium. This system has been used to overproduce specific proteins as well as to achieve the full repression of specific essential genes in a time-regulated manner.

The *pMET3* promoter turns off the expression of *pMET3*-fused genes after the addition of fresh methionine (usually 8 mM) to cells growing in media lacking methionine (4 mM methionine was re-added every hour to keep the promoter weaken). This system has been used to down-regulate the expression of essential genes in a time-regulated manner.

The AID system allowes rapid degradation of target proteins in response to auxin hormones, through the SCF degradation pathway. To use this system (i) cells must

ectopically express the F-box transport inhibitor response 1 (Tir1) protein and (ii) the target protein has to be fused with the AID degron. To induce the degradation of the protein of interest 500μM of indole-3-acetic acid (IAA; a natural auxin) was added to the medium so that IAA binding to Tir1 promotes the interaction between Tir1 and the AID degron. This interaction is required to the further activation of the SCF degradation pathway against the AID-fused protein.

2.5.3 Regulation of conditionally mutant genes

Temperature sensitive alleles were inactivated by incubating cells at the restrictive temperature (usually 37°C).

cdc5-as1 ATP-analogue sensitive allele (Zhang, Kenski et al. 2005) was inactivated by 5 μM CMK inhibitor (Accenda Tech) (Snead, Sullivan et al. 2007) dissolved in DMSO added to the medium.

2.5.4 Tubulin staining via *in situ* indirect immunofluorescence (IF)

1 ml of a cell culture at OD_{600} = 0.2 - 0.4 were collected by centrifugation for 1' at 13000 rpm at RT and incubated ON at 4°C in 1 ml fixative solution. Cells were then 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 35°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 for 60'-90', in a humid dark incubation chamber, with primary antibody: 1:100 rat anti-tubulin (Oxford-Biotechonology) in PBS-BSA. After incubation, cells were washed 5 times with PBS-BSA and incubated with secondary antibody: 1:100 FITC-conjugated anti-rat in PBS-BSA for 60'. Cells were then washed 5 times with PBS-BSA and then treated with a DAPI mount solution. The slide was covered with a coverslip and sealed with nail polish. IF was analysed with a Leica DMR HC BIOMED fluorescence microscope using a 100X immersion-oil objective.

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 ddH_2O
Fixative solution:	3.7% formaldehyde in 0.1 M KPi pH 6.4
Sorbitol-citrate 1.2 M:	17.4 g Anhydrous KH_2PO_4 7 g Citric acid 218.64 g Sorbitol up to 1l with ddH_2O
Digestion solution:	1.2 M sorbitol-citrate 10% glusulase 0.1 mg/ml zymolase 100T (Seikagaka Biobusiness)

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

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

2.5.5 Nuclei staining (DAPI staining)

1 ml of a cell culture at OD_{600} = 0.2 - 0.4 was 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.

2.5.6 GFP-signals fixation

750 μl of a cell culture at OD_{600} = 0.2 - 0.4 were fixed via addition of 250 μl of 2% paraformaldehyde (final concentration 2%). After 1' of incubation at RT cells were pelleted and washed twice with 1 ml of 0.1 M K-phosphate buffer pH 6.6. After washes cells were resuspended in 500 μl of 0.1 M K-phosphate buffer pH 7.

0.1 M K-phosphate buffer pH 6.6: 62.5 ml of 0.2 M mono-potassium salt
37.5 ml of 0.2 M di-potassium salt

0.1 M K-phosphate buffer pH 7: 39 ml of 0.2 M mono-potassium salt
61 ml of 0.2 M di-potassium salt

0.2 M mono-potassium salt:	27.2 g KH_2PO_4 up to 1l with ddH_2O
0.2 M di-potassium salt:	34.8 g K_2HPO_4 up to 1l with ddH_2O

2.5.7 Scoring of indirect immunofluorescence samples

Cell cycle progression was scored by analysis of nulcear and spindle morphologies and dividing cells into three categories:

1) Interphase cells (which includes cells in the G1, S, and G2 phases of the cell cycle): these cells are typically unbudded cells (or cells with a small bud) with one nucleus, one single SPB (or two side-by-side SPBs) and 3-5 short cytoplasmic microtubules emanating from each SPB.

2) Metaphase cells: these cells are typically medium or large budded cells with an undivided nucleus closed to the bud-neck, two separated SPBs and a short and thick bipolar spindle.

3) Anaphase cells (including both anaphase and telophase cells): these cells are typically large budded cells with two nuclear masses (one in the mother cell and the other in the daughter cell), one SPB associated to each nucleus and an elongated spindle.

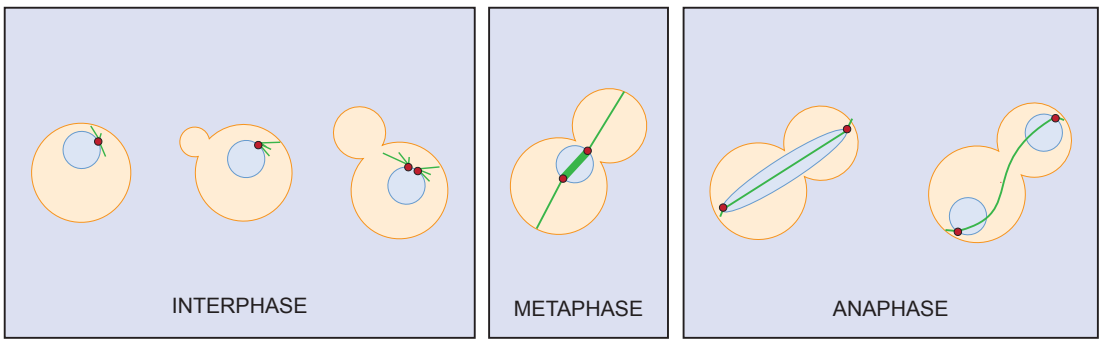

Figure 2.1. Cell morphological classification.

2.5.8 Images acquisition and analysis

Fluorescence microscopy images of **Fig. 3.2c**, **3.16d**, **3.26b**, **3.27b** and **3.28d** were acquired from IF slides with a DeltaVision Elite deconvolution microscope (Applied Precision) equipped with an Olympus IX71 inverted microscope and a CoolSnap HQ2 (Photometrics) CCD camera and driven by SoftWoRx software Images were acquired with a UPlan SApo 100x oil immersion objective (NA 1.4) as z-stacks (0.8μm step). Stacks were deconvoluted by Delta Vision SoftWoRx program (Applied Precision) and converted into maximal intensity projections using ImageJ 1.43u software. No manipulations were performed other than adjustments in brightness and contrast.

Fluorescence microscopy images of **Fig. 3.13** were acquired with the same DeltaVision system from cells fixed as described in section 2.5.6 and put under the microscope.

Images of IF for spindles length analysis were taken using an upright AX70 Olympus Provis microscope with a 100X/1.40 oil UPlanSApo ∞/0.17/FN 26.5 Olympus objective, and images were captured with a Photometrics Coolsnap Black & White 12 bit camera using MetaMorph 7.5.6.0 software (MDS Analytical Technologies). ImageJ 1.43u software was used to analyze the images and for spindles length measurements.

2.5.9 Fluorescense-activating cell sorting (FACS)

1.5 ml of a cell culture at OD_{600} = 0,2 - 0,4 were collected by centrifugation for 1’ at 13000 rpm at RT 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 RNaseA. 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.

Tris-HCl/Ethanol solution:	250 mM Tris-HCl pH 7.5
	70% ethanol
FACS buffer:	200 mM Tris-HCl pH 7.5
	200 mM NaCl
	78 mM $MgCl_2$

Table 2.1 Plasmids used in this study

Plasmid	Description	Origin
Rp89	pFA6a-His3MX6	(Longtine, McKenzie et al. 1998)
Rp173	YCplac22	(Gietz R.D. et al., 1993)
pVF9	pRS305 containing the C-terminal part of cin8-F467A tagged with 3HA	(Movshovich, Fridman et al. 2008)
Mp1	pUC57 with WT *CIN8* coding sequence cloned BamHI-SalI	GenScript
Mp2	pUC57 with *cin8-4A* coding sequence cloned BamHI-SalI	GenScript
Mp3	pUC57 with *cin8-11A* coding sequence cloned BamHI-SalI	GenScript
Mp4	Rp173 with WT *CIN8* coding sequence cloned BamHI-SalI	This study
Mp5	Rp173 with *cin8-4A* coding sequence cloned BamHI-SalI	This study
Mp6	Rp173 with *cin8-11A* coding sequence cloned BamHI-SalI	This study
Mp7	Mp4 with 841bp CIN8 promoter cloned KpnI-BamHI	This study
Mp8	Mp5 with 841bp CIN8 promoter cloned KpnI-BamHI	This study
Mp9	Mp6 with 841bp CIN8 promoter cloned KpnI-BamHI	This study

Table 2.2 Primers used in this study

Primer	Sequence (5'-3')	Purpose
SgoD_F	CACACGCATATATATGTTTAATTGGGTATAGAGGGGTTATTGTTTGACCCGGATCCCCGGGTTAATTAA	*SGO1* deletion
SgoD_R	GCAAAAATATAGAAATTATTAAGGAACACCAGGGCAAAAAGACTATATATCGAATTCGAGCTCGTTTAAAC	*SGO1* deletion
F_SgoC2	GGGTATAGAGGGGTTATTGTTTG	Check *SGO1* deletion
R_SgoC	CCAGAAATCCAAGACCATTCC	Check *SGO1* deletion
PcinN_F	CGCCGGTACCTCAATGGCTTCCCC	*CIN8* promoter amplification (841bp)
PcinN_R	CGGCGGATCCCAAACAAATTCTTTCTTGTTGTATTTTTTGCGC	*CIN8* promoter amplification (841bp)

Table 2.3 Bacterial strains used in this study

Strain	Genotype
TOP10 *E. coli*	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ

Table 2.4 Yeast strains used in this study

Strain (Ry)	Relevant genotype	Origin
1	*MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+*	Visintin lab
60	*MATa, cdc14-3*	Visintin lab
72	*MATa* (mating type tester)	Fink lab
73	*MATα* (mating type tester)	Fink lab
133	*MATa, cdc5-1*	Visintin lab
1029	*MATa, cdc14-1, cdc5::pGAL-URL-3HA-CDC5::KanMX*	Visintin lab
1223	*MATa, pMET3-CDC20::URA3, CDC14-3HA*	Visintin lab
1387	*MATa, pGAL-clb2dBΔ::URA3*	Visintin lab
1574	*MATa, cdc14-1*	Visintin lab
1575	*MATa, cdc14-1, pGAL-clb2dBΔ::URA3*	Visintin lab
1602	*MATa, cdc14-1, cdc5-as1(L158G)*	Visintin lab
1932	*MATa, cdc23-1*	Visintin lab
2169	*MATa, cdc14-1, cdc5-as1(L158G), rad9::URA3*	This thesis
2275	*MATa, cdc14-1, cdc5-as1(L158G), sgo1::HIS3*	This thesis
2277	*MATa, cdc14-1, sgo1::HIS3*	This thesis
2279	*MATa, cdc5-as1(L158G), sgo1::HIS3*	This thesis
2446	*MATa, cdc5-as1(L158G)*	This thesis
2580	*MATa, cdc14-1, cdc5-as1(L158G), rad53-K227A::KanMX*	This thesis
2597	*MATa, cdc14-1, cdc5-as1(L158G), mad1::URA3*	This thesis

Strain (Ry)	Relevant genotype	Origin
2612	*MATa, cdc14-1, cdc5-as1(L158G), pds1::URA3*	This thesis
2626	*MATa, cdc14-1, cdc5-as1(L158G), PDS1-3HA-LEU2::pds1*	This thesis
2629	*MATa, cdc5-as1(L158G), PDS1-3HA-LEU2::pds1*	This thesis
2633	*MATa, cdc14-1, PDS1-3HA-LEU2::pds1*	This thesis
2725	*MATa, cdc14-1, cdc5-as1(L158G), SCC1-18MYC::TRP1, PDS1-3HA-LEU2::pds1*	This thesis
2743	*MATa, cdc14-1, SCC1-18MYC::TRP1, PDS1-3HA-LEU2::pds1*	This thesis
2747	*MATa, cdc5-as1(L158G), SCC1-18MYC::TRP1, PDS1-3HA-LEU2::pds1, CDC14-3HA*	This thesis
2774	*MATa, cdc14-3, cdc5-2(msd2-1)::URA3*	This thesis
2776	*MATa, cdc14-3, cdc5-as1(L158G)*	This thesis
2780	*MATa, cdc14-1, cdc5-as1(L158G), slk19::SLK19-13MYC::KanMX6*	This thesis
2785	*MATa, cdc14-1, slk19::SLK19-13MYC::KanMX6*	This thesis
2790	*MATa, cdc5-as1(L158G), slk19::SLK19-13MYC::KanMX6*	This thesis
2795	*MATa, cdc14-1, cdc5-as1(L158G), scc1::HIS3, SCC1-TEV268-HA::LEU2, pGAL-NLS-9MYC-TEV Protease-NLS::TRP1*	This thesis
2875	*MATa, cdc14-3, cdc5-1*	This thesis
2882	*MATa, cdc14-1, cdc5-1*	This thesis
3015	*MATa, cdc14-1, cdc5-as1(L158G), swe1::LEU2*	This thesis
3020	*MATa, cdc14-1, swe1::LEU2*	This thesis
3025	*MATa, cdc5-as1(L158G), swe1::LEU2*	This thesis
3030	*MATa, cdc14-1, cdc5-as1(L158G), pGAL-ESP1::TRP1*	This thesis
3108	*MATa, cdc14-1, cdc5-as1(L158G), ndc10-1*	This thesis
3136	*MATa, cdc14-1, cdc5-as1(L158G), cdc6::hisG, URA3::pGAL-ubiR-CDC6, mad1::URA3*	This thesis
3141	*MATa, cdc14-1, cdc6::hisG, URA3::pGAL-ubiR-CDC6, mad1::URA3*	This thesis
3145	*MATa, cdc5-as1(L158G), cdc6::hisG, URA3::pGAL-ubiR-CDC6, mad1::URA3*	This thesis
3166	*MATa, cdc14-1, cdc5-as1(L158G), mad1::URA3, mad2::KanMX6*	This thesis

Strain (Ry)	Relevant genotype	Origin
3186	*MATa, cdc14-1, cdc5-as1(L158G), mad2::KanMX6*	This thesis
3203	*MATa, cdc14-1, cdc5-as1(L158G), pMET3-CDC20::URA3*	This thesis
3204	*MATa, cdc14-1, pMET3-CDC20::URA3*	This thesis
3209	*MATa, cdc5-as1(L158G), pMET3-CDC20::URA3, CDC14-3HA*	This thesis
3212	*MATa, cdc14-1, cdc5-as1(L158G), scc1::HIS3, SCC1-TEV268-HA::LEU2, pGAL-NLS-9MYC-TEV Protease-NLS::TRP1, pMET3-CDC20::URA3*	This thesis
3216	*MATa, cdc14-1, scc1::HIS3, SCC1-TEV268-HA::LEU2, pGAL-NLS-9MYC-TEV Protease-NLS::TRP1, pMET3-CDC20::URA3*	This thesis
3220	*MATa, cdc5-as1(L158G), scc1::HIS3, SCC1-TEV268-HA::LEU2, pGAL-NLS-9MYC-TEV Protease-NLS::TRP1, pMET3-CDC20::URA3, CDC14-3HA*	This thesis
3541	*MATa, cdc14-1, cdc5-as1(L158G), sml1::TRP1, mec1::URA3*	This thesis
3548	*MATa, cdc14-1, cdc5-as1(L158G), top2-1*	This thesis
3555	*MATa, cdc5-as1(L158G), top2-1*	This thesis
3606	*MATa, cdc14-1, cdc5-as1(L158G), ase1::HIS3MX6, trp1::GFP-TUB1::TRP1, ura3::ASE1-7A-6HA-NT2::URA3*	This thesis
3726	*MATa, cdc14-1, cdc5-as1(L158G), scc1::SCC1-GFP-KanMX6, SPC110-mcherry::hphMX3*	This thesis
3729	*MATa, cdc14-1, scc1::SCC1-GFP-KanMX6, SPC110-mcherry::hphMX3*	This thesis
3732	*MATa, cdc5-as1(L158G), scc1::SCC1-GFP-KanMX6, SPC110-mcherry::hphMX3*	This thesis
3771	*MATa, cdc14-1, cdc5-as1(L158G), mad2::URA3, rad9::LEU2*	This thesis
4103	*MATa, cdc14-1, top2-1*	This thesis
4105	*MATa, top2-1*	This thesis
4126	*MATa, cdc14-1, cin8::CIN8(F467A)-3HA::LEU2*	This thesis
4128	*MATa, cdc14-1, cdc5-as1(L158G), cin8::CIN8(F467A)-3HA::LEU2*	This thesis
4130	*MATa, cdc5-as1(L158G), cin8::CIN8(F467A)-3HA::LEU2*	This thesis
4542	*MATa, cdc5-as1(L158G), spo12::HIS3, bns1::KanMX*	This thesis
4557	*MATa, cdc5-as1(L158G), spo12::HIS3*	This thesis
4573	*MATa, cdc14-1, cdc5-as1(L158G), pGAL-3HA-STU2::KanMX6*	This thesis

Strain (Ry)	Relevant genotype	Origin
4596	*MATa, cdc14-1, cdc5-as1(L158G), pGAL-CIN8::TRP1*	This thesis
4640	*MATa, cdc14-1, cdc5-as1(L158G), pGAL-3HA-KIP1::KanMX6*	This thesis
4700	*MATa, cdc14-1, cdc5-as1(L158G), dyn1::URA3*	This thesis
4703	*MATa, cdc14-1, dyn1::URA3*	This thesis
4706	*MATa, cdc5-as1(L158G), dyn1::URA3*	This thesis
4709	*MATa, cdc14-1, cdc5-as1(L158G), clb5::URA3*	This thesis
4712	*MATa, cdc14-1, clb5::URA3*	This thesis
4715	*MATa, cdc5-as1(L158G), clb5::URA3*	This thesis
4745	*MATa, cdc5-2(msd2-1)::URA3*	This thesis
4925	*MATa, cdc14-1, CDC20-aid::KanMX, ura3::pADH-OsTIR1-9MYC::URA3*	This thesis
4928	*MATa, cdc5-as1(L158G), CDC20-aid::KanMX, ura3::pADH-OsTIR1-9MYC::URA3*	This thesis

3. Results

Exit from mitosis starts with the down-regulation of cyclin-dependent kinase (CDK) activity. Next, the phosphate groups that CDK added onto its targets must be removed. In budding yeast, the Cdc14 phosphatase is important for both CDK down-regulation and the reversal of the phosphorylation events mediated by this kinase (for review see (Sullivan and Morgan 2007)). Cdc14 activity itself is controlled by changes in its subcellular localization (Stegmeier and Amon 2004). The phosphatase is sequestered in the nucleolus by its inhibitor Cfi1/Net1 for much of the cell cycle (Stegmeier and Amon 2004). At anaphase, two regulatory networks, the Cdc Fourteen Early Anaphase Release network (FEAR) and the Mitotic Exit Network (MEN), sequentially release Cdc14 from Cfi1 (Stegmeier and Amon 2004). This sequence of Cdc14 activation triggers, in a wave-like manner, the timely dephosphorylation of distinct populations of CDK substrates and thus mitotic events (Sullivan and Morgan 2007, Bouchoux and Uhlmann 2011). Central to the anaphase release of Cdc14 is the polo-like kinase Cdc5 (Hu, Wang et al. 2001, Stegmeier, Visintin et al. 2002, Yoshida and Toh-e 2002, Geymonat, Spanos et al. 2003, Visintin, Stegmeier et al. 2003, Visintin, Tomson et al. 2008), the yeast orthologue of the polo-like kinase 1 (Plk1) (Llamazares, Moreira et al. 1991). In the absence of Cdc5 activity Cdc14 remains tethered in the nucleolus in assumed inactive state (Stegmeier, Visintin et al. 2002, D'Amours and Amon 2004, Stegmeier and Amon 2004).

Next to its essential role in releasing Cdc14, as a component of both the FEAR and MEN networks (Visintin, Tomson et al. 2008, Manzoni, Montani et al. 2010) the polo-like kinase Cdc5 also contributes to anaphase by phosphorylating the cohesin subunit Scc1, thereby promoting its cleavage by the Esp1 protease (Alexandru, Uhlmann et al.

2001, Hornig and Uhlmann 2004). Cdc5 is required for proper spindle microtubule dynamics (Lee, Park et al. 2005, Park, Park et al. 2008) and influences CDK activity by antagonizing the Swe1 kinase, which inhibits CDK (Liang, Jin et al. 2009). Similarly, it has become clear that Cdc14 not only promotes mitotic CDK inactivation and exit from mitosis, but also that it contributes to anaphase by regulating a variety of other cellular events, including rDNA and telomere segregation (D'Amours, Stegmeier et al. 2004, Pereira and Schiebel 2004, Sullivan, Higuchi et al. 2004, Wang, Yong-Gonzalez et al. 2004, Geil, Schwab et al. 2008, Clemente-Blanco, Mayan-Santos et al. 2009, Clemente-Blanco, Sen et al. 2011), mitotic spindle dynamics (Higuchi and Uhlmann 2005, Khmelinskii, Lawrence et al. 2007, Khmelinskii, Roostalu et al. 2009) and cytokinesis (Sanchez-Diaz, Nkosi et al. 2012). Remarkably, the execution of these diverse events relies on Cdc14, activated by different regulatory networks. To establish whether additional and/or overlapping roles between Cdc14 and Cdc5 exist we analyzed the consequences of combining loss-of-function alleles of both enzymatic activities.

A possible genetic synthetic interaction between both their encoding genes was assessed by measuring the growth of yeast cells carrying double mutant combinations of the two genes by spotting serial dilutions of liquid cultures onto YEPD plates. Specifically, temperature-sensitive alleles of *CDC14* (*cdc14-1* or *cdc14-3*) were combined with temperature-sensitive alleles of *CDC5* (*cdc5-1* or *cdc5-2*) or with an ATP analogue sensitive allele of *CDC5* (*cdc5-as1,* (Zhang, Kenski et al. 2005)) that can be specifically inhibited with the CMK inhibitor (Snead, Sullivan et al. 2007). The growth of serially diluted wild type, *cdc14* and *cdc5* single mutants cells on YEPD plates incubated for 48 hours at different temperatures (23°C, 25°C, 28°C and 30°C) were compared with the growth of double mutant strains carrying different combinations of loss-of-function alleles of *cdc14* and *cdc5*: *cdc14-3 cdc5-1*, *cdc14-3 cdc5-2*, *cdc14-1 cdc5-as1*, *cdc14-3 cdc5-as1* and *cdc14-1 cdc5-1* double mutant cells. Four out of the five double mutant

strains tested (*cdc14-3 cdc5-1*, *cdc14-3 cdc5-2*, *cdc14-1 cdc5-as1* and *cdc14-3 cdc5-as1*) exhibited a reduced fitness when compared to the growth of their single mutant counterparts, already at the permissive temperature (23°C), suggesting that the two genes might cooperate to control an essential cellular function, **Fig. 3.1**.

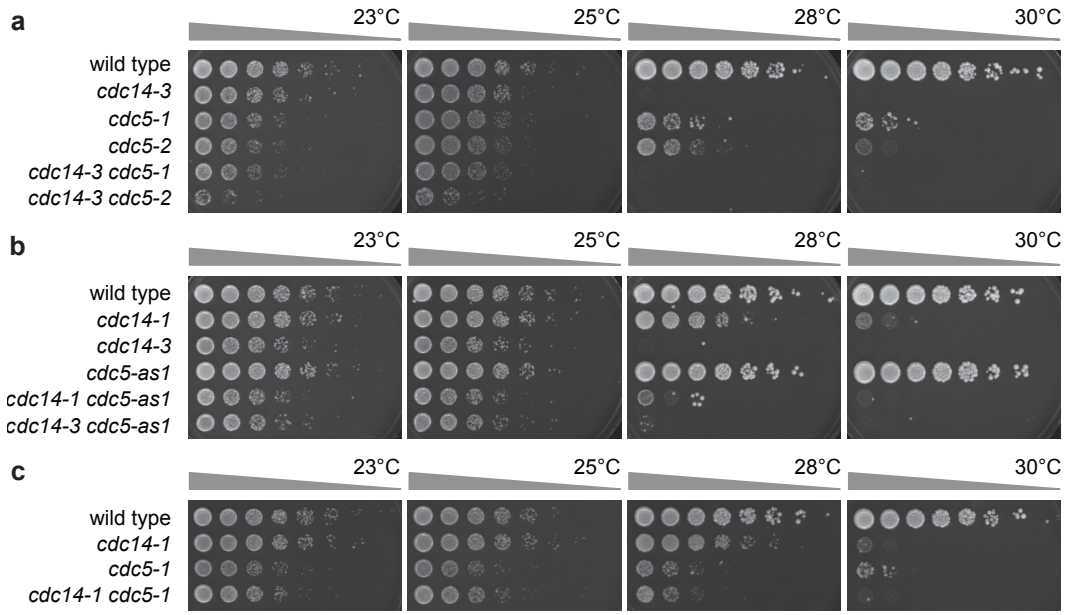

Figure 3.1. Cdc14 phosphatase and Cdc5 kinase show a genetic synthetic interaction. Growth of serially diluted wild type (Ry1), *cdc14-3* (Ry60), *cdc5-1* (Ry133), *cdc5-2* (Ry4745), *cdc14-3 cdc5-1* (Ry2875) and *cdc14-3 cdc5-2* (Ry2774) strains are shown in **(a)**; wild type (Ry1), *cdc14-1* (Ry1574), *cdc14-3* (Ry60), *cdc5-as1* (Ry2446), *cdc14-1 cdc5-as1* (Ry1602) and *cdc14-3 cdc5-as1* (Ry2776) in **(b)**; wild type (Ry1), *cdc14-1* (Ry1574), *cdc5-1* (Ry133) and *cdc14-1 cdc5-1* (Ry2882) in **(c)**. Serial dilutions (1:5) of yeast cells suspensions starting from OD_{600} = 1 were spotted onto YEPD plate and incubated at 23°C, 25°C, 28°C and 30°C for 48 hours **(a-c)**.

3.1 Characterization of the *cdc14 cdc5* phenotype

3.1.1 *cdc14 cdc5* double mutants are defective in anaphase entry

The reduced fitness of the *cdc14 cdc5* double mutant cells when compared to the single mutant counterparts, led us to hypothesize that the two genes may cooperatively control an essential function. To gain insight into this process we combined the temperature-sensitive *cdc14-1* allele with the *cdc5-as1* ATP analogue sensitive allele (Zhang, Kenski et al. 2005), and examined the phenotype of *cdc14-1 cdc5-as1* cells undergoing a synchronous cell cycle at 37°C (restrictive temperature for the *cdc14-1* allele) in the presence of the CMK inhibitor (Snead, Sullivan et al. 2007) (restrictive condition for the *cdc5-as1* allele (Zhang, Kenski et al. 2005). Wild type, *cdc14-1*, *cdc5-as1* and *cdc14-1 cdc5-as1* cells were arrested in G1 by α-factor at the permissive conditions and synchronously released into the next cell cycle at the restrictive conditions. Progression through the cell cycle was tracked by FACS (DNA content) and by nuclear and spindle morphological analysis (anti-Tub1 indirect immunofluorescence), **Fig. 3.2**. Nuclear and spindle morphologies allow us to discriminate metaphase from anaphase cells, thereby pinpointing the exact time during mitotic progression: cells with an undivided nucleus and a short, thick bipolar spindle are usually metaphase cells, while cells with bilobed nuclei and an elongated spindle are in anaphase or telophase. The FACS profiles showed that all the strains entered into and progressed through S phase with similar kinetics, suggesting that the double mutant is not impaired in DNA replication, **Fig. 3.2a**. In contrast, progression through mitosis was different in the four strains **Fig. 3.2b**. Consistent with previous studies, the *cdc14-1* and *cdc5-as1* single mutants arrested in anaphase, but *cdc14-1 cdc5-as1* double mutant cells accumulated as large budded cells with short bipolar spindles and undivided nuclei, **Fig. 3.2b** and **Fig. 3.2c**, suggesting that anaphase spindle elongation did not occur in the double mutant. We concluded that

Cdc14 and Cdc5 redundantly control a process that allows for the metaphase-to-anaphase transition.

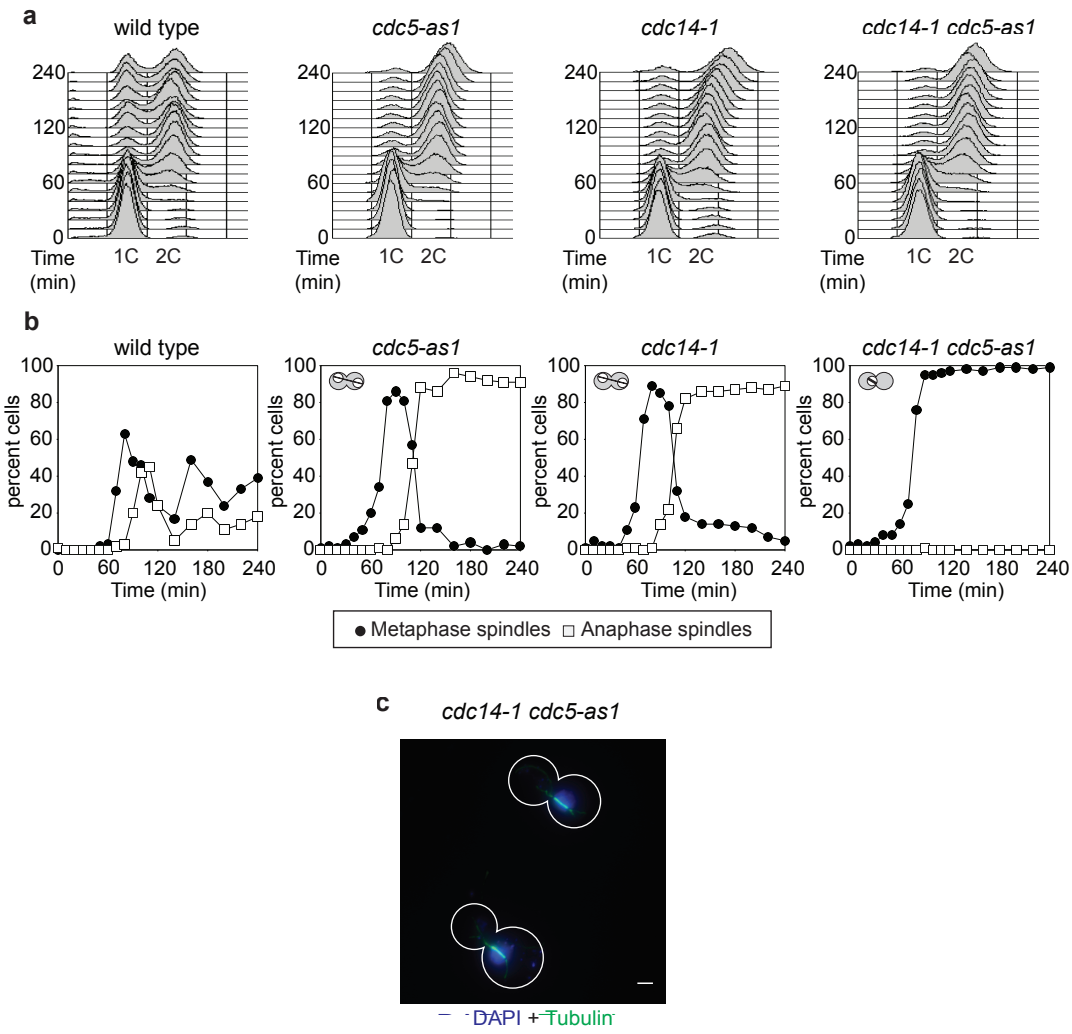

Figure 3.2. *cdc14 cdc5* double mutant cells arrest with undivided nuclei and short bipolar spindles. (a-c) Wild type (Ry1), *cdc5-as1* (Ry2446), *cdc14-1* (Ry1574) and *cdc14-1 cdc5-as1* (Ry1602) cells were arrested in G1 by α-factor in YEPD at 23°C and then released in fresh YEPD media supplemented with the *cdc5-as1* inhibitor (CMK; (Snead, Sullivan et al. 2007)) at 37°C to inactivate the *cdc5-as1* and *cdc14-1* allele, respectively. At the indicated time-points, cells were collected to analyse the cellular DNA content by FACS analysis **(a)** and to determine the percentages of cells with metaphase spindles (closed circles) and anaphase spindles (open squares) **(b)**. Representative image for the *cdc14-1 cdc5-as1* (Ry1602) arrested phenotype (at 240 min. time-point) is shown in **(c)**. Dapi is shown in blue and tubulin in green. Bars, 2 μm **(c)**.

3.1.2 *cdc14-1 cdc5-as1* mutant phenotype is not allele specific

Before proceeding with dissecting the cell cycle defect characterizing the *cdc14-1 cdc5-as1* cells we asked whether the phenotype observed was a peculiarity of the double mutant combination used or common to all *cdc14 cdc5* double mutant pairs. Therefore, we analysed the cell cycle progression of different loss-of-function allelic combinations of *CDC14* and *CDC5*. More specifically, *cdc14-1 cdc5-as1, cdc14-3 cdc5-as1*, *cdc14-1 cdc5-1*, *cdc14-3 cdc5-1* and *cdc14-3 cdc5-2* cells were blocked in G1 and then released from the arrest at restrictive conditions for both *cdc14* and *cdc5*. Progression through the cell cycle was followed looking at nuclear and spindle morphologies, **Fig. 3.3**. We found that all the loss-of-function allele combinations tested arrested as large budded cells with short bipolar spindles and undivided nuclei, **Fig. 3.3**. We concluded that the phenotype of the *cdc14-1 cdc5-as1* double mutant it is not specific to this allele combination and identifies a parallel and/or overlapping function between the two enzymatic activities essential for the completion of mitosis.

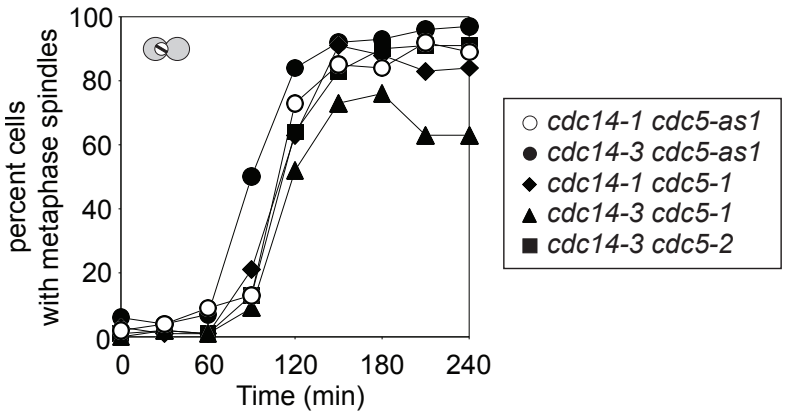

Figure 3.3. *cdc14-1 cdc5-as1* double mutant phenotype is not allele specific. *cdc14-1 cdc5-as1* (Ry1602) (open circles), *cdc14-3 cdc5-as1* (Ry2776) (closed circles), *cdc14-1 cdc5-1* (Ry2882) (closed diamonds), *cdc14-3 cdc5-1* (Ry2875) (closed triangles) and *cdc14-3 cdc5-2* (Ry2774) (closed squares) cells were arrested in G1 by α-factor in YEPD at 23°C. When arrest was complete, cells were released in fresh YEPD medium supplemented with the *cdc5-as1* inhibitor (CMK; (Snead, Sullivan et al. 2007)) and incubated at 37°C, to inactivate the *cdc5-as1* and the temperature sensitive alleles of *CDC14* or *CDC5* (*cdc14-1, cdc14-3, cdc5-1* and *cdc5-2*). Samples were taken at the indicated times to determine the percentage of metaphase spindles.

3.1.3 Restoring favourable conditions allow *cdc14 cdc5* cells to progress into anaphase.

We next asked whether the *cdc14 cdc5* arrest was reversible. To answer this question, we used a *cdc14* and *cdc5* allelic combination that allowed the independent reactivation of each protein. A temperature sensitive allele of *CDC14* (*cdc14-1*) was combined with an allele of *CDC5* (*pGAL-URL-3HA-CDC5*) whose expression is dictated by the carbon source employed (Visintin, Tomson et al. 2008). We could not use the *cdc5-as1* allele for this purpose as the CMK inhibitor (Snead, Sullivan et al. 2007) binds *cdc5-as1* in an irreversible manner. *pGAL-URL-3HA-CDC5 cdc14-1* cells were synchronized in G1 at permissive conditions and released at conditions restrictive for both proteins (raffinose containing medium incubated at 37°C). 180 minutes into the release, when more than 80% of the cells were arrested in metaphase (based on nuclear morphology), the culture was split in three parts. One part was kept at the restrictive conditions for both enzymes, **Fig. 3.4a**, one part was put in a condition that is permissive for Cdc5 (raffinose containing medium supplemented with galactose and incubated at 37°C), **Fig. 3.4b**, and the last part was shifted to a condition that is permissive for Cdc14 (raffinose containing medium incubated at 23°C), **Fig. 3.4c**. Whilst cells impaired for both proteins maintained the arrest up to the end of the experiment, **Fig. 3.4a**, 30 minutes after Cdc14 reactivation, about 50% of cells had progressed into anaphase. The kinetic of anaphase entry was slower when Cdc5 was reactivated indeed to have half of the cells population entered into anaphase we required 60 minutes after Cdc5 reactivation, **Fig. 3.4b**, consistent with the additional requirement of *de novo* protein synthesis for re-accumulating the kinase. In both cases reactivation of one protein was sufficient to re-establish the anaphase arrest characteristic of the single mutants, **Fig. 3.4b** and **Fig. 3.4c**. This data let us to conclude that restoring favourable conditions allows *cdc14 cdc5*

cells to bypass their arrest and also confirmed the requirement of Cdc14 or Cdc5 to progress from metaphase to anaphase.

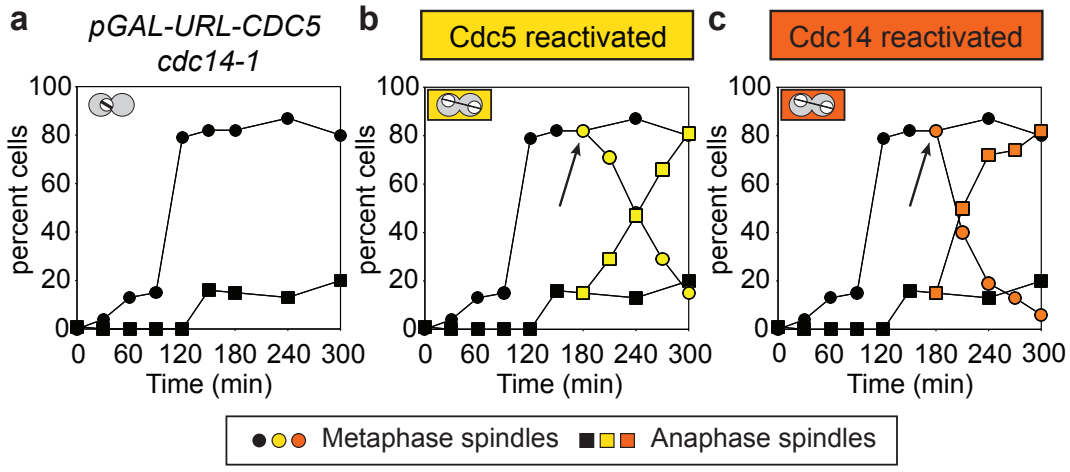

Figure 3.4. The *cdc14 cdc5* arrest is reversible. (a-c) *cdc14-1 GAL-URL-3HA-CDC5* cells (Ry1029) were arrested in G1 with α-factor in YEPR supplemented with 0.5% galactose to induce the expression of *CDC5* at permissive temperature (23°C). When arrest was complete cells were released into fresh YEPR medium lacking galactose at 37°C, to stop the expression of *CDC5* and to inactivate the *cdc14-1* allele of *CDC14*. 180 minutes after the release (arrow) cells were shifted into three different media and incubation conditions: (1) YEPR at 37°C to keep inactive both *cdc5* and *cdc14* **(a)**, (2) YEPR added with 0.5% galactose at 37°C to re-activate *cdc5* **(b)** and (3) YEPR at the permissive temperature of grown (23°C) to re-activate *cdc14* **(c)**. The percentages of cells with metaphase spindles (closed circles) and anaphase spindles (closed squares) were determined at the indicated times. Black symbols are indicative of condition 1, yellow symbols are indicative of condition 2 while orange symbols are indicative of condition 3.

3.1.4 Cdc5 and Cdc14 function after metaphase onset to bring about anaphase entry

Our data suggest that Cdc5 and Cdc14 activities are needed sometime between S phase and early anaphase to promote anaphase progression. To pinpoint their execution point, we assessed the consequences of inactivating both proteins in cells arrested in metaphase. A reversible metaphase arrest can be achieved by placing the *CDC20* gene under the control of the methionine-repressible *pMET3* promoter (Care, Trevethick et al. 1999) as the sole source of the protein. *pMET3-CDC20 cdc5-as1*, *pMET3-CDC20 cdc14-1* and *pMET3-CDC20 cdc14-1 cdc5-as1* cells were synchronized in G1 and next arrested in metaphase by transfer from minimal medium lacking methionine to complete medium containing methionine (Uhlmann, Wernic et al. 2000). When more than 90% of cells were arrested in metaphase, as assessed by nuclear morphology, *cdc14-1* and *cdc5-as1* alleles were inactivated by shifting cells to 37°C in the presence of the CMK inhibitor (Snead, Sullivan et al. 2007), respectively. One hour into the restrictive conditions cells were released from the metaphase block into methionine-free medium, to allow for Cdc20 re-accumulation, while restrictive conditions for *cdc14-1* and *cdc5-as1* were maintained. Cell cycle progression was scored by nuclear and spindle morphologies, **Fig. 3.5**. We noticed that upon methionine removal, *CDC20* rapidly re-accumulated as determined by the kinetics of anaphase entry of *pMET3-CDC20 cdc5-as1* and *pMET3-CDC20 cdc14-1* cells, **Fig. 3.5**. In contrast, entry into anaphase did not occur in *pMET3-CDC20 cdc14-1 cdc5-as1* cells, **Fig. 3.5**. Our results suggest that the essential roles of Cdc5 and Cdc14 in promoting anaphase entry are confined between metaphase and early anaphase.

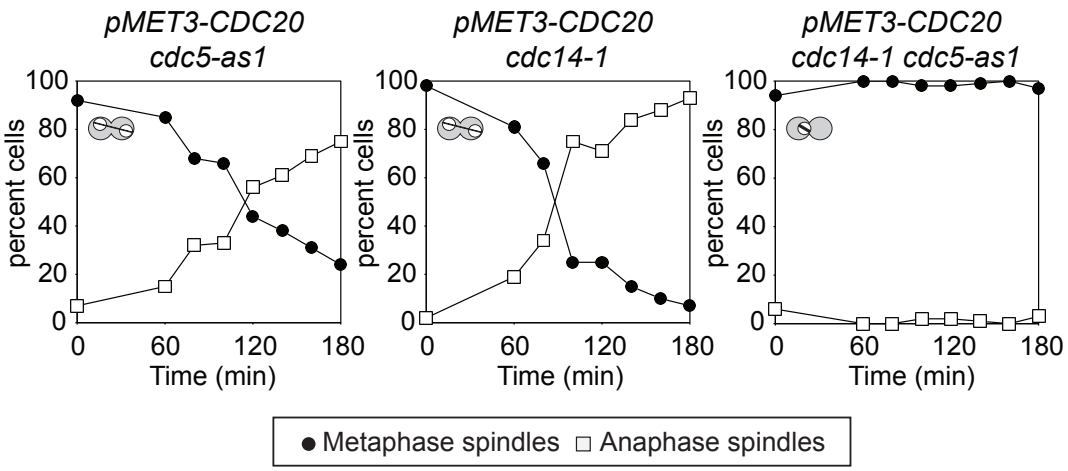

Figure 3.5. Cdc5 and Cdc14 function at or after metaphase to bring about anaphase progression. *pMET3-CDC20 cdc5-as1* (Ry3209), *pMET3-CDC20 cdc14-1* (Ry3204) and *pMET3-CDC20 cdc14-1 cdc5-as1* (Ry3203) cells were arrested in G1 by α-factor in synthetic complete medium lacking methionine (SC-Met) and released in YEPD medium with an added 8 mM methionine to repress the expression of *CDC20* and arrest cells in metaphase. 180 minutes after the release, when >90% of cells were in metaphase (0 time-point), the culture was maintained in the same medium but shifted at the restrictive conditions (37°C and presence of the *cdc5-as1* inhibitor (CMK; (Snead, Sullivan et al. 2007)) to inactivate both *cdc14* and *cdc5*). One hour later cells were released from the metaphase arrest in SC-Met, keeping the restrictive conditions. At the indicated time-points, cells were collected to determine the percentages of cells with metaphase spindles (closed circles) and anaphase spindles (open squares).

3.1.6 *cdc14 cdc5* cells arrest is not dependent on checkpoint activation

The DNA damage checkpoint (DDC) and the spindle assembly checkpoint (SAC) monitor damaged DNA and misaligned spindles, respectively. To probe whether the *cdc14-1 cdc5-as1* mutant arrest was caused by the activation of one or both these surveillance mechanisms we tested the consequences of removing checkpoint activities in our double mutant. Cell cycle progression of *cdc14-1 cdc5-as1* cells carrying additional mutations in essential components of both surveillance mechanisms alone (*rad9Δ*, *rad53-K227A* and *mec1Δ sml1Δ* for the DDC, **Fig. 3.6a**, or *mad1Δ*, *mad2Δ* and *mad1Δ mad2Δ* for the SAC, **Fig. 3.6b**) or in combination (*rad9Δ* plus *mad2Δ*, **Fig.**

3.6c), was followed after a synchronous release from a G1 arrest. Cell cycle progression at the restrictive conditions for both *cdc14* and *cdc5* was scored by nuclear and spindle morphologies, **Fig. 3.6a-c**. We found that neither the abrogation of DDC and SAC alone or in combination bypassed the *cdc14-1 cdc5-as1* terminal phenotype, suggesting that the *cdc14 cdc5* arrest is not due to checkpoint activation.

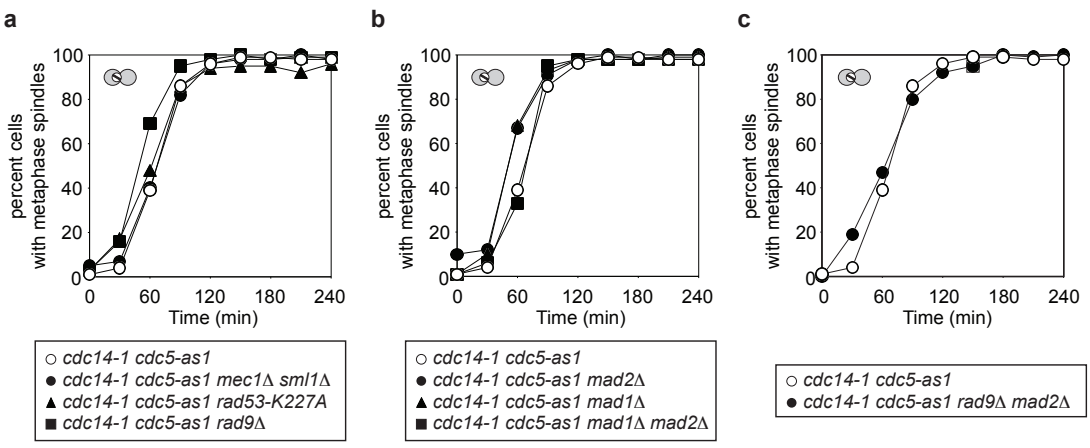

Figure 3.6. *cdc14 cdc5* cells arrest is not checkpoint dependent. *cdc14-1 cdc5-as1* (Ry1602) (open circles) and *cdc14-1 cdc5-as1 mec1Δ sml1Δ* (Ry3541) (closed circles), *cdc14-1 cdc5-as1 rad53-K227A* (Ry2580) (closed triangles) and *cdc14-1 cdc5-as1 rad9Δ* (Ry2169) (closed squares) cells in **(a)**, *cdc14-1 cdc5-as1* (Ry1602) (open circles) and *cdc14-1 cdc5-as1 mad2Δ* (Ry3186) (closed circles), *cdc14-1 cdc5-as1 mad1Δ* (Ry2597) (closed triangles) and *cdc14-1 cdc5-as1 mad2Δ mad1Δ* (Ry3166) (closed squares) cells in **(b)** and *cdc14-1 cdc5-as1* (Ry1602) (open circles) and *cdc14-1 cdc5-as1 rad9Δ mad2Δ* (Ry3771) (closed circles) cells in **(c)** were arrested in G1 by α-factor in YEPD at 23°C. When arrest was complete, cells were released in fresh YEPD medium supplemented with the *cdc5-as1* inhibitor (CMK; (Snead, Sullivan et al. 2007)) at 37°C to inactivate the *cdc5-as1* and *cdc14-1* allele, respectively. Samples were taken at the indicated times to determine the percentages of cells with metaphase spindles.

3.1.7 Sgo1 inactivation did not enable spindle elongation in *cdc14 cdc5* cells.

Yeast cells arrested in metaphase through inhibition of the APC/C^{Cdc20} can elongate their spindles when *SGO1* is inactivated (Kiburz, Amon et al. 2008). Although Sgo1 was originally identified as factor required for preventing the removal of centromeric

cohesin during meiosis I (Kitajima, Kawashima et al. 2004, Xu, Cetin et al. 2009), additional roles in mitotic bi-orientation of sister chromatids and in the spindle assembly checkpoint have been described (Indjeian, Stern et al. 2005, Kiburz, Amon et al. 2008). With this in mind we tested whether the deletion of *SGO1* could elongate the short "metaphase-like" spindles of the double *cdc14 cdc5* mutant. *cdc14-1*, *cdc5-as1* and *cdc14-1 cdc5-as1* cells carrying a deletion in *SGO1* (*sgo1Δ*) were arrested in G1 and synchronously released into the cell cycle at restrictive conditions for both Cdc14-1 and Cdc5-as1. Cell cycle progression was scored by spindle morphology analysis, **Fig. 3.7**. We observed that Sgo1 inactivation did not enable spindle elongation in *cdc14-1 cdc5-as1* cells.

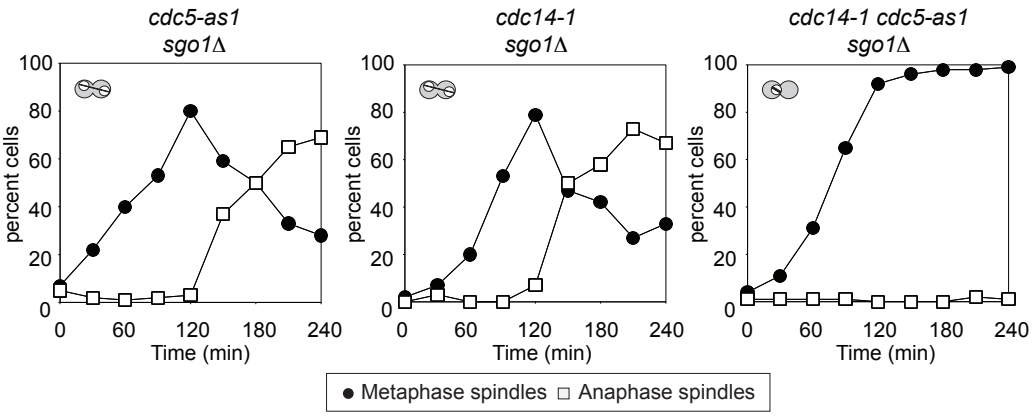

Figure 3.7. Sgo1 inactivation did not enable spindle elongation in *cdc14 cdc5* cells. *cdc5-as1 sgo1Δ* (Ry2279), *cdc14-1 sgo1Δ* (Ry2277) and *cdc14-1 cdc5-as1 sgo1Δ* (Ry2275) cells were arrested in G1 by α-factor in YEPD at 23°C. When arrest was complete, cells were released in fresh YEPD media supplemented with the *cdc5-as1* inhibitor (CMK; (Snead, Sullivan et al. 2007)) at 37°C to inactivate the *cdc5-as1* and the *cdc14-1* alleles, respectively. At the indicated time-points, cells were collected to determine the percentages of cells with metaphase spindles (closed circles) and anaphase spindles (open squares).

3.2 Probing the metaphase-to-anaphase transition in *cdc14 cdc5* cells

3.2.1 Securin is degraded in *cdc14 cdc5* cells

Entry into anaphase starts with the activation of the ubiquitin ligase APC/C^{Cdc20}, which degrades the anaphase inhibitor Pds1 (Cohen-Fix, Peters et al. 1996, Shirayama, Toth et al. 1999). Pds1 degradation releases the separase Esp1 resulting in the cleavage of cohesin subunit Scc1 (Ciosk, Zachariae et al. 1998, Cohen-Fix and Koshland 1999, Tinker-Kulberg and Morgan 1999, Uhlmann, Lottspeich et al. 1999, Uhlmann, Wernic et al. 2000). To establish whether Pds1 degradation is impaired in our double mutant we synchronized *cdc14-1*, *cdc5-as1* and *cdc14-1 cdc5-as1* cells, each carrying a *PDS1-3HA* fusion, in G1 and then synchronously released them into the next cell cycle under conditions that are restrictive for Cdc14-1 and Cdc5-as1. Cell cycle progression was scored by spindle morphology and Pds1 accumulation and degradation was assessed by anti-HA Western blot analysis, **Fig. 3.8**. Pds1 accumulation and degradation occurred with similar kinetics in the three strains, **Fig. 3.8**. Although a slight delay in Pds1 degradation was observed in the double mutant, the cells resulted proficient in securin degradation, **Fig. 3.8**. We concluded that the *cdc14 cdc5* cells arrest for reasons other than an inability to degrade Pds1 and release Esp1.

To further exclude that the arrest of the double mutant was due to defects in Pds1 degradation we probed the consequences of removing Pds1 activity (*pds1Δ*) in our cells. As such, *cdc14-1 cdc5-as1* and *cdc14-1 cdc5-as1 pds1Δ* cells were synchronously released from G1 into restrictive conditions for both Cdc14-1 and Cdc5-as1. Progression through the cell cycle was tracked by spindle morphological analysis, **Fig. 3.9**. Our data showed that deleting *PDS1* in *cdc14 cdc5* cells did not rescue the

arrest characterizing the double mutant, **Fig. 3.9**. These findings support our previous conclusion that the *cdc14 cdc5* arrest is not due to defects in securin degradation.

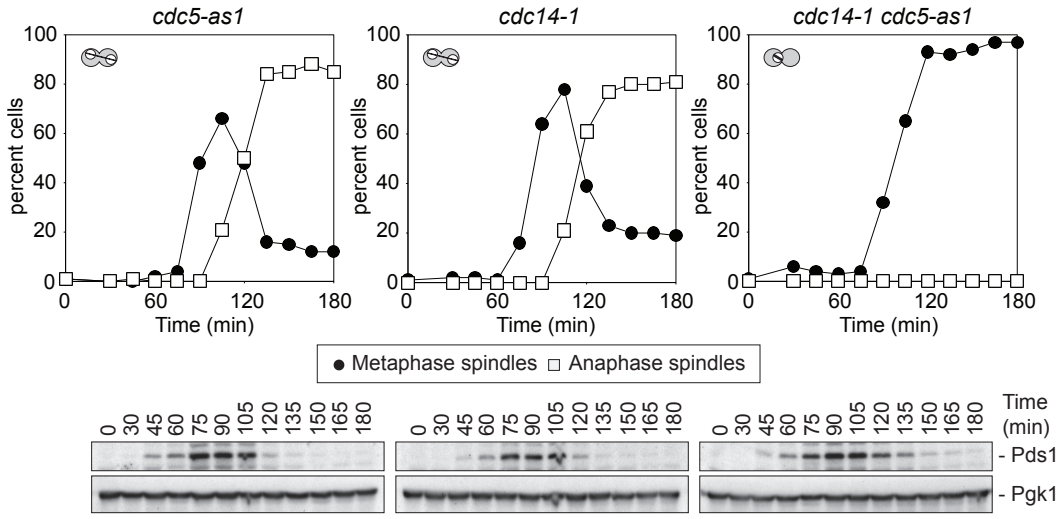

Figure 3.8. *cdc14 cdc5* cells are proficient in securin degradation. *cdc5-as1* (Ry2629), *cdc14-1* (Ry2633) and *cdc14-1 cdc5-as1* (Ry2626) cells carrying a *PDS1-3HA* fusion were arrested in G1 by α-factor in YEPD at 23°C. When arrest was complete, cells were released in fresh YEPD medium supplemented with the *cdc5-as1* inhibitor (CMK; (Snead, Sullivan et al. 2007)) and incubated at 37°C to inactivate the *cdc5-as1* and *cdc14-1* alleles, respectively. Samples were taken at the indicated times to determine the percentages of cells with metaphase spindles (closed circles) and anaphase spindles (open squares) as well as to analyze Pds1 and Pgk1 protein levels. Pgk1 protein was used as an internal loading control in immunoblots.

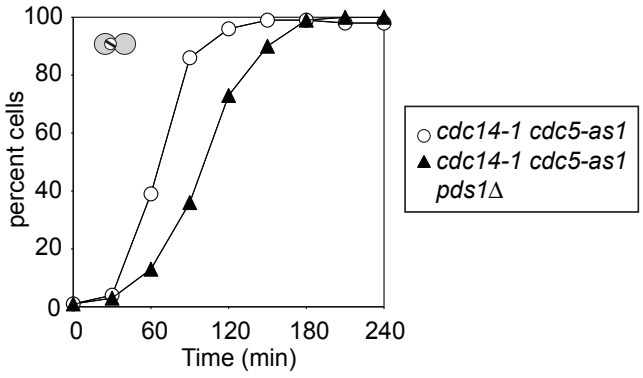

Figure 3.9. The deletion of securin does not bypass the *cdc14 cdc5* arrest. *cdc14-1 cdc5-as1* (Ry1602) (open circles) and *cdc14-1 cdc5-as1 pds1Δ* (Ry2612) (closed triangles) cells were arrested in G1 by α-factor in YEPD at 23°C. When arrest was complete, cells were released in fresh YEPD medium supplemented with the *cdc5-as1* inhibitor (CMK; (Snead, Sullivan et al. 2007)) at 37°C to inactivate the *cdc5-as1* and *cdc14-1* allele, respectively. Samples were taken at the indicated times to determine the percentages of cells with metaphase spindles.

3.2.2 Separase is active in *cdc14 cdc5* cells

Pds1 degradation is a prerequisite for the Esp1-mediated dissolution of the cohesin complexes that hold the sister chromatids together (Michaelis, Ciosk et al. 1997, Ciosk, Zachariae et al. 1998, Uhlmann, Lottspeich et al. 1999, Uhlmann, Wernic et al. 2000). As our double mutant is capable of degrading Pds1, we wondered whether separase was defective in the *cdc14 cdc5* cells. Hence, we tested separase activity by assessing kinetochore protein Slk19, an Esp1 substrate that is cleaved at anaphase onset (Sullivan, Lehane et al. 2001). As such, *cdc14-1*, *cdc5-as1*, and *cdc14-1 cdc5-as1* cells carrying a *SLK19-13MYC* fusion were arrested in G1 at permissive conditions for Cdc14-1 and Cdc5-as1 and then synchronously released into the cell cycle at restrictive conditions for both mutant proteins. Cell cycle progression was scored by nuclear and spindle morphologies, while Slk19 cleavage was probed by anti-Myc Western blot analysis, **Fig. 3.10**. We found that Slk19 was cleaved with similar kinetics in all three mutant strains, **Fig. 3.10**, indicating that Esp1 is active in the *cdc14-1 cdc5-as1* cells.

To further exclude that the *cdc14 cdc5* phenotype was caused by an incomplete activation of the protease Esp1, we examined the effects of ectopically induced *ESP1* once the terminal arrest of the double mutant was achieved. For this purpose we used a *cdc14 cdc5* strain in which the *ESP1* gene was placed under the control of the galactose-inducible and glucose-repressible *pGAL1-10* promoter (West, Chen et al. 1987). *cdc14-1 cdc5-as1 pGAL-ESP1* cells were arrested at the *cdc14 cdc5* terminal phenotype after G1 synchronization in raffinose containing medium, a carbon source that neither repress nor induce the transcriptional activity of *ESP1* (*pGAL1-10* = OFF). When the arrest was complete and more then 90% of cells were in metaphase (as judged by nuclear morphology) the culture was split in two. One-half was maintained in the raffinose-based medium, while the second half of the mother culture was provided with 2% galactose (*pGAL1-10* = ON) to induce *ESP1* expression, **Fig. 3.11**. Samples were

taken at the indicated time-points to determine the percentages of cells with metaphase and anaphase spindles, **Fig. 3.11**. We observed that the ectopic expression of Esp1 did not bypass the arrest of *cdc14-1 cdc5-as1* cells, **Fig. 3.11**, hence concluding that the phenotype of *cdc14 cdc5* cells is not caused by a lack of Esp1 activity.

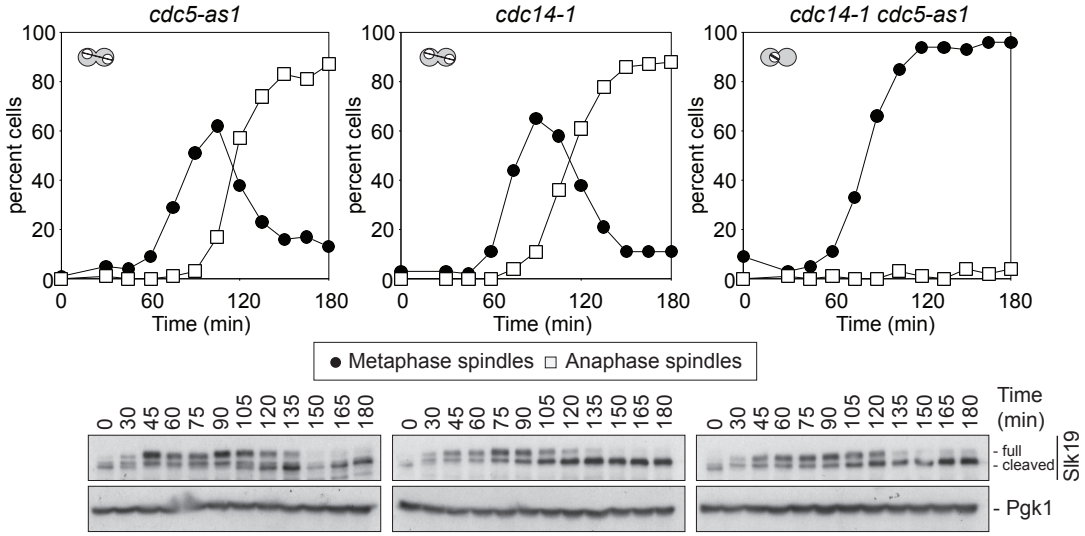

Figure 3.10. Separase is active in *cdc14 cdc5* cells. *cdc5-as1* (Ry2790), *cdc14-1* (Ry2785) and *cdc14-1 cdc5-as1* (Ry2780) cells carrying a *SLK19-13MYC* fusion were arrested in G1 by α-factor in YEPD at 23°C. When arrest was complete, cells were released in fresh YEPD medium supplemented with the *cdc5-as1* inhibitor (CMK; (Snead, Sullivan et al. 2007)) at 37°C to inactivate the *cdc5-as1* and *cdc14-1* allele, respectively. Samples were taken at the indicated times to determine the percentages of cells with metaphase spindles (closed circles) and anaphase spindles (open squares) as well as to analyze Slk19 and Pgk1 protein levels. Pgk1 protein was used as an internal loading control in immunoblots.

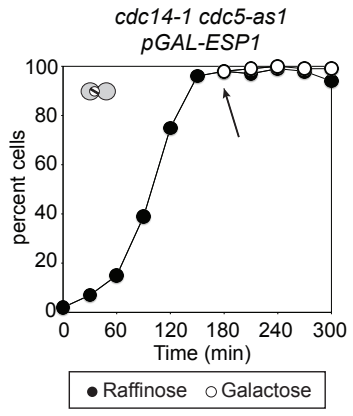

Figure 3.11. Ectopic expression of separase is not sufficient to promote anaphase entry in *cdc14 cdc5* cells. *cdc14-1 cdc5-as1 pGAL-ESP1* (Ry3030) cells were arrested in G1 in YEPR with α-factor at 23°C. When arrest was complete, cells were released in fresh YEPR supplemented with the *cdc5-as1* inhibitor (CMK;(Snead, Sullivan et al. 2007)) at 37°C to inactivate the *cdc5-as1* and *cdc14-1* allele, respectively. Three hours after the release (arrow), when more then 90% of cells entered metaphase, the culture was split in two and one-half was maintained in the same medium (closed circles), whereas 2% galactose was added to the other half to induce *pGAL-ESP1* expression (open circles). The percentages of cells with metaphase spindles was determined at the indicated times.

3.2.3 Cohesin is cleaved in *cdc14 cdc5* cells

Having established that Esp1 is functional in our double mutant, at least towards Slk19, we decided to investigate if Esp1 properly cleaves cohesin in our cells. *cdc14-1*, *cdc5-as1* and *cdc14-1 cdc5-as1* cells carrying a *SCC1-18MYC* fusion were arrested in G1 and synchronously released into the next cell cycle at restrictive conditions for both Cdc14-1 and Cdc5-as1. Cell cycle progression was scored by nuclear and spindle morphologies while Scc1 levels and cleavage were followed by anti-Myc Western blot analysis, **Fig. 3.12**. We found that Scc1 was cleaved in all three mutants. Although cohesin cleavage in the double mutant seemed less efficient when compared with the kinetics of cleavage in the single *cdc14-1* mutant it resembled the cleavage kinetics exhibited by the single *cdc5-as1* mutant, **Fig. 3.12**. However, whilst *cdc5* mutants were able to proceed into anaphase, the *cdc14-1 cdc5-as1* cells arrested with short bipolar spindles and undivided nuclei, **Fig. 3.12**. From these results we concluded that cohesin is cleaved in *cdc14-1 cdc5-as1* cells.

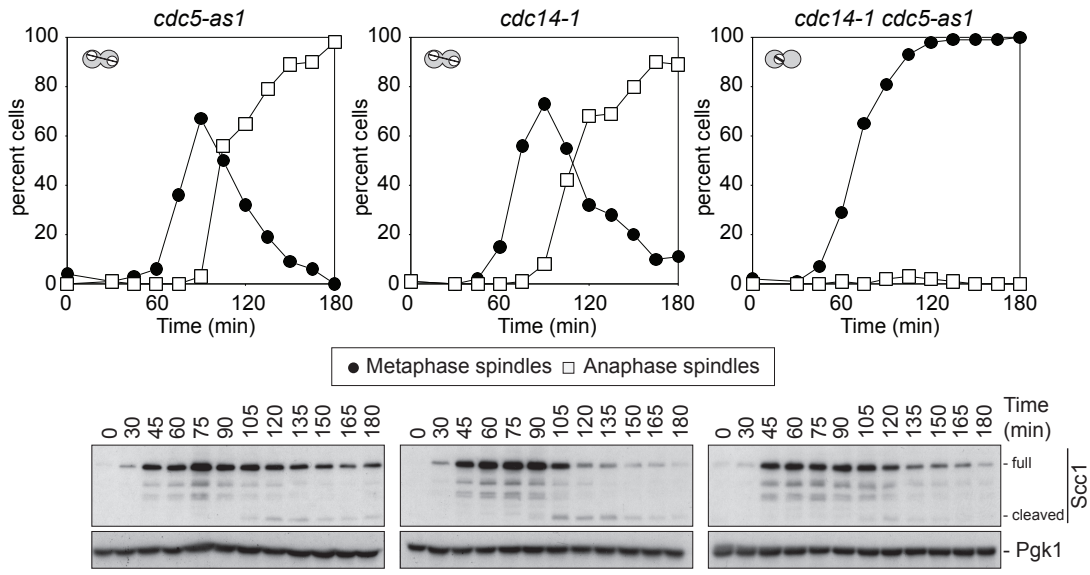

Figure 3.12. Cohesin is cleaved in *cdc14 cdc5* cells. *cdc5-as1* (Ry2747), *cdc14-1* (Ry2743) and *cdc14-1 cdc5-as1* (Ry2725) cells carrying a *SCC1-18MYC* fusion were arrested in G1 by α-factor in YEPD at 23°C. When arrest was complete, cells were released in fresh YEPD medium supplemented with the *cdc5-as1* inhibitor (CMK; (Snead, Sullivan et al. 2007)) at 37°C to inactivate the *cdc5-as1* and *cdc14-1* allele, respectively. Samples were taken at the indicated times to determine the percentages of cells with metaphase spindles (closed circles) and anaphase spindles (open squares) as well as to analyze Scc1 and Pgk1 protein levels. Pgk1 protein was used as an internal loading control in immunoblots.

3.2.4 Inactivation of cohesin cannot suppress the chromosome segregation defect of *cdc14 cdc5* cells

To further exclude that residual cohesion may have caused the arrest of the *cdc14-1 cdc5-as1* cells, we examined: i) cohesin localization at single cell level and determined ii) whether an ectopic cleavage of Scc1 could rescue the requirement for Cdc14 and Cdc5 in promoting chromosome segregation.

For our single cell studies we tracked the cohesin subunit Scc1 tagged with the green fluorescent protein (GFP), Scc1-GFP, in *cdc14-1*, *cdc5-as1* and *cdc14-1 cdc5-as1* cells endogenously expressing mCherry-labeled spindle pole body protein Spc110 (Spc110-mCherry). The three strains were arrested in G1 and synchronously released

into the next cell cycle at restrictive conditions for both Cdc14-1 and Cdc5-as1. Once the cells were in metaphase (80 min. after the G1 release) or at their terminal arrest (240 min. after the G1 release) the cells were fixed and both fluorescence signals analyzed, **Fig. 3.13**. In metaphase, all strains had accumulated cohesin on the chromatin, whereas at the terminal arrest all strains had released cohesin from the DNA. But differently to the *cdc14-1* and *cdc5-as1* single mutants, which progressed through mitosis until late anaphase (arrest with elongated spindles), the *cdc14-1 cdc5-as1* double mutant maintained the short bipolar spindle characteristic of metaphase cells, as judged by the interpolar distance between the two Spc110 signals, **Fig. 3.13**.

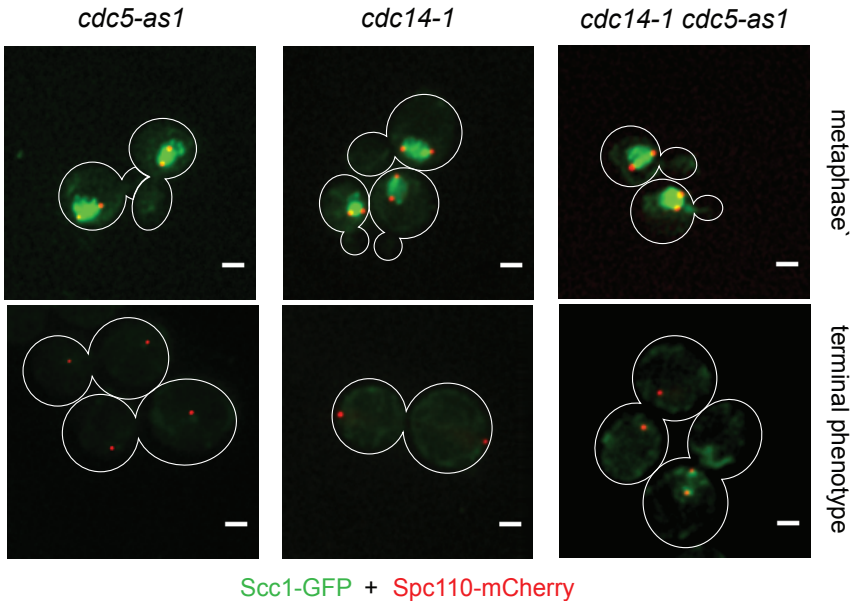

Figure 3.13. In *cdc14 cdc5* cells cohesin is absent from chromatin at the terminal phenotype. *cdc5-as1* (Ry3732), *cdc14-1* (Ry3729) and *cdc14-1 cdc5-as1* (Ry3726) cells carrying a *SCC1-GFP* and a *SPC110*-mCherry fusion were arrested in G1 by α-factor in YEPD at 23°C. When arrest was complete, cells were released into fresh YEPD medium supplemented with the *cdc5-as1* inhibitor (CMK; (Snead, Sullivan et al. 2007)) and incubated at 37°C to inactivate the *cdc5-as1* and *cdc14-1* allele, respectively. When cells were in metaphase (80 min. after the G1 release) and at their terminal arrest (240 min. after the G1 release), samples were taken and treated for a quick fixation for microscopy (see materials and methods). Spc110 is shown in red and Scc1 in green. Bars, 2 µm.

Next, we probed whether the ectopic cleavage of Scc1 would allow *cdc14-1 cdc5-as1* cells to enter anaphase. Specifically, we used the *scc1-TEV268* allele (Uhlmann, Wernic et al. 2000), in which one of the two Esp1 recognition sites (at position 268) was replaced with a target site for the tobacco etch virus (TEV) protease (Dougherty, Parks et al. 1989). This Scc1 variant (Uhlmann, Wernic et al. 2000) supports the viability of cells lacking any other form of Scc1 and can be ectopically cleaved by the TEV protease. Ectopic expression of the TEV protease was achieved by placing it under control of the inducible *pGAL1-10* promoter (West, Chen et al. 1987). *cdc14-1 cdc5-as1 scc1Δ scc1-TEV268-HA pGAL-9MYC-TEV* cells were arrested in G1 in raffinose-based medium at 23°C. When arrest was complete, cells were released in fresh raffinose-based medium at the restrictive conditions to inactivate the *cdc5-as1* and *cdc14-1* alleles. 120 minutes after the release, when about 90% of cells were in metaphase, the culture was split in two. One-half was maintained in the same medium, whereas 2% galactose was added to the other half to induce the expression of the TEV protease, **Fig. 3.14**. Samples were taken at the indicated time-points to determine the percentage of cells with metaphase spindles, **Fig. 3.14a**, and to examine TEV protease induction and Scc1-TEV268 cleavage, **Fig. 3.14b**. We observed that concomitantly with the ectopic induction of the TEV protease, Scc1-TEV268 was cleaved, **Fig. 3.14b**. Nevertheless, the *cdc14-1 cdc5-as1* cells remained arrested with short bipolar spindles and a single DNA mass at the bud-neck, **Fig. 3.14a**. From this set of experiments we conclude that cohesin cleavage is normal in the *cdc14-1 cdc5-as1* cells and that it is not the cause of the observed nuclear division defect.

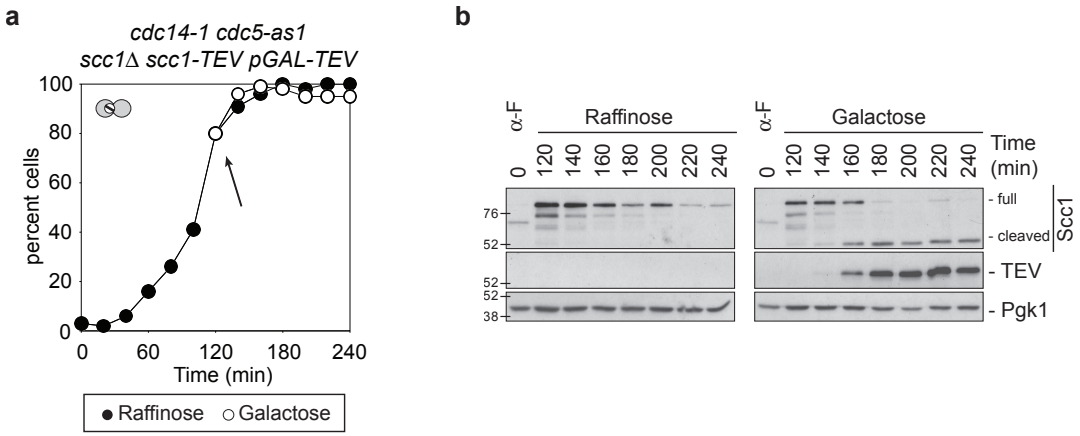

Figure 3.14. The ectopic cleavage of Scc1 by the TEV protease does not suppress the nuclear division defect of *cdc14-1 cdc5-as1* cells. (a-b) *cdc14-1 cdc5-as1 scc1Δ SCC1-TEV268-HA GAL-9MYC-TEV* cells (Ry2795) were arrested in G1 by α-factor in YEPR at 23°C. When arrest was complete, cells were released in fresh YEPR medium supplemented with the *cdc5-as1* inhibitor (CMK; (Snead, Sullivan et al. 2007)) at 37°C to inactivate the *cdc5-as1* and *cdc14-1* allele, respectively. 120 minutes after the release (arrow), when >90% of cells were in metaphase, the culture was split in two. One-half was maintained in the same medium (closed circles), whereas 2% galactose was added to the other half to induce the expression of TEV protease (open circles). Samples were taken at the indicated times to determine the percentage of cells with metaphase spindles **(a)** as well as to analyze Scc1, TEV and Pgk1 protein levels **(b)**. Pgk1 protein was used as an internal loading control in immunoblots **(b)**.

The results concerning Pds1 degradation, Esp1 activity and cohesin cleavage in *cdc14 cdc5* cells indicate that the double mutant, although arresting with metaphase-like spindles, have already entered anaphase. We concluded that a Cdc14 and Cdc5 controlled activity downstream of cohesin cleavage is required to promote progression into anaphase.

3.3 Identification of problems associated with *cdc14 cdc5* cells

3.3.1 *cdc14 cdc5* cells are defective in spindle elongation

The inability of cohesin inactivation to rescue the segregation defect of *cdc14 cdc5* cells suggests that Cdc14 and Cdc5 may contribute to anaphase progression either by resolving cohesin-independent topological linkages (like inter-molecular DNA intertwins or catenates) or by driving spindle elongation. To discriminate between both possibilities we wondered how the absence of sister chromatids pairs affects the cell cycle progression of *cdc14-1 cdc5-as1* mutants. To answer this question we inhibited initiation of DNA replication in our double mutant by preventing pre-replicative complex (pre-RC) assembly through the depletion of the pre-RC essential component Cdc6 (Piatti, Lengauer et al. 1995, Toone, Aerne et al. 1997). Cells whose sole *CDC6* gene is placed under the control of the glucose-repressible and galactose-inducible *pGAL1-10* promoter (West, Chen et al. 1987) fail to replicate their chromosomes in the presence of glucose due to a lack of Cdc6 function (Piatti, Lengauer et al. 1995). Although in the absence of sister chromatids pairs (next referred to as "sister-less" chromatid) these cells are anyway able to form and elongate their mitotic spindles, to segregate their genome and to undergo cytokinesis. However in such situation the un-replicated chromosomes are segregated randomly to one or the other pole of the cell performing an anaphase that has been called "reductional" (Piatti, Lengauer et al. 1995). To avoid SAC activation under these conditions, we deleted SAC component Mad1 (*mad1Δ*) in our *pGAL-CDC6* strains. *cdc14-1 mad1Δ* , *cdc5-as1 mad1Δ* and *cdc14-1 cdc5-as1 mad1Δ* cells, in which *CDC6* was under the control of *pGAL1-10* were first grown in the presence of galactose and hydroxyurea, to obtain a synchronous arrest in S phase with pre-replicative complexes at origins of DNA replication. Cells were then released in glucose medium containing α-factor, to completely repress *pGAL-CDC6*

and arrest cells in G1 in the absence of the pre-replicative complexes. When the G1 arrest was complete, the cells were released in fresh glucose medium under conditions restrictive for Cdc14-1 and Cdc5-as1, **Fig. 3.15**. In cells carrying "sister-less" chromatids we observed that anaphase spindle elongation occurred only if either Cdc14 or Cdc5 was active. Spindle elongation did not happen in the "sister-less" double mutant, indicating that the anaphase spindle elongation process is defective in the *cdc14-1 cdc5-as1* cells, **Fig. 3.15**.

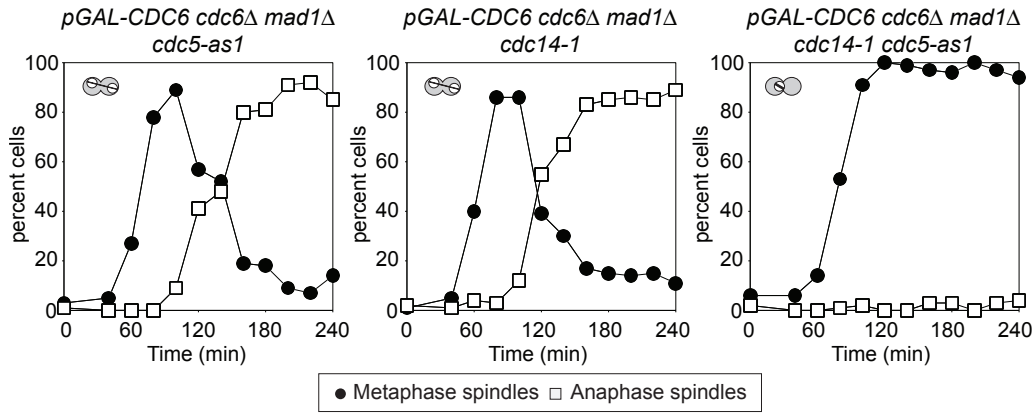

Figure 3.15. *cdc14 cdc5* cells are defective in the spindle elongation process. *cdc5-as1 cdc6Δ mad1Δ pGAL-ubiR-CDC6* (Ry3145), *cdc14-1 cdc6Δ mad1Δ pGAL-ubiR-CDC6* (Ry3141) and *cdc14-1 cdc5-as1 cdc6Δ mad1Δ pGAL-ubiR-CDC6* (Ry3136) cells were first grown at 23°C in YEPR media supplemented with 2% galactose and HU to induce the expression of *CDC6* and to obtain a synchronous arrest in S phase. When the S phase arrest was complete, cells were released in fresh YEPD media containing α-factor to arrest cells in G1 at 23°C. When G1 arrest was complete cells were then released in fresh YEPD medium supplemented with the *cdc5-as1* inhibitor (CMK; (Snead, Sullivan et al. 2007)) at 37°C to inactivate the *cdc5-as1* and *cdc14-1* allele, respectively. Samples were taken at the indicated times to determine the percentages of cells with metaphase spindles (closed circles) and anaphase spindles (open squares).

3.3.2 Analysis of spindle length in *cdc14 cdc5* cells

Anaphase spindle elongation occurs in two steps: an initial rapid phase in which the spindle elongates to 4-6 μm, followed by a phase in which the spindle slowly elongates to a final length of 8-10 μm (Straight, Sedat et al. 1998, Movshovich, Fridman et al.

2008). To characterize the spindle elongation defect in *cdc14 cdc5* mutant cells we measured spindle length in our mutants. *cdc14-1*, *cdc5-as1* and *cdc14-1 cdc5-as1* cells were arrested in G1 at the permissive conditions and were then synchronously released into the cell cycle at the restrictive conditions. Cell cycle progression was followed by nuclear and spindle morphologies, **Fig. 3.16a**, while the spindle length were measured for 100 cells from the 90 min. time-point to the 240 min. time-point, **Fig. 3.16b**. As expected we found that both *cdc14-1* and *cdc5-as1* single mutant cells elongate their spindles but, although they both arrested in anaphase (as indicated by nuclear morphology; two separated DNA masses) the *cdc5-as1* mutants showed spindles with a mode spanning from 4-6 μm while almost all the *cdc14-1* cells elongated their spindles to more than 6 μm **Fig. 3.16b** e **Fig. 3.16d**. On the contrary, the *cdc14-1 cdc5-as1* double mutant exhibited a broad distribution of spindle length with a mean at 2-4 μm, **Fig. 3.16b**. This spindle length is typical for cells arrested in metaphase (Winey and O'Toole 2001). We indeed compared the spindle length distribution profile of *cdc14 cdc5* cells at their terminal phenotype with the same profiles of mutant cells known to arrest in metaphase. To this aim we have chosen two strains in which the ubiquitin ligase APC/C, whose function is required for the metaphase-to-anaphase transition to occur (Cohen-Fix, Peters et al. 1996), can be conditionally inactivated. *cdc14-1 cdc5-as1*, *pMET-CDC20* (in which it is possible to deplete the metaphase cofactor of the APC/C, Cdc20) and *cdc23-1* (in which it is possible to inactivate the essential subunit of the APC/C, Cdc23) cells were arrested at their terminal phenotype after a G1 synchronization, **Fig. 3.16c**. Four hours after the release from G1, when the terminal phenotype of the mutated strains was achieved, the spindle length (in micrometers) was determined for 100 cells per strains, **Fig. 3.16c**. From the graphic it is possible to appreciate that *cdc14-1 cdc5-as1* cells arrest with a “metaphase-like” spindle length distribution, almost identical to the ones characteristic for metaphase-arrested cells.

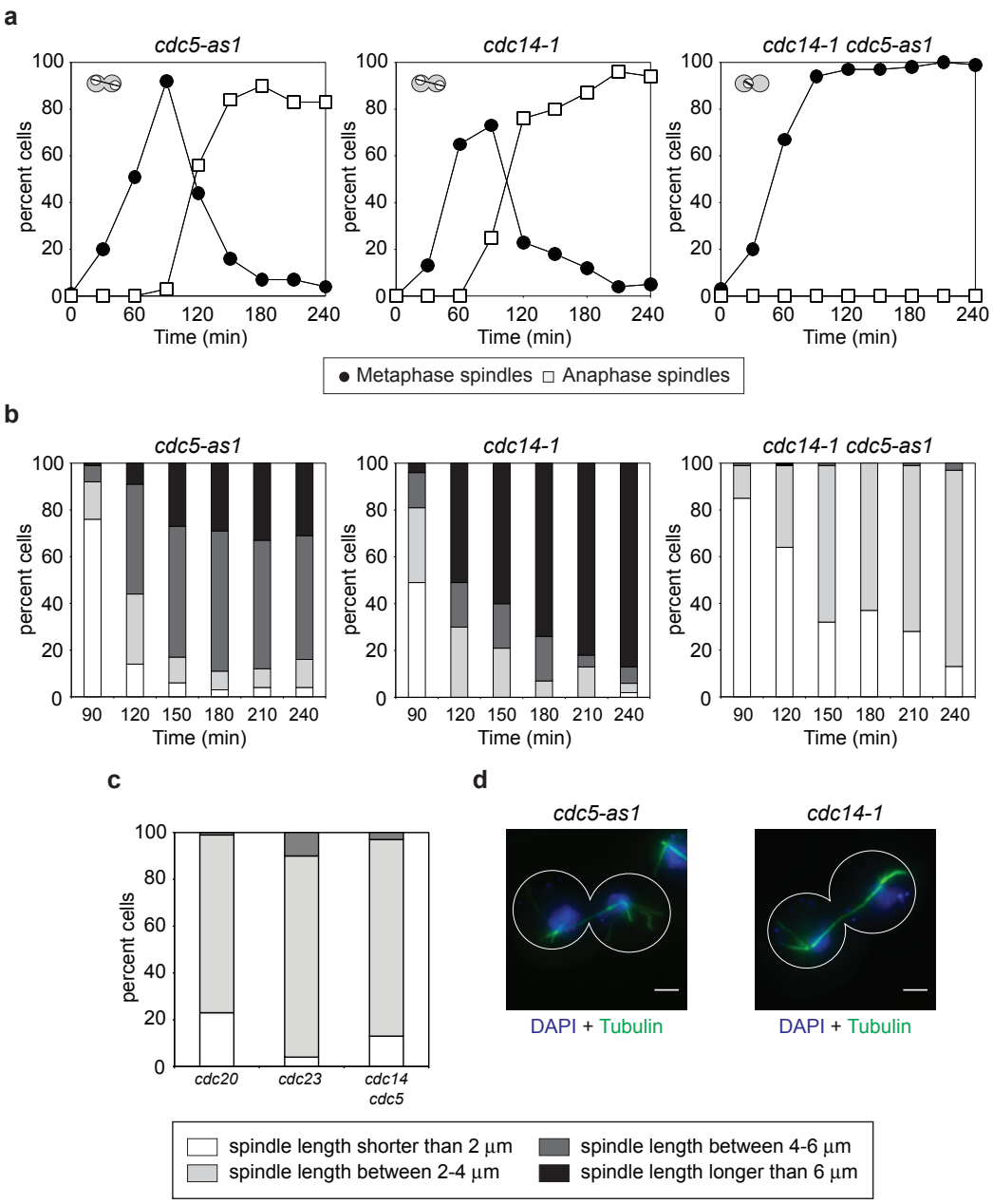

Figure 3.16. Spindle length analysis of *cdc14 cdc5* cells. (a-b) *cdc5-as1* (Ry2446), *cdc14-1* (Ry1574) and *cdc14-1 cdc5-as1* (Ry1602) cells were arrested in G1 by α-factor in YEPD at 23°C. When arrest was complete, cells were released in fresh YEPD at conditions restrictive for both Cdc14-1 and Cdc5-as1. At the indicated time-points, cells were collected to determine the percentages of cells with metaphase spindles (closed circles) and anaphase spindles (open squares) **(a)**. The spindle length (in micrometers) was measured from the 90 min. to the 240 min. time-points (n=100) **(b)**. In **(c)** are shown the length distribution profiles (spindles measured in micrometers) of *pMET3-CDC20* (Ry1223), *cdc23-1* (Ry1932) and *cdc14-1 cdc5-as1* (Ry1602) cells at their terminal phenotype after a G1 synchronization and release at the restrictive conditions (YEPD media at 23°C supplemented with 8 mM methionine for *pMET3-CDC20* cells, YEPD media at 37°C for *cdc23-1* cells and YEPD media at 37°C supplemented with the *cdc5-as1* inhibitor (CMK; (Snead, Sullivan et al. 2007)) for *cdc14-1 cdc5-as1* cells).

3.3.3 *cdc14 cdc5* cells are defective in the slow phase of spindle elongation

As we observed a transient spindle elongation in "sister-less" cells we decided to measure the spindle length at different time-points also for *cdc14-1 cdc5-as1 cdc6Δ mad1Δ pGAL-CDC6* cells. Specifically the spindle length at the 120 min. (soon after metaphase) and at the 240 min. (terminal phenotype) (n=100) were compared to the same time-points in our *cdc14 cdc5* double mutant, **Fig. 3.17a** and **Fig. 3.17b**. We observed that the two length distribution profiles are significantly different at the 120 min. time-point, **Fig. 3.17a**, but they are similar at the 240 min. time-point, **Fig. 3.17b**. While at the 120 min. time-point the majority of the *cdc14 cdc5* cells showed spindles between 2-4 μm, the distribution of spindle length in *cdc14-1 cdc5-as1 cdc6Δ mad1Δ pGAL-CDC6* cells was slightly broader and centered around 4-6 μm, **Fig. 3.17a**. This suggests that other forms of linkages between sister chromatids may persist and contribute to the shorter spindle length observed in *cdc14-1 cdc5-as1* cells, yet not even "sister-less" cells fully elongate their spindles over 6 μm and the slight elongation observed is transient. Indeed, at later time points (240 min.) *cdc14-1 cdc5-as1* cells maintained their spindle length with a mode at 2-4 μm. In contrast, an increasing number of *cdc14-1 cdc5-as1 cdc6Δ mad1Δ pGAL-CDC6* cells shortened their spindle from 4-6 μm to 2-4 μm, **Fig. 3.17b**. Taken together our data identify an impairment in the second, slow phase of spindle elongation as the prime mitotic defect in *cdc14-1 cdc5-as1* cells, although some residual cohesin-independent cohesion between *cdc14 cdc5* sister chromatids seems to exist.

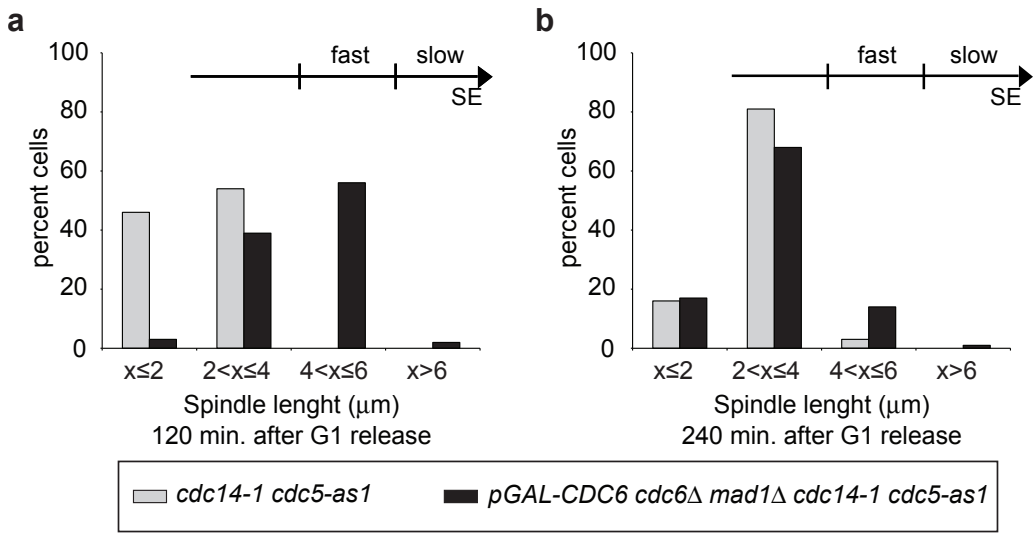

Figure 3.17. *cdc14 cdc5* cells are defective in the slow phase of spindle elongation. The spindle length (in micrometers) was determined for 100 cells at the 120 min. **(a)** and 240 min. **(b)** time-points from a G1 arrest and release for *cdc14-1 cdc5-as1* (Ry1602) and *cdc14-1 cdc5-as1 cdc6Δ mad1Δ pGAL-ubiR-CDC6* (Ry3136) cells. Refer to **Fig. 3.15** for the experiment description. SE stands for spindle elongation; x≤2 means 2 μm spindles or spindles shorter than 2 μm (corresponding to early metaphase spindles), 2<x≤4 means spindles between 2 and 4 μm or 4 μm spindles (corresponding to metaphase spindles), 4<x≤6 means spindles between 4 and 6 μm or 6 μm spindles (corresponding to early anaphase spindles) and x>6 means spindles longer than 6 μm (corresponding to fully elongated anaphase spindles) **(a-b)**.

3.3.4 *cdc14 cdc5* double mutant cells are defective in anaphase B

To determine whether Cdc14 and Cdc5 activities are required for spindle elongation independently from sister chromatid separation, we probed the consequences of simultaneously inactivating Cdc14 and Cdc5 activities in *ndc10-1* kinetochore mutants. The *NDC10* gene encodes for a core kinetochore component (Goh and Kilmartin 1993). Kinetochores are proteinaceous structures that attach sister chromatids to mitotic spindle and couple spindle elongation with sister chromatid separation (Westermann, Drubin et al. 2007, DeLuca and Musacchio 2012). Although this attachment occurs in metaphase, spindle elongation does not take place prior to anaphase because the pulling forces exerted by kinetochore microtubules are opposed by sister chromatid cohesion.

On the contrary, at the restrictive temperature *ndc10-1* cells elongate their spindles without chromosome segregation (since the centrosomes of the chromosomes are not attached to microtubules in *ndc10-1* cells) (Goh and Kilmartin 1993), indicating that in kinetochore mutants spindle elongation is uncoupled from the process of chromosome segregation. *cdc14-1 cdc5-as1 ndc10-1* cells were hence released from G1 at 37°C (restrictive temperature for Cdc14-1 and Ndc10-1) in the presence of the CMK inhibitor (Snead, Sullivan et al. 2007) (restrictive for Cdc5-as1). The spindle length was determined for 100 cells from the 120 min. to the 240 min. time-points, **Fig. 3.18b**. The length distribution profiles were compared with those of *cdc14-1 cdc5-as1 cdc6Δ mad1Δ pGAL-CDC6* cells (refer to **Fig. 3.15** for the experiment description), **Fig. 3.18a**. The *cdc14-1 cdc5-as1* mutant contains spindles that are significantly shorter than those of the *cdc14-1 cdc5-as1 ndc10-1* and *cdc14-1 cdc5-as1 cdc6Δ mad1Δ pGAL-CDC6* cells at all time-points examined. *cdc14-1 cdc5-as1* spindles never extended beyond 2-4 μm, while strains with detached chromosomes (*ndc10-1* background) or containing "sister-less" chromatids (*pGAL-CDC6* cells) allowed for a minor elongation of the spindle, **Fig. 3.18a** e **Fig. 3.18b**. Indeed, 20 to 40 % of *cdc14-1 cdc5-as1 ndc10-1* cells extended their spindles up to 4-6 μm, but similarly to Cdc6 depleted cells they did not fully elongate, **Fig. 3.18a** e **Fig. 3.18b**. As *ndc10-1* mutants have sister chromatids not bound to the mitotic spindle, we speculate that Cdc14 and Cdc5 activities are required for anaphase B spindle elongation.

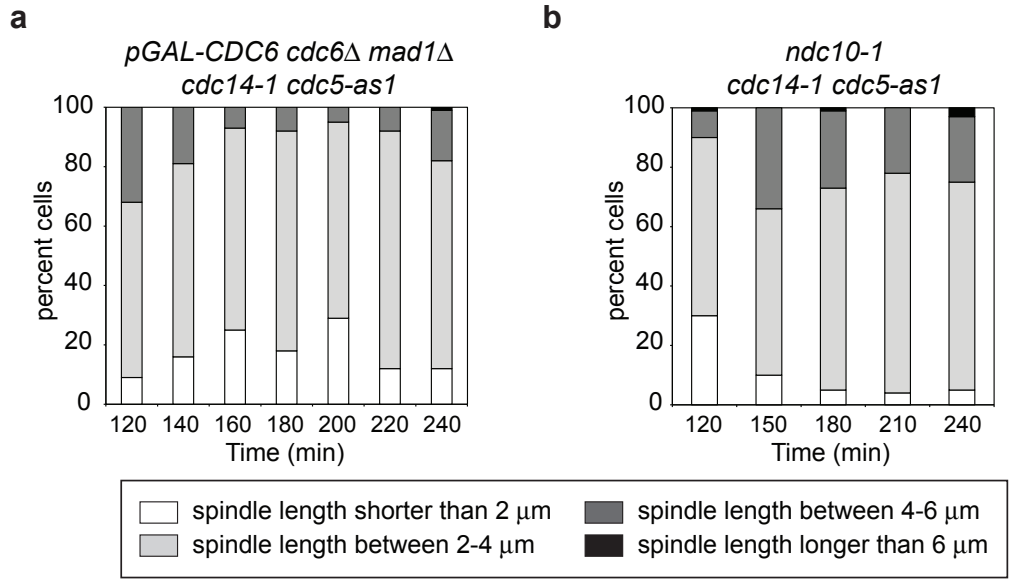

Figure 3.18. Lack of functional kinethocores allows only a minor elongation of the spindles in *cdc14 cdc5* cells. The spindle length distribution profile for *cdc14-1 cdc5-as1 cdc6Δ mad1Δ pGAL-ubiR-CDC6* (Ry3136) cells (n=100) from the 120 min. to the 240 min. time-point (refer to Fig. 3.15 for the experiment description) is shown in **(a)**. The spindle length distribution profile of *cdc14-1 cdc5-as1 ndc10-1* (Ry3108) cells is shown in **(b)**. *cdc14-1 cdc5-as1 ndc10-1* (Ry3108) cells were arrested in G1 by α-factor in YEPD at 23°C. When arrest was complete, cells were released in fresh YEPD media supplemented with the *cdc5-as1* inhibitor (CMK; (Snead, Sullivan et al. 2007)) to inactivate the *cdc5-as1* allele, and at 37°C to inactivate the *cdc14-1* and *ndc10-1* alleles. The spindle length (in micrometers) was determined for 100 cells from the 120 min. to the 240 min. time-point **(b)**.

3.4 Investigating the problem of residual cohesion between sister chromatids in *cdc14 cdc5* cells

3.4.1 Lack of topoisomerase II activity is not the reason for the *cdc14 cdc5* arrest

Next to the cohesin ring complex, sister chromatids cohesion can also in part be obtained by topological intertwinings between the two sisters formed during DNA replication (Murray and Szostak 1985, Peter, Ullsperger et al. 1998, Bermejo, Branzei et al. 2008). In a wild type cell these inter-molecular linkages are resolved by the essential topoisomerase II enzyme, Top2 (Lucas, Germe et al. 2001, D'Amours, Stegmeier et al. 2004, Pereira and Schiebel 2004, Sullivan, Higuchi et al. 2004, Wang, Yong-Gonzalez et al. 2004). Having established that cohesin has been removed from chromosomes in *cdc14-1 cdc5-as1* cells but yet some cohesion between sisters seems to persist, we decided to investigate Top2 activity in our double mutant. We introduced a temperature sensitive allele of *TOP2* (*top2-1*) in the single *cdc14-1* or *cdc5-as1* mutants to examine whether these combinations mimicked the *cdc14-1 cdc5-as1* double mutant phenotype. Next, the *top2-1*, *cdc14-1 top2-1*, and *cdc5-as1 top2-1* strains we arrested in G1 at permissive conditions for all the mutant alleles and then released at the restrictive conditions. Cell cycle progression was scored by spindle morphology analysis, **Fig. 3.19**. In agreement with our data identifying a spindle elongation defect as the primary cause for the "early-anaphase arrest" of the *cdc14-1 cdc5-as1* mutant, we found that single *cdc14-1* or *cdc5-as1* mutants in which Top2 activity was impaired were still able to elongate their spindles, **Fig. 3.19**. Even though a little effect was observed in the *top2-1 cdc5-as1* cells we conclude that inhibition of Top2 activity is not the reason why *cdc14 cdc5* cells arrest with an "early-anaphase phenotype".

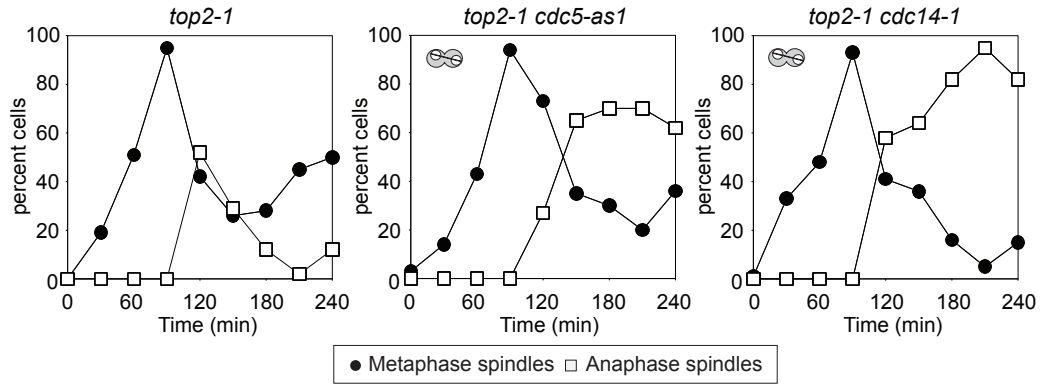

Figure 3.19. *cdc14* or *cdc5* cells lacking Top2 activity are still able to elongate their spindles. *top2-1* (Ry4105), *cdc5-as1 top2-1* (Ry3555) and *cdc14-1 top2-1* (Ry4103) cells were arrested in G1 by α-factor in YEPD at 23°C. When arrest was complete, cells were released in fresh YEPD media supplemented with the *cdc5-as1* inhibitor (CMK; (Snead, Sullivan et al. 2007)) at 37°C to inactivate the *cdc5-as1* and the temperature sensitive alleles (*cdc14-1* and *top2-1*), respectively. At the indicated time-points, cells were collected to determine the percentages of cells with metaphase spindles (closed circles) and anaphase spindles (open squares).

Further evidence for this conclusion came from the growth analysis of strains carrying different combinations of the *top2-1*, *cdc14-1* and *cdc5-as1* alleles. The growth of serially diluted wild type, *cdc14-1*, *cdc5-as1* and *top2-1* single mutants cells on YEPD plates incubated for 48 hours at different temperatures (23°C, 25°C, 28°C and 30°C) were compared with the growth of double mutant strains carrying different combinations of the three loss-of-function alleles (*cdc14*, *cdc5* and *top2)*, **Fig. 3.20**. No synthetic genetic interactions were observed between Top2 and Cdc14 and/or Cdc5, **Fig. 3.20**.

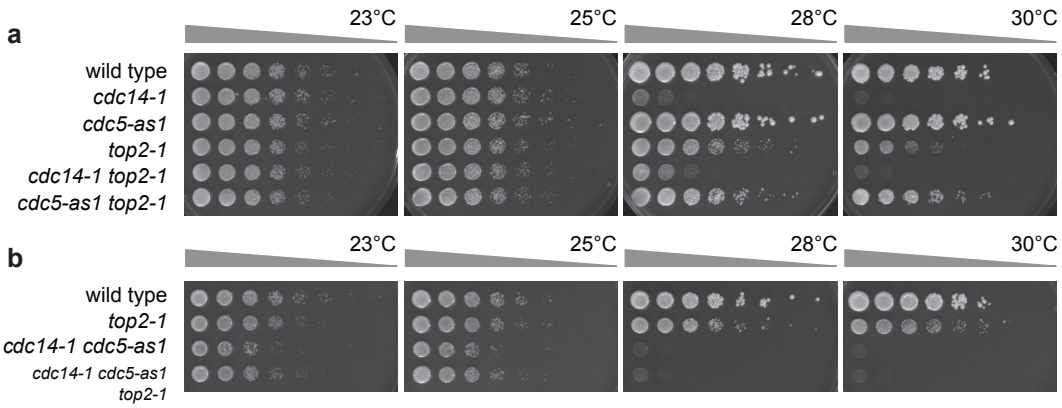

Figure 3.20. No synthetic genetic interactions are observed between Top2 and Cdc14 and/or Cdc5. Growth of serial dilutions from wild type (Ry1), *cdc14-1* (Ry1574), *cdc5-as1* (Ry2446), *top2-1* (Ry4105), *cdc14-1 top2-1* (Ry4103) and *cdc5-as1 top2-1* (Ry3555) cells is shown in **(a)**; wild type (Ry1), *top2-1* (Ry4105), *cdc14*-1 *cdc5-as1* (Ry1602) and *cdc14-1 cdc5-as1 top2-1* (Ry3548) cells is shown in **(b)**. Serial dilutions (1:5) of yeast cells suspensions starting from OD_{600} = 1 were spotted onto YEPD plate and incubated at 23°C, 25°C, 28°C and 30°C for 48 hours **(a-b).**

3.4.2 Defect in silencing transcription seems not the reason why *cdc14 cdc5* cells cannot progress into anaphase

Cdc14 has functions in repressing the transcription activity of both RNA polymerase I (RNAP-I) at ribosomal DNA (rDNA) repeats (Clemente-Blanco, Mayan-Santos et al. 2009) and RNA polymerase II (RNAP-II) at telomeres (Clemente-Blanco, Sen et al. 2011), to allow condensin loading at these loci (Clemente-Blanco, Sen et al. 2011). Cdc14-mediated condensin enrichment at these loci in anaphase (D'Amours, Stegmeier et al. 2004, Sullivan, Higuchi et al. 2004, Clemente-Blanco, Sen et al. 2011) is a pre-requisite for the subsequent recruitment of Top2, ultimately required to disentangle the sister DNA molecules (Lucas, Germe et al. 2001). Consistent with this, *cdc14* mutants fail to segregate repetitive regions. Given these findings, we examined whether the *cdc14 cdc5* phenotype was caused by an inability of the cells to repress RNAP-II. We

therefore analyzed the effect of chemically blocking the RNAP-II in double mutant cells arrested at their terminal phenotype treating them with α-amanitin, a specific RNAP-II inhibitor. *cdc14-1 cdc5-as1* cells were synchronously released from a G1 arrest at the restrictive conditions for both the *cdc14-1* and *cdc5-as1* alleles. Three hours after the release, when their "early-anaphase arrest" was achieved, the culture was split into two. Only one half was treated with α-amanitin, **Fig. 3.21**. Every 5 minutes for 30 minutes after the split, samples of both treated, **Fig. 3.21b**, and un-treated cells, **Fig. 3.21a**, were collected to determine spindle length (n=100). Our data did not evidence a significant or transient elongation of the spindles in the treated or in the un-treated population of *cdc14-1 cdc5-as1* cells, **Fig. 3.21**. This experiment indicates that a defect in silencing transcription is not the reason why *cdc14 cdc5* cells cannot progress into anaphase.

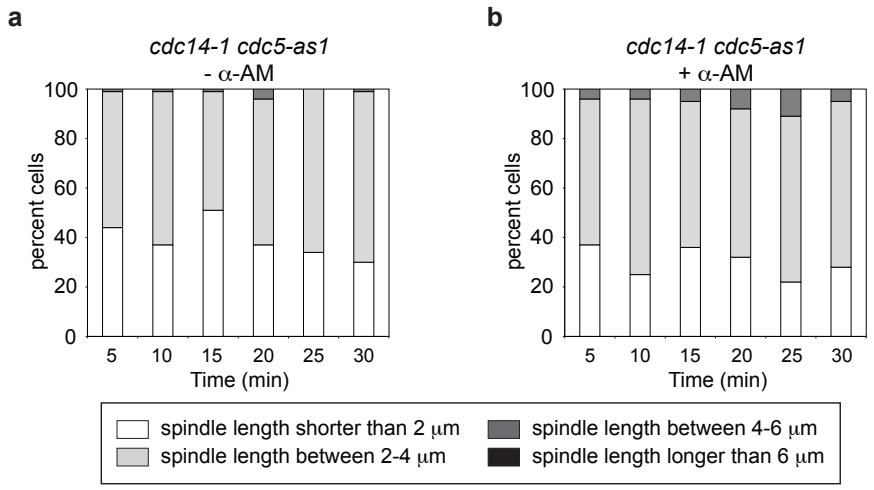

Figure 3.21. RNAP-II inhibition does not allow spindle elongation in *cdc14 cdc5* cells. (a-b) *cdc14-1 cdc5-as1* cells (Ry1602) were arrested in G1 by α-factor in YEPD at 23°C. When arrest was complete, cells were released in fresh YEPD medium supplemented with the *cdc5-as1* inhibitor (CMK; (Snead, Sullivan et al. 2007)) at 37°C to inactivate the *cdc5-as1* and *cdc14-1* allele, respectively. 180 minutes after the release, when cells were in metaphase, the culture was split in two. One-half was maintained in the same medium (- α-AM, **(a)**), whereas 66 μg/ml of α-amanitin (+ α-AM, **(b)**) was added to the other half to inhibit RNAP-II and hence transcription. **(a-b)** The spindle length (in micrometers) was determined for 100 cells every 5 minutes for 30 minutes from the split. The resulting length distribution profiles are shown **(a-b)**.

3.5 Investigating the spindle elongation problem of *cdc14 cdc5* cells

3.5.1 Clb5 is degraded in *cdc14 cdc5* cells

One of the requirements for spindle elongation to occur is the stabilization of microtubule dynamics (Winey and O'Toole 2001, Winey 2012). Microtubule turnover increases as cells progress through metaphase and this event is fundamental for the correction of erroneous sister chromatid attachments. At the onset of anaphase, microtubule turnover decreases and shifts to polymerization as the spindle starts to elongate (Higuchi and Uhlmann 2005). This change in spindle behaviour correlates with a shift from a phosphorylated to a dephosphorylated status of multiple proteins and with a phospho-proteome change dictated by the concomitant inactivation of CDKs and activation of CDKs-counteracting phosphatases (Pereira and Schiebel 2003, Crasta, Huang et al. 2006, Khmelinskii, Lawrence et al. 2007, Woodbury and Morgan 2007, Khmelinskii, Roostalu et al. 2009, Chee and Haase 2010, Avunie-Masala, Movshovich et al. 2011, Nakajima, Cormier et al. 2011). Most of these proteins are negatively phosphorylated by S phase CDKs, with Clb5-CDK being the predominant species (Liang, Richmond et al. 2013). In budding yeast, anaphase spindle elongation requires Clb5 degradation, triggered by the APC/C in conjunction with its co-activator Cdc20 (Liang, Richmond et al. 2013). In parallel, Cdc14 is activated, which was shown to dephosphorylate several proteins implicated in spindle formation/elongation (Liang, Richmond et al. 2013). As such, we wished to probe i) Clb5 levels in our cells, and ii) the consequences of deleting Clb5 in our mutants. First, *cdc14-1*, *cdc5-as1*, and *cdc14-1 cdc5-as1* cells were arrested in G1 and released into the cell cycle at restrictive conditions. Cell cycle progression was scored by spindle morphology analysis, while Clb5 levels were examined by anti-Clb5 Western blot analysis, **Fig. 3.22**. We found that

Clb5 was degraded with similar kinetics in *cdc14-1*, *cdc5-as1* and *cdc14-1 cdc5* cells, **Fig. 3.22**.

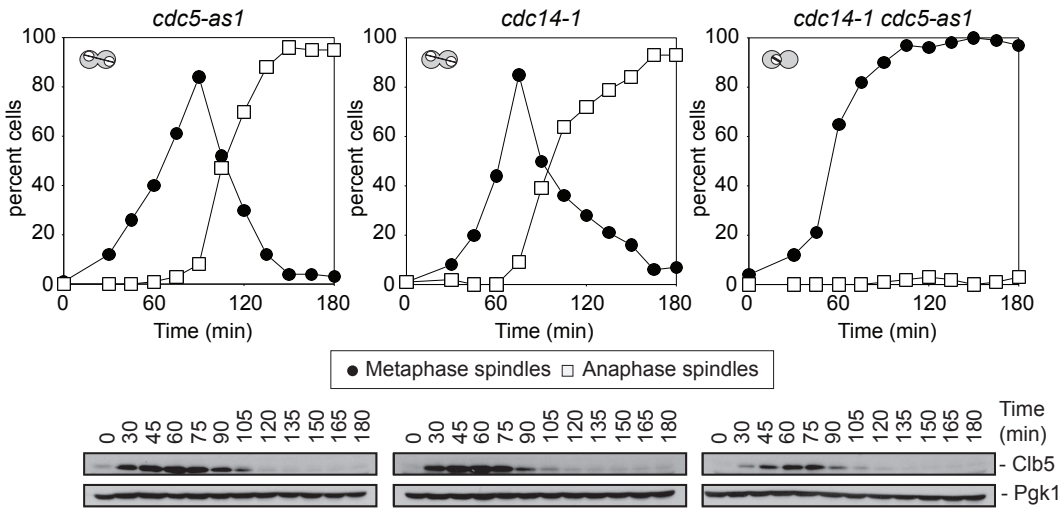

Figure 3.22. Clb5 is properly degraded in *cdc14 cdc5* cells. *cdc5-as1* (Ry2446), *cdc14-1* (Ry1574) and *cdc14-1 cdc5-as1* (Ry1602) cells were arrested in G1 by α-factor in YEPD at 23°C. When arrest was complete, cells were released in fresh YEPD medium supplemented with the *cdc5-as1* inhibitor (CMK; (Snead, Sullivan et al. 2007)) at 37°C to inactivate the *cdc5-as1* and *cdc14-1* allele, respectively. Samples were taken at the indicated times to determine the percentages of cells with metaphase spindles (closed circles) and anaphase spindles (open squares) as well as to analyze Clb5 and Pgk1 protein levels. Pgk1 protein was used as an internal loading control in immunoblots.

We next tested the effects of deleting Clb5 in the *cdc14-1*, *cdc5-as1*, and *cdc14-1 cdc5-as1* cells, **Fig. 3.23**. In agreement with the finding that Clb5 is properly degraded at the metaphase-to-anaphase transition in *cdc14-1 cdc5as-1* cells, **Fig. 3.22**, we found that deletion of Clb5 cannot rescue the double mutant phenotype, **Fig. 3.23**. Taken together our findings suggest that inhibitory phosphorylation events mediated by Clb5-CDK are not the cause of the spindle elongation defect observed in our double mutant.

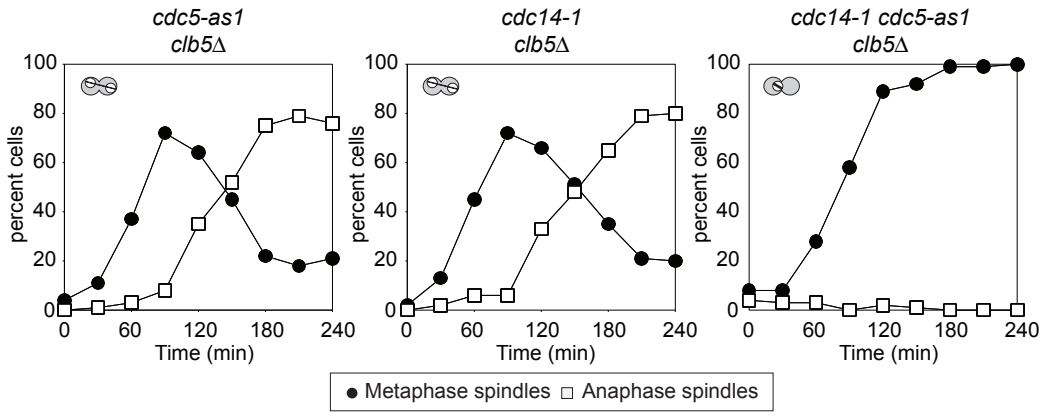

Figure 3.23. The deletion of Clb5 does not bypass the *cdc14 cdc5* arrest. *cdc5-as1 clb5Δ* (Ry4715), *cdc14-1 clb5Δ* (Ry4712) and *cdc14-1 cdc5-as1 clb5Δ* (Ry4709) cells were arrested in G1 by α-factor in YEPD at 23°C. When arrest was complete, cells were released in fresh YEPD medium supplemented with the *cdc5-as1* inhibitor (CMK; (Snead, Sullivan et al. 2007)) at 37°C to inactivate the *cdc5-as1* and *cdc14-1* allele, respectively. Samples were taken at the indicated times to determine the percentages of cells with metaphase spindles (closed circles) and anaphase spindles (open squares).

3.5.3 Clb2 is active in *cdc14 cdc5* cells

In contrast to the inhibitory role of S phase cyclins, mitotic cyclins promote spindle elongation (Liang, Richmond et al. 2013). A recent study showed that cells lacking the mitotic kinases Clb1-CDK and Clb2-CDK displayed a phenotype reminiscent to the one exhibited by our *cdc14-1 cdc5-as1* mutant (Rahal and Amon 2008). As Cdc5 has been implicated in Clb2-CDK activation by promoting Swe1 degradation (Liang, Jin et al. 2009) we examined whether a defect in CDK activity could account at least partially for the *cdc14-1 cdc5-as1* phenotype. Swe1 is the *S. cerevisiae* orthologue of the Wee1 kinase (Booher, Deshaies et al. 1993), which phosphorylates Cdc28 (the sole CDK in budding yeast) thereby inhibiting it. To assess whether Swe1-dependent inhibition of Cdc28 could account for the *cdc5 cdc14* arrest we deleted *SWE1* in our double mutant. *cdc14-1*, *cdc5-as1* and *cdc14-1 cdc5-as1* cells carrying a deleted *SWE1* gene (*swe1Δ*),

were synchronously released from G1 under conditions that are restrictive for *cdc14-1* and *cdc5-as1*. Cell cycle progression was tracked by spindle morphological analysis, **Fig. 3.24**. We found that deleting *SWE1* did not bypass the *cdc14-1 cdc5-as1* arrest suggesting that mitotic kinases are active in our mutant, **Fig. 3.24**.

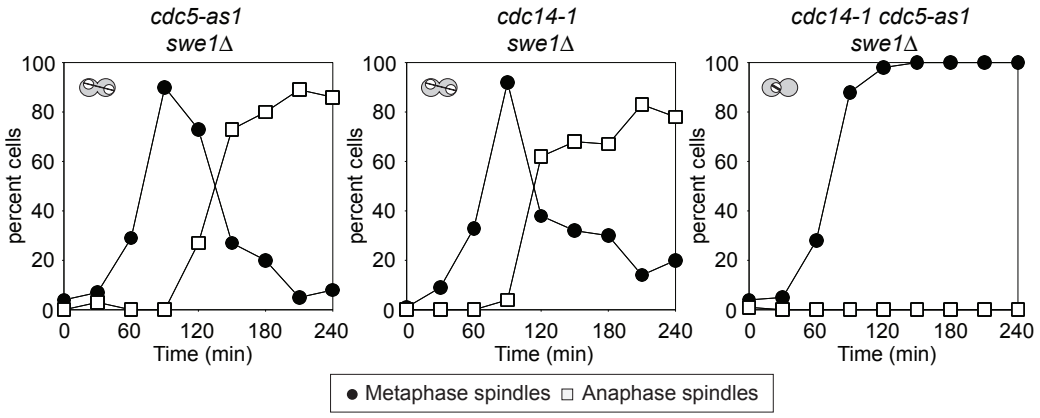

Figure 3.24. The deletion of Swe1 did not bypass the *cdc14 cdc5* arrest. *cdc5-as1 swe1Δ* (Ry3025), *cdc14-1 swe1Δ* (Ry3020) and *cdc14-1 cdc5-as1 swe1Δ* (Ry3015) cells were arrested in G1 by α-factor in YEPD at 23°C. When arrest was complete, cells were released in fresh YEPD medium supplemented with the *cdc5-as1* inhibitor (CMK; Snead et al., 2007 (Snead, Sullivan et al. 2007)) at 37°C to inactivate the *cdc5-as1* and *cdc14-1* allele, respectively. Samples were taken at the indicated times to determine the percentages of cells with metaphase spindles (closed circles) and anaphase spindles (open squares).

To directly assess mitotic CDK activities we probed Clb2 levels and associated kinase activity at histone H1 of *cdc14-1*, *cdc5-as1*, and *cdc14-1 cdc5-as1* synchronously released from G1 at restrictive conditions for *cdc14-1* and *cdc5-as1*, **Fig. 3.25**. Our results show that Clb2-CDK complexes are active in *cdc14 cdc5* double mutant cells. Moreover, while *cdc14-1* cells undergo the canonical metaphase-anaphase decrease both in Clb2 levels and associated kinase activity (Baumer, Braus et al. 2000, Yeong, Lim et al. 2000), *cdc5-as1* cells are defective in this process. These cells accumulated

Clb2 and arrested with high levels of the mitotic kinase, both in the presence or absence of the *cdc14-1* allele, **Fig. 3.25**. This data suggests that low mitotic CDK activity is not the reason why *cdc14-1 cdc5-as1* cells cannot progress through anaphase and raises the possibility that a well-defined ratio of kinase/phosphatase activity is essential for proper spindle formation and that APC/C^{Cdc20} mediated degradation of Clb2 at the metaphase-to-anaphase transition requires Cdc5.

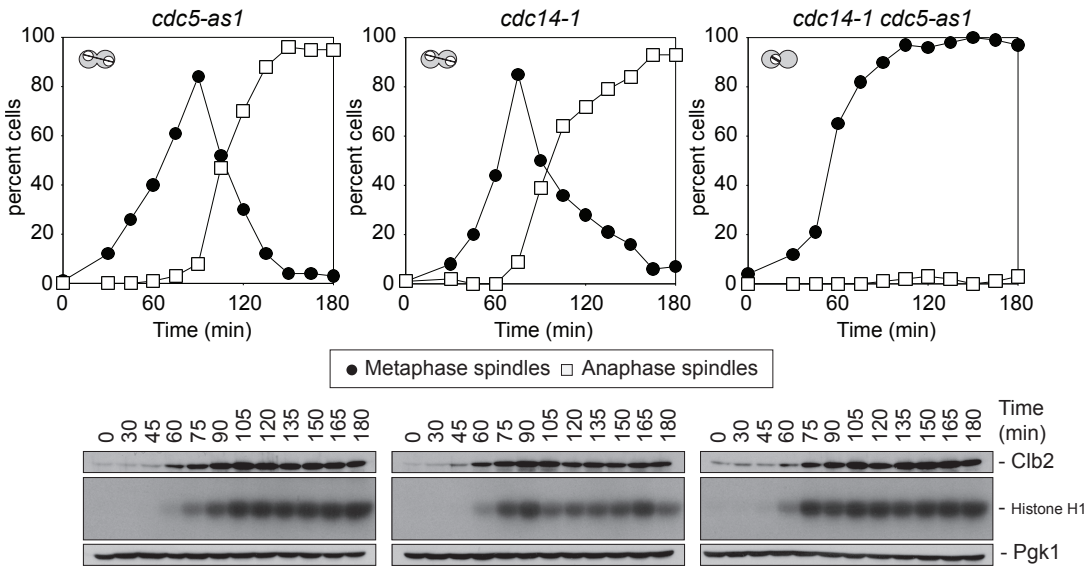

Figure 3.25. Clb2 is active in *cdc14 cdc5* cells. *cdc5-as1* (Ry2446), *cdc14-1* (Ry1574) and *cdc14-1 cdc5-as1* (Ry1602) cells were arrested in G1 by α-factor in YEPD at 23°C. When arrest was complete, cells were released in fresh YEPD medium supplemented with the *cdc5-as1* inhibitor (CMK; (Snead, Sullivan et al. 2007)) at 37°C to inactivate the *cdc5-as1* and *cdc14-1* allele, respectively. Samples were taken at the indicated times to determine the percentages of cells with metaphase spindles (closed circles) and anaphase spindles (open squares), to analyse Clb2 and Pgk1 protein levels and to perform a Clb2-associated kinase assay using Histone H1 as substrate. Pgk1 protein was used as an internal loading control in immunoblots.

3.5.4 Raising Clb2 levels in *cdc14* mutants results in spindle elongation defects

The finding that *cdc5* mutants are defective in CDK inactivation raises the possibility that the arrest of our double mutant is caused in part by excess Clb2-CDK activity. To assess whether raising CDK activity in a defective phosphatase background results in mitotic spindle elongation defects, we examined the consequences of over-expressing a non-degradable allele of Clb2 (*CLB2dBΔ*) in a *cdc14-1* mutant background. To over-express *CLB2dBΔ* its open reading frame was placed under the control of the inducible *pGAL1-10* promoter (West, Chen et al. 1987). *pGAL-clb2dBΔ, cdc14-1,* and *pGAL-clb2dBΔ cdc14-1* cells were synchronized in G1 and released in medium containing galactose at the restrictive temperature for *cdc14-1*. The spindle length was determined for 100 cells once strains reached their terminal phenotype (240 min. after the G1 release), **Fig. 3.26**. We found that over-expressing Clb2 in the wild-type background did not alter the kinetics of spindle elongation. Indeed, 240 minutes after the release from a G1 arrest about 90 percent of cells had a spindle length greater than 6 μm, **Fig. 3.26**. Interestingly, when Clb2 levels were raised in the *cdc14-1* mutant spindle elongation defects were observed in about 40 percent of the cells, **Fig. 3.26**. Our data suggest that one possible role of Cdc5 is to contribute to the decrease in Clb2 levels observed at the metaphase-to-anaphase transition thereby maintaining a kinase/phosphatase ratio required for correct spindle elongation.

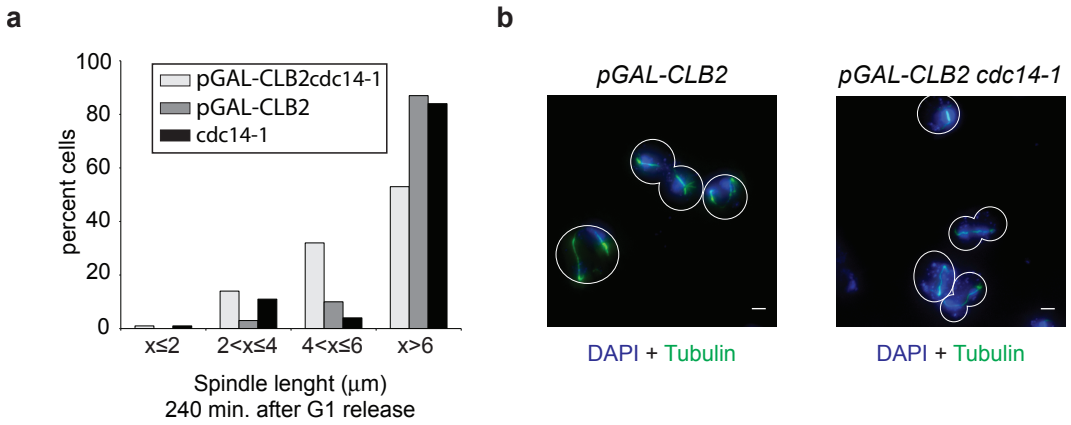

Figure 3.26. Raising Clb2 levels in *cdc14-1* mutant cells results in spindle elongation defects. (a-b) *pGAL-clb2dBΔ cdc14-1* (Ry1575), *pGAL-clb2dBΔ* (Ry1387) and *cdc14-1* (Ry1574) cells were arrested in G1 in YEPR at 23°C. Cell were then released from the arrest in fresh YEPR at 37°C to inactivate the *cdc14-1* allele. 30 minutes after the release 2% galactose was added to the media to over-express *pGAL-clb2dBΔ*. Length distribution profiles (spindles measured in micrometers; n=100) of the three strains at their terminal phenotype (240 min. time-point) after the release are shown in **(a)**. x≤2 means 2 μm spindles or spindles shorter than 2 μm (corresponding to early metaphase spindles), 2<x≤4 means spindles between 2 and 4 μm or 4 μm spindles (corresponding to metaphase spindles), 4<x≤6 means spindles between 4 and 6 μm or 6 μm spindles (corresponding to early anaphase spindles) and x>6 means spindles longer than 6 μm (corresponding to fully elongated anaphase spindles) **(a)**. Representative images of *pGAL-clb2dBΔ* (Ry1387) and *pGAL-clb2dBΔ cdc14-1* (Ry1575) cells at their terminal phenotype (240 min. time-point) are shown in (b). Dapi is shown in blue and tubulin in green. Bars, 2 μm (b).

3.5.5 The motor protein Cin8 is a member of the Cdc5 branch of the pathway controlling spindle elongation

The defect in the slow elongation step of the mitotic spindle could reflect changes in microtubule motor functions or those of microtubule-associated proteins (MAPs). We tested this hypothesis by overexpressing factors that promote spindle elongation such as the kinesin-5 proteins Kip1 and Cin8 (Hildebrandt and Hoyt 2000) or the MAP, Stu2 (Severin, Habermann et al. 2001). *cdc14-1 cdc5-as1* cells in which either *KIP1*, *CIN8* or *STU2* were placed under *pGAL1-10* control (*cdc14-1 cdc5-as1 pGAL-STU2*, *cdc14-1*

cdc5-as1 pGAL-KIP1 and *cdc14-1 cdc5-as1 pGAL-CIN8*) were arrested in G1 in raffinose-based medium and then released in galactose-containing medium to overexpress Stu2, Kip1 or Cin8 at restrictive conditions for both Cdc14-1 (37°C) and Cdc5-as1 (CMK inhibitor, (Snead, Sullivan et al. 2007)), **Fig. 3.27**. Spindle length was measured for 100 cells between the 120 min. and 240 min. time-point, **Fig. 3.27**. We found that over-expressing *STU2* or *KIP1* did not rescue the spindle elongation defect of the *cdc14-1 cdc5-as1* mutant. However, enhancing the levels of *CIN8* allowed the majority of *cdc14-1 cdc5-as1* cells to elongate their spindle: 40% of cells reached a spindle length of 4-6 µm whereas about 20 percent of the cells elongated their spindle to lengths greater that 6 µm, **Fig. 3.27**.

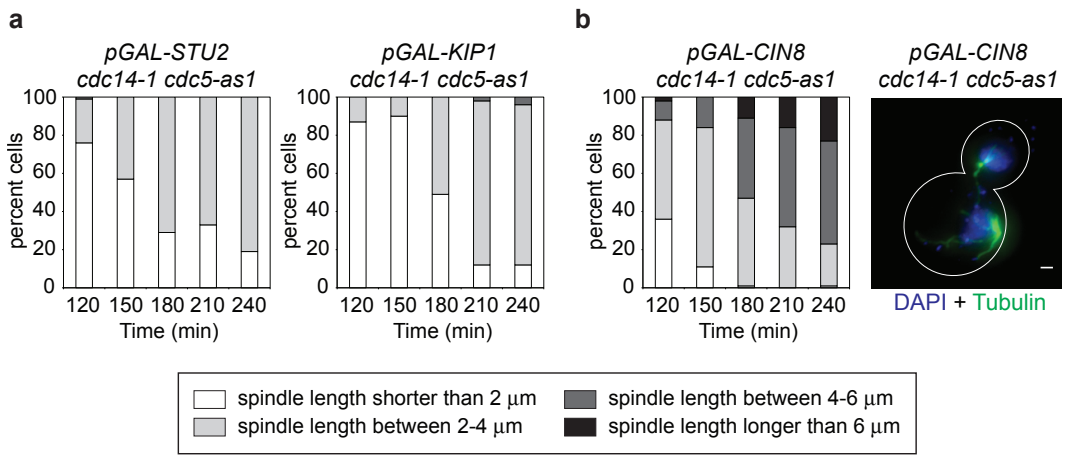

Figure 3.27. Raising the levels of *CIN8* allowed the majority of *cdc14-1 cdc5-as1* cells to complete the first phase of spindle elongation. (a-b) *cdc14-1 cdc5-as1 pGAL-STU2* (Ry4573), *cdc14-1 cdc5-as1 pGAL-KIP1* (Ry4640) and *cdc14-1 cdc5-as1 pGAL-CIN8* (Ry4596) cells were arrested in G1 by α-factor in YEPR at 23°C. When arrest was complete, cells were released in fresh YEPR media supplemented with 2% galactose at the restrictive conditions for both *cdc14* and *cdc5*. The spindles length (in micrometers) was determined for 100 cells from the 120 min. to the 240 min. time-point. The resulting length distribution profiles are shown **(a-b)**. A representative image of *cdc14-1 cdc5-as1 pGAL-CIN8* (Ry4596) cells at their terminal phenotype (240 min. time-point) is shown in **(b)**. Dapi is shown in blue and tubulin in green. Bars, 2 µm **(b)**.

The observation that high levels of Cin8 partially suppress the spindle elongation defect caused by the concomitant inactivation of Cdc14 and Cdc5 indicates that Cin8 acts downstream or in parallel to both enzymes. To discriminate between both possibilities we compared the kinetics of anaphase progression of single *cdc14-1* and *cdc5-as1* mutant cells carrying an allele of *CIN8* that abrogates the interaction between Cin8 and the spindle microtubules, *cin8-F467A* (Movshovich, Fridman et al. 2008). As such, the *cdc5-as1 cin8-F467A*, *cdc14-1 cin8-F467A* and *cdc5-as1 cdc14-1 cin8-F467A* cells were synchronously released from G1 in conditions restrictive for Cdc14-1 and Cdc5-as1. Cell cycle progression was tracked by spindle morphology analysis, **Fig. 3.28a**. The spindle length distribution profiles of the strains as measured between the 120 min. and the 240 min. time-point (n=100) were then compared, **Fig. 3.28b**. We also followed the kinetics of Pds1 accumulation and degradation by Western blot analysis, **Fig. 3.28c**. We found that the *cin8-F467A* allele did not alter the spindle kinetics of the *cdc5-as1* single mutant but enhanced the defect in spindle elongation of the *cdc14-1* mutant, **Fig. 3.28a**, **3.28b** and **3.28d**. These cells, similarly to the *cdc14-1 cdc5-as1* double mutant, the *cdc14-1 cin8-F467A* mutant arrested with undivided nuclei and short bipolar spindles, **Fig. 3.28a**, **3.28b** and **3.28d**. Significantly, similar to the *cdc14-1 cdc5-as1* cells, also the *cdc14-1 cin8-F467A* mutant was proficient in Pds1 degradation, **Fig. 3.28c**. These data identify Cin8 as a critical component of the pathway that controls spindle elongation and indicate that Cdc5 and Cin8 function in the same pathway with Cin8 acting downstream of Cdc5, **Fig 3.28**. Thus, one essential function of Cdc5 is to directly or indirectly regulate Cin8 activity, **Fig 3.28**.

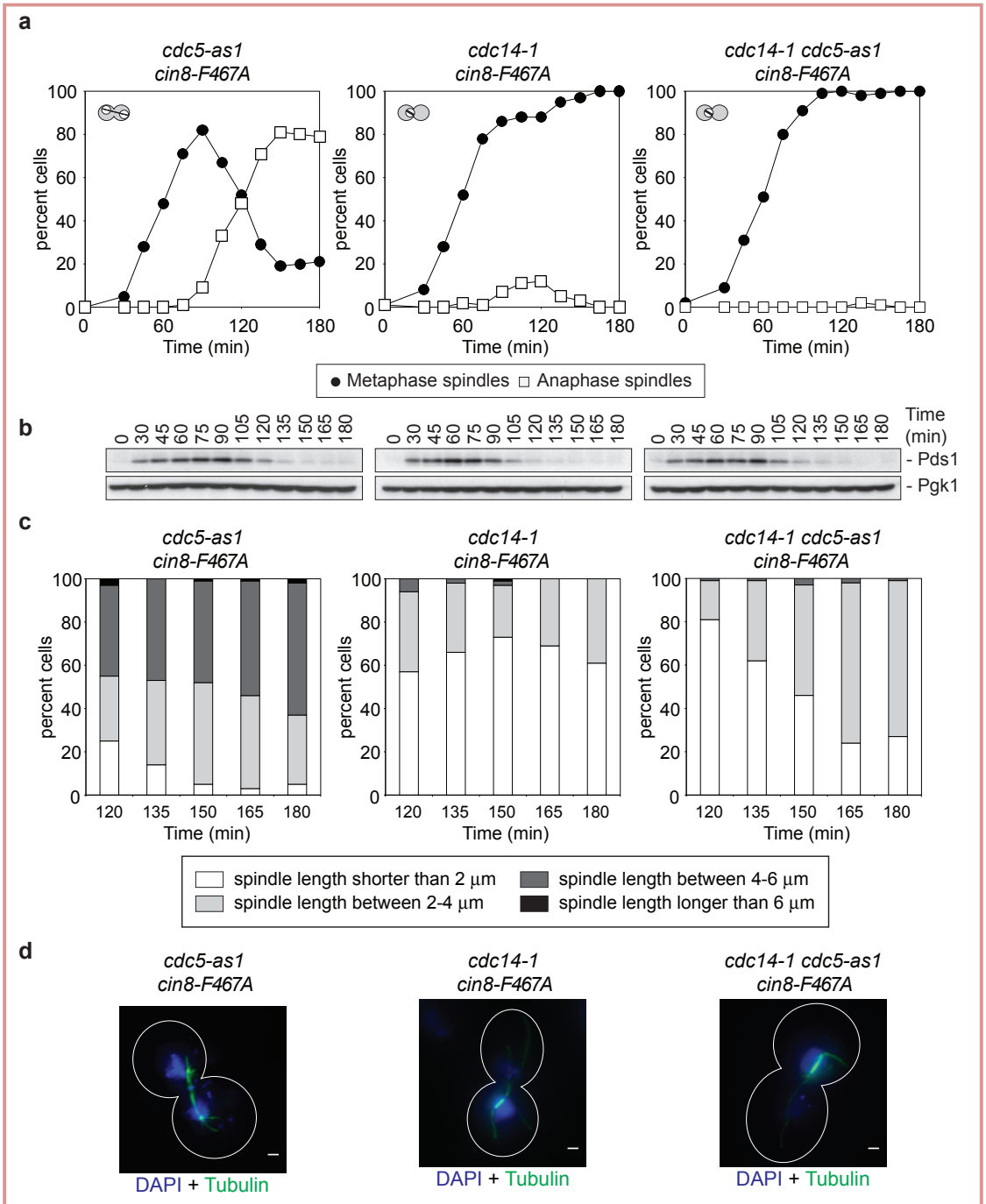

Figure 3.28. Cin8 is a member of the Cdc5 pathway involved in the regulation of spindle elongation. (a-d) *cdc5-as1 cin8-F467A* (Ry4130), *cdc14-1 cin8-F467A* (Ry4124) and *cdc5-as1 cdc14-1 cin8-F467A* (Ry4126) cells were arrested in G1 by α-factor in YEPD at 23°C. When arrest was complete, cells were released in fresh YEPD at the restrictive conditions for both *cdc14* and *cdc5*. Samples were taken at the indicated times to determine the percentages of cells with metaphase spindles (closed circles) and anaphase spindles (open squares) **(a)**. The spindle length (in micrometers) was determined for 100 cells from the 120 min. to the 180 min. time-point and the resulting length distribution profiles are shown in **(b)**. Pds1 and Pgk1 protein levels were analysed by Western blot **(c)**. Pgk1 protein was used as an internal loading control in immunoblots **(c)**. Representative images of *cdc5-as1 cin8-F467A* (Ry4130), *cdc14-1 cin8-F467A* (Ry4124) and *cdc5-as1 cdc14-1 cin8-F467A* (Ry4126) cells at their terminal phenotype (180 min. time-point) are shown in **(d)**. Dapi is shown in blue and tubulin in green. Bars, 2 µm **(d)**.

3.5.6 Dynein

In yeast both Cin8 and dynein (*DYN1*) are required for proper anaphase spindle elongation (Hoyt, He et al. 1992, Saunders, Koshland et al. 1995). *cin8Δ dyn1Δ* double mutants can not proceed through the slow phase of anaphase spindle elongation (Hoyt, He et al. 1992, Saunders, Koshland et al. 1995, Gerson-Gurwitz, Movshovich et al. 2009). Having identified Cin8 as a member of the Cdc5 pathway we wondered whether the Cdc14 branch might regulate dynein as their combined inactivation could phenocopy the phenotype of the *cdc14-1 cdc5-as1* mutant. To test this hypothesis, *cdc5-as1*, *cdc14-1* and *cdc14-1 cdc5-as1* cells deleted in *DYN1* (*dyn1Δ)* were released from G1 in conditions restrictive for both *cdc14-1* and *cdc5-as1* alleles. Spindle length was measured in 100 cells between the 120 and the 240 min. time-point, **Fig. 3.29**. We found that deleting dynein did not alter the spindle elongation kinetics of either the single or the double *cdc14 cdc5* mutants, suggesting that dynein is not part of the Cdc14 branch of the pathway.

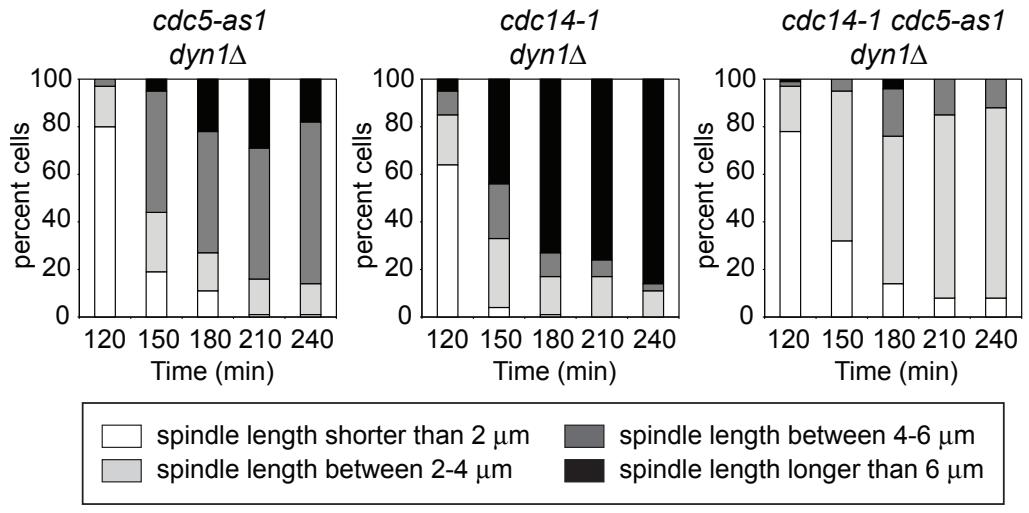

Figure 3.29. Dynein deletion does not alter the spindle behaviour of *cdc5* and *cdc14* single and double mutants. *cdc5-as1 dyn1Δ* (Ry4706), *cdc14-1 dyn1Δ* (Ry4703) and *cdc5-as1 cdc14-1 dyn1Δ* (Ry4700) cells were arrested in G1 by α-factor in YEPD at 23°C. When arrest was complete, cells were released in fresh YEPD at the restrictive conditions for both *cdc14* and *cdc5*. The spindle length (in micrometers) was determined for 100 cells from the 120 min. to the 240 min. time-point. The resulting length distribution profiles are shown.

3.6 Characterization of the Cdc5-mediated regulation of Cin8

3.6.1 Analysis of *cin8* phospho-mutants in a *cdc14-1* background

To understand whether the Cdc5-mediated regulation of Cin8 occurs directly through phosphorylation of this kinesin we examined whether a *cin8* mutant that is not phosphorylable by Cdc5, in combination with the *cdc14-1* allele, reproduces the *cin8-F467A cdc14-1* phenotype. To identify putative Cdc5 phosphorylation sites in Cin8, we probed the Cin8 protein sequence with the Cdc5 consensus sequence [E/D]-X-[S/T]-[F/L/I/Y/V/W/M] (Nakajima, Toyoshima-Morimoto et al. 2003). We identified four putative consensus sites: S52, S71, T761 and S820. Next, we mutated each residue into alanine thereby preventing its phosphorylation (alanine doesn't have the -OH group needed for substitution with phosphate group in the phosphorylation reaction): S52A, S71A, T761A and S820A. The resulting *cin8* phospho-mutant allele (*cin8-4A*), was cloned in a centromeric plasmid (CEN) under the control of the endogenous *CIN8* promoter (841bp long). The *cin8-4A*-CEN plasmid was then transformed in a *cin8Δ cdc14-1* strain to express the Cin8-4A protein as unique type of the motor. Once obtained the strains of interest we followed the cell cycle progression of *cdc14-1 cin8Δ cin8-4A* and *cdc14-1 cin8Δ* cells synchronously released from a G1 in conditions restrictive for Cdc14-1. Of note, the deletion of *CIN8* in an otherwise wild type strain is not essential at 23°C but becomes essential at 37°C (Hoyt, He et al. 1992). Indeed, after G1 synchronization and subsequent release at 37°C *cin8Δ* cells do not form bipolar spindles but arrest as large budded cells with two duplicated but not separated SPBs and elaborated astral MTs extending from a single region in each cell, reflecting problems in the formation of the mitotic spindle (Hoyt, He et al. 1992). According to this, at 37°C the double mutant *cdc14-1 cin8Δ* arrested with the *cin8Δ* phenotype, not able to

assemble the spindle. On the contrary, *cdc14-1 cin8Δ cin8-4A* cells went beyond the *cin8Δ* phenotype and arrested with fully elongated spindles (like a single *cdc14* mutant). The late anaphase/telophase arrest of *cdc14-1 cin8Δ cin8-4A* cells suggests that mutating the four identified residues to alanine does not affect Cin8 activity both in spindle formation and elongation. Indeed, the Cin8-4A protein behaves like a wild type Cin8 protein and rescues the deletion of *CIN8*. A less stringent search for candidate Cdc5-target residues in Cin8 using a less restrictive Cdc5-consensus sequence: [E/N/D/Q]-X-[S/T]-[F/L/I/Y/V/W/M] (Santamaria, Wang et al. 2011) led us to identify seven additional candidate target residues (T38, T123, S409, S441, T497, S654 and S859), with four out of these seven that lie in the motor domain (Hildebrandt, Gheber et al. 2006) of the kinesin (T123, S409, S441 and T497). Next, we mutated all eleven Cdc5-putative phosphoryation sites into alanine (*cin8-11A*) and placed *cin8-11A* in a centromeric plasmid under the control of the endogenous *CIN8* promoter. The plasmid was then transformed into a *cin8Δ cdc14-1* strain to examine the effects of the mutated motor protein. *cdc14-1 cin8Δ cin8-11A* and *cdc14-1 cin8Δ* cells were synchronously released from G1 at 37°C, to inactivate *cdc14*. We found that at 37°C *cdc14-1 cin8Δ cin8-11A* cells arrested before spindle formation exhibiting the same phenotype of single *cin8Δ* mutants when placed at high temperature. The data that the *cin8-11A* behaves as a *cin8Δ* suggests that this allele is likely a loss-of-function mutant. Our strategy is to obtain a less sick mutant of Cin8 reducing the total number of mutated Cdc5-putative phosphorylation sites. We are currently making two mutant versions of Cin8, one keeping only the mutations falling into the motor domain (important for the ATPase activity and the sliding of anti-parallel MTs) and one keeping only the mutations present in the tail of the motor (required for the homo-tetramerization of the motor and for its binding to MTs) (Hildebrandt, Gheber et al. 2006).

3.7 Characterization of the Cdc14 branch involved in the *cdc14 cdc5* phenotype

3.7.1 The *cdc14 cdc5* arrest seems to involve an early anaphase pool of Cdc14

In budding yeast, the artificial cleavage of cohesin in metaphase-arrested cells is sufficient to trigger disjunction of the majority of the sisters, to induce spindle elongation and to proceed into anaphase (Uhlmann, Wernic et al. 2000). Our double mutant phenotype implies that Cdc14 or Cdc5 must be active for spindle elongation to occur. To address whether this is true also when entry into anaphase is ectopically triggered by cleavage of Scc1 in metaphase-arrested cells, we analyzed the consequences of inactivating Cdc5 and Cdc14 alone or in combination in the same experimental set up (Uhlmann, Wernic et al. 2000). As such, *pMET3-CDC20 cdc5-as1 scc1Δ scc1-TEV268-HA pGAL-9MYC-TEV*, *pMET3-CDC20 cdc14-1 scc1Δ scc1-TEV268-HA pGAL-9MYC-TEV* and *pMET3-CDC20 cdc14-1 cdc5-as1 scc1Δ scc1-TEV268-HA pGAL-9MYC-TEV* cells were synchronized in G1 in raffinose medium and next arrested in metaphase by adding methionine to fresh raffinose medium. When ~ 80% of cells reached the arrest, *cdc14-1* and *cdc5-as1* were inactivated (180 minutes after the release). 1 hour after their inactivation the synthesis of the TEV protease was induced by adding galactose (240 minutes after the release), **Fig. 3.30**. We found that cleavage of Scc1 in metaphase-arrested cells was sufficient to trigger anaphase in cells lacking Cdc5 or Cdc14, **Fig. 3.30**. On the contrary, the *cdc14 cdc5* double mutant maintained short bipolar spindles and unseparated DNA masses regardless of TEV protease induction, **Fig. 3.30**. The finding that cells impaired in Cdc5 activity can elongate their spindle supports the idea that a pool of Cdc14 is active already in

metaphase and that its activity is essential to promote anaphase entry and progression in the absence of Cdc5 activity.

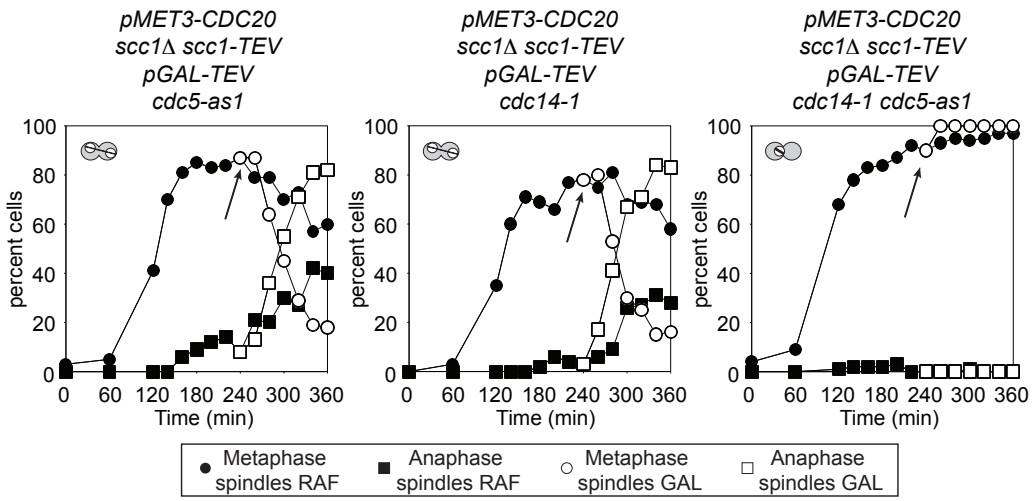

Figure 3.30. Cohesin cleavage is sufficient to promote anaphase spindle elongation only if one between Cdc14 or Cdc5 is active. *cdc5-as1 pMET3-CDC20 scc1Δ scc1-TEV268-HA pGAL-9MYC-TEV* (Ry3220), *cdc14-1 pMET3-CDC20 scc1Δ scc1-TEV268-HA pGAL-9MYC-TEV* (Ry3216) and *cdc14-1 cdc5-as1 pMET3-CDC20 scc1Δ scc1-TEV268-HA pGAL-9MYC-TEV* (Ry3212) cells were arrested in G1 by α-factor in synthetic medium lacking methionine (SC-Met) supplemented with 2% raffinose and released in YEPR medium with an added 8 mM methionine to repress the expression of Cdc20 and arrest cells in metaphase. 180 minutes after the release, when >80% of cells were in metaphase, the culture was maintained in the same medium but shifted at the restrictive conditions (37°C and presence of the Cdc5-as1 inhibitor (CMK; (Snead, Sullivan et al. 2007))), to inactivate both Cdc14-1 and Cdc5-as1. 1 hr later (arrow) the culture was split in two and one-half was maintained in the same medium (closed symbols = RAF), whereas 2% galactose was added to the other half to induce the expression of TEV protease (open symbols = GAL), keeping the restrictive conditions. Samples were taken at the indicated times to determine the percentages of cells with metaphase spindles (circles) and anaphase spindles (squares).

The experiment shown in **Fig. 3.30** tells us about the existence of a pool of Cdc14 already active in metaphase that is required for spindle elongation to occur. This conclusion derives from the observation that *cdc5* cells kept arrested in metaphase elongate their spindles after the ectopic cleavage of cohesion. Nevertheless we noticed that the half of the cells arrested in metaphase without cohesin cleavage slowly

elongated their spindles, **Fig. 3.30**. In that experimental set up the metaphase-arrest of the cells hinged upon the control of the *pMET3* promoter on the *CDC20* gene. However, the *pMET3* promoter is a weak promoter, especially in case of long lasting experiments, so we cannot exclude that *cdc5* cells in **Fig. 3.30** were elongating their spindles after cohesin cleavage only because Cdc20 re-accumulated (circumstance that will explain also why they elongate their spindles even without TEV induction). We hence decided to repeat the same experiment using a stronger, more prolonged metaphase arrest. Unfortunately, we were not able to obtain viable strains carrying both the loss-of-function alleles of *CDC14* and *CDC5* concomitantly with temperature sensitive alleles of essential components of the APC/C (like the *cdc20-1* or *cdc23-1* alleles). Since we cannot put the *CDC20* gene under the control of the strong *pGAL1-10* promoter (already used to control the expression of the TEV protease) we tagged Cdc20 taking advantage of the auxin-inducible degron system (Freeman, Aragon-Alcaide et al.), which allowed the degradation of target proteins in response to auxin (Nishimura, Fukagawa et al. 2009). Although also in this case we could not obtain a *cdc14 cdc5* double mutant carrying the Cdc20-AID system we decided nevertheless to test the single *cdc5* mutant to ask which is the pool of Cdc14 involved in the *cdc14 cdc5* phenotype. *cdc5-as1 scc1Δ scc1-TEV268-HA pGAL-9MYC-TEV CDC20*-AID and *cdc14-1 scc1Δ scc1-TEV268-HA pGAL-9MYC-TEV CDC20*-AID cells were synchronized in G1 in raffinose medium and then released in fresh raffinose medium containing auxin to deplete cells of Cdc20 and hence achieve a metaphase arrest. Following the metaphase arrest, cells were shifted in conditions restrictive for Cdc14-1 or Cdc5-as1 and 30 minutes later the culture was splitted into two. One-half was maintained in raffinose medium, **Fig. 3.31a**, whereas 2% galactose was added to the other half to induce the expression of the TEV protease, **Fig. 3.31b**. Spindle length was measured in 100 cells every 30 minutes for 2 hours starting from the moment of TEV induction (from 180 to 240 min. time-point),

Fig. 3.31a-b. Surprisingly, we found that although cohesin was cleaved only *cdc14* cells were able to elongate their spindles while *cdc5* cells remained arrested in metaphase, **Fig. 3.31a-b**, suggesting the requirement of an anaphasic pool of Cdc14 to spindle elongation to occur.

However, further experiments are required to solve these controversial results. For example it would be important to repeat the *pMET3-CDC20* experiments in the presence of a tagged Pds1 to asses whether those cells that elongate their spindles are the one in which Pds1 is already degraded.

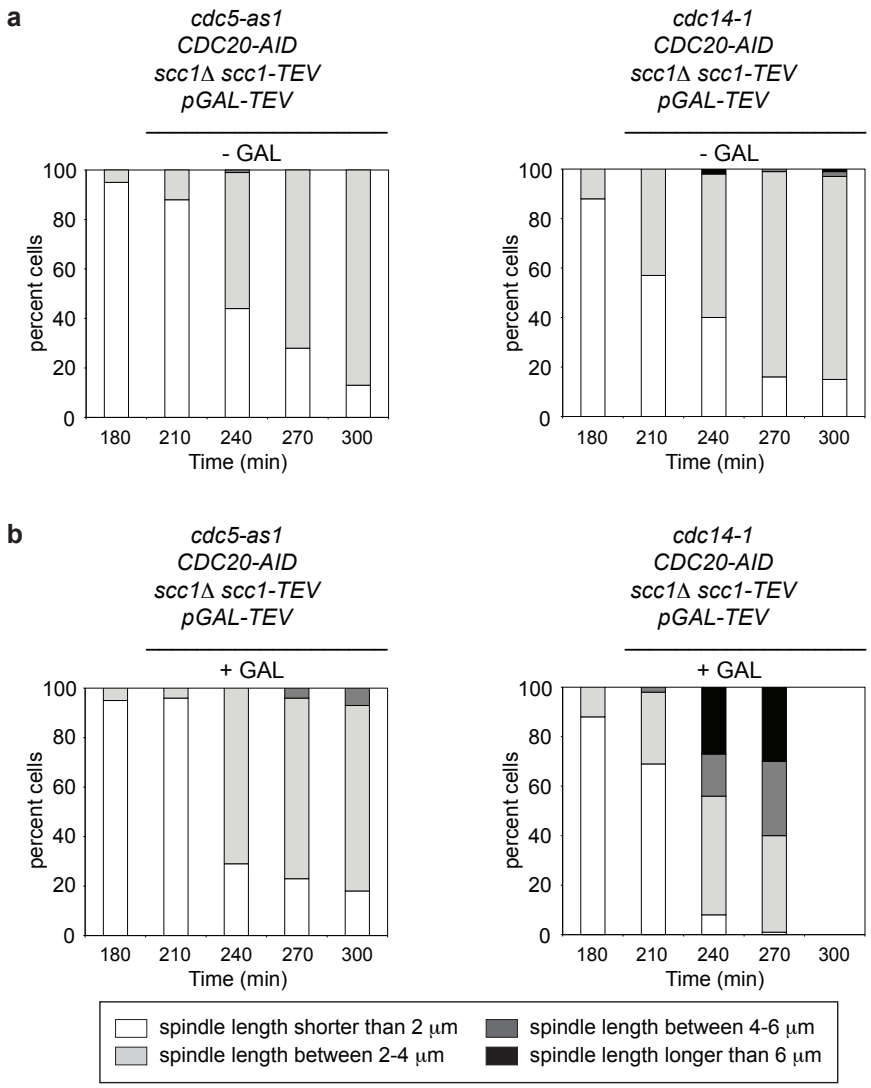

Figure 3.31. The *cdc14 cdc5* arrest seems to involve an early anaphase pool of Cdc14. (a-b) *cdc5-as1 CDC20-AID scc1Δ scc1-TEV268-HA pGAL-9MYC-TEV* (Ry4928) and *cdc14-1 CDC20-AID scc1Δ scc1-TEV268-HA pGAL-9MYC-TEV* (Ry4925) cells were arrested in G1 by α-factor in raffinose (YEPR) medium and released in fresh YEPR medium with an added 500 μM auxin to degrade Cdc20 and arrest cells in metaphase. 150 min. after the release, when cells were in metaphase, the culture was shifted at the restrictive conditions for *cdc14* and *cdc5*. 30 min. later (180 min. time-point) the culture was split in two and one-half was maintained in the same medium (- GAL; **(a)**), whereas 2% galactose was added to the other half to induce the expression of TEV protease (+ GAL; **(b)**), keeping the restrictive conditions. The spindle length (in micrometers) was determined for 100 cells from the 180 min. to the 300 min. time-point. The resulting length distribution profiles are shown in **(a-b)**.

3.7.2 The *cdc14 cdc5* arrest seems to involve a Cdc5-independent but FEAR-related pool of Cdc14

One set of our data suggests that an early-anaphase pool of Cdc14 independent of Cdc5 exists. Because during early anaphase Cdc14 is released by the FEAR network we wondered whether this pool of Cdc14 was Cdc5-independent but FEAR-dependent. The FEAR network is composed of at least two branches, one encompassing Spo12 and a parallel one including Esp1, Slk19 and Cdc5 (with Cdc5 the most downstream component). To examine whether the Spo12 branch was responsible for this Cdc5-independent release of Cdc14 we combined the *cdc5-as1* allele with the deletion of the FEAR component Spo12 alone or in association with Bns1 (Stegmeier, Visintin et al. 2002). *cdc5-as1*, *cdc5-as1 spo12Δ bns1Δ* and *cdc5-as1 spo12Δ* cells were synchronously released from G1 in conditions restrictive for *cdc5-as1*. Spindle length was measured for 100 cells at the terminal phenotype (240 min. time-point) of the three strains, **Fig. 3.32**. We found that impairing the Spo12/Bns1 branch of the FEAR network did not mimic the *cdc14-1 cdc5-as1* double mutant phenotype but it nevertheless shortened the *cdc5* spindles in an appreciable way, **Fig. 3.32**. We are now trying to inactivate both the Spo12/Bns1 and the Slk19 branch of the FEAR network in a *cdc5* defective background to better understand if the Cdc14 pool involved in the *cdc14 cdc5* phenotype is really related to the FEAR network.

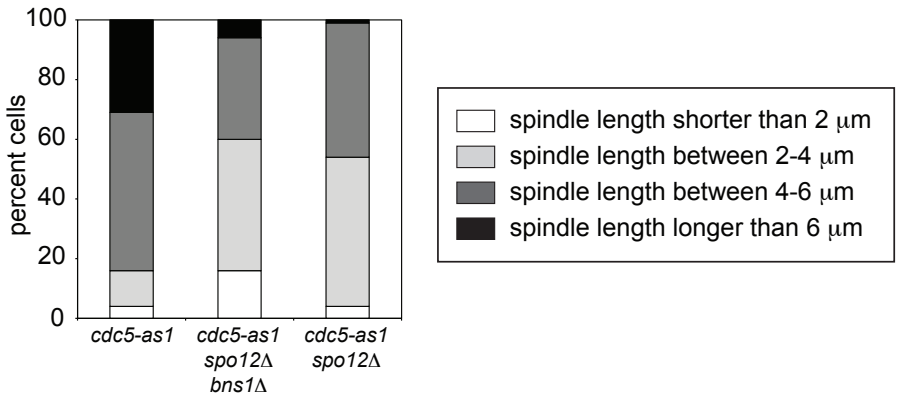

Figure 3.32. Inactivation of the Spo12/Bns1 branch of the FEAR network reduces the *cdc5* spindle length. *cdc5-as1* (Ry2446), *cdc5-as1 spo12Δ bns1Δ* (Ry4542) and *cdc5-as1 spo12Δ* (Ry4557) cells were arrested in G1 in YEPD at permissive conditions for *cdc5*. Cells were then released from the arrest in fresh YEPD at the restrictive conditions for *cdc5*. The length distribution profiles (spindles measured in micrometers; n=100) of the three strains at their terminal phenotype (240 minutes after the G1 release) are shown.

3.7.3 The missing role of Cdc14 affecting *cdc14 cdc5* cells could be in spindle microtubule stabilization

FEAR-released Cdc14 is key for spindle microtubules stabilization and thus is required for spindle elongation (Pereira and Schiebel 2003, D'Amours and Amon 2004, Stegmeier and Amon 2004, Khmelinskii, Lawrence et al. 2007, Khmelinskii, Roostalu et al. 2009). Interestingly, it has been published that the midzone organizer protein Ase1 has to be dephosphorylated by Cdc14 at anaphase onset to allow the recruitment to the midzone of all the proteins necessary for midzone stabilization and spindle elongation (among which also Cin8) (Khmelinskii, Roostalu et al. 2009). We hence decided to test the consequences of introducing in our *cdc14-1 cdc5-as1* mutant a non-phosphorylatable allele of *ASE1* (*ase1-7A*) (Khmelinskii, Roostalu et al. 2009). Next, the *cdc14-1 cdc5-as1 ase1Δ ase1-7A* cells were released form G1 at conditions restrictive for Cdc14-1 and Cdc5-as1. Cell cycle progression was tracked via spindles morphology analysis, **Fig. 3.33a**. We noticed a transient and minor elongation of the

spindles soon after metaphase entry followed by a rapid shortening later on, **Fig. 3.33a**. More interestingly, we found that at the terminal phenotype (240 minutes after the G1 release) although we do not have spindles longer than those of *cdc14 cdc5* cells, the 100% of them are collapsed and almost half of the cells population exhibit bilobated nuclei, **Fig. 3.33b**. We conclude that a non-phosphorylatable allele of Ase1 only partially rescues the defect associated with the *cdc5 cdc14* double mutant suggesting that multiple Cdc14 substrates may cooperate to promote spindle elongation in the absence of Cdc5.

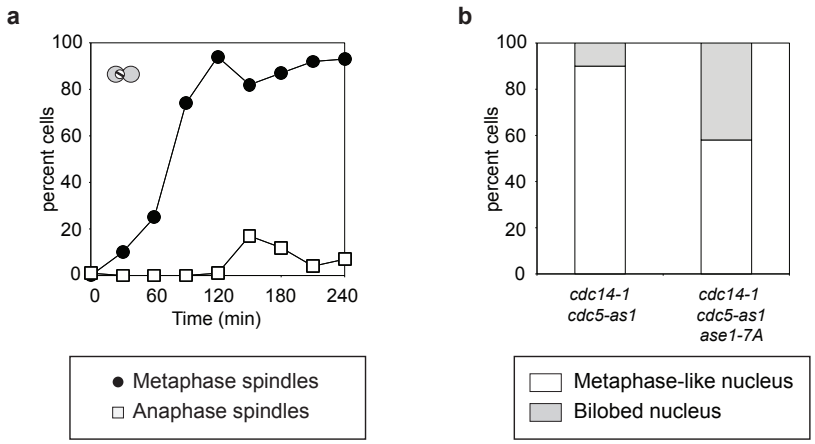

Figure 3.33. The missing role of Cdc14 affecting *cdc14 cdc5* cells could be in spindle microtubule stabilization. (a-b) *cdc14-1 cdc5-as1 ase1Δ ase1-7A TUB1-GFP* (Ry3606) cells were arrested in G1 in YEPD at permissive conditions. Cells were then released from the arrest in fresh YEPD at the restrictive conditions for both Cdc14-1 and Cdc5-as1. Samples were taken at the indicated times to determine the percentages of cells with metaphase spindles (closed circles) and anaphase spindles (open squares) **(a)**. In **(b)** the nuclear morphologies of *cdc14-1 cdc5-as1* (Ry1602) cells 240 min. after the release (at their terminal phenotype) is compared with the nuclear morphologies of *cdc14-1 cdc5-as1 ase1Δ ase1-7A TUB1-GFP* (Ry3606) cells at the same time-point (n=100).

4. Discussion

4.1 Chromosome segregation

A pre-requisite for the faithful segregation of chromosomes is the correct establishment of the linkages between replicated chromosomes (sister chromatids) from the moment of their duplication to the time of their segregation. Next, proper segregation of both chromatids requires that each sister chromatids pair attach to the mitotic spindle microtubules, emanating from the opposite (spindle) poles of the cell. Sister chromatids align on the spindle via protein complexes named kinetochores, one of which assembles on the centromeric sequence of each chromosome. At metaphase, one chromatid is bound to MTs extending from one spindle pole, whereas its sister is attached via its kinetochore to the MTs emanating from the second spindle pole, thereby establishing a so called bipolar state of attachment. Bipolar attachment allows microtubules to exert pulling forces on each chromatid in the direction of the pole to which it is bound. However, these forces are counteracted by cohesion holding sister chromatids pairs together. Upon entry into anaphase, the cohesion between sister chromatids is dissolved, thereby allowing the chromosomes to be pulled apart by the spindle MTs towards opposite poles. Further segregation of the separated chromosomes is achieved firstly by the movement of the spindle pole bodies away from each other and at a later stage by the progressive elongation of the mitotic spindle.

In summary, accurate chromosome segregation requires the (i) correct establishment of linkages between sister chromatids, (ii) proper assembly and activity of kinetochores, (iii) complete resolution of the linkages between the sister chromatids, and (iv) proper regulation of spindle MTs dynamics.

How these processes are coordinated is not yet clear. Our finding that combining loss-of-functions alleles of the polo-like kinase Cdc5 and the phosphatase Cdc14 has a synergistic effect and generates cells that arrest with undivided nuclei and stable short bipolar spindles regardless of having cleaved cohesin, unveils the existence of a new pathway for mitotic regulation that oversee chromosome segregation perhaps by integrating the above-mentioned processes.

4.1.1 Phosphatase Cdc14 and kinase Cdc5 contribute to removal of cohesin-independent cohesion events

Chromosome segregation is conventionally marked at the metaphase-to-anaphase transition by the dissolution of the cohesin linkages that hold sister chromatids together. Indeed, cleavage of cohesin by separase reduces the forces that oppose the pulling forces of the spindle MTs, thereby promoting anaphase spindle elongation in metaphase-arrested cells (Uhlmann, Wernic et al. 2000). At the heart of this process lies the degradation of the anaphase inhibitor Pds1 by the ubiquitin-degradation pathway, and involves the activity of the anaphase promoting complex/cyclosome (APC/C). Indeed, the fidelity of anaphase onset is ensured by several different regulatory mechanisms that modulate the APC/C activity (Peters 2006).

While cohesins are essential to hold sister chromatids together, cohesin-independent forces, such us DNA catenation (Lucas, Germe et al. 2001, Toyoda and Yanagida 2006, Bermejo, Branzei et al. 2008), provide additional linkages that resist the mitotic spindle suggesting that cohesin dissolution may be not enough to ensure chromosome segregation (Higuchi and Uhlmann 2005, Baskerville, Segal et al. 2008). Indeed, in addition to cohesin removal chromosome segregation requires that sister chromatid DNAs are disentangled from each other, a process mediated by topoisomerase II (Lucas, Germe et al. 2001, Wang 2002). Besides topoisomerase II also condensins have

been implicated in chromosome segregation through their action in recoiling chromosome regions during anaphase (Bhalla, Biggins et al. 2002).

Our finding that lack of a sister chromatids ("sister-less" chromosomes) in the *cdc14 cdc5* mutant background resulted in cells with mitotic spindles more fragile and slightly longer than the one observed in *cdc14 cdc5* cells where DNA synthesis proceeded regularly support the idea that Cdc5 and Cdc14 activities besides regulating spindle elongation may be required for (i) resolving cohesin-independent linkages between sister molecules and (ii) coordinating the completion of sister chromatid separation with anaphase spindle elongation. This way Cdc14 and Cdc5 ensure that spindle elongation follows the dissolution of sister chromatid linkages in a timely and orderly fashion. It is worth noting that this residual cohesion can be and is efficiently removed if spindle microtubules generate sufficient pulling forces, as assessed by the observation that high level of kinesin motor Cin8 allow spindle elongation in *cdc14 cdc5* cells.

To understand how Cdc14 and Cdc5 are regulated and how both regulate the complete removal of sister cohesion and coordinate this with anaphase progression remains an important question for the future. Intriguingly, yeast condensins are phosphorylated by polo-like kinase Cdc5 specifically during anaphase. This enhances the DNA supercoiling activity of condensins *in vitro* (St-Pierre, Douziech et al. 2009) may facilitate their action on chromosome recoiling *in vivo* and as such contribute to chromosome segregation.

4.1.2 A model for anaphase spindle elongation: It is all a matter of thresholds

Proper chromosome segregation asks for an orderly sequence of events whereby spindle elongation follows the dissolution of sister chromatid cohesion. The molecular mechanism that guarantees this chain of events remains elusive. Although cohesin is thought to provide the main force counteracting the pulling force of the mitotic spindle,

we have discussed the existence of cohesin-independent mechanisms contributing to controlling the timing of spindle elongation. This was already suggested by the observation that yeast cells lacking either Pds1 or cohesin do not elongate their spindle prematurely. Recently Liang and colleagues have provided a molecular explanation and suggest that the timely control of spindle elongation is based on a balance of mitotic (positive effect) versus S phase (negative effect) CDKs activity (Liang, Richmond et al.). Our work further implicates the involvement of at least the polo-like kinase Cdc5 or the phosphatase Cdc14, suggesting that a more global kinase/phosphatase threshold is required for the timely progression through anaphase. To understand molecular mechanisms underlying spindle dynamics becomes essential to understand how is this threshold obtained.

A steady rise in mitotic CDK activity establishes the sequence of events during early mitosis, with a lower Clb-CDK activity threshold triggering entry into mitosis and a higher one triggering entry into anaphase (Rahal and Amon 2008) and seems to be a general mechanism via which eukaryotes ensure that chromosome segregation occurs only after chromosome condensation and spindle formation (Arion, Meijer et al. 1988, Labbe, Picard et al. 1988, Gautier, Matsukawa et al. 1989, Labbe, Picard et al. 1989, Murray and Kirschner 1989, Lindqvist, van Zon et al. 2007). Upon anaphase entry, however, Clb-CDK activity declines and concomitantly the levels of its counteracting phosphatase Cdc14 increases, thereby initiating mitotic exit (Yeong, Lim et al. 2000) (Baumer, Braus et al. 2000, Yeong, Lim et al. 2000). This initial reduction in Clb-CDK activity it is triggered by the APC/C^{Cdc20}. The periodical expression of cyclins controls the activity and substrate specificity of the CDK, next the conserved protein phosphatase Cdc14 reverses the phosphorylation of these CDK substrates. Cdc14 activity as well relies on the APC/C^{Cdc20}, that by triggering the degradation of the anaphase inhibitor Pds1 liberates the protease Esp1 thereby starting the FEAR network.

However, the synergistic effect obtained by combining loss of function alleles of Cdc14 and Cdc5 invokes for an additional mechanism of Cdc14 activation independent from Cdc5. Whether this mechanism activates Cdc14 in metaphase or in early anaphase is still a matter of debate. Preliminary data from our lab suggest that both Esp1 and Spo12 maybe directly involved in this release. This tightly regulated activity of CDK and phosphatases enables unique temporal phosphorylation kinetics of each CDK substrate during the cell cycle. In agreement with this we found that modulating CDK activity during anaphase impacts anaphase spindle elongation. Overexpression of a non-degradable form of the mitotic cyclin Clb2 leads to high levels of mitotic CDK activity and led to spindle elongation defects in cells also harboring the hypomorphic *cdc14-1* allele, suggesting that raising CDK activity in a phosphatase defective background may be detrimental for anaphase spindle elongation. Furthermore, the observation that inactivation of Cdc5 also causes increased levels of CDK activity and spindle elongation defects in *cdc14-1* mutants, suggests that one possible role for the polo kinase at anaphase is to contribute to the decline in CDK activity. Taken together these data indicate that a transient down-regulation of mitotic CDK activity at the onset of anaphase is critical for decreasing microtubule dynamics, which in turn is critical for anaphase chromosome movement.

In support to this idea is the observation that similarly to CDK activity, microtubule turnover changes throughout mitosis. Microtubule dynamics increase as cells progress through metaphase, which is important for the correction of erroneous sister chromatid attachments and then suddenly decrease at the onset of anaphase, reflecting the shift from a phosphorylated to a dephosphorylated state of multiple proteins important for microtubule dynamics which is dictated not only by the inactivation of Clb-CDK complexes but also by the concomitant activation of CDK-counteracting phosphatases, such as Cdc14.

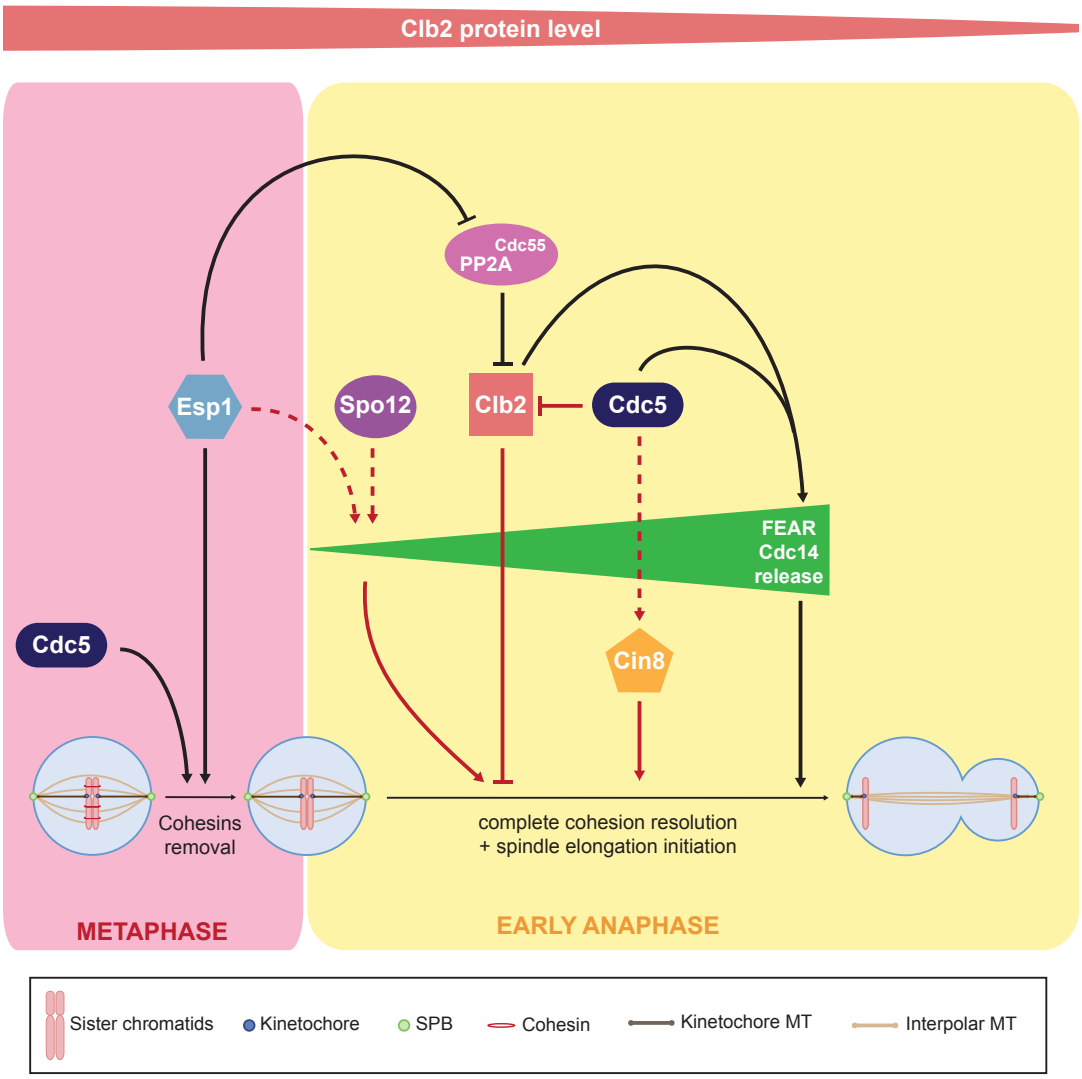

Figure 4.1. Model showing how Cdc14 and Cdc5 overlap in function in regulating the metaphase-to-anaphase transition.

4.1.3 What is the function of these enzymatic activities and how do they contribute to anaphase spindle elongation?

Cell cycle kinases and phosphatases mutually regulate themselves, with Clb-CDKs and Cdc5 contributing to the activation of Cdc14, which in turns culminates first in the reversal of S phase CDKs mediated phosphorylation events, an essential step in spindle elongation and ultimately to triggering complete Clb-CDKs inactivation.

Several lines of evidence, however, suggest that at least one additional function has to be envisioned for proteins including Clb-CDKs, Cdc5 and Cdc14. First of all the

anaphase spindle elongation defect associated with *cdc14* mutants is subtle when compared with that of cells lacking mitotic CDKs suggesting that the mitotic CDKs contribution to spindle elongation cannot be explained only by their role in Cdc14 activation at the metaphase-to-anaphase transition (Azzam, Chen et al. 2004). Secondly, the finding that deletion of S phase cyclin Clb5 does not bypass the terminal phenotype of *cdc5 cdc14* double mutant cells suggests that at least one function other than reversing S phase CDKs mediated phosphorylation events should be envisioned for Cdc14. Interestingly, this function of Cdc14 becomes essential in the absence of the polo kinase Cdc5. Finally, the observation that the phenotype of *cdc14-1* cells overexpressing Clb2-CDK is less severe than that of *cdc14 cdc5* cells suggest an additional function for the polo-like kinase. Interestingly, overexpression of spindle elongation promoting factors such as *CIN8* (Hildebrandt and Hoyt 2001) or *STU2* (Severin, Habermann et al. 2001) and deletion of spindle elongation inhibitors such as *KIP3* (Hildebrandt and Hoyt 2000, Gupta, Carvalho et al. 2006, Varga, Helenius et al. 2006) led us to identify the kinesin 5 Cin8 as an essential effector of the Cdc5 pathway allowing us to speculate that a fine-tuned balance in kinase and phosphatase activities triggers spindle elongation by regulating the activity of multiple motors and/or microtubule-associated proteins, especially those at the spindle midzone. Although the CDK substrates involved in this process remain unknown, possible substrates of the phosphatase Cdc14 include several microtubule-associated proteins, such as Ase1, Fin1, and Ask1, whose phosphorylation status regulates spindle dynamics (Higuchi and Uhlmann 2005, Woodbury and Morgan 2007, Khmelinskii, Roostalu et al. 2009). These findings allow us to suggest that Clb-CDKs dependent recruitment and phosphatase-dependent recruitment and maintenance of proteins to the spindle midzone are important for full anaphase spindle elongation. Along this line of thinking is the finding that mutation of the nine CDK consensus sequences in the chromosomal passenger

protein Bir1 to amino acids that can no longer be phosphorylated reduces the extent of spindle elongation and prevents the localization of proteins such as Ndc10 to the spindle midzone during anaphase (Widlund, Lyssand et al. 2006). Taken together, these observations suggest that anaphase spindle elongation requires a fine-tuned balance of kinase-phosphatase activities. Having identified a novel pathway controlling spindle elongation after cohesin cleavage may prove a valuable tool for identifying the minimal requirement for successful completion of mitosis and ultimately for understanding how the activity of a sophisticated machinery such as the mitotic spindle is regulated and coupled with that of other cell division events. Given the conserved nature of the proteins involved and reinforced by the observation that polo mutants of Drosophila exhibit a phenotype similar to the one observed in our double mutant strain (Donaldson, Tavares et al. 2001). We speculate that elucidating the role of budding yeast Cdc14 and Cdc5 activities in the spindle process will also be instrumental to understand spindle dynamics in higher eukaryotes, including humans.

4.2 The Cdc14 conundrum

The phenotype associated with our double mutant highlighted a Cdc14 puzzle. In budding yeast, the nucleolar phosphatase Cdc14 is sequestered in the nucleolus and considered inactive from G1 until anaphase onset when it is released into the nucleus and cytoplasm to dephosphorylate its substrates and drive progression through anaphase and exit from mitosis (Visintin, Craig et al. 1998, Stegmeier and Amon 2004, Rock and Amon 2009). The release of Cdc14 from the nucleolus requires the polo-like kinase Cdc5 (Hu, Wang et al. 2001, Pereira, Manson et al. 2002, Stegmeier, Visintin et al. 2002, Geymonat, Spanos et al. 2003, Visintin, Stegmeier et al. 2003, Visintin, Tomson et al. 2008) in concert with a partner kinase, whose identity changes in the different

stages of anaphase (Manzoni, Montani et al. 2010). Consistent with this, cells that are impaired in Cdc14 or Cdc5 activity arrest in anaphase (Stegmeier and Amon 2004). Our finding that combining loss-of-functions alleles of Cdc14 and Cdc5 exhibits a synergistic effect supports the idea that a pool of Cdc14 exists whose regulation is Cdc5-independent. Whether this pool of Cdc14 is active throughout the cell cycle or it is an early anaphase pool remains unclear. Both possibilities are equally likely. Recent work suggests that there may be a pool of Cdc14 active prior to anaphase onset, while some confine this activity in the nucleolus (Geil, Schwab et al. 2008, Tomson, Rahal et al. 2009), other propose that this pool maybe active in other cellular compartments (Akiyoshi and Biggins 2010) consistent with the finding that Cdc14 dephosphorylates kinetochore component Dns1 prior to anaphase when CDKs activity is high (Akiyoshi and Biggins 2010). Interestingly the *S. pombe* Cdc14 orthologue Clp1/Flp1 is released from the nucleolus at mitotic entry yet it does not induce mitotic exit (Cueille, Salimova et al. 2001, Trautmann, Wolfe et al. 2001, Wolfe, McDonald et al. 2006). Clp1 localizes to KTs and plays important roles in promoting bi-orientation during prometaphase (Trautmann, Rajagopalan et al. 2004). KTs localization of budding yeast Cdc14 was also reported during anaphase (Pereira and Schiebel 2003, Stoepel, Ottey et al. 2005). Thus elucidating which pool of Cdc14 is involved in the here described spindle elongation process and understanding how it is regulated will reveal a novel conserved regulatory mechanism that contributes to maintaining genomic stability.

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