
# New substrates and functions of PP2A-Cdc55 phospatase in the mitotic exit

Bàrbara Baró Sastre

*Be curious,*
*and however difficult life may seem,*
*there's always something you can do*
*and succeed at.*
*It matters that you don't just give up.*

*Stephen Hawking*

*A la meva família*
*i a l'Oleguer*

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# *Index*

# *Summary*

## Nous substrats i funcions de la fosfatasa PP2A$^{Cdc55}$ durant la sortida de mitosis.

### Antecedents

La sortida de mitosis comprèn tots els processos de la mitosis que van des de la segregació de les cromàtides germanes a l'obtenció de dues cèl·lules filles genèticament idèntiques. A nivell molecular, les cèl·lules inicien diversos processos per inactivar la Cdk mitòtica, requisit imprescindible per acabar la mitosis i entrar en la següent fase G1. La sortida de mitosis en el llevat de gemmació, *S. Cerevisiae,* s'inicia amb l'activació del complex promotor d'anafase APC$^{Cdc20}$ i l'activació de la fosfatasa Cdc14. Dues rutes complementàries i seqüencials, FEAR (de l'anglès *Cdc14 early anaphase release*) i MEN (de l'anglès *mitotic exit network*), promouen l'alliberament i activació de la fosfatasa Cdc14 durant l'anafase, la qual es considera clau per eliminar l'activitat mitòtica de Cdk1 i sortir de mitosis. La fosfatasa Cdc14 es manté segrestada al nucleol durant la resta del cicle cel·lular mitjançant la seva unió a la proteïna nucleolar Net1. A l'inici de l'anafase, la proteasa separasa hidrolitza el complex de cohesina, desencadenant així la separació de les cromàtides germanes i, alhora, coopera amb Zds1 per promoure la inhibició de la fosfatasa PP2A$^{Cdc55}$. La caiguda d'activitat PP2A$^{Cdc55}$ facilita la fosforilació de Net1 dependent de Cdk1, promovent així que Cdc14 s'alliberi al nucli. La quinasa polo, Cdc5, contribueix en aquest procés. A mesura que l'activitat mitòtica de Cdk1 disminueix, degut a l'acció d'APC$^{Cdc20}$ degradant ciclines mitòtiques i l'activitat de Cdc14, les cèl·lules activen la ruta MEN per mantenir Net1 fosforilat i Cdc14 actiu

fins al final d'anafase. La ruta de MEN comprèn la GTPasa Tem1, i les quinases Cdc15 i Mob1-Dbf2. L'inici de la ruta està regulat pel complex Bfa1-Bub2, que manté Tem1 inactiu fins l'anafase, i per Lte1, que promou l'activació de Tem1. Un cop activa, la ruta de MEN allibera Cdc14 al citoplasma, on permet l'acumulació de l'inhibidor de Cdk1, Sic1, i on també activa el complex $APC^{Cdh1}$, el qual finalitza amb la degradació de les ciclines mitòtiques. Per tant, la completa activació de Cdc14 al final de l'anafase promou la inactivació de Cdk1 i contrarresta les fosforilacions mitòtiques d'aquesta, permetent així la sortida de mitosis i entrada a G1.

Com la cèl·lula coordina la segregació cromosòmica amb la inactivació de la Cdk mitòtica és de gran interès, doncs errors en aquest procés provoquen la mort cel·lular. Així doncs, la descripció detallada dels mecanismes moleculars que regulen la sortida de mitosis tenen un gran potencial pel disseny de futures teràpies contra el càncer. El treball que es presenta en aquesta tesis doctoral aprofundeix en el paper de la fosfatasa $PP2A^{Cdc55}$ en la sortida de mitosis, fosfatasa que està emergent com a regulador clau del cicle cel·lular en organismes superiors. Resultats previs del grup suggereixen la implicació de la fosfatasa $PP2A^{Cdc55}$ en la iniciació de la ruta MEN i d'altres possibles funcions.

**Objectius**

1. Descriure el paper de la fosfatasa $PP2A^{Cdc55}$ en la regulació de la ruta MEN
2. Identificar nous possibles substrats i funcions de la fosfatasa $PP2A^{Cdc55}$ mitjançant un *screening* proteòmic.

## Resultats

La inactivació de la fosfatasa $PP2A^{Cdc55}$ a l'inici d'anafase promou la fosforilació de Bfa1. La quinasa responsable d'aquesta fosforilació és la quinasa polo o Cdc5 i indueix la localització asimètrica de Bfa1 al centrosoma destinat a la cèl·lula filla (*daughter spindle pole body*, *dSPB*). Tant la localització asimètrica com la fosforilació de Bfa1 s'han relacionat amb la pèrdua d'activitat del complex Bfa1-Bub2, la qual facilita l'activació de la ruta de MEN. Tot i així, la inactivació prematura de Bfa1 en absència d'activitat $PP2A^{Cdc55}$ no implica una sortida de mitosis prematura. De fet, la inactivació de la fosfatasa $PP2A^{Cdc55}$ indueix la inactivació de Bfa1-Bub2 i inicia la ruta de MEN, però aquesta només s'activa fins al nivell de la quinasa Cdc15. Així, quan $PP2A^{Cdc55}$ s'inactiva, Cdc15 es localitza prematurament als centrosomes i es defosforila (dos esdeveniments necessaris per la seva activació), però no és totalment activa com a quinasa ja que no és capaç de reclutar el complex Mob1-Dbf2 als centrosomes. Conseqüentment, la proteïna Mob1 tampoc canvia la seva localització ni estat de fosforilació quan s'inactiva la fosfatasa $PP2A^{Cdc55}$. L'activitat Clb2-Cdk1 és la responsable de mantenir Cdc15 i Mob1-Dbf2 inactives fins l'anafase tardana, en què els nivells de Cdk1 ja són més baixos i per tant es requereix l'activació de MEN. De fet, quan s'inactiva artificialment el complex Clb2-Cdk1, mitjançant l'inhibidor 1NM-PP1, Mob1 es defosforila ràpidament. Per tant, la inactivació de $PP2A^{Cdc55}$ a l'inici d'anafase inicia la ruta de FEAR i MEN, i l'activitat decreixent de Clb2-Cdk1 durant l'anafase és el que determina l'acció seqüencial d'ambdues vies. Per altra banda, la fosfatasa $PP2A^{Cdc55}$ es requereix per regular correctament la quinasa Mob1-Dbf2, regulant la defosforilació de Mob1. Mob1 està hiperfosforilat

en metafase en absència de $PP2A^{Cdc55}$, i tampoc es defosforila correctament al final d'anafase en absència de de $PP2A^{Cdc55}$. La recuperació de l'activitat fosfatasa $PP2A^{Cdc55}$ al final d'anafase es requereix per una correcta activació del complex $APC^{Cdh1}$ però no per una correcta acumulació de Sic1. Finalment, la cerca de nous substrats de la fosfatasa $PP2A^{Cdc55}$ mitjançant un *screening* proteòmic basat en la tècnica de SILAC ha permès identificar noves possibles funcions d'aquesta fosfatasa. Els resultats preliminars de l'*screening* suggereixen que la fosfatasa $PP2A^{Cdc55}$ podria contrarestar o regular l'activitat d'altres quinases, diferents de Cdk1 i Cdc5. També s'han identificat nous possibles substrats de la sortida de mitosis, relacionats amb al ruta FEAR, l'elongació dels microtúbuls i la citocinesis.

**Discussió i Conclusions**

El treball que es presenta en aquesta tesis doctoral identifica dos nous substrats de la fosfatasa $PP2A^{Cdc55}$ en la sortida de mitosis, les proteïnes Bfa1 i Mob1. La inactivació de la $PP2A^{Cdc55}$ a l'inici d'anafase inicia la ruta MEN mitjançant la inactivació del complex Bfa1-Bub2, ja que facilita la seva fosforilació i localització asimètrica. Tot i que les rutes FEAR i MEN s'inicien per tant alhora, l'activitat decreixent de Clb2-Cdk1 ordena seqüencialment l'activació de ambdues rutes, restringint l'activitat de les quinases Cdc15 i Mob1 a l'anafase tardana. La reactivació de la fosfatasa $PP2A^{Cdc55}$ al final d'anafase, quan els nivells de Clb2-Cdk1 són baixos, permet la correcta activació del complex Mob1-Dbf2 i també d'$APC^{Cdh1}$. Per tant, l'activitat $PP2A^{Cdc55}$ es requereix per una sortida eficient de mitosis i una correcta G1. Finalment, la fosfatasa $PP2A^{Cdc55}$ podria contrarestar o regular d'altres quinases, a

més de Cdk1 i Cdc5, i tenir funcions addicionals durant la sortida de mitosis.

# *Introduction*

# 1. Cell Cycle in Eukaryotes

The cell cycle comprises a series of sequential and coordinated events that lead to the formation of two or more daughter cells. The cell division is a central biological process for any living organism, required to the generation of a progeny. Cell division is also crucial in adult multicellular organisms to supply with the required cells for repairing and maintaining old or damaged tissue.

In eukaryotes, the cell cycle is usually subdivided into four phases G1, S, G2 and M (Fig. I.1). These phases include two major periods of activity in which the genome is first duplicated (DNA synthesis or S phase) and the two newly replicated genomes are then distributed between two daughter cells (Mitosis or M phase). On the other hand, when diploid organisms generate haploid cells, the cellular division process is called Meiosis, which consists in one S-phase followed by two rounds of division, resulting in four haploid daughter cells. Additional gap periods, G1 (preceding S phase) and G2 (preceding mitosis), are required to coordinate DNA synthesis and segregation with mitogenic signals and to synthesize and assemble the proteins and cellular structures required for the next phase. G1 and G2 are periods of growing and regulation.

Most of the cell divisions in eukaryotics organisms are mitotic cell cycles. Meiosis is restricted to the production of gametes or spores for sexual reproduction, and the rest of cell types come from mitotic cell divisions. Mitosis is subdivided into four major steps: prophase, metaphase, anaphase and telophase. In prophase, chromosomes undergo condensation and sister chromatids bind together by the centromere, forming the mitotic chromosomes. The two centrosomes separate and

migrate to opposite poles of the cell, and the formation of the mitotic microtubules takes place. Nuclear envelope breaks down and the spindle microtubules attach to sister chromatids at the kinetochore, a specialised chromatin structure built on centromeric DNA. This attachment must be bipolar. At this point, cells reach metaphase and the sister chromatids are aligned at the metaphase plate. Anaphase occurs when the cohesion complex holding the sister chromatids together is cleaved, allowing them to separate. The sister chromatids are pulled apart by the shortening of the kinetochore microtubules, moving toward the respective centrosomes to which they are attached. At the same time, the mitotic spindle elongates resulting in the complete segregation of the sister chromatids to opposite poles of the cell. Mitosis is completed in telophase, when the sister chromatids are repackaged into two identical daughter nuclei, the mitotic spindle is disassembled, the nuclear membrane assembled and the chromosomes start to decondense (Morgan, 2007).

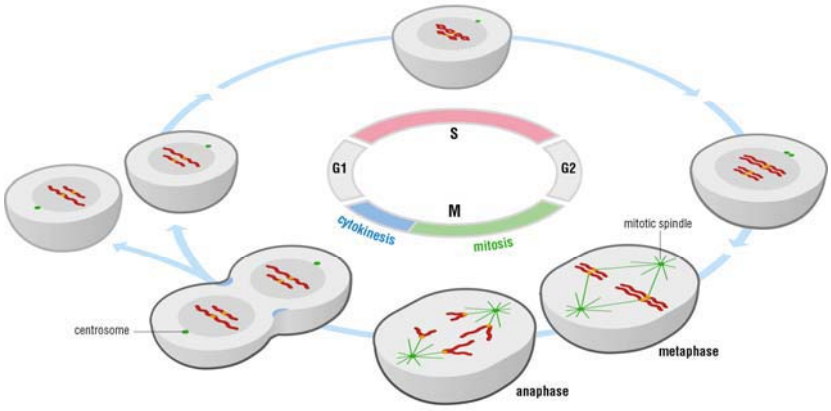



**Figure I.1. The phases of the cell cycle (Morgan 2007)**

To complete cell division cells undergo the process called cytokinesis, which takes place at the end of mitosis. During cytokinesis, the two nuclei and other cellular components are distributed into a pair of daughter cells, each containing a single nucleus, centrosome and roughly equal cytoplasmic content. This is achieved by the contraction of a ring composed of actin filaments at the site of cell division, together with the generation of new cellular membrane (Morgan, 2007).

To ensure proper progression through the cell cycle phases, cells possess restriction points that monitor proper conditions to generate healthy daughter cells (Hartwell LH, 1989). Thus, the cell division cycle can be defined as the process by which cells monitor proper conditions for cell division, activate the required biochemical machineries for DNA replication and chromosome segregation, and monitor these steps to generate two genomically stable daughter cells (Malumbres 2011).

## 2. Budding Yeast Cell Cycle

Regulation of the cell cycle is well conserved among all eukaryotes. The molecular bases of cell cycle regulation were first studied in landmark genetic screens in yeast. Most of the genes identified in these screenings have a mammalian counterpart. However, as it may be expected from the diversity of mammalian cell types, many of these individual yeast genes are represented as complex gene families in mammals. Therefore, it is common practice to use single-cell yeasts to study the basic mechanisms of cell cycle control.

The budding yeast *Saccharomyces cerevisiae* is one of the most commonly used eukaryotic model organisms in cell cycle studies (reviewed in Alberghina et al., 2011). Among its advantages are its genetic versatility and the existence of highly optimized synchronization tools. It also presents different morphological and molecular markers that allow easy monitoring of cell cycle progression. The appearance and size of the bud is a marker of the cell cycle phase. In G1 phase, cells have no bud. In S phase, cells have a small bud as DNA replication and budding begin simultaneously. At the end of G2 and mitosis, the bud has an intermediate size and, at the end of mitosis, the separation of the two daughter cells takes place. The bud does not reach the size of the mother cell, and therefore budding yeast has an asymmetric cell division. During mitosis, it is not possible to differentiate between prophase, metaphase, anaphase and telophase from the size of the bud. However, other markers can be used as the nucleus staining using DAPI (4',6-diamidino-2-phenylindole) or the tubulin staining for visualization of the mitotic spindle.

Another particularity of *S. cerevisiae* is that the nuclear envelope remains intact while mitosis takes place. Shortly before cytokinesis, it is pinched in two, instead of being resynthesised as it is in vertebrates. Thus, *S. cerevisiae* undergoes the so called closed mitosis.

# 3. Cell Cycle Control System

## 3.1. Cdks and cyclins

The cell cycle control has been the subject of numerous studies over the past decades. Early studies in yeast mutants and *Xenopus* oocytes allowed the identification of genes that regulate cell cycle progression (Nurse, 1975) and factors responsible for inducing entry into a particular phase of the cycle (Gautier et al., 1988; Lohka et al., 1988). Later on, it was discovered a highly conserved and specific family of serine/threonine kinases called Cdks (cyclin-dependent kinases), which are the main component of the cell cycle control system. Cdks phosphorylate a broad range of proteins, regulating their enzymatic activity or their ability to bind to other proteins. Cdks target serine and threonine residues, and their activity is dependent on the binding of specific activating subunits, known as cyclins. Cdks protein levels are constant during the cell cycle whereas cyclins levels oscillate, being expressed and degraded specifically at different phases of the cell cycle (Evans et al., 1983). Moreover, each cyclin determines which substrates will be target by the specific cyclin-Cdk complex (reviewed in Murray 2004). Thus, different cyclins are present at different cell-cycle phases, allowing the periodic formation of a specific cyclin-Cdk complex that will trigger different events (Fig. I.2). The deregulation of Cdks can lead to uncontrolled cell division and originate neoplastic processes (Malumbres and Barbacid, 2009).

Eleven genes that encode for different Cdks have been identified in humans and murine models (CDK1-11), and it has been found nine genes encoding for proteins similar to Cdk (reviewed in Malumbres

2005; Malumbres and Barbacid, 2005). However, only Cdk1 (together with the cyclins A2 and B1) is able to drive cell cycle independently of other Cdks. Similarly, the budding yeast, *S. cerevisiae,* possesses just one Cdk essential for cell-cycle regulation, named Cdc28 or Cdk1. *CDC28* gene is constitutively expressed and Cdc28 protein levels are almost constant and non-limiting during all cell cycle (Reed et al., 1982; Reed et al., 1985). However, monomeric Cdk is inactive as a kinase: it requires its phosphorylation and the binding to one cyclin in order to be active. There are nine cyclins in budding yeast that bind to Cdk1 and govern the progression through the different phases of the cell cycle: Cln1-3 and Clb1-6. These cyclins activate Cdk1 and they regulate the target specificity and subcellular localization of each cyclin-Cdk1 complex.

Cln1-3 are known as G1-cyclins because in absence of all three, cells arrest in G1 (Richardson et al., 1989). Clb1-6 are known as B-type cyclins and they are responsible for S and M phases. Clb5 and Clb6 are expressed earlier than the rest of Clb and trigger DNA replication, the main event of S phase. Clb3 and Clb4 increase during mid S phase, at the same time that the spindle pole bodies duplicate. Finally Clb1 and Clb2 levels rise during spindle assembly (Fitch et al., 1992). Clb1/2 - Cdk1 is the main kinase activity during mitosis, triggering chromosome segregation and the isotropic bud growth. *CLB2* seems to be the most important of the *CLB* genes, at least phenotypically. The *Δclb2* mutant presents a hyperpolarized cell growth that does not show other *CLB* deletions; meaning that Clb2-Cdk1 complex is the responsible of promoting isotropic growth of the bud (Lew and Reed, 1993). Moreover, when all possible single and multiple *CLB* deletion mutants were

constructed, all lethal combinations included the *CLB2* deletion, whereas the triple mutant *clb1Δclb3Δclb4Δ* was viable, demonstrating the importance of Clb2 (Fitch et al., 1992).

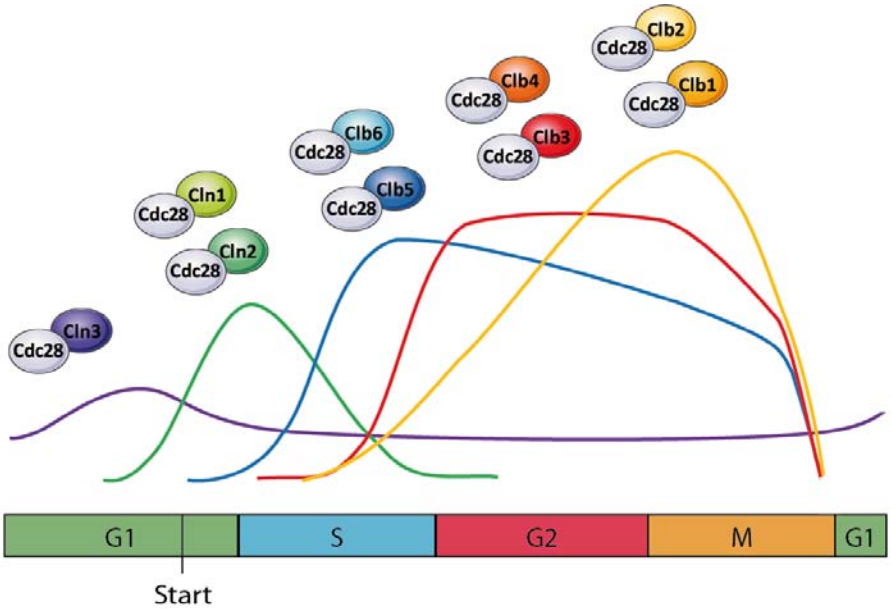



**Figure I.2. The different cyclin-Cdk1 complexes in *S. cerevisiae* and its temporal activation**

G1 and B-type cyclins do not share the same transcriptional machinery, which increases the specificity of their regulation. The *CLN3* gene is transcribed throughout the cell cycle, although its transcription is accentuated during late M phase and early G1. The *CLN1* and *CLN2* genes are transcribed during G1-S transition. This process is mediated by SBF, a heterodimeric transcription factor composed of Swi4 and Swi6. The *CLB5* and *CLB6* genes are transcribed during G1-S as well, but it is mediated by the transcription factor MBF, which is composed of Mbp1 and Swi6. Hence, MBF is closely related to SBF and recent evidence suggests that there is a high degree of functional overlap

between these two transcription factors (Bean et al., 2005). Cln3 has an important role in activating the transcription of Cln1 and Cln2. The Cln3-Cdk1 complex phosphorylates the SBF inhibitor, Whi5, and promotes its export from the nucleus. Consequently, SBF can be activated and the *CLN1* and *CLN2* genes are transcribed. Cln1/2-Cdk1 complexes also phosphorylate Whi5; therefore promoting the activation of their own transcription factor SBF. This positive feed-back loop enhances the upregulation of Cln-Cdk1 complexes in G1 phase. The onset of *CLN1* and *CLN2* transcription determines the timing of START and entry into a new round of division (Tyer 1993). Clb5 and Clb6, and a large number of proteins involved in DNA replication, are also activated at START, but Clb5/6-Cdk1 peak of activity occurs later on, due to the action of the Cdk1 inhibitor, Sic1. Clb3 and Clb4 are also transcribed soon after START. Clb2 transcription is mediated by the transcription factor Mcm1, the forkhead transcription factor Fkh2, and the co-activator Ndd1, all three being directed to the *CLB2* gene promoter. Ndd1 requires Clb2-Cdk1 mediated phosphorylation to be recruited, which means that Clb2 enhances its own transcription as well (Pic-Taylor et al., 2004).

Specificity in cyclin regulation occurs not only at the transcriptional level, but also at the level of protein degradation. Cyclin degradation depends on previous ubiquitylation, which allows the 26S proteasome to target cyclins for degradation. Specificity arises from different cyclins being substrates of different ubiquitin ligases. Cln1, Cln2 and Clb6 are targets of the SCF complex (Skp/Cullin/F-box), while the other B-type cyclins are ubiquitylated by the Anaphase Promoting Complex or Cyclosome (APC/C). The SCF complex that targets Cln1 and Cln2 contains the F-box protein Grr1, while the SCF complex that targets Clb6 contains the

F-box protein Cdc4 (Barral et al., 1995; Skowyra et al., 1997). The APC/C binds with two mutually exclusive activating subunits, Cdc20 and Cdh1, and exhibits a differential degradation of cyclins, depending on which subunit is bound to it. Cdc20 binds first and is responsible for targeting all B-type cyclins with the exception of Clb6. However, degradation of Clb2 is only completed when Cdh1 binds to the APC (Shirayama et al., 1999; Wäsch and Cross, 2002). $APC^{Cdh1}$ remains active during G1, maintaining Clb-Cdk1 in a low activity state. The early Clb kinase complexes Clb3, Clb4 and Clb5, inactivate $APC^{Cdh1}$ in S-phase, which is necessary for the effective expression and accumulation of Clb2, essential for the onset of mitosis.

Despite the range of mechanisms conferring specificity to cyclin regulation, experimental results have shown that cells lacking only one cyclin are viable (Fitch et al., 1992). There is therefore redundancy in the system; although different cyclins peak at different times, cyclins can replace the functions of the missing one.

### 3.2. Control of CDK1 activity

Cyclin concentrations are not the only mechanism by which Cdk1 activity is regulated; Cdk1 is also activated or inactivated by phosphorylation. The presence of Cdk1 inhibiting proteins can also downregulate its activity.

For Cdk1 to be active, its T169 residue must be phosphorylated. In budding yeast, the enzyme responsible for this phosphorylation belongs to the Cdk1-activating kinases (CAKs) family, and is known as Cak1. Cak1 is maintained at a constant level throughout the cell cycle and phosphorylates Cdk1 before cyclin binding (Harper and Elledge, 1998).

Cdk1 can also be inhibited by phosphorylation. This inhibitory step mainly targets the Clb2-Cdk1 complex and occurs during S and G2 phases. The kinase responsible is Swe1, an orthologue of the Wee1 kinase in *Schizosaccharomyces pombe* (fisson yeast), which targets the Y19. Swe1-mediated phosphorylation specifically inhibits the Clb1-4 kinases, while Cln1-3, Clb5 and Clb6 are unaffected. In addition, Cdk1 reciprocally regulates Swe1. (McMillan et al., 2002; Sia et al., 1998). Swe1 is thought to prevent mitosis if there is a malformation in the bud. In this event, Swe1 cannot be degraded and instead will enhance Clb2-Cdk1 inhibition. The counteracting phosphatase is Mih1, an orthologue of the Cdc25 phosphatase in fission yeast (Russell et al., 1989).

Cyclin-dependent Kinase Inhibitors (CKIs) are proteins that can bind to Cyclin-Cdk1 complexes and inactivate their kinase activity. They are present in most, if not all, eukaryotic organisms. In budding yeast, the main CKI is Sic1 (Mendenhall, 1993). Sic1 expression is regulated by the transcription factor Swi5, and is limited to the M/G1 transition. Swi5 is downregulated by Clb-Cdk1 complexes and upregulated by Cdc14, a phosphatase known to be present at the completion of mitosis. Clb-Cdk1 phosphorylation retains Swi5 in the cytoplasm and Cdc14 dephosphorylation allows Swi5 import to the nucleous (Toyn et al., 1997). In this way, Sic1 transcription is activated at the end of mitosis. Sic1 function is related to the prevention of premature S phase. Until Cln-Cdk1 complexes have reached levels high enough to trigger bud initiation and spindle pole duplication, Sic1 levels remain high and inhibit Clb5/6-Cdk1 complexes (Schwob et al., 1994). Sic1 is a substrate of Cln-Cdk1 complexes, and therefore, at the end of G1, Sic1 is phosphorylated and targeted for degradation by the SCF complex

(Schwob et al., 1994; Verma et al., 1997). Sic1 is also important in late anaphase, when it downregulates Clb2-Cdk1 and thereby promotes an efficient exit from mitosis (Toyn et al., 1997).

Cdc6 also behaves like a CKI, although the Cdc6/Clb-Cdk1 complex is less stable than the Sic1/Clb-Cdk1 complex. While its main known function is related to the promotion of DNA replication, its overexpression may cause a G2 delay (Bueno and Russell, 1992). In this way, Cdc6 is responsible for the fine-tuning of Clb5 and Clb6 activity at the origins of replication (Piatti et al., 1995). The regulation of Cdc6 is similar to that of Sic1; it is destroyed at the G1/S transition by an ubiquitination system analogous to the one that targets Sic1 (Basco et al., 1995; Drury et al., 1997).

### 3.3. Substrate targeting by Cyclin-Cdk1 complexes

The different cyclin-Cdk1 complexes show function redundancy. Because of this, it was thought that cyclins were unlikely to confer essential target specificity to Cdk1 activity. Nevertheless, when the S phase cyclin Clb5 is substituted by the M phase cyclin Clb2, the initiation of DNA replication is less accurate (Cross et al., 1999). In addition, overexpression of Clb5 is unable to block cells in mitosis, as has been reported for Clb2 overexpression (Jacobson et al., 2000). This is clear evidence of cyclin target specificity.

Cyclins possess an active T-loop site that interacts with the (S/T)PXK consensus sequence found in the Cdk1 substrates, which is the phosphorylation site. In this way the cyclin-Cdk1 complexes recognise their substrates. S phase cyclins have a hydrophobic patch on their surface that recognises the R/KXL motif present on some substrates,

conferring substrate-specificity to these cyclins (Loog and Morgan, 2005). Subcellular localization is another mechanism that confers target specialization. Some cyclins present sequence information that regulates their localization inside the cell and thus directs the cyclin-Cdk1 complex to a specific location. A good example in budding yeast is the hydrophobic patch on S phase cyclins. This sequence directs Clb5 to the origins of DNA replication where it is required during S phase (Wilmes et al., 2004).

### 3.4. Protein degradation in cell cycle control

Protein degradation is the main factor responsible for irreversibility in the cell cycle. Cyclins, Cdk1 inhibitor proteins, and other cell cycle regulators are degraded in timely fashion, so that the cell cycle engine moves from one state to the next in an orderly and conserved fashion. The degradation of proteins is generally regulated through poliubiquitination, the attachment of an ubiquitin tail to the target protein, making it recognizable to the proteasome. Ubiquitination involves the coordination of three enzymes, generically called E1, E2 and E3, and is carried out in three steps (Hershko and Ciechanover, 1998):

-Ubiquitin activation – ubiquitin is covalently attached to E1;

-Ubiquitin conjugation – ubiquitin is transferred to E2, the ubiquitin-conjugating enzyme;

-Ubiquitin-protein ligation – E3 catalyses the transfer of ubiquitin from E2 to the target protein.

At the G1/S transition, G1 cyclins and CKI are recognised by the ubiquitin protein ligase SCF (Orlicky et al., 2003). They are ubiquitinated

and degraded before cells enter S phase. During mitosis, B-type cyclins are degraded by the ubiquitin ligase APC and its activating subunits Cdc20 and Cdh1 (Visintin et al., 1997). These transitions separate two alternative states in the cell cycle. The first state corresponds to G1 where cells are small and the chromosomes unreplicated. In the cell cycle engine, it corresponds to low levels of B-type cyclins and high levels of G1 cyclins and Cdk1 inhibitors. Before cells initiate the replication-division process, G1 cyclins must target Sic1 for degradation by the SCF complex (Orlicky et al., 2003), allowing B-type cyclins to be upregulated.

The second transition occurs at the metaphase-to-anaphase transition, and is marked by sister-chromatid segregation. At this point, the APC is activated and promotes B-type cyclin degradation, a crucial requirement for the completion of mitosis. This transition is known as EXIT (Tyson and Novak, 2008). Both START and EXIT are irreversible transitions and proteolysis plays a key role in this irreversibility. Nevertheless, recent evidence suggests that proteolysis is not the sole requirement; feedback loops (e.g. double negative feedback loop between Sic1 and Cdh1) in the network further ensure that the cell cycle works as a clock whereby events are irreversible (López-Avilés et al., 2009).

### 3.5. Cell cycle restriction points and checkpoints

The cell cycle control system drives progression through the cell cycle at regulatory transitions called restriction points (Fig I.3). When the cell reaches a restriction point, the cell cycle control system will stop cell cycle progression unless all of the conditions to proceed are fulfilled. Thus, restriction points delay cell-cycle progression to give time to solve

any detected problem. Cell-cycle restriction points and other checkpoints can also drive cell death if the problem cannot be solved (Elledge, 1996; Hartwell and Weinert, 1989).

The first restriction point is called G1/S checkpoint or START in budding yeast (restriction point (R) in animal cells). START regulates the entry into a new cell cycle and occurs in mid to late G1. Progression past this point is prevented if cell growth is insufficient, DNA is damaged or other preparations for cell-cycle entry are not complete. Cells prevented from passing START typically exit the cell cycle into a prolonged non-dividing state (G0 or quiescence). The activation of this restriction point depends on the prevention of Cln-Cdk1 activation, and consequently, on the stabilization of Sic1. Sic1 is a strong Clb-Cdk1 antagonist, and thus the cell cannot enter S phase (Johnston et al., 1977). Thus, at START cells decide between the different development options which in *S. cerevisiae* are: 1) Start a new round of cell division, which is irreversible until the next G1 phase. This occurs whenever the cell has reached a minimum size and environmental factors and nutrients are appropriate. 2) When cells are haploid, they can respond to the presence of pheromone and activate the process of conjugation. 3) Under certain conditions, cells may also begin pseudohifal growth. 4) If cell conditions, nutritional or environmental are not appropriate, cells enter G0 or sporulation (if diploid) depending on nutrient limitation. G0 cells can reenter G1 phase in the presence of specific stimuli.

The second restriction point regulates the G2 to M transition and corresponds to a morphogenetic checkpoint in budding yeast. If bud formation fails or cells lose their polarity, then progression into mitosis is prevented and cells arrest in G2. When morphogenic anomalies are

detected and the checkpoint is activated, Swe1 levels are kept high and Cdk1 is inhibited by tyrosine phosphorylation. The absence of Cdk1 activity prevents cells from entering mitosis (Lew and Reed, 1995; Sia et al., 1998).

The third restriction point occurs in mitosis, during the metaphase-to-anaphase transition and it involves the spindle assembly checkpoint (SAC). The SAC is activated if the mitotic spindle is corrupted or if there is a problem with the bipolar attachment of the sister chromatids at the metaphase plate. Indeed, a single unattached sister chromatid is enough to trigger the SAC activation, and the cell remains arrested in metaphase with sister chromatids bound together. The target of the SAC is the APC subunit Cdc20, which is indirectly responsible for sister chromatid segregation. While SAC is active, Cdc20 is bound to the kinetochores and thus, prevented from binding to and activating APC (De Antoni et al., 2005; Hwang et al., 1998). Once the SAC is satisfied, Cdc20 is released and free to activate APC and initiate the metaphase-to-anaphase transition.

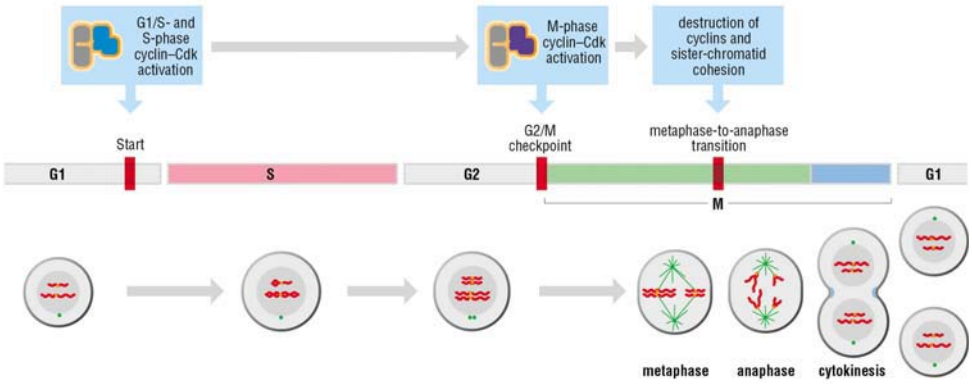



**Figure I.3. Cell-cycle restriction points (Morgan 2007)**

Apart of these three restriction points, other additional control mechanisms can stop cell cycle. In *S. cerevisiae*, the spindle positioning checkpoint (SPOC) can stop anaphase when the spindle is not correctly aligned at the mother-bud axis. The activation of the SPOC triggers the activation of the kinase Kin4, which inhibits the mitotic exit network (MEN) until the spindle is properly aligned (Hu et al., 2001; Pereira and Schiebel, 2005). On the other hand, the NoCut pathway delays the completion of cytokinesis in response to anaphase defects. Retention of chromatin in the plane of cleavage allows interaction of acetylated chromatin with the passenger complex Aurora/Ipl1 kinase at the spindle midzone, triggering NoCut activation (Mendoza et al., 2009).

Finally, there are several DNA checkpoints acting during all cell cycle. DNA replication checkpoint acts during S phase to monitor any defect during DNA replication. Moreover, DNA damage response can stop cell cycle in G1- and S-phase or at the G2/M transition and give time to repair the DNA damage.

# 4. Mitosis

Mitosis is a complex and precise ordered series of events that results in chromosome segregation. As mentioned before, the mitosis is divided into four major phases named prophase, metaphase, anaphase and telophase followed by the cytokinesis, the process that allows cell separation.

## 4.1. General overview of mitosis regulation: mitotic entry and exit.

The key molecular event that initiates mitosis is the activation of Cdk1 by mitotic cyclins. Clb2 cyclin is the main mitotic cyclin in budding yeast and starts to be synthesized in S/G2 transition. A key early mitotic event is a positive feed-back loop in which the first low levels of Clb2-Cdk1 activity are able to stimulate Clb2 transcription, leading to a rapid rise in Clb2 (Amon et al., 1993; Harvey et al., 2011). A second key event is a switch in the pattern of bud growth by mitotic cyclins, changing from polar to isotropic growth. The mitotic cyclins induce this switch by repressing transcription of the G1 cyclins Cln1 and Cln2, which drive polar bud growth (Lew and Reed, 1993; Amon et al., 1994; McCusker et al., 2007).

A gradual increase in Clb2-Cdk1 activity helps ensure mitotic events occur in the proper order. For instance, low threshold of Cdk1 activity is required for initiation of mitotic spindle assembly, and a higher threshold initiates spindle elongation (Rahal and Amon, 2008, Deibler and Kirschner, 2010). G2/M cell-cycle checkpoint plays a crucial role in allowing the initial increase in mitotic Cdk1 activity and entry into mitosis. Wee1 kinase, Swe1 in budding yeast, phosphorylates and inhibits Cdk1 activity delaying entry into mitosis (Gould and Nurse, 1989). On the other

hand, Cdc25 (Mih1, in budding yeast) promotes entry into mitosis by removing the inhibitory phosphorylation (Gautier et al., 1991; Kumagai and Dunphy, 1991). Swe1 is initially phosphorylated by Clb2-Cdk1, which stimulates Swe1 to bind, phosphorylate, and inhibit Cdk1. After the initial phosphorylation of Swe1 in G2/M by Cdk1, subsequent phosphorylation events carried out by Cdc5 polo kinase lead to full hyperphosphorylation of Swe1, which inactivates Swe1 and promotes mitotic entry (Harvey et al., 2005 and 2011). The dramatic cell cycle–dependent changes in phosphorylation of Mih1 and Swe1 likely reflect the action of upstream checkpoint signals that control their activity. Cellular events send checkpoint signals to Mih1 and Swe1, like membrane trafficking and growth, therefore cells can monitor crucial processes that must be coordinated with cell cycle progression (Anastasia SD et al., 2012).

Mitotic entry involves all early mitotic events that take place until chromosmes are prepared for segregation. Chromosomes undergo condensation during prophase and centrosomes duplicate. Budding yeast centrosomes, the spindle pole bodies (SPBs) duplicate at the end of S phase instead. In most multicellular organisms, nuclear envelope breaks down and the growing mitotic spindle binds to sister chromatids during prometaphase. This is called an open mitosis. Yeast do not dismantle their nuclear envelope and the mitotic spindle forms inside the nucleus, what is known as a closed mitosis. During prometaphase, kinetochores assemble in the centromeres. Clb2-Cdk1 activity rises during prophase and prometaphase and reaches a peak of kinase activity in metaphase. During metaphase, chromosomes are aligned at the metaphase plate due to the counterbalance of the pulling forces

generated by the opposing kinetochores. Satisfaction of the SAC allows entry into anaphase.

The completion of mitosis begins with sister-chromatid segregation, when cohesion is abruptly disrupted and sister chromatids are pulled to opposite poles of the spindle. This is known as anaphase A or early anaphase. In anaphase B, or late anaphase, spindle poles themselves move further apart from each other, completing sister chromatids segregation. Mitosis ends with telophase, when chromosomes and other nuclear components are repackaged into identical daughter nuclei and spindle is disassembled. The events leading from sister chromatids separation until the cell division or cytokinesis are known as mitotic exit. During mitotic exit, Clb2-Cdk1 activity is gradually eliminated. Cytokinesis is strongly inhibited by Clb2-Cdk1 activity, thereby cells need to switch off Cdk1 activity to finish cell division and enter into a new G1 phase.

The metaphase to anaphase transition is triggered by the $APC^{Cdc20}$ activation. Cdk1 phosphorylates the APC complex increasing its affinity for its coactivator Cdc20 (Fig. I.4). The SAC keeps APC inactive until sister chromatid are correctly orientated and attached in a bipolar manner (reviewed in Khodjakov and Pines, 2010; Musacchio and Salmon 2007). When this occurs, the SAC signal is silenced and Cdc20 is able to bind and activate the APC complex. $APC^{Cdc20}$ promotes the degradation of several proteins by ubiquitination and targeting to the 26S proteasome. The most important $APC^{Cdc20}$ targets are the protein securin, which inhibits the protease separase, and the B-type cyclins, whose degaration leads to certain Cdk1 inactivation. After securin degradation, separase is active and cleaves Scc1 subunit of the cohesin

complex leading to sister-chromatids segregation (Uhlmann et al., 2000). But separase also performs other non-proteolitic functions, related to spindle stabilization and Cdk1 inactivation. Separase allows the activation of a Cdk1-counteracting phosphatase, Cdc14, which dephosphorylates several Cdk1 substrates. Cdc14 is a key regulator of the anaphase events, and most of its early-anaphase substrates are related to spindle elongation and stabilization, and to the segregation of repetitive DNA. In late anaphase, two important Cdc14 substrates are Cdh1, the second APC coactivator that replaces Cdc20, and the Cdk1 inhibitor, Sic1. $APC^{Cdh1}$ completes destruction of mitotic cyclins and degrades other proteins: Cdc20, Cdc5 polo kinase and factors related to the mitotic spindle like Ase1 (Visintin et al., 2008). $APC^{Cdh1}$ activation together with Sic1 accumulation reset Cdk1 activity level, leading cells to a new G1 phase. Thus, Cdc14 activation is a key step to completely inactivate Cdk1 in anaphase and exit mitosis.

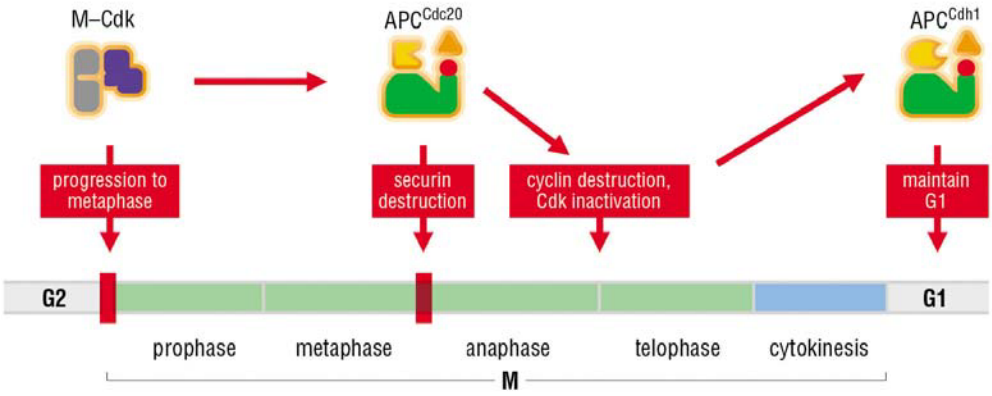



**Figure I.4. APC and the mitotic exit (Morgan 2007)**

## 4.2. Mitotic Exit I: Cdc14 phosphatase

*CDC14* gene was originally identified in LH Hartwell famous screen for genes that regulate the cell cycle in budding yeast (Culotti and Hartwell, 1971). Subsequent work showed that Cdc14 belongs to a family of highly conserved dual-specificity phosphatases (DUSPs) present in a wide range of organisms from yeast to human (reviewed in Mocciaro and Schiebel, 2010). DUSPs are a heterogenous group of phosphatases that belong to the single-subunit protein-tyrosine phosphatase (PTP) superfamily and its unique feature is the ability to dephosphorylate both phosphotyrosine and phosphoserine/phosphothreonine residues within their substrates (Denu and Dixon, 1998).

Cdc14 phosphatase family is highly conserved, and Cdc14 orthologs have been identified and characterized in several organisms. Cdc14 functions in the mitotic exit are well-characterized in yeast but this specific role in metazoans is still controversial. Other functions such as transcription repression have been shown to be conserved (Clemente-Blanco et al., 2011). Cdc14 is essential for mitotic exit in budding yeast, and *cdc14ts* mutants arrest in a telophase-like mitotic state, with an elongated mitotic spindle and a bilobed nulceous (Culotti and Hartwell, 1971). Cells where mitotic Cdk activity cannot be decreased during mitotic exit, by expression of non-degradable mitotic cyclins, show a phenotype similar to *cdc14ts* mutants (Surana et al., 1993), pointing to its essential role in Cdk1 inactivation. The first well-characterized Cdc14 activity was the dephosphorylation of Cdk1 substrates, and hence the activation, of the Cdk1 inhibitor Sic1, the transcription factor Swi5, and the second APC coactivator Cdh1 (Visintin et al., 1998).

During most of the cell cycle, Cdc14 is sequestered in the nucleolus by binding to its inhibitor Net1, also called Cfi. This nucleolar protein anchors Cdc14, and together with Sir2, they form the RENT (*RE*gulator of *N*ucleolar silencing and *T*elophase) complex, which inhibits transcription by RNA polymerase II at the rDNA intergenic spacers (IGS) (Shou et al.,. 1999; Visintin et al., 1999). It has been traditionally thought that Cdc14 is inactive as a phosphatase when part of the RENT complex. However, recent studies demonstrated that Cdc14 has an active role in repression of RNA polymerase II transcription in rDNA and telomeric regions (Clemente-Blanco et al., 2011). Inactivation of Cdc14 causes silencing defects at IGS of rDNA during interphase and at Y' repeats in sub-telomeric regions during mitosis. Cdc14 role in transcription silencing is independent from the RENT deacetylase subunit Sir2. Instead, Cdc14 acts directly on RNA Polymerase II by targeting the C-terminal domain phosphorylation at S2 and S5, which are required for transcription initiation and elongation. This Cdc14 function has been shown to be conserved in humans.

During anaphase, Cdc14 is dissociated from the RENT complex, which is an essential step for Cdc14 functions in mitotic exit. At the anaphase onset, Cdk1-dependent phosphorylation of Net1, with the contribution of Cdc5 kinase, allows Cdc14 release from the nucleolus since Net1 phosphorylated form has lower affinity for Cdc14 (Yoshida and Toh-e, 2002; Azzam et al., 2004). Cdc14 is initially released in the nucleus and soon after, re-distributed throughout the cytoplasm. There are two complementary and consecutive pathways essential for the Cdc14 release in budding yeast. These are the FEAR (Cdc *F*ourteen *E*arly *A*naphase *R*elease) acting in early anaphase, when Cdk1 activity is high

(reviewed in Queralt and Uhlmann, 2008a; Rock and Amon, 2009), and the GTPase-driven kinase cascade called MEN (*M*itotic *E*xit *N*etwork), which is activated in late anaphase, when Cdk1 activity decreases (Jaspersen et al.,. 1998; Stegmeier et al., 2002). The initial activation of Cdc14 by the FEAR pathway is particularly important for dephosphorylation of Cdk1 substrates involved in the anaphase spindle stabilization and elongation, and in the segregation of repetitive DNA regions such as rDNA and telomeres (Sullivan et al., 2004 Higuchi and Uhlmann, 2005; Khmelinskii et al., 2007). Also, the anaphase-specific gene silencing by Cdc14 is thought to be a prerequisite for allowing condensin access to these regions and for their correct segregation (Clemente-Blanco et al., 2011).

The FEAR-released Cdc14 cannot fully activate the $APC^{Cdh1}$ and therefore, it cannot lead to complete inactivation of Cdk1 and exit from mitosis. A second pathway is required to fully release Cdc14 in late anaphase, when Cdk1 activity declines. Thus, cells activate the MEN and its most downstream kinase, Mob1-Dbf2, promotes the cytoplasmic release of Cdc14. Full released and active Cdc14 leads to Cdh1 activation and Sic1 accumulation, and cells achieve the completion of mitosis. FEAR-Cdc14 release also stimulates MEN activation through dephosphorylation of Cdc15 MEN kinase, as a positive feed-forward loop that enhances full release of Cdc14. Recent studies show that Cdc14 also localizes at the bud neck after Cdk1 inactivation, where it dephosphorylates Inn1 allowing Inn1-Cyk3 complex formation, suggesting an additional Cdc14 role in promoting the cytokinesis (Palani et al., 2012). Thus, different localization of Cdc14 phosphatase allows targeting of different substrates during anaphase. Early-anaphase

substrates of Cdc14 like Fin1, Sli15 and Ask1, localize in the nucleus and are activated by FEAR-Cdc14 release; whereas mid-late anaphase substrates of Cdc14 such as Cdh1 and Swi5 are in the cytoplasm, and are activated by MEN-Cdc14 release. In the same way, cytokinesis related substrates are just activated by MEN-Cdc14 release although it would be interesting to explore the mechanism that targets Cdc14 specifically to the site of cell division. In addition, as Cdc14 counteracts Cdk1 phosphorylation, Cdk1 activity towards its substrates during anaphase will determine the net balance of each substrate phosphorylation status. It has been proposed each Cdk1/Cdc14 substrates responds by dephosphorylation to different thresholds of Cdk1 activity (Bouchoux and Uhlmann 2011).

It has been described a non-essential ortholog of Cdc14 In *Saccharomyces pombe*, known as Clp1 or Flp1. Clp1 is not important for mitotic exit, but rather mainly regulates mitotic entry and, in addition, coordinates cytokinesis with the initiation of the next cell cycle. Like its budding yeast ortholog, Clp1 localizes to the nucleolus during G1- and S-phase. In contrast, Clp1 is released at the G2/M transition without the contribution of fission yeast homologs of the FEAR pathway (Chen et al., 2006). Clp1 initially localizes to the mitotic spindle and to kinetochores, which has been linked to accurate chromosome segregation (Trautmann et al., 2004). Later in mitosis, Clp1 localizes to the site of cytokinesis, the medial actomyosin ring at the equator of the cell, where regulates the formation of the septum. Nevertheless, it is not essential for either septation or cytokinesis (Clifford et al., 2008; Cueille et al., 2001; Simanis, 2003; Trautmann et al., 2001).

Cdc14 is also highly conserved in metazoa. However, little is known about its functions. In *Caenorhabditis elegans*, Cdc14 was observed in the cytoplasm in interphase cells, and on centrosomes, spindle microtubules and at the midbody in mitotic cells. In post-mitotic cells, Cdc14 localized to the nucleus and the nucleolus (Saito et al., 2004). Nevertheless, it is still controversial an essential role in mitotic exit or cytokinesis due to different results obtained when depleting Cdc14 by iRNA. On the other hand, *Xenopus laevis* genome encodes two Cdc14 isoforms, *CDC14A* and *CDC14B*, but just Cdc14A has been related to any physiological function until now. Cdc14A can dephosphorylate Cdc25, pointing to a role in the mitotic entry, and it has been shown to inhibit the recruitment of important factors for abscission, at the end of cytokinesis (Krasinska et al., 2007). Recently, the avian orthologs of Cdc14, Cdc14A and Cdc14B, have been identified in chicken DT40 cells. None of the avian Cdc14 proteins was found to be essential for cell viability, and the lack of one isoform alone does not cause obvious defects in cell-cycle progression, mitotic entry, chromosome segregation, mitotic exit or cytokinesis (Mocciaro et al., 2010). In humans, three Cdc14 isoforms have been identified: Cdc14A, Cdc14B and Cdc14C. Both Cdc14A and Cdc14B isoforms can rescue Clp1-deficient fission yeast strains and, furthermore, Cdc14B is able to fulfil all essential functions of Cdc14 in *S. cerevisiae* (Vázquez-Novelle et al., 2005). Human Cdc14 isoforms have been shown to localize at cell-cycle related structures such as centrosomes and the spindle midzone. However, there are non-conclusive results from Cdc14 silencing or depletion studies and few putative targets have been validated. Therefore, Cdc14 functions in higher eukaryotes remain to be established.

### 4.3. Mitotic Exit II: Separase and the FEAR pathway

In *S. cerevisiae*, the FEAR pathway induces Cdc14 release from the nucleolus in early anaphase, when Cdk1 activity is high. The existence of the FEAR network became apparent after the identification of the MEN, through the observation that Cdc14 was still released from the nucleolus in cells lacking MEN activity. This release occurs transiently during early anaphase (Sullivan and Uhlmann 2003) and all factors required for this transient release of Cdc14 were collectively referred to as the FEAR network. The FEAR includes several proteins, some functioning in a positive manner and others having an inhibitory role. However, our knowledge of the relationship among FEAR components is still very limited.

The main component of the FEAR is the protease called separase (Esp1 in budding yeast), which is responsible for sister chromatid segregation (Fig. I.5). Separase is an essential cistein-protease that cleaves Scc1, a subunit of the cohesin complex, triggering loss of cohesion and segregation. In addition, separase possesses a non-proteolytic function that allows Cdc14 release from the nucleolus (Sullivan and Uhlmann 2003). Separase is kept inactive until the anaphase onset by binding to its inhibitor securin (Pds1 in budding yeast). Securin is a target of the $APC^{Cdc20}$ and therefore its degradation in anaphase depends on the SAC inactivation. Securin inhibits both separase's protease function in promoting sister chromatid separation and its non-proteolytic (FEAR network-related) function (Sullivan and Uhlmann 2003). For this reason, the SAC is able to inhibit both chromosome segregation and Cdc14 early-anaphase release (Stegmeier et al.,. 2002; Yoshida et al.,. 2002).

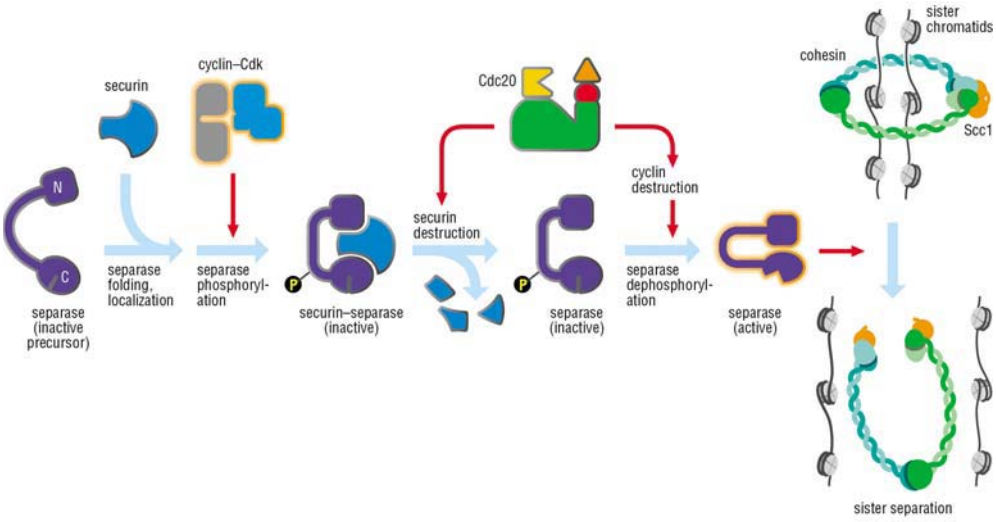



**Figure I.5. Separase activation and its proteolitic activity (Morgan 2007)**

*esp1* mutants arrest in metaphase and the ectopic release of Cdc14 rescues this phenotype. Although chromosome segregation fails, cells are able to exit from mitosis indicating an essential role of separase in Cdc14 release and mitotic exit (Queralt et al., 2006). Separase cooperates with Zds1 and Zds2 proteins to allow the Cdk1-dependent phosphorylation of Net1, which provides the first wave of Cdc14 release. Clb1/2-Cdk1 complex phosphorylates Net1 in at least 6 sites promoting its dissociation from Cdc14 (Azzam et al., 2004). Net1 phosphorylation is counteracted by $PP2A^{Cdc55}$ phosphatase, which keeps Net1 dephosphorylated until anaphase. Separase and Zds proteins downregulate $PP2A^{Cdc55}$ phosphatase at the anaphase onset, thereby promoting the accumulation of phosphorylated forms of Net1 and releasing Cdc14. Recently, the C-terminal region of Zds proteins, the Zds1_C motif, have been shown to be essential for $PP2A^{Cdc55}$ downregulation and Cdc14 release (Calabria et al., 2012; Rossio et al.,

2011). However, the molecular mechanism that promotes $PP2A^{Cdc55}$ downregulation by Zds proteins and separase remains unknown. Thus, the FEAR release of Cdc14 takes place at anaphase onset, upon separase activation and $PP2A^{Cdc55}$ downregulation, and this release is restricted to the nucleous.

Another negative regulator of FEAR-Cdc14 release is the nucleolar protein Fob1. Fob1 binds to Net1 and prevents Cdc14 release, although the molecular mechanism of its regulation remains unclear. Other factors of the FEAR pathway are the kinetochore/spindle protein Slk19, and Spo12 and its close homolog Bns1. Slk19 is cleaved by separase in an anaphase-specific manner, but its cleavage is not required for Cdc14 release. Instead, the ability of Slk19 to target separase to the spindle midzone and vice versa may be important for FEAR functions (Sullivan et al.,. 2001; Sullivan and Uhlmann 2003). On the other hand, Spo12 and Bns1 are both small proteins, which its enzymatic activity is not known. Spo12 is a nucleolar phosphoprotein that binds to the inhibitor of the FEAR network Fob1. Because Fob1 also binds to Net1 and prevents Cdc14 release, it has been proposed that Spo12 can induce an anaphase-specific conformational switch in Fob1 that would reduce its ability to inhibit Cdc14 release (Stegmeier et al.,. 2004).

Finally, the polo kinase Cdc5 has been related to the FEAR network function, as several studies suggest that Cdc5 promotes the phosphorylation of Net1 and Cdc14 (Shou et al.,. 2002; Yoshida and Toh-e 2002; Visintin et al.,. 2003). However, cells depleted for the mitotic cyclins Clb1 and Clb2 enter anaphase (although with a great delay) but fail to release Cdc14 from the nucleolus and arrest prior to exit from mitosis. As Cdc5 is activated by Clb2-Cdk1, and it has been shown

to act occasionally as a Cdk1 primase kinase, Cdc5 direct contribution to Cdc14 release remains unclear.

Although the specific function of several FEAR components is still poorly understood, FEAR-Cdc14 functions have been extensively studied. FEAR-Cdc14 release stimulates MEN activation through dephosphorylation of Cdc15 kinase, as a positive feed-forward loop to increase Cdc14 activation. Cdc15 is transiently dephosphorylated during anaphase (Visintin and Amon 2001). FEAR mutants do not show Cdc15 dephosphorylation during early anaphase (Stegmeier et al.,. 2002) and show a delay progression through the cell cycle, due to a delay in Cdc14 release. In addition, FEAR pathway controls anaphase spindle stability, having an important role Esp1, Cdc14 and Slk19, and the Cdc14-target Fin1 and Ask1. Although the molecular mechanism is not clear, FEAR components target the evolutionarily conserved Ipl1-Sli15-Bir1 complex to the spindle midzone, where it then promotes the activity of spindle-stabilizing factors (Pereira and Schiebel 2003). Cdc14 promotes the localization of microtubule-stabilizing proteins, such as Fin1, to the anaphase spindle, and dephosphorylation of the kinetochore component Ask1 contributes to both the silencing of microtubule turnover and successful anaphase A (Higuchi 2005). The FEAR network not only regulates spindle stability, but also forces exerted by cytoplasmic microtubules (cMTs) during anaphase. These FEAR network-dependent cMT-directed forces play an important role in positioning the dividing nucleus so that the mother and daughter cells will each receive their proper half of the duplicated genome. The contribution of Cdc14 and other FEAR components to nuclear positioning is still poorly understood. Finally, as mentioned before, FEAR-Cdc14 is required for correct

segregation of repetitive DNA, playing a role in chromosome condensation and organization.

### 4.4. Mitotic Exit III: The mitotic exit network (MEN)

In budding yeast, mitotic exit and cytokinesis require full release and activation of Cdc14 by the mitotic exit network (MEN). The MEN was initially outlined by identifying genes required for mitotic exit and by establishing several genetic interactions between these genes (Jaspersen et al., 1998). This pathway is activated in mid-late anaphase, when Clb2-Cdk1 activity has declined and is not enough to keep Cdc14 released. The Cdc14 release driven by the FEAR and the MEN pathways differ in two important aspects: (1) MEN-Cdc14 release occurs throughout the cell, but FEAR-Cdc14 release is restricted to the nucleus, and (2) FEAR-Cdc14 cannot promote Cdk1 complete inactivation and exit from mitosis, while MEN-Cdc14 release does. However, these differences should not be misunderstood, as timely Cdc14 release and exit from mitosis is only achieved under the activation of both pathways. Moreover, progression through anaphase in the absence of FEAR pathway activity is associated with a significant loss in viability (D'Amours et al.,. 2004).

The MEN is a GTPase-driven signaling cascade that is associated with the Spindle Pole Body (SPB), the yeast centrosome. MEN components changes in localization during anaphase suggest a tight temporal and spatial regulation of the pathway (Shirayama et al., 1994; Luca and Winey, 1998; Cenamor et al., 1999; Gruneberg et al., 2000; Xu et al., 2000; Menssen et al., 2001; Pereira and Schiebel, 2001; Stegmeier and Amon, 2004). The main switch of this cascade is the Ras-like GTPase

protein, Tem1. Tem1 is controlled by two factors: the putative guanine nucleotide exchange factor Lte1, which activates it; and the GTPase-activating (GAP) complex Bfa1-Bub2, which keeps Tem1 inactive (Shirayama et al., 1994; Bardin et al., 2000; Pereira et al., 2000). Once active, Tem1 interacts with the Pak-like kinase Cdc15 (Asakawa et al., 2001), which in turn activates the Mob1-Dbf2 kinase via phosphorylation of the kinase subunit Dbf2 (Mah et al., 2001). The activated Mob1-Dbf2 complex translocates to the nucleus where it comes into contact with Cdc14 phosphatase (Stoepel et al., 2005). It is thought that Mob1-Dbf2 phosphorylates Net1 (or an unknown protein) to dislodge Cdc14 from Net1. Net1 was identified in an *in vitro* proteomic screen for Mob1-Dbf2 substrates (Mah et al., 2005) but still remains to be demonstrated whether Mob1-Dbf2 phosphorylates Net1. Mob1-Dbf2 also phosphorylates Cdc14 on sites flanking its NLS sequence, thereby inhibiting the NLS. Phosphorylated Cdc14 cannot efficiently return to the nucleus and thus linger in the cytoplasm where it can dephosphorylate substrates such as Cdh1 and Swi5. Although phosphorylated Cdh1 and Swi5 are retained in the cytoplasm (Jans et al., 1995; Knapp et al., 1996; Jaquenoud et al., 2002), the dephosphorylated forms can now gain access to the nucleus, where they activate APC and Sic1 expression, respectively (Visintin et al., 1998; Jaspersen et al., 1999). $APC^{Cdh1}$ activation and Sic1 accumulation extinguish mitotic Cdk1 activity, thereby enabling a return to G1 phase.

During an unperturbed cell cycle, Bfa1-Bub2 complex keeps the MEN inactive until anaphase, when Cdc5-dependent phosphorylation of Bfa1 inactivates the Bfa1-Bub2 complex. Bfa1-Bub2 localizes asymmetrically during anaphase to the daughter SPB, dSPB (Bardin et al., 2000;

Pereira et al., 2000) and this asymmetry is required for recruiting MEN components to the SPBs (Monje-Casas and Amon, 2009). Bfa1 phosphorylation during anaphase induces its asymmetric localization onto the dSPB (Hu et al., 2001; Pereira et al., 2001; Kim et al., 2012). In contrast, when the spindle is misaligned and the spindle position checkpoint (SPOC) is activated, Bfa1 is phosphorylated by the kinase Kin4 and localizes symmetrically to the SPBs (Pereira and Schiebel, 2005; Caydasi and Pereira, 2009). Kin4 phosphorylates Bfa1 in sites that unable Cdc5 phosphorylation. Thereby, Kin4 effectively locks Bfa1-Bub2 in an active state, keeping the MEN inactive until abrogation of the SPOC signal (Hu et al., 2001; Geymonat et al., 2003; Fraschini et al., 2006; Maekawa et al., 2007). Kin4 is predominantly present in the mother cell, and thus its positive effect on Bfa1-Bub2 is strongly reduced when Bfa1-Bub2 migrates to the bud during anaphase (D'Aquino et al., 2005; Pereira and Schiebel, 2005). On the other hand, the guanine nucleotide exchange factor (GEF) that positively regulates Tem1 is thought to be Lte1, a protein that resides in the bud cortex (Bardin et al., 2000; Jensen et al., 2002). Lte1 also participates in controlling Bfa1 asymmetric localization and cell polarization (Geymonat et al., 2009). Hence, after correct orientation of the mitotic spindle and entry into anaphase, Cdc5 phosphorylates Bfa1 inhibiting Bfa1-Bub2 complex. This, together with Lte1 stimulation, allows Tem1 activation and initiation of the MEN (Fig. I.6).

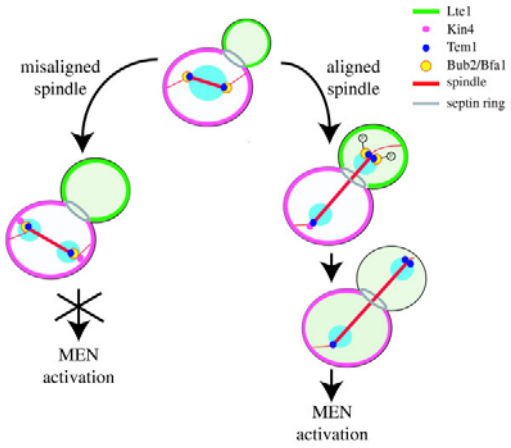



**Figure I.6. Spatial regulation of the MEN (Piatti et al., 2006)**

Both Bfa1-Bub2 complex and Tem1 are bound to the scaffold protein Nud1, and localizes asymmetrically to the dSPB during anaphase. Tem1 association to the SPBs is essential for mitotic exit (Bardin et al., 2000; Geymonat et al., 2003; Pereira et al., 2000; Molk et al., 2004; Valerio-Santiago and Monje-Casas, 2011). Tem1 recruits Cdc15, as increased residence time of Tem1 on the SPBs leads to premature Cdc15 loading (Valerio-Santiago and Monje-Casas, 2011). Cdc15 is also bound to the scaffold protein Nud1 (Gruneberg et al., 2000). Cdc15 kinase recruits Mob1-Dbf2 to the SPB by phosphorylation of Nud1 (Rock et al., 2013). Consistently, *nud1* mutants are defective for MEN signalling (Gruneberg et al., 2000). Cdc15 is further regulated through phosphorylation. Cdc15 contains seven Cdk1-consensus sites and is highly phosphorylated during G1 and S phases. At anaphase onset, Cdc15 is dephosphorylated by the FEAR-dependent release of Cdc14 (Cenamor et al., 1999; Gruneberg et al., 2000; Jaspersen and Morgan, 2000; Xu et al., 2000; Menssen et al., 2001). Cellular viability is restored to *cdc5-1*,

*dbf2-2* and *tem1-2* MEN mutant cells when Cdc15-7A is expressed, a Cdc15 version immune to Cdk1 phosphorylation. However, the Cdc15 kinase activity remains the same whether Cdc15 is phosphorylated or not (Jaspersen and Morgan, 2000, Stegmeier et al., 2002). Therefore, Cdk1-dependent phosphorylation of Cdc15 may regulate its localization to the SPB rather than directly its activity. In fact, Cdc15 and Cdk1 show a mutual regulation. Cdc15 kinase recruits Cdk1 to the mSPB in early-anaphase, and conversely, Cdk1 negatively regulates binding of Cdc15 to the mSPB (König et al., 2010).

Cdk1 also restrains MEN activity through Mob1 phosphorylation, which inhibits the complex Mob1-Dbf2. Mob1 is strongly phosphorylated by Cdk1 and is hyperphosphorylated when cells reach metapahse. S36 and T85 are the two major Cdk1 phosphorylation sites of Mob1 protein *in vivo*, being inhibitory for Mob1-Dbf2 activity. In contrast, abrupt Mob1 dephosphorylation takes place during mitotic exit, coincident with Cdc14 release and Cdk1 inactivation. In addition, Cdc14 overexpression in metaphase-arrested cells promotes Mob1 dephosphorylation, suggesting Cdc14 could dephosphorylate Mob1 during mitotic exit. Therefore, high Cdk1 activity inhibits Cdc15 and Mob1-Dbf2, preventing MEN activation and the mitotic exit, and Cdc14 would counteract this inhibition (König et al., 2010).

In summary, the MEN activation scheme closely follows changes in phosphorylation and loading of the MEN components onto the SPBs. Bfa1-Bub2 needs to be phosphorylated by Cdc5 and asymmetrically located onto the dSPB to recruit Tem1. SPB binding of Cdc15 requires Tem1, and Mob1-Dbf2 only associates with the SPBs when Tem1 and Cdc15 are functional. On the other hand, Cdk1/Cdc14 ratio activity

controls the downstream MEN kinases Cdc15 and Mob1-Dbf2. Hence, later components of the MEN become active when Cdk1 activity has declined, and are stimulated by FEAR-Cdc14 release.

MEN activation and cytoplasmic Cdc14 have been recently linked to cytokinesis initiation and polarized growth inhibition during late mitosis (Sanchez-Diaz et al., 2012). Cdc14 dephosphorylation of Cdk1 targets appears to be the central mechanism. For instance, Cdc14 counteracts Cdk1 phosphorylation of Inn1 to facilitate Inn1-Cyk3 complex formation and promote cytokinesis as mentioned before (Palani et al., 2012). Inn1 targeting to the contractile actomyosin ring is crucial for ingression of the plasma membrane during cytokinesis (Sanchez-Diaz et al., 2008). Other MEN components have been linked to cytokinesis. Recruitment of Dbf2 to the bud neck is dependent upon actomyosin ring assembly and correlates with Hof1 phosphorylation (Corbett et al., 2006). It has been recently shown that Mob1-Dbf2 phosphorylates Hof1 to relocate Hof1 from septins to the actomyosin ring (Meitinger et al., 2011). The MEN homologue in fission yeast, the Septation Initiation Network (SIN), is extensively known for controlling cytokinesis instead of mitotic exit and Cdk1 inactivation. Interestingly, the most downstream SIN component, the kinase Sid2, maintains Clp1 in the cytoplasm in late mitosis. Mutation of the Sid2 phosphorylation sites on Clp1 disrupts the Clp1-Rad24 interaction and causes Clp1 to return prematurely to the nucleolus during cytokinesis. Loss of Clp1 from the cytoplasm in telophase renders cells sensitive to perturbation of the actomyosin ring but does not affect other Clp1 functions (Chen et al., 2008). Moreover, human Mob1 proteins have been recently related to regulation of microtubule stability at the midbody structure during exit from mitosis. Mob1A and Mob1B,

members of the Hippo pathway, are needed for cell abscission in human cells (Florindo et al., 2012). All the components and regulatory mechanisms of the MEN and SIN pathway seem to be conserved; therefore its characterization in yeast is a step forward for MEN-related pathways study in higher eukaryotes.

# 5. PP2A$^{Cdc55}$ phosphatase

## 5.1. Phosphatases general overview

Protein phosphorylation is one of the most prevalent and versatile mechanisms for protein function regulation, playing critical roles in regulating many cellular processes including cell cycle, growth, apoptosis and signal transduction pathways. Approximately one-third of the cellular proteins are phosphorylated, with the vast majority of the modifications occurring on serine residues, 12% on threonines and 2% on tyrosine (Olsen et al.,. 2006). Protein phosphorylation is a reversible post-translational modification catalyzed by protein kinases and reversed by protein phosphatases. Thus, the phosphorylation status of a protein, at a given moment in time, is the net result of the opposing activities of the relevant kinase(s) and phosphatase(s).

The human genome encodes 518 kinases but only ~137 protein phosphatases (Alonso et al., 2004; Manning et al., 2002). There are two major groups of protein phosphatases: protein serine/threonine phosphatases (PSPs) and single-subunit protein-tyrosine phosphatase (PTPs). These two phosphatases families counteract the activity of 428 serine/threonine kinases (PSKs) and 90 tyrosine kinases (PTKs),

respectively. The small number of catalytic phosphatase subunits compared to kinases, and the *in vitro* promiscuity of the isolated phosphatase catalytic subunits, has been thought to signify that phosphatases were generally promiscuous enzymes. However, unlike kinases, phosphatases complexity does not lie in the number of genes encoding phosphatase catalytic subunits. Most PSPs are obligate multimeric enzymes *in vivo*, assembled from only a small number of catalytic subunits (30 aproximately) combining with many hundreds of regulatory subunits. The combinatorial and regulatory complexity of PSPs creates unambiguously targeted enzymes that coordinate and control highly regulated biochemical events (Virshup and Shenolikar 2009). On the other hand, number of PTKs and PTPs roughly match each other, but larger PTPs diversity is achieved through the use of alternative promoters to control their expression, the presence of multiple splicing variants and post-translational modifications (Tonks, 2006). In addition, the completion of the human genome has demonstrated that (1) there are more PTPs than PTKs (2) the possible number of PSPs holoenzymes, generated by a combinatorial mechanism, far exceeds the number of all protein kinases, and (3) there are additional large families of protein phosphatases, such as the haloacid dehalogenase (HAD) family, and possibly others. Thus, one could argue that phosphatases are even more important than kinases in setting the levels of protein phosphorylation (reviwed in Mustelin 2007). What is becoming clear at least is that phosphatases are specific and tightly regulated ezymes, and phosphatase regulation is nowadays a trendy topic in biomedicine research.

Based on structure, rather than function, the protein phosphatases can be classified into several completely separate families (see Table 1) that do not share any structural similarities and apparently evolved independently from different ancestral folds (reviewed in Mustelin 2007). It is important to note that this newer structural classification overlaps, but does not coincide, with the older classification of protein phosphatases by substrate specificity into serine/threonine specific (PSPs), tyrosine-specific (PTSs), and dual-specific phosphatases (DUSPs). The most abundant phosphatases are the PSPs and they have been classified into three structurally distinct families: (1) the PPM family of Mg2+-dependent phosphatases, including PP2C, (2) the Mg2+-dependent FCP family, and (3) the PPP family, which is the largest and contains the well-known enzymes PP1, PP2A, PP2B (calcineurin), PP5, and many others. PP1 and PP2A are the most abundant PSPs in eukaryotic cells.

**Phosphatase Families**

| Phosphatase families | Examples of members |
|---|---|
| 1. PPM family | PP2C |
| 2. FCP family | FCP |
| 3. PPP family | PP1, PP2A, calcineurin, PP5 |
| 4. HAD family (Asp-based) | Eya, CTD, cronophin |
| 5. Class I Cys-based PTPs | |
| 5.1. Classical PTPs | |
| 5.1.1. Transmembrane PTPs | PTPα, CD45, CD148, IA-2, GLEPP1 |
| 5.1.2. NRPTPs | PTP1B, TCPTP, SHP1, LYP, MEG2 |
| 5.2. DSPs or VH1-like PTPs | |
| 5.2.1. MKPs | MKP1–5, MKP7, PAC1 |
| 5.2.2. Atypical DSPs | VHR, PIR, Laforin, VHZ, STYX |
| 5.2.3. Slingshots | SSH1, SSH2, SSH3 |
| 5.2.4. PRLs | PRL-1, PRL-2, PRL-3 |
| 5.2.5. CDC14s | CDC14A, KAP, PTP9Q22 |
| 5.2.6. PTENs | PTEN, TPIP, tensin, C1ten |
| 5.2.7. Myotubularins | MTM1, MTMR1—15 |
| 6. Class II Cys-based PTPs | CDC25A, CDC25B, CDC25C |
| 7. Class III Cys-based PTPs | LMPTP |

**Table 1. Phosphatase Structural Families (Mustelin 2007)**

A key defining feature of all PPPs, including the most abundant PP1 and PP2A, is that they are multimeric enzymes. While only 13 human genes encode PPP catalytic subunits, these are associated with numerous PPP regulatory subunits (Cohen, 2009). The full complement of PPP regulators remains unknown, with new ones being discovered on a regular basis. Unlike phosphatase catalytic subunits and kinases, the phosphatase regulators do not share extensive sequence conservation. Instead, they are identified by their physical association and function. It is these regulators, rather than the catalytic subunits, that provide the essential determinants for subcellular localization, substrate specificity, and fine-tuning of phosphatase activity. Added to these regulatory subunits, an increasing number of PPP inhibitory proteins also control the cell's dephosphorylation capacity.

Given the importance of protein phosphorylation in regulating protein function, subtle alterations in many kinases and phosphatases can cause human disease. As well as some kinases, phosphatases have been related to cancer and tumorogenesis. Phosphatases are also implicated in several inherited genetic diseases, as well as multifactor diseases such as Alzheimer's disease.

### 5.2. PP2A phosphates structure and regulation

PP2A, like PP1, works solely as a multimeric enzyme. Cellular PP2A exists in two general forms: a heterodimeric core enzyme and a heterotrimeric holoenzyme. The PP2A core enzyme consists of a scaffold subunit (also known as the A or PR65 subunit) and a catalytic subunit (C subunit). The heterodimeric complex interacts with a variable regulatory subunit (B subunit) to assemble into a holoenzyme (Fig. I.8).

The regulatory subunits comprise four families in mammals: B (also known as B55 or PR55), B′ (B56 or PR61), B″ (PR48/PR72/PR130) and B‴ (PR93/PR110). Each family consists of two to five isoforms that are encoded by different genes and some isoforms have multiple splice variants. Although highly conserved within the same family, these regulatory subunits share little sequence similarity across families, and their expression levels vary greatly in different cell types and tissues (Shi 2009). The exquisite substrate specificity of different PP2A holoenzymes *in vivo* is achieved by the different PP2A regulatory subunits. For example, the B′ subunit, but not B or B″, is specific for interacting with shugoshin (Kitajima et al., 2006; Riedel et al., 2006; Tang et al., 2006), a centromeric protein required for proper genome segregation (Gregan et al., 2008). In contrast, the B, but not B′ or B″, subunit was responsible for dephosphorylation of the microtubule-binding protein Tau (Drewes et al., 1993; Gong et al., 1994; Xu et al., 2008), a key player in Alzheimer's disease.

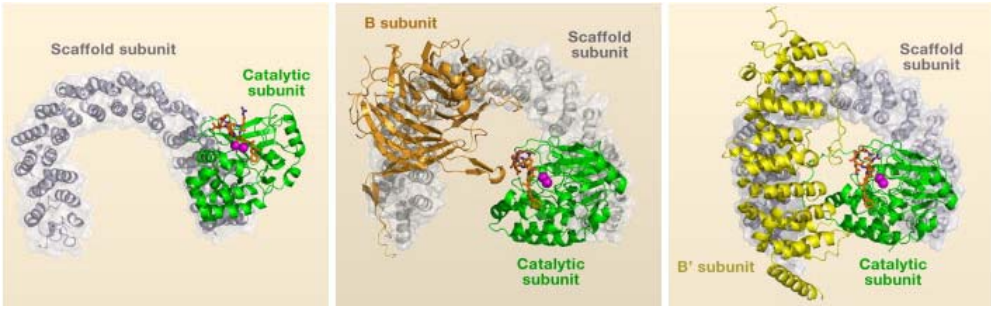



**Figure I.7. PP2A core enzyme and holoenzymes structure (Shi 2009)**

PP2A is highly conserved from yeast to humans. PP2A is an essential enzyme, and knockdown of the catalytic or a subset of regulatory subunit

genes results in unviable cells (Gotz et al., 1998; Kong et al., 2004; Li et al., 2002; Silverstein et al., 2002; Strack et al., 2004). In *S. cerevisiae*, PP2A scaffold subunit is known as Tpd3. The catalytic subunit of the core enzyme is either Pph21 or Pph22, two highly similar proteins sharing 95% of sequence identity (Sneddon et al., 1990, Ronne et al., 1991). Mutation of both *PPH21* and *PPH22* eliminates most of the PP2A activity in the cell and drastically reduces growth. Strains lacking *PPH21*, *PPH22*, and a third related gene, *PPH3*, are completely inviable (Ronne et al., 1991). The regulatory subunits comprise Cdc55 (B-type in mammals), Rts1 (B'-type in mammals) and the predicted B-subunit Rts3. In this project we refer to Tpd3, Pph21 or Pph22, and Cdc55 holoenzyme as $PP2A^{Cdc55}$.

A major determinant of PP2A holoenzyme composition *in vivo* is the availability of specific regulatory subunits, which in turn depends on their spatially and temporally regulated expression. Another important determinant is the stoichiometric balance of C, A, and B-type subunits. PP2A subunits are stabilized in the holoenzyme, but are instable as monomeric. Consequently, native PP2A isolated from various tissues consists of dimeric and trimeric complexes, but does not contain free monomeric C subunit. In addition, cells coordinate the assembly of the PP2A holoenzime with the activation of the C subunit in order to avoid active PP2A core enzyme complexes (Fig. I.9). Reversible methylation of the PP2A C subunit plays a key role in the assembly and activation of the PP2A holoenzime. In *S. cerevisiae*, Ppe1 methilesterase keeps Pph21 and Pph22 inactive. The demethylated C subunits forms interact with the dimmer Rrd2/Tpd3, which promotes the methylation of the carboxyterminal leucine of the C subunits. This methylation is carried out

by the methyltransferase Ppm1 and increases the affinity of the C subunits for the A and B subunits. Thus, assembly of PP2A holoenzyme is coupled with the catalytic activation of PP2A.

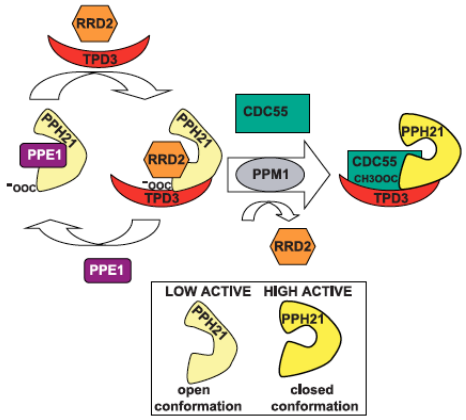



**Figure I.8. Model of PP2A holoenzyme assembly and activation (Hombauer et al., 2007)**

## 5.3. Mitosis regulation by PP2A$^{Cdc55}$

PP2A$^{Cdc55}$ phosphatase has been extensively related to cell cycle regulation in budding yeast. PP2A$^{Cdc55}$ has been recently linked to activation of the quiescent cell state, after endosulfin-mediated inhibition in response to certain stimuli during G1 (Bontron et al., 2013). Endosulfin mediated regulation of PP2A$^{Cdc55}$ has been also reported in mammals (Yamamoto et al., 2011), showing the strong conservation of PP2A regulatory mechanisms across eukaryotes. But certainly, most of the identified and studied PP2A$^{Cdc55}$ cell-cycle functions are related to mitosis execution, having several roles at both the entry and the exit from mitosis.

One of the first known $PP2A^{Cdc55}$ function in the cell-cycle regulation was its key role at the G2/M transition by affecting Swe1 and Mih1 activity (Wee1 and Cdc25 in mammals; Minshull et al., 1996, Wang and Burke, 1997, Yang et al., 2000). Swe1 phosphorylates Cdk1 preventing entry into mitosis and Mih1 opposes Swe1 inhibitory phosphorylation, thereby promoting entry into mitosis. Swe1 is initially phosphorylated by Clb2-Cdk1, which stimulates Swe1 inhibitory activity towards Cdk1 (Harvey et al., 2005, 2011). The Cdk1-dependent phosphorylation of Swe1 is opposed by $PP2A^{Cdc55}$ phosphatase, setting a threshold that limits activation of Swe1 by Cdk1, thereby allowing a low level of Cdk1 activity to escape Swe1 inhibition in early mitosis (Harvey et al., 2011). Swe1 is further phosphorylated by Cdc5 polo kinase as Clb2-Cdk1 activity arises, leading to its degradation and a rapid increase in Cdk1 activity during mitotic entry. $PP2A^{Cdc55}$ also dephosphorylates Mih1. Mih1 is hyperphosphorylated early in the cell cycle by casein kinase 1, restraining Mih1 activity. Cdk1-dependent phosphorylation of Mih1 promotes dephosphorylation of Mih1 by $PP2A^{Cdc55}$ and cells enter into mitosis. Because casein kinase 1 is associated with sites of polar growth, it may regulate Mih1 as part of a signaling mechanism that links successful completion of growth-related events to cell cycle progression (Pal et al.,. 2008). In addition, blocking membrane traffic causes a Swe1-dependent G2/M arrest. The arrest is triggered via concerted effects on the regulation of both Swe1 and Mih1. Signals regarding the status of membrane traffic are relayed to $PP2A^{Cdc55}$ via a signaling axis that includes Rho1, Pkc1, and Zds1/2. Pkc1 binds to $PP2A^{Cdc55}$-Zds1/2, which directly controls the phosphorylation states of Mih1 and Swe1 (Uetz et al., 2000; Pal et al., 2008; Yasutis et al., 2010; Wicky et al., 2011; Anastasia et al., 2012).

$PP2A^{Cdc55}$ also participates in the mitotic exit (Fig. I.9). $PP2A^{Cdc55}$ counteracts Cdk1-dependent phosphorylation of Net1, which is crucial for Net1-Cdc14 dissociation (Queralt et al., 2006). Net1 is sequestering Cdc14 in the nucleolus during most of the cell cycle and activation of separase at anaphase onset promotes the downregulation of $PP2A^{Cdc55}$, allowing Cdk1-dependent phosphorylation of Net1 and the Cdc14 release. Zds1/2 proteins cooperate with separase to downregulate $PP2A^{Cdc55}$ (Queralt and Ulhmann 2008). Thus, Zds1/2 are common $PP2A^{Cdc55}$ modulators, participating in both entry and exit from mitosis. It was also suggested that $PP2A^{Cdc55}$ downregulation in early-anaphase could initiate MEN activity by facilitating Cdc5-dependent phosphorylation of Bfa1, thereby unlocking mitotic exit (Queralt et al., 2006). Although this idea has not been further explored, $PP2A^{Cdc55}$ was also reported as a negative regulator of mitotic exit (Wang and Ng 2006), proposing a role of $PP2A^{Cdc55}$ regulating Tem1 activation. In addition, $PP2A^{Cdc55}$ downregulation at anaphase onset facilitates separase proteolitic activity towards Scc1, which triggers sister-chromatid segregation. Cdc5 polo kinase stimulates cleavage of the cohesin subunit Scc1 by its phosphorylation (Alexandru et al., 2001; Hauf et al., 2005; Hornig and Uhlmann, 2004). $PP2A^{Cdc55}$ counteracts Cdc5 polo kinase phosphorylation of Scc1, thereby inhibiting cohesin cleavage. Thus, separase acts directly on Scc1 and also indirectly, through downregulation of $PP2A^{Cdc55}$, to stimulate cohesin cleavage, providing a feed-forward loop that may contribute to a robust and timely anaphase (Yaakov et al., 2012).

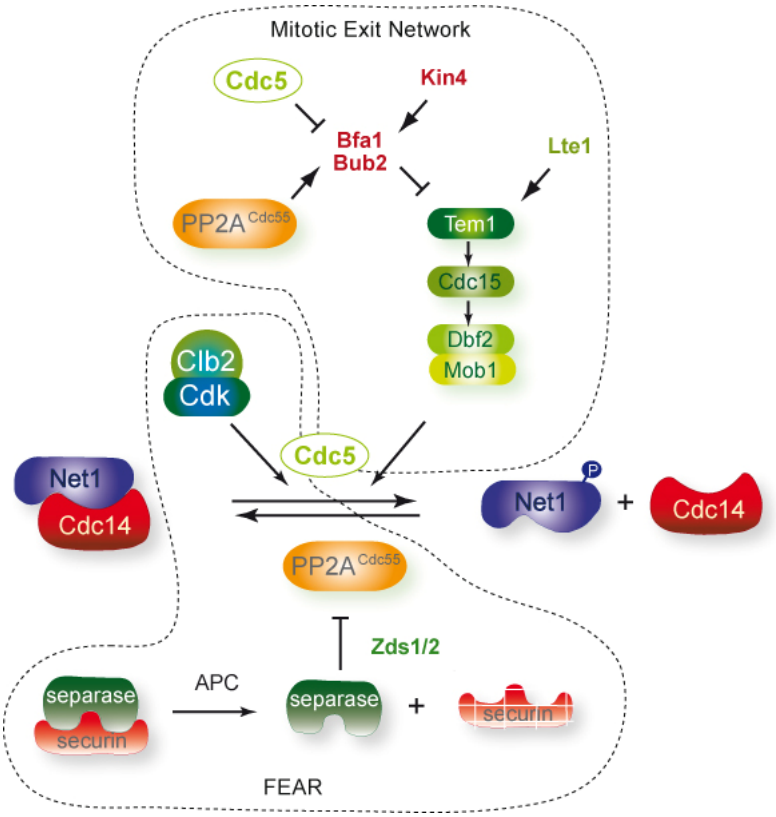



**Figure I.9. Model of mitotic exit (Queralt and Uhlmann, 2008a)**

$PP2A^{Rts1}$ was described to associate with shugoshin Sgo1, playing a crucial role in preventing cohesion dissociation at centromers; a PP2A function that was also described in human cells (Riedel et al., 2006; Tomoya et al., 2006). It was recently shown that $PP2A^{Cdc55}$ also interacts with shugoshins. Budding yeast has a single Shugoshin, Sgo1, that protects centromeric cohesin during meiosis I and monitors the biorientation of sister chromatids during mitosis (Kitajima et al., 2004; Indjeian et al., 2005). It has been recently described that Sgo1 can inhibit separase cleavage of Scc1 independently of securin estabilization

through estimulation of $PP2A^{Cdc55}$ activity, when sister chromatids are not under tension (Clift et al., 2009). In addition of $PP2A^{Cdc55}$-mediated separase inhibition, characterization of adenovirus E4orf4 cell toxicity revealed a functional link between $PP2A^{Cdc55}$ and $APC^{Cdc20}$ function. Adenovirus E4orf4 protein leads to an impaired $APC^{Cdc20}$ function and it has been suggested it is due to an increase of $PP2A^{Cdc55}$ targeting to the APC. It remains unclear whether $APC^{Cdc20}$ inhibition mechanism is exclusive of E4orf4 protein or, in contrast, virus protein E4orf4 takes advantatge of a common $APC^{Cdc20}$ regulation mechanism. The second option is more likely as E4orf4-induced $APC^{Cdc20}$ inactivation mechanism is conserved in mammals, pointing to a conserved $PP2A^{Cdc55}$ function in modulating $APC^{Cdc20}$ activation. In fact, Cdc6 plays a role in mitosis in addition to its function as a component of the pre-replication complex (pre-RC). Cdc6 has been shown to inhibit $APC^{Cdc20}$ and this effect is dependent on $PP2A^{Cdc55}$. Cdc6-Cdc55 interaction is required to inhibit the activity of the APC. $PP2A^{Cdc55}$ targeted to the $APC^{Cdc20}$ would counteract Cdk1-dependent activation of $APC^{Cdc20}$, keeping the $APC^{Cdc20}$ inactive (Boronat 2007, Mui et al., 2010).

# *Material and Methods*

# 1. Strains and protocols for culture and sinchronization

## 1.1. Plasmids

Plasmids used in this work, its origin and DNA content, is detailed in the following table.

| Insert | Vector | Origin |
|---|---|---|
| *$HA_3$-HCT1* | YIplac204 | W. Seufert |
| *GFP-Mob1* | pRS306 | F. Luca |
| *SPC42-eYFP* | pRS303 | R. Tsien |
| *$HA_3$-Cdc55* | pRS306 | our group |
| *$PK_3$-Cdc55* | pRS306 | our group |
| *MET3-$HA_3$-CDC20* | YIp22 | our group |
| *GAL1-$HA_3$-CDC20* | YIp22 | our group |
| *GAL1-$Flag_3$-Zds1* | YIp204 | our group |
| *GAL-$Flag_3$-ESP1-CBD* | YIp204 | our group |

**Table 2. Plasmids used in this work**

## 1.2. Yeast strains

All yeast strains used in this work were derivatives of W303 strain and are described in table 3. Culture medium used was either YPD (1% yeast extract, 2% peptone, 2% glucose), YPRaf (1% yeast extract, 2% peptone, 2% raffinose, 0.01% glucose), YPGal/Raf (1% yeast extract, 2% peptone, 2% galactose, 2% raffinose, 0.01% glucose) or minimum

medium (0.67% yeast nitrogen base, 2% glucose or raffinose) supplemented with appropiate aminoacids 40 µg/ml for selection. Strains resistant to geneticine were selected in YPD with 200 mg/l of geneticine (G418). Sporulation of diploids was performed in KSM medium plates. Cells were grown at 25ºC.

| Strain | Genotype | Origin |
|---|---|---|
| W303 | *MATa ade2-1 trp1-1 can1-100 leu2-3 his3-11 ura3* | |
| Y513 | *MATa MET-CDC20 CDC14-Pk$_9$ BFA1-HA$_6$* | this work |
| Y514 | *MATa MET-CDC20 cdc55Δ CDC14-Pk$_9$ BFA1-HA$_6$* | this work |
| Y528 | *MATa MET-CDC20 cdc55Δ CDC14-Pk$_9$ CDC15-HA$_6$* | this work |
| Y547 | *MATa MET-CDC20 CDC14-Pk$_9$ HA$_3$-CDH1* | this work |
| Y548 | *MATa MET-CDC20 cdc55Δ CDC14-Pk$_9$ HA$_3$-CDH1* | this work |
| Y559 | *MATa MET-CDC20 CDC14-Pk$_9$ BFA1-eGFP* | this work |
| Y560 | *MATa MET-CDC20 cdc55Δ CDC14-Pk$_9$ BFA1-eGFP* | this work |
| Y597 | *MATa MET-CDC20 GAL1-Flag$_3$-ZDS1 CDC14-Pk$_9$ BFA1-HA$_6$* | this work |
| Y603 | *MATa MET-CDC20 GAL1-Flag$_3$-ZDS1 CDC14-Pk$_9$ CDC15-HA$_6$* | this work |
| Y807 | *MATα MET-CDC20 GAL1-Flag$_3$-ZDS1 CDC14-Pk$_9$ MOB1-HA$_6$* | this work |
| Y870 | *MATa CDC14-Myc$_9$ CDC28F19 BFA1-mCherry* | this work |
| Y875 | *MATα MET-CDC20 GAL1-Flag$_3$-ZDS1 CDC14-Pk$_9$ BFA1-mCherry SPC42-YFP* | this work |

| | | |
|---|---|---|
| Y876 | *CDC28F19 CDC14-9myc BFA1-mCherry SPC42-YFP* | this work |
| Y913 | *MATα MET-CDC20 GAL1-Flag$_3$-ZDS1 CDC14-Pk$_9$ BFA1-mCherry MOB1-eGFP* | this work |
| Y951 | *MATa, MET-CDC20 CDC28F19 CDC14-9myc BFA1-mCherry SPC42-YFP* | this work |
| Y966 | *MATa MET-CDC20 cdc55Δ CDC14-9myc CDC15-eGFP BFA1-mCherry* | this work |
| Y967 | *MATa MET3-CDC20 CDC28F19 cdc55Δ CDC14-9myc Cdk1-eGFP BFA1-mCherry* | this work |
| Y984 | *MATa MET-CDC20 CDC14-9myc CDC15-eGFP BFA1-mCherry* | this work |
| Y985 | *MATa MET3-CDC20 CDC28F19 CDC14-9myc Cdk1-eGFP BFA1-mCherry* | this work |
| Y1006 | *MATa CDC28F19 cdc55Δ CDC14-9myc BFA1-mCherry SPC42-YFP* | this work |
| Y1014 | *MATα MET-CDC20 GAL1-Flag$_3$-ZDS1 CDC14-Pk$_9$ CDC15-eGFP BFA1-mCherry* | this work |
| Y1017 | *MATa, MET-CDC20 CDC28F19 cdc55Δ CDC14-9myc BFA1-mCherry SPC42-YFP* | this work |
| Y1020 | *MATa pph3Δ pph21Δ pph22-172 BFA1-HA$_6$* | this work |
| Y1021 | *MATa Pk$_3$-CDC55 BFA1-HA$_6$* | this work |
| Y1023 | *MATa MET-CDC20 CDC28F19 CDC149-myc MOB1-eGFP BFA1-mCherry* | this work |
| Y1033 | *MATa MET-CDC20 GAL1-Flag$_3$-ZDS1 cdc5Δ CDC5-14-HA$_3$ CDC14-Pk$_9$ BFA1-HA$_6$* | this work |
| Y1034 | *MATa MET-CDC20 GAL1-Flag$_3$-ZDS1 CDC14-Pk$_9$ BFA1-HA$_6$* | this work |
| Y1063 | *MATα BFA1-HA$_6$* | this work |

| | | |
|---|---|---|
| Y1076 | *MATa MET-CDC20 CDC28F19 cdc55Δ CDC149-myc MOB1-eGFP BFA1-mCherry*) | this work |
| Y1104 | *MATa MOB1-HA$_6$* | this work |
| Y1106 | *MATa Pk$_3$-CDC55 MOB1-HA$_6$* | this work |
| Y1109 | *MATa MET-CDC20 CDC14-Pk$_9$ eGFP-MOB1* | this work |
| Y1110 | *MATa MET-CDC20 cdc55Δ CDC14-Pk$_9$ eGFP-MOB1* | this work |
| Y1643 | *MATα CDC28-as1* | Jeff Ubersax /Dave |
| Y2961 | *MATa MET-CDC20 CDC14-Pk$_9$ CDC15-HA$_6$* | this work |

**Table 3. Strains used in this work**

### 1.3. Protocols for culture and synchronization

Cells were grown in the appropriate medium at 25ºC. For microscopy experiments, appropriate minimum media supplemented with casaminoacids was used.

Metaphase arrest by Cdc20 depletion.

Metaphase arrest by Cdc20 depletion was obtained in cells with Cdc20 under the control of the *MET3* or the *GAL1* promoter. When using MET-Cdc20 strains, cells were grown in media without methionine and 2% of the appropriate carbon source. Methionine was added at 2 mM final concentration, and metaphase arrest was checked by morphology of the cells, considering they are correctly sinchronized when 90% of the cells present a big bud (dumbbell shape), usually after 3-4h. For release from the metaphase arrest, cells were washed by filtration and transferred to media without methionine. When using GAL-Cdc20 strains, cells were grown in media containing 2% of raffinose and 2% of galactose and

shifted to medium containing raffinose only to arrest them. For release from the arrest, 2% of galactose was added back to the culture.

Metaphase arrest using Nocodazole

15mg/ml final concentration of nocodazole dissolved in 1% DMSO was added to cycling cells. After 2.5-3h cells were visualized under the microscope to verify the metaphase arrest.

G1 arrest using mating pheromone

All strains used for G1 arrest experiments were *MAT a*. G1 arrest was achieved by adding 1mg/ml final concentration of the pheromone α-factor every 30-45min during 2-3h. Cells were periodically checked under the microscope, using the presence of the bud as a marker of cycling cells. When 80% of the cells showed no bud, synchronous release into the cell cycle was induced by washing cells and transferring them into medium with no pheromone.

Protein overexpression

Zds1 and Esp1 ectopic expression were under the *GAL1* promoter. Cells were grown in medium containing 2% raffinose as the only carbon source, and GAL1 promoter was induced by adding 2% final concentration of galactose to the medium.

Protein inactivation

Inactivation of Cdk1 was performed in cells bearing the *cdc28-as1* allele and was obtained adding 1μm final concentration of 1NM-PP1.

Inactivation of *pph21-ts* and *cdc5-ts* mutants was achieved by shifting to 37ºC during 3h.

# 2. Genetic techniques

## 2.1. Yeast transformation

Epitope tagging of endogenous genes and gene deletions were performed by gene targeting using polymerase chain reaction (PCR) products. For N-terminal tagging of endogenous *CDC55*, plamids *$HA_3$-Cdc55* and *$PK_3$-Cdc55* were used (Queralt et al., 2006). Plasmids were integrated into the yeast genome after linearization with MscI. For tagging of Cdh1, Mob1 and SPC42, linearized plasmids were used. Plasmids were linearized using Bgl2, Plm1 and AflII respectively.

Strains containing *$CDC28^{Y19F}$* were obtained by crossing with the strain Y870. Strains containing the *CDC28-as1* allele were obtained by crossing with the strain Y1643. *MET-CDC20* and *GAL-CDC20* strains were obtained by genomic integration at the *CDC20* locus of the corresponding plasmids previously linearized with MscI restriction enzyme.

Yeast transformation using plasmids and PCR products were performed following lithium acetate protocol. After the heat shock, cells were plate in the appropriate selection medium. Transformations using geneticine resistance as selection marker were performed using the same protocol but, after the heat shock, cells were incubated during 3-4h in YPD before plating the cells in geneticine-supplemented plates.

## 2.2. Diploids obtaining and analysis

To obtain diploid strains, strains of opposed sexual type (*MAT a* and *MAT* $\alpha$) were crossed and incubated at 25ºC during 24h. Diploids were selected in minimum media supplemented with the appropriate aminoacids and were grown in YPD before transferring to sporulation plates. Diploids were incubated in sporulation plates at least for 3 days.

To dissect tetrads, cells were digested with zymolyase 20T 0.1% in 1M sorbitol pH 7.4 buffer. Cells were dissected using a microscope coupled to a micromanipulator MSM System from Singer. Spores were incubated at 25ºC until appearance of colonies.

# 3. Microscopy techniques

## 3.1. Immunofluorescence in situ

Approximately $10^8$ cells were collected for immunofluorescence in situ. Cells were fixed in cold formaldehyde buffer (potassium phosphate 100 mM pH 6.4, MgCl2 0.5 mM containing 3.7% formaldheid), incubating 20 min at 30ºC. After fixation, cells were washed twice with potassium phosphate pH 6.4 buffer. Fixed cells were resuspended in sorbitol buffer ($K_2HPO_4$ 120mM pH 7.4, citric acid 33mM, sorbitol 1.2M) and digested with 0.1mg/ml zymolyase 100T (US Biological) and 1/10 volume of glusulase (PerkinElmer) at 30ºC during 20-30min. Spheroplasts were washed twice with sorbitol buffer before proceeding with antibodies incubation. Spheroplasts were fixed onto the slide using polilysine 0.1%. Blocking was performed using PBS containing BSA 1% (bovine serum albumin) as blocking buffer. Primary and secondary antibodies were diluted at the corresponding concentration in blocking buffer. Blocking buffer was also used for washing between primary and secondary antibodies. Mounting medium containing DAPI (Vectashield) was added before covering with a slide and sealing.

The primary antibodies used for immunofluorescence were α-HA clone 16B12 (Babco), α-Pk clone SV5-Pk1 (Serotec), anti-tubulin clone YOL1/34 (Serotec) and Cdc14 (yE-17) sc-12045 (Santa Cruz Biotechnology). The secondary antibodies were Cy3 labeled α-mouse (GE Healthcare), fluorescein-conjugated α-rat (Millipore) and Cy3

labeled α-goat (Jackson ImmunoResearch). DAPI staining allowed the visualization of the nucleus. Images were acquired with Leica camera DFC 360FX coupled with Leica DM6000B epifluorescence microscope, equipped with HCX PL APO 63x/1.40-0.60 OIL objective and illumination system EL6000 using Leica filtercubes A (Ex: BP 360/40), GFP (Ex: BP 470/40) and Y3 (Ex: BP 545/40) (Leica Microsystems).

### 3.2. Time-lapse microscopy and Image analysis

For the Zds1-induction experiments cells were fixed in absolute ethanol during the time-course and re-hydrated in minimum media before visualization. At least 50 cells were used for the quantification of the SPBs ratios. For the time-lapse microscopy cells were incubated in minimum media during the experiment with an environmental chamber and images were acquired every 5 minutes. A Zeiss Axio Observer Z1 inverted epifluorescence microscope with Apotome system equipped with Fluorescent Lamp HXP 120C and the objective Carl Zeiss Plan-Apochromat 63x N.A 1.40 oil was used. The filters used were EGFP (EX BP 470/40, BS FT 495, EM BP 525/50) and Cy3 (EX BP 550/25, BS FT 570, EM BP 605/70). Z-stacks at 1-µm intervals were taken for each fluorescent channel and projected onto a single image per channel. SPBs ratios were quantified as the signal intensity ratio between both SPBs (dSPB relative intensity/mSPB relative intensity). SPBs ratios equal or lower than 2 were considered as symmetric localization. SPBs ratios higher than 2 were considered as asymmetric localization. For acquisition and quantitative analysis of fluorescent microscopy (quantification of signal intensity and spindle length measurements) we used ZEN 2011 software.

# 4. Protein detection

## 4.1. Western blot

Whole protein extracts for Western blot analysis were obtained by NaOH or TCA protein extraction protocol. NaOH extraction was used for abundant and high stable proteins (Mob1, Clb2, Cdc55, overexpressed Zds1 and Esp1). Cells were collected and kept in cold buffer HEPES 50mM pH7.5. Cells were lysed adding NaOH 2mM and β-mercaptoethanol 80µl/ml final concentration. Cell pellet was resuspended in SDS-PAGE loading buffer and boiled at 95ºC. TCA extraction was used for low abundant and/or unestable proteins (Cdh1, Sic1, Cdc15, Cdc5, Bfa1). Cells were collected and kept in cold TCA 20%. Cells were washed with Tris 1M and resuspended in SDS-PAGE loading buffer. Cells were lysed using glass-beads (Glass beads Sigma) and Precellys 24 for lysis and homogenization (Bertin Technologies). Approximately 5µL of protein extract was used for SDS-PAGE.

Proteins were separated using SDS-PAGE electrophoresis and transferred into nitrocellulose membrane. Primary and secondary antibodies were diluted in blocking buffer, 5% milk PBS-T. Washes between antibodies were done with PBS-T. The antibodies used for western blots were α-HA clone 12CA5 (Roche), α-FLAG clone M2 (Sigma), α-Pk clone SV5-Pk1 (Serotec), Cdc5 (yC-19) sc-6733 (Santa Cruz Biotechnology), Sic1 (FL-284) sc-50441 (Santa Cruz Biotechnology), Clb2 (y-180) sc-907 (Santa Cruz Biotechnology), anti-tubulin clone YOL1/34 (Serotec) and anti-Phosphoglycerate Kinase (Life Technologies).

### 4.2. Co-immunoprecipitation

Co-immunoprecipitation assays were performed using $2x10^9$ cells for Mob1 and $3.2x10^{10}$ cells for Bfa1 analysis. Protein extracts were prepared by mechanical lysis using glass beads. The clarified extracts were incubated with antibody, and the immunocomplexes were adsorbed onto magnetic protein-A Dynabeads® (Life Technologies). The beads were washed in extraction buffer and the protein-bound eluted with SDS-PAGE loading buffer. The antibody used for immunoprecipitation was α-Pk clone SV5-Pk1 (Serotec).

## 5. Fluorescence Flow Cytometry Analysis (FACS)

Fluorescence flow cytometry analysis was performed to determine DNA content of cells during some time-course experiments. Cells were collected in absolute ethanol. Cells were resuspended in cold 50mM sodium citrate pH 7.4 buffer. Cells were first treated with RNAse 0.2mg/ml during 1h at 50ºC. Afterwards, cells were treated with proteinaseK 1mg/ml during 1h at 50ºC. Finally, 50mM sodium citrate pH 7.4 buffer containing 16μg/ml propidium iodide was added to all samples. Flow cytometer used was Gallios Beckman Coulter, with Gallios software. 488 laser, FL3 detector was used. For analysis of the data Kaluza Flow Cytometry was used.

## 6. Proteomics

### 6.1. SILAC

Stable isotope labelling of yeast cells and preparation of the protein extract were performed as previously described (Mascaraque et al., 2012). Cells were grown in minimun media containing either 100 mg/L arginine and 100 mg/L lysine or 100 mg/L $^{13}C_6$-arginine and 100 mg/L $^{13}C_6$-lysine (Cambridge Isotope Laboratories Inc.). Protein extracts were

prepared by mechanical lysis using glass beads. Approximately 250 µg of the mixed heavy/light protein sample was processes for in-solution digestion as previously reported (Monteoliva et al., 2011). Phospho-peptide enrichment by sequential elution from IMAC (SIMAC) was done as previously described (Mascaraque et al., 2012). Peptides were analyzed using a LTQ-Orbitrap Velos LC-MS/MS mass spectometer (Termo Scientific). Peptide identification was performed using MASCOT software and SILAC quantification was done with the ProteomeDiscoverer 1.3 (Thermo Scientific).

### 6.2. Bioinformatic tools for analysis

We used free software Motif-X to look for phosphorylation motifs along phospho-peptides identified in SILAC screening. Motif-X and corresponding literature is available at http://motif-x.med.harvard.edu/ . The methodology relies on the intrinsic alignment of phospho-residues and the extraction of motifs through iterative comparison to a dynamic statistical background. Phospho serine residues were used as central residue. Yeast Data Base was selected for background.

To generate protein-protein interaction map of the whole significative phospho-peptids identified, STRING 9.05 software was used. Classification into functional clusters and gene ontology was obtained using *D*atabase for *A*nnotation, *V*isualization and *I*ntegrated *D*iscovery (DAVID) 6.7 software.

For protein modelling, Protein threading and Homology modelling method were used depending on the availability of homologous protein structures or not at the Protein Data Bank (PDB). For the docking analysis, PyMol software was used for modelling visualization.

# *Objectives*

## 1. Elucidate the PP2A$^{Cdc55}$ putative regulation of the Mitotic Exit Network

Separase activation at anaphase onset promotes the downregulation of PP2A$^{Cdc55}$ and Cdc14 release from the nucleolus. It has been suggested that PP2A$^{Cdc55}$ downregulation could also promote MEN activation, through facilitating Bfa1 phosphorylation. Thus, the first goal of this work is to address whether PP2A$^{Cdc55}$ regulates the MEN.

## 2. Find new putative substrates of PP2A$^{Cdc55}$ in the mitotic exit

While kinases function has been extensively studied in cell cycle regulation, little is known about the opposing phosphatases, which have recently emerged as equally important regulators of the cell cycle. The completion of mitosis depends on Cdk1 inactivation and removal of mitotic phosphorylations from a broad range of substrates. Thus, activation of counteracting phosphatases is a key step for mitotic exit. In budding yeast, Cdc14 phosphatase is a well-characterized Cdk1-counteracting phosphatase, but other phosphatases are currently emerging as important regulators of the mitotic exit such as PP2A$^{Cdc55}$ phosphatase. This work aims to find new putative substrates of PP2A$^{Cdc55}$ phosphatase in the mitotic exit in order to gain insight into PP2A$^{Cdc55}$ regulated processes as well as general phosphatases contribution to mitosis completion.

# *Results*

**1. PP2A$^{Cdc55}$ downregulation promotes Bfa1 phosphorylation.**

Downregulation of PP2A$^{Cdc55}$ phosphatase at anaphase onset is a key event to initiate the mitotic exit because it facilitates the Cdk1-dependent phosphorylation of Net1, which provides the first wave of Cdc14 release (Queralt et al., 2006). In the same study it was suggested that PP2A$^{Cdc55}$ downregulation also promotes the phosphorylation of Bfa1, which contributes to MEN activation in anaphase. In addition, it was also proposed that Cdc55 influences the mitotic exit as part of the MEN (Wang and Ng, 2006). Bfa1-Bub2 complex keeps the MEN inactive by inhibiting Tem1, and the Cdc5-dependent phosphorylation of Bfa1 in anaphase alleviates its inhibitory activity on MEN (Hu *et al.,* 2001; Pereira *et al.,* 2002). However, there is no evidence for Cdc5 upregulation during anaphase and, as mentioned before, preliminary results suggested that downregulation of PP2A$^{Cdc55}$ phosphatase at anaphase onset could facilitate Bfa1 phosphorylation (Queralt 2006). To further investigate the role of PP2A$^{Cdc55}$ regulating the MEN activity, we studied the dephosphorylation of Bfa1 by PP2A$^{Cdc55}$ phosphatase. First, we confirmed that Bfa1 was already phosphorylated in metaphase in a *cdc55*Δ mutant (Fig.R1.1. and Queralt et al., 2006). Wild-type and *cdc55*Δ cells were synchronized at the metaphase to anaphase transition by Cdc20 depletion. Bfa1 electrophoretic mobility and Cdc14 release were analyzed after releasing cells into anaphase. In wild-type cells, Bfa1 decreases its electrophoretic mobility at 10-15 minutes, coincident to the spindle elongation and Cdc14 release from the nucleolus. In contrast, in *cdc55*Δ cells, Bfa1 has a slower migration form already in metaphase. This result indicates that Bfa1 showed an

anaphase-like phosphorylation pattern already in metaphase; suggesting that PP2A$^{Cdc55}$ prevents Bfa1 phosphorylation.

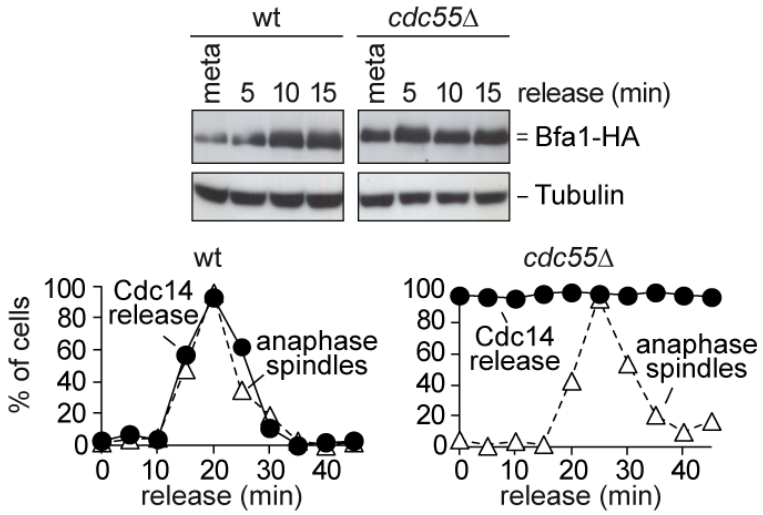



**Figure R1.1. Premature Bfa1 phosphorylation in metaphase in the absence of PP2A$^{Cdc55}$.** Strains Y513 (*MATa MET-CDC20 CDC14-Pk$_9$ BFA1-HA$_6$*) and Y514 (as Y513, but *cdc55Δ*) were arrested in metaphase by Cdc20 depletion and released into synchronous anaphase by Cdc20 reintroduction. Bfa1 phosphorylation was analyzed by western blot. Cdc14 release from the nucleolus and anaphase spindles elongation was monitored by immunofluorescence. At least 100 cells were scored at each time point. Tubulin served as a loading control.

We next studied Bfa1 phosphorylation in absence of PP2A$^{Cdc55}$ phosphatase catalytic activity. We used a strain carrying a deletion of *PPH21* and a temperature-sensitive *pph22-172* allele to inactivate the two PP2A$^{Cdc55}$ catalytic subunits; and also a deletion of the *PPH3* (since Pph3 phosphatase can partly compensate for PP2A$^{Cdc55}$ activity in budding yeast, Evans and Stark, 1997). Cells were arrested in metaphase by nocodazole and shifted to restrictive temperature in order to inactivate PP2A$^{Cdc55}$. We observed a decrease in Bfa1 electrophoretic

mobility in both strains after 1.5-2h of incubation at 37ºC, suggesting that Bfa1 suffers post-transational modifications at high temperatures. Also, Cdc14 is released in approximately 25-30% of the wild-type cells after incubation at 37ºC. For this reason, we compared the Bfa1 electrophoretic mobility just during the first hour of the experiment, when wild-type strain showed no changes in Bfa1 electrophoretic mobility. Bfa1 phosphorylation increased after 20min at restrictive temperature in *pph3Δpph21Δpph22-172* cells during while in wild-type cells did not (Fig.R1.2.). After longer incubation at 37ºC, hyper-phosphorylation of Bfa1 was higher in the *pph3Δpph21Δpph22-172* mutant. Upon treatment at 37ºC, Cdc14 was rapidly released from the nucleolus in the *pph3Δpph21Δpph22-172* mutant, confirming PP2A$^{Cdc55}$ inactivation. This result suggests that PP2A$^{Cdc55}$ catalytic activity is required to keep Bfa1 under-phosphorylated in metaphase.

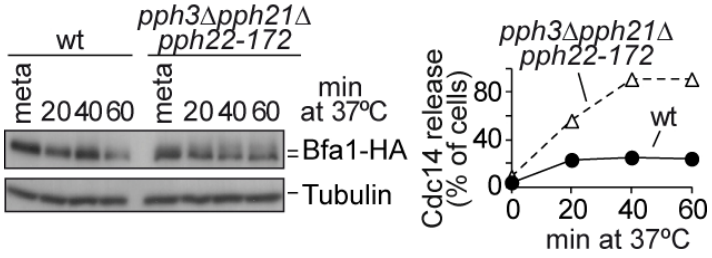



**Figure R1.2. PP2A$^{Cdc55}$ catalytic activity is required to keep Bfa1 under-phosphorylated in metaphase.** Strains Y1021 (*MATa Pk$_3$-CDC55 BFA1-HA$_6$*) and Y1020 (*MATa pph3Δ pph21Δ pph22-172 BFA1-HA$_6$*) were arrested in metaphase by nocodazole treatment and shifted to 37ºC.

To confirm above results, we used another approach to inactivate PP2A$^{Cdc55}$. We determined Bfa1 phosphorylation status under Zds1 over-expression, which promotes PP2A$^{Cdc55}$ inactivation (Queralt and

Uhlmann, 2008b). Cells were arrested in metaphase by Cdc20 depletion and Zds1 ectopic expression was induced by galactose addition. Bfa1 became phosphorylated between 40-60 minutes post-induction when Cdc14 has been released, as a marker of $PP2A^{Cdc55}$ phosphatase inactivation (Fig.R1.3.A). This result indicates that Zds1-dependent inactivation of $PP2A^{Cdc55}$ promotes Bfa1 phosphorylation. We obtained similar results when over-expressing Separase (Fig.R1.3.B), which also leads to $PP2A^{Cdc55}$ inactivation (Queralt and Uhlmann, 2008b), indicating that Separase-dependent inactivation of $PP2A^{Cdc55}$ also promotes Bfa1 phosphorylation. Taking all these results together, we can conclude that $PP2A^{Cdc55}$ phosphatase counteracts Bfa1 phosphorylation.

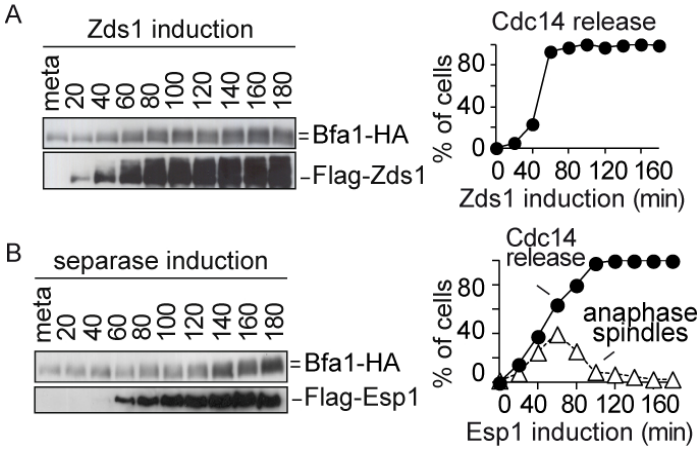



**Figure R1.3. (A) Zds1-dependent inactivation of $PP2A^{Cdc55}$ promotes Bfa1 phosphorylation.** Strain Y597 (*MATa MET-CDC20 GAL1-Flag$_3$-ZDS1 CDC14-Pk$_9$ BFA1-HA$_6$*) was arrested in metaphase by Cdc20 depletion and Zds1 ectopic expression was induced by galactose addition. Bfa1 phosphorylation and Zds1 expression levels were analyzed by western blot. **(B) Esp1-dependent inactivation of $PP2A^{Cdc55}$ promotes Bfa1 phosphorylation**. Strain Y532, (*MATa MET-CDC20 GAL1-Flag3-ESP1 CDC14-Pk9 BFA1-HA6*) was arrested in metaphase by Cdc20 depletion and Esp1 ectopic expression was induced by galactose addition. Bfa1 phosphorylation and Esp1 expression levels were analyzed by western blot. Cdc14 release was monitored by immunofluorescence as control of $PP2A^{Cdc55}$ inactivation.

Finally, to address whether Bfa1 could be a PP2A$^{Cdc55}$ substrate, we examined whether Cdc55 and Bfa1 physically interact. Co-immunoprecipitation experiments showed that Bfa1 co-purified with Cdc55 (Fig.R1.4.). Altogether these results suggest that Bfa1 is likely to be an *in vivo* substrate of PP2A$^{Cdc55}$. Thus, PP2A$^{Cdc55}$ downregulation at anaphase onset would facilitate Cdc5-dependent phosphorylation of Bfa1.

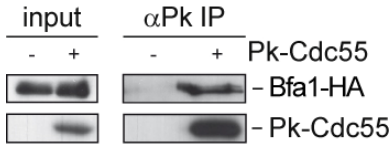



**Figure R1.4. Cdc55 and Bfa1 interact.** Co-immunoprecipitation between Cdc55 and Bfa1 was analyzed in protein extracts from strain Y1021 (*MATa Pk$_3$-CDC55 BFA1-HA$_6$*). Protein extracts from strain Y1063 (*MATα BFA1-HA$_6$*) lacking a Pk epitope on Cdc55 served as a control.

## 2. Cdc5 is the kinase responsible for Bfa1 phosphoryation upon PP2A$^{Cdc55}$ inactivation.

Bfa1 phosphorylation in a normal cell cycle depends on Cdc5 polo-like kinase activity. Therefore, we expected that the premature phosphorylation of Bfa1 observed upon PP2A$^{Cdc55}$ inactivation would depend on Cdc5. To check this possibility, we compared Bfa1 electrophoretic mobility after Zds1 induction in wild-type and *cdc5-14* mutant cells. Cells were arrested in metaphase by Cdc20 depletion, shifted to restrictive temperature during 3h to inactivate Cdc5, and then galactose was added to induce Zds1 ectopic expression. Bfa1 is already phosphorylated in metaphase in both strains due to the prolonged incubation at 37ºC (Fig. R2.1.). However, a Bfa1 hyper-phosphorylation

was observed upon Zds1 induction in wild-type cells. On the contrary, Bfa1 hyper-phosphorylation was not induced in *cdc5-14* mutant cells during the induction time-course. This result suggests that Bfa1 phosphorylation after $PP2A^{Cdc55}$ inactivation depends on the Cdc5 polo kinase.

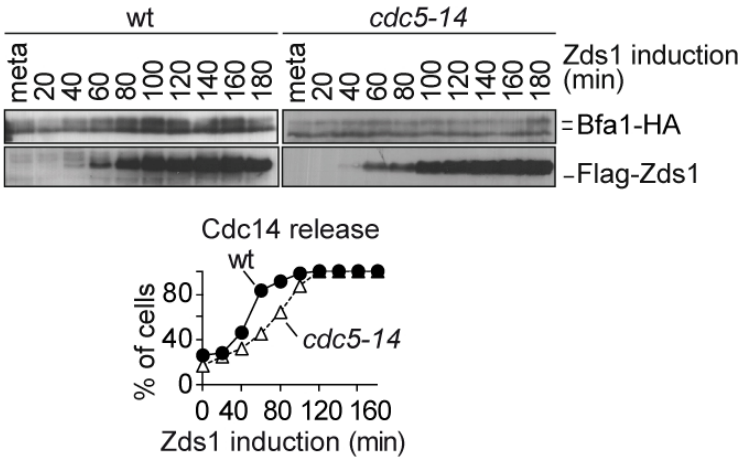



**Figure R2.1. Cdc5 Polo-like kinase dependent Bfa1 phosphorylation is required for Zds1-induced $PP2A^{Cdc55}$ inactivation**. Strains Y1034 (*MATa MET-CDC20 GAL1-Flag$_3$-ZDS1 CDC14-Pk$_9$ BFA1-HA$_6$*) and Y1033 (as Y1034, but *cdc5Δ CDC5-14-HA$_3$*) were arrested in metaphase by Cdc20 depletion and shifted to 37ºC for 180 min before Zds1 induction.

It has been described that Bfa1 is also phosphorylated by the kinase Kin4, depending on the spindle positioning checkpoint (SPOC) activation. When mitotic spindles are misaligned, Kin4 is activated and phosphorylates Bfa1 in specific sites that unable Cdc5-dependent phosphorylation. As a consequence, Bfa1-Bu2 complex is effectively locked in an active state and MEN pathway is kept inactive until abrogation of SPOC signal (Hu *et al.,*. 2001; Geymonat *et al.,*. 2003; Maekawa *et al.,*. 2007).

The SPOC should not be active when PP2A downregulation takes place at anaphase onset, but in order to rule out any possible Kin4 contribution to the Bfa1 phosphorylation we observed upon $PP2A^{Cdc55}$ inactivation, we compared Bfa1 electrophoretic mobility in wild-type and *kin4*Δ cells after

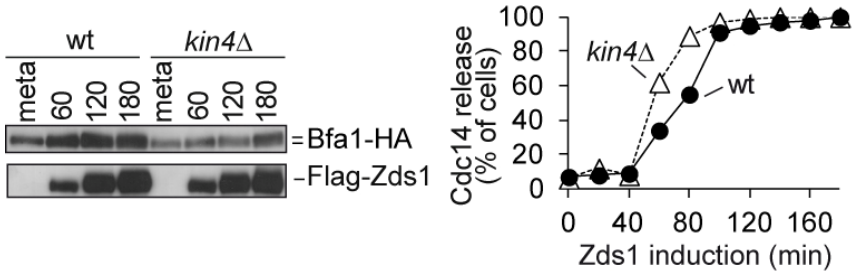



**Figure R2.2. Bfa1 phosphorylation observed upon $PP2A^{Cdc55}$ inactivation doesnot depend on the kinase Kin4.** Strains Y597 (*MATa MET-CDC20 GAL1-Flag$_3$-ZDS1 CDC14-Pk$_9$ BFA1-HA$_6$*) and Y563 (as Y597 but *kin4*Δ) were arrested in metaphase by Cdc20 depletion and Zds1 ectopic expression was induced by galactose addition. Bfa1 phosphorylation and Zds1 expression levels were analyzed by western blot. Cdc14 release was monitored by immunofluorescence as control of $PP2A^{Cdc55}$ inactivation.

Zds1 ectopic expression. Cells were arrested in metaphase by Cdc20 depletion and Zds1 overexpression was induced adding galactose. Both strains showed Bfa1 hyper-phosphorylation as Zds1 accumulated (Fig. R2.2). This result indicates that the Bfa1 phosphorylation observed upon $PP2A^{Cdc55}$ inactivation does not depend on the kinase Kin4. We reproduce this result when treating cells with Nocodazole to induce the SPOC, further confirming Kin4 kinase is not involved in Bfa1 phosphorylation upon $PP2A^{Cdc55}$ inactivation.

Taking all these results together, we propose $PP2A^{Cdc55}$ downregulation at anaphase onset facilitates Cdc5-dependent phosphorylation of Bfa1.

### 3. Bfa1 localizes asymmetrically to the dSPB in absence of $PP2A^{Cdc55}$ function.

MEN components localize to the SPBs and accumulated evidences indicate that their spatial regulation is important for timely MEN activation. In a normal cell cycle, the Bfa1-Bub2 complex localizes asymmetrically at the daughter SPB (dSPB) in anaphase, when Cdc5 polo kinase phosphorylates Bfa1 and allows MEN activation (Bardin et al., 2000; Pereira et al., 2000; Hu et al., 2001; Caydasi and Pereira, 2009). Bfa1 weakly localizes to both SPB until the spindle aligns along the mother-bud axis and the dSPB migrates to the bud neck, the moment when Bfa1 becomes asymmetry located on the dSPB. Bfa1 asymmetric localization is important for the proper recruitment of MEN components (Monje-Casas and Amon, 2009). Bfa1 is a phosphoprotein and its asymmetric localization onto the dSPB is induced upon Cdc5 phosphorylation. On the other hand, Bfa1 shows a symmetric localization when the spindles are misaligned, because of the SPOC activation (Hu et al., 2001, Pereira et al., 2001; Caydasi and Pereira, 2009; Kim et al., 2012).

Our above results indicate that Bfa1 is hyper-phosphorylated in absence of $PP2A^{Cdc55}$ activity and Cdc5 is likely to be involved. Therefore, we next investigated whether Bfa1 also presented a premature asymmetric localization on the dSPB upon $PP2A^{Cdc55}$ inactivation. First, we examined Bfa1 localization in wild-type and *cdc55*Δ metaphase arrested cells containing Bfa1-eGFP (Fig.R3.1). In wild-type cells, Bfa1 is preferentially

located on both SPBs, showing 96% of cells with symmetric or high/low localization onto the SPBs. Conversely, in the *cdc55*Δ mutant 58% of the cells showed Bfa1 located to just one of the SPBs. Therefore, Bfa1 presented mostly an asymmetric localization in absence of Cdc55. In order to distinguish different levels of asymmetry (high/low localization patterns) in the Bfa1 localization, we quantified the signal intensity ratio between both SPBs (dSPB/mSPB relative intensity; hereafter SPBs ratio). In wild-type metaphase arrested cells, the SPBs ratio of Bfa1 is around 2, when is described to be symmetric. In contrast, *cdc55*Δ cells showed an increased Bfa1 SPBs ratio, reaching an average ratio of 5.8, indicating a dramatic asymmetric distribution. In the following experiments we used the SPBs ratio to differentiate between Bfa1 symmetric distribution (SPBs ratio < 2) and Bfa1 asymmetric localization (SPBs ratio > 2).

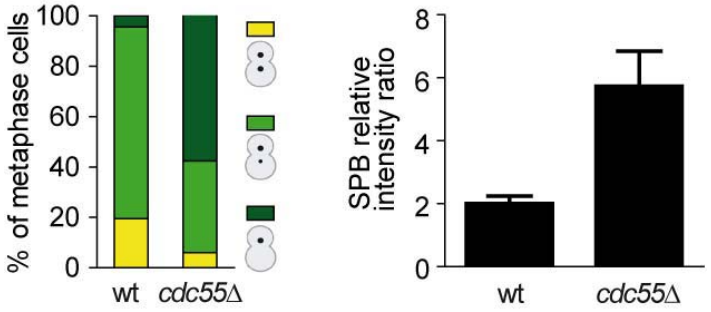



**Figure R3.1. Cdc55 deletion causes premature Bfa1 asymmetric localization at the dSPB in metaphase-arrested cells**. Strains Y559 (*MATa MET-CDC20 CDC14-Pk*$_9$ *BFA1-eGFP*) and Y560 (as Y559, but *cdc55*Δ) were arrested in metaphase by Cdc20 depletion. Percentages of equal, high/low and single distribution of Bfa1-eGFP were determined. At least 100 cells were scored for each strain. Asymmetric Bfa1-mCherry signal was quantified as the SPB relative intensity ratio as described in materials and methods.

To further study Bfa1 localization in absence of PP2A$^{Cdc55}$ activity, we induced Bfa1 phosphorylation by Zds1-dependent inactivation of PP2A$^{Cdc55}$. Cells containing Bfa1-mCherry and Spc42-YFP as a SPB marker were arrested in metaphase by Cdc20 depletion and galactose was added to induce Zds1 ectopic expression. Bfa1 localization onto the SPBs was asymmetric in 75% of the cells after Zds1 induction (Fig.R3.2). Bfa1 SPBs ratio increased from 2 in metaphase to a value of 5.2 at 120 min after Zds1 induction, indicating a Zds1-induced asymmetric distribution.

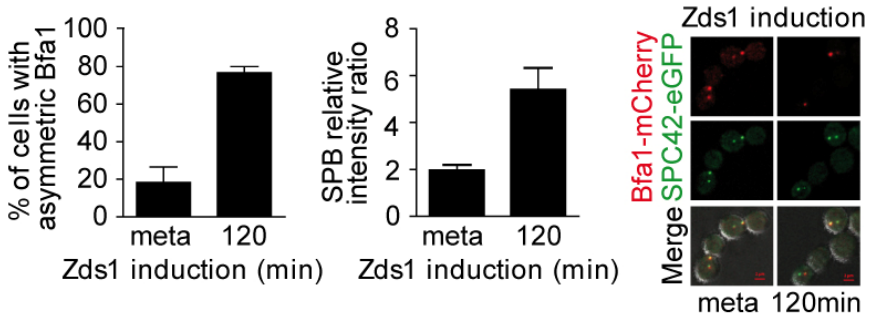



**Figure R3.2. Bfa1 localization becomes asymmetric upon Zds1-dependent inactivation of PP2A$^{Cdc55}$.** Strain Y875 (*MATα MET-CDC20 GAL1-Flag$_3$-ZDS1 CDC14-Pk$_9$ BFA1-mCherry SPC42-YFP*) was arrested in metaphase by Cdc20 depletion and galactose was added to induce Zds1 overexpression. Asymmetric Bfa1-mCherry signal was quantified as the SPB relative intensity ratio as described in materials and methods.

Lastly, we studied the changes of the Bfa1 localization during the cell cycle in absence of PP2A activity. In a first approach, we synchronized cells at the metaphase to anaphase transition by Cdc20 depletion (Fig.R3.3). After release into anaphase by Cdc20 re-addition, the

kinetics of Bfa1 localization confirmed the previous results. In wild type cells, Bfa1 became strongly asymmetric in late anaphase, when the cells had a spindle length longer than 6 μm, reaching a SPBs ratio average of 20. Conversely, *cdc55*Δ mutant cells showed a SPBs ratio average of 8 already in metaphase, which is an asymmetric distribution comparable to wild-type anaphase, with spindle length 3-6μm (SPBs ratio 7).

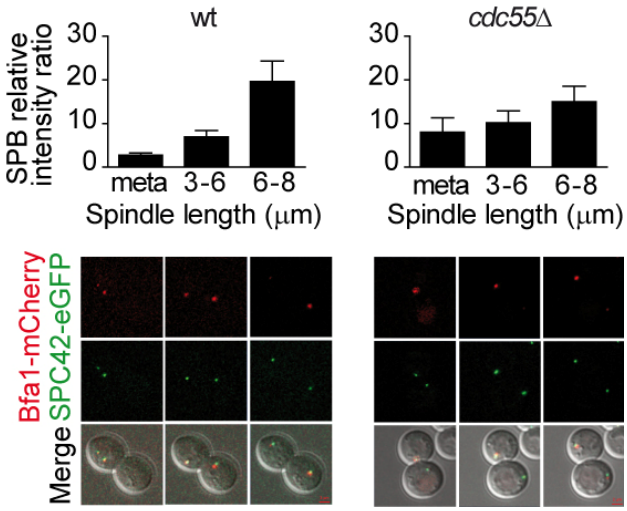



**Figure R3.3. Premature Bfa1 asymmetric localization in the absence of Cdc55 in a metaphase-to-anaphase transition.** Strains Y951 (*MATa MET-CDC20 CDC28F19 CDC14-myc$_9$ BFA1-mCherry SPC42-YFP*) and Y1017 (as Y951, but *cdc55Δ*) were arrested in metaphase by Cdc20 depletion and released into synchronous anaphase by Cdc20 reintroduction. Time-lapse microscopy was used to visualize Bfa1-mCherry localization dynamics. Bfa1 SPB ratios were measured during mitosis progression (n=15 for WT; n=10 for *cdc55Δ*). The distance between the two SPBs was used to calculate the spindle length.

We next analyzed Bfa1 localization in a cell-cycle progression after synchronous release from G1 in wild-type and *cdc55*Δ mutant cells bearing the *CDC28Y19F* allele that is refractory to Cdk1 inhibition

(Fig.R3.4). *cdc55*Δ cells show a delay in progression through mitosis since they have compromised Cdk1 activity because of the inhibitory Cdc28-Y19 phosphorylation (Minshull et al., 1996). The *cdc55*Δ *CDC28Y19F* mutant enter normally into mitosis and shows premature Cdc14 release from the nucleolus in metaphase, typical phenotype of the *cdc55*Δ (Queralt et al., 2006), thus being a useful genetic background to separate the functions of $PP2A^{Cdc55}$ phosphatase at the entry into mitosis from the ones implicated in the exit from mitosis. In wild-type cells, Spc42-YFP signal splits during S phase, indicative of the SPB duplication, however the Bfa1-mCherry signal is hardly detectable until cells enter into anaphase. Cells at the S/G2 transition show a weak Bfa1 signal but symmetrically located (Fig.R3.4 S/G2 column). When the cells had a spindle length longer than 6 μm, Bfa1-mCherry was detected only in the dSPB, consistent with published results (Caydasi and Pereira, 2009).

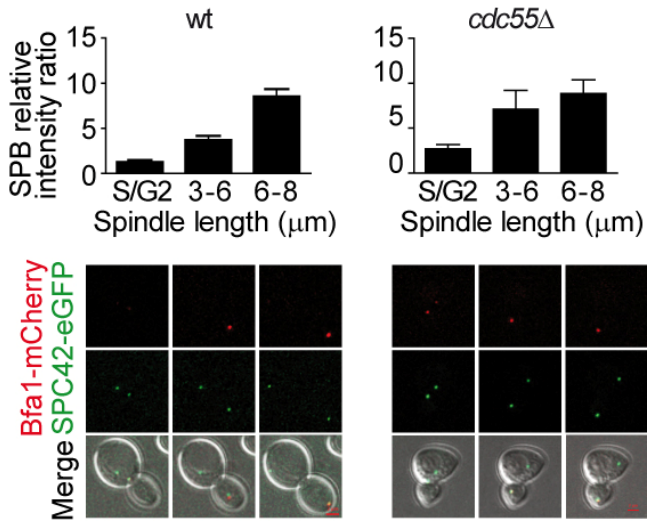


**Figure R3.4. Premature Bfa1 asymmetric localization in the absence of Cdc55 in a synchronous cell cycle after G1 release**. Strains Y876 (MATa CDC28F19 CDC14-myc9 BFA1-mCherry SPC42-YFP) and Y1006 (as Y876, but cdc55Δ) were arrested at G1 with α-factor and released into a synchronous cell cycle. Time-lapse microscopy was performed as in Fig.R3.3 (n=26 for WT; n=25 for cdc55Δ).

On the other hand, in *cdc55Δ CDC28Y19F* mutant cells, Bfa1-mCherry was detected on the SPBs short after the Spc42-YFP signal duplicates. As soon as the Bfa1-mCherry signal was visualized, Bfa1 was asymmetrically loaded onto the dSPB (SPBs ratios average of 3). When the cells reach mitosis, the Bfa1-mCherry signal increases and the asymmetry becomes more evident, reaching SPBs ratios of 8 and 11. These results further confirmed the premature asymmetric Bfa1 localization in absence of $PP2A^{Cdc55}$ function.

Therefore, $PP2A^{Cdc55}$ downregulation at anaphase onset would facilitate Bfa1 inactivation by Cdc5, as indicated by Bfa1 changes in phosphorylation and localization pattern.

### 4. Premature Bfa1 inactivation does not provoke a premature exit from mitosis.

The Bfa1-Bub2 complex inhibits the MEN pathway until Cdc5-dependent phosphorylation of Bfa1 alleviates Tem1 inhibition in anaphase (Hu et al., 2001). Therefore, we would expect that the premature inactivation of Bfa1 observed in absence of $PP2A^{Cdc55}$ activity would result in a premature MEN activation. If so, *cdc55Δ* mutant would present a faster mitotic exit. However, we did not observe any accelerated mitotic exit compared to wild-type cells, as indicated by the anaphase spindle

dynamics (Fig.R1.1). For this reason, we wonder whether the MEN is properly activated in absence of $PP2A^{Cdc55}$ activity.

Cdc15 is a MEN kinase that promotes mitotic exit by directly switching on the kinase activity of Dbf2 (Mah et al., 2001). Cdc15 associates with the cytoplasmic face of the SPBs during anaphase and is dephosphorylated by Cdc14 as a positive feed-forward loop (Cenamor et al., 1999; Gruneberg et al., 2000; Jaspersen and Morgan, 2000; Xu et al., 2000; Menssen et al., 2001). Cdc15 dephosphorylation has been commonly used as a MEN activation marker. To further investigate MEN activation in absence of $PP2A^{Cdc55}$, we synchronized wild-type and *cdc55*Δ mutant cells at the metaphase to anaphase transition by Cdc20 depletion and analyzed Cdc15 phosphorylation pattern by western blot. In wild-type cells, Cdc15 increases its electrophoretic mobility being especially evident between 20 and 40 minutes when cells are in anaphase and Cdc14 is fully released (Fig. R4.1). At these time points, MEN pathway is active (as indicated by the anaphase spindle dynamics) and Cdc15 is dephosphorylated, as previously reported. Interestingly, in the *cdc55*Δ mutant, we can detect a faster migration form of Cdc15 already at metaphase. Note that this could be reminiscent of Cdc14 being released from the nucleolus already in metaphase in the *cdc55*Δ mutant. However, the Cdc15 dephosphorylation does not increase further when the cells reach anaphase (20-40 min). We can conclude that in absence of $PP2A^{Cdc55}$ activity, Cdc15 is prematurely dephosphorylated although partially, and this dephosphorylation is not efficient enough to provoke a premature MEN activation, since mitotic exit is not accelerated.

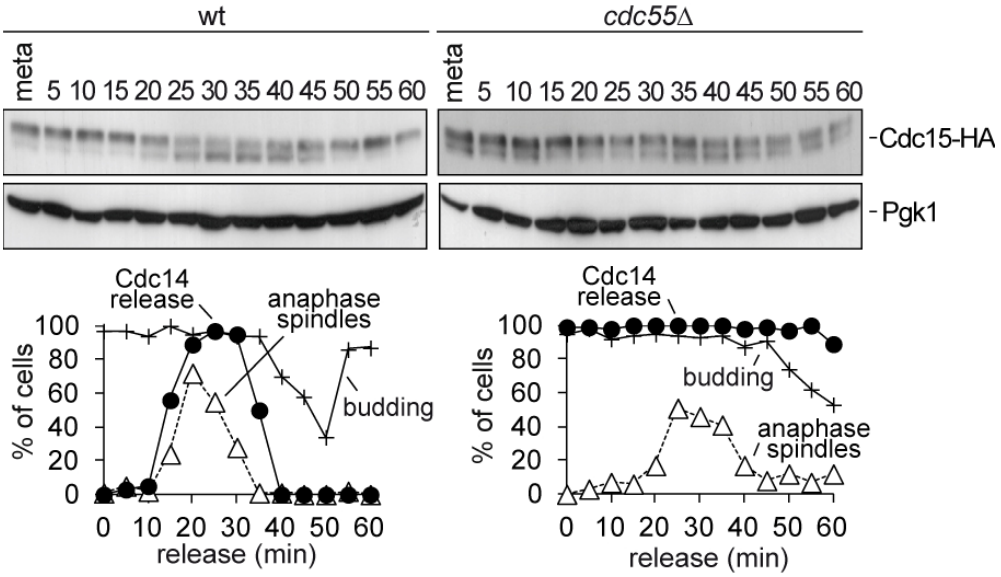



**Figure R4.1. Cdc15 phosphorylation in the absence of Cdc55.** Strains Y2961 (*MATa MET-CDC20 CDC14-Pk$_9$ CDC15-HA$_6$*) and Y528 (as Y2961, but *cdc55Δ*) were released into a synchronous anaphase by Cdc20 depletion and reintroduction. Cdc15 phosphorylation was analyzed by western blot. Pgk1 served as a loading control. Cdc14 release and anaphase spindle dynamics were analyzed by immunofluorescence. The budding index was used to monitor cell cycle progression.

To further study whether the MEN is prematurely activated or not in absence of PP2A$^{Cdc55}$ function, we decided to check late anaphase events that require MEN activation. Cells need to eliminate completely Clb2-Cdk1 activity in order to exit from mitosis and perform the cytokinesis. Cdc14 dephosphorylates Cdh1, an activator of the APC complex in late anaphase, which is responsible for the degradation of mitotic B-type cyclins and the Cdc5 polo kinase among other numerous proteins, including several involved in spindle stability and assembly (Schwab et al., 1997; Charles et al., 1998; Shirayama et al., 1998; Hildebrandt and Hoyt, 2001; Huang et al., 2001; Woodbury and Morgan,

2007; Benanti et al., 2009). Cdc14 also allows Sic1 accumulation, the Cdk1 inhibitor. Cdc14 promotes *SIC1* transcription and the stabilization of Sic1 protein by dephosphorylating Sic1 and its transcription factor Swi5 (Visintin 1999). Therefore, efficient activation of Cdc14 by MEN pathway is required to drive cells into cytokinesis and the next G1 phase. We decided to study Cdh1 dephosphorylation, Cdc5 and Clb2 degradation, and Sic1 accumulation in the *cdc55*Δ mutant (Fig.R4.2). Wild-type and *cdc55*Δ mutant cells were arrested in metaphase by Cdc20 depletion and then released into a synchronous anaphase by Cdc20 re-introduction. Cdh1, Cdc5, Clb2 and Sic1 were analyzed by western blot. Wild-type cells showed a decrease of the Cdh1 slower migration forms at 20-40 minutes when cells are in anaphase, indicating that Cdh1 gets dephosphorylated at that time points. By contrast, *cdc55*Δ mutant cells showed no evident changes in the electrophoretic migration of Cdh1 during anaphase, and only at later time-points, when cells are in G1, a partial dephosphorylation of Cdh1 is observed. This result suggests that Cdh1, a direct target of MEN, is not properly activated in absence of $PP2A^{Cdc55}$ activity. We further confirmed $APC^{Cdh1}$ activation by observing the degradation of its substrates, the Cdc5 polo kinase and Clb2. In wild-type cells, Cdc5 polo kinase and Clb2 degradation started when Cdh1 became active in anaphase (30-40 minutes). However, Cdc5 protein levels were almost constant in the *cdc55*Δ mutant cells in anaphase. On the other hand, Clb2 is only partially degraded at 30 minutes in the *cdc55*Δ mutant, indicative of the first Clb2 degradation by $APC^{Cdc20}$. We can conclude that *cdc55*Δ mutant cells do not show an efficient Cdc5 and Clb2 degradation by $APC^{Cdh1}$.

Finally, we checked the accumulation of Sic1 protein levels, the Cdk1 inhibitor. In wild-type cells, Sic1 protein became detectable during anaphase and persisted until the next G1/S phase. Nevertheless, Sic1 is timely accumulated in *cdc55*Δ mutant cells (note that the *cdc55*Δ mutant released from metaphase with 10 min delayed from the wild-type in this experiment), but remained constant for longer time-points. The *cdc55*Δ mutant does not exit mitosis efficiently as shown by the FACS profile and it presents a longer G1 phase (Queralt et al., 2006). This is consistent with the cells not being able to enter the next S phase until the $APC^{Cdh1}$ substrates are properly degraded during G1. Despite of the slower kinetics in Cdh1 dephosphorylation and Cdc5 polo kinase and Clb2 degradation, Sic1 accumulation is probably enough to inhibit Cdk1 and to exit from mitosis in absence of Cdc55, but not to complete mitosis as efficiently as in the wild-type cells.

All these results together indicate that the premature Bfa1 inactivation in absence of $PP2A^{Cdc55}$ function does not induce a premature MEN activation. On the contrary, absence of $PP2A^{Cdc55}$ activity compromises an efficient mitotic exit.

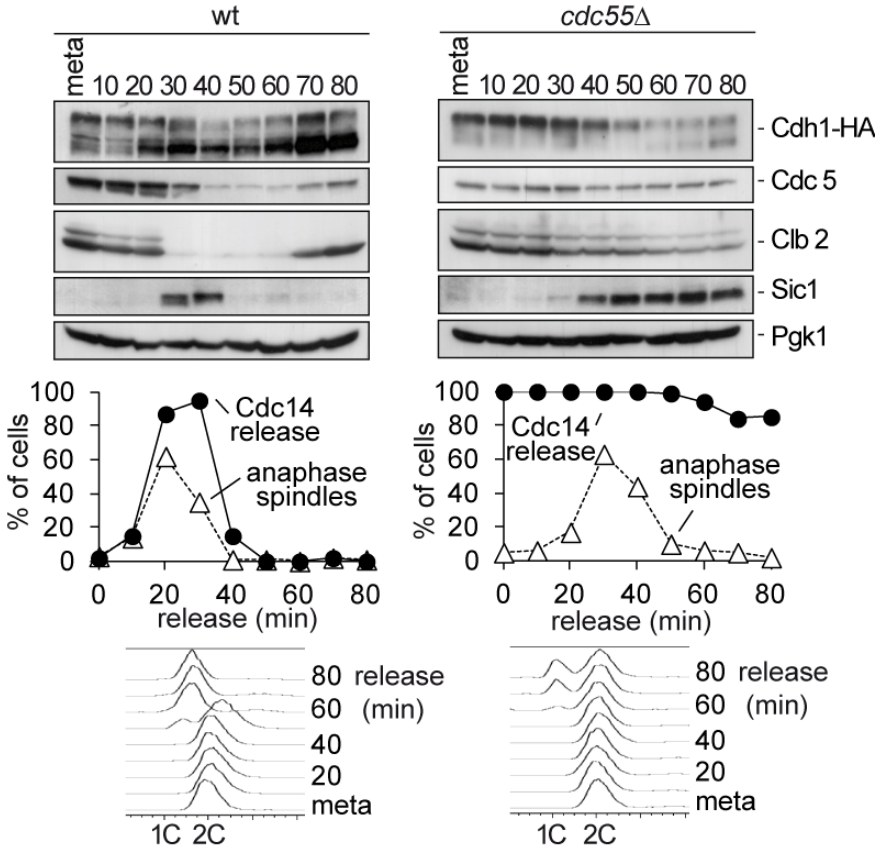



**Figure R4.2. The MEN is not prematurely active in the absence of Cdc55.** Strains Y547 (*MATa MET-CDC20 CDC14-Pk9 HA3-CDH1*) and Y548 (as Y547, but *cdc55Δ*) were released into synchronous anaphase by Cdc20 depletion and reintroduction. Cdh1 phosphorylation and Cdc5, Clb2 and Sic1 proteins levels were analyzed by western blot. Pgk1 served as a loading control. Cdc14 release and anaphase spindle dynamics were analyzed by immunofluorescence. FACS was used to monitor cell cycle progression.

## 5. MEN signalling is transduced until Cdc15 kinase after PP2A$^{Cdc55}$ downregulation.

If PP2A$^{Cdc55}$ counteracts Bfa1 phosphorylation in metaphase and, upon downregulation at anaphase onset, Bfa1 becomes hyper-phosphorylated

and asymmetrically located to the dSPB, why exit from mitosis and MEN activation are not accelerated in *cdc55*Δ cells? Recently, it has been described that increased residence time of Tem1 on SPBs leads to premature Cdc15 loading but does not lead to a premature entry into anaphase (Valerio-Santiago and Monje-Casas, 2011). Moreover, it has been also described that Cdk1 inhibits Cdc15 and Mob1-Dbf2 kinases at the mSPB (König et al., 2010). In order to investigate whether a similar scenario occurs in *cdc55*Δ cells, we examined the Cdc15 and Mob1-Dbf2 activation.

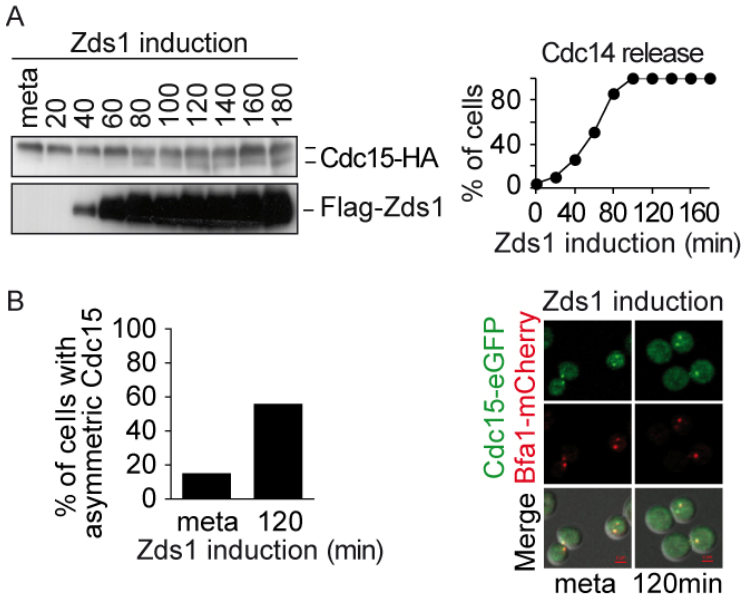



**Figure R5.1. (A) Cdc15 is dephosphorylated upon Zds1-dependent inactivation of PP2A$^{Cdc55}$.** Strain Y603 (*MATa MET-CDC20 GAL1-Flag$_3$-ZDS1 CDC14-Pk$_9$ CDC15-HA$_6$*) was arrested in metaphase by Cdc20 depletion and galactose was added to induce Zds1 expression. Cdc15 phosphorylation and Zds1 expression levels were analyzed by western blot. **(B) Zds1-dependent inactivation of PP2A$^{Cdc55}$ induces Cdc15 asymmetric localization.** Strain Y1014 (*MATα MET-CDC20 GAL1-Flag$_3$-ZDS1 CDC14-Pk$_9$ CDC15-eGFP BFA1-mCherry*) was arrested in metaphase by Cdc20 depletion and Zds1 expression was induced. At least 50 cells were scored for each strain.

Cdc15 kinase is dephosphorylated and activated by early released Cdc14 from the nucleolus, and it has been described to localize preferentially at dSPB in anaphase. Cells were arrested in metaphase by Cdc20 depletion and galactose was added to induce Zds1 ectopic expression ($PP2A^{Cdc55}$ inactivation). Cdc15 phosphorylation status was analyzed by western blot (Fig. R5.1.A). Upon Zds1 induction Cdc15 was dephosphorylated, consistent with the result showed in Fig.R4.1, further confirming Cdc15 is dephosphorylated in absence of $PP2A^{Cdc55}$ activity. We next checked the localization of Cdc15 onto the SPBs upon Zds1-dependent $PP2A^{Cdc55}$ inactivation. After Zds1 induction in metaphase-arrested cells, Cdc15-eGFP asymmetrically located onto one SPB in 53 % of the cells compared to 12% of non-induced cells (Fig. R5.1.B). Therefore, Cdc15 shows an asymmetric localization onto the SPBs upon Zds1-dependent inactivation of $PP2A^{Cdc55}$. In addition, we studied the Cdc15 localization in *cdc55*Δ mutant cells in a metaphase to anaphase transition. Interestingly, in *cdc55*Δ metaphase-arrested cells, Cdc15-eGFP was prematurely asymmetrically located onto the dSPB in 44% of the cells (Fig. R5.2.A). In wild type cells, Cdc15-eGFP was detected preferentially on the dSPB just when cells enter anaphase, as reported previously (Cenamor et al., 1999; Xu et al., 2000; Visintin and Amon, 2001; Menssen et al., 2001; Molk et al., 2004). The quantification of the SPBs ratios confirmed these results (Fig. R5.2.B). In wild type cells, the SPBs ratio increased in anaphase cells with spindle length longer than 6μm, while in *cdc55*Δ mutant cells is already high at metaphase, indicative of asymmetric Cdc15 localization.

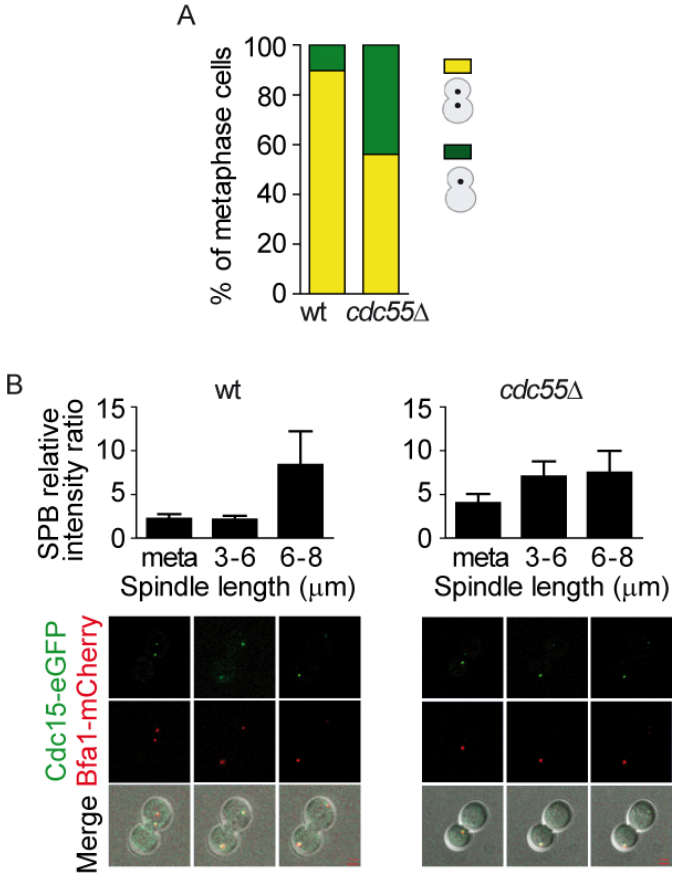



**Figure R5.2. (A) Cdc55 deletion causes a premature Cdc15 asymmetric localization in metaphase.** Strains Y984 (*MATa MET-CDC20 CDC14-myc$_9$ CDC15-eGFP BFA1-mCherry*) and Y966 (as Y984, but *cdc55Δ*) were arrested in metaphase by Cdc20 depletion and the percentage of cells with asymmetric Cdc15-eGFP was quantified. At least 50 cells were scored for each strain. **(B) Cdc15 premature asymmetric localization in the absence of Cdc55 in a metaphase-to-anaphase transition.** The same strains as in (A) were released into a synchronous anaphase by Cdc20 depletion and reintroduction and followed by time-lapse microscopy. (n=14 for WT; n=10 for *cdc55Δ*).

We obtained similar results when looking at Cdc15 localization in a synchronous cell cycle from G1 arrest (Fig. R5.3). At the S/G2 transition,

wild-type cells showed no Cdc15-eGFP loading onto the SPBs, while the *cdc55Δ CDC28Y19F* mutant did. Moreover, when cells enter into anaphase, *cdc55Δ CDC28Y19F* mutant cells showed an increased Cdc15 asymmetric localization onto the dSPB in early-anaphase, comparing to wild-type cells. Therefore, in absence of $PP2A^{Cdc55}$ activity, Cdc15 is prematurely dephosphorylated and loaded onto the dSPB. These results suggest that, upon $PP2A^{Cdc55}$ downregulation at anaphase onset, Tem1 is active and the MEN activation signal is transduced until Cdc15.

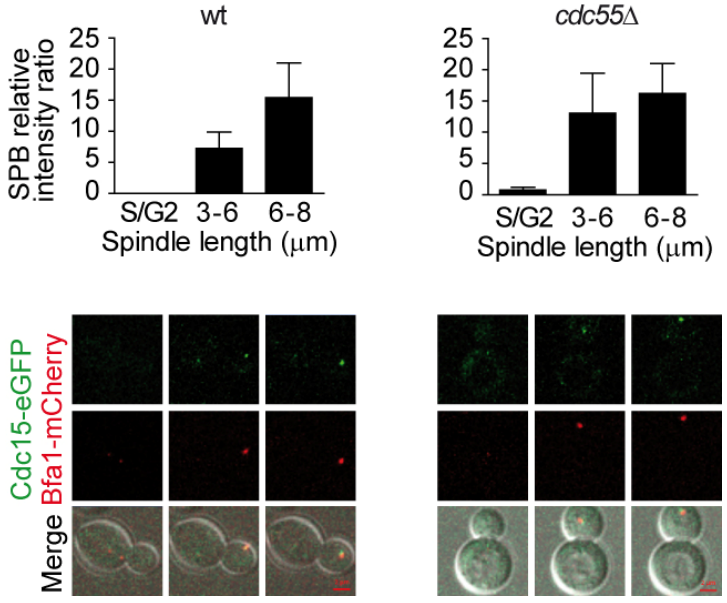



**Figure R5.3. Increased Cdc15 asymmetric localization in absence of Cdc55 in a synchronous cell cycle after G1 release**. Strains Y911 (*MATa CDC28F19 CDC14-9myc CDC15-eGFP BFA1-mCherry*) and Y957 (as Y911, but *cdc55Δ*) were arrested at G1 with α-factor and released into a synchronous cell cycle. Time-lapse microscopy was performed as described in material and methods.

We next studied Mob1-Dbf2 activation upon $PP2A^{Cdc55}$ downregulation. Mob1-Dbf2 complex localizes to both SPBs during anaphase (Visintin and Amon, 2001; Luca et al., 2001); and its localization onto the SPBs is important for Dbf2 kinase activity, but not sufficient (Visintin and Amon, 2001). Dbf2 phosphorylation levels correlate with its kinase activity, being active as a kinase when it is dephosphorylated (Visintin and Amon, 2001). Moreover, it has been described that Cdk1 phosphorylates and inhibits Mob1 (König et al., 2010). Therefore, Mob1-Dbf2 phosphorylation levels and its recruitment into the SPBs are a good marker of Mob1-Dbf2 activity. For this reason, we next studied the Mob1 phosphorylation levels and localization upon Zds1-dependent inactivation of $PP2A^{Cdc55}$. Cells were arrested in metaphase by Cdc20 depletion and Zds1 ectopic expression was induced. Strikingly, Mob1 was not dephosphorylated upon Zds1 induced-$PP2A^{Cdc55}$ inactivation (Fig. R5.4.A). This result suggests that in absence of $PP2A^{Cdc55}$ activity, MEN is not active since the downstream effector Mob1 is not dephosphorylated. Upon Zds1 over-expression, Clb2-Cdk1 kinase activity is still present and maintains Mob1 phosphorylated and inactive. We did not observed any phosphorylation changes in Mob1 upon Esp1-dependent inactivation of $PP2A^{Cdc55}$ (not shown), nor in Dbf2 kinase subunit (Fig. R5.4.B). Consistent with above results, we were not able to detect Mob1-eGFP signal onto the SPBs upon Zds1 induction in metaphase-arrested cells (Fig. R5.4.C). Moreover, Mob1-eGFP signal onto the SPBs increased in anaphase similarly in wild-type and *cdc55*Δ mutant cells (Fig. R5.5). Therefore, we can conclude that in absence of $PP2A^{Cdc55}$ activity, Cdc15 is untimely dephosphorylated and loaded to the dSPB, but a premature mitotic exit is not observe due to Mob1-Dbf2 remaining inactive.

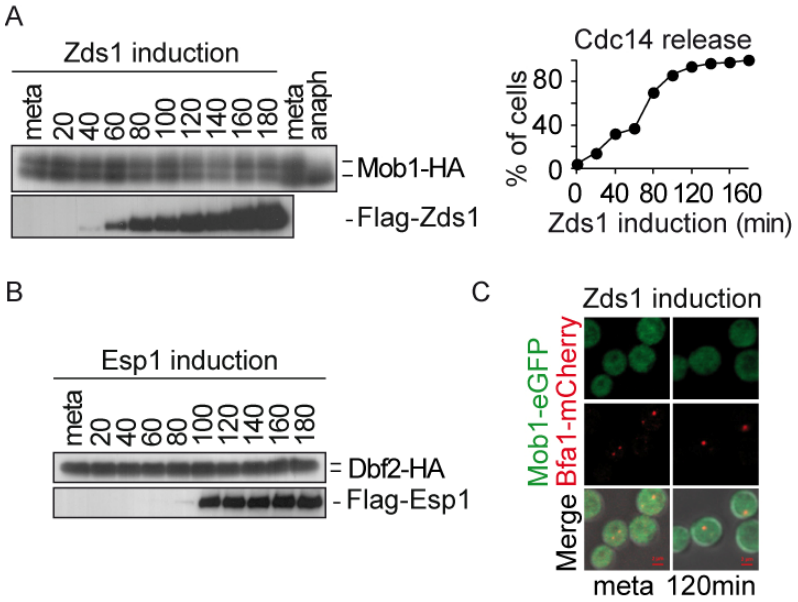



**Figure R5.4. (A) Mob1 phosphorylation is not affected by PP2A$^{Cdc55}$ downregulation after Zds1 induction.** Strain Y807 (*MATα MET-CDC20 GAL1-Flag$_3$-ZDS1 CDC14-Pk$_9$ MOB1-HA$_6$*) was arrested in metaphase by Cdc20 depletion and Zds1 expression was induced. Mob1 phosphorylation and Zds1 expression levels were analyzed by western blot. Anaphase (anaph) sample from a synchronous culture served as a Mob1 dephosphorylation control. Cdc14 release was quantified using immunofluorescence as PP2A$^{Cdc55}$ inactivation control. **(B) Dbf2 phosphorylation is not affected by PP2A$^{Cdc55}$ downregulation after Esp1 induction.** Strain Y781 (*MATα MET-CDC20 GAL1-Flag$_3$-ESP1 CDC14-Pk$_9$ DBF2-HA$_6$*) was arrested in metaphase by Cdc20 depletion and Esp1 expression was induced. Dbf21 phosphorylation and Esp1 expression levels were analyzed by western blot. **(C) Mob1 is not loaded onto the SPB upon Zds1-dependent inactivation of PP2A$^{Cdc55}$.** Strain Y913 (*MATα MET-CDC20 GAL1-Flag$_3$-ZDS1 CDC14-Pk$_9$ BFA1-mCherry MOB1-eGFP*) was arrested in metaphase by Cdc20 depletion and galactose was added to induce Zds1 overexpression. At least 50 cells were scored for each strain.

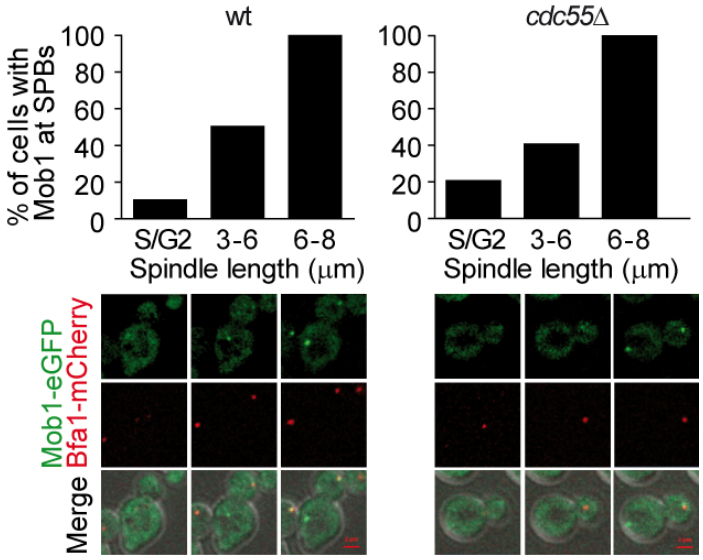



**Figure R5.5. Mob1 loading onto the SPB is restricted to anaphase in the absence of Cdc55.** Strains Y1023 (*MATa MET-CDC20 CDC28F19 CDC14-myc$_9$ MOB1-eGFP BFA1-mCherry*) and Y1076 (as Y1023, but *cdc55Δ*) were released into a synchronous cell cycle after α-factor arrest in G1 and followed by time-lapse microscopy. (n=10 for WT; n=10 for *cdc55Δ*).

## 6. Concerted MEN regulation by Bfa1-Bub2 and Clb2-Cdk1.

The above results suggested that Clb2-Cdk1 inhibitory effect on Cdc15 and Mob1-Dbf2 is the mechanism that restrains full MEN activation in absence of PP2A$^{Cdc55}$ in metaphase-arrested cells. König et al.,. (2010) suggested a concerted MEN regulation by Bfa1-Bub2 and Cdk1. They showed that Cdk1 localizes at the mSPB in anaphase, suggesting that recruitment of Clb2-Cdk1 to the mSPB is an inhibitory mechanism to ensure that the MEN does not become active prematurely. They also showed a mutual regulation of Cdk1 and Cdc15, being Cdc15 required

to recruit Cdk1 to the mSPB, where Cdk1 negatively regulates the binding of Cdc15.

Since Cdc15 showed prematurely asymmetric localization at the dSPB in absence of $PP2A^{Cdc55}$ activity, we next asked whether Cdk1 is prematurely bound to the mSPB upon $PP2A^{Cdc55}$ inactivation. Wild-type and *cdc55*Δ cells were arrested in metaphase by Cdc20 depletion and Cdk1-eGFP localization was analyzed. Cdk1-eGFP was prematurely visualised on the mSPB in 38% of the *cdc55*Δ cells compared to 6% of wild-type cells (Fig. R6.1.A). Note that Cdk1-eGFP signal always opposes Bfa1-mCherry signal. In addition, we studied Cdk1 localization in a metaphase to anaphase transition (Fig R6.1.B). Accordingly to what has been reported, wild-type cells showed an increased Cdk1-eGFP loading onto the SPBs as they progress through anaphase. On the contrary, the *cdc55*Δ mutant showed Cdk1-eGFP prematurely asymmetrically located onto the mSPB in 66% of the metaphase cells compared to 33% in wild-type metaphase cells, consistent with Fig R6.1.A. We also observed that Cdk1-eGFP signal onto the mSPB was reduced during anaphase in *cdc55*Δ mutant cells (38% and 18%), which suggests that Clb2-Cdk1 is not the only mechanism that restrains full MEN activation in the *cdc55*Δ mutant.

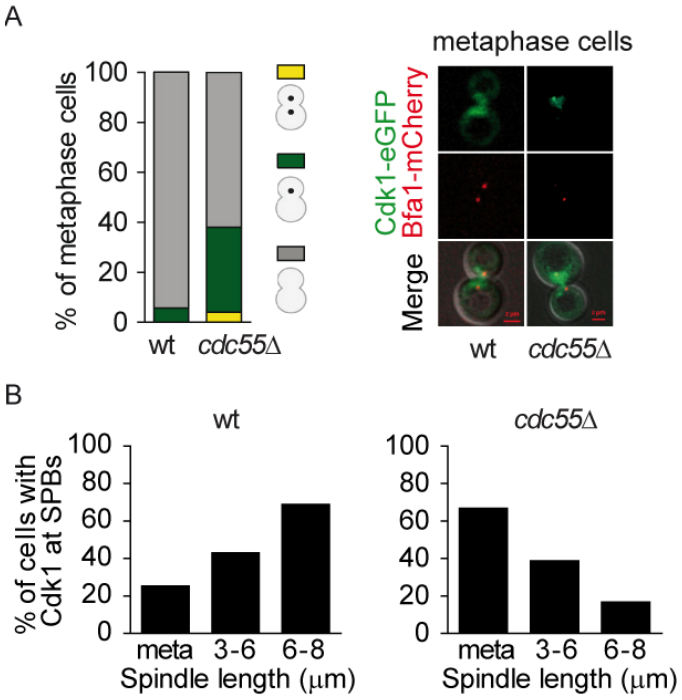



**Figure R6.1. (A) Premature Clb2-Cdk1 localization at the SPB during metaphase in the absence of Cdc55.** Strains Y985 (*MATa MET3-CDC20 CDC28F19 CDC14- myc$_9$ Cdk1-eGFP BFA1-mCherry*) and Y967 (as Y985, but *cdc55Δ*) were arrested in metaphase by Cdc20 depletion. At least 50 cells were scored for each strain. **(B) Premature Clb2-Cdk1 localization at the SPB in a metaphase-to-anaphase transition in the absence of Cdc55.** The same strains as in (A) were released into a synchronous anaphase by Cdc20 depletion and reintroduction and followed by time-lapse microscopy. (n=12 for WT; n=12 for *cdc55Δ*).

To further study Cdk1 inhibitory input on MEN pathway, we inactivate PP2A$^{Cdc55}$ by Zds1 induction and inhibit Cdk1 by adding 1NM-PP1 in cells bearing the ATP analog-sensitive Cdk1 allele, *cdc28-as1*. We arrested cells in metaphase by Cdc20 depletion and induced Zds1 by galactose addition. After 180 min of Zds1 induction, when 80% of cells had released Cdc14 from the nucleolus (indicative of PP2A$^{Cdc55}$

inactivation), 1NM-PP1 drug was added to inhibit Cdk1. Remarkably, upon Cdk1 inactivation Mob1 was dephosphorylated (Fig. R6.2.A). In contrast, when we added DMSO Mob1 phosphorylation status was unchangeable during the experiment, accordingly to results showed in Fig R5.4.A. We can conclude that Cdc14 release from the nucleolus is not enough to promote Mob1 dephosphorylation. Instead, drop in Clb2-Cdk1 kinase activity is prerequisite to dephosphorylate Mob1.

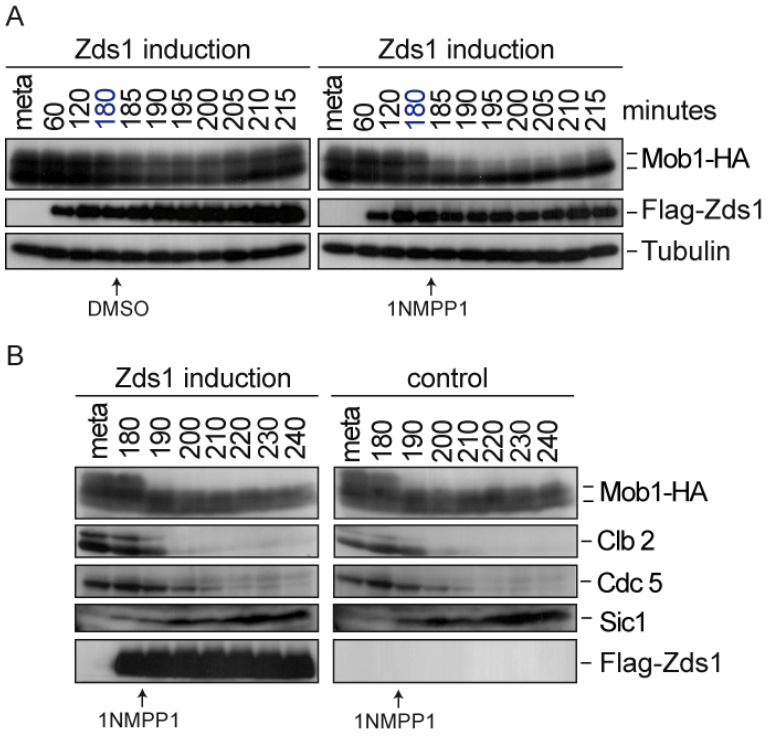


**Figure R6.2. (A). Mob1 dephosphorylation upon Cdk1 inhibition.** Strain Y1032 (*MATa MET3-CDC20 CDC28-as1 GAL1-Flag3-ZDS1 MOB1-HA6*) was arrested in metaphase by Cdc20 depletion and Zds1 was induced. After 180 min of Zds1 induction, 1 μM of 1NM-PP1 drug was added to inhibit Cdk1 to half of the culture and DMSO was added to the other half as a control. **(B). Mob1 dephosphorylation upon Cdk1 inhibition occurs in metaphase-arrested cells.** Strain Y1032 was arrested in metaphase by Cdc20 depletion and 1 μM of 1NM-PP1 drug was added to inhibit Cdk1. We show a Zds1-induced experiment performed in parallel as described in (A).

Surprisingly, Mob1 was also dephosphorylated when we added 1NM-PP1 to metaphase-arrested cells to inactivate Cdk1 (Fig R6.2.B). In this case Cdc14 is not released from the nucleolus when we added 1NM-PP1, strongly supporting Cdc14 release is not required to dephosphorylate Mob1 in these conditions. The fact that Cdc14 is not released during the experiment also suggests that Mob1-Dbf2 complex is not fully active despite Mob1 being dephosphorylated. Moreover, we checked Clb2, Cdc5 and Sic1 protein levels. Upon Cdk1 inactivation, Sic1 was accumulated and Clb2 and Cdc5 were degraded.

Taking all these results together, we conclude that high levels of Clb2-Cdk1 activity maintain Mob1-Dbf2 complex inactive. In this way, $PP2A^{Cdc55}$ downregulation at anaphase onset would initiate both the first wave of Cdc14 release and the MEN pathway. However, the downstream MEN elements would just become active later on, in mid-late anaphase, when Clb2-Cdk1 activity is diminished. This would be a safe mechanism to order the activation of both FEAR and MEN pathways.

## 7. PP2A$^{Cdc55}$ regulates Mob1 phosphorylation.

To screen for new PP2A$^{Cdc55}$ substrates during mitosis, we performed a global study of the PP2A$^{Cdc55}$ phosphoproteome by a quantitative phosphoproteomic analysis based on SILAC labelling (discussed in the next chapter). The SILAC screening revealed a phosphopeptide corresponding to Mob1 protein as being hyper-phosphorylated in *cdc55*Δ mutant cells. The Mob1 peptide contains two S/TP sites: S80 and T85 (Fig. R7.1). Both sites were detected with the highest confidence (pRS site probability around 100% and q-value=0.000168) and the T85 is one of the full Cdk1 consensus sites (S/T-P-x-K/R) previously reported to be phosphorylated by Cdk1 (König et al., 2010). This result suggests that Mob1 could be a PP2A$^{Cdc55}$ substrate.

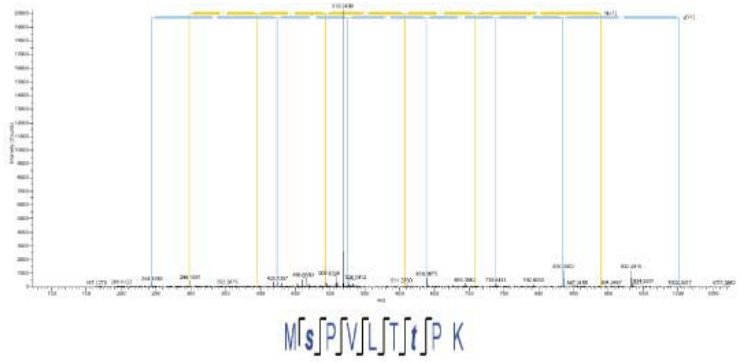



**Figure R7.1. MS$^2$ spectrum of peptide MpSPVLTpTPK, which includes the dually phosphorylated Ser and Thr residues**. Peptide sequence and assigned fragment ions *y* (in blue) and *b* (in yellow) are indicated (pRS site probabilities = 98.8% by Proteome Discoverer Software v 1.3).

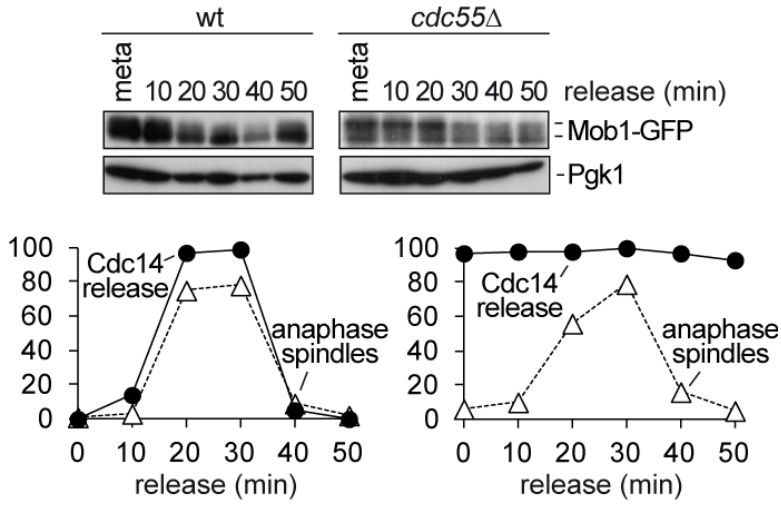



**Figure R7.2. Cdc55 counteracts Mob1 phosphorylation.** Strains Y1109 (*MATa MET-CDC20 CDC14-Pk$_9$ eGFP-MOB1*) and Y1110 (as Y1109, but *cdc55Δ*) were released into a synchronous anaphase by Cdc20 depletion and reintroduction. Mob1 phosphorylation was analyzed by western blot. Cdc14 release and anaphase spindle dynamics were quantified by immunofluorescence.

To further examine the possible role of PP2A$^{Cdc55}$ regulating Mob1 phosphorylation, we compared Mob1 phosphorylation pattern in wild-type and *cdc55*Δ cells, in a metaphase to anaphase transition (Fig. R7.2). We arrested cells in metaphase by Cdc20 depletion and allow them to progress into a synchronous anaphase by Cdc20 re-induction. In wild-type cells, Mob1 decreases its electrophoretic mobility during anaphase, coincident to the spindle elongation and Cdc14 release from the nucleolus. In contrast, Mob1 was hyper-phosphorylated in metaphase in *cdc55*Δ cells, and its high electrophoretic mobility forms were still detected during anaphase. This result indicates that Mob1 dephosphorylation is regulated by PP2A$^{Cdc55}$ activity. Mob1 is dephosphorylated in mid-late anaphase and Cdc14 has been proposed

to contribute to Mob1 dephosphorylation (König et al., 2010). However, our previous results suggested that it must exit another phosphatase capable to dephosphorylate Mob1 (Fig. R5.4.A and R6.2.A). Moreover, we also observed inefficient MEN activation and mitotic exit in the *cdc55*Δ mutant (Fig. R4.2). All these results are in accordance with $PP2A^{Cdc55}$ regulating Mob1 dephosphorylation. $PP2A^{Cdc55}$ is reactivated at the end of mitosis and the next G1 phase (Queralt et al.,. 2006), when Cdk1 activity is very low. In fact, after 1NM-PP1 addition in cells bearing the *cdc28-as1* allele, changes in Net1 phosphorylation pattern suggest a certain recovery of $PP2A^{Cdc55}$ activity (Fig. R7.3.A). Therefore, $PP2A^{Cdc55}$ phosphatase could be dephosphorylating Mob1 in these experiments, independently of Cdc14 release.

Finally, we examined whether Cdc55 and Mob1 physically interact. Co-immunoprecipitation experiments showed that Mob1 co-purified with Cdc55 (Fig. R7.3.B). Altogether these results suggest that Mob1 is likely to be an *in vivo* substrate of $PP2A^{Cdc55}$.

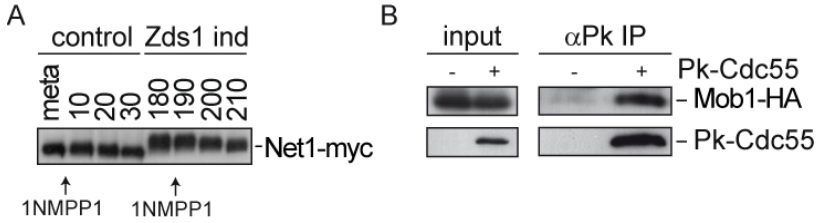



**Figure R7.3. (A) Net1 is dephosphorylated upon Cdk1 inactivation.** Strain Y3407 (*MAT a MET-Cdc20 GAL-3Flag-ZDS1 CDC14-9Pk NET1-myc9 cdc28as-1*) was arrested in metaphase by Cdc20 depletion. 1 µM of 1NM-PP1 drug was added to inhibit Cdk1 to half of the culture. Galactose was added to the other half to induce Zds1 expression and after 180min, 1 µM of 1NM-PP1 drug was added. **(B) Cdc55 and Mob1 interact.** Co-immunoprecipitation between Cdc55 and Mob1 was analyzed in protein extracts from Y1106 (*MATa* $Pk_3$*-CDC55 MOB1-*$HA_6$). Protein extracts from strain Y1104 (*MATa MOB1-*$HA_6$) lacking a Pk epitope on Cdc55 served as a control.

## 8. Screening for new PP2A$^{Cdc55}$ putative substrates

In order to screen for new PP2A$^{Cdc55}$ substrates during mitosis, we performed a global study of the PP2A$^{Cdc55}$ phosphoproteome using a quantitative phosphoproteomic analysis based on *S*table *I*sotope *L*abeling by *A*mino Acids in *C*ell Culture (SILAC) technique (Fig R8.1). Wild-type cells were labelled using $^{13}C_6$-lysine and -arginine (heavy), and *cdc55*Δ mutant cells were grew in presence of unmodified arginine and lysine (light). Cells were arrested in metaphase and protein extracts were prepared as described in material and methods. Phosphopeptide enrichment was performed by SIMAC-based enrichment of phosphopeptides. Phosphopeptide analysis of the heavy/light labelled was done by LC-MS/MS (see material and methods for more details).

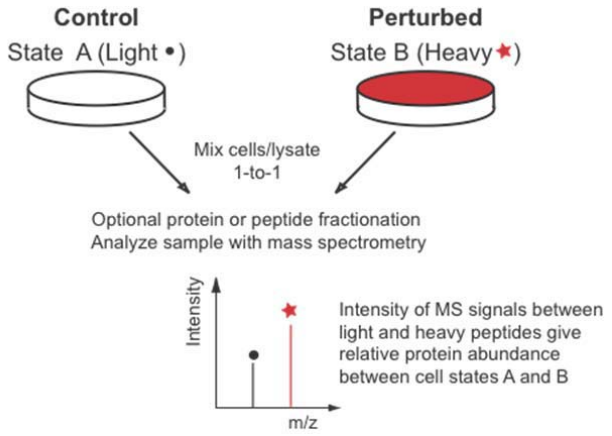



**Figure R8.1. Diagram of the SILAC technique (Ong and Mann, 2007)**

Our screen revealed several phosphopeptides that were significantly increased in the *cdc55*Δ mutant compared to the wild-type. The proteins containing these phosphopeptides are potential substrates of PP2A$^{Cdc55}$.

We performed a protein-protein interaction map of all the putative candidates obtained (Fig. R8.2). Gene onthology classification of all of them was performed using DAVID 6.7 software, and it is summarized in Table 4. We found 22 proteins related to cell cycle (GO Term 0007049), 14 of them corresponding to mitotic cell cycle or M-phase (GO Term 0000278 and 0000279). We also found 11 proteins related to sporulation (GO Term 0043934). Another major category found was cell wall organization (GO Term 0007047), with 10 proteins. Membrane organization and invagination was another major group, form by 11 proteins (GO Terms 0016044 and 0010324), 6 of them from endocytosis (GO Term 0006897). We found 8 proteins related to cell morphogenesis (GO Term 0000902), most of them related to cell polarity establishment (GO Term 0030010) and 5 of them falling into the categories of cytokinesis and bud/cytokinesis site selection (Go Terms 0033205, 0000282 and 0007105). We found 9 proteins of cytoskeleton organization (GO Term 0007010), 4 of them related to the mitotic spindle organization (GO Term 0007052). Establishment of organelle localization was also represented by 5 proteins (GO Term 0051656). Finally, we found proteins related to the cell response to external stimuli and metabolic processes. Response to osmotic stress stands out with 10 proteins (GO Term 0006970), followed by response to nutrients, represented by 5 proteins (GO Term 0031669). We found 5 proteins of carbohydrate/alcohol catabolic process (GO Term 0044275), 4 proteins of amino acids transport (GO Term 0006865) and other 4 proteins related to cofactor transport (GO Term 0051181).

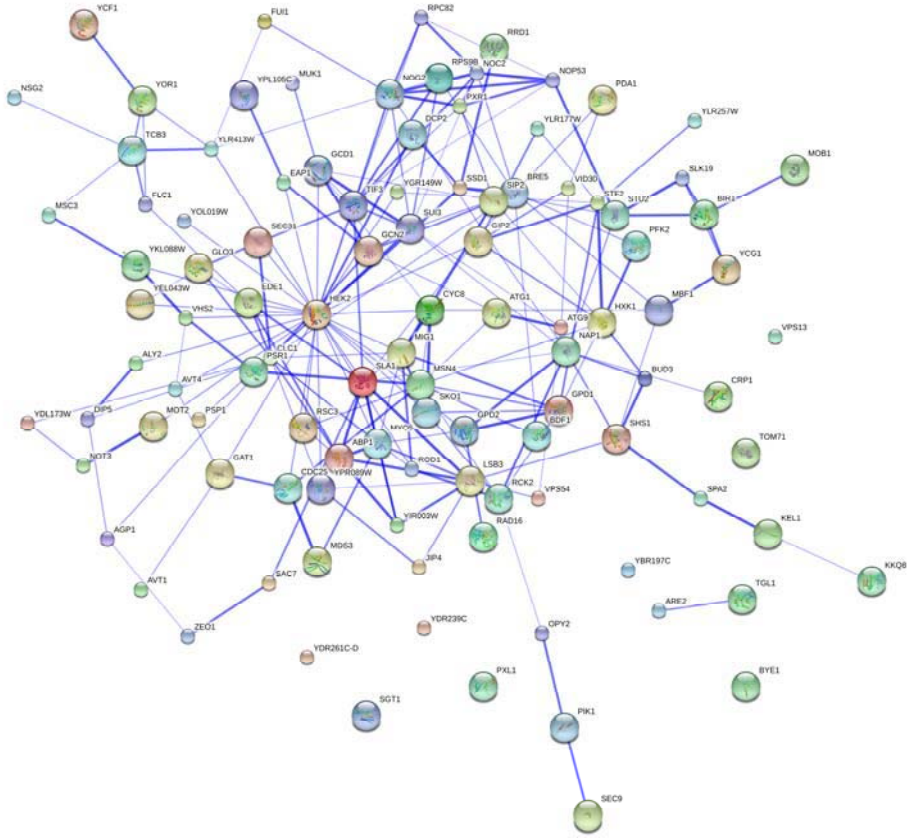



**Figure R8.2. Protein-protein interaction map of SILAC-detected peptides**

| GO Category | | Genes |
|---|---|---|
| Cell cycle | 22 | *MYO5, OPY2, SLA1, SPA2, SHS1, SAC7, SGT1, BUD3* |
| Mitotic cell cycle and M-phase | (14) | *MDS3, BDF1, MOB1, STU2, MSC3, NAP1, BIR1, RRD1, YCG1, SSD1, SLK19, CDC25, RSC3, VHS2* |
| Sporulation | 11 | *MDS3, ABP1, SLA1, GIP2, VPS54, BDF1, EDE1, SEC9, VPS13, ATG1, CDC25* |
| Cell wall organization | 10 | *ZEO1, MYO5, SLA1, VPS54, EDE1, LSB3, SSD1, FLC1, SPA2, SHS1* |
| Membrane organization and invagination | 11 | *ATG9, KEL1, GLO3, SEC9, ATG1* |
| Endocytosis | (6) | *CLC1, MYO5, SLA1, ROD1, EDE1, ALY2* |
| Cell morphogenesis and polarity | 8 | *ABP1, PXL1, NAP1* |
| Cytokinesis and bud/cytokinesis site selection | (5) | *MYO5, SLA1, BUD3, SPA2, SHS1* |
| Cytoskeleton organization | 9 | *ABP1, MYO5, SLA1, SAC7, SPA2* |
| Mitotic spindle organization | (4) | *STU2, BIR1, RRD1, SLK19* |
| Establishment of organelle localization | 5 | *GLO3, NOP53, BIR1, NOG2, NOC2* |
| Response to osmotic stress | 10 | *PRS1, GPD1, ABP1, MYO5, STF2, OPY2, SLA1, MSN4, RCK2, RRD1* |
| Response to nutrients levels | 5 | *ATG9, SIP2, MSN4, MIG1, ATG1* |
| Alcohol catabolic process | 5 | *GPD2, GPD1, PFK2, PDA1, HXK1* |
| Amino acids transport | 4 | *AVT4, DIP5, AVT1, AGP1* |
| Cofactor transport | 4 | *PXL1, LSB3, FLC1, SHS1* |

**Table 4. Gene Onthology classification of PP2A$^{Cdc55}$ substrate candidates**

On the other hand, we were interested in the kinases counteracted by $PP2A^{Cdc55}$. It has been shown that $PP2A^{Cdc55}$ phosphatase can counteract Cdk1 phosphorylation (Queralt et al., 2006) and Cdc5 phosphorylation (Yaakov et al., 2012 and our results). We detected a set of phosphopeptides that contained Cdk1 phosphorylation consensus sites (Table 5). We also screen for the several Cdc5 consensus sites described, obtaining another set of phosphopeptides (Table 6). Taking the rest of the phosphopeptides, which do not have any Cdk1 or Cdc5 consensus site, we scan for other kinases consensus sites using Motif-X software, in order to detect other possible kinases that are counteracted by $PP2A^{Cdc55}$. We found the consensus motif RXXS, being S the phosphoserine and the central residue used for the screening (Fig R8.3). The RXXS motif was also detectable when using the whole set of significantly increased phosphopeptides. The proteins containing RXXS sites are listed in Table 7. Finally, we scan for other putative phosphorylation motifs across the rest of phosphopeptides, which did not show any Cdk1, Cdc5 or RXXS consensus site, but motif-X did not detect any other motif.


**Figure R8.3. RXXS motif logo obtained with Motif-X software.**

| PETIDE SEQUENCE | GENE | MODIFICATION | pRS SITE PROBABILITIES | q-VALUE |
|---|---|---|---|---|
| TEQDDILETEAsPAK | *CLC1* | S12(Phospho) | T(1): 3.5; T(9): 48.2; S(12): 48.2 | 0,000058 |
| AHNVSTSNNsPSTDNDSISK | YDR261C-D | S10(Phospho) | S(5): 32.4; T(6): 32.4; S(7): 32.4; S(10): 2.6; S(12): 0.3; T(13): 0.0; S(17): 0.0; S(19): 0.0 | 0,000058 |
| DVSQITSsPK | *RCK2* | S8(Phospho) | S(3): 0.0; T(6): 0.0; S(7): 0.3; S(8): 99.7 | 0,000058 |
| EEESIEQANAGsPGR | *RRD1* | S12(Phospho) | S(4): 27.1; S(12): 72.9 | 0,000168 |
| FtAPTSPSTSsPK | *LSB3* | T2(Phospho); S11(Phospho) | T(2): 100.0; T(5): 0.0; S(6): 0.4; S(8): 93.5; T(9): 5.6; S(10): 0.4; S(11): 0.0 | 0,000058 |
| GGAGNIIsPK | *PAR32* | S8(Phospho) | S(8): 100.0 | 0,000257 |
| GNNIGsPLGAPK | *MBF1* | S6(Phospho) | S(6): 100.0 | 0,000058 |
| GPEQLKsPEVQR | *SPA2* | S7(Phospho) | S(7): 100.0 | 0,000058 |
| IIEEHEsPIDAEK | YLR413W | S7(Phospho) | S(7): 100.0 | 0,000058 |
| ITQQEVsPDR | *FLC1* | S7(Phospho) | T(2): 0.5; S(7): 99.5 | 0,000168 |
| msPVLTtPK | *MOB1* | M1(Oxidation); S2(Phospho); T7(Phospho) | S(2): 100.0; T(6): 0.1; T(7): 99.9 | 0,000168 |
| NEAtPEAEQVK | *ZEO1* | T4(Phospho) | T(4): 100.0 | 0,000058 |
| NNDEEDDEDPVsPKPVSK | *MDS3* | S12(Phospho) | S(12): 98.8; S(17): 1.2 | 0,000213 |
| NPISSTVSSNQQsPk | *DCP2* | S13(Phospho); K15(Label:13C(6)) | S(4): 7.7; S(5): 7.7; T(6): 16.8; S(8): 43.3; S(9): 16.8; S(13): 7.7 | 0,000058 |
| NQSQQPQQQLsPFR | *SSD1* | S11(Phospho) | S(3): 0.1; S(11): 99.9 | 0,000058 |
| SFTPSKsPAPVSK | *ABP1* | S7(Phospho) | S(1): 0.5; T(3): 5.6; S(5): 5.6; S(7): 87.8; S(12): 0.5 | 0,000213 |
| sPQENTLPR | *CYC8* | S1(Phospho) | S(1): 100.0; T(6): 0.0 | 0,000058 |
| SPsPDPASLSSESEr | *VPS13* | S3(Phospho); R15(Label:13C(6)) | S(1): 50.0; S(3): 50.0; S(8): 0.0; S(10): 0.0; S(11): 0.0; S(13): 0.0 | 0,000058 |
| TEQDDILETEAsPAK | *CLC1* | S12(Phospho) | T(1): 3.5; T(9): 48.2; S(12): 48.2 | 0,000058 |

| | | | | |
|---|---|---|---|---|
| tPTQPIr | *MOT2* | T1(Phospho); R7(Label:13C(6)) | T(1): 99.9; T(3): 0.1 | 0,000306 |
| TSIQNSTLEDFsPSNK | *CDC25* | S12(Phospho) | T(1): 2.1; S(2): 2.1; S(6): 9.7; T(7): 9.7; S(12): 66.7; S(14): 9.7 | 0,000058 |
| TStPTTMLsR | *MIG1* | T3(Phospho); S9(Phospho) | T(1): 3.1; S(2): 3.1; T(3): 92.4; T(5): 1.1; T(6): 1.6; S(9): 98.7 | 0,000899 |

**Table 5. SILAC identified peptides containing Cdk1/MAPK consensus phosphosites (S/TP).**

| PETIDE SEQUENCE | GENE | MODIFICATION | pRS SITE PROBABILITIES | q-VALUE |
|---|---|---|---|---|
| **D/E-X-S/T-φ-X-D/E (Cdc5 full consensus)** | | | | |
| EEAsDDELNAFK | *JIP4* | S4(Phospho) | S(4): 100.0 | 0,000058 |
| KAEAsGEAAEEAEDEE | *RPS9B* | S5(Phospho) | S(5): 100.0 | 0,000058 |
| **E/D-X-S/T** | | | | |
| DDsFAVPDGK | *DIP5* | S3(Phospho) | S(3): 100.0 | 0,000058 |
| DSsTNILIR | *BIR1* | S3(Phospho) | S(2): 33.3; S(3): 33.3; T(4): 33.3 | 0,000058 |
| EEAsDDELNAFK | *JIP4* | S4(Phospho) | S(4): 100.0 | 0,000058 |
| ITQQEVsPDR | *FLC1* | S7(Phospho) | T(2): 0.5; S(7): 99.5 | 0,000168 |
| KAEAsGEAAEEAEDEE | *RPS9B* | S5(Phospho) | S(5): 100.0 | 0,000058 |
| KLEELEQTQDNsK | *DCP2* | S12(Phospho) | T(8): 0.2; S(12): 99.8 | 0,000058 |
| LVQIAEEEsDK | *OPY2* | S9(Phospho) | S(9): 100.0 | 0,000168 |
| NEAtPEAEQVk | *ZEO1* | T4(Phospho); K11(Label:13C(6)) | T(4): 100.0 | 0,000058 |
| NIDLsDVEQYmEk | *NOP53* | S5(Phospho); M11(Oxidation); K13(Label:13C(6)) | S(5): 100.0; Y(10): 0.0 | 0,000257 |
| REStEGVLDGSK | *CRP1* | T4(Phospho) | S(3): 88.9; T(4): 11.1; S(11): 0.0 | 0,000058 |
| SNNEVTEHsDSEDLTEK | *AIM21* | S9(Phospho) | S(1): 14.0; T(6): 63.9; S(9): 14.0; S(11): 4.1; T(15): 4.1 | 0,000058 |
| TEQDDILETEAsPAK | *CLC1* | S12(Phospho) | T(1): 3.5; T(9): 48.2; S(12): 48.2 | 0,000058 |

| | | | | |
|---|---|---|---|---|
| TSIQNSTLEDFsPSNK | *CDC25* | S12(Phospho) | T(1): 2.1; S(2): 2.1; S(6): 9.7; T(7): 9.7; S(12): 66.7; S(14): 9.7 | 0,000058 |

**Table 6. SILAC identified peptides containing Cdc5 consensus phosphosites.**

| PETIDE SEQUENCE | GENE | MODIFICATION | pRS SITE PROBABILITIES | q-VALUE |
|---|---|---|---|---|
| SsAQEDAPIVIR | *BDF1* | S2(Phospho) | S(1): 50.0; S(2): 50.0 | 0,000058 |
| sSAQEDAPIVIR | *BDF1* | S1(Phospho) | S(1): 50.0; S(2): 50.0 | 0,000058 |
| SDsAVSIVHLk | *GPD2* | S3(Phospho); K11(Label:13C(6)) | S(1): 14.0; S(3): 85.9; S(6): 0.1 | 0,000058 |
| sDSAVSIVHLK | *GPD2* | S1(Phospho) | S(1): 1.1; S(3): 98.8; S(6): 0.1 | 0,000058 |
| sFNVGSR | *GCN2* | S1(Phospho) | S(1): 100.0; S(6): 0.0 | 0,000306 |
| sIHDESNFER | *CAB3* | S1(Phospho) | S(1): 99.9; S(6): 0.1 | 0,000058 |
| SDsASFLEEK | *HEK2* | S3(Phospho) | S(1): 2.8; S(3): 11.3; S(5): 85.9 | 0,000058 |
| AAsFQDSTIPDAr | *NOG2* | S3(Phospho); R13(Label:13C(6)) | S(3): 98.1; S(7): 0.9; T(8): 0.9 | 0,000058 |
| SGsNAAASLPSk | *RPC82* | S3(Phospho); K12(Label:13C(6)) | S(1): 2.7; S(3): 96.1; S(8): 0.6; S(11): 0.6 | 0,000058 |
| sAINIETESR | *VID30* | S1(Phospho) | S(1): 99.9; T(7): 0.0; S(9): 0.0 | 0,000058 |
| KGsMADVPK | *HXK1* | S3(Phospho) | S(3): 100.0 | 0,000058 |
| KGsmADVPK | *HXK1* | S3(Phospho); M4(Oxidation) | S(3): 100.0 | 0,000058 |
| NDsFNINTDTLK | *SLK19* | S3(Phospho) | S(3): 98.5; T(8): 1.0; T(10): 0.5 | 0,000058 |
| REsVQDNLPSTIR | *ALY2* | S3(Phospho) | S(3): 100.0; S(10): 0.0; T(11): 0.0 | 0,000058 |
| DDsFAVPDGK | *DIP5* | S3(Phospho) | S(3): 100.0 | 0,000058 |
| RLsAYLESSK | *TGL1* | S3(Phospho) | S(3): 95.9; Y(5): 3.1; S(8): 0.8; S(9): 0.3 | 0,000058 |
| sMENVTPK | *AVT4* | S1(Phospho) | S(1): 100.0; T(6): 0.0 | 0,000213 |
| RQsEAFAGQNEDEADLK | *TOM71* | S3(Phospho) | S(3): 100.0 | 0,000402 |
| KNsAVTTAPAQK | *SKO1* | S3(Phospho) | S(3): 100.0; T(6): 0.0; T(7): 0.0 | 0,000058 |

| | | | | |
|---|---|---|---|---|
| sFSESFK | *KKQ8* | S1(Phospho) | S(1): 98.1; S(3): 1.7; S(5): 0.1 | 0,000058 |
| SmsLLGYr | *MYO5* | M2(Oxidation); S3(Phospho); R8(Label:13C(6)) | S(1): 0.0; S(3): 100.0; Y(7): 0.0 | 0,000058 |
| KLsEDGVTDGDGKPIPESER | *RSC3* | S3(Phospho) | S(3): 85.8; T(8): 14.2; S(18): 0.0 | 0,000058 |
| TGsIDLISNNNK | YEL043W | S3(Phospho) | T(1): 84.6; S(3): 15.1; S(8): 0.3 | 0,000058 |
| VPsLVAtSESPr | *SEC31* | S3(Phospho); T7(Phospho); R12(Label:13C(6)) | S(3): 91.4; T(7): 12.9; S(8): 12.9; S(10): 82.7 | 0,000991 |
| VPSLVAtSESPR | *SEC31* | T7(Phospho) | S(3): 0.0; T(7): 13.1; S(8): 73.8; S(10): 13.1 | 0,000058 |
| NAsTGSLQASVK | *PXL1* | S3(Phospho) | S(3): 79.6; T(4): 12.8; S(6): 3.8; S(10): 3.8 | 0,000058 |
| SSsSVSLK | *GPD1* | S3(Phospho) | S(1): 0.9; S(2): 6.9; S(3): 78.3; S(4): 6.9; S(6): 6.9 | 0,000058 |
| DSsTNILIR | *BIR1* | S3(Phospho) | S(2): 33.3; S(3): 33.3; T(4): 33.3 | 0,000058 |

**Table 7. SILAC identified peptides containing RXXS phosphosite motif.**

| PETIDE SEQUENCE | GENE | MODIFICATION | pRS SITE PROBABILITIES | q-VALUE |
|---|---|---|---|---|
| AADVENLsDDDEHR | *BUD3* | S8(Phospho) | S(8): 100.0 | 0,000058 |
| AGLDNVDAEsk | *SUI3* | S10(Phospho); K11(Label:13C(6)) | S(10): 100.0 | 0,000058 |
| AIsSDQLFGR | *GLO3* | S3(Phospho) | S(3): 14.6; S(4): 85.4 | 0,000058 |
| AQNDsEEEQVK | *NAP1* | S5(Phospho) | S(5): 100.0 | 0,000402 |
| DFIIDLTGsDK | *RAD16* | S9(Phospho) | T(7): 1.2; S(9): 98.8 | 0,000213 |
| GDNNSsHSPISPLK | *SGT1* | S6(Phospho) | S(5): 8.0; S(6): 8.0; S(8): 8.0; S(11): 75.9 | 0,000058 |
| IVVPEGSPsr | *VPS54* | S9(Phospho); R10(Label:13C(6)) | S(7): 98.0; S(9): 2.0 | 0,000058 |
| EsSIPVEGELEQLQK | *YCF1* | S2(Phospho) | S(2): 50.0; S(3): 50.0 | 0,000257 |
| EVDPNIsESNILPSK | YDR261C-D | S7(Phospho) | S(7): 87.3; S(9): 11.8; S(14): 0.9 | 0,000213 |

| | | | | |
|---|---|---|---|---|
| FDIDADsEANLr | YOL019W | S7(Phospho); R12(Label:13C(6)) | S(7): 100.0 | 0,000168 |
| FTNNDmDsIVVK | *AVT1* | M6(Oxidation); S8(Phospho) | T(2): 1.1; S(8): 98.9 | 0,000058 |
| GsFDEAANR | *GLO3* | S2(Phospho) | S(2): 100.0 | 0,000058 |
| GVHQTNSPPsK | *PSR1* | S10(Phospho) | T(5): 0.5; S(7): 2.1; S(10): 97.4 | 0,000058 |
| IEQTHAIsK | *BYE1* | S8(Phospho) | T(4): 0.4; S(8): 99.6 | 0,000058 |
| KLEELEQTQDNsK | *DCP2* | S12(Phospho) | T(8): 0.2; S(12): 99.8 | 0,000058 |
| LGPQsMSr | *GCD1* | S5(Phospho); R8(Label:13C(6)) | S(5): 16.8; S(7): 83.2 | 0,000058 |
| LQPDSDAVIsDAsVNDK | YGR149W | S10(Phospho); S13(Phospho) | S(5): 6.4; S(10): 96.1; S(13): 97.5 | 0,000058 |
| LVQIAEEEsDK | *OPY2* | S9(Phospho) | S(9): 100.0 | 0,000168 |
| NDTYTDLASIAsGR | *SHS1* | S12(Phospho) | T(3): 1.2; Y(4): 1.2; T(5): 1.2; S(9): 3.5; S(12): 93.0 | 0,000402 |
| NFDIIsENSNDVR | *AGP1* | S6(Phospho) | S(6): 95.1; S(9): 4.9 | 0,000058 |
| NIDLsDVEQYmEk | *NOP53* | S5(Phospho); M11(Oxidation); K13(Label:13C(6)) | S(5): 100.0; Y(10): 0.0 | 0,000257 |
| NNsFEHDNLEK | *TAE2* | S3(Phospho) | S(3): 100.0 | 0,000058 |
| NsEVDLNEEPR | *SEC9* | S2(Phospho) | S(2): 100.0 | 0,000058 |
| NsNILTAPAVK | *ATG1* | S2(Phospho) | S(2): 99.6; T(6): 0.4 | 0,000058 |
| NTVSsNNLER | *NSG2* | S5(Phospho) | T(2): 1.8; S(4): 10.5; S(5): 87.7 | 0,000213 |
| NVsFVLPDEK | *YCG1* | S3(Phospho) | S(3): 100.0 | 0,000213 |
| REStEGVLDGSK | *CRP1* | T4(Phospho) | S(3): 88.9; T(4): 11.1; S(11): 0.0 | 0,000058 |
| RQsEAFAGQNEDEADLK | *TOM71* | S3(Phospho) | S(3): 100.0 | 0,000402 |
| sEDNEIYEELK | *NSG2* | S1(Phospho) | S(1): 100.0; Y(7): 0.0 | 0,000058 |
| sEQmELEK | *NOC2* | S1(Phospho); M4(Oxidation) | S(1): 100.0 | 0,000213 |
| SGGFGGsFGGR | *TIF3* | S7(Phospho) | S(1): 15.3; S(7): 84.7 | 0,000862 |
| SIsSSLNR | *MSN4* | S3(Phospho) | S(1): 97.7; S(3): 1.1; S(4): 1.1; S(5): 0.2 | 0,000058 |
| sLADLPGK | YLR177W | S1(Phospho) | S(1): 100.0 | 0,000213 |

| | | | | |
|---|---|---|---|---|
| sLQQVSk | YDR239C | S1(Phospho); K7(Label:13C(6)) | S(1): 100.0; S(6): 0.0 | 0,000257 |
| sLSDLPr | *PSP1* | S1(Phospho); R7(Label:13C(6)) | S(1): 96.5; S(3): 3.5 | 0,000862 |
| SmsSENITVPR | *SAC7* | M2(Oxidation); S3(Phospho) | S(1): 7.0; S(3): 85.9; S(4): 7.0; T(8): 0.1 | 0,000058 |
| SmSsENITVPR | *SAC7* | M2(Oxidation); S4(Phospho) | S(1): 0.5; S(3): 99.0; S(4): 0.5; T(8): 0.0 | 0,000058 |
| SNNEVTEHsDSEDLTEK | *AIM21* | S9(Phospho) | S(1): 14.0; T(6): 63.9; S(9): 14.0; S(11): 4.1; T(15): 4.1 | 0,000058 |
| sQQAISNLFQK | *EAP1* | S1(Phospho) | S(1): 99.9; S(6): 0.1 | 0,000058 |
| SSEsASNIPDAVNTR | *PXR1* | S4(Phospho) | S(1): 13.4; S(2): 13.4; S(4): 13.4; S(6): 59.8; T(14): 0.1 | 0,000058 |
| SsESASNIPDAVNTR | *PXR1* | S2(Phospho) | S(1): 25.0; S(2): 25.0; S(4): 25.0; S(6): 25.0; T(14): 0.0 | 0,000058 |
| SSEsIGDLPHR | *STU2* | S4(Phospho) | S(1): 8.6; S(2): 8.6; S(4): 82.9 | 0,000058 |
| SsIALQIGK | *SIP2* | S2(Phospho) | S(1): 50.0; S(2): 50.0 | 0,000306 |
| SSISNTsDHDGANR | *IBI2* | S7(Phospho) | S(1): 29.6; S(2): 29.6; S(4): 29.6; T(6): 5.6; S(7): 5.6 | 0,000213 |
| SSsQEGNPQLVQLK | YBR197C | S3(Phospho) | S(1): 33.3; S(2): 33.3; S(3): 33.3 | 0,000058 |
| SSSSVsLK | *GPD1* | S6(Phospho) | S(1): 5.9; S(2): 5.9; S(3): 29.4; S(4): 29.4; S(6): 29.4 | 0,000257 |
| SStNLAALPK | *ROD1* | T3(Phospho) | S(1): 33.3; S(2): 33.3; T(3): 33.3 | 0,000058 |
| SYTsVAELNR | YLR257W | S4(Phospho) | S(1): 8.1; Y(2): 8.1; T(3): 75.8; S(4): 8.1 | 0,000058 |
| TITVGDAVSEtELENk | *YOR1* | T11(Phospho); K16(Label:13C(6)) | T(1): 25.0; T(3): 25.0; S(9): 25.0; T(11): 25.0 | 0,000168 |
| TVsNNAANSLSR | *VHS2* | S3(Phospho) | T(1): 7.0; S(3): 92.2; S(9): 0.7; S(11): 0.1 | 0,000058 |
| VHsYTDLAYR | *PFK2* | S3(Phospho) | S(3): 97.4; Y(4): 2.2; T(5): 0.4; Y(9): 0.0 | 0,000058 |
| YGGHsmSDPGTTYR | *PDA1* | S5(Phospho); M6(Oxidation) | Y(1): 2.5; S(5): 94.9; S(7): 2.5; T(11): 0.0; T(12): 0.0; Y(13): 0.0 | 0,000058 |

| | | | | |
|---|---|---|---|---|
| YPAPGTSPSHNEGNsk | *EDE1* | S15(Phospho); K16(Label:13C(6)) | Y(1): 1.1; T(6): 2.8; S(7): 2.8; S(9): 2.8; S(15): 90.4 | 0,000597 |

**Table 8. Other SILAC identified peptides, which does not contain any motif detectable using Motif-X**

## 9. Docking modelling of PP2A$^{Cdc55}$ and its substrates

The focus of molecular docking is to computationally simulate the molecular recognition process between an enzyme and its substrates. The aim of molecular docking is to achieve an optimized conformation for both the protein and the ligand and the relative orientation between both such that the free energy of the overall system is minimized.

We performed a docking modelling of Cdc55 and its new described substrates, Bfa1 and Mob1. We used the available crystal estrcuture of Mob1, and we construct a 3D model structure of Cdc55 and Bfa1. For Cdc55, we used homology modelling taking advantatge of the crystal structures available from other organisms (i.e. B55); and for Bfa1 we used protein threading method of modelling. From the docking modelling we obtained several models ordered by the free-energy of the system. We analyzed the first 10 models of minimum energy of either Cdc55-Bfa1 or Cdc55-Mob1. We discarded the models where the location of Bfa1 or Mob1 could interfere with the interaction between Cdc55 and PP2A catalytic subunit, and Cdc55-PP2A scaffold subunit (Tpd3). Most of the remaining models showed Mob1 and Bfa1 interacting with the groove structure located in Cdc55, which has been proposed to be the region that interacts with the substrate in the human PP2A-B55 structure (Xu et al., 2008). We compared the Cdc55 aminoacids at the interaction region (located at 3.5 Å or less from any Bfa1/Mob1 residue)

of Cdc55-Bfa1 and Cdc55-Mob1 models involving the groove, and we found some aminoacids in common for both substrates (Table 9 and Fig R9.1).

| | |
|---|---|
| Cdc55 residues at 3.5A from Bfa1 and Mob1 | Glu24, Leu27, Ser80, Glu230, Leu232, Thr233, Phe288, Thr289, Glu290, Glu345, Tyr344, Lys497 |

**Table 9. Putative Cdc55 residues that interact with Bfa1 and Mob1**

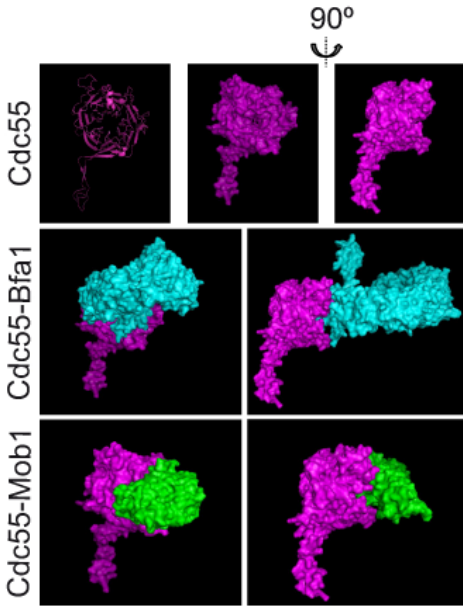



**Figure R9.1. 3D structure of Cdc55. Docking modelling of Cdc55-Bfa1 and Cdc55-Mob1**

Interestingly, most of the Cdc55 aminoacids predicted to interact with Bfa1 and Mob1 are conserved in humans, and some are coincident with the B55 aminoacids that interact with pTau *in vivo* (Fig. R9.2).

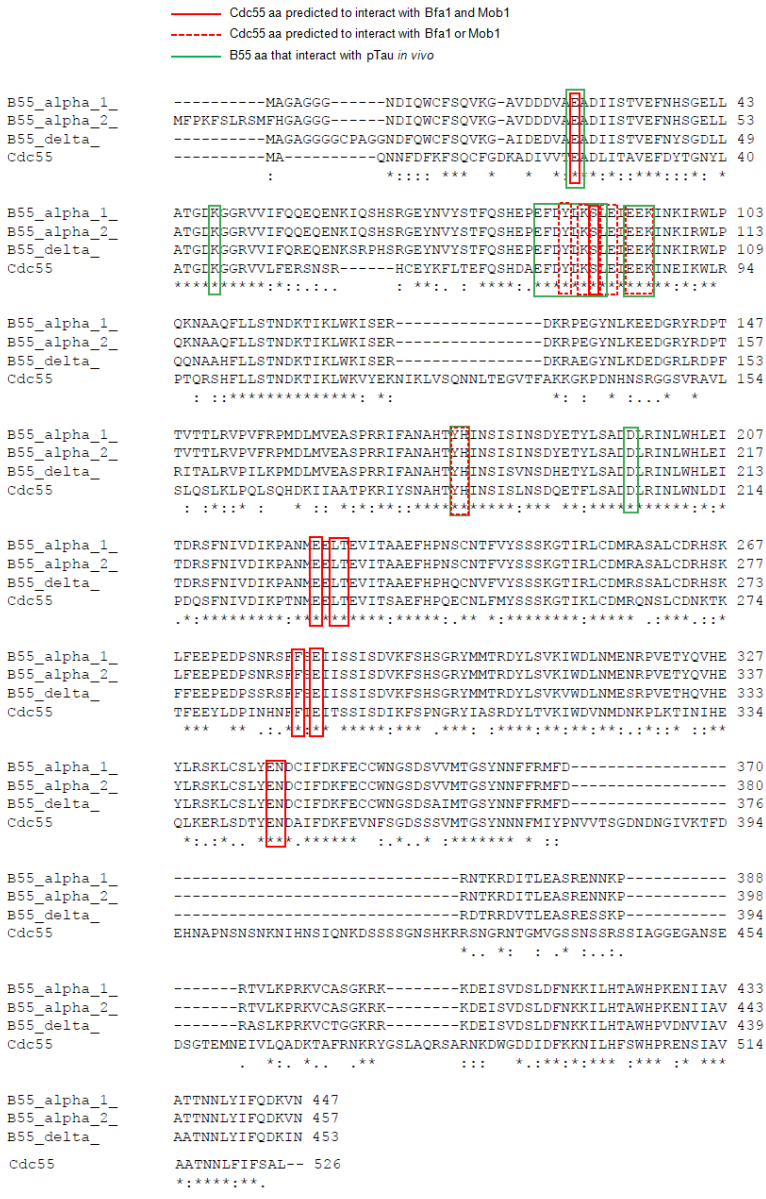



**Figure R9.2. Clustalw alignment of Cdc55 from *S.cerevisiae* and B55 alpha (isoforms 1 and 2) and B55 delta from H.sapiens.** The aminoacids predicted to interact with Bfa1 and Mob1 are indicated in red. B55 aminoacids described to interact with pTau are indicated in green

# Discussion

The cell cycle regulation has been a subject of intense study since chromatin and chromosomes were visualized for the first time, more than one century ago. As technology improved, cell cycle research has tried to elucidate the molecular mechanisms beyond cell division, one of the highest regulated cell processes, which ensures life in all organisms. Protein phosphorylation emerged as a key regulatory mechanism of protein function in the cell cycle. The highly conserved and specific family of serine/threonine kinases, the Cdks (cyclin-dependent kinases), were considered the main component of the cell cycle control system once they were discovered. However, nowadays is clear that the opposing phosphatases play also a key role in setting the phosphorylation state of the proteins, thereby being the other side of the cell cycle control system. It was traditionally thought that phosphatases were generally promiscuous enzymes because of the small number of catalytic phosphatase subunits compared to kinases, and the *in vitro* promiscuity of the isolated phosphatase catalytic subunits. However, the *in vivo* phosphatase complexes have recently shown to be highly specific and regulated, and they even exceed the number of kinases. For this reason, one of the main focuses of interest in the current cell biology research is how phosphatase complexes achieve their specificity and how they are regulated.

The simultaneous separation of all pairs of sister chromatids at the metaphase to anaphase transition is one of the most dramatic events of the cell cycle. Errors during the separation of the duplicated genomes lead to aberrant daughter cells, which can cause several diseases and cell death. In addition, chromosome splitting is an irreversible event and, therefore, it is highly regulated. Cells enter into mitosis when mitotic Cdk

activity increases, having its pick of activity during metaphase. In order to exit mitosis, cells coordinate chromosome segregation with Cdk inactivation processes, which reset Cdk activity levels in order to finish the cell division and enter into a new G1 phase. Thus, mitotic exit is also a dramatic change in the balance of kinase/phosphatase activity of the cell, as cells move from the highest Cdk activity to the lowest. Phosphatase complexes play a key role during this process. For this reason, phosphatase regulation and functions during mitotic exit is a trendy research topic of high impact for Biomedicine applications.

In *S. cerevisiae*, a widely used model organism for cell cycle studies, the activation of the Cdc14 phosphatase is a key step of mitotic exit. Two complementary and consecutive pathways, FEAR (Cdc*14* *e*arly *a*naphase *r*elease) and MEN (*m*itotic *e*xit *n*etwork), activate the Cdc14 phosphatase by promoting its release from the nucleolus. Cdc14 is kept sequestered in the nucleolus by binding to its inhibitor Net1. At anaphase onset, the protease separase becomes active due to $APC^{Cdc20}$-dependent degradation of securin, and triggers sister chromatids separation. In addition, separase cooperates with Zds1 to promote the downregulation of $PP2A^{Cdc55}$ phosphatase, which facilitates the Cdk1-dependent phosphorylation of Net1. $PP2A^{Cdc55}$ downregulation allows the accumulation of Net1 phosphorylated forms, which have lower affinity for Cdc14, and provides the first wave of Cdc14 activity (Cdc14 release into the nucleoplasm). Clb2-Cdk1 complex and Cdc5 polo kinase are both responsible for Net1 phosphorylation in early anaphase. After activation of the FEAR, and the partial Clb2 degradation induced by $APC^{Cdc20}$, Clb2-Cdk1 activity declines and cells activate the MEN to continue with Net1 phosphorylation and fully activate Cdc14 in late

anaphase. Mob1-Dbf2 kinase, the most downstream effector of the MEN, phosphorylates Cdc14 at sites adjacent to its NLS, thereby retaining Cdc14 in the cytoplasm. Cytoplasmic Cdc14 activates $APC^{Cdh1}$ complex, which completes mitotic cyclins degradation among other substrates. Moreover, cytoplasmic Cdc14 also allows Sic1 accumulation, the main Cdk1 inhibitor. $APC^{Cdh1}$ activation and Sic1 accumulation lead to Cdk1 inhibition and completion of mitosis.

How chromosomes segregation is coordinated with sequential Cdk1 inactivation steps during mitosis is a subject of great interest. It is known that separase activation triggers both chromosome segregation and FEAR-Cdc14 release. However, specific FEAR and MEN components regulation, and how these pathways are coordinated during anaphase is not fully understood. It was suggested that $PP2A^{Cdc55}$ downregulation at anaphase onset could facilitate Bfa1 phosphorylation, thereby facilitating MEN activation in anaphase (Queralt et al.,. 2006). In this way, $PP2A^{Cdc55}$ downregulation by separase would unlock mitotic exit, initiating FEAR-Cdc14 release and the MEN. Moreover, there is no evidence of Cdc5 upregulation during anaphase, pointing to a downregulation of the counteracting phosphatase. However, cells lacking the PP2A regulatory subunit Cdc55 did not show any accelerated mitotic exit, despite having Cdc14 already released (Queralt et al., 2006, Fig1A). In addition, FEAR and MEN take place in early and late anaphase respectively. The first objective of this work was to investigate the role of $PP2A^{Cdc55}$ phosphatase in regulating the MEN.

## 1. Study of the Mitotic Exit Network (MEN) regulation by PP2A$^{Cdc55}$ phosphatase.

The MEN is a GTPase driven signalling cascade tightly associated with the SPBs (the yeast centrosomes). The main switch of the cascade is the GTPase Tem1, and its regulators: the Bfa1-Bub2 complex, which inhibits Tem1; and the putative GTP exchange factor, Lte1, which promotes Tem1 activation. Once Tem1 is active, it recruits Cdc15 kinase to the SPBs and promotes its activation. Cdc15 in turn, recruits Mob1-Dbf2 kinase complex and activates it via phosphorylation of the kinase subunit, Dbf2. Mob1-Dbf2 allows Cdc14 activation and release into the cytoplasm during late anaphase. Bfa1-Bub2 complex keeps the whole pathway inactive during most of the cell cycle. In anaphase, Cdc5-dependent phosphorylation of Bfa1 leads to Bfa1-Bub2 inactivation, thereby initiating MEN signalling. We demonstrated that PP2A$^{Cdc55}$ regulates Bfa1 phosphorylation. Bfa1 is hyperphosphorylated in *cdc55*Δ cells, upon inhibition of PP2A$^{Cdc55}$ by Zds1 overexpression, or by inactivation of the catalytic PP2A subunits. Moreover, Bfa1 and Cdc55 physically interact, suggesting that Bfa1 is likely to be an *in vivo* substrate of PP2A$^{Cdc55}$. In a normal cell cycle, Bfa1 inactivation depends on Cdc5 polo kinase phosphorylation. Although Bfa1 may be a substrate for other kinases that have not yet been described, our results indicate that PP2A$^{Cdc55}$ counteracts Cdc5-dependent phosphorylation of Bfa1. Previous results have already shown that PP2A$^{Cdc55}$ can counteract Cdc5-dependent phosphorylation (Yaakov et al., 2012). Therefore, PP2A$^{Cdc55}$ is not only a Clb2-Cdk1 counteracting phosphatase but is also able to dephosphorylate Cdc5 targets.

Bfa1-Bub2 complex have shown to localize to the dSPB in anaphase, which is required for Tem1 activation. In contrast, when the spindle is misaligned and the SPOC is activated, Bfa1 is phosphorylated by the kinase kin4 in sites that avoid Cdc5-dependent phosphorylation, keeping Bfa1-Bub2 complex active and the MEN effectively locked. In this case, Bfa1 localizes symmetrically to both SPBs until the abrogation of the SPOC signal. We demonstrated that Bfa1 phosphorylation upon $PP2A^{Cdc55}$ inactivation does not depend on the kinase Kin4. Indeed, Bfa1 localizes asymmetrically to the dSPB after $PP2A^{Cdc55}$ inactivation, both in the *cdc55*Δ mutant and after Zds1-dependent inactivation of $PP2A^{Cdc55}$. Thus, $PP2A^{Cdc55}$ downregulation at anaphase onset facilitates inactivation of Bfa1-Bub2 complex, by facilitating Cdc5-dependent phosphorylation and inducing its asymmetric localization.

$PP2A^{Cdc55}$ downregulation would initiate the MEN at anaphase onset, but we did not observe premature MEN activation and mitotic exit in cells lacking $PP2A^{Cdc55}$ activity. For this reason we explored the effect of inactivating $PP2A^{Cdc55}$ phosphatase on downstream MEN elements. Cdc15 dephosphorylation pattern have often been used as a marker of MEN activity. Cdc15 is dephosphorylated by Cdc14 phosphatase, as a feed-forward activation loop. We observed that Cdc15 is prematurely dephosphorylated and loaded to the dSPB in *cdc55*Δ mutant cells and after Zds1-induced $PP2A^{Cdc55}$ inactivation, in accordance with Cdc14 being released after $PP2A^{Cdc55}$ inactivation. However, Cdc15 dephosphorylation in such conditions is not as efficient as in a normal anaphase, when MEN is active. Moreover, we did not observe Mob1 recruitment to the SPBs after $PP2A^{Cdc55}$ inactivation. It was recently described that Cdc15 kinase phosphorylates the scaffold SPB protein

Nud1, allowing Mob1-Dbf2 recruitment to the SPBs and its activation. Thereby, our results suggest that Cdc15 is not totally active as a kinase after $PP2A^{Cdc55}$ inactivation (despite being properly recruited to the dSPB) because it is not able to recruit Mob1-Dbf2. The study of Nud1 phosphorylation under $PP2A^{Cdc55}$ inactivation could be an alternative way to confirm this result. In addition, Mob1 and Dbf2 proteins are dephosphorylated in anaphase, which is required for Mob1-Dbf2 activation. We did not observe any change in the phosphorylation pattern of Mob1 nor Dbf2 after $PP2A^{Cdc55}$ inactivation by Zds1 induction (despite having Cdc14 released); further confirming that Mob1-Dbf2 complex is not activated in this condition. Finally, the study of late anaphase events that depend on MEN activation confirmed the previous results. Cdh1, which is a late Cdc14 substrate, is not prematurely dephosphorylated in *cdc55*Δ mutant cells, indicating that the downstream MEN effector, Mob1-Dbf2 is not prematurely activated. We concluded that $PP2A^{Cdc55}$ downregulation at anaphase onset initiates the MEN signalling, which is transduced until Cdc15 kinase. However, full MEN activation is restrain until mid-late anaphase since Mob1-Dbf2 kinase is kept inactive.

Taking together the above results, we hypothesize that there is an inhibitory input into the MEN cascade, downstream of Bfa1, which acts as a break until mid-late anaphase, thereby avoiding the untimely full MEN activation. In fact, Cdk1-dependent inhibition of Cdc15 has been previously postulated (Jaspersen and Morgan, 2000), and in previous publications by our group we proposed a mathematical model to describe the negative regulation of Cdc15 by Clb2-Cdk1 (Attila Toth, 2007; Queralt et al., 2006; Vinod et al., 2011). In addition, the mutual

regulation of Clb2-Cdk1 and Cdc15 has been described more recently (König et al., 2010). These authors also demonstrated that Cdk1 phosphorylates Mob1 protein to inhibit the activity of Mob1-Dbf2 kinase. Finally, there is a genetic evidence of a concerted action by Bfa1 and Clb2 when Tem1 is accumulated in the SPBs, (Valerio-Santiago and Monje-Casas, 2011). We found that Cdk1-eGFP was prematurely located onto the mSPB in metaphase *cdc55*Δ cells, as would be expected if MEN activity was inhibited by Clb2-Cdk1. Moreover, we observed Mob1 dephosphorylation when inactivating Cdk1. Therefore, Clb2-Cdk1 activity acts as break for MEN activation. In this way, cells would initiate both FEAR and MEN at anaphase onset, unlocking Cdk1 counteracting processes, and the decreasing Clb2-Cdk1 activity would be the mechanism to sequentially activate both pathways (see Fig. D2). The model we propose fits perfectly with the fact that the upstream MEN complex Bfa1-Bub2 plays a role in early anaphase, monitoring the spindle orientation through the mother-bud axis; but in contrast, downstream MEN components play a role in $APC^{Cdh1}$ activation and also cytokinesis, thereby being active in late anaphase and telophase. In fact, a recent study of how Cdc14 discriminates between its early and late anaphase substrates demonstrated that each Cdc14 substrate has a different Cdk1 inhibitory threshold. Indeed, we observed that Cdc15 is highly sensitive to FEAR-Cdc14 release whereas Mob1 is not. Cdc15 seems to be the MEN sensor for the Cdc14/Cdk1 relative activity. Thus, after unlock of mitotic exit at anaphase onset by $PP2A^{Cdc55}$ downregulation, Cdc14/Cdk1 relative activity drives the sequential activation of different processes during anaphase, as Cdk1 activity progressively declines.

It is possible to reproduce a correct anaphase by overexpressing Separase and inactivating Cdk1 artificially (Fig D1). Suprisingly, Zds1 overexpression combined with Cdk1 inhibition is not able to efficiently drive exit from mitosis. In fact, although MEN upstream elements are activated after Zds1 induction, the result in terms of cell division and efficient mitotic exit resembles the results obtained by solely Cdk1 inhibition in metaphase-arrested cells. Actually, solely by inhibiting Cdk1 in metaphase-arrested cells, we are reseting Cdk1 activity to G1-like levels independently of Cdc14 release, which facilitates initiation of budding and S phase programme (metaphase-arrested cells re-bud and form chains). After some time, cells manage to separate somehow, but they have not performed a proper anaphase since the daughter cells carry an aberrant DNA content. Although MEN is probably active after Zds1 overexpression combined with Cdk1 inhibition, chromosomes are still placed in the metaphase plate, avoiding cytokinesis. This could be the reason why Zds1 overexpression combined with Cdk1 inhibition does not entail efficient mitotic exit as separase overexpression does. In contrast, separase overexpression combined with Cdk1 inhibition is able to reproduce a proper anaphase. This is easily explained by the fact that chromosomes are segregated and the spindle is elongated under separase overexpression, which does not occur when overexpressing Zds1 or a protease-inactive version of separase (Sullivan and Uhlmann 2003). This highlights the importance of separase proteolitic activity for chromosome segregation and spindle elongation, key features of anaphase. In summary, separase activation at anaphase onset initiates chromosome segregation, the spindle elongation, and downregulates $PP2A^{Cdc55}$ phosphatase (activating the FEAR and initiating the MEN), thereby unlocking mitotic exit. Later on, when Clb2-Cdk1 activity

declines, downstream MEN components are activated and cells perform MEN-Cdc14 functions, achieve full Cdk1 inhibition, perform the cytokinesis and enter into a new G1 phase.

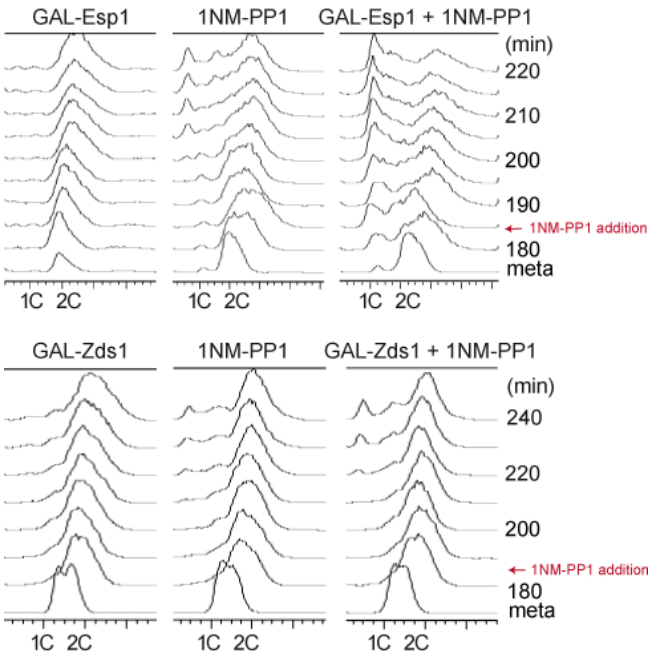
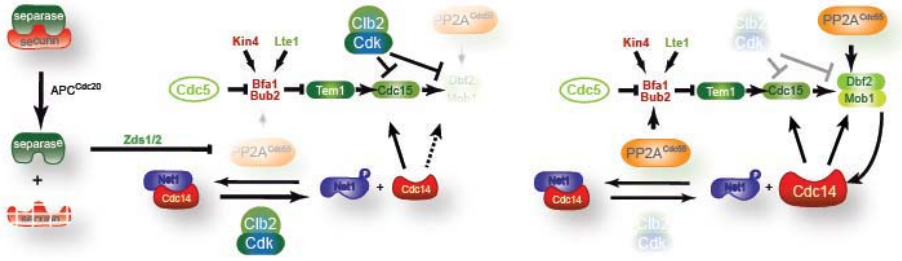



**Figure D1. Separase overexpression combined with Cdk1 inhibition allows a proper exit from mitosis in metaphase-arrested cells.** Strains Y1046 (*MATa MET3-CDC20 CDC28-as1 GAL1-Flag3-ESP1 MOB1-HA6*) and Y1032 (as Y1046, *GAL1-Flag3-ZDS1)* was arrested in metaphase by Cdc20 depletion and Esp1 or Zds1 was induced. After 180 min of induction, 1 μM of 1NM-PP1 drug was added to inhibit Cdk1. We show the FACS of both controls (overexpression + DMSO, non-overexpressed + 1NM-PP1) and the combined effect of the overexpression and Cdk1 inhibition (third graphs).

The second goal of this work was to identify new $PP2A^{Cdc55}$ putative substrates by a global phosphoproteome analysis of the *cdc55*Δ mutant (discussed below). We identified a phosphopeptide corresponding to Mob1 protein, which was highly increased in absence of $PP2A^{Cdc55}$

function. We validate Mob1 as an *in vivo* substrate of $PP2A^{Cdc55}$. We demonstrated that Mob1 is hyperphosphorylated in *cdc55*Δ metaphase-arrested cells, and that Mob1 is not efficiently dephosphorylated during anaphase in the *cdc55*Δ mutant. In addition, we demonstrated that Cdc55 and Mob1 physically interact *in vivo*, suggesting that Mob1 is likely to be an *in vivo* substrate of $PP2A^{Cdc55}$ phosphatase. This fits well with our observations that Mob1 phosphorylation state was not affected by Cdc14 release (in presence of high levels of Clb2-Cdk1 activity), and more importantly, Mob1 dephosphorylation was independent of Cdc14 release after Cdk1 inhibition; pointing to additional phosphatase(s) dephosphorylating Mob1. Therefore, $PP2A^{Cdc55}$ downregulation at anaphase onset would increase Cdk1-dependent phosphorylation of Mob1, helping to keep Mob1-Dbf2 complex inactive during early anaphase. Later, when Clb2-Cdk1 activity declines, $PP2A^{Cdc55}$ gain of activity would dephosphorylate Mob1, contributing to full activation of the Mob1-Dbf2 complex. $PP2A^{Cdc55}$ is reactivated at the end of mitosis, when Cdk1 activity is low (Fig. D2). Strikingly, when artificially inhibiting Cdk1, we were also reactivating $PP2A^{Cdc55}$ (at least partially), as shown by the dephosphorylation of the well-characterized $PP2A^{Cdc55}$ substrate, Net1 (Fig R7.3A). Thus, $PP2A^{Cdc55}$ seems to be also regulated by Cdc14/Cdk1 relative activity. Mathematical modelling of $PP2A^{Cdc55}$ positive contribution to mitotic exit would give hints to uncover new $PP2A^{Cdc55}$ functions and to better understand the regulation loops between $PP2A^{Cdc55}$ and Cdk1 during mitosis.




**EARLY ANAPHASE**


**LATE ANAPHASE**

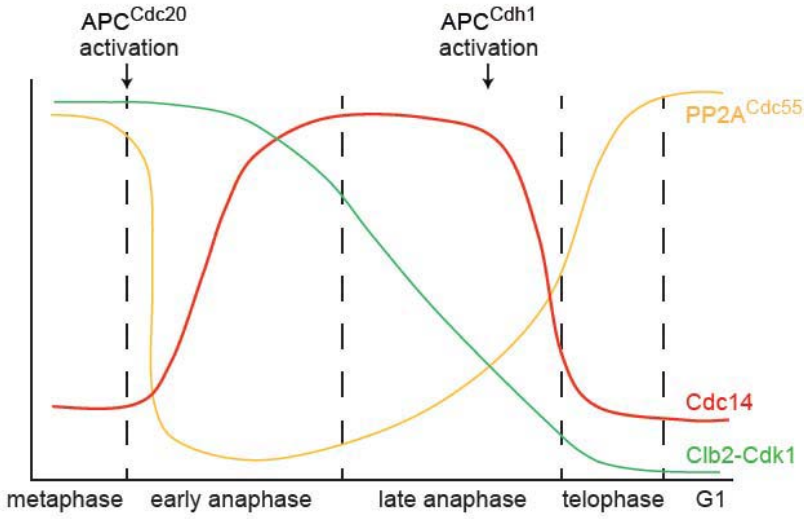



**Figure D2. Mitotic Exit model.** At anaphase onset, active $APC^{Cdc20}$ allows separase activation, which triggers chromosome segregation and unlocks mitotic exit. Separase cooperates with Zds1 to downregulate $PP2A^{Cdc55}$ phosphatase, providing the first wave of Cdc14 release (FEAR-Cdc14 activity) and initiating MEN signalling until Cdc15 kinase. High levels of Clb2-Cdk1 activity keep Cdc15 and Mob1-Dbf2 kinases inactive. $PP2A^{Cdc55}$ downregulation also contributes to keep Mob1-Dbf2 inactive. Progressive decline of Clb2-Cdk1 activity during anaphase allows Cdc15 activation and certain recovery of $PP2A^{Cdc55}$ activity, which leads to full activation of the Mob1-Dbf2 kinase. Full MEN activation in late anaphase allows $APC^{Cdh1}$ activation, which completely eliminates Clb2-Cdk1 activity and shuts-down Cdc14 release mechanisms.

Mob1 dephosphorylation by $PP2A^{Cdc55}$ phosphatase, and in turn its contribution to efficiently activate Mob1-Dbf2, is in accordance with an inefficient mitotic exit in the *cdc55*Δ mutant. Full MEN activation allows Cdc14 release into the cytoplasm, where Cdc14 dephosphorylates Cdh1 and Swi5. We observed that $APC^{Cdh1}$ is not efficiently activated in absence of $PP2A^{Cdc55}$ function (despite being Cdc14 prematurely released), as shown by the defective degradation of its substrates Cdc5 polo kinase and Clb2. The inefficient activation of Mob1-Dbf2 complex and, in turn, Cdc14 cytoplasmic phosphatase, would be a reasonable explanation for $APC^{Cdh1}$ defective activation. However, we cannot discard a possible direct contribution of $PP2A^{Cdc55}$ to Cdh1 dephosphorylation. Although having a defective $APC^{Cdh1}$ complex, the *cdc55*Δ mutant eventually exits from mitosis, albeit with slower kinetics. Sic1 accumulates timely in the *cdc55*Δ mutant and its accumulation is probably sufficient to inhibit Cdk1 and exit from mitosis in the absence of Cdc55. This is consistent with *GAL-SIC1*-db being sufficient to drive exit from mitosis in MEN mutants (Jaspersen et al., 1998; Luca et al., 2001). Independently of a putative direct $PP2A^{Cdc55}$ contribution to Cdh1

dephoshorylation, $PP2A^{Cdc55}$ activity is required for an effective $APC^{Cdh1}$ activation by Cdc14. This would be the shutt off mechanism of the MEN. $APC^{Cdh1}$ is responsible for Cdc5 polo kinase degradation, which is required for Cdc14 resequestration into the nucleolus. $PP2A^{Cdc55}$ is required to keep Cdc14 sequestred and the MEN inactive, by dephosphorylating Net1 and Bfa1 respectively. Mob1 is rapidily dephosphorylated upon Cdk1 inhibition, therefore, robust Mob1-Dbf2 activation, and Cdc14 fully activation, starts with its own inactivation mechanism by stimulating $APC^{Cdh1}$ activity. As Cdc5 polo kinase and Clb2-Cdk1 activity drops, $PP2A^{Cdc55}$ would be able to totally recover its activity and return Cdc14 into the nucleolus and reactivate Bfa1-Bub2 complex. In accordance, the *cdc55*Δ mutant shows a delayed in Cdc14 resequestration, prolonged Sic1 accumulation and a longer G1 phase, as $APC^{Cdh1}$ does not efficiently degrade mitotic proteins. Thus, $APC^{Cdh1}$ allows the complete Clb2-Cdk1 inactivation, the shut off of Cdc14-release mechanisms and, at the same time, induces the degradation of anaphase-related proteins (Cdc20, Ase1, Cin8 and Fin1) driving cells into G1. Surprisingly, Sic1 accumulates timely in the *cdc55*Δ mutant whereas Cdh1 is not efficiently dephosphorylated. Despite being both cytoplasmic Cdc14 susbtrates, Cdh1 and Swi5 could be Cdc14 substrates responding to different Cdk1 inhibition thresholds. In addition, we cannot discard a putative direct $PP2A^{Cdc55}$ contribution to Cdh1 dephoshorylation, or other indirect mechanisms of $PP2A^{Cdc55}$ regulating $APC^{Cdh1}$. Therefore, $PP2A^{Cdc55}$ reactivation could contribute to Cdc14 activity towards Cdh1, but not Swi5. It would be interesting to test whether hyperactivation of $PP2A^{Cdc55}$ phosphatase in late anaphase is able to overcome MEN mutants to exit mitosis.

Mob1-Dbf2 and cytoplasmatic Cdc14 activity have been recently linked to cytokinesis initiation (Sanchez-Diaz et al., 2012). In fact, several MEN components localize to the bud neck and were found to be required for the contraction of the actomyosin ring (Frenz et al., 2000; Luca et al., 2001; Menssen et al., 2001; Visintin and Amon, 2001; Yoshida and Toh-e, 2001). Therefore, MEN downstream components and MEN-Cdc14 activity would link complete Cdk1 inactivation and cytokinesis. $PP2A^{Cdc55}$ reactivation at the end of anaphase could play a role in efficiently promote Mob1-Dbf2 and MEN-Cdc14 functions in cytokinesis. Interestingly, we identified several proteins related to cytokinesis in our SILAC screening, mostly related to the actin cytoskeleton organization and the septin ring. $PP2A^{Cdc55}$ reactivation in telophase could shut down anaphase processes and promote cytokinesis while returning cells into G1 phase. The MEN homologue in fission yeast, the Septation Initiation Network (SIN), controls cytokinesis rather than mitotic exit and Cdk1 inactivation. Interestingly, a physical interaction between fission yeast Pab1 (B-regulatory subunit of PP2A in *S. pombe*) and Sid2 (Mob1 orthologous) has recently been described (Lahoz et al., 2010). PP2A-Pab1 regulates SIN activity at different levels, suggesting a conserved function for $PP2A^{Cdc55}$ regulating the MEN and SIN pathways. More recent evidences suggest that Mob1-Dbf2 regulates the cytokinetic components Chs2, Hof1 and Inn (Meitinger et al., 2011; Meitinger et al., 2010; Oh et al., 2012). It would be interesting to see whether these cytokinetic functions of Mob1-Dbf2 are affected in the absence of $PP2A^{Cdc55}$ function.

Cdk1 substrates dephosphorylation during mitotic exit depends on phosphatase activation in all organisms studied so far. In budding yeast,

the main mitotic exit phosphatase was considered Cdc14, which mediates both completion of Cdk1 inactivation (by upregulating Sic1 and Cdh1), and dephosphorylation of Cdk1 substrates. Other organisms, however, seem to rely on distinct mitotic exit phosphatases, like PP2A and PP1, despite the presence of genes that are homologous to *CDC14*. In addition, the budding yeast $PP2A^{Cdc55}$ phosphatase was described as a negative regulator of mitotic exit, because it counteracts the phosphorylation of the Cdc14 inhibitor Net1. Resolving this apparent evolutionary diversity in phosphatase regulation in the context of generally highly conserved cell cycle control system is a current important challenge in the field. Here we showed that $PP2A^{Cdc55}$ activity at the end of mitosis is required for efficient mitotic exit, by allowing full-Cdc14 and $APC^{Cdh1}$ activation. Therefore, $PP2A^{Cdc55}$ plays also a positive role in mitotic exit in budding yeast, moving closer the different findings obtained in this model organism and mammalian cells. In mammalian cells, PP2A-B55 is highly active during G1 (as well as in budding yeast) and it is shut off during mitosis (at the entry, in mammals; and at the metaphase to anaphase transition in budding yeast). Depletion of B55$\delta$ isoform in interphase frog-eggs extracts accelerates entry into mitosis and blocks exit from mitosis. However, when PP2A-B55$\delta$ was depleted from mitotic extracts, exit from mitosis was hardly delayed, showing that other phosphatase(s) are also required for mitotic exit (Mochida 2008). It is tempting to think that some of the mammalian Cdc14 orthologs could be some of these additional phosphatases required for mitotic exit in mammalian cells.

One of the biggest differences between budding yeast and mammalian mitosis is the fact that the budding yeast undergoes an asymmetric cell

division. This fact has been used to reason that MEN functions might not be conserved in mammals. However, mammalian cells also determine a division axis and several member of the Hippo pathway (the MEN orthologus in mammals) are conserved. In fact, human Mob1 proteins have been recently related to regulation of microtubule stability at the midbody during exit from mitosis, and are needed for cell abscission in human cells (Florindo et al., 2012). All the components and regulatory mechanisms of the MEN and SIN pathway could probably be conserved in higher eukaryotes, despite organism-specific specialization of these pathways. Therefore, studies of the regulatory mechanisms of the Hippo-related pathways in organisms like yeast have an important value as a starting point for its study in higher eukaryotes. Another major difference between budding yeast and mammalian cell cycle is that the nuclear envelope is not dissasembled. It is reasonable to think that Cdc14 regulation in mammals does not relay in the sequential Cdc14 release into the nucleoplasm and cytoplasm. However, this does not mean that mammalian Cdc14 cannot participate in the budding yeast Cdc14 described processes. In fact, mammalian Cdc14B is able to fulfil all essential functions of Cdc14 in *S. cerevisiae* (Vazquez-Novelle et al., 2005). Although different organisms will show specific differences in certain processes, the global regulation and functions of mitotic exit phosphatases could be highly conserved, as it has been previously shown for kinases.

## 2. Investigation of new putative substrates of PP2A$^{Cdc55}$ phosphatase in the mitotic exit.

Large-scale study of phosphoproteins, like the SILAC screening we have performed, can give useful information of general functions of the kinases or phosphatases studied. We depleted PP2A$^{Cdc55}$ function in metaphase-arrested cells and screened for phosphopeptides enriched in these conditions versus control. Although the increased phosphorylation of the different proteins identified is either a direct or indirect effect of PP2A$^{Cdc55}$ depletion, new regulated PP2A$^{Cdc55}$–processes can be discovered. It is also possible to study the different kinases counteracted or regulated by PP2A$^{Cdc55}$ through the screening of kinase phosphorylation motifs in the phosphopeptides identified. Interestingly, we identified a set of proteins containing S/TP motifs, corresponding to Cdk1 or MAP kinases consensus site. We also identified a group of proteins containing some of the proposed Cdc5 polo kinase consensus sites. Strikingly, we identified a third group of peptides containing the RXXS consensus motif. This motif corresponds to $Ca^{2+}$/calmodulin-dependent protein kinases (CaM kinases) and Chk1 kinase. CaM kinases are regulated by $Ca^{2+}$/Calmodulin complex and are involved in many signalling cascades. CaM kinases have been shown to be an important mediator of learning and memory in mammals and its misregulation has been related to diseases such as Alzheimer's disease, Angelman Syndrome and heart arrhythmia. Some specific CaM kinase forms are also altered in cancer. On the other hand, Chk1 has been shown to regulate cell cycle and DNA damage response, and it has a clear role in the G2/M transition. The RXXS motif logo showed a high number of peptides containing RRXS motif, which would correspond to

RSK1, AMPK and PAK kinases. Therefore, $PP2A^{Cdc55}$ could be the counteracting phosphatase of other kinases different to Cdk1 and Cdc5. Another possibility is that $PP2A^{Cdc55}$ regulates directly the activity of some kinase(s) from these groups. For instance, Rck2 kinase was identified in the screening, which is a CaM-like kinase related to oxidative and osmotic stress response. In addition, the yeast casein kinases, Yck1 and Yck2, showed genetic interaction with Cdc55 (Robinson 1993). Yck2 shows cell cycle-specific localization to sites of polarized growth and it is required for proper septin organization (Robinson 1999). Thus, $PP2A^{Cdc55}$ could regulate other kinases, like it has been shown for Swe1.

We identified several proteins in our SILAC screening corresponding to different functional categories. Focusing on specific cell cycle functions, we found several proteins related to sporulation, M-phase and cytokinesis. $PP2A^{Cdc55}$ has been shown to have functions during meiosis and, in addition, sporulation impinges a dramatic re-arrangement of the cell structure, cell membrane and wall. Interestingly, we identified a FEAR components, Slk19, which was hyperphosphorylated in the *cdc55*Δ mutant (despite having Cdc14 prematurely released in metaphase). Remarkably, Slk19 undergoes separase cleavage in anaphase, like the subunit of the cohesin complex Scc1. It was recently shown that $PP2A^{Cdc55}$ downregulation enhances Scc1 cleavage by separase. Moreover, Slk19 has been shown to be required for kinetochore protein clustering, thereby facilitating chromosome bipolar attachment (Richmond 2013). We also identified Bir1 protein, another anaphase-related protein. Bir1 is a subunit of the chromosome passenger complex formed by Ipl1-Sli15-Bir1-Nbl1, which regulates

chromosome segregation. Bir1 is required for chromosome bi-orientation as well as for the SAC activation upon reduced sister kinetochore tension (Makrantoni 2009). Thus, $PP2A^{Cdc55}$ downregulation at anaphase onset could modulate Slk19 and Bir1 activity. Another interesting candidate to be substrate of $PP2A^{Cdc55}$ is the microtubule-associated protein Stu2. Stu2 regulates microtubule dynamics during spindle orientation and metaphase chromosome alignment. In addition, Stu2 interacts with the SPB, where other $PP2A^{Cdc55}$ substrates are localized. The nucleosome assembly protein, Nap1, has been shown to interact with Clb2 and it is also required for regulation of spindle dynamics during mitosis and also regulates centromere structure, among other functions. Other proteins related to DNA remodelling, like Rsc3, have also been identified. *RSC3* is an essential gene required for maintenance of proper ploidy and regulation of rDNA. We also identified a subunit of the condensin complex, Ycg1, required for the establishment and maintenance of chromosome condensation, for chromosome segregation and binding of the condensin complex to the chromatin. Interestingly, the mammalian PP2A-B55 has been related to disassembly of the mitotic spindle and chromosome decondensation during telophase. Therefore, all these proteins look like promising candidates to be regulated by $PP2A^{Cdc55}$ activity during mitotic exit. In addition, we also identified proteins required for cytokinesis like Shs1 protein, which is a component of the septin ring. Bud3 was also identified in the screening, which localizes with septins to the bud neck in mitosis and it is involved in bud-site selection and axial budding pattern. Proteins involved in the general organization of the actin cytoskeleton like Myo5 and Sla1, were also identified. Considering all these proteins as substrate candidates of $PP2A^{Cdc55}$ phosphatase, looks like $PP2A^{Cdc55}$

downregulation at anaphase onset and its reactivation at the end of mitosis have much more implications than what is currently known. Just few of the specific functions of $PP2A^{Cdc55}$ in the mitotic exit have been identified during the last years, and this kind of screening pave the way to new functions discovery. Finally, we also identified Rrd1 protein. Strikingly, Rrd1 is an activator of $PP2A^{Cdc55}$ holoenzyme. It has been involved in G1 phase progression, microtubule dynamics and bud morphogensis; all processes linked to $PP2A^{Cdc55}$ function. Exploring the effect of Rrd1 phosphorylation state in the $PP2A^{Cdc55}$ activation mechanism, and the possible feed-back loop of $PP2A^{Cdc55}$ regulating its own activation, would bring important clues of how $PP2A^{Cdc55}$ holoenzymes are regulated.

$PP2A^{Cdc55}$ have been recently shown to monitor membrane trafficking and bud growth, integrating several cues to the mitotic entry regulators Swe1 and Mih1 (Wee1 and Cdc25 in mammals). Interestingly, we found components of the cell wall integrity pathway in our screening for $PP2A^{Cdc55}$ related functions. We also found proteins related to membrane organization and endocytosis, as well as polar growth control. Although mammalians undergo symmetric cell division, control of membrane structures, cell membrane trafficking and cell growth may be conserved. Indeed, PP2A-B55 activity in telophase has been linked to the reformation of the nuclear envelope and the Golgi apparatus, because it removes mitotic phosphorylations of these structures. Probably, the downregulation of PP2A-B55 during mitotic entry would promote disassembling of these structures. We have also found in our screening proteins related to the establishment of organelle localization and cytoskeleton organization. Cell wall organization, membrane

organization and trafficking, polarized growth and organelle localization are generally active cell processes, which should be monitored and coordinated with cell cycle. Interestingly, we also identified proteins related to osmotic stress and nutrient response. Thus, $PP2A^{Cdc55}$ phosphatase seems to play a key role sensing several clues of the environmental conditions, cell growth and cell structure, and integrate it to the cell cycle regulation. Having a list of putative candidate substrates can help as a starting point for elucidating specific regulatory mechanisms of these pathways and its crosstalk with cell cycle machinery.

We are currently repeating the phosphopeptide enrichment and the following analysis by LC-MS/MS of the SILAC experiments. An improved phosphopeptide purification step has been used this time, as the previously used titanium dioxide ($TiO_2$) purification is efficient for multiphosphorylated peptides purification but not for monophosphorylated peptides, which are also of our interest. Once we get a second list of candidate peptides, it would be interesting to see common peptides detected in both LC-MS/MS analysis. Later, with a more reliable list of candidate substrates, any interesting candidate will be validated *in vivo* before exploring the functional implication of this new $PP2A^{Cdc55}$-regulated substrate.

Molecular modelling such as docking analysis can be used as a tool for hit identification. We performed a docking modelling of the two new *in vivo* $PP2A^{Cdc55}$ substrates described in this work, Bfa1 and Mob1. Interestingly, we found in both cases a model of interaction of minimized free energy involving the Cdc55 “groove” in the binding to the substrate. The groove that Cdc55 presents in its surface has been proposed as the

binding site for its substrates. Remarkably, some of the Cdc55 aminoacids that interact with Bfa1 and Mob1 are the same and localize in the borders of the mentioned groove. Most of these common residues are conserved in humans and some are coincident with the aminoacids described to interact with pTau. Further study of the Cdc55 groove composition and the relevance of certain aminoacids to bind to the substrates *in vivo*, could bring insight to the mechanism of how Cdc55 achieve its specificity. Moreover, docking analysis combined with a scoring function can be used to screen large datasets of potential substrates *in silico* to identify the ones more likely to bind to Cdc55. In fact, this kind of analysis is a useful tool to screen for $PP2A^{Cdc55}$ inhibitors or modulators, for therapeutic purpouses.

This work attempts to bring new insight into the mitotic exit regulation picture. We highlight the existence of specific phosphatases functions and phosphatases regulation during cell cycle, with an intense crosstalk with Cdks. A profound understanding of the mitotic exit regulation could set the stage for new therapeutic strategies, since failure to progress normally through mitotic exit can induce cell death and may be exploited to kill hyperproliferating cancer cells. The study of phosphatase holoenzymes, and especially, the regulatory phosphatase subunits such as Cdc55, provides valuable information for the development of new pharmacological inhibitors or modulators that selectively target specific phosphatase holoenzymes.

# *Conclusions*

1. The MEN components, Bfa1 and Mob1, are *in vivo* substrates of $PP2A^{Cdc55}$ phosphatase.

2. $PP2A^{Cdc55}$ downregulation at anaphase onset facilitates MEN activation by allowing inactivation of Bfa1-Bub2 complex.

3. Premature Bfa1 inactivation in absence of $PP2A^{Cdc55}$ activity does not entail a premature exit from mitosis, since Clb2-Cdk1 activity restrains Cdc15 and Mob1-Dbf2 activities.

4. $PP2A^{Cdc55}$ activity is required for efficient $APC^{Cdh1}$ activation at the end of mitosis and effectively entry into a new G1.

5. $PP2A^{Cdc55}$ is a Cdk1 and Cdc5-counteracting phosphatase and possibly counteracts or regulates other kinases such as MAPK, CaM, Chk1, RSK1, AMPK and PAK kinases, as suggested by the phosphoproteome changes found in absence of Cdc55.

6. Cdc55 aminoacids located at the groove conformation are the predicted aminoacids to interact with Bfa1 and Mob1 substrates.

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