

Mitosis exit regulation by Cdc5 and PP2A- Cdc55

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*Mas no habiendo patria que me conmueva,
es el olor de sus rincones,
extravagantes especias y jazmín,
lo que a mi mente se lleva.*

*Que es su extensa tierra,
el olivar con sus raíces,
los almendros bañados al sol,
la que una más anhela.*

*Y es su bella imagen,
sierra blanca manchada de oro,
callejas estrechas y empinadas,
la que no sacaran del corazón, ¡ahí me muera!*

-Yolanda Moyano

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ABSTRACT

Cell cycle progression is regulated by different mechanisms which are mostly dependent on kinases and phosphatases action. Clb2-Cdc28 is the major cyclin-Cdk complex controlling mitosis in budding yeast. Clb2-Cdc28 activity peaks at metaphase and is inactivated to exit from mitosis mostly by the Cdc14 mitotic phosphatase. Two pathways contribute to Cdc14 activation: FEAR (Cdc14 early anaphase release) and MEN (mitotic exit network). Clb2-Cdk1 dependent Net1 phosphorylation provokes the FEAR-Cdc14 activation. However, it is not the unique kinase contributing to mitosis. Polo-like kinase Cdc5 was also described to be involved in the regulation of Cdc14. Cdc5 function in mitotic exit and Cdc14 activation requires sequential activation by Clb2-Cdk1. Nevertheless, the *in vivo* Net1 phosphorylation sites phosphorylated by Cdc5 have not been described, obscuring the Cdc5 role in the Cdc14 activation. Here, we described the Cdc5 involvement in the Net1 phosphorylation by the identification of some Cdc5-dependent phosphorylation sites on Net1. On the other hand, phosphatases also contribute to the regulation of mitosis. Cdc14 triggers mitosis exit and cytokinesis; while PP2A^{Cdc55} has several functions during mitosis. In this thesis we described the new PP2A^{Cdc55} role in cytokinesis. We demonstrated that PP2A^{Cdc55} dephosphorylates pivotal proteins for proper cytokinesis completion and their deregulation promote defects during cytokinesis. Taking all together, this thesis contributes to the knowledge of the kinases and phosphatases roles during mitosis and cytokinesis.

La progresión del ciclo celular está regulada por diferentes mecanismos que dependen esencialmente de la acción de quinasas y fosfatasas. Clb2-Cdc28 es el principal complejo Cdk-ciclina que controla la mitosis en la levadura de gemación. El pico de actividad máxima de Clb2-Cdc28 aparece en metafase y su inactivación por la fosfatasa mitótica Cdc14 es esencial para la salida de mitosis. Dos vías alternativas participan en la activación de Cdc14: FEAR (Cdc14 early anaphase release) y MEN (mitotic exit network). La fosforilación de Net1 por Clb2-Cdk1 promueve la activación de Cdc14-FEAR. Sin embargo, no es la única quinasa que contribuye a la regulación de la mitosis. También se ha descrito que la quinasa Polo Cdc5 regula la activación de Cdc14. Cdc5 se activa durante la mitosis de forma secuencial a través de su fosforilación por Clb2-Cdk1. No obstante, los sitios de Net1 fosforilados *in vivo* por Cdc5 no se han descrito hasta la fecha, contrariando la implicación de Cdc5 en la activación de Cdc14. En el trabajo realizado en esta tesis doctoral hemos identificado algunos sitios de fosforilación de Net1 dependientes de la fosforilación por Cdc5; confirmando la regulación de la activación de Cdc14 por Cdc5. Por otro lado, las fosfatasas también participan en la regulación de la mitosis; como es el caso de la fosfatasa Cdc14 implicada en la salida de mitosis y en la citocinesis, o la fosfatasa PP2A^{Cdc55} con múltiples funciones durante la mitosis. En esta tesis doctoral describimos un nuevo papel de la fosfatasa PP2A^{Cdc55} regulando la citocinesis. Hemos demostrado que PP2A^{Cdc55} defosforila proteínas claves reguladoras de la citocinesis cuya desregulación promueve defectos o impide la progresión de la citocinesis. En definitiva, esta tesis contribuye a la generación de conocimiento sobre las funciones de las quinasas y fosfatasas que regulan la mitosis y la citocinesis.

PREFACE

Mitosis has been long studied since it is an essential step for chromosome segregation and maintenance of genome stability. Any mistake has direct clinical implications since chromosome missegregation is a leading cause of miscarriages and birth defects and is linked to malignant tumor progression. Understanding the mechanisms governing cell division is crucial for managing the different pathologies it is related to.

Mitosis is composed by an extensive web of proteins interconnections that hinders the bases of each protein contribution to the process. For this reason, the use of model organisms, such as budding yeast, emerged to facilitate the understanding of these processes. The molecular bases of the mitosis in *Saccharomyces cerevisiae* is quite similar, although simpler, to mammal's and the proteins and pathways have been conserved through evolution.

Mitosis is regulated by the concerted action of kinases and phosphatases. The main kinases are the Cyclin-Cdk complexes, that control the progression throughout the cell cycle. Cdc14 is the main mitotic phosphatase counteracting the Cyclin-Cdk phosphorylations in order to overcome mitosis. However, they are not the unique kinase/phosphatase involved in mitosis regulation. PP2A^{Cdc55} phosphatase and the Polo-like Cdc5 kinase are also required for mitosis progression. PP2A^{Cdc55} was extensively studied because of its implication in the G2 to M phase transition and in the Cdc14 release from the nucleolus. Cdc5 best-known roles are the activation of the MEN pathway, the mitotic spindle regulation and the sister chromatids segregation.

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However, there is still long way to understand the whole role of these two proteins in mitosis regulation. During this thesis, we have contributed to the knowledge of two new roles associated to these proteins.

First, we describe the PP2A^{Cdc5} role in cytokinesis, dephosphorylating the cytokinesis proteins Hof1 and Chs2, and controls the phosphorylation state of other members of the ingression progression complex (IPC). This dephosphorylation contributes to the correct actomyosin ring constriction and primary septum formation, key processes for cytokinesis completion.

Second, we extend our knowledge in the Cdc5 role during mitosis exit by an independent pathway to the mitotic exit network (MEN). We narrow the possible Net1 residues candidate to be phosphorylated by Cdc5 in order to satisfy the mitotic exit and find out that Cdc5 regulates Cdc14 activation through the Net1 phosphorylation and by an alternative mechanism, probably by competing with the Cdc14-Net1 association.

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INTRODUCTION

1. The cell cycle

1.1. Cell cycle in eukaryotes

Cell division became an essential process to maintain the continuity of life since the first cell appeared. The cell cycle is the result of a series of regulated events that determines the physical separation between the new two cells after the duplication of the genetic material. Along the cell cycle we can distinguish 4 differentiated phases: G1, S, G2 and M. G1 phase is established as the initial gap phase and comprises the time from the previous division until the next S phase. During G1, coordination between cell growth and division takes place and the cell decides among the different cell fates: (1) a new round of cell division when a critical cell size is reached and the environmental and the nutrients conditions are appropriate; (2) to enter into G0 or sporulate upon lack of nutrients; (3) to conjugate during mating in presence of pheromones. Next, the DNA content is duplicated during S phase (Synthesis phase). During G2, the cell follows its growth until it acquires a minimum volume to be divided into two daughter cells. In addition, the big organelles (such as the endoplasmic reticulum (ER)) are duplicated. Mitosis, the last phase, drives cells to the physical separation of the two new cells ensuring the correct genetic material segregation and cell septation. Mitosis is divided into prophase, metaphase, anaphase and telophase. During prophase the centrosomes (called spindle pole bodies (SPB) in budding yeast) separate and migrate to opposite poles of the cell, the mitotic microtubules are assembled, and the DNA is condensed. The nuclear envelope breaks down, the sister

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chromatids attach to the kinetochore and the chromosomes are mobilized and driven by the mitotic microtubule until they are definitely aligned at the metaphase plate during metaphase. After their alignment, sister chromatids are separated and directed to the opposite poles of the cell in anaphase. Finally, in telophase nuclear envelope is formed again, chromosomes decondensed and the mitotic spindle is disassembled. At that point, cytokinesis mediates the physical separation of the two new cells by the generation of new cellular membrane (Fig. I1) (Morgan, 2007).

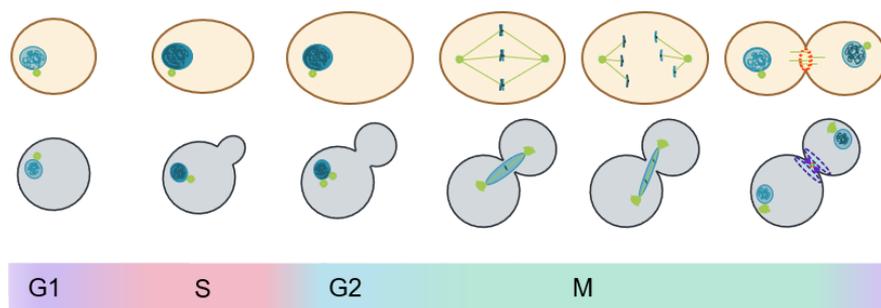


Figure I1. The phases of the cell cycle in animal cells (beige cells) and budding yeast (grey cells).

1.2. Cell cycle in *Saccharomyces cerevisiae*

Despite the fact that cells have evolved in different ways, the bases of cell division have been conserved among eukaryotes. The similarities among the different organisms allowed the study of the molecular mechanisms regulating the cell cycle in simpler organisms such as budding yeast. *Saccharomyces cerevisiae* is an example of how single-cell organisms can help us to understand the biology of the cell cycle.

The budding yeast, *Saccharomyces cerevisiae*, was originally discovered by Louis Pasteur in the synthesis of alcohol through fermentation (Barnett, 2000). Since then, its use as eukaryotic model has been expanded. *Saccharomyces cerevisiae* is a unicellular organism that can be found as haploid or diploid cells and it is characterized by having both sexual and asexual reproduction. Both diploid and haploid cells can reproduce asexually during the mitotic cell cycles. In addition, haploid cells can reproduce sexually by conjugation, where two different mating type cells fuse in order to become a diploid cell. The sex of budding yeast is characterized by the mating type, consisting in the expression of the *MAT* gene which can be found in two splicing forms: **a** or **α**. The expression of the *MAT* allele determines the sexual pheromone and the receptor for the opposite pheromone that will be synthesized (Bender & Sprague, 1989; Duntze, Mackay, & Manney, 1970; Hawthorne, 1963; Lipke, Taylor, & Ballou, 1976). The resultant diploid cell upon conjugation can enter the cell cycle or sporulate by the meiotic cell cycle under unfavorable conditions (as nitrogen and glucose starvation) (Freese, Chu, & Freese, 1982). This ability to sporulate is an evolutionary advantage to survive upon limiting conditions and it has become a genetically versatile invaluable tool to use in a wide variety of genetic studies in the laboratory.

For cell cycle studies, there are additional advantages that makes budding yeast an attractive model. It has a quick cell cycle (about 90 min), simpler regulatory pathways compared to higher eukaryotes and high conservation in the processes. In addition, the existence of highly optimized synchronization methods and the different morphological and cell molecular markers that facilitates

monitoring the cell cycle progression made the budding yeast one of the most commonly used eukaryotic model organism in cell cycle studies. While in humans the cell prepares to divide by growing until it reaches a minimum volume to be divided in two in the mid-plane where the midbody is formed; in *S. cerevisiae*, the new cell grows as a bud and the cell size of the nascent cell will be smaller than the mother cell in an asymmetric cell cycle (Fig. I1). Therefore, the appearance and the size of the bud is commonly used as a marker of the cell cycle progression. Moreover, *S. cerevisiae* presents a closed mitosis; the nuclear envelope remains intact and keeps growing to allow the separation of the duplicated genetic material before cytokinesis (Fig. I1). Finally, since budding yeast has cell wall (CW), there is an additional step during cytokinesis for the synthesis of the new CW synthesis in the place of abscission (Morgan, 2007).

1.3. Cell cycle regulation

Cell cycle requires a tight regulation of the progression through the different phases. The cell cycle progression is controlled by two main interlaced regulatory mechanisms: the Cyclins-Cdk and the cell cycle control systems.

a) The Cyclins-Cdk regulation

Cyclin dependent kinases (Cdk) are serine/threonine kinases conserved along eukaryotes that regulate progression through the cell cycle by the association to different cyclins (Fig. I2). In budding yeast, there is a unique Cdk: the Cdc28 (Hartwell, Mortimer,

Culotti, & Culotti, 1973; K. A. Nasmyth & Reed, 1980). Cdc28 is the homolog to the Cdk1 in humans and can be associated to the nine cyclins present in *S. cerevisiae*: the three cyclins N (Cln1-3) and the six cyclins B (Clb1-6). Cdk1 protein levels are mostly constant during the cell cycle whereas cyclin levels oscillate, being expressed and degraded specifically at different phases of the cell cycle (Fig. 12) (Evans, Rosenthal, Youngblom, Distel, & Hunt, 1983). Cyclins confer specificity to the Cdk1 determining the cell cycle event to regulate by Cyclins-Cdk complexes, although some cyclins have redundant activity (Bloom & Cross, 2007; Cross, 1990). During G1, Cdc28 forms complex with Cln1-3. The G1 cyclins are homologues to cyclins D in humans and have different subcellular localization. Cln3 is retained at the ER before its export to the nucleus once Start machinery is switch on (Vergés, Colomina, Garí, Gallego, & Aldea, 2007). Cln1 and Cln2 are localized at the cytoplasm during early G1, until they translocate into the nucleus upon Cln3-Cdc28 phosphorylation. Then, in late G1, Cln2 is exported from the nucleus to the cytoplasm in order to promote budding (M. E. Miller & Cross, 2000, 2001; Quilis & Igual, 2012). The differential cell localization has been related to the functional diversity of the cyclins, although they are redundant in some processes (M. E. Miller & Cross, 2000, 2001; Quilis & Igual, 2012). For instance, all G1 cyclins can regulate the transcription program required for the execution of Start and the response to the mating pheromone (Cross, 1990; Oehlen & Cross, 1994; Richardson, Wittenberg, Cross, & Reed, 1989). However, only Cln2 initiates the budding process at the cytoplasm or Cln3 regulates the expression of Cln1 and Cln2 during late G1 (De Bruin, McDonald, Kalashnikova, Yates, & Wittenberg, 2004; Dirick & Nasmyth, 1991; Queralt & Igual, 2004).

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Apart from their differential localization, their gene expression is also differentially regulated. *Cln3* is expressed in M/G1 (Fig. I2) under the regulation of the early cell cycle box (ECB) element recognized by the Mcm1 and Fkh2 transcription factors (TF) and

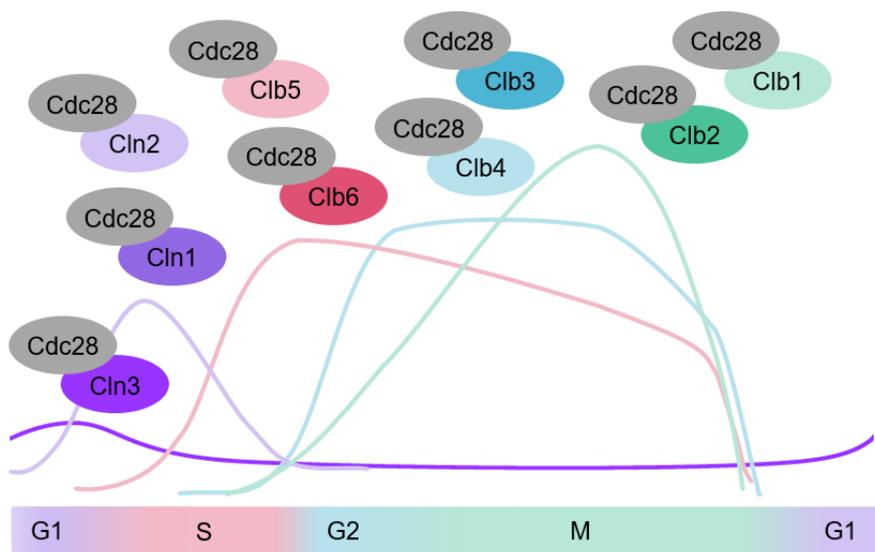


Figure I2. The Cyclin-Cdc28 complexes oscillations through the cell cycle.

the co-factor Ndd1. The ECB element regulates also the gene expression of the G1 transcription factor Swi4 (MacKay, Mai, Waters, & Breeden, 2001; Mai, Miles, & Breeden, 2002; McInerney, Partridge, Mikesell, Creemer, & Breeden, 1997). Swi4 with Swi6 form a heterodimer called SCB binding factor (SBF) responsible for the transcription of *Cln1* and *Cln2* in late G1. SBF recognizes the sequence Swi4/Swi6 cell cycle box (SCB) at the promoter of the *Clns* (Andrews & Herskowitz, 1989; Breeden & Nasmyth, 1987; Koch, Moll, Neuberg, Ahorn, & Nasmyth, 1993; K. Nasmyth & Dirick, 1991). By contrast, its transcription is counteracted by the SBF inhibitors *Whi3*, *Whi5* and *Whi7* (Adames et al., 2015;

Colomina, Ferrezuelo, Vergés, Aldea, & Garí, 2009; Gomar-Alba, Méndez, Quilis, Bañó, & Igual, 2017; Travesa et al., 2013). Whi5 binds to the SBF through Swi4 and avoids its association to the SCB elements (Travesa et al., 2013). This inhibition prevents premature Start activation and is maintained until Cln3-Cdc28 phosphorylates Whi5 promoting its dissociation of the complex and its nuclear export (Costanzo et al., 2004; De Bruin et al., 2004). The *CLN1-2* transcription mediates a feedback loop inducing SBF and cyclins expression (Dirick & Nasmyth, 1991; Harris, Lee, Farmer, Lowndes, & de Bruin, 2013; Skotheim, Di Talia, Siggia, & Cross, 2008). The onset of *CLN1* and *CLN2* transcription determines the timing of START and entry into a new round of cell division (Tyers, Tokiwa, & Futcher, 1993).

During S phase, Clb5-6 replace the Clns (Fig. I2). The Clbs are transcribed by the interaction of the MCB binding factor (MBF) with the Mlu1 cell cycle box elements (MCB) at the promoters of its regulated genes. The MBF is a heterodimer composed by Mbp1 and Swi6 (Dirick, Moll, Auer, & Nasmyth, 1992). The *CLB5* and *CLB6* genes are transcribed during G1/S similar to the Clns, but the Clb5,6-Cdk1 peak of activity occurs later on due to the action of the Cdk1 inhibitor, Sic1 (Tyers et al., 1993). Clb5-6 are required for DNA replication. They are localized at the nucleus to determine the replication start points and phosphorylate several components of the replication machinery (Epstein & Cross, 1992; Jackson, Reed, & Haase, 2006; Loog & Morgan, 2005; E. Schwob & Nasmyth, 1993). Moreover, they are responsible of maintaining DNA cohesion during replication by avoiding cohesin cleavage (Hsu et al., 2011).

CLB3 and *CLB4* are also transcribed after Start while *CLB2* transcription occurs during S/G2 phases (Fig. I2) (Epstein & Cross, 1992; Richardson, Lew, Henze, Sugimoto, & Reed, 1992). *CLB2* transcription is mediated by the transcription factor Mcm1, the forkhead transcription factor Fkh2 and the co-activator Ndd1 (Fitch et al., 1992; Linke et al., 2017; Reynolds et al., 2003). Once expressed, they will mediate their own expression by a positive-feedback loop mediated by Clb2-Cdc28 complex (Amon, Tyers, Futcher, & Nasmyth, 1993; Pic-Taylor, Darieva, Morgan, & Sharrocks, 2004). Clb3,4-Cdk1 peaks during mid S phase, at the same time that the spindle pole bodies duplicate (Fitch et al., 1992). Clb2-Cdk1 is the main Cdk complex during mitosis, responsible of triggering chromosome segregation and the isotropic bud growth (Fitch et al., 1992; Lew & Reed, 1993). Clb1, instead, seem more important for meiosis (Dahmann & Futcher, 1995; Grandin & Reed, 1993). Clb1-4 inhibit the transcription of G1/S cyclins by preventing the SBF interaction with the SCB element at the promoters of their regulated genes (Amon et al., 1993; Koch, Schleiffer, Ammerer, & Nasmyth, 1996; Siegmund & Nasmyth, 1996).

Oscillation of the cyclin protein levels does not occur only at the transcriptional level. Cyclin degradation is also tightly regulated. Cyclins are ubiquitinated by different ubiquitin ligases and targeted for degradation by the 26S proteasome. Cln2 and Cln3 are ubiquitinated by SCF^{Grr1} and SCF^{Cdc4} E3 ubiquitin ligases, while Cln1 is regulated just by SCF^{Grr1} after been phosphorylated by Clb-Cdc28 (Barral, Jentsch, & Mann, 1995; Quilis & Igual, 2012, 2017). Clb6 is target of the SCF^{Cdc4} E3 ubiquitin ligase (Drury, Perkins, & Diffley, 2000). Later on, both S phase-Clbs and Clb2 are degraded

by the anaphase promoting complex or cyclosome with the Cdc20 cofactor (APC/C^{Cdc20}) (Shirayama, Tóth, Gálová, & Nasmyth, 1999; S. Y. Wu, Kuan, Tzeng, Schuyler, & Juang, 2016). Clb2 degradation is completed at the end of mitosis by the APC/C^{Cdh1} (Schwab, Lutum, & Seufert, 1997; Wäsch & Cross, 2002). The Clb3,4,5-Cdk1 complexes inactivate APC/C^{Cdh1} during late S phase allowing the accumulation of Clb2 (Yeong, Lim, Padmashree, & Surana, 2000).

As mentioned above, the cyclins have different localizations and oscillations; but the Cyclin-Cdk complexes are also activated or inactivated by phosphorylation and by the Cdk inhibitors (CKIs). Cdk1 is phosphorylated, and activated, by the Cdk1-activating kinase (CAKs family) (Harper & Elledge, 1998). In addition, Clb1/4-Cdk1 are inhibited by the Swe1 kinase (Wee1 in humans) phosphorylation (McMillan, Theesfeld, Harrison, Bardes, & Lew, 2002; Sia, 1998). CKI's are proteins that bind to the Cyclin-Cdk complexes and inhibit their kinase activity (Örd, Venta, Möll, Valk, & Loog, 2019). In budding yeast, the main inhibitor during the cell cycle is Sic1 (Mendenhall, 1993). Sic1 transcription is activated at the end of mitosis and inhibits the Clb-Cdk complex during anaphase contributing to the exit from mitosis (Toyn, Johnson, Donovan, Toone, & Johnston, 1997). In G1/S, Cln-Cdc28 phosphorylates Sic1 for being ubiquitinated by the E3 ubiquitin ligase SCF^{Cdc4} and quickly degraded by the proteasome (Schwob, Böhm, Mendenhall, & Nasmyth, 1994; Verma et al., 1997; Verma, Feldman, & Deshaies, 1997). Sic1 degradation allows the initiation of S phase by the activation of the Clb5,6-Cdc28.

b) The cell cycle control systems

INTRODUCTION

In parallel to the Cyclin-Cdk regulation, there are specific control points during the cell cycle that drives and regulates its progression. They are called cell cycle control systems (restriction points in mammals) and are related to the cyclin-Cdk regulation. The first control system is called Start in budding yeast (restriction point R in animal cells) and takes place at the end of G1. This is the most important control system in *S. cerevisiae*. At that point, cell has to decide among the different development options: 1) start a new round of cell division when the cell reach a minimum size and the environmental factors and nutrients are appropriate; 2) haploids cells can respond to pheromones and activate conjugation; 3) under certain conditions cells may begin pseudohifal growth or 4) resting in G0 or sporulate (diploids cells) when the environmental conditions are not appropriate. In addition, cell will check that there is not DNA damage and the machinery for DNA replication is prepared.

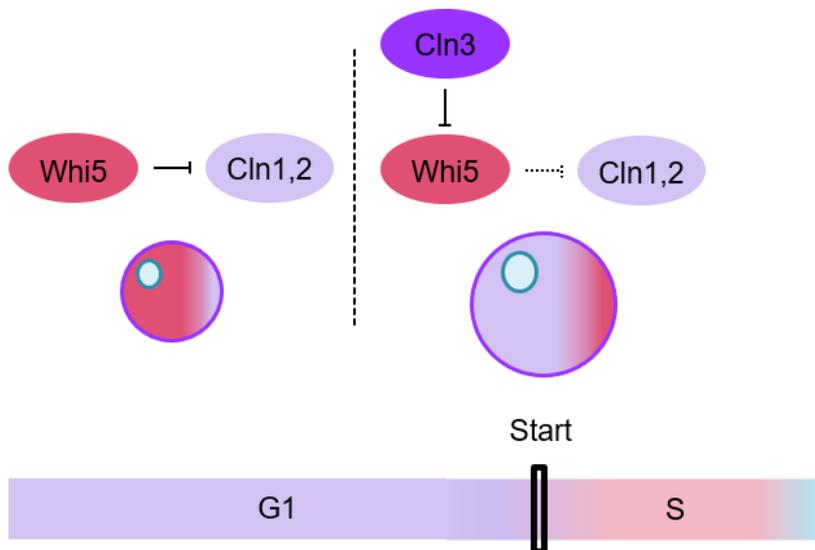


Figure I3. Start control system in *S. cerevisiae*. Cln1 and Cln2 expression regulation depends on Whi5 inhibitor and Cln3 nuclear concentration in late G1.

The activation of Start depends on the activation of Cln-Cdk1 and stabilization of Sic1. During G1, cell performs a cell size control through the Cln-Cdk1 and Whi5 regulation. At early G1 Whi5 inhibits Cln1,2-Cdk1. When the cell acquires a minimal required size, Cln3-Cdk1 inhibits Whi5 in order to activate Cln1,2-Cdk1. Therefore, the cell size control is critical since regulates Whi5 inhibition. While cell grows, Whi5 becomes diluted in the cell and Cln3 synthesis is extended proportional to cell growth (Fig. I3). This turns over the balance of Cln3 and Whi5, favoring the Cln1,2-Cdk1 activation and the execution of Start (Schmoller, Turner, Köivomägi, & Skotheim, 2015).

The availability of nutrients has also a role in regulating Start. The presence of nutrients activates pathways such as Ras and TOR that inhibits Whi3 (inhibitor of Clns transcription) by the Ras/cAMP-dependent protein kinase (PKA) phosphorylation (Mizunuma et al., 2013), or activating *CLN3* and *CDC28* expression while inhibiting Sic1 respectively (Moreno-Torres, Jaquenoud, & De Virgilio, 2015; Newcomb, Diderich, Slattery, & Heideman, 2003).

In absence of pheromones, Far1 is phosphorylated by Cln-Cdk1 for its degradation (Gartner et al., 1998; McKinney, Chang, Heintz, & Cross, 1993). In response of pheromones, Far1 protein is phosphorylated by a mitogen activated protein kinase (MAPK) cascade and has a critical role delaying Start and promoting the conjugation by inhibiting Cln2-Cdc28 (Gartner et al., 1998; Jeoung, Oehlen, & Cross, 1998; Peter & Herskowitz, 1994; Tyers & Futcher, 1993).

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Once Start is executed, the next restriction point occurs at the G2/M transition. The morphogenetic control regulates the nucleus division upon actin depolarization and the change from apical to isotropic bud growth. It regulates the action of Clb2-Cdc28 through Cdc28 phosphorylation. Clb2-Cdc28 regulates the actin depolarization promoting the change from apical to isotropic bud growth (Fig. I4) (Lew & Reed, 1993). But, the expression of the cyclin Clb2 and Clb2-Cdc28 complex formation occurs earlier in the cell cycle. Therefore, the cell maintains inhibited the Clb2-Cdc28

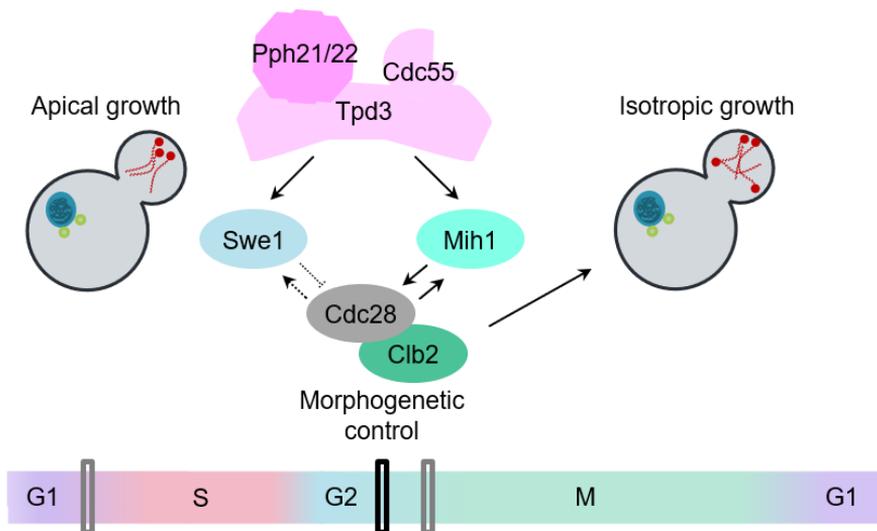


Figure I4. Morphogenetic control system. Cdc28 regulation by Swe1 kinase and Mih1 phosphatase during the G2 to M transition.

complex activity until metaphase. The inhibition is acquired via the phosphorylation of Cdc28 at the tyrosine 19 (Y19) (Y15 in humans) by the Swe1 kinase (Wee1 in mammals) (Booher, Deshaies, & Kirschner, 1993; F. Hu, Gan, & Aparicio, 2008). This phosphorylation is counteracted by Mih1 phosphatase (the Cdc25 homolog), but during G2 the Swe1 inhibiting phosphorylation predominates (Fig. I4) (Sia, Herald, & Lew, 1996). The presence of

a small pool of Cdc28 dephosphorylated is required for the activation of Swe1 by a low rate of phosphorylation (Harvey et al., 2011). Once cell is prepared to enter mitosis, the protein phosphatase 2 A (PP2A) with the regulatory subunit Cdc55 (B55 in humans) dephosphorylates and activates the Mih1 phosphatase that dephosphorylates the Cdc28-Y19. Active Cdc28 promotes a positive feedback loop that mediates the Mih1 activation (priming the phosphatase to be dephosphorylated) (Fig. I4) (Pal, Paraz, & Kellogg, 2008). In parallel, Swe1 is degraded to complete Cdc28 activation. Its degradation is induced by the hyperphosphorylation of Swe1 by Cla4 (p21-activated kinase (PAK) protein) and Cdc5 (the Polo-like kinase) and by its inhibitors Hsl1/7, as well as Cdc28 (Asano et al., 2005; Sakchaisri et al., 2004). Swe1 degradation and septins restructuration mediates the change from apical to isotropic growth (Barral, Parra, Bidlingmaier, & Snyder, 1999; King, Kang, Jin, & Lew, 2013; H. Yang, Jiang, Gentry, & Hallberg, 2000).

The final restriction point takes place in mitosis and it involves the spindle assembly checkpoint (SAC) (Fig. I5). The spindle assembly checkpoint regulates the metaphase to anaphase transition. SAC ensures mitotic spindles are attached to the kinetochores, the chromosomes are correctly aligned to the metaphase plate and the tension due to the bipolar attachment of the sister chromatids at the metaphase plate is produced. The process is tightly conserved, but the localization of the proteins differs among organisms. The closed mitosis in budding yeast restricts the SAC regulation to the nucleus (Heasley, DeLuca, & Markus, 2019). The main complex regulating this process is the mitotic checkpoint complex (MCC). MCC is formed by Mad2, Mad3 (BubR1 in vertebrates), Bub3 and Cdc20 (Fig. I5). These proteins regulate the accessibility of Cdc20

cofactor to the APC/C, preventing sister chromatids segregation. The APC/C^{Cdc20} is an E3 ubiquitin ligase and its activity is crucial to enter to mitosis. The APC/C E3 ligases ubiquitinate the proteins containing the destruction box (D box) in the case of APC/C^{Cdc20} or KEN box sequence for APC/C^{Cdh1} (Glotzer, Murray, & Kirschner, 1991; Pflieger & Kirschner, 2000). APC/C^{Cdc20} is activated by the Clb2-Cdc28 phosphorylation (Rudner & Murray, 2000) and is counteracted by PP2A^{Cdc55} during SAC activation (Rossio et al., 2013; Vernieri, Chirolì, Francia, Gross, & Ciliberto, 2013).

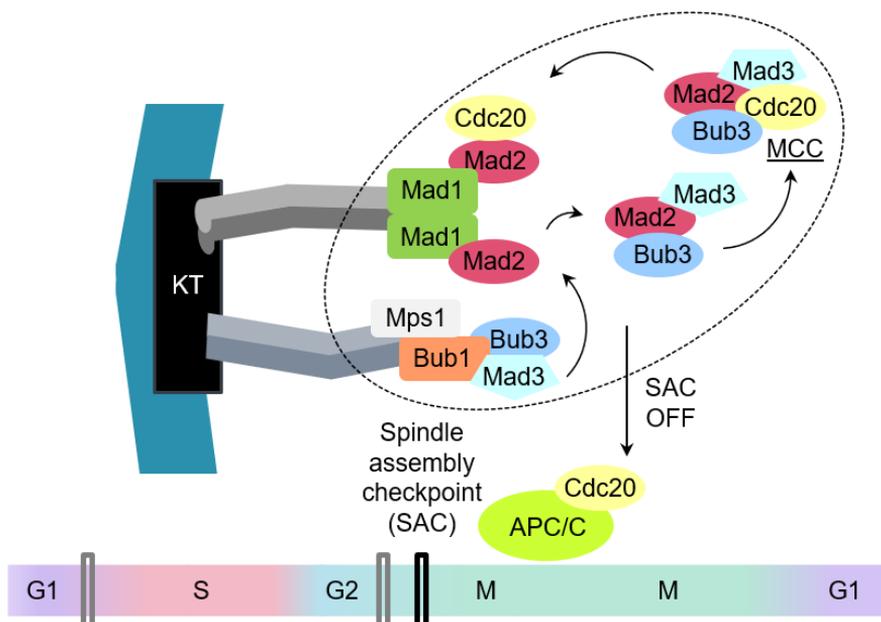


Figure I5. Spindle assembly checkpoint (SAC). Mitotic checkpoint complex (MCC) formation and Cdc20 inhibition until SAC is satisfied.

The kinetochore Ncd80 protein interacts with the spindle protein Mps1 to recruit the MCC (Hiruma et al., 2015; Ji, Gao, & Yu, 2015). Bub1 and Mad3 are the main interactors with the kinetochores and mediate the interaction with the rest of the MCC elements. Both proteins interact directly with Bub3 modulating the complete

formation of MCC (London & Biggins, 2014; Overlack et al., 2017). The MCC formation is completed by the Mad1 dimer and Mad2 interaction (Fig. I5) (Kulukian, Han, & Cleveland, 2009; Nezi et al., 2006). Mad1 interacts with the two conformations of Mad2 (open and closed) and facilitates the Mad2-Cdc20 interaction (De Antoni et al., 2005). Cdc20 inhibition is mediated by its recruitment to the kinetochore (E. M. J. King, van der Sar, & Hardwick, 2007; Kulukian et al., 2009; J. V. Shah et al., 2004). Once the SAC is satisfied, Cdc20 is released from the kinetochores and is free to activate APC (Fig. I5). Finally, the autoubiquitination of Cdc20 by APC/C complex inactivates the SAC (Foster & Morgan, 2012; Mansfeld, Collin, Collins, Choudhary, & Pines, 2011).

Eukaryotes have also mechanisms to detect errors during the cell cycle that stops progression in order to solve the problems before continuing with cell division: the checkpoints. Cells are able to response to the DNA damage, to different stress signals (i.e. osmotic stress) or to the disruption of any of the regulatory elements of the cell cycle described.

2. Mitosis

Dividing cells have the purpose of becoming two daughter cells and preserve the genetic material upon completion of the cell cycle. Mitosis comprises the mechanisms regulating the sister chromatids segregation in order to generate two daughter cells with identical genetic dotation after cell division.

2.1. General Overview

The key molecular event that initiates mitosis is the activation of the Clb2-Cdk1 ensuring that mitotic events occur in the proper order. Clb2-Cdc28 activity peaks at metaphase as consequence of Cdc28 activation upon Swe1 degradation and the positive feedback loop of Clb2-Cdc28 activating Clb2 expression (Amon et al., 1993; Harvey, Charlet, Haas, Gygi, & Kellogg, 2005; Harvey et al., 2011; section cell cycle control systems). Lower level of Cdk1 activity is required for the mitotic spindle assembly while higher activity is required for spindle elongation (Chee & Haase, 2010; Deibler & Kirschner, 2010; Fitch et al., 1992; Rahal & Amon, 2008; Richardson et al., 1992).

Mitotic entry involves all early mitotic events that take place until sister chromatids are prepared for segregation. DNA is condensed by the condensing complex forming the mitotic chromosomes and, then, aligned to the metaphase plate (Hassler et al., 2019; Robellet et al., 2015). When the chromosomes are correctly attached, aligned and the bipolar tension forces are present; the SAC is satisfied and cell enters anaphase (Mirchenko & Uhlmann, 2010; Teichner et al., 2011). The metaphase to anaphase transition is triggered by the APC/C^{Cdc20} activation. After being activated by Clb2-Cdc28, APC/C^{Cdc20} ubiquitinates several proteins promoting their degradation by the proteasome (Lu et al., 2014). The most important APC/C^{Cdc20} targets are the securin (the separase inhibitor; Pds1 in budding yeast) (Fig. I6) and the B-type cyclins (Marangos & Carroll, 2008). Initial cyclin B degradation leads to the first wave of Cdk1 inactivation (Imniger, Piatti, Michaelis, &

Nasmyth, 1995; Sudakin et al., 1995; Wäsch & Cross, 2002; Yeong et al., 2000). APC/C^{Cdc20} recognizes Pds1's D box and ubiquitinates

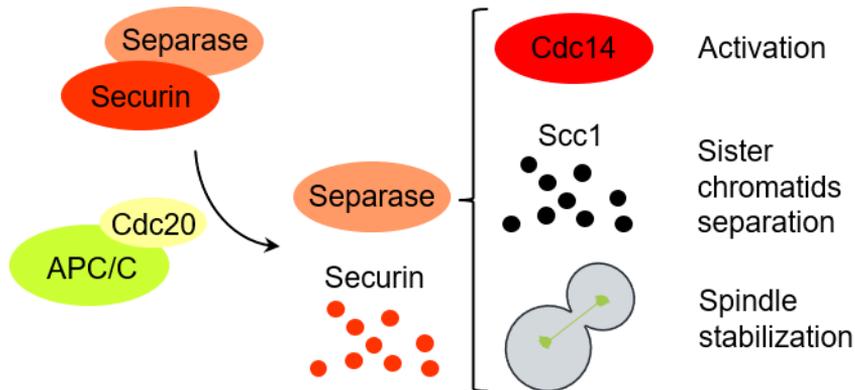


Figure I6. Separase activation and functions. Separase is activated by APC/C^{Cdc20}-dependent securin degradation. Active separase mediates the activation of the pathways that mediate Cdc14 activation, separation of the sister chromatids and the spindle stabilization and elongation.

it for its destruction by the 26S proteasome (Cohen-Fix, Peters, Kirschner, & Koshland, 1996; Hilioti, Chung, Mochizuki, Hardy, & Cohen-Fix, 2001; Lim, Goh, & Surana, 1998). Moreover, later in anaphase, the APC/C^{Cdh1} fully degrades Pds1 (Hatano, Naoki, Suzuki, & Ushimaru, 2016). Securin is bound to separase (Esp1 in budding yeast) most of the cell cycle and inhibits it until anaphase (Fig. I6) (Ciosk et al., 1998). Separase must be kept inactive until the chromosomes are aligned and attached to the microtubules to prevent precocious sister chromatids segregation. Separase is a cysteine like-caspase protease that has a proteolytic and a non-proteolytic function, both required during mitosis (Sullivan & Uhlmann, 2003; Uhlmann, Wernic, Poupart, Koonin, & Nasmyth, 2000). Active separase cleaves the Sccl subunit of the cohesin complex (Fig. I6) (Uhlmann, Lottspeich, & Nasmyth, 1999a) upon its phosphorylation by Cdc5 (Alexandru, Uhlmann, Mechtler,

Poupart, & Nasmyth, 2001; Pakchuen, Ishibashi, Takakusagi, Shirahige, & Sutani, 2016; Uhlmann, Lottspeich, & Nasmyth, 1999b) promoting sister chromatids separation (Ciosk et al., 1998; Tanaka et al., 2002). At the same time, separase promotes Cdc14 activation and spindle stabilization by its non-proteolytic function (Fig. 16) (Stegmeier, Visintin, & Amon, 2002; Sullivan & Uhlmann, 2003).

2.2. Cdc14 release

Cdc14 belongs to a family of highly conserved dual-specificity phosphatases (DUSP's) conserved from yeast to humans (Mocciaro & Schiebel, 2010) which unique feature is the ability to dephosphorylate both tyrosine and serine/threonine residues (Bremmer et al., 2012; Patterson, Brummer, O'Brien, & Daly, 2009). Cdc14 phosphatase orthologues have been identified and characterized in several eukaryotes.

Cdc14 is an essential gene in yeast and is a key mitotic exit component. Cdc14 has an essential role in Cdk1 inactivation, a critical step to exit from mitosis and to start a new cell cycle; that was the first well characterized Cdc14 activity (Visintin et al., 1998). Cdc14 phosphatase dephosphorylates Sic1, the Cdk1 inhibitor stabilizing it; and Swi5, the transcription factor that regulates the expression of Sic1, promoting its accumulation (Jaspersen, Charles, Tinker-Kulberg, & Morgan, 1998; Visintin et al., 1998). Furthermore, Cdc14 also induces Clb2 degradation by APC/C upon the dephosphorylation and activation of the second APC cofactor: Cdh1 (Jaspersen et al., 1998; Simpson-Lavy, Zenvirth, & Brandeis,

2015; Visintin et al., 1998). Therefore, Cdc14 counteracts Clb2-Cdc28 activity promoting exit from mitosis through the accumulation of Sic1, the activation of APC^{Cdh1} and the dephosphorylation of Cdk1 substrates (Kataria & Yamano, 2019; Knapp, Bhoite, Stillman, & Nasmyth, 1996; Powers & Hall, 2017; Tzeng, Huang, Schuyler, Wu, & Juang, 2011; Visintin et al., 1998).

Cdc14 is kept sequestered at the nucleolus during most of the cell cycle by binding to its inhibitor, Net1 (also called Cfi1) (Traverso et al., 2001). They both, together with Sir2 and Fob1, form the RENT (regulator of nucleolar silencing and telophase) complex, which inhibits transcription by RNA polymerase II at the ribosomal DNA (rDNA). RENT interacts with the rDNA and mediates its silencing and segregation (Clemente-Blanco et al., 2011; Shou et al., 1999; Straight et al., 1999; Torres-Rosell, Machin, Jarmuz, & Aragon, 2004; Visintin, Hwang, & Amon, 1999). Cdc14 role in transcription silencing is conserved also in humans (Clemente-Blanco et al., 2011).

During anaphase, Cdc14 is dissociated from the RENT complex, released from the nucleolus and activated as phosphatase (Shou et al., 1999). Mitosis exit is achieved through two complementary and consecutive pathways that collaborate for the activation of Cdc14: the cdcFourteen early anaphase release (FEAR) and the mitotic exit network (MEN) (Fig. I7) (Stegmeier et al., 2002). FEAR acts in early anaphase, when Cdk1 activity is high and promotes the first wave of Cdc14 release from the nucleolus to the nucleus (reviewed in Queralt & Uhlmann, 2008b). MEN, a GTPase-driven kinase cascade, is activated in late anaphase when Cdk1 activity decreases (Jaspersen et al., 1998; Stegmeier et al., 2002), and

completes Cdc14 release to the cytoplasm, making it available to the cytosolic Cdk-substrates (S L Jaspersen et al., 1998a; Mohl, Huddleston, Collingwood, Annan, & Deshaies, 2009).

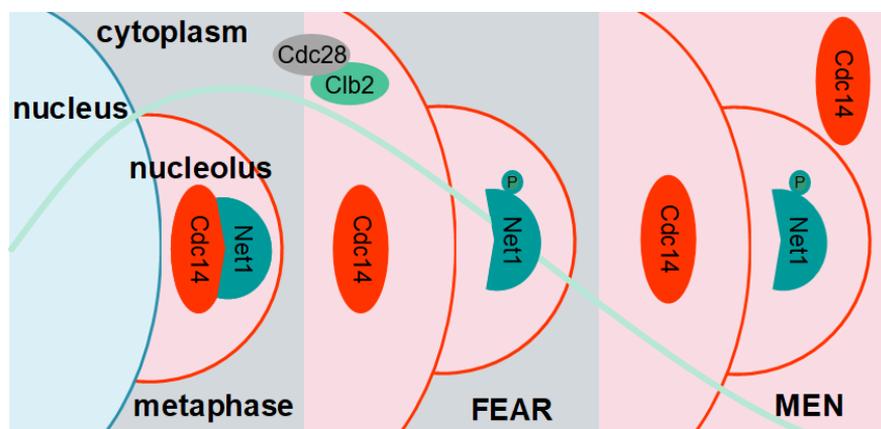


Figure 17. Cdc14 release through mitosis. Cdc14 is released from the nucleolus to the nucleus during early anaphase by FEAR when the Clb2-Cdc28 has a peak of activity, and from the nucleus to the cytoplasm in late anaphase by MEN promoting the Clb2-Cdc28 inhibition.

At anaphase onset, Cdk1-dependent phosphorylation of Net1, with the contribution of the polo-like kinase, Cdc5, allows Cdc14 release from the nucleolus since Net1 phosphorylated form has lower affinity towards Cdc14 (Fig. 17) (Azzam et al., 2004; Yoshida & Toh-e, 2002). The initial FEAR-Cdc14 activation is important for the dephosphorylation of Cdk1 substrates involved in the: 1) anaphase spindle stabilization and elongation (Higuchi & Uhlmann, 2005; Villoria et al., 2017), 2) the segregation of repetitive DNA regions such as the rDNA and the telomeres (D'Amours, Stegmeier, & Amon, 2004; Sullivan, Higuchi, Katis, & Uhlmann, 2004), 3) the recruitment of condensin to the rDNA (Clemente-Blanco et al., 2009; Clemente-Blanco et al., 2011; de los Santos-Velázquez, de Oya, Manzano-López, & Monje-Casas, 2017) and 4) the full Cdc14 activation by a positive feedback loop activating MEN by Cdc15

dephosphorylation (Godfrey, Kuilman, & Uhlmann, 2015; Pereira, Manson, Grindlay, & Schiebel, 2002).

The Cdc14 released by FEAR cannot fully activate the APC^{Cdh1}; and therefore, cell cannot complete Cdk1 inactivation and exit from mitosis. A second pathway, MEN, is required to fully activate Cdc14 in late anaphase when Cdk1 activity declines (Fig. 17). The downstream MEN kinases keep Cdc14 phosphorylated and released from the nucleus (Mohl et al., 2009). Fully released and active Cdc14 leads to Cdh1 activation and Sic1 accumulation, promoting Clb2-Cdk1 inactivation and exit from mitosis. Activated Cdc14 also regulates the disassembly of the mitotic spindle at the end of mitosis (Visintin et al., 1998), and cytokinesis (Kuilman et al., 2015; Miller et al., 2015; Palani, Meitinger, Boehm, Lehmann, & Pereira, 2012; Tamborrini, Juanes, Ibanes, Rancati, & Piatti, 2018). Different localization of the Cdc14 phosphatase allows targeting of its different substrates during anaphase progression; i.e. localizing at the bud neck for cytokinesis regulation (Tamborrini et al., 2018). In addition, the net balance of Cdk1 and Cdc14 activities towards their substrates also regulates their phosphorylation status. Different substrates respond to different thresholds of Cdk1/Cdc14 also contributing to the order of substrates dephosphorylation (Bouchoux & Uhlmann, 2011).

a) FEAR pathway

In budding yeast, the FEAR pathway initiates the Cdc14 release from the nucleolus in early anaphase when Cdk1 activity is high. The FEAR pathway was discovered studying the phenotypes of the

MEN mutants. In MEN mutants, Cdc14 was still released from the nucleolus transiently in early anaphase (Sullivan & Uhlmann, 2003). In addition, MEN mutants are synthetic lethal with FEAR mutants, indicating that both pathways act in parallel. All factors required for the transient release of Cdc14 in early anaphase and presenting synthetic lethal interactions with MEN mutants are commonly considered as FEAR components. To date, several proteins have been described as FEAR components: Slk19, Fob1, Spo12, Clb2, Cdc5, Zds1, PP2A^{Cdc55}, separase (Esp1) and Hit1 (Fig. 18) (Azzam et al., 2004; Calabria et al., 2012; de los Santos-Velázquez et al., 2017; Queralt, Lehane, Novak, & Uhlmann, 2006; Queralt & Uhlmann, 2008; Stegmeier et al., 2004; Stegmeier et al., 2002; Tomson et al., 2009). Nevertheless, the mechanism of how they contribute to the Cdc14 activation in early anaphase is not fully understood.

The most studied FEAR component and the only essential is the protease Esp1 (separase). Separase cleaves a subunit of the cohesin complex, Scc1, allowing the segregation of the sister chromatids during anaphase (Uhlmann et al., 1999a). In addition, separase presents a non-proteolytic function that allows the release of Cdc14 from the nucleolus (Sullivan & Uhlmann, 2003). Separase degron mutants (*esp1-dg*) arrest in early mitosis and has impaired Cdc14 release from the nucleolus; while ectopic release of Cdc14 rescues the phenotype and promotes exit from mitosis (Queralt et al., 2006). These results indicate that separase has an essential role in Cdc14 activation and mitotic exit. It was also described that separase cooperates with Zds1 and Zds2 to promote Cdk1-dependent phosphorylation of Net1 (Azzam et al., 2004) by downregulating the PP2A^{Cdc55} phosphatase (Queralt et

al., 2006; Queralt & Uhlmann, 2008a). PP2A^{Cdc55} counteracts Cdk1-dependent Net1 phosphorylation, keeping Cdc14 sequestered at the nucleolus from G1 to mitosis. At anaphase onset, separase and Zds1 promote the inactivation of PP2A^{Cdc55} phosphatase activity (Fig. I8) (Queralt et al., 2006; Queralt & Uhlmann, 2008a). Zds1

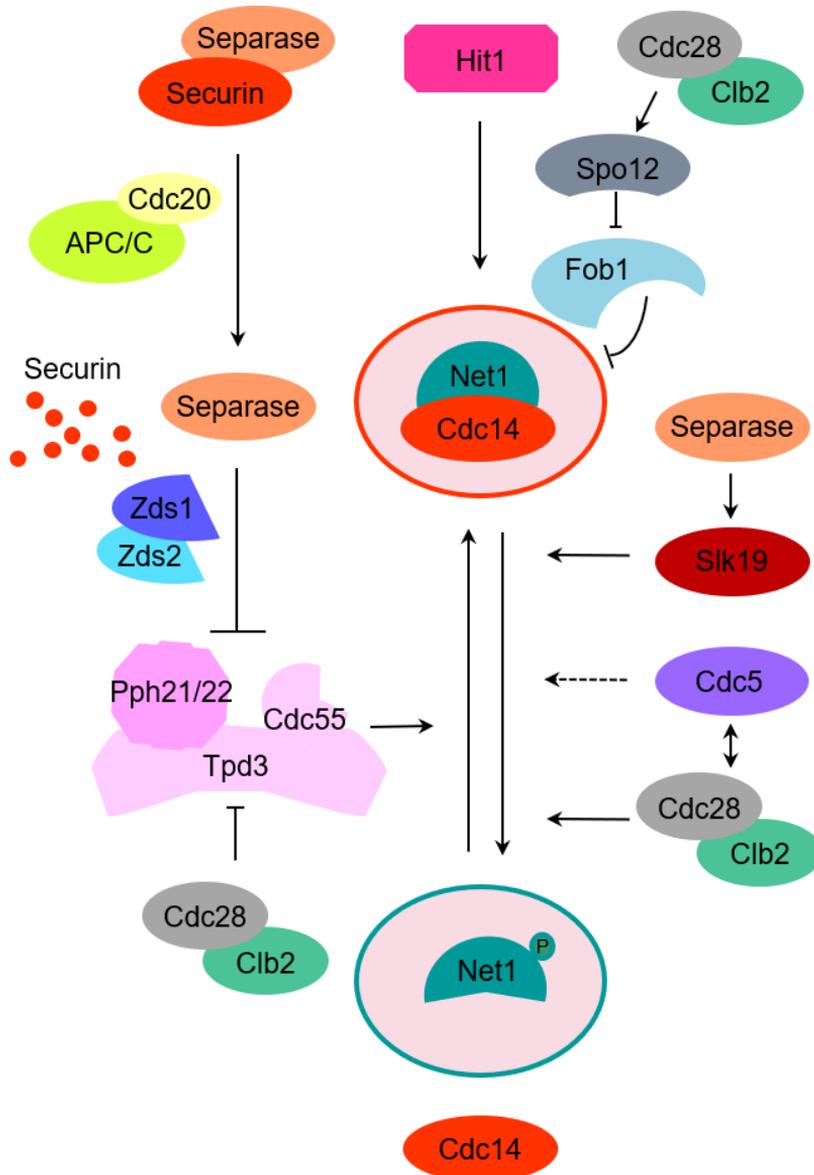


Figure I8. cdcFourteen early anaphase release (FEAR) pathway.

physically interacts with Cdc55 and regulates its localization at the nucleolus (Calabria et al., 2012). Recently, it has been described that Clb2-Cdk1 phosphorylates Cdc55 during anaphase, promoting the inhibition of the PP2A^{Cdc55} phosphatase activity and the Cdc14 release from the nucleolus (Fig. I8) (Játiva, Calabria, Moyano-Rodriguez, Garcia, & Queralt, 2019). Moreover, separase cleaves and regulates the kinetochore-associated protein Slk19 (Fig. I8). Cleaved-Slk19 changes its localization from kinetochores to the midzone spindles promoting spindle elongation (Richmond, Rizkallah, Liang, Hurt, & Wang, 2013; Sullivan, Lehane, & Uhlmann, 2001a). Slk19 has multiple roles: 1) clusters kinetochores and facilitates chromosome bipolar attachment (Richmond et al., 2013); 2) stabilizes kinetochore proteins by inhibiting separase-dependent Scc1 cleavage (Liang et al., 2018; T. Zhang, Lim, Cheng, & Surana, 2006); 3) regulates spindle elongation (Sullivan, Lehane, & Uhlmann, 2001b; Zeng et al., 1999); and 4) contributes to keep Cdc14 at the nucleus until late anaphase (Faust, Wong, Yates, Drubin, & Barnes, 2013).

Spo12, a protein of unknown biochemical activity, is a nuclear protein related to mitosis and meiosis regulation (Grether & Herskowitz, 1999; Parkes & Johnston, 1992; Stegmeier et al., 2002). Spo12 is phosphorylated by Cdk1 during early anaphase promoting the dissociation of Cdc14 from Fob1 (Fig. I8) (a RENT interacting protein) (Stegmeier et al., 2004; Tomson et al., 2009). In addition, Spo12 levels are also regulated by APC^{Cdh1}-dependent degradation and by the HECT-type ubiquitin ligase Tom1 (Nakatsukasa, Sone, Alemayehu, Okumura, & Kamura, 2018; R. Shah, Jensen, Frenz, Johnson, & Johnston, 2001). The nucleolar protein Fob1 protein recruits RENT components to the rDNA

(Huang & Moazed, 2003) and stabilizes Cdc14 and Net1 physical interaction acting as a negative regulator of FEAR (Stegmeier et al., 2004; Tomson et al., 2009).

Recently, it has been described that Hit1, a small nucleolar ribonucleoproteins (snoRNP) assembly factor also participates in the activation of Cdc14 (de los Santos-Velázquez et al., 2017). Hit1 is necessary to promote an efficient nucleolar release of Cdc14, linking rRNA maturation with mitotic exit (Fig. I8).

The polo-like kinase, Cdc5, was originally proposed to be involved in Cdc14 release from the nucleolus acting as FEAR and MEN component (Stegmeier et al., 2002). The best-studied role of Cdc5 is the phosphorylation of the MEN inhibitor Bfa1 (F. Hu et al., 2001). However, the specific role of Cdc5 in Cdc14 activation has been controversial for many years. Ectopic expression of Cdc5 is able to promote Cdc14 and Net1 phosphorylation and release Cdc14 from the nucleolus (Shou & Deshaies, 2002; Visintin, Stegmeier, & Amon, 2003; Yoshida & Toh-e, 2002). Nevertheless, the *in vivo* Cdc5 role phosphorylating Net1 or Cdc14 and its physiological relevance has not been demonstrated (Fig. I8). Later on, it was described that Cdk1-dependent phosphorylation of Net1 is the critical step to release Cdc14 from the nucleolus during early anaphase (Azzam et al., 2004; Queralt et al., 2006). Recently in our laboratory, it has been described that Cdc5 contributes to Cdc14 activation by promoting Net1 phosphorylation in the presence of Clb2-Cdk1 activity (J.-A. Rodríguez-Rodríguez, Moyano, Játiva, & Queralt, 2016). FEAR-dependent Cdc14 is first released upon Net1 phosphorylation by Cdk1-dependent during early anaphase. At the same time, Cdk1 phosphorylates Cdc5 at

residues T238 and T242 activating its kinase activity. Active Cdc5 sustains Net1 phosphorylation. In late anaphase, Cdc5 also helps to maintain Cdc14 activation upon Cdk1 phosphorylation mostly at residue T70 (J.-A. J.-A. Rodriguez-Rodriguez et al., 2016). Cdc5 has additional roles during the cell cycle regulating spindle elongation (C. J. Park et al., 2008; Rocuzzo, Visintin, Tili, & Visintin, 2015) and modulating Cdk1 activation by Mih1 phosphorylation during G2/M transition (F. Liang, Jin, Liu, & Wang, 2009; Raspelli, Facchinetti, & Fraschini, 2018).

b) MEN pathway

MEN pathway is an essential GTPase-driven signaling pathway, closely related to the Hippo pathway in mammals that is involved in mitotic exit regulation, the spindle position checkpoint (SPoC) and cytokinesis. The core of the MEN cascade consists of two serine/threonine kinases, Cdc15 (the Hippo-related kinase) and Dbf2-Mob1 (NDR/LATS-related MEN kinases) (Fig. I9). They are activated in mid-late anaphase to promote full activation of Cdc14 (Mah, Jang, & Deshaies, 2001; Visintin & Amon, 2001; Yoshida & Toh-e, 2001). After the first wave of FEAR-Cdc14 release, Clb2-Cdc28 starts to be inactivated by Clb2 APC/C^{Cdc20}-dependent degradation (Bäumer, Braus, & Irniger, 2000; Lim et al., 1998; Wäsch & Cross, 2002); therefore, additional kinases are needed to sustain Net1 phosphorylation and Cdc14 activation. At that point, the Net1 phosphorylation required for the Cdc14 release to the cytoplasm will be driven by MEN kinases (Fig. I9). MEN is regulated by changes in the activity of its components, their ability to interact with each other and to localize to different structures.

Most of the MEN components undergo changes in their phosphorylation status, being Cdk1 kinase and the phosphatases Cdc14 and the PP2A^{Cdc55} the main regulators (Baro et al., 2013; Jones et al., 2011; Seshan, Bardin, & Amon, 2002; Visintin et al., 1998).

The upstream effector of MEN is Tem1, a small Ras-like GTPase that is localized at the SPBs (M Shirayama, Matsui, & Toh, 1994).

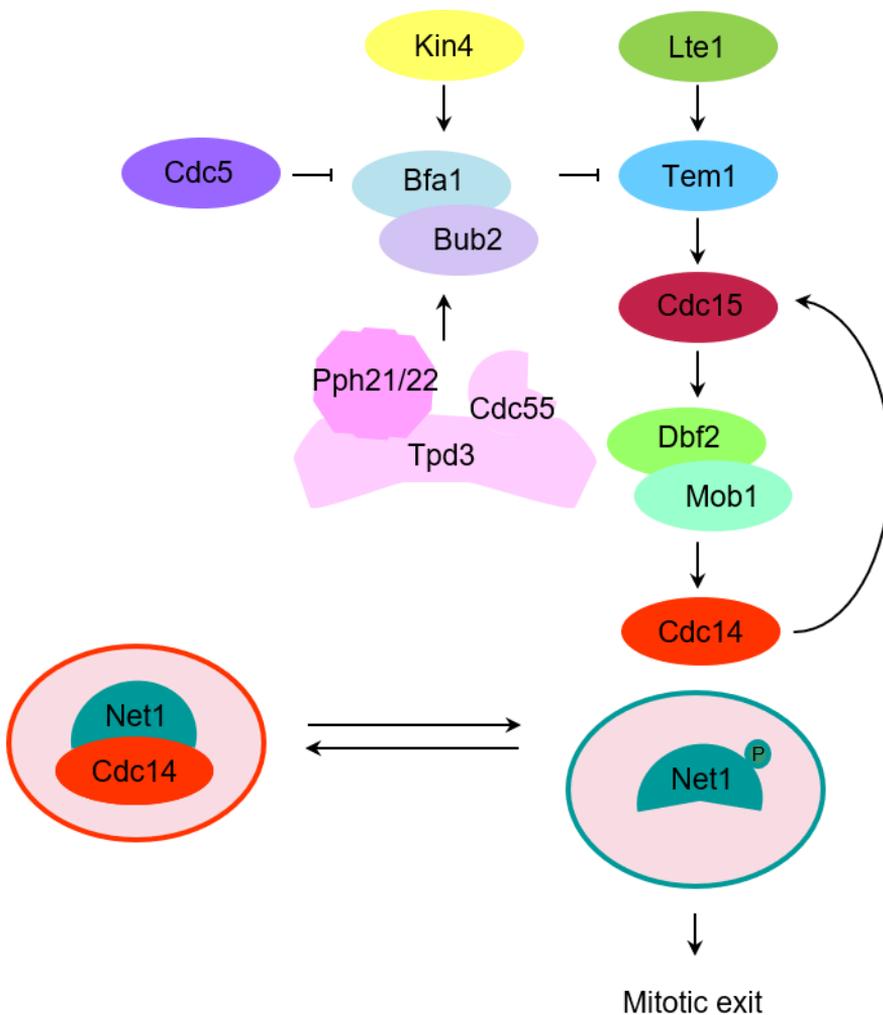


Figure I9. Mitotic exit network (MEN) pathway.

Its localization is very dynamic and depends on the interaction with the two-component GTPase-activating protein (GAP) Bub2/Bfa1 (Whalen, Sniffen, Gartland, Vannini, & Seshan, 2018). During an unperturbed cell cycle, Bub2/Bfa1 keeps Tem1 inactive until anaphase. PP2A^{Cdc55} contributes to the activation of Bub2/Bfa1 dephosphorylating Bfa1 in metaphase (Baro et al., 2013). When cells reach anaphase with a correct aligned mitotic spindle, Cdc5 phosphorylates Bfa1 and inactivates the Bub2/Bfa1 GAP activity (Fig. I9) (Geymonat, Spanos, Walker, Johnston, & Sedgwick, 2003; F. Hu et al., 2001). In addition, Cdk1-dependent PP2A^{Cdc55} inhibition at early anaphase also promotes the accumulation of the Cdc5-dependent Bfa1 phosphorylated isoforms (Játiva et al., 2019; Queralt et al., 2006). In addition, localization of Tem1 to the SPBs is essential for proper MEN activation. Interestingly, the localization of Tem1 and Bub2/Bfa1 to the SPBs is asymmetric during anaphase, being located preferentially to the old SPB (also daughter SPB (dSPB) since is the one that translocated to the daughter during anaphase) (Molk et al., 2004; Pereira, Hofken, Grindlay, Manson, & Schiebel, 2000; Scarfone et al., 2015; Whalen et al., 2018). Bfa1 phosphorylation by Cdc5 also regulates its localization (Baro et al., 2013; Botchkarev, Garabedian, Lemos, Paulissen, & Haber, 2017; Hu et al., 2001; Kim, Luo, Bahk, & Song, 2012; Pereira, Tanaka, Nasmyth, & Schiebel, 2001). Apart of the Bub2/Bfa1 GAP regulation, the GEF Lte1 also activates Tem1 (Fig. I9) (Falk, Chan, & Amon, 2011) helping to restrict the activation of Tem1 at the dSPB (Bertazzi, Kurtulmus, & Pereira, 2011; Falk et al., 2011; Geymonat, Spanos, de Bettignies, & Sedgwick, 2009; Jensen, Geymonat, Johnson, Segal, & Johnston, 2002). Lte1 also controls Bfa1 asymmetric localization, possibly because of its

asymmetric activation by Cla4 (Geymonat et al., 2009; Hofken & Schiebel, 2002).

Active Tem1 interacts with the Pak-like kinase Cdc15 (Asakawa, Yoshida, Otake, & Toh-e, 2001; Gruneberg, Campbell, Simpson, Grindlay, & Schiebel, 2000; Rock & Amon, 2011). Cdc15 is activated by Tem1 and by the Cdc14 (Fig. I9) (Jaspersen & Morgan, 2000; Rock & Amon, 2011). Cdc15 is inhibited by Cdk1 phosphorylation during most of the cell cycle (Konig, Maekawa, & Schiebel, 2010). Instead, FEAR-Cdc14 dephosphorylates Cdc15 during anaphase which facilitates Cdc14 activation in a positive feedback loop (Menssen, Neutzner, & Seufert, 2001). Bub2/Bfa1, Tem1 and Cdc15 bind to the scaffold protein Nud1, an integral component of the SPB (Rock et al., 2013). Once active, Cdc15 phosphorylates Nud1 facilitating the recruitment of the LATS-related Mob1/Dbf2 to the SPB (Rock et al., 2013; Visintin & Amon, 2001). Then, Dbf2/Mob1 are activated; Dbf2 is phosphorylated by Cdc15 and the phosphatases Cdc14 and PP2A^{Cdc55} dephosphorylate Mob1 alleviating its Cdk1 inhibitory phosphorylation (Baro et al., 2013; Konig et al., 2010; Mah et al., 2001). Dbf2 kinase and its regulator Mob1, as the final effectors of the MEN pathway, mediate the Cdc14 release from the nucleus keeping Net1 phosphorylation (Fig. I9) (Bembenek et al., 2007; Campbell, Zhou, & Amon, 2019; Mah et al., 2005; Manzoni et al., 2010; Mohl et al., 2009). Moreover, the Dbf2/Mob1 kinase phosphorylates Cdc14 inhibiting its nuclear localization signal (NLS) and retaining Cdc14 in the cytoplasm (Mohl et al., 2009; Yoshida, Asakawa, & Toh-e, 2002).

Cytoplasmic Cdc14 release promotes the activation of APC^{Cdh1} and the accumulation of Sic1, the Cdk1 inhibitor. Cdk1 inactivation at the end of mitosis promotes cytokinesis and MEN is inactivated at the next G1. APC^{Cdh1} induces the degradation of Cdc5 contributing to MEN inactivation (Visintin et al., 2008). Cdc14 dephosphorylates Bfa1 restoring Bub2/Bfa1 GAP activity (Pereira et al., 2002). In addition, Cdc14 also dephosphorylates Lte1 promoting its delocalization from the bud cortex (Jensen et al., 2002; Seshan & Amon, 2005; Seshan et al., 2002). Finally, Cdc14 mediates the dissociation of Cdc5 from the SPBs (Botchkarev et al., 2017). All these processes contribute to finally inactive MEN during the next G1.

3. Cytokinesis

Upon mitosis exit and chromosomes segregation, the two new cells must be physically separated during a process called cytokinesis. Although cytokinesis is the final event of the cell cycle, cell starts the regulation early in the cell cycle (Fig. I10).

3.1. Cell polarity: determining the cytokinesis site

In budding yeast, bud formation and cytokinesis are coordinated with progression through the cell cycle. Upon Start commitment, actin is polarized toward the bud site and the septin ring is assembled simultaneously (Fig I10) (Lew & Reed, 1993). These cytoskeletal reorganizations are dependent on Cdc42, a highly conserved Rho-like GTPase that is the master regulator of cell polarity during the cell cycle. *S. cerevisiae* cells have pre-localized

transmembrane “landmark” proteins at the proximal and distal pole of the cell in bipolar growth cells, and at the dividing pole in axial

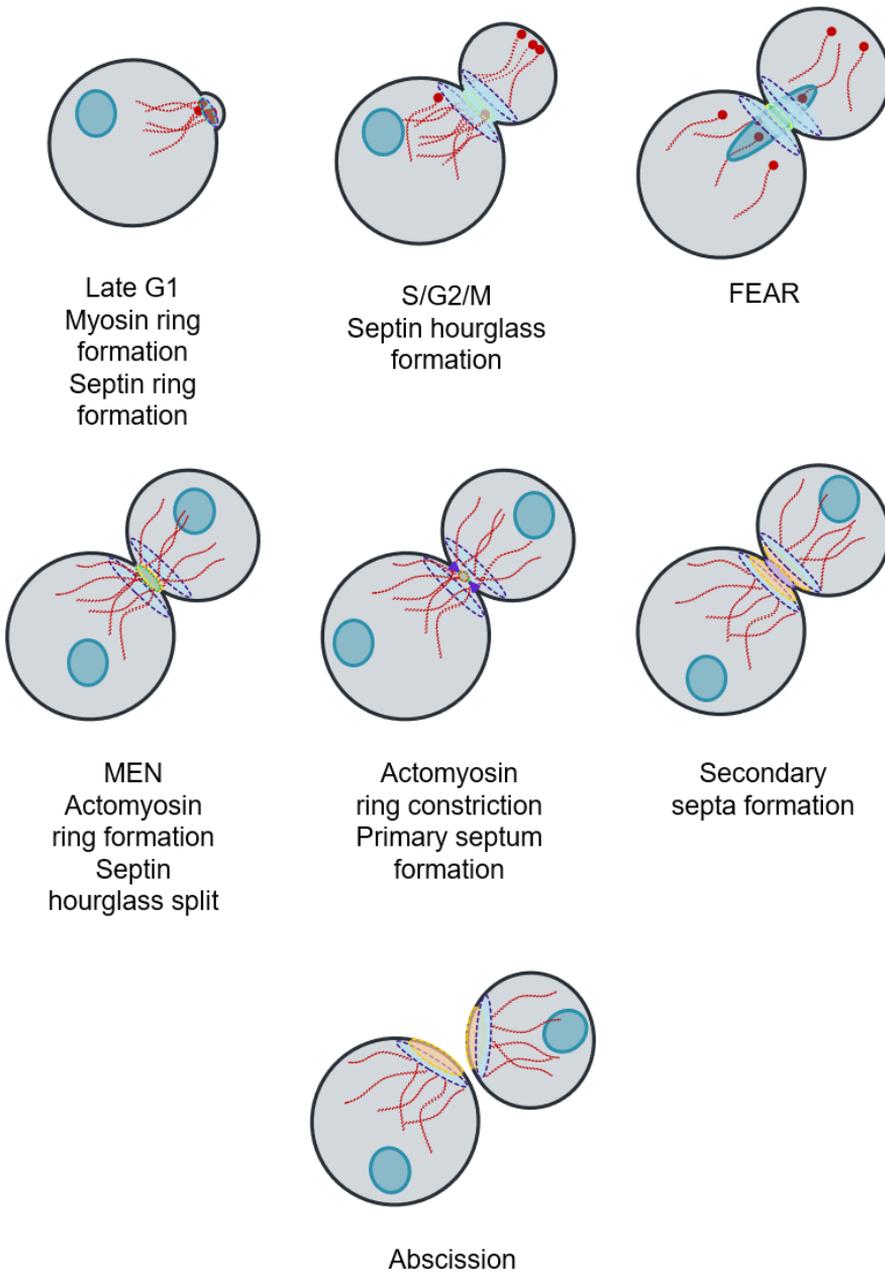


Figure 110. Cytokinesis components during the cell cycle in *Saccharomyces cerevisiae*.

growth cells (P. J. Kang, Angerman, Jung, & Park, 2012; P. J. Kang, Miller, Guegueniat, Beven, & Park, 2018; Krappmann, Taheri, Heinrich, & Mösch, 2007). Landmark proteins can localize Bud5, a guanine-nucleotide exchange factor (GEF) for the Ras-family GTPase Rsr1 (P. J. Kang et al., 2012). Active Rsr1 recruit Cdc24, the major GEF for Cdc42 promoting Cdc42 activation near the landmarks (Kozubowski et al., 2008). Cdc42 activation occurs concomitantly with Start activation at late G1 and is bound to the guanosine triphosphate (GTP) (Cvrčková, De Virgilio, Manser, Pringle, & Nasmyth, 1995). Cdc42 activation is achieved in different ways that promote Cdc42-GTP accumulation (Kozubowski et al., 2008; M. E. Lee, Lo, Miller, Chou, & Park, 2015): 1) alleviating Cdc24 (Cdc42 guanine-nucleotide exchange factor (GEF)) inhibition by Far1 (Shimada, Gulli, & Peter, 2000) and 2) activating Cdc24 by phosphorylating its activator, the Cla4 kinase (Gulli et al., 2000; Witte, Strickland, & Glotzer, 2017). Once Cdc42 is active, it recruits the septins and the exocyst machinery. Cdc42 also interacts with Gic1-2 (Borgs in mammals), Pak kinases Ste20 and Cla4, Bni1 and Iqg1 proteins to regulate actin polarization and septin organization (Evangelista et al., 1997; Howell et al., 2012; Iwase et al., 2006; P. J. Kang et al., 2018; Okada et al., 2013; Osman & Cerione, 1998; Sadian et al., 2013); and Sec3 to promote exocytosis (Chen et al., 2012; Okada et al., 2013; X. Zhang et al., 2001, 2008).

Active Cdc42 prevents cytokinesis; failure to downregulate Cdc42 during mitotic exit impairs the normal localization of key cytokinesis regulators Iqg1 and Inn1 at the division site and results in an abnormal septum (Atkins et al., 2013). Local inhibition of Cdc42 at the bud neck is, also, required to establish the site of bud growth in

the subsequent cell cycle (Meitinger, Richter, et al., 2013). Rga1 GTPase activating protein (GAP) and Gps1 protein inhibit Cdc42 before the formation of the secondary septa (SS; described deeply at section 3.5) (Meitinger, Richter, et al., 2013). This bud neck inhibition prompts to landmark proteins to activate the Cdc42 next to the bud neck for next cell cycle (Bi et al., 2000; Meitinger & Pereira, 2017; Meitinger, Richter, et al., 2013; Tong et al., 2007).

3.2. Septins

The polarisome complex provides the machinery required for bud growth at the bud emergence site. Moreover, the complex promotes the septins mobilization and assembly. The septins are GTP-binding proteins whose GTPase activity is required for septin-septin interaction (Sirajuddin et al., 2007; Weems & McMurray, 2017). They were discovered in yeast by Hartwell LH at 1971 and have been described to be conserved in eukaryotes, excluding plants (Reviewed at Bridges & Gladfelter, 2015; Smertenko et al., 2017). The number of septins genes is different in each organism, ranging from 17 septins in zebrafish to 2 in nematodes (Lihuan Cao et al., 2007). In human, there are 13 septin coding genes (*SEPT1-12,14* divided in groups: *SEPT2*, *SEPT3*, *SEPT6* and *SEPT7*) (Valadares, d' Muniz Pereira, Ulian Araujo, & Garratt, 2017), while in budding yeast there are 7 (*CDC3*, *CDC10*, *CDC11*, *CDC12*, *SHS1/SEPT7*, *SPR3* and *SPR28*) (Bhavsar-Jog & Bi, 2017; De Virgilio, DeMarini, & Pringle, 1996; Hartwell, 1971; Mino et al., 1998; Ozsarac, Bhattacharyya, Dawes, & Clancy, 1995); being *Spr3* and *Spr28* the meiotic septins (De Virgilio et al., 1996; Ozsarac et al., 1995).

a) Septins organization

Septins are organized in nonpolar rod-shaped heterooligomers (hexamers and octamers usually). This conformation in budding yeast, also conserved in humans (Kim, Froese, Estey, & Trimble, 2011; Sellin, Sandblad, Stenmark, & Gullberg, 2011; Sirajuddin et al., 2007; Valadares et al., 2017; Vissa et al., 2019), consists on the palindromic combination of Cdc3, Cdc10, Cdc12 and Cdc11; being the last interchangeable by Shs1 (Bertin et al., 2008; Garcia et al., 2011).

The nonpolar nature of the septins oligomer favor the binding to the phosphatidylinositol biphosphate (PIP₂), a phospholipid present at the cell membrane (Bertin et al., 2010; Bridges et al., 2014; J. Zhang et al., 1999).

Septin oligomers are recruited to the bud site after Cdc42 activation during late G1 (Iwase et al., 2006; Kim, Haarer, & Pringle, 1991). Cdc42, together with the activation of Gic1/2 (Borgs in human), promotes the recruitment of the septins (Cvrckova, De Virgilio, Manser, Pringle, & Nasmyth, 1995; Iwase et al., 2006; Okada et al., 2013). Septins oligomer formation depends on the GTP availability (Khan, Newby, & Gladfelter, 2018; Sirajuddin et al., 2007; Weems & McMurray, 2017). Cdc10 rapid GTP hydrolysis induces the formation of the Cdc10 homodimer, which assemble with Shs1/Cdc11-Cdc12-Cdc3 heterotrimers formed by Cdc12 slow GTP hydrolysis. Then, the Cdc3 N-terminal binds to Cdc12, and the Cdc3 heterotrimer interacts with Cdc10, forming the octamer (Weems & McMurray, 2017).

The formation of the septin oligomers depends on the activities of Cdc42 and Gic1/2 (P. J. Kang et al., 2018), and PIP₂ (Bertin et al., 2010; J. Zhang et al., 1999). In addition, Gic1/2 mediates the assembly of the filaments formed by septin oligomers (Joberty et al., 2001; Sadian et al., 2013; Sheffield et al., 2003). Gic1/2 also regulate septins organization indirectly by the activation of formin, Bni1 in budding yeast (Gao, Liu, & Bretscher, 2010; Kadota, Yamamoto, Yoshiuchi, Bi, & Tanaka, 2004b). Formins are proteins that nucleate actin and mediate the endosomal transport to the bud neck during polarized growth (Evangelista, Pruyne, Amberg, Boone, & Bretscher, 2002; Kadota, Yamamoto, Yoshiuchi, Bi, & Tanaka, 2004a; Kozubowski, Larson, & Tatchell, 2005; Sagot, Rodal, Moseley, Goode, & Pellman, 2002). Furthermore, the formins are, in turn, activated by septins (Buttery, Kono, Stokasimov, & Pellman, 2012). The septin oligomers assemble as paired filaments of Cdc11-ended oligomers or rings in Shs1-ended (Bertin et al., 2008; Garcia et al., 2011; Khan et al., 2018). Finally, several kinases contribute to the septins assembly. For instance, the Pak kinase Cla4 and Gin4 (the Nim1-related kinase) are required for septin GTP hydrolysis and organization, respectively (Kadota et al., 2004b; C. R. Li, Yong, Wang, & Wang, 2012; Mark S. Longtine, Fares, & Pringle, 1998; Eric M. Mortensen, McDonald, Yates, & Kellogg, 2002; Versele & Thorner, 2004).

Upon assembly, septins form a ring-like structure at the bud neck. This structure has a dynamic septin organization that becomes stabilized when the definitive collar or hourglass-like structure is acquired during late anaphase (Fig. I10) (DeMay, Noda, Gladfelter, & Oldenbourg, 2011; Ong, Wloka, Okada, Svitkina, & Bi, 2014). The septins collar consist on double filaments composed by Cdc11

heterooligomers that are disposed parallel to the mother-daughter axis, covering the inner face of the bud neck plasma membrane. In perpendicular to the double filaments, there are disposed simple septin circular filaments composed by the Shs1 heterooligomers that stabilizes the collar (Garcia et al., 2011; Khan et al., 2018; Ong et al., 2014). This stabilization depends also on the Shs1 phosphorylation (Dobbelaere, Gentry, Hallberg, & Barral, 2003; Eric M. Mortensen et al., 2002; Versele & Thorner, 2004). This collar structure becomes reorganized in two rings (one in the mother side and one in the daughter side) at the onset of cytokinesis (Fig. I10), in a process regulated by F-Bar proteins Hof1 (Lippincott & Li, 1998a; Meitinger, Palani, Hub, & Pereira, 2013) and Bud4 (P. J. Kang, Hood-DeGrenier, & Park, 2013; McQuilken et al., 2017; H. Wu, Guo, Zhou, & Gao, 2015). The rings are structured by the initial simple filaments and by the reassembly of disassembled double filaments into simple circular ones in parallel to Shs1-filaments (DeMay et al., 2011; Ong et al., 2014). Upon cytokinesis completion, and just before new septin recruitment, septins are cleared from the bud neck (H. B. Kim et al., 1991; Ong et al., 2014). It occurs in a G1 Cyclin-Cdc28 and Cdc42 dependent manner. Cln-Cdc28 complex phosphorylates Cdc3 and primes septins for the recycling of the subunits by Cdc42, being inhibited the septin assembly in Cdc42-GDP regions (corresponding to the old bud neck) and recruited by Cdc42-GTP at the new bud site (Eluère, Varlet, Bernadac, & Simon, 2012; H. Lai et al., 2018; Tang & Reed, 2002).

b) Septins function

Septin collar and rings act as scaffold platforms for many proteins and impose a diffusion barrier for the regulation of polarity, cell remodeling and cytokinesis.

Septins serve as anchoring for the polarisome complex (Spa2, Bni1, Pea2 and Bud6 in budding yeast) that regulates the apical bud growth (H. Chen et al., 2012; Kadota et al., 2004b; Sheu, Barral, & Snyder, 2000) and polarizes actin by the interaction with formins (Buttery et al., 2012; Evangelista et al., 2002). Moreover, different regulators of the morphogenetic cell cycle control system are bound to septins. Some of these regulators are the proteins Elm1 kinase and the Hsl1 and Hsl7 kinases that regulate the Swe1 degradation promoting the bud isotropic growth during G2 (Barral et al., 1999; H. Kang, Tsygankov, & Lew, 2016; M. S. Longtine et al., 2000). The septin net wrapping the membrane at the bud neck also limits the transport of growing elements to the bud during isotropic growth (Barral, Mermall, Mooseker, & Snyder, 2000).

During cytokinesis, septins interact with the anillin-like Bni5 (in budding yeast) recruiting myosin (Myo1 in budding yeast) to the bud neck (Booth, Sterling, Dovala, Nogales, & Thorner, 2016; Fang et al., 2010; Kechad, Jananji, Ruella, & Hickson, 2012). Myo1 is an element of the contractile actomyosin ring (CAR), required for membrane evagination (see below). Another cytokinesis protein related to septins is the Hof1 F-bar protein, that regulates collar splitting and cytokinesis (Lippincott & Li, 1998a; Meitinger, Palani, et al., 2013; Oh, Schreiter, Nishihama, Wloka, & Bi, 2013; Vallen, Caviston, & Bi, 2000).

In human, septins are also involved in ciliogenesis during dendrification in neurons through the compartmentalization by the diffusion barrier properties of septins structures (Ageta-Ishihara et al., 2013; Hu et al., 2012) and in spermatozoids formation (Lai et al., 2016; Yeh et al., 2019).

3.3. Actomyosin ring

The actin filaments and myosin-II (Myo1 in budding yeast) interact to form an actomyosin ring (AMR). The actomyosin ring (AMR) regulates the membrane evagination through its contraction. In animal and yeast cells, the AMR (or contractile ring (CR) in animals) constricts centripetally—it is bound to the cell membrane from its formation—and mediates the stretching of the plasma membrane (PM). At the same time, actin cables are polarized towards the bud neck to deliver post-Golgi vesicles which fuse to the plasma membrane to increase the surface and release cargoes such as the chitin synthase-II (Chs2) to drive primary septum (PS) formation (Fig. 110) (Chuang & Schekman, 1996). In plants the separation of the two new cells do not require a contractile ring. By contrast, a structure of microtubules and actin filaments, called phragmoplast, located at the cell plate drives cell separation. The phragmoplast grows centrifugally until the two cells are completely separated by the cell wall (Müller & Jürgens, 2016).

a) AMR formation

Upon bud emergence, the septin ring expands to an hourglass (or collar) structure and together with the myosin marks the bud neck.

The septin collar serves as platform for the AMR formation where the first protein recruited is Bni5 (anillin in animal cells) that, in turn, recruits Myo1 to the septin collar (Fang et al., 2010; Finnigan, Booth, Duvalyan, Liao, & Thorner, 2015; S. R. Lee, Jo, Namgoong, & Kim, 2016). Bni5 promotes the septin restructuration in the central part of the bud neck in order to prepare myosin deposition (Booth et al., 2016; Patasi et al., 2015). Anillin-like proteins are removed from the bud neck in late anaphase before mitosis exit (Finnigan et al., 2015; P. R. Lee et al., 2002).

Anillin-like proteins are phosphorylated by Gin4 kinases, and this phosphorylation promotes their interaction with septins (Liang et al., 2017; Mortensen et al., 2002). Once Bni5 is localized to the bud neck, it recruits Myo1 through its Myo1's targeting domain 1 (TD1) (Fang et al., 2010).

At anaphase, actin filaments and myosin-II interact and form the AMR. The formation of the AMR requires the Iqg1 function (Lippincott & Li, 1998b). Iqg1 protein is a GAP protein with a cluster of IQ motifs (Lippincott & Li, 1998b; Shannon & Li, 1999). The IQ motifs are required for its localization at the bud neck during mitosis by the direct interaction with Mlc1 (Boyne, Yosuf, Bieganowski, Brenner, & Price, 2000; Shannon & Li, 1999, 2000; Tian, Wu, & Johnsson, 2014). The Mlc1 (or essential light chain (ELC) from Myo1) protein is recruited by Myo1 and localized at the septin collar during anaphase (Feng, Okada, Cai, Zhou, & Bi, 2015; Luo et al., 2004). In turn, Mlc1 anchors Iqg1 to the bud neck upon activation of the MEN pathway (Lippincott & Li, 1998b). Iqg1 is also phosphorylated at the calponin homolog domain (CHD) by Cdk1 inhibiting its recruitment to the bud neck before Cdc14 activation

(Naylor & Morgan, 2014). Cdc14 dephosphorylates Iqg1 contributing to its recruitment to the bud neck (D. P. Miller et al., 2015). At the bud neck, Iqg1 binds to Mlc1 by the CHD domain, but also to Myo1 and Hof1 by its GAP related domain (GRD) (Naylor & Morgan, 2014; Tian et al., 2014). Myo1 interaction keeps Iqg1 at the bud neck and stabilizes it (Fang et al., 2010; Wloka et al., 2013). Then, Iqg1 promotes the AMR formation by the engagement of actin filaments to the myosin ring (Bashour, Fullerton, Hart, & Bloom, 1997; Epp & Chant, 1997; D. P. Miller et al., 2015; Shannon & Li, 1999).

Actin filaments are formed and nucleated before its recruitment to the bud neck. Tropomyosins, profilins and formins are involved in filament formation and nucleation. These proteins are regulated by the Rho1 GTPase (RhoA in animals) —and Rac GTPase in animal cells (Zhao & Fang, 2005). Rho1 is mobilized by its GEF Tus1 (Ect2 in animals) and activated by Tus1 and Rom2. The GEFs are in turn regulated and activated at anaphase by Cdc5 kinase (Wagner & Glotzer, 2016; Yoshida et al., 2006). Then, Rho1 activates the formins, Bni1 and Bnr1 (Dong, Pruyne, & Bretscher, 2003; Yoshida et al., 2006). Bni1 and Bnr1 formins are associated to actin at different times. Bnr1 is present from G1 to telophase (Gao et al., 2010), while Bni1 is present from telophase to cytokinesis (Vallen et al., 2000). Moreover, while Bnr1 has a focused function in actin nuclearization for bud neck association, Bni1 has multiple roles in polarization and cytokinesis (Liu, Santiago-Tirado, & Bretscher, 2012; Sagot et al., 2002). Interestingly, they are also differentially regulated (apart from the Rho1 GTPase); Bni1 is regulated by Cdc42 (H. Chen et al., 2012; Evangelista et al., 1997), and Bnr1 is regulated by the septin Shs1

(Buttery et al., 2012). Finally, formins are inhibited by Hof1 which restrain actin nucleation to stabilize the AMR (Garabedian et al., 2018).

The completion of actin filaments is performed by profilins and tropomyosin. The profilins mediate the addition of the actin monomers to the filaments (Sherer, Zweifel, & Courtemanche, 2018). Tropomyosin direct contribution has not been determined, although it is required for actin nucleation (Alioto, Garabedian, Bellavance, & Goode, 2016).

Once actomyosin ring is formed, it triggers the mobilization of the proteins involved in AMR contraction and primary septum formation (Fig. I11) (Foltman et al., 2016; Naylor & Morgan, 2014; Tian et al., 2014; Wloka et al., 2013).

In animal cells, no new actin nucleation is required and, therefore, it has not been described any protein with a similar role to Iqg1 (Balasubramanian, Bi, & Glotzer, 2004). Instead, they mobilize the actin distributed across the plasma membrane (L. G. Cao & Wang, 1990). Moreover, as they do not have cell wall, they do not need PS formation.

b) AMR contraction

Actomyosin ring contraction triggers membrane evagination in both animal and yeast cells. The AMR is attached to the inner side of the plasma membrane at the site of division. The binding of the AMR to the plasma membrane is required to trigger membrane

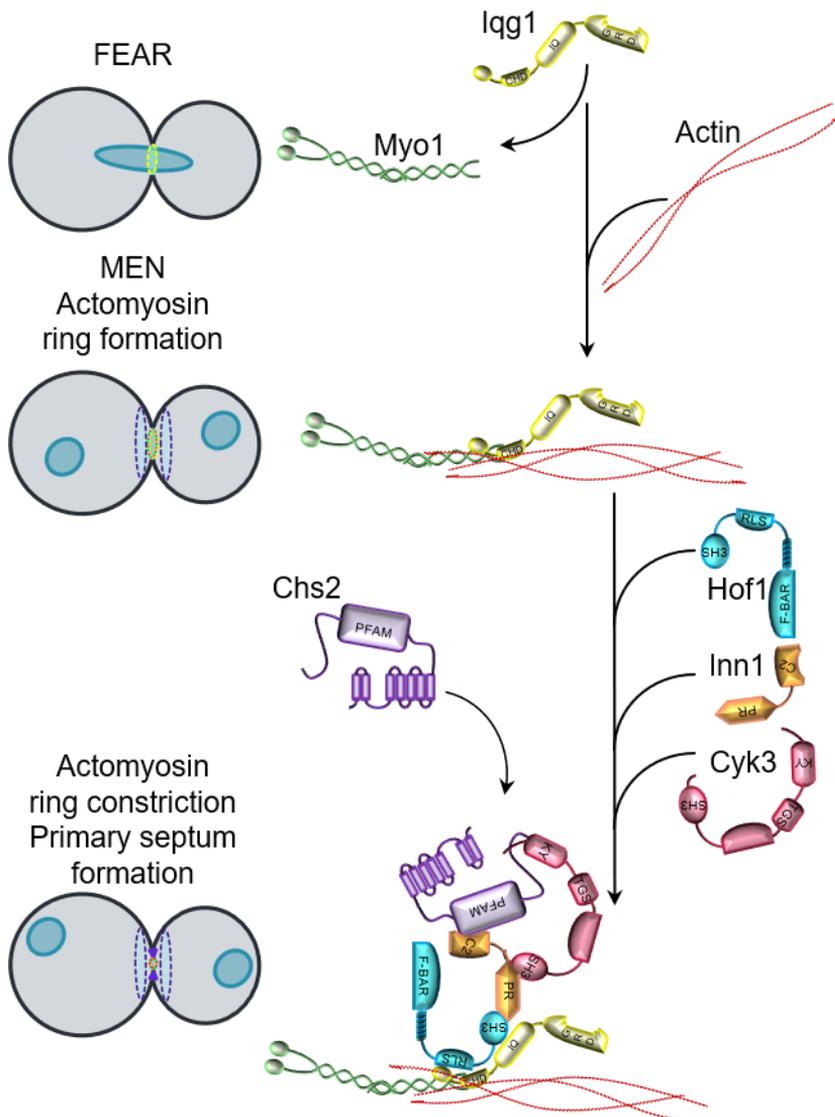


Figure I11. Ingression progression complex (IPC) and cytokinesis.

evagination. In budding yeast, it is achieved through Hof1 (or Cdc15 in fission yeast). Hof1 interacts with Iqg1 and Myo1 at the AMR through its ring location sequence (RLS) (Meitinger et al., 2011; Tian et al., 2014) and with the plasma membrane. Hof1 binds to the plasma membrane directly or/and through the interaction with PS regulators Inn1 and Cyk3 by its SRC homolog 3 (SH3)

domain (Jendretzki, Ciklic, Rodicio, Schmitz, & Heinisch, 2009; Moravcevic et al., 2015; Nishihama et al., 2009a; Oh et al., 2013). By contrast, the interaction is mediated by cadherins and β 1,3-glucans in animal cells (Guillot & Lecuit, 2013; Muñoz et al., 2013). There are some other particularities that make them different. The bud neck of the budding yeast cells has 1-3 μ m of diameter, while animal cells have 10-30 μ m of diameter. Despite the size difference, the velocity of AMR contraction is similar and takes about 5 minutes (Carvalho, Desai, & Oegema, 2009; Mendes Pinto, Rubinstein, Kucharavy, Unruh, & Li, 2012; Wollrab, Thiagarajan, Wald, Kruse, & Riveline, 2016). In addition, in yeast cells the AMR is dispensable although its absence perturb normal cytokinesis (Bi et al., 1998; Fang et al., 2010; Wloka et al., 2013); while animal cells are not able to perform cytokinesis in absence of AMR or microtubules (Wheatley & Wang, 1996; Zhao & Fang, 2005).

The mechanism of AMR constriction is not fully understood, but contraction seems to be coupled to AMR disassembly. Actin filament depolymerization and the motor domain of Myo1 play a major role in AMR contraction and disassembly. This depolymerization promotes the tension forces required for membrane evagination and is triggered by cofilin (Cof1) (Chew et al., 2017; Mendes Pinto et al., 2012). Cofilin binds to tropomyosin and mediates the disassembly of actin filaments (Bertling et al., 2004; Q. Chen, Courtemanche, & Pollard, 2015; de la Cruz, 2009). Myosin motor function contributes to the buckling of actin promoting its disassembly (Murrell & Gardel, 2012; Wollrab et al., 2019). In budding yeast, Myo1 motor function is not essential for

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AMR constriction, although contributes to the turnover of actin (Lord, Laves, & Pollard, 2005; Mendes Pinto et al., 2012).

Yeast cells also need to assemble a septum since they have cell wall. Primary septum (PS) deposition is mediated by vesicles fusion at the site of division that allow deposition of a specialized chitin structure. The AMR serves as bounding module for the regulators of the primary septum formation. It has been described that the disturbance of whichever of the process affects the viability of the other (Lippincott & Li, 1998a; Rodriguez & Paterson, 1990; Schmidt, Bowers, Varma, Roh, & Cabib, 2002). After primary septum formation, a secondary septum (SS) made of glucans and mannans is assembled at both sides of the primary septum.

Membrane evagination requires a driving force for evaginating: the actin filaments and the motor myosin II of the AMR form contractile bundles that invaginate the membrane. Moreover, addition of new membrane is required to increase the surface area and to seal the connection between the two daughter cells. Exocytic vesicles are transported to the AMR through the actin cables. The responsible players are the Rab GTPases (Ypt32/33 and Sec4 Rab in yeast, or Rab11 in animals) and the exocyst complex. Sec4 interacts with Myo2 (type V myosin) for its transport through the actin cables (Jin et al., 2011). In turn, Sec4 interacts with the post-golgi secretory vesicle containing its GEF Sec2 (Ortiz, Medkova, Walch-Solimena, & Novick, 2002). This interaction is mediated by the Ypt31/32 GTPases that recruit Sec2 to the vesicle (Casavola et al., 2008; Ortiz et al., 2002). The Sec4 GTPase facilitates the fusion of the vesicles through its interaction with the exocyst protein Sec15 (Heger, Wrann, & Collins, 2011; Lepore, Spassibojko, Pinto, &

Collins, 2016). The exocyst is a multiprotein complex required for vesicle tethering at the plasma membrane. This mechanism ensures that membrane expansion at the division site is coupled with the AMR constriction and septum deposition in time and space (VerPlank & Li, 2005). In addition, some cargoes are delivered to do specific functions and promote abscission. The chitin synthase II, Chs2 responsible for PS formation is one of the relevant cargoes and requires the exocyst complex for its normal localization of the bud neck (VerPlank & Li, 2005; G. Zhang, Kashimshetty, Kwee, Heng, & Foong, 2006).

Deletion-mutants yeast cells for Myo1 (*myo1Δ*) do not form a proper AMR. Instead, secretory vesicles are delivered to the bud neck fusing with the plasma membrane originating the remedial septum. Therefore, AMR assembly defects can be bypassed by the remedial septum formation (Schmidt et al., 2002; Tolliday, Pitcher, & Li, 2003).

3.4. Primary Septum

Actomyosin ring contraction and primary septum formation are mutually coordinated events required for budding yeast cytokinesis. The AMR serves as scaffold for the proteins that regulates cytokinesis, as Hof1 (Meitinger et al., 2011; Tian et al., 2014). Hof1 F-bar protein is essential for cytokinesis since it regulates septins splitting, AMR constriction and PS formation. Primary septum deposition separates the mother and bud cytoplasm concurrently to the AMR contraction. The PS is mainly composed of chitin, a

polymer of N-acetylglucosamine synthesized by the chitin synthase II, Chs2.

a) PS machinery activation

Hof1 is recruited to the bud neck upon bud emergence and localizes at the bud cortex via its F-BAR domain (Oh et al., 2013; Vallen et al., 2000). Later on, it is mobilized to the septin collar where it regulates its stability through the direct interaction of the Hof1's coiled coil 2 (CC2) domain and the Cdc10 septin (Meitinger, Palani, et al., 2013; Oh et al., 2013). Once in late anaphase, Hof1 is phosphorylated by Cdc5 and Dbf2 kinases (Meitinger et al., 2011; Meitinger, Palani, et al., 2013). These phosphorylations mediate its translocation to the

AMR, where it interacts with Myo1, Iqg1 and Mlc1 (Foltman et al., 2016; Naylor & Morgan, 2014; Oh et al., 2013; Tian et al., 2014). Hof1 presence at the AMR mediates three different events required for PS formation: 1) the septin collar splitting into two rings at both sides of the AMR, 2) the AMR contraction, and 3) the PS formation.

Septin collar splitting occurs at late anaphase, after Hof1 localization at the AMR. It has been described that Hof1 stabilizes the septins in the collar structure (Lippincott & Li, 1998a) and inhibits septin splitting (Oh et al., 2013). However, the molecular mechanism describing how Hof1 regulates septin splitting is not known.

Hof1 role regulating AMR contraction is better studied, although some features remain unknown. It was described that the absence of Hof1 destabilizes and disassembles the AMR (P. R. Lee et al., 2002; Lippincott & Li, 1998a). In contrast, excessive Hof1 at the AMR avoids the ring constriction and its removal from the AMR promotes constriction failures (Wolken, McInnes, & Pon, 2014; Blondel et al., 2005; W. J. Li et al., 2006; Meitinger et al., 2010; Stockstill et al., 2013). The Hof1 clearance is dependent on SCF^{Grr1} ubiquitin E3 ligase (Blondel et al., 2005; W. J. Li et al., 2006; Stockstill et al., 2013). Moreover, after AMR constriction, the resting Hof1 is translocated to the septin rings until the septins removal upon septation (Lippincott & Li, 1998a; Vallen, Caviston, & Bi, 2000). These observations indicate that Hof1 localization at the AMR is timely regulated. AMR constriction starts after Hof1 localization; however, the molecular mechanism how Hof1 promotes AMR contraction is not well defined. Hof1 could induce AMR constriction by its actin disassembly function (Garabedian et al., 2018; Graziano et al., 2014) or by the activation of primary septum formation (Foltman et al., 2016; Jendretzki et al., 2009; Korinek et al., 2000; Nishihama et al., 2009b; M. Wang, Nishihama, Onishi, & Pringle, 2018).

PS formation is dependent on the activation of chitin synthase II (CSII), which catalytic subunit is Chs2 (Sburlati & Cabib, 1986). Hof1 interacts with Chs2 regulating also PS formation. Chs2 is activated by the Cyk3 and Inn1 proteins (Fig. I11) (components of the IPC (Ingression promoting complex); Foltman et al., 2016). Inn1 protein is recruited to the bud neck in late anaphase, when AMR starts to be constricted (Sanchez-Diaz et al., 2008b) and was proposed to depend on its phosphorylation state (Meitinger et al.,

2010; Palani, Meitinger, Boehm, Lehmann, & Pereira, 2012). Inn1 is dephosphorylated at Cdk1-dependent sites, probably by Cdc14 (Meitinger et al., 2010; Palani et al., 2012). At the bud neck, Inn1 interacts with Iqg1 and Hof1 at the AMR (Nkosi, Targosz, Labib, & Sanchez-Diaz, 2013). Then, Inn1 interacts with Hof1 and the Cyk3 transglutaminase-like protein (Foltman et al., 2016; Nkosi et al., 2013; Palani et al., 2012). Cyk3 also requires MEN activity to be recruited (Meitinger et al., 2010; Oh et al., 2012; Palani et al., 2012). Cyk3-Hof1-Inn1 complex activates Chs2, both by Inn1 and Cyk3 direct interaction (Devrekanli, Foltman, Roncero, Sanchez-Diaz, & Labib, 2012; Foltman et al., 2016; Oh et al., 2012; Oh et al., 2013; Wang et al., 2018).

Strikingly, Cyk3 also has been proposed to activate Chs2 independently to Hof1 and Inn1 (Foltman, Filali-Mouncef, Crespo, & Sanchez-Diaz, 2018; Ko et al., 2007; Nishihama et al., 2009b; M. Wang et al., 2018). Some reinforcing arguments are: 1) Cyk3 can be also recruited by the Spa2 protein (Foltman et al., 2018), 2) Cyk3 overexpression rescues PS formation in *inn1* Δ (Nishihama et al., 2009b; M. Wang et al., 2018), and 3) regulates the PS formation by inhibiting Rho1 that activates the SS formation (Atkins et al., 2013; Onishi, Nolan, Nishihama, & Pringle, 2013).

b) PS formation by Chs2

There are 7 different chitin synthases (Chs1-6) separated into 2 families: family 1 (Chs1-2) and family 2 (Chs3-7) (reviewed in (Ruiz-Herrera, Manuel González-Prieto, & Ruiz-Medrano, 2002). They are grouped depending on its chitin synthase (CS) activity.

The differences between the chitin synthases, confers them specific functionality (reviewed in Roncero, 2002). Chitin synthase I (CSI) activity depends on Chs1 and regulates the repair of the cell wall at the cytokinesis site, since the chitinases sometimes degrade excessive cell wall (CW) that need to be restored (Cabib, Sburlati, Bowers, & Silverman, 1989). CSIII depends on Chs3 enzyme and is responsible for the secondary septa formation representing the 90% of the chitin synthesis present at the CW (Shaw et al., 1991). Chs3 has been also involved in the formation of the remedial septum, a chitin structure that allows the cytokinesis in absence of PS (Cabib & Schmidt, 2003; Schmidt et al., 2002). Chs4-7 are regulators of the CSIII activity by interacting and recruiting Chs3 (Oh et al., 2017; Ono et al., 2000; Santos & Snyder, 1997; Trilla, Durán, & Roncero, 1999; Trilla, Cos, Duran, & Roncero, 1997; Ziman, Chuang, Tsung, Hamamoto, & Schekman, 1998).

Chs2 is the chitin synthase responsible of primary septum formation (Sburlati & Cabib, 1986; Shaw et al., 1991). Chs2 protein levels fluctuate along the cell cycle, suggesting the regulation of the protein at the synthesis/degradation level (Choi, Sburlati, & Cabib, 1994; Chuang & Schekman, 1996). Moreover, Chs2 localization changes during cell cycle and depends on the kinases and phosphatases activities (Martínez-Rucobo, Eckhardt-Strelau, & Terwisscha van Scheltinga, 2009). Chs2 expression peaks during G2/M and is maintained at the ER upon synthesis (Choi et al., 1994). The ER retention depends on the Chs2 phosphorylation by Clb2-Cdc28, that inhibits its release from the ER (Chin, Bennett, Ma, Hall, & Yeong, 2012; Teh, Chai, & Yeong, 2009). In late anaphase, Chs2 is translocated from the ER when the Clb2-Cdc28 activity levels decline (VerPlank & Li, 2005; G. Zhang et al., 2006),

is phosphorylated by Cdc5 (Jakobsen et al., 2013) and dephosphorylated by Cdc14 phosphatase (Chin et al., 2012). Dephosphorylated Chs2 is packaged into the COPII vesicles (Jakobsen et al., 2013). These vesicles are transported to the bud neck by the exocyst machinery where they fused to the cell wall (VerPlank & Li, 2005; G. Zhang et al., 2006). Once located at the bud neck, Chs2 will be activated by Inn1 and Cyk3 (Devrekanli et al., 2012; Foltman et al., 2016; M. Wang et al., 2018). Active Chs2 catalyzes the chitin synthesis reaction that consists on the formation and linkage of the β 1,4-linked N-acetylglucosamine to the plasma membrane. Two groups of N-acetylglucosamine (GlcNAc) from 2 uridine diphosphate N-acetylglucosamine form the β 1,4-linked N-acetylglucosamine (Gyore et al., 2014; Orlean, 1987).

Chs2 is an essential protein. In absence of Chs2 activity, abnormal septa are formed and the AMR is assembled but contracts asymmetrically (Schmidt et al., 2002; VerPlank & Li, 2005). This asymmetric AMR contraction is characteristic of AMR mutants, such as *hof1* and *inn1* (Lippincott & Li, 1998a; Sanchez-Diaz et al., 2008b), and support the idea of mutual dependency between PS formation and AMR contraction.

Upon PS formation, the Chs2 must be disassociated from the bud neck in a process that requires both the Dbf2-dependent phosphorylation (Oh et al., 2012) and the endocytosis machinery with the Pep4 protease, that will degrade the Chs2 inside the vacuole (Chin et al., 2012; Chuang & Schekman, 1996).

3.5. Secondary Septum

After primary septum formation, cell starts the synthesis of two secondary septa (SS) at both sides of the primary septum. The secondary septa are composed by the same elements than the cell wall, being the β -1,3-glucan and mannoproteins the main components. The chitin is also part of the SS, providing additional structural reinforcement although just represent 1-2% of the total components (Lesage & Bussey, 2006). The synthesis of these elements is dependent on chitin synthase III –formed by the catalytic subunit Chs3 and the regulatory subunit Chs4–, glucanases, and mannosyltransferases (Cabib et al., 1989; Douglas et al., 1994; Yamamoto, Guacci, & Koshland, 1996). The main glucanase in budding yeast is the integral membrane protein Fks1 (Douglas et al., 1994). Interestingly, glucanases and CSIII are regulated by the Rho1 GTPase (Qadota et al., 1996; Shaw et al., 1991). Rho1 regulates the hydrolases until Lrg1 (Rho1 GAP) inactivates it (Jonasson et al., 2016; Svarovsky & Palecek, 2005).

Rho1 is localized at the cell wall regulating two different processes at different moments of the cell cycle: budding and cytokinesis. During cytokinesis, Rho1 is first involved in actomyosin ring assembly regulating formin-mediated actin cable assembly (Kamada et al., 1996; Kohno et al., 1996; Yoshida, Bartolini, & Pellman, 2009). Rho1 is inhibited by Cyk3 during primary septum formation and reactivated afterwards to promote secondary septum deposition (Onishi et al., 2013). During septation, Rho1 activation is regulated by the PIP2 interaction and Tus1 GEFs association (Onishi et al., 2013; Yoshida et al., 2009). Once activated, Rho1 recruits both Fks1 and Chs3 (Qadota et al., 1996; Shaw et al., 1991; Valdivia & Schekman, 2003). Rho1 regulates Chs3 recruitment through the activation of the cell wall integrity pathway

components *Slr2* and *Pkc1* (Kamada et al., 1996; Valdivia & Schekman, 2003).

Chs3 is retained at the ER upon synthesis (similar to *Chs2*) until *Chs7* allows its translocation to the chitosomes before cytokinesis (Flores Martinez & Schwencke, 1988; Leal-Morales, Bracker, & Bartnicki-Garcia, 1988; Valdivia, Baggott, Chuang, & Schekman, 2002). Chitosomes are between the trans-Golgi network (TGN) and early endosomes (Trilla, Durán, & Roncero, 1999). *Chs3* transport to the bud neck during budding and cytokinesis depends on *Bni4* protein (DeMarini et al., 1997; Kozubowski et al., 2003). *Bni4* is associated to the septins and phosphorylated by two kinases, the Cdk *Pho85* (Zou et al., 2009) and the MAPK *Slr2* (Pérez, Arcones, Gómez, Casquero, & Roncero, 2016), to promote the *Chs3* transport through *Chs4*. Phosphorylated *Bni4* activates *Chs4* (Larson et al., 2008; Sanz, Castrejón, Durán, & Roncero, 2004) which, in turn, activates *Chs3*. *Chs4-Chs3* interaction is important for the *Chs3* activation, but also to maintain *Chs3* at the bud neck avoiding the degradation and recycling of *Chs3* (Arcones, Sacristán, & Roncero, 2016; Ono et al., 2000; Reyes, Sanz, Duran, & Roncero, 2007). In addition, *Chs4* is also phosphorylated by *Gin4*, which is required for its proper localization at the bud neck (Gohlke, Heine, Schmitz, & Merzendorfer, 2018). Once at the bud neck, active *Chs3* mediates the chitin synthesis by the same reaction than *Chs2*. Surprisingly, both *Chs2* and *Chs3* are delivered to the division site at the onset of cytokinesis (VerPlank & Li, 2005; Yoshida et al., 2009), but *Chs3*-mediated chitin synthesis is inhibited by *Hof1* interaction with *Chs4* (Oh et al., 2017). The SS synthesis is stopped once the secondary septa acquire the appropriate thickness. At that point, the *Lrg1* *Rho1*-GAP turns off

Rho1 promoting the inactivation of the Chs3 activating pathway (Meitinger, Richter, et al., 2013; Svarovsky & Palecek, 2005).

Secondary septum deposition is a very robust process and it can take place even in the absence of primary septum formation and AMR constriction by the formation of the “remedial septum” (Cabib & Schmidt, 2003). The remedial septum is essentially a disorganized secondary septum deposited at the bud neck that is enough to separate the mother and daughter cells avoiding cell lethality. Also, in absence of chitin synthase activity, deposition of other polymers in the remedial septum occurs (Schmidt, 2004). Remedial septum is formed in *chs2* Δ and *myo1* Δ mutants (Schmidt et al., 2002). This reparatory mechanism allows cell to bypass the defects in AMR constriction and PS formation.

3.6. Abscission

To complete the physical separation of the mother and daughter cells, the primary septum and part of the secondary septum are degraded. The endochitinase Cts1 (Kuranda & Robbins, 1991) and the glucanases Eng1 and Egt2 (Baladrón et al., 2002; Kovacech, Nasmyth, & Schuster, 1996) are the hydrolases responsible for PS degradation.

The chitinase Cts1 and glucanases expression depends on the transcription factor Ace2 at the daughter side. Asymmetric localization and activation of Ace2 acts as the daughter specific factor that regulates the hydrolases synthesis only at the daughter side (Kovacech et al., 1996; Kuznetsov, Váchová, & Palková, 2016;

O'Conalláin, Doolin, Taggart, Thornton, & Butler, 1999). Swi5 transcription factor is also required for cell separation. Swi5 regulates the transcription of the Cdk1 inhibitor, (Jaspersen, Charles, Tinker-Kulberg, & Morgan, 1998b; Knapp, Bhoite, Stillman, & Nasmyth, 1996b; Toyn, Johnson, Donovan, Toone, & Johnston, 1997; Visintin et al., 1998) but also the expression of genes involved in cell septation and polarity together with Ace2 (Toyn et al., 1997; Voth, Olsen, Sbia, Freedman, & Stillman, 2005).

Ace2 transcription and activation is regulated by the RAM pathway, a conserved signaling network involved in polarized cell growth and cell separation (homolog to the Hippo pathway in animals) (Brace, Hsu, & Weiss, 2011; Racki, 2000). RAM pathway is asymmetrically activated at the daughter cell and is composed by: Tao3, Hym1, Kic1, Sog1, Mob2 and Cbk1 proteins; being the two last the final effectors (Nelson et al., 2003; Racki, 2000; Voth et al., 2005). Cbk1/Mob2 localization at the daughter nucleus is regulated positively by MEN via the Cdc14-dependent dephosphorylation at Cdk1 sites; and negatively by the activation of the Cbk1-inhibitor, Lre1 (Brace et al., 2011; Mancini Lombardi et al., 2013; Versele & Thevelein, 2001). Active Cbk1 phosphorylates Ace2 promoting its activation and avoiding its nuclear export from the daughter nucleus (Bourens et al., 2008; Mazanka et al., 2008). Upon septa hydrolysis and cell separation, the daughter cell inhibits Ace2 by Pho85 and Cdc28-Cln dependent phosphorylation (Mazanka & Weiss, 2010). Phosphorylated Ace2 is translocated to the cytoplasm and remains there until the next cell division. Swi5 inhibition is mediated by its low stability at the nucleus, being degraded upon transcription of its targets (Tebb, Moll, Dowzer, & Nasmyth, 1993).

In animal cells there is a specific regulation mediated by the endosomal sorting complex required for transport II (ESCRT-III). The last joining region between the two new cells is called midbody. The midbody is an electron dense structure that must be removed, and the initial step is the removal of actin and microtubules (Green et al., 2013; Schiel et al., 2012). The ESCRT-III machinery that mediates the abscission is probably delivered by the exocytosis vesicles present at the midbody (Goss & Toomre, 2008; Kumar et al., 2019). In fact, Rab35 GTPase, a component of the exocyst, is responsible of the removal of the microtubules; together with actin depolymerases (Dambournet et al., 2011; Klinkert & Echard, 2016; Neto, Balmer, & Gould, 2013). The ESCRT-III and the membrane tension generated by the transmembrane glycoproteins integrins coordinate the abscission by the membrane remodeling (N. Elia, Fabrikant, Kozlov, & Lippincott-Schwartz, 2012; Green et al., 2013; Gupta, Du, Kamranvar, & Johansson, 2018).

3.7. MEN and cytokinesis regulation

The MEN pathway is essential for mitotic exit, but it has also been involved in cytokinesis regulation (Jiménez, Castelao, González-Novo, & Sánchez-Pérez, 2005; Jiménez et al., 1998). The fine coordination between mitosis exit and cytokinesis is essential to ensure the proper completion of cytokinesis.

During late anaphase, MEN is involved in the AMR formation by the regulation of actin recruitment. Dephosphorylation of Iqg1 by Cdc14 is required for the recruitment of actin (D. P. Miller et al.,

2015) and Cdc5 is involved in actin nucleation by the phosphorylation of the Tus1 Rho1 GEFs that, in turn, activates the formins (Yoshida et al., 2006).

MEN is also intimately related to the AMR contraction. Cdc5 and Dbf2 phosphorylate Hof1 promoting its translocation to the AMR (Meitinger et al., 2011; Meitinger, Palani, et al., 2013). It has been also suggested that Cdc14 could be involved in this process by Cdk1-dependent dephosphorylation of Hof1 (Meitinger et al., 2010). In addition, the MEN-dependent Hof1 localization at the AMR regulates indirectly the septin collar splitting.

During primary septum formation, MEN regulates the process at different levels. PS formation requires of Hof1-Inn1-Cyk3 interaction for Chs2 activation (Foltman et al., 2016). The formation of the Hof1-Inn1-Cyk3 complex depends on the Cdc14-dependent dephosphorylation of Inn1 in order to be able to interact with Cyk3 (Meitinger et al., 2010; Palani et al., 2012). The Cyk3 protein in turn depends on MEN indirectly by Inn1 and Iqg1 regulation and Cdc14 is required for proper Cyk3 localization (Meitinger et al., 2010; Oh et al., 2012; Palani et al., 2012). Finally, Chs2 is phosphorylated by Cdc5 and dephosphorylated by Cdc14 to regulate its proper localization at the bud neck and activation (Chin et al., 2012; Jakobsen et al., 2013). After primary septum formation, Chs2 is phosphorylated by Dbf2 to be dissociated from the bud neck (Oh et al., 2012).

3.8. Cytokinesis and disease

Cytokinesis is a quick process with multiple successive steps that must be finely coordinated in order to succeed in cell separation. The disturbance of some of the cytokinetic components in human cells provokes different pathologies.

Septins have been involved in neurological disorders and cancer. Septins play a role in neurons ramifications and dendrites formation (Tada et al., 2007), being septin7 the most abundant septin at the forebrain (Peng et al., 2004). Defective septin7 functions have been related to neural pathologies such as the Alzheimer disease (Cruz & Tsai, 2004). The upregulation of the Cdk5 kinase disturbs the correct neuronal spines formation by an alteration of the septin levels (Engmann et al., 2011). In addition, septin7 is involved in ciliary defects during spermiogenesis related to asthenospermia pathology (Chao et al., 2010). Reduced levels of septin7 are linked to abnormal ciliary morphology and lower motility (Chao et al., 2010). Septin7 upregulation has been also related to cancerogenic cells growth in gliomas (Xu et al., 2010). Finally, upregulation of septin2 and septin11 has been related to renal cell carcinoma (RCC) (Craven et al., 2006).

The depletion of the tumor suppressor BRCA2 at the midbody in breast cancer cells promotes the disorganization of myosin II and, as consequence, cytokinesis failure (Daniels, Wang, Lee, & Venkitaraman, 2004). Other tumor suppressor is the large tumor suppressor 1, LATS1, which downregulation mediates the instability of the contractile ring by cofilin (actin depolymerization protein) inhibition (X. Yang et al., 2004).

Remodeling of the extracellular matrix (ECM) which might be analogous to septation in yeast is also a hallmark of animal cytokinesis (reviewed in Bi & Park, 2012). Defective glycosaminoglycan synthesis causes embryonic lethality and cytokinetic failure in *C. elegans* and mice (Izumikawa et al., 2010; Mizuguchi et al., 2003).

4. Polo-like kinases and Cdc5

Polo like kinases is a family of serine/threonine (S/T) protein kinases conserved along eukaryotes. Polo-like kinases were first described in *Drosophila*, where the unique representative is Polo (Sunkel & Glover, 1988). While in yeast we find also just one representative, Cdc5 in *S. cerevisiae* and Plo1 in *S. pombe*; in higher eukaryotes as *Xenopus* and *H. sapiens* have been identified 5 homologues (*PLX1-5* and *PLK1-5* respectively) divided in 3 different subfamilies (reviewed in De Cárcer, Manning, & Malumbres, 2011).

All polo-like kinases are monomeric kinases that contain a conserved protein structure that consists on the presence of the S/T kinase domain at the N-terminal and one or more polo-box domain (PBD) at the C-terminal (Cheng, Lowe, Sinclair, Nigg, & Johnson, 2003). The PBD determines the substrate specificity and the subcellular localization in the cell (Kyung S. Lee, Grenfell, Yarm, & Erikson, 1998; Song, Grenfell, Garfield, Erikson, & Lee, 2000). Usually, the PBD recognizes their interactors by a phosphorylated or “primed” sequence. It was described that the polo-like kinases have preference for the S/pS/pT-P/X sequences,

characteristic of the Cdk1 consensus sequence, acting the Cdk1 as a primase for Cdc5 (A. E. H. Elia, Cantley, & Yaffe, 2003; A. E. H. Elia, Rellos, et al., 2003). Although the most accepted Cdc5 consensus sequence is the Paulson consensus site D-E-N/X/S-T, included in the best-studied substrates Bfa1 and Scc1 (Nakojima, Toyoshima-Morimoto, Taniguchi, & Nishida, 2003). The catalytic domain at the N-terminal mediates the kinase activity and is dependent on the asparagine 209 (N209) in the active center of the enzyme. Finally, Polo kinases contain the destruction box or KEN box that is recognized by the APC/C^{Cdh1} for its ubiquitination and degradation at the end of mitosis (Arnold, Höckner, & Seufert, 2015; Lindon & Pines, 2004).

Cdc5 is activated by the phosphorylation of the activation loop at the onset of mitosis. In *S. cerevisiae* the activation depends on the Cdk1 phosphorylation mainly at the threonine 242 (T242), and the threonine 70 and 238 to activate late mitotic functions of Cdc5 (Mortensen, Haas, Gygi, Gygi, & Kellogg, 2005; Rodriguez-Rodriguez, Moyano, Játiva, & Queralt, 2016). The activation loop containing the T238 and T242 is highly conserved in evolution (K S Lee & Erikson, 1997). The Plk1 (human homologue of Cdc5) also have an Aurora A-dependent mitotic regulation by the phosphorylation at T210 and S137 (Bruinsma, Macůrek, Freire, Lindqvist, & Medema, 2014; Macůrek et al., 2008); suggesting that the mechanism have diverged during evolution.

Cdc5, as its homologues, is involved in multiple processes during the cell cycle. It regulates cell polarity, cell fate, meiosis, G2/M transition, cohesin cleavage, centrosomes separation, centromeres and kinetochores function, chromosome condensation, spindle

assembly, mitosis exit and cytokinesis (reviewed in Van De Weerd & Medema, 2006). Moreover, is critical in the repairing process of DNA damage response (Pakchuen et al., 2016; Ratsima, Serrano, Pascariu, & D'Amours, 2016; Rawal et al., 2016). Nevertheless, the Cdc5 direct phosphorylation targets in most of these processes have not been described. The best-studied Cdc5 substrates are the MEN inhibitor, Bfa1 during anaphase (Baro et al., 2013; Botchkarev et al., 2017; J. Kim et al., 2012) and the Scc1 subunit of the cohesin complex (Alexandru, Uhlmann, Mechtler, Poupart, & Nasmyth, 2001b; Pakchuen et al., 2016).

Cdc5 was also involved in the regulation of the Cdc14 release from the nucleolus (Shou, Azzam, Chen, Huddleton, et al., 2002; Visintin et al., 2003) although the exact mechanism and its direct target have not been described. Net1 sequesters Cdc14 at the nucleolus until the downregulation of PP2A^{Cdc55} at anaphase onset allows the increase of the Cdk1-dependent phosphorylation of Net1 (Queralt et al., 2006). Nevertheless, it was previously seen that the Cdc5 ectopic expression promotes Cdc14 release from the nucleolus (Manzoni et al., 2010; Visintin et al., 2003) and increases the Cdc14 and Net1 phosphorylation levels (Shou, Azzam, Chen, Huddleton, et al., 2002; R Visintin et al., 2003). Moreover, it was described that Cdc5 interact with Cdc14 and Esp1 FEAR component *in vivo* (Rahal & Amon, 2008). *CDC5* is an essential gene and the difficulties to have a complete null mutant introduced more controversy in the Cdc5 role regulating Cdc14 activation. Different thermosensitive, degron mutants of Cdc5 and the drug-inhibited *cdc5-as1* allele present distinct levels of Cdc14 release from the nucleolus introducing more debate to the Cdc5 role regulating Net1 phosphorylation (Hancioglu & Tyson, 2012; Liang

et al., 2009; Rodriguez-Rodriguez et al., 2016; Stegmeier et al., 2002; Yoshida & Toh-e, 2002). Recently, in our laboratory we demonstrated that Cdc14 release was defective in several Cdc5 phospho-mutants, being impaired the FEAR-Cdc14 release in the *cdc5-T242A* mutant and MEN-Cdc14 release in the *cdc5-T70A* mutant (Rodriguez-Rodriguez et al., 2016). In addition, although Net1 was phosphorylated *in vitro* by Cdc5 (Rodriguez-Rodriguez et al., 2016; Wenying Shou, Azzam, Chen, Huddleton, et al., 2002), the *in vivo* Cdc5 phosphorylation sites has not been identified yet. Altogether, we envisage that Cdc5 has a role in FEAR by phosphorylating Net1, albeit it has to be characterized the specific phosphorylated sites and the specific role of Cdc5 at early anaphase. This is the topic under study in the objective 2 of this thesis.

5. PP2A^{Cdc55}

5.1. PP2A phosphatases

The PP2A phosphatase is part of a family of serine/threonine phosphatases conserved along eukaryotes. It is a multimeric enzyme that can be structured as a heterodimeric or heterotrimeric holoenzyme. This multimeric structure is constituted by a scaffold subunit A (or PR65), a catalytic subunit C and a regulatory subunit B. The A and C subunits form the dimeric core enzyme that can bind to different regulatory subunits. In budding yeast there is just a scaffold subunit Tpd3 (van Zyl et al., 1992) and 2 catalytic subunits Pph21 and Pph22 (Sneddon, Cohen, & Stark, 1990). The regulatory B subunit is divided into 4 families in mammals B (B55

or PR55), B' (B56 or PR61), B'' (PR48/PR72/PR130) and B''' (PR93/PR110). In humans, this classification can be subdivided into up to 5 isoforms that in turns have different splice variants (reviewed in Shi, 2009). In budding yeast there are 2 regulatory subunits: Rts1 and Cdc55 (Healy et al., 1991b; Shu, Yang, Hallberg, & Hallberg, 1997). The regulatory subunit confers to the PP2A holoenzyme the specificity towards the substrates and determines the localization of the PP2A phosphatase.

The assembly of all the subunits is determined by the post-translational modifications. The catalytic subunits Pph1 and Pph22 are methylated at the C-terminal lysine309 by Ppm1 in budding yeast or Pmt1 in mammals in order to interact with the regulatory subunit and be functional as a holoenzyme (Wei et al., 2001). Counteracting the methylation, there is the methylesterase Pme1, which was described to be also protecting the degradation of the C subunit (Yabe et al., 2015, 2018). The phosphorylation of the Pph21 protein at the residue threonine364 or tyrosine367 is also required for the assembly to the Cdc55 regulatory subunit (Wei H et al, 2001). A mechanism for inhibiting the activity of the PP2A is the phosphorylation of the tyrosine307 (J. Chen, Martin, & Brautigan, 1992).

5.2. Cdc55 regulatory subunit

In this study we will consider PP2A^{Cdc55} as the heterotrimer composed by Tpd3, Pph21/Pph22 catalytic subunits and Cdc55. Cdc55 is a 55KDa protein found to be the regulatory B subunit of PP2A in *Saccharomyces cerevisiae* (Healy et al., 1991). The

coding gene is localized at the left arm of chrVII and it is constitutively expressed. It was first described as the regulatory subunit of the PP2A phosphatase in mammalian cells, where B55 α subunit has a 53% of homology with Cdc55 (Healy et al., 1991; Z. Zhang et al., 2011).

Cdc55 and Tpd3 are found in the same subcellular localizations. They are localized at the cytoplasm and the nucleus throughout the cell cycle. During G1/S, they are also detected at the cortex of the new bud upon bud emergence. At cytokinesis they are mobilized to the bud neck. At the bud neck, their localization was seen to be dynamic: first at the daughter side, followed by two rings that converge in one after septation (Gentry & Hallberg, 2002).

Deletion mutant cells for *CDC55* show elongated morphology (Healy et al., 1991) that was described to be a consequence of a delay in Cdc28 activation during G2/M (Minshull et al., 1996; H. Yang, Jiang, Gentry, & Hallberg, 2000). In addition, *cdc55 Δ* is cold sensitive and resistant to nocodazole arrest at G2/M (Y. Wang & Ng, 2006).

PP2A^{Cdc55} was described to be involved in the regulation of cell polarization (Jonasson et al., 2016), meiosis (Kerr, Wong, & Arumugam, 2016), Start activation (McCourt, Gallo-Ebert, Gonghong, Jiang, & Nickels, 2013; Moreno-Torres et al., 2015), DNA replication (K. Yamamoto et al., 2018), the morphogenetic control at the G2 to M transition (Pal et al., 2008), the spindle assembly checkpoint (Vernieri et al., 2013), cohesin regulation (Yaakov, Thorn, & Morgan, 2012), Cdc14 activation (Calabria et al.,

2012; Játiva et al., 2019; Queralt et al., 2006), mitosis exit network regulation (Baro et al., 2013), and cytokinesis (Healy et al., 1991).

5.3. PP2A^{Cdc55} regulation

The best-studied functions of the PP2A^{Cdc55} are the regulation of mitosis entry and the mitotic exit. The PP2A^{Cdc55} phosphatase is regulated by the Zds1/2 paralogues proteins and by the endosulfines (Igo1/2 in budding yeast) (Rossio, Kazatskaya, Hirabayashi, & Yoshida, 2014). Similar sequences to Zds proteins were found in the pseudouridine synthase Pus10 in humans (Yasutis et al., 2010).

The *IGO1/2* genes belong to the *ENSA* family 4 of endosulfines and are homologues to the *ARPP19* in animals. The endosulfines are activated by the Greatwall kinase (Rim15 in *S. cerevisiae*) (Talarek et al., 2010). Once active, the endosulfines bind to and inhibit the PP2A^{Cdc55} phosphatase (Bontron et al., 2013). It was described that this inhibition allows the cell to prevent premature Start activation (Bontron et al., 2013; McCourt et al., 2013; Sarkar, Dalgaard, Millar, & Arumugam, 2014).

PP2A^{Cdc55} is also regulated by the endosulfines depending on the availability of nutrients. Upon nutrients sensing, TOR pathway promotes the PP2A^{Cdc55} activity and, as consequence, the Start activation for G1/S progression. TORC1 kinase inhibits Rim15 alleviating the inhibitory signal on PP2A^{Cdc55} through the Igo1/2 (Moreno-Torres, Jaquenoud, & De Virgilio, 2015). However, under poor nutrients conditions, where TORC1 and PKA levels are low,

Rim15/Greatwall inhibitory pathway is activated and PP2A^{Cdc55} blocked. This inhibition promotes the entry to G₀, quiescence conditions in G₁ cells (Bontron et al., 2013; Sarkar et al., 2014), or quick entry into mitosis in G₂ cells, as consequence of Clb2-Cdc28 activation (Bontron et al., 2013; Chica et al., 2016; Moreno-Torres, Jaquenoud, & De Virgilio, 2015; Moreno-Torres, Jaquenoud, Péli-Gulli, Nicastro, & De Virgilio, 2017; Pal et al., 2008). Recently, it has been also described the implication of Rim15 pathway and PP2A^{Cdc55} in Whi5 phosphorylation during G₁ (Talarek, Gueydon, & Schwob, 2017).

PP2A^{Cdc55} was described to be involved in the regulation of Cdk1 activity during G₂/M by dephosphorylating Mih1 (Cdc25 in mammals) and Swe1 (Wee1 in mammals) (F. Hu et al., 2008; Pal et al., 2008). Surprisingly, it was also described that Igo1/2 have a positive role in PP2A^{Cdc55} activation promoting G₂/M transition and regulating PP2A^{Cdc55} nuclear export (Juanes et al., 2013).

PP2A^{Cdc55} function in the G₂/M transition is also dependent on a Zds1/2 dual-function. Zds1/2 proteins regulate the phosphatase activity through the direct interaction between Cdc55 and Zds1 C-terminal (Calabria et al., 2012; Yasutis et al., 2010). This interaction is required for inhibiting PP2A^{Cdc55} during G₂/M (Yasutis et al., 2010). Zds1/2 inhibition avoids the phosphatase-dependent dephosphorylation of Swe1. Swe1 hyperphosphorylation allows its own inhibition and, consequently, Cdc28 phosphorylation on Y19 is reduced promoting mitotic entry (Yasutis et al., 2010).

Recently, it was reported that PP2A^{Cdc55} has a role in cell polarity and cell wall synthesis by inhibiting the Rho1 GTPase during the

G2/M transition (Jonasson et al., 2016). Interestingly, it was also described that Pkc1 (*PKC α - β* homolog in mammals) phosphorylates and activates PP2A^{Cdc55} mediating the dissociation of Igo1/2 and promoting its activation (Thai et al., 2017). This allows the cell to coordinate mitotic entry with new cell wall synthesis.

Moreover, PP2A^{Cdc55} regulates exit from mitosis. At metaphase, it inhibits the mitosis progression by dephosphorylating Net1 and keeping Cdc14 sequestered at the nucleolus (Queralt et al., 2006). At anaphase onset, Zds1/2 proteins and separase cooperatively trigger mitosis exit by the downregulation of PP2A^{Cdc55} (Queralt & Uhlmann, 2008b). This inhibition is mediated by the Zds1 C-terminal interaction with Cdc55 (Calabria et al., 2012). In addition, PP2A^{Cdc55} is phosphorylated by Clb2-Cdc28 at anaphase onset (Játiva et al., 2019). Upon Cdc55 phosphorylation mediated by Zds1 and separase, PP2A^{Cdc55} activity is inhibited promoting Cdc14 release from the nucleolus and exit from mitosis (Játiva et al., 2019).

5.4. PP2A^{Cdc55} in cytokinesis

This project is focused on the study of the PP2A^{Cdc55} role during cytokinesis. Previous observations suggested a putative role of PP2A^{Cdc55} in cytokinesis but has not been further studied. In 1991, it was described for the first time the *cdc55* Δ mutant phenotype. It was seen that the *CDC55* deletion mutant has elongated cells with multiple nuclei under low temperature conditions (Healy et al., 1991). The authors proposed that PP2A^{Cdc55} protein could be

involved in the regulation of cytokinesis and/or septation due to the multi-nuclei phenotype. A few years later, PP2A^{Cdc55} phosphatase was described to be present at the bud neck during cytokinesis (Gentry & Hallberg, 2002). This finding further supports the Healy's hypothesis of the PP2A^{Cdc55} during cytokinesis.

Moreover, Hof1 protein was found hyperphosphorylated in *cdc55Δ* mutant compared to WT cells (Y. Wang & Ng, 2006). The authors assume that the Hof1 hyperphosphorylation was due to an hyperactivation of MEN kinases. Later on, it was actually described that the *CDC55* deletion promotes Bfa1 premature asymmetric localization at the SPB (characteristic of MEN activation), but the downstream MEN kinases are not activated under these conditions. Indeed, König and colleagues at 2010 described that Clb2-Cdc28 inactivates Cdc15 and Dbf2 MEN kinases. Therefore, the increase in Hof1 phosphorylation levels in the *cdc55Δ* mutant cannot be a consequence of hyperactive MEN. To our knowledge, no other work has dealt with the PP2A^{Cdc55} implication in cytokinesis up to now in this project.

OBJECTIVES

1. Study of the PP2A^{Cdc55} role in cytokinesis

Early studies about the phenotype of the *cdc55*Δ deletion mutant suggested a putative role of PP2A^{Cdc55} in the regulation of cytokinesis. However, this role of PP2A^{Cdc55} was not further studied. Nonetheless, recent proteomic analysis showed that *cdc55*Δ deletion increased the phosphorylation state of several proteins involved in cytokinesis. These findings prompted us to study the PP2A^{Cdc55} role in cytokinesis. Our first objective is to investigate the contribution of PP2A^{Cdc55} dependent dephosphorylation on the progression through cytokinesis.

2. To investigate the Cdc5 regulation on Net1 phosphorylation

Cdc5 contributes to mitosis exit promoting the Cdc14 release from the nucleolus in different ways. The best-studied Cdc5's role is the activation of the MEN pathway in late anaphase through the Bfa1 phosphorylation. However, although it has been proposed by *in vitro* studies, its direct role phosphorylating Net1 has not been proved. Our second objective is to characterize the specific and *in vivo* Cdc5- phosphorylated Net1 residues and the explicit role of Cdc5 in the Cdc14 activation.

MATERIALS AND METHODS

1. Plasmids, yeasts strains and growth condition

1.1. Plasmids

Plasmids used during this thesis are described in the table 1. The plasmids used were amplified and extracted from *Escherichia coli* DH5 α competent cells. The *E. coli* transformants were grown and selected in LB (0.5% yeast extract, 1% tryptone, 1% NaCl) medium containing the appropriate bactericidal antibiotic (100 μ g/mL Ampicillin, 10 μ g/mL Kanamycin or 100 μ g/mL Spectinomycin) at 37°C overnight before the DNA extraction. This extraction was performed with the NucleoSpin® Plasmid Kit (Macherey-Nagel) following the manufacturer's protocol.

The plasmids constructed during this project for *NET1* mutant versions and *NET1-CDC5* fusions were performed by DNA Assembly of PCR products using the Gibson Assembly® or NEBuilder® HiFi assembly kits (New England Biolabs). The DNA's containing the Net1 phosphorylation mutants were obtained by gene synthesis (Life Technologies manufacturing). The amplification of DNA regions by PCR for cloning or DNA assembly, were performed using Phusion polymerase (Qiagen) or Pfu Plus polymerase (Cultek) following the manufacturer's instructions.

To obtain the p*GAL1-CDC55* construct, the DNA fragment containing the *GAL1* promoter was cut with *SpeI* and introduced into the *CDC55* containing plasmid digested with *NheI* through traditional ligation (Quick Ligation™ NEB).

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Plasmid	Insert	Vector	Origin
E362	<i>promCDC55-HA₃-CDC55</i>	pRS316	This laboratory
E377	<i>promCDC55-HA₃-Cdc55_T174E_S301D</i>	pRS316	This laboratory
E442	<i>Primer annealing site F-LoxP- HA₃STOP- Net1 terminator- URA₃- LoxP- Linker (10)- CDC5- HA3STOP-CDC5 terminator- Primer annealing site R</i>	pYIplac128	This study
E448	<i>promNET1-MYC₉-net1_5A</i>	pRS306	This study
E449	<i>promNET1-MYC₉-net1_14A</i>	pRS306	This study
E454	<i>Primer annealing site F-LoxP- HA3STOP- Net1 terminator- URA₃- LoxP- Linker (10)- cdc5_T242A- HA₃STOP-CDC5 terminator- Primer annealing site R</i>	pYIplac128	This study
E473	<i>promNET1-MYC₉-net1_5A_S447A</i>	pRS306	This study
E474	<i>promNET1-MYC₉-net1_5A_T534A</i>	pRS306	This study
E482	<i>promNET1-MYC₉-net1_5A_Nterm</i>	pRS306	This study
E483	<i>promNET1-MYC₉-</i>	pRS306	This study

	<i>net1_4A_Cterm</i>		
E485	<i>GAL1-CDC5-MYC₉</i>	pYIplac128	This study
E486	<i>Primer annealing site F- LoxP- HA₃STOP- Net1 terminator- URA₃- LoxP- Linker (10)- cdc5_N209A- HA₃STOP-CDC5 terminator- Primer annealing site R</i>	pYIplac128	This study
E499	<i>₆HIS-INN1</i>	pET28c	Alberto Sánchez- Díaz
E501	<i>STREPIII-chs2-1-629</i>	pET100	Alberto Sánchez- Díaz
E510	<i>₆HIS-HOF1</i>	pET28c	Alberto Sánchez- Díaz

Table M1. Plasmids used in this thesis.

1.2. Yeast strains

Yeast strains used in this thesis are summarized in table 2. All strains are derivative from W303. The strains were obtained by yeast DNA transformation to introduce DNA fragments from plasmids or PCR products, or by tetrads dissection of diploids cells (see below). Cells were grown in yeast rich medium YP (1% yeast extract, 2% peptone) supplemented with the appropriate carbon source, YPD (YP, 2% glucose), YPRaf (YP + 2% raffinose, 0.01% glucose), YPGal/Raf (YP + 2% galactose, 2% raffinose, 0.01%

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glucose); or minimum medium YNB (0.67% yeast nitrogen base, 40 µg/mL supplementary aminoacids and nucleotides) containing the required carbon source (same concentrations than YP). The strains with resistance to geneticin were selected using 200 µg/mL G418 (Formedium). For the sporulation of diploids cells, diploids were grown in sporulation medium (90 mM NaOAc, 20 mM NaCl, 25 mM KCl, 2 mM MgSO₄).

Strain	Genotype	Origin
W303	<i>MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL psi+</i>	F. Uhlmann's lab
Y564	<i>MATα MET-HA₃-CDC20 HA₃-CDC55</i>	This laboratory
Y571	<i>MATa MET-HA₃-CDC20 CDC14-PK₉ BFA1-HA₆ GAL1-CDC5-MYC₉ NET1-MYC₉</i>	This laboratory
Y688	<i>MATa MET-HA₃-CDC20 GAL1-FLAG-ESP1-CBD-C1531A CLB2-PK₃ BFA1-HA₆</i>	This laboratory
Y824	<i>MATα MET-HA₃-CDC20</i>	This laboratory
Y844	<i>MATα cdc55Δ</i>	This laboratory
Y968	<i>MET-HA₃-CDC20 GAL1-CDC5-MYC₉ net1(1-600)-PrA Cdc14-PK₉</i>	This laboratory
Y1306	<i>MATα MET-HA₃-CDC20 MYO1-TOMATOE SPC42-GFP HOF1-GFP</i>	This study
Y1314	<i>MATa MET-HA₃-CDC20 HOF1-HA₆</i>	This study

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Y1315	<i>MATα MET-HA₃-CDC20 CHS2-HA₆ cdc55Δ</i>	This study
Y1318	<i>MATα MET-HA₃-CDC20 CHS2-HA₆</i>	This study
Y1354	<i>MATα MET-HA₃-CDC20 net1Δ::MYC₉-Net1</i>	This study
Y1356	<i>MATα MET-HA₃-CDC20 net1Δ::net1-14A</i>	This study
Y1357	<i>MATα MET-HA₃-CDC20 cdc5-as NET1- MYC₉ CDC5 (5' UTR)::CDC5-MYC9 DBF2- PK₆</i>	This study
Y1380	<i>MATα MET-HA₃-CDC20 net1Δ::MYC₉- net1-5A_S447A</i>	This study
Y1386	<i>MATα MET-HA₃-CDC20 net1Δ::MYC₉- net1-5A_T534A</i>	This study
Y1367	<i>MATα MET-HA₃-CDC20 CDC14-PK₉ GAL1-CDC5 net1Δ::MYC₉-net1-14A</i>	This study
Y1394	<i>MATα MET-HA₃-CDC20 HOF1-HA₆ cdc55Δ</i>	This study
Y1395	<i>MATα MET-HA₃-CDC20 net1Δ::MYC₉- net1-5A</i>	This study
Y1396	<i>MATα MET-HA₃-CDC20 CDC14-PK₉ NET1-CDC5-HA₃</i>	This study
Y1398	<i>MATα MET-HA₃-CDC20 GAL1-CDC5 net1Δ::MYC₉-net1-5A</i>	This study
Y1423	<i>MATα MET-HA₃-CDC20 HOF1-HA₆ PK₃- CDC55</i>	This study
Y1434	<i>MATα MET-HA₃-CDC20 MYO1-GFP cdc55Δ</i>	This study

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Y1435	<i>MATa MET-HA₃-CDC20 MYO1-GFP</i>	This study
Y1437	<i>MATa MET-HA₃-CDC20 CYK3-HA₆</i>	This study
Y1438	<i>MATa MET-HA₃-CDC20 CYK3-HA₆ cdc55Δ</i>	This study
Y1454	<i>MATa MET-HA₃-CDC20 MYO1-TOMATOE SPC42-GFP INN1-GFP</i>	This study
Y1491	<i>MATa MET-HA₃-CDC20 MYO1-FLAG IQG1-HA₆ HOF1-MYC₉</i>	This study
Y1497	<i>MATα MET-HA₃-CDC20 MYO1-FLAG IQG1-HA₆ cdc55Δ</i>	This study
Y1512	<i>MATa MET-HA₃-CDC20 MYO1-TOMATOE SPC42-GFP cdc55Δ</i>	This study
Y1516	<i>MATa MET-HA₃-CDC20 CHS2-GFP SPC42-GFP</i>	This study
Y1528	<i>MATα CYK3-HA₆ BFA1-HA₃ GAL1-CDC55</i>	This study
Y1531	<i>MATa CDC14-PK₉ MET-HA₃-CDC20 NET1-cdc5-T242A-HA₃</i>	This study
Y1535	<i>MATα MET-HA₃-CDC20 GAL1-CDC5 net1Δ::MYC₉.net1-5A_Nterm</i>	This study
Y1536	<i>MATα MET-HA₃-CDC20 GAL1-CDC5 net1Δ::MYC₉.net1-4A_Cterm</i>	This study
Y1555	<i>MATa MET-HA₃-CDC20 HOF1-HA₆ GAL1- CDC55</i>	This study
Y1565	<i>MATa MET-HA₃-CDC20 CYK3-HA₆ PK₃- CDC55</i>	This study
Y1566	<i>MATa MET-HA₃-CDC20 INN1-HA₆ PK₃- CDC55</i>	This study

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Y1567	<i>MATa MET-HA₃-CDC20 CHS2-HA₆ PK₃-CDC55</i>	This study
Y1572	<i>MATa MET-HA₃-CDC20 MYO1-TOMATOE SPC42-GFP yE-GFP-IQG1</i>	This study
Y1574	<i>MATa MET-HA₃-CDC20 MYO1-TOMATOE SPC42-GFP CYK3-GFP</i>	This study
Y1575	<i>MATa MET-HA₃-CDC20 MYO1-TOMATOE CHS2-GFP cdc55Δ</i>	This study
Y1576	<i>MATα MET-HA₃-CDC20 MYO1-TOMATOE SPC42-GFP CHS2-GFP</i>	This study
Y1578	<i>MATa MET-HA₃-CDC20 MYO1-TOMATOE HOF1-GFP cdc55Δ</i>	This study
Y1580	<i>MATα MET-HA₃-CDC20 GAL1-CDC5 net1Δ::MYC₉.net1-5A_T447A</i>	This study
Y1588	<i>MATa MET-HA₃-CDC20 SHS1-HA₆</i>	This study
Y1589	<i>MATa MET-HA₃-CDC20 SHS1-HA₆ cdc55Δ</i>	This study
Y1590	<i>MATα MET-HA₃-CDC20 GAL1-CDC5 net1Δ::MYC₉.net1-5A_T534A</i>	This study
Y1596	<i>MATα MET-HA₃-CDC20 MYO1-TOMATOE chs3Δ cdc55Δ</i>	This study
Y1604	<i>MATa MET-HA₃-CDC20 MYO1-TOMATOE SPC42-GFP CYK3-GFP cdc55Δ</i>	This study
Y1605	<i>MATα MET-HA₃-CDC20 MYO1-TOMATOE chs3Δ</i>	This study
Y1606	<i>MATa MET-HA₃-CDC20 MYO1-</i>	This study

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	<i>TOMATOE SPC42-GFP yE-GFP-IQG1 cdc55Δ</i>	
Y1608	<i>MATa MET-HA3-CDC20 MYO1- TOMATOE SPC42-GFP INN1-GFP cdc55Δ</i>	This study
Y1623	<i>MATa CDC14-PK9 MET-HA3-CDC20 NET1-cdc5-N209A-HA3</i>	This study
Y1614	<i>MATa ADH1-OsTIR-Myc9 chs2-aid</i>	Alberto Sánchez- Díaz
Y1626	<i>MATa MET-HA3-CDC20 CHS2-HA6 GAL1-CDC55</i>	This study
Y1628	<i>MATa ADH1-OsTIR-Myc9 chs2-aid cdc55Δ</i>	This study
Y1639	<i>MATa MET-HA3-CDC20 CYK3-HA6 INN1- Myc9</i>	This study
Y1640	<i>MATa MET-HA3-CDC20 CYK3-HA6 INN1- Myc9 cdc55Δ</i>	This study
Y1643	<i>MATa MET-HA₃-CDC20 MYO1-FLAG IQG1-HA₆ INN1-MYC₉ GAL1-CDC55</i>	This study
Y1652	<i>MATa MET-HA₃-CDC20 MYO1-TOMATOE cdc55Δ URA3::HA₃-CDC55</i>	This laboratory
Y1653	<i>MATa MET-HA₃-CDC20 MYO1-TOMATOE cdc55Δ URA3::HA₃-CDC55-T174E_S301D</i>	This laboratory
Y1708	<i>MATa MET-HA₃-CDC20 MYO1-TOMATOE ₃GFP-RAS2 cdc55Δ</i>	This study
Y1717	<i>MATa MET-HA₃-CDC20 MYO1-TOMATOE ₃GFP-RAS2</i>	This study

Y1725	<i>MATa MET-HA₃-CDC20 MYO1-TOMATOE CDC15-eGFP cdc55Δ cdc28-Y19F</i>	This study
Y1728	<i>MATa ADH1-AtTIR1-MYC₉ hof1-aid</i>	Alberto Sánchez- Díaz
Y1730	<i>MATa GAL1-UBR1 ADH1-AtTIR1-MYC₉ td- cyk3-aid</i>	Alberto Sánchez- Díaz
Y1747	<i>MATa GAL1-UBR1 ADH1-AtTIR1-MYC₉ td- cyk3-aid cdc55Δ</i>	This study
Y1749	<i>MATa ADH1-AtTIR1-MYC₉ hof1-aid cdc55Δ</i>	This study
Y2231	<i>MATa MET-HA₃-CDC20 CDC5-PK₃ CDC14-HA₆</i>	This laboratory
Y2388	<i>MATa MET-HA₃-CDC20 CDC14-HA₆ NET1-PK₃ GAL1-CDC5-PrA</i>	This laboratory
Y2299	<i>MATα MET-HA₃-CDC20 CDC14-HA₆</i>	This laboratory

Table M2. Yeast strains used in this thesis.

1.3. Growth conditions and cells synchronization

Cell cultures were grown overnight at 25°C until exponential phase (0.4-0.6 OD₆₀₀ (Optical Density at 600nm wavelength)).

a) Metaphase arrest by Cdc20 depletion

Cells were arrested in metaphase by the depletion of Cdc20. The endogenous *CDC20* promoter was replaced by the repressive *MET3* promoter. Cells with *MET3-CDC20* were grown overnight in absence of methionine to allow the expression of Cdc20. Cell cultures at exponential growing phase were arrested in metaphase by transferring the cells to medium containing 2 mM methionine and incubated for 3-4 hours (1-2 duplication times). Cells were considered arrested when 85% population presented buds with almost the same size than mother cells.

To synchronously release cells at anaphase, the cells were collected, extensively washed by filtration and resuspended in medium without methionine to allow Cdc20 expression.

b) G1 arrest with alpha factor

The strains used in the laboratory are haploids cells and do not have the ability to change mating since are mutated for the endonuclease gene (*HO*) (Winge, & Roberts, 1949; Klar, Fogel, & Radin, 1979). The expression of *MATa* allows to arrest the cell cycle at the end of G1 by the addition of the alpha factor pheromone. The pheromone promotes the cell cycle arrest at Start, the moment of preparation for conjugation (Throm & Duntze, 1970). This arrest is transient and depends on the amount of pheromone present in the cell culture (Udden & Finkelstein, 1978).

Cells in early exponential phase were arrested with 1 µg/mL α-factor (0.2-0.3 OD₆₀₀). After 45 minutes, 0.5 µg/mL α-factor was added to the medium. Cells are normally arrested after incubation of 90-120 minutes in presence of α-factor and was determined by the accumulation of cells without buds in (at least) 90% of the cell population. Cell cycle progression was recovered after removal of the pheromone by cell filtration, extensively wash of the cells and resuspending the cells in medium free of pheromone.

c) Protein overexpression

Overexpression of *CDC5* and *CDC55* was performed by the introduction of the *GAL1* inducible promoter to the respective genes. For the ectopic expression of *CDC5* in metaphase arrested cells, cells were grown overnight in presence of 2% raffinose as carbon source and were arrested in metaphase by the addition of methionine. After 3-4 hours of incubation to arrest the cells, 2% galactose was added to the medium.

For *CDC55* ectopic expression, cells were arrested in metaphase as before and after 1 hour of arrest, 2% galactose was added to the cell culture to induce Cdc55 overexpression. The cells were arrested 3 hours more before the release from metaphase. The release into free methionine medium was performed in minimum medium with 2% raffinose and galactose, and 0.02% glucose.

d) Protein degradation

Chs2, Inn1, Hof1 and Cyk3 proteins degradation were induced by the introduction of an auxin-inducible-degron (AID) sequence at the C-terminal of the corresponding proteins. The expression of the auxin degron promotes the activation of the SCF^{Tir1} E3 ligase and subsequent protein degradation. The Tir1 cofactor from plants is introduced to the yeast genome for SCF^{Tir1} complex formation. The degradation of the proteins is induced when auxin or indole-3-acetic acid (IAA) is added to the medium and the Tir1 recognized the auxin (IAA) bound to the AID sequence (Nishimura, Fukagawa, Takisawa, Kakimoto, & Kanemaki, 2009). To study the viability of the essential genes, cells were grown in presence of 0.2 mM, 0.5 mM, 1 mM and 1.5 mM IAA, and incubated for 2-3 days.

2. Strains preparation

2.1. Yeast transformation

Epitope tagging of endogenous genes and gene deletions were obtained through homologue recombination after DNA transformation with polymerase chain reaction (PCR) products containing 40-50 nucleotides of the gene of interest. Oligonucleotides to amplify the cassettes were based on Knop M et al 1999 for protein tagging and Wach A et al 1994 for gene deletions. N-terminal tagging of endogenous *CDC55* was performed using the plasmids pPK₃-CDC55-(1-178) and integrated at the endogenous locus upon digestion with MscI (Queralt et al., 2006). For the integration of plasmids at the auxotrophs marker

genes (i.e. *URA3* or *TRP1*), restriction enzymes were used to linearize the plasmids before yeast transformation and integration by recombination at the endogenous gene locus.

Transformations were carried out following the lithium acetate protocol. For DNA transformation with selection to geneticin, the cells were grown in YPD for 3-4 hours before plating to allow the expression of the resistance KanMX gene. All strains were plated in the appropriated selective plates for the selection of the transformants.

2.2. Conjugation and tetrad dissection

For the conjugation of haploid cells, two strains of different mating type (*MATa* and *MAT α*) were crossed in non-selective plates at 25°C for 48 hours. Diploids were selected in the appropriate medium and then transfer to the sporulation plates at 25°C for 3-5 days. For the tetrad dissection, the ascus containing the spores were digested with 0.1% zymolyase 20T in 1 M sorbitol pH 7.4 buffer for 3 minutes. Spores were dissected using a micromanipulator MSM System (Singer) and were incubated at 25°C in non-selective plates for 3-4 days. The four segregant were genotyped by replica plating in the appropriate selection media.

3. Microscopy techniques

3.1. Immunofluorescence *in situ*

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For immunofluorescence in situ, cells collected during the time-course experiments were fixed in cold phosphate buffer pH 6.4 (100 mM potassium phosphate, pH 6.4, 0.5 mM MgCl₂) containing 3.7% formaldehyde, up to 24 hours at 4°C or 1 hour at 25°C. Fixed cells were washed once with phosphate buffer pH 6.4 and once with phosphate buffer pH 7.4 (120 mM potassium phosphate, pH 7.4, 0.5 mM MgCl₂) containing 1.2 M sorbitol. Then, cells were resuspended in sorbitol buffer pH 7.4 containing 0.1 mg/mL zymolyase 100T and 0.2% β-Mercaptoethanol and were digested at 30°C for 20-40 minutes. After digestion, spheroplasts were washed once with sorbitol buffer without zymolyase and resuspended again in sorbitol buffer without zymolyase. Spheroplasts were fixed over a multitest slide containing separated wells with 0.1% poli-L-lysine. Cells were blocked with phosphate buffered saline (PBS) complemented with 1% bovine serum albumin (BSA) for 20 minutes. After blocking, cells were incubated with PBS-BSA containing the corresponding primary and secondary antibodies for 1 hour and 40 minutes, respectively. After each antibody's incubation, cells were washed 3 times with PBS-BSA. Mounting medium containing DAPI (Vectashield) was added, cells were covered with a coverslip and sealed with nail polish.

Primary antibodies used were α-Myc clone 9e10 (Santa Cruz Biotechnology), α-HA clone 12CA5 (Roche), α-tubulin YOL1/34 (Serotec), Cdc11 (Santa Cruz Biotechnologies) and α-Cdc14 (Santa Cruz Biotechnology). Secondary antibodies used were Cy3 tagged α-goat (GE Healthcare), Cy3 tagged α-mouse (GE Healthcare), red TEXAS α-rabbit (Jackson Laboratories), 488 α-mouse (INVITROGEN) and fluorescein-conjugated α-rat (Millipore). Images were acquired with a Zeiss Axio Observer Z1 inverted

epifluorescence microscope with Fluorescent Lamp HXP 120C with the oil immersion objective Carl Zeiss Plan-Apochromat 63x N.A 1.40. The filters used were Cy3, GFP and DAPI. Images acquisition was done using the ZEN software. The quantitative analysis of signal intensity and images processing were done using the Fiji software.

3.2. Time-lapse microscopy

GFP-Cyk3, Iqg1-GFP, Chs2-GFP, Inn1-GFP, Hof1-GFP, Myo1-GFP and Myo-Tomatoe proteins localization during cytokinesis were observed by time-lapse microscopy. For time-lapse experiments, cells were grown in minimum medium complemented with the appropriate aminoacids and nucleotides and synchronized in metaphase or G1 following the synchronization methods explained in sections 1.3.a and 1.3.b. Upon release from the arrest, cells were incubated in agitation at 25°C for up to 15 minutes in minimum medium. Then, cells were sedimented into slides containing chambers. The chambers were previously filled with a PBS solution containing concanavalin A 1 mg/mL for generating the fixing surface. After cells fixation, images were taken every 2 minutes. Different z-stacks at 0.7 μ m intervals were taken and projected onto a single image per channel. The microscope used was the same as for in situ immunofluorescences but using the Apotome system to avoid photobleaching. For the GFP labeled Cyk3 time-lapses, the microscope Confocal Microscope Carl Zeiss LSM880, objective 63x N.A was used. Images acquisition was done using the ZEN software. Image quantification and processing was done using Fiji software.

3.3. Calcofluor staining

The analysis of chitin deposition during cytokinesis was done by chitin staining with calcofluor white MR2 (Fluorescent brightener 28, Sigma) in living cells. Cells were synchronized in metaphase as previously described and 50 µg/mL calcofluor was added upon release from the metaphase arrest. Images were taken from cells at cytokinesis (Myo1 signal was used as internal control). Images were acquired with a Zeiss Axio Observer Z1 inverted epifluorescence microscope with Fluorescent Lamp HXP 120C with the oil immersion objective Carl Zeiss Plan-Apochromat 63x N.A 1.40. The filters used were GFP, Cy3 and DAPI. Images acquisition was done using the ZEN software. Image quantification and processing was done using Fiji software.

3.4. Phalloidin staining

The visualization of actin rings and actin patches were done through the staining of actin with phalloidin on fixed cells. Cells collected during the time-course experiments were pre-fixed with PBS containing 3.7% formaldehyde and 0.1% Triton X-100 at 25°C for 10 minutes. Then, cells were washed with PBS and fixed with 3.7% formaldehyde at 25°C for 1 hour. After fixation, cells were washed twice with PBS and sedimented onto a multi-well slide previously incubated with poly-L-lysine. Cells were stained with a PBS solution containing 50 U/mL rhodamine phalloidin R415 (Life Technologies) for 2 hours. Cells were washed twice with PBS, mounting medium containing DAPI (Vectashield) was added, cells were covered with a coverslip and sealed with nail polish. Images

were acquired with a Zeiss Axio Observer Z1 inverted epifluorescence microscope with Fluorescent Lamp HXP 120C with the oil immersion objective Carl Zeiss Plan-Apochromat 63x N.A 1.40. The filters used were Cy3 and GFP. Images acquisition was done using the ZEN software. Image quantification and processing was done using Fiji software.

3.5. Electron microscopy

The analyses of primary and secondary septa structures were observed with a transmission electron microscope. Cells were arrested in metaphase by Cdc20 depletion and then released into anaphase by Cdc20 re-addition. A time-course experiment was performed, and cells samples were fixed with 0.2 M phosphate buffer (without salts) pH 7.4, containing 2.5% glutaraldehyde at 25°C for 60 minutes. Then, cells were washed 3 times with phosphate buffer without glutaraldehyde. Cells were stored at 4°C until the sections were performed. Cells were rinsed with milliQ water and post-fixed with 1% osmium tetroxide for 2 hours. Then, they were rinsed with milliQ water and dehydrated in acetone series (10%, 20%, 30%, 40%, 60%, 80% and 100%) for 15-20 minutes.

Ultrathin sections of 60nm in thickness were obtained using a UC6 ultramicrotome (Leica Microsystems, Austria) and were stained with 2% uranyl acetate and lead citrate. Sections were observed in a Jeol EM J1010 (Jeol, Japan) and images were acquired at 80 kV with a 1k x 1k CCD Megaview camera. The samples dehydration

and sections preparation were performed in the CMRB-IDIBELL electron microscopy unit and the CCIT electron microscopy unit.

4. Proteins techniques

4.1. Western blot

Protein extracts were obtained by the trichloride acetic acid (TCA) method. Collected cells were fixed with cold 20% TCA for 20 minutes or overnight at 4°C. Cells were washed with 1 M tris base and resuspended in loading buffer (100 mM Tris-HCl pH 6.8, 0.2 M DTT, 4% glycerol, 0.002% bromophenol blue) at 95°C. Then, 1 volume of glass beads (425-600 µm of diameter) were added and cells were broken by mechanical lysis with a disrupter (Precellys 24 homogenizer; Bertin technologies) for 6 cycles at 5000 rpm. Protein extracts were clarified at 12.000 rpm for 5 min and stored at -20°C. For western blot analysis, samples were boiled at 95°C for 5 minutes, centrifuged 5 min at maximum speed and loaded to acrylamide SDS-PAGE gels. Common 6%, 8%, 10% and 12% acrylamide gels and phos-tag gels of 5%, 6%, 7% and 8% acrylamide were used. Phos-tag gels contained 10 µM phos-tag® and 10 mM MnCl₂. After protein separation by electrophoresis, proteins were transferred to PVDF membranes (Immobilon® PVDF, Millipore) previously activated with 100% methanol. Membranes were blocked with PBS-Tween (PBS, 0.02% Tween-20) containing 5% dry milk (Panreac). Protein membranes were incubated with corresponding primary antibodies from 1 hour to overnight, and secondary antibodies for 40 minutes. After each antibody incubation, membranes were washed with PBS-Tween 3

times. For the chemiluminescent detection, membranes were incubated with ECL (Millipore) (or ECL Select™ for low signal proteins (GE Healthcare)) and proteins were visualized with an Amersham imager 600 (GE Healthcare).

The primary antibodies used were: α -HA clone 12CA5 (Roche), α -HA rabbit (Sigma), α -Myc clone 9e10 (Babco), α -FLAG polyclonal (Sigma), α -Pk clone SV5-Pk1 (Serotec), α -Pgk1 (Invitrogen), α -Clb2 (y-180) sc-907 (Santa Cruz Biotechnology) and α -Chs2 (a gift from Alberto Sánchez-Díaz lab); and the secondary antibodies: α -Mouse-HRP (GE Healthcare), α -Rabbit-HRP (GE Healthcare), and α -Goat-HRP (GE Healthcare).

4.2. Co-Immunoprecipitation experiments

Approximately 10^8 cells were collected and washed with PBS1X. Cells were resuspended in lysis buffer (50 mM HEPES-KOH, pH 7.5, 70 mM KOAc, 5 mM Mg(OAc)₂, 10% glycerol, 0.1% Triton X-100, 8 μ g/mL protease inhibitors, 20 mM β -glycerophosphate) and lysates obtained by mechanical lysis using glass beads in a Bertin disrupter (6 cycles of 10 sec at 5.000 rpm). Protein extracts were clarified by centrifugation at maximum speed and incubated with primary antibody for 1 hour. Then, the protein extracts were incubated 1 hour with protein A conjugated dynabeads (Life technologies). After incubation, beads were washed with lysis buffer with incremental KOAc concentration (100 mM, 120 mM, 150 mM and 60 mM NaOAc). Co-immunoprecipitated proteins were eluted with loading buffer and protein co-purification was visualized by western blot.

4.3. Recombinant protein purifications

All recombinant protein purifications were done from BL21 *E. coli* fresh transformants to obtain the protein substrate for the activity assays. Cells were grown in LB medium and protein expression was induced with 0.1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) at 25°C overnight. Collected cells were washed with PBS1X and frozen for at least 30 minutes. Cells were resuspended in lysis buffer and purified with the corresponding beads: ϵ His-Hof1, ϵ His-Inn1 and ϵ His-Cyk3 with Ni-NTA magnetic beads (ThermoFisher); and StrpIII-Chs2_1-629 with Strep-tactin® Superflow® resin (Iba Lifescience).

For His-tagged proteins (Cyk3, Inn1 and Hof1), cells were resuspended in cold lysis buffer (30 mM Tris-HCl, pH 8, 300 mM NaCl, 30 mM imidazole, 0.1% NP40, 10 mM β -mercaptoethanol, 1 mM PMSF, complete EDTA-free tablet (Roche)) and sonicated 6 cycles of 1 min at 25 microns amplitude. Protein extracts were clarified and incubated with Ni-NTA magnetic beads (ThermoFisher) at 4°C for 1 hour. Beads were washed with 10X lysis buffer and protein eluted in PBS, 5mM EDTA, 5mM DTT, 0.1% NP40, 500 mM imidazole at 4°C for 30-60 minutes.

For streptavidine epitopes (StrpIII-Chs2_1-629), cells were resuspended in cold lysis buffer (50 mM Tris-HCl, pH 8.0, 10% Glycerol, 0.1% NP-40, 10 mM MgCl₂, 300 mM NaCl, 5 mM β -mercaptoethanol, 10% BugBuster® (Millipore), 1 mM PMSF, complete EDTA-free tablet (Roche)) containing 5 U/mL of nuclease (Pierce) and incubated in a rotatory wheel at 25°C for 20 minutes.

Protein extracts were clarified and incubated with Strep-tactin® Superflow® resin (Iba Lifescience) at 4°C for 1 hour. Beads were washed with 10X lysis buffer (without BugBuster®) and protein eluted in 50 mM Tris-HCl, pH 8.0, 10% Glycerol, 0.1% NP-40, 10 mM MgCl₂, 150 mM NaCl, 5 mM β-mercaptoethanol, 2.5 mM desthiobiotin at 4°C for 1 hour.

4.4. Kinase assays

The Pk-epitope tagged proteins Cdc5 and Clb2 were collected from metaphase-arrested cells and Dbf2 and Cdc15 were previously released from the arrest and sample taken 30 minutes after the release from metaphase when cells are at anaphase. All cells were washed with PBS1X and resuspended in lysis buffer (50 mM HEPES/KOH, pH 7.5, 100 mM KCl, 2.5 mM MgCl₂, 0.25% Triton X-100, 1 mM DTT). Then, cells were broken by mechanic lysis as previously described and protein extracts clarified by centrifugation. Protein extracts were incubated with Pk antibody at 4°C for 1 hour and with protein-A conjugated dynabeads for an additional hour. Beads were washed with 10X lysis buffer and twice with the kinase reaction buffer (50 mM Tris-HCl, pH7.4, 10 mM MgCl₂, 1 mM DTT). Kinase reaction (50 mM Tris-HCl, pH7.4, 10 mM MgCl₂, 1 mM DTT, 5 mM β-glycerophosphate, 25 μM ATP, 10 mCi/mL ATP³² (2mM EGTA in Cdc5 kinase assay) and 2 ng-1 μg of protein substrate) was incubated at 30°C for 30 minutes for 6Hys-Inn1, 6Hys-Hof1 and Strp-Chs2, and 1 hour for 6Hys-Cyk3. Kinase assays was stopped by incubated the tubes on ice. The supernatant containing the phosphorylated substrate was separated from the magnetic beads and store at -80°C. An aliquot of the kinase assay was

mixed with SDS-PAGE loading buffer, proteins were separated by electrophoresis, transferred to nitrocellulose membranes and radioactivity detected in a Typhoon FLA950 (GE healthcare). Then, a western blot with the membrane was performed to analyzed and quantify the amount of the protein immunoprecipitated. Finally, the membrane was stained with Coomassie to detect the recombinant substrate. Quantification of proteins was performed using the FIJI software.

4.5. Phosphatase assays

For the phosphatase assays, cells containing HA-epitope tagged *cdc55_ED* and Cdc55 were collected after arresting them in metaphase, while cells containing Cdc14-HA were previously released from the arrest and sample taken 30 minutes after the release when cells are in anaphase. Immunoprecipitation were performed following the same protocol for the kinase assays. After the immunoprecipitation, beads were washed twice with phosphatase buffer. Beads were incubated with phosphatase reaction buffer (500 mM Tris-HCl pH7.4, 1 mM EGTA, 10 mM β -mercaptoethanol, 10mg/mL BSA; and the phosphorylated substrate) at 30°C for 30 minutes. Reaction was terminated by adding SDS-PAGE loading buffer. Proteins were separated by electrophoresis, transferred to nitrocellulose membranes and radioactivity detected in a Typhoon FLA950 (GE healthcare). Then, a western blot with the membrane was performed to analyzed and quantify the amount of protein immunoprecipitated. Finally, the membrane was stained with Coomassie to detect the recombinant

substrate. Quantification of proteins was performed using the FIJI software.

4.6. Peptide array

The *in vitro* analysis of Net1 phosphorylation by Cdc5 was done using a peptide array. A library of tiling Net1 peptides was incubated with yeast immunopurified Cdc5. Protein extracts were prepared from a strain containing a *GAL1-CDC5* to overexpress the protein. Cells were arrested in metaphase by Cdc20 depletion and Cdc5 expression was induced by galactose addition for 3 hours. Then, cells were collected and immunoprecipitation of Cdc5 was prepared following the kinase assay protocol. Purified protein was quantified using the Bradford reagent (Bio-Rad) and a BSA standard curve. 8 µg/mL Cdc5 was sent to LC sciences for the hybridization of the peptide array. An array with 8 aminoacids tiling Net1 peptides was prepared. The chip surface was blocked and washed with TBS buffer, pH 7.0, 0.05% Tween-20, 0.05% Triton X-100 at 4 °C overnight. Once blocked, the chip is washed with kinase reaction buffer (50 mM Tris-HCl, pH7.4, 10 mM MgCl₂, 150 µM ATP, 2 mM DTT, 5 mM β-glycerophosphate, pH 7.4) at 25 °C for 20 min. Kinase reaction was done with 8 µg/mL Cdc5 in kinase reaction buffer at 30 °C for 1 hour. After the kinase assay, the array was washed once with kinase reaction buffer, once with TBS buffer and once with deionized water for 5 min. After the washes, the arrays were incubated with ProQ staining buffer at 25 °C for 30 min to stain the phosphorylated residues. Then, the peptide arrays were washed with ProQ destaining buffer at 25 °C for 30 min and

with deionized water for 5 min. Finally, the Cy3 signal was scanned with a Cy3 filter.

5. Fluorescence flow cytometry analysis (FACS)

DNA content of cells from time-course experiments were measured by FACS. Collected cells were fixed in absolute ethanol and fixed for at least 5 min. Then, cells were centrifuged 5 min at maximum speed and resuspended in 50 mM sodium citrate pH7.4 containing 0.2 mg/ml RNase. Cells were incubated at 50°C for 1 hour, 1mg/ml proteinase K was added, and incubated for an additional hour. Then, the same volume of 50 mM sodium citrate pH7.4 containing 16 µg/mL propidium iodide was added. DNA content analysis was done with a flow cytometer (Gallios Beckman Coulter). Analysis was performed with Kaluza flow cytometry software.

RESULTS

1. PP2A^{Cdc55} role in cytokinesis

1.1. PP2A^{Cdc55} dephosphorylates IPC's proteins during cytokinesis

The PP2A^{Cdc55} phosphatase was suggested to be involved in cytokinesis based on the multi-nuclei phenotype of the *cdc55*Δ deletion mutant at cold temperature (Healy et al., 1991b). However, the PP2A^{Cdc55} role during cytokinesis has not been studied. Recently, our lab published a SILAC-based phosphoproteomics study which identified putative PP2A^{Cdc55} targets (Barbara Baro et al., 2018). In this study, it was compared the phosphorylation state of around 3000 phosphopeptides in presence and absence of Cdc55, the PP2A^{Cdc55} regulatory subunit. Among the phosphopeptides 1262 were found hyperphosphorylated (corresponding to 282 proteins) in absence of Cdc55; and, therefore were considered putative target of PP2A^{Cdc55}. A gene ontology analysis identified 23 proteins related to cytokinesis. Among these proteins, 2 are part of the ingression progression complexes (IPC's): Inn1 and Iqg1. In addition, other IPC's proteins, Chs2, Cyk3 and Hof1, were detected in the analysis with lower confidence scores (and therefore, not included in the publication). Since many IPC's proteins were detected in the phosphoproteomic study we envisaged that the IPC's proteins could be the possible link between PP2A^{Cdc55} and cytokinesis.

To study whether PP2A^{Cdc55} regulates the dephosphorylation of IPC's proteins, we analyzed the phosphorylation state of the

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different IPC's components in absence of Cdc55 during cytokinesis; to validate the SILAC results.

The phosphorylation state of the IPC's proteins Myo1, Iqg1, Hof1, Cyk3, Inn1 and Chs2 were analyzed in synchronized cells upon release from metaphase until they reach the next G1. To determine the influence of PP2A^{Cdc55} in the phosphorylation levels of these proteins, it was compared the phosphorylation in presence and absence of Cdc55 by western blot using Phos-tag gels. The phos-tag specifically associate to the phosphates groups of the phosphorylated proteins promoting a shift in its mobility since it increases the size of the phospho-proteins (Nagy, Comer, & Smolenski, 2018). Strains containing the APC/C cofactor *CDC20* under the regulation of the methionine-repressible *MET3* promoter were arrested in metaphase by Cdc20 depletion by adding 2mM methionine to the culture media. To release the cells from the metaphase-arrest, the cell cultures were filtered and extensively washed to remove the methionine before being resuspended in fresh media without methionine. Time-course experiments were performed to collect cells during mitosis and cytokinesis. Samples were taken for protein analysis to follow the phosphorylation levels of the proteins. In addition, cells were also collected to study the DNA content by flow-cytometry and the progression throughout the cell cycle by mitotic markers such as nuclei staining by DAPI and mitotic spindle staining by tubulin immunofluorescence.

First, the phosphorylation levels of Myo1 were analyzed. This protein is involved in AMR formation and interacts directly with Mlc1, Iqg1 and Hof1 (Boyne et al., 2000; Foltman et al., 2016; Tian et al., 2014). In both WT and *cdc55*Δ cells, a unique Myo1 band

was resolved in the protein gel (Fig. R1a). Therefore, no phosphorylation isoforms were detected for Myo1 in this condition, at least perceptible by western blot analysis.

Next, we studied the phosphorylation level of the Iqg1 protein. Iqg1 was suggested to be phosphorylated by Cdk1 and its localization and function depends on the Cdk1-phosphorylation sites (D. P. Miller et al., 2015; Naylor & Morgan, 2014). However, changes in the Iqg1 phosphorylation levels during the cell cycle were not previously reported. Here, we showed how Iqg1 is phosphorylated and dephosphorylated during progression through mitosis. In WT cells, Iqg1 was phosphorylated in metaphase, became dephosphorylated during anaphase (20-30 minutes; Fig. R1a) and was quickly phosphorylated in telophase/cytokinesis (40 minutes; Fig. R1a). In addition, the dephosphorylated fraction was maintained unchanged during the last time points when cells enter the next cell cycle (60-90 minutes; Fig. R1a). In *cdc55*Δ mutant cells, the dephosphorylation event in anaphase was observed although with a bit lower efficiency than WT cells (40 minutes; Fig. R1a). Later on, when the cells reach G1, the amount of the faster migration bands were lower compared to WT cells (60-90 minutes; Fig. R1a); although the poor definition of the bands in the mutant prevented visualization of further differences. The slight differences observed between WT and *cdc55*Δ deletion cells are not conclusive enough to support an increase in the Iqg1 phosphorylation in absence of Cdc55.

If PP2A^{Cdc55} dephosphorylates Iqg1, the ectopic expression of Cdc55 should promote its dephosphorylation. To further analyze whether PP2A^{Cdc55} is involved in the dephosphorylation of Iqg1, we

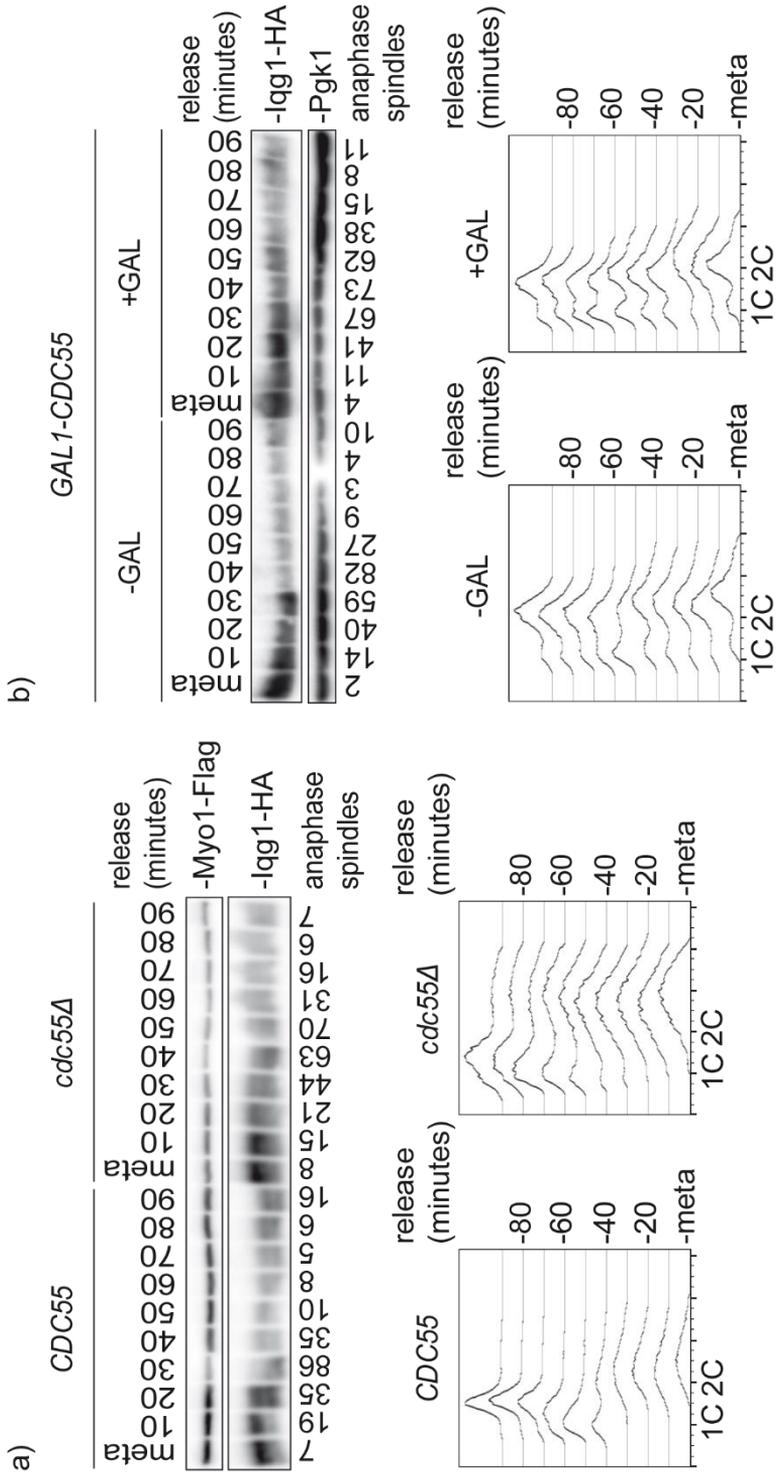


Figure R1. PP2A^{Cdc55} does not regulate Myo1 and Iqg1 phosphorylation. **(A)** Myo1 and Iqg1 phosphorylation in absence of Cdc55. Strains Y1491 (*MATa MET-HA₃-CDC20 MYO1-FLAG IQG1-HA₆*) and Y1497 (as Y1491, but *MATa* and *cdc55Δ*) were arrested in metaphase by Cdc20 depletion and released into anaphase by Cdc20 re-addition. Proteins phosphorylation were analyzed by Western blotting in Phos-tag gels. Myo1 protein levels also served as loading control. Mitosis progression was followed by FACS analysis of DNA content and anaphase spindle elongation by in situ immunofluorescences. At least 100 cells were scored at each time point. **(B)** Ectopic expression of *CDC55* does not reduce the phosphorylation levels of Iqg1. Strain Y1643 (*MATa MET-HA₃-CDC20 MYO1-FLAG IQG1-HA₆ INN1-MYC₉ GAL1-CDC55*) was arrested in metaphase by Cdc20 depletion and 2% galactose was added to induce the *GAL1-CDC55* ectopic expression for three hours before the release into anaphase. Half of the culture without galactose addition was used as control. The analysis of the Iqg1 phosphorylation was done as in **(A)**. Pgk1 levels were used as loading control.

investigated its phosphorylation levels upon expression of Cdc55 under the control of the galactose inducible *GAL1* promoter. We synchronized cells at metaphase by Cdc20 depletion, added 2% galactose to induce Cdc55 expression for 3 hours and released cells into synchronous anaphase by Cdc20 re-induction. Cell cultures without galactose addition were used as control. Under these conditions, we observed that the non-induced *GAL1-CDC55* cells showed similar phosphorylation pattern than WT cells at Figure R1a, although the definition of the bands for Iqg1 phosphorylation was different in these gels. In addition, the induction of the *CDC55* ectopic expression did not affect the phosphorylation levels of Iqg1 compared to the non-induced control or the WT cells (Fig. R1a and b). Therefore, this result suggests that the Iqg1 phosphorylation does not change upon Cdc55 induction. In conclusion, we cannot discard the influence of PP2A^{Cdc55} over Iqg1 phosphorylation, since the phosphorylation levels of the IQGAP protein were increased in the *cdc55Δ* mutant

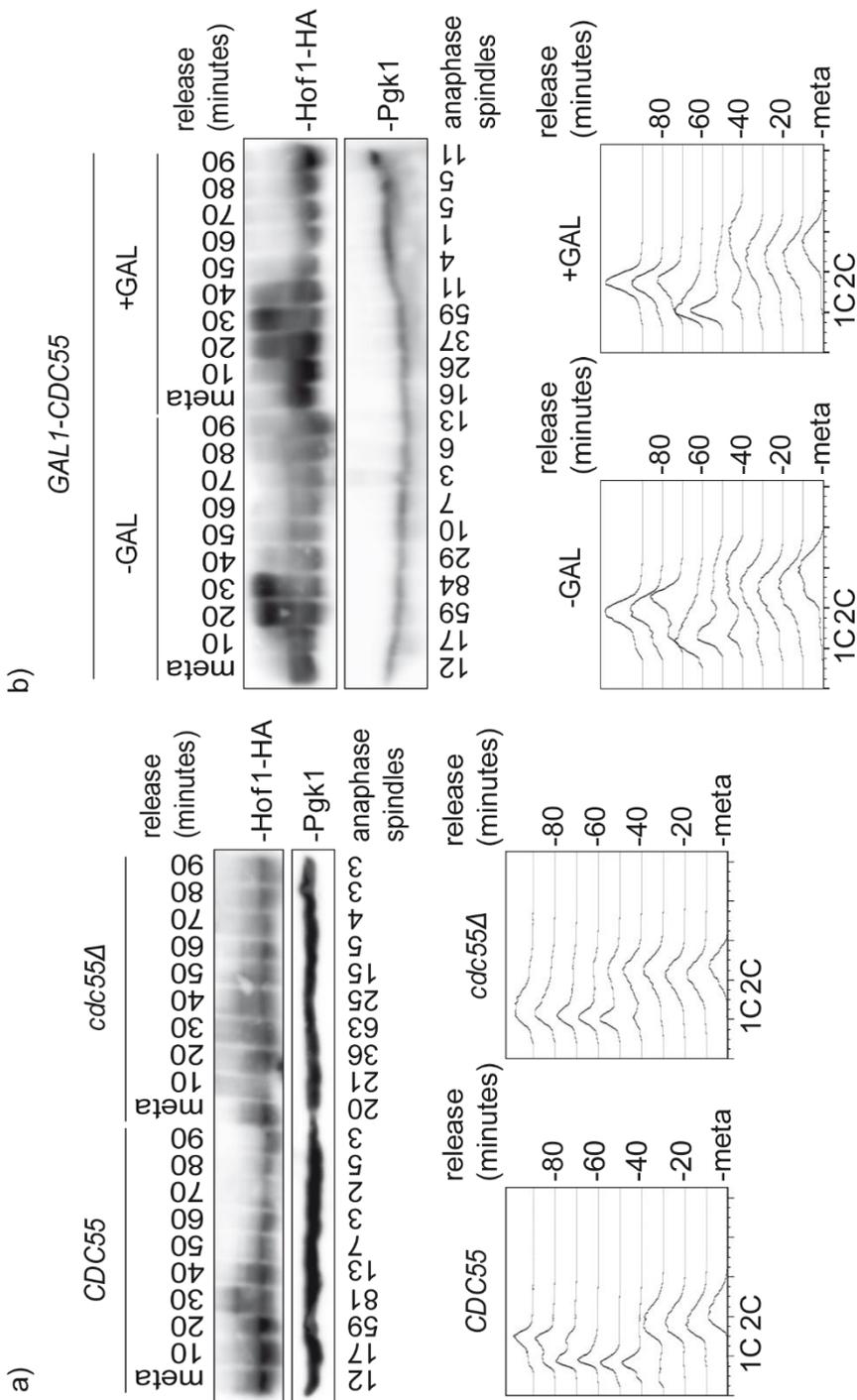


Figure R2. PP2A^{Cdc55} promotes the dephosphorylation of Hof1 during anaphase and cytokinesis. (A) Hof1 is hyperphosphorylated in absence of Cdc55, being more pronounced during cytokinesis. Strains Y1314 (*MATa MET-HA₃-CDC20 HOF1-HA₆*) and Y1394 (as Y1314, but *MATa* and *cdc55Δ*) were arrested in metaphase and released into anaphase by Cdc20 depletion and re-addition, respectively. Protein phosphorylation was analyzed by Western blotting in Phos-tag gels. Pgc1 levels were used as loading control. Mitosis progression was followed by FACS analysis of DNA content and anaphase spindle elongation by in situ immunofluorescences. At least 100 cells were scored at each time point. (B) Ectopic expression of *CDC55* reduces the phosphorylation levels of Hof1 during anaphase and cytokinesis. Strain Y1555 (*MATa MET-HA₃-CDC20 HOF1-HA₆ GAL1-CDC55*) was arrested in metaphase by Cdc20 depletion and 2% galactose was added to induce the *GAL1-CDC55* ectopic expression for three hours before the release into anaphase. Half of the culture without galactose addition was used as control. The analysis of the phosphorylation was done as in (A).

cells. However, this could be a secondary effect associated to the *cdc55Δ* deletion mutant.

Myo1 and Iqg1 are involved in AMR formation and interact with Hof1. In WT cells, several phosphorylated isoforms of Hof1 were detected at metaphase and became hyperphosphorylated at anaphase (30 minutes; Fig. R2a), as previously described (E. A. Vallen et al., 2000). The hyperphosphorylation was very transient and Hof1 was mostly dephosphorylated when cells enter cytokinesis (60-90 minutes; Fig. R2a), being the phosphorylation state in G1 lower than in metaphase. By contrast, in *cdc55Δ* cells the phosphorylation levels were quite high during the entire time-course experiment. In metaphase, *cdc55Δ* cells presented already slower migration forms of Hof1 than WT cells (meta; Fig. R2a). At

RESULTS

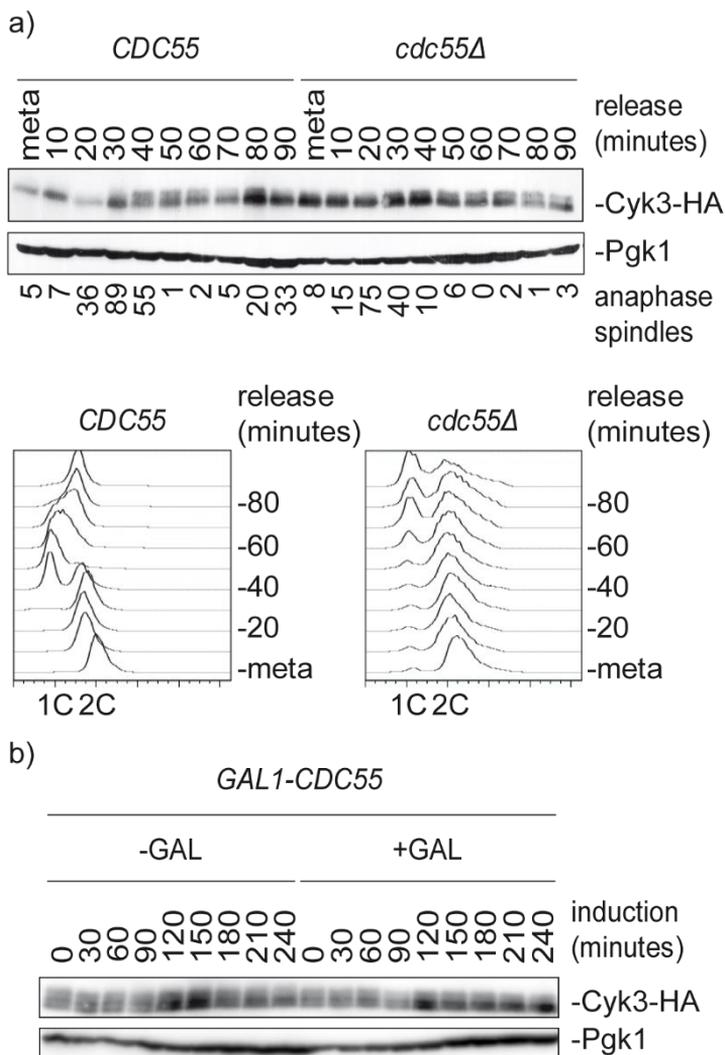


Figure R3. PP2A^{Cdc55} induces the dephosphorylation of Cyk3 during metaphase and early anaphase. (A) Cyk3 is hyperphosphorylated in absence of Cdc55. Strains Y1437 (*MAT α MET-HA₃-CDC20 CYK3-HA₆*) and Y1438 (as Y1437, but *cdc55Δ*) were arrested in metaphase and released into anaphase by Cdc20 depletion and re-addition, respectively. Protein phosphorylation was analyzed by Western blotting in Phos-tag gels. Pgk1 levels were used as loading control. Mitosis progression was followed by FACS analysis of DNA content and anaphase spindle elongation by in situ immunofluorescences. At least 100 cells were scored at each time point. (B) Ectopic expression of *CDC55* diminishes the phosphorylation levels of Cyk3. In exponentially growing cells of the strain Y1528 (*MAT α CYK3-HA₆ BFA1-HA₃ GAL1-CDC55*), 2% galactose was

anaphase, the peak of Hof1 phosphorylation was also detected (40 minutes); but later on, the depletion of the phosphorylations did not occur (60-90 minutes; Fig. R2a). These results showed that Hof1 is hyperphosphorylated in absence of Cdc55, being more pronounced during cytokinesis, suggesting that Hof1 could be a direct PP2A^{Cdc55} target.

Next, to confirm the Hof1 regulation by PP2A^{Cdc55} we decided to observe the Hof1 phosphorylation under the ectopic expression of Cdc55 expression as we did for Iqg1. As described before for WT cells (Fig. R2a), Hof1 was hyperphosphorylated at anaphase and quickly dephosphorylated in control cells without galactose addition (Fig. R2b). Upon *CDC55* overexpression in general the Hof1 phosphorylation levels were reduced (Fig. R2b). The peak of Hof1 phosphorylation during anaphase was still detected upon ectopic expression of Cdc55, suggesting that PP2A^{Cdc55} does not greatly affect Hof1 phosphorylation at that moment (Fig. R2b; 20-40 minutes). Nevertheless, during late anaphase and cytokinesis, Hof1 slower migrating isoforms were not detected indicating that Hof1 was dephosphorylated (50-60 minutes; Fig. R2b). These results, together with the previous experiment, indicate that the PP2A^{Cdc55} regulates the Hof1's phosphorylation state; being the effect of PP2A^{Cdc55} over Hof1 phosphorylation mainly after the peak of anaphase. IPC's proteins are also composed by Cyk3 and Inn1, the activators of Chs2. Recently, it was described that Cyk3 was

added to induce the *CDC55* ectopic expression. Half of the culture without galactose addition was used as control. The analysis of the phosphorylation was done as **(A)**. P_{gk1} levels were used as loading control.

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phosphorylated by MEN kinases concomitantly with Hof1 (M. Wang et al., 2018). In our control cells, Cyk3 started to be phosphorylated in anaphase (Fig. R3a; 30 min) and this phosphorylation was maintained until G1. On the other hand, in *cdc55* Δ mutant cells the phosphorylation was already present in metaphase and increased at anaphase (30-40 minutes; Fig. R3a); indicating that Cyk3 is slightly hyperphosphorylated in absence of Cdc55. In this case, the effect of the ectopic expression of *CDC55* over Cyk3 phosphorylation was analyzed in asynchronous cells since WT cells in metaphase were not phosphorylated. Time-course experiment from asynchronous cells with or without galactose addition were followed for 3 hours. Cyk3 phosphorylation remained unchanged in control cells without galactose addition. By contrast, upon *CDC55* ectopic expression Cyk3 faster migration bands accumulated, indicating that Cyk3 phosphorylation levels were reduced (120-240 minutes; Fig. R3b). These results suggest that PP2A^{Cdc55} controls the Cyk3 phosphorylation, especially during mitosis.

In wild-type strains, we observed that Inn1 was phosphorylated at 30 min, corresponding to cells in anaphase (30 minutes; Fig. R4 a). This phosphorylation has been previously described to be involved in the regulation of Inn1 (F Meitinger et al., 2010; Saravanan Palani et al., 2012). Then, the Inn1 phosphorylation was reduced to an intermediate state (seen as a doublet) that was maintained during the last points of the time-course (40-90 minutes; Fig. R4a). In *cdc55* Δ mutant cells, Inn1 phosphorylation appeared already as a doublet in metaphase and early anaphase (meta to 30 minutes; Fig. R4a). Interestingly, while anaphase hyperphosphorylation occurred normally (40 minutes; Fig. R4a), Inn1 remained

hyperphosphorylated during late anaphase and cytokinesis, contrary to control cells (50-90 minutes; Fig. R4a). Moreover, the ectopic expression of *CDC55* mostly abrogates the hyperphosphorylation of Inn1 at anaphase (40 minutes; Fig. R4b), although the intermediate phosphorylation levels were maintained as in WT cells. Surprisingly, the Inn1 doublet was also observed upon ectopic expression similar to *cdc55* Δ mutant cells (meta-30 minutes; Fig. R4b). We can conclude that PP2A^{Cdc55} regulates Inn1 dephosphorylation during late anaphase, cytokinesis and next G1.

Finally, Chs2 phosphorylation state was analyzed as the final effector of IPC's. Chs2 protein presented slow migration isoforms during metaphase and early anaphase in control cells (meta-20 minutes; Fig. R5a). This Chs2 phosphorylation was described to be important to retain Chs2 to the ER (Teh et al., 2009). During anaphase until early G1 (30-50 min) Chs2 faster migrations isoforms were detected and, later on, slower migrating isoforms were again accumulated (60-90 minutes; Fig. R5a). The dephosphorylation of Chs2 by Cdc14 was described to be important to release Chs2 from the ER (Chin et al., 2012). Remarkably, in *cdc55* Δ mutant cells the slower migrating isoforms appeared in all the time points, indicating that Chs2 was hyperphosphorylated during metaphase and was not efficiently dephosphorylated during late anaphase/cytokinesis (40-70 minutes; Fig. R5a). We can conclude that Chs2 phosphorylation levels are altered in absence of Cdc55. Moreover, the phosphorylation state of Chs2 was also analyzed after Cdc55 overexpression. Upon Cdc55 ectopic expression, Chs2 presented

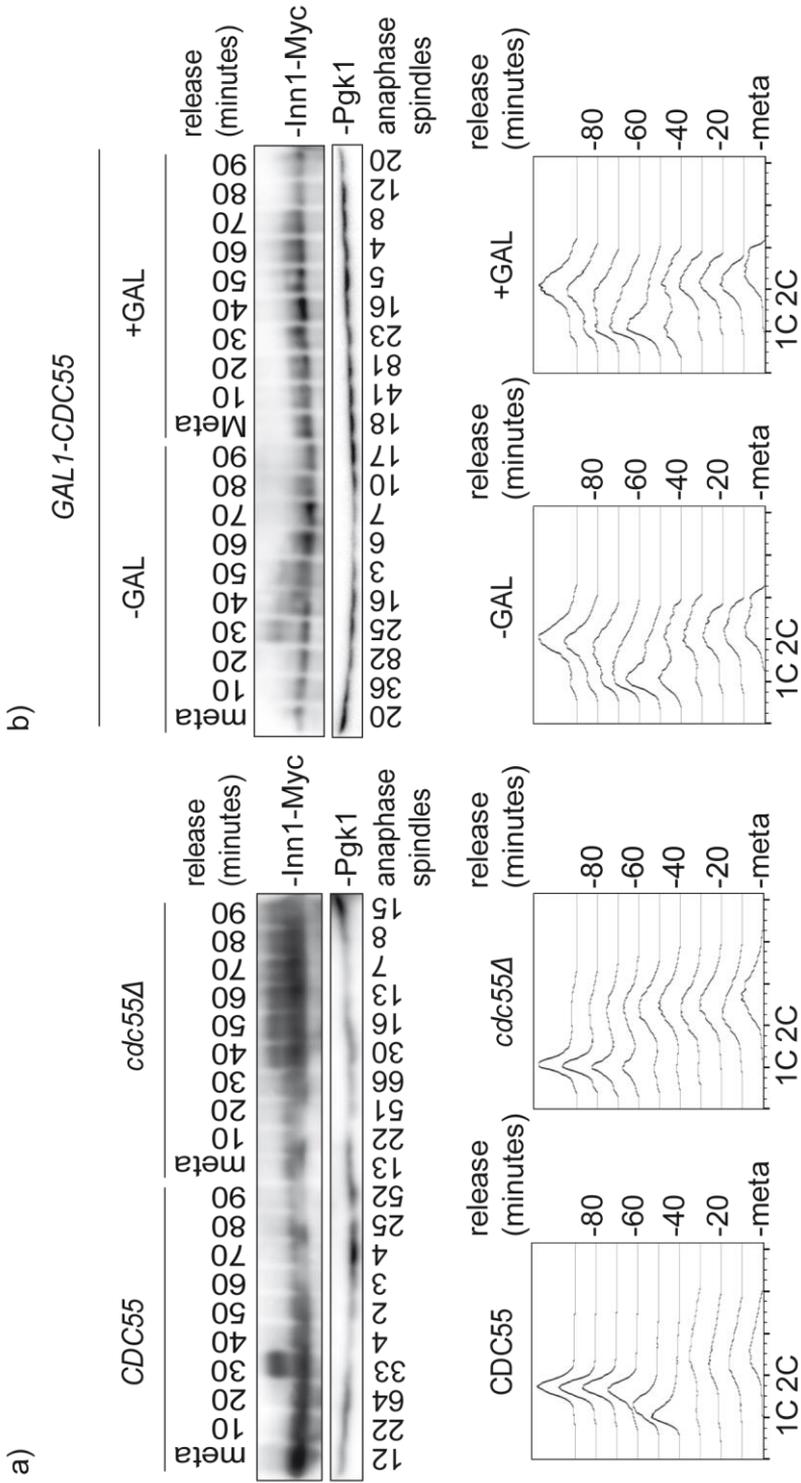


Figure R4. PP2A^{Cdc55} promotes the dephosphorylation of Inn1 during cytokinesis. (A) Anaphase-hyperphosphorylation of Inn1 is maintained in absence of Cdc55. Strains Y1639 (*MATa MET-HA₃-CDC20 CYK3-HA₆ INN1-MYC₉*) and Y1640 (as Y1639, but *cdc55Δ*) were arrested in metaphase and released into anaphase by Cdc20 depletion and re-addition, respectively. Protein phosphorylation was analyzed by Western blotting in Phos-tag gels. Pgk1 levels were used as loading control. Mitosis progression was followed by FACS analysis of DNA content and anaphase spindle elongation by in situ immunofluorescences. At least 100 cells were scored at each time point. (B) Ectopic expression of *CDC55* abrogates Inn1 hyperphosphorylation. Strain Y1643 (*MATa MET-HA₃-CDC20 MYO1-FLAG IQG1-HA₆ INN1-MYC₉ GAL1-CDC55*) was arrested in metaphase by Cdc20 depletion and 2% galactose was added to induce the *GAL1-CDC55* ectopic expression for three hours before the release into anaphase. Half of the culture without galactose addition was used as control. The analysis of the phosphorylation was done as in (A).

more faster migrating isoforms, consistent with Chs2 being dephosphorylated (Fig. R5b 50-90 min). The result indicates that the ectopic expression of PP2A^{Cdc55} promotes the dephosphorylation of Chs2 during cytokinesis.

The previous results suggest that PP2A^{Cdc55} phosphatase counteracts Inn1, Hof1, Cyk3 and Chs2 phosphorylation. To determine whether these IPC's proteins are PP2A^{Cdc55} substrates, we examined whether they physically interact with Cdc55 by co-immunoprecipitation experiments. In Cdc55's immunoprecipitates, from cells released into synchronous anaphase by Cdc20 depletion and re-induction, we detected co-purification of Inn1, Hof1, Cyk3 and Chs2 during anaphase and cytokinesis (Fig. R6a). These results indicate that PP2A^{Cdc55} and Inn1, Hof1, Cyk3 and Chs2 physically interact and those interactions are conserved throughout the progression through mitosis and cytokinesis. Taken together,

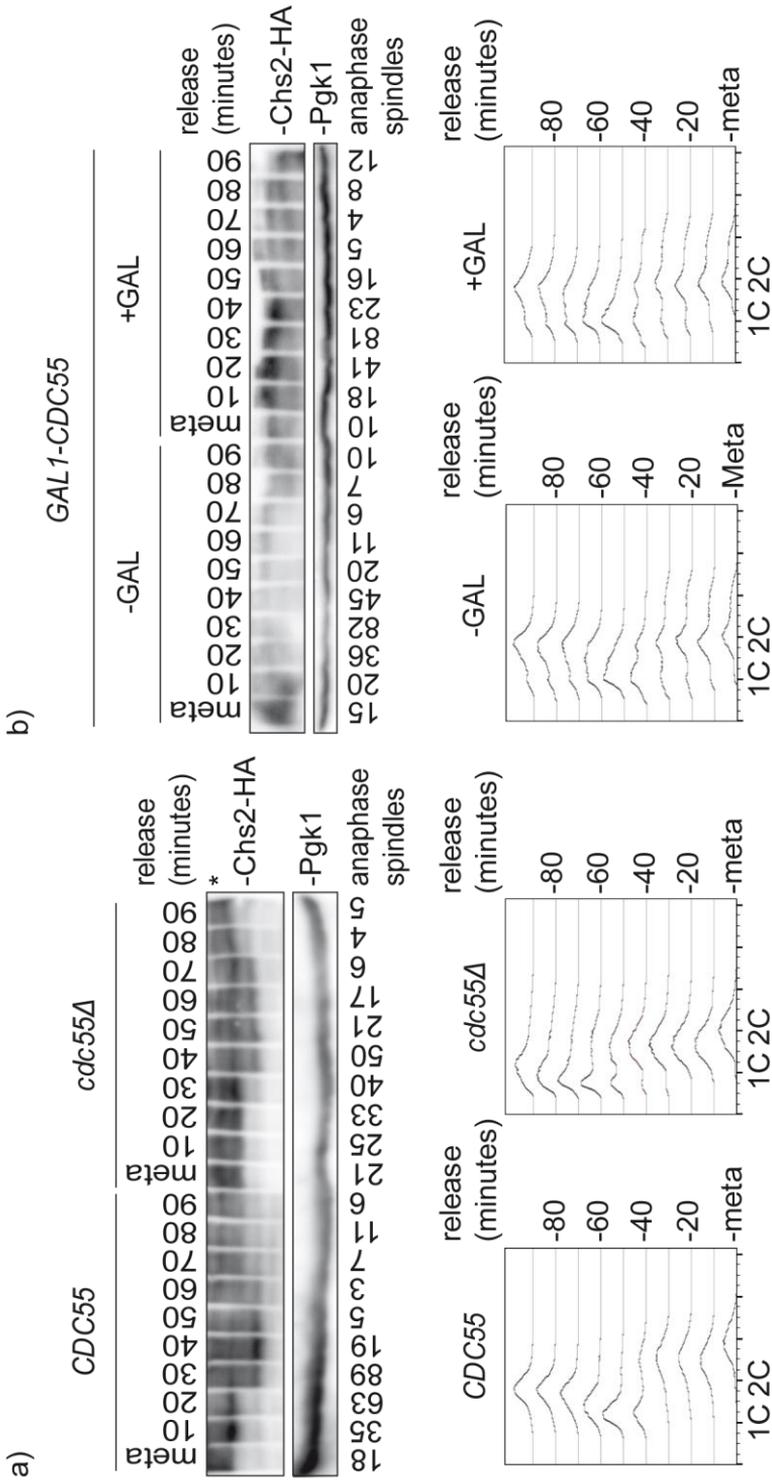


Figure R5. PP2A^{Cdc55} induces the dephosphorylation of Chs2 during cytokinesis. (A) Chs2 is not dephosphorylated during cytokinesis in *cdc55Δ* cells. Strains Y1318 (*MATa MET-HA₃-CDC20 CHS2-HA₆*) and Y1315 (*MATa MET-HA₃-CDC20 CHS2-HA₆ cdc55Δ*) were arrested in metaphase and released into anaphase by Cdc20 depletion and re-addition, respectively. Protein phosphorylation was analyzed by Western blotting in Phos-tag gels. Pgk1 levels were used as loading control. Mitosis progression was followed by FACS analysis of DNA content and anaphase spindle elongation by in situ immunofluorescences. At least 100 cells were scored at each time point. * denotes an unspecific band. (B) Ectopic expression of *CDC55* reduces Chs2 phosphorylation. Strain Y1626 (*MATa MET-HA₃-CDC20 CHS2-HA₆ GAL1-CDC55*) was arrested in metaphase by Cdc20 depletion and 2% galactose was added to induce the *GAL1-CDC55* ectopic expression for three hours before the release into anaphase. Half of the culture without galactose addition was used as control. The analysis of the phosphorylation was done as in (A).

these results suggest that Inn1, Hof1, Cyk3 and Chs2 are likely to be *in vivo* substrate of PP2A^{Cdc55}.

Next, to examine the physical interactions of IPC's protein and Cdc55 in other cell cycle stages, cells were arrested at G1 by alpha-factor addition and released synchronously into the cell cycle. Cdc55 was immunoprecipitated at G1, S/G2 and mitosis. We observed that in the case of Cyk3 no co-purification was detected at the S/G2 transition (Fig. R6b), indicating the interaction is cell cycle regulated.

To explicitly test whether Inn1, Cyk3, Hof1 and Chs2 are PP2A^{Cdc55} direct dephosphorylation targets we measured PP2A^{Cdc55} *in vitro* phosphatase activity assays after Cdc55 immunopurification from metaphase-arrested cells when PP2A^{Cdc55} is active. The substrates were recombinant fragments of Inn1 (full length), Cyk3 (full length), Hof1 (full length) and Chs2 (aa1 to aa629) purified from *E. Coli*. To perform the phosphatase assays, first the substrates need to be

RESULTS

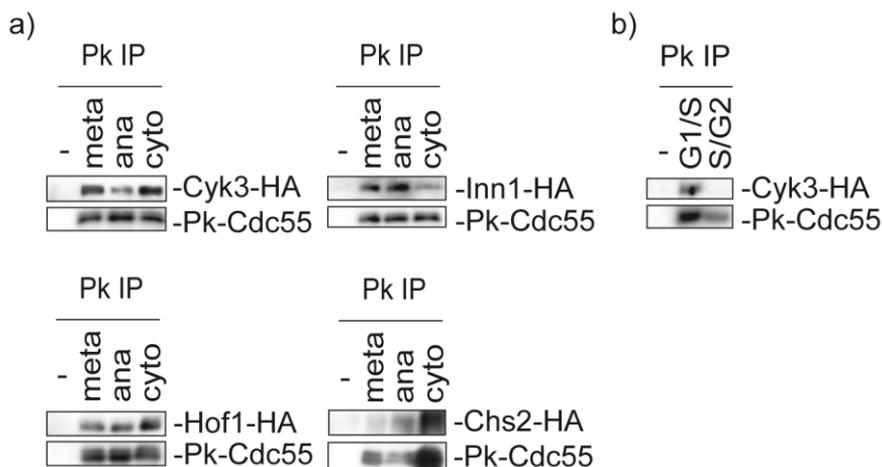


Figure R6. PP2A^{Cdc55} physically interacts with Cyk3, Inn1, Hof1 and Chs2 during anaphase *in vivo*. (A) PP2A^{Cdc55} form a complex with IPC's. Strains Y1565 (*MATa MET-HA₃-CDC20 CYK3-HA₆ PK₃-CDC55*), Y1566 (*MATa MET-HA₃-CDC20 INN1-HA₆ PK₃-CDC55*), Y1423 (*MATa MET-HA₃-CDC20 HOF1-HA₆ PK₃-CDC55*), and Y1567 (*MATa MET-HA₃-CDC20 CHS2-HA₆ PK₃-CDC55*) were synchronized into anaphase progression by Cdc20 depletion and re-addition. Strains Y1437, Y1312 (as Y1566, but without *PK₃-CDC55*), Y1314 and Y1318 were used as negative controls. Protein extracts were prepared at metaphase, anaphase and cytokinesis and Cdc55 was purified by immunoprecipitation with Pk antibody. The analysis of the co-purification of Cyk3-HA₆, Inn1-HA₆, Hof1-HA₆ and Chs2-HA₆ was done by western blotting. Anaphase progression was monitored by tubulin staining. (B) PP2A^{Cdc55} physical interaction with IPC's is cell cycle regulated. Y1565 cells were arrested in G1 with α -factor and release into a synchronous cell cycle. Protein extracts from cells in S/G1 and G2/M were prepared. Strain Y1437 without Pk epitope was used as negative control. Co-immunoprecipitation of Cyk3 and Cdc55 was investigated by western blotting.

phosphorylated. Cdk1 and MEN kinases were used, as are the most relevant regulatory kinases of the IPC's (F Meitinger et al., 2011, 2010; E. A. Vallen et al., 2000). Clb2, Cdc5, Cdc15 and Dbf2 were purified from yeast cells when they are active; Clb2-Cdk1 and Cdc5 in metaphase, and Cdc15 and Dbf2 in anaphase.

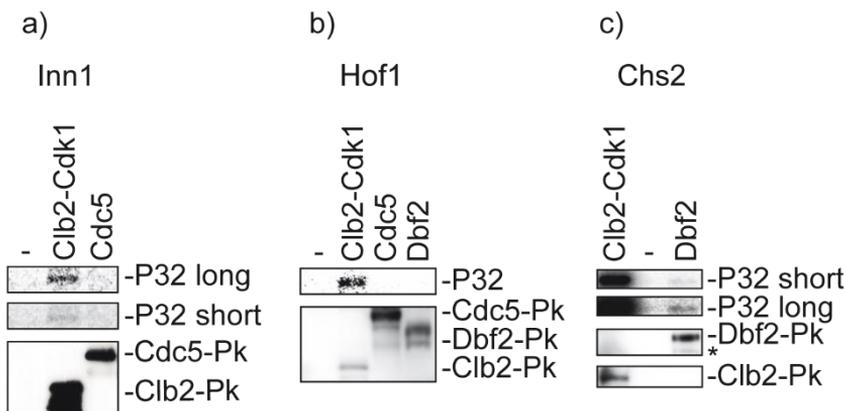


Figure R7. Inn1, Hof1 and Chs2 phosphorylation by mitotic kinases *in vitro*. Strains Y824 (*MAT α MET-HA₃-CDC20*), Y688 (*MAT α MET-HA₃-CDC20 GAL1-FLAG-ESP1-CBD-C1531A CLB2-PK₃ BFA1-HA₆*), Y2231 (*MAT α MET-HA₃-CDC20 CDC5-PK₃ CDC14-HA₆*) and Y1357 (*MAT α MET-HA₃-CDC20 cdc5-as1 NET1-MYC₉ CDC5 (5'UTR):CDC5-Myc₉ DBF2-PK₆*) were arrested in metaphase by Cdc20 depletion. Strain Y1357 was released into anaphase by Cdc20 re-addition and collected samples at 30 min when cells were in anaphase. Protein extracts were prepared, and the kinases were purified by immunoprecipitation with Pk antibody. 6His-Hof1, 6His-Inn1 and Strep-tag-Chs2-1-629 purified from *E. coli* were used as substrates. **(A)** Inn1 was phosphorylated by Clb2-Cdk1; **(B)** Hof1 was phosphorylated by Clb2-Cdk1; and **(C)** Chs2 was phosphorylated by Clb2-Cdc28 and Dbf2. Representative images of the kinase assays are shown. The amount of the kinases immunoprecipitated were analyzed by western blotting. Radioactive signal was detected using a multi-purpose imaging plate (GE healthcare) in a Typhoon FLA950. Quantification of the protein levels were done using the ImageJ software.

Inn1 was incubated with Clb2-Cdk1 and Cdc5 kinases, as Cdk1 was described to be the kinase responsible for Inn1 phosphorylation (Saravanan Palani et al., 2012) and Cdc5 is the second most abundant kinase-consensus sequence present at Inn1. We detected a faint Inn1 phosphorylation in Clb2-Cdk1 kinase assays after very long exposure times (Fig. R7a). However, this Inn1 phosphorylated substrate did not have enough signal to proceed with the phosphatase assay (Fig. R7a).

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Cyk3 kinase assays were performed using Clb2-Cdk1, Cdc5, Cdc15 and Dbf2 since no kinase has been previously described to phosphorylate the protein. Unfortunately, no Cyk3 phosphorylation was observed in any of the kinases used. Therefore, no phosphatase assay could be performed for Cyk3.

Clb2-Cdk1, Cdc5 and Dbf2 are described to phosphorylate Hof1 (Meitinger et al., 2011; Meitinger, Palani, Hub, & Pereira, 2013), and we decided to perform the Hof1 kinase assays with the three kinases. Nevertheless, we only detected Hof1 phosphorylation in Clb2-Cdk1 kinase assays (Fig. R7b) and used this Hof1 phosphorylated protein in the phosphatase assays (see below).

Finally, Chs2 kinase assays were performed with Clb2-Cdk1 and Dbf2-Mob1 both previously described to be responsible of Chs2 phosphorylation (Teh et al., 2009) or phosphorylate Chs2 *in vitro* (Martínez-Rucobo et al., 2009; Y Oh et al., 2012). Here, we observed that Chs2 was indeed phosphorylated by Dbf2-Mob1 and Clb2-Cdk1 (Fig. R7c).

For the phosphatase assays, Cdc55 and Cdc14 immunoprecipitates were incubated with the phosphorylated substrates and the reduction in the ³²P-phosphorylated levels were determined. Clb2-Cdk1-phosphorylated Hof1 and Chs2, and Dbf2-phosphorylated Chs2 were incubated with the Cdc55 immunoprecipitates from control Cdc55 and *cdc55_ED* inactive version of PP2A^{Cdc55} (Játiva et al., 2019) from metaphase-arrested cells, and with Cdc14 immunoprecipitates from anaphase cells. Cdc14 was previously described to dephosphorylate Chs2 (Chin et al., 2012), while no phosphatase has been related to Hof1.

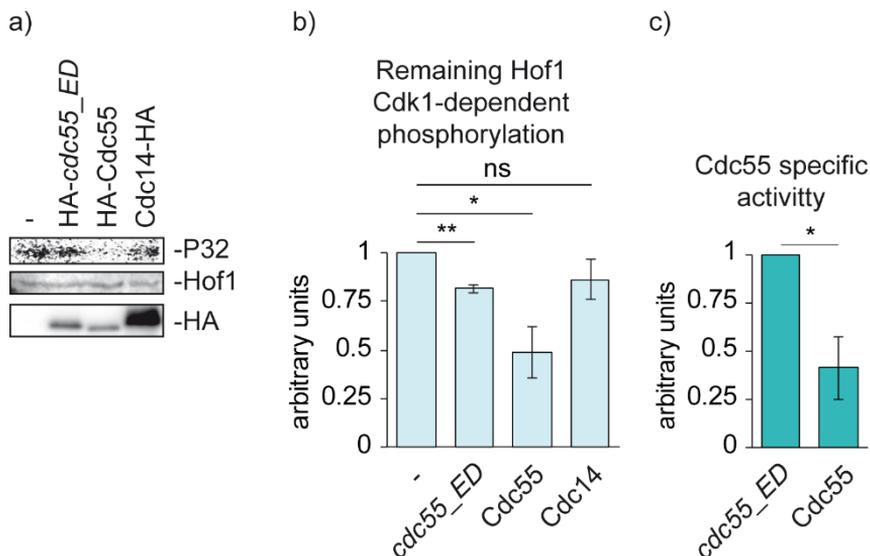


Figure R8. PP2A^{Cdc55} dephosphorylates Hof1 *in vitro*. Strains Y824, Y564 (*MAT α MET-HA₃-CDC20 HA₃-CDC55*), Y1653 (*MAT α MET-HA₃-CDC20 MYO1-TOMATOE cdc55 Δ URA3::HA₃-CDC55-T174E_S301D*) and Y2299 (*MAT α MET-HA₃-CDC20 CDC14-HA₆*) were arrested in metaphase by Cdc20 depletion. Strain 2299 was released into anaphase by Cdc20 re-addition and collected samples at 30 min when cells were in anaphase. Protein extracts were prepared, and the phosphatases were purified by immunoprecipitation with HA antibody. ³²P-Hof1 obtained from the Clb2-Cdk1 kinase assays was used as substrate for the phosphatase assays. Phosphatase activity of immunopurified phosphatases was measured as described in materials and methods. Representative images of the phosphatase assays are shown in **(A)**. Measurement of the ³²P-Hof1 signal from the different phosphatase assays performed **(B)** and quantification of the specific PP2A^{Cdc55} activity by normalizing the phosphorylation signal to the amount of HA-Cdc55 or HA-cdc55_ED **(C)**. Means and SEM of three/two phosphatase assays are represented.

Using Hof1 as a substrate, the Hof1 phosphorylation signal intensity was reduced to half in the assay with PP2A^{Cdc55} (Fig. R8a and b). By contrast, in PP2A^{cdc55_ED} and Cdc14 phosphatase assays no biological significant decrease of Hof1 phosphorylation was detected (Fig. R8a and b). In addition, when we calculated the

RESULTS

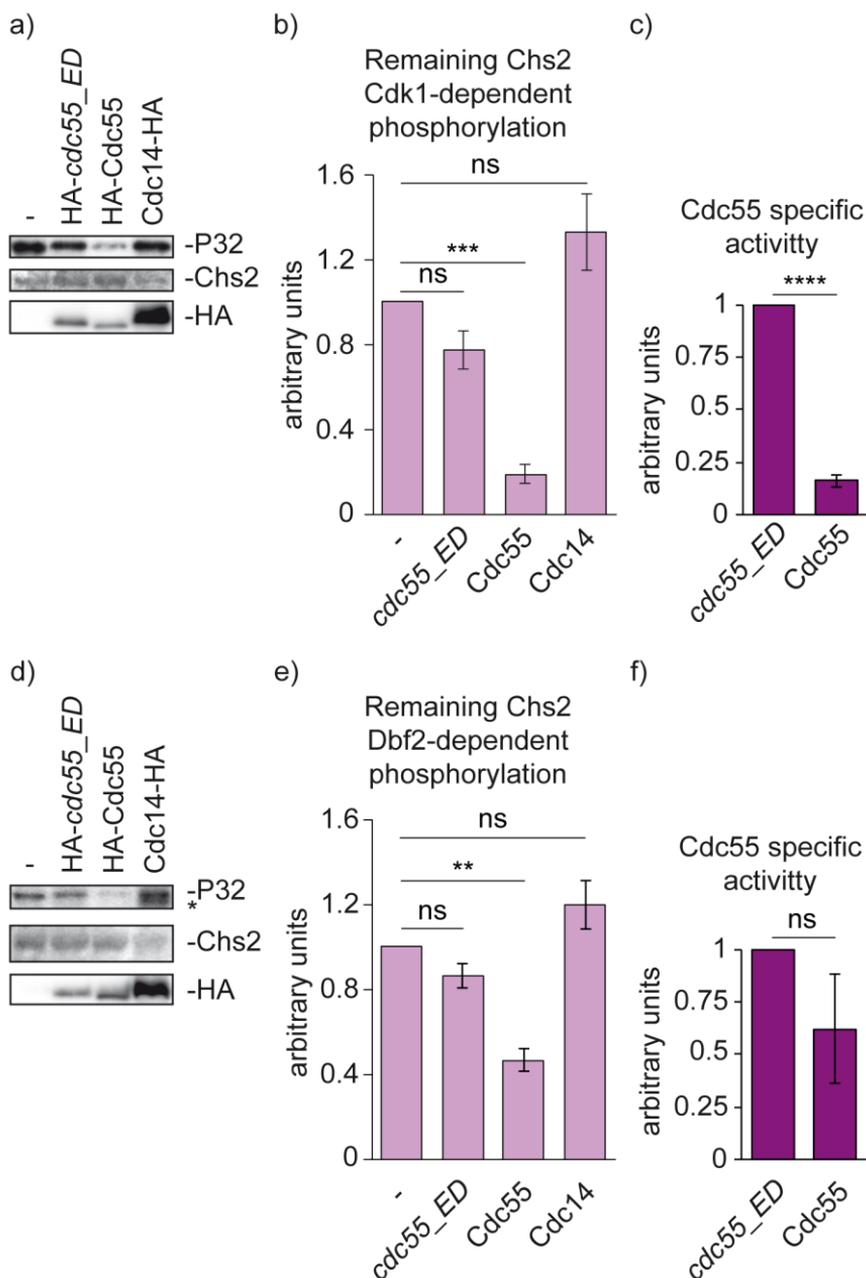


Figure R9. PP2A^{Cdc55} dephosphorylates Chs2 *in vitro*. Strains Y824, Y564 (*MAT α MET-HA₃-CDC20 HA₃-CDC55*), Y1653 (*MAT α MET-HA₃-CDC20 MYO1-TOMATOE cdc55 Δ URA3::HA₃-CDC55-T174E_S301D*) and Y2299 (*MAT α MET-HA₃-CDC20 CDC14-HA₆*) were arrested in metaphase by Cdc20 depletion. Strain Y2299 was released into anaphase by Cdc20 re-addition and collected samples at 30 min when cells were in

Cdc55 specific activity (normalizing to the amount of phosphatase) over Hof1 comparing Hof1 *in vitro* phosphorylation in *cdc55_ED* and Cdc55, we observed that the Hof1 phosphorylation is reduced significantly in Cdc55 respect to *cdc55_ED* (Fig. R8c). These results clearly demonstrate that PP2A^{Cdc55} dephosphorylates Hof1 *in vitro*, confirming that Hof1 is its substrate. Moreover, Hof1 dephosphorylation depends on active PP2A^{Cdc55} further supporting that Hof1 is a PP2A^{Cdc55} substrate.

When using Chs2 as a substrate, the PP2A^{Cdc55} phosphatase assays for both Cdk1- and Dbf2-phosphorylated Chs2 showed a strong decrease in the Chs2 phosphorylation signal (Fig. R9). A reduction of 81% was measured for the Chs2 phosphorylation signal in the case of Cdk1-phosphorylated Chs2, and 53% reduction for Dbf2-phosphorylated Chs2 respect to the sample without phosphatase, indicating higher dephosphorylating ratios for Cdk1-phosphorylated residues (Fig. R9b and e). Remarkably, the *cdc55_ED* mutant version was not able to dephosphorylate significantly any phosphorylated Chs2 (Fig. R9b and e); 23% and 14% reduction in Chs2 phosphorylation levels. As occurred with Hof1, we observed that after normalizing the Chs2 phosphorylation

anaphase. Protein extracts were prepared, and the phosphatases were purified by immunoprecipitation with HA antibody. ³²P-Chs2 obtained from Clb2-Cdk1 and Dbf2 kinase assays were used as substrates for the phosphatase assays. Phosphatase activity of immunopurified phosphatases was measured as described in materials and methods. Representative images of the phosphatase assays are shown in **(A)**. Measurement of the ³²P-Hof1 signal from the different phosphatase assays performed **(B)** and quantification of the specific PP2A^{Cdc55} activity by normalizing the phosphorylation signal to the amount of HA-Cdc55 or HA-*cdc55_ED* **(C)**. Means and SEM of three/two phosphatase assays are represented.

RESULTS

intensity to the amount of purified phosphatase, there is a significant reduction in the Chs2 phosphorylation intensity in presence of Cdc55 compared to the inactive *cdc55_ED* (Fig. R9c and f). The difference in the Chs2 substrate phosphorylated by Dbf2 is not statistically significant, although the tendency to reduce Chs2 phosphorylation was detected. These results strongly support that Chs2 is dephosphorylated *in vitro* by PP2A^{Cdc55}, further ratifying that Chs2 is also a PP2A^{Cdc55} substrate.

It was previously described that Cdc14 dephosphorylate Chs2 (Chin et al., 2012); however, we were not able to see a decrease in the intensity of the phosphorylation signal of Chs2 substrates in the Cdc14 phosphatase assays (Fig. R9). Upon quantification, we observed an increase in the phosphorylation levels in presence of Cdc14 phosphatase in our assay conditions, even though it was not statistically significant. This increase in the phosphorylation levels was probably as consequence of a kinase co-precipitated with the Cdc14 in the immunoprecipitation.

In conclusion, the phosphatase assays indicate that PP2A^{Cdc55} counteracts phosphorylations in Hof1 and Chs2, reinforcing the idea that they are PP2A^{Cdc55} direct substrates. It has been previously described that PP2A^{Cdc55} counteracts Cdk1 phosphorylation (Godfrey et al., 2017; Harvey et al., 2011), but it is the first time, to our knowledge, that a Dbf2 substrate is shown to be dephosphorylated by PP2A^{Cdc55}. Furthermore, it was described by the first time a phosphatase, concretely PP2A^{Cdc55}, dephosphorylating Hof1. Nevertheless, we could not check whether Inn1 and Cyk3 are direct PP2A^{Cdc55} substrates due to the technical problems with those substrates in the *in vitro* kinase assays.

1.2. PP2A^{Cdc55} modulates AMR contraction at the bud neck

Once we established that at least Hof1 and Chs2 are direct PP2A^{Cdc55} targets, we wondered whether the changes in their phosphorylation levels altered their bud neck localization; and, therefore, PP2A^{Cdc55} regulates the IPC proteins localization to the bud neck. Myosin type II protein (Myo1) is the first of the IPC's proteins localized at the bud neck as part of the myosin ring. Myo1 recruitment to the bud neck occurs early in the cell cycle, but it is not until late anaphase when the actin is recruited and forms the AMR (D. P. Miller et al., 2015; Naylor & Morgan, 2014). We examined the Myo1-Tomatoe localization at the bud neck and the AMR behavior during constriction in WT and *cdc55*Δ cells by time-lapse experiments. Myo1 localizes to the bud neck immediately after budding; therefore, Myo1-Tomatoe was already localized at the bud neck in metaphase-arrested cells in both wild-type and *cdc55*Δ strains (Fig. R10). During cytokinesis there was a reduction in the signal size, which reflects the contraction of the AMR, until the signal became a single dot and finally disappeared (Fig. R10). The AMR contraction took 5 min of mean in both strains (4.63 ±0.16 in *CDC55* cells and 4.6 ±0.22 in *cdc55*Δ cells); but, interestingly, the “dot” signal collapsed to one side of the bud neck in mostly all the *cdc55*Δ cells (Fig. R10). We observed that the AMR contraction was asymmetric to the centripetal axis in the 96% (N=30) of *cdc55*Δ cells (Fig. R10).

RESULTS

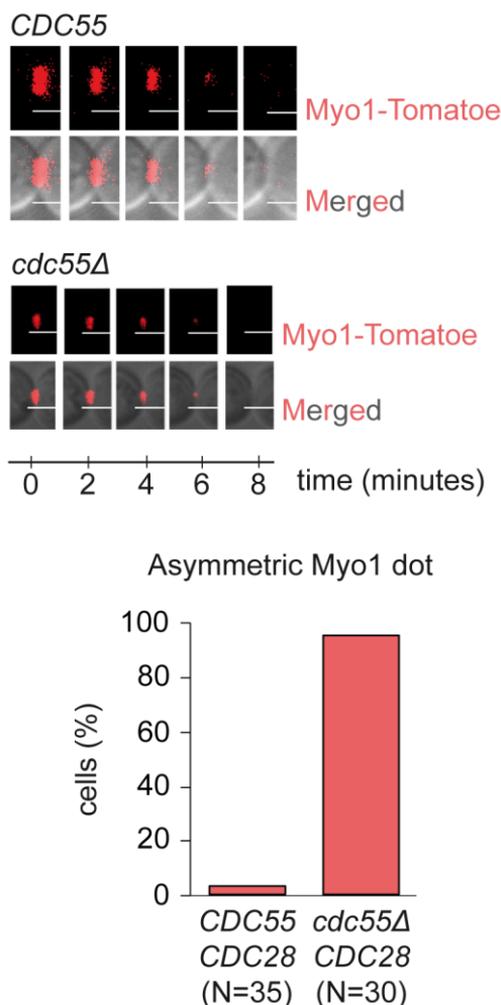


Figure R10. Absence of Cdc55 promotes the asymmetry of Myo1-Tomatoe signal upon AMR contraction. Strains Y1306 (*MAT α MET-HA₃-CDC20 MYO1-TOMATOE SPC42-GFP HOF1-GFP*) and Y1578 (as Y1306, but *MAT α* and *cdc55Δ*) were arrested in metaphase and released into anaphase by Cdc20 depletion and re-addition, and the time-lapse experiment was performed taking images every 2 minutes. Spc42-GFP (spindle pole bodies protein) was used as control of anaphase progression. Quantification of the population of cells with asymmetric Myo1 constriction is shown (lower panel). Representative images of Myo1-Tomatoe signal in *CDC55* (N=30) and *cdc55Δ* (N=30) are shown (upper panel). Images were taken with an Axio Observer.Z12 microscope (Carl Zeiss, Thornwood, NY). GFP and Cy3 filters were used. Fiji software was used to analyze the images. Scale bar of 2 μ m.

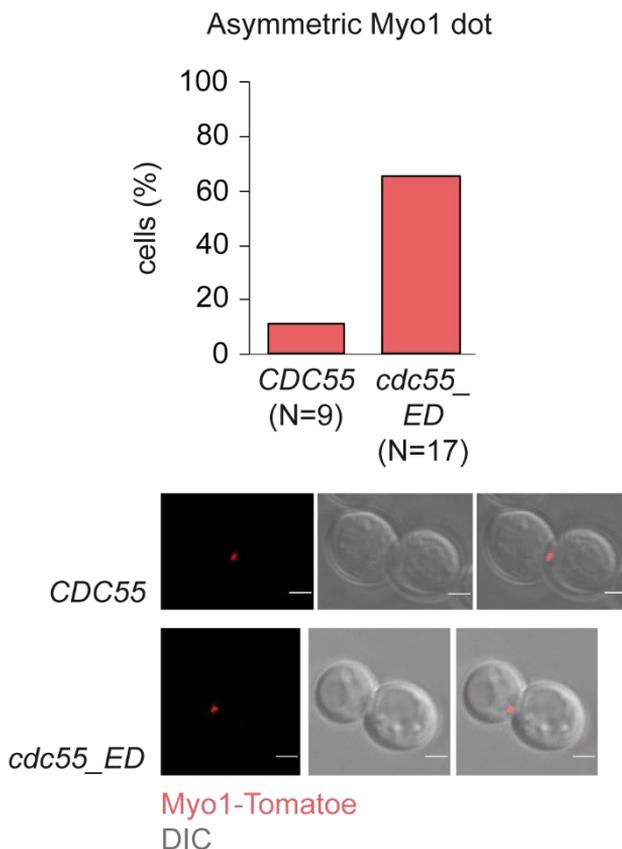


Figure R11. PP2A^{Cdc55_ED} inactive version promotes the Myo1 asymmetry similar to the *cdc55* Δ deletion mutant. Strain Y1652 (*MATa MET-HA₃-CDC20 MYO1-TOMATOE cdc55 Δ URA3::HA₃-CDC55*) and Y1653 (*MATa MET-HA₃-CDC20 MYO1-TOMATOE cdc55 Δ URA3::HA₃-cdc55-T174E_S301D*) were arrested in metaphase and released into anaphase by Cdc20 depletion and re-addition, and the time-lapse experiment was performed taking images every 2 minutes. Quantification of the cell population with asymmetric Myo1 constriction is shown (upper panel). Representative images of Myo1-Tomatoe signal from Cdc55 (N=9) and *cdc55_ED* (N=17) cells (lower panel). Images were taken with an Axio Observer.Z12 microscope (Carl Zeiss, Thornwood, NY). Cy3 filter was used. Fiji software was used to analyze the images. Scale bar of 2 μ m.

Next, we wondered whether the Myo1 asymmetry could be also detected in the inactive version of Cdc55 (*cdc55_ED*). We synchronized cells at the metaphase-anaphase transition by Cdc20

RESULTS

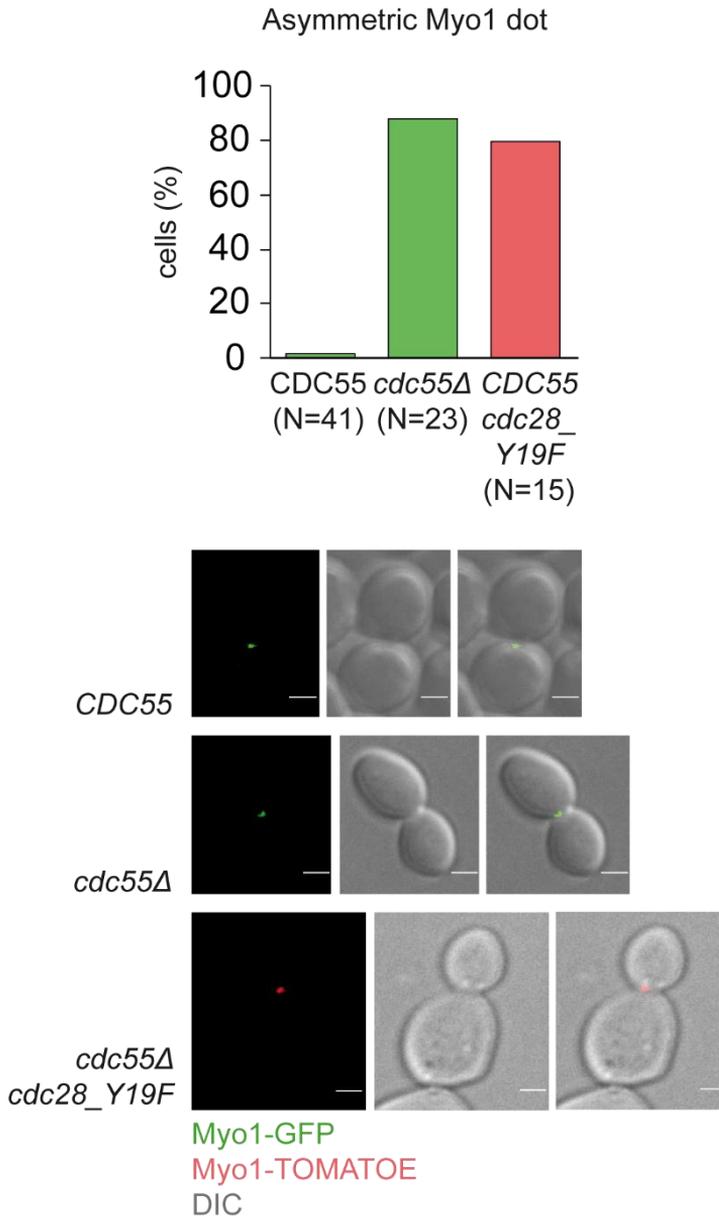


Figure R12. Myo1 asymmetric signal in *cdc55Δ* cells is not a consequence of its tagging or the synchronization method used. Strains Y1434 (*MATa MET-HA₃-CDC20 MYO1-GFP cdc55Δ*) and Y1435 (*MATa MET-HA₃-CDC20 MYO1-GFP*) were arrested in metaphase and released into anaphase by Cdc20 depletion and re-addition. Strain Y1725 (*MATa MET-HA₃-CDC20 MYO1-TOMATOE CDC15-eGFP cdc55Δ cdc28-Y19F*) was arrested in G1 by α -factor addition and released into cell cycle

depletion and analyzed the contraction of the Myo1-Tomatoe. We observed that 65% of *cdc55_ED* mutants cells showed asymmetric Myo1-Tomatoe signal upon contraction (Fig. R11). This result indicates that, similarly to the absence of Cdc55, the non-functional Cdc55 promotes a failure in AMR contraction. Altogether, we conclude that Myo1 recruitment and residence time at the bud neck is not altered in absence of Cdc55; interestingly, AMR contraction is defective in absence of PP2A^{Cdc55} activity. This asymmetric Myo1 signal has been previously observed in some IPC mutants, such as *inn1-td* (Sanchez-Diaz, 2008) and suggests a dysfunctional AMR. These results demonstrate that PP2A^{Cdc55} is required for the correct AMR constriction.

To further confirm that the asymmetric AMR contraction phenotype was not affected by either the Tomatoe-tagging or the synchronization method, we observed fixed *cdc55Δ* cells tagged with Myo1-GFP upon release from metaphase-arrested cells by Cdc20 depletion and *cdc55Δ* cells tagged with Myo1-Tomatoe synchronizing cells with alpha factor in G1. The *cdc55Δ* Myo1-GFP tagged cells showed an asymmetry of the 87.5% of the population (N=23) (Fig. R12); quite similar phenotype penetrance to Myo1-Tomatoe in *cdc55Δ* cells (Fig. R10). This asymmetry was not observed in WT cells (1 asymmetric cell (2.4%) in the 41 cells

progression by pheromone removal. Cells were fixed with formaldehyde before taking images. Quantification of the cell population with asymmetric Myo1 signal is shown (upper panel). Representative images of the Myo1-GFP signal from *CDC55* (N=41) and *cdc55Δ* (N=23) cells, and Myo1-Tomatoe from *CDC55 cdc28-Y19F* (N=15) cells are shown (lower panel). Images were taken with an Axio Observer.Z12 microscope (Carl Zeiss, Thornwood, NY). GFP and Cy3 filter was used. Fiji software was used to process images. Scale bar of 2 μm.

analyzed; Fig. R12).

Next, we performed the assays synchronizing cells in G1 by alpha factor in *cdc55Δ cdc28_Y19F* cells. *cdc55Δ* cells enter mitosis with a delay due to compromised Cdk1 activity because of inhibitory Cdc28-Y19 phosphorylation (Minshull et al., 1996). To correct for this mitotic entry delay we introduced the *cdc28_Y19F* allele that is refractory to Cdk1 inhibition. The cells containing *cdc28_Y19F* progressed through the cell cycle normally (Queralt et al., 2006). Again, we observed that Myo1-Tomatoe constriction was asymmetric in the 80% of *cdc55Δ cdc28_Y19F* population (Fig. R12; N=15). Therefore, the asymmetric Myo1 signal in absence of Cdc55 was observed independently of the epitope and the synchronization method used.

1.3. PP2A^{Cdc55} regulates the residence time of the IPC's at the bud neck

Since Myo1-Tomatoe has no difference in protein recruitment nor residence time at the bud neck in absence of Cdc55, we decided to use the Myo1 dynamics as inner control for the dynamic of AMR constriction when studying the localization of the others IPC's proteins.

First, we analyzed the Iqg1 protein localization using Iqg1 GFP-tagged protein. Iqg1 localization has been described to depend on Cdc14 dephosphorylation of the Cdk1-phosphorylated residues (Miller et al., 2015; Naylor & Morgan, 2014). To analyze whether

PP2A^{Cdc55} also regulates its localization, we used Iqg1-GFP tagged strains in controls and *cdc55*Δ cells and visualized their localization by time-lapse microscopy. We arrested cells in metaphase by Cdc20 depletion and we took images every 2 minutes after synchronously release into anaphase by Cdc20 re-induction. In metaphase, Iqg1 was already present at the bud neck in WT and *cdc55*Δ mutant cells (not shown). We observed that in 74% of *cdc55*Δ mutant cells Iqg1-GFP contracted asymmetrically as Myo1-Tomatoe (Fig. R13a), and this contraction was mostly simultaneous to Myo1-Tomatoe in both strains (Fig. R13b and c). In addition, in WT cells the Iqg1-GFP contraction to disappear signal took 8 minutes in 80% of the cells (Fig. R13d 3rd column). By contrast, the Iqg1-GFP signal was observed during longer times in absence of Cdc55. In 43% of *cdc55*Δ cells the signal lasted for 11 minutes on average (Fig. R13d, marked in yellow). These results indicate that Iqg1 signal was also asymmetric in absence of Cdc55, and its residence time at the bud neck was longer compared to WT cells. These results suggest that the lack of PP2A^{Cdc55} activity provokes a collapse of the AMR that also alter the residence time of the Iqg1, indicating that the cytokinesis is altered.

Then, we observed the Hof1 localization dynamics at the bud neck. Hof1 localization was described to depend on its phosphorylation state (Vallen, Caviston, & Bi, 2000), being localized at the AMR after Cdc5 and Dbf2-Mob1 phosphorylation (Meitinger et al., 2011). We repeated the *in vivo* time-lapse microscopy using Hof1-GFP Myo1-Tomatoe cells. Hof1-GFP was already localized at the bud neck in metaphase cells as expected (not shown). Hof1 contraction was also seen to be asymmetric to the bud neck in *cdc55*Δ mutant

RESULTS

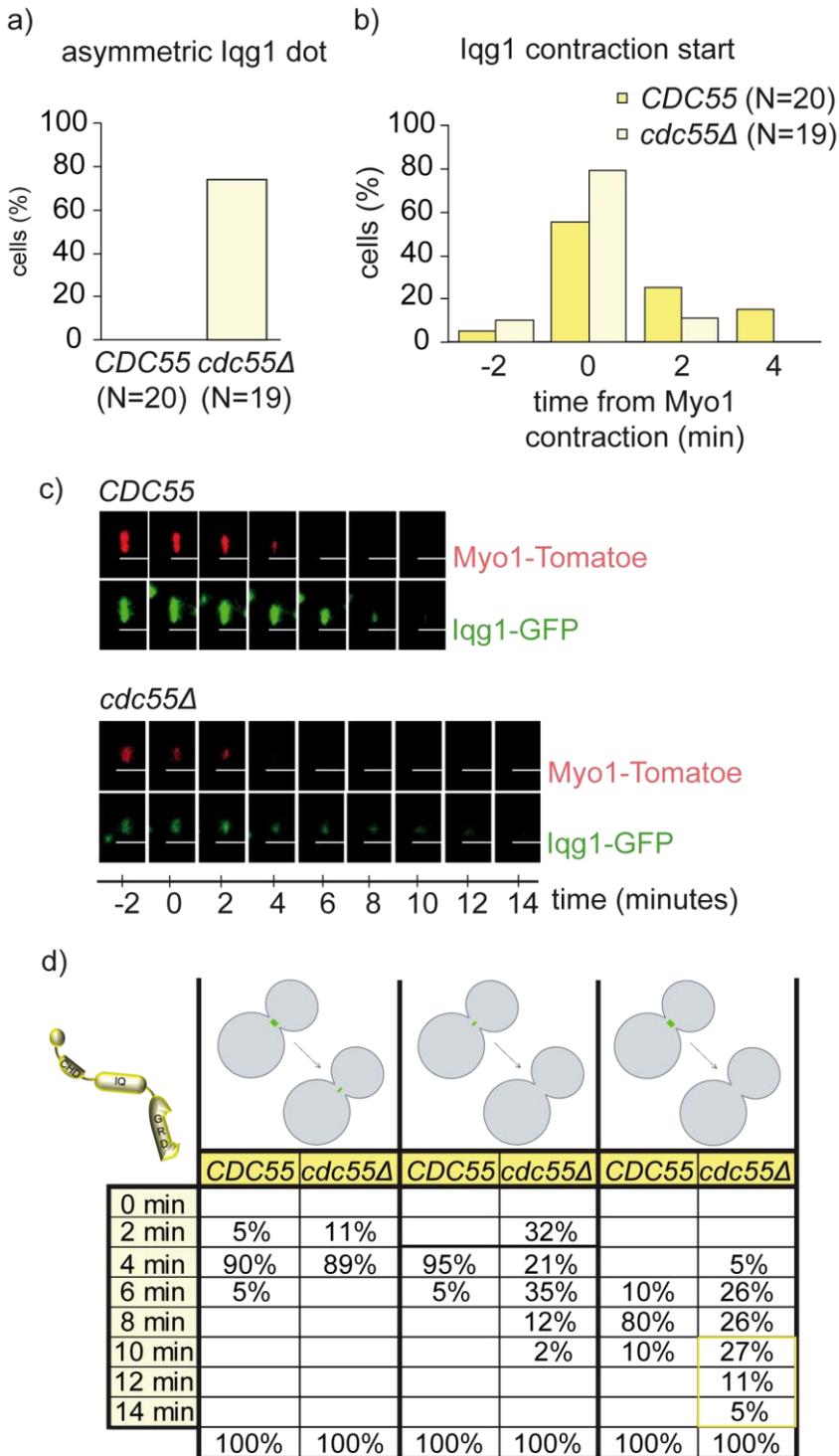


Figure R13. Iqg1 maintenance and contraction at the bud neck is altered in absence of Cdc55. Strains Y1572 (*MATa MET-HA₃-CDC20 MYO1-TOMATOE SPC42-GFP yE-IQG1- GFP*) and Y1606 (as Y1572, but *cdc55Δ*) were synchronized into anaphase by Cdc20 depletion and re-addition, and the time-lapse experiment was performed taking images every 2 minutes. Spc42-GFP and Myo1-Tomatoe were used as control of cytokinesis progression. **(A)** Quantification of Iqg1 asymmetric signal is shown. **(B)** Quantification of the Iqg1-GFP onset of contraction respect to the Myo1 signal contraction. **(C)** Representative images of Iqg1-GFP and Myo1-Tomatoe signals from *CDC55* (N=20) and *cdc55Δ* (N=19) cells. **(D)** Analysis of the residence time of Iqg1-GFP. The yellow square marks the cells with longer Iqg1 residence time. Images were taken with an Axio Observer.Z12 microscope (Carl Zeiss, Thornwood, NY). GFP and Cy3 filters were used. FiJi software was used to analyze the images. Scale bar of 2 μm.

cells (75% cells; Fig. R14a). In the main population of WT and *cdc55Δ* cells, Hof1-GFP starts to contract 0-2 minutes after Myo1-Tomatoe does (Fig. R14b and c). When we analyzed the dynamics of Hof1 localization, we observed that the Hof1-GFP signal from its constriction until the signal disappeared was longer in *cdc55Δ* than WT cells. Surprisingly, when look carefully we observed that the duration of the Hof1-GFP constriction to dot lasted 2 minutes in *cdc55Δ* cells (83% cells; N=12) while 4 minutes in WT cells (92%; N=24); indicating that the Hof1 contraction was actually faster in the *cdc55Δ* mutant cells (Fig. R14d, 1st column). Nevertheless, the Hof1 clearance from dot is longer in *cdc55Δ* mutants (8-10 minutes in 67% of the cells; Fig. R14d second column *cdc55Δ* marked in blue) than in WT cells (2-4 minutes in 80% of the cells; Fig. R14d second column *CDC55* marked in blue). We conclude that the impaired Hof1 phosphorylation in absence of Cdc55 increases the residence time of Hof1 signal at the bud neck; demonstrating again that cytokinesis is affected.

Later on, we investigated the Chs2 activators Cyk3 and Inn1 using

RESULTS

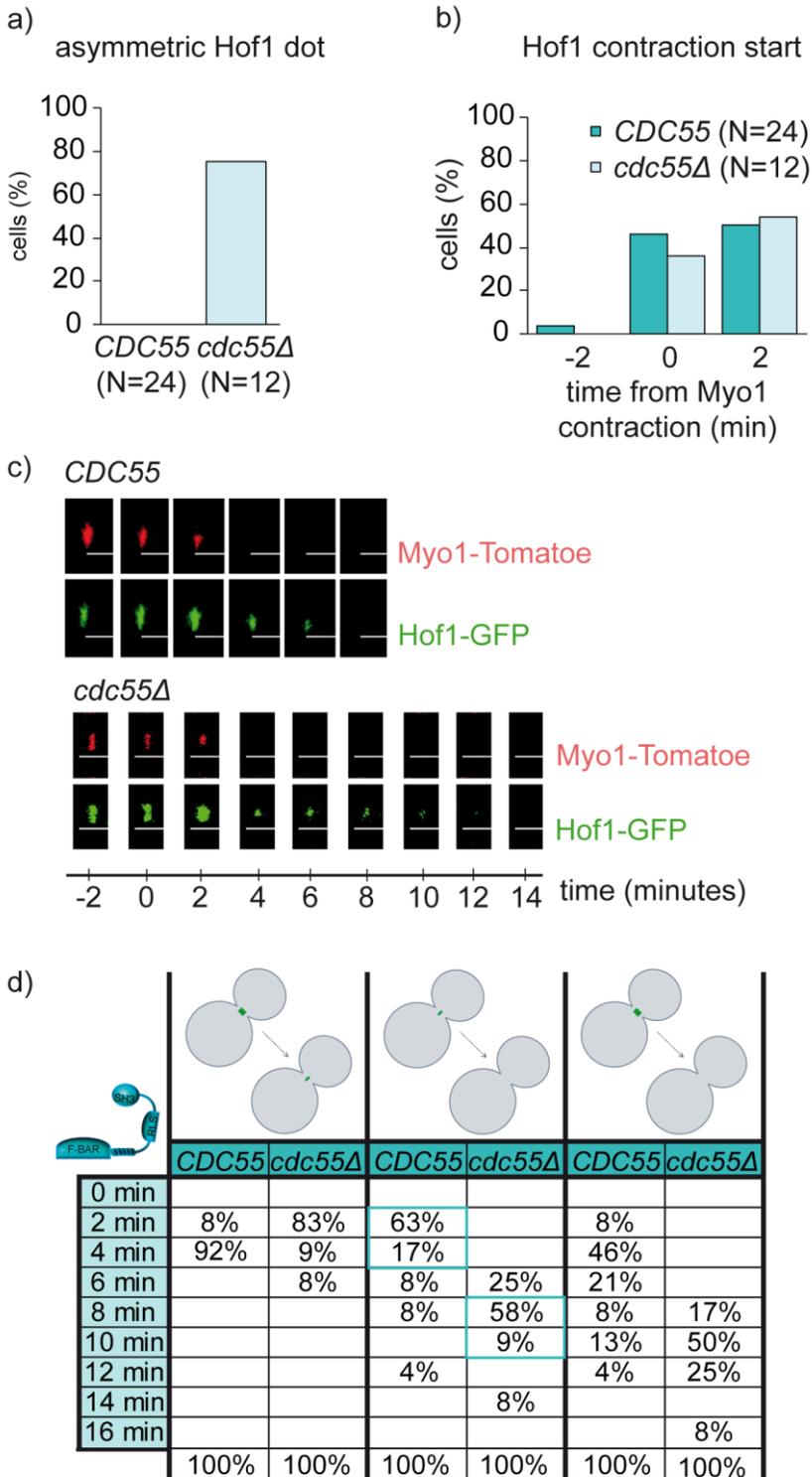


Figure R14. PP2A^{Cdc55} is required for proper Hof1 localization and contraction at the bud neck. Strains Y1306 (*MAT α MET-HA₃-CDC20 MYO1-TOMATOE SPC42-GFP HOF1-GFP*) and Y1578 (as Y1306, but *MAT α* and *cdc55* Δ) were synchronized into anaphase by Cdc20 depletion and re-addition, and the time-lapse experiment was performed taking images every 2 minutes. Spc42-GFP and Myo1-Tomatoe were used as control of cytokinesis progression. **(A)** Quantification of Hof1 asymmetric signal is shown. **(B)** Quantification of the Hof1-GFP onset of contraction respect to the Myo1 signal contraction. **(C)** Representative images of Hof1-GFP and Myo1-Tomatoe signals from *CDC55* (N=24) and *cdc55* Δ (N=12) cells. **(D)** Analysis of the residence time of Hof1. The blue squares mark the main population of contracting *CDC55* cells and cells with longer Hof1 residence time in *cdc55* Δ . Images were taken with an Axio Observer.Z12 microscope (Carl Zeiss, Thornwood, NY). GFP and Cy3 filters were used. FiJi software was used to analyze the images. Scale bar of 2 μ m.

the GFP tagged proteins under the same time lapse conditions. In accordance with the other IPC's proteins, Cyk3 and Inn1 were contracted asymmetrically to the bud neck in 86 and 84% of *cdc55* Δ cells, respectively (Fig. R15a and R16a). Cyk3-GFP signal appeared at the bud neck 0-2 minutes after Myo1-Tomatoe signal started to contract in 96% of WT cells (N=28) and 2 minutes after in 86% of *cdc55* Δ cells (N=7) (Fig. R15b and c). In addition, Cyk3 signal also stayed longer at the bud neck in absence of Cdc55. In WT cells, Cyk3 signal took 2 min to disappear once contracted in 82% of the cells (Fig. R15d, 2nd column). By contrast, in *cdc55* Δ cells Cyk3 signal expended 4-6 minutes from contraction start to disappearance in 72% of the cells (Fig. R15d, 2nd column marked in pink). These results indicate that Cyk3 localization dynamics is regulated by the PP2A^{Cdc55} dephosphorylation of IPC's substrates.

On the other hand, Inn1-GFP appeared at the bud neck when Myo1-Tomatoe started to be contracted in 85% (N=13) of WT cells

RESULTS

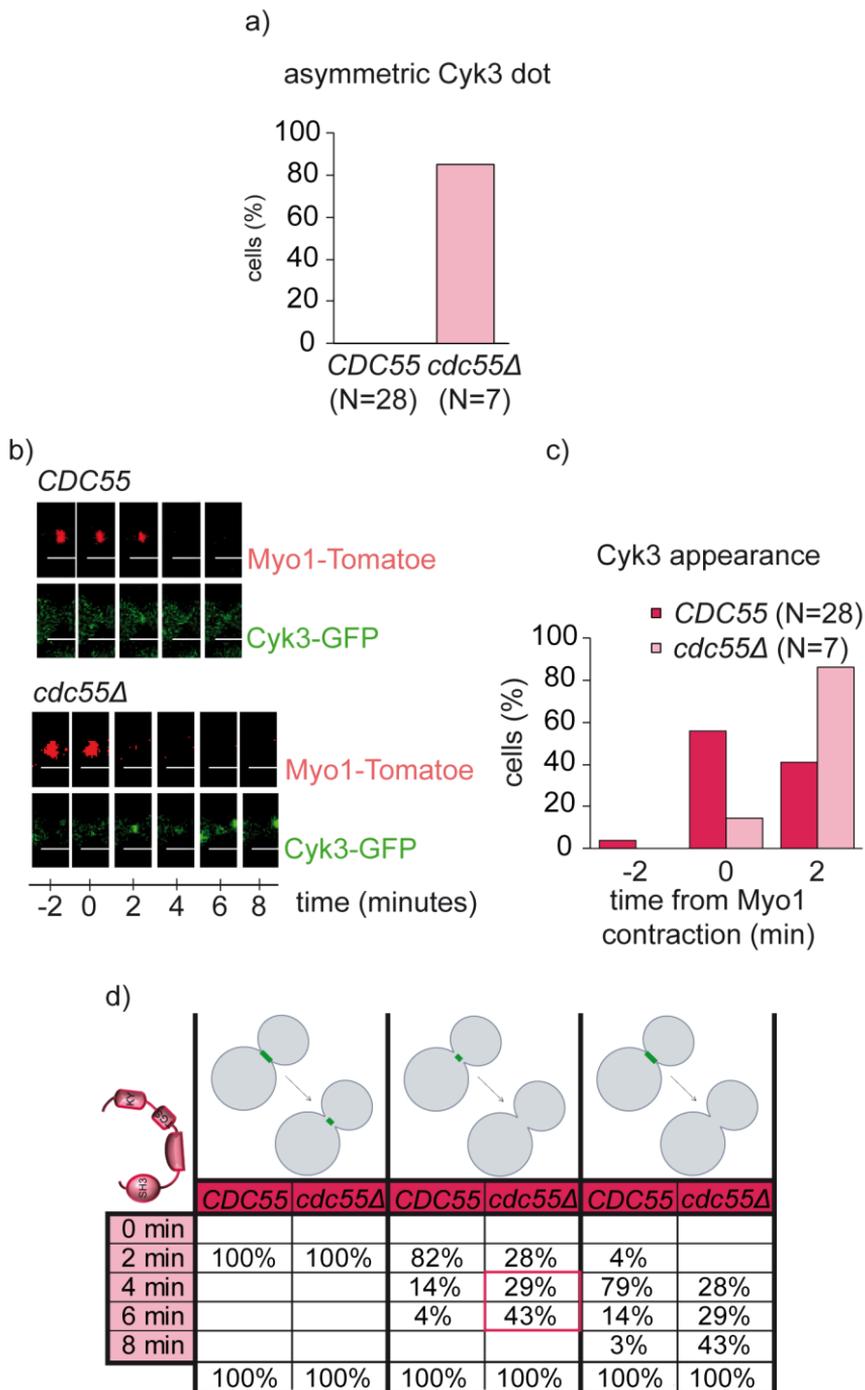


Figure R15. Cyk3 symmetric contraction and residence time at the bud neck depends on PP2A^{Cdc55}. Strains Y1574 (*MATa MET-HA₃-CDC20 MYO1-TOMATOE SPC42-GFP CYK3-GFP*) and Y1604 (as Y1574, but *cdc55Δ*) were synchronized into anaphase by Cdc20 depletion and re-addition, and the time-lapse experiment was performed taking images every 2 minutes. Spc42-GFP and Myo1-Tomatoe signals were used as control of cytokinesis progression. **(A)** Quantification of Cyk3 asymmetric signal is shown. **(B)** Representative images of Cyk3-GFP and Myo1-Tomatoe signals from *CDC55* (N=28) and *cdc55Δ* (N=7) cells. **(C)** Quantification of the Cyk3-GFP recruitment to the bud neck respect to the initiation of Myo1 signal contraction. **(D)** Analysis of the residence time of Cyk3. The red square marks the cells with longer Cyk3 residence time. Images were taken with an Axio Observer.Z12 microscope (Carl Zeiss, Thornwood, NY). GFP and Cy3 filters were used. Fiji software was used to analyze the images. Scale bar of 2 μm.

(Fig. R16b and c). By contrast, in *cdc55Δ* mutant cells, Inn1-GFP signal appeared 2 min earlier to Myo1-Tomatoe contraction in 24% (N=41) of the cells (Fig. R16b and c). However, no significant difference was observed in the Inn1-GFP residence time upon its contraction between WT and *cdc55Δ* cells (Fig. R16d). Therefore, in those *cdc55Δ* cells Inn1-GFP stayed longer at the bud neck as consequence of their early recruitment (Fig. R16d). We can conclude that PP2A^{Cdc55} regulates Inn1 recruitment at the bud neck.

Finally, we analyzed Chs2 localization at the bud neck. It was previously described that Chs2 transport to the bud neck depends on Cdc14 dephosphorylation (Chin et al., 2012). To analyze the possible influence of PP2A^{Cdc55} in Chs2 localization we used Chs2 tagged with GFP in control and *cdc55Δ* cells and visualized again their localization by time-lapse microscopy. Similar to the other IPC's components, in *cdc55Δ* mutant cells the Chs2-GFP signal showed a displacement to one side of the bud neck upon contraction in 81% of cells (Fig. R17a). Moreover, Chs2-GFP

RESULTS

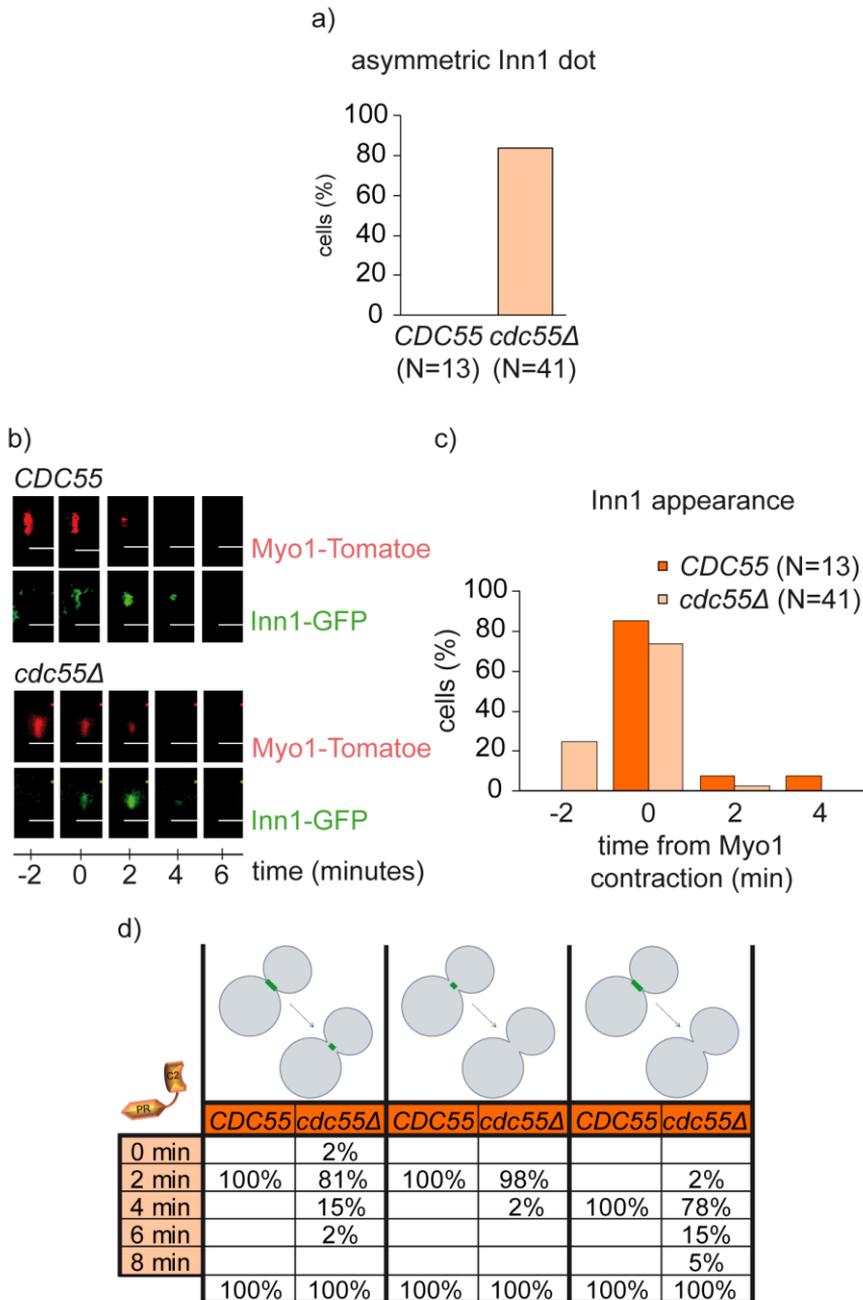


Figure R16. Inn1 timely recruitment and contraction depends on PP2A^{Cdc55} activity. Strains Y1454 (*MATa MET-HA₃-CDC20 MYO1-TOMATOE SPC42-GFP INN1-GFP*) and Y1608 (as Y1454, but *cdc55Δ*) were synchronized into anaphase by Cdc20 depletion and re-addition,

residence time was also longer in absence of Cdc55 (Fig. R17.d). Chs2-GFP was recruited at the bud neck when the AMR started to be contracted in both WT and *cdc55Δ* cells (Fig. R17b and c). In control cells, Chs2-GFP started to contract just after the Myo1-Tomatoe signal becomes a dot and Chs2 contraction took an average of 3 min in the whole population (N=20) (Fig. R17d, second column marked in purple). By contrast, in *cdc55Δ* cells, Chs2-GFP started to contract with a delay of 2 min on average in 31% of the cells (N=35) compared to control cells (Fig. R17d, first column marked in purple). As consequence of the delayed in Chs2-GFP onset of contraction, Chs2 signal stayed longer (2-4 more min) at the bud neck in 43% of *cdc55Δ* cells (Fig. R17d; third column marked in purple). Therefore, the decrease on dephosphorylation of Chs2 in absence of Cdc55 interferes with Chs2 contraction and its residence time at the bud neck. We can conclude that timely Chs2 dephosphorylation by PP2A^{Cdc55} is required for proper Chs2 localization dynamics.

All the time-lapses experiments of the IPC's components showed a common asymmetric AMR signal in absence of PP2A^{Cdc55} activity, indicating that the *cdc55Δ* mutant has a dysfunctional AMR. In

and the time-lapse experiment was performed taking images every 2 minutes. Spc42-GFP and Myo1-Tomatoe signals were used as control of cytokinesis completion. **(A)** Quantification of the Inn1 asymmetric signal is shown. **(B)** Representative images of Inn1-GFP and Myo1-Tomatoe signals from *CDC55* and *cdc55Δ* cells. **(C)** Quantification of the Inn1-GFP recruitment to the bud neck respect to the initiation of Myo1 signal contraction. **(D)** Analysis of the residence time of Inn1. The orange square marks the cells with longer Inn1 residence time. Images were taken with an Axio Observer.Z12 microscope (Carl Zeiss, Thornwood, NY). GFP and Cy3 filters were used. Fiji software was used to analyze the images. Scale bar of 2 μ m.

RESULTS

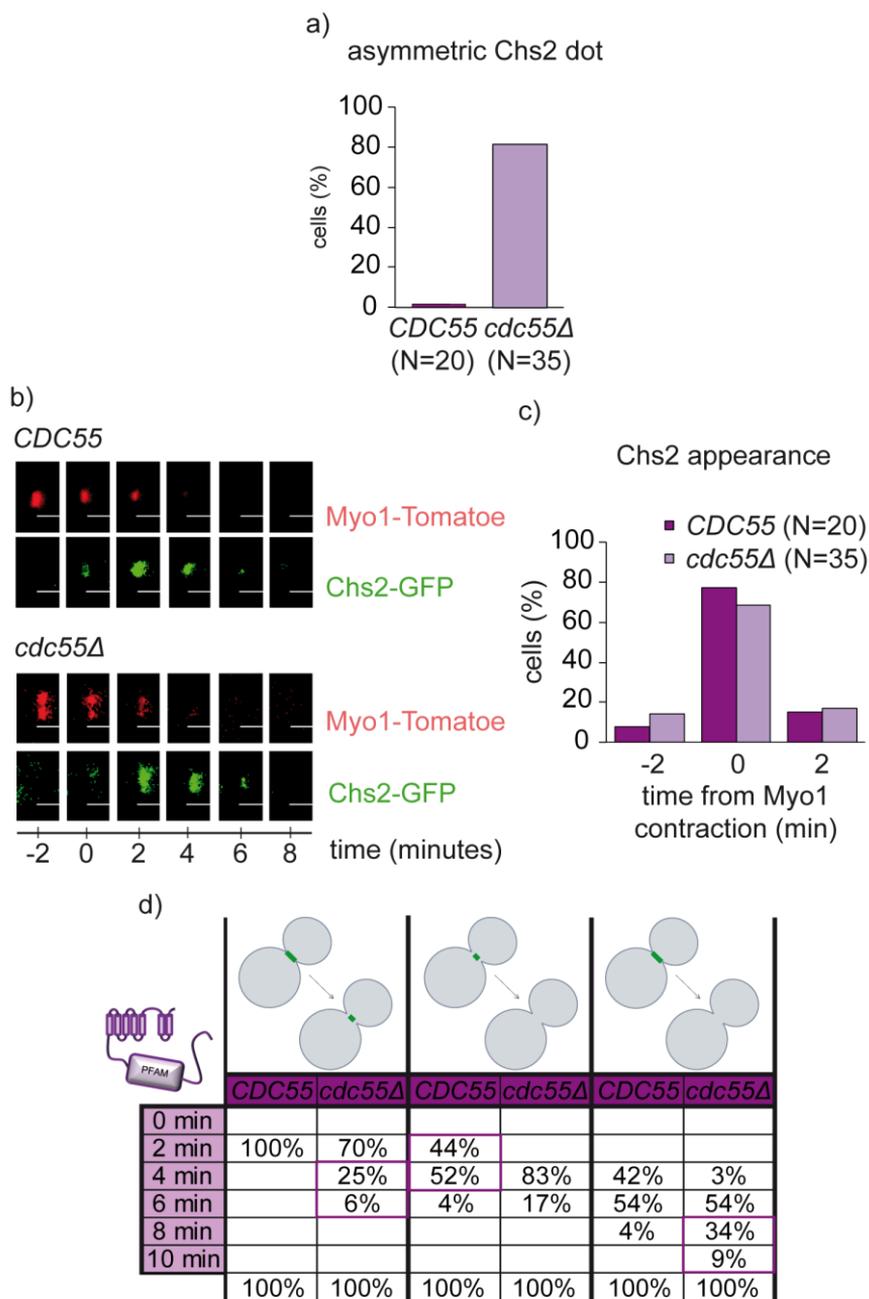


Figure R17. PP2A^{Cdc55} regulates Chs2 maintenance and contraction at the bud neck. Strains Y1576 (*MATa MET-HA₃-CDC20 MYO1-TOMATOE SPC42-GFP CHS2-GFP*) and Y1575 (as Y1576, but *MATα* and *cdc55Δ*) were synchronized into anaphase by Cdc20 depletion and re-addition, and the time-lapse experiment was performed taking images

addition, a longer residence time at the bud neck has been observed in the different IPC's components revealing a delay in the progression through cytokinesis in absence of Cdc55.

1.4. Impaired primary septum formation in absence of Cdc55

The above experiments showed that PP2A^{Cdc55} is required for proper AMR contraction and proper IPC's components localization dynamics at the bud neck. The asymmetric AMR contraction phenotype was also associated to some IPC's mutants such as *hof1Δ* and *inn1-td* (Lippincott & Li, 1998a; Sanchez-Diaz et al., 2008a). This prompted us to study the cytokinesis phenotypes of the *cdc55Δ* mutant cells.

PP2A^{Cdc55} was described to be involved in bud morphology regulating the Rho1 activation, responsible of actin polarization and cell wall synthesis (Jonasson et al., 2016). Actin polarization and recruitment to the bud neck is required for the AMR formation.

every 2 minutes. Spc42-GFP and Myo1-Tomatoe signals were used as control of cytokinesis progression. **(A)** Quantification of the Chs2 asymmetric signal is shown. **(B)** Representative images of Chs2-GFP and Myo1-Tomatoe signals from *CDC55* and *cdc55Δ* cells. **(C)** Quantification of the Chs2-GFP recruitment to the bud neck respect to the initiation of Myo1 signal contraction. **(D)** Analysis of the residence time of Chs2. The purple square marks the main population of contracting *CDC55* cells and cells with longer Chs2 residence time in *cdc55Δ*. Images were taken with an Axio Observer.Z12 microscope (Carl Zeiss, Thornwood, NY). GFP and Cy3 filters were used. FiJi software was used to analyze the images. Scale bar of 2 μm.

RESULTS

Therefore, we envisage the possibility that PP2A^{Cdc55} could affect actin cytoskeleton polarization, not only during the period of apical growth, but also at the end of mitosis. For this reason, we wondered whether actin polarization was defective in *cdc55Δ* cells. To test this possibility, actin filaments were stained with labelled phalloidin in cells progressing through mitosis and cytokinesis. Actin signal was detected depolarized in metaphase-arrested cells both in the wild-type and *cdc55Δ* strains, as expected (Fig. R18 metaphase). When cells completed mitosis, wild-type cells normally polarized the actin to the bud neck (Fig. R18 anaphase). In *cdc55Δ* mutants, actin was also timely polarized at the bud neck during cytokinesis (Fig. R18 cytokinesis). In the next S phase, actin polarization in the new bud was clearly observed in WT and *cdc55Δ* cells (Fig. R18 next S phase). However, in *cdc55Δ* cells actin polarization at the new bud was already observed before cytokinesis was completed. We observed both actin signal, at the bud neck during cytokinesis and at the new bud site, simultaneously, in 12.5% of *cdc55Δ* mutant cells (Fig. R18 actin premature polarization). This result suggests that cytokinesis is delayed in absence of Cdc55 and actin polarization to the new-born cells started before cytokinesis is completed. In wild-type cells this premature actin repolarization in the new bud site was not observed. We conclude that actin polarization during cytokinesis is not affected in absence of Cdc55; nevertheless, PP2A^{Cdc55} regulates that completion of cytokinesis is accomplished before actin is polarized during budding in the next cell cycle.

As far as actin staining can indicate, actin was correctly attached to the myosin II ring. However, other cytokinetic processes could be affected in absence of Cdc55. We wondered whether septins

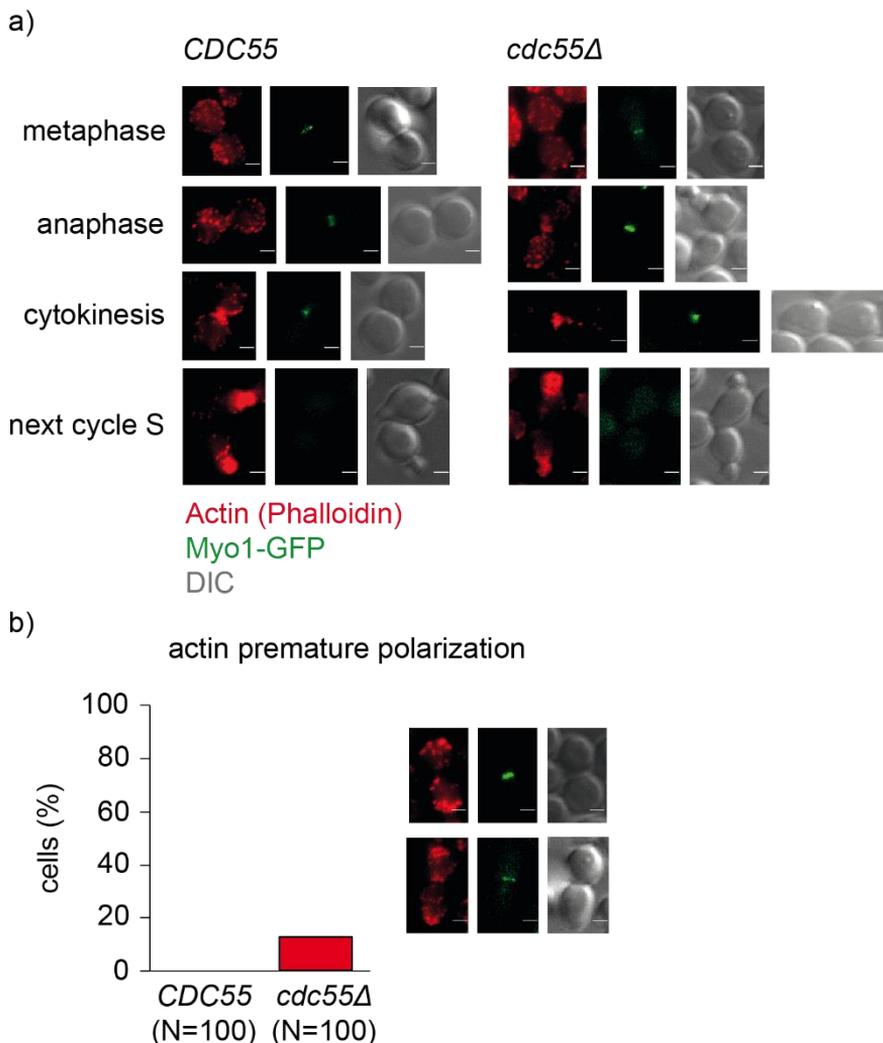


Figure R18. Actin is polarized to the new bud site before cytokinesis completion in *cdc55Δ* cells. Strains Y1434 (*CDC55*) and Y1435 (*cdc55Δ*) were arrested in metaphase by Cdc20 depletion and released into anaphase by Cdc20 re-induction. Formaldehyde-fixed cells were stained with 50 U/mL of rhodamine phalloidin (R415; Life Technologies). Representative images of the actin staining and Myo1-GFP signals from *CDC55* and *cdc55Δ* at different cell cycle stages (metaphase, anaphase, cytokinesis and next cycle S) are shown in **(A)**. Quantification and representative images of the cells with re-polarized actin to the new bud site are shown in **(B)**. Images were taken with an Axio Observer.Z12 microscope (Carl Zeiss, Thornwood, NY). GFP and Cy3 filters were used. Fiji software was used to analyze the images. Scale bar of 2 μ m.

RESULTS

structures were defective in absence of Cdc55. Septins serve as scaffold for AMR proteins as Hof1, linking septin and actomyosin ring (Lippincott & Li, 1998a). Moreover, septin function has been

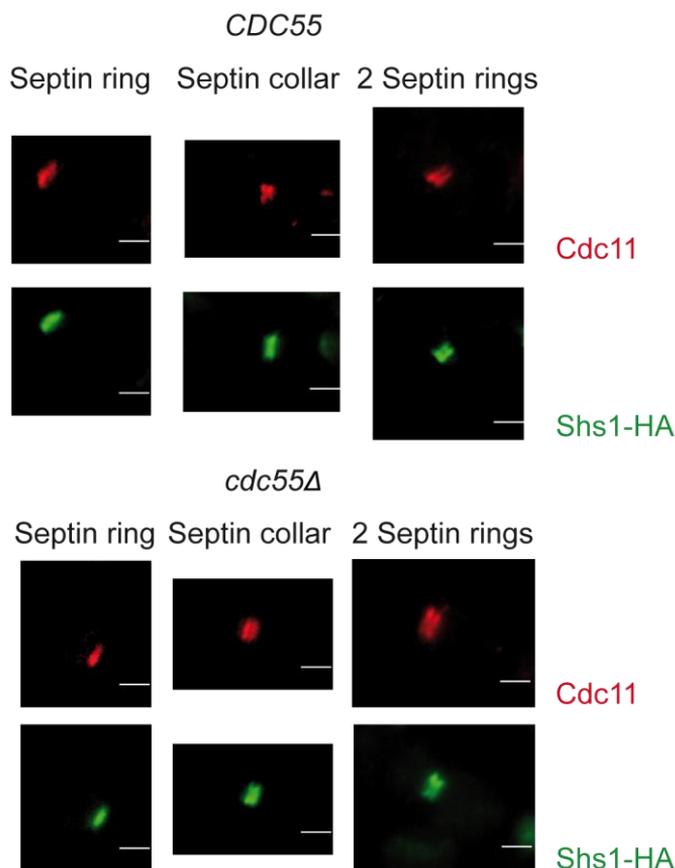


Figure R19. Septin structures formation are independent of PP2A^{Cdc55}. Cycling cells from strains Y1588 (*MATa MET-HA₃-CDC20 SHS1-HA₆*) and Y1589 (as Y1588, but *cdc55Δ*) were fixed and immunofluorescence *in situ* performed for septins Cdc11 and Shs1-HA visualization. α -Cdc11 and α -HA clone 12CA5 antibodies were used. Representative images of Cdc11 and Shs1-HA septin structures from *CDC55* and *cdc55Δ* cells are shown. Images were taken with an Axio Observer.Z12 microscope (Carl Zeiss, Thornwood, NY). GFP and Cy3 filters were used. FiJi software was used to analyze the images. Scale bar of 2 μ m.

described to be involved in correct AMR constriction (Tamborrini et al., 2018). Septins were visualized by immunofluorescence *in situ* staining of Cdc11 and Shs1-HA in asynchronous cells. We observed the different septin structures: single ring, collar and double rings in control and *cdc55* Δ cells (Fig. R19). This finding suggests that septin dynamics are not greatly affected in absence of Cdc55.

Cytokinesis in budding yeast is accomplished by the concerted action of the actomyosin contractile ring (AMR) and the formation of the septum by polarized secretion to the bud neck. Since AMR contraction is defective in absence of Cdc55 we wonder whether septum formation was also impaired. Primary septum formation depends on Chs2, which is responsible of chitin engagement to the bud neck (Sburlati & Cabib, 1986; Schmidt et al., 2002). The chitin deposition in the neck can be visualized by the chitin staining with Calcofluor White, a molecule that binds to chitin while it is being incorporated to the cell wall. We arrested cells at metaphase by Cdc20 depletion, released them into mitosis and observed the fluorescence intensity of the incorporated calcofluor on living cells containing Myo1-Tomatoe as control of cytokinesis progression. We took images between 30-45 min after the release into anaphase when we found cells at cytokinesis (Myo1 contracted). Calcofluor intensity was then measured and quantified in wild-type and *cdc55* Δ cells. A reduction of 45% in the intensity of the calcofluor staining was observed in absence of Cdc55 compared to control cells (Fig. R20a). Chitin is incorporated in both primary (PS) and secondary septa (SS). Therefore, we cannot distinguish whether the reduction occurs at the primary or the secondary septa. For this reason, we repeated the calcofluor staining in cells

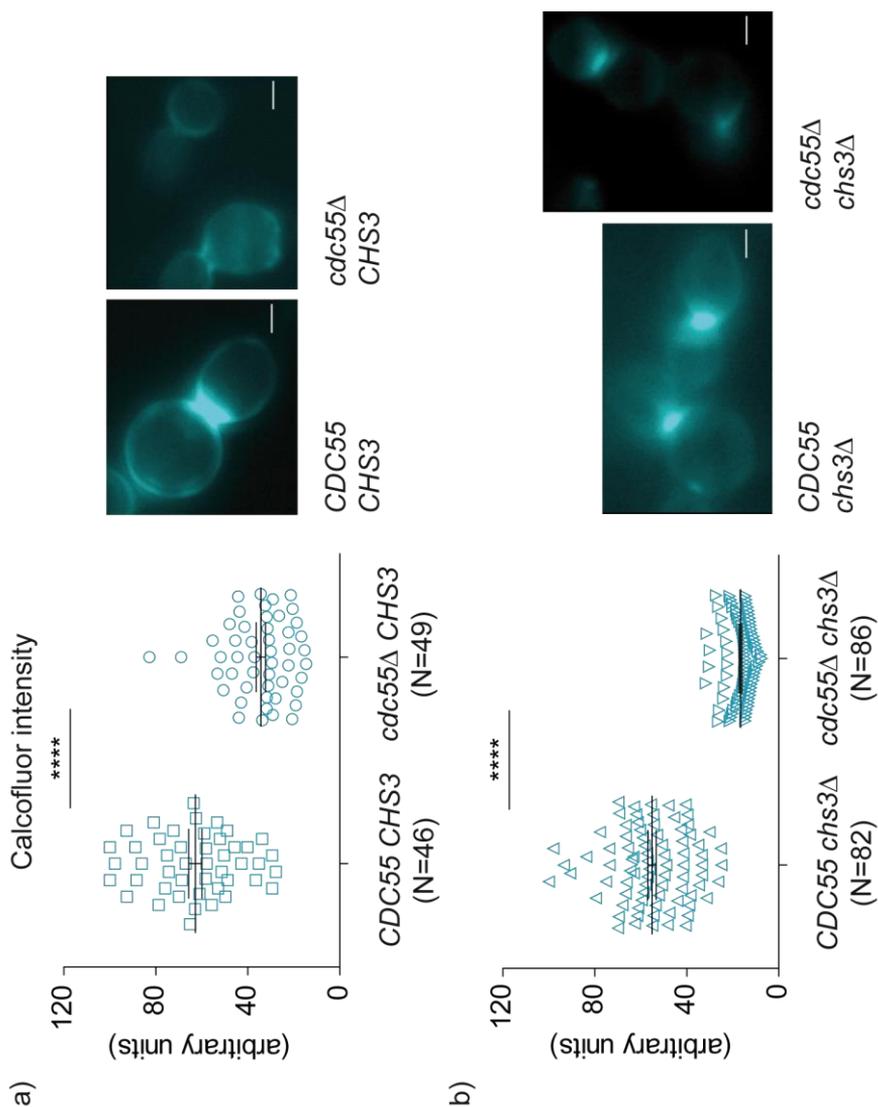


Figure R20. Chitin deposition at primary and secondary septa is regulated by PP2A^{Cdc55}. Strains Y1516 (*MATa MET-HA₃-CDC20 CHS2-GFP SPC42-GFP*), Y1512 (*MATa MET-HA₃-CDC20 MYO1-TOMATOE SPC42-GFP cdc55Δ*), Y1605 (*MATα MET-HA₃-CDC20 MYO1-TOMATOE chs3Δ*) and Y1596 (as Y1605, but *cdc55Δ*) were arrested in metaphase by Cdc20 depletion, released into anaphase by Cdc20 re-induction and 50 μg of calcofluor (Fluorescent brightener 28, Sigma) was added to visualize **(A)** both septa (representative images at right panel) and **(B)** primary septum (representative images at right panel). Images were taken with an Axio Observer.Z12 microscope (Carl Zeiss, Thornwood, NY) using the DAPI filter. Fiji software was used to analyze the images. Scale bar of 2

containing the deletion of *CHS3*, the chitin synthase responsible of the secondary septum formation (Shaw et al., 1991). This time, a reduction of 70% in the calcofluor intensity was observed in absence of *Cdc55* compared to control cells (Fig. R20b). Remarkably, chitin deposition was observed asymmetrically to one side of the bud neck in absence of *Cdc55*, indicating that not only AMR is asymmetric but also the septum signal. Altogether, these experiments indicate that both primary and secondary septa formation are reduced in cells lacking $PP2A^{Cdc55}$ activity. IPC's regulate the coordination between AMR contraction and septa formation (Foltman et al., 2016; Meitinger et al., 2011; Nishihama et al., 2009; Sanchez-Diaz et al., 2008; Wang et al., 2018); therefore, regulation of the IPC's components by $PP2A^{Cdc55}$ dephosphorylation is also required for both processes, indicating that $PP2A^{Cdc55}$ has a role coordinating AMR contraction with septa formation.

The decrease of calcofluor signal at the bud neck, the asymmetric actomyosin ring and the increase in the residence times of IPC's subunits prompted us to postulate that abscission (resolution of the plasma membrane) could be delayed in *cdc55Δ* cells. The membrane continuity was determined using a 3GFP-Ras2 marker

μm . The relative signal of chitin staining was measured for **(A)** both septa (*CDC55* N=46, *cdc55Δ* N=49; left pannel) and **(B)** just primary septum (*chs3Δ* N=82, *chs3Δ cdc55Δ* N=86; left pannel). Statistical analysis of the T-student with a Pvalue of 0.01 of significance has been performed using Prism5 program.

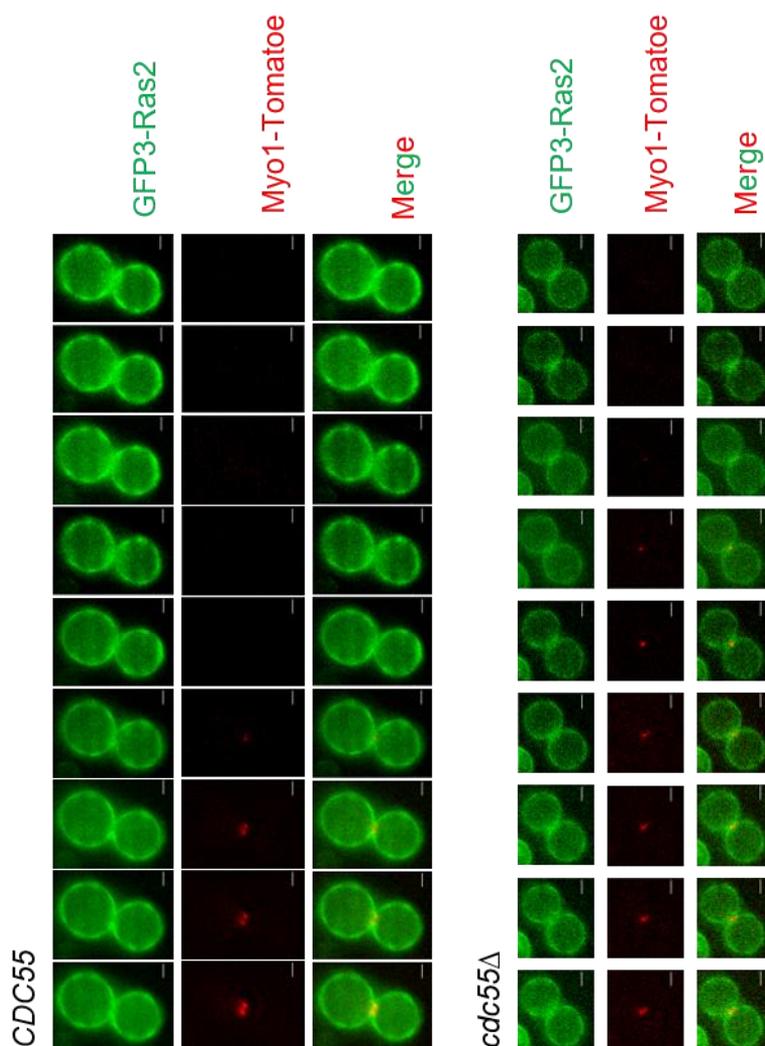


Figure R21. Proper cytoplasm separation during membrane abscission in absence of Cdc55. Y1717 (*MATa MET-HA₃-CDC20 MYO1-TOMATOE₃GFP-RAS2*) and Y1708 (as Y1717, but *cdc55Δ*) were arrested in metaphase by Cdc20 depletion, released into anaphase by Cdc20 re-induction and the time-lapse were performed taking images every 2 minutes. Membrane abscission was followed by *GFP-Ras2* signal and Myo1-Tomatoe was used as control of cytokinesis progression. Representative images from *CDC55* and *cdc55Δ* cells are shown. Images were taken with an Axio Observer.Z12 microscope (Carl Zeiss, Thornwood, NY). GFP and Cy3 filters were used. FiJi software was used to analyze the images. Scale bar of 2 μ m.

(Foltman & Sanchez-Diaz, 2017) to follow abscission. Ras2 is a membrane associated GTPase protein that is permanently bound to the plasma membrane, allowing the observation of membrane behavior throughout the cell cycle. We synchronized cells containing Myo1-Tomatoe and 3GFP-Ras2 at the metaphase to anaphase transition and followed cytokinesis based on Myo1-Tomatoe contraction. The visualization of the cell membranes revealed no cytoplasm connection between mother and daughter cells by the end of cytokinesis, confirming that cytoplasmic division had been completed properly in control and *cdc55Δ* cells (Fig. R21). In conclusion, *cdc55Δ* cells manifest a defect in AMR contraction and in septation, although finally the cells manage to complete cell division during abscission.

To finally confirm the defects in septa formation we investigated the cytokinetic structure by transmission electron microscopy (TEM). We synchronized cells at the metaphase to anaphase transition by Cdc20 depletion and took images from 40-50 minutes after metaphase release. In wild-type cells, we observed that the PS was formed at both sides of membrane invagination (Fig. R22a), as expected. Once finished the PS formation, the two SS were formed at both sides of the PS (Fig. R22b). Finally, the primary septum was degraded, and cells were physically separated (Fig. R22c). On the contrary, in *cdc55Δ* cells the PS was formed just in one side of the bud neck (Fig. R22d). In fact, some cells did not even present the single incipient PS. Instead of, they showed aberrant, thicker structures with diverse morphologies called remedial septa (Fig. R22e and f). The remedial septum was first described in IPC's mutant cells, i.e. *myo1Δ* and *chs2Δ* (Schmidt et al., 2002) and is a

RESULTS

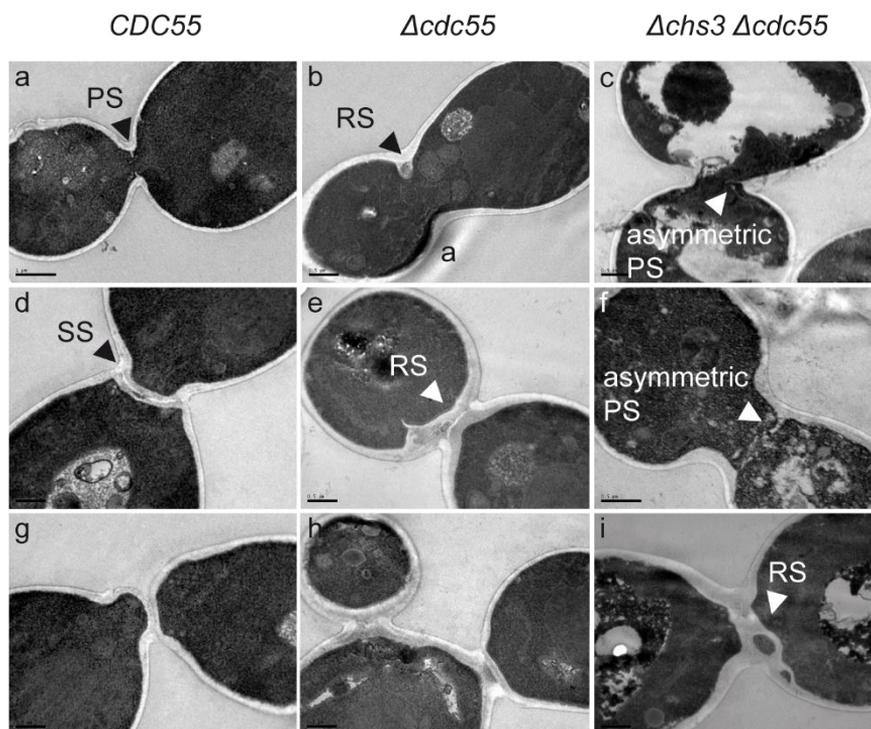


Figure R22. Asymmetric primary septum formation and appearance of the remedial septum in absence of Cdc55. Strains Y1315 (WT), Y1318 (*cdc55* Δ) and Y1605 (*cdc55* Δ *chs3* Δ) were arrested in metaphase and released into anaphase by Cdc20 depletion and re-addition. Cells were fixed at cytokinesis and sections were observed in a Jeol EM J1010 (Jeol, Japan) transmission electron microscope and images (a-i) were acquired at 80 kV with a 1k x 1k CCD Megaview camera. Representative images from TEM are shown. Scale bar of 0.5 μ m.

chitin structure reminiscent to SS that allows cells to finish cytokinesis upon defective PS (Schmidt et al., 2002; Schmidt, Varma, Drgon, Bowers, & Cabib, 2003). Strikingly, in *cdc55* Δ cells, the mother and daughter cells managed to finally separate physically upon formation of the remedial septum (Fig. R22e and f). To further confirm that *cdc55* Δ cells can synthesize a remedial septum as a rescue mechanism to complete cell division, we repeated the experiments in a *chs3* Δ background where no

secondary septa are formed. Similar results to control cells with Chs3 were obtained, the *chs3* Δ cells had asymmetric PS formation and remedial septum was formed (Fig. R22g- i). This result confirms that remedial septum formation allows the cytoplasm separation and cell division in absence of Cdc55. The asymmetry seen in PS formation in *cdc55* Δ by TEM and the asymmetric AMR contraction further confirm that PP2A^{Cdc55} regulates correct coordination of the AMR contraction and PS formation.

1.5. PP2A^{Cdc55} regulates cytokinesis in a parallel pathway to Hof1 and Cyk3

The results obtained indicate that the mutant *cdc55* Δ cells have cytokinesis defects in AMR contraction and PS formation. This phenotype was first described for mutants in the IPC's components. It has been previously reported that some mutants in the IPC's subunits are synthetic lethal (SL). For instance, *hof1* Δ and *cyk3* Δ mutants are SL (Korinek et al., 2000). The genetic interaction studies can give us a hint whether PP2A^{Cdc55} acts in parallel or in the same pathway than the IPC's proteins. For this reason, we investigated the genetic interaction among *cdc55* Δ and IPC's mutants. First, we tried to prepare double deletion mutants with *cdc55* Δ and the non-essential IPC's genes, *hof1* Δ and *cyk3* Δ . However, the diploids cells did not sporulate and we could not obtain tetrads. For this reason, as a second approach we prepared double mutants with *cdc55* Δ and degron conditional mutants for the IPC's subunits (Foltman et al., 2016). For Hof1 we used the auxin

RESULTS

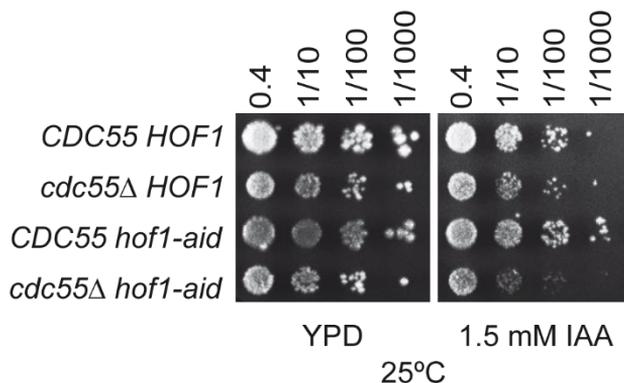


Figure R23. *cdc55Δ* and *hof1-aid* mutants are synthetic sick. Serial dilutions of WT (W303), Y844 (*MAT α cdc55Δ*), Y1730 (*MAT α GAL1-UBR1 ADH1-AtTIR1 td-cyk3-aid*) and Y1747 (*MAT α GAL1-UBR1 ADH1-AtTIR1 td-cyk3-aid cdc55Δ*) were spotted in YPD plates with and without 1 mM and 1.5 mM auxin (IAA). Cells were grown at 25°C for 2-3 days.

degron *hof1-aid* and for Cyk3 we used the double thermosensitive and auxin degron *td-cyk3-aid*. In control plates (YPD and YPGAL/SUC at 25°C) we didn't observe any difference in cell growth among the different mutant strains compared to control cells (Fig. R23 and R24); except for the *cdc55Δ* single mutant that grows slightly slower in presence of galactose as previously reported (our observation and Rudner paper) (Fig. R24). However, the viability of the double mutants *cdc55Δ hof1-aid* and *cdc55Δ td-cyk3-aid* were impaired at restrictive conditions (presence of auxin for *hof1-aid* and auxin plus high temperature and presence of galactose for *td-cyk3-aid*) compared to the single mutants (Fig. R23 and R24). The synthetic sick interactions found among *cdc55Δ* and *hof1-aid* or *td-cyk3-aid* degron mutants indicate that Cdc55 is functionally related to the IPC's subunits Hof1 and Cyk3; and suggest that Cdc55 acts in a parallel pathway to Hof1 and Cyk3.

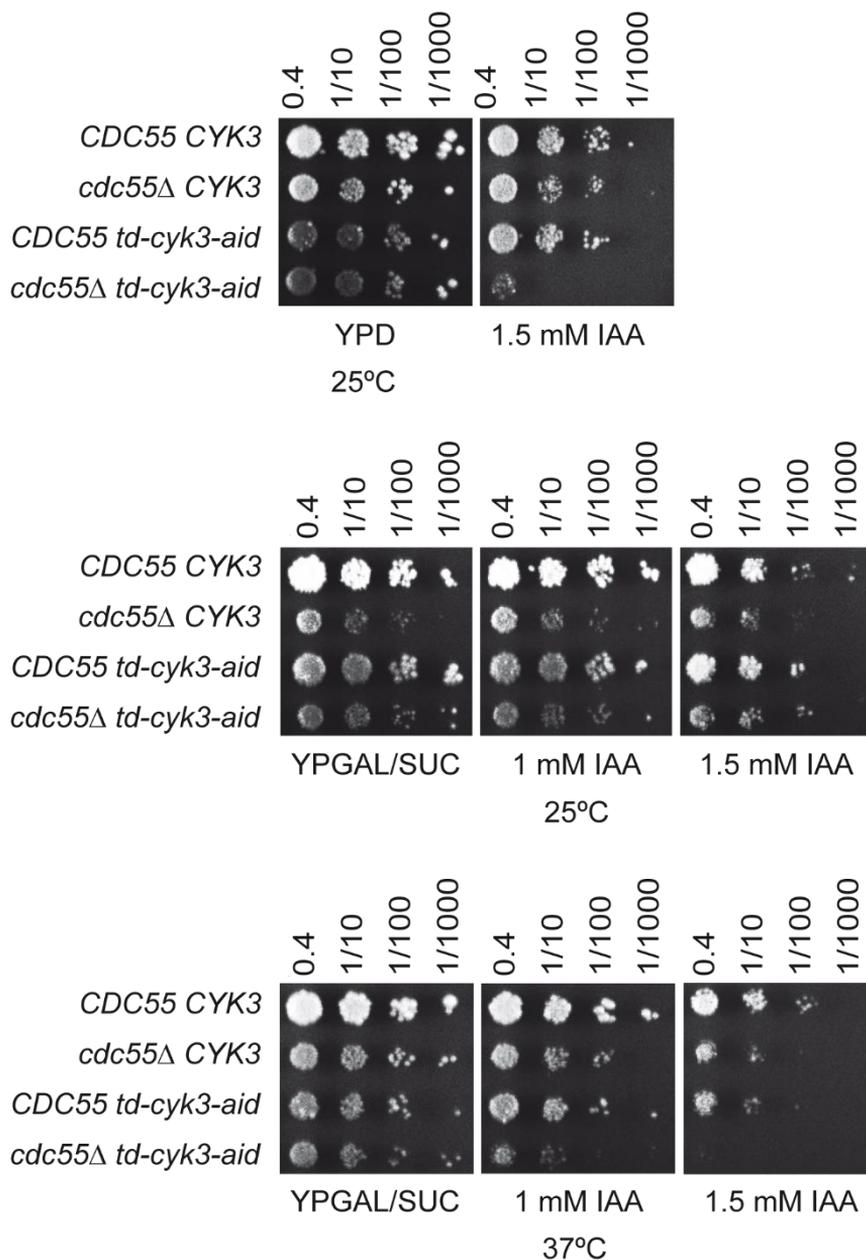


Figure R24. *cdc55Δ* and *td-cyk3-aid* mutants are sick. Serial dilutions of WT (W303), *cdc55Δ* (Y844), *GAL1-UBR1 ADH1-AtTIR1 td-cyk3-aid* (Y1730) and *GAL1-UBR1 ADH1-AtTIR1 td-cyk3-aid cdc55Δ* (Y1747 and Y1748) were spotted in YPD and YPGAL/SUC plates with and without 1 mM and 1.5 mM auxin (IAA). Cells were grown at 25°C and 37°C for 2-3 days.

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In the case of the *chs2-aid* mutant, we observed, that in control plates (YPD) all strains grew normally (Fig. R25). In contrast, when cells were grown at restrictive conditions, in presence of auxin to inhibit the AID degron, different viabilities were obtained. The single mutant *CDC55 chs2-aid* strain viability was impaired compared to control cells, as expected (Foltman et al., 2016) (Fig. R25). Strikingly, the double mutant, *cdc55Δ chs2-aid* rescued the cell unviability of the *chs2-aid* single mutant. This result indicates that $PP2A^{Cdc55}$ has an opposing role to Chs2. $PP2A^{Cdc55}$ could be involved in an additional mechanism to inhibit septa formation. Alternatively, the *cdc55Δ* deletion mutant might have evolved a system to develop a remedial septum and the appearance of the remedial septum in the *cdc55Δ chs2-aid* double mutant allowed its viability.

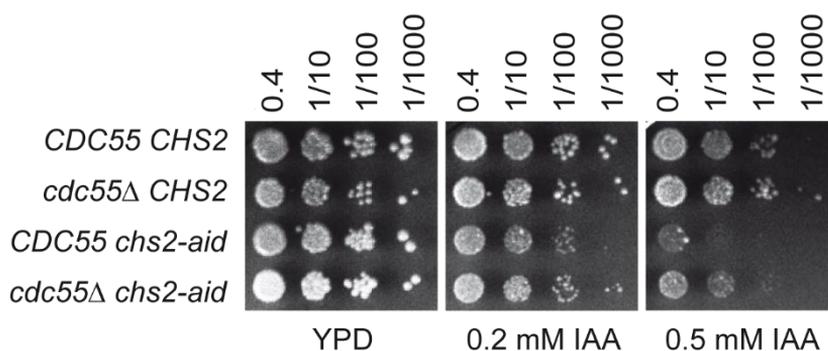


Figure R25. *cdc55Δ* rescues *chs2-aid* lethality. Serial dilutions of WT (W303), Y844 (*MAT α cdc55Δ*), Y1614 (*MAT α ADH1-OsTIR1 chs2-aid*) and Y1628 (*MAT α ADH1-OsTIR1 chs2-aid cdc55Δ*) were spotted in YPD plates with and without 0.2 mM and 0.5 mM auxin (IAA). Cells were grown at 25°C for 3 days.

2. To investigate the Cdc5 regulation on Net1 phosphorylation.

2.1. Study of the Net1 residues phosphorylated by Cdc5.

Cdc5 best-studied role in mitotic exit is the phosphorylation of Bfa1, the inhibitor of the MEN pathway, promoting the activation of MEN and, indirectly, contributing to the Cdc14 activation (Botchkarev et al., 2017; F. Hu et al., 2001). But, it was also suggested to be regulating Cdc14 release from the nucleolus as part of the FEAR pathway, indirectly through Clb2-Cdc28 activation (F. Liang et al., 2009; Raspelli et al., 2018) or directly by phosphorylating Net1 (the Cdc14's inhibitor) (J.-A. J.-A. Rodriguez-Rodriguez et al., 2016; Wenying Shou, Azzam, Chen, Huddleston, et al., 2002a). However, Net1 *in vivo* residues phosphorylated by Cdc5 has not been identified yet, preventing the demonstration of Cdc5 direct regulation of the Cdc14 activation. For this reason, our goal was the identification and characterization of the Net1 residues that are phosphorylated by Cdc5 *in vivo*.

Previously in the laboratory a mass spectrometry analysis of Net1 phosphorylations was performed in cells arrested in metaphase by Cdc20 depletion and in cells arrested in metaphase upon ectopic expression of *CDC5* (induction of *GAL1-CDC5*) in order to determine the Cdc5-dependent phosphorylated Net1's residues. We obtained a series of residues (Table R1) of which 16 (S23, S69, S166, S171, T248, S259, T297, T304, S332, T341, S433, S437, S511, T534, S573, T584) were just phosphorylated upon

Metaphase arrested cells		
Endogenous Cdc5	Ectopic Cdc5 (1)	Ectopic Cdc5 (2)
S31, S43, S48, S56, S60, T62, S64, S101, S115, S148, S228, S231, S252, S269, S270, S278, T302, S385, S388, S417, S418, S439, S440, S447, S452, S497, T500, S506, S611, S613, S614, S615	S43, S56, S60, T62, S69, S228, S231, T248, S252, S269, S270, S278, T297, T304, S332, T341, S385, S433, S437, S439, S440, S447, S452, S497, T500, S611, S613, S614, S615	Y23, S64, S166, S171, S228, S231, S252, S259, S385, S439, S447, S497, S506, S511, T534, S573, T584, S611, S613, S614

Table R1. Detected phosphorylated residues from Net1(1-600) by mass spectrometry analysis. Y968 (MET-HA₃-CDC20 GAL1-CDC5-MYC₉ net1(1-600)-PrA Cdc14-PK₉) was arrested in metaphase by Cdc20 depletion. GAL1-CDC5 expression was induced for 3 hours during the arrest. Half of the culture without galactose addition was used as control in metaphase arrested cells. net1(1-600)-PrA was immunoprecipitated, digested by trypsin, and peptides enriched with TiO₂.

ectopic expression of Cdc5 (Table R1, marked in green).

To further narrow the Net1 residues phosphorylated by Cdc5, we performed a Net1 peptide array. The array is composed by Net1 tiling peptides of 8 amino acids alternated with Net1 peptides containing serines mutated to alanine. The Cdc5 was immunopurified from yeast cells in anaphase and used for the kinase assays. We considered positives the residues with a 30% increase in signal intensity compared to the adjacent peptides that do not contain the residue (absence) and/or peptides with serine mutated to alanine. We identified 36 residues with increased phosphorylation in the peptide array (Fig. R26). Among these residues, 15 were detected also in the mass spectrometry analyses (Table R2).

Next, we grouped the Cdc5 residues containing the Paulson Cdc5 consensus sequence identified in both approaches, mass spectrometry analysis and/or the peptide array (Table R3). The residues summarized in Table R3 were considered the most probable to be phosphorylated by Cdc5 since they contain the kinase consensus sequence. We can observe that in the case of the residues detected by the peptide array, from the initial 36 residues (Table R2), just 9 (Table R3) contain the Cdc5 consensus sequence. This is consequence of the high non-specificity of the Cdc5 kinase when is used for *in vitro* assays.

After the analysis of the Net1 residues detected, we choose some to validate them *in vivo*. We focused in the Net1 residues containing the Cdc5 consensus sequence.

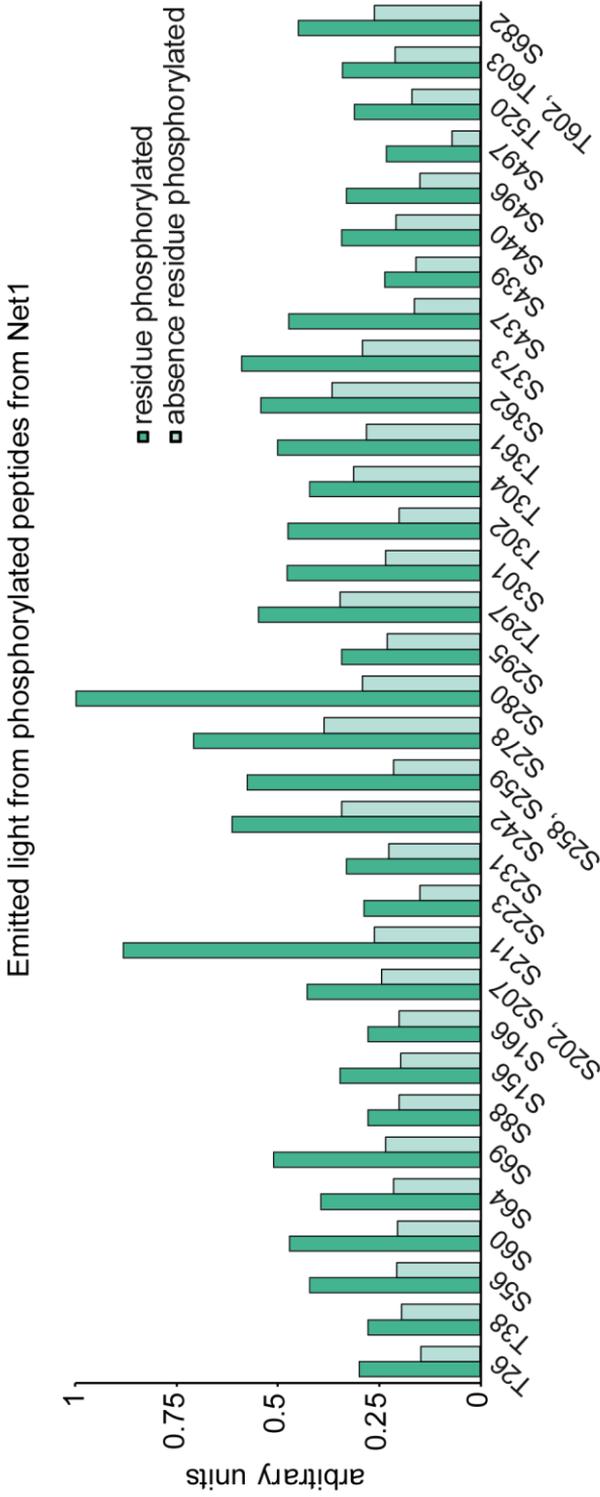


Figure R26. Luminescent intensity emitted by the phosphorylated residues in the Net1 peptide array. Strain Y2388 (*MATa MET-HA3-CDC20 CDC14-HA6 NET1-PK₃ GAL1-CDC5-PrA*) was arrested at metaphase by Cdc20 depletion and *GAL1-CDC5-PrA* expression was induced for 3 hours. Cdc5 was immunoprecipitated with IgG beads. Immunopurified-Cdc5 was used for the kinase assay in the Net1 peptide array. Emitted luminescence from the kinase assay was scanned using a Cy3 filter. Peptides containing the phosphorylated residue in dark green and peptides lacking the residue or with the residue mutated to alanine in light green.

Net1 residues detected by Peptide array	
Unique	Common Mass spectrometry
T26, T38, S88, S156, S202, S207, S211, S223, S242, S258, S280, S295, S301, T361, S362, S373, S496, T520, T602, T603, S682	S56, S60, S64, S69, S166, S231, S259, S278, T297, T302, T304, S437, S439, S440, S497

Table R2. Net1 phosphorylated residues detected in the Net1 peptide array. Net1 residues detected in the peptide array as unique detection or common to the mass spectrometry analysis are represented.

Previously, the *net1-6cdk* mutant containing 6 Net1 residues with Cdk consensus sequence mutated to alanine, was described to present impaired Cdc14 release from the nucleolus (Azzam et al., 2004). Interestingly, one of the *net1-6cdk* residues, S166, has overlapping Cdc5 and Cdk1 kinase consensus sequences (D/E/NXS/T and T/SP respectively), and was detected at the peptide array and at the mass spectrometry analysis (Table R3). Another residue S447, also contain a double consensus site for Cdc5 and Cdk1. Because of this, we consider them to be interesting, even ambiguous, candidates. We mutated to alanine some candidate Net1 residues (Table R4) and analyzed the Cdc14 release from the nucleolus. First, we prepared *net1 Δ* mutant strains with the *net1_5A* and *net1_14A* mutant proteins integrated at the *URA3* locus; and determined the mitosis progression and Cdc14 release from the nucleolus in cells synchronized in the metaphase to anaphase transition by Cdc20 depletion and re-introduction. In *net1_5A* mutant we observed that Cdc14 was released concomitantly to the anaphase progression, in a similar way to the WT cells (Fig. R27). By contrast, *net1_14A* mutant showed reduced Cdc14 release during anaphase (Fig. R27a). In order to determine whether the impaired Cdc14 release corresponds

Table R3

	D/E/N X S/T X Y/D	D/E/N X S/T F/M	D/E/N X S/T
Detected Mass spectrometry	S231, T534	S60	S31, S48, S69, S115, S148, S166, S447, S511, T584, S614
Detected Peptide array	S231	S60, S207, S242	T38, S69, S88, S166, T603
Do not detected			T288, S415, S416, S474, S561, T564, S579, T589

Table R4

Mutant strains	Detected by Mass spectrometry	Detected by Peptide array	Detected at both	Do not detected with consensus sequence
<i>net1_5A</i>	T248, S614		S60, S69, S231	
<i>net1_14A</i>	S115, S511, T534, T584	S242, T603	S166	T288, S415, S416, S561, T564, S579, T589
<i>net1_5A_Nterm</i>	S31, S48, S115, S148		S166	
<i>net1_4A_Cterm</i>	S447, S511, T534, T584			
<i>net1_5A_S447A</i>	T248, S614, S447		S60, S69, S231	
<i>net1_5A_T534A</i>	T248, S614, T534		S60, S69, S231	

Table R3. Identified Net1 residues containing the Cdc5 consensus sequence. Net1 residues from table R2 that contain the complete –D/E/N X S/T X Y/D or D/E/N X S/T F/M– or the simple –D/E/N X S/T– Cdc5 consensus sequence are represented. Common residues detected by mass spectrometry and the Net1 peptide array are marked in green.

Table R4. Representation of the Net1 residues mutated to alanine in the *net1* mutant strains.

to early or late anaphase, we analyzed the delay in Cdc14 release from the nucleolus with respect to the anaphase spindle length (Fig. R27b). Metaphase cells have mitotic spindles of 2 μm , early anaphase cells have spindle lengths from 2 to 5 μm , and late anaphase cells with spindles from 5 to 11 μm . We observed that WT and *net1_5A* showed around 70% of cells with Cdc14 released in early anaphase cells and almost complete release in cells in late anaphase. In contrast, *net_14A* cells showed no Cdc14 release in early anaphase cells and only 20% of cells, on average, released Cdc14 in late anaphase cells (Fig. R27b); indicating that there is not any FEAR-Cdc14 release and the MEN-Cdc14 release is greatly impaired. The results show that the phosphorylations of Net1 residues included in the *net1_5A* mutant are not required for the Cdc14 release. In contrast, residues included in the *net1_14A* mutant are required for the release of Cdc14 from the nucleolus.

To study whether the Net1 phosphorylation residues depend on Cdc5, we repeated the experiment upon the ectopic expression of Cdc5 in metaphase-arrested cells by Cdc20 depletion. Cells were arrested in metaphase by Cdc20 depletion and after the arrest the *CDC5* overexpression was induced by galactose addition. We

RESULTS

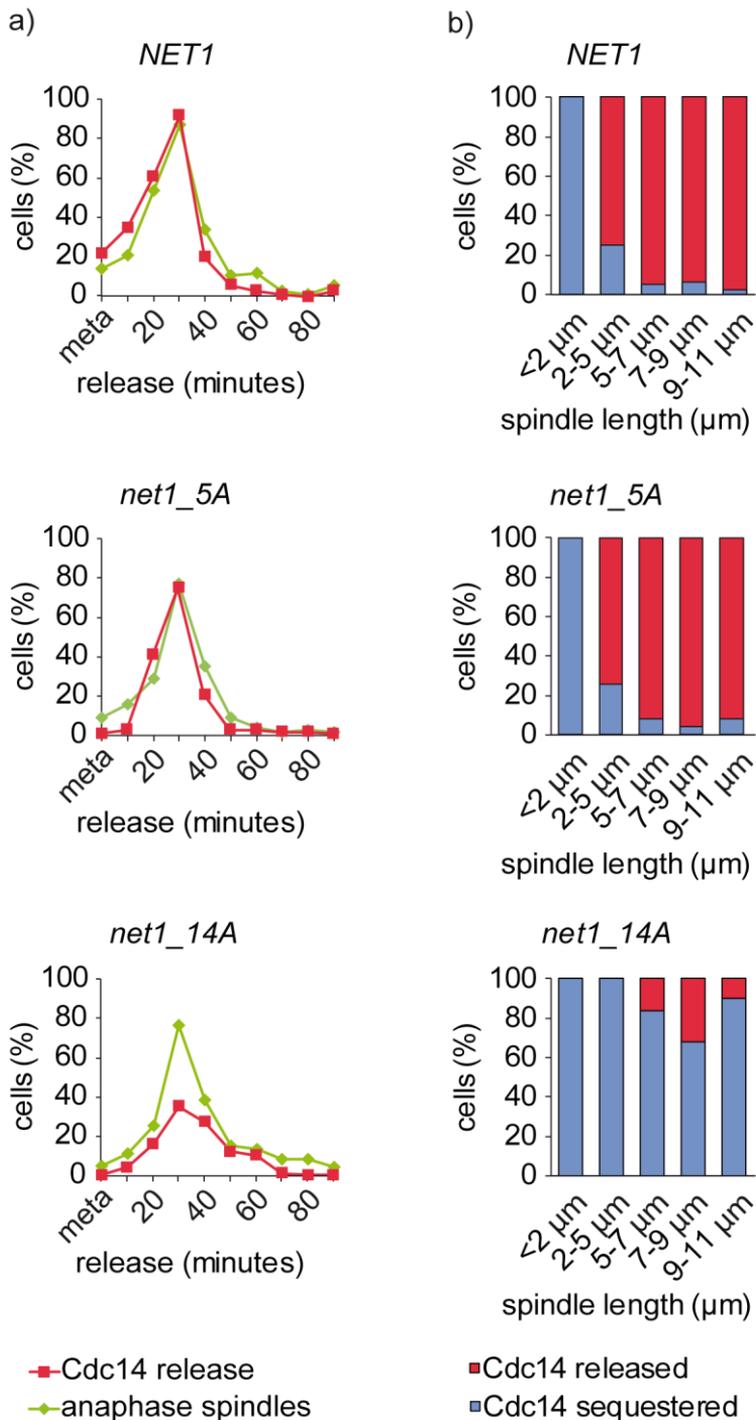


Figure R27. Defective Cdc14 release from the nucleolus in *net1_14A* cells. Strains Y1354 (*MAT α MET-HA₃-CDC20 net1 Δ ::MYC₉-Net1*), Y1395 (*MAT α MET-HA₃-CDC20 net1 Δ ::MYC₉-net1-5A*) and Y1356 (*MAT α MET-HA₃-CDC20 net1 Δ ::net1-14A*) were arrested in metaphase by Cdc20 depletion and released into synchronous anaphase by Cdc20 re-introduction. Cdc14 release from the nucleolus and anaphase spindles were observed by immunofluorescence *in situ*. At least 100 cells were scored for each condition. Cdc14 release during the time course is shown in (A) and respect to the spindle length in (B).

observed that around 43% of *net1_5A* cells released Cdc14 from the nucleolus upon 180 min of Cdc5 ectopic expression compared to 100% of WT cells (Fig. R28); suggesting that the *net1_5A* mutant is still able to release Cdc14 although with lower kinetics. On the contrary, Cdc14 release was greatly impaired (less than 20% of cells) in the *net1_14A* mutant upon Cdc5 induction (Fig. R28 and Table R5). These results further confirm the importance of

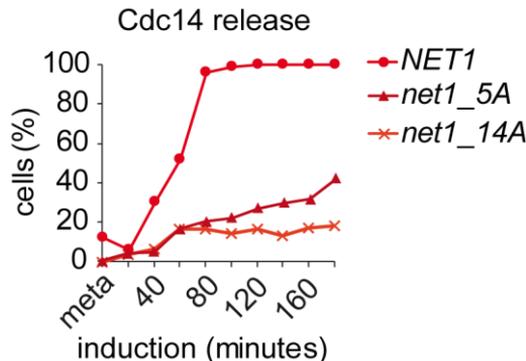


Figure R28. Cdc14 release from the nucleolus after Cdc5 ectopic expression is compromised in *net1_14A*. Strains Y571 (*MAT α MET-HA₃-CDC20 CDC14-PK₉ BFA1-HA₆ GAL1-CDC5-MYC₉ NET1-Myc₉*), Y1398 (*MAT α MET-HA₃-CDC20 GAL1-CDC5-MYC₉ net1 Δ ::MYC₉-net1-5A*) and Y1367 (*MAT α MET-HA₃-CDC20 CDC14-PK₉ GAL1-CDC5-MYC₉ net1 Δ ::MYC₉-net1-14A*) were arrested in metaphase and *GAL1-CDC5* was induced with 2% galactose for 3 hours. Cdc14 release from the nucleolus was observed by immunofluorescence *in situ*. At least 100 cells were scored for each condition.

RESULTS

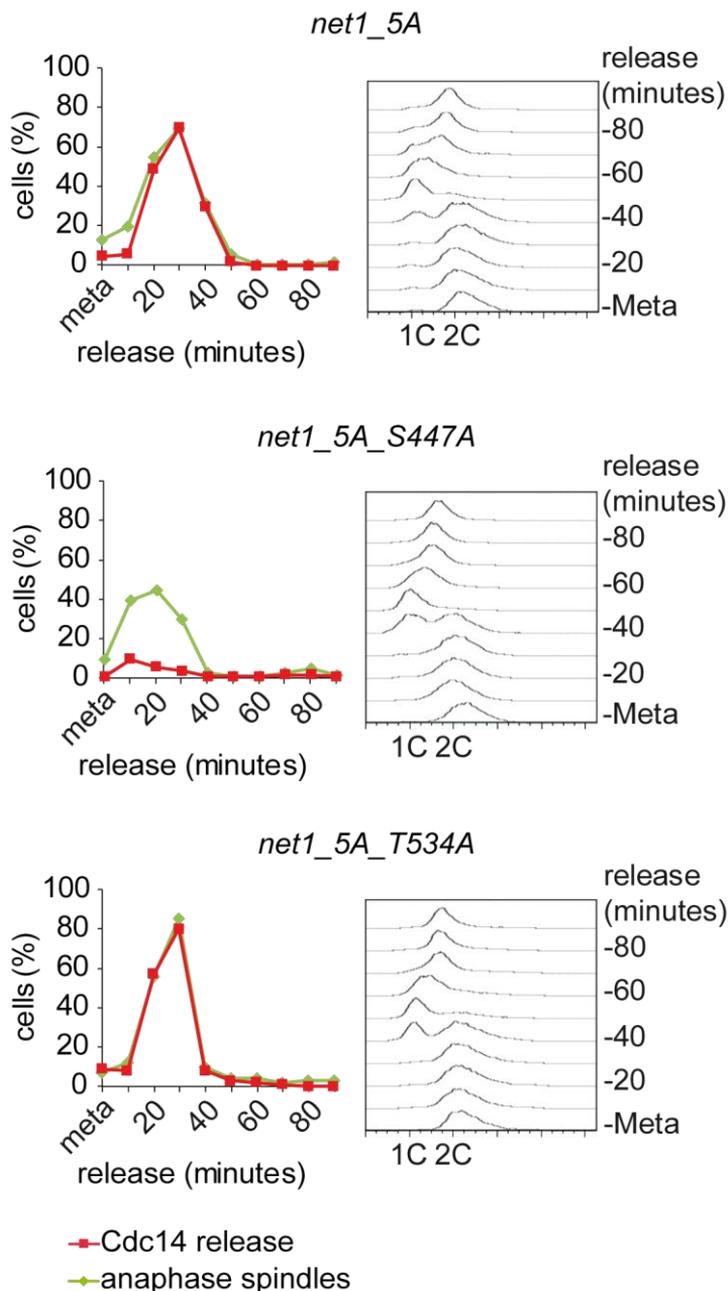


Figure R29. Impaired Cdc14 release from the nucleolus in *net1_5A_S447A* cells during anaphase. Strains Y1395 (*MAT α MET-HA₃-CDC20 net1 Δ ::MYC₉-net1_5A*), Y1380 (*MAT α MET-HA₃-CDC20 net1 Δ ::MYC₉-net1-5A_S447A*) and Y1386 (*MAT α MET-HA₃-CDC20 net1 Δ ::MYC₉-net1-5A_T534A*) were arrested in metaphase by Cdc20

the residues mutated in the *net1_14A* for the Cdc5-dependent Cdc14 release from the nucleolus.

Next, we investigated the influence of two more Net1 candidate residues, S447 and T534, in the Cdc14 release from the nucleolus. As mentioned before, the S447 residue contains the Cdc5 consensus sequence D/E/N X S/T (NGSP) and the Cdk1 consensus sequence (NGSP), and it was detected in the mass spectrometry analysis. The T534 residue is one of the two residues (together with S231 included in the *net1_5A*) within a full consensus sequence D/E/N X S/T X Y/D (EDTND) for Cdc5 kinase and it was also detected in the mass spectrometry analysis. In this case we studied whether the combination of these residues with *net1_5A* has an additive effect in the Cdc14 activation. We prepared the strains containing the *net1_5A_S447A* and *net1_5A_T534A* mutations and analyzed the Cdc14 activation during anaphase progression synchronizing cells at the metaphase to anaphase by Cdc20 depletion and re-addition. The *net1_5A_T534A* strain released Cdc14 from the nucleolus with similar kinetics to WT cells (Fig. R29), indicating that the phosphorylation at T534 is also dispensable for the Cdc14 release. By contrast, we observed that in the *net1_5A_S447A* mutant the Cdc14 release from the nucleolus was greatly impaired, although it was enough to promote mitotic exit as we can observe by the flow

depletion and released into synchronous anaphase by Cdc20 re-introduction. Cdc14 release from the nucleolus and anaphase spindles were observed by immunofluorescence *in situ*. At least 100 cells were scored for each condition. Cell cycle progression was followed by FACS analysis of DNA content.

RESULTS

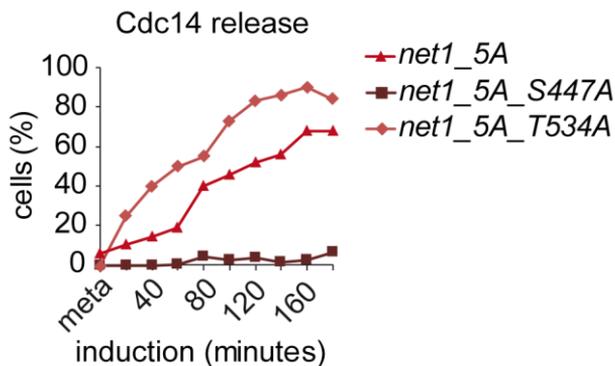


Figure R30. Cdc14 release from the nucleolus upon Cdc5 ectopic expression is reduced in *net1_5A_S447A* cells. Strains Y1398 (*MAT α MET-HA₃-CDC20 GAL1-CDC5-MYC₉ net1 Δ ::MYC₉-net1-5A*), Y1580 (*MAT α MET-HA₃-CDC20 GAL1-CDC5-MYC₉ net1 Δ ::MYC₉-net1-5A_S447A*) and Y1590 (*MAT α MET-HA₃-CDC20 GAL1-CDC5-MYC₉ net1 Δ ::MYC₉-net1-5A_T534A*) were arrested in metaphase and *GAL1-CDC5* was induced with 2% galactose for 3 hours. Cdc14 release from the nucleolus was observed by immunofluorescence *in situ*. At least 100 cells were scored for each condition.

cytometry analysis (Fig. R29). This result indicates that Net1 phosphorylation at S447 is required to timely release Cdc14 from the nucleolus during anaphase.

Next, we analyzed the Cdc14 release from the nucleolus upon ectopic expression of Cdc5 in the *net1_5A_S447* and *net1_5A_S534* mutants. In the *net1_5A_T534A* mutant upon Cdc5 induction (Fig. R30), Cdc14 was released in around 60% of the cells, similarly to the *net1_5A* mutant. On the contrary, we observed almost null Cdc14 release in the *net1_5A_S447* mutant upon Cdc5 induction (Fig. R30), indicating that phosphorylation at S447 residue is required for the Cdc5-dependent Cdc14 activation. These results suggest that phosphorylation by Cdc5 at S447 Net1 residue is required to release Cdc14 from the nucleolus, or Cdc5 induction indirectly regulate the kinase involved in the Net1

phosphorylation at S447. Further experiments will be required to distinguish these possibilities and to narrow down the others Net1 phosphorylation residues involved in the Cdc5-dependent Cdc14 activation.

In addition, in preliminary experiments we checked the Cdc14 activation in two other mutants (Table R4), *net1_5A_N-term* and *net1_4A_C-term*, upon Cdc5 induction. We observed that Cdc14 release from the nucleolus is impaired upon ectopic expression of Cdc5 in both mutants (Table R5). Remarkably, *net1_4A_C-term*

Net1 strains	Cells with Cdc14 released (%)
<i>NET1</i>	+++
<i>net1_5A</i>	++
<i>net1_14A</i>	+
<i>net1_5A_S447A</i>	+
<i>net1_5A_T534A</i>	++
<i>net1_5A_Nterm</i>	+
<i>net1_4A_Cterm</i>	+

Table R5. Summary of the Cdc14 release in the *Net1* mutant strains. Strains Y571 (*MATa MET-HA₃-CDC20 CDC14-PK₉ BFA1-HA₆ GAL1-CDC5-MYC₉ NET1-MYC₉*), Y1398 (*MATa MET-HA₃-CDC20 GAL1-CDC5-MYC₉ net1Δ::MYC₉-net1-S60A_S69A_S231A_T248A_S614A*) and Y1367 (*MATa MET-HA₃-CDC20 CDC14-PK₉ GAL1-CDC5-MYC₉ net1Δ::MYC₉-net1-14A*), Y1535 (*MATa MET-HA₃-CDC20 GAL1-CDC5-MYC₉ net1Δ::MYC₉-net1-5A_Nterm*), Y1536 (*MATa MET-HA₃-CDC20 GAL1-CDC5-Myc₉ net1Δ::MYC₉-net1-4A_Cterm*), Y1580 (*MATa MET-HA₃-CDC20 GAL1-CDC5-MYC₉ net1Δ::MYC₉-net1-5A_S447A*) and Y1590 (*MATa MET-HA₃-CDC20 GAL1-CDC5-MYC₉ net1Δ::MYC₉-net1-5A_T534A*) were arrested in metaphase by Cdc20 depletion. *GAL1-CDC5* was induced for 3 hours with 2% galactose. Cdc14 release from the nucleolus was observed by immunofluorescence *in situ*. At least 100 cells were scored for each condition.

containing S447 has the stronger defects in the Cdc14 release from the nucleolus, suggesting that, so far, is the most relevant Cdc5-dependent Net1 residue. In addition, Net1 residues included in the *net1_5A_N-term* are also required for the Cdc5-dependent Cdc14 released.

2.2. Net1-Cdc5 fusion promotes Cdc14 release from the nucleolus.

To further investigate the Cdc5 contribution to Net1 phosphorylation, we decided to examine the Cdc5 activity over Net1 independently to the Cdk1 contribution. Cdc5 is required for the activation of Cdk1 in G2/M (F. Liang et al., 2009; Raspelli et al., 2018); and, in turn, Cdk1 is necessary for Cdc5 activation during mitosis (J.-A. J.-A. Rodriguez-Rodriguez et al., 2016). This Cdc5-Cdk1 interdependence increase the difficulty to study the individual contribution of the two kinases. For instance, Cdc5 kinase activity is impaired in the *cdc28-as1* mutant, and, therefore it behaves as a double *cdc5 cdc28* mutant (J.-A. J.-A. Rodriguez-Rodriguez et al., 2016). For this reason, we decided to prepare a Net1-Cdc5 fusion strain inspired in a model previously described for protein fusion with Clb2 (Kuilman et al., 2015). It consists on the inducible fusion of the proteins depending on the Cre recombinase system (Fig. R31). We initially tried to obtain the Net1-Clb2 and the Net1-Cdc5 fusion. However, we did not manage to obtain the Net1-Clb2 fusion upon Cre recombinase treatment, although the Net1-Clb2 Δ Cdk mutant protein fusion, unable to interact with Cdc28 and therefore acting as a negative control protein fusion, was obtained. Net1-Clb2 fusion was found to be lethal for the cells, even by induction of

the Cre recombinase during a time-course experiment. Unfortunately, Net1-Clb2 fusion cells were unable to divide during the arrest with the Cdc20 depletion, just being viable during the assay the cells whose fusion was not successful upon Cre induction (results not shown). This result suggests that continuous Net1 phosphorylation by Clb2-Cdk1 is deleterious for the cells affecting is viability. This reinforced the importance of the Net1 phosphorylation by Clb2-Cdk1.

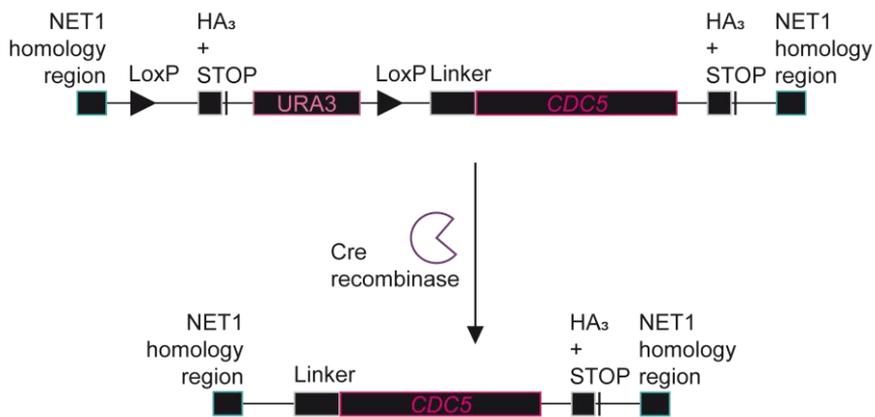


Figure R31. Illustration of the Net1-Cdc5 fusion by the Cre-Lox system.

Contrary to the Net1-Clb2 fusion, the Net1-Cdc5 fusions were stable and viable. We prepared three different fusion proteins, one with the Cdc5 control and two with mutant versions: the inactive *cdc5_T242A* and the kinase-death *cdc5_N209A*. T242 residue was described to be responsible of Cdc5 activation in FEAR and MEN by Cdk1 phosphorylation (J.-A. J.-A. Rodriguez-Rodriguez et al., 2016). The N209 residue is at the active site of the Cdc5 kinase, becoming kinase death when mutated to alanine (Hardy & Pautz, 1996). We used both Cdc5 mutant versions as negative controls for

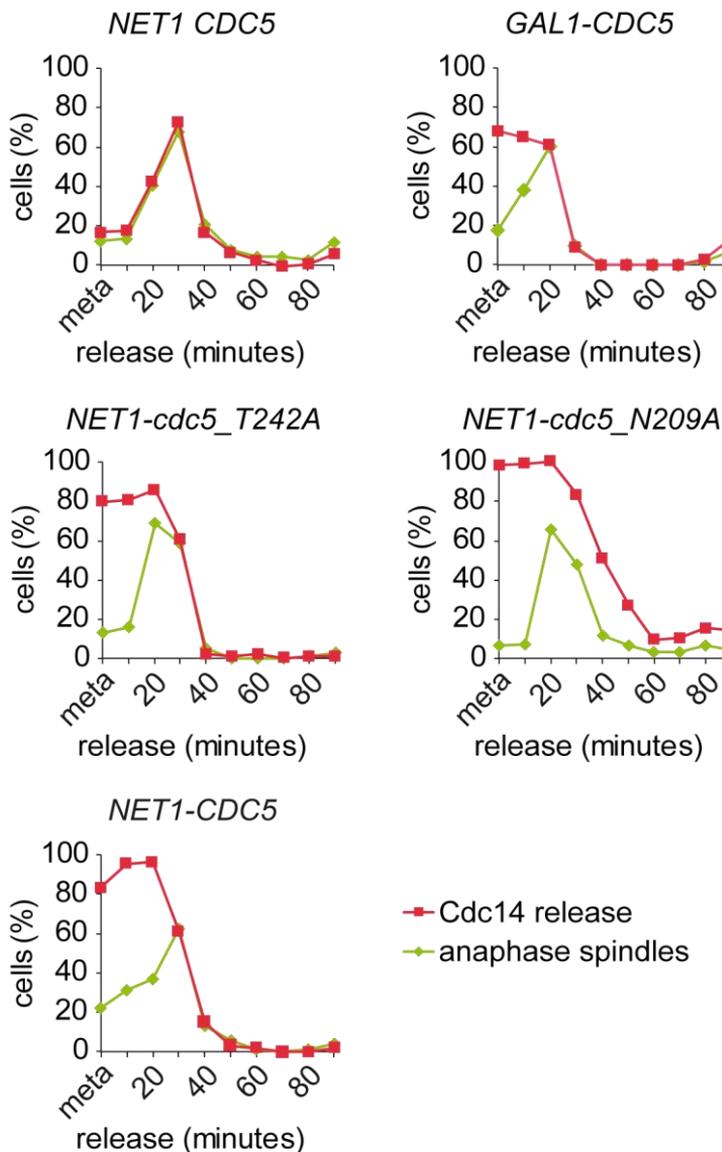


Figure R32. Premature Cdc14 release from the nucleolus in Net1-Cdc5 fusions. Strains Y1555 (*MATa MET-HA₃-CDC20 HOF1-HA₆ GAL1-CDC55*), Y571 (*MATa MET-HA₃-CDC20 CDC14-PK₉ BFA1-HA₆ GAL1-CDC5-MYC₉ NET1-MYC₉*), Y1396 (*MATa MET-HA₃-CDC20 CDC14-PK₉ NET1-CDC5-HA₃*), Y1531 (*MATa CDC14-PK₉ MET-HA₃-CDC20 NET1-cdc5-T242A-HA₃*) and Y1623 (*MATa CDC14-PK₉ MET-HA₃-CDC20 NET1-cdc5-N209A-HA₃*) were arrested in metaphase by Cdc20 depletion and released into synchronous anaphase by Cdc20 re-introduction. Cdc14 release from the nucleolus and anaphase spindles were observed

the fusion proteins. In addition, in another parallel strain we overexpressed Cdc5 for 2 hours before releasing the cells from the metaphase arrest by Cdc20 depletion as a control for the hyperactivation of Cdc5.

In WT cells, Cdc14 is released during anaphase as expected; while the *GAL1-CDC5* strain showed prematurely Cdc14 released already at metaphase (Fig. R32). Later on, Cdc14 was normally re-sequestered in both strains.

In the Net1-Cdc5 fusion, we observed that Cdc14 was prematurely released during metaphase, compared to the WT cells (Fig. R32). Strikingly, in the non-functional Cdc5 fusions, *net1-cdc5_T242A* and *net1-cdc5_N209A*, Cdc14 was also released from the nucleolus at metaphase. These results indicate that the Net1-Cdc5 fusion promotes the Cdc14 release from the nucleolus independently to the Cdc5 kinase activity. We hypothesize that Cdc5 compete with Cdc14 to form a complex with Net1. This will explain why the ectopic expression of Cdc5 induces Cdc14 activation, promoting the Cdc14 dissociation from the nucleolar Net1.

by immunofluorescence *in situ*. At least 100 cells were scored for each condition.

DISCUSSION

1. PP2A^{Cdc55} role in cytokinesis

The final physical separation of the two new cells during cell division takes place during cytokinesis. Cytokinesis is a process mainly regulated by the GTP exchange and the phosphorylation of the different proteins involved. GTP is necessary for the activation of the different GTPases regulating cytokinesis, such as the septins organization by Cdc42 (Iwase et al., 2006; P. J. Kang et al., 2018) or the actin recruitment to the actomyosin ring (AMR) by Rho1 in yeast (Dong et al., 2003; Satoshi Yoshida et al., 2006a).

However, phosphorylation has a wider range of action; regulating the activation and localization of many proteins involved in cytokinesis. Phosphorylation events occur in any cytokinesis step, from septins organization to RAM pathway activation in abscission.

The main kinases regulating cytokinesis are the Cdk1, Cdc5 and Dbf2. They were described to regulate the localization of the proteins required for AMR formation, constriction and primary septum (PS) formation, the IPC's. Iqg1, Myo1, Hof1, Cyk3, Inn1 and Chs2 proteins are the IPC's subunits which coordinate the AMR contraction and the PS formation. Myo1 is the only IPC protein that has not been described to be phosphorylated. In the opposite site, Hof1 is phosphorylated by multiple kinases, Cdk1, Db2 and Cdc5 (Meitinger et al., 2011; Meitinger, Palani, Hub, & Pereira, 2013b).

Not only the kinases phosphorylation, but also the phosphatases dephosphorylation events are key for cytokinesis progression. So

DISCUSSION

far, only two phosphatases Cdc14 and PP2A^{Rts1} were described to have a role during cytokinesis; although, just Cdc14 was found to dephosphorylate some of the IPC's proteins (Chin et al., 2012; Dobbelaere et al., 2003; D. P. Miller et al., 2015; Saravanan Palani et al., 2012). During this thesis, we have found that PP2A^{Cdc55} regulates AMR contraction and septum formation, and the dephosphorylation of IPC's proteins. We demonstrated that PP2A^{Cdc55} dephosphorylates Hof1 and Chs2 *in vivo* and *in vitro*; indicating that they are direct PP2A^{Cdc55} targets. In addition, PP2A^{Cdc55} participates in the regulation of Cyk3 and Inn1 phosphorylation. Although we did not manage to validate Cyk3 and Inn1 as PP2A^{Cdc55} direct substrates by the *in vitro* phosphatase assays, we proved that their phosphorylation levels are increased and their residence time at the bud neck are also affected in absence of Cdc55. In conclusion, PP2A^{Cdc55} regulates the dephosphorylation of IPC's and is involved in AMR contraction and septum formation. Since IPC's coordinate the AMR constriction with the septum formation, we propose that PP2A^{Cdc55} is also involved in this coordination mechanism through IPC's dephosphorylation. Unfortunately, we could not detect Cdc55 localization at the bud neck; and, therefore, it was not possible to perform co-localization experiments with the IPC's at the bud neck. GFP-Cdc55 bud neck localization during cytokinesis was described previously (Gentry & Hallberg, 2002). However, in our strains containing HA-Cdc55 and mCherry-Cdc55 we were not able to visualize Cdc55 at the bud neck. Despite that, our results are consistent with PP2A^{Cdc55} regulating cytokinesis through the IPC's phosphorylation.

1.1. PP2A^{Cdc55} regulates IPC's phosphorylation and localization at the bud neck

The Iqg1 protein was described to be phosphorylated by Cdk1 (Naylor & Morgan, 2014). Iqg1 phosphorylation prevents its recruitment to the myosin ring. The Cdc14 phosphatase mediates the Iqg1 dephosphorylation for its proper recruitment and function during cytokinesis (D. P. Miller et al., 2015). In the case of Iqg1, we observed a small increase in its phosphorylation in the *cdc55Δ* mutant (Fig. R1a) but did not detect a decrease in its phosphorylation upon ectopic expression of Cdc55 (Fig. R1b). However, the protein dynamics at the bud neck was perturbed in absence of Cdc55. The Iqg1 residence time at the bud neck upon Iqg1 contraction is longer in *cdc55Δ* mutant cells (Fig. R15). This increase in the Iqg1 residence time was already observed in *cdh1Δ* cells, when Iqg1 degradation was defective (Tully, Nishihama, Pringle, & Morgan, 2009). Nonetheless, we did not detect an increase in the Iqg1 protein levels in *cdc55Δ* mutant cells (see Fig. R1). Conversely, Iqg1 protein levels were reduced during anaphase in all our time-courses. Therefore, we do not think that Cdh1-dependent degradation of Iqg1 is affected in *cdc55Δ* cells. Moreover, Cdh1 is activated upon Cdc14 dephosphorylation in late anaphase (Sue L. Jaspersen, Charles, & Morgan, 1999; Rosella Visintin et al., 1998). Cdc14 is prematurely released in *cdc55Δ* mutant cells (Queralt et al., 2006). In spite of this, Cdh1 is timely activated during late anaphase/telophase in absence of Cdc55 (B. Baro et al., 2013). In agreement, Cdc14-dependent dephosphorylation of Iqg1 is still visualized in absence of Cdc55 during anaphase (Fig. 1a 40 min). Therefore, we can conclude that

DISCUSSION

the slight difference on the Iqg1 phosphorylation in absence of Cdc55 could be an indirect effect of the *cdc55* Δ deletion. The phosphorylation levels of the recruiting proteins of Iqg1 at the myosin ring, Mlc1 and Myo1 (Boyne et al., 2000; Shannon & Li, 2000; Tian et al., 2014) or the effect on Cdc14 involved in their dephosphorylation (D. P. Miller et al., 2015) could be the link between PP2A^{Cdc55} and Iqg1.

Despite the absence of different migrating isoforms of Myo1 even in Phostag gels; and, therefore, the lack of detection of Myo1 phosphorylation and dephosphorylation, we observed that its localization was affected in absence of Cdc55. Myo1 was constricted and unanchored timely, but the constriction was displaced from the central axis becoming asymmetric in the *cdc55* Δ deletion mutant (Figs. R10, R11 and R12). This phenotype is characteristic of IPC's mutants (Lippincott & Li, 1998a; Sanchez-Diaz et al., 2008a; Tully et al., 2009; VerPlank & Li, 2005; Wloka et al., 2013) and denotes a dysfunctional AMR. In agreement, Iqg1, Cyk3, Hof1, Inn1 and Chs2 localization were also asymmetric upon AMR constriction (Figs. R13-R17). We suggest that PP2A^{Cdc55}-dependent IPC's dephosphorylation mediates the correct regulation of the AMR constriction, perturbed when *CDC55* is deleted.

Myo1 and Iqg1 physical interaction is required to keep Iqg1 located at the bud neck and to stabilize it (Fang et al., 2010; Wloka et al., 2013). Therefore, the correct Myo1 constriction could be necessary for the regulation of the Iqg1 residence time at the bud neck, which is affected in *cdc55* Δ mutant cells (Fig. R13). In turn, it was also described that Iqg1 regulates the AMR formation and contraction (Shannon & Li, 1999), becoming the two proteins mutually

dependent. Iqg1 mediates the actin recruitment to the bud neck required for AMR formation (D. P. Miller et al., 2015; Shannon & Li, 1999). Nevertheless, the actin recruitment to the AMR was not affected in *cdc55* Δ cells (Fig. R20), indicating that Iqg1 is still able to recruit actin in absence of Cdc55. In conclusion, the PP2A^{Cdc55} regulation over the other IPC's subunits could be essential for proper symmetric AMR contraction and functional AMR, but do not compromises the Iqg1 capability to recruit actin and, as a consequence, AMR formation is normal.

Cyk3 was described to be phosphorylated during anaphase (M. Wang et al., 2018), probably by mitotic exit network (MEN) kinases Dbf2 and Cdc5; and to our knowledge the phosphatase involved in its dephosphorylation has not been described. Cyk3 was more phosphorylated in *cdc55* Δ mutant cells than in WT cells already at metaphase and early anaphase (Fig. R3). Later on, in mid and late anaphase, Cyk3 is phosphorylated in WT and *cdc55* Δ mutant cells consistently with being phosphorylated by MEN kinases. Therefore, PP2A^{Cdc55} could be regulating Cyk3 dephosphorylation from metaphase to the next G1. Unfortunately, we couldn't determine the direct PP2A^{Cdc55} dephosphorylation of Cyk3 by *in vitro* phosphatase assays. However, we observed that IPC's, including Cyk3, from a complex with Cdc55 by co-immunoprecipitation experiments (Fig. R6), suggesting that Cyk3 might be a substrate of PP2A^{Cdc55}. But, given that the IPC's proteins already form a complex independently of Cdc55, we cannot discard the possibility that the Cdc55 and Cyk3 co-purification detected is due to the pulling-down of the entire IPC complex.

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In addition, we demonstrated that PP2A^{Cdc55} is required for the normal Cyk3 maintenance at the bud neck (Fig. R17). We showed that the lack of Cyk3 dephosphorylation or the indirect effect of the other IPC's dephosphorylation in absence of Cdc55 promotes longer residence time of Cyk3 at the bud neck. Moreover, the constriction of Cyk3 was also affected being asymmetric (Fig. R17). The asymmetric Cyk3 localization was also described in *hof1Δ* mutants (M. Wang et al., 2018). This result indicates that Hof1 dephosphorylation by PP2A^{Cdc55} (Figs. R2 and R8) could be contributing to the Hof1 functionality; and, therefore, to the Cyk3 residence time and asymmetry at the bud neck.

Cdk1 phosphorylates and inhibits Inn1, avoiding its premature interaction with Cyk3 (Saravanan Palani et al., 2012). Cdc14 dephosphorylates Inn1 allowing the correct Cyk3-Inn1 interaction and activation (Meitinger et al., 2010; Palani et al., 2012). Here, we described that Inn1 phosphorylation was increased in *cdc55Δ* cells during anaphase-cytokinesis and it was decreased upon ectopic expression of Cdc55 (Fig. R4). Interestingly, the reduction of the anaphase-specific phosphorylation of Inn1 was the most dramatic effect that we did observe upon Cdc55 ectopic expression in any of the IPC's proteins under study. For this reason, we envisaged that Inn1 could be a direct PP2A^{Cdc55} substrate. Disappointingly, we could not obtain the results of the PP2A^{Cdc55} phosphatase assays *in vitro*, despite we managed to phosphorylate it by Cdk1. But, the Inn1 phosphorylation signal obtained was too low and the dilution of the substrate during the following phosphatase assay prevented the detection and quantification of the Inn1 remaining phosphorylation. Similar to Cyk3, we co-purified Cdc55 and Inn1

by co-immunoprecipitation, but again, this could be due to the co-purification of the entire IPC's.

In WT cells, Inn1 phosphorylation peaks in late anaphase (Fig. R4) when Clb2-Cdk1 starts to decrease. This late anaphase-phosphorylation was also detected in absence of Cdc55, indicating that other kinases might be involved in Inn1 phosphorylation. We suggest that MEN kinases may also phosphorylate Inn1, since MEN kinases are timely activated in absence of Cdc55 (B. Baro et al., 2013). In addition, it was proposed that MEN regulates Inn1 recruitment at the bud neck (Meitinger et al., 2010) suggesting that MEN kinases could phosphorylate Inn1. It was previously described in our lab that the Dbf2-Mob1 are dephosphorylated by PP2A^{Cdc55} for its complete activation (B. Baro et al., 2013); suggesting that Dbf2-Mob1 could be the MEN kinase responsible to phosphorylate Inn1. This hypothesis is reinforced by the observation that Inn1 localization at the bud neck is reduced in *dbf2-2* mutants (Meitinger et al., 2010). Therefore, PP2A^{Cdc55} could participate indirectly in Inn1 dephosphorylation by the regulation of Dbf2-Mob1, regulating its localization to the bud neck. The results are also in agreement with PP2A^{Cdc55} being the Dbf2-counteracting phosphatase of Inn1 and would imply that the anaphase Dbf2-dependent Inn1 phosphorylation is required for its localization to the bud neck. In this case, *dbf2-2* mutants are expected to have reduced Inn1 localization and *cdc55*Δ increased localization.

Curiously, we observed an increase in Inn1 phosphorylation earlier to anaphase when *CDC55* was overexpressed (Fig. R4b). This increase in phosphorylation is difficult to explain but could be

consequence of Cdk1 hyperactivation due to altered Swe1 and Mih1 activities upon Cdc55 ectopic expression.

Strikingly, Inn1 was recruited prematurely at the bud neck in *cdc55* Δ cells (Fig. R18). It was described that Inn1 maintenance at the bud neck depends on the dephosphorylation of the Cdk1-phosphorylated Inn1 residues mediated by Cdc14 (Saravanan Palani et al., 2012). Cdc14 is prematurely released in metaphase in *cdc55* Δ cells, indicating that premature Inn1 dephosphorylation by Cdc14 could be the responsible of the earlier Inn1 localization at the bud neck.

Moreover, Inn1 recruitment is avoided completely in absence of Myo1 and Iqg1 (Sanchez-Diaz, 2008) or reduced in the double mutant *cyk3* Δ *hof1* Δ (Meitinger et al., 2010; Nishihama et al., 2009). Cyk3 overexpression is able to rescue Inn1 recruitment in the *myo1* and *iqg1* mutants (Jendretzki et al., 2009). Therefore, any alteration in the phosphorylation levels of the others IPC's subunits, could also indirectly affect the Inn1 recruitment to the bud neck.

Finally, we also showed that the constriction of Inn1 signal at the bud neck was asymmetric (Fig. R18) and, Inn1 asymmetry was also observed in *hof1* Δ mutants, similar to Cyk3, and in *myo1* Δ mutants (Nishihama et al., 2009a). This result further confirms that *cdc55* Δ has an AMR mutant phenotype similar to *myo1* Δ and *hof1* Δ .

Hof1 protein has multiple functions in cytokinesis, from septins organization (Norden, Liakopoulos, & Barral, 2004) to Chs3 activation at secondary septa formation (Younghoon Oh et al., 2017). Its multiple functions make Hof1 a key regulator of

cytokinesis. Hof1 was seen to be phosphorylated by Cdk1, Dbf2 and Cdc5 (F Meitinger et al., 2011; Meitinger et al., 2013b). The phosphorylation by the different kinases regulates the Hof1 localization at the septins ring or at the AMR. We detected Hof1 hyperphosphorylation during cytokinesis in absence of Cdc55 (Fig. R2a) and Hof1 phosphorylation was decreased upon Cdc55 ectopic expression (Fig. R2b); suggesting that Hof1 is dephosphorylated by PP2A^{Cdc55}. Furthermore, we demonstrated that Hof1 is dephosphorylated *in vitro* by PP2A^{Cdc55} (Fig. R8), indicating that Hof1 is a direct PP2A^{Cdc55} target. To our knowledge, is the first time a phosphatase is described to dephosphorylate Hof1.

Hof1 maintenance at the bud neck and its constriction was affected in absence of Cdc55 (Fig. R14). Moreover, Hof1 signal constriction was delayed in *cdc55Δ* cells. Hof1 protein translocates from the septin ring to the AMR during cytokinesis (Lippincott & Li, 1998a; E. a Vallen et al., 2000). On the other hand, Hof1 was described to regulate the septins organization (Lippincott & Li, 1998; Meitinger et al., 2013). Therefore, the defects in Hof1 localization could be a consequence or a cause of defective septins function in *cdc55Δ* cells. Nevertheless, the preliminary results obtained from immunofluorescence *in situ* showed no alterations in septins ultrastructures in *cdc55Δ* cells. These experiments cannot distinguish small differences in the amounts of septins recruited to the bud neck or little alteration in their structure. In order to refine the results, we should perform further analysis using fluorescence-tagged septins. Moreover, we did not detect differences in protein levels or phosphorylation state in Cdc11 and Shs1 in absence of Cdc55 (results not shown). In addition, it was previously described

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that the dephosphorylation of septins by PP2A^{Rts1} is required for septins ultrastructure stability (Dobbelaere et al., 2003); suggesting that septins are regulated by the other PP2A regulatory subunit, Rts1.

Similar to the other IPC's, Hof1 was also asymmetric in absence of Cdc55. Hof1 asymmetry was already seen in *chs2Δ* (Younghoon Oh et al., 2013); but not vice versa, although Chs2 localization was reduced at the bud neck and was more dynamic in *hof1Δ* cells (Foltman et al., 2016; Younghoon Oh et al., 2013). Therefore, PS formation influences Hof1 constriction (Younghoon Oh et al., 2013) and, strikingly, it was described that Hof1 constricts independently to the actomyosin ring (Lippincott & Li, 1998a). Consequently, PP2A^{Cdc55} could be regulating Hof1 constriction directly and/or through the PS regulation, since both Hof1 and Chs2 are PP2A^{Cdc55} substrates.

Finally, Chs2 was described to be phosphorylated by Cdk1, Cdc5 and Dbf2. Cdk1 phosphorylation keeps Chs2 away from the bud neck (Chin et al., 2012; Teh et al., 2009), while Cdc14 dephosphorylation and Cdc5 phosphorylation mediate its translocation from the ER to the bud neck (Chin CF, 2011; Jakobsen MK, 2013). We showed that anaphase-Chs2 phosphorylation is reduced in WT cells and is maintained in *cdc55Δ* cells (Fig. R5). In accordance, Chs2 dephosphorylation was increased upon *CDC55* overexpression (Fig. R5). We demonstrated that Chs2 form a complex with Cdc55 and the chitin synthase is dephosphorylated *in vitro* by PP2A^{Cdc55} (Figs. R6 and R9). Thus, PP2A^{Cdc55} regulates Chs2 residence time and symmetry at the bud neck. Chs2 is the chitin synthase responsible of PS

formation; hence, defective PS formation could provoke the asymmetry seen in the IPC's proteins.

1.2. PP2A^{Cdc55} is required for proper AMR constriction

The defects seen in IPC's residence time and asymmetric localization in absence of Cdc55, can be a direct consequence of its own phosphorylation regulation by PP2A^{Cdc55} or a secondary effect due to the others IPC's dephosphorylation by PP2A^{Cdc55}. In fact, both scenarios might cooperate in the PP2A^{Cdc55} regulation of IPC's, since we demonstrated that at least two proteins, Hof1 and Chs2, are direct targets of PP2A^{Cdc55}, and Cyk3 and Inn1 are also likely to be its substrates.

IPC's proteins were described to form a complex which function is the coordination of the AMR contraction with PS formation (Foltman et al., 2016; M. Wang et al., 2018). The formation of the IPC depends on the phosphorylation state of its subunits. For instance, the *inn1-K31A* non-phosphorylatable mutant does not interact with Chs2 (Devrekanli et al., 2012); suggesting that Inn1 phosphorylation is required for its interaction with Chs2. Therefore, the changes in the phosphorylation levels of the different IPC's subunits observed in absence of Cdc55 could affect the formation of the IPC. However, the IPC's subunits still co-purify among them in presence and absence of Cdc55 (results not shown). These results show that the dephosphorylation mediated by PP2A^{Cdc55} do not greatly affect the association among the IPC subunits. To discriminate which of the IPC's proteins is directly interacting with

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PP2A^{Cdc55} we should repeat the co-immunoprecipitation assays in single IPC's mutants. Nevertheless, since PP2A^{Cdc55} interaction with Hof1 and Chs2 occur in the phosphatase *in vitro* assays, we envisage that more than one IPC subunit interact directly with PP2A^{Cdc55} and the aforementioned co-immunoprecipitation assays would not give us any clear conclusion.

Actomyosin ring formation also requires the recruitment of actin. As we mentioned before, actin was timely and correctly localized to the AMR, indicating that myosin and actin, the two basic components of the AMR are correctly attached to the bud neck. However, we observed an aberrant actin polarization phenotype to the tips of the two dividing cells during cytokinesis (Fig. R18). This premature actin localization to the new bud site indicates that actin polarization of the next cell cycle is advanced before cytokinesis completion. In a normal cell cycle, Rho1 GTPase regulates the secondary septa formation and the polarization of the actin for the next cell cycle (Meitinger et al., 2013; Onishi, Nolan, Nishihama, & Pringle, 2013; Tolliday, Pitcher, & Li, 2003; Yoshida, Bartolini, & Pellman, 2009). Therefore, PP2A^{Cdc55} could act preventing the premature Rho1 activation until cytokinesis is completed. Lrg1 GAP inactivates Rho1 preventing the actin polarization (Jonasson et al., 2016; Svarovsky & Palecek, 2005). Interestingly, in the previous SILAC proteomics analysis performed in the lab (B. Baro et al., 2013), we found Pop2 protein to be more phosphorylated in *cdc55Δ* cells. These findings suggest that Pop2 could be a target of PP2A^{Cdc55}. The Pop2 RNase is part of the Ccr4-Not complex that deadenylates the 3' to 5'mRNA promoting mRNA degradation. Pop2 was described to promote the degradation of Lrg1 mRNA (X. Li et al., 2016). We propose that PP2A^{Cdc55} would be negatively

regulating the Lrg1 mRNA degradation, maybe inhibiting Pop2, stabilizing the protein to prevent actin polarization to the new bud site until the next G1. Other authors also proposed a link between PP2A^{Cdc55} and the Lrg1 (Jonasson et al., 2016). The other proteins involved in cytoplasmic mRNA degradation found in the SILAC analysis (Not3, Not5, Dcp2, Pat1, Edc3) could also contribute to the regulation of Lrg1 mRNA levels. Curiously, Mss4 protein, responsible of PIP2 formation (Desrivières, Cooke, Parker, & Hall, 1998; Homma et al., 1998) was detected in our SILAC analysis, as well (B. Baro et al., 2013).

1.3. PP2A^{Cdc55} and septum formation

The asymmetric localization of the Iqg1, Cyk3 Inn1, Hof1 and Chs2 IPC's in absence of Cdc55 point out to a PP2A^{Cdc55} role in AMR constriction and/or PS formation. In addition, we saw a decrease in the chitin signal by calcofluor staining in *cdc55Δ* and *cdc55Δ chs3Δ* cells; indicating a defective incorporation of chitin during septa formation (Fig. R20). These results suggest that Chs2 activity might be impaired in absence of Cdc55. However, the electron microscopy study of septa formation demonstrated that PS is still formed in *cdc55Δ* cells, being predominantly asymmetric in one side of the bud neck. Therefore, we do not expect big changes in Chs2 enzymatic activity.

After PS formation, SS synthesis and cell abscission have to be completed to finish cytokinesis. We described that, while abscission was apparently not affected, (Figs. R21 and R22), the secondary septum was defective in our *cdc55Δ* mutant. We saw a

decrease in the primary septum but also in the secondary septa staining with calcofluor (Fig. R21), which indicate a decrease in the chitin incorporation during SS formation; suggesting a PP2A^{Cdc55} role regulating Chs3, the chitin synthase responsible of the SS formation. Consistently to the AMR asymmetric signal, we demonstrated that PS formation occurred asymmetrically in some *cdc55Δ* mutant cells by electron microscopy (Fig. R22c and f); while the most penetrance *cdc55Δ* mutant cells did not have neither PS nor SS (Fig. R22e), instead a remedial septum was formed containing even cytosol fractions embedded in the septa (Fig. R22e and i). This remedial septum formation has been previously seen when PS formation is disturbed in IPC's mutants (Nishihama et al., 2009a; Y Oh et al., 2012; Onishi et al., 2013; S. Y. Park, Cable, Blair, Stockstill, & Shannnon, 2009; M. Wang et al., 2018) and MEN mutants (Meitinger et al., 2010; Oh et al., 2012; Park et al., 2009).

The lower calcofluor staining of the septa in *cdc55Δ* mutant cells would indicate that it incorporates lower amounts of chitin. Chs3 is the chitin synthase responsible of the SS formation but also for the construction of remedial septum (Enrico Cabib & Schmidt, 2003); however, the *chs3Δ* mutant is still viable and capable to divide; suggesting that additional chitin synthases might be able to incorporate chitin in absence of Chs3. This will explain why the remedial septum is still form in the *cdc55Δ chs3Δ* double mutant. In chitin synthases mutants, undetermined suppressors mutations altered the synthesis and/or degradation of cell wall components (Schmidt, 2004).

1.4. Model of PP2A^{Cdc55} function during cytokinesis

Our genetic interaction studies support the PP2A^{Cdc55} role in septa formation and AMR contraction. PP2A^{Cdc55} and Chs3 are from the same pathway since no additive effects were observed in *cdc55Δ chs3Δ* double mutants compared to the single mutants (Fig. D1); consistent with Chs3 and PP2A^{Cdc55} promoting remedial septum formation.

Remarkably, Chs2 conditional mutant (*chs2-aid*) compromises cell viability that was rescued in the *chs2-aid cdc55Δ* double mutant (Fig. R25). It indicates that PP2A^{Cdc55} and Chs2 have opposite roles. This rescue phenotype was also seen in *cdc55Δ td-inn1-aid* double mutant in preliminary analysis (results not shown). In addition, *cyk3* and *hof1* mutants showed synthetic sickness with *cdc55Δ* (Figs. R23 and R24), indicating that the phosphatase and the IPC's regulates the same process in parallel pathways. Our results demonstrated that PP2A^{Cdc55} is required for AMR contraction and PS formation, similarly to IPC's, consistently with the genetic interactions observed.

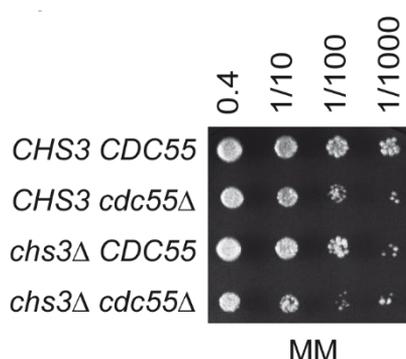


Figure D1. CDC55 deletion does not compromise the survival of *chs3Δ* cells. Serial dilutions of WT (W303), Y844 (*MATα cdc55Δ*), Y1605 (*MATα MET-HA₃-CDC20 MYO1-TOMATOE chs3Δ*) and Y1596 (as Y1605, but *cdc55Δ*) were spotted in minimum medium (MM) plates. Cells were grown at 25°C for 2-3 days.

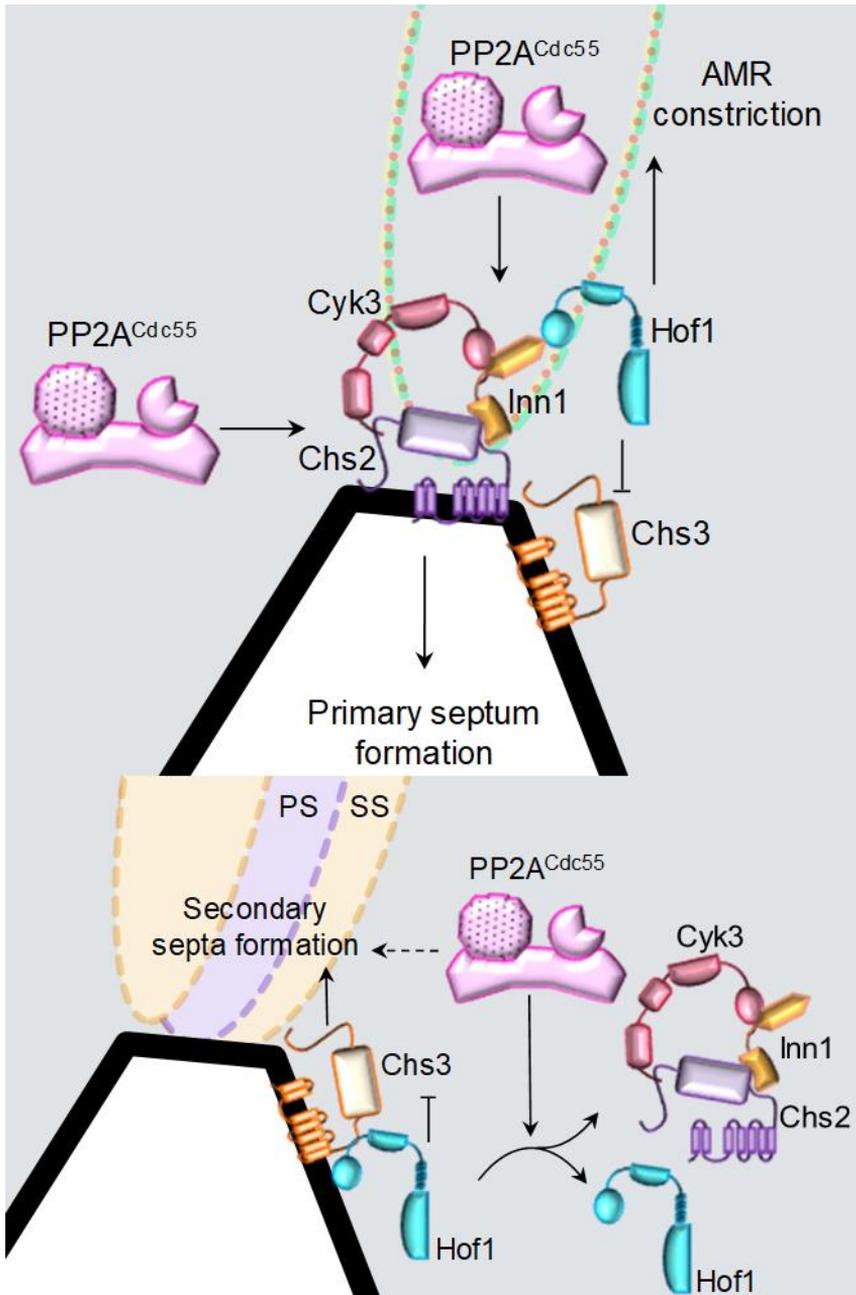


Figure D2. Model for PP2A^{Cdc55} regulation of actomyosin ring (AMR) contraction, primary septum (PS) and secondary septa (SS) formation.

Taking into account our results we can propose several PP2A^{Cdc55} mode of action regulating cytokinesis. PP2A^{Cdc55} as regulator of IPC's dephosphorylation, at least demonstrated for Hof1 and Chs2, could positively control the IPC's function in order to promote the correct AMR constriction and PS formation in probably three parallel ways: 1) by the activation of Hof1, and maybe Inn1, to activate Chs2; 2) activating Cyk3 for Chs2 activation and 3) directly activating Chs2 (Fig. D2). These would explain that the double mutants *td-cyk3-aid cdc55Δ* and *hof1-aid cdc55Δ* are synthetic sick suggesting that Hof1 and Cyk3 act in parallel regulating Chs2; and it is in accordance to the *cyk3Δ hof1Δ* synthetic lethal phenotype (Jendretzki et al., 2009; Korinek et al., 2000; Meitinger et al., 2010; Wang et al., 2018). In the case of Chs2, PP2A^{Cdc55} could be regulating positively Inn1 and Chs2 but, probably, also being involved in other mechanism with opposing roles to Inn1 and Chs2. Another possibility to justify the viability rescue in *td-inn1-aid cdc55Δ* and *chs2-aid cdc55Δ* double mutants, is that *cdc55Δ* mutation promotes the formation of the remedial septum in *td-inn1-aid* and *chs2-aid* mutants. Deletion mutants for *inn1Δ* and *chs2Δ* are lethal (Sanchez-Diaz et al., 2008a; Silverman, Sburlati, Slater, & Cabib, 1988), while other authors worked with viable *inn1Δ* and *chs2Δ* mutant capable to form a remedial septum (Bulawa & Osmond, 1990; Nishihama et al., 2009a). We suggest that the remedial septum seen in the *inn1Δ* (Nishihama et al., 2009a) and *chs2Δ* (Schmidt et al., 2002) was due to accumulation of suppressors mutations.

We further discarded that PP2A^{Cdc55} negatively regulate Chs2 since the double mutant *chs3Δ cdc55Δ* is viable. If Chs2 is inactive in *cdc55Δ* cells, the double *chs3Δ cdc55Δ* should be lethal as the

chs3Δ chs2Δ double mutant (Schmidt et al., 2002). We are currently studying the viability of the *cdc55Δ chs2-aid chs3Δ* triple mutant. The lack of Chs3 could compromise the remedial septum formation that together with the absence of PS will compromise the cell viability.

In addition, it was described that Hof1 regulates the CSIII function inhibiting Chs3 activation through the interaction with Chs4 (Oh et al., 2017). Hof1 removal from the bud neck also dissociates Chs4, allowing Chs3 activation. These authors showed that in *hof1Δ* cells the calcofluor intensity is increased. Therefore, the increased Hof1 residence time in absence of Cdc55 would predict an inactive Chs3 at the bud neck; and, as consequence less SS formation in agreement with our calcofluor staining in *cdc55Δ* mutant cells (Fig. D2).

Taking together all the results, we propose that the PP2A^{Cdc55} is responsible of the regulation of AMR constriction and PS formation during cytokinesis through the dephosphorylation of the IPC's. Parallely, SS formation would be activated through the removal of Hof1 from the bud neck upon PP2A^{Cdc55} dephosphorylation.

1.5. PP2A^{Cdc55} cytokinesis in higher eukaryotes

Most of the cytokinesis events and proteins are conserved in higher eukaryotes. Our results could be translated to the mechanism governing cytokinesis regulation in human cells. PP2A^{Cdc55} homologue in human, PP2A^{B55}, could be regulating the proteins

involved in the cleavage furrow and cell membrane integrity helping to understand the human diseases with alter cytokinesis.

Recently, it was published that PP2A^{B55} is inhibited upon MASTL/Greatwall overexpression in breast cancer (Rogers et al., 2018) or in hepatocarcinogenesis (Liye Cao et al., 2019). This inhibition prevents correct mitotic exit, and, consequently, cytokinesis fails (Rogers et al., 2018). In addition, the PP2A^{B55}/ENSA/Greatwall pathway regulates temporal order of mitotic events, ensuring that cytokinesis follows chromosome segregation (Cundell et al., 2013). These observations suggest that PP2A^{B55} could be also involved in cytokinesis and that our results in budding yeast could be extrapolated to human cells.

On the other hand, cytokinesis regulation is also important for the control of fungal infections. Some fungal therapies are directed to the perturbation of the yeast cell wall formation. The mechanism regulating secondary septum formation are conserved among fungal species. The knowledge of the proteins regulating cytokinesis will allow to improve those targeted therapies.

The cytokinetic proteins studied during this thesis have been also related to human disease. The Hof1 protein homolog in mammals, PSTPIP1 is highly expressed in hematopoietic tissues and lungs (Spencer et al., 1997). This protein is involved in actin organization during mammal's cytokinesis interacting with phosphatases (Spencer et al., 1997). PSTPIP1 is related to the inflammatory response by the association to a receptor of the T cells (Badour et al., 2003). In fact, it has been related to an autoimmune syndrome called pyogenic arthritis, pyoderma gangrenosum, and acne

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(PAPA) where the loss of the PEST region and, in consequence, its dephosphorylation promotes the autoimmune response (Shoham et al., 2003).

In addition, it was described that PP2A^{B56} (the PP2A^{Rts1} homolog) activates the RhoA GTPase and cleavage furrow ingression (C. G. Wu et al., 2017). PP2A^{B56} has been also involved in abscission opposing Aurora B phosphorylations (Fung, Kitagawa, Liao, Wong, & Lee, 2017).

In summary, studying the PP2A role in model organisms will help us to advance in our knowledge of cytokinesis in mammalian cells.

2. Cdc5 regulation on Net1 phosphorylation.

Mitosis exit requires the activation of the Cdc14 phosphatase. The phosphatase is kept sequestered at the nucleolus during most of the cell cycle by binding to the nucleolar protein Net1 (Visintin, 1999). During anaphase, two complementary pathways promote the Cdc14 release from the nucleolus and activation: the FEAR (Cdc14 early anaphase release) and the MEN (mitosis exit network). The release of Cdc14 through these pathways involves the phosphorylation of Net1 protein which reduces its affinity towards Cdc14. This phosphorylation is dependent on Clb2-Cdc28 in FEAR (Azzam et al 2004; Queralt 2006) and Dbf2-Mob1 in MEN (Mohl Da, 2009). However, it was suggested that other kinases, such as Cdc5, could be also involved in the Net1 phosphorylation. Cdc5 regulates indirectly the Net1 phosphorylation through the activation of Clb2-Cdc28 in FEAR (Liang F, 2009; Raspelli E, Facchinetti S and Fraschini R, 2018) and by the activation of MEN pathway (Kim J 2012; Baro 2013; Botchkarev VV Jr et al, 2017). In addition, ectopic Cdc5 expression promotes the Net1 phosphorylation and Cdc14 activation in pre-anaphase cells (Shou W, 2002), metaphase-arrested cells (Rodriguez-Rodriguez JA, 2016; Fig. R28), and FEAR-mutant cells (Visintin R, 2003). For this reason, in this thesis we studied the Cdc5 role in the MEN-independent phosphorylation of Net1.

Cdc5 was proposed to phosphorylate Net1 directly in a MEN independent pathway. It was described that Cdc5 phosphorylate *in vitro* 19 Net1 residues: T16, S30, S31, S48, S60, S64, T194, T195, T196, T197, S223, S224, S228, S231, S242, S295, S301, T302,

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S335 (Loughrey CS, 2002). The Net1(19m) mutant has reduced affinity towards Cdc14 *in vitro*, but only minor effects in Cdc14 release from the nucleolus *in vivo* (Shou W, 2002). In our study we performed a mass spectrometry analysis allowing the identification of several Net1 residues phosphorylated upon Cdc5 ectopic expression in metaphase (Table R1). 6 of these Net1 residues (S31, S48, S60, S64, S228 and S231) were already found in the *in vitro* assays (Shou 2002); and 7 were coincident to the detected by our peptide array (Fig. R26; S60, S64, S223; S242, S295, S301 and T302). In our analysis we obtained more candidate Net1 residues than in this first approach from CS Loughrey and colleagues (Figs. R26 and Table R1). The biggest difference is that we purified Net1 *in vivo* from yeast cells and the mass spectrometers have increased sensitivity over the years. However, the lack of *in vivo* relevance of the *net1(19'm)* mutant, indicates that the *in vitro* assays failed (Loughrey et al and our Net1 peptide assays) to identify the real Net1 phosphorylated residues. In our mass spectrometry analysis, Net1 was purified from yeast cells upon phosphorylated conditions. Therefore, proper Net1 folding is required for the identification of Net1 phosphorylations.

To study the Cdc5-dependent Net1 phosphorylation contribution to the Cdc14 activation we analyzed the Net1 residues containing the Cdc5 kinase consensus sequence (Tables R3 and R4). From our experiments we determined that the residues contained in the *net1_14A* and the S447 are candidates to be responsible of the Net1 phosphorylation required for Cdc5-dependent Cdc14 release. We observed that both strains have impaired Cdc14 release from the nucleolus during anaphase progression, showing the *net1_5A_S447* more severe phenotype (Figs. R27 and R29). This

would indicate that the Net1 residues included in the *net1-14A*, or part of them, and the S447 are required for Net1 phosphorylation in anaphase. However, further analysis must be done to demonstrate whether this phosphorylation is MEN-independent and if it is part of FEAR or a new parallel pathway. Genetic interaction analysis with the *net1* phosphorylation mutant will allow us to determine its influence as FEAR and/or MEN. However, we want first to narrow down the Net1 residues phosphorylated by Cdc5.

Upon the *CDC5* ectopic expression, we observed that the two *net1* mutants, *net1-14A* and *net1-5A_S447A* compromises the Cdc14 release from the nucleolus (Figs. R28 and R30). This phenotype further confirms the requirement of the residues found in *net1_14A* and the S447 for the Cdc14 release and suggests that they depend on the Cdc5 function. In the lab, we previously described that the Dbf2-Mob1 is inhibited by Clb2-Cdc28 (König C, Maekawa H and Schiebel E, 2010) dependent phosphorylation at metaphase despite the early release of the Cdc14 in *cdc55Δ* mutant cells (Baro B, 2013). Moreover, upon *CDC5* overexpression in *dbf2-2* cells Net1 is still phosphorylated in metaphase-arrested cells (Rodriguez-Rodriguez JA, 2016); indicating that Cdc5 ectopic expression promotes Net1 phosphorylation in different residues and independently of Dbf2-Mob1. By contrast, Net1 phosphorylation was reduced upon Cdc5 ectopic expression in separate mutants, *esp1-2* (Rodriguez-Rodriguez 2016). However, we cannot discriminate whether Cdc5 ectopic expression requires the FEAR pathway to phosphorylate Net1 on the Net1 14 residues and S447. We should repeat the experiments combining our *net1* mutant strains with separate mutant to see the phosphorylation under these conditions.

DISCUSSION

In preliminary results with the *net1_5A_Nterm* and *net1_4A_Cterm*, we showed that the *net1_5A_Nterm* mutant have a mild effect (Table R5), while *net1_4A_Cterm* mutant show a more pronounced effect over Cdc14 release. This is consistent with the *net1_4A_Cterm* mutant containing the S447 residue. Further analysis should be done to finally identify the Net1 residues phosphorylated *in vivo* by Cdc5 and to characterize its role during anaphase progression.

In parallel to the characterization of the Net1 residues phosphorylated by Cdc5, we wanted to study the Cdc5 contribution independently to Cdk1-Clb2. We prepared Net1-Cdc5 fusions in order to see the effect of the permanent Net1 phosphorylation by Cdc5 on Cdc14 regulation. However, the fact that the Net1-Cdc5 fusion strains, with and without active Cdc5, showed premature Cdc14 release from metaphase (Fig. R32) argues against Net1 phosphorylation by Cdc5. By contrast, a competition for the Net1 binding between Cdc14 and Cdc5 is suggested by the results obtained with the Net1-Cdc5 fusion. Further experiments will be required to prove this hypothesis. We cannot rule out that the phenotype is exacerbated in the Net1-Cdc5 fusions due to changes in the Net1 conformation in the fusions.

Despite the Cdc14 premature release in all fused strains, we found slight differences. *cdc5_N209A* showed, together with early Cdc14 release, a delay in the re-sequestration, too. Premature Cdc14 release and a delay in the Cdc14 re-sequestration was already seen in *net1Δ* mutants. Therefore, Cdc5 could be also necessary for timely release of Cdc14. Nevertheless, both possibilities can be embedded if we consider different roles for Cdc5. The first is the

influence over Net1 phosphorylation to promote Cdc14 release during anaphase. And, the second is the possibility that Cdc5 ensures the Cdc14 nucleolar localization before and after mitotic exit through the opposite role, the dephosphorylation of Net1 by the activation of a phosphatase. A candidate would be the PP2A^{Cdc55}, already described to dephosphorylate Net1. In addition, Cdc5 protein levels are tightly regulated during the cell cycle. Cdc5 protein appears at the end of the S phase and its degradation is mediated by Cdc14 activation of APC^{Cdh1} in late anaphase which facilitates the re-sequestration of Cdc14. Therefore, the competition between Cdc14 and Cdc5 for Net1 binding in a normal cell cycle is prevented by regulating the Cdc5 protein levels and restricting Cdc5 to mitosis.

Taking all together, we hypothesize that the Cdc5 kinase regulates the Cdc14 release from the nucleolus positively phosphorylating Net1 directly, competing with Cdc14 to bind to Net1, and promoting the activation of the MEN pathway, and negatively promoting the nucleolar localization of Cdc14 by activating the PP2A^{Cdc55}.

CONCLUSIONS

1. PP2A^{Cdc55} role in cytokinesis

1. PP2A^{Cdc55} dephosphorylates Hof1 and Chs2 *in vivo* and *in vitro*, indicating that they are new PP2A^{Cdc55} substrates.
2. PP2A^{Cdc55} contributes to the regulation of the dephosphorylation of Cyk3 and Inn1, *in vivo*.
3. PP2A^{Cdc55} forms a complex with Hof1, Cyk3, Inn1 and Chs2.
4. The actomyosin ring and the IPC's proteins present asymmetric signal upon AMR constriction in absence of Cdc55, indicating a dysfunction in the AMR.
5. PP2A^{Cdc55} regulates the residence time at the bud neck of the IPC's proteins.
6. The lack of PP2A^{Cdc55} activity provokes a reduction in primary and secondary septum formation.
7. The remedial septum formation allows abscission and cytokinesis completion in absence of Cdc55.
8. PP2A^{Cdc55} prevents actin re-polarization at the new bud site until cytokinesis is completed.
9. Our genetic studies indicate that PP2A^{Cdc55} has an opposite role to Chs2, and acts in parallel to Hof1 and Cyk3 in cytokinesis.

2. Cdc5 regulation on Net1 phosphorylation

1. The Net1 S447 residue and some included in the *net1-14A* (S115A, S166A, S242A, T288A, S415A, S416A, S511A, T534A, S561A, T564A, S579A, T584A, T589A and T603A) are the most probable residues phosphorylated by Cdc5 required to promote Cdc14 activation.
2. The Net1 peptide array experiments identify the same unspecific residues than previous studies using Net1 phosphorylated *in vitro* by Cdc5; suggesting that the presence of a proper folded Net1 protein is required for the identification of the Net1 residues phosphorylated by Cdc5 *in vivo*.
3. The Net1-Cdc5 fusion promotes the Cdc14 release from the nucleolus in an independent way to its kinase activity.

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ANNEX I: SUPPLEMENTARY ARTICLES

Rodriguez-Rodriguez, J. A., Moyano, Y., Játiva, S., & Queralt, E., (2016). [Mitotic Exit Function of Polo-like Kinase Cdc5 Is Dependent on Sequential Activation by Cdk1.](#) *Cell Reports*, 15(9), 2050-2062.

Játiva, S., Calabria, I., Moyano-Rodriguez, Y., Garcia, P., & Queralt, E., (2019) [Cdc14 activation requires coordinated Cdk1-dependent phosphorylation of Net1 and PP2A–Cdc55 at anaphase onset.](#) *Cellular and Molecular Life Sciences*, 76 (18), 3601-3620.

ANNEX II: LIST OF ABBREVIATIONS

AID: Auxin inducible degron
AMR: Actomyosin ring
APC/C: Anaphase promoting complex or Cyclosome
CAK: Cdk1-activating kinase
CAR: Contractile actomyosin ring
CC2: Coiled coil 2
Cdk: Cyclin-dependent kinase
CHD: Calponin homolog domain
CKI: Cyclin-dependent kinase inhibitor
Clb: Cyclin B
Cln: Cyclin N
COPII: Conserved coat protein complex II
CR: Contractile ring
CS: Chitin synthase
CW: Cell wall
CWI: Cell wall integrity
dSPB: daughter Spindle pole body
ECB: Early cell cycle box
ECM: Extracellular matrix
ELC: Essential light chain of Myo1
ER: Endoplasmic reticulum
ESCRT-III: Endosomal sorting complex required for transport III
FEAR: cdcFourteen early anaphase release
GAP: GTPase activating protein
GEF: Guanine-nucleotide exchange factor
GlcNAc: N-acetylglucosamine
GRD: GAP related domain
GTP: Guanosine triphosphate
IAA: Indole-3-acetic acid or auxin
IPC: Ingression progression complex

MAPK: Mitogen activated protein kinase
MBF: MCB binding factor
MCB: Mlu1 cell cycle box
MCC: Mitotic checkpoint complex
MEN: Mitotic exit network
MM: Minimum medium
mSPB: mother Spindle pole body
NTS: Non-transcribed spacer
PAK: p21-activated kinase
PBD: Polo-box domain
PIP₂: Phosphatidylinositol biphosphate
PKA: Ras/cAMP-dependent protein kinase
PM: Plasma membrane
PP2A: Protein phosphatase 2 A
PS: Primary septum
RCC: Renal cell carcinoma
rDNA: ribosomic DNA
RENT: Regulator of nucleolar silencing and telophase
RLS: Ring location sequence
RS: Remedial septum
SAC: Spindle assembly checkpoint
SBF: SCB binding factor
SCB: Swi4/Swi6 cell cycle box
SCF: Skp1–Cul1–F-box-protein ubiquitin ligase
SH3: SRC homolog 3
SL: Synthetic lethal
snoRNP: small nucleolar Ribonucleoproteins
SPB: Spindle pole body
SPoC: Spindle position checkpoint
SS: Secondary septa

TD1: Targeting domain 1

TF: Transcription factor

TGN: Trans-Golgi network

WT: Wild type

