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Cell cycle regulation of septins: implications for cytokinesis and the spindle position checkpoint

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…to myself and to those
who believed in me…

"…on ne voit bien
qu'avec le Cœur.
L'essentiel est invisible
pour les yeux...»

-Le Petit Prince-
A.dSE

Contents

Abstract

In most eukaryotic cells, cytokinesis is driven by a contractile actomyosin ring, which forms at the site of cell division and drives furrow ingression. In metazoans and fungi cytokinesis requires also other cytoskeletal proteins called septins. Septins are evolutionarily conserved proteins that can hydrolyse GTP and form polymers that assemble into higher order structures, such as filaments and rings. They can interact both with the actin and microtubule cytoskeleton and with membranes. Besides being involved in cytokinesis, septins are involved in many other functions, such as polarized growth, vescicle trafficking, cellular morphology and the creation of diffusion barriers.

In budding yeast the first step towards cytokinesis is the assembly of a rigid septin ring composed of five different septins (Cdc3, Cdc10, Cdc11, Cdc12 and Shs1) at the bud neck, the constriction between the mother cell and the bud where cytokinesis takes place. The septin ring, which is formed in G1 at the site of future bud emergence, expands into a broader hourglass structure as cells bud and enter S phase, and it splits into two separate rings just before cytokinesis. These structural changes are accompanied by dynamic transitions. In fact, septins are static (during the "frozen" state) throughout most of the cell cycle and dynamic (during the "fluid" state) in late G1 and prior to cytokinesis. The transition from fluid to frozen state is promoted by protein kinases, such as Gin4 and the PAK kinase Cla4, which localize at the bud neck and directly phosphorylate septins. Conversely, the Tem1 GTPase and the PP2A protein phosphatase likely regulate the reverse transition.

The yeast septin ring is also involved in proper spindle positioning, which is in turn crucial for balanced chromosome partitioning. Whenever budding yeast cells experience spindle positioning defects, they undergo anaphase within the mother cell, and then hold on in telophase with elongated spindles and high levels of mitotic CDKs. This cell cycle delay is imposed by the spindle position checkpoint (SPOC), which prevents mitotic exit and cytokinesis until errors are corrected, thus avoiding the generation of anucleate and binucleate cells. The SPOC target is the Tem1 GTPase, whose active GTP-bound form promotes a signal transduction cascade called Mitotic Exit Network (MEN) that ultimately drives cells out of mitosis by leading to inactivation of mitotic CDKs. The dimeric GTPase-activating protein (GAP) Bub2/Bfa1 keeps Tem1 inhibited until the spindle is properly aligned, thus coupling mitotic exit with nuclear division. The Kin4 protein kinase is also involved in the SPOC, by keeping Bfa1

active and by regulating the dynamics of Bub2/Bfa1 at spindle poles. In addition, the Elm1 kinase and the PP2A phosphatase contribute to the SPOC at least partly through Kin4 activation.

Tem1 and several downstream MEN components are found at SPBs in a cell cycle-regulated manner and are thought to promote mitotic exit from this location. The Bub2/Bfa1 complex is found predominantly on the SPB that is pulled towards the bud, while it is present on both SPBs of misaligned spindles. Conversely, the Lte1 protein, which positively regulates Tem1, is confined in the bud from the G1/S transition to telophase, when it spreads throughout the cytoplasm of both mother cell and bud. Septins participate in the SPOC by preventing the unscheduled diffusion of Lte1 into the mother cell.

Our laboratory had previously implicated the two functionally redundant ubiquitin-ligases Dma1 and Dma2 in proper septin ring positioning, cytokinesis and SPOC regulation. During my PhD, I have been trying to gain insights into the molecular mechanisms by which Dma proteins regulate these processes.

Lack of both Dma1 and Dma2 compromises septin ring assembly and has additive effects with the deletion of the PAK kinase *CLA4*. Indeed, we found that Dma proteins are essential together with Cla4 for septin ring stabilization throughout the cell cycle. Consistently, FRAP (Fluorescence Recovery After Photobleaching) analyses revealed that concomitant deletion of *DMA1* and *DMA2* increases septin turnover at the bud neck, thus destabilizing the septin ring. Conversely, overexpression of *DMA2* stabilizes the septin ring and delays its disassembly at the end of mitosis. Genetic analyses showed that lack of both Dma proteins is lethal when combined with the deletion or mutation in septin genes or genes involved in septin ring assembly and/or stabilization, underlying the importance of these two proteins in the control of septin ring dynamics.

We also showed that the role of Dma1 and Dma2 in the SPOC is not due to Lte1 spreading in the mother cell and seems to be also independent of Bub2/Bfa1. Rather, Dma1 and Dma2 appear to control localization of the Elm1 kinase at the bud neck, thus providing a mechanistic explanation for their role in both septin dynamics and SPOC.

Being Dma proteins ubiquitin ligases, we hypothesised that they could target for ubiquitylation a regulator of cytokinesis. Therefore, we carried out a genetic screen for extragenic suppressors of the lethality of *dma1*Δ *dma2*Δ *cla4Δ* cells. We got 44 mutants that could potentially identify targets of Dma1/2. We initially focused our

attention on the dominant mutations, because some of them suppressed very efficiently the cytokinesis and septin deposition defects of *dma1*Δ *dma2*Δ *cla4*Δ cells. Remarkably, cloning and sequencing revealed that one of the suppressors corresponds to *RHO1*, encoding for the yeast counterpart of metazoan RhoA. Rho1/RhoA is a conserved GTPase that is required for the assembly and the contraction of the actomyosin ring. The *RHO1* mutant allele that we have isolated in our screen is novel (*RHO1-D72N*) and, strikingly, three additional suppressors carried exactly the same *RHO1* mutation. The *RHO1-D72N* allele is likely hyperactive, since we could also suppress the lethality of *dma1*Δ *dma2*Δ *cla4*Δ cells using known *RHO1* hyperactive alleles, such as *RHO1-G19V*, but not by dominant-negative alleles, such as *rho1-D125A*. Since protein kinase C (PKC) is a major target of yeast Rho1, we tested whether a hyperactive *PKC1* allele (*PKC1-R398P*) could also suppress the lethality of the *dma1*Δ *dma2*Δ *cla4*Δ triple mutant and found that this was indeed the case. In addition, both *RHO1-D72N* and *PKC1-R398P* alleles were able to suppress partially the temperature-sensitivity of *cdc12* mutants, which undergo septin ring disassembly at high temperature. These data strongly argue that Rho1/Pkc1 hyperactivation stabilizes the septin ring. Indeed, genetic and FRAP analyses showed that the septin ring is more stable in *RHO1* and *PKC1* hyperactive mutants, while lack of these proteins destabilized it. Finally we also found that the deletion of *RTS1*, which promotes septin ring disassembly at the end of the cell cycle, suppresses the lethality and septin ring defects of *dma1Δ dma2Δ cla4Δ* cells. Altogether, our data suggest that Dma1/2 might promote septin stabilization by activating Elm1, the Rho1/Pkc1 pathway and/or inhibiting $PP2A^{Rts1}$, directly or indirectly. Whether these proteins are direct ubiquitylation targets of Dma1 and Dma2 is an important issue to be addressed in the future.

Riassunto

Nella maggior parte delle cellule eucariotiche, la citochinesi è guidata da un anello contrattile di actomiosina, che si forma al sito di divisione cellulare. Sia nei metazoi che nei funghi la citochinesi richiede inoltre la presenza di altre proteine citoscheletriche chiamate septine. Le septine sono una famiglia di proteine evolutivamente conservate che idrolizzano il GTP e formano polimeri che si assemblano in strutture complesse, come filamenti e anelli. Le septine sono in grado di interagire sia con il citoscheletro di actina e i microtubuli, che con le membrane. Oltre ad essere implicate nella citochinesi, le septine sono coinvolte in molte altre funzioni, come la crescita polarizzata, il traffico vescicolare, la morfologia cellulare e la creazione di barriere di diffusione.
Nel lievito gemmante il primo passaggio che porta alla citochinesi è l'assemblaggio di un anello rigido di septine, composto da 5 subunità di septine diverse (Cdc3, Cdc10, Cdc11, Cdc12 e Shs1) al collo della gemma (bud neck), la costrizione tra cellula madre e gemma dove ha luogo la citochinesi. L'anello di septine, che si forma nella fase G1 del ciclo cellulare al futuro sito di emergenza della gemma, si espande in una struttura a clessidra non appena le cellule gemmano ed entrano in fase S, e si separa in due anelli distinti appena prima della citochinesi. Questi cambiamenti strutturali sono accompagnati da transizioni dinamiche. Infatti, le septine sono statiche (durante lo stato "rigido") per la maggior parte del ciclo cellulare, e dinamiche (durante lo stato "fluido") in tarda fase G1 e prima della citochinesi. La transizione dallo stato fluido a quello rigido è promossa da protein chinasi, come Gin4 e la PAK chinasi Cla4, che si localizzano al bud neck e fosforilano direttamente le septine. Al contrario, la GTPasi Tem1 e la proteina fosfatasi PP2A regolano probabilmente la transizione inversa.
In lievito l'anello di septine è anche implicato nel posizionamento del fuso mitotico, che è a sua volta cruciale per una ripartizione cromosomica bilanciata. Ogni volta che cellule di lievito mostrano difetti nel posizionamento del fuso, vanno incontro ad anafase nella cellula madre, e si arrestano in telofase con fusi allungati e alti livelli di CDKs mitotiche. Questo ritardo nel ciclo cellulare è garantito dall'attivazione del checkpoint da posizionamento del fuso (SPOC), che impedisce l'uscita dalla mitosi e la citochinesi finchè gli errori non sono corretti, impedendo così la generazione di cellule anucleate e binucleate. Il bersaglio molecolare dello SPOC è la GTPasi Tem1, la cui forma attiva legata al GTP promuove una cascata di trasduzione del segnale chiamata Mitotic Exit Network (MEN), che, portando all'inattivazione delle CDKs mitotiche, favorisce in ultimo l'uscita

dalla mitosi. La GTPase-activating protein (GAP) dimerica Bub2/Bfa1 inibisce l'attivazione di Tem1 fino a che il fuso mitotico non è correttamente orientato, accoppiando così l'uscita dalla mitosi con la divisione nucleare. Anche la proteina chinasi Kin4 è coinvolta nello SPOC, mantenendo attiva Bfa1 e regolando la dinamica di Bub2/Bfa1 ai poli del fuso (SPBs). Inoltre, la proteina chinasi Elm1 e la proteina fosfatasi PP2A contribuiscono allo SPOC almeno in parte attraverso l'attivazione di Kin4.

Tem1 e molti altri componenti del MEN si localizzano agli SPBs in modo regolato durante il ciclo cellulare e si pensa che questa particolare localizzazione possa promuovere l'uscita dalla mitosi. Il complesso Bub2/Bfa1 si localizza prevalentemente sullo SPB che entra nella gemma, mentre è presente su entrambi gli SPBs di fusi misposizionati. Al contrario, la proteina Lte1, che regola positivamente Tem1, è confinata alla corteccia della gemma dalla transizione G1/S alla telofase, quando diffonde nel citoplasma di cellula madre e gemma. Le septine partecipano allo SPOC prevenendo la prematura diffusione di Lte1 nella cellula madre.

Precedentemente, nel nostro laboratorio, le due ubiquitina-ligasi funzionalmente ridondanti Dma1 e Dma2 erano state implicate nel corretto posizionamento dell'anello di septine, nella citochinesi e nella regolazione dello SPOC. Durante il mio PhD, ho cercato di approfondire i meccanismi molecolari tramite i quali le proteine Dma regolano questi processi.

La contemporanea mancanza di Dma1 e Dma2 compromette l'assemblaggio dell'anello di septine e ha effetti additivi se associata alla delezione della PAK chinasi *CLA4*. Infatti, abbiamo osservato che le proteine Dma sono essenziali, insieme con Cla4 per la stabilizzazione dell'anello di septine durante il ciclo cellulare. Inoltre, esperimenti di FRAP (Fluorescence Recovery After Photobleaching) mostrano che la contemporanea delezione di *DMA1* e *DMA2* aumenta il turnover di septine al bud neck, destabilizzando così l'anello di septine. Al contrario, l'overespressione di *DMA2* stabilizza l'anello di septine e rallenta il suo disassemblaggio alla fine della mitosi. Analisi genetiche mostrano che la contemporanea mancanza delle proteine Dma è letale se abbinata a delezioni o mutazioni in geni che codificano per septine o che sono coinvolti nell'assemblaggio e/o stabilizzazione dell'anello di septine, sottolineando l'importanza di queste due proteine nel controllo della dinamica dell'anello.

Abbiamo inoltre dimostrato che il ruolo di Dma1 e Dma2 nello SPOC non è dovuto alla diffusione di Lte1 nella cellula madre e sembra

essere anche indipendente da Bub2/Bfa1. Al contrario, Dma1 e Dma2 sembrano controllare la localizzazione della proteina chinasi Elm1 al bud neck, fornendo così una possibile spiegazione del loro ruolo sia nella regolazione dell'anello di septine che nello SPOC.
Poichè le proteine Dma sono ubiquitina ligasi, abbiamo ipotizzato che potessero avere come target un regolatore della citochinesi. Per questo abbiamo eseguito uno screening genetico alla ricerca di soppressori extragenici della letalità di cellule *dma1*Δ *dma2*Δ *cla4Δ*. Abbiamo ottenuto 44 mutanti che potevano portare all'identificazione di potenziali bersagli di Dma1/2. Inizialmente abbiamo focalizzato la nostra attenzione sulle mutazioni dominanti, poiché alcune di esse erano in grado di sopprimere in modo efficiente i difetti di citochinesi e di deposizione dell'anello di septine di cellule *dma1*Δ *dma2*Δ *cla4*Δ. Tramite clonaggio e sequenziamento abbiamo scoperto che uno dei soppressori dominanti contiene una mutazione nel gene *RHO1*, che codifica per la controparte in lievito di RhoA nei metazoi. Rho1/RhoA è una GTPasi conservata, richiesta per l'assemblaggio e la contrazione dell'anello di actomiosina. L'allele mutante di *RHO1* che abbiamo isolato nel nostro screening è nuovo (*RHO1-D72N*) e inoltre altri tre soppressori portavano esattamente la stessa mutazione. L'allele *RHO1-D72N* è probabilmente iperattivo, poiché è possibile sopprimere la letalità di cellule *dma1*Δ *dma2*Δ *cla4*Δ usando alleli iperattivi noti di *RHO1*, come *RHO1-G19V*, ma non mediante l'espressione di alleli dominanti-negativi, come *rho1-D125A*. Poiché la proteina chinasi C (PKC) è uno dei maggiori target di Rho1 in lievito, abbiamo verificato e potuto confermare che anche un allele iperattivo di *PKC1* (*PKC1-R398P*) sopprime la letalità del triplo mutante *dma1*Δ *dma2*Δ *cla4*Δ. Inoltre, entrambi gli alleli *RHO1-D72N* e *PKC1-R398P* sono in grado di sopprimere parzialmente la termo-sensibilità di mutanti *cdc12*, che disassemblano l'anello di septine ad alte temperature. Questi dati suggeriscono che l'iperattivazione di Rho1/Pkc1 stabilizzi l'anello di septine. In aggiunta, analisi genetiche e di FRAP mostrano che l'anello di septine è più stabile in mutanti iperattivi di *RHO1* e *PKC1*, mentre la mancanza di queste proteine lo destabilizza. Infine abbiamo anche osservato che la delezione di *RTS1*, che promuove il disassemblaggio dell'anello di septine alla fine del ciclo cellulare, sopprime la letalità e i difetti nell'assemblaggio dell'anello di septine di cellule *dma1Δ dma2Δ cla4Δ*. Complessivamente i nostri dati suggeriscono che Dma1/2 possano promuovere la stabilizzazione dell'anello di septine mediante l'attivazione di Elm1, del pathway di Rho1/Pkc1 e/o inibendo

$PP2A^{Rts1}$, in maniera diretta o indiretta. In futuro sarà interessante investigare se queste proteine siano regolate da Dma1 e Dma2 direttamente mediante ubiquitinazione.

Introduction

Asymmetric cell division and spindle positioning

Asymmetric cell division is widespread in nature and generates unequal daughter cells with different size and/or fate. This modality of cell division is characteristic of several unicellular organisms, either prokaryotic (such as the bacterium *Caulobacter crescentus*) or eukaryotic (such as the budding yeast *Saccharomyces cerevisiae*), and is essential for embryonic development of multicellular eukaryotes. A paradigmatic example of asymmetric cell division is that of stem cells, which generate one daughter cell committed to differentiate and another stem cell that maintains its identity and self-renewing potential. This contributes to organ formation during development and to the homoeostasis of tissues during adulthood. Disrupting asymmetric cell division can lead to uncontrolled cell proliferation and, ultimately, to cancer (reviewed in Gonzalez, 2007; Fraschini et al., 2008). Asymmetric cell division has been studied to a great extent in invertebrate model systems, such as *Drosophila melanogaster* neuroblasts (Fig. 1A) and *Caenorhabditis elegans* embryos. The budding yeast *S. cerevisiae* has also proven to be an excellent model system to investigate the molecular mechanisms governing asymmetric cell division. Budding yeast divides asymmetrically, producing daughter cells (originating from the buds) that at the moment of division are smaller than their mothers. It has been proposed that the bud might be the primitive stem cell equivalent in yeast, in that it can divide for a greater number of generations than its mother (Yamashita and Fuller, 2008). The axis of cell polarity is established during the G1 phase of the cell cycle through localization of determinants that define the site of bud emergence to a particular

site at the cell cortex that depends on the site of the previous cell division. After polarity establishment, the mitotic spindle must be correctly aligned with respect to the polarity axis in order to ensure accurate chromosome segregation. Budding yeast undergoes a close mitosis, without nuclear envelope breakdown. Thus spindle position corresponds to nuclear position. Cytoplasmic (or astral) microtubules (cMTs) emanate from the SPBs (spindle pole bodies), the MT (microtubule)-organizing centres, and are highly dynamic. cMTs are essential for nuclear positioning by connecting the SPB, which is embedded in the nuclear envelope, with the cell cortex, thus promoting the generation of forces that move the nucleus towards and inside the bud neck. Two sequential processes, the Kar9 pathway and the dynein pathway, contribute to spindle positioning (Fig. 1B and C) (reviewed in Fraschini et al., 2008). Either pathway is dispensable for cell viability, whereas inactivation of both is lethal (Miller and Rose, 1998). Kar9 is localized at the SPB and is translocated to MT plus ends through interaction with the plus end directed motor Kip2 and the MT-associated protein Bim1 (Lee et al., 2000; Maekawa et al., 2003). Through these interactions, the Kar9 pathway promotes capture of cMTs with the bud cortex prior to anaphase (Fig. 1B). The second pathway of spindle positioning requires the minus end-directed motor dynein (Yeh et al., 1995) that associates with the cortical anchor Num1 (Farkasovsky and Kuntzel, 2001; Heil-Chapdelaine et al., 2000). The dynein pathway acts predominantly during anaphase and might contribute to the cMT capture by the bud tip through sliding plus-ends of cMTs along the cortex (reviewed in Pearson and Bloom, 2004) (Fig. 1C). These complicated interactions generate forces that

place the nucleus at the bud neck and orientate the mitotic spindle with respect to the division axis.

The accuracy of spindle positioning is partly ensured by an asymmetry of spindle poles. In vertebrate cells, the old centriole migrates to the site of the cytokinetic furrow, indicating the existence of a specific pattern of centrosome inheritance (Piel et al., 2000). In *C. elegans*, some proteins are specifically recruited to one spindle pole (Kirkham et al., 2003; Leidel and Gonczy, 2003), whereas in *Drosophila* germline stem cells, the mother centrosome is segregated into the cell that maintains stem cell identity (Yamashita et al., 2007). Similarly, in budding yeast, the old SPB is inherited by the daughter cell, while the newly synthesized SPB stays in the mother cell (Pereira et al., 2001). SPB asymmetry is established at least at three levels: (I) the activity of S-phase CDK (cyclin-dependent kinase) transiently inhibits MT nucleation from the new SPB until formation of the bipolar spindle (Segal et al., 2000); (II) Kar9 localization is restricted exclusively to the bud-directed SPB (Maekawa et al., 2003; Liakopoulos et al., 2003); (III) dynein is also asymmetrically localized on the SPB moving towards the bud, but only in metaphase (Grava et al., 2006; Shaw et al., 1997).

Homologues of the above proteins regulate asymmetric cell division in higher eukaryotes. Indeed the dynein complex is involved in proper spindle positioning in *Drosophila* neuroblasts (Yamashita and Fuller, 2008). Kar9 shares limited homology with APC (adenomatous polyposis coli), a MT-binding and –regulating protein that is in turn modulated by a protein complex involved in polarity establishment in mammals (Bienz, 2001; Etienne-Manneville and Hall, 2003;

Zumbrunn et al., 2001). APC is required for the control of spindle orientation in *Drosophila* stem cells to drive asymmetric cell division (Yamashita et al., 2003). Altogether, evolutionary conservation of these proteins indicates that the molecular mechanisms at the basis of spindle polarity and positioning might be conserved from yeast to humans.

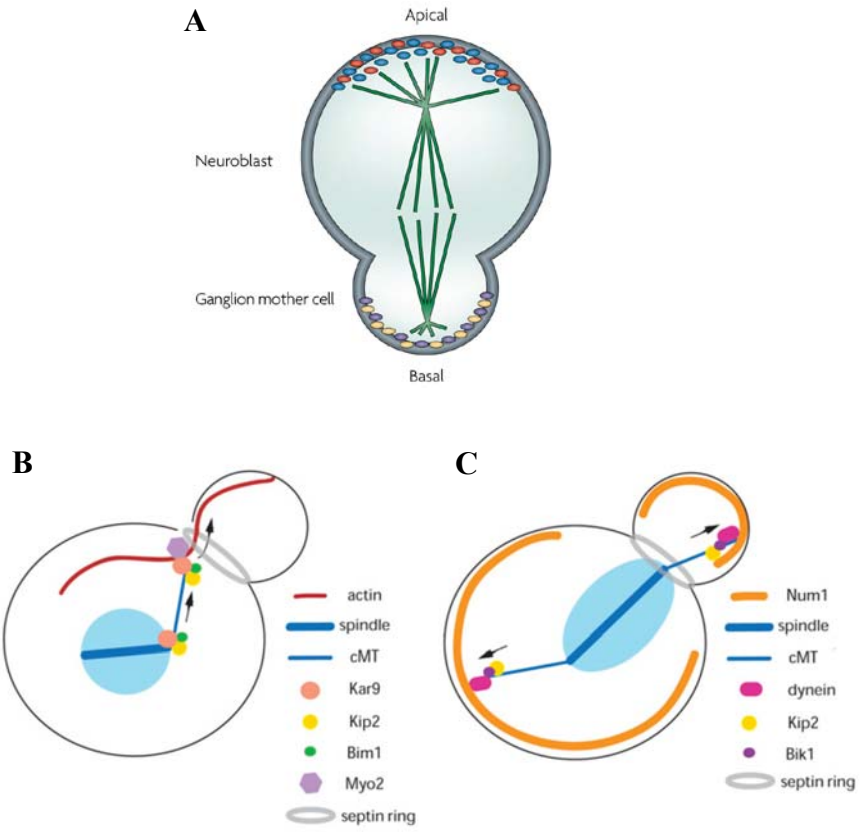


Figure 1. Asymmetric cell division in eukaryotes. A: The asymmetric division of neuroblasts in *Drosophila melanogaster* involves the segregation of basally localized cell-fate determinants, that in turn establishes proper spindle positioning (from Gonzalez, 2007). **B, C**: The two pathways of budding yeast spindle positioning: the Kar9 pathway (B) and the dynein pathway (C) (from Fraschini et al., 2008).

Regulation of mitotic exit

In all eukaryotes mitotic exit takes place when mitotic cyclin-dependent kinases (CDKs) are inactivated, a task that is usually fulfilled by cyclin proteolysis. Mitotic CDKs inactivation is in turn necessary for spindle disassembly, licensing of replication origins and cytokinesis (reviewed in Piatti et al., 2006). Regulation of mitotic exit has been best studied in budding yeast, where the Cdc14 phosphatase is essential for mitotic exit. *cdc14* mutants arrest in a telophase-like mitotic state, with an elongated mitotic spindle and bilobed anaphase nucleus. Experimental analysis placed *CDC14* function after nuclear division but before cytokinesis consistent with a role of the phosphatase in spindle disassembly and cytokinesis during mitotic exit (reviewed in Queralt and Uhlmann, 2008). Budding yeast cells in which mitotic CDK activity cannot be downregulated during mitotic exit, owing to expression of undegradable mitotic cyclin, display an arrest phenotype reminiscent of *cdc14* mutant cells (Surana et al., 1993). Indeed, the first well-characterised role of Cdc14 became its contribution to CDK downregulation by dephosphorylation, through activation of the CDK inhibitor Sic1, its transcription factor Swi5, and the Cdh1 activator of the anaphase promoting complex (APC) that promotes cyclin B proteolysis (Visintin et al., 1998). In addition, the structure of Cdc14 suggests that it reverses phosphorylation events catalysed by proline-directed kinases, such as CDKs (Gray et al., 2003). Consistent with its apparent execution point after nuclear division, circumstantial evidence linked activation of the Cdc14 phosphatase to elongation of the anaphase spindle (Bardin et al., 2000; Pereira et al., 2000). In this way, Cdc14-dependent mitotic exit would

not set in before successful chromosome segregation. Indeed, Cdc14 is kept inactive throughout most of the cell cycle, anchored in the nucleolus by tight binding to the Net1/Cfi1 inhibitor (Shou et al., 1999; Visintin at al., 1999) and in case of spindle mispositioning Cdc14 is maintained sequestered in the nucleolus, thereby preventing mitotic exit (D'Amours and Amon, 2004; Geymonat et al., 2002; Stegmeier and Amon, 2003). Cdc14 is partially released into the nucleoplasm at the metaphase to anaphase transition by the FEAR (Cdc fourteen early anaphase release) pathway, whereas the MEN (mitotic exit network) drives its full release also into the cytoplasm later in anaphase (Fig. 2A), thus allowing it to dephosphorylate its targets (reviewed in Piatti et al., 2006). The ultimate goal of the FEAR and MEN pathways is to bring about Net1/Cf1 phosphorylation that allows its dissociation from Cdc14 (Stegmeier and Amon, 2004). Proteins involved in the FEAR pathway are the Esp1/separase, the polo kinase Cdc5, the kinetochore protein Slk19, the small nuclear protein Spo12 and its homologue Bns1 and the nucleolar protein Tof2 (Stegmeier et al., 2002; Visintin et al., 2003; Geil et al., 2008; Waples et al., 2009). In addition, the early anaphase release of Cdc14 is negatively regulated by the $PP2A^{Cdc55}$ phosphatase (Queralt et al., 2006) and the replication fork block protein Fob1, which binds to Spo12 and presumably inhibits it (Stegmeier et al., 2004). $PP2A^{Cdc55}$ counteracts CDK-dependent (and perhaps polo-dependent) phosphorylation of Net1/Cf1. Its activity is regulated by Esp1, possibly through the Zds1 and Zds2 proteins that are bound to $PP2A^{Cdc55}$ and likely inhibit it (Queralt et al., 2006; Queralt and Uhlmann, 2008). Less clear is how the other FEAR components

intervene in the pathway. Genetic analyses of this pathway have suggested that *ESP1* and *SLK19* act in parallel to *SPO12* and *BNS1*. It seems that Slk19 forms a complex with Esp1 that is required for Esp1 kinetochore localization (Sullivan and Uhlmann, 2003). Moreover, there are evidences suggesting a possible role of Esp1 and Slk19 in regulating Cdc5 activity during anaphase (Sullivan and Uhlmann, 2003: Rahal and Amon, 2008). In particular, since Cfi1/Net1 phosphorylation is partly dependent on Cdc5 (Shou et al., 2002; Visintin et al., 2003), it is possible that Esp1 guides Cdc5 kinase activity towards these substrates during anaphase.

While the FEAR is dispensable for mitotic exit, the MEN is absolutely required for this process. The MEN is a signal transduction cascade that includes several protein kinases, such as Cdc5, Cdc15, Dbf2 and its associated activator Mob1 (Fig. 2B). A similarly organized pathway, the septation initiation network (SIN), promotes cytokinesis in fission yeast (Simanis, 2003; Bardin and Amon, 2001). Most MEN components are localized during mitosis at microtubule organizing centers, called spindle pole bodies (SPBs) in yeast, whereas they can also be found at the septum just before cytokinesis. SPB localization of MEN components correlates with MEN activation, and mutations that disrupt this localization, like temperature-sensitive mutations in the SPB scaffold component Nud1, lead to a telophase arrest (Visintin and Amon, 2001; Gruneberg et al., 2000). Interestingly, components of the fission yeast SIN, which comprises proteins homologous to those of the MEN, display a similar localization pattern. Polarity proteins, such as Gic1, Gic2, Ste20, Cla4 and Cdc42 also participate to MEN activation (Hoefken and Schiebel, 2002; Seshan et al., 2002;

Chiroli et al., 2003; Hoefken and Schiebel, 2004). The G-protein Tem1 (Spg1 in fission yeast) triggers MEN activation by activating the Cdc15 kinase (Shou et al., 1999; Shirayama et al., 1994) and is the direct target of the spindle position checkpoint (SPOC), which delays mitotic exit in case of spindle mispositioning (Simanis, 2003; Bardin and Amon, 2001). Whenever the spindle is mispositioned relative to the mother-bud axis, Tem1 is maintained inactive, to provide the time for spindle re-orientation. Due to its central role as a trigger of mitotic exit, Tem1 activity is finely tuned by positive (Lte1) and negative (Bub2, Bfa1 and Kin4) regulators, which all contribute to coupling correctly oriented nuclear division with mitotic exit. Lte1 was first identified as a positive mitotic exit regulator because the failure of *lte1* mutants to grow at low temperature occurs with an arrest at telophase (Shirayama et al., 1994). Lte1 shares homology with the guanosine nucleotide exchange domain of the Ras-GEF Cdc25 (Shirayama et al., 1994). Thus, when *TEM1* was isolated as a high copy number suppressor of the cold sensitivity of *lte1* mutants, it was proposed that Lte1 might be a GEF for this small GTPase (Keng et al., 1994; Shiryama et al., 1994). More recent data have challenged this notion, since the GEF domain of Lte1 seems dispensable for its MEN-promoting activity (Yoshida et al., 2003). In addition, Lte1 does not display in vitro GEF activity towards Tem1 but rather regulates Bub2/Bfa1 localization at SPBs (see below, Geymonat et al., 2009). Bfa1 and Bub2 are negative regulators of the MEN (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999) that together act as a two component GTPase Activating Protein (GAP) for Tem1 *in vitro* (Geymonat et al., 2003; Fraschini et al., 2006). The

GAP activity resides on Bub2 (Fraschini et al., 2006; Geymonat et al., 2003), which carries a GAP TBC domain (Tre-2, Bub2 and Cdc16, Neuwald, 1997), whereas Bfa1 is required for Bub2 interaction with Tem1 (Ro et al., 2002). When the mitotic spindle is properly aligned along the mother-bud axis Tem1 activation is promoted by inhibition of Bub2/Bfa1 through phosphorylation by the Polo-like kinase Cdc5 (Bardin et al., 2000; Pereira et al., 2000; Hu et al., 2001). Circumstantial evidence suggests that Bfa1 might inhibit Tem1 also independently of Bub2; indeed, Bfa1 interferes with Tem1 binding to its effector kinase Cdc15 and its overexpression inhibits mitotic exit independently of Bub2 (Ro et al., 2002; Kim et al., 2004).

The localization of Tem1 and its regulators is tightly controlled during the cell cycle. Tem1, which is present on the single SPB in G1, gets distributed with roughly equal amounts on both SPBs at the time of SPB duplication, but later on, at the metaphase to anaphase transition, it is found more concentrated on the bud-directed SPB (Pereira et al., 2000; Molk et al., 2004; Bardin et al., 2000). However, a small fraction of Tem1 can still be found on the mother cell SPB also after anaphase (Pereira et al., 2000; Molk et al., 2004). Bub2/Bfa1 subcellular localization follows essentially the same pattern, with the notable exception that the complex completely disappears from the SPB remaining in the mother cell at the onset of anaphase (Pereira et al., 2000; Molk et al., 2004; Bardin et al., 2000). Bub2 and Bfa1 are actually required for Tem1 SPB localization during interphase and mitosis, but no longer in late anaphase (Bardin et al., 2000). Lte1 also shows a peculiar localization pattern, as it is confined in the bud (mostly at the bud cortex) throughout most of the cell cycle, spreading

into the cytoplasm of both mother and daughter after the nucleus has migrated into the bud (Pereira et al., 2000; Molk et al., 2004; Bardin et al., 2000). The spatial segregation of Lte1 and Tem1 has led to an attractive model that explains how nuclear division might be coupled with mitotic exit. According to this model, Tem1 is kept inactive at SPBs by Bub2/Bfa1 until the nucleus is pulled into the bud at the time of anaphase onset. Only in this case would Tem1 encounter its activator Lte1, thus leading to MEN activation and mitotic exit. However, Lte1 is neither required for cell viability at physiological temperatures (Shirayama et al., 1994), nor for the unscheduled mitotic exit of mutants defective in spindle positioning (Adames et al., 2001). Conversely, deletion of either *BUB2* or *BFA1* is sufficient to neutralize completely the SPOC (Pereira et al., 2000; Molk et al., 2004; Bardin et al., 2000), suggesting that inactivation of the Bub2/Bfa1 complex might be the actual trigger of mitotic exit. How the Bub2/Bfa1 complex is regulated during the cell cycle and upon spindle mispositioning will be discussed in a separate chapter (see below). In conclusion, there are several mechanisms contributing to Tem1 activation in anaphase: the inhibitory phosphorylation of Bfa1 by the polo kinase Cdc5 (Hu et al., 2001); the asymmetric disappearance of Bub2/Bfa1 from the mother-bound SPB (Fraschini et al., 2006); the encounter between Tem1 and its activator Lte1 in the bud (Bardin et al., 2000; Chan and Amon, 2010). However, recent data indicate that Lte1 might regulate Tem1 by promoting the loss of Bfa1 from the maternal SPB (Geymonat et al., 2009), suggesting that the encounter between Tem1 and Lte1 and the disappearance of

Bub2/Bfa1 from one SPB might actually be two aspects of the same mechanism.

Because several MEN (and SIN) components can also be found in higher eukaryotic systems, from plants to human, similar pathways might also exist in multicellular eukaryotes to survey the correct pattern of asymmetric cell division during development (reviewed in Bedhomme et al., 2008).

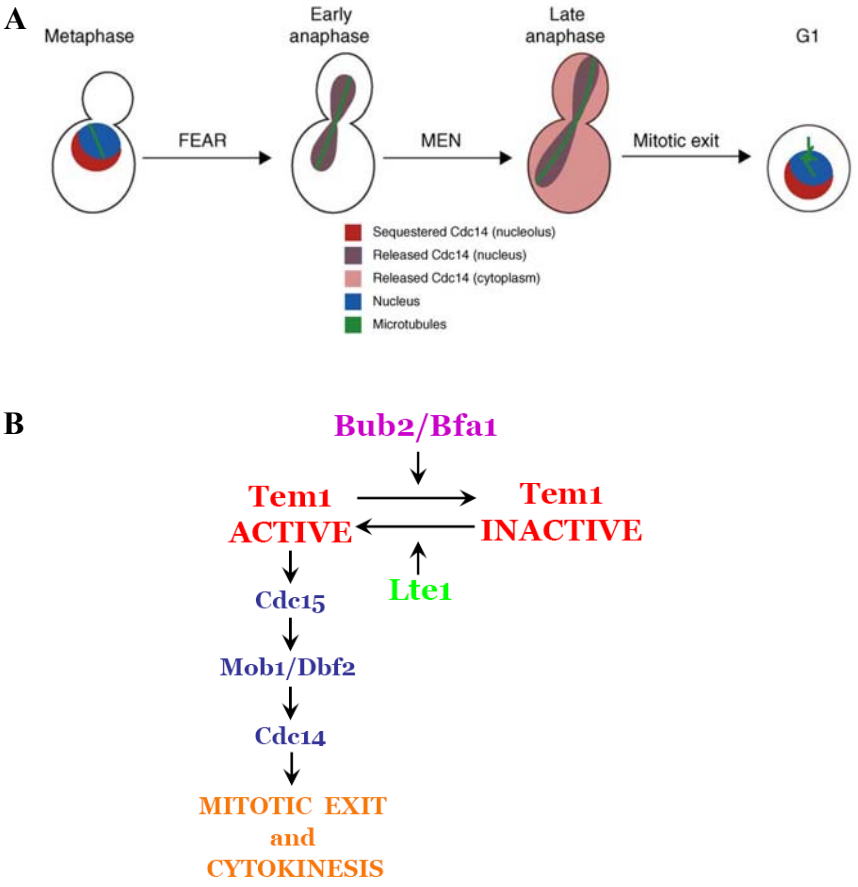


Figure 2. Mitotic exit in *S. cerevisiae*. **A**: Cdc14 partial and total release from the nucleolus depends on the activation of the FEAR and MEN pathways (from DeWulf et al., 2009). **B**: Schematic representation of the MEN pathway (modified from Piatti et al., 2006).

Regulation of cytokinesis

Once mitotic exit has been completed, cytokinesis can occur. Cytokinesis is the complex process by which two daughter cells physically separate at the end of cell division. Understanding this process is a very important goal, not only for its basic biological significance, but also for its practical implications. Indeed, cytokinesis failure can lead to polyploid cells that are genetically unstable and undergo more frequently to chromosome missegregation and aneuploidy, eventually leading to cancer development (reviewed by Ganem and Pellman, 2007). In most animal cells, the plane of cell division is specified immediately following chromosome segregation to a region on the equatorial cortex midway between the separated chromosomes. In contrast, in other eukaryotes such as yeast and plants, the plane of division is determined prior to chromosome segregation (Fig. 3) (Balasubramanian et al. 2004). Indeed in *S. cerevisiae* the cell division plane is defined early in the cell-cycle, in late G1 phase, as the site where the bud will emerge during S-phase. However, in most eukaryotic cells, cytokinesis is driven by a contractile actomyosin ring, which forms at the site of cell division and drives furrow ingression (reviewed by Glotzer, 2005). The mechanisms that regulate the assembly and contraction of the actomyosin ring are conserved from fungi to animal cells. Specifically, in higher eukaryotes, the anaphase microtubule cytoskeleton positions the small GTPase RhoA at the equatorial cortex, where it induces assembly of the actomyosin-based contractile ring just underneath the plasma membrane at the future division site. The constriction of this contractile ring during cytokinesis pulls the

overlying plasma membrane inward toward the cell center to cleave the cell in two (reviewed by Wolfe and Glotzer, 2009). Because mislocalization of the contractile ring can have deleterious effects on genome stability, one of the many hallmarks of cancer, the positioning of the RhoA signal is under stringent cell cycle control both spatially and temporally. Indeed, in animal cells, redundant mechanisms collaborate to precisely define the narrow region on the cortex to which RhoA associates during anaphase (Dechant and Glotzer 2003; Bringmann and Hyman 2005; Werner et al. 2007; Chen et al. 2008; Murthy and Wadsworth 2008). In budding yeast, similar to the situation in higher eukaryotes, a contractile ring (CAR, cytokinetic actin ring) containing actin, myosin, and associated proteins forms at the bud neck following chromosome segregation and contributes to the ingression of the membrane material as well as the deposition of septal material to facilitate cytokinesis. Whereas the actomyosin ring is absolutely essential for cell division in most eukaryotes, such a structure is not absolutely required for cytokinesis in budding yeast (Vallen et al. 2000), which possesses an alternative, CAR-independent pathway, that relies on the construction of a secondary septum (reviewed by Wolfe and Glotzer, 2009). Consistent with its broad-based requirement for assembly of the CAR in eukaryotes, budding yeast Rho1 (the counterpart of mammalian RhoA) becomes concentrated at the bud neck during late mitosis and is indispensable for CAR assembly and cytokinesis (Tolliday et al., 2002; Yoshida et al., 2006). Analogous to the mechanism in higher eukaryotes (Takaki et al., 2008), in budding yeast Rho1 concentration at the bud neck requires the activity of the Polo-like kinase Cdc5 (Yoshida et al.,

2006). In addition, septins, a family of conserved GTP-binding proteins, have been implicated in cytokinesis in many eukaryotic organisms, even if their role in this process remains elusive. Studies in budding yeast indicate that septins participate to cytokinesis by recruiting specific factors to the site of cell division and regulating the actomyosin ring contraction (Bi et al., 1998; Lippincott and Li, 1998).

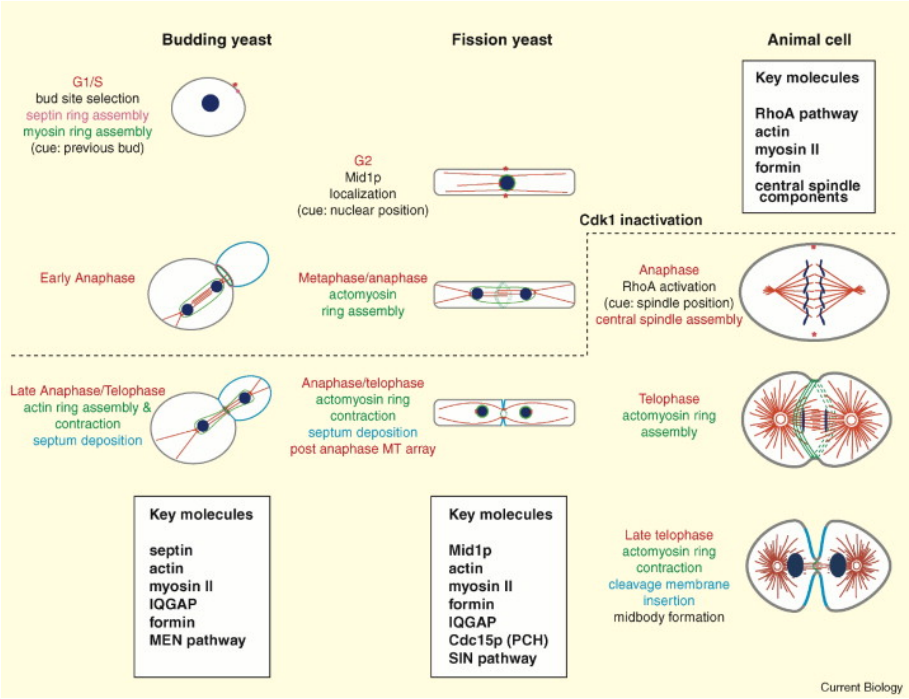


Figure 3. Cytokinesis in eukaryotes. Comparative representation of cytokinesis in budding yeast, fission yeast and animal cells (from Balasubramanian et al., 2004).

Septins in eukaryotes

Septins are a family of conserved GTP-binding proteins that act as dynamic, regulatable scaffolds for the recruitment of other proteins in yeast and metazoans (Field and Kellogg, 1999; Longtine and Bi, 2003; Versele and Thorner, 2005; Kinoshita, 2006; Spiliotis and Nelson, 2006). The septin family was originally discovered in 1971 through genetic screens for budding yeast mutants defective in cell-cycle progression (Hartwell, 1971; Byers and Goetsch, 1976). Over the next 2 decades, septins were found in all animals and fungi, but not in plants (Leipe et al., 2002; Kinoshita, 2003). Septins are required for cytokinesis in most organisms where they have been found. However, their requirement in this process varies tremendously from one organism to another. For example, septins play an essential role in cytokinesis in *S. cerevisiae*, presumably by functioning as a scaffold that tethers cytokinetic components, such as those involved in the formation of the actomyosin contractile ring and of the septum at the bud neck (Bi et al., 1998; Vallen et al., 2000). In contrast, deletion of all four vegetatively expressed septins in the fission yeast *S. pombe* causes only a mild defect in cell separation (Berlin et al., 2003; Tasto et al., 2003). In budding yeast, septins usually assemble as rings at the bud neck. Above their function in cytokinesis (Longtine and Bi, 2003; Bi et al., 1998), the yeast septin ring has been involved in diverse cellular processes such as bud site selection (Longtine and Bi, 2003; Sanders and Herskowitz, 1996), chitin deposition (DeMarini et al., 1997), mitotic spindle positioning (Kush et al., 2002; Grava et al., 2006), polarized growth (Barral et al., 1999; Longtine et al., 2000), the morphogenetic checkpoint (Barral et al., 1999; Shulewitz et al., 1999)

and the formation of membrane diffusion barriers (Barral et al., 2000; Dobbelaere and Barral, 2004). In addition, septins were also implicated in the coordination of the DNA-damage response and cellular morphology (Enserink et al., 2006; Smolka et al., 2006). In contrast to yeast septins, which polymerize mainly at cortical sites of the mother–bud neck, mammalian septins localize not only to the plasma membrane, but also throughout the cytoplasm together with the microtubule and actin cytoskeleton (Kinoshita, 2006). Human septins have been implicated in many cellular processes, including microtubule and actin function (Nagata et al., 2003; Kartmann and Roth, 2001), DNA damage-related checkpoint response (Kremer, 2007), cytokinesis (Kinoshita et al., 1997; Nguyen et al., 2000; Spiliotis et al., 2005), membrane associated cell movement (Hsu et al., 1998; Finger et al., 2003) and vesicle trafficking (Finger et al., 2003). Septins share a common domain organization: a conserved and distinct N-terminal GTP-binding (G) domain and, with few exceptions, a C-terminal domain that is predicted to form a coiled-coil. Many septins possess additional extensions at their N and C termini, which are predicted to be disordered (Fig. 4A) (reviewed in Weirich et al., 2008). Individual septins form small, core complexes both *in vivo* and *in vitro* that contain, depending on the organism, two, three or four septins, each present in two copies (Frazier et al., 1998; Field et al., 1996; Versele et al., 2004; Sirajuddin et al., 2007; John et al., 2007). These core tetra-, hexa- or octameric complexes are thought to define the building blocks that are further assembled into various higher-ordered structures, including filaments, gauzes and rings (Kinoshita et al., 2002; Rodal et al., 2005). Studies in fungi and

animals have shown the importance of complex formation for septin function, both for core heteromers and for higher-order assemblies. Indeed, the biological functions of septins are generally thought to stem from their intrinsic ability to assemble into oligomeric complexes and highly ordered polymers. In fact purified septins have been observed to polymerize *in vitro* into filaments composed of two or more different septins (reviewed in Cao et al., 2009). *C. elegans* has a minimal two-component septin system (UNC-59 and UNC-61) and therefore proved to be an ideal system to determine the relative orientation and assembly mode of septins in a core complex using electron microscopy (EM) (John et al., 2007). Indeed UNC-59 and UNC-61 are aligned in a striking linear pattern (Fig. 4B and C). In addition, experiments using N-terminal green fluorescent protein (GFP) tagged UNC-59 and UNC-61 showed that the septin core structure is composed of the G domains of these proteins in the order UNC-59–UNC-61–UNC-61–UNC-59, and it is therefore non-polar (Fig. 4B and C). Analysis of filaments formed *in vitro* by the *C. elegans* septin proteins revealed that filament formation occurs by end-to-end joining of the core tetramers, and that the resulting filaments must also be non-polar along the direction of filament extension. Interestingly, the coiled-coil domains are flexible and extend away from the tetramer (John et al., 2007). Like the *C. elegans* UNC-59–UNC-61–UNC-61–UNC-59 tetramer, a complex of recombinant *S. cerevisiae* septins — Cdc3, Cdc10, Cdc11 and Cdc12 — forms an elongated linear octamer, detectable by EM (Sirajuddin et al., 2007) (Fig. 4B and C). More recently, EM imaging of wild-type and mutant yeast septin oligomers have revealed that the order of

subunits within the yeast core octamer is Cdc11–Cdc12–Cdc3–Cdc10– Cdc10–Cdc3–Cdc12–Cdc11 (McMurray and Thorner, 2008).

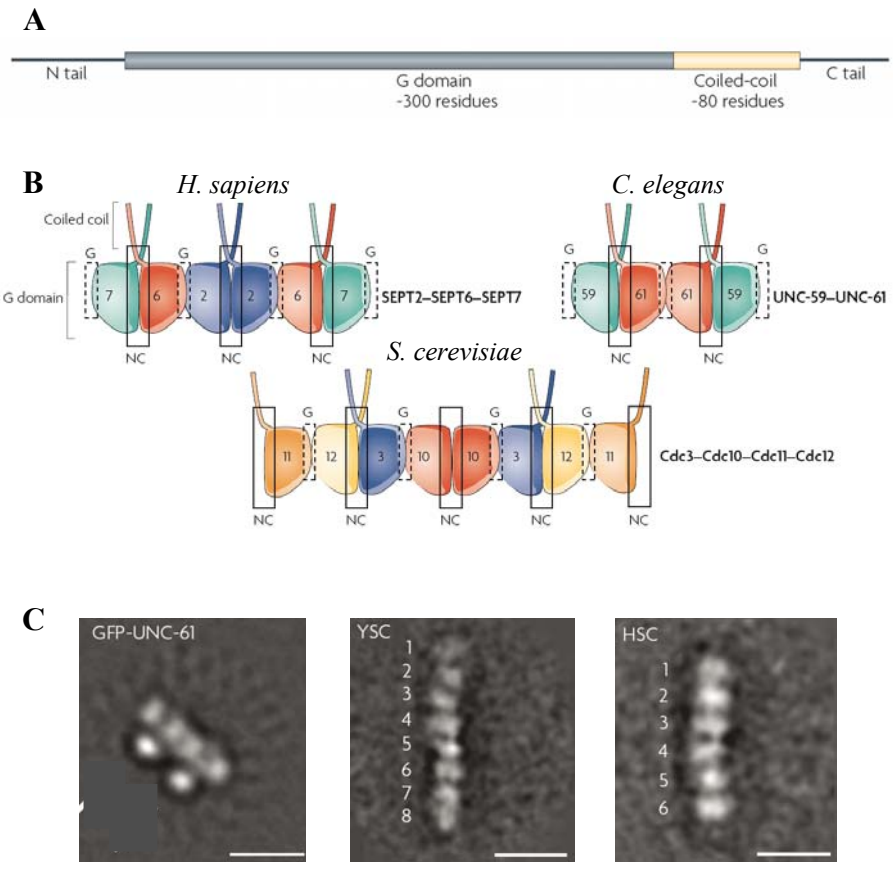


Figure 4. The structure of septin complexes. A: Septins share a conserved GTP-binding (G) domain and a predicted coiled-coil domain at the C terminus. **B**: Core architectures of septin complexes in metazoan and *Saccharomyces cerevisiae*. **C**: Representative images of the nematode septin complex (GFP-UNC-61) showing the four subunits, *Saccharomyces cerevisiae* septin complex (YSC), showing the eight subunits, and the humans septin complex (HSC), showing the six subunits as visualized by electron microscopy (EM) (from Weirich et al., 2008).

Similarly, an EM reconstruction of the complex of mammalian SEPT3–SEPT5–SEPT7 demonstrates that this complex forms an elongated hexamer (Lukoyanova et al., 2008) (Fig. 4B and C), indicating that this architecture is common to all septin complexes. The crystal structure of the human SEPT2–SEPT6–SEPT7 complex revealed that complex and filament formation is dependent on the GTPase domains rather than, as had been previously proposed, on the coiled-coil domains (Versele and Thorner, 2005). Indeed, similar to what was observed in studies of the *C. elegans* septins, the coiled-coil domains of SEPT2, SEPT6 and SEPT7 appear to be flexible and are therefore disordered in the crystal lattice (reviewed in Weirich et al., 2008).

In vivo, septins interact with the actin and microtubule cytoskeletons, as well as with membranes. Although the precise functions of septins remain unclear, current data suggest that they coordinate changes in cytoskeletal and membrane organization by acting as scaffolds that recruit factors to specific sites in a cell and/or as barriers that segregate membrane areas into distinct domains. The plasma membrane of many cell types displays domains with distinct composition and properties. This polarization allows for instance vectorial transport of solutes across epithelia and unidirectional propagation of signals by neurons. Association of septins with phospholipids is crucial to the formation and maintenance of membrane domains (Casamayor and Snyder, 2003). Mammalian septins associate with biological membranes through a highly conserved polybasic region at the N-terminus of the GTP-binding domain. Through this region, recombinant SEPT4 specifically binds

phosphatidylinositol (4,5)-*bis*phosphate (PtdIns(4,5)P_2) (Zhang et al., 1999; Casamayor and Snyder, 2003). In addition phosphatidylinositol (4,5)-*bis*phosphate is enriched at sites of septin functions (Yu et al., 2004; Saul et al., 2004; Field et al., 2005) and required for normal SEPT4 localization (Zhang et al., 1999) as well as for budding yeast filament assembly and localization (Casamayor and Snyder, 2003; Bertin et al., 2010). Furthermore, forced wholesale conversion of PtdIns(4,5)P_2 in PtdIns(3,4,5)P_3 in budding yeast, causes the detachment of septins from the bud neck and the formation of coils and rings in the cytosol (Rodriguez-Escudero et al., 2005). Septins interaction with membranes seems to be important to their function as diffusion barriers. Diffusion barriers help to laterally compartmentalize cellular membranes into separate domains. Such structures are found in continuous membranes such as the plasma and endoplasmic reticulum and restrict the lateral diffusion of membrane-associated proteins. As a result, these proteins freely diffuse in the membrane domain where they act but are prevented from diffusing into other membrane areas (reviewed by Caudron and Barral, 2009). Indeed, yeast septins form a diffusion barrier at the mother-bud neck, to prevent the diffusion of specific proteins between the mother and the bud through the bud neck (Fig. 5). As septins are conserved from yeast to higher eukaryotes, their role in the formation of diffusion barriers seems also to be conserved. In fact septins are present at the bases of dendritic spines (Tada et al., 2007; Xie et al., 2007), in the annuli of spermatozoa (Ihara et al., 2005; Kissel et al., 2005; Steels et al., 2007) and at cytokinetic barriers (Schmidt and Nichols, 2004). In addition recent data suggest that septins contribute to the formation

and maintenance of vertebrate cilia (Kim et al., 2010; Hu et al., 2010) (Fig. 5). Interestingly, there are many morphological similarities between the annulus, the late cytokinetic bridge, the spine neck and the yeast bud neck (Barral and Mansuy, 2007), suggesting more profound connections. For example, similar to yeast, septins depletion or overexpression interferes with spine morphogenesis and complexity (Tada et al., 2007; Xie et al., 2007). Indeed, the downregulation of *SEPT7* expression causes defects in dendritic spine morphology. This suggests that septins may be important in regulating growth and morphology of these structures (Xue et al., 2000; Xie et al., 2007; Tada et al., 2007). In spermatozoa, septins are thought to be the major constituent of the annulus at the boundary of the anterior and posterior tail compartments (Ihara et al., 2005; Kissel et al., 2005; Steels et al., 2007). Accordingly, the depletion of Sept4 affects sperm maturation and leads to male infertility in mice. Furthermore, septins have been linked to several human pathologies, including tumorigenesis (Hall and Russel, 2004), Parkinson's disease (Ihara et al., 2003; Ihara et al., 2007) and hereditary neuralgic amyotrophy (Kuhlenbaumer et al., 2005). A better understanding of how the assembly of septin complexes and dynamics are regulated in yeast can help to have a better knowledge of septins also in higher eukaryotes.

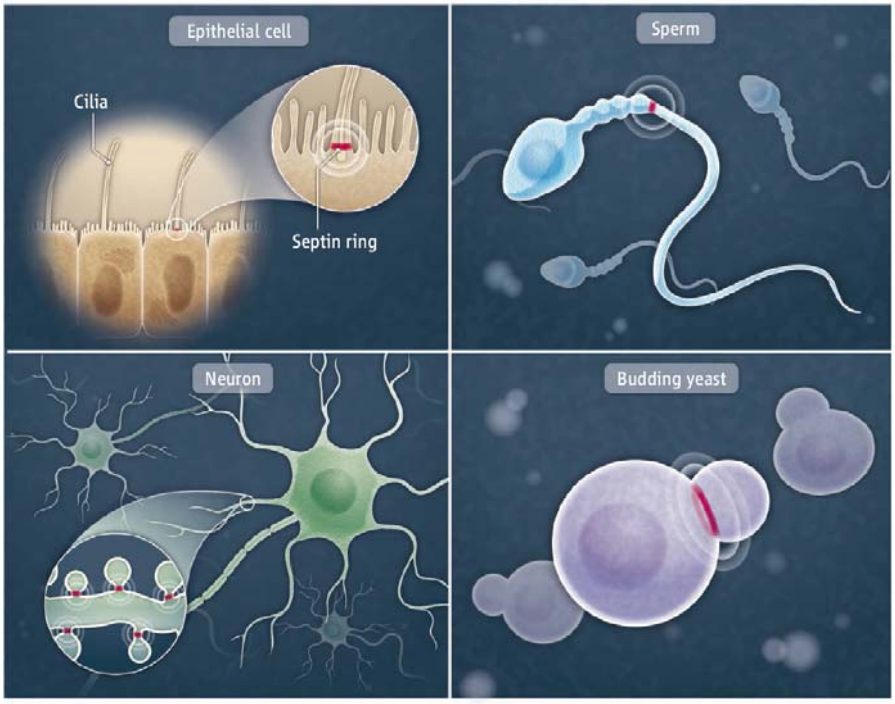


Figure 5. Septins in the formation of diffusion barriers. A ring of septin proteins assembles at the base of the cellular appendages shown, creating a barrier that restricts the diffusion of membrane proteins (from Barral, 2010).

Septins in budding yeast

In *S. cerevisiae* the mitotic septins Cdc3, Cdc10, Cdc11, Cdc12 and Shs1 form complexes that assemble into a tubular collar of highly ordered filaments at the cortex of the mother-bud neck throughout the cell cycle, except for disassembly and reassembly during G1 (Fig. 6A). In mitotic cells, these septins are fundamental for cytokinesis and are also implicated in bud site selection, in the establishment and maintenance of polarized bud growth, in the switch from polarized to isotropic bud growth, in spindle positioning and in the formation of diffusion barriers (reviewed in Versele and Thorner, 2005). In meiotic cells, two sporulation-specific septins (Spr3 and Spr28) replace Cdc12 and Shs1, respectively, and form an alternative complex with Cdc3, Cdc10 and Cdc11 that localizes to the prospore envelope as it forms (Kusch et al., 2002; DeVirgilio et al., 1996).

Budding yeast septins are first recruited to the presumptive bud site as unorganized septin clouds or patches, which are then transformed into the septin ring within minutes (Kinoshita, 2003). A cortical septin ring forms in the late G1 phase of the cell cycle, approximately 15 min before bud emergence. Around emergence of the bud, the septin ring expands into a collar "hourglass structure", spanning the whole bud neck. During cytokinesis, the collar is split into two distinct rings, marking the division site at both the mother and daughter sides (Fig. 6A and B) (Lippincott et al., 2001). FRAP (Fluorescence Recovery After Photobleaching) studies indicate that the septin structures prior to bud emergence are highly dynamic (the so called "fluid state"). Upon bud emergence, the septin collar at the bud neck is stabilized (the so called "frozen" state), while, during cytokinesis, the split

septin rings become dynamic again (Caviston et al., 2003; Dobbelaere et al., 2003) (Fig. 6B). Interestingly, using polarized fluorescence microscopy, the septin filaments inside the ring were found to be arranged in parallel arrays and aligned along the mother-bud axis, whereas upon cytokinesis, when the septin ring splits, they undergo a 90° rotation (Vrabioiu and Mitchison, 2006; Vrabioiu and Mitchison, 2007).

The binding of GTP contributes to septin ring assembly at the bud neck. Indeed, Cdc3, Cdc10, Cdc11 and Cdc12 have all been reported to bind GTP, with GTP-binding by all four septins needed for formation of the septin ring (Nagaraj et al., 2008). Nucleotide binding is important both for septin–septin interactions and for complex formation. In the absence of complete complexes, septins do not locate to the cortex, suggesting septin localization factors interact only with complete complexes. However, there is no evidence demonstrating that nucleotide binding is specifically involved in the interaction of septins with septin-associated proteins (Nagaraj et al., 2008).

The small Rho-family GTPase, Cdc42, also plays a crucial role in septin ring assembly in yeast (Caviston et al., 2003; Gladfelter et al., 2002; Smith et al., 2002). Cdc42 is a highly conserved Rho-type small GTPase, required for polarized growth in *S. cerevisiae* (Park and Bi, 2007). Indeed mutations in *CDC42* or in its activator *CDC24* result in defects in the polarized localization of both the actins and the septins to the presumptive bud site (Gladfelter et al., 2001; Jeong et al., 2001). Cdc42-GTP was suggested to be needed for the whole process of septin collar formation. After septin recruitment, Cdc42, its GAPs

(Rga1, Rga2, and Bem3) and the PAK kinase Cla4, an effector of Cdc42, are thought to play a role in septin ring formation. Mutations in *CDC42* that affect the cycling of Cdc42 between its GDP- and GTP-bound states confer profound defects in septin ring assembly (Gladfelter et al., 2002), while the deletion of all Cdc42 GAPs, *rga1Δ rga2Δ bem3Δ*, delays septin ring formation without affecting septin recruitment (Iwase et al., 2006; Gladfelter et al., 2002). In mammalian cells, Cdc42 also seems to have dual roles in promoting the organization of the actin cytoskeleton and the septin complexes (Joberty et al., 2001). Several yeast kinases, such as Cla4, Gin4 (Longtine et al., 1998) and Elm1 (Bouquin et al., 2000), as well as septin-interacting proteins such as Bni5 (Lee et al., 2002), locate to the bud neck in a septin-dependent manner and are also involved in septin collar formation. Cla4 is activated through binding to Cdc42–GTP. Because Cdc42 function is implicated in bud emergence, stimulation of Cla4 activity by Cdc42 provides a mechanism to link the timing of this event to the assembly of the septin collar (Versele and Thorner, 2004). Several effectors of Cdc42 have been identified above Cla4: the PAK kinases Ste20 and Skm1, the proteins Gic1 and Gic2 and the formin family protein Bni1 (Pruyne and Bretscher, 2000), a component of the polarisome complex, which is in turn important for apical growth (Sheu et al., 2000). *cla4* mutant cells fail to assemble a normal septin ring (Versele and Thorner, 2004; Gladfelter et al., 2005; Kadota et al., 2004); in addition, in yeast strains carrying both *bni1* and *cla4* mutations septins are recruited to the incipient budding site, but the septin ring is not assembled, and septins remain at the polarized growing sites (Kadota et al., 2004).

Septin ring stabilization in S phase is promoted by phosphorylation events (Fig. 6B). Specifically, Cla4 phosphorylates Cdc10, Cdc3 and Cdc11. Mutation of at least one of the Cla4 sites in Cdc10 (Ser256 to Ala) has readily discernible effects on cell morphology and septin architecture (Versele and Thorner, 2004). Gin4 phosphorylates the yeast septin Shs1 in vitro and was found to be directly phosphorylated by the neck-associated Ser/Thr kinase Elm1. Thus, septin assembly can also be regulated by Elm1 directly phosphorylating and activating the Gin4-dependent pathway. Gin4 can then regulate septin ring formation through Shs1 phosphorylation (Mortensen et al., 2002; Asano et al., 2006). Cdc42 and Cla4 are required for the activation of the Gin4 kinase, indicating another route through which Cla4 regulates septin collar assembly (Park and Bi, 2007).

At the end of the cell cycle, during cytokinesis, the septin-collar scission into two separate rings correlates with dephosphorylation of Shs1 by protein phosphatase 2A (PP2A), linked with its B-type regulatory subunit Rts1 (Dobbelaere et al., 2003). In addition also the phosphorylation of Cdc3 at its C-terminus by Cdc28 (Tang and Reed, 2002) and the activity of the small GTPase Tem1 have been implicated in septin ring disassembly (Lippincott et al., 2001, Fig. 6B).

Septin SUMOylation has also been linked to septin ring disassembly. Septins are the most abundant substrates for SUMOylation in budding yeast. Indeed, at least three septins, Cdc3, Cdc11 and Shs1, become sumoylated specifically during mitosis. This modification occurs before anaphase and is removed at cytokinesis, suggesting a possible role in the disassembly of the septin ring (Johnson and Blobel, 1999;

Takahashi et al., 1999). Interestingly, just septins that localize at the mother side after ring splitting were found to be sumoylated (Johnson and Blobel, 1999). However, while preventing SUMO attachment to septins Cdc3 and Cdc11 by mutagenesis of the relevant Lys residue to Ala causes a stabilization of the septin ring, preventing SUMO attachment to the same septins by inactivation of the major SUMO ligase Siz1 does not cause the same phenotype (Johnson and Gupta, 2001). Thus, the role of septin sumoylation in promoting septin ring destabilization has not yet been clarified.

Whether disassembly of the septin ring involves the degradation of septin subunits or whether septins are recycled has not been determined. However, using fluorescence-based pulse-chase methods to visualize the fate of pre-existing (old) and newly synthesized (new) molecules of two septins, recent data suggest that Cdc10 and Cdc12 are recycled through multiple mitotic divisions, because old and new molecules were incorporated indistinguishably into the collar (McMurray and Thorner, 2008).

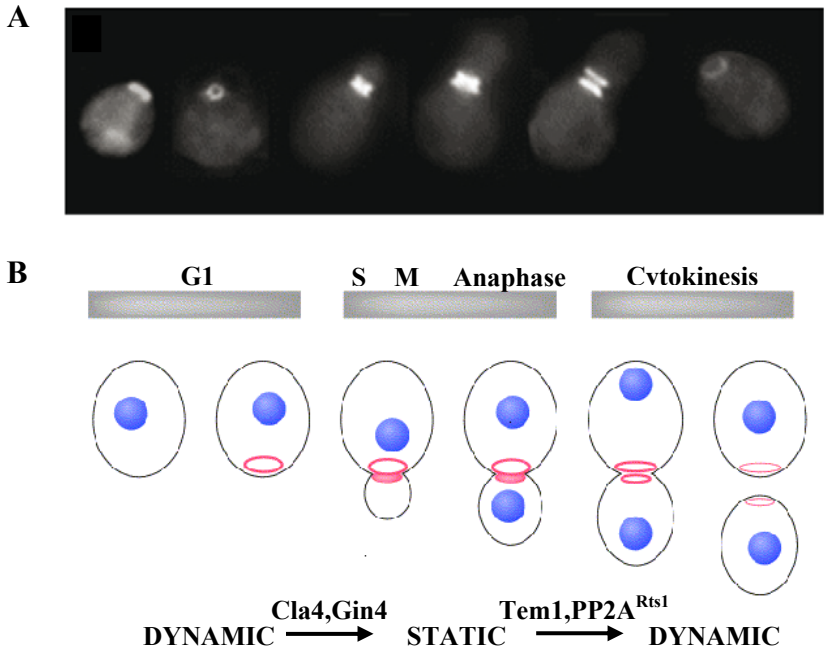


Figure 6. Septin dynamics in budding yeast. A: Septin localization throughout the *Saccharomyces cerevisiae* cell cycle as visualized by fluorescence microscopy (from Weirich et al., 2008). **B**: Late in G1, septins localize to the incipient bud site as a cortical ring. As the bud emerges, the septins are organized into a cortical hourglass structure, which is maintained until the time of cytokinesis, when it is split into two distinct rings. Cortical septin rings remain at the division site after cell separation where they mark the old division sites. Phosphorylation events catalyzed by the kinase Gin4 and PAK Cla4 promote septin ring stabilization, while dephosphorylation events catalyzed by $PP2A^{Rts1}$ and the action of Tem1 promote septin ring destabilization at the end of the cell cycle (modified from Longtine and Bi, 2003). Nuclei are shown in blue and the septin ring in red.

The mitotic checkpoints

Cells must accurately replicate their chromosomes during S phase and then equally segregate the genetic material during mitosis. Failure to correctly replicate and segregate chromosomes results in the gain or loss of genetic information, which may cause lethality or promote the genetic changes that predispose higher eukaryotic cells to cancer (reviewed in Wassmann and Benezra 2001; Kops et al., 2005).

To ensure faithful chromosome transmission and genome stability, progression through mitosis is controlled by surveillance mechanisms called mitotic checkpoints that prevent or delay the onset of anaphase, mitotic exit and cytokinesis in response to different kinds of injuries. Cell cycle checkpoints are surveillance pathways that monitor key events of the cell cycle and provide time for error correction when execution of those events is delayed (reviewed in Lew and Burke, 2003). The Spindle Assembly Checkpoint (SAC) monitors the sister chromatid tension/attachment to the mitotic spindle via a specialized chromatid structure called kinetochore. Even when a single kinetochore is not correctly attached to the spindle, the SAC delays the cell cycle prior to anaphase, thus preventing chromosome segregation until each duplicated chromosome has established a bipolar attachment to spindle fibres coming from opposite poles (reviewed in Musacchio and Salmon, 2007). As a consequence, cells with defective SAC do not arrest the cell cycle in the presence of unattached kinetochores, but proceed through the cell cycle leading to chromosome missegregation (Li and Murray, 1991; Hoyt and Roberts, 1991) (Fig.7A).

As already discussed, during asymmetric cell division, spindle positioning is critical for ensuring the unequal segregation of polarity factors and generating daughter cells with different sizes or fates. In budding yeast, the mother-bud neck determines the cleavage plane, and a correct nuclear division between mother and daughter cell requires orientation of the mitotic spindle along the mother-bud axis. A surveillance device called the Spindle Position/Orientation Checkpoint (SPOC) oversees this process and delays mitotic exit and cytokinesis until the spindle is properly oriented along the division axis, thus ensuring genome stability (reviewed in Bardin and Amon, 2001; Piatti et al., 2006) (Fig. 7B). Experimental evidence suggests that pathways similar to the SPOC might control cell cycle progression in response to spindle mispositioning in other eukaryotic cells that divide asymmetrically (Cheng et al., 2008).

Budding yeast has a highly polarized actin cytoskeleton (Adams and Pringle, 1984), which is responsible for polarized bud growth (Pruyne and Bretscher, 2000). However, many environmental perturbations result in actin depolarization, causing significant delay in bud formation. If cell cycle would continue when budding is delayed, cells would become binucleate. Indeed, this delay does not lead to uncoupling of the budding from the nuclear cycle, suggesting that delayed bud formation is accompanied by a delayed nuclear division. This implies the existence of a mechanism, which has been called morphogenesis checkpoint (reviewed in Lew, 2003; Keaton and Lew, 2006), sensing the delay in the budding cycle and consequently transiently arresting nuclear division (Fig. 7C).

Below, the SPOC and the morphogenesis checkpoints that are more relevant than the SAC to this thesis are discussed in details.

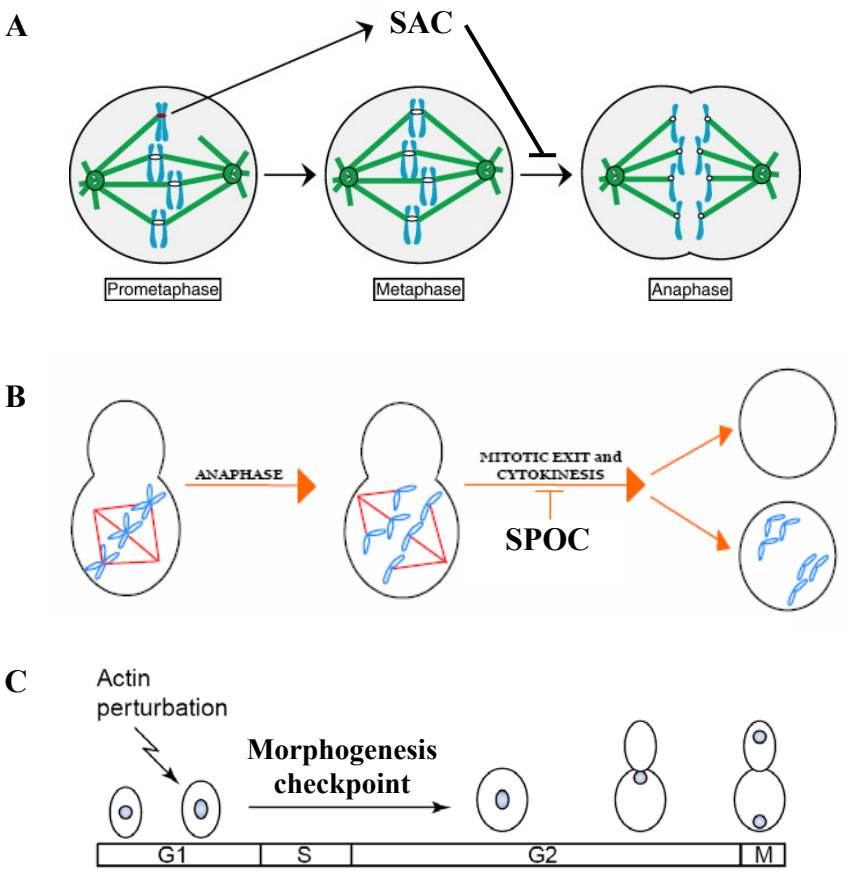


Figure 7. The mitotic checkpoints in *S. cerevisiae*. **A**: The spindle assembly checkpoint (SAC) delays the onset of anaphase until each chromosome has established a bipolar attachment (from Suijkerbuijk and Kops, 2008). **B**: the spindle position/orientation checkpoint (SPOC) delays mitotic exit and cytokinesis until the mitotic spindle is properly oriented. **C**: Bud formation defects delay the G2/M cell cycle transition through the morphogenesis checkpoint (from Lew, 2003).

The spindle position checkpoint

In case of spindle orientation defects, in budding yeast mitotic exit and cytokinesis are delayed to provide the time necessary for error correction (reviewed in Fraschini et al., 2008) and this is crucial to prevent unbalanced chromosome partitioning. The SPOC is responsible for this cell cycle delay by inhibiting the MEN (mitotic exit network) signal transduction cascade, thus avoiding the generation of anucleate and binucleate cells (Bardin and Amon, 2001; Lew and Burke, 2003; Piatti et al., 2006). As already mentioned, the SPOC target is the Tem1 GTPase, whose active GTP-bound form promotes MEN activation that ultimately drives cells out of mitosis by leading to inactivation of mitotic CDKs (Bardin and Amon, 2001; McCollum and Gould, 2001; Simanis, 2003; Surana et al., 2002). The regulation of Tem1 is complex, with multiple regulatory factors impacting on its activity through several partially redundant pathways. The dimeric GTPase-activating protein (GAP) Bub2/Bfa1 keeps Tem1 inhibited until the spindle is properly aligned, thus coupling mitotic exit with nuclear division (Fig. 8A) (Bardin et al., 2000; Bloecher et al., 2000; Pereira et al., 2000). The Kin4 protein kinase is also involved in the SPOC (D'Aquino et al., 2005; Pereira and Schiebel, 2005), by keeping Bfa1 active through direct phosphorylation, thereby counteracting Bfa1 inhibitory phosphorylation by Cdc5 (Maekawa et al., 2007). In addition, Kin4 also regulates the dynamics of Bub2/Bfa1 at spindle poles (Caydasi and Pereira, 2009). The Elm1 kinase (Caydasi et al., 2010; Moore et al., 2010) and the protein phosphatase 2A (PP2A) (Chan and Amon, 2009) contribute to the SPOC at least partly through Kin4 activation. In particular, the localization of Kin4

to the cortex and SPBs seems to be enhanced by the activity of the PP2A subunit Rts1 (Chan and Amon, 2009), even if the underlying molecular mechanisms remain unclear. Elm1 is required for Kin4 kinase activity. Indeed, in *elm1Δ* cells the kinase activity of immunoprecipitated Kin4 is drastically reduced and Kin4 turns out to be phosphorylated at the conserved Threonine T209 in an Elm1-dependent manner (Caydasi et al., 2010). However, since Kin4 phosphorylation on T209 does not change during the cell cycle or upon spindle mispositioning, Elm1 might control the SPOC also through additional mechanism(s). Accumulation of Elm1 at the bud neck seems to promote its function in the SPOC, because Elm1 is enriched at the bud neck when the checkpoint is active (Moore et al., 2010) and Elm1 kinase activity was found to be necessary for its own association with the bud neck (Thomas et al., 2003). Tem1 and several downstream MEN components are found at SPBs in a cell cycle-regulated manner and are thought to promote mitotic exit from this location (Stegmeier and Amon, 2004). The Bub2/Bfa1 complex is found predominantly on the SPB that is pulled towards the bud, while it is present on both SPBs of misaligned spindles (Fig. 8A and B) (Caydasi and Pereira, 2009; Molk et al., 2004; Monje-Casas and Amon, 2009; Pereira et al., 2000; Pereira et al., 2001). Kin4 localizes to the mother-bound SPB in anaphase and binds to both SPBs in case of spindle misalignment (Fig. 8A and B) (Pereira and Schiebel, 2005). Conversely, the Lte1 protein, which positively regulates Tem1, is confined in the bud from the G1/S transition to telophase, when it spreads throughout the cytoplasm of both mother cell and bud (Bardin et al., 2000). The turnover rate of Tem1 at the SPBs is high and is

independent of the status of the spindle orientation (Molk et al., 2004; Caydasi and Pereira, 2009). In contrast, Bub2 and Bfa1 bind stably to the daughter cell SPB (dSPB) when the spindle is properly aligned. However, upon checkpoint activation, the dynamics with which Bub2-Bfa1 turns over at the SPBs increase dramatically (Caydasi and Pereira, 2009; Monje-Casas and Amon, 2009). This change in binding dynamics seems to be triggered by Kin4-dependent phosphorylation of Bfa1 and plays a key role in SPOC function (Caydasi and Pereira, 2009). Disappearance of Bub2/Bfa1 from the mother-bound SPB during anaphase requires Bub2 GAP activity and a functional septin ring (Fraschini et al., 2006). The septin ring is also required for Lte1 localization in the bud: lack of septins causes mislocalization of Lte1 to the mother cell and abolishes the SPOC (Castillon et al., 2003). Indeed, during telophase of unperturbed cell cycles, Lte1 asymmetry is lost and the protein diffuses from the bud cortex to the cytoplasm of both mother cell and bud (Bardin et al., 2000; Bloecher et al., 2000), thus perhaps contributing to Tem1 activation in the mother cell. Altogether, these data suggest that key regulatory events preventing the MEN upon spindle mispositioning take place within the mother cell. In addition, the involvement of the septin ring in this process would provide an explanation for the finding that mitotic exit is signalled by passage of one SPB through the bud neck (Molk et al., 2004).

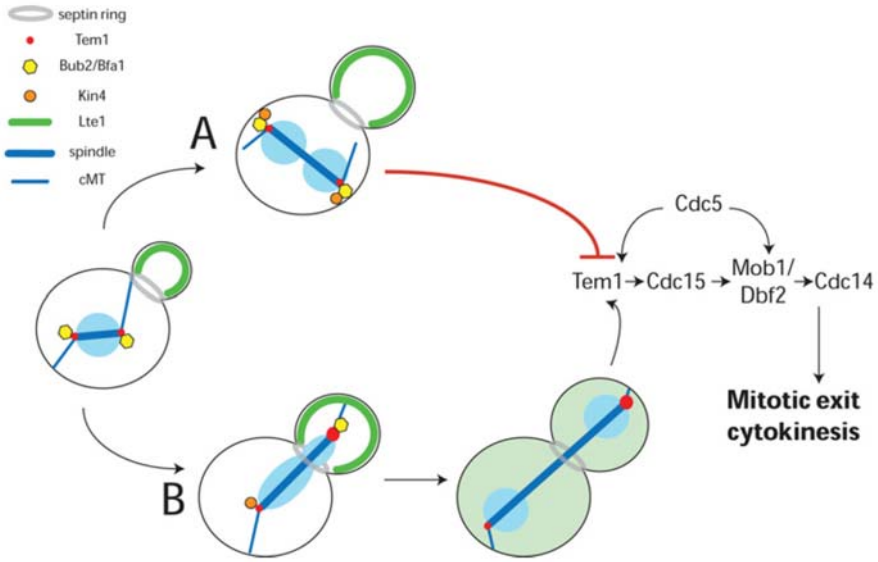


Figure 8. The spindle position checkpoint in *S. cerevisiae*. The SPOC is activated in response to spindle misalignment (**A**) and is switched off when the spindle is properly oriented along the mother-bud axis (**B**). Upon spindle misalignment (A) Tem1 is present on both SPBs along with its negative regulators Bub2/Bfa1 and Kin4, while its activator Lte1 is confined on the bud cortex. During properly oriented anaphase (B) Bub2/Bfa1 disappears from the mother-bound SPB while it accumulates, along with Tem1 on the bud-directed SPB. In telophase Bub2/Bfa1 and Kin4 finally disappear from both SPBs, while Lte1 spreads into the cytoplasm of both mother cell and bud. Both events likely contribute to full MEN activation and mitotic exit (from Fraschini et al., 2008).

The morphogenesis checkpoint

Budding yeast contains a highly polarized actin cytoskeleton (Adams and Pringle, 1984) which is responsible for the polarized growth of the bud (Pruyne and Bretscher, 2000). However many environmental perturbations (including mild heat shock and osmotic shock) result in actin depolarization, causing significant delays in bud formation. Nevertheless, these delays do not lead to uncoupling of the budding cycle from the nuclear cell cycle: the cells remain fully viable and mononucleate, suggesting that a delay in bud formation is accompanied by a delay in the nuclear cell cycle. Indeed, a mechanism that senses the delay in the budding cycle and consequently transiently arrests entry into mitosis exists and is called "morphogenesis checkpoint" (Lew and Reed, 1995). The activity of cyclin-CDK1 complexes required to enter mitosis can be inhibited by phosphorylation of a tyrosine residue (Y19) in the CDK, catalyzed by the Wee1 family of protein kinases. Swe1, the *Saccharomyces cerevisiae* homolog of Wee1, inhibits Cdk1 by phosphorylating on Y19 (Booher et al., 1993). Mutants lacking Swe1 display a normal cell cycle in the absence of any perturbation (Booher et al., 1993), but fail to restrain mitosis when actin polarization and bud formation are impared (McMillan et al., 1998). The action of Swe1p is counteracted by the phosphatase Mih1p (Russell et al., 1989). Swe1 accumulates during late G1 and S phase, and it is then degraded prior to nuclear division. Both transcription of *SWE1* gene and degradation of the corresponding protein are regulated, but the latter process seems to have a dominant role (Sia et al., 1996; Sia et al., 1998). When the morphogenesis checkpoint is activated, Swe1 is stabilized and

accumulates, suggesting that checkpoint activity blocks its degradation (Sia et al., 1998). Swe1 degradation requires the Nim1-family protein kinase Hsl1, which acts together with the protein methyltransferase Hsl7 (McMillan et al., 1999). It is known that Hsl7 interacts directly with Hsl1 and Swe1 and mutations that impair either of these interactions stabilize Swe1, suggesting that formation of Swe1-Hsl1-Hsl7 complex is necessary for Swe1 degradation (McMillan et al., 1999; Shulewitz et al., 1999; Cid et al., 2001; McMillan et al., 2002). Hsl1 and Hsl7 are localized to the septin ring on the bud side of the mother-bud neck, and targeting of Hsl7 to that location requires Hsl1 (Fig. 9) (Barral et al., 1999; Shulewitz et al., 1999; Longtine et al., 2000). Besides being involved in the SPOC, Elm1 also functions in the morphogenesis checkpoint through direct activation of Hsl1 (Edgington et al., 1999) and its inactivation causes a Swe1-dependent mitotic delay (Sreenivasan and Kellogg, 1999). When Swe1 first accumulates, in late G1, it is localized to the nucleus, but after bud emergence a subpopulation of Swe1 is recruited to the bud side of the neck by Hsl1 and Hsl7 (Fig. 9) (Longtine et al., 2000). In mutant strains that misorganize septins, localization of Hsl1 and Hsl7 is disrupted. These strains exhibit Swe1-dependent G2 delays in the cell cycle, suggesting that localization of Hsl1 and Hsl7 to the neck is important for down-regulation of Swe1 (Barral et al., 1999; Longtine et al., 2000). Although Hsl1 recruitment to the septin cortex can occur in unbudded cells, its activation and the recruitment of Hsl7 are absolutely dependent on bud formation (Theesfeld et al., 2003; reviewed in Keaton and Lew, 2006), suggesting that these two proteins can somehow detect the presence or absence of the bud,

ensuring that Swe1 degradation does not begin until a bud has formed. Swe1 is hyperphosphorylated before its degradation (Asano et al., 2005; McMillan et al., 2002). In particular Cdc5-dependent phosphorylation of Swe1 is essential for Swe1 degradation and Hsl1 and Hsl7 seems to be important also for Cdc5 targeting to the bud neck (Asano et al., 2005). In addition to Cdc5, CDK activity is important for Swe1 degradation (Fig. 9) (Harvey et al., 2005). Although a mechanism to monitor bud formation is inapplicable to cells that do not grow by budding, there is evidence for the existence of similar mechanisms that monitor aspects of cytoskeletal tension or cell morphology in other cells. For example, in fission yeast, Wee1 is part of a cell-size checkpoint that prevents entry into mitosis before cells have reached a critical size (Kellogg, 2003). Indeed loss of Wee1 activity causes cells to enter mitosis before sufficient growth has occurred and cytokinesis therefore produces two abnormally small daughter cells (Nurse, 1975). Conversely, increasing the gene dosage of *wee1* causes delayed entry into mitosis and an increase in cell size, indicating that the levels of Wee1 activity determine the timing of entry into mitosis and can have strong effects on cell size (Russell and Nurse, 1987). In mammalian cells a pathway regulated by the availability of monomeric actin, in which unpolymerized actin binds directly to a transcription cofactor, has been identified (Posern et al., 2002; Miralles et al., 2003). This mechanism might link stress- or drugs- induced actin depolymerization to a biological response, as observed in the yeast morphogenesis checkpoint.

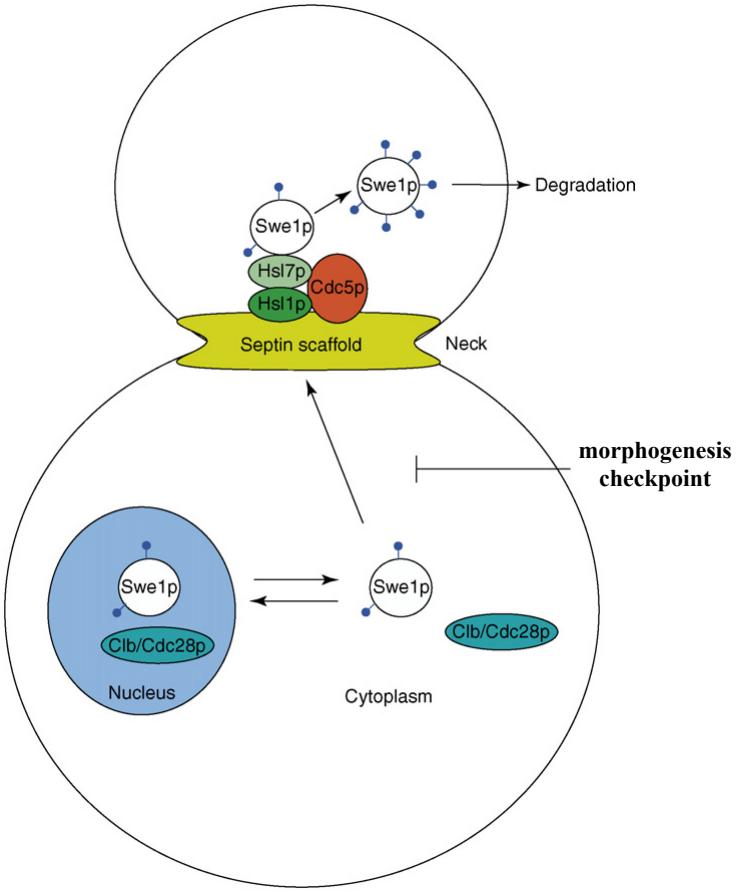


Figure 9. The morphogenesis checkpoint in *S. cerevisiae*. Swe1 first accumulates in the nucleus of unbudded cells. Following bud emergence, Hsl1 becomes activated at the septin cortex. This enables it to recruit Hsl7, which in turn recruits Swe1 and Cdc5 (polo kinase) to the septin collar. Swe1 is phosphorylated by Cdc28p (CDK) that leads to its degradation. Morphogenetic defects prevent activation of Hsl1p, precluding neck targeting and/or degradation of Swe1p and enabling Swe1p to return to the nucleus (from Keaton and Lew, 2006).

Dma proteins in *S. cerevisiae*

S. cerevisiae Dma1 and Dma2 proteins are 58% homologous to each other and have redundant function(s) (Fraschini et al., 2004). Dma1 and Dma2 are E3 ubiquitin-ligases (Loring et al., 2008). They share the same structural organization as *S. pombe* Dma1 and human Chfr and Rnf8, which are all involved in checkpoint mechanisms (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Murone and Simanis, 1996; Scolnick and Halazonetus, 2000; Wang and Elledge, 2007). Indeed, Dma proteins, like their homolog Chfr and Rnf8, have a forkhead-associated domain (FHA), which is a protein interaction motif that binds to phosphoepitopes (Durocher et al., 2000) and a Ring-finger domain typical of E3 ubiquitin-ligases (Lorick et al., 1999) (Fig. 10).

In budding yeast Dma proteins have been implicated in proper septin ring positioning, cytokinesis and in the SPOC (Fraschini et al., 2004). In particular, lack of both Dma1 and Dma2 compromises septin ring assembly and have additive effects with *cla4* mutations. Conversely, overexpression of *DMA2* delays septin ring disassembly at the end of mitosis (Fraschini et al., 2004). The double deletion of *DMA1* and *DMA2* allows unscheduled mitotic exit of dynein mutants, suggesting that these proteins are involved in the SPOC (Fraschini et al., 2004). Intriguingly, both *S. pombe* Dma1 and human Rnf8 have been found to localize at the cell division site and to regulate mitotic exit (Guertin et al., 2002; Plans et al., 2007; Tuttle et al., 2007). Specifically, in *S. pombe* Dma1 localizes at the site of cell division and inhibits the SIN during spindle checkpoint activation. This effect appears to be obtained by preventing the SIN activator, Plo1 (Polo-like) kinase,

from localizing to the SPB. It seems that microtubule depolymerization triggers Dma1-dependent inhibition of Plo1 kinase localization at SPBs, thus preventing SIN activation and cytokinesis (Guertin et al., 2002). Human Rnf8 localizes to the midbody and its depletion compromises mitotic arrest in cells exposed to nocodazole (Tuttle et al., 2007). Furthermore, the ectopic expression of *RNF8* causes a delay in the final steps of cytokinesis, accompanied with frequent aberrant mitoses (Plans et al., 2007).

The other human homolog of Dma proteins, Chfr, is instead part of a mitotic stress checkpoint that delays metaphase entry upon transient treatment with drugs interfering with microtubule dynamics (Scolnick and Halazonetis, 2000; Kang et al., 2002). Interestingly, Chfr has been found either mutated or not expressed in several tumour cell lines, suggesting that it might act as a tumour suppressor (Scolnick and Halazonetis, 2000) and that also Dma proteins could be crucial to maintain genome stability.

In this thesis we used different approaches to unravel the role of Dma proteins both in septin dynamics and SPOC regulation. We show that the Dma proteins control septin ring stability throughout the cell cycle and appear to function in the SPOC independently of Bub2/Bfa1, suggesting that the MEN may be subject to different regulatory mechanisms under spindle mispositioning. Strikingly, the bud neck localization of the Elm1 kinase, which controls both septin deposition and the SPOC, is impaired by deletion of *DMA1* and *DMA2*. In addition we propose that Dma proteins might regulate septin dynamics through multiple pathways, by alternatively activating Rho1 and Elm1 and/or inhibiting $PP2A^{Rts1}$ directly or indirectly. Altogether, our data

highlight important roles for the Dma proteins in coordinating nuclear division and cytokinesis, thus contributing to equal chromosome partitioning.

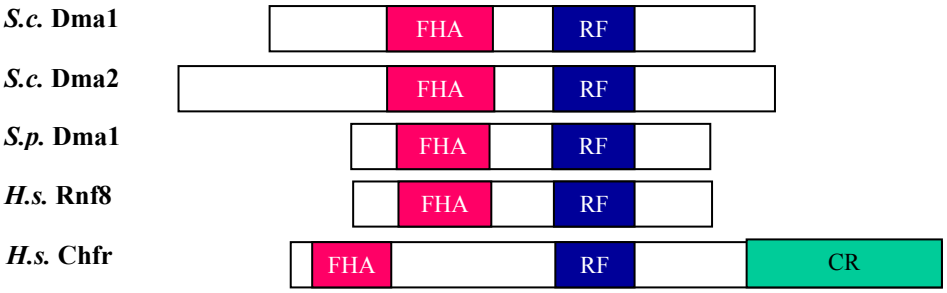


Figure 10. Dma proteins and their human homolog. *S. cerevisiae* Dma1 and Dma2 are 58% identical to each other and are homologs of *S. pombe* Dma1 and human Chfr and Rnf8. All these proteins are ubiquitin ligases implicated in mitotic checkpoints and in the maintenance of genome stability. (FHA, forkhead associated domain; RF, ring finger domain; CR, cistein rich domain).

Results

Dma proteins control septin ring deposition and maintenance

Simultaneous lack of Dma1 and Dma2 causes cell lethality in the absence of the PAK kinase Cla4. Moreover, a *dma1Δ dma2Δ cla4Δ cla4-75* conditional mutant exhibits severe defects in bud neck morphology and septin ring localization under restrictive conditions, indicating that Dma proteins might be involved in septin dynamics (Fraschini et al., 2004).

To further investigate this issue, we analysed the effects of the *dma1Δ dma2Δ* double deletion in septin mutants, such as *cdc12-1*, *cdc12-6* and *shs1Δ*, as well as in mutants lacking protein kinases that regulate septin dynamics, such as *kcc4Δ*, *gin4Δ*, *elm1Δ* and *hsl1Δ* (Barral et al., 1999). Interestingly, the lack of Dma proteins caused cell lethality when combined with conditional septin mutations or with *GIN4*, *HSL1* and *ELM1* deletion (Table1), supporting the notion that Dma1 and Dma2 might regulate septin ring dynamics. Conversely, they were not essential in the absence of the Kcc4 protein kinase, which was shown to play a minor role in this process (Longtine et al., 2000).

GENOTYPE	PHENOTYPE AT 25°C
dma1Δ dma2Δ kcc4Δ	HEALTHY
dma1Δ dma2Δ hsl1Δ	LETHAL/VERY SICK
dma1Δ dma2Δ gin4Δ	LETHAL/VERY SICK
dma1Δ dma2Δ elm1Δ	LETHAL
dma1Δ dma2Δ cla4Δ	LETHAL
dma1Δ dma2Δ cdc12-1	LETHAL
dma1Δ dma2Δ cdc12-6	LETHAL
dma1Δ dma2Δ shs1Δ	LETHAL

Table I. Synthetic effects between the lack of Dma proteins and mutations affecting septin dynamics. Strains carrying *DMA1* and *DMA2* deletions were crossed with mutants defective in septin dynamics. The resulting diploids were induced to sporulate and meiotic segregants were assayed for their ability to grow on rich medium at 25°C.

Dma proteins could control either the assembly or the maintenance of the septin ring. To distinguish between these possibilities, we analysed septin ring assembly during a synchronous release from G1 of *dma1Δ dma2Δ cla4-75* conditional mutant cells (Fraschini et al., 2004). Cultures of wild type, *dma1Δ dma2Δ*, *cla4-75* and *dma1Δ dma2Δ cla4-75* cells all carrying a *CDC3-GFP* construct to visualize the septin ring, were arrested in G1 by α factor at 25°C and released in the cell cycle at 37°C, to inactivate the temperature-sensitive *cla4-75* allele. Samples were taken at different time points to monitor the kinetics of DNA replication (Fig. 11A), budding, nuclear division and septin ring assembly (Fig. 11B). Both *dma1Δ dma2Δ* and *cla4-75* cells underwent proper deposition of the septin ring with kinetics similar to wild type, although with somewhat reduced efficiency. Conversely, *dma1Δ dma2Δ cla4-75* cells were virtually unable to assemble the septin ring at the bud neck throughout the duration of the experiment, suggesting that Dma proteins and Cla4 share overlapping function(s) in septin ring deposition. In addition, *dma1Δ dma2Δ cla4-75* cells did not undergo nuclear division and arrested in G2 as budded mononucleate cells.

The septin interacting protein Bni5 is involved in septin ring stabilization, but not in the initial septin ring recruitment or assembly, since it localizes to the bud neck only after the septins are already there (Lee et al., 2002). *BNI5* overexpression from either a multicopy plasmid (Fig. 11C) or the *GAL1* promoter (data not shown) did not rescue *dma1Δ dma2Δ cla4-75* cell lethality at 37°C, consistently with a role of Dma proteins and Cla4 in septin ring deposition.

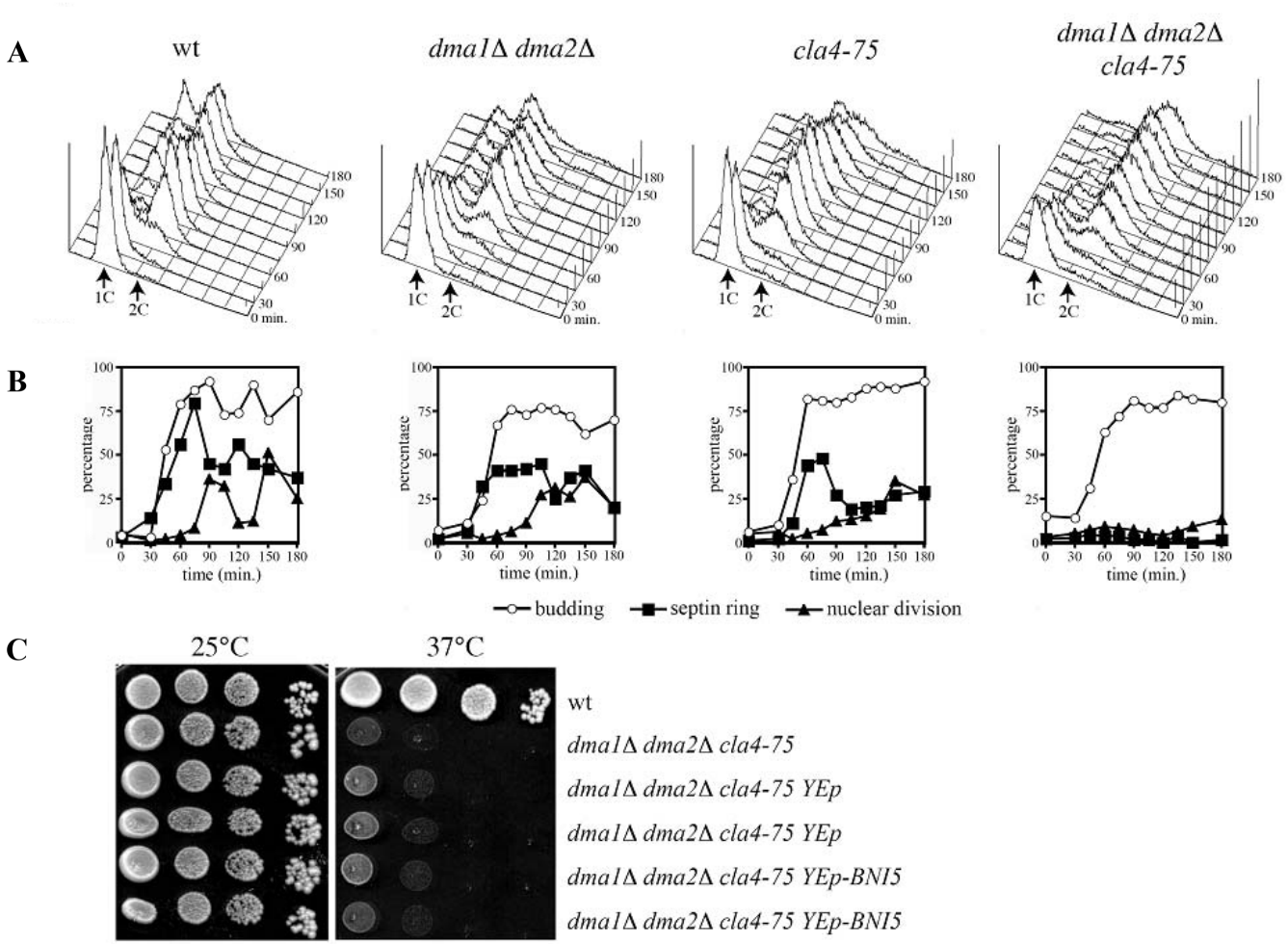


Figure 11. Dma proteins are required together with Cla4 for septin ring deposition. A, B: Logarithmically growing cultures of wild type (ySP3070), *dma1Δ dma2Δ* (ySP4319), *cla4-75* (ySP4320) and *dma1Δ dma2Δ cla4-75* (ySP4321) cells, all expressing Cdc3-GFP, were arrested in G1 by alpha factor at 25°C and released into the cell cycle at 37°C. At the indicated times, cell samples were taken for FACS analysis of DNA contents (A) and for scoring budding, nuclear division and septin ring deposition (B). **C**: Serial dilutions of stationary phase cultures of the indicated strains were spotted on YEPD and incubated for 2 days at 25°C and 37°C.

To assess whether Dma1 and Dma2, together with Cla4, control also the maintenance of the septin ring, we analysed the latter in *dma1Δ dma2Δ cla4-75* cells that were shifted to the restrictive temperature at various cell cycle stages. Elutriated wild type and *dma1Δ dma2Δ cla4-75* small unbudded cells were resuspended in fresh medium at 25°C. Aliquots of the synchronous cultures were then shifted to 37°C after 120, 130 and 140 minutes, and the stability of the septin ring was followed by in situ immunofluorescence at different time points after the temperature shift. A slight delay in septin ring assembly, relative to budding and to the wild type control, was detectable in *dma1Δ dma2Δ cla4-75* cells already at 25°C (Fig. 12A), consistently with a role of Dma proteins in this process. The septin ring was fairly stable in wild type cells after shift to 37°C, and showed cell cycle-dependent kinetics. In contrast, it rapidly mispositioned and eventually disassembled in *dma1Δ dma2Δ cla4-75* cells at the restrictive temperature, irrespective of the cell cycle stage at which cells were shifted to 37°C (Fig. 12A and B). We then asked whether *dma1Δ dma2Δ cla4-75* cells would maintain a properly assembled septin ring if shifted to high temperature in mitosis, when the septin ring is very stable (Caviston et al., 2003; Dobbelaere et al., 2003). To this end, cells were arrested in mitosis by nocodazole treatment, followed by shift to 37°C and septin ring analysis at different time points. As shown in Figure 13 (A and B), the septin ring was maintained at the bud neck in a high fraction of wild type cells, whereas it progressively disassembled in *dma1Δ dma2Δ cla4-75* cells, although not as fast as in *cdc12-6* cells, where it was previously shown to be extremely unstable (Dobbelaere et al., 2003).

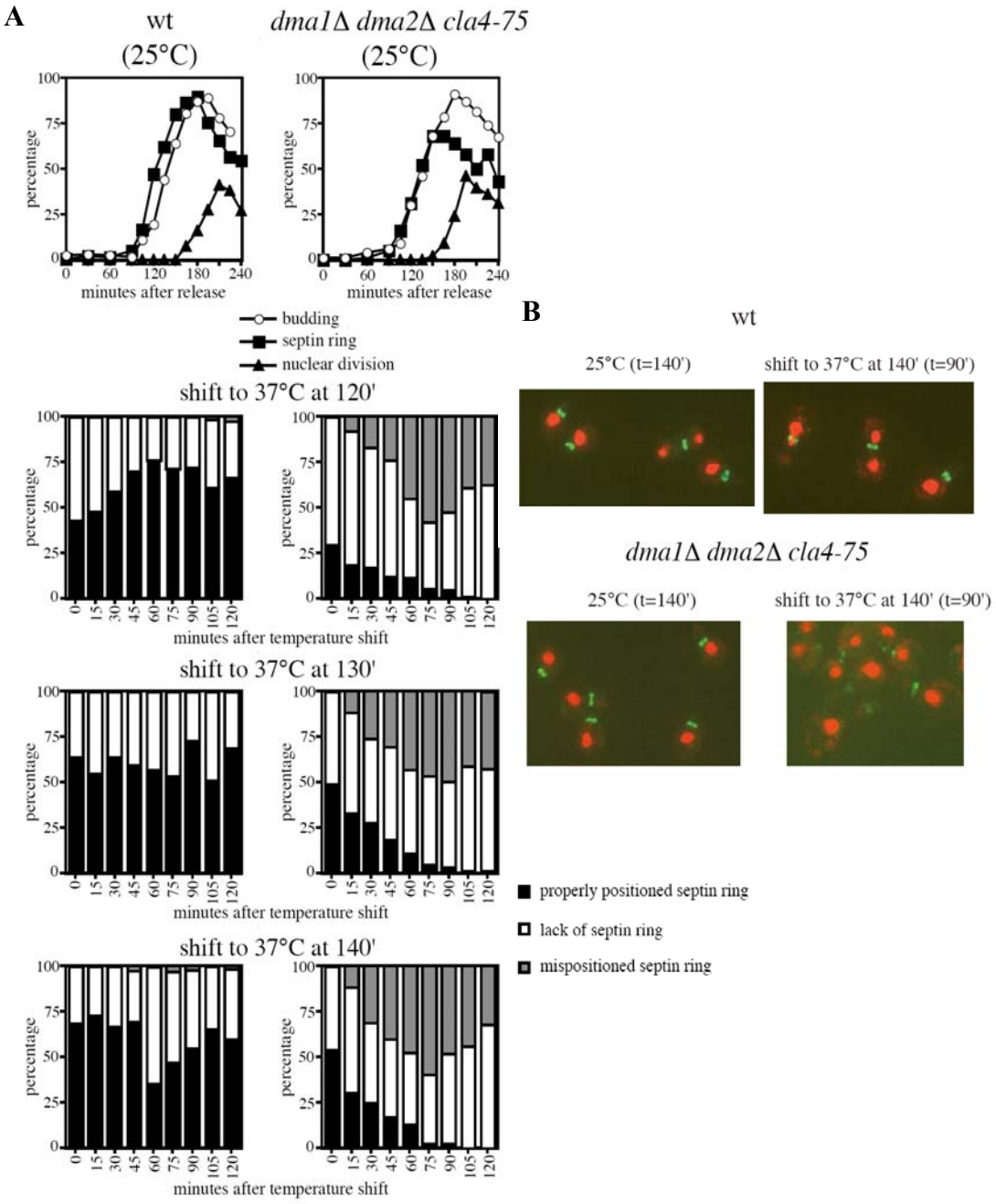


Figure 12. Septin ring maintenance throughout the cell cycle requires Cla4 and the Dma proteins. **A**: Small unbudded cells of wild type (W303) and *dma1Δ dma2Δ cla4-75* (ySP5247) strains were isolated by centrifugal elutriation and resuspended in fresh medium at the permissive temperature (25°C) at time 0 (upper graphs). Aliquots of these cultures were then shifted to 37°C after 120, 130 and 140 minutes (bottom panels). At the indicated times, cell samples were taken for FACS analysis of DNA contents (not shown), for scoring budding and nuclear division (shown only at 25°C) and for in situ immunofluorescence of the septin ring with anti-Cdc11 antibodies. **B**: Micrographs of cells either growing at 25°C (t=140') or 90' after cells were shifted to 37°C (at t=140').

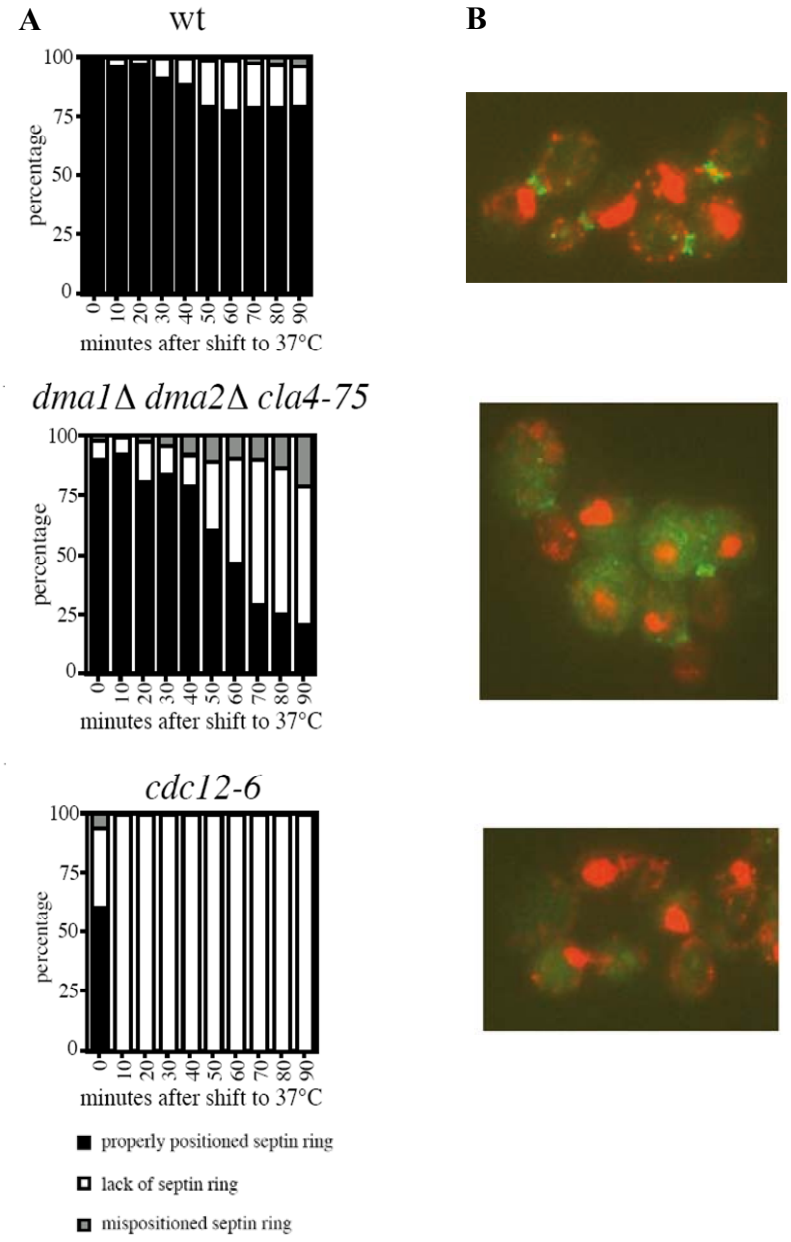


Figure 13. Septin ring maintenance throughout the cell cycle requires Cla4 and the Dma proteins. A: Logarithmically growing cultures of wild type (W303), *dma1Δ dma2Δ cla4-75* (ySP5264) and *cdc12-6* (ySP5182) cells were arrested in mitosis by nocodazole treatment for 2.5 hours and then shifted to 37°C, followed by FACS analysis of DNA contents (not shown) and in situ immunofluorescence of the septin ring with anti-Cdc11 antibodies. We confirmed that the mitotic arrest was maintained throughout the time course in all strains. **B**: Micrographs of the nocodazole-arrested cells after 70' of incubation at 37°C.

Thus, Dma1 and Dma2 are required, together with Cla4, for septin ring stability throughout the cell cycle.

In order to investigate in deeper detail the role of Dma1 Dma2 and Cla4 in the regulation of septin dynamics, we decided to study septin dynamics by fluorescence recovery after photobleaching (FRAP) on yeast cells that express the septin Cdc12 fused to GFP. This technique has been used to study the regulation of septin dynamics during the cell cycle and has allowed to discover that the septin ring oscillates between a frozen and a fluid state (Dobbelaere et al., 2003). Upon bleaching of half of the septin ring, fluorescence recovery can only be observed if septin complexes redistribute inside the ring from the unbleached semi-ring (Dobbelaere et al., 2003). We did the half bleaching in large budded cells (rigid state) of wild type, *dma1*Δ *dma2*Δ*, cla4-75* and *dma1*Δ *dma2*Δ *cla4-75* at 37°C, to inactivate the *cla4-75* temperature-sensitive allele. Recovery curves showed that *dma1*Δ *dma2*Δ and *cla4-75* cells recover fluorescence after photobleaching more efficiently than wild type cells (29,6% and 23,9%, respectively, versus 14,4% of wild type at the end of the experiment), and in the *dma1*Δ *dma2*Δ *cla4-75* triple mutant the percentage of recovery reaches 41,1% (Fig. 14). Therefore, consistent with the interpretation of our genetic data, lack of Dma1/2 as well as Cla4 inactivation destabilizes the septin ring in cell cycle stages where the septin ring is rigid. In addition, since the septin ring is even more fluid when the three proteins are simultaneously inactivated, Cla4 and Dma1/2 likely regulate independently septin dynamics.

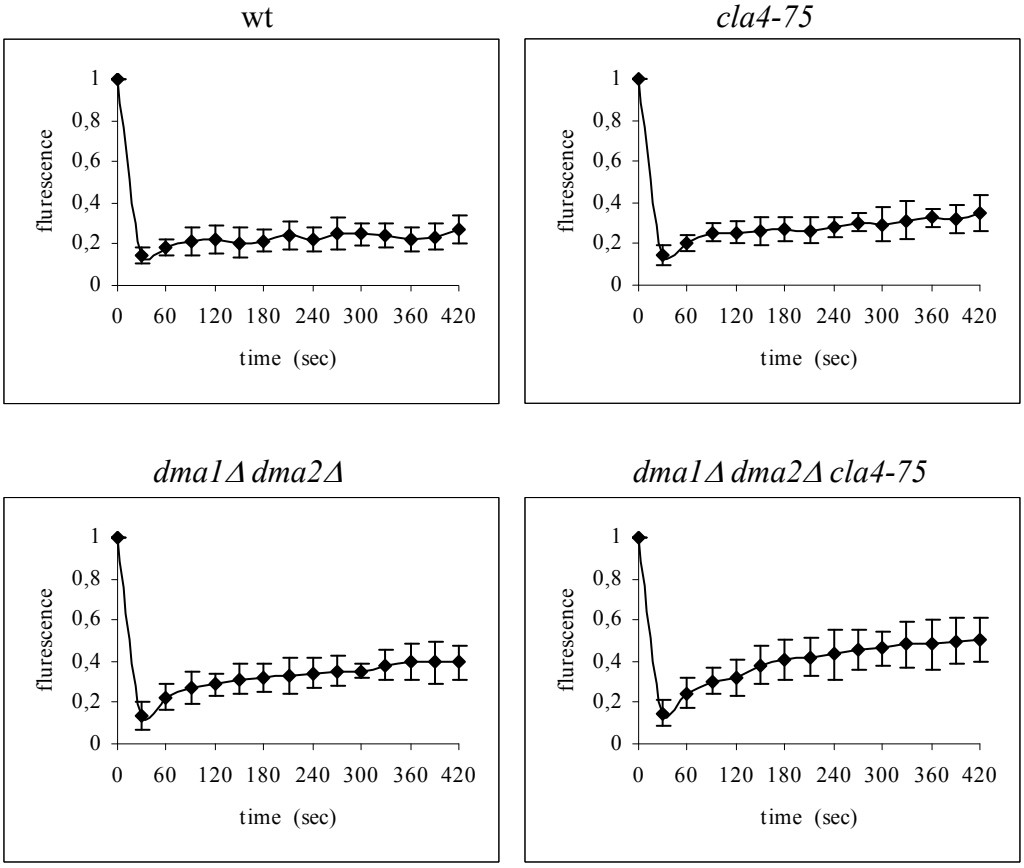


Figure 14. Septin ring stability is affected in *dma1 dma2* and *cla4* mutants. FRAP analysis of Cdc12-GFP in wild type (ySP8176), *dma1Δ dma2Δ* (ySP8173), *cla4-75* (ySP8296), *dma1Δ dma2Δ cla4-75* (ySP8193). Half of the septin ring was bleached in large budded cells at 37°C and recovery of fluorescence was recorded over time. The graphs represent relative average fluorescence intensities with bars showing standard errors (N=13 and N=8 for wt and *dma1Δ dma2Δ* respectively; N=9 for both *cla4-75* and *dma1Δ dma2Δ cla4-75*).

The FHA and RING domains are required for Dma proteins' function in septin ring deposition and stabilization

Dma1 and Dma2 contain a central FHA domain, usually involved in binding Thr-phosphorylated proteins (Durocher et al., 2000), and a C-terminal RING finger domain, typical of E3 ubiquitin ligases (Joazeiro and Weissman, 2000). We asked if these domains are essential for Dma function in septin ring deposition, by analyzing *dma1* and *dma2* mutant alleles altering specifically either the FHA domain (*dma1*FHA1: dma1-S220A, H223L; dma1*FHA2: dma1-G192E*) or the RING domain (*dma2*RING: dma2-C451S, H456A*) (Bieganowski et al., 2004). As shown in Figure 15A, wild type *DMA1* or *DMA2* expressed from the galactose-inducible *GAL1* promoter on a centromeric plasmid rescued the lethality of *dma1Δ dma2Δ cla4-75* cells at 37°C whereas none of the above mutant alleles could do so. In addition, these alleles caused aberrant cell shapes and inability to assemble a normal septin ring at the bud neck at the restrictive temperature (Fig. 15B). In fact, 53% of *dma1Δ dma2Δ cla4-75* cells overexpressing either *GAL1-DMA1* or *GAL1-DMA2* showed properly deposited septin rings 3 hours after temperature shift, when only 5-6% of *dma1Δ dma2Δ cla4-75* cells overexpressing any of the mutant alleles had normally positioned septin rings, suggesting that both FHA and RING domains are required for the function of Dma proteins in septin dynamics.

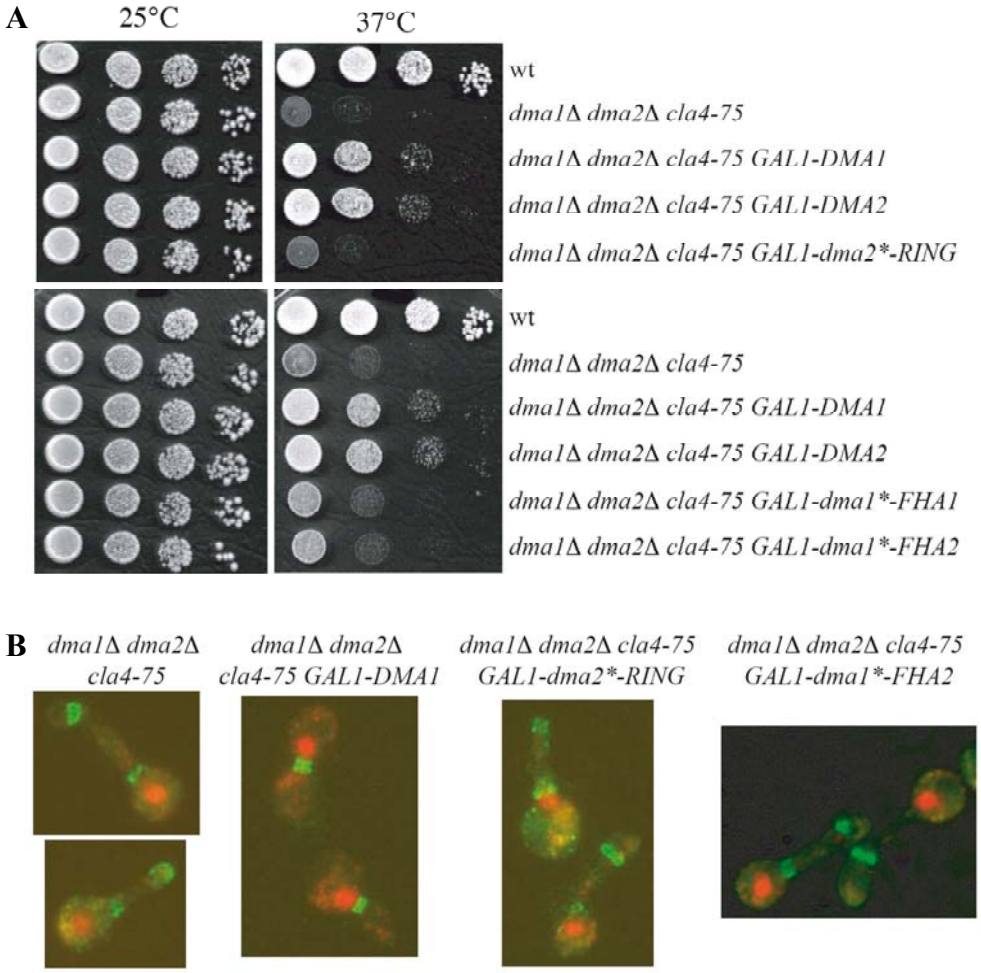


Figure 15. The FHA and RING domains are required for Dma proteins' function in septin ring organization. A: Serial dilutions of stationary phase cultures of the strains with the indicated genotypes were grown in synthetic raffinose medium lacking leucine at 25°C, spotted on YEPRG and incubated at the indicated temperatures for 2 days. **B**: Strains with the indicated genotypes were grown in synthetic medium lacking leucine at 25°C and arrested in G1 by α-factor. 1% galactose was added 30 minutes before the release in YEPRG at 37°C. The septin ring was stained by in situ immunofluorescence with anti-Cdc11 antibodies and micrographs were taken after 3 hours at 37°C. An asterisk indicates the missense mutations in the Dma RING and FHA domains that are detailed in the text

The lethal effects of *DMA2* overexpression are suppressed by septin ring destabilization

Yeast cells carrying multiple copies of a *GAL1-DMA2* construct integrated in the genome have cytokinesis defects when shifted to galactose-containing medium. Nonetheless, they enter a new cell cycle, accumulating binucleate cells that re-bud and assemble a new septin ring at the site of bud emergence without disassembling that formed in the previous cell cycle (Fraschini et al., 2004). As Dma proteins are involved in septin ring deposition, the cytokinesis defects caused by high levels of Dma2 might be due to excessive stabilization of the septin ring. To test this hypothesis, we asked whether mutations that destabilize the septin ring, such as *cdc12-1* and *shs1Δ*, could rescue the lethality caused by *DMA2* overexpression and restore normal septin dynamics. Indeed, *GAL1-DMA2* toxic effects on galactose were partially suppressed by both *cdc12-1* and *shs1Δ* alleles at 25°C (Fig. 16A), suggesting that septin ring destabilization allows *GAL1-DMA2* cells to undergo proper cytokinesis. We then analysed directly septin ring formation and disassembly in *GAL1-DMA2* and *GAL1-DMA2 cdc12-1* cells during a synchronous release from G1 in galactose-containing medium at 25°C. In parallel, we monitored other cell cycle parameters, such as spindle formation and elongation by *in situ* immunofluorescence, as well as the protein levels of the polo kinase Cdc5, which is degraded during mitotic exit. *GAL1-DMA2* cells exited mitosis more slowly than wild type cells, as shown by the delayed spindle disassembly (Fig. 16B) and Cdc5 proteolysis (Fig. 16D). Later on, a fraction of *GAL1-DMA2* cells rebudded and formed new septin rings before dividing and disassembling the old septin

rings, as expected (Fig. 16B, C). In sharp contrast, such cytokinesis defects were suppressed in *GAL1-DMA2 cdc12-1* cells. In addition, these cells displayed wild type kinetics of spindle elongation and Cdc5 degradation. Altogether, these data support the notion that high levels of Dma2 cause septin ring hyperstabilization that leads to cytokinesis failure and mitotic exit defects.

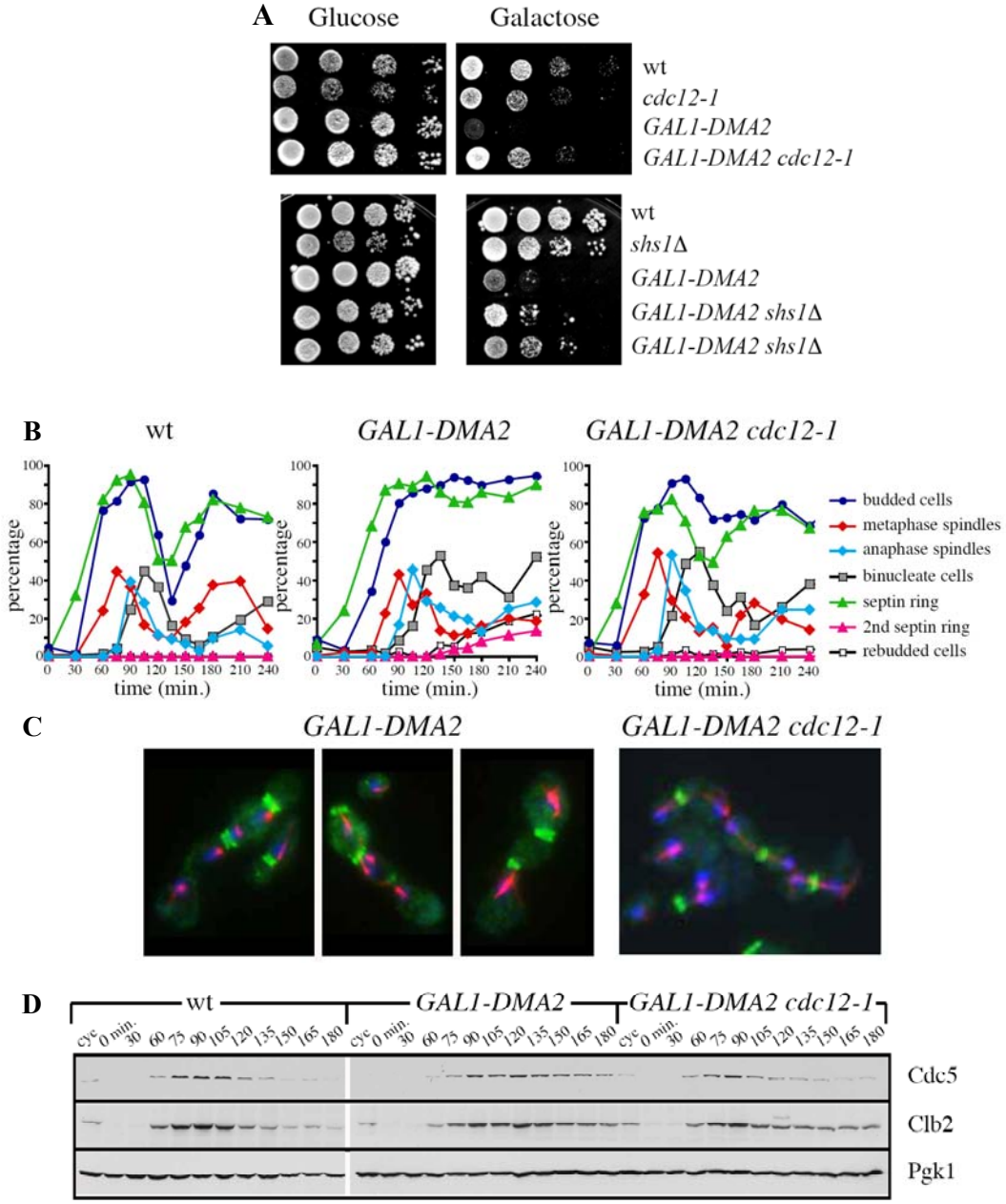


Figure 16. Destabilization of the septin ring rescues the cytokinesis defects caused by high levels of Dma2. A: Serial dilutions of stationary phase cultures of strains with the indicated genotypes were spotted on YEPD (glucose) or YEPRG (galactose) plates and incubated at 25°C for 2 days. **B-D**: Wild type (W303), *GAL1-DMA2* (ySP3018) and *GAL1-DMA2 cdc12-1* (ySP5244) cells grown in YEPR medium were arrested in G1 by α factor and released in YEPRG at 25°C. α factor (10 μg/ml) was re-added at t=105' to the half of the culture used to make protein extracts (D) to prevent cells from entering a second cell cycle. At the indicated times, samples were taken for FACS analysis of DNA contents (not shown) and for scoring budding, nuclear division, spindle formation/elongation and septin ring deposition by in situ immunofluorescence with anti-tubulin and anti-Cdc11 antibodies, respectively (B). Representative micrographs were taken at t=240' (C). Protein extracts were analysed by western blot with anti-Cdc5 and anti-Pgk1 antibodies (D).

***dma1 dma2 cla4* lethality is suppressed by *RTS1* deletion**

The transition of the septin ring from a rigid to a dynamic state that allows the splitting of the septin ring just before cytokinesis is mediated by Rts1, a B type regulatory subunit of the protein phosphatase PP2A. Specifically, Rts1 is localized at the bud neck in telophase (Dobbelaere et al., 2003) and the septin Shs1 is dephosphorylated by Rts1 at the beginning of cytokinesis (Shu et al., 1997, Dobbelaere et al., 2003). Since Rts1 is a negative regulator of septin ring stability whereas Dma1, Dma2 and Cla4 promote the stabilization of septin ring, we asked whether *RTS1* deletion could suppress the lethality of *dma1 dma2 cla4* cells. As shown in Figure 17A, *RTS1* deletion did not suppress the thermo-sensitivity of a *dma1 dma2 cla4-75* triple mutant. However, as previously shown by others (Artiles et al., 2009), we found that *RTS1* deletion confers a temperature-sensitive phenotype. In contrast, *RTS1* deletion was sufficient to rescue the lethality of *dma1 dma2 cla4* cells at 25°C (data not shown). We therefore carried out a phenotypic characterization of the *dma1Δ dma2Δ cla4Δ rts1Δ* quadruple mutant, especially for what concerned septin ring deposition at the bud neck. Elutriated wild type and *dma1Δ dma2Δ cla4Δ rts1Δ* small unbudded cells were resuspended in fresh medium at 25°C and at different time points after release we monitored DNA contents by FACS analysis (Fig. 17B) and the kinetics of budding, nuclear division and septin ring assembly (Fig. 17C). Interestingly, *dma1Δ dma2Δ cla4Δ rts1Δ* cells were able to properly assemble septin rings, albeit with a slight delay (15 minutes) and less efficiently with respect to wild type cells. These results

suggest that the role of Dma1, Dma2 and Cla4 in septin ring stabilization is counteracted by $PP2A^{Rts1}$.

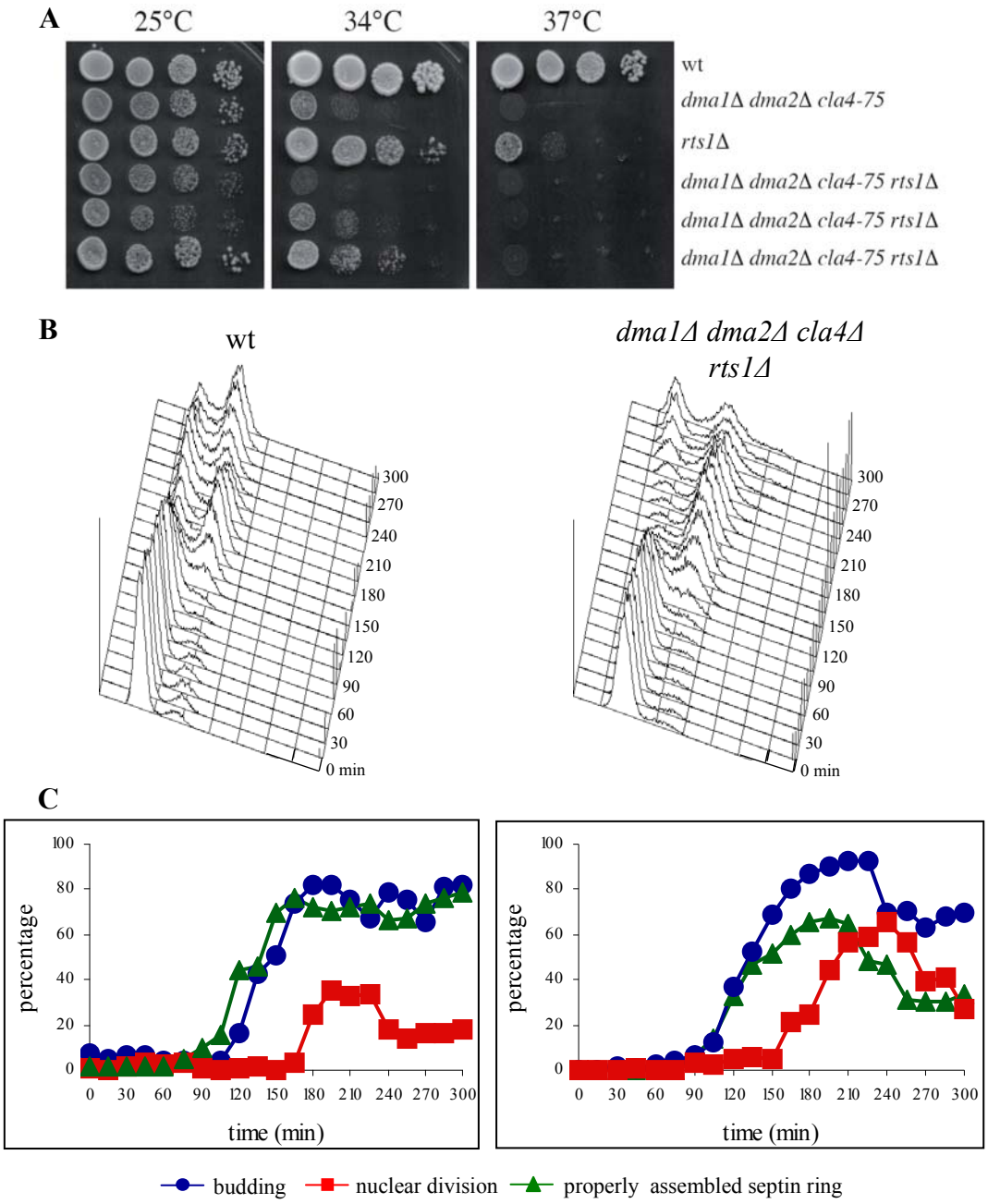


Figure 17. The cytokinesis defects of *dma1 dma2 cla4* cells are suppressed by *RTS1* deletion. A: Three independent *dma1Δ dma2Δ cla4-75 rts1Δ* meiotic segregants were obtained by genetic crosses. Serial dilutions of stationary phase cultures of strains with the indicated genotypes were spotted on YEPD plates and incubated for 2 days at the indicated temperatures. **B**, **C**: Small unbudded cells of wild type (W303) and *dma1Δ dma2Δ cla4Δ rts1Δ* (ySP8679) strains were isolated by centrifugal elutriation and resuspended in fresh medium at 25°C (time 0). At the indicated times, cell samples were collected for FACS analysis of DNA contents (B), for scoring budding and nuclear division and for the septin ring analysis by in situ immunofluorescence with anti-Cdc11 antibodies (C).

Simultaneous lack of Dma1, Dma2, Cla4 and Swe1 causes spindle mispositioning without activation of the spindle position checkpoint

Because *dma1Δ dma2Δ cla4-75* cells are unable to properly deposit septin rings at the restrictive temperature, their G2 arrest is likely due to activation of the morphogenesis checkpoint that responds to septin defects by delaying mitotic entry (Barral et al., 1999; Lew, 2003). We then knocked-out Swe1, the direct target of this checkpoint (Sia et al., 1996), in cells lacking Dma1, Dma2 and Cla4. Wild type, *dma1Δ dma2Δ cla4-75* and *dma1Δ dma2Δ cla4-75 swe1Δ* cells were arrested in G1 by α factor at 25°C and then released into the cell cycle at 37°C. Indeed, deletion of *SWE1* allowed *dma1Δ dma2Δ cla4-75* cells to undergo nuclear division at the restrictive temperature (Fig. 18A), indicating that septin defects activate the morphogenesis checkpoint in these cells. Interestingly, nuclear division took place in the mother cell in a high fraction of *dma1Δ dma2Δ cla4-75 swe1Δ* cells (40% at 180 minutes) (Fig. 18A, B), which is consistent with the known role of the septin ring in spindle positioning (Kusch et al., 2002). In spite of that, cells exited mitosis and underwent a new round of DNA replication (Fig. 18A), suggesting that the spindle position checkpoint, which normally prevents mitotic exit and cytokinesis in cells with mispositioned spindles (reviewed in Fraschini et al., 2008), is inactive in these cells. This is likely caused by unscheduled Tem1 activation, which in turn can be triggered by either Lte1 or Bub2/Bfa1 mislocalization. In particular, both septins and Cla4 are required to restrict Lte1 localization to the bud (Castillon et al., 2003; Chiroli et al., 2003; Hofken and Schiebel, 2002; Seshan et al., 2002).

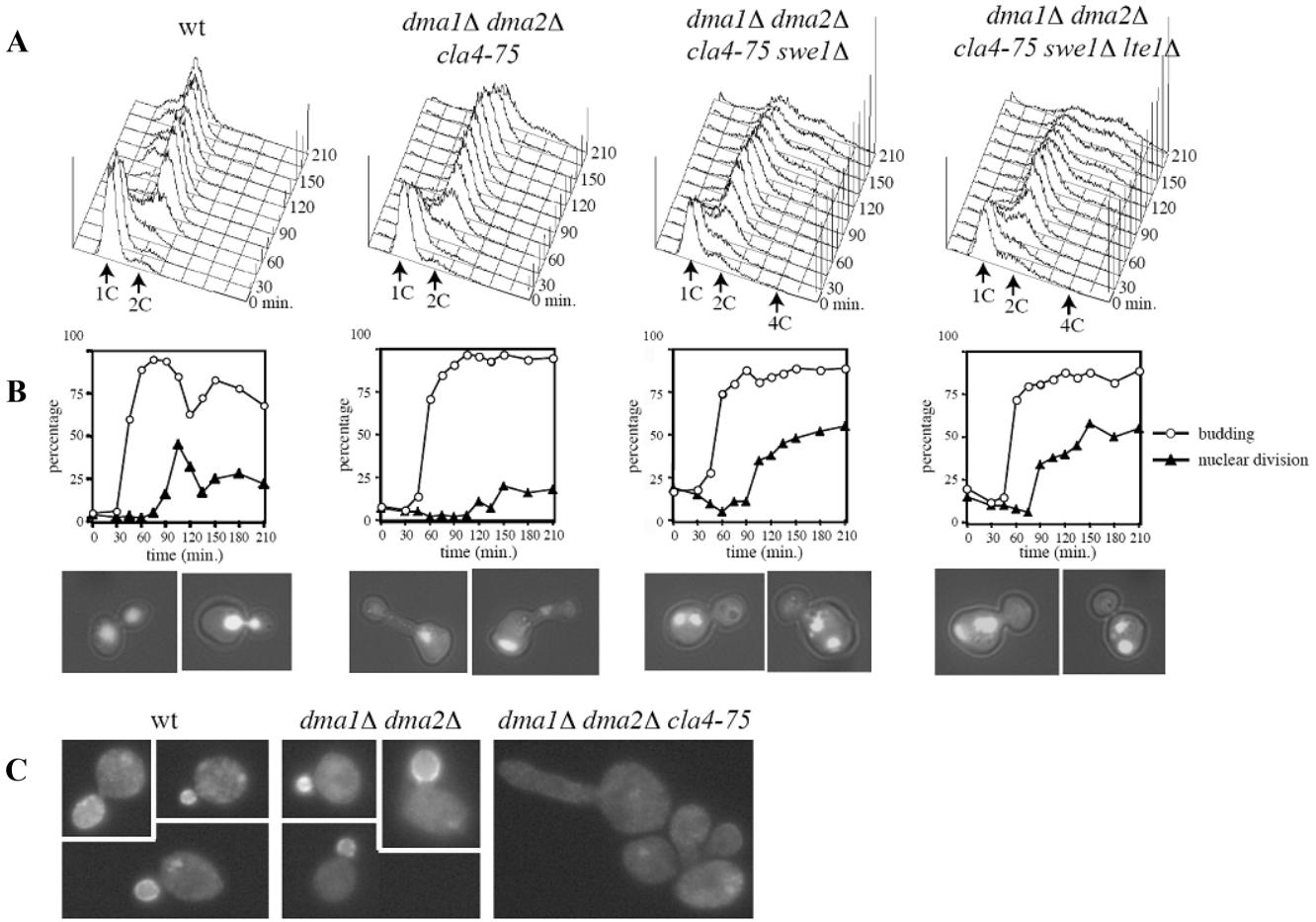


Figure 18. Cells lacking Dma proteins and Cla4 are arrested by the morphogenesis checkpoint and are defective in SPOC response to spindle mispositioning. A: Logarithmically growing cultures of wild type (W303), *dma1Δ dma2Δ cla4-75* (ySP5247), *dma1Δ dma2Δ cla4-75 swe1Δ* (ySP6238) and *dma1Δ dma2Δ cla4-75 swe1Δ lte1Δ* (ySP7672) cells were arrested in G1 by α factor at 25°C and released into the cell cycle at 37°C at time 0. Cells were collected at the indicated times for FACS analysis of DNA contents (upper histograms) and scoring of budding and nuclear division (bottom graphs). **B**: Micrographs of cells in (A) were taken at 120 minutes after release. **C**: Cultures of wild type (ySP3333), *dma1Δ dma2Δ* (ySP4380) and *dma1Δ dma2Δ cla4-75* (ySP7766) cells expressing Lte1-GFP from the *GAL1* promoter were grown in YEPR medium, induced with 1% galactose for 1 hour and then shifted to 37°C for 3 hours.

Consistently, Lte1 was homogeneously distributed in *dma1Δ dma2Δ cla4-75* cells in all cell cycle stages (Fig. 18C), raising the possibility that mitotic exit in *dma1Δ dma2Δ cla4-75 swe1Δ* cells with mispositioned spindles was caused by Lte1 spreading into the mother cell. However, *LTE1* deletion did not affect the ability of *dma1Δ dma2Δ cla4-75 swe1Δ* cells to escape from mitosis and enter into a new round of DNA replication upon spindle mispositioning (Fig. 18A), indicating that Dma proteins and/or Cla4 can control mitotic exit in ways that do not involve Lte1 localization/activation.

We then analysed the subcellular distribution of Bub2, Bfa1 and Tem1, all tagged with HA epitopes at the C-terminus, in *dma1Δ dma2Δ cla4-75 swe1Δ* cells that underwent anaphase in the presence of mispositioned spindles. After 3 hours at the non-permissive temperature, about 30-40% of the cells had undergone anaphase within the mother cell due to spindle misalignment. Tem1-HA3 localized on both SPBs in 72% of these cells (Fig. 19C), as expected, whereas Bub2-HA3 and Bfa1-HA6 were retained on both SPBs only in 21% and 24% of cells undergoing anaphase within the mother cell, respectively (Fig. 19A, B). To exclude that asymmetric SPB localization of Bub2/Bfa1 under these conditions was due to the unscheduled mitotic exit of *dma1Δ dma2Δ cla4-75 swe1Δ* cells, we double stained Bfa1-HA6 and tubulin by immunofluorescence in *dma1Δ dma2Δ cla4-75 swe1Δ* cells released from G1 arrest at 37°C (Fig. 19D). Under these conditions, Bfa1-HA6 was present only on one SPB in 77% of the cells undergoing anaphase with mispositioned spindle before spindle disassembly, i.e. before mitotic exit. Thus, Dma

proteins and/or Cla4 might be required to maintain the Bub2/Bfa1 complex on both SPBs upon spindle mispositioning.

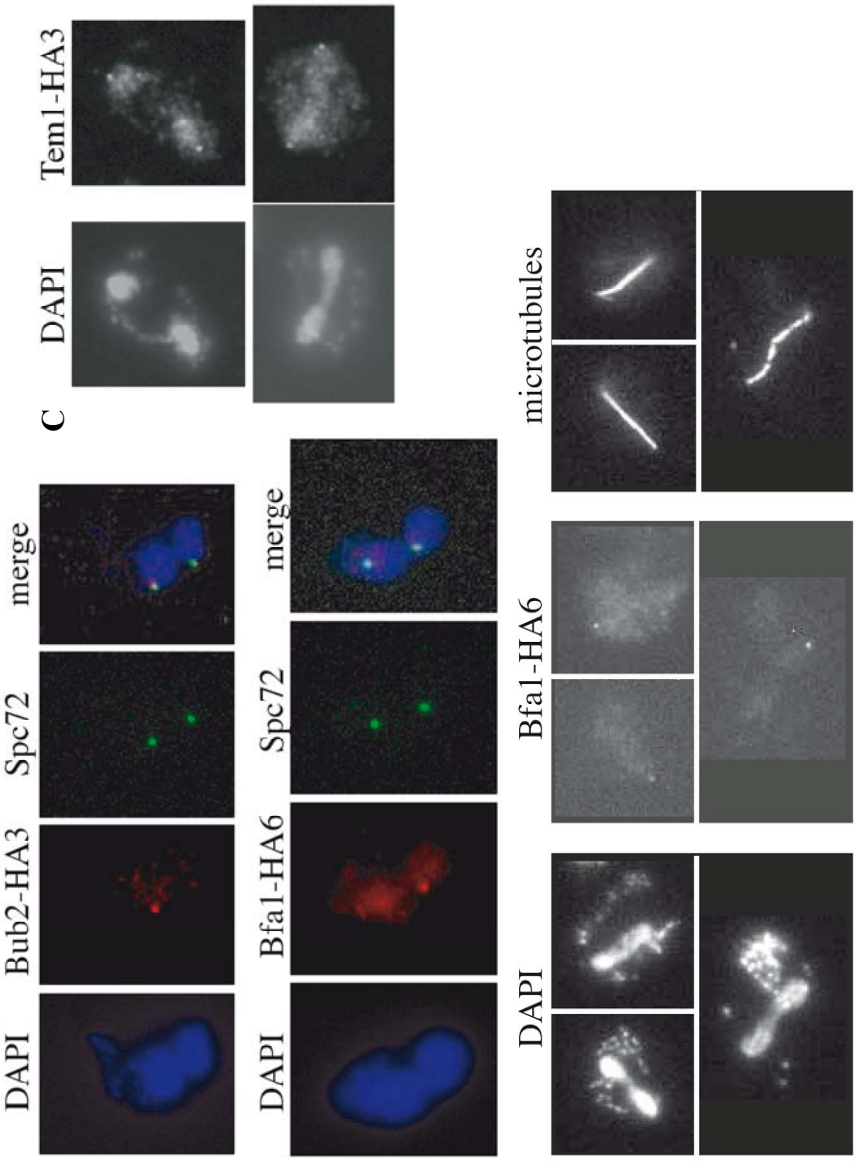


Figure 19. Asymmetric SPB localization of Bub2/Bfa1 in *dma1Δ dma2Δ cla4-75 swe1Δ* cells with mispositioned spindle. A-C: Cells were grown in YEPD at 25°C and shifted to 37°C for 3 hours, followed by DAPI staining of nuclei and visualization of the indicated proteins and of the Spc72 SPB component (Knop and Schiebel, 1998) by indirect immunofluorescence. Micrographs of cell with mispositioned spindles are shown. **A**: *dma1Δ dma2Δ cla4-75 swe1Δ* cells (ySP7771) expressing HA-tagged Bub2 (Bub2-HA3). **B**: *dma1Δ dma2Δ cla4-75 swe1Δ* cells (ySP7819) expressing HA-tagged Bfa1 (Bfa1-HA6). **C**: *dma1Δ dma2Δ cla4-75 swe1Δ* cells (ySP7789) expressing HA-tagged Tem1 (Tem1-HA3). **D**: *dma1Δ dma2Δ cla4-75 swe1Δ* cells (ySP7819) expressing Bfa1-HA6 were grown in synthetic medium lacking uracil at 25°C, synchronized in G1 by α factor and released at 37°C, followed by nuclei DAPI staining and visualization of Bfa1-HA6 and microtubules by indirect immunofluorescence. Micrographs were taken at 90 minutes after release.

Thus, we decided to investigate whether re-establishment of the Bub2/Bfa1 symmetric localization at SPBs by expression of the Bub2-myc9 variant (Fraschini et al., 2006) could rescue the SPOC defect of these cells. Wild type, *dma1Δ dma2Δ cla4-75*, *dma1Δ dma2Δ cla4-75 swe1Δ* and *dma1Δ dma2Δ cla4-75 swe1Δ BUB2-myc9* cells were synchronized in G1 with α factor at 25°C and released in the cell cycle at 37°C. Progression through the cell cycle was followed by cytofluorimetric analysis of DNA contents (Fig. 20A) and the symmetric localization of Bub2-myc9 at SPBs was checked by indirect immunofluorescence (Fig. 20B). As shown in Figure 20, the re-establishment of Bub2/Bfa1 symmetric localization at SPBs did not suppress the SPOC defect of *dma1Δ dma2Δ cla4-75 swe1Δ* cells. These data suggest that loss of Bub2/Bfa1 from one SPB is unlikely the only reason for cells to escape mitosis in the presence of mispositioned spindles. Therefore, in these conditions the MEN must be activated by other means than mislocalization of Lte1 and Bub2/Bfa1.

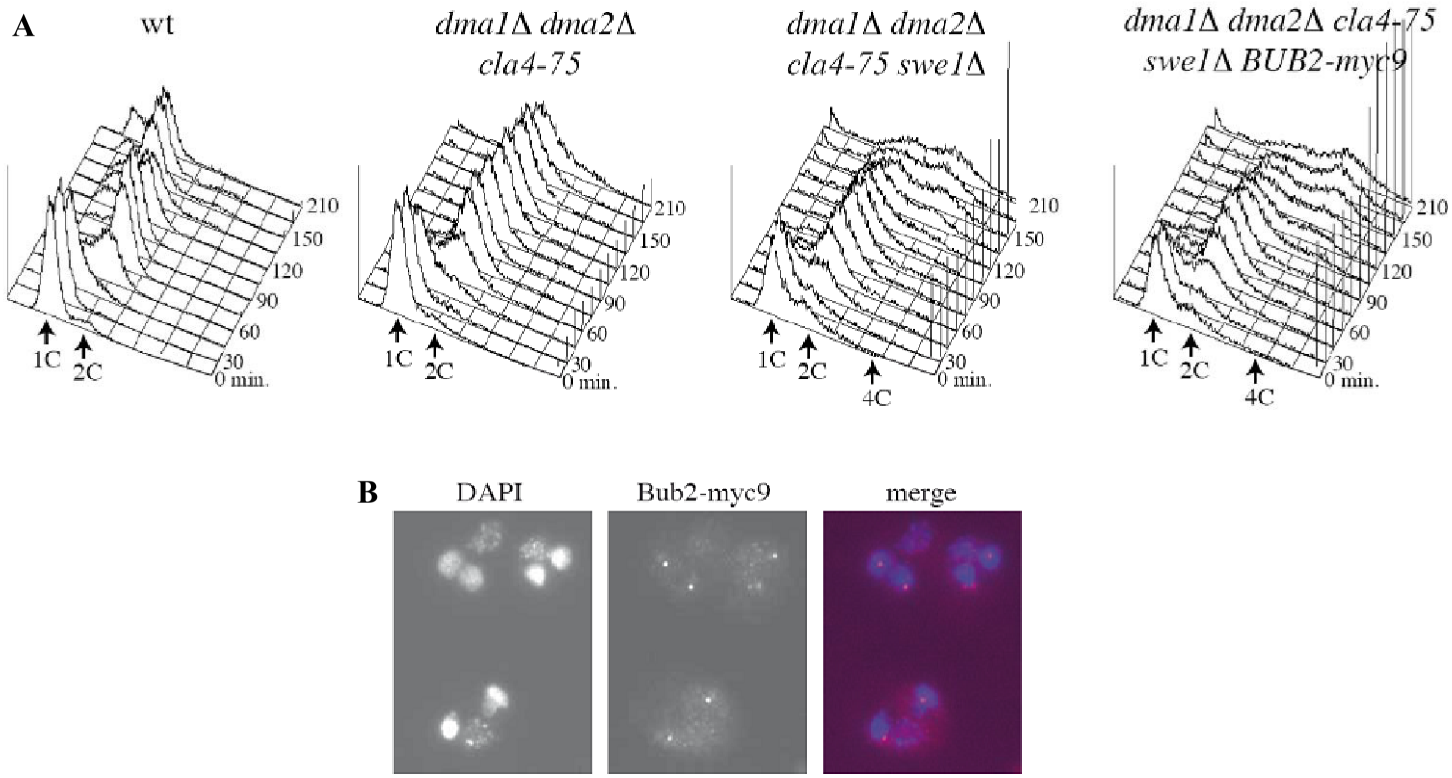
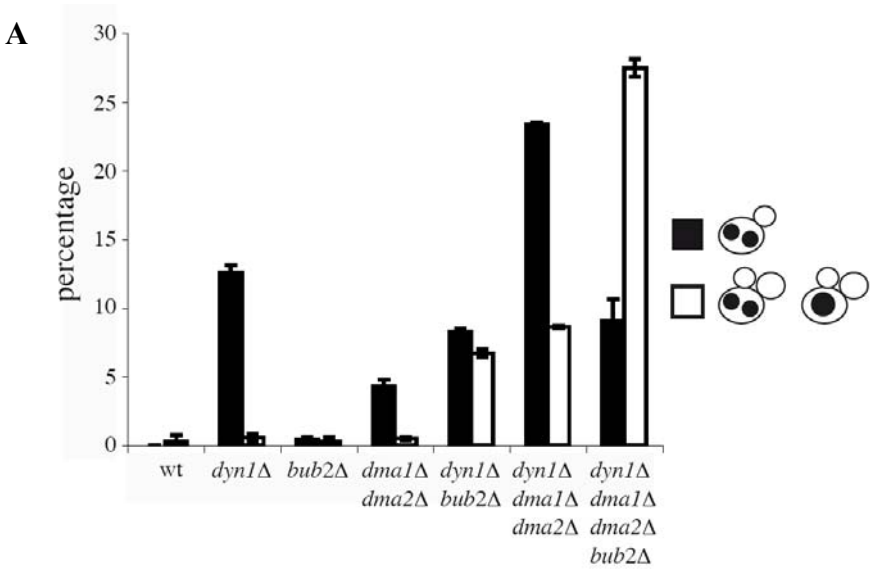


Figure 20. Symmetric recruitment of Bub2 at SPBs does not suppress the SPOC defect of *dma1Δ dma2Δ cla4-75 swe1Δ* mutants. A, B: Logarithmically growing cultures of wild type (W303), *dma1Δ dma2Δ cla4-75* (ySP5247), *dma1Δ dma2Δ cla4-75 swe1Δ* (ySP6238) and *dma1Δ dma2Δ cla4-75 swe1Δ BUB2-myc9* (ySP7868) cells were arrested in G1 by α factor at 25°C and released into the cell cycle at 37°C at time 0. Cells were collected at the indicated times for FACS analysis of DNA contents (A) and immunostaining of Bub2-myc9 with anti-myc antibodies (B). Micrographs were taken after 3 hours at 37°C.

Dma1 and Dma2 control the SPOC independently of Bub2/Bfa1

To test whether Dma proteins act in the same pathway of Bub2/Bfa1 in controlling mitotic exit upon spindle mispositioning, we deleted *DMA1* and *DMA2* along with *BUB2* or *BFA1* in *dyn1Δ* cells, which show a relatively high fraction of spindle position defects at low temperatures (Yeh et al., 1995). As a consequence, cells undergo anaphase and nuclear division within the mother cells. Cells were grown at 30°C and shifted to 14°C for 16 hours. Nuclear division in the mother cell was scored as indicative of spindle position defects, while re-budding of these cells indicated the failure to activate the SPOC. Under these conditions, about 15% of *dyn1Δ* cells had undergone nuclear division in the mother cell, but only a minor fraction of them re-budded (Fig. 21A, B), indicating that the SPOC efficiently prevented mitotic exit. Deletion of *BUB2* (Fig. 21A), *BFA1* (Fig. 21B) or *DMA1* and *DMA2* (Fig. 21A, B) allowed a significant fraction of *dyn1Δ* cells to exit mitosis before proper spindle positioning, confirming the involvement of the corresponding proteins in the SPOC. It should be noted, however, that deletion of *DMA1* and *DMA2* also enhanced the spindle position defect caused by *dyn1Δ* (Fig. 21A, B), in agreement with our previous data (Fraschini et al., 2004). Therefore, the SPOC defect of *dyn1Δ dma1Δ dma2Δ* cells relative to the spindle position defect was similar, if not slightly less prominent, to that of *dyn1Δ bub2Δ* and *dyn1Δ bfa1Δ* cells. Interestingly, the concomitant lack of Dma proteins and Bub2 or Bfa1 had additive effects on the SPOC failure of *dyn1Δ* cells (Fig. 21A, B), suggesting that Bub2/Bfa1 and Dma proteins control mitotic exit independently of each other upon spindle mispositioning. To support

this hypothesis, we evaluated the effects of the combined deletion of *DMA1*, *DMA2* and *BUB2* or *BFA1* in cells lacking Kar9, which are also defective in spindle positioning (Beach et al., 2000; Miller and Rose, 1998). Concomitant lack of Dma proteins and Bub2 or Bfa1 had synthetic effects in *kar9Δ* cells in our genetic background, causing growth defects at 25°C and a marked temperature-sensitivity at 37°C (Fig. 21C). Moreover, *dma1Δ dma2Δ bub2Δ* and *dma1Δ dma2Δ bfa1Δ* cells were more sensitive than *dma1Δ dma2Δ, bub2Δ* or *bfa1Δ* cells to sublethal doses of the microtubule-depolymerizing compound benomyl (Fig. 21D). Thus, Dma proteins and Bub2/Bfa1 seem to control cell response to microtubule perturbations through different pathways.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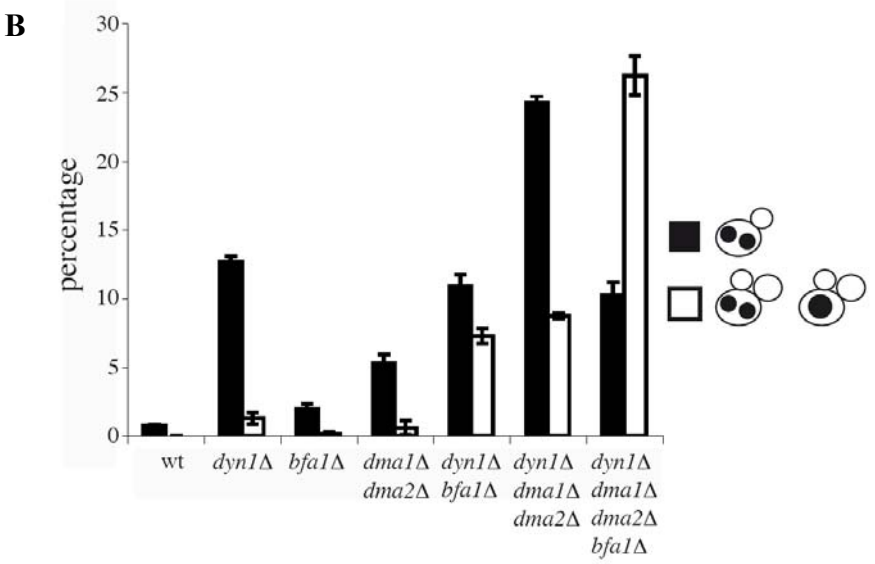
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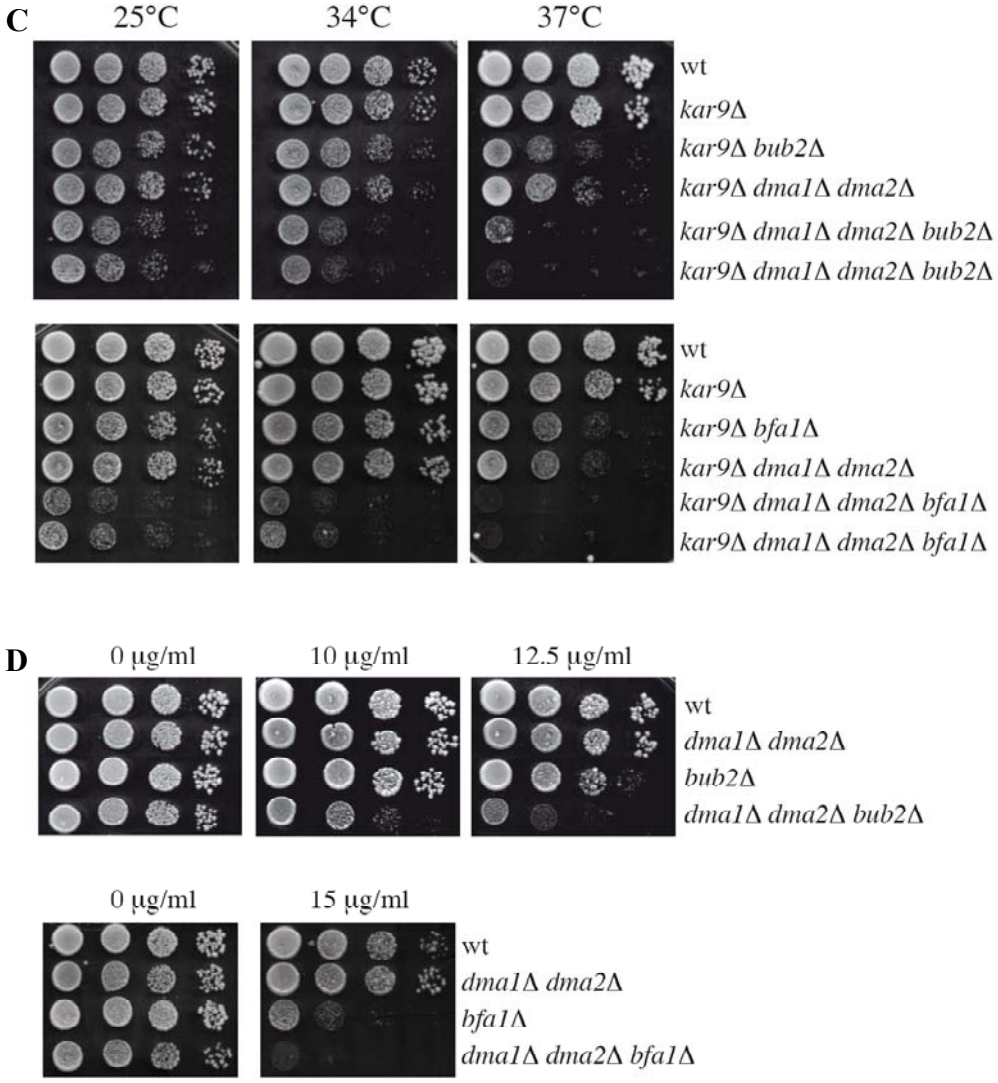


Figure 21. Dma proteins regulate mitotic exit independently of Bub2/Bfa1. A: Exponentially growing cultures of *dyn1Δ* (ySP6292), *bub2Δ* (ySP3138), *dma1Δ dma2Δ* (ySP1569), *dyn1Δ bub2Δ* (ySP6680), *dyn1Δ dma1Δ dma2Δ* (ySP7454) and *dyn1Δ bub2Δ dma1Δ dma2Δ* (ySP7855) cells were shifted to 14°C for 16 hours, followed by scoring cell morphology and nuclear division. Histograms represent average values of three independent experiments, with bars showing standard errors. **B**: The experiment was performed as in A, but the *bub2Δ* strains in A were replaced by the following strains: *bfa1Δ* (ySP1243), *dyn1Δ bfa1Δ* (ySP8450) and *dyn1Δ bfa1Δ dma1Δ dma2Δ* (ySP8444). **C:** Serial dilutions of stationary phase cultures of strains with the indicated genotypes were spotted on YEPD plates and incubated for 2 days at the indicated temperatures. **D**: Serial dilutions of stationary phase cultures of strains with the indicated genotypes were spotted on YEPD plates containing the indicated benomyl concentrations and incubated for 2 days either at 25°C (upper pictures) or at 30°C (lower pictures).

Dma1 and Dma2 control the bud neck localization of the Elm1 kinase

The recent finding that the Elm1 kinase, besides controlling septin localization (Bouquin et al., 2000; Thomas et al., 2003), participates in the SPOC by activating Kin4 (Caydasi et al., 2010, Moore et al., 2010), prompted us to investigate whether its bud neck localization is regulated by the Dma proteins. Upon release from G1 arrest, wild type and *dma1Δ dma2Δ* cells progressed similarly through the cell cycle, as shown by their kinetics of DNA replication (not shown), budding and nuclear division (Fig. 22A). In spite of that, Elm1-eGFP localization at the bud neck was severely impaired in *dma1Δ dma2Δ* cells compared to wild type cells. Indeed, the fraction of cells with Elm1-eGFP at the bud neck was much lower in *dma1Δ dma2Δ* than in wild type cells throughout the experiment (Fig. 22A, B). In addition, the fluorescence intensity of bud neck-localized Elm1-eGFP was dramatically reduced by lack of Dma1 and Dma2 (469 ± 288 in *dma1Δ dma2Δ* vs. 1097 ± 426 in wild type at time 80', Fig. 22B). Similarly, upon release of G1 cells in the presence of hydroxyurea (HU) or nocodazole the bud neck localization of Elm1-eGFP was dramatically compromised in the absence of the Dma proteins (Fig. 22B and C; average fluorescence intensity in HU: 674 ± 335 in *dma1Δ dma2Δ* vs. 928 ± 485 in wild type cells). The reduced localization of Elm1 at the bud neck is not simply a consequence of the septin defects caused by the lack of Dma proteins, because the latter did not impair bud-neck localization of Cdc5 (data not shown). In a similar experiment to the one above, we monitored bud neck localization of Elm1-eGFP in wild type versus *DMA2*-overexpressing cells.

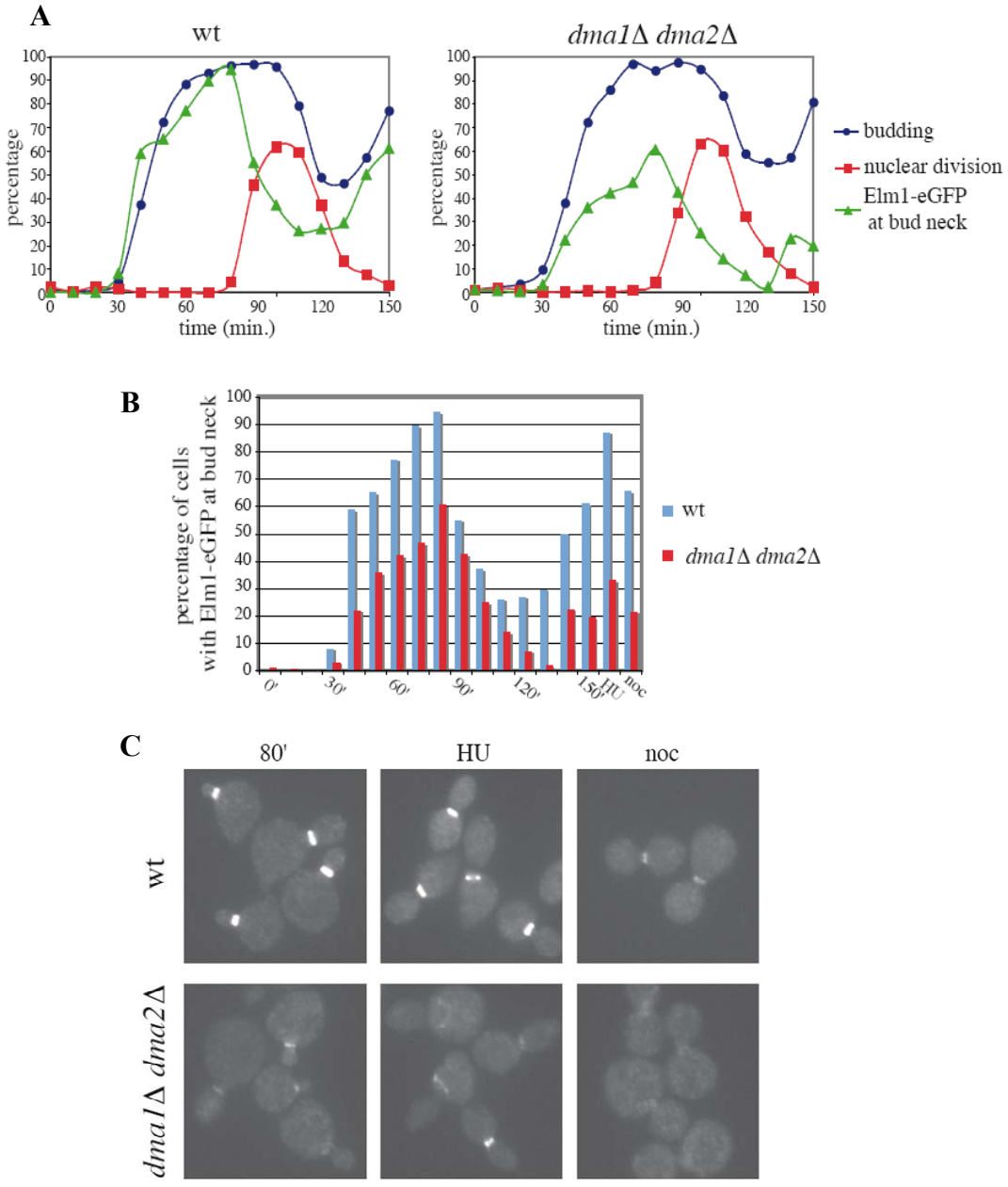


Figure 22. Dma proteins are required for efficient recruitment of Elm1 to the bud neck. A, B, C: Wild type (ySP8813) and *dma1Δ dma2Δ* (ySP8829) cells expressing Elm1-eGFP were arrested in G1 by α factor and released into the cell cycle at 25°C (time 0). The culture was split in three parts: one part was left untreated, while the other two were incubated for 150 minutes with HU (120 mM) and nocodazole (15 µg/ml), respectively. Cell samples from the untreated culture were withdrawn at the indicated times for FACS analysis of DNA contents (not shown) and to score budding, nuclear division and bud neck localization of Elm1-eGFP (A and B). Representative micrographs were taken for all cultures (C).

Consistent with our previous results (Fraschini et al., 2004), *GAL1-DMA2* cells started budding slightly later than wild type cells (Fig. 23A) and, accordingly, slightly delayed the appearance of Elm1-eGFP at the bud neck. However, disappearance of Elm1-eGFP from the bud neck at the end of the cell cycle was markedly delayed upon *DMA2* overexpression (Fig. 23A, B). Thus, lack of Dma proteins destabilizes Elm1 at the bud neck, whereas *DMA2* overexpression stabilizes it. We then monitored the levels of a HA-tagged Elm1 variant (Elm1-HA3) in wild type and *dma1Δ dma2Δ* cells during a synchronous release from G1. In contrast to previous data (Bouquin et al., 2000), we did not detect significant fluctuations in Elm1 protein levels during the cell cycle. In addition, Elm1 seemed to be expressed to similar levels in wild type and *dma1Δ dma2Δ* cells (Fig. 23C). However, the latter contained also a slowly migrating form of Elm1-HA3 that was undetectable in wild type cells (Fig. 23C). Although the post-translational modification appended to this electrophoretic variant remains to be identified, it might be related to Elm1 localization defects.

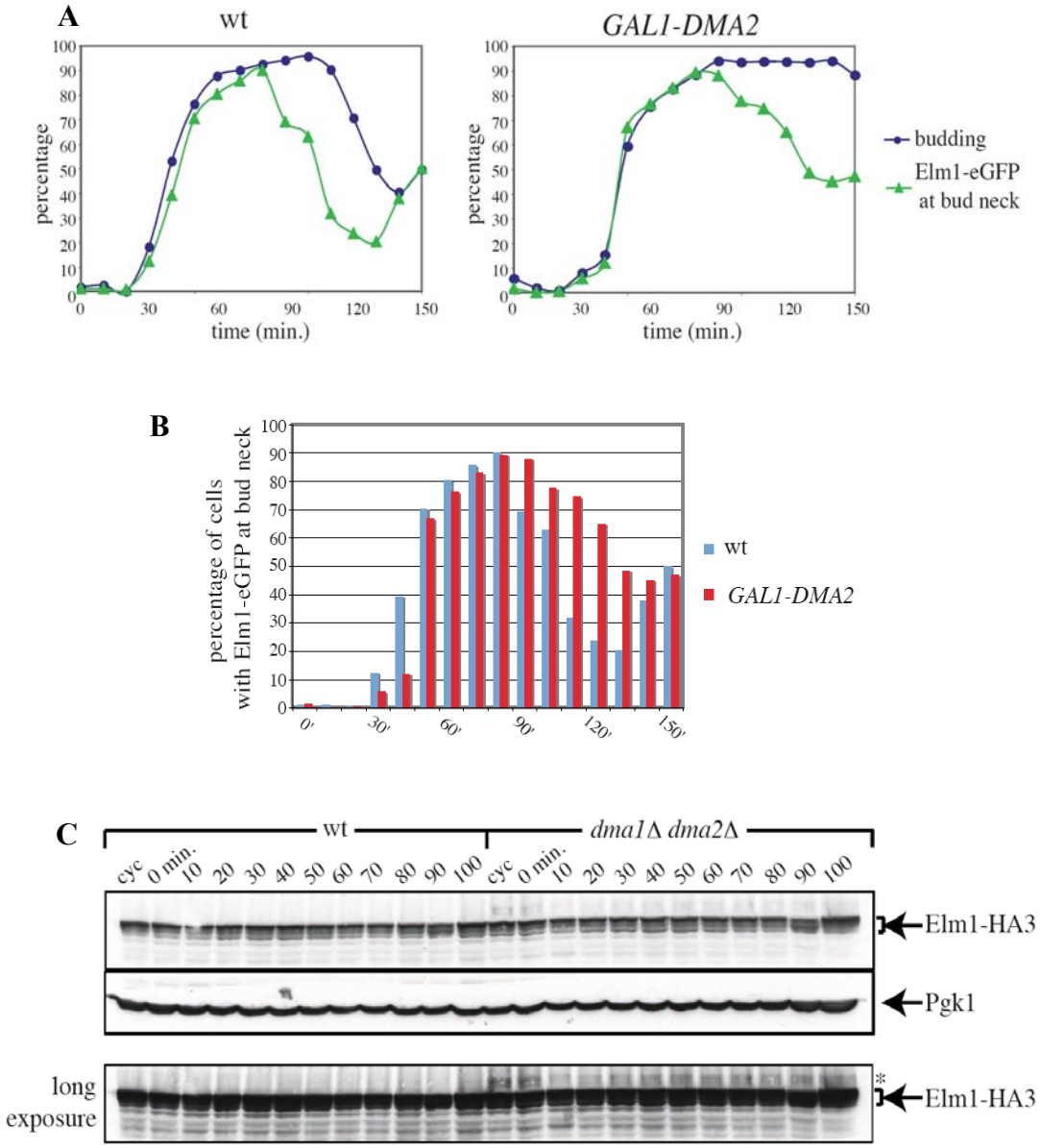


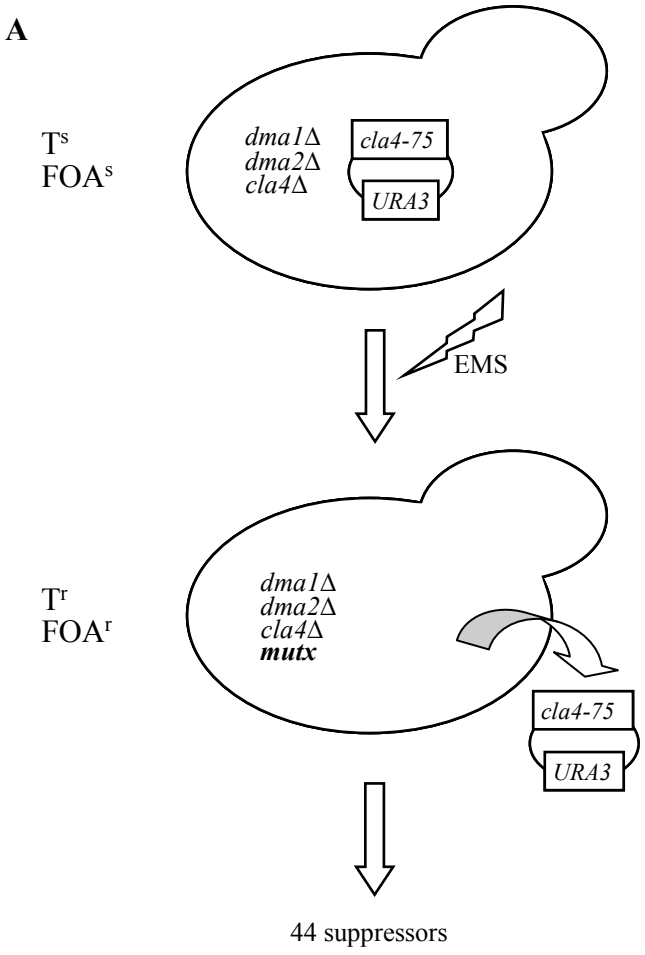
Figure 23. *DMA2* overexpression leads to persistent bud neck localization of Elm1 . A, B: Wild type (ySP8813) and *GAL1-DMA2* (ySP8814) cells expressing Elm1-eGFP were grown in YEPR, arrested in G1 by α factor, induced by galactose for 30' and released in YEPRG at 25°C (time 0). At the indicated time points cell samples were withdrawn to score budding and Elm1-eGFP localization. **C**: Wild type (ySP8820) and *dma1Δ dma2Δ* (ySP8821) cells expressing Elm1-HA3 were arrested in G1 by α factor and released in fresh YEPD at 25°C (time 0). Cell samples were collected at the indicated time points for FACS analysis of DNA contents (not shown) and western blot analysis of Elm1-HA3. Pgk1 levels are used as loading control. Asterisk marks a post-translationally modified variant of Elm1-HA3.

A genetic screen to find extragenic suppressors of the lethality *of dma1 dma2 cla4* cells

Dma1 and Dma2 are E3 ubiquitin-ligases (Loring et al., 2008). Since *dma1Δ dma2Δ cla4-75* cells do not undergo cytokinesis at the restrictive temperature, we hypothesised that Dma proteins could target for ubiquitylation a regulator of cytokinesis.

We therefore embarked in a genetic screen to search for extragenic suppressors of the lethality of *dma1 dma2 cla4* cells. To this end, *MATa* and *MATα dma1Δ dma2Δ cla4Δ cla4-75* cells were mutagenized with EMS (ethyl-methan-sulphonate), which introduces random point mutations in the genome, and incubated at the restrictive temperature after plating (see Materials and Methods for details). In order to discard all possible revertents of *cla4-75*, temperature-resistant clones were then assessed for their ability to lose the *cla4-75* allele carried on an episomal centromeric plasmid (Fig. 24A). After this secondary screen, we recovered 44 suppressors (30 MATa and 14 MATα) that retained viability in face of the simultaneous lack of Dma1, Dma2 and Cla4. We first analysed the dominance/recessivity of the suppressing mutations (see Materials and Methods for details), from which 11 mutants were classified as dominants, 10 as semi-dominants and 19 as recessives (Fig. 24B). We also checked by FACS analysis the ability of the isolated suppressors to undergo cytokinesis and indeed most of them were able to do so (data not shown). An example of this is shown in Figure 25A, where cells of a dominant suppressor (*MUT-135*) displayed a FACS profile similar to a wild type at 25°C. The ability of these cells to divide could be assessed by the presence of cells with unreplicated DNA contents (1C), while the

parental strain (*dma1Δ dma2Δ cla4-75*) at 37°C was arrested in G2 with 2C DNA contents as previously shown.



B

RECESSIVES	DOMINANTS	SEMI-DOMINANTS	NOT CROSSING
19	11	10	4

Figure 24. Genetic screen to find extragenic suppressors of the lethality of *dma1 dma2 cla4* cells. Mutagenesis of *dma1Δ dma2Δ cla4Δ cla4-75* cells (ySP5247 and ySP5264) by ethylmethansulfonate (EMS) and preliminary characterization of the isolated extragenic suppressors.

We then decided to analyze the ability of the *MUT-135* suppressor to properly assemble a septin ring. Wild type and *dma1Δ dma2Δ cla4Δ MUT-135* cells were arrested in G1 by α factor at 25°C and then released into the cell cycle. Samples were taken at different time points to monitor the kinetics of DNA replication and cell division by FACS analysis of DNA contents (data not shown), budding, nuclear division and septin ring assembly (Fig. 25B). As indicated by the graphs, mutant cells showed a delay of about 15 minutes both in bud emergence and in septin ring assembly, but, interestingly, they were able to properly position the septin ring at the bud-neck, although less efficiently than wild type cells. These data suggest that some mutations found in our genetic screen effectively rescue the viability of *dma1Δ dma2Δ cla4Δ* cells by suppressing their cytokinesis and septin defects.

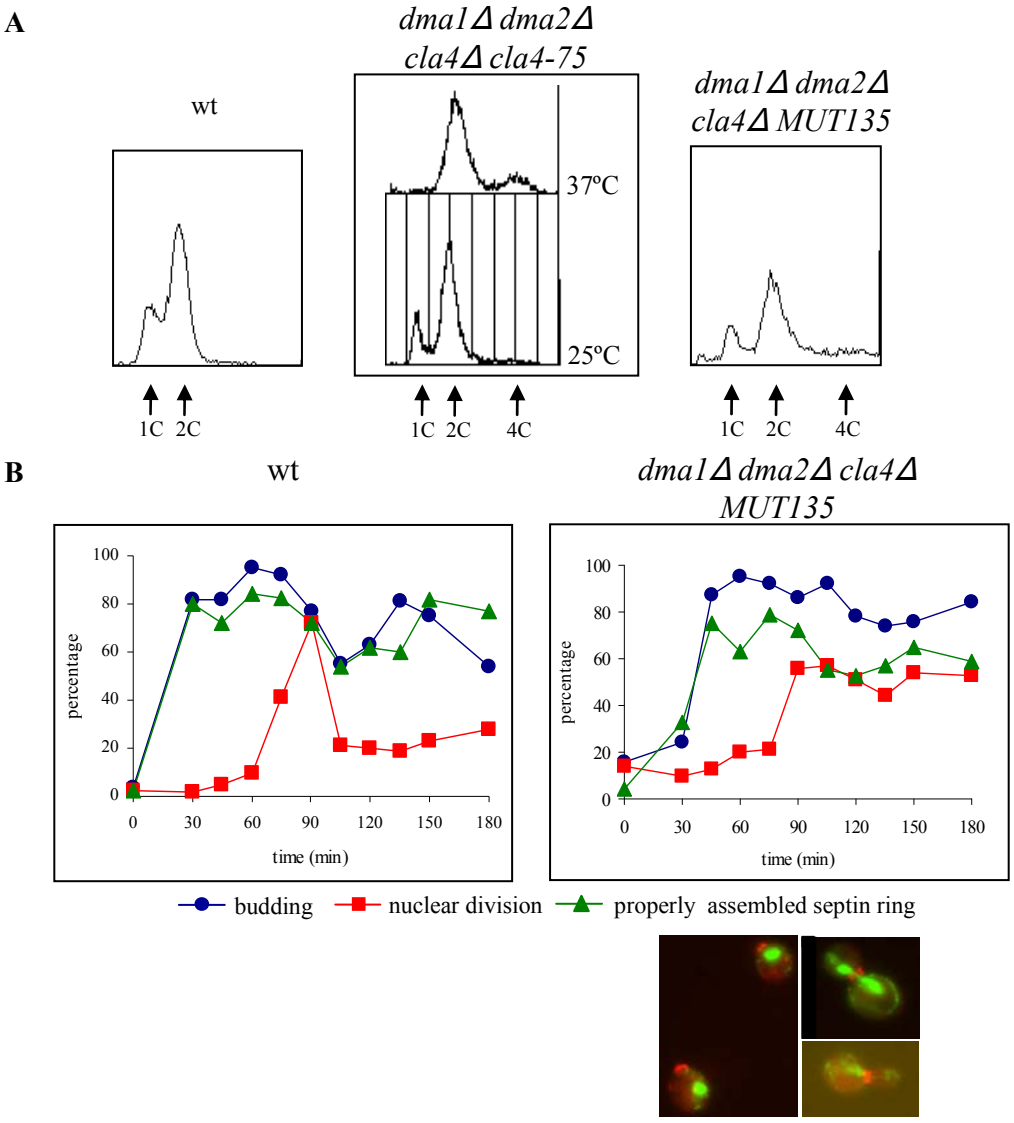


Figure 25. A dominant suppressor able to rescue the cytokinesis and septin ring defects of *dma1 dma2 cla4* cells. A: Logarithmically growing cultures of wt (W303), *dma1Δ dma2Δ cla4-75* (ySP5264) and *dma1Δ dma2Δ cla4Δ MUT-135* (ySP8922) were collected for FACS analysis of DNA contents at 25°C or 37°C (*dma1Δ dma2Δ cla4-75* only). **B:** Logarithmically growing cultures of wild type (W303) and *dma1Δ dma2Δ cla4Δ MUT-135* (ySP8922) cells, were arrested in G1 by α factor at 25°C and released into the cell cycle at 25°C. At the indicated times, cell samples were taken for FACS analysis of DNA contents (data not shown) and for scoring budding, nuclear division and septin ring deposition. Micrographs of *dma1Δ dma2Δ cla4Δ MUT-135* cells were taken at t=90' after the release.

MUT-135 is mutated in *RHO1*

Since *MUT-135* was able to suppress both the cytokinesis and the septin defects caused by the simultaneous lack of *DMA1 DMA2* and *CLA4*, we decided to clone the gene carrying the dominant mutation responsible for the suppression. To this end, we constructed a genomic library from *dma1Δ dma2Δ cla4Δ MUT-135* cells. The library construction is schematically shown in Figure 26. We partially digested the genomic DNA with Sau3A and ligated the digested DNA in a centromeric plasmid, followed by *E. coli* transformation. We obtained about 100,000 *E. coli* transformants that should be representative of 3,5 genome equivalents, thus providing a good coverage of the genome. We then re-transformed *dma1Δ dma2Δ cla4-75* cells with the genomic library to isolate clones able to grow at the restrictive temperature (37°C). Subsequent plasmid recovery, DNA sequencing and BLAST search revealed that all the suppressing plasmids carried a mutated allele in the *RHO1* gene, which encodes for the yeast counterpart of the RhoA GTPase that is involved in cytokinesis in most eukaryotic organisms (reviewed in Glotzer, 2009; Pollard, 2010). More precisely, *MUT-135* carried a single nucleotidic substitution that caused Asp72 of Rho1 to be replaced by Asn (*RHO1-D72N*), a mutation that has not been described before. We confirmed that *RHO1-D72N* was responsible for the suppression of the lethality of *dma1 dma2 cla4* cells by cloning *RHO1-D72N* in a centromeric plasmid and re-transforming the *dma1Δ dma2Δ cla4-75* strain used for our screen (Fig. 27). This finding prompted us to sequence *RHO1* in all the other dominant suppressors isolated in our screen. Strikingly, three additional suppressors carried exactly the same *RHO1* mutation.

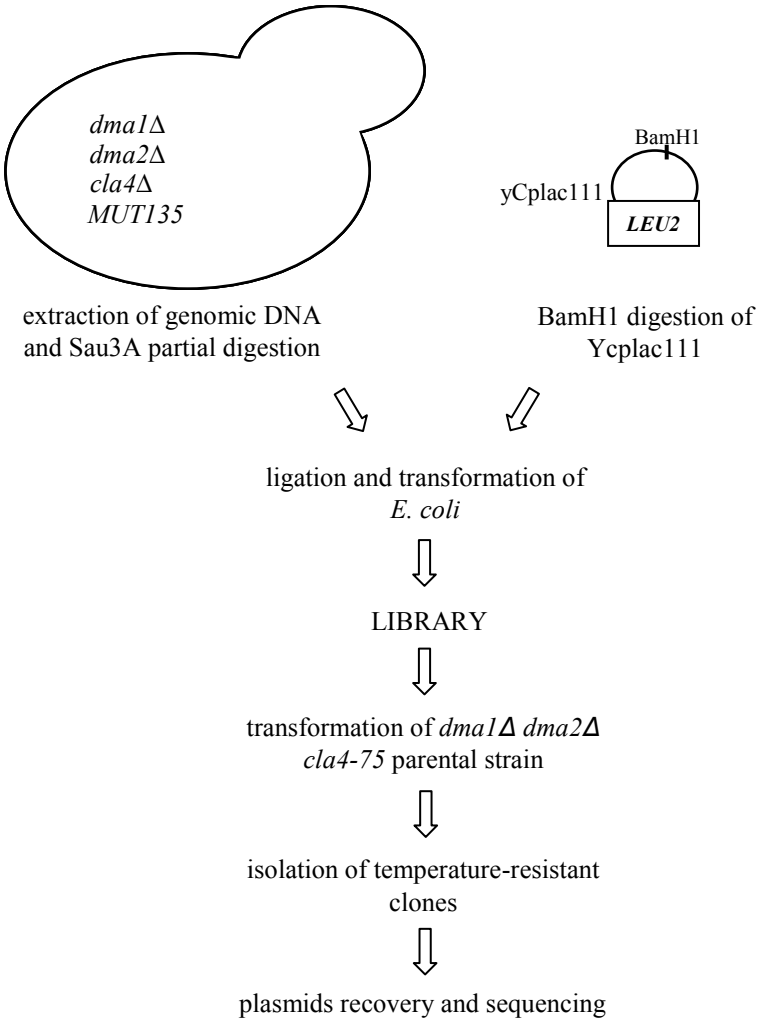


Figure 26. Construction of a genomic DNA library from the *dma1Δ dma2Δ cla4Δ MUT-135* suppressor. Schematic representation of genomic library construction from *dma1Δ dma2Δ cla4Δ MUT-135* (ySP8922) cells.

In contrast, we did not identify any other mutation in *RHO1*, suggesting that *RHO1-D72N* might have some peculiarities that will be further explored in the future.

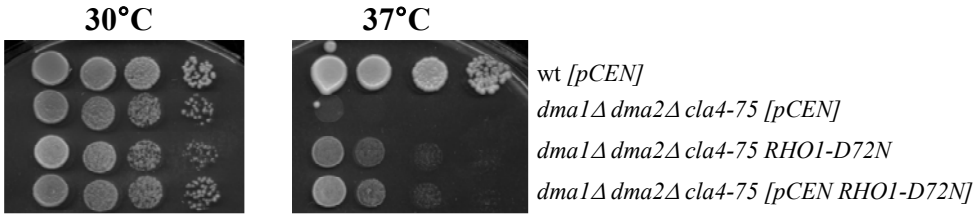


Figure 27. The *RHO1-D72N* allele corresponds to the suppressing mutation of *MUT-135*. Serial dilutions of stationary phase cultures of the strains with the indicated genotypes were grown in synthetic medium lacking leucine at 25°C, spotted on YEPD and incubated at the indicated temperatures for 2 days.

Lethality of *dma1 dma2 cla4* cells is suppressed by Rho1 and Pkc1 hyperactivation

Rho1 is essential for cell viability in *S. cerevisiae*. Like mammalian RhoA, it has a function in the deposition and contraction of the actomyosin ring during cytokinesis (reviewed by Wolf and Glotzer, 2009). Rho1 also controls the cell wall integrity signalling, regulates protein kinase C (Pkc1) activation, β1,3-glucan synthase (GS) activity, actin cytoskeleton organization and plays a role in polarized secretion (reviewed by Levin, 2005). Like other G-proteins, Rho1 cycles between the active GTP-bound state and the inactive GDP-bound state. The Rho1 cycle is regulated positively by GTPase-Activating Proteins (GAPs) and negatively by Guanosine nucleotide Exchange Factors (GEFs) acting in opposition. Among the eleven Rho-GAPs identified in *S. cerevisiae*, four have been shown to inhibit Rho1 activity both in vitro and in vivo: Bem2, Sac7, Bag7 and Lrg1 (Cid et al., 1998, Roumanie et al., 2001, Schmidt et al., 2002, Watanabe et al., 2001). Rho1 is activated by the GEFs Rom1, Rom2 and Tus1 (Ozaki et al., 1996, Schmelzle et al., 2002). Five effectors of Rho1 have been described: the Pkc1 protein kinase (Kamada et al., 1996), the β1,3-glucan synthase (Mazur and Baginsky, 1996), the Bni1 and Bnr1 formins (Kohno et al., 1996), the Skn7 transcription factor (Alberts et al., 1998) and the Sec3 exocyst component (Guo et al., 2001).

The D72N *RHO1* mutation that we found in our screen falls in a region conserved in all GTPases called switch II. Together with the switch I this part of the protein is responsible for the binding of GEFs, GAPs, effectors and nucleotides (Vetter and Wittinghofer, 2001). Since *RHO1-D72N* is a dominant allele and usually dominant

mutations result in the gain of function, we hypothesised that *RHO1-D72N* might be a hyperactive allele of *RHO1*. We therefore asked if a known hyperactive allele of *RHO1* (*RHO1-G19V*) (Tiedje et al., 2008) was able to suppress the temperature-sensitivity of *dma1Δ dma2Δ cla4-75* cells. We therefore transformed *dma1Δ dma2Δ cla4-75* cells with integrative plasmids carrying wild type *RHO1*, *RHO1-G19V* or the *RHO1-D125A* dominant negative allele (Tiedje et al., 2008) and we checked single integrations by southern blot analysis (data not shown). Strains were then inoculated in YEPD medium and serial dilutions of the cultures were spotted on YEPD plates at different temperatures. Interestingly, the hyperactive *RHO1-G19V* allele was sufficient to rescue the viability of *dma1Δ dma2Δ cla4-75* cells at 37°C, while the dominant negative allele *RHO1-D125A* did not (Fig. 28A).

If the suppression of the temperature-sensitivity of *dma1Δ dma2Δ cla4-75* cells by *RHO1-D72N* requires Rho1 hyperactivation, deletion of Rho1 GAPs could also rescue the lethality of the triple mutant at 37°C. We separately deleted in *dma1Δ dma2Δ cla4-75* cells three of the four genes encoding for Rho1 GAPs (*SAC7*, *LRG1* and *BAG7;* deletion of *BEM2* is synthetic lethal with *cla4-75* allele (data not shown)). Serial dilution of the various mutants were spotted on YEPD plates and incubated at different temperatures. As shown in Figure 28B, deletion of *LRG1* and *SAC7* suppressed the lethality of a *dma1Δ dma2Δ cla4-75* strain at 34°C and partially at 37°C, while deletion of *BAG7* had no effect. These results confirm that Lrg1 and Sac7 are the main GAPs to regulate Rho1 activity toward cytokinesis (Yoshida et al., 2009) and support the notion that Rho1 hyperactivation is

sufficient to rescue the septin and cytokinesis defects of *dma1 dma2 cla4* cells. We can therefore reasonably conclude that *RHO1-D72N* might be a hyperactive allele of Rho1, at least for what concerns activation of specific targets.

Pkc1 is a major target of Rho1 (Kamada et al., 1996). Pkc1 associates with and is activated by GTP-bound Rho1 (Nonaka et al., 1995; Kamada et al., 1996), which confers to the protein kinase the ability to be stimulated by phosphatidylserine as a cofactor (Kamada et al., 1996). Pkc1 is larger than any of the mammalian PKCs due to an extended regulatory region (reviewed by Levin, 2005). It possesses two homologous regions (HR1A and B) at its N-terminus (Mellor and Parker, 1998), which are found in proteins that bind to RhoA (Shibata et al., 1996). Pkc1 also contains a cysteine-rich domain (C1), which is the site of dyacilglycerol and phorbol ester binding (Kaibuchi et al., 1989; Szallasi et al., 1996), as well as a C2 domain, responsible for binding phospholipids in a Ca^{2+}-dependent manner (Bazzi and Nelsestuen, 1990). Finally Pkc1, like all PKCs, possesses a pseudosubstrate site, typically positioned immediately N-terminal to the C1 domain (Newton, 1995). This site resembles a PKC phosphorylation site except that it has an alanine at the position corresponding to the phosphorylated target serine or threonine. Under conditions in which the PKC is not active, the pseudosubstrate site inhibits protein kinase activity through an intramolecular interaction with the active site. Mutations in the Pkc1 psudosubstrate site, like *PKC1-R398P*, yield to a constitutively active kinase (Pears et al., 1990; Watanabe et al., 1994) that can suppress the growth defects of some conditional *rho1* mutants (Nonaka et al., 1995).

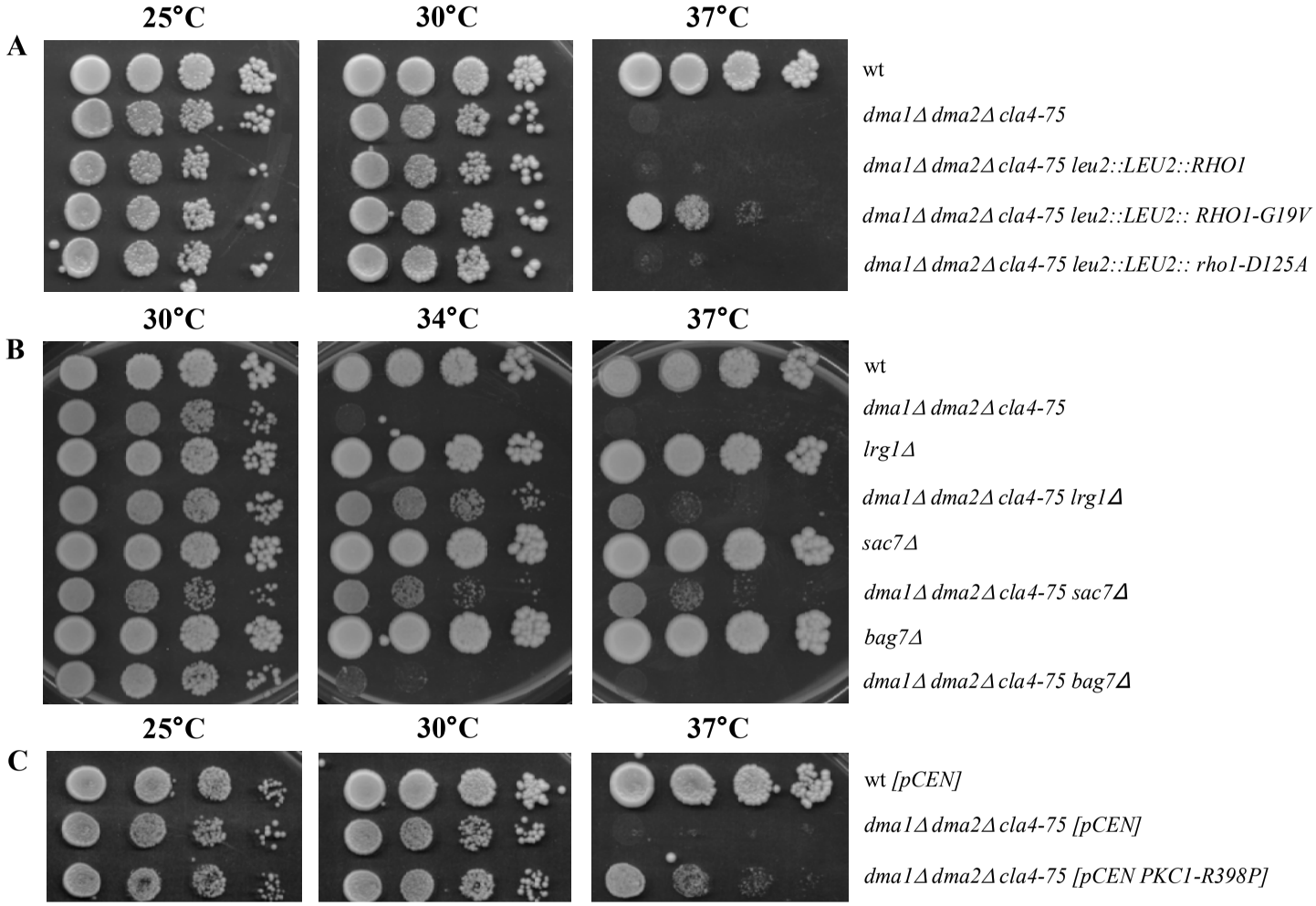


Figure 28. Hyperactivation of the Rho1-Pkc1 pathway suppresses the temperature-sensitivity of *dma1Δ dma2Δ cla4-75* cells. A, B, C: Serial dilutions of stationary phase cultures of the strains with the indicated genotypes were grown in YEPD (A and B) or in synthetic medium lacking uracil (C) at 25°C, spotted on YEPD and incubated at the indicated temperatures for 2 days.

We therefore tested whether hyperactive *PKC1-R398P* could also suppress the lethality of the *dma1 dma2 cla4* triple mutant. We transformed wild type and *dma1*Δ *dma2*Δ *cla4-75* cells with an empty plasmid or a plasmid carrying *PKC1-R398P* allele. The isolated transformants were grown in selective medium and serial dilutions of the cultures were spotted on YEPD plates at different temperatures. The expression of *PKC1-R398P* suppressed the lethality of *dma1Δ dma2Δ cla4-75* cells both at 34°C and 37°C (Fig. 28C). Thus, hyperactivation of the Rho1/Pkc1 pathway can suppress the lethality of *dma1 dma2 cla4* triple mutants. Having found that Rho1 and Pkc1 hyperactivation suppresses the lethality of *dma1 dma2 cla4* triple mutants raised the question of whether it suppressed the defects caused by Dma1/2 or Cla4 inactivation. Since diploid strains carrying the deletion of *DMA1* and *DMA2* in homozygosis do not sporulate (our unpublished data), we investigated whether the *PKC1-R398P* allele could suppress these sporulation defects. To this purpose, we transformed the *dma1Δ/dma1Δ dma2Δ/dma2* diploid strain with a centromeric plasmid carrying *PKC1-R398P*. After incubation of the selected transformants on sporulation medium, we found that *dma1Δ/dma1Δ dma2Δ/dma2* cells carrying *PKC1-R398P,* but not the empty vector, were perfectly able to sporulate. Since Pkc1 has been extensively involved in the regulation of membrane dynamics and cell wall biosynthesis, this result is consistent with the recent finding that *S. pombe dma1* homozygous mutants fail to sporulate due to defects in forespore membrane formation (Krapp et al., 2010; Li et al., 2010).

If Rho1 hyperactivation suppresses the lethality of *dma1 dma2 cla4* cells because it suppresses the defects caused by lack of Cla4, *RHO1-*

D72N should also suppress the temperature-sensitivity of a *ste20Δ cla4-75* mutant, which shows defects in septin ring deposition and an aberrant bud neck at 37°C (Cvrckova et al., 1995). We transformed *ste20Δ cla4-75* cells with a centromeric plasmid carrying *RHO1-D72N* allele to evaluate the ability to grow at different temperatures. Cells were grown in selective medium and serial dilutions were spotted on YEPD plates at 30°C and 37°C. As we can see in Figure 29, expression of *RHO1-D72N* is not sufficient to suppress *ste20Δ cla4-75* lethality at 37°C. Altogether, these data suggest that hyperactivation of the Rho1/Pkc1 pathway specifically suppresses the defects caused by lack of Dma1/2. Thus, Rho1 and Pkc1 might act downstream of Dma1/2 in the regulation of septin dynamics and cytokinesis.

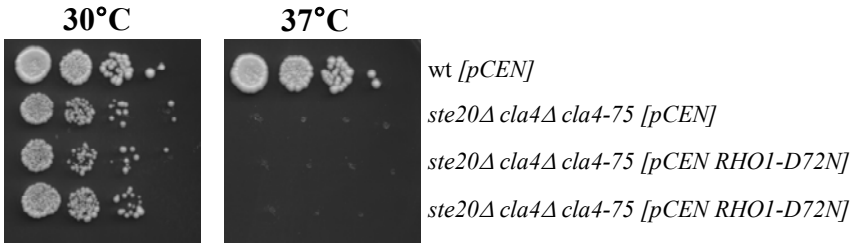


Figure 29. *RHO1-D72N* does not suppress the temperature-sensitivity of *ste20Δ cla4-75* cells. Serial dilutions of stationary phase cultures of the strains with the indicated genotypes were grown in synthetic medium lacking leucine at 25°C, spotted on YEPD and incubated at the indicated temperatures for 2 days.

Septin dynamics is affected by *RHO1* mutations

Despite the vast literature linking Rho1/RhoA to the assembly and contraction of the actomyosin ring, no data connect the Rho1/Pkc1 pathway with septins. Interestingly both Rho1 and Pkc1 appear to be localized at the bud neck in some cell cycle phases (Yamochi et al., 1994, Denis and Cyert, 2005), suggesting a possible link with septins. Since *RHO1-D72N* rescues the ability of *dma1Δ dma2Δ cla4Δ* cells to properly positioned the septin ring at the bud neck, we decided to investigate directly a possible role for Rho1 in the regulation of septin dynamics.

First we asked whether *RHO1-D72N* could stabilize the septin ring in a phase of the cell cycle where it is usually unstable. We expressed *CDC12-GFP* construct in wild type and *RHO1-D72N* cells and we investigated by FRAP the stability of the septin ring in G1 cells, immediately prior to bud emergence. As expected (Caviston et al., 2003), after total bleaching of the ring in G1, septin ring is unstable in wild type cells (60,6% of fluorescence recovery at the end of the experiment). In contrast, the septin ring is more stable in *RHO1-D72N* cells (24,7% of recovery) (Fig 30A and B).

If the expression of a hyperactive allele of *RHO1* stabilizes the septin ring we could predict that, on the contrary, *RHO1* inactivation could destabilize the septin ring. Since *RHO1* is an essential gene, we analysed septin ring dynamics in temperature sensitive *rho1* mutants at the restrictive temperature of 37°C, to inactivate Rho1 function. The *rho1-5* mutant, in particular, is specifically defective in Pkc1 activation because its temperature-sensitivity can be suppressed by

expression of the constitutively active *PKC1-R398P* allele (data not shown).

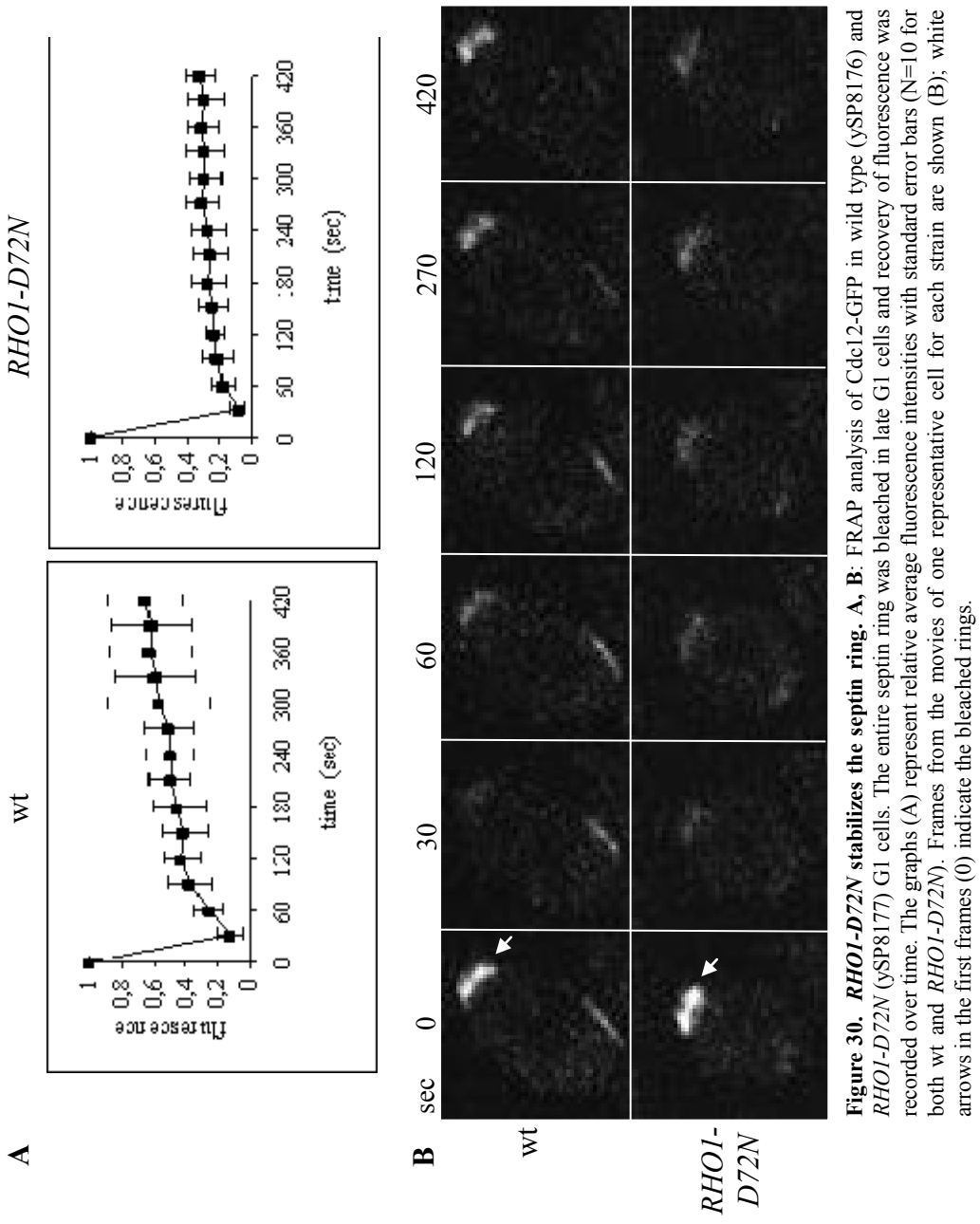


Figure 30. *RHO1-D72N* stabilizes the septin ring. A, B: FRAP analysis of Cdc12-GFP in wild type (ySP8176) and *RHO1-D72N* (ySP8177) G1 cells. The entire septin ring was bleached in late G1 cells and recovery of fluorescence was recorded over time. The graphs (A) represent relative average fluorescence intensities with standard error bars (N=10 for both wt and *RHO1-D72N*). Frames from the movies of one representative cell for each strain are shown (B); white arrows in the first frames (0) indicate the bleached rings.

In contrast, *PKC1-R398P* cannot suppress the temperature-sensitivity of *rho1-1* cells, which carry a point mutation in a region of Rho1 that is not implicated in Pkc1 activation (Nonaka et al., 1995).

We bleached half of the septin ring in large budded wild type and *rho1-5* cells expressing septin Cdc12 fused with GFP. Subsequent FRAP analysis showed that *rho1-5* cells recovered fluorescence after photobleaching more efficiently than wild type cells both at 37°C (29,8% versus 10% of wild type at the end of the experiment, Fig. 31A and B) and 25°C (data not shown) indicating that Rho1 inactivation impairs septin ring stability. In contrast, the septin ring remained stable under the same conditions in *rho1-1* cells (data not shown), thus strengthening the notion that the role of Rho1 in septin ring stability involves Pkc1 activation.

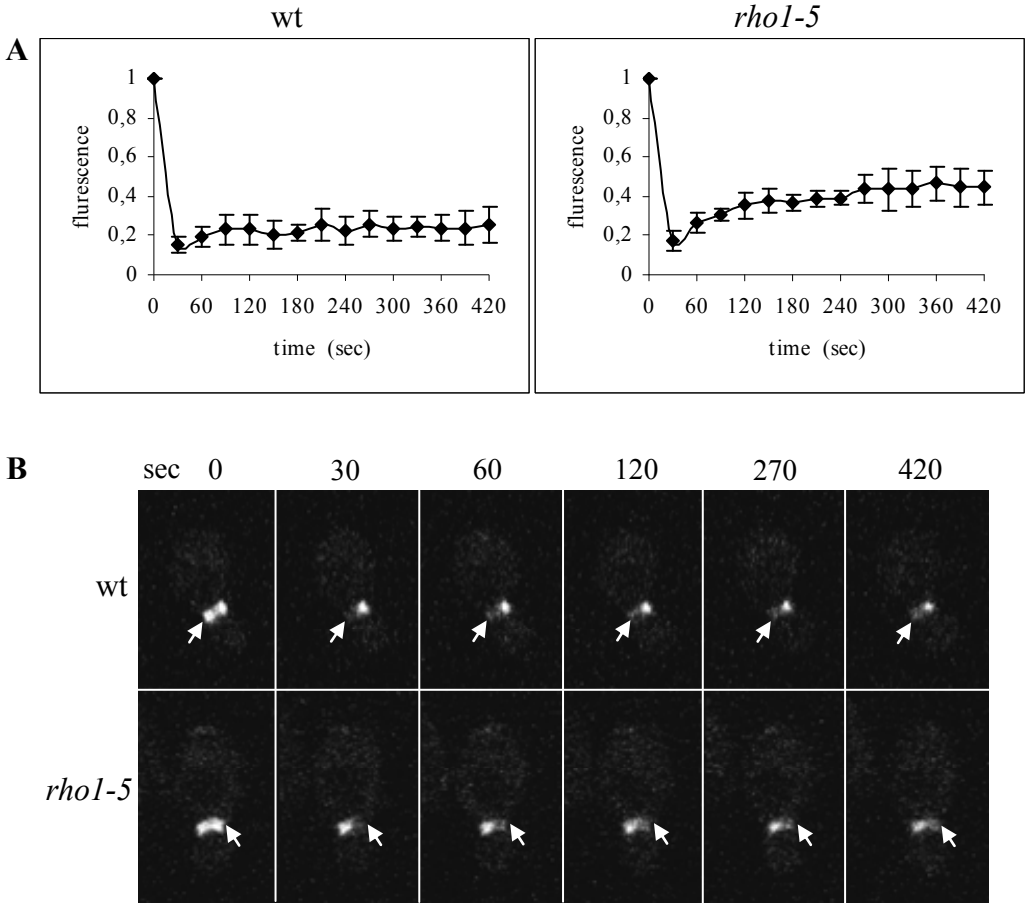


Figure 31. *RHO1* inactivation destabilizes the septin ring. A, B: FRAP analysis of Cdc12-GFP in wild type (ySP8176) and *rho1-5* (ySP8321) cells**.** Half of the septin ring was bleached in large budded cells at 37°C and recovery of fluorescence was recorded over time. The graphs (A) represent relative average fluorescence intensities (N=7 for both wt and *rho1-5*). Frames from the movies of one representative cell for each strain are shown (B); white arrows indicate the side of the ring that was bleached.

Dominant *RHO1* and *PKC1* mutations advance septin ring assembly during the cell cycle

Since *dma1Δ dma2Δ cla4-75* cells are defective in septin ring deposition in late G1 and the expression of *RHO1-D72N* and *PKC1-R398P* is sufficient to suppress the lethality of *dma1 dma2 cla4* strains, we analysed the timing of septin ring deposition at the beginning of the cell cycle and the timing of septin ring disassembly prior to cytokinesis in *RHO1-D72N* and *PKC1-R398P* cells. Elutriated wild type, *RHO1-D72N* and *PKC1-R398P* small unbudded cells were resuspended in fresh medium at 25°C. The kinetics of DNA replication (Fig. 32A), increase in cell size, budding, nuclear division and septin ring assembly (Fig. 32B) were followed at different time points. Both *RHO1-D72N* and *PKC1-R398P* cells budded, entered S phase and divided nuclei with kinetics similar to wild type (Fig. 32A and B). In contrast, septin ring deposition was anticipated by about 15 minutes in *RHO1-D72N* and *PKC1-R398P* cells relative to the wild type and to the increase in cell size. In addition, septin ring seemed to be slightly more stable in *RHO1-D72N* and *PKC1-R398P* cells than in the wild type at the end of the cell cycle.

Time lapse video microscopy to assess the timing of septin ring assembly relative to disassembly of the septin ring from the previous cell cycle confirmed that *RHO1-D72N* cells deposited the septin ring at the future bud emergence site on average 14 min before wild type cells (N=105 for wt and N=101 for *RHO1-D72N*). An example is shown in Figure 33: after septin ring splitting (t=3 min), the septin ring was rapidly disassembled in a representative wild type cell and the new septin ring appeared in the mother cell at the future site of bud

emergence about 36 min after splitting of the previous ring. On the contrary, in *RHO1-D72N* the septin ring was more stable after splitting and a new septin ring was rapidly assembled in the mother cell (about 21 min after splitting of the old ring).

These data strongly implicate Rho1/Pkc1 in the regulation of septin ring deposition in late G1 and of septin ring stability at the end of the cell cycle.

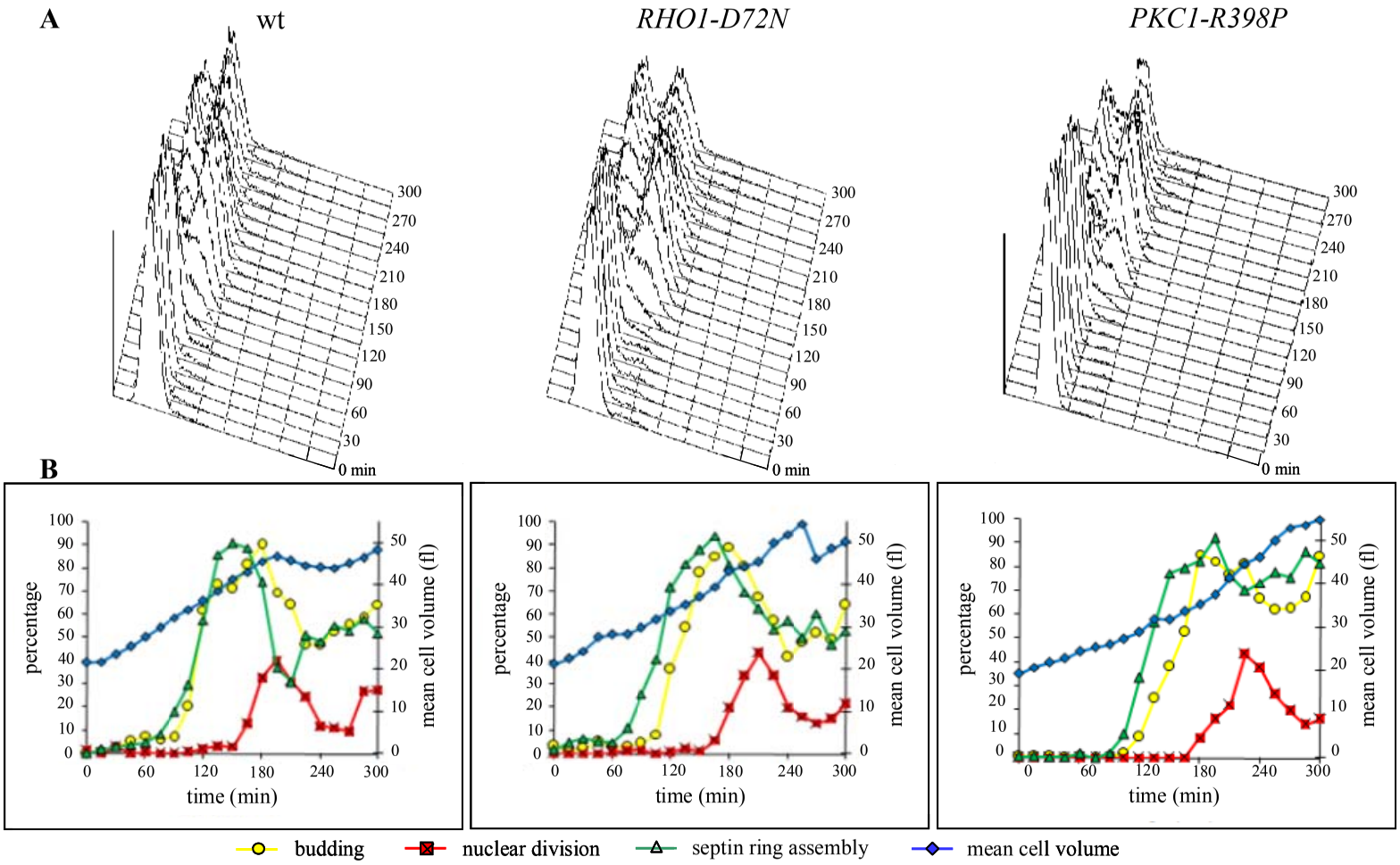


Figure 32. Dominant *RHO1* and *PKC1* mutations advance septin ring assembly during the cell cycle. A, B: Small unbudded cells of wt (W303), *RHO1-D72N* (ySP7933) and *PKC1-R398P* (ySP8400) strains were isolated by centrifugal elutriation and released in YEPD at 25°C at time zero. At the indicated times, cell samples were taken for FACS analysis of DNA contents (A) and to score budding, nuclear division, cell size and septin ring deposition by in situ immunofluorescence with anti-Cdc11 antibodies(B).

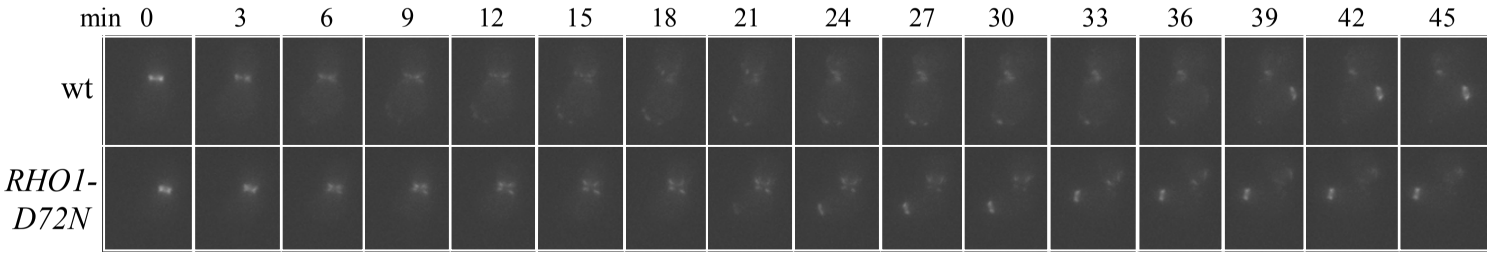


Figure 33. *RHO1-D72N* advances septin ring assembly and stabilizes the septin ring at the end of the cell cycle. Time lapse analysis of Cdc12-GFP in wild type (ySP8176) and *RHO1-D72N* (ySP8177) cells. Micrographs of one representative cell for each strain were taken at the indicated time points.

Dominant *RHO1* and *PKC1* mutations suppress the temperature-sensitivity of septin mutants

In budding yeast genes encoding for septins are essential for cell viability (except *SHS1*). Strains carrying temperature-sensitive mutations in these genes, like *cdc12-1* and *cdc12-6*, quickly disassemble the septin ring upon shift to the restrictive temperature and show defects in septin ring deposition/stabilization also at permissive temperatures (Barral et al., 2000; Dobbelaere et al., 2003).

Since *RHO1-D72N* allele seems to stabilize the septin ring, we asked whether this allele could suppress the temperature-sensitivity of septin mutants. To this purpose, we crossed *cdc12-1* and *cdc12-6* strains with the *RHO1-D72N* strain to obtain double mutants after meiotic recombination. Mutant and control strains were inoculated in YEPD at 23°C and serial dilutions were spotted on YEPD plates at different temperatures. Strikingly, the *RHO1-D72N* allele rescued the lethality of *cdc12-1* and *cdc12-6* mutants at 30°C (Fig. 34A). We also transformed *cdc12-1* and *cdc12-6* cells with a centromeric plasmid either empty or carrying the *PKC1-R398P* allele and analysed growth of the various strains at different temperatures. Similarly to *RHO1-D72N*, also *PKC1-R398P* suppressed the temperature-sensitivity of septin mutants at 30°C (Fig. 34B). Thus, these data further support a role for Rho1 and Pkc1 in the regulation of septin assembly and stability.

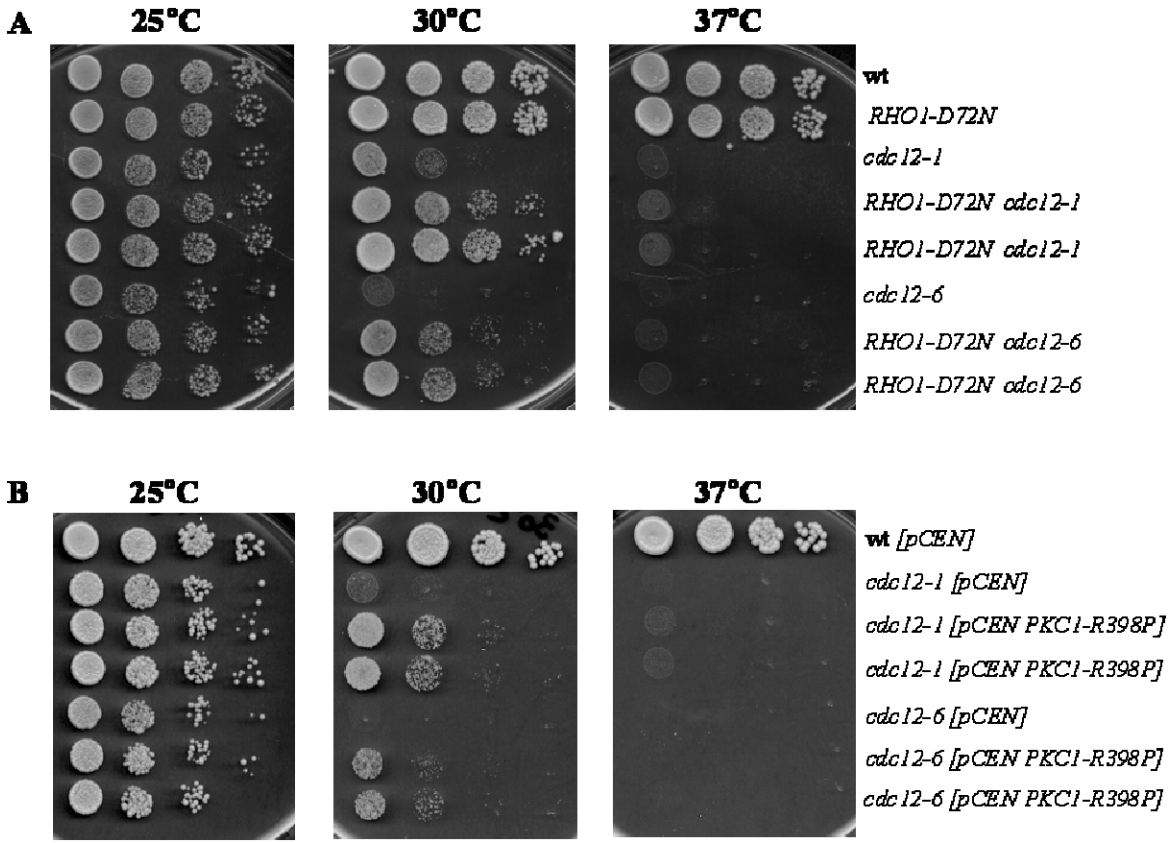


Figure 34. Dominant *RHO1* and *PKC1* mutations suppress the temperature-sensitivity of septin mutants. A, B: Serial dilutions of stationary phase cultures of the strains with the indicated genotypes were grown in YEPD (A) or in synthetic medium lacking uracil (B) at 23°C, spotted on YEPD and incubated at the indicated temperatures for 2 days.

The lack of Pkc1 affects septin ring stability

Loss of *PKC1* results in a more severe growth defect than that displayed by deletion of any other members of the MAP kinase cascade downstream of Pkc1, suggesting that Pkc1 regulates multiple pathways (Lee and Levin, 1992). In addition, an intracellular localization study of Pkc1 revealed that it resides at sites of polarized cell growth (Andrews and Stark, 2000). Specifically, early in the cell cycle, Pkc1 was detected at the pre-bud site and at bud tips, a pattern that is very similar to that of Rho1 (Qadota et al., 1996; Yan and Lennarz, 2002). Later in the cell cycle it becomes delocalized and finally relocalized at the mother-bud neck. The neck localization of Pkc1 requires an intact septin ring (Denis and Cyert, 2005). Each Pkc1 domain seems to be responsible for localizing a pool of Pkc1 to various subcellular sites. For example, the HR1 domain is implicated in the localization of Pkc1 to the bud tip and neck, consistently with a role for this domain in mediating Rho1-Pkc1 interaction (Denis and Cyert, 2005).

Since *PKC1* hyperactivation advances septin ring deposition during the cell cycle and stabilizes it, we asked whether the lack of *PKC1* could affect septin ring assembly. To this purpose, wild type and *pkc1Δ* cells were grown in the presence of sorbitol as osmotic stabilizer, arrested in G1 by α factor and released into the cell cycle at 30°C in the presence of nocodazole, to arrest cells in a cell cycle stage where the septin ring is stable. Cell cycle progression was followed at different time points after the release by cytofluorimetric analysis of DNA contents (data not shown), while the assembly and stabilization of the septin ring was monitored by in situ immunofluorescence of the

Cdc11 septin. In wild type cells septin ring started to assemble at the future site of bud emergence 30 min after the release and it was rapidly stabilized in an hourglass structure, which persisted until the end of the experiment (Fig. 35A). In *pkc1Δ* cells the appearance of the septin ring was markedly delayed and limited to a small fraction of cells. In addition, in the small perecentage of cells where the septin ring was deposited at the bud neck it mostly failed to be converted into the hourglass structure and persisted as a single ring until the end of the experiment (Fig. 35A and B). Therefore Pkc1, like Rho1, seems to be involved both in septin ring assembly and stabilization. To support this conclusion we also analyzed septin ring stabilization in 2 temperature sensitive mutants of *PKC1*, *pkc1-2* and *pkc1-3*, which have point mutations inside the catalytic domain (Levin and Bartlett-Heubusch, 1992). Exponentially growing cell cultures of wild type, *pkc1-2* and *pkc1-3* cells were synchronized in G1 by α-factor and released into the cell cycle at 37°C in the presence of nocodazole, to arrest cells in the following mitosis. Cell cycle progression was monitored at different time points after the release by cytofluorimetric analysis of DNA contents (data not shown), whereas assembly/stabilization of the septin ring was analysed by in situ immunofluorescence of Cdc11. As shown in the graphs in Figure 35C both *pkc1* mutants show severe defects in septin ring stabilization at 37°C. Like *pkc1Δ* cells, these mutants are defective in forming the stable hourglass structure, confirming that Pkc1 is involved in septin ring stabilization.

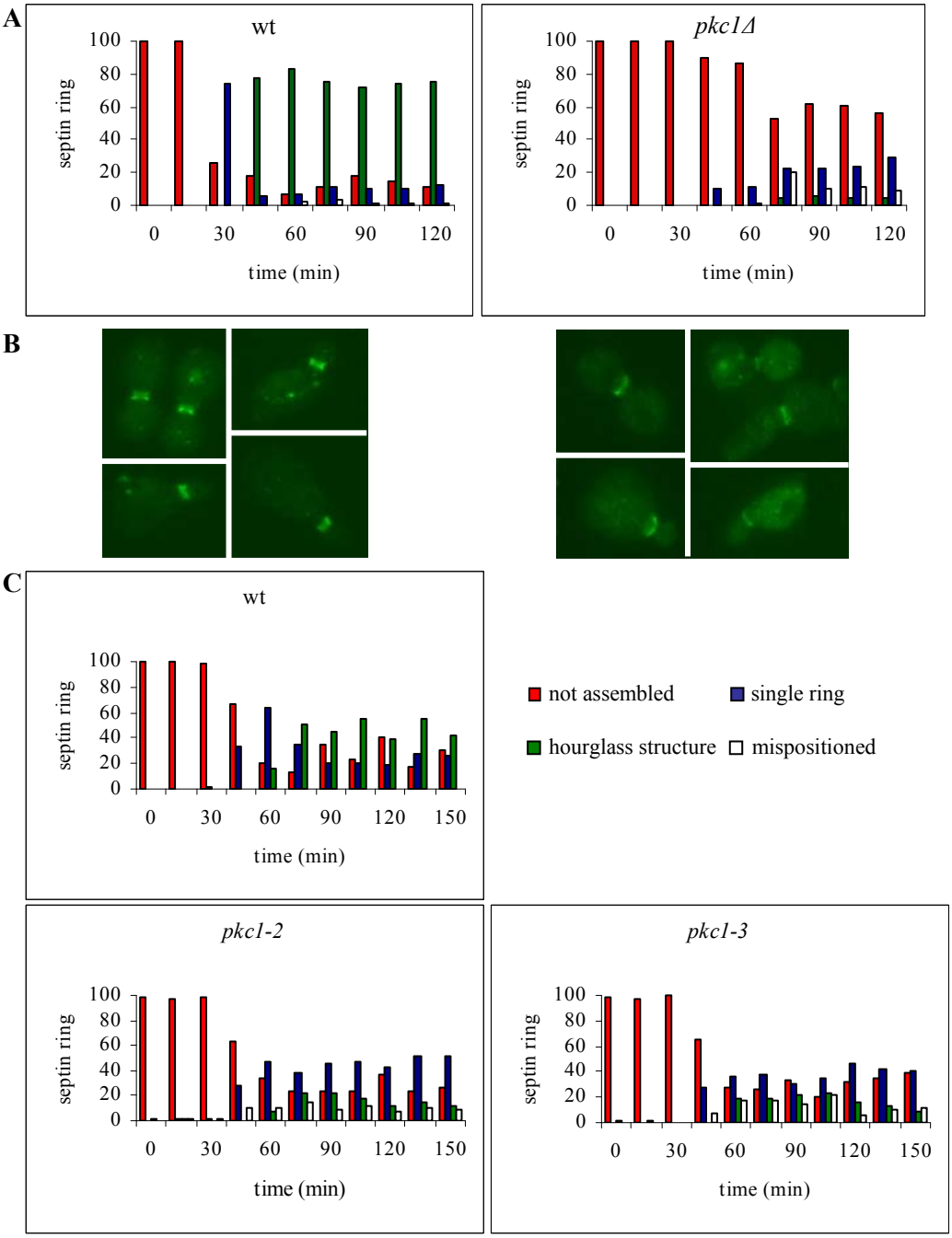


Figure 35. The lack of *pkc1* affects septin ring stability. A, B: Logarithmically growing cultures of wild type (W303) and *pkc1Δ* (ySP8491) cells were synchronized in G1 by α factor at 30°C (in YEPD + 1M sorbitol) and released into the cell cycle at 30°C in the presence of nocodazole to arrest cells in mitosis. At the indicated times, cell samples were taken for FACS analysis of DNA contents (data not shown) and in situ immunofluorescence of the septin ring with anti-Cdc11 antibodies (A). Micrographs were taken 90 min after the release (B). **C:** Logarithmically growing cultures of wild type (W303), *pkc1-2* (ySP8472) and *pkc1-3* (ySP8470) cells were synchronized in G1 by α factor at 25°C (in YEPD + 1M sorbitol) and released into the cell cycle at 37°C in the presence of nocodazole to arrest cells in mitosis. At the indicated times, cell samples were analysed as in (A-B).

The *RHO1-D72N* and *RHO1-G19V* alleles do not hyperactivate the cell wall integrity signalling pathway

Pkc1 has been implicated in the cell wall integrity signalling pathway (CWI) (Levin et al., 1990). Deletion of *PKC1* is lethal under normal growth conditions, but the viability of *pkc1Δ* mutant cells can be rescued by addition of an osmotic support to the medium (Levin et al., 1990; Levin and Bartlett-Heubusch, 1992; Paravicini et al., 1992). Although Pkc1 likely has several intracellular substrates, only its regulation of the Bck1-Mkk1/2-Mpk1 MAP kinase cascade has been studied in detail. Briefly, the MAP kinase cascade for CWI signalling is a linear pathway comprised of Pkc1 (Levin et al., 1990), a MEKK (Bck1) (Costigan et al., 1992; Lee and Levin, 1992), a pair of redundant MEKs (Mkk1/2) (Irie et al., 1993) and a MAP kinase (Mpk1/Slt2) (Lee et al., 1993; Martin et al., 1993). A combination of genetic and biochemical studies has established that Pkc1 activates Bck1, which activates Mkk1/2, which in turn activate Mpk1. Loss of function of any of these protein kinases results in cell lysis at elevated growth temperature; the growth defects of these mutants are osmoremediable (e.g. with 1M sorbitol), consistent with a primary defect in cell wall biogenesis (reviewed by Levin, 2005). Several stress factors, like heat shock, can induce the activation of CWI signalling pathway (Kamada et al., 1995). Since it was formally possible that the suppression of *dma1 dma2 cla4* lethality by *RHO1-D72N* and *RHO1-G19V* was due to hyperactivation of the CWI pathway, we tested this directly by monitoring the levels of Mpk1 phosphorylation after heat shock. Wild type, *mpk1Δ*, *rho1-1* and *rho1-5* strains were also used as controls. Cells were grown in YEPD

medium at 25°C and shifted to 37°C for 0.5h and 3h. Protein extracts were the analysed by western blot with anti-Mpk1 antibodies to monitor the total levels of Mpk1 and with anti-phosphoMpk1 antibodies to assess Mpk1 phosphorylation in different conditions. Activation of the MAP kinase cascade was stimulated in wild type cells after 0.5h at 37°C, as shown by the appearance of the phosphorylated form of Mpk1, while phosphorylation levels decreased after 3h at 37°C (Fig. 36, upper panel), consistently with previously published data (Kamada et al., 1995). A similar induction of phosphorylated Mpk1 was observed in *RHO1-D72N* and *RHO1-G19V* cells, suggesting that these alleles do not hyperactivate the CWI signalling pathway. In contrast, in *rho1-1* cells the MAP kinase pathway was hyperactive, as shown by the presence of phosphorylated Mpk1 already at 25°C, while cells expressing the *rho1-5* allele were not able to induce Mpk1 phosphorylation in any conditions, as previously published (Nonaka et al., 1995).

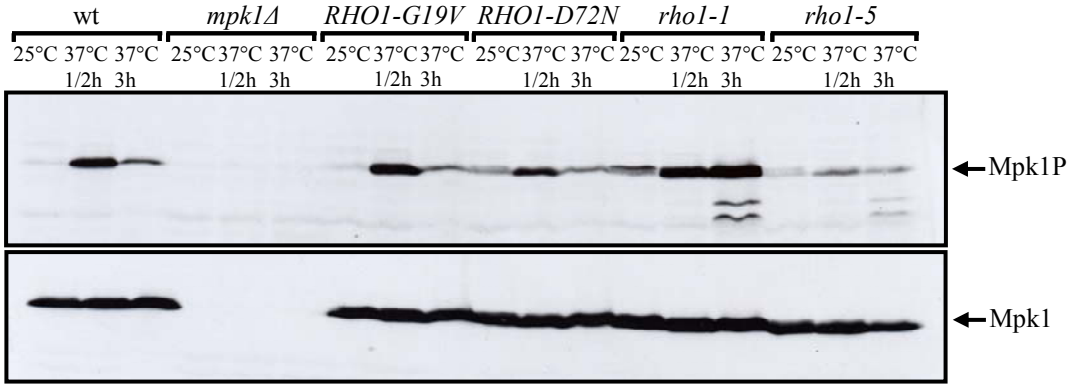


Figure 36. *RHO1-D72N* and *RHO1-G19V* do not hyperactivate the cell wall integrity signalling pathway. Logarithmically growing cultures of wt (W303), *mpk1Δ* (ySP8463), *RHO1-G19V* (ySP7901), *RHO1-D72N* (ySP7933), *rho1-1* (ySP8150) and *rho1-5* (ySP8153) were shifted from 25°C to 37°C for either 1/2h or 3h. Samples were collected for western blot ansalysis of protein extracts with anti-Mpk1 and anti-phosphorylatedMpk1 (Mpk1P) antibodies.

Sorbitol suppresses the temperature-sensitivity of *dma1Δ dma2Δ cla4-75* cells and of septin mutants

Based on our data, we have hypothesised that Rho1/Pkc1 are downstream effectors of Dma1 and Dma2 in the regulation of septin ring dynamics. Since osmotic supports rescue the viability of *pkc1Δ* cells (Levin et al., 1990; Levin and Bartlett-Heubusch, 1992; Paravicini et al., 1992), and Dma proteins and septins seem to be implicated in the same pathway of Pkc1, we asked whether the temperature-sensitivity of *dma1Δ dma2Δ cla4-75* cells and of septin mutants could be suppressed by adding sorbitol into the growth medium.

As shown in Figure 37A and B, *pkc1Δ* cells died on YEPD plates and were viable on YEPD/sorbitol plates at all temperatures tested. The presence of the osmotic support in the growth medium suppressed also the lethality of *dma1Δ dma2Δ cla4-75* cells at 37°C (Fig. 37A) and the temperature-sensitivity of *cdc12-1* and *cdc12-6* mutants at 30°C and 34°C (Fig. 37B). Thus, the lethality of these strains is likely to be partly due to cell lysis. Since septins interact with membranes, these data suggest that they might be required for membrane integrity.

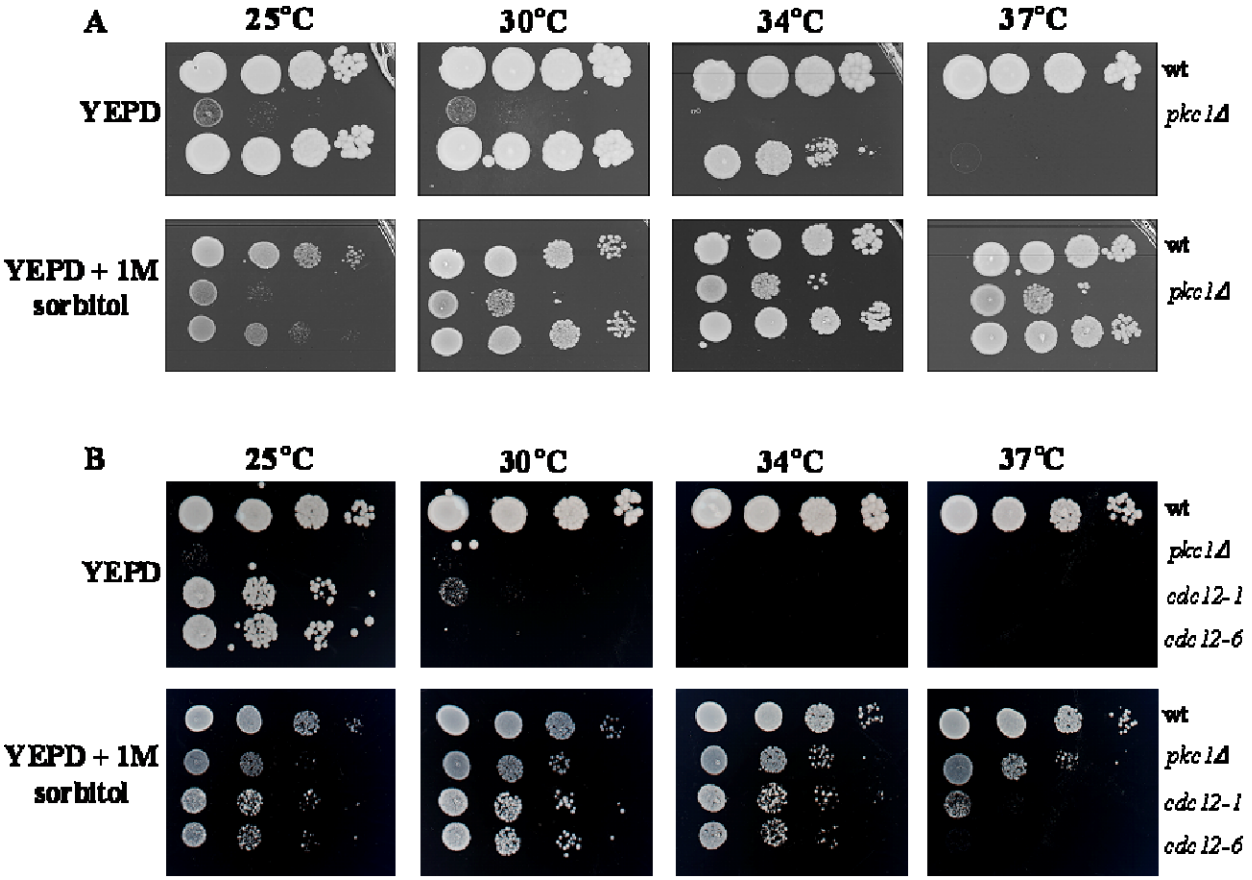


Figure 37. Sorbitol suppresses the temperature-sensitivity of *dma1Δ dma2Δ cla4-75* cells and septin mutants. A, B: Serial dilutions of stationary phase cultures of the strains with the indicated genotypes were grown in YEPD or in YEPD + 1M sorbitol (for *pkc1Δ* cells) at 25°C (A) or 23°C (B), spotted on YEPD or on YEPD + 1M sorbitol and incubated at the indicated temperatures for 2-3 days.

The Syp1 membrane protein: a possible link between the Rho1/Pkc1 pathway and septins

Phosphorylation events are known to stabilize the septin ring (Dobbelaere et al., 2003). It is therefore possible that Pkc1 directly phosphorylates septins and/or its regulators. A recent work (Breitkreutz et al., 2010) shows that among other cellular targets yeast Pkc1 phosphorylates Syp1. Syp1 was originally identified as a high dosage suppressor of profilin deletion mutants (Marcoux et al., 2000) and later as a suppressor of *arf3Δ*, the yeast homolog of Arf6, a mammalian regulator of endocytosis (Lambert et al., 2007). Indeed, several data have implicated Syp1 in endocytosis (Boettner et al., 2009; Lambert et al., 2007; Reider et al., 2009; Stimpson et al., 2009). Interestingly, Syp1 was also found to interact with septin proteins at the bud neck and to regulate septin dynamics (Qiu et al., 2008). Indeed, Syp1 colocalizes with septin filaments throughout most of the cell cycle and physically interacts with the septin subunit Cdc10 *in vitro*. Both loss of function and overexpression of Syp1 affect septin assembly and disassembly at specific cell cycle stages. In particular, in *syp1Δ* cells both septin ring formation at the beginning of the cell cycle and septin ring disassembly at the daughter side of the neck in late stages of the cell cycle are delayed (Qiu et al., 2008). FRAP and overexpression analyses suggest that, even if Syp1 seems to participate to septin ring assembly, it most probably functions as a negative regulator of septin stability. In fact *SYP1* overexpression accelerates septin ring disassembly and is lethal for septin mutants. Furthermore, turnover of septins shows a significant delay in *syp1Δ* cells in both early and late cell cycle stages (Qiu et al., 2008). In

addition, deletion of *SYP1* is lethal when combined with deletion of *RTS1*, another negative regulator of septin stability, suggesting that these two proteins share certain essential functions *in vivo* (Qiu et al., 2008). Syp1 localization at the bud neck resembles septin localization throughout most of the cell cycle except for a 15 minutes period prior to cytokinesis in which Syp1 is delocalized from the bud neck (Qiu et al., 2008). Syp1 is therefore an excellent candidate to be the critical target of Pkc1 in septin regulation. Since we hypothesise that Dma1/2 are upstream regulators of the Rho1/Pkc1 pathway and Pkc1 could regulate septin dynamics through Syp1, we first asked whether Syp1 localization at the bud neck during the cell cycle could be altered in cells lacking *DMA1* and *DMA2* or *PKC1*. In two different experiments, wild type and *dma1Δ dma2Δ* cells or wild type and *pkc1Δ* cells all expressing Syp1 tagged at the C-terminus with 3 HA epitopes were arrested in G1 by α factor and released in the cell cycle at 25°C (wild type and *dma1Δ dma2Δ ,* Fig. 38A) or 30°C (wild type and *pkc1Δ,* Fig. 38B). Progression through the cell cycle was followed by cytofluorimetric analysis of DNA contents (data not shown) and kinetic of budding, nuclear division and localization of Syp1-HA at the bud neck were analyzed at different time points after release. As previously shown (Qiu et al., 2008), Syp1-HA started to localize at the bud neck just before (or contemporarily with) budding. At anaphase, Syp1-HA localization at the bud neck decreased, and a stronger signal appeared again after nuclear division (Fig. 38A and B). No main differences in Syp1-HA localization at the bud neck could be observed between the wild type and *dma1Δ dma2Δ* or *pkc1Δ* strains. These results suggest that if Dma1/2 and the Rho1-Pkc1 pathway regulate

septin ring dynamics through Syp1, they do not do so by regulating Syp1 localization at the bud neck.

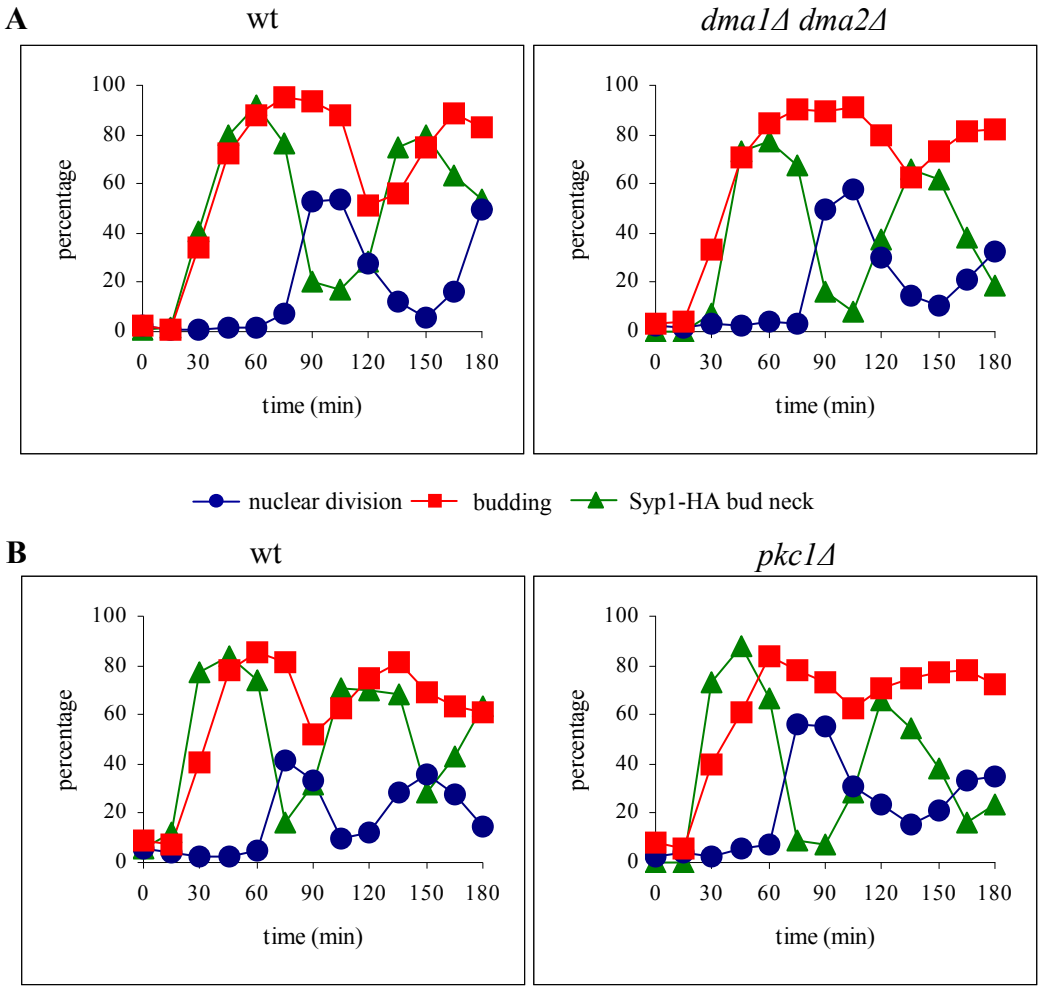


Figure 38. The localization of Syp1 at the bud neck is not impaired in *dma1Δ dma2Δ* and *pkc1Δ* cells. A: Exponentially growing cultures of wild type (ySP8725) and *dma1Δ dma2Δ* (ySP8784) cells expressing Syp1-3HA were arrested in G1 by α factor at 25°C and released into the cell cycle in YEPD at 25°C (time 0). Cell samples were withdrawn at the indicated times for FACS analysis of DNA contents (not shown) and to score budding, nuclear division and bud neck localization of Syp1-3HA by in situ immunofluorescence. **B**: Exponentially growing cultures of wild type (ySP8725) and *pkc1Δ* (ySP8799) cells expressing Syp1-3HA were arrested in G1 by α factor at 30°C and released into the cell cycle in YEPD + 1M sorbitol at 30°C (time 0). Cell samples were analysed at the indicated times as in (A).

Expression of a mutated form of Syp1 in the Pkc1 phosphorylation sites destabilizes the septin ring

Syp1 is an F-BAR protein homologous to mammalian FCHo1 and FCHo2 (Fig. 39). F-BAR proteins bridge the cytoskeleton and cell membrane in a wide variety of cellular contexts, including endocytosis, establishment or maintenance of cellular morphology, neurotransmission, motility and cytokinesis (Aspenström, 2009; reviewed by Roberts-Galbraith and Gould, 2010). The first characterization of F-BAR domains established their ability to bind phospholipids (Tsujita et al., 2006) with a preference for phospholipid bilayers rich in phosphatidylserine and/or phosphatidylinositol (4,5)-biphosphate (Itoh et al., 2005; Tsujita et al., 2006; Henne et al., 2010). The F-BAR domains dimerize into a curved structure that binds to liposomes and either senses or induces the curvature of the membrane bilayer to cause biophysical changes in the shape of the bilayer and to recruit other trafficking factors (Tsujita et al., 2006). Syp1 also contains at the C-terminus a conserved μHD domain (Fig. 39), which is usually involved in cargo selection (Ohno et al., 1995; Owen and Evans, 1998). The region of Syp1 between the F-BAR and the μHD domains contains a Proline Rich Domain (PRD) and a Serine rich region, which is subjected to phosphorylation by different kinases. Indeed, Syp1 is phosphorylated at Thr577 and Thr 588 by the endocytic protein kinase Prk1 (Huang et al., 2009). In addition the middle domain turned out to be phosphorylated also in other F-BAR proteins and importantly this phosphorylation events often appear to be inhibitory (reviewed in Roberts-Galbraith and Gould, 2010).

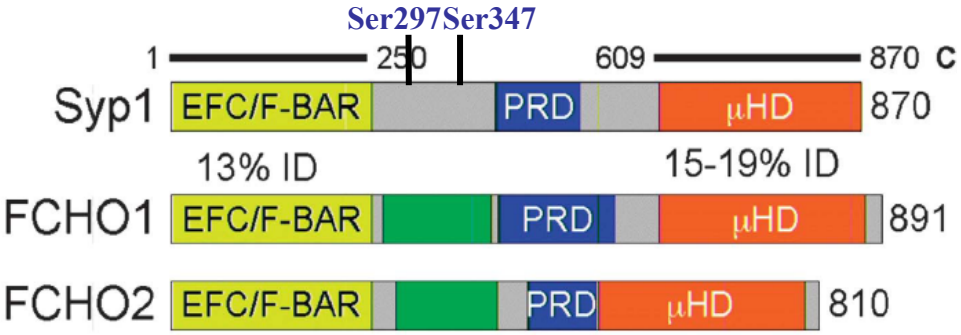


Figure 39. Syp1 domains. Schematic representation of the the protein domains of Syp1 and its mammalians homolog FCHO1 and FCHO2. % of identity between the Syp1 domains and the human domains are indicated. EFC/F-BAR, extended FCH/FCH-BAR domain; PRD, proline-rich domain; μHD, μ-subunit homology domain; green box, region of homology shared by human proteins. (from Reider et al., 2009). The putative Pkc1-dependent phosphorylated serines are shown.

For instance, phosphorylation at dozen of sites within this domain causes *S. pombe* Cdc15 to adopt an autoinhibited conformation in interphase (Roberts-Galbraith et al., 2010) and the phosphorylation of *S. cerevisiae* Hof1 leads to its ubiquitin-mediated degradation (Corbett et al., 2006; Blondel et al., 2005). The central domain of Syp1 is likely to be disordered and seems to be primarly implicated in the interaction with phosphatidylinositol (4,5)-biphosphate (Kalthoff et al., 2002; Reider et al., 2009), suggesting that the two structurally defined domains of Syp1 are separated by a linker whose primary function is to strongly interact with membranes. The central region of Syp1 contains two phosphorylation sites, Ser 297 and 347 (Bodenmiller et al., 2008; Breitkreutz et al., 2010), that fall in similar motifs (**S**AFG and **S**IFG) and within two lysine-rich consensus motifs for binding phosphatidylinositol (4,5)-biphosphate (Reider et al., 2009). In addition, Ser297 has been shown to be phosphorylated by Pkc1 (Fig.

39, Breitkreutz et al., 2010). We therefore generated *SYP1* mutants where Ser297 and Ser347 were changed into Ala, to abolish phosphorylation, and into Asp, to mimic constitutive phosphorylation. These alleles were integrated at the *SYP1* locus and single integrations were checked by southern blot analysis (data not shown).

If these 2 residues were important for Syp1-dependent regulation of septin dynamics, the *SYP1-AA* mutant should ricapitulate the septin defects of *rho1* and *pkc1* mutants (i.e. unstable septin ring), while the phosphomimetic *SYP1-DD* mutant should behave similarly to *RHO1* and *PKC1* hyperactive mutants. We performed a preliminary experiment by comparing septin ring assembly in wild type and *SYP1-AA* cells. Cell cultures were synchronized in G1 with α factor at 25°C and released into the cell cycle at 25°C in the presence of nocodazole to arrest cells in mitosis. The nocodazole arrest was confirmed by cytofluorimetric analysis of DNA contents (data not shown) and the assembly of the septin ring was followed at different time points after the release by in situ immunofluorescence of Cdc11 (Fig. 40). As shown by the graphs in Figure 40, *SYP1-AA* cells underwent septin ring deposition with a delay of about 15 min with respect to wild type cells. In addition, the septin ring seemed to be less stable than in wild type cells, as in a fraction of cells the septin ring remained single until the end of the experiment and did not expand into the hourglass structure. However, this septin ring destabilization was not as marked as in *pkc1* mutants, suggesting that either additional Syp1 residues or additional proteins are targeted by Pkc1 for its regulation of septin dynamics.

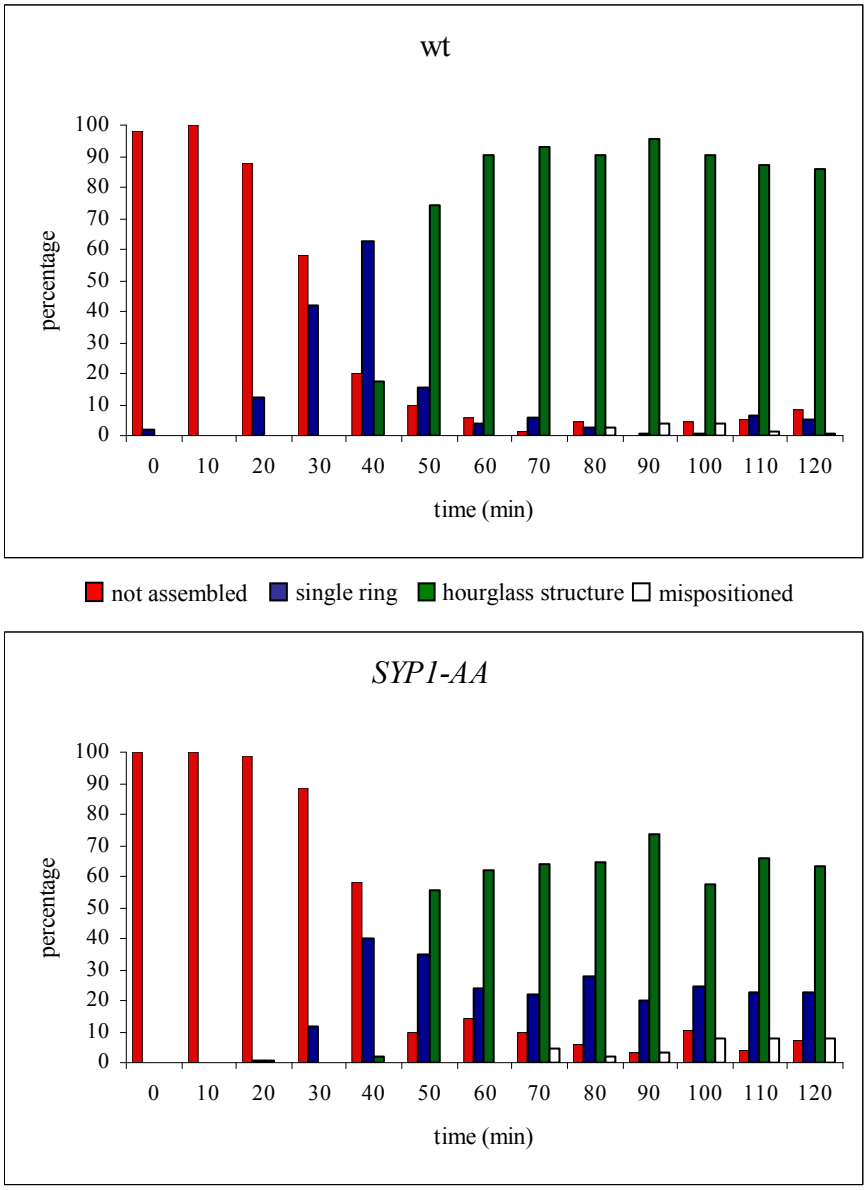


Figure 40. Expression of Syp1-AA destabilizes the septin ring. Logarithmically growing cultures of wild type (W303) and *SYP1-AA* (ySP8837) cells were synchronized in G1 by α factor and released in cycle at 25°C in the presence of nocodazole to arrest cells in mitosis. At the indicated times, cell samples were taken for FACS analysis of DNA contents (data not shown) and in situ immunofluorescence analysis of the septin ring with anti-Cdc11 antibodies.

Materials and Methods

Abbreviations

BFB	Bromophenol Blue
BSA	Bovine Serume Albumine
EDTA	Ethylenediaminetetracetate
kb	Kilobase
kDa	Kilodalton
OD	Optical Density
PEG	Polyethylene glycol
rpm	rounds per minute
SDS	Sodium Dodecyl Sulphate
TRIS	Tris-(hydroximethyl)-aminomethane
YNB	Yeast Nitrogen Base
NOC	Nocodazole
FRAP	Fluorescence Recovery After Photobleaching
aa	Aminoacid
O/N	Overnight
SPBs	Spindle Pole Bodies
MEN	Mitotic Exit Network
FEAR	cdc Fourteen Early Anaphase Release
GAP	GTPase Activating Protein
GEF	Guanine-nucleotide Exchange Factor

Strains and plasmids

Bacterial strains

E. coli DH5α^{TM} (F^-,Φ 80dlacZΔM15, ΔlacZTA-argF) U169, deoR, recA1, endA1, hsdR17, (rK^-, mK^+) supE44, λ-, thi-1, gyrA96, relA1 strain was used as bacterial host for plasmids construction and amplification. Bacterial cells competent for transformation were provided by Invitrogen.

Yeast strains

All yeast strains were derivatives of W303 (MATa or MATα, ade2-1, can1-100, trp1-1, leu2-3,112, his3-11,15, ura3).

Name	**Relevant genotype**
ySP294	*MATa, cdc12-1*
ySP1243	*MATa, bfa1::TRP1*
ySP1569	*MATa, dma1::KlLEU2, dma2::KlTRP1*
ySP1603	*MATa, dma1::KlLEU2, dma2::KlTRP1, bub2::HIS3*
ySP3018	*MATa, ura3::4X URA3::GAL1-DMA2*
ySP3070	*MATa, [CEN-URA3-CDC3-GFP]*
ySP3138	*MATa, bub2::HIS3*
ySP3333	*MATa, GAL1-GFP-LTE1::KanMX4*
ySP4319	*MATa, dma1::KlLEU2, dma2::KlTRP1, [CEN-URA3-CDC3-GFP]*
ySP4320	*MATa, cla4::KanMX4, [CEN-TRP1-cla4-75], [CEN-URA3-CDC3-GFP]*
ySP4321	*MATa, dma1::KlLEU2, dma2::KlTRP1, cla4::KanMX4, [CEN-TRP1-cla4-75], [CEN-URA3-CDC3-GFP]*
ySP4380	*MATa, dma1::KlLEU2, dma2::KlTRP1, GAL1-GFP-LTE1::KanMX4*

ySP5060	*MATa, rts1::kanmX6*
ySP5182	*MATα, cdc12-6*
ySP5244	*MATa, ura3::4X URA3::GAL1-DMA2, cdc12-1*
ySP5247	*MATa, dma1::KlTRP1, dma2::HPHMX, cla4::KanMX4, [CEN-URA3-cla4-75]*
ySP5264	*MATα, dma1::KlTRP1, dma2::HPHMX, cla4::KanMX4, [CEN-URA3-cla4-75]*
ySP6238	*MATa, dma1::KlTRP1, dma2::HPHMX, cla4::KanMX4, [CEN-URA3-cla4-75], swe1::LEU2*
ySP6270	*MATa, kar9::KanMX4*
ySP6292	*MATa, dyn1::KanMX4*
ySP6394	*MATα, cla4::kanMX4, dma1::TRP1, dma2::HPHMx, [CEN-URA3-cla4-75], RHO1-D72N*
ySP6678	*MATa, kar9::KanMX4, bub2::HIS3*
ySP6680	*MATa, dyn1::KanMX4, bub2::HIS3*
ySP7342	*MATa, shs1::natNT2*
ySP7343	*MATα, cla4::kanMX4, dma1::TRP1, dma2::HPHMx, MUT-135*
ySP7454	*MATa, dyn1::KanMX4, dma1::KlLEU2, dma2::KlTRP1*
ySP7554	*MATα, ura3::4X URA3::GAL1-DMA2, shs1::natNT2*
ySP7555	*MATa, ura3::4X URA3::GAL1-DMA2, shs1::natNT2*
ySP7672	*MATa, dma1::KlTRP1, dma2::HPHMX, cla4::KanMX4, swe1::LEU2, [CEN-URA3-cla4-75] , lte1::SpHIS5*
ySP7681	*MATa, dma1::KlTRP1, dma2::HPHMX, cla4::KanMX4, [CEN-LEU2-GAL1-DMA2-2HA], [CEN-URA3-cla4-75]*
ySP7682	*MATa, dma1::KlTRP1, dma2::HPHMX, cla4::KanMX4, [CEN-LEU2-GAL1-DMA1-2HA], [CEN-URA3-cla4-75]*

ySP7684	*MATa, dma1::KlTRP1, dma2::HPHMX, cla4::KanMX4, [CEN-LEU2-GAL1-DMA2- C451S,H456A], [CEN-URA3-cla4-75]*
ySP7685	*MATa, dma1::KlTRP1, dma2::HPHMX, cla4::KanMX4, [CEN-LEU2-GAL1-DMA1- G192E-2HA], [CEN-URA3-cla4-75]*
ySP7686	*MATa, dma1::KlTRP1, dma2::HPHMX, cla4::KanMX4, [CEN-LEU2-GAL1-DMA1- S220A, H223L-HA], [CEN-URA3-cla4-75]*
ySP7766	*MATa, dma1::KlTRP1, dma2::HPHMX, cla4::KanMX4, [CEN-URA3-cla4-75], GAL1-GFP-LTE1::KanMX4*
ySP7771	*MATa, dma1::KlTRP1, dma2::HPHMX, cla4::KanMX4, [CEN-URA3-cla4-75], swe1::LEU2, BUB2-HA3::KlTRP1*
ySP7789	*MATa, dma1::KlTRP1, dma2::HPHMX, cla4::KanMX4, [CEN-URA3-cla4-75], swe1::LEU2, TEM1-HA3::KlURA3*
ySP7819	*MATa, dma1::KlTRP1, dma2::HPHMX, cla4::KanMX4, [CEN-URA3-cla4-75], swe1::LEU2, BFA1-HA6::HIS3MX6*
ySP7855	*MATa, dyn1::KanMX4, dma1::KlLEU2, dma2::KlTRP1, bub2::HIS3*
ySP7862	*MATα, cla4::kanMX4, dma1::TRP1, dma2::HPHMx, [CEN-URA3-cla4-75], leu2::LEU2::3HA-RHO1* (s. c.)
ySP7863	*MATα, cla4::kanMX4, dma1::TRP1, dma2::HPHMx, [CEN-URA3-cla4-75], leu2::LEU2::3HA-RHO1-G19V* (s. c.)
ySP7864	*MATα, cla4::kanMX4, dma1::TRP1, dma2::HPHMx, [CEN-URA3-cla4-75], leu2::LEU2::3HA-rho1-D125A* (s. c.)
ySP7868	*MATa, dma1::KlTRP1, dma2::HPHMX, cla4::KanMX4, [CEN-URA3-cla4-75], swe1::LEU2, BUB2-9myc::TRP1*
ySP7873	*MATa, kar9::KanMX4, dma1::KlTRP1, dma2::HPHMX*
ySP7876	*MATa, kar9::KanMX4, dma1::KlTRP1, dma2::HPHMX, bub2::HIS3*

ySP7901	*MATa, leu2::LEU2::3HA-RHO1-G19V* (s. c.)
ySP7933	*MATa, RHO1D72N::LEU2Kl* (integarated 6000 bp downstream RHO1 stop codon)
ySP7986	*MATa, cdc12-6, RHO1D72N::LEU2Kl* (integrated 6000 bp downstream RHO1 stop codon)
ySP7987	*MATa, cdc12-1, RHO1D72N::LEU2Kl* (integarated 6000 bp downstream RHO1 stop codon)
ySP8107	*MATα, sac7::NAT*
ySP8123	*MATa, cla4::kanMX4, dma1::TRP1,[CEN-URA3-cla4-75, dma2::HPHMx, sac7::NAT*
ySP8150	*MATa, rho1::HIS3, ade3::rho1-1::LEU2*
ySP8153	*MATa, rho1::HIS3, ade3::rho1-5::LEU2*
ySP8173	*MATa, dma2::LEU2, dma1::TRP1, GFP-CDC12::URA3*
ySP8176	*MATa, GFP-CDC12::URA3*
ySP8177	*MATa, RHO1D72N::LEU2Kl* (integrated 6000 bp downstream RHO1 stop codon), *GFP-CDC12::URA3*
ySP8193	*MATa, dma2::LEU2, dma1::TRP1, cla4::kanMX4, [CEN-TRP1-cla4-75], GFP-CDC12::URA3*
ySP8259	*MATα, lrg1::LEUKl*
ySP8296	*MATa, cla4::kanMX4, ,[CEN-TRP1-cla4-75], GFP-CDC12::URA3*
ySP8309	*MATα, cla4::kanMX4, dma1::TRP1, [CEN-URA3-cla4-75], dma2::HPHMx, lrg1::LEUKl*
ySP8321	*MATα, rho1::HIS3, ade3::rho1-5::LEU2, GFP-CDC12::URA3*
ySP8334	*MATa, bag7::NAT*
ySP8400	*MATa, [CEN-URA3-PKC1-R398P]*
ySP8435	*MATα, cla4::kanMX4, dma1::TRP1, [CEN-URA3-cla4-75], dma2::HPHMx, bag7::NAT*
ySP8442	*MATa, bfa1::TRP1, dma1::KlLEU2, dma2::KlTRP1*

ySP8444	*MATa, bfa1::TRP1, dma1::KlLEU2, dma2::KlTRP1, dyn1::KanMX4*
ySP8448	*MATα, bfa1::TRP1, dma1::KlLEU2, dma2::KlTRP1, kar9::KanMX4*
ySP8449	*MATa, bfa1::TRP1, dma1::KlLEU2, dma2::KlTRP1, kar9::KanMX4*
ySP8450	*MATa, bfa1::TRP1, dyn1::KanMX4*
ySP8458	*MATα, bfa1::TRP1, kar9::KanMX4*
ySP8463	*MATa, mpk1::URA3*
ySP8470	*MATa, pkc1::LEU, [CEN-URA3- pkc1-3]*
ySP8472	*MATa, pkc1::LEU, [CEN-URA3- pkc1-2]*
ySP8473	*MATa, cla4::kanMX4, dma1::TRP1 [CEN-URA3-cla4-75], dma2::HPHMx, rts1::kanmX6*
ySP8483	*MATa, cla4::kanMX4, dma1::TRP1, [CEN-TRP1-cla4-75], dma2::HPHMx, [CEN-URA3-PKC1-R398P]*
ySP8491	*MATa, pkc1::kanMX4*
ySP8679	*MATa, cla4::kanMX4, dma1::TRP1, dma2::HPHMx, rts1::kanmX6*
ySP8725	*MATa, SYP1-HA3-KITRP1*
ySP8784	*MATa, dma2::LEU2, dma1::TRP1, SYP1-HA3-KITRP1*
ySP8799	*MATa, pkc1::kanMX4, SYP1-HA3-KITRP1*
ySP8810	*MATa, ELM1::natNT2*
ySP8813	*MATa, ELM1-eGFP::kanMX4*
ySP8814	*MATa, ELM1-eGFP::kanMX4, ura3::4X URA3::GAL1-DMA2*
ySP8820	*MATa, ELM1-HA3::KIURA3*
ySP8821	*MATa, dma2::LEU2, dma1::TRP1, ELM1-HA3::KIURA3*
ySP8829	*MATa, dma2::LEU2, dma1::TRP1, ELM1-eGFP::kanMX4*
ySP8837	*MATa, syp1::NAT:: SYP1 (S297A, S347A)* (s. c.)
ySP8909	*MATa, cdc12-1[CEN-URA3]*

ySP8910	*MATa, cdc12-1, [CEN-URA3-PKC1-R398P]*
ySP8911	*MATa, cdc12-6, [CEN-URA3]*
ySP8912	*MATa, cdc12-6, [CEN-URA3-PKC1-R398P]*
ySP8913	*MATa, [CEN-URA3]*
ySP8922	*MATa, cla4::kanMX4, dma1::TRP1, dma2::HPHMx, RHO1-D72N*

Table 2. Yeast strains used in this work. (s. c. single copy integration)

Plasmids

pSP211	*cla4-75* in *YCplac22 (TRP1)*
pSP340	*cla4-75* in *YCplac33 (URA3)*
pSP524	*YCp50 (URA3)* (empty plasmid)
pSP634	genomic library derived from ySP7343 in *Ycplac111 (LEU2)*
pSP670	*pRS305-3HA-RHO1*
pSP671	*pRS305-3HA-RHO1-G19V*
pSP672	*pRS305-3HA-rho1-D125A*
pSP675	*RHO1-D72N* in *Ycplac111 (LEU2)*
pSP677	*PKC1-R398P* in *YCp50 (URA3)*
pSP795	*SYP1-(S297A, S347A) in YIplac128*

Growth media

All media are autoclave-sterilized and stored at room temperature.

Media for *E. coli*

LD	1% Bactotryptone
	0.5% Yeast extract
	0.5% NaCl (pH 7.25)
LD amp	LD + ampicillin (2.5 g/L)

Agar to 1% was added in order to obtain solid *E. coli* media

Media for *S. cerevisiae*

YEP 1% Yeast extract
2% bactopeptone
50 mg/l adenine (pH 5.4)

Before using, YEP medium was supplemented with different carbon source: 2% glucose (YEPD), 2% raffinose (YEPR), 2% raffinose and 1% galactose (YEPRG), 2% glucose and 1M sorbitol (YEPD + sorbitol).

Synthetic medium (S) 0.7% Yeast nitrogen base (YNB)
without aminoacids (pH 5)

Before using, S medium was supplemented with 2% glucose (YEPD), 2% raffinose (YEPR) or 2% raffinose and 1% galactose (YEPRG) and 25 g/ml of all aminoacids and nitrogen bases (SC medium), unless differently indicated.

5-FOA medium was obtained by adding 1 g/L 5-FOA, 50 mg/L uracil to SC medium.

Sporulation medium (VB) 1.36% CH3COONa3H2O
0.19% KCl
0.0035% MgSO47H2O
0.12% NaCl (pH 7)

Agar to 2% was added in order to obtain solid yeast media.

Buffers

SDS-PAGE running buffer 5X: 2M Glycin, 0.25M TRIS, 0.02M SDS, pH8.3.

SSC20X: 3M NaCl, 0.3M Na cytrate, pH 7.5.

STET: 8% sucrose, 5% triton X100, 50mM EDTA, 50mM TRIS-HCl, pH 8.

TAE: 0.04M Tris acetate, 0.001M EDTA.

TE: 10mM TRIS-HCl, 1mM EDTA, pH 7.4.

Laemly bf: 0.62M TRIS, 2% SDS, 10% Glycine, 0.001% BFB, 100mM DTT.

TBS 10X: 1.5M NaCl, 0.5M TRIS-HCl, pH 8.

BLUE 6X: 0,2% bromofenol blue in 50% glycerol

Generation of diploid strains and sporulation

Diployd strains were generated by crossing the appropriate haploid strains on YEPD plates. When diploid cell selection was possible, crosses were transferred after 24 hours at 25°C to selective media and/or temperatures allowing only diploid cells growth. Diploid cells were allowed to sporulate by transferring them onto VB sporulation medium. These plates were then incubated for 2 days at 25°C. After zymoliase digestion of the cell wall, tetrads were dissected with an optical micromanipulator on the appropriate agar medium.

Yeast transformation

Cells were inoculated o/n at 25°C YEP medium containing the appropriate sugar, allowing them to reach stationary phase. Cell cultures were then diluted and allowed to grow for at least 2 hours, until they reached a concentration between $1x10^6$ and $1x10^7$ cells/ml. 10 ml of each culture were then centrifuged for 5 minutes at room temperature and pellets were washed with 1 ml of LiAc 1 M to completely eliminate the growth medium. Each pellet was then

resuspended in 200 µl of 1 M LiAc, and 1-5 µl of DNA containing solution were added to cells suspension (sufficient for one transformation), together with 5 µl of carrier DNA (salmon sperm DNA) and 45 µl of PEG 4000 50%. After gently mixing, the tubes were incubated 30'- 60' at 25°C. Subsequently, 6 µl of glycerol 60% were added to the cell suspension, followed by incubation at 25°C for 30'- 60'. After a 5'heat shock at 42°C, cells were finally plated on selective medium.

Mutagenesis with EMS

A cellular suspension of *dma1Δ dma2Δ cla4-75* cells was treated with 3% EMS for 60 minutes. Cells were plated on YEPD plates both at 25°C to determine the percentage of cells that were viable after the treatment and at 37°C to isolate thermo-resistant suppressors. In these conditions about 50% of the cells were viable at 25°C. In order to discard all possible revertants of *cla4-75*, temperature-resistant clones were then assessed for their ability to lose the *cla4-75* allele carried on an episomal centromeric plasmid. Thus cells were replica-plated on 5-FOA (5-Fluoro-Orotic-Acid) plates to select their derivatives lacking the *cla4-75* construct, to be sure that the isolated clones were effectively extragenic suppressors of *dma1Δ dma2Δ cla4Δ* lethality and not revertents of the *cla4-75* allele (Fig. 14A). After this secondary screen we recovered 44 suppressors (30 MATa and 14 MATα) than retained viability in face of the simultaneous lack of Dma1, Dma2 and Cla4.

Dominance test

To analyse the dominance/ recessivity of the suppressing mutations we crossed each clone containing an extragenic mutation with the parental strain (*dma1Δ dma2Δ cla4-75*). We selected the derivative diploids that were analyzed for their ability to growth at 37ºC and on 5-FOA. If the mutation was recessive the diploid should lose the ability to growth at the restrictive temperature and to form colonies on 5-FOA plates; instead a dominant mutation should allow diploid to growth both at 37°C and on 5-FOA. Finally 11 mutants were classified as dominants, 9 as semi-dominants and 20 as recessives (figure 14B).

Library construction

To construct the library we partially digested the genomic DNA with Sau3A and ligated the digested DNA in BamHI site of the centromeric plasmid Ycplac111. Ligation of 8.0-12.0 Kb DNA fragments was performed using a high efficiency kit (Takara DNA-ligase). After *E. coli* transformation we obtained about 100,000 *E. coli* transformants that should be representative of 3,5 genome equivalents, thus providing a good coverage of the genome (Fig. 26).

***E. coli* transformation**

DH5αTM-GIBCO BRL competent cells, kept at 80°C, were defrozen in ice, and 50-100 µl of cell suspension were used for each reaction. After incubation in ice for 30 minutes, 1-10 ng of DNA were added to the cells. After a further incubation in ice for 30 minutes, cells were subjected to a 30 sec heat shock at 37°C, followed by incubation in ice

for 2 minutes. Finally, 900 µl of LD medium were added to each reaction tube. Cell suspension was then shacked for one hour at 37°C before plating on selective medium and incubation at 37°C.

Preparation of plasmid DNA from *E. coli*

Two different techniques were used, depending on the amount and the quality of the DNA to be obtained.

(I): MINIPREPS BOILING. *E. coli* cells (2ml overnight culture) are harvested by centrifugation and resuspended in 500 µL STET buffer (8% sucrose, 5% TRITON X-100, 50mM EDTA, 50mM Tris-HCl, pH 8). Bacterial cell wall is digested boiling the sample for 2 minutes with 1 mg/ml lysozyme. Cellular impurities are removed by centrifugation and DNA is precipitated with isopropanol and resuspended in the appropriate volume of water or TE 1X.

(II): QIAGEN COLUMNS. This protocol allows the purification of up to 20 g high copy plasmid DNA from 1-5 ml overnight *E. coli* culture in LD medium. Cells are pelleted by centrifugation and resuspended in 250 µl buffer P1 (100 g/mL RNase, 50mM Tris HCl pH 8, 10mM EDTA pH 8). After addition of 250 µl buffer P2 (200mM NaOH, 1% SDS) the solution is mixed thoroughly by inverting the tube 4-6 times, and the lysis reaction occur in 5 minutes at RT. 350 µl N3 buffer (QIAGEN) are added to the solution, which is then centrifuged for 10 minutes. The supernatant is applied to a QIAprep spin column which is washed once with PB buffer (QIAGEN) and once with PE buffer (QIAGEN). The DNA is eluted with EB buffer (10mM Tris HCl pH 8.5) or water.

Preparation of genomic DNA from yeast

Yeast cells of the desired strain were grown in 10 ml YEP containing the appropriate sugar to lag phase. Cells were collected by centrifuging and then washed with 1 ml of a solution containing 0.9 M sorbitol, 0.1 M EDTA pH 7.5. Pellet was then transferred into 0.4 ml of a solution containing 0.9 M sorbitol, 0.1 M EDTA pH 7.5 and 14mM β-mercaptoethanol. After mixing, 0.1 ml of a 2 mg/ml solution of Zimolyase 100T were added, and the tubes were incubated at 37°C up to spheroplasts formation (20'-30'), checked by optical microscopy. After 30' of centrifugation the pellet was carefully resuspended in 0.4 ml TE 1X. After addition of 90 µl of a solution containing 1.5 ml of EDTA pH 8.5, 0.6 ml of Tris base and 0.6 ml 10% SDS (SDS solution), the tubes were mixed and incubated 30' at 65°C. 80 µl of potassium acetate 5 M was added and then the tubes were incubated in ice for at least 1 hour. The tubes were then centrifuged and the supernatant was then precipitated and washed with 70% ethanol. Dried pellet was carefully resuspended in 0.5 ml of TE 1X. 25 µl of RNAse 1 mg/ml was added to the tube and the solution was incubated 30' at 37°C. DNA was then precipitated by addiction of 0.5 ml of isopropanol and then centrifuged. Pellet was washed with cold 70% ethanol, dried and finally resuspended in 20-50 µl of TE 1X, to obtain a final concentration of 100 g/l of yeast genomic DNA.

Synchronization with α-factor

MATa cells were inoculated in YEP medium supplemented with the appropriate sugar, allowing them to reach a concentration of $5x10^6$ cells/ml. α-factor was then added to a final concentration of 2 g/ml,

and the percentage of budded cells was scored 2 hours later. When more then 95% of cells arrested as unbudded (G1-arrested cells), the pheromone was removed and cells were washed once with fresh medium and then re-suspended and incubated in fresh medium. Unless otherwise stated, syncronizations were performed at 25°C and galactose, when requested, was added half an hour before the release from α-factor.

Nocodazole response

Nocodazole allows yeast cell synchronization in G2 by causing microtubule depolymerization. For nocodazole response, cells were released from G1 arrest in the presence of 15 μg/ml nocodazole (US Biolabs) that was dissolved in DMSO. The final DMSO percentage in the noc-treted cell cultures was 1%.

DNA digestions with restriction endonucleases and agarose gels

DNA samples were digested with the appropriate restriction enzymes, using the conditions described by Maniatis et al. (1989), or the own provider's instructions. 1/5 volumes of a BFB solution (6X stock solution: 0.2% BFB in 50% glycerol) were added to the digested samples, before loading onto 0,6-2% agarose gels. Fragments were separated in virtue of their molecular weight, performing electrophoresis in TAE 1X buffer. DNA was visualized adding 10 μg/ml Ethydium Bromide directly to the gel, and then by analysis of the gel with UV radiations of 260 nm wavelength. To determine the size of the DNA fragments loaded onto the gel, a marker containing

DNA fragments of known length (provided by Biolabs) was loaded in parallel with the samples.

DNA purification from agarose gel

DNA was loaded into an agarose gel and then separated by electrophoresis. The gel was cut just downstream of the DNA fragment of interest. Purification of DNA from the gel slice was done using Agarose GelExtract Mini kit (5 PRIME).

Ligation

Appropriate amounts of the isolated fragment and of the vector, cut with restriction enzymes, were ligated in the following conditions:

T4 DNA ligase buffer 10X	1 µl
T4 DNA ligase (Promega)	1 µl

The reaction volume is corrected with water to a final volume of 10 µl and the resulting mix is incubated O/N at 16°C.

Southern blot analysis

Yeast DNA was prepared according to standard methods and digested with the appropriate endonuclease. The resulting DNA fragments were separated by gel electrophoresis in 0.6% agarose gel and transferred (Magnatis et al., 1989) to a GeneScreen nylon membrane (Roche), followed by hybridization with an appropriate probe, DIG labelled. Standard hybridization conditions were used. After appropriate washings, the membrane is treated with peroxydase that, together with other reagents, catalyses a reaction which produces light, detectable on the film after treatment with developing and fixing solutions.

PCR (Polymerase Chain Reaction)

PCR was performed on plasmidic DNA preparation (Qiagen) or on genomic yeast DNA. The DNA fragment amplification requires two oligonucleotides flanking the interesting region, working as primers for the DNA polymerase. Several DNA polymerase were used: Taq polymerase (Roche, Genespin), Vent polymerase (Biolabs), Phusion polymerases (Finnzymes), depending on the kind of DNA template and fidelity of the amplificated fragment.

The reaction mix was:

DNA	100 ng
10X Buffer	5 µl
Primers	250 ng x 2
dNTPs	0.2 mM x 4
H_2O	to 25µl

DNA amplification was performed with a Hybrid Omn-E device, with the following parameters:

1.	heat shock step	2’	at 95°C
2.	denaturation step	30”	at 95°C
3.	annealing step	30”	at primers T_m
4.	extension step	depending on polymerase	
5.	repeat steps from 2 to 4 25/30 times		
6.	extension step	10’	at 72°C
7.	end		

A primer’s melting temperature (T_m) was calculated with the following formula:

$T_m = 16.6 * \log(M) + 0.41\ (P_{GC}) + 81.5 - P_m - B/L - 0.65\ P_f$

Where:

- M: Na^+ concentration
- P_{GC}: GC percent content in primer olygonucleotyde
- P_m: primer/template mismatch in percent
- P_f: formaldeyde percent in the reaction
- B: 675 for oligonucleotides < 100 bp
- L: oligonucleotide length

Protein extracts

Cells were collected by centrifugation, washed once with 1ml Tris 10 mM pH 7.5 cold and then resuspended in two volumes of breaking buffer (Tris 50 mM pH 7,5 and protease inhibitor cocktail by Boehringer Mannheim), using Eppendorf tubes that were always kept in ice. Equal amounts of acid-treated glass-beads were then added to each suspension, and cells were broken by vortexing for 5 min at 4°C. Samples were then transferred into new tubes and clarified by centrifugation. 1 µl of each protein extract was diluted in 1 ml of Biorad Protein Assay (Biorad) for spectrophotometric protein quantification at 595 nm UV wavelength. Clarified extracts were resuspended in 50 µl Laemmli buffer 3X (0.62M Tris, 2% SDS, 10% glycine, 0.001% Bfb, 100mM DTT), boiled for 3 minutes, and loaded on polyacrylamide gel.

Western blot analysis

Proteins were separated based on their molecular weight on 10% or 12.5% polyacrylamide gel in SDS-PAGE. Proteins were transferred on nitrocellulose filters by 200mA o/n. In order to preliminarily quantify the total amount of transferred proteins, the filters were stained with Ponceau S (Sigma). After destaining with TBS 1X, filters were incubated for one hour at room temperature in non-fat 4% or 5% dust milk in TBS 1X and 0.2% Triton X-100. Filters were then incubated 2 hours with primary antibodies directly diluted in 4% or 5% milk. To detect HA tagged proteins, we used 1:3000 dilutions of monoclonal antibodies 12CA5; to detect Mpk1 we used 1:1000 dilution of α-Mpk1 polyclonal antibodies (Santa Cruz); to detect the phosphorylated form of Mpk1 we used 1:1000 dilution of α-P-Mpk1 polyclonal antibodies (New England Biolabs). Filters were then washed three times in TBS 1X for 10', before incubating them for an hour at room temperature with properly diluted secondary antibodies (1:10000 anti-mouse IgG, anti-rabbit IgG or anti-goat IgG). These secondary antibodies, provided by Amersham in the kit ECL, are conjugated with the peroxydase enzyme. Filters were finally washed three times for ten minutes, dried on 3MM paper and carefully dipped in a mix composed by equal volumes of the two ECL solutions. After a new drying, the filters were exposed for different times to a film; peroxydase, together with its reagents on the film, catalyses a reaction which emits light, which is detectable on the film filter, after treatment with developing and fixing solution.

FACS analysis, budding and nuclear division

For FACS (Fluorescence-Activated Cell Sorter) analysis, $5x10^6$ cell samples were collected by centrifugation and then resuspended in 1 ml of 70% Ethanol, prior to 1 hour of incubation at room temperature. Cells were then washed once with 1 ml of Tris 50 mM pH 7.5 and the pellet was then resuspended in 0.5 ml of Tris 50 mM pH 7.5 containing 1 mg/ml RNAse. After incubation over night at 37°C, cells were collected by centrifugation, and pellets were resuspended in 0.5 ml of pepsin 5 mg/ml, dissolved in 55 mM HCl, and incubated 30' at 37°C. Cells were then washed once with 1 ml of FACS Buffer (200 mM Tris pH 7.5, 200 mM NaCl, 78 mM $MgCl_2$) and resuspended in the same buffer containing 5s mg/ml Propidium Iodide. Samples were finally analysed with a FACS-Scan device provided by Becton Dickinson. Budding and nuclear division were scored microscopically on FACS samples.

Fluorescence Microscopy

Cells were fixed in 1ml IF buffer (0.1M K+/PO43- buffer pH6.4, 0.5mM $MgCl_2$) containing 3.7% formaldehyde at 4°C overnight (for anti-Cdc11 and anti-tubulin staining) or 30'- 60' (for anti-HA and anti-myc staining). Samples were washed three times in IF buffer and once in IF buffer containing 1.2 M sorbitol. Cells were spheroplasted by incubating for 5-60 minutes at room temperature in 0.2 ml spheroplasting buffer (1.2M sorbitol, 0.1M K+/PO43- buffer pH6.4, 0.5mM $MgCl_2$, 250g/ml zymoliase, 0.2% β-mercaptoethanol (only for o/n incubation)). Spheroplasting was monitored by mixing a drop of cells in spheroplasting buffer with an equal amount of 10% SDS.

When cells lysed under these conditions the spheroplasts were washed once in IF buffer containing 1.2 M sorbitol and resuspended in 20-200 µl of the same solution depending on the amount of cells. A 30-well slide was coated with 0.1% polylysine, rinsed with mQ H_2O and dried. A drop of cell suspension of medium density was added to each well, and cells were allowed to attach to the slide surface for 10-20 minutes. The cell suspension was then removed, and slides were put in a methanol bath at -20°C for 6 minutes, and in an acetone bath at -20°C for 30 seconds. Next, the slides were warmed up and dried. Primary antibody was added to each well and incubated for two hours at RT. Then the primary antibody was aspirated off, each well was washed three times with BSA-PBS, followed by addition of secondary antibody. Slides were incubated in the dark for 2 hours, then the secondary antibody was aspirated off, and each well was washed four times with BSA-PBS. For DAPI staining, a drop of pd-DAPI (0.25 g/ml DAPI (4,6 diamino-2-Phenylindolo), 0.1% p-phenylenediamine, and 10% PBS pH 8.0 in glycerol) was added to each well, a coverslip was placed over, and the coverslip was sealed using nail hardener. Slides were stored at -20°C in the dark until use. Immunostaining of α-tubulin was performed with the YOL34 mAb (Serotec) that was diluted 1:100 in BSA-PBS (1% crude BSA, 0.04M K2HPO4, 0.01M KH2PO4, 0.15M NaCl), followed by indirect immunofluorescence using rhodamine-coniugated anti-rat Ab (1:100 Pierce Chemical Co.). Immunostaining of α-HA was performed with the 16B12 mAb (Babco) diluted 1:500 in BSA-PBS (1% crude BSA, 0.04M K_2HPO_4, 0.01M KH_2PO_4, 0.15M NaCl), followed by indirect immunofluorescence using CY3-conjugated goat anti–mouse Ab

(1:1000; GE Healthcare). Immunostaining of α-myc was performed with the 9E10 mAb diluted 1:500 in BSA-PBS (1% crude BSA, 0.04M K_2HPO_4, 0.01M KH_2PO_4, 0.15M NaCl), followed by indirect immunofluorescence using CY3-conjugated goat anti–mouse Ab (1:1000; GE Healthcare). Immunostaining of α-Cdc11 was performed with α-Cdc11 antibodies (Santa Cruz) that were diluted 1:200 in BSA-PBS (1% crude BSA, 0.04M K2HPO4, 0.01M KH2PO4, 0.15M NaCl), followed by indirect immunofluorescence using Alexa Fluor 488-conjugated anti-rabbit antibody (1:100, Invitrogen).

Detection of Cdc3-GFP, Lte1-GFP and Elm1-eGFP was carried out on 100% cold ethanol-fixed cells, upon wash with TRIS 10mM pH 8,5 and sonication.

Digital images were taken with a Leica DC350F charge-coupled device camera mounted on a Nikon Eclipse 600 and controlled by the Leica FW4000 software or with the MetaMorph imaging system software on a fluorescent microscope (Eclipse 90i; Nikon), equipped with a charge-coupled device camera (Coolsnap, Photometrics) with an oil 100X 0,5-1.3 PlanFluor oil objective (Nikon). Fluorescence intensity of Elm1-eGFP at the bud neck was measured with ImageJ on a plane that had the bud neck in focus.

FRAP analysis

FRAP experiments were performed on a Zeiss LSM 510 confocal microscope. Cells expressing Cdc12-GFP were grown on YPD plates overnight. Cells were resuspended in synthetic complete medium and spread on a 1.6% agarose pad. The entire or half the septin ring was bleached with a sequence of 20 to 25 irradiations at 50% of laser

intensity. Pictures were taken at least every 30 s for 10 min. Fluorescence intensities were analyzed with NIH image. In each picture we scored the average values of the bleached part. To correct for general bleaching, al least two reference cells were present in each movie.

Time Lapse analysis

Cells were mounted in low autofluorescence selective drop-in medium shortly before viewing. Objects were subjected to time-lapse microscopy for a maximum of 2,5 h. To visualize Cdc12-GFP full-frame pictures of fields of cells were taken at 9 focal planes (0.2 μm step size) using an Olympus BX50 fluorescence microscope, a piezo motor, and the TILLVision software (TILLphotonics, Martinsried, Germany). The pictures were projected on one single plane using the maximum method. Z-stacks of 6 focal planes (0.3 μm step size) were taken at intervals of 30 sec for a total time of 2,5 h. Z-stacks were projected by the maximum method. Before each movie a single transmission image was taken at the middle focal plane.

Drop test

For drop test analysis, cell cultures were grown overnight to a 10^8 cells/ml concentration and then diluted to 1.25×10^6, 1.25×10^5, 1.25×10^4, 1.25×10^3 cells/ml, respectively. For each dilution, 5μl of suspension were spotted on plates that were incubated at the appropriate temperatures.

Discussion

Role of the Dma proteins in septin ring dynamics

The septin ring has been shown to oscillate between a rigid state, which persists throughout most of the cell cycle, and a highly dynamic state that is established in late G1 and during septin ring splitting, prior to cytokinesis (Weirich et al., 2008). The transition between the dynamic and the static state is promoted by the Cla4 and Gin4 kinases, which directly phosphorylate septins (Dobbelaere et al., 2003; Versele and Thorner, 2004). We previously implicated Dma1 and Dma2 in the regulation of septin ring organization (Fraschini et al., 2004), and we now extended our initial observations by showing that Dma1 and Dma2 likely promote septin ring stabilization. In fact, *DMA1* and *DMA2* double deletion is synthetically lethal with mutations that destabilize the septin ring. Conversely, the toxic effects of *DMA2* overexpression, which causes the formation of a new septin ring at the incipient bud site before the old one has been disassembled, can be relieved by mutations destabilizing the septin ring, such as *cdc12-1*. Moreover, a conditional *dma1Δ dma2Δ cla4-75* triple mutant is unable to properly deposit a septin ring at the bud neck in late G1, as well as to maintain it after its formation. Our FRAP analysis indicates that Dma1 and Dma2 regulate septin turnover independently from Cla4, which is also consistent with the synthetic lethality observed by the simultaneous inactivation of the three proteins.

How Dma proteins control septin dynamics remains to be established. Being Dma1 and Dma2 E3 ubiquitin ligases (Loring et al., 2008), they might directly ubiquitylate septins or their regulators to control septin turnover within the ring. However, septins were not found to be ubiquitylated in a previous study (Johnson and Gupta, 2001) and our

efforts in this direction did not provide reproducible results (data not shown). Our finding that Elm1 localization at the bud neck is defective in cells lacking the Dma proteins and the appearance in these cells of an Elm1 isoform that is absent in wild type cells suggests a possible mechanism for the regulation of septin dynamics by these proteins. Indeed, similarly to Dma1 and Dma2, Elm1 is required for cytokinesis and proper localization of septins at the bud neck (Bouquin et al., 2000; Thomas et al., 2003). In addition, its inactivation causes a Swe1-dependent mitotic delay (Sreenivasan and Kellogg, 1999). Interestingly, the bud neck localization of Elm1 requires its own kinase activity (Thomas et al., 2003), raising the possibility that the latter might be regulated by Dma proteins. Moreover, accumulation of Elm1 at the bud neck seems to promote its function in the SPOC, because Elm1 is enriched at the bud neck when the checkpoint is active (Moore et al., 2010). At a first glance, the synthetic lethality between *ELM1* and *DMA1/DMA2* deletion seems at odds with the proposal that Elm1 might act in the same pathway of Dma proteins. However, Elm1 was found to regulate both Gin4 and Hsl1, which in turn become essential in the absence of Dma1 and Dma2. Therefore, Dma proteins might regulate a subset of Elm1 functions that are independent of Gin4 and Hsl1 regulation. Alternatively, a recent study suggests that Elm1 has a primary role in the SPOC which is separated from its role in septin organization, since it contributes a specific function in the checkpoint which is independent of its known roles in the regulation of Swe1 (Moore et al., 2010). It is also possible that Dma1/2 control the localization of Elm1 at the bud neck in relationship to its SPOC activity, but the three

proteins act independently in the regulation of septin ring stability, thus explaining the synthetic lethality caused by simultaneous deletion of *DMA1 DMA2* and *ELM1*. However, we favor a model in which Dma proteins regulate both septin ring assembly/stabilization and the spindle position checkpoint by recruiting Elm1 at the bud neck.

Interestingly, we have also found that deletion of *RTS1,* which encodes a regulatory subunit of the PP2A phosphatase that was previously involved in septin destabilization (Dobbelaere et al., 2003), rescues the viability of *dma1Δ dma2Δ cla4Δ* cells. Rts1 has also been recently involved in the SPOC (Chan and Amon, 2009). Although the molecular mechanism by which Rts1 participates in the SPOC has not been defined, the observation that hyperphosphorylation of the SPOC kinase Kin4 observed in the absence of Rts1 depends on Elm1 (Caydasi et al., 2010) suggests that $PP2A^{Rts1}$ might antagonize Elm1. How two antagonizing functions both contribute to the SPOC remains to be established. However, our genetic data strengthen the above conclusion: Dma1/2 might promote septin stabilization by activating Elm1 and/or inhibiting $PP2A^{Rts1}$ directly or indirectly. Whether Elm1 and/or Rts1 are direct ubiquitylation targets of Dma proteins is an important issue to be addressed in the future.

Dma-dependent regulation of the SPOC

The failure of *dma1Δ dma2Δ cla4-75 swe1Δ* cells to activate the SPOC in the presence of mispositioned spindles is likely to be ascribed to the sole lack of Dma1 and Dma2 for two main reasons: i) lack of Dma proteins is sufficient to cause re-budding of *dyn1* and *kar9* mutant cells that undergo spindle mispositioning (Fraschini et al.,

2004); ii) Cla4 promotes, rather than inhibits, timely mitotic exit and MEN activation independently of Swe1 (Chiroli et al., 2003; Hofken and Schiebel, 2002; Seshan et al., 2002). On the other hand, the SPOC defect of *dma1Δ dma2Δ cla4-75 swe1Δ* cells does not seem to be an indirect consequence of their septin ring instability. In fact, septin mutants were shown to be defective in the SPOC presumably as a consequence of Lte1 spreading into the cytoplasm, since their ability to escape mitosis in the presence of mispositioned spindles depends on Lte1 (Castillon et al., 2003). In contrast, *dma1 dma2 cla4 swe1* cells exit mitosis under these conditions independently of Lte1. We also show that *dma1Δ dma2Δ cla4-75 swe1Δ* cells display asymmetric localization of the Bub2/Bfa1 complex at SPBs in cells undergoing anaphase within the mother cell, where normally Bub2/Bfa1 is present on both SPBs (Caydasi and Pereira, 2009; Molk et al., 2004; Monje-Casas and Amon, 2009). This is consistent with the mislocalization of Lte1 in the mother cell that has been recently shown to cause loss of Bfa1 from one SPB in cells with a misaligned spindle (Geymonat et al., 2009). However, we think this is not the main reason for the SPOC defect of *dma1Δ dma2Δ cla4-75 swe1Δ* cells, because *DMA1/2* deletion and *BUB2* or *BFA1* deletion cause additive SPOC defects, suggesting that Dma proteins and Bub2/Bfa1 control mitotic exit independently of each other. Moreover, defective SPOC in *dma1Δ dma2Δ cla4-75 swe1Δ* cells cannot be rescued by expression of the hyperactive Bub2-myc9 variant that we previously showed to be constitutively present on both SPBs throughout mitosis (Fraschini et al., 2006). The asymmetric localization of Bub2/Bfa1 at SPBs in *dma1Δ dma2Δ cla4-75 swe1Δ* cells with misaligned spindles could

stem from defects in Elm1 activity in cells lacking the Dma proteins. Indeed, Elm1 activates the SPOC kinase Kin4 (Caydasi et al., 2010; Moore et al., 2010), which is in turn required to maintain symmetric localization of Bub2/Bfa1 on misaligned spindles (Caydasi and Pereira, 2009). The novel links between Dma proteins and Elm1 raise the possibility that Dma1 and Dma2 operate in the same SPOC pathway as Kin4. On the other hand, several data indicate that Kin4 is an upstream regulator of Bub2/Bfa1 through Bfa1 phosphorylation, which counteracts its inhibition by the polo kinase (D'Aquino et al., 2005; Maekawa et al., 2007; Pereira and Schiebel, 2005). In contrast, our genetic data suggest that the Dma proteins act in the SPOC independently of Bub2/Bfa1. It is possible that not all SPOC functions of Elm1 are to be ascribed to Kin4 regulation. Indeed, Elm1-dependent Kin4 activation does not seems to depend on Elm1 localization at the bud neck (Caydasi et al., 2010), suggesting that bud-neck localized Elm1 has a different role in the SPOC and/or in other processes.

In conclusion, Dma1 and Dma2 might regulate both septin ring dynamics and the SPOC by controlling the bud neck localization of Elm1, thus coordinating the timing of mitotic exit and cytokinesis. Interestingly, the human Dma homolog Rnf8 localizes at the spindle midbody and has been proposed to antagonize the human mitotic exit network (Tuttle et al., 2007), suggesting that it might be the functional human counterpart of Dma proteins. The identification of the ubiquitylation targets of Dma proteins will be a challenging task for future research and will help to clarify the mechanism(s) through which they control cytokinesis.

Regulation of septin dynamics by the Rho1/Pkc1 pathway

We have shown that hyperactivation of the Rho1/Pkc1 pathway suppresses the lethality of *dma1 dma2 cla4* triple mutants. Indeed, expression of *RHO1-D72N*, *RHO1-G19V* and *PKC1-R398P* hyperactive alleles rescues the viability of *dma1Δ dma2Δ cla4-75* cells at 37°C. In addition, the *RHO1-D72N* allele that we have found in our genetic screen suppresses the lethality of *dma1Δ dma2Δ cla4Δ* cells also at 25°C and rescues their cytokinesis and septin ring defects. Rho1 and its mammalian counterpart RhoA have been largely implicated in cytokinesis. In animal cells the assembly of the contractile ring depends on the concentration of RhoA around the cell equator, i.e. the future site of the cleavage furrow (reviewed by Glotzer, 2009; Pollard, 2010). Unlike in animal cells, where the cell division site is determined at the timing of chromosome segregation by spindle positioning, in budding yeast the plane of division is defined at the beginning of the cell cycle as the site of bud emergence, i.e. much earlier than a bipolar spindle is formed (Balasubramanian et al., 2004). Nevertheless, the mechanism that promotes the formation and contraction of the actomyosin ring is conserved. In fact, also in *S. cerevisiae* Rho1 is required for the formation and contraction of the actomyosin ring (Tolliday et al. 2002), it becomes concentrated at the bud neck during late mitosis and is essential for cytokinesis (Yoshida et al., 2006). Our finding that hyperactive alleles of *RHO1* suppress the septin ring deposition/stabilization defects of cells lacking *DMA1 DMA2* and *CLA4* highlights a new cytokinesis fuction for Rho1 in septin regulation. It is worth noting that, although our genetic analysis shows that Rho1 hyperactivation through the *RHO1-G19V* allele can

suppress the lethality of *dma1 dma2 cla4* mutants, no other *RHO1* allele beside *RHO1-D72N* was identified in our genetic screen. The D72N mutation of Rho1 falls in an Asp residue that is conserved in all the GTPases of the Rho family and located inside the switch II. The switch I and the switch II are the domains of GTPases that undergo the most dramatic structural rearrangements upon GTP binding and hydrolysis and together cooperate to promote GTPase activation. In addition, these regions modulate the interactions of GTPases with their GAPs and effectors (Vetter and Wittinghofer, 2001). Thus, the D72N amminoacid substitution could modify Rho1 structure, leading to a Rho1 hyperactive variant that either stabilizes the interaction with GTP (active form), or promotes the interaction with a specific GEF, or inhibits the binding to a GAP. Otherwise, Rho1-D72N could bind with higher affinity some effectors, such as Pkc1. It will be important in the future to analyse the catalytic activity of this new Rho1 variant and its ability to interact with regulators and effectors.

Since we found that the classic hyperactive *RHO1-G19V* can suppress the lethality of *dma1 dma2 cla4* cells, the question arises as to why we did not find this and/or other hyperactive *RHO1* alleles in our screen. The type of mutagen used (the alkylating agent EMS) might be partly responsible for the specificity. However, a more likely explanation is that we found the *RHO1-G19V* allele to be lethal unless expressed along with wild type *RHO1*. It is therefore possible that Rho1 must cycle between the GTP- and the GDP-bound state to maintain cell viability, and therefore classic hyperactive alleles of *RHO1* that lock the GTPase in the GTP-bound form could not be recovered in our screen. Thus, the *RHO1-D72N* allele might be hyperactive only for

what concerns the activation of some, but not all, effectors. Interestingly, neither *RHO1-D72N* nor *RHO1-G19V* hyperactivates the cell wall integrity signalling pathway, as judged by the induction of Mpk1 phosphorylation. A detailed biochemical characterization of the different Rho1 mutants will be necessary to uncover their specific defects.

Since we found that deletion of the Rho-GAPs Lrg1 and Sac7, as well as Pkc1 hyperactivation, also suppressed the lethality of *dma1 dma2 cla4* mutants, we sequenced *LRG1* and *SAC7* open reading frames in the recessive suppressors found in our screen and *PKC1* in the dominant suppressors. Interestingly, this analysis revealed that one of the recessive mutants carries a nonsense mutation in the open reading frame of *LRG1*, while no mutations in *SAC7* were identified; moreover, we found five dominant suppressors to carry mutations in *PKC1*. Although we are still analyzing the biological effects of these mutations, these data underscore once more that hyperactivation of the Rho1/Pkc1 pathway suppresses the lethality, and therefore likely also the cytokinesis defects, of *dma1 dma2 cla4* cells.

Both Rho1 and Pkc1 are localized at the prebud site and at bud tips in the first steps of the cell cycle, (Yamochi et al., 1994; Denis and Cyert, 2005), while later in the cell cycle they localize at the mother-bud neck (Denis and Cyert, 2005; Yoshida et al., 2006). Thus, there is a time window during the cell cycle in which these 2 proteins share the same localization pattern of septins. Strikingly, expression of *RHO1-D72N* and *PKC1-R398P* also partially suppresses the temperature-sensitivity of *cdc12* mutants, which rapidly disassemble the septin ring at restrictive temperatures (Dobbelaere et al., 2003),

suggesting that Rho1 and Pkc1 promote septin ring stability. Indeed, we found Rho1 and Pkc1 hyperactivation to advance septin ring deposition relative to bud emergence. In addition, our time lapse analysis of *RHO1-D72N* cells shows that the septin ring is more stable in this mutant after splitting, when it is usually rapidly disassembled (Caviston et al., 2003; Dobbelaere et al., 2003), and formation of a new ring in the mother cell is sped up compared to the kinetics observed in wild type cells. Our FRAP analysis of *RHO1* mutants expressing Cdc12-GFP directly proves that Rho1 stabilizes the septin ring. In fact, fluorescence recovery is faster (i.e. septin turnover at the ring is higher) upon Rho1 inactivation in cell cycle phases where the ring is normally stable, whereas it is slower in *RHO1-D72N* cells in late G1, when the ring is normally highly dynamic. In addition, we have found that *rho1-5* cells, which are specifically compromised in their ability to activate Pkc1 (Nonaka et al., 1995), at the restrictive temperature undergo septin ring destabilization faster than *rho1-1* cells (data not shown), which instead seem to hyperactivate Pkc1 and the cell integrity signalling pathway (Nonaka et al., 1995). Finally, our cell cycle analysis of *pkc1* mutants shows that lack of Pkc1 delays septin ring deposition and likely impairs septin ring stabilization, since the septin ring fails to be converted into the hourglass structure. The observation that, like the lethality of *pkc1Δ* cells, the temperature-sensitivity of *dma1Δ dma2Δ cla4-75* and *cdc12* mutant cells is partially suppressed by the presence of an osmotic support in the medium further supports the idea that Dma proteins, Pkc1 and septins are implicated in the same pathway.

How does hyperactivation of the Rho1/Pkc1 pathway suppress the septin defects of *dma1 dma2 cla4* cells? In principle, it could suppress the defects caused by the lack of Cla4 or of Dma1/2 or both. Having found that expression of *PKC1-R398P* suppresses the sporulation defects of *dma1Δ dma2Δ* homozygous diploids, combined with the observation that *RHO1-D72N* does not rescue the temperature-sensitivity of a *ste20Δ cla4-75* mutant, strongly argues that Rho1/Pkc1 act downstream or in parallel to Dma1 proteins, rather than to Cla4, in septin regulation. Our working model (Fig. 41B) envisions that Dma1 and Dma2 might regulate septin ring assembly and/or dynamics either through direct activation of Rho1 or by promoting the degradation of its GAPs, thus leading to Pkc1 activation. Western blot analysis of Lrg1 and Sac7 during the cell cycle revealed that these two proteins are stable throughout the cell cycle (data not shown), making the hypothesis of their eventual degradation by Dma1 and Dma2 less likely. However, it remains possible that Dma proteins inhibit Lrg1 and Sac7 activity through non-degradative mono- or poly-ubiquitination events. Indeed, Dma1 and 2 were shown to be capable of generating K63 poly-ubiquitin linkages (Loring et al., 2008) that are typically non-degradative. Rho1 itself is also a likely target of Dma proteins since it has been found to be ubiquitylated *in vivo* (Kleijnen et al., 2007). In addition, since we found that *RTS1* deletion can suppress the lethality and septin ring defects of *dma1Δ dma2Δ cla4Δ* cells, it is also possible that Dma proteins downregulate $PP2A^{Rts1}$ to stabilize the septin ring. In this scenario Rho1/Pkc1 hyperactivation could simply counteract for the excessive $PP2A^{Rts1}$ activity in the absence of Dma1 Dma2 and Cla4. Interestingly, Rts1

was found to be ubiquitylated with a large-scale proteomic approach (Peng et al., 2003). Finally, Dma proteins control bud neck localization of Elm1, which is in turn involved in the septin network organization and in cytokinesis (Bouquin et al., 2000; Gladfelter et al., 2004). At present, no data link the Rho1/Pkc1 pathway to the activity of Elm1 or $PP2A^{Rts1}$. Therefore, we hypothesise that they control in parallel septin stability and/or the SPOC. Dma1/2 could in turn intervene in these processes by regulating one or more of these independent pathways. Indeed, Dma proteins might favour septin stabilization by alternatively activating Rho1 and Elm1 and/or inhibiting $PP2A^{Rts1}$ directly or indirectly (Fig. 41A). The biological meaning of Rho1 and Rts1 ubiquitylation, as well as their possible dependence on Dma1/2 are important issues that we plan to investigate in the future. It will also be important to assess whether Elm1 is directly or indirectly regulated by Dma proteins. An effort in this direction will come from the identification of the Elm1 post-translational modification that is induced in *dma1Δ dma2Δ* cells, as well as from *ELM1* sequencing in the dominant suppressors that we have found in our screen.

How could the Rho1/Pkc1 pathway regulate septin dynamics? Since septins are regulated by phosphorylation events, Pkc1 could directly phosphorylate septins or regulate septin ring dynamics indirectly by promoting the activation/inactivation of septin ring regulators (Fig. 41B). Pkc1 function has been well studied for what concerns its role in the CWI signalling pathway, but the fact that *PKC1* deletion gives rise to more severe defects than deletion of any other CWI component suggests that Pkc1 has additional molecular targets. No data in

literature link budding yeast Pkc1 to septin phosphorylation, but phosphorylation events, mainly by Cla4 and Gin4, are thought to stabilize the septin ring (Dobbelaere et al., 2003). In addition, the human septin SEPT2 is phosphorylated by the cGMP-dependent Protein Kinase I (Xue et al., 2004). We have tried to investigate septin phosphorylation in wild type as well as in *pkc1Δ* and *PKC1-R398P* cells by studying the electrophoretic mobility of all mitotic septins tagged with HA tags, but since many kinases are likely to phosphorylate septins, the interpretation of the result was not straightforward (data not shown). The recent finding that Syp1, a known regulator of septin dynamics (Qiu et al., 2008), is phosphorylated by Pkc1 raises the possibility that Pkc1 promotes septin ring stability only indirectly (see below).

Is Syp1 the critical target of Pkc1 in the regulation of septin dynamics?

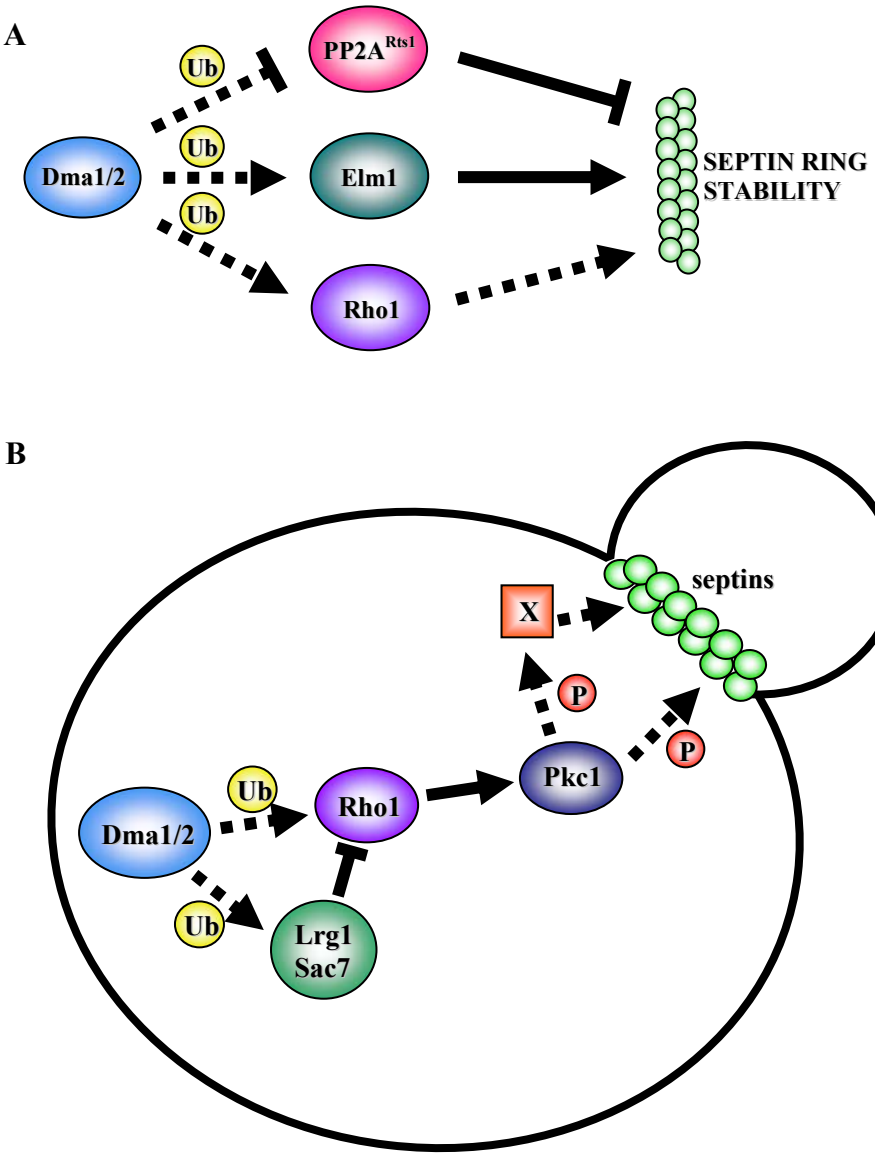
Septin complexes and filaments interact directly with membranes; this interaction could influence the ultrastructure of septin filaments and may facilitate or preclude interactions between any given septin in a filament and other proteins. In addition, membrane-associated septins can act as barriers to the diffusion of integral membrane proteins (Takizawa et al., 2000; Dobbelaere and Barral, 2004). Indeed, in the past few years, mammalian septins have been found at the neck of dendritic spines, where they insulate each spine to make it respond individually to external signals, and on sperm flagella, where they confine mitochondria and other polarity factor in the proximal part of the flagellum (Tada et al., 2007; Xie et al., 2007; Kissel et al., 2005;

Ihara et al., 2005). Septins were also recently shown to localize at the base of the ciliary membrane to create a diffusion barrier that retains ciliary membrane proteins in the primary cilium (Hu et al., 2010; Kim et al., 2010). In budding yeast, septins, thanks to their localization at the bud neck, govern the compartmentalization of membranes, restricting the exchange of cellular material between mother and bud (reviewed by Barral, 2010). Direct phospholipid binding has been reported for human SEPT4 (Zhang et al., 1999) and budding yeast Cdc3, Cdc10, Cdc11 and Cdc12 (Casamayor and Snyder, 2003). Specifically phosphatidylinositol (4,5)-*bis*phosphate (PtdIns[4,5]P_2) is enriched at sites of septin functions (Saul et al., 2004; Field et al., 2005), is required for normal SEPT4 localization (Zhang et al., 1999) and for budding yeast filament assembly and localization (Bertin et al., 2010). Beside septins, other proteins, like proteins containing F-BAR domains, specifically interact with membranes through their binding to PtdIns[4,5]P_2. F-BAR proteins are able to induce and/or sense membrane curvature and connect cell membranes with the cytoskeleton in a wide variety of cellular contexts, including endocytosis and cytokinesis (reviewed by Roberts-Galbraith and Gould, 2010). In *S. cerevisiae,* septins recruit the F-BAR protein Syp1 to the bud neck (Qiu et al., 2008), where it plays a role in endocytic site formation and placement (Reider et al., 2009; Stimpson et al., 2009). In addition, Syp1 physically interacts with septins and promotes septin turnover at the bud neck (Qiu et al., 2008). Consistently, we found that *SYP1* deletion rescues the viability of *cdc12* mutants at 30°C (data not shown), confirming that Syp1 normally contributes to septin ring destabilization. Syp1 could act as

an adapter, recognizing the septin ring and directing endocytic site formation to the bud neck. It could then promote endocytosis at the bud neck, which might allow turnover of membrane components that regulate septin stability and are important for cytokinesis. Indeed, advances in the past few years clearly led to the idea that membrane traffic plays a critical role in the late stages of cytokinesis in animal cells, as previously demonstrated for plant cells (Jurgens, 2005). In particular, it seems that complex bidirectional movements of vesicles are important for the mechanism of abscission (reviewed by Montagnac et al., 2008). Syp1 has been recently shown to be phosphorylated by Pkc1 on Ser297 (Breitkreutz et al., 2010). In addition, a second phosphorylated Ser is present at position 347 within an aminoacid context similar to Ser297 (SAFG and SIFG, respectively), suggesting that it might also be target of Pkc1. Ser297 and 347 fall in the central region of Syp1, which in other F-BAR proteins is most subject to phosphorylation and regulation (Roberts-Galbraith et al., 2010). In addition, the domain of Syp1 for binding to liposomes has been mapped between aminoacid 265 and 365, which contains two lysine-rich consensus motifs for binding to PtdIns[4,5]P_2. Altogether, these data make Syp1 an excellent candidate for being the critical target of Rho1/Pkc1 in the regulation of septin ring stability. Pkc1 could phosphorylate and inhibit Syp1 activity on septins, thus stabilizing the septin ring (Fig. 41C). We have mutagenised Ser297 and Ser347 into both Ala (generating a non-phosphorylatable form of Syp1) and Asp (generating a phospho-mimic form of Syp1), expecting the Syp1-AA mutant to display an unstable septin ring and the Syp1-DD mutant a hyperstable septin ring, perhaps able to rescue the septin

defects of *pkc1Δ* cells. Our data indicate that indeed the septin ring is less stable in *SYP1-AA* than in wild type cells, but defects are not as striking as in the absence of Pkc1. Since the central region of Syp1 contains other phosphorylation sites in addition to the two that we have mutagenized (Bodenmiller and Aebersold, 2010), it is possible that other important residues are subjected to Pkc1 phosphorylation for septin regulation. It will be interesting in the next future to eventually identify and mutagenize other Syp1 residues that are phosphorylated by Pkc1. It will also be important to investigate whether the expression of the *SYP1-DD* phospho-mimetic mutant is able to suppress at least partially the septin ring defects of *pkc1* mutants. Finally, Syp1 recruits the endocytic protein Ede1 to the bud neck (Stimpson et al., 2009) and there seems to be a competition between Ede1 and septins for binding to Syp1 (Reider et al., 2009). We are therefore in the process of deleting *EDE1* in *SYP1-AA* cells to investigate a possible additive effect in septin ring destabilization.

Of course, Pkc1 might have additional targets, beside Syp1, in the regulation of septin dynamics. Unfortunately, very little is known about the physiological targets of Pkc1. The recent large-scale phospho-proteomic approaches to map phosphorylated residues by SILAC and mass-spectrometry in kinase-defective mutants (Deutsch et al., 2008; Pan et al., 2009) promise fast advances in this respect and hopefully will help us uncovering the molecular details of septin regulation by Pkc1.


A
Ub
Ub
Ub
Dma1/2
PP2A Rts1
Elm1
Rho1
SEPTIN RING
STABILITY
B
septins
X
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P
Pkc1
Ub
Ub
Dma1/2
Rho1
Lrg1
Sac7

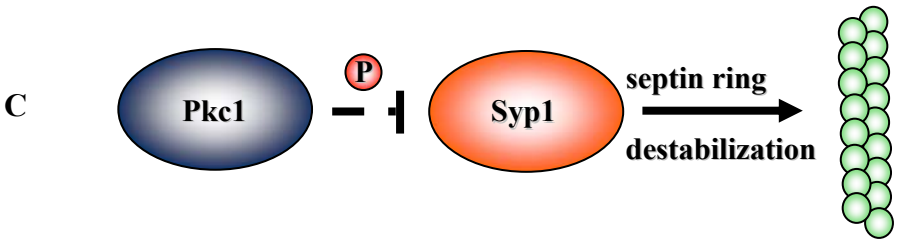

Figure 41. Models for septin ring dynamics regulation by Dma proteins. A: Our working model envisions that Dma1 and Dma2 might regulate septin ring stabilization by regulating either one of three parallel pathways: one involving Rho1 and Pkc1, one dependent on Elm1 activation and a third one linked to $PP2A^{Rts1}$. **B**: For what concerns the Rho1/Pkc1 pathway, Dma proteins might regulate septin ring assembly and/or dynamics either directly through Rho1 activation, or by promoting degradation of its GAPs, thus leading to Pkc1 activation. Since septins are regulated by phosphorylation events, Pkc1 could directly phosphorylate septins or regulate septin ring dynamics indirectly by promoting the activation/inactivation of septin ring regulators. **C**: We propose that Pkc1 could indirectly promote septin ring stabilization by inhibiting Syp1 activity on septins through its phosphorylation.

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