Functional Characterization of Dma1 and Dma2, the Budding Yeast Homologues of *Schizosaccharomyces pombe* Dma1 and Human Chfr[□]

Roberta Frascini, Denis Bilotta, Giovanna Lucchini, and Simonetta Piatti*

Dipartimento di Biotecnologie e Bioscienze, Piazza della Scienza 2, 20126 Milan, Italy

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Proper transmission of genetic information requires correct assembly and positioning of the mitotic spindle, responsible for driving each set of sister chromatids to the two daughter cells, followed by cytokinesis. In case of altered spindle orientation, the spindle position checkpoint inhibits Tem1-dependent activation of the mitotic exit network (MEN), thus delaying mitotic exit and cytokinesis until errors are corrected. We report a functional analysis of two previously uncharacterized budding yeast proteins, Dma1 and Dma2, 58% identical to each other and homologous to human Chfr and *Schizosaccharomyces pombe* Dma1, both of which have been previously implicated in mitotic checkpoints. We show that Dma1 and Dma2 are involved in proper spindle positioning, likely regulating septin ring deposition at the bud neck. *DMA2* overexpression causes defects in septin ring disassembly at the end of mitosis and in cytokinesis. The latter defects can be rescued by either eliminating the spindle position checkpoint protein Bub2 or overproducing its target, Tem1, both leading to MEN hyperactivation. In addition, *dma1Δ dma2Δ* cells fail to activate the spindle position checkpoint in response to the lack of dynein, whereas ectopic expression of *DMA2* prevents unscheduled mitotic exit of spindle checkpoint mutants treated with microtubule-depolymerizing drugs. Although their primary functions remain to be defined, our data suggest that Dma1 and Dma2 might be required to ensure timely MEN activation in telophase.

INTRODUCTION

Faithful chromosome segregation requires the mitotic spindle to be correctly positioned with respect to the cell division axis. Cytokinesis must take place only after this task is achieved and the two sets of chromosomes are sufficiently far apart. Unscheduled cytokinesis before proper chromosome segregation would, in fact, lead to formation of anucleate and polyploid cells. Eukaryotic cells prevent these harmful events by a surveillance mechanism, called spindle position checkpoint, that delays cytokinesis in the presence of mispositioned or misoriented spindles until errors are corrected. This control is particularly critical for organisms, such yeasts and higher plants, that specify the site of cytokinesis before spindle assembly.

In the budding yeast *Saccharomyces cerevisiae*, the mitotic spindle first gets positioned close to the bud neck, the constriction where cytokinesis will take place later on, and then crosses the neck to enter the bud, dragging the nucleus along. Spindle positioning and orientation involve a search-and-capture process and occur in two steps, which depend on two partially overlapping pathways (Schuyler and Pellman, 2001). During the first step cytoplasmic microtubules (cMTs) try to find and interact with specific sites on the bud neck cortex. The EB1/Bim1 protein, localized at the plus end of cMTs, is directly involved in searching cortical sites by promoting cMTs dynamicity (Tirnauer *et al.*, 1999). Nuclear

positioning also requires the cortical protein Kar9, with which Bim1 interacts (Miller and Rose, 1998; Korinek *et al.*, 2000; Lee *et al.*, 2000); the septin ring, which also specifies the site for cytokinesis (Kusch *et al.*, 2002); the kinesin Kip3 (Cottingham and Hoyt, 1997; DeZwaan *et al.*, 1997); and other proteins required for Kar9 localization, such as actin (Cottingham *et al.*, 1999), the formin Bni1 (Lee *et al.*, 1999), and myosin V (Beach *et al.*, 2000; Yin *et al.*, 2000). During the second step, dynein (Dyn1) and its regulators dynactin and *Pac1* mediate lateral interactions between cMTs and the cortical protein Num1, thus helping the nucleus to be pulled inside the bud (Adames and Cooper, 2000; Heil-Chapdelaine *et al.*, 2000; Lee *et al.*, 2003; Sheeman *et al.*, 2003).

The spindle position checkpoint is well characterized in *S. cerevisiae* and requires the Bub2 and Bfa1 proteins (Bardin *et al.*, 2000; Bloecher *et al.*, 2000; Pereira *et al.*, 2000), which form a two-component GTPase-activating protein inhibiting the G protein Tem1 (Geymonat *et al.*, 2002), which is in turn required to activate the mitotic exit network (MEN) in telophase (Simanis, 2003). Tem1-dependent activation of the MEN leads to the release from the nucleolus of the Cdc14 protein phosphatase, which is crucial to promote inactivation of cyclinB-dependent CDKs at the end of mitosis (Visintin *et al.*, 1998). Inhibition of such CDKs is an essential prerequisite for spindle disassembly and cytokinesis at the end of the cell cycle and is obtained in budding yeast by both anaphase promoting complex (APC)-mediated proteolysis of their cyclin subunits and accumulation of the CDK inhibitor Sic1 (Zachariae and Nasmyth, 1999). In addition to its role in MEN activation, however, Tem1 also has been implicated in a MEN-independent cytokinetic process, leading to septin ring splitting into an hourglass-shaped structure (Lippincott *et al.*, 2001). The MEN has a similar structural organization to fission yeast septation initiation network (SIN),

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* Corresponding author. E-mail address: simonetta.piatti@unimib.it.

which is required for cytokinesis but not for mitotic exit (Simanis, 2003). Unlike budding yeast Bub2, its fission yeast homologue Cdc16 performs an essential cell cycle function, and its inactivation leads to unscheduled formation of septa, generating multinucleate cells. In addition, Cdc16, like Bub2, is involved in the spindle checkpoint (Fankhauser *et al.*, 1993).

Several aspects of MEN regulation and its connections with mitotic exit and cytokinesis control are still obscure, and new players in these important processes still await discovery. In this article, we provide the first characterization of the budding yeast open reading frames *YHR115c* and *YNL116w*, which we renamed *DMA1* and *DMA2*, based on the homology of their gene products with that of the fission yeast *dma1⁺* gene, which was originally isolated as multicopy suppressor of the temperature-sensitive growth phenotype caused by *cdc16* mutations (Murone and Simanis, 1996). *Schizosaccharomyces pombe* Dma1 is required for SIN inhibition in the presence of spindle damage (Murone and Simanis, 1996; Guertin *et al.*, 2002) and is homologous, like *S. cerevisiae* Dma1 and Dma2, to human Chfr, which is missing in several cancer cell lines and has been shown to be a component of a stress-induced mitotic checkpoint (Scolnick and Halazonetis, 2000). All these proteins share a forkhead-associated domain and a ring finger motif, the latter being typical of ubiquitin ligase subunits (Joazeiro and Weissman, 2000). We show here that *S. cerevisiae* Dma1 and Dma2, which seem to be functionally redundant, are involved in proper septin ring positioning and cytokinesis. The simultaneous lack of Dma1 and Dma2 leads to spindle mispositioning and defects in the spindle position checkpoint. Conversely, overexpression of *DMA2* causes cytogenetic defects that are partially suppressed by either *BUB2* deletion or *TEM1* overexpression, consistently with a lack of proper Tem1 activation.

MATERIALS AND METHODS

Strains, Media, and Reagents

All yeast strains (Table 1) were derivatives of or were backcrossed at least three times to W303 (*ade2-1, trp1-1, leu2-3112, his3-11,15, ura3, ssd1*). Cells were grown in YEP medium (1% yeast extract, 2% bacto-peptone, 50 mg/l adenine) supplemented with 2% glucose (YEPR), 2% raffinose (YEPR), or 2% galactose (YEPRG). Unless otherwise stated, α -factor was used at 2 μ g/ml, nocodazole at 15 μ g/ml, and hydroxyurea at 100 mM. Synchronization experiments were carried out at 25°C, and unless otherwise stated, galactose was added 0.5 h before release from either α -factor or nocodazole.

Plasmid Constructions and Genetic Manipulations

Standard techniques were used for genetic manipulations (Sherman, 1991; Maniatis *et al.*, 1992). To clone *DMA2* under the *GAL1-10* promoter (plasmid pSP185), a *XbaI* PCR product containing the *DMA2* coding region was cloned in the *XbaI* site of a *GAL1-10*-bearing Yiplac211 vector. pSP185 integration was directed either to the *URA3* locus by *EcoRV* digestion (strains ySP3016 and ySP3018) or to the *DMA2* locus by *HpaI* digestion (strains ySP3656 and ySP3657). Copy number of the integrated plasmid was verified by Southern blot analysis. Because this analysis did not allow to assess the precise number of integrated plasmids for all the transformants, we generically indicated as multiple integrants the strains with an integrated plasmid copy number higher than three (ySP3018 and ySP3657). All strains carrying multiple copies of the *GAL1-DMA2* construct (*GAL1-DMA2 m*) at either the *URA3* or *DMA2* locus are meiotic segregants derived from the parental strains ySP3018 or ySP3657, respectively, and therefore carry the same number of pSP185 integrated copies as the corresponding parental strain. Gene deletions were generated by one-step gene replacement (Wach *et al.*, 1994).

Protein Extracts, Western Blot Analysis, and Kinase Assays

For Western blot analysis, protein extracts were prepared according to Surana *et al.* (1993). For Clb2/Cdk1 kinase assays, protein extracts were prepared as described previously (Schwob *et al.*, 1994), and 50 μ g of extract was used for measuring kinase activity on histone H1 (Surana *et al.*, 1993) and for Western

blot analysis. Proteins transferred to Protran membranes (Schleicher & Schuell, Keene, NH) were probed with 12CA5 monoclonal antibodies for hemagglutinin (HA)-tagged Cdc5, and with polyclonal antibodies against Swi6, Clb2, and Sic1 (kind gifts from K. Nasmyth (Institute of Molecular Pathology, Vienna, Austria), W. Zachariae (Max Planck Institute & Molecular Cell Biology, Dresden, Germany), and Mike Tyers (University of Toronto, Toronto, ON, Canada), respectively). Secondary antibodies were from Amersham Biosciences (Piscataway, NJ), and proteins were detected by an enhanced chemiluminescence system according to the manufacturer.

Fluorescence Microscopy, Image Analysis, and Measurements

Nuclear division was scored with a fluorescent microscope on cells stained with propidium iodide. In situ immunofluorescence of microtubules was performed according to Fraschini *et al.* (1999) by using the YOL34 monoclonal antibody (Serotec, Oxford, United Kingdom) followed by indirect immunofluorescence with rhodamine-conjugated anti-rat antibody (1:100; Pierce Chemical, Rockford, IL). Detection of Cdc3-green fluorescent protein (GFP), Spc42-GFP, and tetR-GFP dots to score sister chromatid separation was carried out on ethanol-fixed cells, upon wash with water 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. To measure distance between spindle and bud neck, sets of three to four fluorescent images in Z-focal planes 0.3 μ m apart were combined into single two-dimensional projection. Distances were measured between the proximal end of either metaphase spindles or spindle poles (on Spc42-GFP-expressing cells) and the bud neck on 100–200 cells by using the Leica FW4000 software. Comparisons of statistical significance were done by Student's *t*-test.

Other Techniques

Flow cytometric DNA quantitation was determined according to Fraschini *et al.* (1999) on a BD Biosciences FACScan. Percentage of cells with 2C DNA contents was measured with the CELLQuest software.

RESULTS

Lack of Dma1 and Dma2 Affects Spindle Positioning

Two homologues of human Chfr, which we renamed Dma1 and Dma2, are found to be encoded by the *S. cerevisiae* uncharacterized open reading frames *YHR115c* and *YNL116w*, respectively. They show 58% of amino acid identity with each other and >40% with *S. pombe* Dma1, whereas homology with Chfr is less extensive but still significant. To functionally characterize Dma1 and Dma2, we first deleted each single open reading frame and found no detectable effects of the single deletions on either cell cycle progression, cell viability, or spindle checkpoint response (our unpublished data). Because these results, together with the high degree of homology between the two gene products, suggested a possible functional redundancy, we constructed a double *dma1 Δ dma2 Δ* strain, which turned out to be viable and therefore suitable for further analysis. To investigate the role of Dma1 and Dma2 in the yeast cell cycle, we then deleted both genes in a haploid yeast strain carrying the tetO/tetR-GFP constructs to monitor sister chromatid separation at arms of chromosome V (Michaelis *et al.*, 1997). Small unbudded cells were purified by elutriation and released into fresh medium at 25°C to monitor at different time points the kinetics of budding, DNA replication, bipolar spindle assembly and elongation, and sister chromatid separation. Despite that we did not find major differences in any of these parameters between *dma1 Δ dma2 Δ* cells and wild type (Figure 1A), we noticed that two GFP-separated dots, indicative of chromosome V sister separation, could be found within the same cell body in a higher fraction of *dma1 Δ dma2 Δ* cells than wild type (Figure 1A). This suggests that the lack of Dma1 and Dma2 might affect, among other processes, spindle positioning, because the presence of mispositioned spindles does not prevent the onset of anaphase in budding yeast (Sullivan and Huffaker, 1992). Accordingly, when we measured directly the average distance be-

Table 1. Strains used in this study

Name	Relevant genotype
ySP324	<i>MATa, cdc5-2</i>
ySP601	<i>MATa, leu2::LEU2::tetR-GFP, ura3::URA3::336XtetO</i>
ySP939	<i>MATa, cdc5::CDC5-HA3::URA3</i>
ySP1034	<i>MATa, his3::HIS3::GAL1-CDC5</i>
ySP1125	<i>MATa, pds1::PDS1-myc18::LEU2, leu2::LEU2::tetR-GFP, ura3::URA3::336XtetO</i>
ySP1152	<i>MATa, leu2::LEU2::tetR-GFP, ura3::URA3::336XtetO, mad2::TRP1, bub2::HIS3</i>
ySP1241	<i>MATa, bni1::URA3</i>
ySP1242	<i>MATa, bim1::URA3</i>
ySP1370	<i>MATa, sve1::LEU2</i>
ySP1402	<i>MATa, dyn1::URA3</i>
ySP1569	<i>MATa, dma1::KITR1, dma2::KILEU2</i>
ySP1605	<i>MATa, dma1::KITR1, dma2::KILEU2, leu2::LEU2::tetR-GFP, ura3::URA3::336XtetO</i>
ySP1778	<i>MATa, spc42::LEU2, his3::3X HIS3-SPC42-GFP</i>
ySP1791	<i>MATa, his3::HIS3-TUB1-GFP</i>
ySP1958	<i>MATa, leu2::LEU2::tetR-GFP, BMH1::336XtetO::URA3</i>
ySP1978	<i>MATa, mad2::TRP1, leu2::LEU2::tetR-GFP, BMH1::336XtetO::URA3</i>
ySP2402	<i>MATα, dma1::KITR1, dma2::KILEU2, bni1::URA3</i>
ySP2403	<i>MATa, dma1::KITR1, dma2::KILEU2, dyn1::URA3</i>
ySP2406	<i>MATa, dma1::KITR1, dma2::KILEU2, bim1::URA3</i>
ySP2529	<i>MATa, dma1::KITR1, dma2::KILEU2, pds1::PDS1-myc18::LEU2, leu2::LEU2::tetR-GFP, ura3::URA3::336XtetO</i>
ySP2925	<i>MATa, dma1::KITR1, dma2::KILEU2, his3::HIS3-TUB1-GFP</i>
ySP2940	<i>MATa, dma1::KITR1, dma2::KILEU2, spc42::LEU2, his3::3X HIS3-SPC42-GFP</i>
ySP3016	<i>MATa, ura3::1X URA3::GAL1-DMA2</i>
ySP3018	<i>MATa, ura3::4X URA3::GAL1-DMA2</i>
ySP3076	<i>MATa, cla4::kanMX4</i>
ySP3138	<i>MATa, bub2::HIS3</i>
ySP3174	<i>MATa, ura3::URA3::GAL1-DMA2(s), bub2::HIS3</i>
ySP3175	<i>MATa, ura3::URA3::GAL1-DMA2(m), bub2::HIS3</i>
ySP3188	<i>MATa, ura3::URA3::GAL1-DMA2(m), cdc5::CDC5-HA3::URA3</i>
ySP3355	<i>MATa, dma1::KITR1, dma2::KILEU2, cdc5::CDC5-HA3::URA3</i>
ySP3402	<i>MATa, ura3::URA3::GAL1-DMA2(s), mad2::TRP1, leu2::LEU2::tetR-GFP, BMH1::336XtetO::URA3</i>
ySP3413	<i>MATa, dyn1::URA3, bub2::HIS3</i>
ySP3417	<i>MATa, tem1::URA3, tem1-3 (on Ycplac111)</i>
ySP3423	<i>MATa, ura3::URA3::GAL1-DMA2(m), his3::HIS3::GAL1-CDC5</i>
ySP3428	<i>MATa, ura3::URA3::GAL1-DMA2(m), leu2::LEU2::tetR-GFP, BMH1::336XtetO::URA3</i>
ySP3433	<i>MATa, ura3::URA3::GAL1-DMA2(s), leu2::LEU2::tetR-GFP, BMH1::336XtetO::URA3</i>
ySP3524	<i>MATa, cla4::kanMX4, cla4-75 (on Ycplac22)</i>
ySP3535	<i>MATa, lte1::kanMX4</i>
ySP3616	<i>MATa, ura3::URA3::GAL1-TEM1</i>
ySP3656	<i>MATa, DMA2::GAL1-DMA2(s)::URA3</i>
ySP3657	<i>MATa, DMA2::GAL1-DMA2(m)::URA3</i>
ySP3721	<i>MATa, DMA2::GAL1-DMA2(m)::URA3, ura3::URA3::GAL1-TEM1</i>
ySP3748	<i>MATa, DMA2::GAL1-DMA2(m)::URA3, bub2::HIS3</i>
ySP3779	<i>MATa, cdc5::kanMX4, trp1::TRP1::cdc5-1-HA3</i>
ySP3833	<i>MATa, amn1::kanMX4</i>
ySP3962	<i>MATa, ura3::URA3::GAL1-DMA2(m), amn1::kanMX4</i>
ySP4000	<i>MATa, ura3::URA3::GAL1-DMA2(s), cla4::kanMX4, bub2::HIS3</i>
ySP4001	<i>MATα, ura3::URA3::GAL1-DMA2(s), cla4::kanMX4</i>
ySP4006	<i>MATa, ura3::URA3::GAL1-DMA2(s), lte1::kanMX4, bub2::HIS3</i>
ySP4008	<i>MATa, ura3::URA3::GAL1-DMA2(s), lte1::kanMX4</i>
ySP4010	<i>MATa, ura3::URA3::GAL1-DMA2(s), cdc5::kanMX4, trp1::TRP1::cdc5-1-HA3, bub2::HIS3</i>
ySP4011	<i>MATa, ura3::URA3::GAL1-DMA2(s), cdc5::kanMX4, trp1::TRP1::cdc5-1-HA3</i>
ySP4013	<i>MATa, ura3::URA3::GAL1-DMA2(s), cdc5-2, bub2::HIS3</i>
ySP4014	<i>MATa, ura3::URA3::GAL1-DMA2(s), cdc5-2</i>
ySP4023	<i>MATa, ura3::URA3::GAL1-DMA2(m), sve1::LEU2</i>
ySP4133	<i>MATα, ura3::URA3::GAL1-DMA2(s), tem1::URA3, tem1-3 (on Ycplac111)</i>
ySP4154	<i>MATα, dma1::KITR1, dma2::KILEU2, cla4::kanMX4, cla4-75 (on Ycplac22)</i>
ySP4155	<i>MATa, dma1::KITR1, dma2::KILEU2, cla4::kanMX4, cla4-75 (on Ycplac22)</i>
ySP4156	<i>MATα, ura3::URA3::GAL1-DMA2(s), tem1::URA3, tem1-3 (on Ycplac111), bub2::HIS3</i>

tween bud necks and the proximal end of metaphase spindles at the time of their highest percentage (135' for wild-type and 180' for *dma1Δ dma2Δ* cells), we found a slight but statistically significant increase in the value found for *dma1Δ dma2Δ* spindles (1.35 ± 0.39 vs. $1.14 \pm 0.36 \mu\text{m}$, $p < 0.0001$). To confirm that lack of Dma1 and Dma2 affects spindle positioning, we also measured the average distance between

the bud neck and the proximal spindle pole body (SPB), as well as the average angle between the short bipolar spindle and the mother-bud axis, in either cycling wild-type or *dma1Δ dma2Δ* cells expressing the structural SPB component Spc42 fused to GFP and displaying two GFP dots facing each other in the same cell body, indicative of a preanaphase stage. Again, the average distance between the proximal SPB

Figure 1. Dma1 and Dma2 are required for proper spindle positioning. (A) Small unbudded wild-type (ySP1125) and *dma1Δ dma2Δ* cells (ySP2529) were purified by elutriation and allowed to progress in the cell cycle in fresh YEPD medium at 25°C (time 0). At the indicated time points cell samples were collected for fluorescence-activated cell sorting analysis of DNA contents (top) and to score microscopically kinetics of budding, sister chromatid separation, bipolar spindle assembly, and elongation (bottom). (B) Percentage of correctly positioned and mispositioned short metaphase spindles was scored in logarithmically growing cell cultures of wild-type and *dma1Δ dma2Δ* strains expressing a *TUB1-GFP* fusion ($n \geq 200$). The photograph shows examples of short metaphase spindles in *dma1Δ dma2Δ* cells. (C) Serial dilutions of strains with the indicated genotypes were spotted on YEPD plates and incubated for 2 d at either 25 or 37°C. (D) Logarithmically growing cultures of strains with the indicated genotypes were shifted from 25 to 37°C for 4 h. The percentage of binucleate cell bodies over total number of cells was scored after sonication and nuclear staining with propidium iodide ($n \geq 500$). (E) The uninucleate budded cells grown at 25°C from D) were scored for nuclear position with respect to the middle (dotted line) of the mother cell ($n \geq 200$).

and the bud neck was higher in *dma1Δ dma2Δ* than in wild-type cells (0.8 ± 0.39 vs. 0.65 ± 0.37 μm , $p = 0.004$), whereas the mean angle seemed to be not significantly affected (38.6 ± 24.1 vs. $35.6 \pm 22.7^\circ$, $p = 0.3$). When we

compared spindle orientation in *dma1Δ dma2Δ* versus wild-type cells expressing α tubulin fused to GFP (Tub1-GFP), we found that indeed the lack of Dma1 and Dma2 caused a slight increase (13%) in the percentage of cells that displayed

Table 2. Nuclear distribution in HU-arrested cells

	or			n
			%		
Wild type	6	53	39.5	1.5	283
<i>dma1Δdma2Δ</i>	4.8	81.6	11.8	1.8	331

The position of the nucleus relative to the bud and neck was determined on propidium iodide-stained cells. n, cells scored.

short bipolar spindles pointing away from the bud neck (Figure 1B).

Wild-type cells treated with hydroxyurea (HU) display penetration of the nucleus into the bud, whereas the spindle is confined in the mother cell. This process is defective when spindle positioning is compromised (Yeh *et al.*, 1995). We therefore asked what was the behavior of *dma1Δdma2Δ* cells in these conditions and found that, whereas in HU a significant fraction of wild-type cells (39.5%) displayed stretched nuclei across the neck, only 11.8% of *dma1Δdma2Δ* cells did so (Table 2), suggesting that proper spindle positioning requires Dma1 and/or Dma2. Conversely, spindle alignment along the mother-bud axis was similar in HU-treated wild-type and *dma1Δdma2Δ* cells (mean angle $39.9 \pm 25.4^\circ$ for wild type and $39.9 \pm 25^\circ$ for *dma1Δdma2Δ*).

We therefore looked for synthetic effects between the double *DMA1/DMA2* deletion and mutations in genes implicated in nuclear positioning and/or spindle orientation, such as *kip1Δ*, *kip3Δ*, *kar3Δ*, *bim1Δ*, *kar9Δ*, *dyn1Δ*, *bni1Δ*, and *cla4Δ*. The *dma1Δdma2Δcla4Δ* combination turned out to be lethal at 25°C (see below), whereas *dma1Δdma2Δbim1Δ*, *dma1Δdma2Δbni1Δ*, and *dma1Δdma2Δdyn1Δ* strains were temperature sensitive for growth at 37°C (Figure 1C), although to different extents. No obvious synthetic growth defects were found by combining *dma1Δdma2Δ* with the remaining tested mutations (our unpublished data). We then asked whether the observed synthetic growth defects correlated with additive effects caused by the above-mentioned mutations on spindle positioning. For this purpose, we measured the percentage of binucleate cell bodies, arising from anaphase taking place in the mother cell, in strains lacking *Bim1*, *Bni1*, or *Dyn1* either alone or in combination with *dma1Δdma2Δ*. As shown in Figure 1D, the absence of Dma1 and Dma2 caused by itself a modest increase in the percentage of binucleate cell bodies, which was additive with that caused by deletion of *BIM1*, *BNI1*, or *DYN1*. The increment in the fraction of binucleate cell bodies in the triple mutants compared with the single mutants was already apparent at 25°C and became more evident after incubation for 4 h at 37°C, due to the stronger effect of *BIM1*, *BNI1*, and *DYN1* deletions on spindle positioning at this temperature (Figure 1D). Finally, we analyzed nuclear positioning in some of the above-mentioned mutants grown at 25°C, by measuring the amount of budded cells where the nucleus was away from the bud neck with respect to those where the nucleus was correctly positioned. We excluded from this analysis *bni1Δ* mutants because of their marked clumpiness, which made very difficult to establish the position of the bud necks. *DMA1/DMA2* deletion increased by itself the amount of cells with mispositioned nuclei by more than threefold compared with wild type, and slightly increased nuclear positioning defects of *bim1Δ* and *dyn1Δ* mutants (Figure 1E). Together, these data suggest that Dma1 and Dma2 participate to spindle positioning.

Dma1 and Dma2 Are Required for Correct Septin Ring Assembly

The synthetic lethality caused by the simultaneous lack of Cla4, Dma1, and Dma2 suggested that these proteins might be involved in the same process and prompted us to test whether Dma1 and Dma2 could be involved in septin ring deposition. In fact, the Cla4 PAK kinase has been implicated in the correct assembly of the septin ring at the bud neck at the onset of budding (Cvrckova *et al.*, 1995) and the septin ring is in turn required for correct nuclear positioning (Kusch *et al.*, 2002). By using the temperature-sensitive *cla4-75* allele (Cvrckova *et al.*, 1995), we first constructed a *cla4-75 dma1Δdma2Δ* triple mutant, which was viable at 25°C but could not grow at 37°C, whereas *cla4-75* cells grew normally at 37°C (Figure 2A) due to the functional redundancy between Cla4 and the PAK kinase Ste20 (Cvrckova *et al.*, 1995). Cultures of wild-type, *dma1Δdma2Δ*, *cla4-75*, and *cla4-75 dma1Δdma2Δ* cells, all expressing the septin ring component Cdc3 fused to GFP (Cdc3-GFP), were grown at 25°C and then shifted to 37°C for 3 h. As shown in Figure 2B, upon temperature shift the lack of either Cla4 or Dma1 and Dma2 reduced the percentage of cells with a correctly assembled septin ring from 60 to 23 and 32% respectively, whereas it markedly increased the fraction of cells with either mislocalized rings or diffuse unlocalized GFP signal, despite that Cdc3-GFP protein levels remained unchanged (Figure 2C). Strikingly, the simultaneous lack of all the above-mentioned proteins dramatically compromised septin ring deposition, allowing this process to take place correctly in only 8% of the cells. In addition, *cla4-75 dma1Δdma2Δ* cells were often misshapen or with wide bud necks (Figure 2B) and mostly arrested with 2C DNA contents and undivided nuclei (our unpublished data), suggesting that the morphogenesis checkpoint might be active in these conditions (Lew and Reed, 1995). Thus, Dma1 and Dma2 participate, like Cla4, to proper septin ring deposition.

Dma1 and Dma2 Participate to the Spindle Position Checkpoint

Both *S. pombe* Dma1 and human Chfr have been shown to be involved in mitotic checkpoint responses (Murone and Simanis, 1996; Scolnick and Halazonetis, 2000). To verify whether Dma1 and Dma2 could themselves function in the spindle assembly checkpoint, we arrested in G1 by α factor and released in the presence of nocodazole *dma1Δdma2Δ* cells, as well as wild-type and *mad2Δbub2Δ* double mutant cells, all carrying the tetO/tetR-GFP constructs to monitor sister chromatid separation at arm regions of chromosome V (Michaelis *et al.*, 1997). Whereas *mad2Δbub2Δ* cells separated sister chromatids and rereplicated their genome very efficiently, being completely checkpoint deficient (Alexandru *et al.*, 1999; Fesquet *et al.*, 1999; Fraschini *et al.*, 1999), wild-type and *dma1Δdma2Δ* mutant cells arrested mostly in metaphase with 2C DNA contents (Figure 3A), suggesting that Dma1 and Dma2 are not required for the checkpoint activated by spindle disassembly.

We then asked whether Dma1 and Dma2 could instead be involved, like Bub2 and Bfa1, in the spindle orientation checkpoint, that delays MEN activation in response to spindle mispositioning. To address this question, we deleted *DMA1* and *DMA2* in a strain lacking the dynein-encoding gene *DYN1*. In the absence of dynein spindles are mispositioned, and as a consequence, a small fraction of cells undergo anaphase and nuclear division in the mother cell (Yeh *et al.*, 1995). In spite of that, cells fail to exit mitosis and divide, due to a Bub2/Bfa1-dependent checkpoint (Figure

Figure 2. Synthetic effects between $dma1\Delta dma2\Delta$ and $cla4-75$ on cell growth and septin ring deposition. (A) Serial dilutions of logarithmically growing cultures of strains with the indicated genotypes were spotted on YEPD plates and incubated for 2 d at 25 and 37°C. Two independent $cla4-75 dma1\Delta dma2\Delta$ meiotic segregants from the same heterozygous diploid are shown. (B) Cultures of the indicated strains, all carrying a $CDC3-GFP$ -bearing low copy number plasmid, were grown in selective medium to maintain the plasmid at 25°C and then shifted to 37°C for 3 h. The percentage of cells with either correctly deposited or mispositioned septin ring, as well as diffuse unlocalized signal, was scored by monitoring the septin ring marker Cdc3-GFP (graph, $n \geq 300$). The photographs show examples of Cdc3-GFP localization at 37°C in wild-type (wt), $dma1\Delta dma2\Delta$, $cla4-75$, and $cla4-75 dma1\Delta dma2\Delta$ cells. (C) Same amounts of protein extracts from the cell cultures used for B were analyzed by Western blot with anti-GFP antibodies to monitor protein levels of Cdc3-GFP. 1, wt 25°C; 2, wt 37°C; 3, $dma1\Delta dma2\Delta$ 25°C; 4, $dma1\Delta dma2\Delta$ 37°C; 5, $cla4-75$ 25°C; 6, $cla4-75$ 37°C; 7, $cla4-75 dma1\Delta dma2\Delta$ 25°C; and 8, $cla4-75 dma1\Delta dma2\Delta$ 37°C. C, loading control.

3B; Bardin *et al.*, 2000). Because $dyn1\Delta$ mutants accumulated binucleate cell bodies at 37°C (Figure 1D) and the $dma1\Delta dma2\Delta dyn1\Delta$ triple mutant was temperature sensitive (Figure 1C), we scored failures in the spindle position checkpoint after 7 h at 37°C, by monitoring the percentage of cells

with more than one bud, which had either one undivided nucleus or two separated nuclei within the same cell body and therefore had not undergone a proper anaphase. In fact, the emergence of a second bud in such cells is a marker of unscheduled mitotic exit, no longer coupled to nuclear di-

A

B

Figure 3. Dma1 and Dma2 are involved in the spindle position checkpoint. (A) Isogenic strains with the indicated genotypes, all carrying the tetO/tetR-GFP constructs, were arrested in G1 by α factor and released in the presence of nocodazole to measure at different time points DNA contents by fluorescence-activated cell sorting analysis (left) and sister chromatid separation at arms of chromosome V (right). (B) Cultures of strains with the indicated genotypes were grown at 25°C and shifted at time 0–37°C for 7 h. Percentages of the different cell types, drawn on the right side of the figure, were determined after sonication and nuclear staining with propidium iodide.

vision. Under these conditions, 14% of *dyn1* cells underwent anaphase in the mother cell due to spindle misorientation, but neither rebudded (Figure 3B) nor rereplicated DNA (our unpublished data). Similarly to *BUB2* deletion, the lack of Dma1 and Dma2 caused 16% of *dyn1* cells to bud again before proper nuclear division (Figure 3B) and to enter a new round of DNA replication, accumulating with DNA contents higher than 2C (our unpublished data), suggesting that these proteins are required to delay mitotic exit in the presence of mispositioned spindles.

DMA2 Overexpression Prevents Mitotic Exit in Nocodazole-treated *bub2Δ* and *mad2Δ* Cells

Because *S. pombe dma1⁺* was isolated as multicopy suppressor of the temperature sensitivity of *cdc16* mutant cells (Murone and Simanis, 1996), we asked whether overexpression of one of its budding yeast homologues could rescue the checkpoint defects of cells lacking Bub2, the budding yeast counterpart of Cdc16. To address this question, the *DMA2* coding sequence was cloned under the control of the galactose-inducible *GAL1* promoter (*GAL1-DMA2*) and integrated in single copy into the yeast genome. We first compared the kinetics of mitotic exit in *GAL1-DMA2* versus

wild-type cells in synchronized cultures grown in raffinose-containing medium (*GAL1* promoter off), arrested in G1 with α factor and then released in the presence of galactose (*GAL1* promoter on). No major differences could be seen between the two strains in the kinetics of Clb2 degradation, Sic1 accumulation, Clb2/Cdk1 kinase inactivation, and spindle disassembly (Figure 4A), suggesting that overproduction of Dma2 from the *GAL1* promoter does not interfere with mitotic exit during an unperturbed cell cycle. To ask whether overexpression of *DMA2* could rescue spindle checkpoint defects, wild-type and *bub2Δ* cells, either lacking or carrying the *GAL1-DMA2* fusion, were treated as described above and then released from the G1 arrest in the presence of galactose and nocodazole, to depolymerize microtubules. As expected, in these conditions wild-type cells arrested in metaphase with 2C DNA contents, irrespective of the presence of *GAL1-DMA2*, and *bub2Δ* cells exited mitosis and entered into a new round of budding and DNA replication, accumulating 4C DNA contents. Conversely, *DMA2* overexpression in *bub2Δ* cells mostly prevented them from rereplicating their DNA (Figure 4B).

Whereas Bub2 and Cdc16 inhibit mitotic exit and cytokinesis through similar pathways (Simanis, 2003), Mad2 be-

Figure 4. Overexpression of *DMA2* prevents mitotic exit in nocodazole treated spindle checkpoint mutants. (A) Cultures of wild-type cells either lacking (wt) or carrying (*GAL1-DMA2*) one single copy of the *GAL1-DMA2* fusion integrated at the *URA3* locus were grown in YEPR, arrested in G1 by α factor, and released in YEPRG. When the percentage of budded cells was >90% ($t = 105'$) α factor was readded at the concentration of 10 $\mu\text{g/ml}$. Cells were harvested at the indicated times for fluorescence-activated cell sorting analysis (FACS) of DNA contents, kinetics of budding, nuclear division, and spindle elongation after immunostaining of α tubulin. At the same time points, cell samples were collected also for Western blot analysis with anti-Clb2 and anti-Sic1 (Swi6, loading control) and for Clb2/Cdk1 immunoprecipitations followed by kinase assays by using histone H1 as substrate. (B) Cultures of isogenic strains with the indicated genotypes, either lacking or carrying one copy of the *GAL1-DMA2* fusion integrated at the *URA3* locus and logarithmically growing in YEPR were arrested in G1 by α factor and released in YEPRG containing nocodazole (time 0). At the indicated times, DNA contents were analyzed by FACS. (C) Wild-type, *GAL1-DMA2* (our unpublished data), *mad2Δ*, and *mad2Δ GAL1-DMA2* strains, all carrying the tetO/tetR-GFP constructs to measure sister chromatid separation at subtelomeric regions, were treated as in A. At the indicated times, cell samples were collected for FACS analysis of DNA contents (left) and kinetics of sister chromatid separation (right).

longs to a different branch of the mitotic checkpoint, which prevents sister chromatid separation and MEN activation via a different route (Gardner and Burke, 2000). It was therefore interesting to verify whether *DMA2* overexpression also could suppress the checkpoint defects of cells

lacking Mad2. To this end, wild-type, *GAL1-DMA2*, *mad2Δ*, and *mad2Δ GAL1-DMA2* strains carrying the tetO/tetR-GFP constructs to monitor separation of sister chromatids at subtelomeric regions (Alexandru *et al.*, 2001) were treated as described above. Upon spindle disassembly by nocodazole,

Figure 5. Overexpression of *DMA2* interferes with cytokinesis and septin ring disassembly. (A) Cultures of wild-type cells, either untransformed (wt) or carrying one (s) or multiple (m) copies of the *GAL1-DMA2* fusion integrated at the *DMA2* locus, were grown in YEPR, synchronized in G1 with α factor, and released into YEPRG at time 0. Samples were withdrawn at the indicated time points for fluorescence-activated cell sorting analysis (FACS) analysis of DNA contents (top) and to score kinetics of budding, nuclear division and spindle elongation (bottom). Cell cycle progression of a *bub2Δ GAL1-DMA2(m)* cell culture under the same conditions was followed in parallel by FACS analysis. (B) Serial dilutions of the indicated cell cultures, either lacking or carrying a single (s) or multiple (m) copies of the *GAL1-DMA2* fusion integrated at the *DMA2* locus were spotted on YEPR (Glu) and YEPRG (Gal) plates and incubated for 3 d at 23°C. (C) Wild-type (W303) and *GAL1-DMA2(m)* (ySP3018) cells expressing a Cdc3-GFP fusion were grown in YEPR, synchronized with α factor, and released into YEPRG. At the time cells entered S phase of the second cell cycle (150', as determined by FACS analysis), cells with two Cdc3-GFP rings or mispositioned GFP signals were scored (see text for details). Photographs show examples of *GAL1-DMA2* cells that either deposit a new septin ring before disassembly of the previous one (left) or show mislocalized Cdc3-GFP signal (right).

wild-type and *GAL1-DMA2* cells arrested with unseparated sister chromatids (Figure 4C) and 2C DNA contents (our unpublished data), whereas *mad2Δ* cells, as expected, lost sister chromatid cohesion and underwent a second round of DNA replication (Figure 4C). Overexpression of *DMA2* in the latter cells could not prevent the onset of anaphase, but mostly inhibited rereplication (Figure 4C), suggesting that *Dma2* does not influence the ability of *Mad2* to inactivate Cdc20/APC, which in turn promotes sister chromatid separation (Musacchio and Hardwick, 2002), but rather intervenes more downstream in the pathway. Thus, high levels of *Dma2* can prevent both *mad2Δ* and *bub2Δ* cells from exiting mitosis and entering into a new cell cycle, indicating that they might affect MEN activation under certain conditions.

Overexpression of *DMA2* Causes Cytokinetic Defects Partially Suppressed by *BUB2* Deletion

To gain insights into the molecular mechanisms leading to suppression of mitotic checkpoint defects by *DMA2* overexpression, we analyzed the effects of *Dma2* high dosage dur-

ing an unperturbed cell cycle. For this purpose, we monitored cell cycle progression under galactose-induced conditions of otherwise wild-type cells carrying either none, one single (s) or multiple tandem copies (m) of *GAL1-DMA2* integrated in the genome. G1 synchronized cell cultures were released in the presence of galactose, and kinetics of DNA replication, budding, spindle assembly/breakdown, and nuclear division were monitored on fixed cells at different times after release. As shown in Figure 5A, wild-type cells could complete one division cycle within 120 min, as indicated by the reappearance of cells with 1C DNA contents after nuclear division and disassembly of anaphase spindles. Expression of *GAL1-DMA2(s)*, as observed previously (Figure 4A), had only modest effects on cell cycle progression, causing at most a 15-min delay in spindle disassembly and cell division. However, compared with wild type, a higher percentage of *GAL1-DMA2(s)* cells with 2C DNA contents persisted throughout the course of the experiment and a small fraction of undivided cells occurred at later time points, accumulating as chains of cells with DNA contents higher than 2C (Figure 5A). These effects were

magnified by expression of *GAL1-DMA2(m)*, which allowed very few cells with 1C DNA contents to reaccumulate, whereas ~30% of the cells occurred as rebudded, multinucleated cells with DNA contents higher than 2C at 240 min after induction (Figure 5A). Most of these cells could not be separated after cell wall digestion with zymolase (our unpublished data), suggesting that they arose from a cytokinetic failure. These cytokinetic defects did not correlate with inability to exit mitosis. In fact, *GAL1-DMA2(m)* cells could not only rebud and enter into a new round of DNA replication but also could disassemble their spindles, albeit more slowly than wild-type cells (Figure 5A), suggesting that high levels of Dma2 can, at most, delay mitotic exit, while more severely affecting cytokinesis.

By analogy to SIN inhibition by *S. pombe* Dma1 (Guertin *et al.*, 2002), it was possible that the phenotypic effects caused by *DMA2* overexpression were due to partial MEN inactivation. We therefore asked whether deletion of *BUB2*, encoding a well-characterized MEN inhibitor (Simanis, 2003), could restore normal cell cycle progression in cells expressing high Dma2 levels. Strikingly, the cytokinetic defects of *GAL1-DMA2(m)* cells could be almost totally rescued by inactivation of Bub2. In fact, *GAL1-DMA2(m) bub2Δ* cells were almost completely able to divide and reaccumulate with 1C DNA contents (Figure 5A). Furthermore, deletion of *BUB2* partially rescued the lethality caused by *GAL1-DMA2(m)* overexpression (Figure 5B). We also analyzed whether the cytokinetic defects of *GAL1-DMA2(m)* cells could be rescued by inactivation of other mitotic inhibitors, such as the mitotic CDK inhibitory kinase Swe1 (Boohar *et al.*, 1993) and the Tem1 inhibitor Amn1 (Wang *et al.*, 2003). In contrast to *BUB2* deletion, neither *AMN1* nor *SWE1* deletion could alleviate the cytokinetic defects caused by *DMA2* overexpression (Figure S1A, supplementary data).

To investigate in further detail the reasons for the cytokinetic failure observed upon overexpression of *DMA2*, we analyzed whether the septin ring was correctly positioned in *GAL1-DMA2(m)* cells compared with wild-type. In fact, a septin ring, which is an essential prerequisite for cytokinesis, is assembled at the G1/S transition at the site of bud emergence (Longtine *et al.*, 1996) and is normally disassembled during mitotic exit in a Tem1-dependent manner (Lippincott *et al.*, 2001). We found that indeed overproduction of Dma2 caused remarkable differences in the kinetics of septin ring disassembly compared with wild type. In fact, after release in the presence of galactose of G1 synchronized cultures of wild-type and *GAL1-DMA2(m)* cells carrying the Cdc3-GFP fusion, no cells with two septin rings were seen among >300 wild-type cells, whereas 17% of *GAL1-DMA2(m)* cells showed a new septin ring at the site of bud emergence before disassembly of the old one (Figure 5C, left) at 150 min after release from G1, when cells entered into a new round of budding and DNA replication. Interestingly, these cells rebudded only from the previous bud and never from the mother cell, consistently with a role of the septin ring in creating a physical barrier to the diffusion of cell polarity determinants through the bud neck (Barral *et al.*, 2000). A fraction of *GAL1-DMA2(m)* cells (14 vs. 1.2% of wild-type cells) showed also aberrant septin ring deposition (Figure 5C, right). Together, these data suggest that overexpression of *DMA2* affects normal septin ring deposition and disassembly, thus causing cytokinetic defects.

Because we found that spindle disassembly was somewhat delayed in *DMA2*-overexpressing cells (Figure 5A), we also analyzed more accurately to what extent mitotic exit could be compromised in such cells by checking directly MEN activation in late mitosis. As a readout, we analyzed

by Western blot degradation of two APC substrates, the Polo kinase Cdc5 and the main mitotic cyclin Clb2, as well as reappearance of the CDK inhibitor Sic1, both events depending on MEN-dependent activation of the Cdc14 phosphatase at the end of mitosis (Shirayama *et al.*, 1998; Visintin *et al.*, 1998). Wild-type and *GAL1-DMA2(m)* cells were arrested in metaphase by nocodazole treatment and then released in the presence of galactose and α factor, to arrest them in the next G1 phase. Consistently with a cytokinetic defect, cell division kinetics was very slow in *GAL1-DMA2(m)* cells, and *DMA2* overexpression caused accumulation of cells with long anaphase spindles compared with wild type, suggesting that spindle disassembly was transiently delayed (Figure 6). However, spindles eventually disassembled between 90 and 120 min. Similarly, Clb2 was degraded and Sic1 reappeared with ~15-min delay with respect to wild type (Figure 6). Proteolysis of a HA-tagged version of Cdc5 was more dramatically affected and was not completed by the end of the experiment (150 min) in *GAL1-DMA2(m)* cells, whereas it was already apparent at 105 min in wild-type cells under the same conditions. Therefore, Dma2 high levels seem to interfere with some aspects of mitotic exit.

Overexpression of *DMA2* Interferes with Tem1 Activation

Because the data so far suggested that Dma1 and Dma2 could impinge on the same pathway as Bub2, we asked whether their target might be the MEN, and looked for genetic interactions between MEN components and Dma2. To this end, we analyzed the effects of *GAL1-DMA2(s)* expression, which has only minor effects on cell proliferation of otherwise wild-type cells, in some mutants defective at the top of the MEN cascade, such as *cdc5-1*, *cdc5-2*, *lte1Δ*, *cla4Δ*, and *tem1-3*. In all mutants, except for *lte1Δ*, we could see a toxic effect on cell proliferation caused by ectopic *DMA2* expression even at 25°C, permissive temperature for the above-mentioned mutants. Therefore, high levels of Dma2 are deleterious when the MEN is partially compromised (Figure 7). In two mutants, *cdc5-1* and *cla4Δ*, synthetic growth effects caused by *GAL1-DMA2* could be partially rescued by *BUB2* deletion. The *tem1-3* mutant was the most sensitive to *DMA2* overexpression, and upon galactose induction accumulated cells rebudding from the previous bud and eventually containing multiple nuclei and mitotic spindles (Figure 7, B and C). This phenotype can also be seen in a low percentage of *tem1-3* cells at the permissive temperature (Figure 7B), allowing to speculate that Dma2 might act close to Tem1 in the MEN. If this were the case, we would expect overexpression of *TEM1* to counteract the cytokinetic defects caused by high levels of Dma2, like *BUB2* deletion does (Figure 5A). Indeed, *TEM1* overexpression from the *GAL1* promoter allowed cells expressing *GAL1-DMA2(m)* to undergo cytokinesis with wild-type kinetics (Figure 8A), suggesting that Tem1 might become limiting for promoting mitotic exit and cytokinesis in *DMA2*-overexpressing cells. In addition, *GAL1-TEM1* expression also could restore cell viability in Dma2 overproducing cells more efficiently than *BUB2* deletion (Figure 8B). Because *S. pombe* Dma1 was proposed to antagonize the polo kinase (Guertin *et al.*, 2002), we wondered whether overproduction of the polo kinase Cdc5 could restore proper cell division in cells containing high Dma2 levels. However, overexpression of *CDC5*, unlike that of *TEM1*, did not suppress either cytokinetic defects or cell lethality of *GAL1-DMA2(m)* cells (Figure S1, A and B, supplementary data).

Figure 6. Mitotic exit upon *DMA2* overexpression. Wild-type (wt, ySP939) and *GAL1-DMA2m* (ySP3318) cells expressing HA-tagged Cdc5 (Cdc5-HA3) were grown in YEPR, synchronized in metaphase by nocodazole treatment (5 μ g/ml), and released at time 0 into YEPRG containing α factor to arrest cells in the next G1. Cell samples were withdrawn at the indicated times for fluorescence-activated cell sorting analysis of DNA contents (top); kinetics of budding, nuclear division, and spindle elongation (middle); and Western blot analysis of Cdc5-HA3, Clb2, and Sic1 (bottom).

DISCUSSION

A Role for *Dma1* and *Dma2* in Septin Ring Assembly

The first step in nuclear positioning occurs through migration of the spindle toward the bud neck and requires a Kar9-dependent pathway that involves, besides the cortical determinant Kar9 (Miller and Rose, 1998), the plus-end microtubule binding protein Bim1, that interacts with Kar9 (Korinek *et al.*, 2000; Lee *et al.*, 2000); the Bni1 protein, required for Kar9 proper localization (Miller *et al.*, 1999); and

the microtubule motor Kip3 (Cottingham and Hoyt, 1997; DeZwaan *et al.*, 1997). A second pathway, leading to insertion of the spindle through the bud neck, depends on cytoplasmic dynein (Cottingham and Hoyt, 1997; DeZwaan *et al.*, 1997) and on the cortical protein Num1 (Heil-Chapdelaine *et al.*, 2000). The two pathways are presumably functionally redundant, because inactivation of any gene of one pathway in the absence of any component of the second pathway is often lethal or severely deleterious. Recently, septins also

Figure 7. DMA2 overexpression is toxic for MEN mutants. (A) Serial dilutions of the indicated cell cultures, lacking or carrying one copy of the *GAL1-DMA2* fusion integrated at the *URA3* locus, were spotted on either YEPD (Glu) or YEPRG (Gal) plates and incubated for two days at 25°C. (B) Cultures of the indicated strains were grown in YEPR at 25°C and induced with 1% galactose at time 0. Cell samples were withdrawn every 2 h for fluorescence-activated cell sorting analysis of DNA contents (our unpublished data), kinetics of accumulation of multinucleate cells (graph) and in situ immunostaining of α tubulin (our unpublished data). (C) Examples of nuclei and microtubules of *tem1-3 GAL1-DMA2* multinucleated cells from the experiment in B) after 4 h of galactose induction are shown.

have been implicated in spindle positioning by providing a cortical cue for microtubule capture, and based on genetic interactions have been proposed to act in the nuclear positioning Kar9 pathway (Kusch *et al.*, 2002). We now show that Dma1 and Dma2 might have a direct role in septin ring deposition and might therefore control spindle positioning. In fact, lack of both Dma1 and Dma2 compromise septin ring assembly and have additive effects with *cla4* mutations. In addition, overexpression of Dma2 delays septin ring disassembly at the end of mitosis. We find that the lack of Dma1 and Dma2 does not cause synthetic growth defects when combined with deletion of either *KAR9* or *KIP3* (our unpublished data), whereas it confers a temperature-sensitive growth phenotype to *dyn1Δ* cells, which would suggest that Dma1 and Dma2 might act in the Kar9-dependent pathway. However, the finding that *DMA1/DMA2* deletion causes additive defects on spindle positioning when combined with

the lack of either Bim1 or Bni1 argues against this simple view, suggesting that Dma1 and Dma2 might act in a novel spindle positioning pathway. This is still an open question, since genetic interactions do not always reflect the complex functional interactions taking place between components of the spindle positioning/orientation pathways, because other exceptions to the above-mentioned simple genetic rule have been previously observed. For example, deletion of *BIM1* is lethal in *kip3Δ* cells, despite Bim1 and Kip3 supposedly belong to the same spindle positioning pathway (Tong *et al.*, 2001). In addition, despite that Cla4 also should participate to the Kar9-dependent pathway by virtue of its involvement in septin ring organization (Cvrckova *et al.*, 1995), it is essential in a *bni1Δ* background (Goehring *et al.*, 2003).

How Dma1 and Dma2 regulate septin ring organization is unknown at the moment. Septins undergo different post-translational modifications, including covalent attachment of the ubiquitin-related peptide SUMO between the onset of anaphase and cytokinesis (Johnson and Blobel, 1999). Multiple mutations in septin sumoylation sites prevents the disappearance of septin rings from old bud sites, leading to the proposal that sumoylation of septins promotes disassembly of the septin ring, either directly or indirectly, by preventing/inducing other posttranslational modifications, such as ubiquitination (Johnson and Blobel, 1999). Based on the failure of *DMA2*-overexpressing cells to timely disassemble septin rings, and on the notion that Dma1 and Dma2 are RING finger-containing proteins, and therefore possibly part of an E3 ubiquitin-ligase (Joazeiro and Weissman, 2000), an attractive hypothesis that is worth further investigation is that Dma1 and Dma2 might directly mediate septins' ubiquitination or sumoylation.

The MEN as a Target of Dma1/Dma2

Fission yeast Dma1 has been found to prevent activation of the SIN, thereby inhibiting cytokinesis, and to interfere with the correct localization of the Polo kinase Plt1 and other SIN regulators (Guertin *et al.*, 2002). In addition, its human homologue Chfr is a ubiquitin ligase that ubiquitinates the Polo-like kinase Plk1 in *Xenopus* cell extracts (Kang *et al.*, 2002). The following lines of evidence suggest that the mitotic exit network could be a target of Dma1 and Dma2 regulation in budding yeast: 1) moderate *DMA2* overexpression can suppress the ability of spindle checkpoint mutants to exit mitosis in the presence of nocodazole; 2) increased levels of Dma2 that are perfectly tolerated by wild-type cells are extremely toxic specifically for some MEN mutants; and 3) strong *DMA2* overexpression causes mitotic exit and cytokinesis defects that can be rescued by either deletion of *BUB2* or simultaneous overexpression of *TEM1*. Indeed, all these data could be consistent with a model where Dma1 and Dma2 antagonize the activity of the Polo kinase Cdc5, because one important function of Cdc5 in the MEN is to down-regulate the GTPase-activating protein Bub2/Bfa1, thus allowing Tem1 activation (Hu *et al.*, 2001). However, so far we have been unable to find any correlation between Dma1/Dma2 activation and Cdc5 regulation. In fact, Cdc5 protein levels are unaltered in *dma1Δ dma2Δ* cells (Figure S2A, supplementary material) and more stable, rather than more labile, in *DMA2*-overexpressing cells (Figure 6). Moreover, high levels of Dma2 do not interfere with separation of sister telomeres (Figure S2B, supplementary material), which depends upon Cdc5 activity (Alexandru *et al.*, 2001). Finally, cytokinetic defects caused by *DMA2* overexpression are not rescued by high Cdc5 levels (Figure S1A, supplementary material).

Figure 8. TEM1 overexpression rescues cytokinetic and growth defects caused by Dma2 high levels. (A) Cultures of isogenic strains with the indicated genotypes (ySP3616, ySP3657, and ySP3721), logarithmically growing in YEPR, were arrested in G1 by α factor and released in YEPRG at time 0. Cell samples were withdrawn at the indicated times for fluorescence-activated cell sorting analysis of the DNA contents. (B) Serial dilutions of the indicated cell cultures were spotted on YEPD (Glu) or YEPRG (Gal) plates and incubated for 3 d at 23°C.

Another possibility is that the MEN inhibitory function of Dma1/Dma2 is to inhibit Tem1 through the GAP Bub2/Bfa1. However, we think this is not the case, because *BUB2* deletion only partially counteracts the deleterious effects on cytokinesis of Dma2 high levels and mostly does not suppress their lethal effects in MEN mutants. Moreover, *DMA1/DMA2* deletion dramatically increases the benomyl sensitivity of *bub2Δ* cells (our unpublished observations), suggesting that Dma1/Dma2 and Bub2 might operate in different pathways. Accordingly, *DMA2* overexpression can suppress the spindle checkpoint defects of *bub2Δ* cells. Therefore, we rather suspect that Dma1 and Dma2 might impinge more directly on Tem1 activation. In fact, *TEM1* overexpression can partially counteract the inhibitory effects on cytokinesis and the toxicity on growth caused by high levels of Dma2. In addition, depletion of Tem1 in conditions that allow mitotic exit causes defects in septin ring disassembly (Lippincott *et al.*, 2001) that are remarkably similar to those observed in *DMA2*-overexpressing cells.

Despite our efforts, how Tem1 inhibition by Dma1/Dma2 could be achieved has not yet been elucidated. In fact, Tem1 protein levels, localization at SPBs or interaction with Cdc15 (another MEN component) seemed to be unaffected by either *DMA1/DMA2* deletion or *DMA2* overexpression (our unpublished data). Accordingly, deletion of *AMN1*, which inhibits Tem1/Cdc15 interaction and MEN activation (Wang *et al.*, 2003), did not rescue the deleterious effects caused by high levels of Dma2 (Figure S1A/B, supplementary material), suggesting that Dma2

acts independently of Amn1. Because *DMA2* overexpression seems to affect cytokinesis more than mitotic exit, it is also possible that only a special pool of Tem1 is regulated by Dma1/Dma2 or that the latter proteins and Tem1 antagonistically act in parallel to regulate downstream MEN components. It is now well established that MEN components have cytokinetic functions independent of Cdc14 release from the nucleolus and inactivation of mitotic CDKs (Shou *et al.*, 1999; Lippincott *et al.*, 2001; Park *et al.*, 2003). Further experiments will be required to clarify these points. Being Dma1 and Dma2 possibly part of an E3 ubiquitin ligase, the identification of their substrates will be critical to understand their possible role in MEN regulation. Interestingly, a high-throughput mass spectrometric analysis indicates that Dma1 and Dma2, besides interacting with each other, physically associate with a F-box protein (the gene product of *YNL311*), which in turn interacts with ubiquitin (Ho *et al.*, 2002), further supporting their possible involvement in a ubiquitination pathway.

Dma1/Dma2 and the Spindle Position Checkpoint

S. pombe Dma1 is required to delay mitotic exit and septum formation when spindle function is compromised (Murone and Simanis, 1996). Its human homologue 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). Accordingly, Chfr was also found to

inhibit the Cdc2 kinase at the G2/M transition by prolonging its inhibitory tyrosine 15 phosphorylated state (Kang *et al.*, 2002). We showed that DMA2 overexpression interferes with mitotic exit and cytokinesis, but apparently not with the onset of anaphase. In addition, the deleterious effects caused by high Dma2 levels are not rescued, but rather enhanced, by deletion of *SWE1* (Figure S1A, supplementary material) that encodes the Wee1-like inhibitory kinase responsible for tyrosine 15 phosphorylation of mitotic CDKs (Booher *et al.*, 1993). Therefore, Dma1 and Dma2 seem to have functions closer to their fission yeast, rather than human, counterpart.

The concomitant lack of Dma1 and Dma2 impairs the spindle position checkpoint. A striking correlation can be drawn between the involvement of Dma1/Dma2 in this checkpoint and their role in septin ring assembly. In fact, septins have recently been shown to prevent inappropriate mitotic exit when cytoplasmic microtubules do not interact properly with the bud neck (Castillon *et al.*, 2003). Unlike *S. pombe* Dma1, *S. cerevisiae* Dma1 and Dma2 are not required to delay cell cycle progression in the presence of nocodazole. In addition, whereas lack of Dma1 and Dma2 allows unscheduled mitotic exit in dynein mutants, it does not have any effect in the absence of Kar9 (our unpublished observations), suggesting that Dma1 and Dma2 are required to respond only to some kinds of spindle misposition signals. In this respect, Dma1 and Dma2 share remarkable similarities with Bim1, which is involved in spindle positioning/orientation (Tirnauer *et al.*, 1999), and also has been implicated in a checkpoint that delays cytokinesis in the presence of mispositioned spindles specifically caused by dynactin mutation (Muhua *et al.*, 1998). The surprising observation that *bim1Δ* cells do not necessarily undergo premature cytokinesis in the presence of spindles mispositioned by other means (Tirnauer *et al.*, 1999; Adames *et al.*, 2001), together with our similar observation on *dma1Δ dma2Δ* cells, suggests that defects in dynein/dynactin might generate different signals from those arising by other kinds of spindle positioning errors.

Interestingly, human Chfr is missing in several tumor cell lines (Scolnick and Halazonetis, 2000), whereas the Bim1 homologue EB1 is associated with the tumor suppressor adenomatous polyposis coli protein (Su *et al.*, 1995), suggesting that perhaps these proteins are crucial to maintain genome stability. A detailed characterization of the errors they detect might therefore help to shed light on the processes at the basis of tumor progression.

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REFERENCES

Adames, N.R., and Cooper, J.A. (2000). Microtubule interactions with the cell cortex causing nuclear movements in *Saccharomyces cerevisiae*. *J. Cell Biol.* 149, 863–874.

Adames, N.R., Oberle, J.R., and Cooper, J.A. (2001). The surveillance mechanism of the spindle position checkpoint in yeast. *J. Cell Biol.* 153, 159–168.

Alexandru, G., Uhlmann, F., Mechtler, K., Poupart, M., and Nasmyth, K. (2001). Phosphorylation of the cohesin subunit Scc1 by Polo/Cdc5 kinase regulates sister chromatid separation in yeast. *Cell* 105, 459–472.

Alexandru, G., Zachariae, W., Schleiffer, A., and Nasmyth, K. (1999). Sister chromatid separation and chromosome re-duplication are regulated by different mechanisms in response to spindle damage. *EMBO J.* 18, 2707–2721.

Bardin, A.J., Visintin, R., and Amon, A. (2000). A mechanism for coupling exit from mitosis to partitioning of the nucleus. *Cell* 102, 21–31.

Barral, Y., Mermall, V., Mooseker, M.S., and Snyder, M. (2000). Compartmentalization of the cell cortex by septins is required for maintenance of cell polarity in yeast. *Mol. Cell.* 5, 841–851.

Beach, D.L., Thibodeaux, J., Maddox, P., Yeh, E., and Bloom, K. (2000). The role of the proteins Kar9 and Myo2 in orienting the mitotic spindle of budding yeast. *Curr. Biol.* 10, 1497–1506.

Bloecher, A., Venturi, G.M., and Tatchell, K. (2000). Anaphase spindle position is monitored by the BUB2 checkpoint. *Nat. Cell Biol.* 2, 556–558.

Booher, R.N., Deshaies, R.J., and Kirschner, M.W. (1993). Properties of *Saccharomyces cerevisiae* wee1 and its differential regulation of p34CDC28 in response to G1 and G2 cyclins. *EMBO J.* 12, 3417–3426.

Castillon, G.A., Adames, N.R., Rosello, C.H., Seidel, H.S., Longtine, M.S., Cooper, J.A., and Heil-Chapdelaine, R.A. (2003). Septins have a dual role in controlling mitotic exit in budding yeast. *Curr. Biol.* 13, 654–658.

Cottingham, F.R., Gheber, L., Miller, D.L., and Hoyt, M.A. (1999). Novel roles for *saccharomyces cerevisiae* mitotic spindle motors. *J. Cell Biol.* 147, 335–350.

Cottingham, F.R., and Hoyt, M.A. (1997). Mitotic spindle positioning in *Saccharomyces cerevisiae* is accomplished by antagonistically acting microtubule motor proteins. *J. Cell Biol.* 138, 1041–1053.

Cvrckova, F., De Virgilio, C., Manser, E., Pringle, J.R., and Nasmyth, K. (1995). Ste20-like protein kinases are required for normal localization of cell growth and for cytokinesis in budding yeast. *Genes Dev.* 9, 1817–1830.

DeZwaan, T.M., Ellingson, E., Pellman, D., and Roof, D.M. (1997). Kinesin-related KIP3 of *Saccharomyces cerevisiae* is required for a distinct step in nuclear migration. *J. Cell Biol.* 138, 1023–1040.

Fankhauser, C., Marks, J., Raymond, A., and Simanis, V. (1993). The *S. pombe* cdc16 gene is required both for maintenance of p34cdc2 kinase activity and regulation of septum formation: a link between mitosis and cytokinesis? *EMBO J.* 12, 2697–2704.

Fesquet, D., Fitzpatrick, P.J., Johnson, A.L., Kramer, K.M., Toyn, J.H., and Johnston, L.H. (1999). A Bub2p-dependent spindle checkpoint pathway regulates the Dbf2p kinase in budding yeast. *EMBO J.* 18, 2424–2434.

Fraschini, R., Formenti, E., Lucchini, G., and Piatti, S. (1999). Budding yeast Bub2 is localized at spindle pole bodies and activates the mitotic checkpoint via a different pathway from Mad2. *J. Cell Biol.* 145, 979–991.

Gardner, R.D., and Burke, D.J. (2000). The spindle checkpoint: two transitions, two pathways. *Trends Cell Biol.* 10, 154–158.

Geymonat, M., Spanos, A., Smith, S.J., Wheatley, E., Rittinger, K., Johnston, L.H., and Sedgwick, S.G. (2002). Control of mitotic exit in budding yeast. In vitro regulation of Tem1 GTPase by Bub2 and Bfa1. *J. Biol. Chem.* 277, 28439–28445.

Goehring, A.S., Mitchell, D.A., Tong, A.H., Keniry, M.E., Boone, C., and Sprague, G.F., Jr. (2003). Synthetic lethal analysis implicates Ste20p, a p21-activated protein kinase, in polarisome activation. *Mol. Biol. Cell* 14, 1501–1516.

Guertin, D.A., Venkatram, S., Gould, K.L., and McCollum, D. (2002). Dma1 prevents mitotic exit and cytokinesis by inhibiting the septation initiation network (SIN). *Dev. Cell* 3, 779–790.

Heil-Chapdelaine, R.A., Oberle, J.R., and Cooper, J.A. (2000). The cortical protein num1p is essential for dynein-dependent interactions of microtubules with the cortex. *J. Cell Biol.* 151, 1337–1344.

Ho, Y., *et al.* (2002). Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. *Nature* 415, 180–183.

Hu, F., Wang, Y., Liu, D., Li, Y., Qin, J., and Elledge, S.J. (2001). Regulation of the Bub2/Bfa1 GAP complex by Cdc5 and cell cycle checkpoints. *Cell* 107, 655–665.

Joazeiro, C.A., and Weissman, A.M. (2000). RING finger proteins: mediators of ubiquitin ligase activity. *Cell* 102, 549–552.

Johnson, E.S., and Blobel, G. (1999). Cell cycle-regulated attachment of the ubiquitin-related protein SUMO to the yeast septins. *J. Cell Biol.* 147, 981–994.

- Kang, D., Chen, J., Wong, J., and Fang, G. (2002). The checkpoint protein Chfr is a ligase that ubiquitinates Plk1 and inhibits Cdc2 at the G2 to M transition. *J. Cell Biol.* 156, 249–260.
- Korinek, W.S., Copeland, M.J., Chaudhuri, A., and Chant, J. (2000). Molecular linkage underlying microtubule orientation toward cortical sites in yeast. *Science* 287, 2257–2259.
- Kusch, J., Meyer, A., Snyder, M.P., and Barral, Y. (2002). Microtubule capture by the cleavage apparatus is required for proper spindle positioning in yeast. *Genes Dev.* 16, 1627–1639.
- Lee, L., Klee, S.K., Evangelista, M., Boone, C., and Pellman, D. (1999). Control of mitotic spindle position by the *Saccharomyces cerevisiae* formin Bni1p. *J. Cell Biol.* 144, 947–961.
- Lee, L., Tirnauer, J.S., Li, J., Schuyler, S.C., Liu, J.Y., and Pellman, D. (2000). Positioning of the mitotic spindle by a cortical-microtubule capture mechanism. *Science* 287, 2260–2262.
- Lee, W.L., Oberle, J.R., and Cooper, J.A. (2003). The role of the lissencephaly protein Pac1 during nuclear migration in budding yeast. *J. Cell Biol.* 160, 355–364.
- Lew, D.J., and Reed, S.I. (1995). A cell cycle checkpoint monitors cell morphogenesis in budding yeast. *J. Cell Biol.* 129, 739–749.
- Lippincott, J., Shannon, K.B., Shou, W., Deshaies, R.J., and Li, R. (2001). The Tem1 small GTPase controls actomyosin and septin dynamics during cytokinesis. *J. Cell Sci.* 114, 1379–1386.
- Longtine, M.S., DeMarini, D.J., Valencik, M.L., Al-Awar, O.S., Fares, H., De Virgilio, C., and Pringle, J.R. (1996). The septins: roles in cytokinesis and other processes. *Curr. Opin. Cell Biol.* 8, 106–119.
- Maniatis, T., Fritsch, E.F., and Sambrook, J. (1992). Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Michaelis, C., Ciosk, R., and Nasmyth, K. (1997). Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. *Cell* 91, 35–45.
- Miller, R.K., Matheos, D., and Rose, M.D. (1999). The cortical localization of the microtubule orientation protein, Kar9p, is dependent upon actin and proteins required for polarization. *J. Cell Biol.* 144, 963–975.
- Miller, R.K., and Rose, M.D. (1998). Kar9p is a novel cortical protein required for cytoplasmic microtubule orientation in yeast. *J. Cell Biol.* 140, 377–390.
- Muhua, L., Adames, N.R., Murphy, M.D., Shields, C.R., and Cooper, J.A. (1998). A cytokinesis checkpoint requiring the yeast homologue of an APC-binding protein. *Nature* 393, 487–491.
- Murone, M., and Simanis, V. (1996). The fission yeast *dma1* gene is a component of the spindle assembly checkpoint, required to prevent septum formation and premature exit from mitosis if spindle function is compromised. *EMBO J.* 15, 6605–6616.
- Musacchio, A., and Hardwick, K.G. (2002). The spindle checkpoint: structural insights into dynamic signalling. *Nat. Rev. Mol. Cell Biol.* 3, 731–741.
- Park, C.J., Song, S., Lee, P.R., Shou, W., Deshaies, R.J., and Lee, K.S. (2003). Loss of CDC5 function in *Saccharomyces cerevisiae* leads to defects in Swe1p regulation and Bfa1p/Bub2p-independent cytokinesis. *Genetics* 163, 21–33.
- Pereira, G., Hofken, T., Grindlay, J., Manson, C., and Schiebel, E. (2000). The Bub2p spindle checkpoint links nuclear migration with mitotic exit. *Mol. Cell* 6, 1–10.
- Schuyler, S.C., and Pellman, D. (2001). Search, capture and signal: games microtubules and centrosomes play. *J. Cell Sci.* 114, 247–255.
- Schwob, E., Bohm, T., Mendenhall, M.D., and Nasmyth, K. (1994). The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in *S. cerevisiae* [see comments] [published erratum appears in *Cell* 1996 Jan 12;84(1):following 174]. *Cell* 79, 233–244.
- Scolnick, D.M., and Halazonetis, T.D. (2000). Chfr defines a mitotic stress checkpoint that delays entry into metaphase. *Nature* 406, 430–435.
- Sheeman, B., Carvalho, P., Sagot, I., Geiser, J.R., Kho, D., Hoyt, M.A., and Pellman, D. (2003). Determinants of *S. cerevisiae* dynein localization and activation: implications for the mechanism of spindle positioning. *Curr. Biol.* 13, 364–372.
- Sherman, F. (1991). Getting started with yeast. *Methods Enzymol.* 194, 3–21.
- Shirayama, M., Zachariae, W., Ciosk, R., and Nasmyth, K. (1998). The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/fizzy are regulators and substrates of the anaphase promoting complex in *Saccharomyces cerevisiae*. *EMBO J.* 17, 1336–1349.
- Shou, W., Seol, J.H., Shevchenko, A., Baskerville, C., Moazed, D., Chen, Z.W., Jang, J., Charbonneau, H., and Deshaies, R.J. (1999). Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. *Cell* 97, 233–244.
- Simanis, V. (2003). Events at the end of mitosis in the budding and fission yeasts. *J. Cell Sci.* 116, 4263–4275.
- Su, L.K., Burrell, M., Hill, D.E., Gyuris, J., Brent, R., Wiltshire, R., Trent, J., Vogelstein, B., and Kinzler, K.W. (1995). APC binds to the novel protein EB1. *Cancer Res.* 55, 2972–2977.
- Sullivan, D.S., and Huffaker, T.C. (1992). Astral microtubules are not required for anaphase B in *Saccharomyces cerevisiae*. *J. Cell Biol.* 119, 379–388.
- Surana, U., Amon, A., Dowzer, C., McGrew, J., Byers, B., and Nasmyth, K. (1993). Destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. *EMBO J.* 12, 1969–1978.
- Tirnauer, J.S., O'Toole, E., Berrueta, L., Bierer, B.E., and Pellman, D. (1999). Yeast Bim1p promotes the G1-specific dynamics of microtubules. *J. Cell Biol.* 145, 993–1007.
- Tong, A.H., *et al.* (2001). Systematic genetic analysis with ordered arrays of yeast deletion mutants. *Science* 294, 2364–2368.
- Visintin, R., Craig, K., Hwang, E.S., Prinz, S., Tyers, M., and Amon, A. (1998). The phosphatase Cdc14 triggers mitotic exit by reversal of Cdk-dependent phosphorylation. *Mol. Cell* 2, 709–718.
- Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994). New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* 10, 1793–1808.
- Wang, Y., Shirogane, T., Liu, D., Harper, J.W., and Elledge, S.J. (2003). Exit from exit: resetting the cell cycle through Amn1 inhibition of G protein signaling. *Cell* 112, 697–709.
- Yeh, E., Skibbens, R.V., Cheng, J.W., Salmon, E.D., and Bloom, K. (1995). Spindle dynamics and cell cycle regulation of dynein in the budding yeast, *Saccharomyces cerevisiae*. *J. Cell Biol.* 130, 687–700.
- Yin, H., Pruyne, D., Huffaker, T.C., and Bretscher, A. (2000). Myosin V orientates the mitotic spindle in yeast. *Nature* 406, 1013–1015.
- Zachariae, W., and Nasmyth, K. (1999). Whose end is destruction: cell division and the anaphase-promoting complex. *Genes Dev.* 13, 2039–2058.