

Budding yeast PAK kinases regulate mitotic exit by two different mechanisms

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We report the characterization of the dominant-negative *CLA4t* allele of the budding yeast *CLA4* gene, encoding a member of the p21-activated kinase (PAK) family of protein kinases, which, together with its homologue *STE20*, plays an essential role in promoting budding and cytokinesis. Overproduction of the *Clat* protein likely inhibits both endogenous *Clat* and *Ste20* and causes a delay in the onset of anaphase that correlates with inactivation of Cdc20/anaphase-promoting complex (APC)-dependent proteolysis of both the cyclinB *Clb2* and securin. Although the precise mechanism of APC

inhibition by *Clat* remains to be elucidated, our results suggest that *Clat* and *Ste20* may regulate the first wave of cyclinB proteolysis mediated by Cdc20/APC, which has been shown to be crucial for activation of the mitotic exit network (MEN). We show that the Cdk1-inhibitory kinase *Swe1* is required for the *Clat*-dependent delay in cell cycle progression, suggesting that it might be required to prevent full Cdc20/APC and MEN activation. In addition, inhibition of PAK kinases by *Clat* prevents mitotic exit also by a *Swe1*-independent mechanism impinging directly on the MEN activator *Tem1*.

Introduction

In eukaryotic cells, entry into mitosis is driven by both activation of cyclinB-dependent Cdks and ubiquitin-dependent proteolysis triggered by the ubiquitin–ligase anaphase-promoting complex (APC).^{*} At the metaphase–anaphase transition, the APC targets the *Esp1* separase inhibitor securin for degradation, leading in turn to cleavage of the bonds between sister chromatids (Peters, 2002). APC-dependent proteolysis is also required at the end of mitosis to promote mitotic exit and cytokinesis. In particular, degradation of B-type cyclins seems to be crucial for Cdk inactivation, leading to spindle disassembly, cytokinesis, and entry into a new round of DNA replication (Zachariae and Nasmyth, 1999). To display full ubiquitin–ligase activity, the APC must bind to the Cdc20 and Cdh1 accessory factors that are responsible for securin and cyclinB degradation, respectively (Peters, 2002).

As in other eukaryotic systems, the onset of anaphase in budding yeast requires activation of the Cdk Cdc28 bound to any of four mitotic B-type cyclins, *Clb1–4*. Although the

role of *Clb1–4/Cdc28* kinases during this cell cycle transition can be attributed primarily to their essential function in spindle assembly (Surana et al., 1991; Fitch et al., 1992), some evidence suggests that they also stimulate the binding between Cdc20 and APC, thereby allowing mitotic exit (Rudner et al., 2000; Rudner and Murray, 2000). In this way, at the onset of anaphase, *Clb1–4/Cdc28* kinases sow the seeds for their own destruction.

Work in budding yeast has shown that the Cdc14 protein phosphatase is critical to inactivate cyclinB/Cdks at the end of mitosis by dephosphorylating and activating both Cdh1 and the cyclinB/Cdk inhibitor Sic1 (Visintin et al., 1998). Cdc14 must be released from the nucleolus in order to be active during mitotic exit, and this requires a signal transduction cascade called mitotic exit network (MEN), involving the *Tem1* GTPase, its GTPase exchange factor *Lte1*, several protein kinases and associated factors (Cdc5, Cdc15, Mob1/Dbf2, Dbf20), and a scaffold protein (Nud1; Bardin and Amon, 2001; Geymonat et al., 2002). Recently, an early anaphase release of Cdc14 from the nucleolus has been shown to be transiently driven by the Polo kinase Cdc5 and separase independently of the MEN, and to contribute to full MEN activation, thus generating a positive feedback loop (Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002).

Although Cdc20/APC reaches its maximal activity at the onset of anaphase, it is also necessary for inactivation of cyclinB/Cdks at the end of mitosis in different ways: (1) by triggering

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^{*}Abbreviations used in this paper: APC, anaphase-promoting complex; GAP, GTPase-activating protein; MEN, mitotic exit network; PAK, p21-activated kinase.

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securin degradation, which in turn promotes separase activation and Cdc14 early release from the nucleolus; (2) by promoting degradation of the S-phase cyclin Clb5, whose associated Cdk activity prevents Cdc14 activation (Shirayama et al., 1999); and (3) by inducing directly a first wave of cyclinB degradation during anaphase (Baumer et al., 2000; Yeong et al., 2000), which is important for subsequent Cdh1 and MEN activation. Furthermore, a recent report has challenged the current view on mitotic exit regulation by highlighting the essential role of Cdc20/APC-driven degradation of the mitotic cyclin Clb2 (Wasch and Cross, 2002).

To ensure faithful chromosome transmission and genomic stability, progression through mitosis is tightly regulated by surveillance mechanisms (called checkpoints) that prevent or delay specific cell cycle transitions in response to different kinds of injuries, thus allowing correction of errors (Hartwell and Weinert, 1989). In particular, the metaphase to anaphase transition can be delayed by checkpoint mechanisms that respond to DNA damage (Weinert et al., 1994), spindle disassembly/misorientation (Hoyt, 2000; Gorbsky, 2001; Wassmann and Benezra, 2001), or faulty cytoskeletal organization (Lew, 2000). In budding yeast, the spindle assembly checkpoint is divided in two different branches, one monitoring kinetochore/microtubule attachment and/or kinetochore tension, and the other sensing spindle mispositioning. The kinetochore checkpoint, which involves Mad1, -2, and -3, Bub1 and -3, Mps1, Ndc10, and Ipl1, leads to inhibition of Cdc20/APC, thereby preventing both the anaphase onset and exit from mitosis (for review see Musacchio and Hardwick, 2002). Conversely, the spindle position checkpoint inhibits the Tem1 GTPase at the top of the MEN through the Bub2/Bfa1 two-component GTPase-activating protein (GAP), and therefore controls primarily mitotic exit and cytokinesis (Hoyt, 2000). Tem1 activation requires either inactivation of the GAP by the Cdc5 Polo kinase (Hu et al., 2001) or exposure to the GTPase exchange factor Lte1 (Shirayama et al., 1994). Strikingly, Tem1 resides at SPBs, whereas Lte1 is asymmetrically localized at the bud cortex, thus coupling spindle pole and nuclear migration in the bud with mitotic exit (Bardin et al., 2000; Pereira et al., 2000). Finally, the morphogenesis checkpoint delays the G2/M transition, specifically in response to perturbations in the actin cytoskeleton or in septin deposition, and depends on the Swe1-mediated phosphorylation and inactivation of Cdc28 (Lew, 2000).

Whether there are crosstalks between different checkpoints and how all the outputs of checkpoint controls are integrated in order to ensure the right progression of mitotic events are critical points still under investigation. In this paper, we present data that link cytokinesis, the spindle checkpoint, and the morphogenesis checkpoint by describing a dominant-negative *CLA4* allele (*CLA4t*) that likely inhibits both endogenous Cla4 and Ste20. These two proteins share overlapping essential functions in controlling budding and cytokinesis (Cvrckova et al., 1995; Holly and Blumer, 1999; Weiss et al., 2000), and are members of the p21-activated kinase (PAK) family of protein kinases, which are effectors of the Cdc42 GTPase, playing a crucial role in regulating cell polarity in all eukaryotic cells (Johnson, 1999). From our data, PAK kinases appear to contribute to promote mi-

totic exit by regulating the MEN in two different ways; by allowing the first burst of cyclinB/Cdk inactivation that occurs at the onset of anaphase, and more directly, by establishing the conditions for Tem1 activation.

Results

High copy number of a truncated *CLA4* gene can suppress the benomyl sensitivity of spindle checkpoint mutants

To gain insights into the Bub2/Bfa1 signal transduction cascade, we screened a budding yeast genomic library for high dosage suppressors of the sensitivity of a *bub2Δ* mutant to the microtubule-depolymerizing drug benomyl (see Materials and methods). Among the isolated clones, we found a plasmid carrying the whole promoter region and 92% of the *CLA4* coding sequence (*CLA4t*). The Cla4t protein lacks 67 amino acids at its COOH-terminal region, where the essential kinase domain resides, but retains unaltered pleckstrin homology and PAK domains, the latter being involved in the association with Cdc42 (Benton et al., 1997; Fig. 1 A). Subcloning of both full-length and truncated *CLA4* in the YEpl3 2μ vector revealed that only high copy number *CLA4t* can suppress the benomyl sensitivity of *bub2Δ*, although to a lesser extent than wild-type *BUB2* (Fig. 1 B). Moreover, 2μ *CLA4t* could also suppress the benomyl sensitivity of other spindle checkpoint mutants, like *mad2Δ* and *bfa1Δ* (Fig. 1 C), but not that of the β-tubulin mutant *tub2-405* (unpublished data), suggesting that the suppression mechanism might involve differences in cell cycle progression rather than in microtubule stability.

Expression of *CLA4t* suppresses spindle checkpoint defects

To better control the expression of *CLA4t*, we cloned it under the galactose-inducible *GAL1-10* promoter, and integrated the *GAL-CLA4t* fusion at the *URA3* chromosomal locus. Galactose-induced expression of *GAL-CLA4t* had a strong dose effect on growth, with 4X *GAL-CLA4t* integrated copies almost totally inhibiting cells' ability to form colonies, whereas a single copy had little effect (Fig. 1 D). Therefore, we asked whether expression of *GAL-CLA4t* in *bub2Δ*, *mad2Δ*, and *mad2Δ bub2Δ* strains could suppress the checkpoint defects of these mutants. Synchronized G1 cultures of wild-type and the above mutants, either lacking or carrying a single copy of *GAL-CLA4t*, were released in the presence of galactose and the microtubule-depolymerizing drug nocodazole, to follow their kinetics of cell cycle progression by FACS® analysis (Fig. 2 A). As expected, in these conditions *bub2Δ*, *mad2Δ*, and *mad2Δ bub2Δ* mutants, unlike the wild-type strain, accumulated cells with DNA contents higher than 2C and *mad2 bub2* double mutants re-replicated their genome much faster than either single mutant, as previously shown (Alexandru et al., 1999; Fesquet et al., 1999; Fraschini et al., 1999; Li, 1999). Strikingly, expression of *GAL-CLA4t* prevented *bub2Δ* and *mad2Δ* cells from entering a new round of DNA replication and restrained also a fraction of *mad2Δ bub2Δ* cells from doing so.

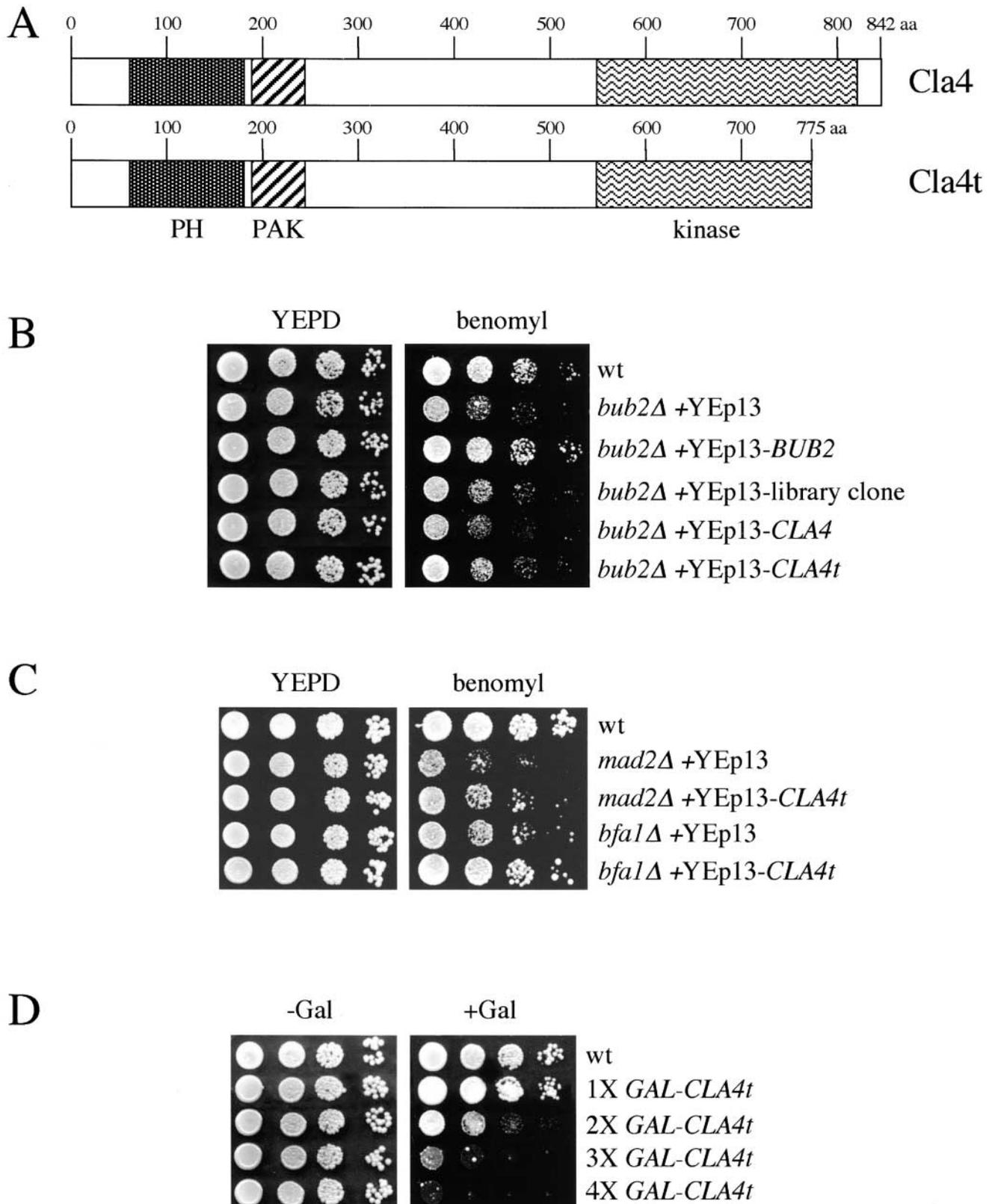


Figure 1. Effects of *CLA4t* overexpression on benomyl sensitivity and cell growth. (A) Schematic representation of wild-type Cla4 and its truncated Cla4t variant. PH, pleckstrin homology domain; PAK, p21-activated kinase family domain. (B and C) Serial dilutions of wild-type (W303), *bub2Δ* (ySP1071), *mad2Δ* (ySP1070), and *bfa1Δ* (ySP1243) strains carrying the indicated 2 μ plasmids were spotted on YEPD plates with or without benomyl and incubated for two days at 25°C. (D) Serial dilutions of wild-type (W303) and isogenic strains carrying the indicated numbers of integrated *GAL-CLA4t* fusions were spotted on YEPD (-Gal, *GAL1* promoter off) and YEPRG (+Gal, *GAL1* promoter on) plates and incubated for two days at 30°C.

Figure 2. GAL-CLA4t expression suppresses the spindle checkpoint defects of *mad2* and *bub2* mutants.

(A) Cultures of wild-type, *bub2* Δ , *mad2* Δ , and *mad2* Δ *bub2* Δ mutants, either lacking (W303, ySP3138, ySP1070, and ySP1086) or carrying one integrated copy of *GAL-CLA4t* (ySP2622, ySP2626, ySP2752, and ySP3068) were arrested in G1 by α -factor treatment and then released into YEPRG medium containing nocodazole ($t = 0$). (B) The same strains and procedure as in A were used, but 10 μ g/ml α -factor was re-added to all cultures at $t = 100'$ after release (at least 90% of budded cells) to prevent them from entering a new cell cycle. At the indicated times cells were collected for FACS[®] analysis of DNA contents (A) and for Western blot analysis of Clb2 and Sic1 protein levels (B). Swi6 was used as loading control. Cyc, cycling cells.

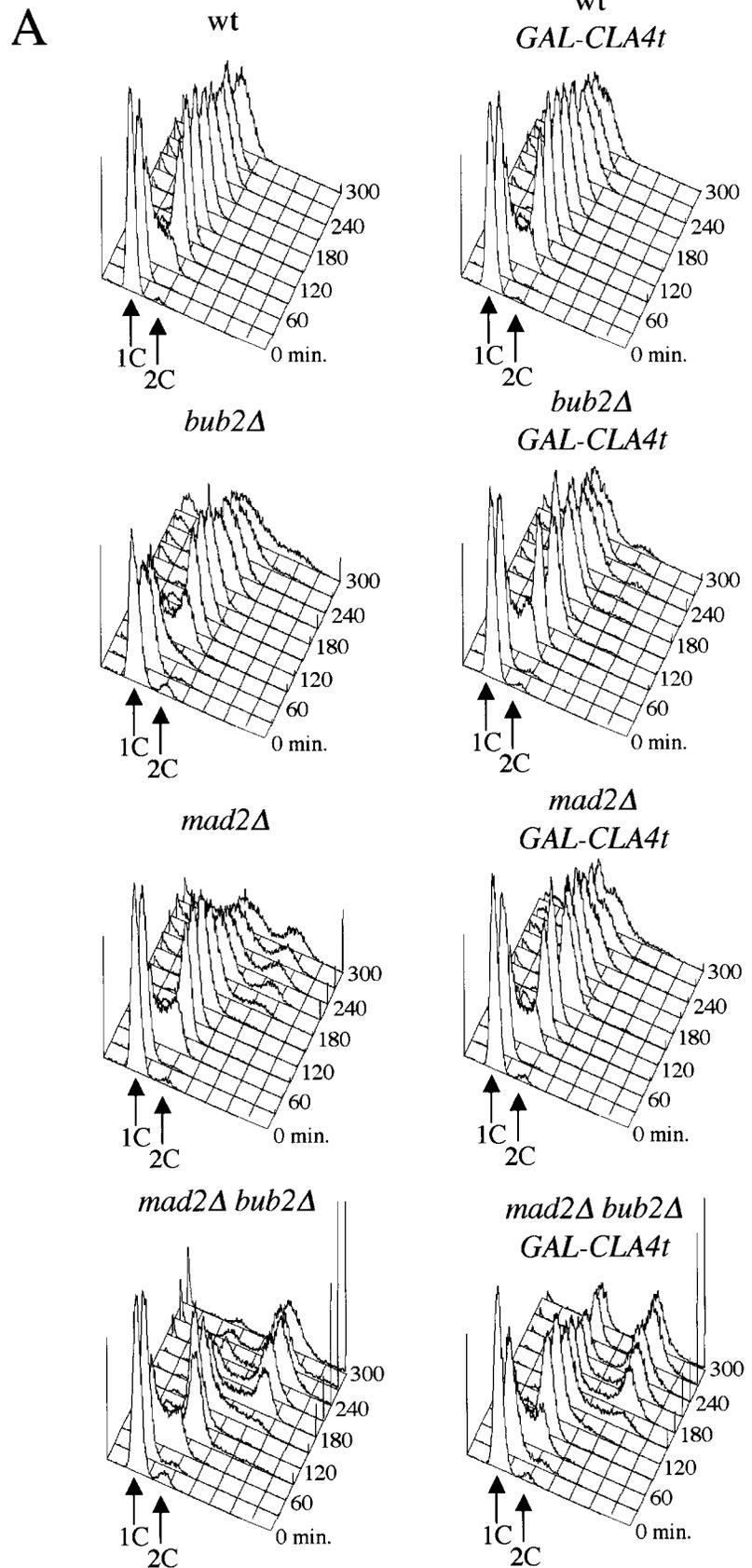
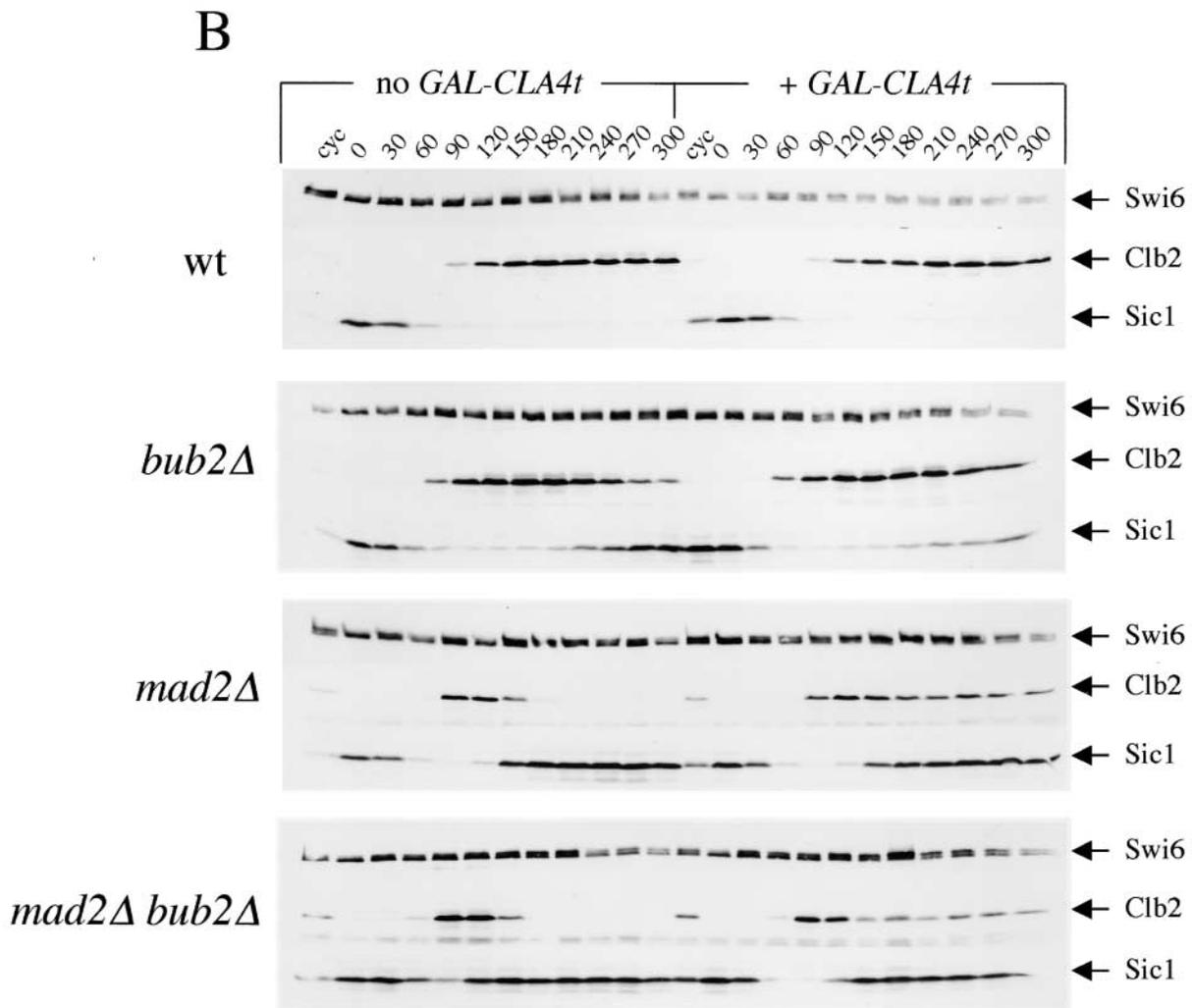


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Because re-replication in the presence of nocodazole depends on inactivation of cyclinB-dependent Cdks by both APC-mediated proteolysis of cyclins and accumulation of

Cdk inhibitors, we analyzed the levels of the main mitotic cyclinB (Clb2) and the Clb/Cdk inhibitor Sic1 in an experiment similar to the one above (Fig. 2 B). Although *bub2* Δ ,



mad2Δ, and *mad2Δ bub2Δ* cells underwent Clb2 proteolysis in the presence of nocodazole as described previously (Alexandru et al., 1999; Fraschini et al., 1999), expression of *GAL-CLA4t* in these mutants protected a fraction of Clb2 from degradation. In addition, Cla4t strongly reduced reaccumulation of Sic1 in *bub2Δ* cells, but only slightly (if at all) in *mad2Δ* and *mad2Δ bub2Δ* cells. This suggests that in the latter mutants, the phosphatase Cdc14, which promotes Sic1 transcription and activation (Visintin et al., 1998), might be active at least for what concerns accumulation of Sic1.

***CLA4t* inhibits PAK kinases and causes Swe1-dependent cell cycle delay**

To gain insights into the molecular mechanism by which Cla4t might prevent inactivation of Clb/Cdk1 kinases in spindle checkpoint mutants, we investigated its effects when four copies of *GAL-CLA4t* were expressed in otherwise wild-type cells, where they almost completely inhibit cell proliferation (Fig. 1 D). Synchronized G1 wild-type cells, either lacking or carrying four integrated *GAL-CLA4t* copies (4X *GAL-CLA4t*), were released in the presence of galactose and analyzed at different time points for DNA contents, kinetics of nuclear division, and cell morphology. 4X *GAL-CLA4t* cells arrested with 2C DNA contents (Fig. 3 A) and undi-

vided nuclei (Fig. 3, B and C), suggesting that not only mitotic exit, but also the onset of anaphase, failed to take place. Indeed, the securin Pds1 was stabilized in these cells (unpublished data), suggesting that Cdc20/APC could be inactive. In addition, cells displayed a dramatic cytokinetic defect due to a failure to form a proper bud neck (Fig. 3 C). This peanut-shaped cell phenotype closely resembled that observed after simultaneous inactivation of *CLA4* and *STE20* (Cvrckova et al., 1995), suggesting that these two redundant PAK kinases might be inactive in galactose-induced 4X *GAL-CLA4t* cells. In agreement with this hypothesis, a reduced fraction of these cells was able to assemble or maintain a proper septin ring at the bud neck (Fig. 3 E), as shown by localization of the Cdc3 septin fused to the GFP (Cdc3-GFP), similarly to what was shown for *cla4 ste20* double mutants (Cvrckova et al., 1995; Holly and Blumer, 1999). In addition, overexpression of *GAL-CLA4t* disrupted localization of both myc-tagged Cla4 at the bud tip and myc-tagged Ste20 on polarized cell surface projections of pheromone-treated cells (Fig. 3 F), despite that it did not affect distribution of cortical actin (unpublished data; see Fig. 6 C). High levels of Cla4t also reduced the ability of endogenous Cla4 to phosphorylate myelin-basic protein in vitro (Fig. 3 D), suggesting that they could compromise Cla4 ki-

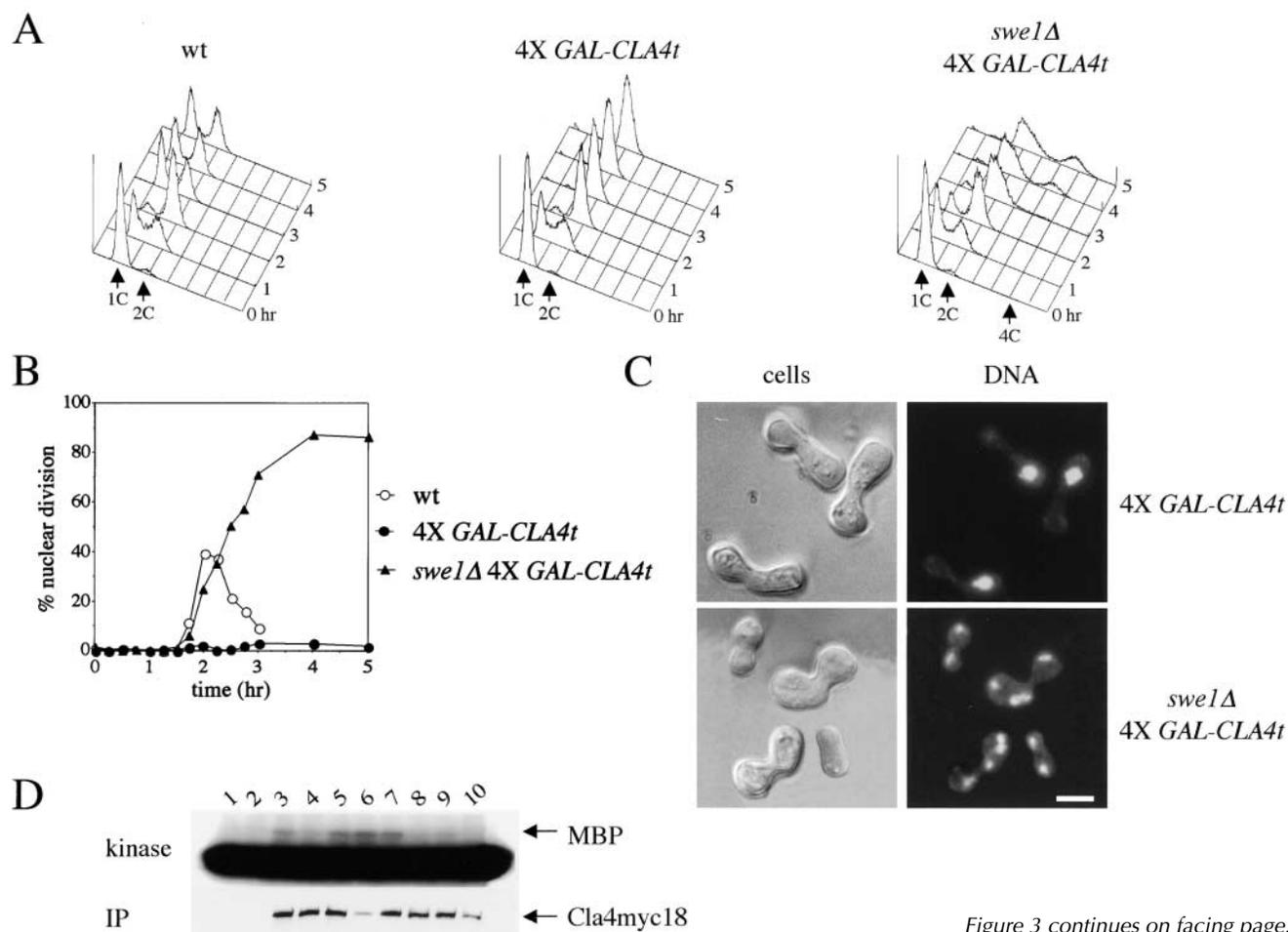


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nase activity in vivo. Finally, deletion of either *CLA4* or *STE20* reduced, but did not abolish, the ability of *bub2Δ* cells to re-replicate in the presence of nocodazole (Fig. 3 G).

We also noticed that the Hsl1 protein kinase, responsible for targeting the Swe1 kinase to degradation (McMillan et al., 1999), was localized at the bud neck in virtually all wild-type budded cells from the onset of budding until late anaphase, but it was homogeneously distributed throughout the cells of the 4X *GAL-CLA4t* strain (Fig. 3 H) and might thus be inactive. Because deletion of *CLA4* was shown to activate the morphogenesis checkpoint, resulting in a G2/M delay due to the Swe1-dependent phosphorylation of Cdc28 (Longtine et al., 2000; Weiss et al., 2000; Mitchell and Sprague, 2001), we asked whether the cell cycle delay caused by overproduced Cla4t could also depend on *SWE1*. Indeed, deletion of *SWE1* allowed synchronized 4X *GAL-CLA4t* cells to undergo nuclear division with nearly wild-type kinetics in the presence of galactose (Fig. 3, B and C), suggesting that Swe1 is responsible for preventing anaphase in these cells. Conversely, lack of Swe1 did not rescue their cytokinetic defects (Fig. 3 C) and was only partially able to drive them out of mitosis and into a new round of DNA replication, as shown by the appearance of cells with 4C DNA contents (Fig. 3 A). Thus, Swe1 restrains mitotic exit in Cla4t-overproducing cells, but additional mechanisms are also involved.

Cla4t ability to suppress spindle checkpoint defects partially depends on Swe1

The data above indicate that Cla4t high levels delay the metaphase–anaphase transition in a Swe1-dependent manner, suggesting that Cla4t and Swe1 could prevent the onset of anaphase by inhibiting more directly the activity of Cdc20/APC. Therefore, we investigated whether expression of one copy of *GAL-CLA4t*, which has only mild effects on cell proliferation (Fig. 1 D) but suppresses spindle checkpoint defects (Fig. 2), might affect Cdc20/APC-dependent proteolysis of Pds1 and Clb2 during an unperturbed cell cycle. As shown in Fig. 4 A, Pds1 was stabilized in Cla4t expressing cells, and this correlated with a slight nuclear division delay (Fig. 4 B). Cla4t also protected a fraction of Clb2 from degradation, whereas no major effects were found on the levels of Sic1 accumulation. Thus, Cdc20/APC, which triggers both Pds1 and Clb2 degradation, might not be fully active in Cla4t expressing cells. Remarkably, deletion of *SWE1* accelerated proteolysis of both Pds1 and Clb2 in these cells, although not to wild-type levels (Fig. 4 A), and restored normal kinetics of nuclear division (Fig. 4 B), suggesting that Swe1 is required for Cla4t to delay the onset of anaphase. The *CDC28^{Y19F}* allele, encoding an unphosphorylatable Cdc28 protein, had an intermediate effect because it reduced Pds1 levels but did not rescue the delay in either nuclear division or Clb2 degradation. However, we could not

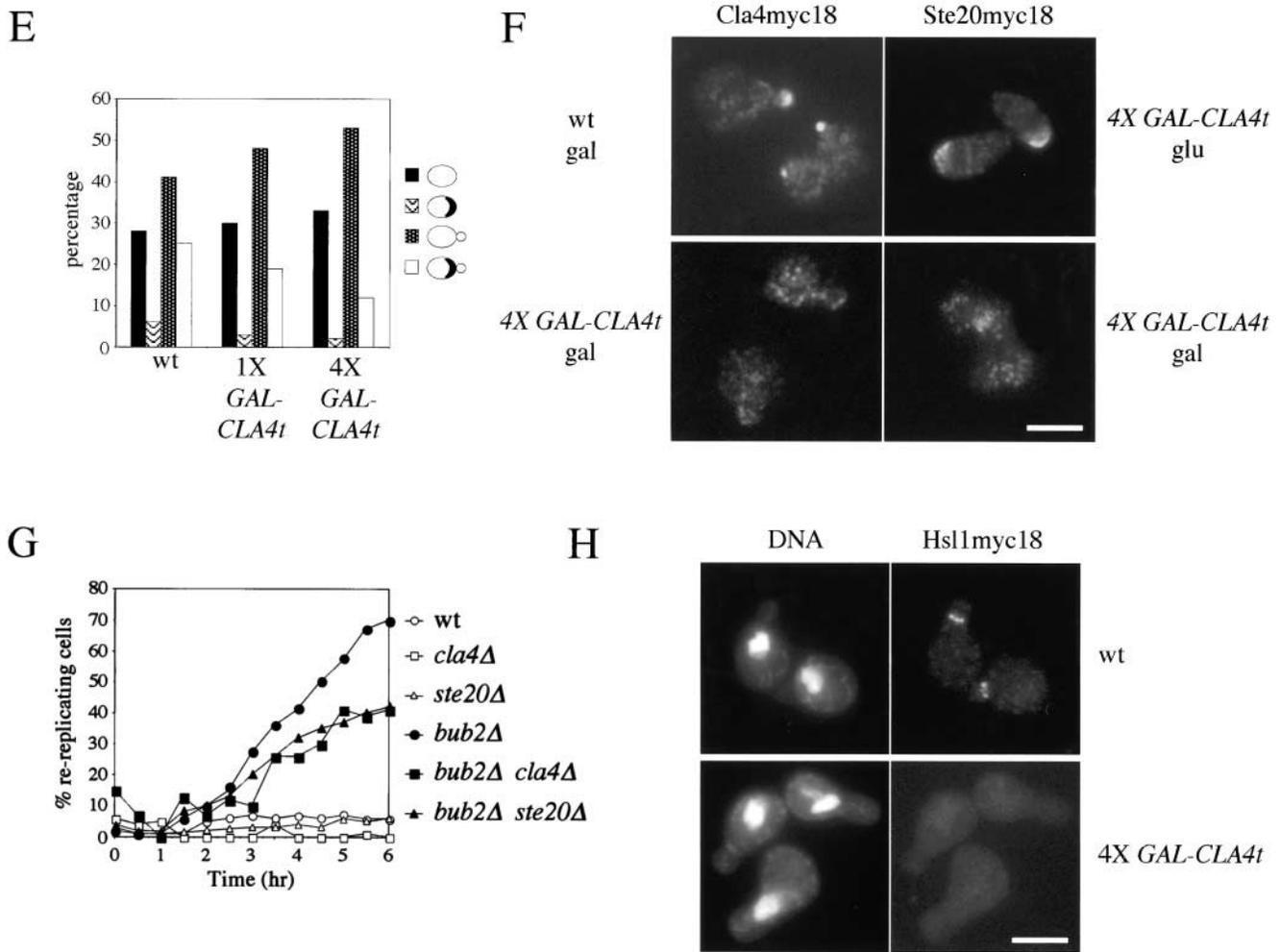


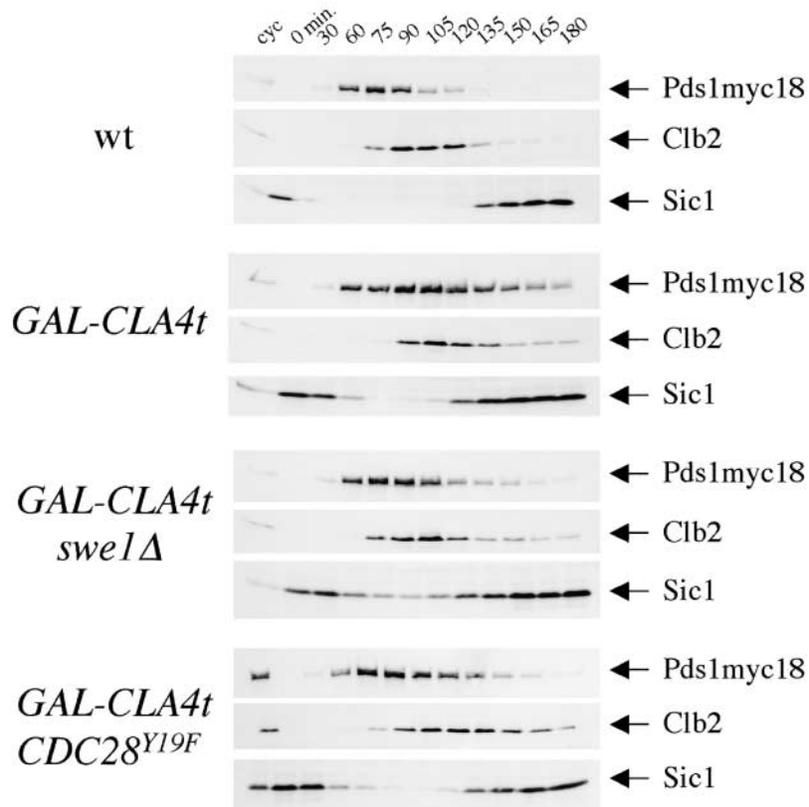
Figure 3. *CLA4t* overexpression inhibits endogenous Cla4 and Ste20 PAK kinases and causes a Swe1-dependent G2/M arrest. (A–C) Wild-type (W303), *4X GAL-CLA4t* (ySP2625) and *swe1Δ 4X GAL-CLA4t* (ySP2711) cells were grown in YEPR, arrested in G1 with α -factor and then released in YEPRG medium ($t = 0$). Samples were collected at the indicated times for FACS[®] analysis of DNA contents (A) and to follow kinetics of nuclear division (B). Photographs were taken at $t = 5$ h after release (C). Bar, 5 μ m. (D) wild-type (ySP3086, lanes 3–6) and *4X GAL-CLA4t* cells (ySP3088, lanes 7–10), expressing myc-tagged Cla4 (Cla4myc18), as well as an untagged strain (W303, lanes 1 and 2) were grown in YEPR (lanes 1, 3, and 7) and then shifted to YEPRG for 2 h (lanes 4 and 8) and 3 h (lanes 5 and 9), or to YEPRG with nocodazole for 2.5 h (lanes 2, 6, and 10). Anti-myc immunoprecipitates from the corresponding cell extracts were subjected to Western blot analysis with anti-myc antibodies (IP) and to kinase assays using myelin basic protein (MBP) as substrate. (E) Wild-type (W303), *1X GAL-CLA4t* (ySP2622) and *4X GAL-CLA4t* (ySP2625) cells carrying a pRS316 plasmid with a *GFP-CDC3* fusion (Vallen et al., 2000) were grown in YEPR and then shifted to YEPRG medium. After 3 h, GFP-Cdc3 was scored in unbudded and budded cells ($n = 500$) to monitor the presence of a septin ring. (F) Left; wild-type (ySP3086) and *4X GAL-CLA4t* (ySP3088) cells, expressing Cla4myc18, were grown in YEPR, arrested in G1 with α -factor, and released in YEPRG ($t = 0$). Samples were collected every 10 min for 2 h for FACS[®] analysis of DNA contents (not depicted) and immunostaining of Cla4myc18. Photographs were taken at $t = 50'$, when wild-type cells reached a peak of small budded cells with cortical Cla4myc18 (30% of total cells). The fraction of *4X GAL-CLA4t* cells with cortical Cla4myc18 staining remained below 1% throughout the time course. Right; *4X GAL-CLA4t* cells expressing myc-tagged Ste20 (Ste20myc18, ySP3091) were grown in YEPR, arrested in G1 with α -factor, and then shifted to either YEPD (glu) or YEPRG (gal) medium containing α -factor for 2 h to maintain the G1 arrest. The fraction of cells displaying polarized localization of Ste20 was 55% in YEPD and 3% in YEPRG ($n = 100$). Bar, 5 μ m. (G) Wild-type (W303), *cla4Δ* (ySP3076), *ste20Δ* (ySP3078), *bub2Δ* (ySP3138), *bub2Δ cla4Δ* (ySP3186), and *bub2Δ ste20Δ* (ySP3198) cells were grown in YEPD, shifted to YEPD containing nocodazole ($t = 0$), and collected at the indicated times for FACS[®] analysis of DNA contents. The percentage of re-replicating cells at each time point was calculated as described in Materials and methods. (H) Wild-type (ySP3157) and *4X GAL-CLA4t* (ySP3202) cells expressing myc-tagged Hsl1 were grown in YEPR, arrested in G1 with α -factor, and released in YEPRG. Samples were collected at different times for immunostaining of Hsl1myc18. Photographs were taken 90' after release. Bar, 5 μ m.

exclude that Swe1 exerts its inhibitory function through Cdc28 phosphorylation because our results could be biased by the fact that the *CDC28^{Y19F}* mutant displays, by itself, reduced Cdc20/APC activity (Rudner et al., 2000).

Next, we asked whether deletion of *SWE1* could also revert the Cla4t-dependent suppression of spindle checkpoint de-

fects. For this purpose, we analyzed the ability of *swe1Δ mad2Δ* or *swe1Δ bub2Δ* strains carrying a single copy of *GAL-CLA4t* to support Clb2 degradation and Sic1 accumulation, as well as to re-replicate DNA, on nocodazole treatment. In a first experiment *GAL-CLA4t*, *mad2Δ*, *mad2Δ GAL-CLA4t*, *mad2Δ swe1Δ GAL-CLA4t*, and *mad2Δ CDC28^{Y19F} GAL-*

A



B

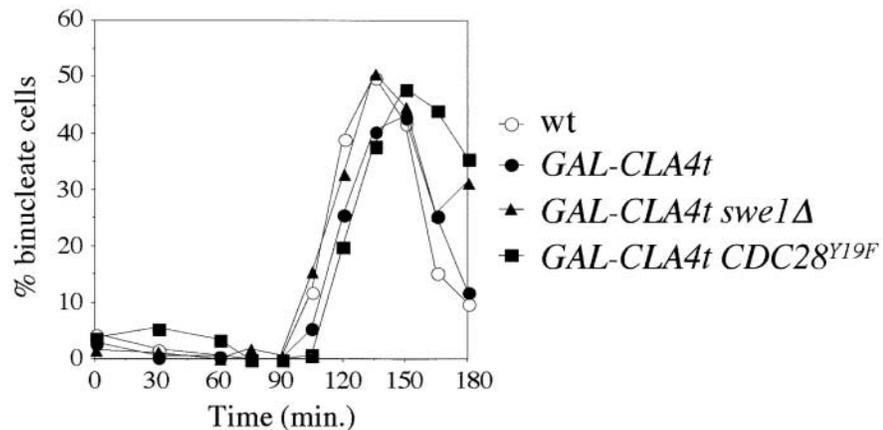


Figure 4. **Expression of *GAL-CLA4t* delays degradation of Cdc20/APC substrates with a mechanism partially dependent on *Swe1*.** Wild-type (ySP1969), 1X *GAL-CLA4t* (ySP3073), *swe1*Δ 1X *GAL-CLA4t* (ySP3098), and *CDC28*^{Y19F} 1X *GAL-CLA4t* (ySP3074) cells expressing myc-tagged securin (Pds1myc18) were grown in YEPR, arrested in G1 with α -factor, and released in YEPRG (t = 0). Samples were collected at the indicated times for Western blot analysis (A) to score nuclear division (B) and for FACS[®] analysis of DNA contents (not depicted). 10 μ g/ml α -factor was added back to the cultures at t = 110'.

CLA4t pheromone-treated G1 cells were released into the cell cycle in the presence of both galactose and nocodazole. As shown in Fig. 2, expression of *CLA4t* prevented a fraction of Clb2 from being degraded, slightly delayed Sic1 accumulation

and prevented most *mad2*Δ cells from rebudding (Fig. 5 A) and re-replicating (unpublished data). Deletion of *SWE1* in these conditions antagonized the effects of Cla4t, in that it accelerated kinetics of Clb2 degradation and Sic1 accumulation

A

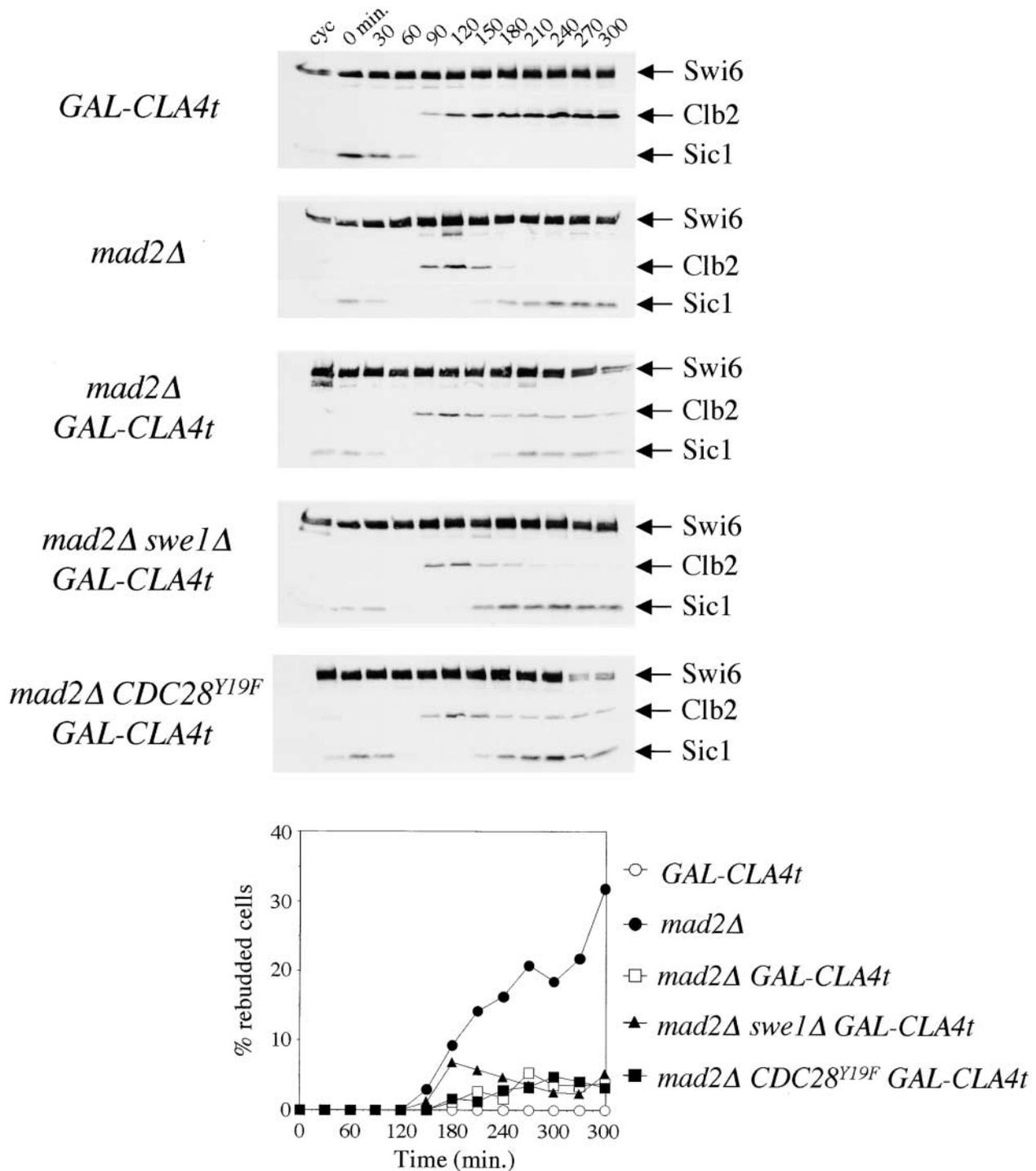


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of *mad2Δ GAL-CLA4t* cells, although not to the levels observed for *mad2Δ* cells (Fig. 5 A). Furthermore, *mad2Δ swe1Δ GAL-CLA4t* rebudded and re-replicated only poorly in these conditions, suggesting that Cla4t prevents Clb2 degradation and re-replication in *mad2Δ* cells both by activating Swe1 and through an additional mechanism. Again, we found that the

CDC28^{Y19F} allele had no major effect on either Clb2 and Sic1 protein levels (Fig. 5 A) or re-replication efficiency of *mad2Δ GAL-CLA4t* cells (unpublished data). Because Mad2 and Bub2 activate the spindle checkpoint via different pathways, we then performed the same analysis on *bub2Δ* cells. As shown in Fig. 5 B, knocking out Swe1 function in *bub2Δ*

B

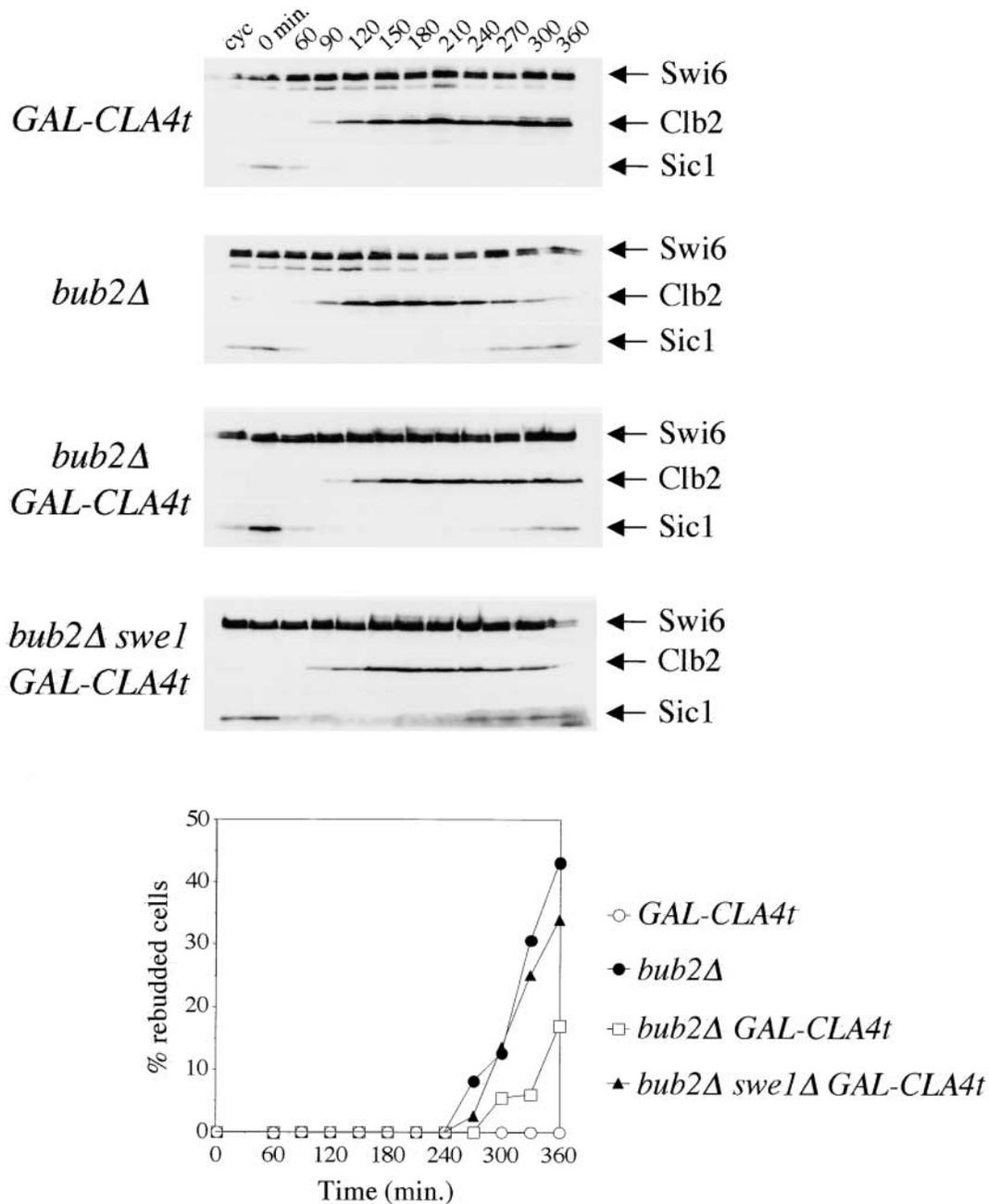


Figure 5. **Swe1 is required for Cla4t-dependent stabilization of Clb2 in nocodazole-treated spindle checkpoint mutants.** (A) *1X GAL-CLA4t* (ySP2622), *mad2Δ* (ySP1070), *mad2Δ 1X GAL-CLA4t* (ySP2752), *mad2Δ swe1Δ 1X GAL-CLA4t* (ySP3145), and *mad2Δ CDC28^{V19F} 1X GAL-CLA4t* (ySP3148) cells (A) or *1X GAL-CLA4t* (2622), *bub2Δ* (ySP3138), *bub2Δ 1X GAL-CLA4t* (ySP2626), and *bub2Δ swe1Δ 1X GAL-CLA4t* (ySP2726) cells (B) were grown in YEPR, arrested in G1 with α -factor, and released in YEPRG ($t = 0$). Samples were collected at the indicated times for Western blot analysis of Clb2 and Sic1 and for FACS[®] analysis of the DNA contents (not depicted). 10 μ g/ml α -factor was added back to the cultures at $t = 120'$. Swi6 was used as loading control. Cyc, cycling cells.

GAL-CLA4t cells restored a checkpoint defect similar to that of *bub2Δ* cells, in terms of Clb2 degradation, Sic1 re-accumulation, and kinetics of rebudding, erasing completely the effects of Cla4t. Thus, Swe1 can cooperate with either the Mad2- or the Bub2-dependent pathways in preventing exit from mitosis. Moreover, the different effects of Cla4t on the

ability of *mad2Δ* and *bub2Δ* cells to enter a new cell cycle on checkpoint activation suggest that Cla4t can inhibit mitotic exit in at least two different ways, one dependent and another independent on Swe1; *mad2Δ* cells would be susceptible to both inhibitory mechanisms, whereas the lack of *BUB2* antagonizes the Swe1-independent mechanism.

PAK kinases contribute to proper MEN activation

Because Cla4t prevents mitotic exit when heavily overproduced in wild-type cells or when cell cycle progression is partially compromised by spindle checkpoint activation, we wondered whether it could affect MEN activation. Indeed, the temperature-sensitive *men* mutants *cdc15-2* and *cdc14-3* were more sensitive than wild type to expression of a single copy of *GAL-CLA4t* at the permissive temperature (Fig. 6 A). Moreover, deletion of *SWE1* did not rescue, but eventually accentuated, the lethality of *cdc15-2 GAL-CLA4t* and *cdc14-3 GAL-CLA4t* cells on galactose-containing plates, suggesting that Cla4t might directly affect MEN activation independently of Swe1. In the attempt to correlate with specific cell cycle defects the poor tolerance of *cdc15-2* and *cdc14-3* mutants to moderately high levels of Cla4t, we arrested in G1 wild-type, *cdc15-2*, and *cdc14-3* cells, either carrying or lacking one copy of *GAL-CLA4t*, and followed the appearance and disappearance of binucleate anaphase cells on release from the G1 block in the presence of galactose at 25°C. Although Cla4t did not seem to affect the kinetics of nuclear division, comparison of the kinetics of disappearance of binucleate cells indicated that it delayed cell division by ~30 min in both *cdc15-2* and *cdc14-3* cells (Fig. 6 B) independently of Swe1 (unpublished data), suggesting that PAK kinases contribute to MEN activation under these conditions. This is not achieved by promoting Bfa1 phosphorylation, which in turn correlates with inhibition of Bfa1/Bub2 GAP activity (Hu et al., 2001) because we found no major differences in the kinetics of Bfa1 phosphorylation between wild-type and *swe1Δ GAL-CLA4t* cells (unpublished data). We then verified whether high levels of Cla4t might displace and therefore inactivate Lte1, as wild-type Cla4 has been recently implicated in the correct localization of the Tem1 activator Lte1 at the bud cortex (Hofken and Schiebel, 2002; Seshan et al., 2002). Indeed, overexpression of *CLA4t* in 4X *GAL-CLA4t* cells, where we assume Cla4 and Ste20 are completely inactive, abolished polarized Lte1 but not actin distribution at the bud cortex (Fig. 6 C). Again, deletion of *SWE1* did not reestablish proper Lte1 localization (unpublished data). Expression of a single copy of *GAL-CLA4t* slightly decreased the percentage of small- and medium-size budded cells with polarized Lte1 (70% in small/medium budded *GAL-CLA4t* cells versus 90% of wild-type cells). Thus, moderately high levels of Cla4t, although not completely inhibiting endogenous Cla4 and Ste20, might be sufficient to delay Tem1 activation at the end of mitosis in cells whose MEN activity is partially compromised. If partial Tem1 inactivation due to PAK kinases inhibition were the only reason for the mitotic exit delay caused by Cla4t in the absence of Swe1, one would predict that Tem1 activation could be rescued by eliminating its GAP Bfa1/Bub2, thereby counteracting the effect of Lte1 displacement from the bud cortex. To test this notion, we analyzed the effects of *BUB2* deletion on cell cycle progression of *swe1Δ* 4X *GAL-CLA4t*, which we previously showed to be only partially able to exit mitosis (Fig. 3 A). Deletion of *BUB2* by its own had negligible effects on the cell cycle block of galactose-induced 4X *GAL-CLA4t* cells, that arrested with 2C DNA contents (Fig. 7 A), undivided nuclei and metaphase spindles (Fig. 7 B). Strikingly, *bub2Δ swe1Δ*

4X *GAL-CLA4t* cells exited mitosis much faster than *swe1Δ* 4X *GAL-CLA4t* cells, entering a new round of DNA replication (Fig. 7 A) and disassembling their anaphase spindles with nearly wild-type kinetics, thus accumulating as multinucleate cells (Fig. 7 B). We obtained similar results by analyzing the effects of *SWE1* and *BUB2* deletion in the temperature-sensitive *cla4-75 ste20* double mutant (Cvrckova et al., 1995), although Bub2 seemed to have a predominant role over Swe1 in preventing mitotic exit at 37°C (unpublished data). Therefore, PAK kinases appear to control MEN activation by at least two different mechanisms; one operating at the metaphase to anaphase transition and that can be inhibited by Swe1, and the other impinging more directly on Tem1 activation.

Discussion

Inhibition of PAK kinases by Cla4t

We have shown that overexpression of the truncated dominant-negative *CLA4t* allele prevents cell cycle progression of nocodazole-treated spindle checkpoint mutants and causes a mitotic arrest very reminiscent of the one reported for *cla4 ste20* double mutants (Cvrckova et al., 1995). In fact, cells have duplicated chromosomes, but fail to undergo nuclear division and mitotic exit, and display a characteristic peanut-shaped morphology due to their inability to form a proper bud neck constriction in preparation to cytokinesis. This correlates with a slight defect in assembling the septin ring, but presumably other as yet unidentified cytokinetic processes are also impaired. Importantly, polar localization of both endogenous Cla4 and Ste20 is disrupted in *CLA4t*-overexpressing cells, and the in vitro kinase activity of immunoprecipitated endogenous Cla4 is reduced under the same conditions. The *CLA4t* gene product, which lacks part of the COOH-terminal kinase domain, but still contains protein-protein interaction motifs in the NH₂-terminal part, might directly bind to endogenous Cla4 and Ste20 and therefore inhibit them. In fact, mammalian PAK kinases have been shown to dimerize and autoinhibit in trans, with the NH₂-terminal portion of one subunit inhibiting the catalytic domain of the other (Parini et al., 2002). Although immunoprecipitation assays did not reveal physical interactions between wild-type Cla4 and either itself or Ste20 (unpublished data), we cannot exclude that interaction is labile and stabilized when Cla4t acts as a partner in the dimers. Cla4t might also act by sequestering Cdc42 in an inactive form, resulting in the lack of activation of Cdc42 targets other than Cla4 and Ste20. If this were the case, we would expect to alleviate the effects of Cla4t overproduction by replacing wild-type *CDC42* with the *cdc42^{V36A}* allele, which impairs more efficiently interaction of Cdc42 with Cla4 than with other effectors (Gladfelter et al., 2001), and should therefore reduce the capacity of Cla4t to titrate Cdc42 out, allowing it to activate other targets. Conversely, we found that not only did the *cdc42^{V36A}* allele not restore the ability to progress through the cell cycle in *bub2Δ GAL-CLA4t* cells treated with nocodazole, but also decreased by its own the ability of *bub2Δ* cells to re-replicate under these conditions (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200209097>). In addition, concomitant overexpression of *CDC42* from the *GAL1* promoter does not counteract the

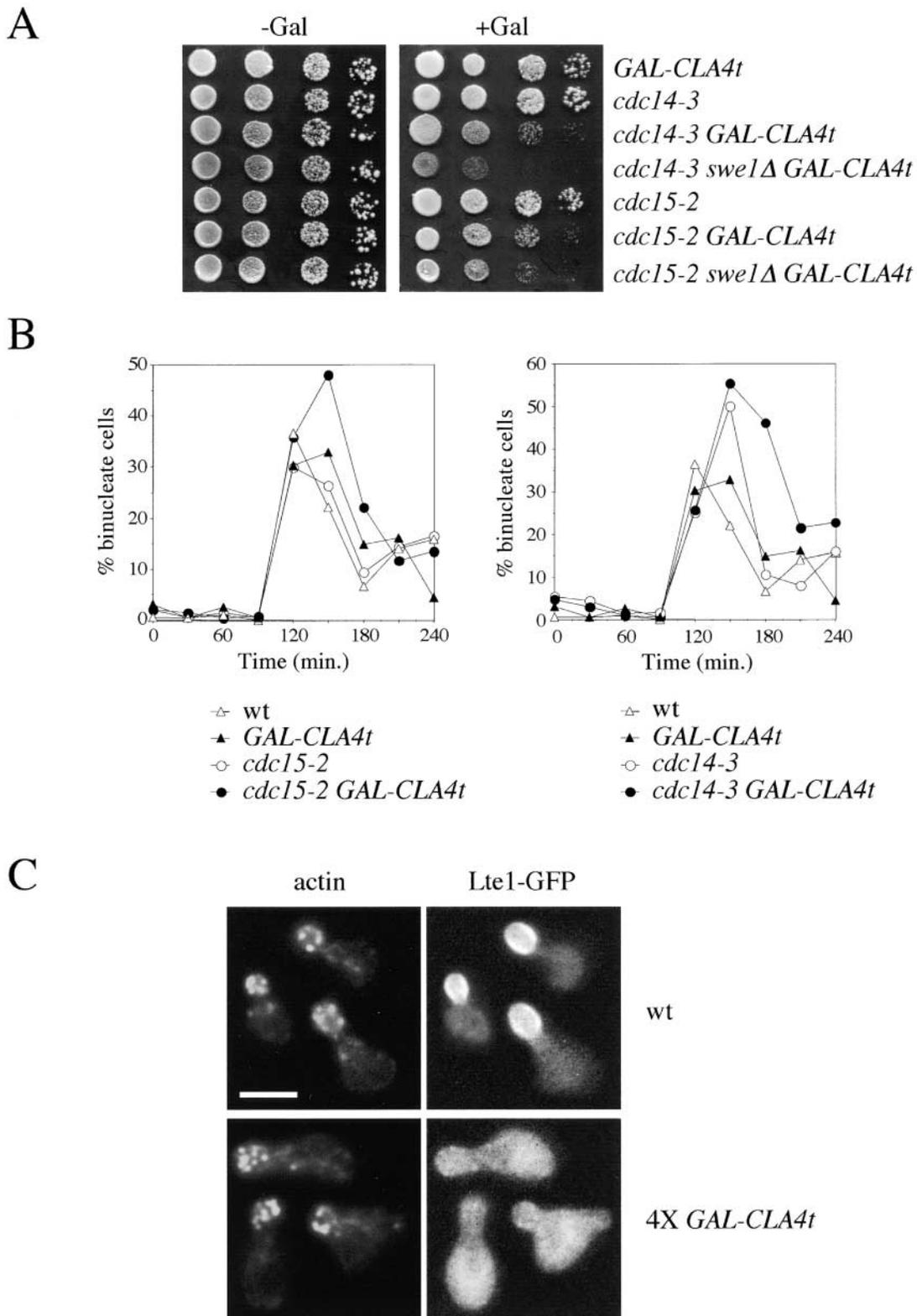


Figure 6. ***GAL-CLA4t* expression delays MEN activation and prevents proper localization of Lte1 at the bud cortex.** (A) Serial dilutions of 1X *GAL-CLA4t* (ySP2622), *cdc14-3* (ySP284), *cdc14-3* 1X *GAL-CLA4t* (ySP3028), *cdc14-3 swe1Δ* 1X *GAL-CLA4t* (ySP3047), *cdc15-2* (ySP51), *cdc15-2* 1X *GAL-CLA4t* (ySP3022), and *cdc15-2 swe1Δ* 1X *GAL-CLA4t* (ySP3023) strains were spotted on either YEPR (–Gal) or YEPRG (+Gal) plates and incubated for 2 d at 26°C. (B) Wild-type, *cdc15-2*, and *cdc14-3* cells either lacking (W303, ySP51, and ySP284) or carrying (ySP2622, ySP3022, and ySP3028) one copy of *GAL-CLA4t* were grown in YEPR, arrested in G1 with α -factor, and released in YEPRG ($t = 0$). Samples were collected every 30' for FACS[®] analysis of the DNA contents (not depicted) and to score nuclear division. (C) Wild-type (ySP3222), 1X *GAL-CLA4t* (ySP3220, not depicted), and 4X *GAL-CLA4t* (ySP3221) cells were treated as in B. Samples were collected every 15' for 150' for FACS[®] analysis of the DNA contents (not depicted) and to score localization of GFP-Lte1 and actin by rhodamine-phalloidin. Photographs were taken 75' after release. Bar, 5 μ m.

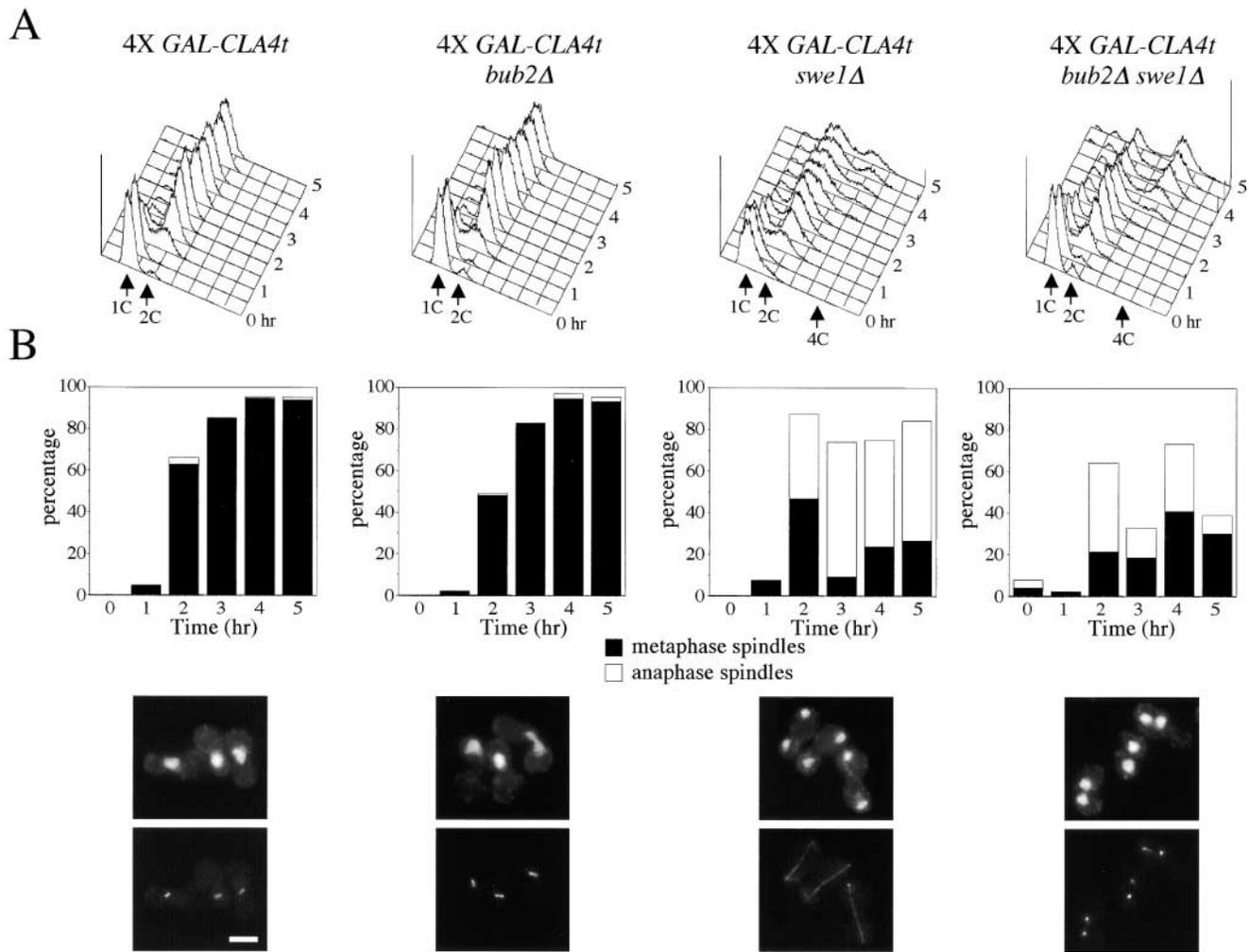


Figure 7. **Bub2 and Swe1 act synergistically in regulating mitotic exit.** 4X *GAL-CLA4t* (ySP2625), *bub2*Δ 4X *GAL-CLA4t* (ySP2630), *swe1*Δ 4X *GAL-CLA4t* (ySP2711), and *bub2*Δ *swe1*Δ 4X *GAL-CLA4t* (ySP2728) cells were grown in YEPR, arrested in G1 with α -factor, and released in YEPRG medium ($t = 0$). Samples were collected at the indicated times for FACS[®] analysis of DNA contents (A) and to follow kinetics of spindle assembly/disassembly by α -tubulin immunostaining (B). Photographs were taken 3 h after release. Bar, 5 μ m.

toxicity caused by high levels of Cla4t (unpublished data). It is also worth noting that polarized actin distribution, which is affected in cells lacking either functional Cdc42 (Adams et al., 1990) or Cdc42 effectors other than Cla4 and Ste20, such as Gic1 and Gic2 (Brown et al., 1997; Chen et al., 1997), is not disrupted by either overexpression of Cla4t (Fig. 6 C) or inactivation of both Cla4 and Ste20 (Cvrckova et al., 1995). Therefore, we propose that even if Cla4t can sequester Cdc42, its effects on cell cycle progression might be at least partially attributed to inhibition of wild-type Cla4 and Ste20. Consistently, we show that deletion of either *CLA4* or *STE20* can reduce the fraction of *bub2*Δ cells that re-replicate in the presence of nocodazole (Fig. 3 G). Therefore, we think it is reasonable to assume that endogenous Cla4 and Ste20 are inactive in cells overproducing Cla4t, although we have not formally proven it.

PAK kinases and Swe1 regulate both the onset of anaphase and mitotic exit

In agreement with previous data (Longtine et al., 2000; Weiss et al., 2000; Mitchell and Sprague, 2001), we have

shown that inactivation of Cla4 and Ste20 by Cla4t induces a Swe1-dependent G2/M delay, whose extent depends on Cla4t levels. This is likely the consequence of morphogenesis checkpoint activation (Lew and Reed, 1995) caused by septin ring defects and mislocalization of Hsl1 (Fig. 3, E and H), which normally targets Swe1 to degradation (McMillan et al., 1999; Longtine et al., 2000). Swe1 inactivates Clb1–4/Cdks by inhibitory phosphorylation of their catalytic subunit Cdc28 on tyrosine 19 (Booher et al., 1993). How do Clb1–4/Cdc28 kinases bring about the onset of anaphase? One process they definitely regulate is spindle assembly because inactivation of all mitotic cyclin genes *CLB1–4*, as well as overexpression of *SWE1* or the presence of a *CDC28* allele mimicking constitutive tyrosine 19 phosphorylation, inhibit separation of the duplicated SPBs and bipolar spindle assembly (Surana et al., 1991; Fitch et al., 1992; Lim et al., 1996). However, this is probably not the only anaphase mechanism controlled by Clb1–4/Cdks. In fact, we have shown that Swe1 is responsible for stabilizing the securin Pds1 on moderate *CLA4t* overexpression, when cells undergo normal cycles of

spindle assembly/disassembly. In addition, Swe1 protects a fraction of Clb2 from degradation in spindle checkpoint mutants treated with nocodazole, i.e., in conditions where a spindle is not formed anyway. Both observations might be explained if Swe1 had a role in controlling Cdc20/APC activity. In fact, Cdc20/APC not only drives Pds1 (Visintin et al., 1997; Lim et al., 1998) and a first wave of Clb2 degradation (Baumer et al., 2000; Yeong et al., 2000; Wasch and Cross, 2002), but Cdc28 kinase activity is also necessary for full Cdc20/APC activation (Rudner et al., 2000; Rudner and Murray, 2000). Therefore, Swe1, by phosphorylating Cdc28, might prevent proper Cdc20/APC-dependent degradation of both Pds1 and Clb2, which is a prerequisite for full MEN activation at the end of mitosis (Cohen-Fix and Koshland, 1999; Tinker-Kulberg and Morgan, 1999; Yeong et al., 2000; Wasch and Cross, 2002). This hypothesis (depicted in Fig. 8) is consistent with our finding that Cla4t can delay mitotic exit of *men* mutants at permissive temperature and of nocodazole-treated spindle checkpoint mutants through a mechanism partially dependent on Swe1. Despite that the only known target of Swe1 thus far is Cdc28, we observed only a minor restoration of Clb2 degradation in cells expressing *CLA4t* in the presence of the *CDC28^{Y19F}* allele, which relieves the inhibitory function of Swe1. Thus, either Swe1 phosphorylates targets other than Cdc28, or Swe1-dependent inhibition of Clb2 proteolysis might still be mediated by Cdc28 phosphorylation, but we cannot properly see it because unphosphorylatable Cdc28 displays reduced kinase levels and Cdc20/APC activity (Rudner et al., 2000). Because a *CDC28^{T18VY19F}* allele impairs the physical association between Cdc20 and APC in a *cdc15* telophase arrest (Rudner et al., 2000), we also investigated whether the *CDC28^{Y19F}* allele or overexpression of either *SWE1* or *CLA4t* might similarly affect Cdc20/APC interaction. We found no major differences with a wild-type control (unpublished data), suggesting that other mechanisms, like phosphorylation of

APC subunits, might influence the activity of the complex. A reduced Cdc20/APC activity could also explain why expression of a single copy of *GAL-CLA4t*, which barely delays the onset of anaphase in wild-type cells, can almost completely prevent *mad2Δ* and *bub2Δ* mutants from overriding a checkpoint arrest. In fact, Cla4t-dependent inhibition of Cdc20/APC would counteract its activation due to the lack of Mad2, thus reinforcing MEN inhibition operated by Bub2/Bfa1 (Fig. 8). Less intuitive is how reduced Cdc20/APC activity could prevent *bub2Δ* and *bfa1Δ* cells from exiting mitosis in the presence of nocodazole because Cdc20 should be kept inactive by Mad2 and Mad3 (Hwang et al., 1998; Fraschini et al., 2001; Hoyt, 2001). Remarkably, we have found that the ability of *bub2Δ* cells to separate sister chromatids and re-replicate in nocodazole requires Cdc20 function (Fig. S2), indicating that there might exist a pool of Cdc20 refractory to inhibition by Mad2 and Mad3.

Despite that the deletion of *SWE1* accelerates Clb2 degradation and mitotic exit in cells expressing *GAL-CLA4t*, it does not rescue wild-type kinetics of mitotic exit either on heavy overproduction of Cla4t or in nocodazole-treated *mad2Δ* cells moderately overproducing Cla4t, suggesting that inhibition of PAK kinases by Cla4t might prevent MEN activation also by Swe1-independent means. We find that overexpression of *CLA4t* prevents Lte1 cortical localization, thus down-regulating the MEN, consistent with the recent finding that Cla4 phosphorylates and targets Lte1 to the bud cortex, thereby allowing timely activation of Tem1 (Hofken and Schiebel, 2002; Seshan et al., 2002). However, this cannot be the only level of MEN regulation exerted by PAK kinases, as *LTE1* is not essential at the temperatures we performed our experiments (Adames et al., 2001). Indeed, deletion of *BUB2*, that allows Tem1 activation in the absence of Lte1, although not sufficient by its own to drive *CLA4t* overexpressing cells out of mitosis, greatly stimulates mitotic exit of *swe1Δ GAL-CLA4t* cells, indicating that *SWE1* deletion and *TEM1* activation act synergistically to fully activate the MEN, as proposed in Fig. 8. Interestingly, Hofken and Schiebel (2002) have reported a role for Ste20 in MEN activation unrelated to Lte1 association with the bud cortex. An intriguing hypothesis that would match our data is that Ste20 is implicated in down-regulating Swe1, thereby allowing full activation of Cdc20/APC at the onset of anaphase and the early release of Cdc14 from the nucleolus.

Based on our data, the PAK kinases Cla4 and Ste20 might be considered new members of the MEN. Remarkably, the *Schizosaccharomyces pombe* septation initiation network, which has similar organization to the MEN (Bardin and Amon, 2001; McCollum and Gould, 2001), includes the Sid1 PAK kinase (Guertin et al., 2000), and overexpression of the Sid1-interacting factor Cdc14 (unrelated to its *Saccharomyces cerevisiae* namesake) causes a G2 arrest that likely depends on the Swe1-like kinase Wee1 (Fankhauser and Simanis, 1993).

Interdependency between mitotic exit and cytokinesis

In budding yeast, the site of cell division is already established at the G1/S transition, concomitantly with bud emer-

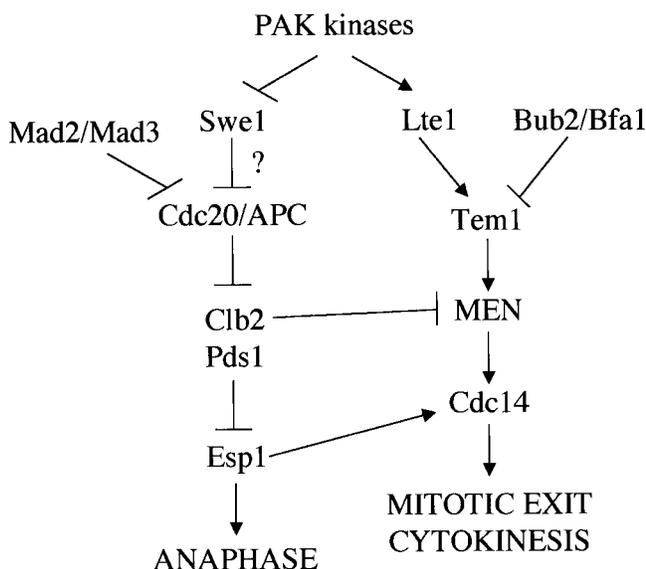


Figure 8. A model for the role of PAK kinases in controlling mitotic progression. See text for details.

gence. During this process, the actin cytoskeleton undergoes profound rearrangements, so that actin patches get concentrated at the tip of the bud and actin cables orient toward it. At this cell cycle stage, the septin ring is also formed (Cooper and Kiehart, 1996; Longtine et al., 1996) and a contractile actomyosin ring starts assembling (Bi et al., 1998).

A dependency of cytokinesis on mitotic exit and inactivation of cyclinB/Cdks has long been established both in yeast and in other eukaryotic systems (Field et al., 1999; Surana et al., 2002). In addition, the requirement of Cdc20/APC-dependent proteolysis for MEN activation dictates a hierarchical order of events, where nuclear division occurs before mitotic exit, which is in turn a prerequisite for cytokinesis. Our data lead to the unforeseen conclusion that cytokinetic

events can influence the kinetics of mitotic exit by allowing full activation of Cdc20/APC and by promoting cortical localization of *Lte1* (Fig. 8). In summary, to exit mitosis, two conditions must be satisfied: (1) Cdc20/APC must be fully active to promote Pds1 and the first wave of Clb1–4 degradation. In fact, Pds1 inhibits the early release of Cdc14 from the nucleolus (Stegmeier et al., 2002; Yoshida et al., 2002), which together with the reduction in Clb/Cdks contributes to activation of Cdc15, and in turn, Cdc14 itself (Jaspersen and Morgan, 2000). Moreover, PAK and Clb1–4/Cdc28 kinases, as well as extinction of checkpoint signals, are necessary for complete activation of Cdc20/APC; and (2) nuclear division must take place along the mother-bud axis, thus allowing Tem1, which is localized at SPBs, to access *Lte1* con-

Table I. Table of strains

Name	Relevant genotype
ySP51	<i>MATa, cdc15-2</i>
ySP284	<i>MATa, cdc14-3</i>
ySP1070	<i>MATa, mad2::TRP1</i>
ySP1071	<i>MATa, bub2::HIS3</i>
ySP1086	<i>MATa, mad2::TRP1, bub2::HIS3</i>
ySP1243	<i>MATa, bfa1::KITRP1</i>
ySP1969	<i>MATa, pds1::PDS1-myc18::LEU2</i>
ySP2457	<i>MATa, cdc20::MET3::HA3-CDC20::TRP1, his3::HIS3::tetR-GFP, ura3::URA3::336XtetO</i>
ySP2622	<i>MATa, ura3::1X URA3::GAL1-CLA4t</i>
ySP2623	<i>MATa, ura3::2X URA3::GAL1-CLA4t</i>
ySP2624	<i>MATa, ura3::3X URA3::GAL1-CLA4t</i>
ySP2625	<i>MATa, ura3::4X URA3::GAL1-CLA4t</i>
ySP2626	<i>MATa, bub2::HIS3, ura3::1X URA3::GAL1-CLA4t</i>
ySP2630	<i>MATa, bub2::HIS3, ura3::4X URA3::GAL1-CLA4t</i>
ySP2631	<i>MATa, cdc20::MET3::HA3-CDC20::TRP1, bub2::HIS3, his3::HIS3::tetR-GFP, ura3::URA3::336XtetO</i>
ySP2634	<i>MATa, bub2::HIS3, his3::HIS3::tetR-GFP, ura3::URA3::336XtetO</i>
ySP2711	<i>MATa, swe1::LEU2, ura3::4X URA3::GAL1-CLA4t</i>
ySP2726	<i>MATa, bub2::HIS3, swe1::LEU2, ura3::1X URA3::GAL1-CLA4t</i>
ySP2728	<i>MATa, bub2::HIS3, swe1::LEU2, ura3::4X URA3::GAL1-CLA4t</i>
ySP2752	<i>MATa, mad2::TRP1, ura3::1X URA3::GAL1-CLA4t</i>
ySP3022	<i>MATa, cdc15-2, ura3::1X URA3::GAL1-CLA4t</i>
ySP3023	<i>MATa, cdc15-2, swe1::LEU2, ura3::1X URA3::GAL1-CLA4t</i>
ySP3028	<i>MATa, cdc14-3, ura3::1X URA3::GAL1-CLA4t</i>
ySP3047	<i>MATa, cdc14-3, swe1::LEU2, ura3::1X URA3::GAL1-CLA4t</i>
ySP3068	<i>MATa, mad2::TRP1, bub2::HIS3, ura3::1X URA3::GAL1-CLA4t</i>
ySP3073	<i>MATa, ura3::1X URA3::GAL1-CLA4t, pds1::PDS1-myc18::LEU2</i>
ySP3074	<i>MATa, cdc28::CDC28^{Y19F}, ura3::1X URA3::GAL1-CLA4t, pds1::PDS1-myc18::LEU2</i>
ySP3076	<i>MATa, cla4::kanMX4</i>
ySP3078	<i>MATa, ste20::kanMX4</i>
ySP3086	<i>MATa, cla4::CLA4-myc18::KITRP1</i>
ySP3088	<i>MATa, cla4::CLA4-myc18::KITRP1, ura3::4X URA3::GAL1-CLA4t</i>
ySP3091	<i>MATa, ste20::STE20-myc18::KITRP1, ura3::4X URA3::GAL1-CLA4t</i>
ySP3098	<i>MATa, swe1::LEU2, ura3::1X URA3::GAL1-CLA4t, pds1::PDS1-myc18::LEU2</i>
ySP3138	<i>MATa, bub2::HIS3</i>
ySP3145	<i>MATa, mad2::TRP1, swe1::LEU2, ura3::1X URA3::GAL1-CLA4t</i>
ySP3148	<i>MATa, mad2::TRP1, cdc28::CDC28^{Y19F}, ura3::1X URA3::GAL1-CLA4t</i>
ySP3157	<i>MATa, hsl1::HSL1-myc18::KITRP1</i>
ySP3186	<i>MATa, bub2::HIS3, cla4::kanMX4</i>
ySP3198	<i>MATa, bub2::HIS3, ste20::kanMX4</i>
ySP3202	<i>MATa, ura3::4X URA3::GAL1-CLA4t, hsl1::HSL1-myc18::KITRP1</i>
ySP3220	<i>MATa, ura3::1X URA3::GAL1-CLA4t, kanMX6::GAL1-GFP-LTE1</i>
ySP3221	<i>MATa, ura3::4X URA3::GAL1-CLA4t, kanMX6::GAL1-GFP-LTE1</i>
ySP3222	<i>MATa, kanMX6::GAL1-GFP-LTE1</i>

strained in the bud (Bardin et al., 2000; Pereira et al., 2000) with a mechanism dependent on Cla4 (Hofken and Schiebel, 2002; Seshan et al., 2002; this work). Alternatively, inhibition of Bub2/Bfa1 by Cdc5 is sufficient to allow Tem1 activation (Adames et al., 2001; Hu et al., 2001). Such an intricate network of controls would guarantee the correct order of mitotic events and the dependency of mitotic exit on both nuclear division and cytokinesis. This latter feature might play a particularly relevant role in organisms like budding yeast, which specify the site of cytokinesis early in the cell cycle, before spindle assembly and orientation. Interestingly, a checkpoint control involving both Wee1 and the septation initiation network monitors proper accomplishment of early cytokinetic events in fission yeast (Liu et al., 2000), suggesting that it might be a common feature in eukaryotic cells.

Materials and methods

Strains, media, and reagents

All yeast strains (Table I) were derivatives of or were back-crossed at least three times to W303 (*ade2-1, trp1-1, leu2-3,112, his3-11,15, ura3, ssd1*), except for ySP3220, ySP3221, and ySP3222, which are congeneric with each other. Cells were grown in YEP medium (1% yeast extract, 2% bacto-peptone, 50 mg/l adenine) supplemented with 2% glucose (YEPD), 2% raffinose (YEPR), or 2% raffinose and 1% galactose (YEPRG). Unless otherwise stated, α -factor, nocodazole, and benomyl were used at 2 μ g/ml, 15 μ g/ml, and 12.5 μ g/ml, respectively. Synchronization experiments were performed at 25°C, and unless otherwise stated, galactose was added half an hour before release from α -factor.

Screen for high copy number suppressors of *bub2* Δ

A *leu2 bub2* Δ mutant (ySP1071) was transformed with a genomic library constructed in the *LEU2* YEp13 2 μ plasmid (from K. Nasmyth, Institute of Molecular Pathology, Vienna, Austria). 26,000 *Leu*⁺ transformants were replica-plated twice on YEPD plates containing benomyl 20 μ g/ml. Plasmids were recovered from benomyl-resistant clones and used to transform again the original *bub2* Δ strain. Sequencing of both junctions of the inserts of 48 plasmids that confirmed suppression allowed to group them into 23 different classes. We then transformed with one plasmid for each class the *tub2-405* mutant (Huffaker et al., 1988), whose benomyl sensitivity is due to defective β -tubulin. Plasmids increasing the benomyl resistance of *tub2-405* cells were discarded, thus leaving eight plasmids specifically rescuing the benomyl sensitivity of *bub2* Δ cells.

Plasmid constructions and genetic manipulations

Standard techniques were used for genetic manipulations (Sherman, 1991; Maniatis et al., 1992). Wild-type and truncated *CLA4* were amplified by PCR from genomic DNA and subcloned in the BamHI site of Yep13, to generate pSP150 and pSP164, respectively. To clone *CLA4t* under the *GAL1-10* promoter (plasmid pSP176) a BglII PCR product containing the *CLA4t* coding region was cloned in the BamHI site of a *GAL1-10*-bearing Ylplac211 vector. pSP176 integration was directed to the *URA3* locus by Apal digestion. Copy number of the integrated plasmid was verified by Southern analysis. *CLA4* and *STE20* chromosomal deletions were generated by one-step gene replacement (Wach et al., 1994). *CLA4*, *STE20*, *HSL1*, and *BFA1* were tagged immediately before the stop codon by one-step gene tagging (Knop et al., 1999). The *GAL-GFP-LTE1* construct (Pereira et al., 2002) is a gift from E. Schiebel (The Paterson Institute for Cancer Research, Manchester, UK). The *cdc42^{V36A}, swe1::LEU2*, and *tub2-405* mutants were provided by D. Lew (Duke University Medical Center, Durham, NC), M.L. Agostoni Carbone (Dipartimento di Genetica, Milano, Italy), and T. Huffaker (Cornell University, Ithaca, NY), respectively.

Immunoprecipitations, kinase assays, and Western blot analysis

Cla4-myc18 was immunoprecipitated from 1 mg of total extract by protein A-sepharose beads cross-linked to anti-myc antibodies, and kinase assays were performed as described previously (Benton et al., 1997). For Western blot analysis, protein extracts were prepared according to Surana et al. (1993). Proteins transferred to Protran[®] membranes (Schleicher and Schuell) were probed with 9E10 mAb for myc-tagged Pds1, and with pAbs

against Clb2, Sic1, and Swi6. Anti-Clb2 and anti-Sic1 antibodies were provided by W. Zachariae (Max Planck Institute of Molecular Cell Biology, Dresden, Germany) and M. Tyers (Department of Molecular and Medical Genetics, Toronto, Canada), respectively. Secondary antibodies were purchased from Amersham Biosciences, and proteins were detected by an ECL system according to the manufacturer.

Other techniques

Flow cytometric DNA quantitation was determined according to Fraschini et al. (1999) on a Becton Dickinson FACScan[™]. Due to a dramatic cytotoxic defect caused by *CLA4* deletion in our genetic background, it was hard to distinguish on the FACS[®] histograms the fraction of *cla4* Δ cells that displayed DNA contents higher than 2C due to the ability to re-replicate in the presence of nocodazole from the fraction of multinucleate cells resulting from a cytokinesis failure. Therefore, the values plotted in Fig. 3 G were obtained by subtracting at each time point the percentage of microscopically scored cell clusters from the percentage of cells with DNA contents higher than 2C measured with CELLQuest[™] software. Nuclear division was scored with a fluorescent microscope on cells stained with propidium iodide. In situ immunofluorescence was performed according to Fraschini et al. (1999). Immunostaining of α -tubulin was performed with the YOL34 mAb (Serotec) followed by indirect immunofluorescence using rhodamine-conjugated anti-rat Ab (1:100; Pierce Chemical Co.). Immunostaining of Cla4myc18, Ste20myc18, and Hsl1myc18 was detected by incubation with the 9E10 mAb followed by indirect immunofluorescence using CY3-conjugated goat anti-mouse Ab (1:500; Amersham Biosciences). Detection of Cdc3-GFP and Lte1-GFP was performed on ethanol-fixed cells, on wash with water and sonication. Cells were similarly fixed to stain actin with 20 U/ml rhodamine-phalloidin (Sigma-Aldrich). Digital images were taken with a CCD camera and software (CoolSNAP; Photometrics).

Online supplemental material

Supplemental figures correspond to Figs. S1 and S2. Online supplemental material available at <http://www.jcb.org/cgi/content/full/jcb.200209097>.

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