The RSC chromatin-remodeling complex influences mitotic exit and adaptation to the spindle assembly checkpoint by controlling the Cdc14 phosphatase

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Upon prolonged activation of the spindle assembly checkpoint, cells escape from mitosis through a mechanism called adaptation or mitotic slippage, which is thought to underlie the resistance of cancer cells to antimitotic drugs. We show that, in budding yeast, this mechanism depends on known essential and nonessential regulators of mitotic exit, such as the Cdc14 early anaphase release (FEAR) pathway for the release of the Cdc14 phosphatase from the nucleolus in early anaphase. Moreover, the RSC (remodel the structure of chromatin) chromatin-remodeling complex bound to its accessory subunit Rsc2 is involved in this process as a novel component of the FEAR pathway. We show that Rsc2 interacts physically with the polo kinase Cdc5 and is required for timely phosphorylation of the Cdc14 inhibitor Net1, which is important to free Cdc14 in the active form. Our data suggest that fine-tuning regulators of mitotic exit have important functions during mitotic progression in cells treated with microtubule poisons and might be promising targets for cancer treatment.

Introduction

Chromosome segregation during anaphase requires the attachment of kinetochores to the mitotic spindle and removal of sister chromatid cohesion (Peters et al., 2008). In particular, cohesin must be cleaved by separase (Esp1 in yeast), which is kept in check by securin (Pds1 in yeast) until anaphase onset (Uhlmann, 2001). The ubiquitin ligase anaphase-promoting complex (APC) bound to its activator Cdc20 drives securin proteolysis and cohesin cleavage by separase at the metaphase-to-anaphase transition, thereby allowing sister chromatid separation (Nasmyth, 2002; Peters, 2006). Separase also contributes to mitotic exit and cyclin B proteolysis by acting in the Cdc14 early anaphase release (FEAR) pathway for nucleolar release and activation of the Cdc14 phosphatase. Indeed, Cdc14 is kept inactive in the nucleolus for most of the cell cycle as part of the regulator of nucleolar silencing and telophase exit (RENT) complex, which includes the Cdc14 inhibitor Net1/Cfi1 and the silencing protein Sir2 (Stegmeier and Amon, 2004). Besides separase, FEAR involves the polo kinase Cdc5, the Slk19 kinetochore protein, Spo12, and Bns1 (Stegmeier et al., 2002) and is negatively regulated by protein phosphatase 2A (Queralt et al., 2006), the replication fork block protein Fob1 (Stegmeier et al., 2004), and Tof2 (Waples et al., 2009). By promoting a first wave of partial Cdc14 release from the nucleolus in early anaphase, FEAR allows activation of the mitotic exit network (MEN), which leads to complete Cdc14 release and activation, thereby triggering cyclin B proteolysis and mitotic exit (Visintin et al., 1998).

The spindle assembly checkpoint (SAC) is a ubiquitous safety device ensuring the fidelity of mitotic chromosome segregation. During the process of microtubule capture by kinetochores in

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Abbreviations used in this paper: APC, anaphase-promoting complex; ChIP, chromatin immunoprecipitation; FEAR, Cdc14 early anaphase release; MCC, mitotic checkpoint complex; MEN, mitotic exit network; PBD, polo-box domain; rDNA, recombinant DNA; RENT, regulator of nucleolar silencing and telophase exit; RSC, remodel the structure of chromatin; SAC, spindle assembly checkpoint; tetO/tetR, tetracycline operator/repressor.

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prophase and prometaphase, the SAC proteins Bub3, Mad2, and Mad3/BubR1 form the mitotic checkpoint complex (MCC), which inhibits the activity of Cdc20–APC, thereby preventing sister chromatid separation and mitotic exit until all chromosomes reach proper bipolar attachment to the mitotic spindle. Other SAC proteins, such as Mad1, Bub1, Mps1, and Ipl1/AuroraB, amplify the signal and regulate the rate of MCC formation (Musacchio and Salmon, 2007). Most SAC proteins accumulate at unattached kinetochores during prophase and prometaphase and generate from this location the stop anaphase signal leading to Cdc20–APC inhibition, possibly by accelerating the rate of MCC formation (Musacchio and Salmon, 2007).

Cells do not arrest indefinitely upon SAC activation, but they escape mitosis after a variable amount of time in the presence of unattached kinetochores. The process by which cells leak through the SAC-induced cell cycle arrest when the checkpoint is not satisfied is called adaptation or mitotic slippage (Rieder and Maiato, 2004). This process is largely responsible for the failure to efficiently block tumor progression with chemotherapeutic compounds targeting the mitotic spindle, such as taxanes and vinca alkaloids. In mammalian cells, mitotic slippage depends on progressive degradation of cyclin B, with SAC proteins being retained at kinetochores (Brito and Rieder, 2006; Gascoigne and Taylor, 2008). In yeast, inhibitory phosphorylation of cyclin B/Cdks has been proposed to accelerate adaptation to prolonged SAC activation (Minshull et al., 1996).

Here, we report a role for the budding yeast RSC (remodel the structure of chromatin) chromatin-remodeling complex in timely mitotic exit and adaptation to the SAC as a novel component of the FEAR network. The Rsc2-bound form of RSC appears to influence the rate of mitotic slippage by facilitating the nucleolar release of Cdc14, which then brings about cyclin B proteolysis and mitotic exit. Furthermore, our data suggest that Rsc2 regulates the FEAR function of the polo kinase Cdc5 in conditions that activate the SAC, but independently of SAC components, and provide a link between chromatin structure and the regulation of mitotic exit.

Results

MAD2 overexpression as a tool to study adaptation to the SAC

To study adaptation to the SAC, we set up conditions that lead to SAC hyperactivation without perturbing kinetochore attachment to the mitotic spindle. We cloned *MAD2* behind the strong galactose-inducible *GAL1* promoter (*GAL1-MAD2*) and integrated this construct in multiple copies in the yeast genome. We estimated that the levels of overexpressed Mad2 after 2 h in galactose are 20-fold higher than those of endogenous Mad2 (unpublished data). *GAL1-MAD2* cells released from G1 in the presence of galactose arrested transiently as large-budded cells with undivided nuclei, metaphase spindles, and high levels of nuclear Pds1 (Fig. 1 A). This metaphase arrest was caused by SAC hyperactivation as it was bypassed by *MAD1* and *MAD3* deletions (not depicted), by *PDS1* deletion (Fig. S1 C), and by expression of the dominant *CDC20-107* allele (Fig. S1, A and B), which is refractory to SAC inhibition (Hwang et al., 1998).

GAL1-MAD2 cells remained arrested for ∼4–5 h and then started to escape mitosis and enter in the next cycle, forming microcolonies of four or more cells on galactose-containing plates 6–8 h after release from G1 (Fig. 1 B) and eventually generating visible colonies (Fig. S1 B). Thus, Mad2-overproducing cells undergo mitotic slippage.

Characterization of SAC adaptation in yeast

In vertebrate cells, adaptation to the SAC takes place with SAC components still bound to kinetochores and is accompanied by cyclin B proteolysis (Brito and Rieder, 2006). As shown in Fig. 2 A, yeast *GAL1-MAD2* cells slipped out of mitosis and started reaccumulating in G1 7 h after release from G1 in the presence of galactose, with concomitant decrease of securin (Pds1) and cyclin B (Clb2) levels, whereas Mad2 levels remained constantly high. A similar independent experiment showed that *GAL1-MAD2* cells carrying the tetracycline operator/repressor (tetO/tetR)–GFP system to monitor sister chromatid separation (Michaelis et al., 1997) also started separating sister chromatids around the same time (Fig. 2 B). We then analyzed mitotic slippage in other conditions that engage the SAC by releasing G1-arrested wild-type cells carrying the aforementioned tetO/tetR-GFP system in the presence of the microtubule-depolymerizing drugs nocodazole or benomyl. Bipolar spindles did not assemble in either condition, although a fraction of benomyl-treated cells displayed cytoplasmic microtubules 4 and 6 h after release (see next paragraph). In spite of the complete absence of spindles, both nocodazole- and benomyl-treated cells underwent Pds1 and Clb2 degradation, separated sister chromatids, and slipped out of mitosis, although cells seemed to adapt faster in benomyl than in nocodazole (Fig. 2 C). In fact, benomyl-treated cells underwent almost complete Pds1 and Clb2 degradation, which resulted in cell division and reaccumulation of unbudded cells within 10 h after release. At the same time, a considerable fraction of nocodazole-treated cells was still arrested as large-budded cells with relatively high levels of Clb2 (Fig. 2 C).

To assess if adaptation in yeast correlates with silencing of SAC signaling, we monitored the levels of Mad1–Bub3 interaction, which takes place only in the presence of unattached kinetochores (Brady and Hardwick, 2000; Fraschini et al., 2001b) and therefore is a good readout for SAC signaling. G1-arrested cells expressing HA-tagged Bub3 (Bub3-HA3) were released in the presence of benomyl or nocodazole, followed by monitoring cell cycle progression by FACS analysis and Mad1–Bub3 interaction by coimmunoprecipitation. Again, 4 and 6 h after G1 release, a fraction of benomyl-treated cells (10 and 50%, respectively) displayed cytoplasmic microtubules (Fig. 2 E), which, in some cases, could drive an abnormal chromosome segregation (not depicted), but no bipolar spindles were detectable. Mad1–Bub3 interaction was stable up to 8 h after the G1 release in nocodazole-treated cells that were still arrested with 2C DNA content, whereas it started decreasing in the presence of benomyl after 4 h and was undetectable by 8 h, when most cells had exited mitosis (Fig. 2 D). The total levels of Mad1, but not of Bub3, also decreased in benomyl during the course of the

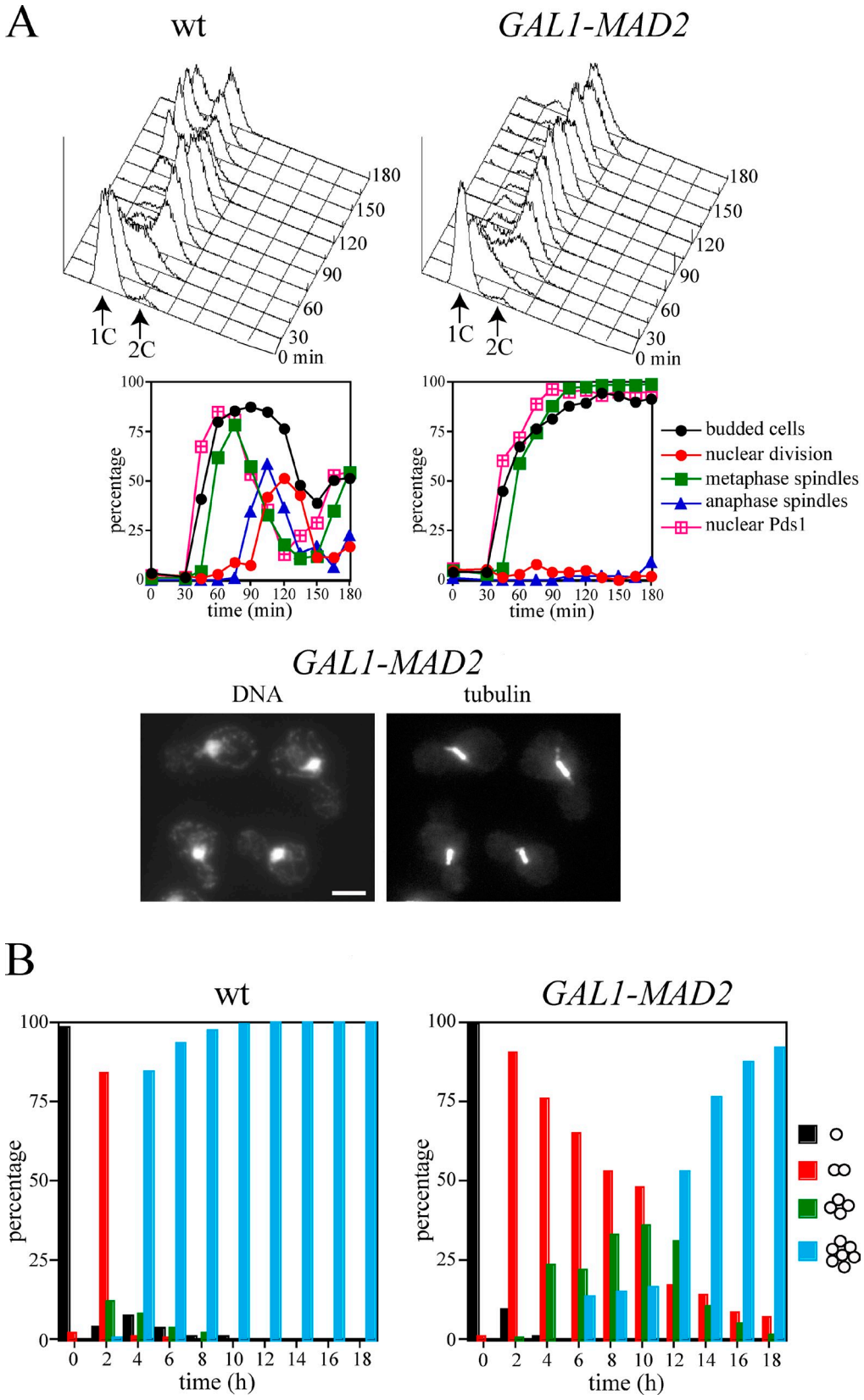

Figure 1. ***MAD2* overexpression induces a transient metaphase arrest.** (A) Wild-type (wt; ySP4806) and *GAL1-MAD2* (ySP8526) cells were grown in YEPR, arrested in G1 with α-factor, and then released in YEPRG medium (t = 0). Samples were collected at the indicated times for FACS analysis of DNA contents and kinetics of budding, nuclear division, mitotic spindle formation/elongation, and Pds1 nuclear accumulation. Micrographs show examples of nuclear and microtubule staining (t = 150 min after release; bar, 5 μm). (B) Wild-type (W303) and *GAL1-MAD2* (ySP6170) cells were grown in YEPR, arrested in G1 with α-factor (unbudded cells), and spotted on YEPRG plates (t = 0). At the indicated times, 200 cells for each strain were scored to determine the frequency of single cells and of microcolonies of two, four, or more than four cells.

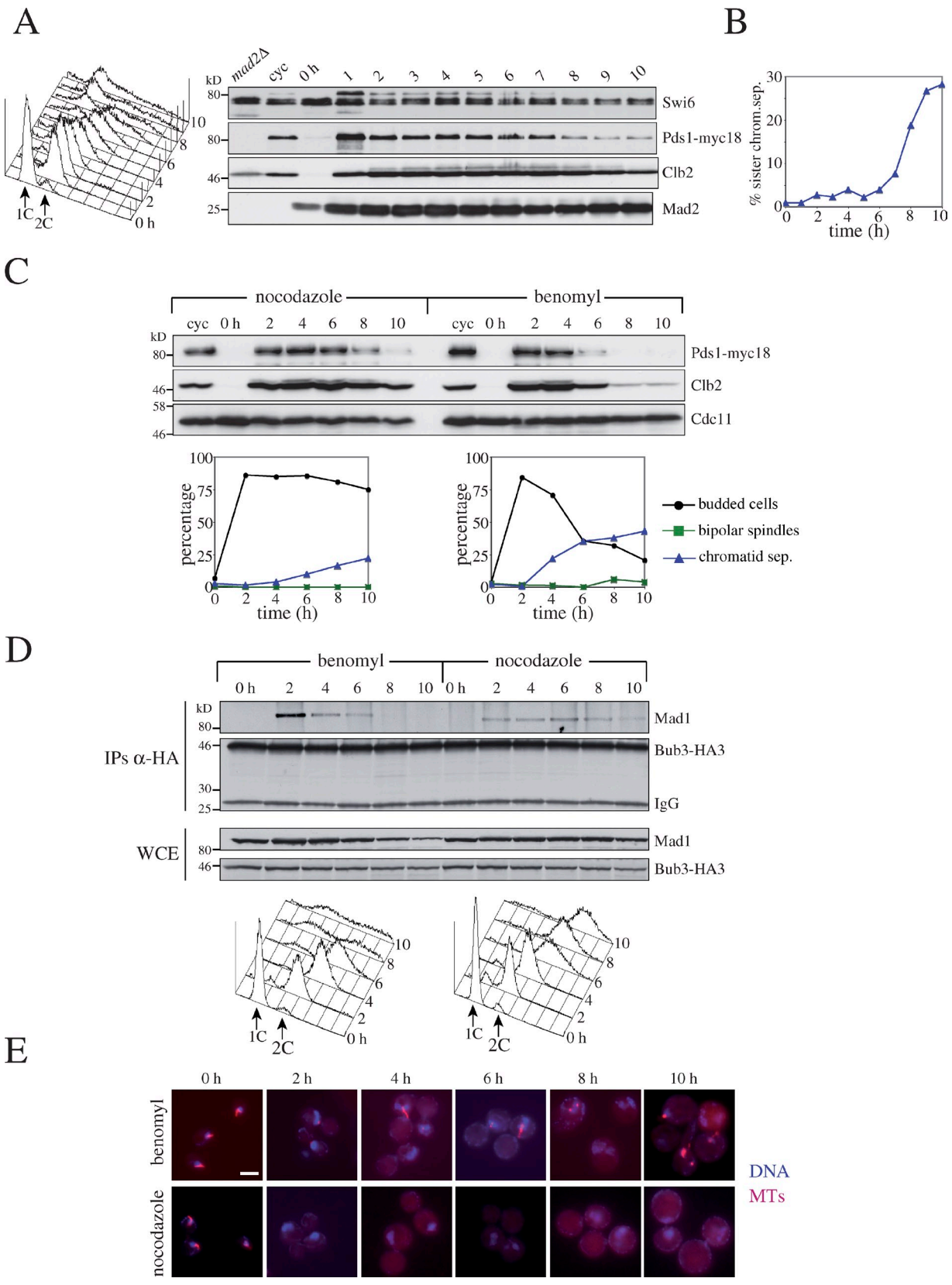

Figure 2. **Mitotic slippage upon prolonged treatment with microtubule destabilizers correlates with degradation of APC substrates and dissociation between Mad1 and Bub3.** (A) α-factor–arrested *GAL1-MAD2* (ySP8599) cells were released in YEPRG at 30°C (t = 0). α-factor was readded at 3 μg/ml after 2 h. Samples were collected at the indicated times for Western blot analysis of Pds1-myc18, Clb2, Mad2, and Swi6 (loading control). Cyc, cycling cells. (B) G1-arrested *GAL1-MAD2* cells carrying the tetO/tetR-GFP markers to score sister chromatid separation (ySP6699; Michaelis et al., 1997) were released in YEPRG at t = 0. (C) α-factor–arrested wild-type cells (ySP8534) were released in the presence of nocodazole or benomyl at t = 0. α-factor was readded at 3 μg/ml after 2 h, and samples were collected at the indicated times for Western blot analysis (top) of Pds1-myc18, Clb2, and Cdc11 (loading control),

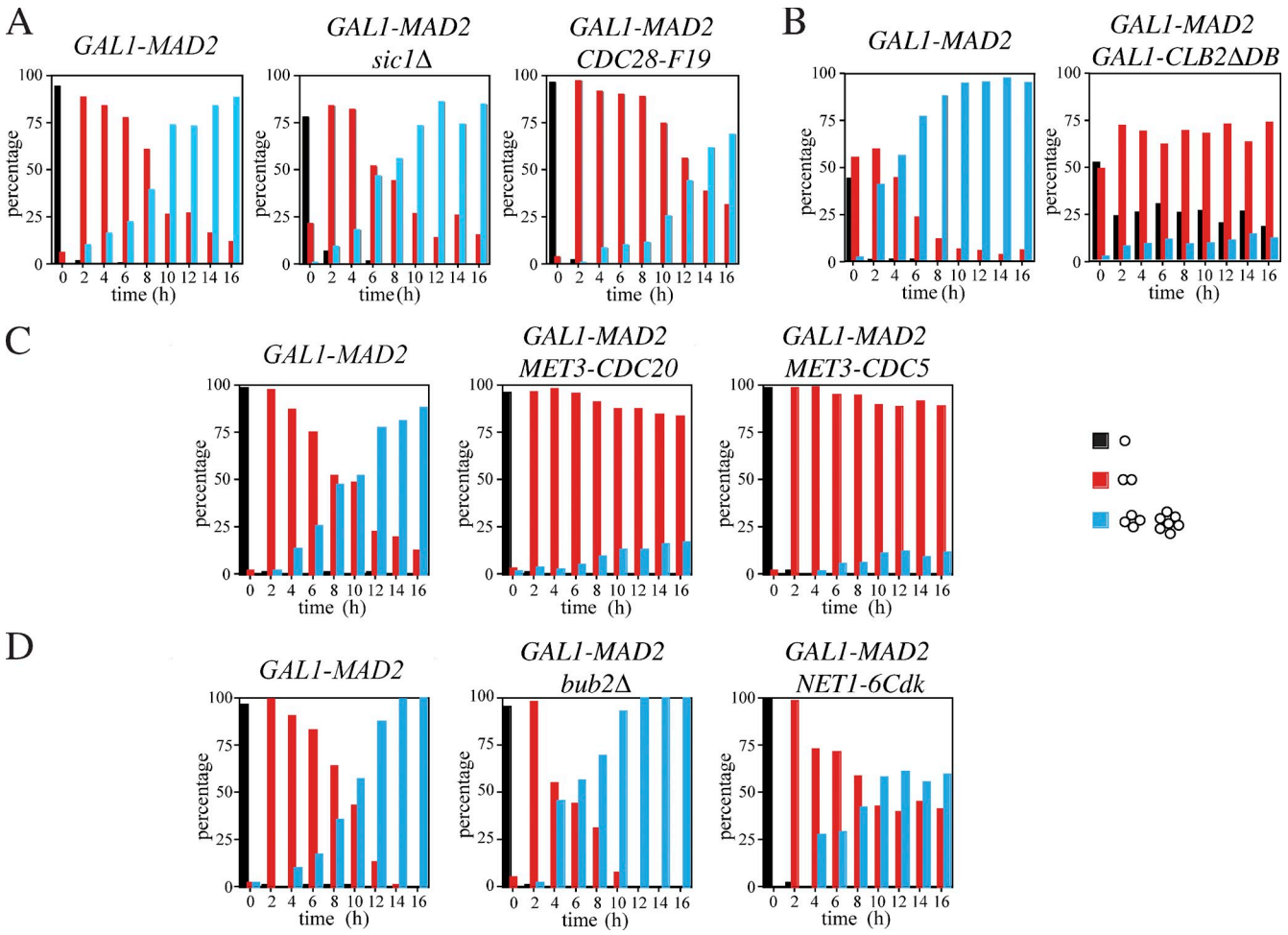

Figure 3. **SAC adaptation requires mitotic exit regulators.** (A) *GAL1-MAD2* (ySP6170), *GAL1-MAD2 sic1Δ* (ySP8706), and *GAL1-MAD2 CDC28-F19* (ySP8704) cells were grown in YEPR, arrested in G1 with α-factor (unbudded cells), and spotted on YEPRG plates (t = 0) at 30°C. 200 cells were scored at each time point for microcolony formation. (B) Cycling cultures of *GAL1-MAD2* (ySP3344) and *GAL1-MAD2 GAL1-CLB2ΔDB* (ySP8710) cells grown in YEPR were spotted on YEPRG plates (t = 0) at 30°C to follow microcolony formation. A fraction of *GAL1-MAD2 GAL1-CLB2ΔDB* cells remained unbudded because Clb2ΔDB inhibits budding (Surana et al., 1993). (C) *GAL1-MAD2* (ySP6170), *GAL1-MAD2 MET3-CDC20* (ySP8138), and *GAL1-MAD2 MET3-CDC5* (ySP8226) cells were grown in raffinose-containing medium lacking methionine, arrested in G1 with α-factor (unbudded cells), and spotted on YEPRG supplemented with 2 mM methionine (t = 0) to follow microcolony formation. (D) *GAL1-MAD2* (ySP6170), *GAL1-MAD2 bub2Δ* (ySP7677), and *GAL1-MAD2 NET1-6Cdk* (ySP7958) cells were treated as in A.

experiment but not as dramatically as in the Bub3 immunoprecipitates. Therefore, adaptation to the SAC in yeast is accompanied by silencing of checkpoint signaling.

Adaptation to the SAC requires cyclin B degradation, Cdc20, the polo kinase Cdc5, and Cdc14 nucleolar release

As SAC adaptation involves Clb2 proteolysis, we asked whether cyclin degradation, Cdk inhibitory phosphorylation, and/or Cdk inhibitors were required for mitotic slippage upon *MAD2* overexpression. As shown in Fig. 3 (A and B), microcolony formation of *GAL1-MAD2* cells on galactose plates was effectively delayed by expression of either the Cdk1 variant Cdc28-F19, which cannot undergo Tyr19 inhibitory phosphorylation, or nondegradable Clb2. In contrast, deletion of the cyclin B/Cdk inhibitor Sic1 (Mendenhall, 1993; Schwob et al., 1994) had no effect.

We then asked whether SAC adaptation depends on cell cycle regulators that modulate mitotic exit and proteolysis of mitotic cyclins. Indeed, *CDC20* repression from the *MET3* promoter markedly prolonged the metaphase arrest of *GAL1-MAD2* cells (Fig. 3 C), suggesting that high levels of Mad2 are not sufficient to maintain Cdc20–APC inhibition for a long time. Inactivation of the polo kinase Cdc5 through a *MET3-CDC5* fusion yielded similar results (Fig. 3 C).

Adaptation to the SAC upon *MAD2* overexpression might also be influenced by advancing or delaying activation of the

as well as to monitor kinetics of budding, bipolar spindle formation, and sister chromatid separation (bottom graphs). (D and E). Cells expressing Bub3-HA3 (ySP8709) were treated as in C. At the indicated times, interaction between Bub3-HA3 and Mad1 was assessed by coimmunoprecipitation using anti-HA antibodies followed by Western blotting with anti-Mad1 and anti-HA antibodies. At the same times, DNA contents were measured by FACS analysis (D, histograms) and for tubulin staining by immunofluorescence (E). MTs, microtubules. WCE, whole cell extract. Bar, 5 μm.

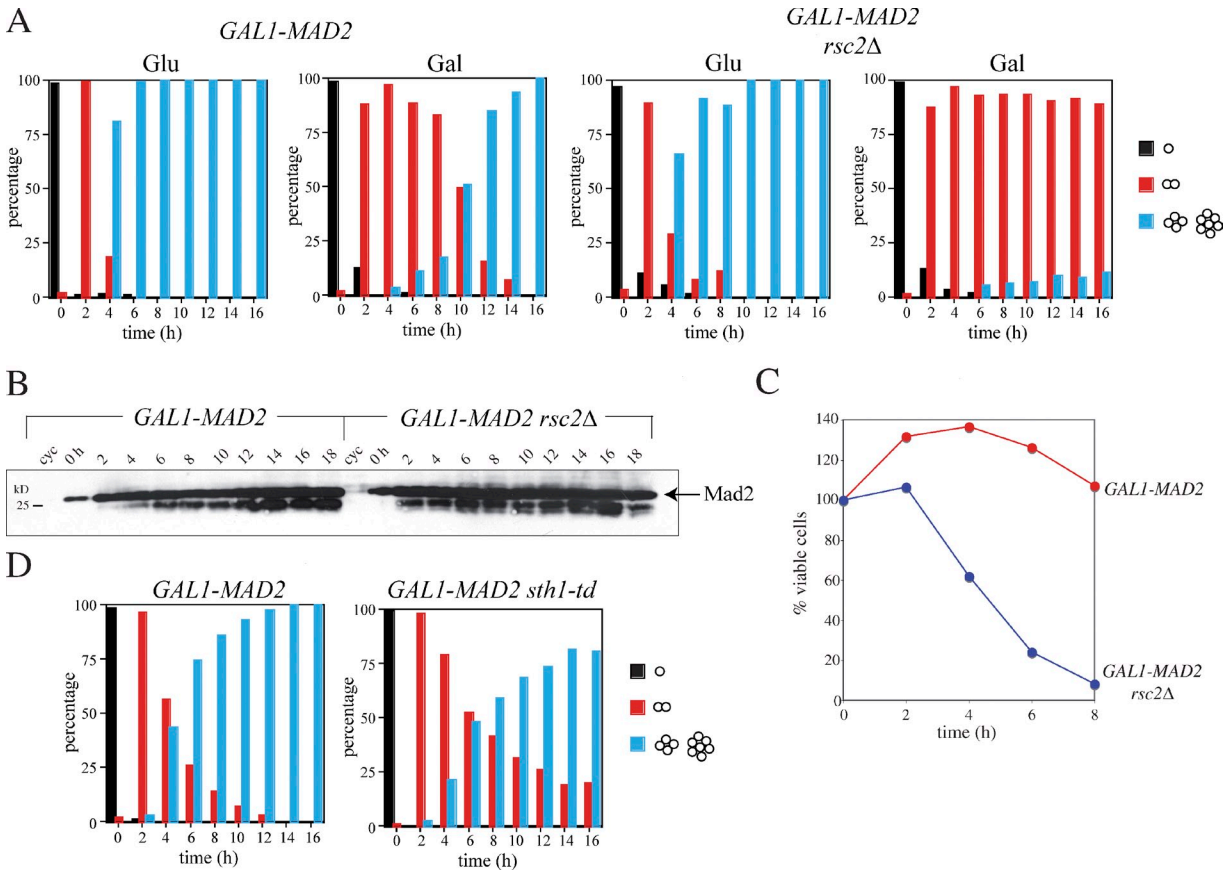

Figure 4. **The RSC complex is involved in SAC adaptation.** (A) *GAL1-MAD2* (ySP6170) and *GAL1-MAD2 rsc2Δ* (ySP6850) cells were grown in uninduced conditions (YEPR), arrested in G1 with α-factor (unbudded cells), and spotted on YEPD (Glu) and YEPRG (Gal) plates (t = 0) at 30°C. 200 cells were scored at each time point for microcolony formation. (B) The same strains as in A were grown in uninduced conditions, arrested in G1 with α-factor at 25°C, and released on YEPRG medium (t = 0) at 30°C. Samples were collected at the indicated times for Western blot analysis of Mad2 levels (arrow). Cyc, cycling cells. (C) The same strains as in A were treated as in B and plated at different times on YEPD plates to assess cell viability. Percentages represent mean values of three independent experiments. (D) *GAL1-MAD2* (ySP6170) and *GAL1-MAD2 GAL1-UBR1 CUP1-sth1*^{td} (ySP7808) cells were grown in YEPR supplemented with 0.1 mM $CuSO_4$, arrested in G1 with α-factor (unbudded cells), and spotted on YEPRG plates (t = 0) at 37°C to follow microcolony formation. Data are representative of three independent repeats.

Cdc14 phosphatase that is necessary for mitotic exit. We thus forced unscheduled activation of the MEN and subsequent Cdc14 nucleolar release by eliminating the MEN inhibitor Bub2 (Piatti et al., 2006). Conversely, we delayed Cdc14 activation by expression of a nonphosphorylatable Net1 variant (Net1-6Cdks) that does not allow the transient release of Cdc14 from the nucleolus in early anaphase (Azzam et al., 2004). Notably, *BUB2* deletion accelerated microcolony formation of *GAL1-MAD2* cells on galactose plates, whereas *NET1-6Cdk* expression slowed it down (Fig. 3 D), suggesting that Cdc14 release from the nucleolus might be important for SAC adaptation.

The chromatin-remodeling RSC complex is involved in adaptation to the SAC

Because *MAD2* overexpression provides a good experimental setup to study the molecular bases of SAC adaptation in the absence of spindle/kinetochore defects, we used transposon mutagenesis of *GAL1-MAD2* cells to identify factors involved in adaptation and/or in fine tuning of mitotic exit. To this end, we screened for clones that were hypersensitive to *MAD2* overexpression and likely prolonged their cell cycle arrest under these conditions. We found that several clones with this phenotype carried the transposon insertion 3′ to the *RSC2* gene, encoding an accessory subunit of the chromatin-remodeling complex RSC (Cairns et al., 1999). Indeed, the Rsc2-containing RSC complex seemed a good candidate for adaptation to the SAC because it had been previously implicated in chromosome segregation, mitotic progression, and regulation of sister chromatid separation (Hsu et al., 2003; Baetz et al., 2004; Huang and Laurent, 2004). Moreover, *RSC2* deletion was shown to have synthetic effects with mutations altering kinetochore components or cohesin (Baetz et al., 2004).

The latter observations were extended by analyzing the effects of *RSC2* deletion in a set of mutants in kinetochore components (Dam1 and Cep3) or microtubule-binding proteins (Stu2 and Cin8; Fig. S2 A). Besides confirming genetic interactions previously reported by others, *RSC2* deletion caused hypersensitivity to benomyl and decreased the maximal permissive temperature of the kinetochore mutants *dam1-11*, *cep3-10*, and *stu2-10*, as well as that of *cin8Δ* cells lacking the BimC family kinesin Cin8, which has a major role in spindle assembly (Hoyt et al., 1992). Because the aforementioned mutations and benomyl treatment engage the SAC, the deleterious effects of *RSC2* deletion in these conditions might be caused by prolonged

SAC activation. Indeed, *RSC2* deletion turned out to be lethal for *GAL1-MAD2* cells in the presence of galactose (Fig. S2 B and Fig. 4 C). We then scored microcolony formation of *GAL1-MAD2* and *GAL1-MAD2 rsc2Δ* cells upon plating G1-synchronized cells on media containing either glucose (*GAL1-MAD2* off) or galactose (*GAL1-MAD2* on). Deletion of *RSC2* slightly delayed cell cycle progression on glucose plates compared with otherwise wild-type cells in the presence of galactose (Fig. 4 A). Strikingly, the presence of galactose caused *GAL1-MAD2 rsc2Δ* cells to remain arrested in mitosis as large-budded cells for a longer time than *GAL1-MAD2* cells (Fig. 4 A), in spite of comparable levels of Mad2 (Fig. 4 B). This behavior paralleled with the dramatic lethal effect of *GAL1-MAD2* overexpression in *rsc2Δ* cells (Fig. 4 C).

Deletion of *RSC1*, encoding an RSC subunit alternative to Rsc2 (Cairns et al., 1999), had no effect on the mitotic escape of *GAL1-MAD2* cells on galactose plates (Fig. S3 A), suggesting that the Rsc2-containing form of RSC (RSC^{Rsc2}) is specifically implicated in this process. The lack of Rsc2 also prolonged the mitotic arrest of *MPS1*-overexpressing cells (Fig. S3 B), which transiently hyperactivate the SAC and eventually adapt (Hardwick et al., 1996), and of benomyl-treated cells (Fig. S3 C).

We then asked whether Rsc2 has a role in SAC adaptation as part of the RSC complex or independently of it. This was not trivial because all core RSC subunits are essential and must be inactivated by temperature-sensitive mutations, whereas the *GAL1* promoter required to overexpress *MAD2* is very inefficient at high temperatures. Indeed, *GAL1-MAD2* cells showed only a modest cell cycle arrest at 37°C, as almost 50% of the cells had escaped from the arrest and formed microcolonies of four or more cells on galactose within 4 h after plating (Fig. 4 D). However, RSC inactivation by the temperature-sensitive degron allele of *STH1* (*sth1^{td}*; Parnell et al., 2008), which encodes the RSC catalytic subunit, delayed adaptation of *GAL1-MAD2* cells by ∼2 h, suggesting that the whole RSC complex is involved in this process.

RSC^{Rsc2} inactivation prevents mitotic exit of SAC-deficient mutants in the presence of microtubule-depolymerizing drugs

As RSC inactivation might delay escape from mitosis by prolonging the SAC-dependent cell cycle arrest, we investigated its effects in SAC-deficient mutants treated with microtubule-depolymerizing drugs. To this end, wild-type, *mad2Δ*, *rsc2Δ*, and *mad2Δ rsc2Δ* cells were arrested in G1 by α-factor and released in the presence of nocodazole. As expected, *mad2Δ* cells rereplicated their DNA efficiently and accumulated DNA contents higher than 2C under these conditions, which instead caused the double *mad2Δ rsc2Δ* mutant to arrest in mitosis similarly to wild-type and *rsc2Δ* cells (Fig. 5 A). Deletion of *RSC2* prevented mitotic exit also of nocodazole-treated *mad1Δ*, *mad3Δ*, *bub1Δ*, *bub3Δ*, *cdc55Δ*, and *CDC20-107* cells (unpublished data). Moreover, rereplication of *mad2Δ* cells upon microtubule disruption was inhibited also by Sth1 inactivation through the *sth1^{td}* allele (Fig. 5 B), whereas it was not affected by *RSC1* deletion (Fig. S4 A). Altogether, these data suggest that RSC^{Rsc2} is required for the unscheduled mitotic exit of SAC mutants in the presence of spindle defects.

RSC2 deletion could prevent mitotic exit and rereplication of nocodazole-treated SAC mutants by either restoring Cdc20–APC inhibition or impinging on pathways controlling mitotic exit, such as the FEAR or MEN pathways for Cdc14 nucleolar release. In fact, whereas Cdc20–APC is required for degradation of securin and a fraction of cyclin B, Cdc14 triggers Cdh1/APC activation, which completes cyclin B degradation and drives accumulation of the Cdk inhibitor Sic1 (Visintin et al., 1998). To distinguish between these two possibilities, we first analyzed Pds1 and Clb2 degradation, as well as Sic1 accumulation, in wild-type, *mad2Δ*, *rsc2Δ*, and *mad2Δ rsc2Δ* cells that were released from G1 in the presence of nocodazole. As shown in Fig. 5 C, Pds1 was degraded in both *mad2Δ* and *mad2Δ rsc2Δ* cells, whereas a fraction of Clb2 was stabilized and Sic1 did not accumulate in *mad2Δ rsc2Δ* cells, in contrast to *mad2Δ* cells. These results are consistent with the role of RSC in the regulation of mitotic exit and, in particular, of Cdc14 nucleolar release (see next paragraph), rather than in Cdc20–APC activation. Like RSC mutations, mutations affecting the FEAR pathway, such as *esp1-1* (Fraschini et al., 2001a), *spo12Δ bns1Δ*, *slk19Δ* (Fig. S4 B), and *NET1-6Cdk* (not depicted) prevented rereplication of nocodazole-treated *mad2Δ* cells. In addition, simultaneous deletion of *SLK19*, *SPO12*, and *BNS1* retarded microcolony formation of *GAL1-MAD2* cells on galactose plates (Fig. S4 C). Similarly to FEAR mutations, *RSC2* deletion only modestly delayed mitotic exit both in unperturbed conditions (Fig. 6 A) and during recovery from nocodazole arrest (Fig. 6 B), as judged by the kinetics of spindle disassembly relative to spindle elongation and nuclear division. Conversely, lack of Rsc2 delayed the onset of anaphase (i.e., spindle elongation and nuclear division) relative to bipolar spindle assembly (Fig. 6, A and B), which is consistent with previous observations (Hsu et al., 2003; Baetz et al., 2004). Thus, RSC^{Rsc2} might regulate mitotic exit in a way similar to the FEAR pathway in conditions of SAC hyperactivation or in the presence of kinetochore/microtubule defects.

Lack of Rsc2 impairs Cdc14 release from the nucleolus at the metaphase-to-anaphase transition

The persistence of Clb2 and the lack of Sic1 accumulation in nocodazole-treated *mad2Δ rsc2Δ* cells, together with the similar effects caused by RSC and FEAR inactivation in SAC mutants upon microtubule disruption, suggested that RSC^{Rsc2} might be involved in the control of Cdc14 release from the nucleolus. We therefore analyzed Cdc14 nucleolar release in *mad2Δ*, *rsc2Δ*, and *mad2Δ rsc2Δ* cells released from G1 in the presence of nocodazole. Although *mad2Δ* cells transiently released Cdc14, all other strains retained it in the nucleolus (Fig. 7 A), suggesting that RSC^{Rsc2} is required for Cdc14 release in these conditions. Strikingly, expression of the $Cdc14^{TAB6\text{-}1}$ dominant variant that associates loosely to its inhibitor Net1 (Shou et al., 2001) restored the ability of nocodazole-treated *mad2Δ rsc2Δ* cells to rereplicate DNA (Fig. 7 B), whereas it was not sufficient by itself to promote mitotic exit in these conditions (not depicted). These data support the notion that RSC^{Rsc2} inactivation interferes with Cdc14 nucleolar release and activation, prompting

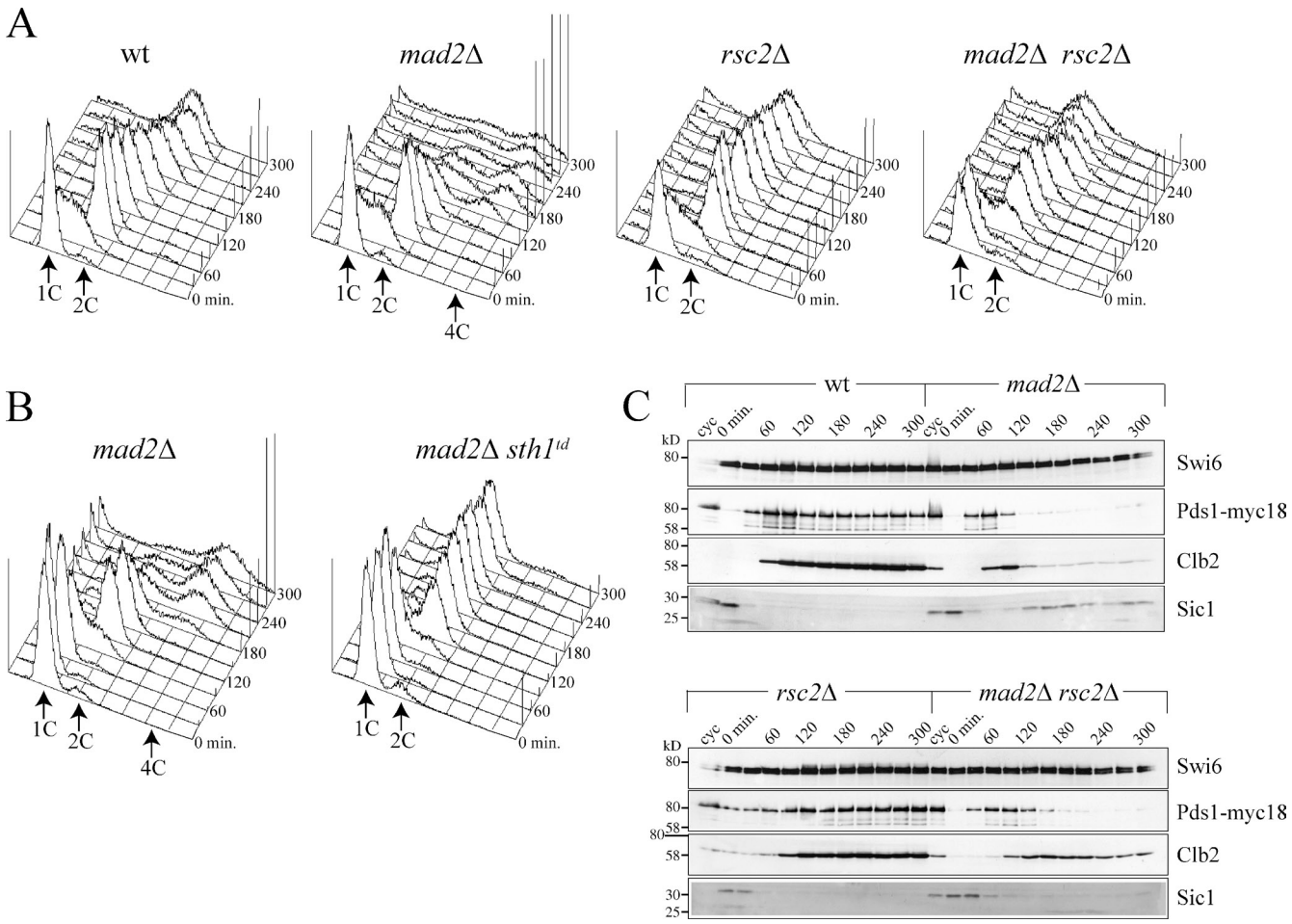

Figure 5. **Lack of the RSC complex prevents mitotic exit of nocodazole-treated SAC-defective cells.** (A) Cultures of wild-type (wt), *mad2Δ*, *rsc2Δ*, and *mad2Δ rsc2Δ* cells (ySP4806, ySP1084, ySP6997, and ySP7543) were grown in YEPD, arrested in G1 by α-factor, and then released into medium containing nocodazole (t = 0). At the indicated times, cell samples were withdrawn for FACS analysis of DNA contents. (B) Cultures of *mad2Δ* (ySP1070) and *GAL1-UBR1 CUP1-sth1*^{td} *mad2::TRP1* (ySP7869) cells were grown in YEPR containing 0.1 mM $CuSO_4$ and arrested in G1 with α-factor at 27°C. 1 h after 2% galactose addition, cells were released in nocodazole-containing YEPRG at 37°C (t = 0), followed by FACS analysis of DNA contents at the indicated times. (C) The same strains and procedure as in A were used, but 10 μg/ml α-factor was readded to all cultures at t = 100 min after release (>90% of budded cells). At the indicated times, cells were collected for FACS analysis of DNA contents (not depicted) and for Western analysis of Pds1, Clb2, Sic1, and Swi6 (loading control). Cyc, cycling cells.

us to directly compare the kinetics of Cdc14 release from the nucleolus in *rsc2Δ* cells versus wild type and the FEAR mutant *spo12Δ bns1Δ*. To monitor only the partial Cdc14 release at the anaphase onset, we prevented MEN activation by overexpressing *BFA1* from the *GAL1* promoter (Li, 1999). Wild-type, *GAL1-BFA1*, *GAL1-BFA1 rsc2Δ*, and *GAL1-BFA1 spo12Δ bns1Δ* cells were synchronized in G1 and released in galactose-containing medium. We then followed partial and total Cdc14 release from the nucleolus during the cell cycle. As expected, wild-type cells started releasing Cdc14 after metaphase spindles had been assembled and concomitant to spindle elongation (Fig. 7 C). Nuclear division immediately followed, and Cdc14 was completely released into the nucleoplasm and cytosol before cytokinesis. Consistent with MEN inhibition, *GAL1-BFA1* cells arrested in telophase as large-budded cells with 2C DNA contents, divided nuclei, and elongated spindles. As expected, Cdc14 total release was abolished in these cells, and only the partial release in anaphase could be observed (Fig. 7 C). Like *GAL1-BFA1* cells, *GAL1-BFA1 rsc2Δ* and *GAL1-BFA1 spo12Δ bns1Δ* cells arrested in telophase and showed no sign of total Cdc14 release. Moreover, Cdc14 partial release was abolished in *GAL1-BFA1 spo12Δ bns1Δ* cells and severely compromised in *GAL1-BFA1 rsc2Δ* cells (Fig. 7 C). Thus, Rsc2 and presumably the whole RSC^{Rsc2} complex contribute to the early anaphase release of Cdc14 from the nucleolus.

Deletion of *RSC2* has synthetic effects with mutations affecting the MEN

We analyzed the relationships between RSC and the FEAR or the MEN cascades by combining *RSC2* deletion with FEAR or MEN mutations. Deletion of *RSC2* caused little or no synthetic growth defects when combined with the FEAR mutations *slk19Δ*, *spo12Δ bns1Δ*, and *esp1-1* (unpublished data), suggesting that RSC^{Rsc2} works together with or in parallel to the FEAR pathway.

Inactivation of the FEAR pathway is known to be lethal for cells lacking the nonessential MEN activator Lte1 (Stegmeier et al., 2002). Similarly, *RSC2* deletion was found to be lethal with *LTE1* deletion (Ye et al., 2005). In fact, *rsc2Δ lte1Δ* cells

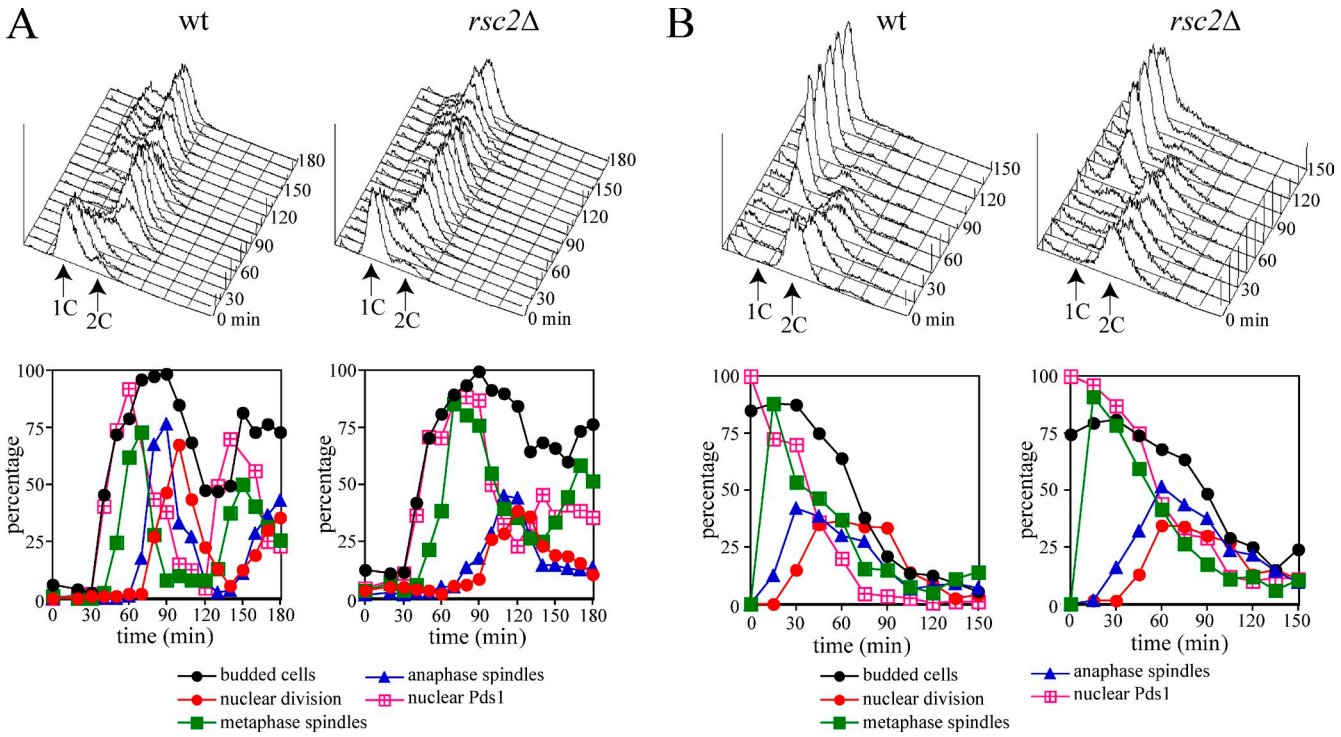

Figure 6. **Cell cycle progression of *rsc2Δ* cells and their recovery from SAC activation.** (A) Cultures of wild-type (wt; ySP4806) and *rsc2Δ* (ySP6997) cells were grown in YEPD, arrested in G1 by α-factor, and then released in fresh medium (t = 0). At the indicated times, samples were analyzed as in Fig. 1 A. (B) The same strains as in A were grown in YEPD, arrested in mitosis by 5 µg/ml nocodazole treatment, and then released (t = 0) in 10 µg/ml YEPD containing α-factor, followed by the same analyses as in Fig. 1 A.

were, in most cases, inviable or extremely sick also in our genetic background (Fig. 8 A), and this lethality could be rescued by *BUB2* deletion (not depicted), suggesting that it was caused by constitutive trapping of Cdc14 in the nucleolus. *RSC2* deletion also caused sickness and lethality when combined with the temperature-sensitive alleles *cdc5-2*, affecting polo kinase, and *cdc14-3*, respectively (Fig. 8 A). In addition, it decreased the maximal permissive temperature of the *tem1-3*, *cdc15-2*, *dbf2-2*, and *cdc14-1* MEN mutants (Fig. 8 B), supporting the notion that RSC^{Rsc2} regulates Cdc14 release from the nucleolus. Accordingly, *RSC2* overexpression suppressed *cdc15-2* lethality at 32°C (Fig. 8 C). Thus, RSC^{Rsc2} controls Cdc14 release from the nucleolus at the metaphase/anaphase transition independently of MEN and in concert with the FEAR pathway.

Rsc2 interacts with the polo kinase Cdc5 and contributes to timely Net1 phosphorylation

FEAR components have been recently found to interact with the polo kinase Cdc5 (Rahal and Amon, 2008), which has a key role in Cdc14 nucleolar release acting in both the FEAR and the MEN pathways (Stegmeier and Amon, 2004). The *Xenopus laevis* homologue of Rsc2, polybromo-1/BAF180, was found to interact with polo kinase (Yoo et al., 2004), and Rsc2 itself was predicted to be a likely binding partner of Cdc5 (Snead et al., 2007). To investigate whether Rsc2 interacts with Cdc5, we expressed Flag-tagged Cdc5 (Cdc5-Flag3) in cells expressing either untagged Rsc2 or HA-tagged Rsc2 (Rsc2-HA3). Rsc2-HA3 immunoprecipitates from both cycling and nocodazole-arrested cells contained Cdc5-Flag3, which was instead absent in the immunoprecipitates from the untagged Rsc2 strain (Fig. 9 A). Rsc2 could also bind the polo-box domain (PBD) of Cdc5, which normally binds substrates previously primed by phosphorylation by another kinase (Elia et al., 2003a). Indeed, Rsc2-HA3 bound to a recombinant GST-PBD fusion protein (Miller et al., 2009) but not to GST alone (Fig. 9 B). Surprisingly, this binding was not disrupted by mutating the critical $W^{517}V^{518}L^{530}$ residues (Elia et al., 2003b) into FAA, suggesting that it might be independent of preliminary phosphorylation.

Because Rsc2 binds to Cdc5 and is required for timely release of Cdc14 from the nucleolus, we evaluated whether *RSC2* deletion affected Net1 phosphorylation, which depends on Cdc5 and is required to release Net1-Cdc14 association (Shou et al., 2002; Yoshida and Toh-e, 2002). As shown in Fig. 9 D, a slow-migrating band corresponding to phosphorylated Net1 (Visintin et al., 2003; Queralt et al., 2006) appeared during anaphase in wild-type cells (80–90 min after release from G1 arrest; Fig. 9 C), whereas it was barely detectable in the absence of Rsc2, suggesting that the FEAR function of Cdc5 might require the RSC^{Rsc2} complex.

RSC was previously involved in sister chromatid cohesion (Baetz et al., 2004; Huang and Laurent, 2004), and Cdc5 facilitates cohesin cleavage and sister chromatid separation besides promoting Cdc14 activation (Alexandru et al., 2001). We then asked whether Cdc5 distribution along chromosomes was altered in the absence of Rsc2 by studying Cdc5-Flag3 chromosomal distribution by ChIP (chromatin immunoprecipitation)-on-chip on the whole genome of yeast cells arrested in mitosis.

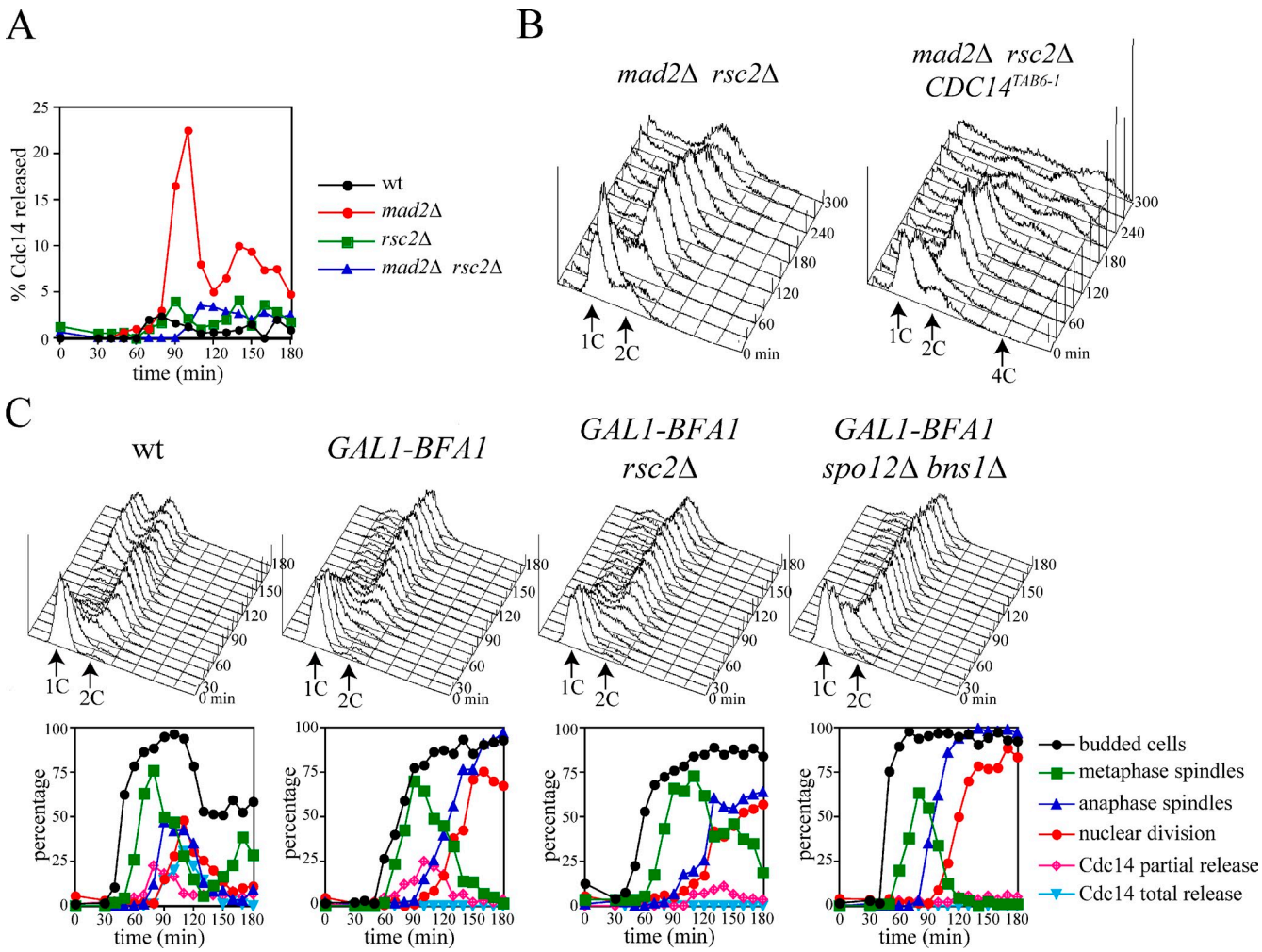

Figure 7. **Rsc2 controls the early release of Cdc14 from nucleolus.** (A) α-factor–arrested wild-type (wt; W303), *mad2Δ* (ySP1070), *rsc2Δ* (ySP6858), and *mad2Δ rsc2Δ* (ySP7088) cells were released in medium containing nocodazole (t = 0). At the indicated times, cells were collected for FACS analysis of DNA contents (not depicted) and for detecting Cdc14 release by immunofluorescence. (B) Cultures of *mad2Δ rsc2Δ* (ySP7088) and *mad2Δ rsc2Δ CDC14*$^{TAB6\text{-}1}$ (ySP7645) were treated as in A. At the indicated times after release (t = 0), cells were collected for FACS analysis of DNA contents. (C) Cultures of wild-type, *GAL1-BFA1*, *GAL1-BFA1 rsc2Δ*, and *GAL1-BFA1 spo12Δ bns1Δ* (W303, ySP1283, ySP7764, and ySP7803) cells were grown in YEPR, arrested in G1, and then released in YEPRG (t = 0). Samples were collected at the indicated times for FACS analysis of DNA contents and to follow the kinetics of budding, nuclear division, mitotic spindle formation/elongation, and Cdc14 partial/total release.

Cdc5 localized at centromeres and discrete sites along chromosome arms corresponding to cohesin-binding sites (see the left arm of chromosome VI as an example; Fig. 10, A and B), and it could be found also at recombinant DNA (rDNA; not depicted). *RSC2* deletion did not affect Cdc5 chromosomal distribution at any locus (Fig. 10 A and not depicted), suggesting that Rsc2 might regulate Cdc5 at levels other than its recruitment to specific chromosomal regions.

Discussion

Adaptation to the SAC depends on regulators of mitotic exit

Eukaryotic cells ultimately adapt to persistent SAC signaling and exit from mitosis, eventually leading to unbalanced chromosome segregation or cell death (Rieder and Maiato, 2004). Mitotic exit under these conditions is linked to a progressive decline in cyclin B/Cdk activity that, after reaching a threshold level, drives cells out of mitosis (Brito and Rieder, 2006; Gascoigne and Taylor, 2008). We show here that, similar to vertebrate cells, mitotic slippage in budding yeast, either in the presence of microtubule inhibitors or upon SAC hyperactivation in the absence of spindle damage, is accompanied by securin and cyclin B degradation and is delayed by expression of nondegradable cyclin B. As in mammalian cells (Brito and Rieder, 2006; Gascoigne and Taylor, 2008), the timing of mitotic slippage is highly variable depending on the conditions, ranging from ∼4 to 5 h in benomyl, 5 to 6 h upon *MAD2* overexpression, and 8 to 10 h in nocodazole. We also find that, as recently shown in mammalian cells (Lee et al., 2010), Cdc20 and other canonical regulators of cyclin B proteolysis and mitotic exit, such as the polo kinase Cdc5, are involved in SAC adaptation. In addition, the unphosphorylatable Cdc28-F19 variant delays mitotic slippage upon Mad2 overexpression consistently with the older proposal that inhibitory phosphorylation of cyclin B/Cdks accelerates adaptation to prolonged SAC activation (Minshull et al., 1996).

A

	LTE1	*lte1Δ*	*CDC5*	*cdc5-2*	*CDC14*	*cdc14-3*
RSC2	31/30	24/30	49/53	43/53	36/34	30/34
rsc2Δ	28/30	4/30*	48/53	32/53*	32/34	0/34

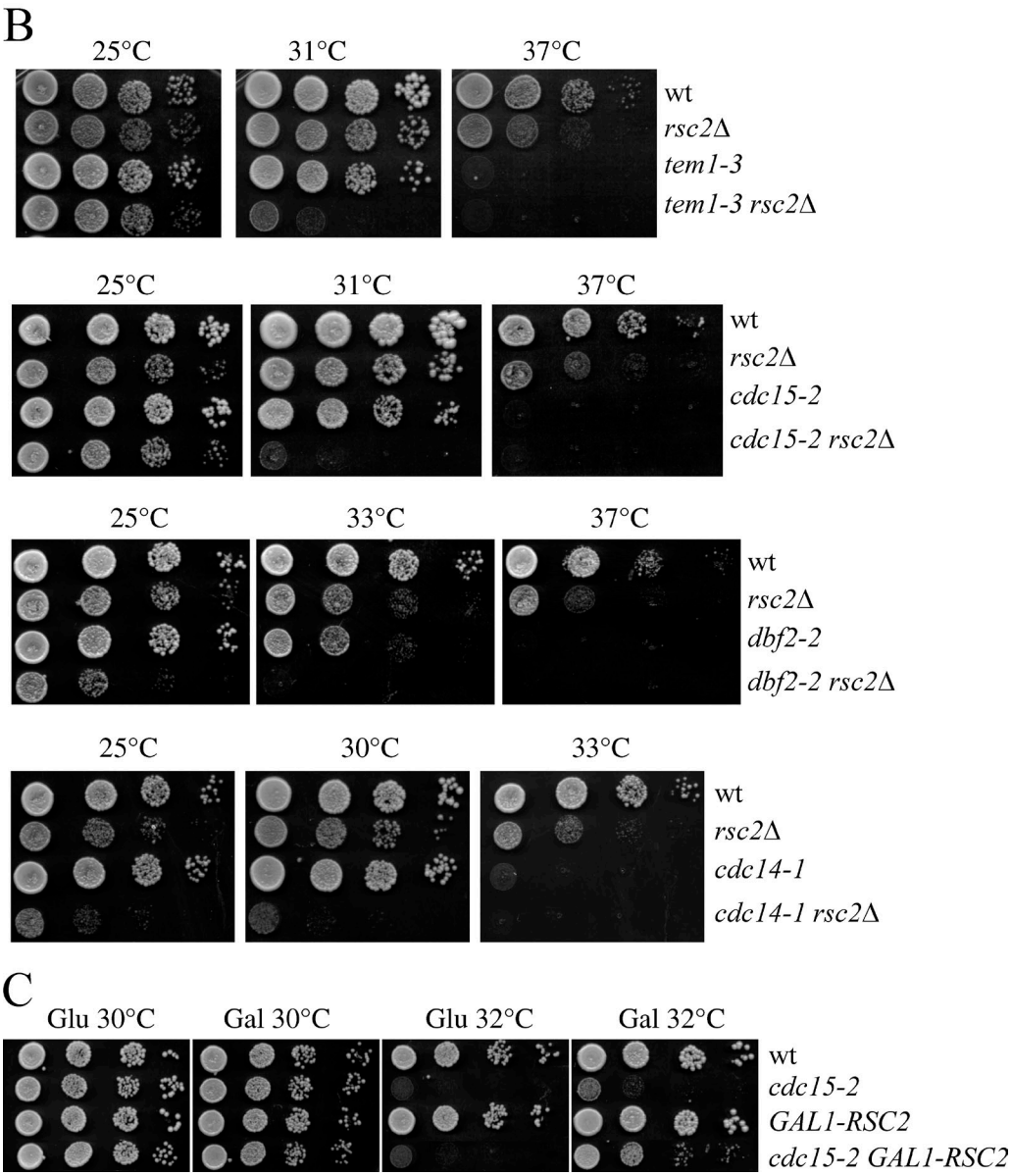

Figure 8. **Functional interactions between *RSC2* and MEN genes.** (A) Ratio of found/expected segregants observed over expected numbers of viable spores with the indicated genotypes after dissection of meiotic tetrads generated from diploid strains heterozygous for the *rsc2Δ* (ySP6859) and *lte1Δ* (ySP3418) alleles, the *cdc5-2* (ySP324) and *rsc2Δ* (ySP6859) alleles, or the *rsc2Δ* (ySP6859) and *cdc14-3* (ySP284) alleles. *, very sick viable spores. (B) Serial dilutions of strains with the indicated genotypes were spotted on YEPD plates and incubated at the indicated temperatures. (C) Serial dilutions of strains with the indicated genotypes were spotted on YEPD (Glu, *GAL1* promoter off) and YEPRG (Gal, *GAL1* promoter on) plates and incubated for 2 d at 30°C and 32°C. wt, wild type.

Cells expressing Cdc28-F19 were previously shown to be defective in Cdc20–APC activation (Rudner et al., 2000), thereby explaining their ability to retard adaptation to the SAC. All these data indicate that mitotic slippage requires conventional regulators of mitotic exit and are consistent with the proposal that it relies on the inability of the SAC to inhibit all Cdc20–APC complexes inside the cell (Brito and Rieder, 2006). Presumably, a fraction of Cdc20–APC remains active upon SAC activation and promotes cyclin B destruction until cyclin B/Cdk activity drops below a threshold level sufficient to drive cells out of mitosis.

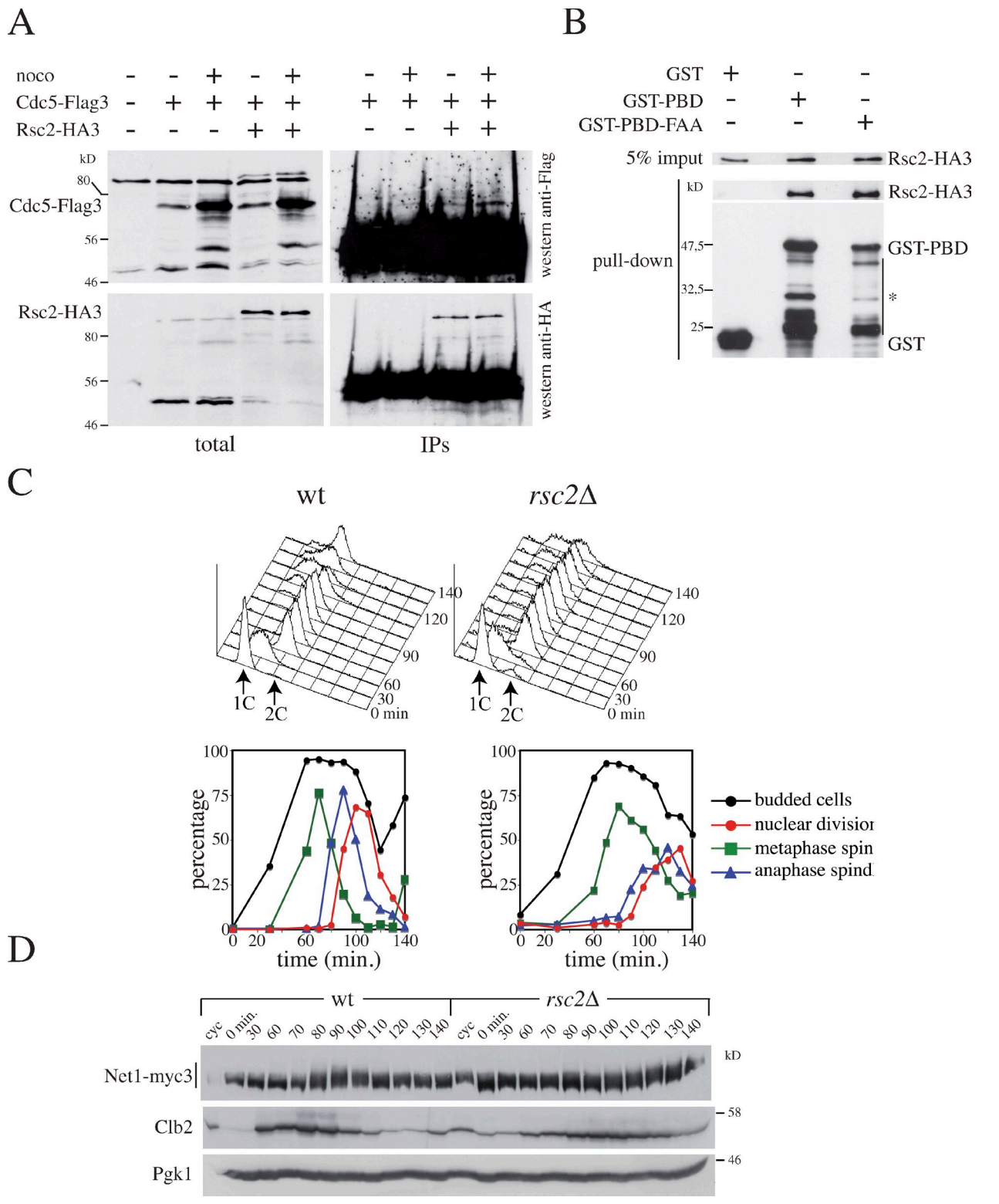

Figure 9. **Rsc2 interacts physically with Cdc5 and is required for timely Net1 and Cdc14 phosphorylation.** (A) Wild type (wt; W303), *CDC5-FLAG3* (ySP7797), and *RSC2-HA3 CDC5-FLAG3* (ySP7814) were grown exponentially or arrested in nocodazole for 3 h. Protein extracts were analyzed by Western blotting with anti-HA (Rsc2) or anti-Flag (Cdc5) antibodies either directly (total) or after Rsc2 immunoprecipitation with anti-HA antibodies (IPs). (B) A protein extract prepared from nocodazole-arrested cells expressing Rsc2-3HA (ySP7092) was incubated with glutathione-Sepharose beads carrying GST, GST-PBD, or mutated GST-PBD-FAA. Input and pull-down samples were analyzed by Western blotting with anti-HA or anti-GST antibodies. The bar with an asterisk denotes truncated forms of GST-PBD. (C and D) α-factor–arrested wild-type (ySP8573) and *rsc2Δ* (ySP8596) cells expressing Net1-myc3 were released in fresh medium at 25°C (t = 0). At the indicated times, cell samples were collected for FACS analysis of DNA contents (C, histograms), to measure the kinetics of budding, spindle formation/elongation, and nuclear division (C, graphs), and for Western blot analysis (D) of Net1-myc3, Clb2, and Pgk1 (loading control).

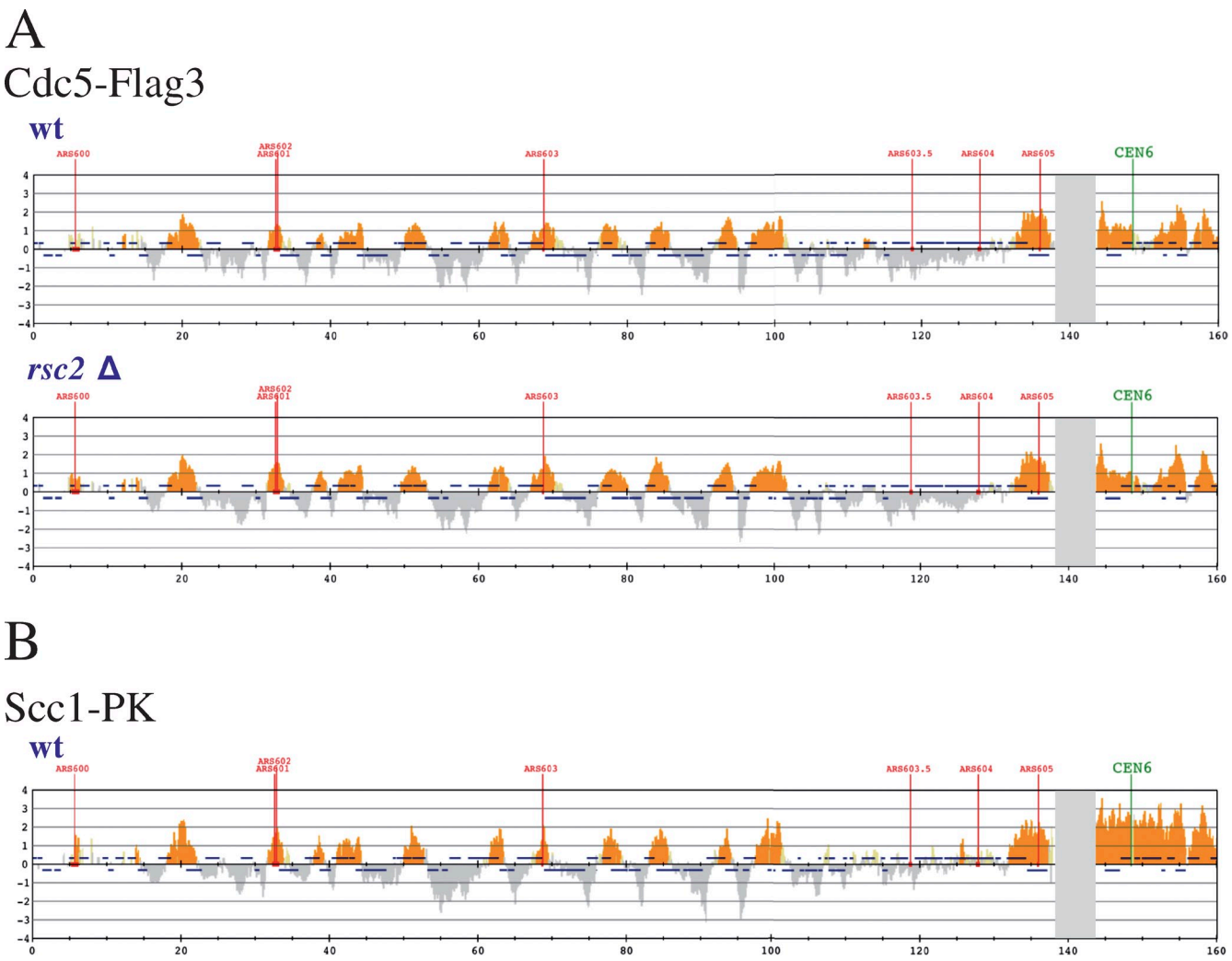

Figure 10. ***RSC2* deletion does not affect Cdc5 chromosomal distribution.** Wild-type (wt; ySP7797) and *rsc2*Δ cells (ySP8200) expressing FLAG-tagged Cdc5 (A) and wild-type cells expressing PK-tagged Scc1 (B) were arrested in mitosis with benomyl and treated for ChIP-on-chip analysis. Enrichment of DNA fragments in the immunoprecipitate relative to a whole-genome DNA sample is shown along the first 160 kb (left arm and centromere) of chromosome VI. The y-axis scale is log2. Orange signals represent significant binding as previously described (Katou et al., 2003). The used statistical algorithm is identical to that for the GeneChip Operating Software (Affymetrix). The greenish signal indicates the centromere. Blue bars above and below the midline represent ORFs transcribed from left to right and opposite, respectively. A region around 140 kb masked by a gray box corresponds to Ty retrotransposon, which exists in multiple copies in the genome and was omitted from the analysis.

Upon prolonged treatment with nocodazole, adaptation in vertebrate cells takes place with SAC proteins still at kinetochores, leading to the proposal that it occurs through SAC signaling override (Brito and Rieder, 2006). We show that adaptation to the SAC in budding yeast coincides with Mad1 dissociation from Bub3, suggesting that the SAC is silenced. Microtubule-binding proteins, such as dynein and spindly, are involved in vertebrate SAC silencing through poleward transport of SAC proteins along microtubules (Howell et al., 2001; Wojcik et al., 2001; Gassmann et al., 2010). Therefore, it is likely that spindle disruption by nocodazole impairs this mechanism, thus accounting for the persistence of SAC proteins at unattached kinetochores during adaptation. In addition, Cdk activity is required to sustain the SAC (Li and Cai, 1997; Kitazono et al., 2003; Yamaguchi et al., 2003), and it drops during adaptation, suggesting that SAC signaling is likely to decline during mitotic slippage. In any case, whether silencing of SAC signaling is a cause or a consequence of adaptation remains to be established.

A role for the RSC complex in the early anaphase release of Cdc14 from the nucleolus and in mitotic exit regulation

We provide experimental evidence of a novel role for the chromatin-remodeling complex RSC in regulation of Cdc14 nucleolar release and mitotic exit. Remarkably, histone post-translational modifications have been recently implicated in the regulation of Cdc14 release from nucleolar chromatin in early anaphase (Hwang and Madhani, 2009), suggesting that multiple chromatin modifiers cooperate in this process.

The RSC complex regulates transcription mainly at PolII and PolIII promoters (Parnell et al., 2008) and has been implicated in several cell cycle processes, such as kinetochore function (Hsu et al., 2003) and sister chromatid cohesion (Baetz et al., 2004;

Huang and Laurent, 2004). However, transcriptional regulation of several classes of mitotic genes seems unaffected by RSC inactivation (Cao et al., 1997), suggesting that this complex might have additional and perhaps more direct functions in cell cycle progression. Other chromatin regulators have been involved in cell cycle processes unrelated to their transcriptional function. For example, chromatin-remodeling proteins were also found at human centrosomes, where they regulate the recruitment of centrosomal proteins, microtubule organization, and cytokinesis (Sillibourne et al., 2007).

Budding yeast RSC associates with two alternative and closely related subunits, Rsc1 and Rsc2 (Cairns et al., 1999), which were previously found to be differentially involved in mitotic processes, such as sister chromatid cohesion and 2-µm plasmid partitioning (Wong et al., 2002; Baetz et al., 2004). However, Rsc1 and Rsc2 bind to the same chromosomal regions (Ng et al., 2002), raising the possibility that differences in their abundance might account for their unique properties. Our data indicate that RSC^{Rsc2}, and not RSC^{Rsc1}, is specifically implicated in Cdc14 activation and adaptation to the SAC. The involvement of RSC^{Rsc2} in the control of mitotic exit is particularly apparent in conditions that activate the SAC, such as upon microtubule disruption or *MAD2* overexpression. Indeed, RSC impairment through *RSC2* deletion delays mitotic exit under these conditions but not during the unperturbed cell cycle. In this respect, RSC mutants behave similarly to FEAR mutants, which show a marked mitotic exit defect only when the MEN is partially inactive (Stegmeier et al., 2002). This raises the interesting possibility that RSC is itself part of the FEAR or acts in a parallel pathway. Indeed, *RSC2* deletion, like FEAR mutations (Stegmeier et al., 2002; Queralt and Uhlmann, 2008), impairs Net1 phosphorylation and prevents the partial nucleolar release of Cdc14 in early anaphase. Furthermore, it is lethal for *lte1Δ* cells and causes synthetic lethality/sickness to several MEN mutants. How RSC^{Rsc2} might regulate Cdc14 release from the nucleolus remains an open question, but our finding that Rsc2, like other FEAR components (Rahal and Amon, 2008), interacts physically with Cdc5 provides a possible mechanistic explanation. The Rsc2–Cdc5 interaction does not seem to require the critical residues in the PBD that are involved in phosphoepitope recognition (Song et al., 2000; Elia et al., 2003b), suggesting that it might be independent of prior Rsc2 phosphorylation and follow unconventional rules. Interestingly, the homologue of Rsc2 in higher eukaryotes, Baf180, interacts with the polo-like kinase in *X. laevis* (Yoo et al., 2004).

How could RSC regulate the FEAR function of Cdc5? Because RSC was found at numerous PolII and PolIII promoters (Ng et al., 2002) as well as at centromeres (Hsu et al., 2003), we wondered whether RSC might regulate Cdc5 recruitment to specific chromosomal regions. However, our ChIP-on-chip data rule out this possibility. We found that Cdc5 binds to the rDNA, where it might interact with the RENT complex and promote Cdc14 release, but this chromosomal location is also unaffected by *RSC2* deletion (unpublished data). In addition, deletion of the whole rDNA region from chromosome XII did not rescue the ability of *mad2Δ rsc2Δ* cells to rereplicate DNA in the presence of nocodazole (unpublished data), suggesting that the control of Cdc14 nucleolar release by RSC might be exerted at levels different from the rDNA. Several other possibilities can be envisioned: for example, RSC could have roles independent from its binding to chromatin, or it could locally regulate Cdc5 kinase activity and/or access to its substrates. Alternatively, because Cdc14 and Net1 bind to different sequences within the rDNA (Huang and Moazed, 2003; Stegmeier et al., 2004), and their binding is regulated by Cdc5 (Shou et al., 2002), changes in chromatin structure might affect interactions within the RENT complex and/or make it more susceptible to Cdc5-dependent regulation. Interestingly, sister chromatid cohesion at the transcriptionally silent mating type loci requires both Sir2, which is also part of the RENT complex (Shou et al., 1999), and RSC^{Rsc2} (Chang et al., 2005), suggesting that functional interactions between RSC and Sir2 may take place at other chromosomal locations.

Knowing the exact function of Cdc5 in the FEAR network and Cdc14 nucleolar release will certainly help addressing the role of RSC^{Rsc2} in Cdc5 regulation. The FEAR function of Cdc5 has been recently attributed primarily to Cdc5's ability to stimulate degradation of Swe1, the Wee1-like Cdk inhibitory kinase (Liang et al., 2009). However, *SWE1* deletion could not bypass the mitotic arrest of nocodazole-treated *mad2Δ rsc2Δ* cells (unpublished data), whereas the $CDC14^{TAB6\text{-}1}$ allele could do so, indicating that Cdc5 targets other substrates besides Swe1 to carry out its FEAR function. Interestingly, Cdc5 was recently shown to interact with Cdc14 (Snead et al., 2007; Rahal and Amon, 2008), suggesting that it might directly regulate its binding to Net1 and/or its phosphatase activity.

Budding yeast as a tool for the discovery of fine-tuning regulators of mitotic exit and candidate targets in cancer therapy

Recent data showed that cancer cells undergo two alternative and competing pathways after prolonged treatment to microtubule toxins: either they die by apoptosis or slip out of mitosis (Gascoigne and Taylor, 2008). Both the apoptotic and slippage pathways have thresholds, and the fate of the cell is dictated by which threshold is breached first. Importantly, inhibiting the cell death pathway by caspase inactivation commits cells to slip out of mitosis, whereas interfering with cyclin B degradation and mitotic exit channels cells into the apoptotic pathway. Thus, discovering the factors that influence the rate of adaptation to microtubule toxins in different organisms is clearly a crucial issue in cancer research. For example, the efficacy of antimitotic drugs could be markedly increased by inhibiting factors involved in mitotic slippage, thus favoring cell death.

Our data indicate that the molecular bases for adaptation to chronic SAC activation are likely conserved in all eukaryotic cells, making budding yeast a good model system to identify factors influencing the rate of mitotic slippage. Indeed, *MAD2*-overexpressing cells have proven to be a valuable tool to find novel factors involved in fine-tuning regulation of mitotic exit and SAC adaptation, which are potential targets for cancer treatment. Strikingly, mitotic exit has recently been proposed to be a better cancer therapeutic target than spindle assembly because Cdc20 inhibition efficiently kills cancer cells, preventing mitotic slippage and providing more time for apoptosis

(Huang et al., 2009). Targeting essential regulators of mitotic exit during cancer treatment would have the drawback of killing also normally proliferating cells. Our finding that nonessential tuners of mitotic exit, such as the RSC complex, dramatically influence SAC adaptation opens important therapeutic perspectives that will be worth addressing in the future.

Materials and methods

Strains, media, and reagents

All yeast strains (Table S1) were derivatives of or were backcrossed at least three times to W303 (*ade2-1*, *trp1-1*, *leu2-3,112*, *his3-11,15*, *ura3*, and *ssd1*). Cells were grown in synthetic complete–selective medium (6.7 g/liter yeast nitrogen base supplemented with the appropriate nutrients and sugar) to maintain selective pressure or YEP (yeast extract, peptone) medium (1% yeast extract, 2% bactopeptone, and 50 mg/liter adenine) supplemented with 2% glucose (YEPD), 2% raffinose (YEPR), or 2% raffinose and 1% galactose (YEPRG). Unless otherwise stated, α-factor was used at 2 µg/ml for *BAR1* and 0.2 µg/ml for *bar1* strains. Nocodazole was used at 15 µg/ml for prolonged mitotic arrests and 5 µg/ml for nocodazole washout experiments. Benomyl was used at 12.5 µg/ml to test the sensitivity of strains or at 80 µg/ml for adaptation experiments. For galactose induction of α-factor–synchronized cells, galactose was added 0.5 h before release.

Plasmid constructions and genetic manipulations

To clone *MAD2* under control of the *GAL1-10* promoter (plasmid pSP493), a BamHI PCR product containing the *MAD2* coding region and 200 bp of downstream sequence was cloned into the BamHI site of a *GAL1-10*–bearing YIplac211 vector; pSP493 integration was directed to the *URA3* locus by StuI digestion. To clone *RSC2* behind the *GAL1-10* promoter (plasmid pSP679), a PstI PCR product containing the *RSC2* coding region and 200 bp of downstream sequence was cloned in the PstI site of a *GAL1-10*–bearing YIplac128 vector. pSP679 integration was directed to the *LEU2* locus by AflII digestion. Copy number of the integrated plasmids was verified by Southern analysis. *RSC2*, *LTE1*, and *RSC1* chromosomal deletion were generated by one-step gene replacement (Wach et al., 1994). *RSC2* was tagged immediately before the stop codon by one-step gene tagging (Knop et al., 1999). *CDC5-3Flag* was a gift from E. Schwob (Institute of Molecular Genetics, Montpellier, France).

Screen for mutants hypersensitive to *MAD2* overexpression

MATa and *MATα GAL1-MAD2* strains (ySP6170 and ySP6273) were transformed with an *mTn-lacZ/LEU2*–mutagenized yeast library (Kumar et al., 2002). 3.2×10^4 Leu^+ transformants were then replica plated on synthetic complete–Leu galactose medium to identify slow-growing clones. To discard the clones that were slow growing because of defects in galactose metabolism, we streaked out the selected clones on 5-fluoro-orotic acid plates to select for their derivatives that had lost the *GAL1-MAD2* construct marked *URA3*. The transposons were recovered and sequenced as previously described (Kumar et al., 2002) to identify their chromosomal insertion sites.

Immunoprecipitations, pull-downs, and Western blot analysis

For Rsc2–Cdc5 coimmunoprecipitation, cells were lysed with Zymolyase 20T at 30°C (1.2 M sorbitol, 0.1 M K-phosphate, pH 6.4, 0.5 mM $MgCl_2$, 0.6% β-mercaptoethanol, and 600 µg/ml Zymolyase). Spheroplasts were washed twice with the same buffer and incubated in immunoprecipitation buffer (50 mM Hepes, pH 7.4, 75 mM KCl, 1 mM $MgCl_2$, 1 mM sodium orthovanadate, 60 mM β-glycerophosphate, 1 mM EGTA, pH 8, 0.1% Triton X-100, and 1 mM DTT supplemented with a cocktail of protease inhibitors [Complete; Boehringer Ingelheim]) at 4°C for 30 min. 1–2 mg of cleared extracts were incubated for 30 min with protein A–Sepharose and 1 h with anti-HA antibodies (12CA5). Protein A–Sepharose was then added to the immunoprecipitations and incubated for 30 min. The slurry was washed four times with immunoprecipitation buffer and twice with PBS before loading. Mad1–Bub3 coimmunoprecipitations and pull-downs were performed as previously described (Brady and Hardwick, 2000; Donnianni et al., 2010). TCA protein extracts were prepared as previously described (Fraschini et al., 1999). Nondenaturing protein extracts were prepared according to Chiroli et al. (2003). Proteins transferred to Protran membranes (Schleicher and Schuell) were probed with 9E10 mAb for myc-tagged proteins, with 12CA5 or 16B12 mAb (Babco) for HA-tagged proteins, with anti-FLAG M2 mAb (Sigma-Aldrich) for FLAG-tagged proteins, or with polyclonal antibodies against Mad2, Clb2, Cdc11 (Santa Cruz Biotechnology, Inc.), Pgk1 (Invitrogen), Sic1, and Swi6. Secondary antibodies were obtained from GE Healthcare, and proteins were detected by an enhanced chemiluminescence system (ECL; GE Healthcare) according to the manufacturer.

Other techniques

Flow cytometric DNA quantitation and in situ immunofluorescence were performed according to Fraschini et al. (1999). Nuclear division was scored with a fluorescence microscope (Eclipse E600; Nikon) on cells stained with propidium iodide. To detect spindle formation and elongation, β-tubulin immunostaining was performed with the YOL34 mAB (AbD Serotec) followed by indirect immunofluorescence using rhodamine-conjugated anti–rat antibody (Thermo Fisher Scientific). Cdc14 immunostaining was performed with sc-12045 polyclonal antibodies (Santa Cruz Biotechnology, Inc.) followed by indirect immunofluorescence using CY3-conjugated anti–goat antibody (GE Healthcare). Immunostaining of Pds1-myc18 was performed by incubation with the 9E10 mAb followed by indirect immunofluorescence using CY3-conjugated anti–mouse antibody (GE Healthcare). ChIP-on-chip analysis was performed as previously described (Sutani et al., 2009). Digital images were acquired at room temperature on a fluorescence microscope equipped with a charge-coupled device camera (DC350F; Leica) with an oil 100× 1.3 NA Plan Fluor objective (Nikon) using FW4000 software (Leica).

Online supplemental material

Fig. S1 shows that the mitotic arrest induced by *MAD2* overexpression depends on SAC proteins and securin. Fig. S2 shows genetic interactions obtained combining *RSC2* deletion with mutations in kinetochore components or microtubule-binding proteins. Fig. S3 shows the effects of *RSC2* deletion on adaptation to the SAC upon *MAD2* or *MPS1* overexpression, as well as upon microtubule depolymerization by benomyl. Fig. S4 shows that FEAR components, but not Rsc1, are required for mitotic exit of *mad2Δ* cells treated with nocodazole, as well as for adaptation upon *MAD2* overexpression. Table S1 contains the list of yeast strains used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201007025/DC1.

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