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Yeast response to prolonged activation of the spindle assembly checkpoint

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CONTENTS

ABSTRACT

Faithful chromosome segregation during mitosis is fundamental for cell viability and genome stability. For a correct division, all kinetochores must be attached to the mitotic spindle and cohesion must be timely removed. Anaphase is triggered by the Anaphase Promoting Complex bound to its regulatory subunit Cdc20 (APC-Cdc20) that polyubiquitylates securin (Pds1 in budding yeast), whose role is to maintain inactive the protease separase (Esp1 in budding yeast) until anaphase onset. Once active, separase cleaves cohesin, thus triggering sister chromatid separation. Separase also promotes cyclinB proteolysis and mitotic exit due to its involvement in the Cdc14-early anaphase release (FEAR) pathway that promotes a partial activation of the Cdc14 phophatase, which is in turn key for CDK inactivation and mitotic exit. Cdc14 is maintained inactive throughout most of the cell cycle bound to its inhibitor Net1/Cfi1 and trapped in the nucleolus. At the beginning of anaphase Cdc14 is released from the nucleolus into the nucleus by the FEAR pathway; subsequently, Cdc14 is released also in the cytoplasm by the MEN (Mitotic Exit Network) pathway. In this way Cdc14 is fully active and can trigger mitotic exit by cyclinB-CDK inactivation.

The Spindle Assembly Checkpoint (SAC) is a surveillance mechanism conserved in all eukaryotic organisms that ensures the correct segregation of the genetic material. In fact, it inhibits the metaphase to anaphase transition until all kinetochores are properly attached to the mitotic spindle by inactivating the APC-Cdc20 complex, thus providing the time for error correction.

Cells do not arrest indefinitely upon SAC activation. After a variable period of time cells escape from the metaphase arrest also in the presence of a damaged mitotic spindle or faulty kinetochore attachments to spindle microtubules. This process is referred to as adaptation or mitotic slippage and is often involved in the resistance to chemotherapeutic compounds that target the mitotic spindle. In spite of its importance, the adaptation process is still little known.

Within this context, the goals of my Ph.D. were: (1) to characterize the molecular mechanisms underlying SAC adaptation and (2) to search for factors involved in this process. For these purposes we used the yeast *Saccharomyces cerevisiae* as a model organism.

(1) We characterized the adaptation process in either the presence or the absence of mitotic spindle perturbations. We depolymerized spindles by using two different drugs that alter microtubule dynamics, i.e. nocodazole and benomyl, whereas we induced SAC hyperactivation without spindle damage by overproducing Mad2 (*GAL1-MAD2* cells), one of the key proteins for SAC signal generation and maintenance. We observed that in all the conditions cells are able to adapt, but with different kinetics. In particular, cells adapt faster in benomyl, while in nocodazole and with high levels of Mad2 cells need more time to slip out of mitosis. The few data available about SAC adaptation in higher eukaryotes indicate that SAC adaptation is accompanied by chromatid separation, a decrease in mitotic CDK activity and mitotic exit. Indeed, like in mammalian cells, yeast securin and cyclinB are degraded and sister chromatids are separated during adaptation. In addition, cyclinB stabilization, as well as Cdc20 and Cdc5 (polo kinase) inactivation, markedly delay

adaptation, while the only yeast CKI (Sic1) is not involved in this process. Finally, when yeast cells adapt the SAC is likely to be turned off, as shown by the disassembly of the Mad1/Bub3 checkpoint complex.

(2) To search for factors involved in SAC adaptation, we performed a genetic screen using *GAL1-MAD2* cells. In particular, we screened for mutants that would remain arrested for prolonged times in mitosis upon *MAD2* overexpression. We identified Rsc2, a non-essential component of the RSC chromatin remodelling complex, as a regulator of SAC adaptation in yeast. We demonstrated that RSC^{Rsc2} is involved in fine tuning mitotic exit during the unperturbed cell cycle. Its activity becomes particularly important in conditions that would activate the SAC, as it contributes to cyclinB degradation. In the absence of Rsc2 Net1 phosphorylation and the early anaphase release of Cdc14 from the nucleolus are impaired, whereas expression of a dominant allele of *CDC14* that loosens Net1 inhibition ($CDC14^{TAB6\text{-}1}$) is sufficient to restore mitotic exit in conditions where Rsc2 becomes essential for this process. We further demonstrated that the ATPase activity of RSC is required for mitotic exit regulation, suggesting that its chromatin-remodelling activity is involved in this process. By studying possible genetic interactions between the *RSC2* deletion and FEAR or MEN mutations, we found that *RSC2* deletion confers synthetic lethality or sickness to MEN but not to FEAR mutants. Altogether, our data suggest that RSC^{Rsc2} is a novel component of the FEAR pathway. Finally, we demonstrated that Rsc2 interacts in vivo and in vitro with the polo kinase Cdc5, which controls mitotic exit at different levels.

Since RSC binds to acetylated histone tails, it is possible that histone transacetylases are also involved in SAC adaptation. We tested if the SAGA (Spt-Ada-Gcn5 Acetyltransferase) complex is involved in SAC adaptation by deleting *ADA2* or *GCN5* in yeast. Indeed, SAGA seems involved in adaptation, although the contribution of Ada2 and Gcn5 in the process differs depending on the conditions used to activate the SAC.

Finally, since we found that upon treatment with benomyl (a microtubule destabilizer) cells adapt dividing nuclei, we wondered if SAC adaptation could be linked to the presence of cytoplasmic microtubules that are still partially detectable in these conditions. We therefore asked whether motor proteins and microtubule regulators are involved in mitotic slippage. Indeed, we found that in the absence of Kip2 and Bik1, which specifically bind to cytoplasmic microtubules, cells divide nuclei and exit mitosis slower than wild type cells, demonstrating that cytoplasmic microtubules and associated proteins could accelerate SAC adaptation.

In conclusion, SAC adaptation is a very complex process whose timing probably depends on the interplay between different mechanisms. An important aim for a complete comprehension of this process, as well as for the development of new and more efficient cancer therapies, will be to identify novel factors implicated in adaptation and clarify how their function might be linked to one another.

RIASSUNTO

La corretta segregazione dei cromosomi durante la mitosi è fondamentale per la vitalità cellulare e la stabilità del genoma. Al fine di avere una corretta divisione, tutti i cinetocori devono essere correttamente attaccati al fuso mitotico e la coesione deve essere tempestivamente rimossa. L'anafase è promossa dall'Anaphase Promoting Complex legato alla suo regolatore Cdc20 (APC-Cdc20) che poliubiquitina la securina (Pds1 nel lievito), che ha il ruolo di mantenere inattiva la proteasi separasi (Esp1 nel lievito) fino all'inizio dell'anafase. Una volta attiva, la separasi taglia la coesina, promuovendo così la separazione dei cromatidi fratelli. Inoltre, la separasi è in grado di promuovere la proteolisi delle cicline mitotiche e l'uscita dalla mitosi come componente nel FEAR pathway, una via di trasduzione del segnale che controlla il rilascio parziale di Cdc14 dal nucleolo al nucleo che permette un'attivazione parziale di Cdc14. Cdc14 è la fosfatasi chiave che promuove l'inattivazione delle CDK e l'uscita dalla mitosi; essa è mantenuta inattiva e segregata nel nucleolo per la maggior parte del ciclo cellulare grazie all'interazione col suo inibitore Net1/Cfi1. All'inizio dell'anafase, Cdc14 viene rilasciata dal nucleolo al nucleo grazie all'attivazione del FEAR pathway; successivamente, Cdc14 viene rilasciato anche nel citoplasma dal MEN (Mitotic Exit Network) pathway. In questo modo Cdc14 è completamente attiva e quindi in grado di promuovere l'uscita dalla mitosi mediante l'inattivazione delle cicline mitotiche.

Lo Spindle Assembly Checkpoint (SAC) è un meccanismo di sorveglianza del ciclo cellulare altamente conservato in tutte le cellule eucariotiche che permette la corretta segregazione dei cromosomi. Infatti, esso è in grado di inibire la transizione metafase-anafase fino alla formazione dell'attacco bipolare tra il fuso mitotico e cinetocori inibendo il complesso APC-Cdc20. Ciò nonostante, il SAC non è in grado di mantenere le cellule arrestate in metafase per un tempo indefinito; infatti, dopo un certo periodo di tempo le cellule progrediscono nel ciclo cellulare sia in presenza di un fuso mitotico danneggiato che in assenza di un attacco corretto tra fuso mitotico e cinetocori. Questo processo è noto col nome di adattamento o "mitotic slippage" ed è spesso responsabile del fenomeno di resistenza ai chemioterapici che hanno come bersaglio il fuso mitotico. Nonostante la sua importanza dal punto di vista biologico, il processo di adattamento è ancora poco caratterizzato.

In questo contesto, i principali obiettivi del mio dottorato di ricerca sono stati: (1) caratterizzare i meccanismi molecolari alla base dell'adattamento e (2) cercare fattori coinvolti in questo processo. Per fare questo, abbiamo deciso di utilizzare l'organismo modello *Saccharomyces cerevisiae*.

(1) Abbiamo caratterizzato l'adattamento sia in presenza sia in assenza del fuso mitotico. In particolare, abbiamo depolimerizzato il fuso mitotico mediante l'utilizzo di due droghe che alterano la dinamica dei microtubuli, il nocodazolo e il benomyl, mentre abbiamo iperattivato il SAC senza danneggiare il fuso mitotico mediante la sovraespressione di *MAD2* (cellule *GAL1-MAD2*), una delle proteine fondamentali per la generazione e il mantenimento del segnale del SAC. Abbiamo osservato che le cellule sono in grado di adattarsi in tutte le condizioni analizzate, ma con cinetiche differenti. In particolare, le cellule si adattano più velocemente in benomyl, mentre in nocodazolo e in presenza di alti livelli di Mad2 le cellule hanno bisogno di più tempo. I dati presenti in letteratura sul processo di adattamento al SAC nelle cellule di mammifero indicano che l'adattamento è correlato con la separazione dei cromatidi fratelli, con una diminuzione dell'attività delle CDK mitotiche e con l'uscita dalla mitosi. In lievito abbiamo verificato che durante l'adattamento avviene la degradazione della securina e della ciclina B e che i cromatidi fratelli vengono separati; inoltre, la stabilizzazione della ciclina B (Clb2) e l'inattivazione di Cdc20 e della polo chinasi Cdc5 ritarda notevolmente l'adattamento, mentre l'inibitore delle CDK mitotiche (Sic1) non è coinvolto in questo processo. Infine, durante il processo di adattamento le cellule di lievito spengono il SAC; infatti, in queste cellule il complesso Mad1/Bub3 viene disassemblato.

(2) Per cercare fattori coinvolti nell'adattamento al SAC, abbiamo eseguito uno screening genetico utilizzando cellule *GAL1-MAD2*. In particolare, eravamo alla ricerca di mutanti che rimanevano arrestati in mitosi più a lungo. Dallo screening abbiamo identificato Rsc2, una componente non essenziale del complesso RSC, coinvolto nel rimodellamento della cromatina. Abbiamo dimostrato che RSC^{Rsc2} è coinvolto nella regolazione dell'uscita dalla mitosi durante un ciclo cellulare normale. Infatti, in assenza di Rsc2 la fosforilazione di Net1 e il rilascio parziale di Cdc14 dal nucleolo sono compromessi; è però sufficiente l'espressione di un allele dominante di *CDC14* che si lega più labilmente all'inibitore Net1 ($CDC14^{TAB6-1}$) per ripristinare l'uscita dalla mitosi in condizioni in cui Rsc2 risulta essenziale per questo processo. Abbiamo inoltre dimostrato che l'attività ATPasica del

complesso RSC è importante per la regolazione dell'uscita dalla mitosi; per questo, abbiamo ipotizzato che l'attività di rimodellamento della cromatina è richiesta per questo processo. Studiando le possibili interazioni genetiche tra la delezione di *RSC2* e mutazioni che affliggono il FEAR o il MEN, abbiamo dimostrato che la delezione di *RSC2* provoca letalità sintetica o forti difetti di crescita se combinata a mutazioni che affliggono il MEN, ma non il FEAR. In conclusione, i nostri dati suggeriscono che RSC^{Rsc2} potrebbe essere una nuova componente del FEAR pathway. Infine, abbiamo dimostrato che Rsc2 interagisce in vivo e in vitro con la polo chinasi Cdc5, una proteina che controlla l'uscita dalla mitosi a diversi livelli.
Dato che il complesso RSC si lega in particolare a regioni di DNA con istoni acetilati, è possibile che anche le acetilasi siano coinvolte nel processo di adattamento al SAC. Abbiamo testato se il complesso SAGA (Spt-Ada-Gcn5 Acetyltransferase) fosse coinvolto nell'adattamento mediante la delezione dei geni *ADA2* e *GCN5*. Dai dati preliminari ottenuti sembra che il complesso SAGA possa regolare l'adattamento, anche se il contributo di Ada2 e Gcn5 dipende dalle condizioni analizzate.
Infine, dato che abbiamo osservato che cellule trattate col benomyl (una droga che destabilizza i microtubuli) si adattano dividendo i nuclei, abbiamo ipotizzato che l'adattamento potrebbe dipendere dalla presenza di microtubuli citoplasmatici che sono parzialmente presenti in queste condizioni. Per questo motivo, ci siamo domandati se proteine motrici o proteine associate ai microtubuli potessero essere coinvolte nel processo di adattamento. In assenza di Kip2 e Bik1, che si legato specificamente ai microtubuli citoplasmatici, le cellule dividono i nuclei ed escono dalla mitosi più lentamente delle cellule selvatiche, dimostrando che i microtubuli citoplasmatici accelerano il processo di adattamento.
In conclusione, l'adattamento al SAC è un processo molto complesso che viene probabilmente regolato a diversi livelli.

INTRODUCTION

Correct segregation of duplicated chromosomes to daughter cells during cellular division is fundamental for the faithful inheritance of genetic material. This process needs that all chromosomes achieve bi-orientation (also called amphitelic attachment), i.e. they attach in a bipolar way to microtubules emanating from opposite spindle poles through their kinetochores, which are protein assemblies that reside at the centromere of chromosomes. As microtubule attachment is a trial and correction process, incorrect attachments can occur. If cells were to undergo anaphase in the presence of erroneous attachments, chromosomes would be segregated randomly, thus generating cells with aberrant chromosome numbers known as aneuploidies. In order to avoid this dramatic event, cells evolved a surveillance mechanism, the spindle assembly checkpoint (SAC) that delays cell cycle progression in mitosis until all chromosomes have reached a bipolar attachment and are aligned on the metaphase plate. Dysfunction in this machinery has been implicated in genetic diseases, such as the Down syndrome, and in tumor progression. (from Tanaka and Hirota, 2009).

Kinetochore-microtubule attachment

The segregation of sister chromatids during mitosis mainly depends on the forces generated by microtubules (MTs) that attach to kinetochores (Tanaka and Desai, 2008). For proper chromosome segregation, kinetochores must capture spindle MTs and properly align on the mitotic spindle before anaphase onset. Cells undergo these processes in a step-wise manner as follows (Tanaka, 2008): (1) the kinetochore initially interacts with the lateral surface of a single

MT extending from a spindle pole (spindle-pole MT) (fig. 1, step 1); (2) the kinetochore is transported along the spindle-pole MT towards a spindle pole (fig. 1, step 2); (3) the kinetochore is tethered at the plus end of a spindle-pole MT (conversion from the lateral to the end-on attachment) (fig. 1, step 2); (4) sister kinetochores attach to MTs extending from opposite spindle poles (sister kinetochore bi-orientation). If sister kinetochores attach to MTs with aberrant orientation, such errors must be corrected by turnover of the kinetochore-microtubule attachment (error correction), before anaphase onset (fig. 1, steps 3 and 4). All these steps, that are conserved in all eukaryotes (from yeast to human), are crucial to ensure high-fidelity chromosome segregation in the anaphase (fig. 1, step 5).

Here is the detailed description of the first two steps of the process:

- Step 1, initial kinetochore-microtubule interaction: this event occurs in a different cell cycle stage depending on the organism. In fact, in metazoan cells in interphase the MT-organizing centres (MTOCs) are outside of the nucleus and MTs can interact with kinetochores only when the nuclear envelope is broken down at the onset of mitosis; on the other hand, in budding yeast the kinetochores are connected to MTOCs (called spindle-pole bodies or SPBs) by MTs throughout most of the cell cycle (Winey and O'Toole, 2001). In all eukaryotic cells, however, kinetochores initially attach to the lateral side of a single microtubule (Hayden et al., 1990; Tanaka et al., 2005) because the lateral side provides a much larger surface compared with microtubule tips, thus providing a more efficient initial attachment.

- Step 2, conversion from lateral to end-on attachment: once bound to the microtubule lateral face (lateral attachment), kinetochores are transported towards a spindle pole along the microtubule (fig. 1, step 2). The kinetochore sliding along microtubule is promoted by minus end-directed motor proteins, i.e. dynein in vertebrate cells (Yang et al., 2007) and Kar3 in budding yeast (Tanaka et al, 2005). While the kinetochore is associated with the microtubule lateral face, the plus end of the shrinking microtubule often catches up with the kinetochore, leading the kinetochore to become tethered at the microtubule plus end (end-on attachment) and being pulled further towards a spindle pole as the microtubule shrinks (Tanaka et al., 2007) (fig. 1, step 2). (from Tanaka, 2010)

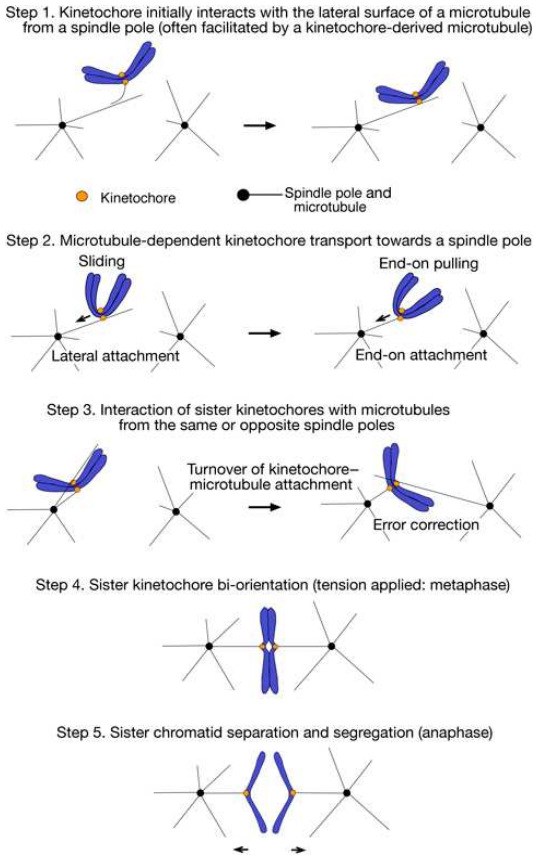

Figure 1. Kinetochore-microtubule interaction in eukaryotic cells.
The figure shows kinetochore–microtubule interactions during prometaphase (steps 1–3), metaphase (step 4) and anaphase (step 5) (from Tanaka, 2010).

Since kinetochore-microtubule attachment is a stochastic mechanism, erroneous attachments can be generated. Different kinds of attachments are possible (fig. 2): (1) amphitelic arrangement with the two sister kinetochores bi-oriented is the only correct attachment and allows proper chromosome segregation; (2) monotelic attachment, in which only one kinetochore is attached to mitotic spindle; (3) syntelic attachment, in which both kinetochores are attached to microtubules arising from the same spindle pole; (4) merotelic attachment, in which one kinetochore is correctly attached while the other one is attached to microtubules arising from the opposite poles of the mitotic spindle. Merotelic attachments are only possible in organisms where kinetochores contact simultaneously several microtubules (e.g. not in budding yeast). Finally, since the pulling forces exerted by spindle microtubules on bi-oriented kinetochores are counteracted by the cohesive forces that maintain sister chromatids together until the onset of anaphase, in the amphitelic configuration kinetochores are under tension. Establishment of kinetochore tension is indeed a key feature of chromosome segregation. (from Musacchio and Salmon, 2007)

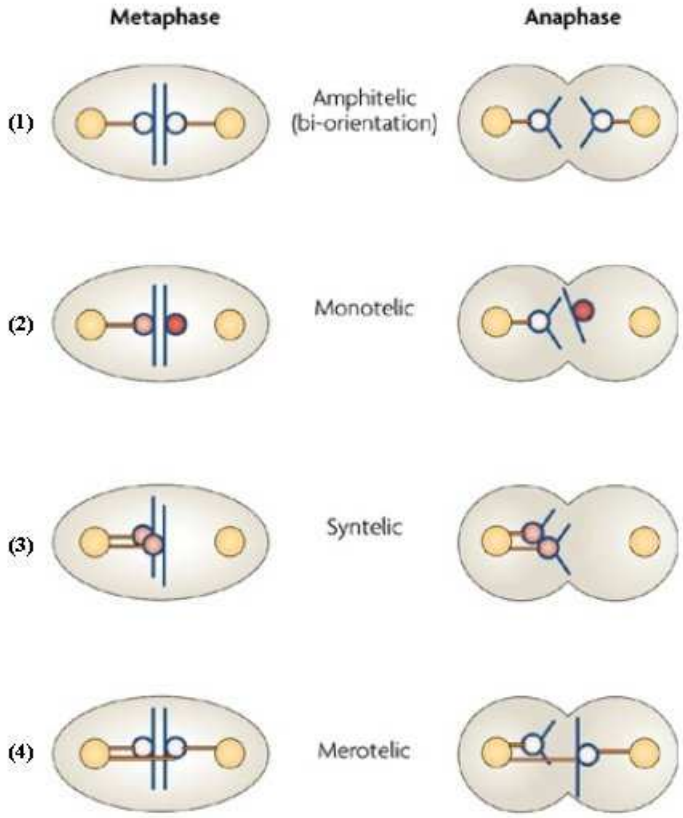

Figure 2. Different kind of kinetochore-microtubule attachments
Correct and incorrect attachments can occur during mitosis. Monotelic attachment is a normal condition during prometaphase before bi-orientation. In syntelic attachment, both sisters in a pair connect to the same pole. Merotelic attachment occurs quite frequently. The SAC is able to sense monotelic syntelic attachment, but it is unable to detect merotelic attachment, since in these condition kinetchores are under tension (from Musacchio and Salmon, 2007).

THE SPINDLE ASSEMBLY CHECKPOINT

The spindle assembly checkpoint (or SAC) inhibits the metaphase-to-anaphase transition until all kinetochores are correctly bi-oriented on the spindle. It is known that the SAC signal originates from unattached kinetochores and that one single unattached kinetochore is sufficient to sustain the checkpoint signal (Rieder at al, 1995). The

target of the SAC is the anaphase promoting complex (APC) bound to its coactivator Cdc20. The APC is an E3 ubiquitin ligase that is absolutely necessary for sister chromatid separation in anaphase and for mitotic exit by targeting some key proteins to degradation (Peters, 2006). When all chromosomes achieve bi-orientation, the activity of the SAC is silenced and the APC triggers degradation of its substrates, including cyclinB and securin: this event promotes anaphase (Clute and Pines, 1999; Hagting et al, 2002). Degradation of securin allows activation of the protease separase, which cleaves cohesion, thereby triggering sister chromatid separation. On the other hand, degradation of cyclinB causes inactivation of CDK activity and induces mitotic exit (Peters, 2006). (from Tanaka and Hirota, 2009)

How the SAC inhibits the APC

The key components of the SAC (the Mad and Bub proteins) were discovered by genetic screens in budding yeast (Li and Murray, 1991; Hoyt et al, 1991). As Mad2 is enriched at unattached kinetochores and it binds directly to Cdc20, Mad2 is thought to play a crucial role in SAC activation and maintenance (Musacchio and Salmon, 2007; Yu, 2006; Sudakin et al., 2001). Consistent with this idea, suppression of Mad2 depletes the checkpoint signal. The finding that only a fraction of Mad2 is stably bound to unattached kinetochores, whereas the other fraction is highly dynamic and turns over with the cytoplasmic pool, led to the idea that Mad2 is activated by cycling through unattached kinetochores (Howell et al, 2000; Howell et al, 2004; Shah et al, 2004). Structural analysis has identified two different conformations of Mad2 (O for open and C for closed), with different activities

(Sironi et al, 2002). The current model for Mad2 regulation is that Mad2 stably binds to Mad1 at unattached kinetochores (C-Mad2) and the resulting C-Mad2-Mad1 complex works as a receptor that binds and converts cytoplasmic free O-Mad2 from an inactive conformation (open) to an active form (closed), which is able to bind and inhibit Cdc20 and, consequently, the APC. The Cdc20-bound C-Mad2 probably activates other molecules of O-Mad2, thereby amplifying the signal (fig. 3A and B). This mechanism, called "template model", explains how the SAC signal is created and amplified and how a single unattached kinetochore can generate a strong SAC signal (Musacchio and Salmon, 2007).

Does Mad2 directly inhibit Cdc20? First, it is known that another checkpoint protein called BubR1 (or Mad3 in yeast) can directly bind Cdc20 and inhibit APC/C activity without Mad2 (Tang et al, 2001). Second, the MCC (mitotic checkpoint complex), composed by Mad2, BubR1 (or Mad3), Bub3 and Cdc20, is a stronger inhibitor for APC than Mad2 alone (Sudakin et al, 2001). Thus, a plausible hypothesis is that BubR1/Mad3, and not Mad2, is the direct inhibitor of the APC and might act through the MCC. Indeed, budding yeast Mad3 was shown to inhibit the activity of APC/Cdc20 by acting as pseudosubstrate (Burton and Solomon, 2007). In addition, it promotes Cdc20 degradation (King et al, 2007). Therefore, the most recent models for SAC signalling envision BubR1/Mad3 as direct inhibitor of Cdc20, with BubR1 binding to Cdc20 significantly enhanced by Mad2-Cdc20 (fig. 3C), which takes into account the key role of Mad2 in the SAC and the old observation that Mad2 is required for Mad3 association with Cdc20 (Hwang et al., 1998). Binding of BubR1 to

Cdc20 inhibits APC activity by acting as a pseudosubstrate and/or by promoting the ubiquitilation and degradation of Cdc20 (Nilsson et al, 2008). (from Tanaka and Hirota, 2009)

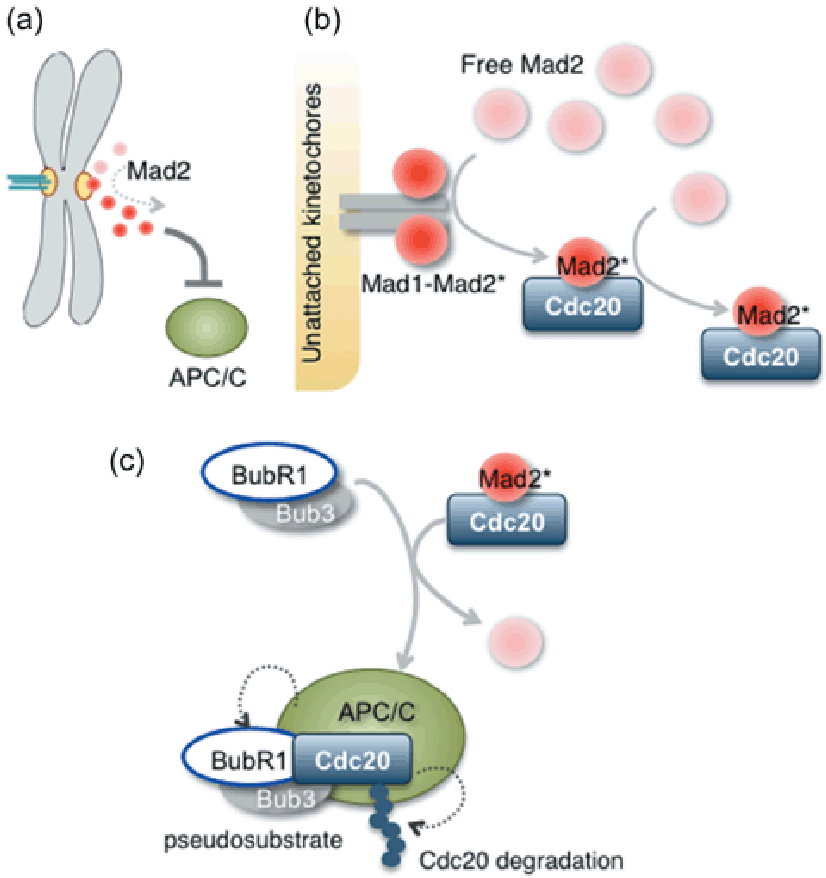

Figure 3. The molecular basis of the SAC
(a) The SAC 'wait anaphase' signal is generated at unattached kinetochores. By binding to unattached kinetochores, Mad2 molecules become active (denoted by a colour change from light to dark red), eventually leading to APC inhibition. (b) Two different conformations of Mad2 are shown in dark and light red circles. Most of the cytoplasmic, free Mad2 (open conformer, light red) is converted to an alternative conformer that can bind Cdc20 (closed conformer, dark red). The template model predicts that the Mad1–Mad2 complex at unattached kinetochores as well as the Cdc20–Mad2 complex can both catalyze this conformational change. (c) Cdc20 is handed over from Mad2 to the BubR1-Bub3 complex. BubR1 inhibits APC activity by acting as a pseudosubstrate, and/or by mediating Cdc20 ubiquitylation and degradation (as denoted by the dotted arrows). It is not entirely clear when the APC recruits Cdc20 in this cascade. (from Tanaka and Hirota, 2009).

Turning-off the SAC

Several mechanisms contribute to the inactivation of the SAC after chromosome bi-orientation during normal cell cycle.

First, one of the key processes that allows SAC inactivation is the "stripping" of Mad1, Mad2 and other SAC proteins from kinetochores, which results in their redistribution to the spindle poles (fig. 4). In metazoans, this process depends on dynein-dependent motility along microtubules (Howell et al, 2001; Wojcik et al, 2001). While Mad1-Mad2 are inactivated by dynein that removes this complex from attached kinetochores, the regulation of BubR1 kinase activity seems to depend on the kinetochore motor protein CENP-E (Mao et al, 2005). However, this second mechanism is less defined at the molecular level, also because BubR1 kinase activity does not seem to be necessary for the SAC (Elowe et al., 2010). Both these "SAC inactivation pathways" were identified in metazoans and depend on kinetochore-microtubule interactions and motor activity. No nuclear dynein or clear CENP-E homologue exist in yeast *S. cerevisiae*, and therefore whether similar mechanisms act also in yeast is unknown at the moment. In the yeasts *S. cerevisiae* and *S. pombe* dephosphorylation events carried out by protein phosphatase PP1 seem necessary for SAC silencing (Pinsky et al., 2009; Vanoosthuyse and Hardwick, 2009).

Another mechanism of SAC inactivation in metazoans is based on the protein $p31^{comet}$. $p31^{comet}$ works as a brake for the positive-feedback loop based on C-Mad2 (Vink et al, 2006) and it is possible (but has not been yet demonstrated) that kinetochores modify this protein to temporarily prevent it from carrying out its function. As part of the

Mad2-template model, it is speculated that the reactivation of $p31^{comet}$ upon disappearance of unattached kinetochores silences the SAC by turning down the catalytic activation of Mad2 (fig. 4). In practice, $p31^{comet}$ competes with free Mad2 for binding either to Mad1/Mad2 at unattached kinetochores or to Cdc20/Mad2.

Another mechanism of SAC inactivation is based on Mad2/Cdc20 dissociation. Although the Mad2/Cdc20 dissociation occurs spontaneously, this process is slow; probably, a source of energy is required to trigger this dissociation during the cell cycle. A non-degradative ubiquitylation of Cdc20 has been proposed as a possible active mechanism for dissociation of the Mad2-Cdc20 complex (Reddy et al., 2007; Stegmeier et al., 2007). Consistently, the de-ubiquitylating (DUB) enzyme "protectin" antagonizes this reaction and is required to sustain the SAC (Reddy et al., 2007; Stegmeier et al., 2007) (fig. 4). Therefore, SAC maintenance might be a dynamic process in which the MCC and Mad2–Cdc20 subcomplexes are continuously actively dissociated and recreated by unattached kinetochores.

Another mechanism that contributes to maintain the SAC inactive in anaphase is APC-induced proteolysis itself. In anaphase, tension at kinetochores is lost but the SAC is not reactivated, probably because the SAC is inhibited in anaphase and this event is important for cell cycle progression. Proteolysis of cyclinB and subsequent CDK inactivation probably play an important role in SAC inhibition (D'Angiolella et al, 2003). In fact, high levels of cyclinB/CDK are required to sustain the SAC in many organisms (Kitazono et al, 2003; Li and Cai, 1997).

Finally, Mad2 phosphorylation has been proposed to contribute to SAC silencing by preventing its conformational change from open into close configuration (Wassmann et al., 2003; Kim et al., 2010). Altogether, these mechanisms contribute to turn the SAC off when the checkpoint is satisfied. (from Musacchio and Salmon, 2007)

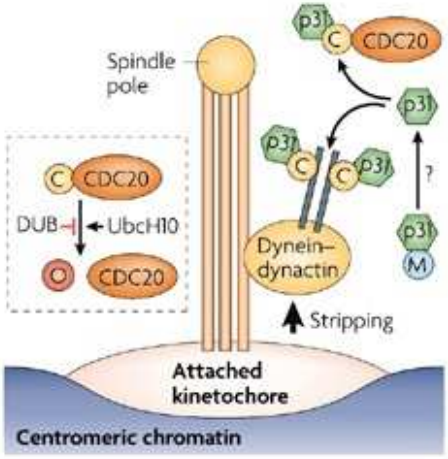

Figure 4. How the SAC is inactivated
Only three regulatory aspects that might favour Mad2–Cdc20 dissociation at anaphase are depicted. First, disappearance of unattached kinetochores might result in reactivation of the capacity of $p31^{comet}$ to inhibit the closed-Mad2/open-Mad2 interaction and thereby to inhibit the catalytic amplification of the SAC signal that is predicted by the "template model". Second, non-degradative ubiquitylation of Ccd20 in a reaction that involves the E2 enzyme UbcH10 and the de-ubiquitylating protein (DUB) protectin might accelerate the dissociation of Mad2–Ccd20. Third, the dynein–dynactin complex 'strips' Mad1–Mad2 and other proteins from kinetochores on formation of kinetochore microtubules, decreasing the ability to form new Mad2–Cdc20 complexes. A forth mechanism based on Mad2 phosphorylation is not depicted (see text) (from Musacchio and Salmon, 2007).

Adaptation to the SAC

As described above, the SAC is activated when at least one kinetochore is not correctly attached to mitotic spindle and it is inactivated when a bipolar attachment between kinetochores and the mitotic spindle is generated. Satisfying the SAC is not always

possible. Upon prolonged SAC activation a mechanism called adaptation or mitotic slippage allows cell cycle progression also in absence of correct kinetochore-microtubule attachments. Understanding how adaptation to the SAC takes place is of primary importance for cancer research and for developing more efficient drugs for cancer therapy (Rieder and Maiato, 2004; Weaver and Cleveland, 2005). In fact, some chemotherapeutic agents used in clinics called antimitotics, e.g. taxanes, inhibit mitotic spindle dynamics thereby causing a prolonged activation of the SAC. After a prolonged activation of the SAC, cancer cells can undergo apoptosis in mitosis or exit from mitosis by adaptation. Cells that adapt in turn can: (1) begin a new cell cycle; (2) arrest in G1; (2) die by apoptosis (Rieder and Maiato, 2004; Gascoigne and Taylor, 2008). Cancer cells often become resistant to antimitotics, likely because they bypass SAC activation through the adaptation process and fail to activate the cell death pathways.

In the last few years the molecular mechanisms at the basis of mitotic slippage have started to be defined. At the moment, it is known that cells undergo a progressive decrease in cyclinB/CDK activity that leads to adaptation (Gascoigne and Taylor, 2008; Brito and Rieder, 2006) and that this event occurs by cyclinB degradation. It is not clear yet if cells that adapt turn off or override the SAC. It has been proposed that cells undergo adaptation in the presence of continuous SAC signalling, as SAC proteins remain stably bound at kinetochores during mitotic slippage in the presence of microtubule depolymerizers (Brito and Rieder, 2006). However, since CDK activity is required to sustain the SAC, it is possible that during adaptation the SAC is

actually switched-off and the persistence of SAC proteins at kinetochores is simply due to the lack of poleward stripping when microtubules are depolymerized.

What happens when cells are treated for prolonged times with microtubule poisons? Recent data demonstrate that cells can undergo two alternative and competing pathways: (1) die by apoptosis or (2) slip out of mitosis through cyclinB proteolysis. Interfering with either one of the two pathways conveys cells into the second one (Gascoigne and Taylor, 2008). Therefore, the identification of factors influencing SAC adaptation would have a major impact on cancer research, as it would help designing more efficient therapeutic strategies.

Further details on the adaptation process are reported in the following review.

Adapt or die: how eukaryotic cells respond to prolonged activation of the spindle assembly checkpoint

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Abstract

Many cancer-treating compounds used in chemotherapies, the so-called antimitotics, target the mitotic spindle. Spindle defects in turn trigger activation of the SAC (spindle assembly checkpoint), a surveillance mechanism that transiently arrests cells in mitosis to provide the time for error correction. When the SAC is satisfied, it is silenced. However, after a variable amount of time, cells escape from the mitotic arrest even if the SAC is not satisfied through a process called adaptation or mitotic slippage. Adaptation weakens the killing properties of antimitotics, ultimately giving rise to resistant cancer cells. We summarize here the mechanisms underlying this process and propose a strategy to identify the factors involved using budding yeast as a model system. Inhibition of factors involved in SAC adaptation could have important therapeutic perspectives by potentiating the ability of antimitotics to cause cell death.

The SAC (spindle assembly checkpoint): a safety device against aneuploidy

Mitosis carries the intrinsic potential of generating genome instability by giving rise to daughter cells with unbalanced genetic material. A number of processes must be executed precisely and correctly to ensure proper chromosome segregation: a bipolar mitotic spindle must be assembled and sister chromatids must attach via their kinetochores to microtubules that associate with opposite poles of this spindle. Since the splitting of sister chromatids during anaphase is one of the major anaphase cell cycle points of no return, it is mandatory that all chromosomes are bipolarly attached to the mitotic spindle before anaphase. This task is overseen by the SAC, a ubiquitous safety device that ensures the fidelity of chromosome segregation during mitosis. In the presence of kinetochores that are either unattached to spindle fibres or mono-oriented with respect to spindle poles, the SAC sends an inhibitory signal that delays the onset of anaphase and mitotic exit until bipolar attachment is achieved, thus preventing aneuploidy occurrence (reviewed in [1,2]).

The players involved in SAC signalling are conserved throughout evolution. During the process of microtubule capture by kinetochores during prophase and prometaphase, the SAC proteins Bub3, Mad2 and Mad3/BubR1 form the MCC (mitotic checkpoint complex) that inhibits the activity of the Cdc20/APC (cell division cycle 20/anaphase-promoting complex) ubiquitin ligase, which is essential to target the anaphase inhibitor securin and cyclin B to degradation. By inhibiting Cdc20/APC, the SAC prevents sister chromatid separation and mitotic exit until all chromosomes reach a correct bipolar attachment to the mitotic spindle. Other SAC proteins, such as the Mad1, Bub1, Mps1 and Ipl1/AuroraB kinases amplify the signal and regulate the rate of MCC formation (reviewed in [2]). Most SAC proteins accumulate at unattached kinetochores during prophase and prometaphase and from this location generate the 'wait anaphase' signal that leads to Cdc20/APC inhibition, possibly by accelerating the rate of MCC formation (reviewed in [3]).

Once satisfied by the attainment of bipolar attachment on all chromosomes, the SAC is switched-off by a number of different mechanisms, allowing sister chromatid separation and cell-cycle progression. This coincides with the disappearance of SAC proteins from kinetochores. Two major mechanisms contribute to SAC switch-off in metazoans: (i) poleward transport of SAC proteins and spindly by the minus-end directed motor dynein/dynactin from kinetochores along microtubules [4–6]; and (ii) SAC silencing by $p31^{comet}$, which turns down the ability of Mad2 to inhibit Cdc20/APC [7–10]. In addition, there are conflicting reports as to whether non-degradative ubiquitylation of Cdc20 by APC is required for MCC disassembly and to extinguish SAC signalling [11–13]. Budding and fission yeast, which undergo a closed mitosis in the absence of nuclear envelope breakdown, lack nuclear dynein and a clear homologue of $p31^{comet}$. In these organisms the protein phosphatase PP1 was recently shown to silence the SAC independently of microtubules [14–16]. In addition, degradation of the SAC kinase Mps1 at the onset of anaphase has also been proposed as a mechanism to switch-off the checkpoint [17].

Key words: adaptation, antimitotic, mitotic slippage, spindle assembly checkpoint.
Abbreviations used: cdc20/APC, cell division cycle 20/anaphase-promoting complex; CDK, cyclin-dependent kinase; FEAR, fourteen early anaphase release; MCC, mitotic checkpoint complex; MEN, mitotic exit network; SAC, spindle assembly checkpoint.
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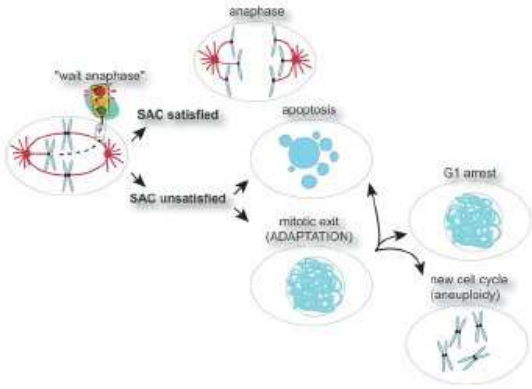
Figure 1 | The different cell fates after SAC activation

Summary of the possible destinies of a cell in which the SAC has been activated by a single unattached kinetochore. The cell-cycle delay imposed by the SAC is normally sufficient to establish a bipolar attachment on all chromosomes and ensure proper anaphase (SAC satisfied). Upon prolonged SAC activation, if errors are not corrected (SAC unsatisfied), the cell can either die in mitosis by apoptosis or slip out of mitosis. This unscheduled mitotic exit can in turn produce different outcomes: (i) drive the cell into the apoptotic pathway, (ii) lead to a stable G_1 arrest or (iii) allow entry into a new cell cycle. The latter fate gives rise to aneuploid cells. In case of treatment with antimitotics that depolymerize microtubules, cells slipping out of mitosis will be either aneuploid (if one or more kinetochores remain attached) or polyploid (if all kinetochores remain unattached).

COLOUR



Adaptation or mitotic slippage: the cell's response to prolonged SAC activation

In spite of its key role in maintaining genome stability, the mitotic delay imposed by the SAC is not permanent, but rather transient. For example, in the continuous presence of microtubule-depolymerizing drugs eukaryotic cells are unable to form proper kinetochore–microtubule connections and to satisfy the checkpoint, but nevertheless can undergo sister chromatid separation and exit mitosis. This process is commonly referred to as 'adaptation' or 'mitotic slippage' [18,19]. Adaptation to the SAC is so far little studied, although premature adaptation to checkpoint signalling might be an important cause of genetic instability and aneuploidy, and have a major impact on cancer treatment [18,19]. Many chemotherapeutic agents used in the clinic, such as taxanes and vinca alkaloids (the so called 'antimitotics'), inhibit spindle function in human cells and are widely used for the treatment of breast and ovarian cancers. Their therapeutic effectiveness relies on their impact on mitotic progression. In fact, their ability to protract mitotic arrest is thought to be a major factor in their cytotoxicity. After many hours of SAC-induced cell-cycle arrest, cancer cells either undergo apoptosis in mitosis, or exit mitosis by slippage into a tetraploid G_1 state, from which they either die, or arrest in G_1, or initiate a new cell cycle (Figure 1) [18,20,21]. The development of resistance to antimitotics correlates with the failure to undergo apoptosis and with the ability of cells to ultimately escape mitosis and enter the next G_1-phase as viable entities [20,22,23]. Although it is quite intuitive that adaptation in the presence of spindle damage can lead to aneuploidy and chromosomal instability, thereby contributing to increase malignancy, more surprising is the finding that escaping the mitotic arrest imposed by *MAD2* overexpression, which does not perturb spindle dynamics, also leads to chromosome gains and losses, as well as chromosome breaks and bridges [24]. This in turn results in the induction of a wide variety of neoplasias, thus accounting for the observation that Mad2 is overexpressed in many human tumours [25–27]. Therefore mitotic slippage to SAC activation seriously harms genome stability, presumably by uncoupling cell-cycle progression and mitotic control.

Whether cells exit from mitosis upon long-term SAC activation through overriding the checkpoint signal or through SAC silencing remains an open question. Adaptation to the DNA-damage checkpoint, for instance, occurs with concomitant extinction of checkpoint signalling. However,

the discovery of mutations (such as the *cdc5-ad* mutation in the budding yeast polo kinase; [28,29]) specifically impairing adaptation to, but not recovery from, the DNA-damage checkpoint argues that adaptation is not only conceptually, but also mechanistically, different from checkpoint silencing. Since upon prolonged treatment with nocodazole (a microtubule-depolymerizing drug) SAC proteins are retained at kinetochores when cells resume cell-cycle progression, adaptation was proposed to occur through SAC signalling override [30]. However, more direct assays on the activity of Cdc20/APC when cells adapt should be applied. In fact, since CDK (cyclin-dependent kinase) activity is required to sustain the SAC [31–33] and it drops during adaptation (see below), it is possible that SAC signalling declines during mitotic slippage. In addition, in light of the involvement of microtubule-binding proteins, such as dynein and spindly, in stripping SAC proteins from kinetochores, it is possible that disruption of the mitotic spindle by nocodazole impairs this mechanism, thus accounting for the persistence of SAC proteins at unattached kinetochores during adaptation. Other ways to silence the SAC, e.g. through $p31^{comet}$, could contribute to adaptation under these conditions.

Recent studies have started to define the molecular mechanisms underlying mitotic slippage. Mitotic exit under these conditions is linked to a progressive decline in cyclinB/CDK activity that, after reaching a threshold level, drives cells out of mitosis [20,30]. The decrease in CDK activity is accompanied by cyclin B degradation and, consistently, expression of non-degradable cyclin B delays mitotic slippage upon prolonged spindle disassembly. Therefore the mechanism for how cells escape mitosis in the presence of functional SAC has been proposed to rely on the inability of the SAC to inhibit all Cdc20/APC complexes inside the cell [30]. Indeed, it has been proposed that the SAC is unlikely to inhibit all Cdc20 within the cell, as this would be hardly reversible [34]. In addition, the APC was recently shown to be activate during mitotic slippage [35].

What is the destiny of adapting cells? Results obtained by treating cancer cells with a KSP (hsEg5, kinesin-5) inhibitor, which leads to a mitotic arrest with monopolar spindles, led to the proposal that persistent spindle damage causes apoptosis coupled to slippage to SAC activation [36]. However, data recently obtained through single-cell analysis rather than on cell populations, 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 [20]. The latter destiny can obviously be detrimental for cancer treatment and is prone to generate cells resistant to antimitotic drugs [22]. Both the apoptotic and slippage pathways have thresholds, and the fate of the cell is dictated by which threshold is breached first. Most importantly, inhibiting the cell-death pathway by caspase inactivation channels cells to slip out of mitosis, whereas interfering with cyclin B degradation and mitotic exit channels cells into the apoptotic pathway [20]. The important implication of these results is that the efficacy of antimitotic agents could be markedly increased by inhibiting the factors involved in mitotic slippage, thus favouring cell death.

Factors influencing adaptation to the SAC: what we can learn from yeast genetics

Surprisingly little is known currently about the factors that influence cell fate upon prolonged exposure to antimitotic drugs.

By using budding yeast as a model system, we started to study the process of adaptation to the SAC upon treatment with microtubule poisons or hyperactivation of the SAC in the absence of spindle damage through *MAD2* overexpression. Both of these conditions temporarily arrest cells in mitosis for a few hours. Afterwards, however, cells slip out of mitosis and keep dividing (V. Rossio, E. Galati and S. Piatti, unpublished work). We find that essential regulators Q1 of mitotic exit, such as Cdc20 and the polo kinase Cdc5, are necessary for mitotic slippage (V. Rossio, E. Galati and S. Piatti, unpublished work), in agreement with the possibility Q1 that a fraction of Cdc20/APC remains active upon SAC activation and promotes cyclin B destruction until their CDK activity drops below a threshold level sufficient to drive cells out of mitosis. Similarly, Cdc20 inactivation by siRNA (small interfering RNA) was recently shown to delay degradation of APC substrate during adaptation to prolonged nocodazole treatment in human cells [35]. Having found that essential regulators of cyclin B proteolysis, i.e. Cdc20 and Cdc5, are necessary for adaptation in yeast, along with the older proposal that inhibitory phosphorylation of cyclin B/CDKs accelerates adaptation to prolonged SAC activation [37], suggests that inactivation of mitotic CDK is the driving force for mitotic slippage. Thus the molecular bases for adaptation to chronic SAC activation might be conserved in all eukaryotic cells, which makes budding yeast a good model system to identify factors influencing the rate of adaptation.

We designed a genetic screen to find yeast mutants defective in adaptation to the SAC. By searching for mutants delaying mitotic exit upon *MAD2* overexpression, we have implicated the conserved RSC chromatin-remodelling complex in the regulation of mitotic exit and adaptation to the spindle checkpoint (V. Rossio, E. Galati and S. Piatti, unpublished work). We find that it does so by controlling the protein Q2 phosphatase Cdc14, which is essential for mitotic exit by promoting cyclin B degradation and accumulation of the cyclinB/CDK inhibitor Sic1 [38]. The yeast Cdc14 phosphatase is activated through its release from the nucleolus, where it is sequestered throughout most of the cell cycle by binding to its inhibitor Net1/Cfi1 as part of the chromatin-bound RENT (regulator of nucleolar silencing and telophase exit) complex [39,40]. Two pathways contribute to the full activation of Cdc14: the FEAR (fourteen early anaphase release) and the MEN (mitotic exit network). By promoting a first wave of partial Cdc14 release from the nucleolus in early anaphase, the FEAR allows full activation of the MEN that leads to complete release of Cdc14 in the nucleus and cytoplasm, thereby triggering cyclin B proteolysis and

mitotic exit [41]. Importantly, whereas MEN inhibition causes a cell-cycle arrest in telophase, FEAR inactivation only moderately delays mitotic exit [42]. We find that the RSC complex impinges specifically on the first wave of Cdc14 nucleolar release, which, although dispensable for mitotic exit under unperturbed conditions, becomes particularly important for mitotic slippage to SAC activation (V. Rossio, E. Galati and S. Piatti, unpublished work). Interestingly, the *cdc5-ad* mutation that delays adaptation to the DNA-damage checkpoint was proposed to be defective in the FEAR network [28], raising the possibility that similar mechanisms underly adaptation to different checkpoints. Besides the different mitotic processes in which it has previously been implicated, the RSC complex has been involved in the detection of double-strand breaks and their repair [43–47]. It will be interesting to analyse its possible involvement also in the adaptation to the DNA-damage checkpoint.

Importantly, RSC mutants not only remain arrested in mitosis for longer times upon *MAD2* overexpression or treatment with microtubule-depolymerizing drugs, but they also die under these conditions (V. Rossio, E. Galati and S. Piatti, unpublished work). We are currently investigating whether these cells die due to apoptosis or to other reasons. Nonetheless, these data suggest that the response to chronic SAC activation might follow the same general rules in budding yeast and mammalian cells and that impairing adaptation could commit cells to death, which has obvious clinical implications. In addition, based on our findings we propose that genetic screens using *MAD2*-overexpressing cells are excellent tools to discover novel fine-tuning regulators of mitotic exit and adaptation to the SAC.

In conclusion, discovering the factors that influence the rate of adaptation to prolonged SAC activation will be an important challenge in cancer research and promises to identify novel targets for therapeutic strategies. Combining inhibition of these proteins with conventional antimitotic agents could prevent adaptation and potentiate cell death. In addition, it would allow use of lower doses of antimitotics in chemotherapy, thus decreasing their neurotoxicity.

Strikingly, mitotic exit has recently been proposed to be a better cancer therapeutic target than spindle assembly. Indeed, Cdc20 inhibition kills efficiently cancer cells, preventing mitotic slippage and providing more time for apoptosis [48]. Targeting essential regulators of mitotic exit during cancer treatment would have the drawback of also killing normally proliferating cells of the human body. Our finding that non-essential tuners of mitotic exit, such as the RSC complex, impair dramatically adaptation to SAC signalling opens important therapeutic perspectives that will be worth pursuing in the future.

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CLOSING THE CELL CYCLE

The central components that coordinate cell cycle progression are CDKs, and their activity is regulated by cyclins, their regulatory subunits. In particular, mitotic CDKs drive the events of early mitosis: chromosome condensation and resolution, nuclear envelope breakdown and assembly of the mitotic spindle. To end the cell cycle, eukaryotic cells must inactivate the mitotic CDKs. This event, referred to as mitotic exit, includes a number of processes that strictly require the inactivation of mitotic CDKs, such as chromosome decondensation, spindle disassembly, formation of pre-replicative complexes at replication origins and cytokinesis. In all eukaryotes mitotic CDK inactivation depends on cyclinB proteolysis promoted by APC/C and this event allows phosphatases to dephosphorylate the CDK substrates. In general, the final stages of mitosis are governed by two mechanisms: (1) dephosphorylation of CDK substrates and (2) ubiquitylation of APC substrates (fig. 5). Out of APC substrates, cyclinB is clearly the most critical protein that needs to be degraded in order for cells to get out of mitosis. (from Sullivan and Morgan, 2007)

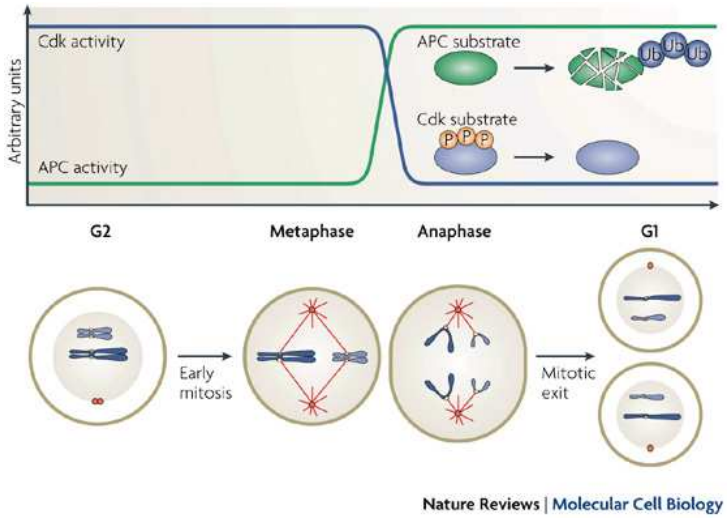

Figure 5. What happens when cells exit from mitosis
Progression through mitosis is shown by the cells along the bottom of the figure. The transition from metaphase to anaphase is triggered by an increase in the activity of the anaphase-promoting complex (APC) (green line; top of figure), a ubiquitin protein ligase that promotes the assembly of chains of ubiquitin (Ub) on its substrates, thereby targeting them for destruction by the proteasome. The main APC targets are securin, the destruction of which leads to sister-chromatid separation, and cyclins, the destruction of which results in a drop in CDK activity (blue line). CDK inactivation allows cellular phosphatases to dephosphorylate CDK substrates during late mitosis. CDK-substrate dephosphorylation is required for the events of anaphase and telophase (not shown) (from Sullivan and Morgan, 2007).

Exit from mitosis in budding yeast: the FEAR and MEN pathways

In budding yeast, where the molecular mechanisms at the basis of mitotic exit have been extensively studied, mitotic exit depends on the key protein phosphatase Cdc14, which promotes directly cyclinB degradation and CDK inactivation and at the same time reverses the phosphorylation events carried out by CDKs in mitosis (Sullivan and Morgan, 2007; Queralt and Uhlmann, 2008). Two pathways contribute to Cdc14 activation: the Cdc-fourteen early anaphase release (FEAR) pathway and the mitotic exit network (MEN).

At the onset of anaphase sister chromatids begin to be separated. This event is triggered by the APC complex (Stegmeier et al., 2002). The FEAR is a non-essential pathway that involves many proteins, such as the separase Esp1, the polo kinase Cdc5, the kinetochore protein Slk19 as well as the homologous proteins Spo12 and Bns1. In addition, the FEAR is negatively regulated by Fob1 and the $PP2A^{Cdc55}$ phosphatase complex (reviewed by D'Amours and Amon, 2004). The FEAR has a dual role: it is required for completion of chromosome segregation (D'Amours et al, 2004) by the separase Esp1 and also causes a partial and transient release of the phosphatase Cdc14 from the nucleolus (fig. 6) (Stegmeier et al., 2002; Azzam et al., 2004;

D'Amours and Amon, 2004). In fact, Cdc14 is kept imprisoned in the nucleolus by binding to the nucleolar protein Net1/Cfi1 until metaphase (reviewed in Queralt and Uhlmann, 2008). At the anaphase onset, separase activation not only leads to proteolytic cleavage of cohesin, but also it utilises a second non-proteolytic activity to activate Cdc14 (Sullivan and Uhlmann, 2003). At this stage, high mitotic CDK activity triggers Cdc14 release by phosphorylating at least 6 CDK recognition sites on Net1 (Azzam et al., 2004). The link between separase and CDK-dependent phosphorylation comes through the PP2A phosphatase bound to its Cdc55 regulatory subunit, which until metaphase counteracts Net1 phosphorylation (Queralt et al., 2006) (fig. 7). Separase-dependent downregulation of $PP2A^{Cdc55}$ at anaphase onset allows Net1 phosphorylation, but the mechanisms by which the separase inhibits $PP2A^{Cdc55}$ are yet not understood. Two proteins that are tightly bound to $PP2A^{Cdc55}$, Zds1 and Zds2, are required downstream of separase for timely phosphorylation of Cdc14 (Queralt and Uhlmann, 2008), suggesting that they might act as inhibitors of $PP2A^{Cdc55}$. The polo kinase Cdc5 also plays a crucial role on the FEAR pathway, likely by contributing to full Net1 phosphorylation (Visintin et al., 2008). The mechanism by which other FEAR proteins promote Cdc14 activation is poorly understood. However, Slk19, as well as separase, binds to Cdc5 and might therefore modulate its FEAR function (Rahal and Amon, 2008).

As mentioned above, FEAR triggers only a transient activation of Cdc14. To exit from mitosis, cells need that another pathway, the MEN, is activated (McCollum and Gould, 2001). Unlike the FEAR, the MEN is an essential pathway for mitotic exit composed by a Ras-

like GTPase called Tem1 and its downstream kinases Cdc15 and Mob1/Dbf2 (Jaspersen et al., 1998; Lee et al., 2001; Visintin et al., 2001) (fig. 7). Tem1 acts at the top of this signalling cascade and is the target of the spindle position checkpoint (SPOC, fig. 8), a surveillance mechanism that delays mitotic exit until the spindle is properly positioned relative to the mother-bud axis. Tem1 is concentrated on the daughter-bound spindle pole in mitosis, and the elongation of the mitotic spindle into the daughter cell brings Tem1 away from its inhibiting kinase Kin4 that is concentrated on the mother-cell cortex and mother-bound spindle pole (fig. 8). For many years, people believed that Lte1 could be the putative GEF of Tem1 (Bardin et al., 2000; Pereira et al., 2000), but during these last years this hypothesis has been disproved (Geymonat et al., 2009). At the moment, how MEN is activated is still unclear. Counteracting Tem1 activation is the GTPase-activating protein (GAP) complex Bfa1/Bub2 (Geymonat et al., 2003), a negative regulator of mitotic exit. Its inhibitory effect on Tem1 is switched off by the phosphorylation of Bfa1 by Cdc5 (Hu et al., 2001; Geymonat et al., 2003), while the Kin4 kinase counteracts this phosphorylation and maintains Bfa1/Bub2 capable of inhibiting Tem1 (Maekawa et al., 2007). Active Tem1 bound to GTP interacts with the Cdc15 kinase to activate the MEN. Cdc15 is in turn able to activate the Mob1/Dbf2 kinase complex (Asakawa et al., 2001; Mah et al., 2001). Besides phosphorylating and inhibiting Bfa1/Bub2, Cdc5 has also a more direct role in the activation of Mob1/Dbf2 (Lee et al., 2001). How Dbf2 finally promotes Cdc14 nucleolar release is not clear, but one hypothesis is that Dbf2 directly phosphorylates Net1 to promote

Cdc14 dissociation (Mah et al., 2005). So, when the spindle is correctly positioned MEN promotes a total activation of Cdc14 (fig. 6). The full activation of Cdc14 leads to mitotic exit through dephosphorylation of Cdh1, another APC coactivator factor, and Sic1, an inhibitor of mitotic CDKs (Prinz and Amon, 1999). APC/Cdh1 and Sic1 together totally inactivate CDK activity, allowing cells to exit from mitosis and enter in a new cell cycle (D'Amours and Amon, 2004).

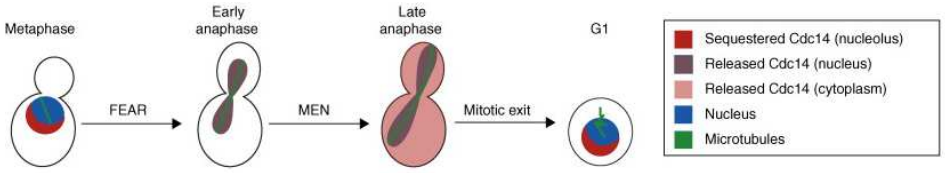

Figure 6. Regulation of the Cdc14 phosphatase in budding yeast
Cdc14 activity is regulated by changes in its intracellular localization. From G1 to metaphase Cdc14 is sequestered in the nucleolus by its inhibitor Net1/Cfi1. Cdc14 release from Net1 requires the action of two consecutive networks, FEAR and MEN. (from De Wulf et al., 2009).

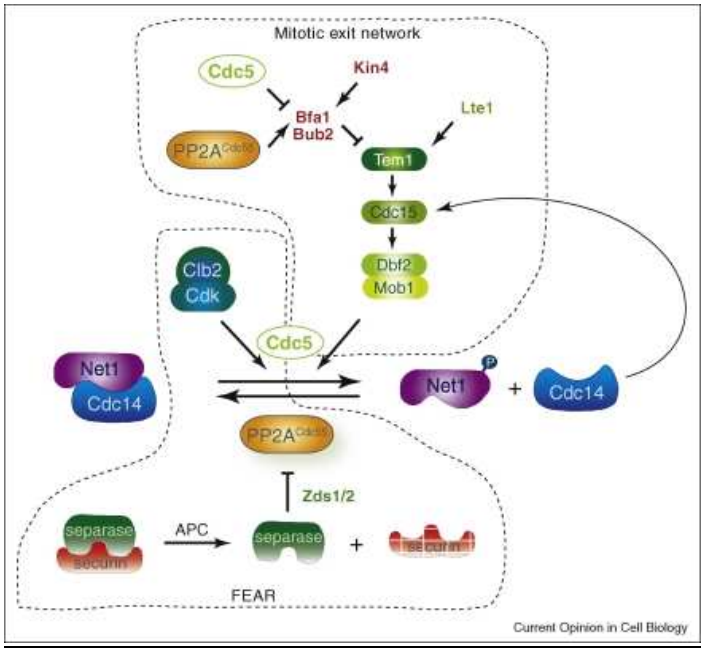

Figure 7. A model for mitotic exit in budding yeast.
At the heart of the scheme is the reversible phosphorylation and dephosphorylation of the Cdc14 phosphatase inhibitor Net1. At anaphase onset, separase-dependent downregulation of $PP2A^{Cdc55}$ promotes CDK-dependent phosphorylation of Net1, amplified by Cdc5. This leads to Cdc14 early anaphase release from nucleolar inhibition by Net1 (FEAR). Inhibition of Bub2/Bfa1, along with Lte1-dependent activation of Tem1, brings about activation of the mitotic exit network (MEN). Kinases of the MEN cascade (e.g. Dbf2) may maintain Cdc14 release by sustaining Cdc5-amplified Net1 phosphorylation. (from Queralt and Uhlmann, 2008)

Cdc5 has therefore emerged as a key factor in mitotic exit regulation, as it is the only protein that is involved in both the FEAR and the MEN pathway (Jaspersen et al., 1998; Stegmeier at al., 2002). One of the best characterized roles of Cdc5 in regulating mitotic exit is the inhibition of Bfa1, thus contributing to Tem1 activation (Geymonat et al., 2003). Bfa1 phosphorylation is counteracted by $PP2A^{Cdc55}$ during metaphase, so the downregulation of $PP2A^{Cdc55}$ at the onset of anaphase promotes not only Net1 phosphorylation but also MEN activation (Queralt et al., 2006). A direct target of Cdc5 could be the inhibitor Net1 (Show et al., 2002; Yoshida and Toh-e, 2002), both early and late in anaphase. In addition, Cdc5 contributes to the build-up of CDK activity during mitotic entry, a pre-requisite for CDK-dependent Net1 phosphorylation (Azzam et al., 2004). To work properly, Cdc5 needs that its target undergoes a priming phosphorylation event by another kinase. This priming phosphorylation is important for substrate specificity (Elia et al., 2003). Because of this peculiarity, Cdc5 might not have a specific function in regulating mitotic exit but could act as a generic phosphorylation amplifier. While Cdc5 may, therefore, not be regulating the onset of Cdc14 activation, it remains a limiting factor for Cdc14 release throughout anaphase, and APC/Cdh1-mediated Cdc5 destruction at the end of mitosis is sufficient to cause Cdc14 re-

sequestration in the nucleolus (Visintin et al., 2008). (from Sullivan and Morgan, 2007; Queralt and Uhlmann, 2008; Bosl and Li, 2005).

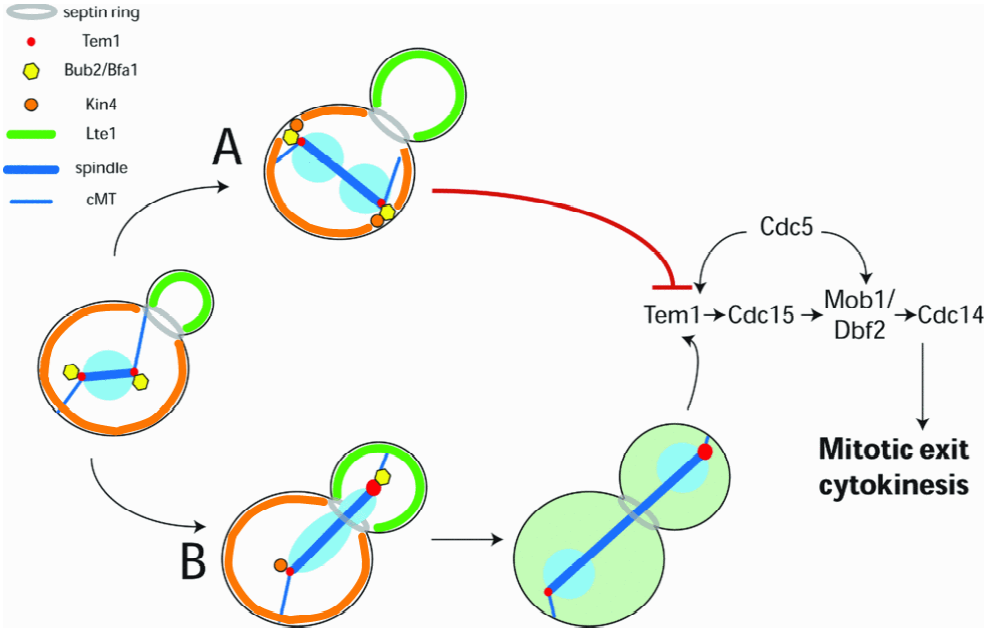

Figure 8. Regulation of the MEN by the SPOC
The MEN proteins and their spatial localization is fundamental for the SPOC. Only when one of the SPBs migrates into the daughter cell, Tem1 can be activated by Lte1 triggering MEN activation, while when the mitotic spindle is mispositioned Tem1 is kept inactive by the Bfa1/Bub2 complex, which is in turn maintained active by the Kin4 kinase (from Fraschini et al., 2008, modified).

Mitotic exit in higher eukaryotes

CDK inhibition and dephosphorylation of CDK substrates promote mitotic exit in all eukaryotes. In fact, although most of the substrates that must be dephosphorylated for triggering mitotic exit are still unknown, persistent CDK activity prevents mitotic exit in all eukaryotes studied. Many of the dephosphorylation-dependent anaphase events take place in a similar manner in yeast and other eukaryotes. These include stabilisation of microtubules at anaphase onset, relocalisation of the Aurora B kinase complex, spindle midzone

assembly and, upon completion of chromosome segregation, spindle disassembly, cytokinesis and cell abscission. Some examples of CDK substrates that are dephosphorylated in higher eukaryotes are the *C. elegans* kinesin ZEN-4 (Mishima et al, 2004), and the human Ase1 orthologue PRC1 (Zhu et al, 2006), both involved in the assembly of the anaphase spindle midzone. It is known that ZEN-4 dephosphorylation requires CDC-14, the non-essential *C. elegans* Cdc14 orthologue. Instead, vertebrates have two Cdc14 orthologues, Cdc14A and Cdc14B. Introduction of both human Cdc14A and Cdc14B into budding yeast rescues *cdc14*Δ yeast mutant phenotypes, suggesting that there is some functional conservation between these phosphatases (Li et al, 1997).

Also fission yeast has a Cdc14 homologue, Clp1, that is not essential for normal cell cycle progression. It is known that fission yeast cells possess a signalling cascade similar to the budding yeast MEN, the Septation Initiation Network (SIN). The SIN controls cytokinesis and its downstream effectors are not yet known. Unlike the MEN, the SIN is not required for Clp1 activation, but only contributes to sustain Clp1 nucleolar release until the completion of cytokinesis (Trautmann et al, 2001). Orthologues of the MEN kinases Cdc15 and Mob1/Dbf2 are also found in higher eukaryotes, while no Cdc14 orthologues have been found encoded in the genomes of higher plants (Kerk et al, 2008). If the processes that control the mitotic exit are partially conserved in eukaryotes, in many organisms the Cdc14 phosphatase is not essential for mitotic exit, so other unknown phosphatases must be required. (from Queralt and Uhlmann, 2008)

RESULTS

The RSC chromatin-remodelling complex influences mitotic exit and adaptation to the SAC by controlling the Cdc14 phosphatase

As mentioned in the introduction, the factors influencing the rate of adaptation to the SAC are currently unknown, in spite of their obvious relevance for cancer research. We decided to use budding yeast as model system to study SAC adaptation. We have verified that yeast cells adapt to SAC activation under different conditions by using the normal regulatory networks that govern mitotic exit in unperturbed conditions, leading to destruction of securin and cyclinB. Unlike previously reported for mammalian cells (Brito and Rieder, 2006), we found that SAC adaptation in yeast is accompanied by silencing of SAC signalling and disassembly of checkpoint protein complexes. By using a yeast strain that hyperactivates the SAC through *MAD2* overexpression and, therefore, in the absence of spindle damage or alterations in kinetochore-microtubule attachments, we carried out a genetic screen for factors involved in SAC adaptation. We found the yeast RSC (Remodel the Structure of Chromatin) complex, and in particular its form bound to the accessory Rsc2 subunit, regulates mitotic slippage. Further analyses showed that RSC^{Rsc2} controls mitotic exit by regulating the early release of the Cdc14 phosphatase from the nucleolus, probably acting as a novel component of the FEAR pathway. In addition, our data indicate that the Rsc2 interacts with the polo kinase Cdc5, which is essential for Cdc14 activation and mitotic exit regulation.

The data obtained from our studies are reported in the following article.

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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

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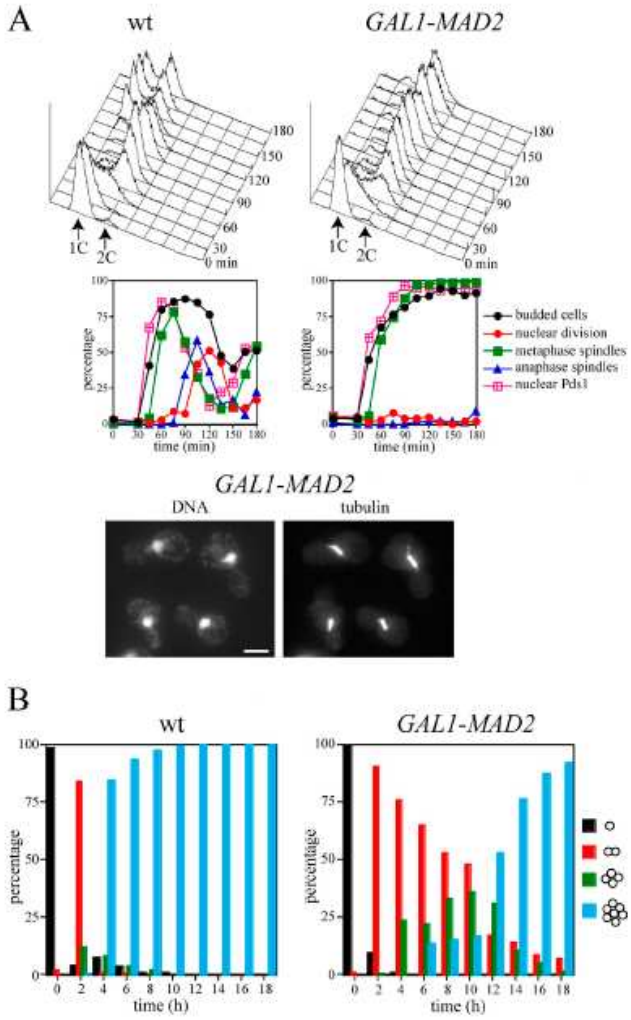

Figure 1. ***MAD2* overexpression induces a transient metaphase arrest.** (A) Wild-type (wt; ySP4806) and *GAL1-MAD2* (ySP8526) cells were grown in YEPR, arrested in G1 with α-factor, and then released in YEPRG medium (t = 0). Samples were collected at the indicated times for FACS analysis of DNA contents 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.

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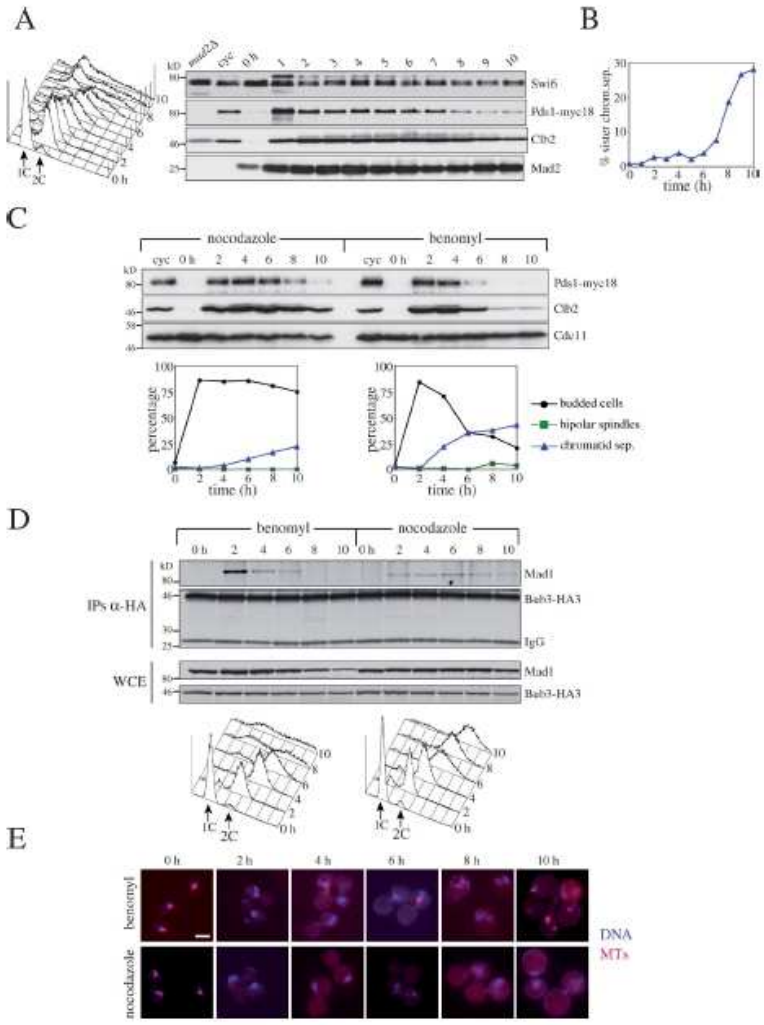

Figure 2. **Mitotic slippage upon prolonged treatment with microtubule destabilizers correlates with degradation of APC substrates and dissociation between Mad1 and Bub3.** (A) α-factor–arrested *GAL1-MAD2* (ySP8599) cells were released in YEPRG at 30°C (t = 0). α-factor was readded at 3 µg/ml after 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).

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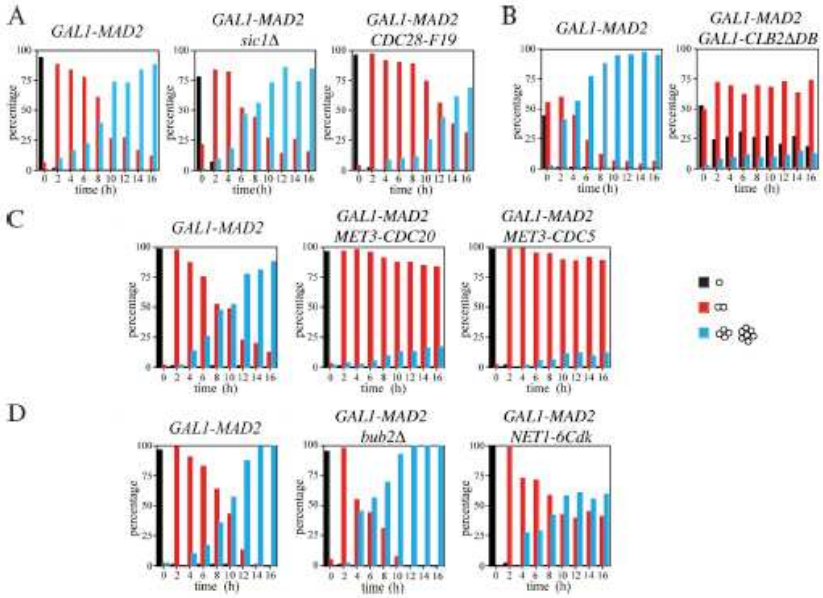

Figure 3. **SAC adaptation requires mitotic exit regulators.** (A) *GAL1-MAD2* (ySP6170), *GAL1-MAD2 sic1Δ* (ySP8706), and *GAL1-MAD2 CDC28-F19* (ySP8704) cells were grown in YEPR, arrested in G1 with α-factor (unbudded cells), and spotted on YEPRG plates (t = 0) at 30°C. 200 cells were scored at each 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.

Published November 22, 2010

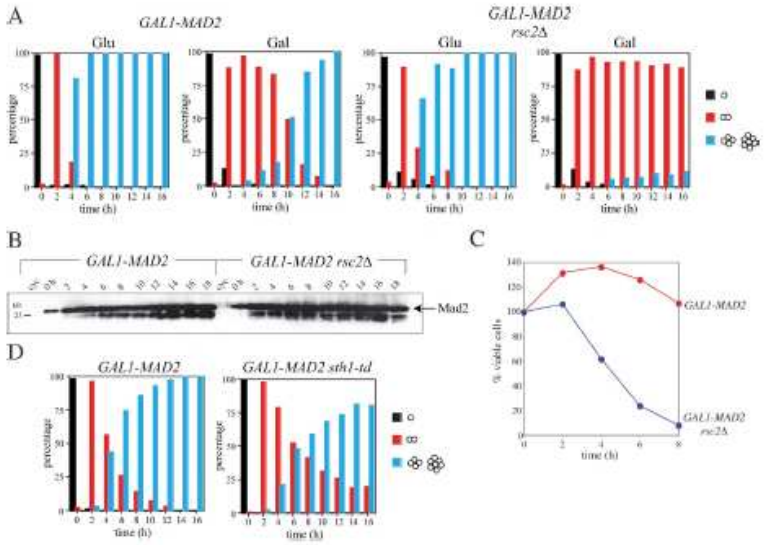

Figure 4. **The RSC complex is involved in SAC adaptation.** (A) *GAL1-MAD2* (ySP6170) and *GAL1-MAD2 rsc2Δ* (ySP6850) cells were grown in uninduced conditions (YEPR), arrested in G1 with α-factor (unbudded cells), and spotted on YEPD (Glu) and YEPRG (Gal) plates (t = 0) at 30°C. 200 cells were scored 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

Published November 22, 2010

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

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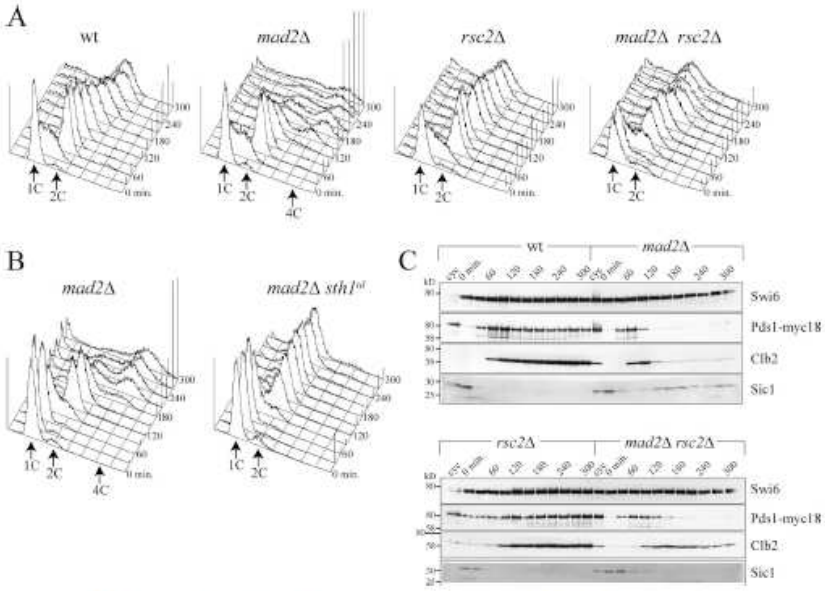

Figure 5. **Lack of the RSC complex prevents mitotic exit of nocodazole-treated SAC-defective cells.** (A) Cultures of wild-type (wt), *mad2Δ*, *rsc2Δ*, and *mad2Δ rsc2Δ* cells (ySP4806, ySP1084, ySP6997, and ySP7543) were grown in YEPD, arrested in G1 by α-factor, and then released into medium containing 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*^{ts} *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

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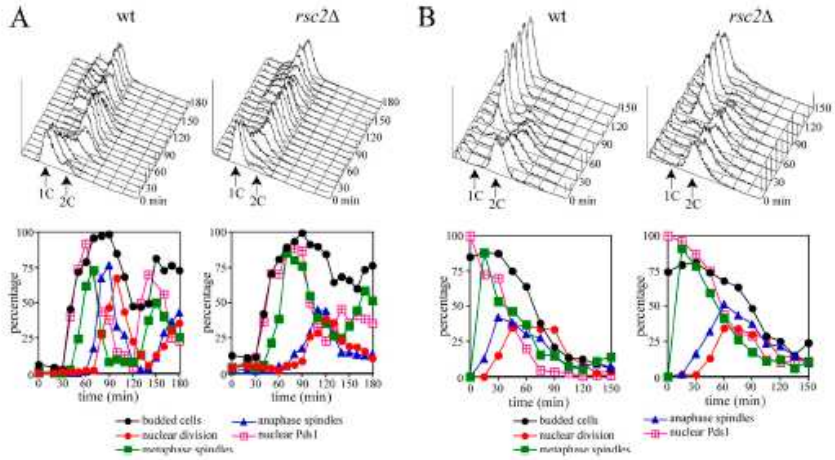

Figure 6. **Cell cycle progression of *rsc2Δ* cells and their recovery from SAC activation.** (A) Cultures of wild-type (wt; ySP4806) and *rsc2Δ* (ySP6997) cells were grown in YEPD, arrested in G1 by α-factor, and then released in fresh medium (t = 0). At the indicated times, samples were analyzed as 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.

Published November 22, 2010

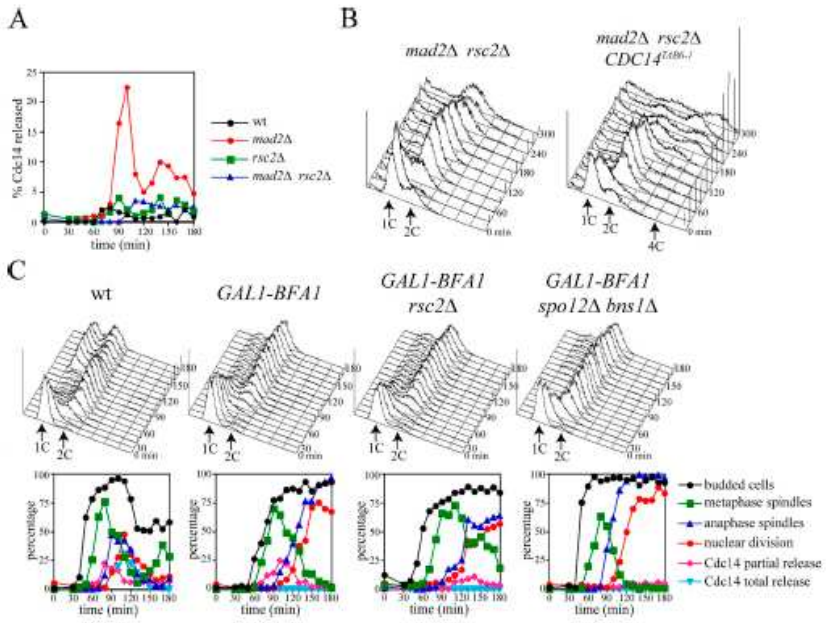

Figure 7. **Rsc2 controls the early release of Cdc14 from nucleolus.** (A) α-factor–arrested wild-type (wt; W303), mad2Δ (ySP1070), rsc2Δ (ySP6858), and mad2Δ rsc2Δ (ySP7088) cells were released in medium containing nocodazole (t = 0). At the indicated times, cells were collected for FACS analysis of 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).

Published November 22, 2010

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

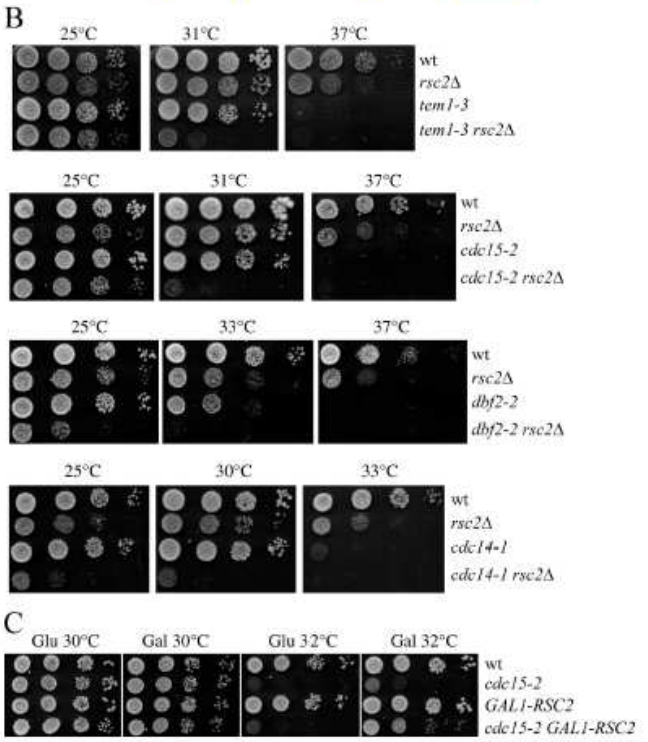

Figure 8. **Functional interactions between *RSC2* and *MEN* genes.** (A) Ratio of found/expected segregants observed over expected numbers of viable spores with the indicated genotypes after dissection of meiotic tetrads generated from diploid strains heterozygous for the *rsc2Δ* (ySP6859) and *lte1Δ* (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.

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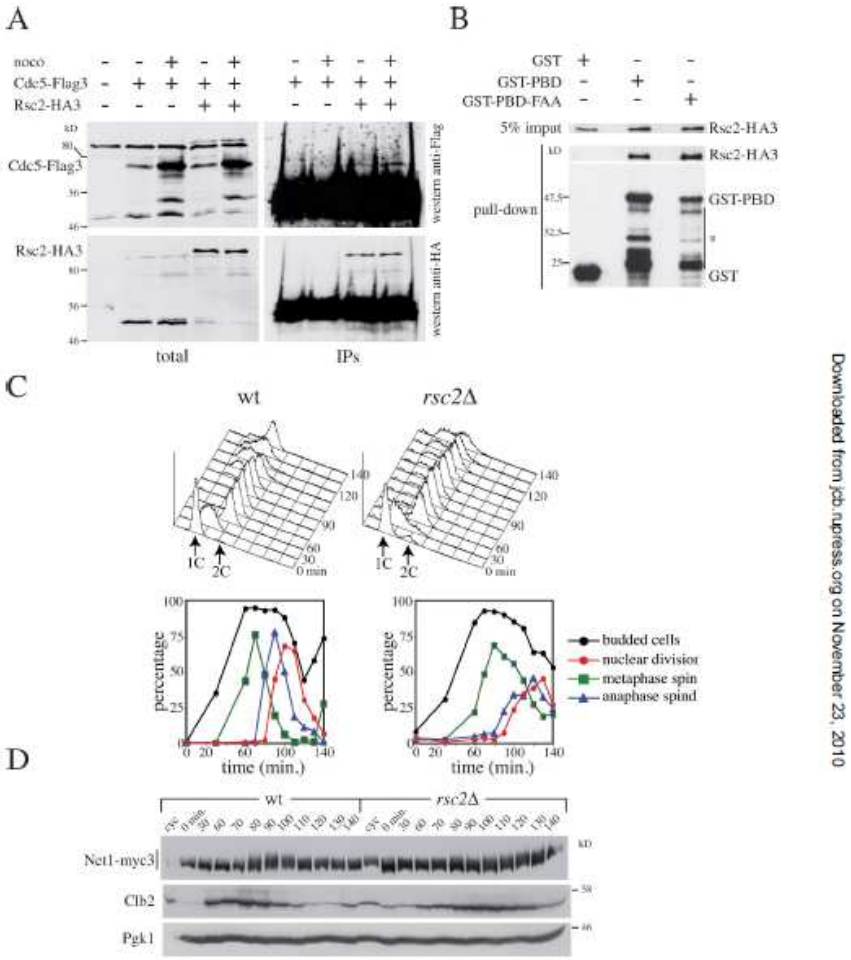

Figure 9. **Rsc2 interacts physically with Cdc5 and is required for timely Net1 and Cdc14 phosphorylation.** (A) Wild type (wt; W303), *CDC5-FLAG3* (ySP7797), and *RSC2-HA3 CDC5-FLAG3* (ySP7814) were grown exponentially or arrested in nocodazole for 3 h. Protein extracts were analyzed by Western blotting 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).

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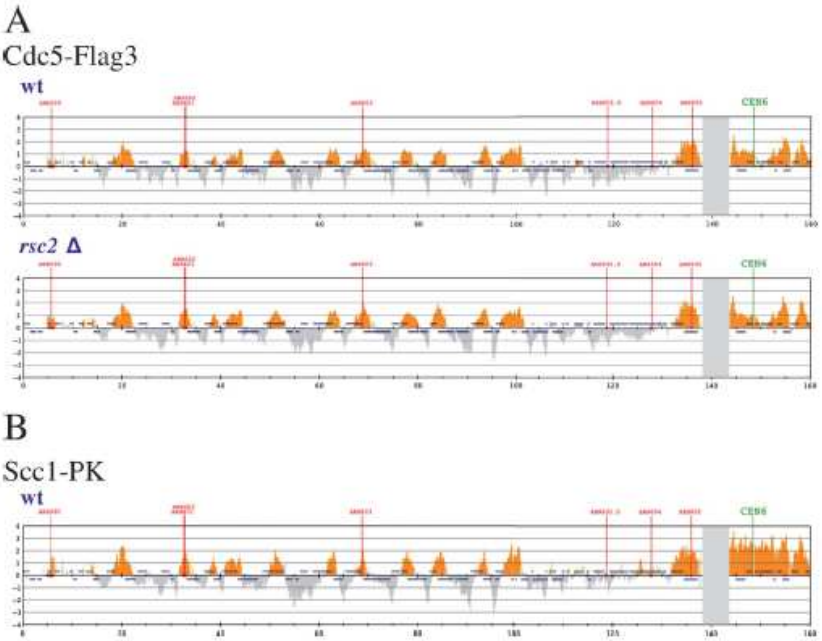

Figure 10. **RSC2 deletion does not affect Cdc5 chromosomal distribution.** Wild-type (wt; ySP7797) and *rsc2*Δ cells (ySP8200) expressing FLAG-tagged Cdc5 (A) and wild-type cells expressing PK-tagged Scc1 (B) were arrested in mitosis with benomyl and treated for ChIP-on-chip analysis. Enrichment of 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;

Published November 22, 2010

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

Published November 22, 2010

(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*, *ITE1*, 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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The ATPase activity of the RSC complex is required for mitotic exit of nocodazole-treated SAC mutant cells

In Rossio et al. (2010) we observed that the RSC^{Rsc2} complex is required for the mitotic exit of SAC-deficient mutants in the presence of microtubule-depolymerizing drugs. To remodel the structure of chromatin the RSC complex uses the ATPase activity provided by the core catalytic subunit Sth1 (Du et al, 1998). To determine if the ATPase activity of the RSC complex is involved in promoting mitotic exit in SAC mutants treated with spindle destabilizers, we introduced in the *mad2*Δ *sth1^{td}* strain (where a temperature-sensitive degron (*sth1^{td}*) inactivates Sth1 at high temperatures) a plasmid carrying the ATPase-defective allele *sth1-K501R* (Laurent et al., 1992; Du et al., 1998). We then analyzed the effects of this mutation on the ability of *mad2Δ* cells to escape from mitosis upon nocodazole treatment and re-replicate their DNA. As expected, *mad2*Δ cells re-replicated their DNA, while most *mad2*Δ *sth1^{td}* cells arrested with 2C DNA contents in these conditions. Similarly, *mad2*Δ *sth1^{td} sth1-K501R* cells arrested in mitosis (fig. 9), demonstrating that the lack of RSC ATPase activity is sufficient to prevent mitotic exit of SAC-defective mutants in presence of nocodazole. We can therefore envision that the role of the RSC^{Rsc2} complex in regulating adaptation and mitotic exit needs chromatin remodelling.

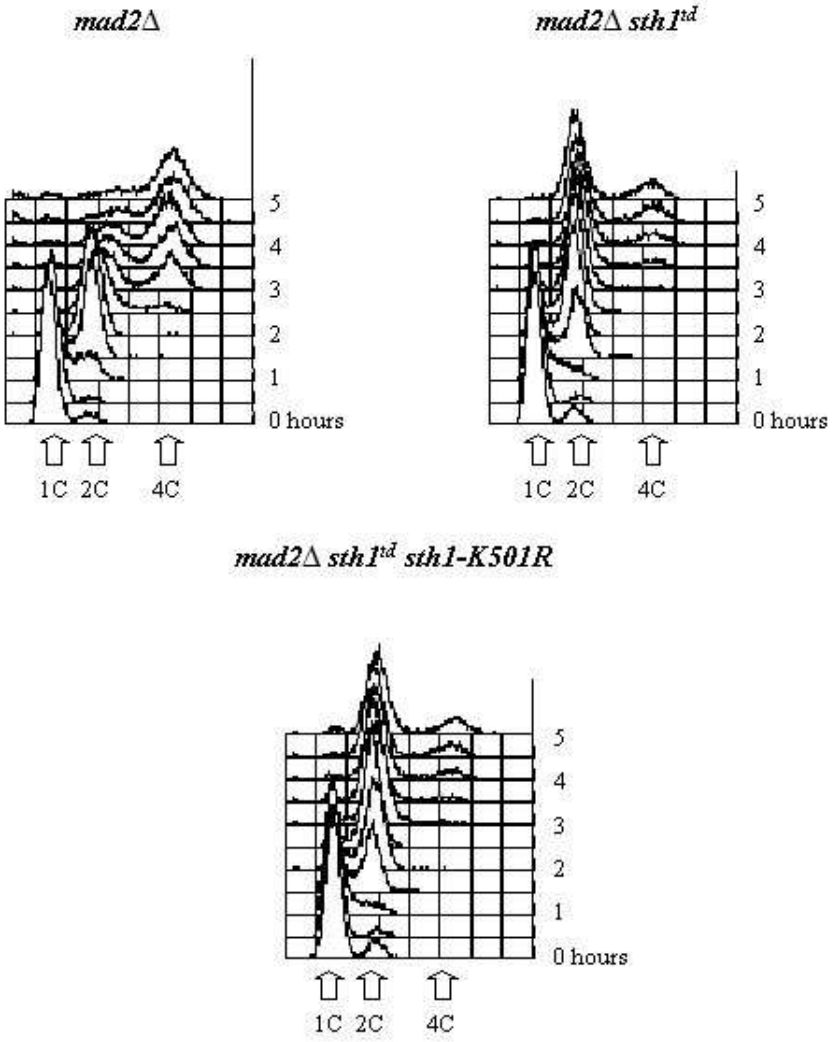


Figure 9. The ATPase activity of RSC is required for *mad2Δ* cells to exit from mitosis upon spindle disruption.
Logarithmically growing cultures of *mad2Δ* and *mad2Δ sth1td cells* carrying the empty vector (*YCplac111*, ySP8789 and ySP8795, respectively) as well as *mad2Δ sth1td* carrying the *sth1-K501R* allele on *YCplac111* (ySP8796) cells were grown overnight in selective (-Leu) raffinose medium supplemented with 0.1 mM $CuSO_4$ and then arrested in G1 with α factor at 27°C in YEP raffinose. Galactose was added 1 hour before release from the G1 arrest, and then cells were released at 37°C in YEP raffinose-galactose medium in the presence of nocodazole (time 0). Samples were collected at the indicated time points for FACS analysis of DNA contents.

Loss of viability of *GAL1-MAD2 rsc2*Δ cells is independent of caspase-dependent cell death pathways

It is known that after a prolonged SAC activation mammalian cells either slip out mitosis or die by apoptosis (Rieder and Maiato, 2004; Gascoigne and Taylor, 2008). These alternative fates are dictated by the speed at which cells undergo apoptosis relative to mitotic exit, the latter of which depends on a progressive decline in cyclinB levels (Brito and Rieder, 2006; Gascoigne and Taylor, 2008). Preventing apoptosis through inhibition of caspase favors mitotic slippage, whereas disabling cyclinB proteolysis channels cells into the apoptotic pathway (Gascoigne and Taylor, 2008). In Rossio et al. (2010) we found that *GAL1-MAD2 rsc2*Δ cells lost viability in inducing conditions (i.e. presence of galactose in the medium). In yeast the only caspase known is encoded by *YCA1*, and its deletion is sufficient to abolish caspase activity (Madeo et al., 2002 Madeo et al., 2009). To understand if the decrease of viability of *GAL1-MAD2 rsc2*Δ cells is due to a caspase-dependent cell death pathway, we analyzed the effects of *YCA1* deletion on the ability of *GAL1-MAD2 rsc2*Δ cells to undergo adaptation using a microcolony assay (see Materials and Methods). *GAL1-MAD2*, *GAL1-MAD2 yca1*Δ, *GAL1-MAD2 rsc2*Δ and *GAL1-MAD2 rsc2*Δ *yca1*Δ cells were grown in uninduced conditions, arrested in G1 with alpha factor (unbudded cells) and spotted on galactose-containing plates, which were incubated at 30°C. Formation of microcolonies was scored with a transmission microscope at different times. In particular, we divided cells in four classes: (1) unbudded cells, (2) budded cells, (3) microcolonies formed by four cells and (4) microcolonies formed by more than four

cells. As expected, *GAL1-MAD2* cells accumulated as budded cells for a few hours and after 8-10 hours from the release some cells adapted and started a new cell cycle. *GAL1-MAD2 yca1*Δ cells behaved similarly. In contrast, most *GAL1-MAD2 rsc2*Δ cells remained arrested as budded cells for all the experiment (fig. 10). Likewise, the *GAL1-MAD2 rsc2*Δ *yca1*Δ triple mutant remained arrested in mitosis, suggesting that *YCA1* is not required for the prolonged mitotic arrest caused by the lack of Rsc2 when the SAC is hyperactive. Thus, the inability of *GAL1-MAD2 rsc2*Δ cells to adapt is not due to the caspase-dependent apoptotic pathways.

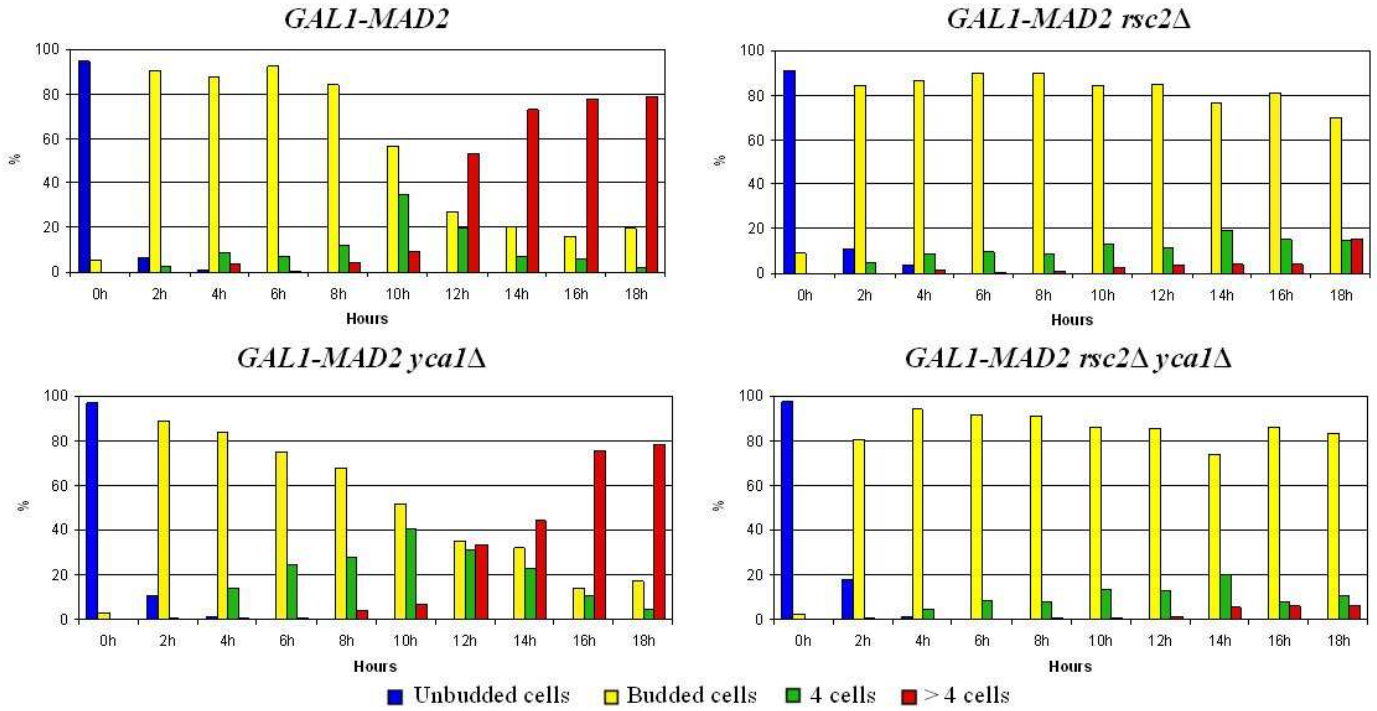


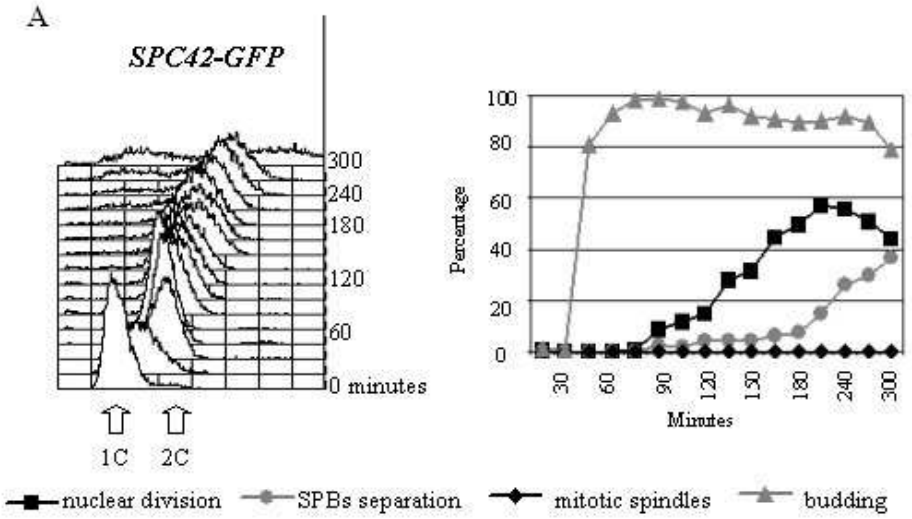
Figure 10. Yca1 is not required for maintaining *GAL1-MAD2 rsc2*Δ cells arrested in mitosis.
GAL1-MAD2 (ySP6170), *GAL1-MAD2 rsc2*Δ (ySP6850), *GAL1-MAD2 yca1*Δ (ySP8737) and *GAL1-MAD2 rsc2*Δ *yca1*Δ (ySP8736) cells were grown in uninduced conditions, arrested in G1 (unbudded cells) and spotted on YEP galactose plates (t=0) at 30°C. Formation of microcolonies was scored at the indicated times with a transmission microscope.

Cells divide nuclei and spindle pole bodies (SPBs) in presence of benomyl in an actin-independent manner.

In Rossio et al. (2010) we found that the time for yeast cells to undergo adaptation is highly variable, depending on the conditions. In particular, we found that mitotic slippage was fastest in the presence of benomyl and this correlated with the ability of cells to form cytoplasmic microtubules and undergo an aberrant nuclear division in the absence of mitotic spindles. In order to better characterize this phenotype, we first decided to verify if these cells also undergo spindle pole separation. To study this process, we expressed a used Spc42-GFP protein fusion in wild type cells (Spc42 is a structural component of the yeast microtubule organizing center or SPB; Adams and Kilmartin, 1999). We synchronized wild type cells in G1 with alpha factor and released them in presence of benomyl at 30°C. We then analyzed DNA contents by FACS analysis at the indicated time points, as well as the kinetics of budding, nuclear division, SPB separation and bipolar spindle formation (fig. 11A). Consistent with our previous observations, cells divided nuclei in absence of mitotic spindles. In these conditions, cells also separated SPBs, albeit with a seemingly slower kinetics than nuclear division. In particular, after 3 hours from the release about 50% of the cells had undergone some kind of nuclear division, while they barely started separating SPBs (fig. 11A and B). Therefore, cells are able to undergo an aberrant nuclear division in the complete absence of bipolar spindles that is also likely independent of spindle pole body separation.

Recently, fission yeast cells have been shown to divide nuclei in the absence of mitotic spindles through an actin-dependent mechanism

(Castagnetti et al., 2010). We therefore decided to test whether the actin cytoskeleton is required for the aberrant nuclear division of budding yeast cells treated with benomyl. To this end, we synchronized wild type cells in G1 with alpha factor and released them in presence of benomyl at 30°C. After 90 minutes we added either DMSO or Latrunculin B (a compound that disassembles the actin cytoskeleton); after 120 minutes alpha factor was re-added to arrest again cells in the next G1 phase. At different time points cell samples were withdrawn for FACS analysis of DNA contents and to measure the kinetics of budding and nuclear division (fig. 12). We found that cells divided nuclei with the same kinetics in the absence and in the presence of Latrunculin B, indicating that the aberrant nuclear division taking place in the presence of benomyl is actin-independent. In spite of that, cells treated with Latrunculin B did not divide and did not re-accumulate with 1C DNA contents because actin is required for cytokinesis.



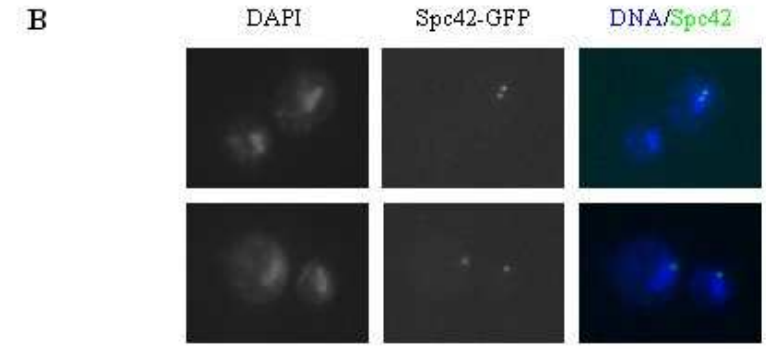


Figure 11. Cells divide nuclei and separate spindle pole bodies in presence of benomyl.
(A) Wild type cells expressing *SPC42-GFP* (ySP6890) were synchronized in G1 with alpha factor and then released in presence of benomyl at 30°C (time=0). Samples were collected at the indicated times for FACS analysis of DNA contents (histograms) and for kinetics of budding, nuclear division, formation of mitotic spindles and SPBs separation (graph). (B) Micrographs of cells at t=300 minutes. Nuclear DNA was stained with DAPI and SPBs were visualized thanks to Spc42-GFP.

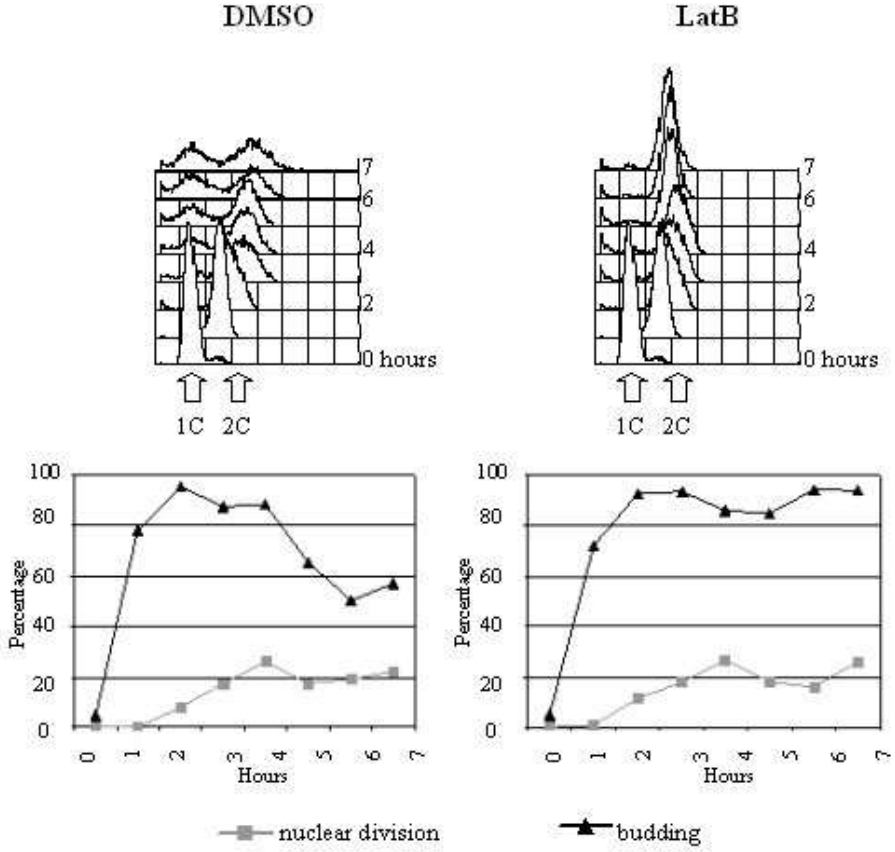


Figure 12. Cells treated with benomyl undergo nuclear division independently of the actin cytoskeleton.
(A) *bar1*Δ (ySP220) cells were arrested in G1 with alpha factor at 25°C and then released in presence of benomyl at 30°C. At 90 minutes after release, either DMSO or Latrunculin-B (Lat-B) were added and at 120 minutes after release alpha factor was re-added to prevent cells from entering a new cell cycle. Samples were collected at the indicated times for FACS analysis of DNA contents (histograms) and for kinetics of budding and nuclear division (graphs).

Involvement of other factors in adaptation to the spindle assembly checkpoint

Our genetic screen for factors involved in the adaptation to the spindle assembly checkpoint was not saturated. While we plan to re-do the screen in the future using a different strategy, we investigated the possible involvement of candidate factors in this process. In particular, we decided to investigate the possible role of three classes of proteins in this process: (1) spindle motors and microtubule-binding proteins; (2) the acetyl-transferase SAGA complex; (3) the polo kinase. The rationale behind this choice is the following. (1) In higher eukaryotic cells, dynein has been shown to silence the spindle assembly checkpoint by mediating the poleward movement of checkpoint proteins from kinetochores to spindle poles using its minus-end directed motor activity (Howell et al, 2001; Wojcik et al, 2001). Budding yeast seems to lack nuclear dynein. However, other motors or microtubule-binding proteins might be involved in a similar process. (2) The RSC complex is known to bind to acetylated histone tails to move nucleosomes along chromatin through its ATPase-dependent remodelling activity (Cairns et al, 1996). Since the SAGA complex is the major acetyltransferase of budding yeast, we asked whether its activity is required, like that of RSC, for adaptation to the SAC; (3) We have shown that the polo kinase Cdc5 is required for adaptation to the SAC as it is for mitotic exit (Rossio et al., 2010). A particular allele of *CDC5*, *cdc5-ad,* is defective in adaptation to the DNA damage checkpoint (Toczyski et al., 1997). However, it is perfectly proficient for mitotic exit during the unperturbed cell cycle.

Thus, we decided to investigate whether *cdc5-ad* delays adaptation to the SAC.

1. Microtubule regulators are involved in SAC adaptation

Like all eukaryotic cells, *S. cerevisiae* has motor proteins that move along microtubules (Hildebrandt et al, 2000). Budding yeast has only a reduced number of motor proteins: six kinesins (Cin8, Kar3, Kip1, Kip2, Kip3, and Smy1) and a single cytoplasmatic dynein heavy chain, Dyn1 (Chervitz et al., 1998). Microtubule motors and microtubule-associated proteins are involved in microtubule dynamics, as well as in mitotic spindle formation, elongation and positioning. In particular, Cin8, Kar3 and Kip1 are involved in bipolar spindles formation and maintenance (Saunders et al., 1992; Saunders et al., 1997; Hoyt et al., 1993), whereas Kip2, Kip3 and Dyn1 are required for spindle positioning (Yeh et al., 1995; Miller et al., 1998; Miller et al., 1998b). Since upon benomyl treatment some cytoplasmic microtubules were still detectable, we hypothesized that they could drive the abnormal chromosome segregation and nuclear division that we observe in these conditions, thus favoring mitotic slippage. We therefore investigated whether adaptation to the SAC was impaired by lack of microtubule motors or microtubule-binding proteins. In particular, we decided to analyze the three microtubule motors localized on cytoplasmic microtubules (Kip2, Kip3 and Dyn1) and Bik1, a protein that forms a complex with Kip2 and associates to the plus-ends of microtubules (Carvalho et al., 2004). Wild type, *bik1*Δ, *kip3*Δ, *dyn1*Δ and *kip2*Δ cells were synchronized in G1 with alpha factor and released in presence of benomyl at 30°C. After 120 minutes

from the release alpha factor was re-added to re-accumulate cells in the next G1. We then analyzed DNA contents by FACS analysis and kinetics of budding and nuclear division at different time points (fig. 13). *dyn1*Δ and *kip3*Δ cells showed no defect in mitotic slippage in these conditions relative to wild type cells, although *kip3*Δ cells underwent nuclear division somewhat more inefficiently. In sharp contrast, *kip2*Δ and *bik1*Δ cells divide nuclei slowly and inefficiently relative to wild type cells and most of them remained arrested as dumbbell-shaped, indicating a strong defect in adaptation. Thus, Kip2 and Bik1 seem to be required for adaptation to the SAC in presence of benomyl.

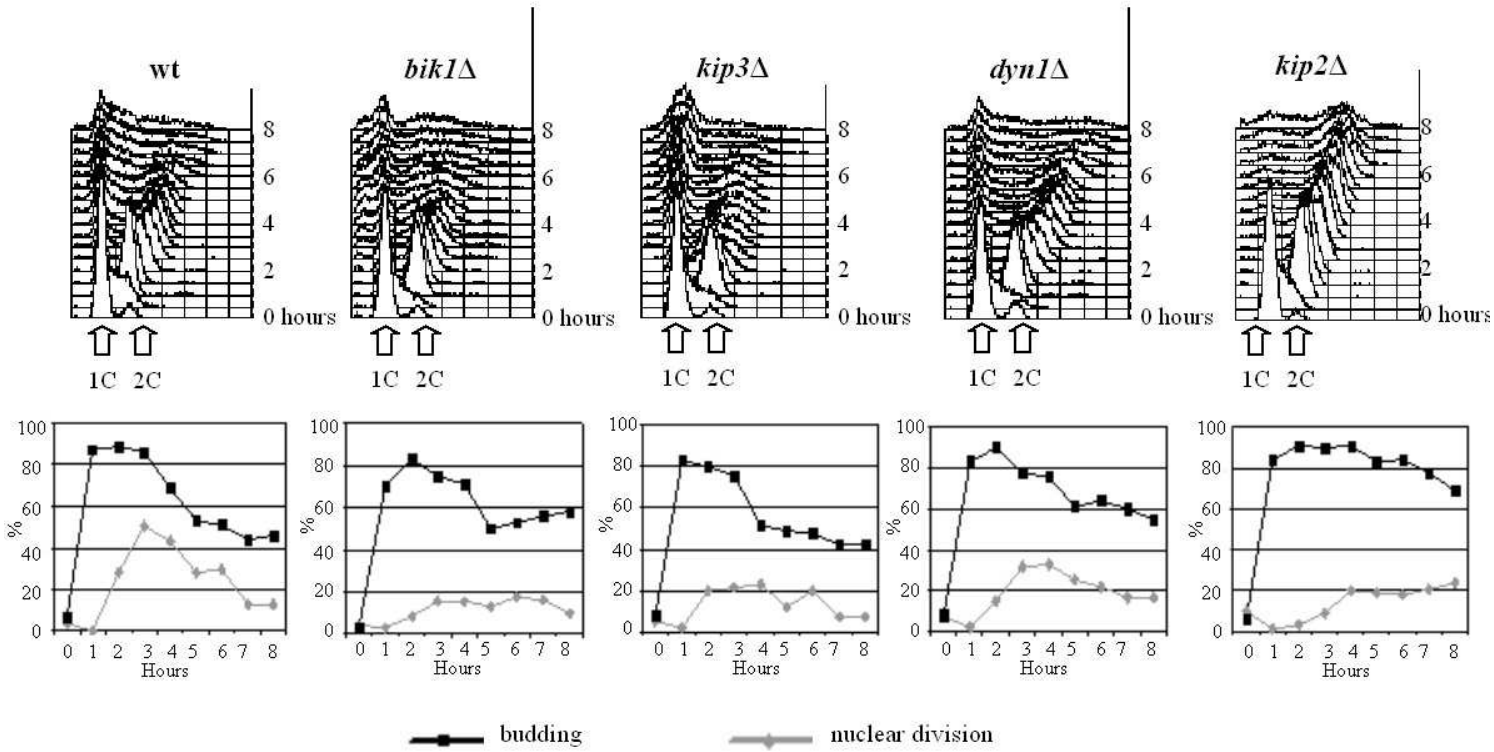


Figure 13. Bik1 and Kip2 are involved in adaptation to the SAC

*bar1*Δ (ySP220), *bik1*Δ *bar1*Δ (ySP8745), *kip3*Δ *bar1*Δ (ySP8746), *dyn11*Δ *bar1*Δ (ySP8750) and *kip2*Δ *bar1*Δ (ySP8770) cells were arrested in G1 with alpha factor at 25°C and then released in presence of benomyl at 30°C. At 120 minutes after release alpha factor was re-added at 2 µg/ml to arrest cells in the next G1 phase. Samples were collected at the indicated times for FACS analysis DNA contents and for kinetics of budding and nuclear division.

2. The SAGA complex plays a role in mitotic slippage

The SAGA complex is a chromatin-modifying complex composed by 21 widely conserved proteins that contains two distinct enzymatic activities, Gcn5 and Ubp8, through which it acetylates and de-ubiquitylates histones, respectively, thus regulating gene expression (Rodriguez-Navarro, 2009).

Since RSC binds to acetylated histone tails (Cairns et al, 1996), we tested if the SAGA complex, like RSC, is involved in SAC adaptation. To assess the effects of SAGA inactivation on adaptation, we deleted either *GCN5* or *ADA2*, the latter of which encodes for another subunit of the complex. We synchronized wild type, *ada2*Δ and *gcn5*Δ cells in G1 with alpha factor and released them in presence of benomyl at 30°C. We then analyzed DNA contents by FACS analysis at different time points, as well as the kinetics of budding and nuclear division (fig. 14). Both *ada2*Δ and *gcn5*Δ cells were delayed in nuclear division and mitotic exit relative to wild type cells.

We also analyzed microcolony formation of *GAL1-MAD2*, *GAL1-MAD2 ada2*Δ and *GAL1-MAD2 gcn5*Δ after release from G1 in the presence of galactose. Surprisingly, under these conditions *ada2Δ* and *gcn5Δ* mutants behaved differently: while *GAL1-MAD2 ada2*Δ cells formed microcolonies with a delay compared to *GAL1-MAD2* cells, *GAL1-MAD2 gcn5*Δ cells formed microcolonies slightly faster than *GAL1-MAD2* cells (fig. 15A and 15B).

These data suggest that in the absence of Ada2 cells delay adaptation in the presence of both microtubule destabilizers and high levels of Mad2, whereas the absence of Gnc5 only delays mitotic slippage in

benomyl. Further analysis will be needed to clarify the role of Gcn5 in this process.

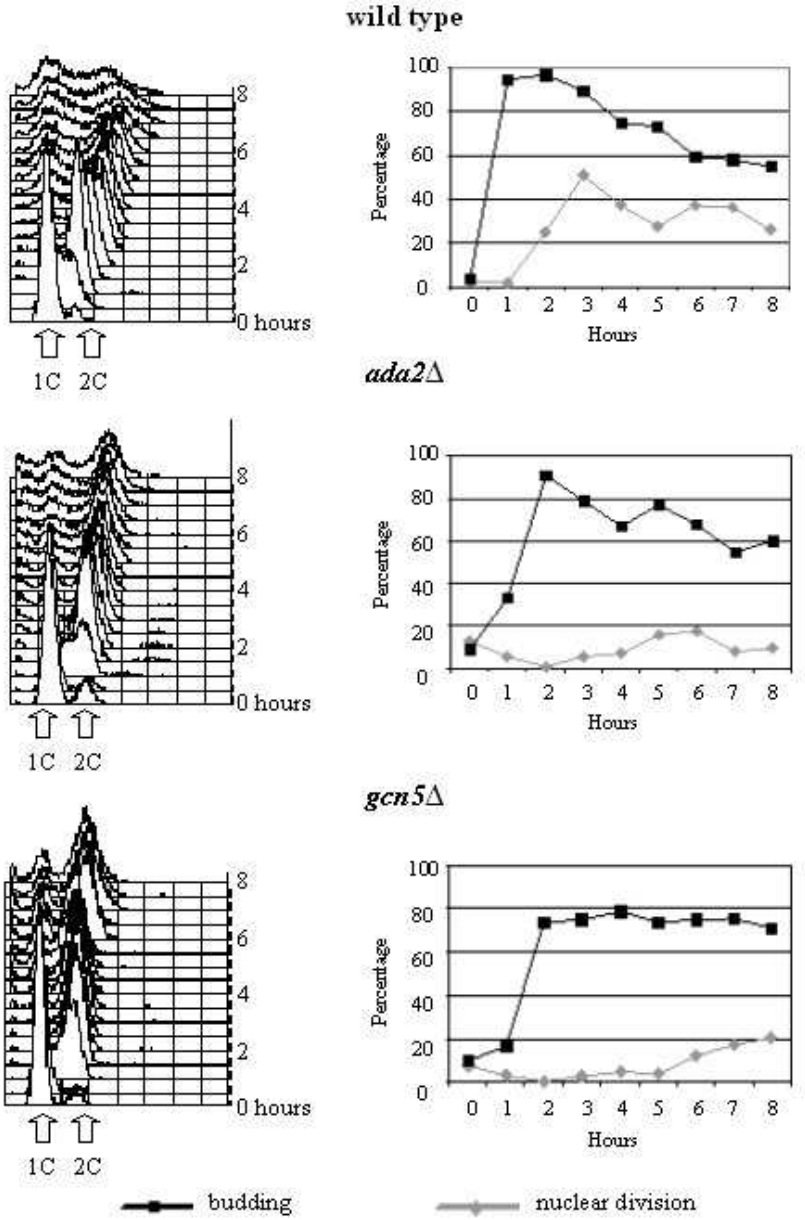


Figure 14. The absence of Ada2 and Gcn5 delays SAC adaptation in the presence benomyl

Wild type (ySP220), *ada2*Δ (ySP8768) and *gcn5*Δ (ySP8752) cells all carrying the *EAR1* deletion were arrested in G1 with alpha factor at 25°C and then released in the presence of benomyl at 30°C. At 120 minutes after release alpha factor was re-added to arrest cells in the following G1. Samples were collected at the indicated times for FACS analysis of DNA contents (left histograms) and for kinetics of budding and nuclear division (right graphs).

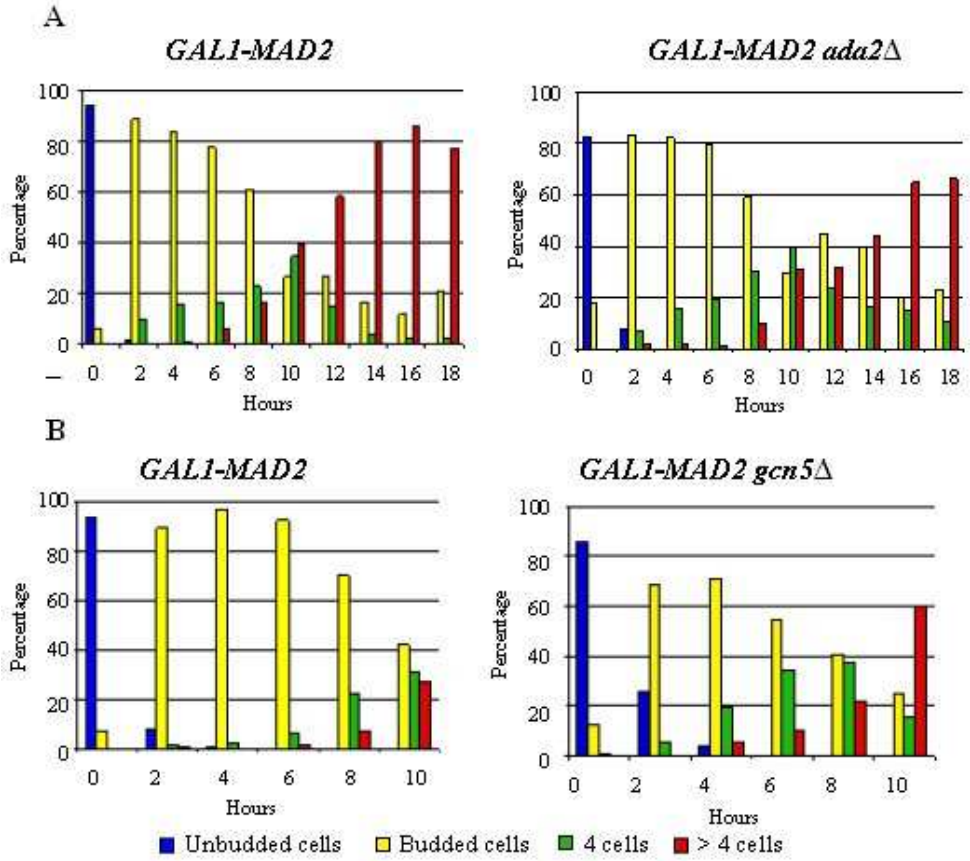


Figure 15. Lack of Ada2 but not of Gcn5 delays adaptation upon *MAD2* overexpression
(A) *GAL1-MAD2* (ySP6170) and *GAL1-MAD2 ada2Δ* (ySP6873) cells were grown in uninduced conditions, arrested in G1 (unbudded cells) at 25°C and then spotted on YEP galactose plates at 30°C. Formation of microcolonies was scored at the indicated times with a transmission microscope. (B) *GAL1-MAD2* (ySP6170) and *GAL1-MAD2 gcn5Δ* (ySP8722) were treated as in (A) but the arrest with alpha factor has been performed at 30°C because the deletion of *GCN5* has a cold-sensitive phenotype.

3. The *cdc5-ad* allele has no effect on SAC adaptation

Recent works demonstrate that there are crosstalks between the SAC and the DNA damage checkpoint (Dotiwala et al, 2010; Kim and Burke, 2008). It is therefore possible that the *cdc5-ad* allele that is defective in adaptation to the DNA damage checkpoint is also defective in adaptation to the SAC. To test this hypothesis, we tested SAC adaptation of *cdc5-ad* and wild type cells in presence of either benomyl or high levels of Mad2 (Rossio et al., 2010). For the experiment in benomyl, we synchronized wild type and *cdc5-ad* cells in G1 with alpha factor and released them in presence of benomyl at 30°C. After 120 minutes from the release we re-added alpha factor to re-accumulate cells in the next G1 phase. We then analyzed DNA contents by FACS analysis and the kinetics of budding and nuclear division at different time points (fig. 16A). Under these conditions, *cdc5-ad* cells divided nuclei and exited mitosis with kinetics similar to wild type cells. To check whether *cdc5-ad* cells slowed down adaptation upon *MAD2* overexpression, *GAL1-MAD2* and *GAL1-MAD2 cdc5-ad* cells were grown in uninduced conditions, arrested in G1 with alpha factor (unbudded cells) and spotted on galactose-containing plates to follow the formation of microcolonies. Surprisingly, *GAL1-MAD2 cdc5-ad* cells escaped from the mitotic arrest and formed colonies even faster than *GAL1-MAD2* cells (fig. 16B). Altogether, these data suggest that the *cdc5-ad* allele does not affect adaptation to the SAC.

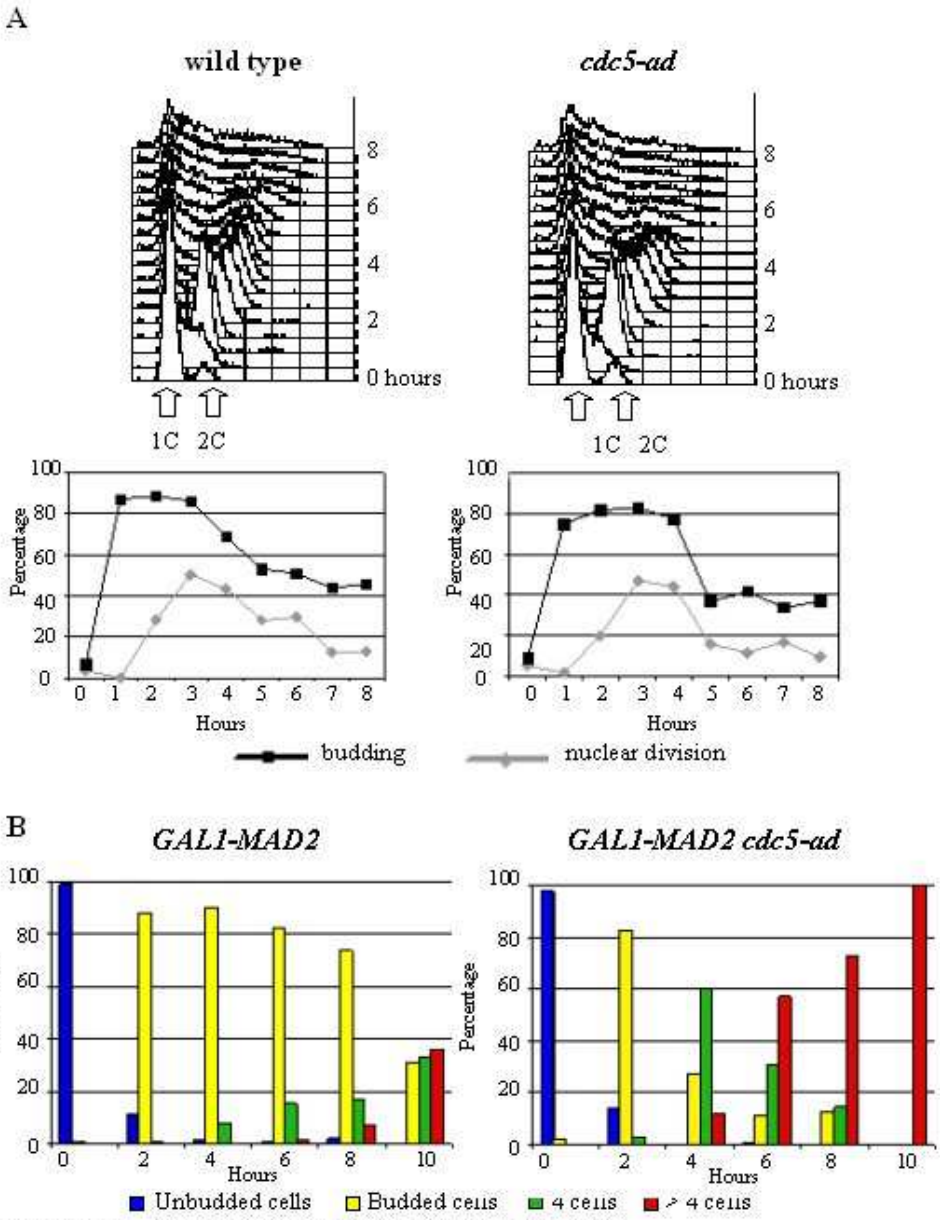


Figure 16. The *cdc5-ad* allele does not affect SAC adaptation

(A) Wild type (ySP220) and *cdc5-ad* (ySP8747) cells carrying the *BAR1* deletion were arrested in G1 with alpha factor at 25°C and then released in presence of benomyl at 30°C. At 120 minutes after release alpha factor was re-added to re-accumulate cells in the next G1. Samples were collected at the indicated times for FACS analysis of DNA contents (histograms) and for kinetics of budding and nuclear division (graphs). (B) *GAL1-MAD2* (ySP6170) and *GAL1-MAD2 cdc5-ad* (ySP8749) cells were grown in uninduced conditions, arrested in G1 (unbudded cells) and spotted on YEP galactose plates (t=0) at 30°C. Formation of microcolonies was scored at the indicated times with a transmission microscope.

MATERIALS AND METHODS

Abbreviations

APC: Anaphase Promoting Complex; CDKs: cyclin-dependent kinases; DMSO: dimethyl sulfoxide; FEAR: cdc-Fourteen Early Anaphase Release; MEN: Mitotic Exit Network; MTs: microtubules; RSC: Remodel the Structure of Chromatin; SAC: Spindle Assembly Checkpoint; SAGA: Spt-Ada-Gcn5 acetyltransferase; SPBs: spindle pole bodies.

Strains and plasmids

Bacterial strains

E. coli DH5α ^{TM} (*F* $^{-}$, *80dlaZΔM15, ΔlacZTA-argF*) *U169, deoR, recA1, endA1, hsdR17,* (*rK*$^{-}$*mK*$^{+}$), *supE44, thi1, gyrA96, relA1*) strain was used as bacterial host for plasmid construction and amplification. Transformation competent bacterial cells were provided by Invitrogen.

Plasmids

- *YCplac111* (Gietz and Sugino, 1998) *LEU2*
- pSP773: *YCplac111* (Gietz and Sugino, 1998) *LEU2* carrying the allele *sth1K501R*

Yeast strains

The genotypes of all the yeast strains used in this study are listed in Table 1. Unless differently stated, all yeast strains were derivatives of W303 (*MATa or MATα ade2-1 can1-100 trp1-1 leu2-3,112 his3-11,15 ura3).*

Name	**Relevant genotypes**
ySP220	*MATa bar1::URA3*
ySP6170	*MATa ura3::URA3::GAL1-MAD2* (m.c.)
ySP6273	*MATalpha ura3::URA3::GAL1-MAD2* (m.c.)
ySP6850	*MATa ura3::URA3::GAL1-MAD2* (m.c.) *rsc2::kanMX*
ySP6873	*MATa ura3::URA3::GAL1-MAD2* (m.c.) a*da2::kanMX*
ySP6890	*MATa TRP1::SPC42-GFP* (m.c.)
ySP8722	*MATa ura3::URA3::GAL1-MAD2* (m.c.) *gcn5::hph*
ySP8736	*MATa ura3::URA3::GAL1-MAD2* (m.c.) *rsc2::kanMX yca1::kanMX*
ySP8737	*MATa ura3::URA3::GAL1-MAD2* (m.c.) *yca1::kanMX*
ySP8745	*MATa bik1::TRP1 bar1::kanMX*
ySO8746	*MATa kip3::HIS3 bar1::kanMX*
ySP8747	*MATa cdc5-ad bar1::kanMX*
ySP8749	*MATa ura3::URA3::GAL1-MAD2* (m.c.) *cdc5-ad*
ySP8750	*MATa dyn1::kanMX bar1::URA3*
ySP8752	*MATa gcn5::hph bar1::kanMX*
ySP8768	*MATa ada2::kanMX bar1::URA3*
ySP8770	*MATa kip2::kanMX bar1::URA3*
ySP8789	*MATa mad2::TRP1 YCplac111*
ySP8795	*MATa mad2::TRP1 GAL1-UBR1::HIS3 sth1::URA3 CUP1-1XHA-STH1^{td} YCplac111*
YSP8796	*MATa mad2::TRP1 GAL1-UBR1::HIS3 sth1::URA3 CUP1-1XHA-STH1^{td} pSP773*

Table 1. Yeast strains used in this work. (m.c.), multiple copy integration.

Growth media

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

Media for E. coli

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

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

Media for *S. cerevisiae*

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

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

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

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

5-FOA medium: it was obtained by adding 1g/l 5-FOA, 50 mg/l uracil to synthetic complete medium.

Sporulation medium (VB)	1.36% $CH_3COONa_3H_2O$
	0.19% KCl
	0.0035% $MgSO_47H_2O$
	0.12% NaCl (pH 7)

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

Buffers

SDS-PAGE running buffer 5X: 2M Glycin, 0.25M TRIS, 0.02M SDS, pH 8.3

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

TAE: 0.04M TRIS acetate, 0.001M EDTA

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

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

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

BLUE 6X: 0.2% bromofenol blue in 50% glycerol

Methods

Generation of diploid strains and sporulation

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

Yeast transformation

Cells were inoculated o/n at 25°C YEP medium containing the appropriate sugar, allowing them to reach stationary phase. Cell

cultures were then diluted and allowed to grow for at least 2 hours, until they reached a concentration between $1x10^6$ and $1x10^7$ cells/ml. 10 ml of each culture were then centrifuged for 5 minutes at room temperature and pellets were washed with 1 ml of LiAc 0.1 M to completely eliminate the growth medium. Each pellet was then resuspended in 500 µl of 0.1 M LiAc, and 1-2 µg of DNA were added to 100 µl of cells suspension (sufficient for one transformation), together with 16 µg of carrier DNA (salmon sperm DNA) and 45 µl of PEG 4000 50%. After gently mixing, the tubes were incubated 30'-60' at 25°C. Subsequently, 6 µl of glycerol 60%was added to the cell suspension, followed by incubation a 25°C for 30'-60'. After a 5' heat shock at 42°C, cells were finally plated on selective medium.

Transposon insertion screen

We mutagenized the yeast genome by random transposon insertions using a yeast genomic DNA library carrying random insertions of a bacterial transposon into the yeast DNA (Kumar et al., 2002) (from M. Snyder, Yale University). The bacterial transposon carries the *lacZ* gene, the yeast marker *LEU2* and the tet^R gene that confers tetracycline resistance to *E. coli*. We digested the mTn3-mutagenized genomic library with *NotI* in order to obtain linear DNA fragments carrying the bacterial transposon flanked by highly recombinant yeast sequences that directed their insertion in the yeast genome by homologous recombination, thereby replacing the corresponding wild type locus. *MATa* and *MATα GAL1-MAD2* cells (ySP6170 and ySP6273) were transformed with the linearized transposon library. After transformation on leucine lacking selective plates containing raffinose, we obtained a collection of $3.2x10^4$ Leu^+ *GAL1-MAD2*

clones carrying the transposon randomly inserted in the genome. These clones were replica-plated on galactose selective medium in order to identify those growing slower than the majority in these conditions. 800 clones were selected in the first step. To discard the clones that were slowly growing due to defects in galactose metabolism, we streaked out the selected clones on 5-FOA (5-fluoro-orotic acid) plates to select for their derivatives which had lost the *GAL1-MAD2* construct marked by the *URA3* gene, based on the toxicity of 5-FOA for *URA3* cells, and chose for further analysis only the clones whose *ura3* derivatives did not show any growth defect on galactose. To identify the transposon insertion site in the clones of interest, we transformed them with the recovery plasmid pRSQ2-*URA3* (Kumar et al., 2002) that replaces part of the transposon. Cutting the genomic DNA from each of the transformed clones with *BamHI*, followed by ligation and *E. coli* transformation, allowed the recover plasmids where the transposon was flanked by the adjacent yeast DNA, whose sequence could be determined using a primer complementary to 5' end of the transposon, in order to identify the precise point of transposon insertion for each clone.

***E. coli* transformation**

DH5α™-GIBCO BRL competent cells, kept at -80°C, were thawed in ice, and 50-100 µl of cell suspension were used for each reaction. After incubation in ice for 30 minutes, 1-10 ng of DNA were added to the cells. After a further incubation in ice for 30 minutes, cells were subjected to a 20-45 seconds heat shock at 37°C, followed by incubation in ice for 2 minutes. Finally, 900-950 µl of LD medium were added to each reaction tube. Cell suspension was then shacked

for one hour at 37°C before plating on selective medium and incubation at 37°C.

Preparation of plasmid DNA from *E. coli*

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

1) Minipreps boiling: *E. coli* cells (2 ml overnight culture) are harvested by centrifugation and resuspended in 500 µl STET buffer. Bacterial cell wall is digested boiling the samples for 2 minutes with 1 mg/ml lysozyme. Cellular impurities are removed by centrifugation and DNA is precipitated with isopropanol and resuspended in the appropriate volume of water or TE.

2) Qiagen columns© kit: this protocol allows the purification of up to 20 µg high copy plasmid DNA from 1-5 ml overnight *E. coli* culture in LD medium. Cells are pelleted by centrifugation and resuspended in 250 µl buffer P1. After addition of 250 µl buffer P2 the solution is mixed thoroughly by inverting the tube 4-6 times, and the lysis reaction occurs. 350 ml N3 buffer are added to the solution, which is then centrifuged for 10 minutes. The supernatant is applied to a QIAprep spin column which is washed once with PB buffer and once with PE buffer. The DNA is eluted with EB buffer or water.

Synchronization with alpha-factor

MATa cells were inoculated in YEP medium supplemented with the appropriate sugar, allowing them to reach a concentration of $5x10^6$ cells/ml. alpha-factor was then added to the final concentration of 2 µg/ml for *BAR1* cells and to the final concentration of 0.2 µg/ml for *bar1*Δ strains, and the percentage of budded cells was scored 2 hours later. When more than 95% of cells arrested as unbudded (G1-arrested

cells), the pheromone was removed and cells were washed once with fresh medium and then re-suspended and incubated in fresh medium. Unless otherwise stated, synchronizations were performed at 25°C and galactose, when required, was added half an hour before the release from alpha-factor.

Nocodazole and benomyl response

Nocodazole and benomyl allow yeast cell synchronization in G2/M transition by causing microtubule depolymerization, thus activating the SAC, which in turn arrests cells at the metaphase to anaphase transition. For nocodazole response, cells were released from G1 arrest in the presence of 15 μg/ml nocodazole (USBio) that was dissolved in DMSO while for benomyl response, cells were released from G1 arrest in the presence of 80 μg/ml benomyl (dissolved in DMSO too). The final DMSO percentage in the growth medium was 1%.

Protein extracts

Cells were collected by centrifugation, washed once with 1 ml TRIS 10 mM pH 7.5 cold and then resuspended in two volumes of breaking buffer (TRIS 50 mM pH 7.5 and protease inhibitor cocktail by Boehringer Mannheim), using Eppendorf tubes that were always kept in ice. Equal amount of acid-treated glass-beads were then added to each suspension, and cells were broken by vortexing for 7 minutes. Samples were then transferred into new tubes and clarified. 1 μl of each protein extract was diluted in 1 ml of Biorad Protein Assay (Biorad) for spectrophotometric protein quantification at 595 nm wavelength. Clarified extracts were resuspended in 50 μl Lammli buffer, boiled for 3 minutes and loaded on polyacrylamide gel.

Coimmunoprecipitation

Cells from 50 ml of a $1x10^7$ cells/ml concentration culture were collected by centrifugation and rinsed. All the immunoprecipitations have been performed with the same Immunoprecipitation buffer (50 mM HEPES pH 7.4, 75 mM KCl, 1 mM $MgCl_2$, 1 mM sodium orthovanadate, 5 mM β-glycerolphosphate, 1 mM EGTA pH 8, 0.1% Triton X-100 and a protease inhibitor cocktail by Boehringer Mannheim). For Mad1-Bub3 coimmunoprecipitations and pull-downs, cells were resuspended in one volume of the Immunoprecipitation buffer; an equal amount of acid-treated glass-beads was then added to the suspension, and cells were broken by 7 cycles of 30'' vortexing. The sample was then transferred into a new tube and then clarified. 2µl of protein extract were taken for spectrophotometric protein quantification at 280 nm UV wavelength. Mad1-Bub3 coimmunoprecipitation were performed as described in Brady and Hardwick (2000), while pull-down were performed as described in Donniani et al.(2010). 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 at 4°C for 30 minutes. 1-2 mg of cleared extracts were incubated for 30 minutes with protein A-Sepharose and 1 hour with anti-HA antibodies (12CA5). Protein A-Sepharose was then added to immunoprecipitation and incubated for 30 minutes. The slurry was washed four times with Immunoprecipitation buffer and twice with

PBS. The pellet is resuspended in 50 µl Laemmly buffer , boiled for 3 minutes and finally clarified by centrifugation before loading.

Western blot analysis

Protein were separated based on their molecular weight on 10% or 12.5% polyacrylamide gel in SDS-PAGE. Proteins were transferred on nitrocellulose filters by 200 mA o/n. In order to preliminarily quantify the total amount of transferred proteins, the filters were stained with Ponceau S (Sigma). After destaining with PBS 1X, filters were incubated for one hour at room temperature in non-fat 4% or 5% dust milk in TBS 1X and 0.2% Triton X-100. Filters were then incubated 2 hours with primary antibodies directly diluted in 4% milk. To detect HA tagged proteins, we used 1:3000 dilutions of monoclonal antibodies 12CA5; to detect myc-tagged proteins, we used 1:2000 dilutions of monoclonal antibodies 9E10; to detect flag-tagged proteins, we used 1:2000 dilutions of anti-flag M2 mAb (Sigma-Aldrich); for Clb2 we used anti-Clb2 polyclonal antibodies kindly supplied by E. Schwob. Filters were then washed three times in TBS 1X for 10 minutes, before incubating them for an hour at room temperature with properly diluted secondary antibodies (anti-mouse IgG against monoclonal antibodies and anti-rabbit IgG for polyclonal antibodies). These secondary antibodies, provided by Amersham in the kit ECL, are conjugated with the peroxydase enzyme. Filters were finally washed three times for 10 minutes, dried on 3MM paper and carefully dipped in a mix composed by equal volumes of the two ECL solutions. After a new drying, the filters were exposed for different times to a film; peroxydase, together with its reagents on the film

catalyses a reaction which emits light, which is detectable on the film filter after treatment with developing and fixing solution.

FACS analysis, budding and nuclear division

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

In situ immunofluorescence and DAPI-staining

Cells were fixed in 1 ml IF buffer (0.1 M K^+/PO_4^{3-} buffer pH 6.4, 0.5 mM $MgCl_2$) containing 3.7% formaldehyde at 4°C overnight. Samples were washed three times in IF buffer and once in IF buffer containing 1.2 M sorbitol. Cells were spheroplasted by incubating for about 45-60 minutes at room temperature in 0.2 ml spheroplasting buffer (1.2 M sorbitol, 0.1 M K^+/PO_4^{3-} buffer pH 6.4, 0.5 mM $MgCl_2$, 0.2% β-mercaptoethanol , 250 μg/ml zymolyase). Spheroplasting was monitored by mixing a drop of cells in

spheroplasting buffer with an equal amount of 10% SDS. When cells lysed under these conditions the spheroplasts were washed once in IF buffer containing 1.2 M sorbitol and resuspended in 20-200 µl of the same solution depending on the amount of cells. A 30-well slide was coated with 0.1% polylysine, rinsed with mQ H_2O and dried. A drop of cell suspension of medium density was added to each well, and cell were allowed to attach to the slide surface for 10-20 minutes. The cell suspension was then removed, and slides were put in a methanol bath at -20°C for 6 minutes, and in an acetone bath at -20°C for 30 seconds. Next, the slide were dried. Primary antibody was added to each well and incubated for two hours at room temperature. Then the primary antibody was aspired off, each well was washed three times with BA-PBS followed by addition of secondary antibody. Slides were incubated in he dark for 2 hours, then the secondary antibody was aspired off, and each well was washed four times with BSA-PBS. For DAPI staining, a drop of pd-DAPI (0.25 µg/ml DAPI (4,6 diamino-2-Phenylindolo), 0.1% p-phenylenediamine, and 10% PBS pH 8.0 in glycerol) was added to each well, a coverslip was placed over, and the coverslip was sealed using nail hardener. Slides were stored at -20°C in the dark until use. Immunostaining of alpha-tubulin was performed with the YOL34 mAb (Serotec) that was diluted 1:100 in BSA-PBS, followed by indirect immunofluorescence using rhodamine-conjugated anti-rat Ab (1:100 Pierce Chemical Co.). Immunostaining of Pds1-myc18 was detected by incubation with the 9E10 mAb followed by indirect immunofluorescence usingCY3-conjugated goat anti-mouse antibody (1:500; GE Healthcare). Immunostaining of Cdc14 was performed with anti-Cdc14 antibody

(supplied by Rosella Visintin) that was diluted 1:300 in ovalbumin-PBS followed by indirect immunofluorescence using CY3-conyugated goat anti-rabbit antibody (1:500).

Digital images were acquired on a fluorescent microscope (Eclipse E600; Nikon) equipped with a change-coupled device camera (DC350F; Leica) at room temperature with an oil 100x 1.3 Plan Fluor objective (Nikon), using FW4000 software (Leica).

Drop test

For drop test analysis, cell cultures were grown overnight to a 10^8 cells/ml concentration and then diluted to 1.6 x 10^6, 1.6 x 10^5, 1.6 x 10^4 and 1.6 x 10^3 cells/ml respectively. For each dilution, 5 µl of cellular suspension were spotted on plates that were incubated at the appropriate temperatures.

Microcolony assay

Cells were grown in uninduced conditions, arrested in G1 with alpha-factor (unbudded cells) and spotted on glucose- or galactose-containing plates, which were then incubated at 30°C (1% of galactose was added to the cultures 30 minutes before the release to pre-induce the *GAL1* promoter). At different time points formation of microcolonies was scored with a transmission microscope. We divided cells into four classes: (1) unbudded cells, (2) budded cells, (3) microcolonies formed by four cells and (4) microcolonies formed by more than four cells.

DISCUSSION

Tools to characterize SAC adaptation in yeast

Adaptation to checkpoint activation is an important process that gives to cells the possibility to proceed into the cell cycle in critical situations, i.e. in the absence of kinetochore-microtubule attachments or in presence of DNA damage. In fact, it provides the cells with a chance to survive in the presence of irreparable damages or errors that might be corrected during the following cell cycle. Adaptation is also a dangerous process as a potential source of aneuploidy if errors and damages are not corrected (Rieder and Maiato, 2004). Since at the moment little is known about the mechanisms that promote SAC adaptation, we chose to use a genetically tractable model system, such as budding yeast, to identify factors influencing the rate of adaptation. We found two conditions suitable to study what happens when yeast cells adapt to the SAC. First, we hyperactivated the SAC overproducing Mad2, one of the key SAC proteins; in this situation cells arrest in metaphase with normal bipolar attachments (i.e. absence of errors). Second, we induced SAC activation by depolymerisation of the mitotic spindle with nocodazole or benomyl. Consistent with previous studies, we found that adaptation to the SAC takes a variable amount of time depending on the conditions. In particular, we found that yeast cells adapt the fastest in benomyl (3-4 hours), somewhat slower in the presence of high levels of Mad2 (4-5 hours) and the slowest in nocodazole (6-8 hours). The reasons for these differences are unclear at the moment. One difference between nocodazole and benomyl treatment is that the former completely depolymerizes both spindle and cytoplasmic microtubules, whereas in the latter some

cytoplasmic microtubules are still detectable in the absence of mitotic spindles. However, since cytoplasmic microtubules should also be unaffected by Mad2 overproduction, this is unlikely to be the main reason for the different adaptation timing between nocodazole and benomyl. Further studies to identify factors that promote adaptation to the SAC in the different conditions will help shedding light on this process.

What happens when yeast cells adapt to the SAC

Like in mammalian cells, we found that yeast cells undergo SAC adaptation with sister chromatid separation and mitotic exit that are accompanied by degradation of the securin Pds1 and the mitotic cyclin Clb2. Unlike in animal cells, however, where adaptation to the SAC takes place in the continuous presence of SAC signalling (Brito and Rieder, 2006), in yeast it occurs concurrently with Mad1/Bub3 dissociation, suggesting that the SAC signal is extinct. Whether SAC silencing is the cause or the consequence of adaptation is unclear at the moment. Beside that, other aspects of SAC adaptation seem to be conserved. For instance, expression of a non-degradable form of Clb2 or inactivation of Cdc20 delay adaptation. In addition, we found that the polo kinase Cdc5, which is a key regulator of cyclin B proteolysis and mitotic exit (Queralt and Uhlmann, 2008; Geymonat et al., 2003; Liang et al., 2009; Yoshida et al., 2006), is involved in SAC adaptation. On the contrary, deletion of the CDK inhibitor Sic1 did not show any effect on the timing of adaptation. Altogether, our data demonstrate that the modality by which cells undergo SAC adaptation is similar from yeast to human, although the molecular mechanisms

underlying this process might differ. In particular, our data raise the possibility that, like for adaptation to DNA damage (reviewed in Clemenson and Marsolier-Kergoat, 2009), SAC adaptation might be indistinguishable from checkpoint silencing.

Adaptation versus apoptosis in yeast: preliminary observations

We observed that the absence of *RSC2* caused a prolonged arrest in mitosis upon engagement of the SAC by either microtubule depolymerization or *MAD2* overexpression. When eukaryotic cells escape from prolonged SAC activation they either undergo adaptation and proceed into the next cell cycle or die by apoptosis (Rieder and Maiato, 2004; Gascoigne and Taylor, 2008). We therefore asked if the persistent cell cycle arrest caused by *RSC2* deletion was due to cell death. We verified that in absence of the only known yeast metacaspase (Yca1) *rsc2Δ* cells remained arrested in mitosis upon SAC hyperactivation. These preliminary data suggest that *rsc2Δ* cells do not die in mitosis by activation of the apoptotic caspase-dependent pathway. However, we cannot exclude that these cells die due to caspase-independent pathways. We are in the process of testing whether in more general terms adaptation to the SAC is accompanied by apoptotic features.

The RSC complex regulates mitotic exit by promoting the early release of Cdc14

The RSC complex is composed by many proteins; in particular, in yeast RSC can bind to two alternative regulatory subunits, Rsc1 and Rsc2 (Cairns et al., 1999). Our experimental data show a new role of

the RSC^{Rsc2}, but not RSC^{Rsc1}, in mitotic exit regulation. In particular, we demonstrated that *RSC2* deletion in cells with hyperactivated SAC by *MAD2* overexpression strongly delays adaptation. Moreover, in the absence of *RSC2* SAC-deficient mutants treated with microtubule drugs are unable to exit from mitosis. These cells degrade Pds1 but maintain a fraction of the mitotic cyclin Clb2 stable and do not accumulate the CDK inhibitor Sic1. These phenotypes have been observed also in absence of the Sth1 catalytic subunit of RSC, or when a ATPase-defective form of Sth1 (*sht1-K501R*) was expressed as sole source of Sth1 in the cells. We found that also during the unperturbed cell cycle, where RSC^{Rsc2} has a minor role in mitotic exit, the early anaphase release of Cdc14 was impaired. In addition, we observed that the early release of Cdc14 from the nucleolus was strongly impaired by the lack of Rsc2 in SAC-deficient mutants in the presence of nocodazole. Expression of a dominant allele of Cdc14 that loosens Net1-dependent inhibition ($CDC14^{TAB6\text{-}1}$, Shou et al, 2001) is sufficient to restore the ability of these cells to escape mitosis in these conditions. Altogether, these data demonstrate that RSC^{Rsc2} is involved in the regulation of Cdc14 and mitotic exit and this role requires its ATPase activity, suggesting that Cdc14 nucleolar release and mitotic exit regulation presumably depend to a certain extent on chromatin-remodelling. Interestingly, a recent report implicated also some histone modifications in the FEAR pathway of Cdc14 activation (Hwang and Madhani, 2009). Our data strongly indicate that RSC^{Rsc2} might be a component of the FEAR pathway. This idea is supported by the fact that deletion of *RSC2* shows synthetic growth defects when combined with mutations affecting the MEN but not the FEAR

pathway. In addition, deletion of FEAR genes in SAC-deficient cells treated with nocodazole shows the same phenotype of *RSC2* deletion, i.e. cells are unable to exit mitosis and re-replicate their DNA.

How could RSC^{Rsc2} affect Cdc14 release from the nucleolus? We showed that Rsc2 interacts with the polo kinase Cdc5 that regulates both the FEAR and the MEN pathway and phosphorylates in vitro the Cdc14 inhibitor Net1 (Shou et al., 2002). Indeed, we found that in the absence of *RSC2* Net1 phosphorylation is impaired. Therefore, RSC^{Rsc2} could stimulate Cdc5 activity for what concerns its function(s) in Cdc14 regulation. Alternatively, it is Cdc5 that modulates the function of RSC^{Rsc2} to allow it to perform its FEAR function. In this scenario, RSC^{Rsc2} could regulate more directly Cdc14 release from the nucleolus, perhaps by loosening the chromatin structure at rDNA and/or by modulating the accessibility of Net1 to CDK phosphorylation.

The SAGA complex regulates SAC adaptation

Since chromatin-remodelling seems important for SAC adaptation, other factors that regulate the structure of chromatin could be involved as well. In particular, since RSC binds to acetylated histone tails to promote nucleosome positioning, it is possible that histone transacetylases are involved together with RSC in adaptation. We decided to test if the SAGA, a protein complex that acetylates histones thereby promoting chromatin restructuring, could have a role in SAC adaptation. Indeed, we observed that elimination of two SAGA components, Ada2 and Gcn5, conferred adaptation defects in presence of benomyl, while the lack of Ada2 only, but not of Gcn5, affected

adaptation in the presence of high levels of Mad2. These preliminary data suggest that the SAGA complex might be involved in SAC adaptation. The fact that Gcn5 and Ada2 do not show the same phenotypes could indicate that these two proteins play different roles in this process. SAGA components have been previously shown to be differentially required for other processes. For example, a recent report demonstrates that Ada2 promotes transcriptional silencing at telomeres and ribosomal DNA in a Gcn5-independent manner (Jacobson and Pillus, 2009). Further investigation will be necessary to better understand the role of the SAGA complex in SAC adaptation.

Microtubule dynamics and SAC adaptation

When we characterised adaptation using benomyl or nocodazole to depolymerise the mitotic spindle we observed that an abnormal nuclear division occurred only in cells treated with benomyl. The main difference between these two drug treatments is that in the presence of nocodazole no microtubules are present in the cells, while in the presence of benomyl some cytoplasmic microtubules can be still detectable. For this reason, we supposed that the nuclear division observed in benomyl could depend on cytoplasmic microtubules. Microtubules dynamics is controlled by motor proteins and microtubule regulators. Among the ones that we tested, we found that in the absence of the kinesin Kip2 or its associated microtubule binding protein Bik1, nuclear division is delayed upon benomyl treatment. In addition, mitotic slippage is also impaired in *kip2Δ* and *bik1Δ* mutants, suggesting that the aberrant nuclear division might be linked to mitotic exit. Since Kip2 and Bik1 regulate the dynamics of

cytoplasmic microtubules, these observations reinforce the hypothesis that nuclear division might be triggered by cytoplasmic microtubules. In the future, it will be interesting to investigate more extensively the role of Kip2 and Bik1 in SAC adaptation, as well as to establish whether other mutants that specifically destabilise cytoplasmic microtubules affect this process.

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