Regulated inactivation of the spindle assembly checkpoint without functional mitotic spindles

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The spindle assembly checkpoint (SAC) arrests mitosis until bipolar attachment of spindle microtubules to all chromosomes is accomplished. However, when spindle formation is prevented and the SAC cannot be satisfied, mammalian cells can eventually overcome the mitotic arrest while the checkpoint is still activated. We find that *Aspergillus nidulans* cells, which are unable to satisfy the SAC, inactivate the checkpoint after a defined period of mitotic arrest. Such SAC inactivation allows normal nuclear reassembly and mitotic exit without DNA segregation. We demonstrate that the mechanisms, which govern such SAC inactivation, require protein synthesis and can occur independently of inactivation of the major mitotic regulator Cdk1/Cyclin B or mitotic exit. Moreover, in the continued absence of spindle function cells transit multiple cell cycles in which the SAC is reactivated each mitosis before again being inactivated. Such cyclic activation and inactivation of the SAC suggests that it is subject to cell-cycle regulation that is independent of bipolar spindle function.

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Introduction

Cells utilize cell-cycle checkpoint regulation to ensure completion of one stage of the cell cycle before the subsequent stage begins. The spindle assembly checkpoint (SAC) ensures that formation of bipolar spindle attachments to all chromosomes is accomplished before the onset of DNA segregation in anaphase (Musacchio and Salmon, 2007). When cells enter mitosis, SAC proteins are recruited to kinetochores which have not formed bipolar attachments to generate a signal which inhibits the anaphase promoting complex/cyclosome (APC/C), causing cells to arrest in a prolonged prometaphase state (Chen *et al*, 1996; Waters *et al*, 1998; Howell *et al*, 2004; Musacchio and Salmon, 2007; De Souza *et al*, 2009). Silencing of the checkpoint

occurs when all kinetochores have formed bipolar attachments to spindle microtubules (Musacchio and Salmon, 2007). The presence of even a single unattached kinetochore is sufficient to maintain mitotic arrest (Rieder *et al*, 1995). However, SAC-mediated arrest is not permanent and cells can eventually escape mitotic arrest in a process termed mitotic slippage or adaptation (Rieder and Maiato, 2004; Brito and Rieder, 2006; Brito *et al*, 2008). Despite the importance of spindle poisons such as taxol as chemotherapeutic agents, the process of mitotic slippage is not well understood. However, it is known that treatment of different cell lines with spindle poisons results in considerable variation in the duration of the mitotic SAC arrest as well as the fate of cells following entry into mitosis without spindle function (Gascoigne and Taylor, 2008). This can in part be explained by the mode of action of the spindle poison. For example, when microtubules are stabilized by taxol mitotic exit occurs when syntelic kinetochore attachments become stabilized in a manner which leads to SAC satisfaction (Yang *et al*, 2009). Contrasting this, when microtubules are depolymerized in PtK2 or RPE1 cells mitotic slippage occurs when the SAC is still active and unsatisfied (Bruto and Rieder, 2006). In this process, the APC/C target Cyclin B is slowly degraded leading to a gradual decrease in the activity of the mitosis promoting kinase Cdk1/Cyclin B. Mitotic slippage then occurs once Cdk1/Cyclin B activity has decreased below a critical threshold required to maintain a mitotic state even though the SAC has not been fulfilled or inactivated (Bruto and Rieder, 2006; Gavet and Pines, 2010b). At present, it is unclear if other mechanisms might exist which allow cells to exit SAC arrest when microtubules are depolymerized.

As in other organisms, the *Aspergillus nidulans* SAC proteins Mad1 and Mad2 localize to nuclear pore complexes (NPCs) during interphase, but function together at kinetochores during mitosis (De Souza *et al*, 2009). Mitosis in *A. nidulans* is characterized by the partial disassembly of its NPCs, at which time Mad1 and Mad2 concentrate in the vicinity of kinetochores (De Souza *et al*, 2004, 2009; Osmani *et al*, 2006a; Liu *et al*, 2009). We have previously shown that when spindle formation is completely prevented in cells which lack Mad2 function, cells transit mitosis with similar timing to a normal mitosis (Ukil *et al*, 2009). Although this occurs without nuclear division, cells complete all aspects of mitotic exit except for those which require spindle function in a process we previously termed spindle-independent mitosis. To more accurately describe this process, we here use the term Spindle-Independent Mitotic Exit, abbreviated to SIME. Notably, post-mitotic NPC reassembly occurs normally during SIME, resulting in the reestablishment of active nuclear transport (Ukil *et al*, 2009). In addition, the mitotic nucleolar cycle, in which the nucleolus is excluded to the cytoplasm during late mitosis before being disassembled and reassembled in early G1 nuclei, also occurs during SIME (Ukil *et al*, 2009). Therefore, at least some cells can complete

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mitotic exit and rebuild functional nuclei in the absence of nuclear segregation.

Here, we have investigated the mechanisms by which *A. nidulans* cells can overcome SAC arrest. This was done by preventing spindle formation and following the localization of SAC proteins and the mitotic status of the nucleus. We find that following a defined period of SAC-mediated mitotic arrest, the SAC is actively turned off allowing cells to undergo SIME and rebuild functional nuclei. These cells then continue through the cell cycle and upon mitotic entry reactivate the previously inactivated SAC. This results in a second mitotic arrest before the SAC is again turned off to allow SIME to occur again. We find that inactivation of the SAC without a functional spindle occurs even when mitotic exit and Cyclin B degradation are prevented by inactivation of the APC/C. The data support a model whereby a previously unrecognized mechanism can inactivate the SAC when it cannot be satisfied by bipolar spindle formation, thus allowing cells to efficiently exit mitosis and reenter interphase.

Results

Cells undergo SAC mitotic arrest for a defined period of time after which functional interphase nuclei are reassembled in the normal manner

To examine how *A. nidulans* responds to an extended mitotic SAC arrest, we treated cells with the microtubule poison benomyl to completely depolymerize microtubules (Oakley and Morris, 1981; Jung *et al*, 1992; Horio and Oakley, 2005). To define when cells entered mitosis we followed the status of NPCs which partially disassemble during mitotic entry and then reassemble as cells return to interphase (De Souza *et al*, 2004; Osmani *et al*, 2006a). Depolymerization of microtubules did not prevent mitotic entry but lead to a mitotic-like arrest which maintained the mitotic dispersal of the NPC proteins Nup49, Nup98, Nup188 and Mlp1 (Figure 1A; Supplementary Figure S1). This mitotic arrest was due to the SAC as it depended on the function of the conserved SAC proteins Mad1, Mad2 and Bub3 (Efimov and Morris, 1998; Prigozhina *et al*, 2004; De Souza *et al*, 2009; Figure 1B). During the period of the SAC arrest, nuclear localization sequence reporter constructs (NLS-DsRed; Suelmann *et al*, 1997; De Souza *et al*, 2004) were released from nuclei, while nuclear export sequence reporter constructs (NES-GFP; Shulga *et al*, 2000) and Ran-GAP (De Souza *et al*, 2004) gained access to nuclei (Figure 1A; Supplementary Figure S1D). This is consistent with the inactivation of nuclear transport and permeabilization of the nuclear envelope which occurs when NPCs are partially disassembled during mitosis (De Souza *et al*, 2004; Osmani *et al*, 2006a). Surprisingly, even though spindle formation was prevented, the SAC only maintained this mitotic state for a defined period of time. For example, Nup49 remained mitotically dispersed for 47.6 ± 8.9 min ($n = 10$) before, along with other mitotically dispersed NPC proteins, it reassembled to NPCs around a single undivided nucleus (Figure 1A and B; Supplementary Figure S1). Similar timing of NPC reassembly was observed when populations of cells were synchronously released from G2 arrest (O'Connell *et al*, 1992; De Souza *et al*, 2000) into mitosis in the presence of benomyl (Figure 1C). Following NPC reassembly, nuclear import and export was reestablished (Figure 1A) and DNA decondensed (Figure 1C), demonstrating cells had effectively

returned to interphase and undergone SIME. Examination of GFP- α -tubulin confirmed that mitotic spindles did not form when benomyl-treated cells transited mitosis (six independent experiments, $n = 27$ cells, e.g., Figure 9B) as also evidenced by the failure of nuclear division.

To ensure that the return of SAC arrested cells to interphase was not due to off target effects of benomyl, we deleted the essential β -tubulin gene *benA* (May, 1989) as a genetic non-pharmacological means to inactivate microtubule functions and followed the phenotype of the null allele by heterokaryon rescue (Osmani *et al*, 2006b). As expected, uni-nucleate *benAΔ* spores underwent limited growth due to lack of the essential β -tubulin. When *benAΔ* cells entered mitosis Nup49-GFP remained mitotically dispersed for 43.3 ± 7 min ($n = 10$) before reassembling to NPCs around a single undivided nucleus (Figure 1D). This transient mitotic SAC arrest is identical to what occurs in benomyl-treated cells.

The defined period of the mitotic arrest in the absence of functional microtubules suggests that the return to interphase is a regulated process, predicting that nuclear reassembly should occur with normal efficiency. During normal mitotic exit, the half maximal accumulation of NLS-DsRed occurred 1.4 ± 0.1 min after the half maximal accumulation of Nup49 at NPCs (Figure 2A and B). When cells underwent SIME from SAC arrest this time was 1.0 ± 0.3 min (Figure 2C and D). Therefore, reassembly of functional NPCs occurs with normal efficiency when cells return to interphase from SAC arrest without spindles. This indicates that the processes, which mediate nuclear reassembly and entry into interphase, are occurring with normal efficiency during SIME.

A. nidulans nuclei contain a single nucleolus which, after segregation of the nucleolar organizing regions (NORs) at anaphase, is expelled into the cytoplasm in late mitosis (Ukil *et al*, 2009). The old nucleolus is then disassembled and its constituent proteins are re-imported into daughter nuclei to form nucleoli around the segregated NORs during G1. Importantly, however, nucleolar disassembly and reassembly does not occur during mitotic SAC arrest (Ukil *et al*, 2009). We reasoned that if SAC arrested cells really exit mitosis without functional mitotic spindles in a regulated manner, then a cycle of nucleolar disassembly and reassembly should also occur during SIME. We, therefore, monitored nucleolar behaviour during SIME. When benomyl-treated cells were arrested in mitosis by the SAC, the nucleolar protein Bop1-GFP remained prominent at the old nucleolus (Figure 3) as we have previously shown (Ukil *et al*, 2009). However, as cells exited the mitotic SAC arrest, the old nucleolus disassembled and a new focus of Bop1 formed within the G1-like nucleus (Figure 3). Notably, as is the case during normal mitotic exit, nucleolar disassembly then reassembly was restricted to the period when cells returned to interphase. This provides further evidence that when normal bipolar spindles cannot form to satisfy the SAC, cells undergo SIME during which nuclear reassembly occurs in an apparently normal regulated manner but without generation of two daughter nuclei.

To further investigate if mitotic exit is occurring in the normal manner after extended mitotic SAC arrest, we next evaluated the status of Cyclin B whose localization and degradation are highly regulated during mitosis (Clute and Pines, 1999; Huang and Raff, 1999; Gavet and Pines, 2010a, b; van Zon *et al*, 2010). Upon entry into *A. nidulans* mitosis,

Figure 1 Mitotic exit and NPC reassembly are delayed by the SAC but occur after a defined period of time without spindle function. **(A)** Representative time-lapse experiments of cells transiting from G2 (top) to G1 (bottom) with or without 2.4 µg/ml benomyl. Kymograph representations (middle) show the intervening period on the same time scale. Shown are Nup49-GFP and NLS-DsRed which disperse from nuclei during mitosis and NES-GFP which is excluded from interphase nuclei but enters nuclei during mitosis. The periods of mitosis or mitotic SAC arrest are indicated by vertical gold lines. **(B)** The functions of Mad1, Mad2 and Bub3 are required to delay NPC reassembly in benomyl ($n \geq 3$). **(C)** Nup188-GFP mitotic dispersal and chromosome condensation during synchronous release from a *nimT23cdc25* G2 arrest in the presence or absence of benomyl. **(D)** As for **(A)** but showing that cells lacking the essential β -tubulin *benA* undergo a reversible mitotic arrest with Nup49-GFP remaining dispersed for 43.3 ± 7 min ($n = 10$) before reassembling around a single undivided nucleus. Scale bar, 5 µm.

some nuclear Cyclin B escapes into the cytoplasm while the remaining Cyclin B concentrates in the region of the spindle pole bodies (SPBs; Wu *et al*, 1998; De Souza *et al*, 2009; Nayak *et al*, 2010). During mitotic exit, nuclear Cyclin B is degraded before cytoplasmic Cyclin B (Supplementary Figure S2), as also occurs in *Drosophila* cells (Huang and Raff, 1999). We reasoned that if exit from SAC arrest without spindles follows the normal regulated process, then the spatial and temporal aspects of Cyclin B behaviour should still occur. We, therefore, followed Cyclin B-GFP in the absence of microtubule function using Nup49-mCherry dispersal as a marker for mitotic SAC arrest. When cells entered SAC arrest, a portion of Cyclin B escaped into the cytoplasm while the remaining nuclear pool concentrated near the SPBs and kinetochores (Figure 4; Supplementary Figure S3A), as occurs in normal prophase (De Souza *et al*, 2009). During SAC arrest, both the SPB/kinetochore pool and cytoplasmic pool of Cyclin B remained fairly constant (Figure 4). Before return of Nup49 to NPCs, Cyclin B levels rapidly decreased in the SPB/kinetochore region while the cytoplasmic pool of Cyclin B persisted (Figure 4; Supplementary Figure S3), as occurs during normal mitosis (Supplementary Figure S2). These studies show that when cells are unable to form bipolar spindles Cyclin B degradation is delayed, but as cells return to interphase during SIME the temporal and spatial regulation of Cyclin B is the same as during normal exit from mitosis.

The SAC can be turned off without functional mitotic spindles

Because of the normal features of mitotic exit observed when cells return to interphase without spindle function we considered the possibility that the SAC is being actively turned off. A defining feature of SAC inactivation is the physical removal of the SAC proteins from kinetochores (Chen *et al*, 1996; Waters *et al*, 1998; Howell *et al*, 2004; Musacchio and Salmon, 2007; De Souza *et al*, 2009), while artificially tethering Mad1 to kinetochores is sufficient to mediate SAC arrest (Maldonado and Kapoor, 2011). Therefore, if the SAC is being inactivated without spindle function, then Mad1 and Mad2 should dissociate from kinetochores and return back to NPCs (Campbell *et al*, 2001; Gillett *et al*, 2004; Scott *et al*, 2005; De Souza *et al*, 2009). During passage into SAC arrest both Mad1 and Mad2 translocated from NPCs and associated with the single cluster of kinetochores present adjacent to the SPBs. We have previously shown that Mad1 in this region is present both on kinetochores and on an Mlp1-dependent spindle matrix-like structure which concentrates near the kinetochores (De Souza and Osmani, 2009; De Souza *et al*, 2009). Both Mad1 and Mad2 remained associated with kinetochores during SAC arrest (Figure 5A and C). Most importantly, during exit from mitotic arrest without spindles, Mad1 and Mad2 relocated from the kinetochore region back to their interphase residence at NPCs (Figure 5A and C). In addition, the SAC kinase Bub1 (Efimov and Morris, 1998) also located

Figure 2 Nuclear reassembly occurs normally during return to interphase without spindle function. (A) Nup49-GFP and NLS-DsRed during normal mitosis. (B) Normalized Nup49-GFP and NLS-DsRed intensities during normal mitotic exit (error bars indicate $\pm$ s.d., $n=4$). Each time series is aligned based on the half maximal intensity for Nup49-GFP ($t_{1/2}$ [Nup49]) during reassembly. (C) Nup49-GFP and NLS-DsRed during return to interphase without spindle function. (D) As for (B) but during return to interphase without spindle function ($n=5$). Scale bars, 5 μ m.

to kinetochores specifically during the period of mitotic SAC arrest, but disappeared from kinetochores as cells returned to interphase (Figure 5E). The removal of these SAC proteins from kinetochores indicates that the SAC has been inactivated even though it cannot be fulfilled.

During the transition from mitotic SAC arrest to interphase, the overall dynamic pattern of Mad1 and Mad2 localization was similar to normal mitotic exit (De Souza *et al*, 2009). First, Mad1 and Mad2 dispersed from the kinetochore region. Next, after NPC reassembly, Mad1 and Mad2 accumulated in the nucleoplasm before finally reassociating with NPCs (Figure 5A and C). Thus, the reassociation of Mad1 and Mad2 with NPCs is a late event in NPC reassembly during SIME, as is the case during normal mitosis. Together, our data indicate that in the absence of normal spindle function SAC activation delays mitotic exit events for a defined period of time before the SAC is inactivated triggering the normal sequence of mitotic exit events to generate a functional G1 nucleus.

Regulated SAC inactivation without spindles allows continued growth and cell-cycle progression into subsequent mitotic SAC arrests

Our data suggest a regulatory system exists that can turn off the SAC without the normal requirement for bipolar

kinetochore spindle attachments. If indeed the SAC is actively turned off, cells should have the capacity to traverse the cell cycle, enter a subsequent mitosis and reengage the SAC. If, however, such cells exit mitosis in a non-physiological manner, then they would not be expected to retain the capacity to cycle normally or reengage the SAC. We, thus, monitored mitosis for the time equivalent of several cell cycles in the presence of benomyl to depolymerize microtubules, and thus prevent spindle function. After reversal of the first SAC imposed mitotic arrest, and a subsequent interphase period, cells entered a second extended mitotic arrest indicated by Nup49 dispersal (Figure 6A and B). As in the first mitotic arrest, the second arrest was not permanent and cells again exited mitosis in a rapid and regulated manner. Remarkably, cells which had undergone two rounds of mitosis without spindle function transited another interphase and entered a third transient mitotic arrest before again exiting mitosis (Figure 6A and B). Similar repeated cell cycles consisting of several rounds of mitotic arrest also occurred when cells lacking the essential β -tubulin *benA* were germinated in the absence of drug treatment (Supplementary Figure S4), indicating that this phenomenon is not specific to benomyl-treated cells.

In these cell cycles without spindle function each reversible mitotic arrest was mediated by the SAC, as benomyl-treated cells lacking Mad2 function also underwent several cell cycles but did not display the mitotic arrests (Figure 6A). Moreover, the Mad1 and Mad2 SAC proteins translocated to kinetochores during each benomyl-induced mitotic arrest before reassociating with NPCs during each mitotic exit

(Figure 6E; Supplementary Figure S5). This demonstrates that the SAC is activated during each mitotic entry, but then inactivated when cells exit mitosis without spindle function.

Further indicating these repeated cell cycles occur in the normal regulated manner, Cyclin B levels oscillated in a temporally and spatially regulated manner during each cycle (Figure 6C). During the interphases, Cyclin B accumulated within the expanding single nucleus and was partially released to the cytoplasm upon mitotic entry. Throughout each SAC arrest, Cyclin B remained concentrated in the SPB/kinetochore region before it was rapidly degraded during reversal of the SAC arrest without spindles (Figure 6C and D; Supplementary Figure S6). In addition, during these cell cycles, the full mitotic nucleolar disassembly and reassembly cycle occurred normally and was restricted to the periods of SIME when the SAC was inactivated (Supplementary Figure S7). Interestingly, following several cell cycles without spindle function, cells had undergone polarized growth and the majority contained a single large nucleolus within an enlarged nucleus (Supplementary Figure S7D).

Together, these data indicate that in the absence of mitotic spindles, the SAC can be turned on and then turned off in a manner allowing cells to transit multiple cell cycles without fulfilment of the SAC. Importantly, such cells remain viable as they continue slow polarized growth.

SAC inactivation can be uncoupled from Cyclin B degradation and mitotic exit

In the above experiments, inactivation of the SAC is coupled with mitotic exit. Thus, it could be argued that exit from mitosis is causing inactivation of the SAC rather than inactivation of the SAC allowing exit from mitosis. We, therefore, determined if the SAC could be inactivated during an extended SAC arrest in cells which are unable to degrade Cyclin B or exit the mitotic state. To this end, we utilized the temperature-sensitive *bimE7^{APC1}* allele to inactivate the APC/C, thus preventing degradation of Cyclin B and exit from the mitotic state (Osmani *et al*, 1988; Ye *et al*, 1998). Cells germinated at permissive temperature were shifted to the *bimE7^{APC1}* non-permissive temperature of 42°C to inactivate the APC/C and the localization of Mad1 and Nup49 followed during the ensuing mitotic arrest. Following mitotic entry, Mad1 only briefly concentrated at kinetochores (Figure 7A and B), consistent with the normal assembly of a metaphase spindle during a *bimE7^{APC1}* arrest (Osmani *et al*, 2003) and satisfaction of the SAC. However, when microtubules were depolymerized and the *bimE7^{APC1}* mitotic arrest imposed, the localization of Mad1 at kinetochore foci was significantly

Figure 3 Nucleolar disassembly and reassembly occurs when cells return to interphase without spindle function. **(A)** Nup49-mCherry together with the Bop1-GFP nucleolar marker in a benomyl-treated cell exiting mitosis without spindle function at 32°C. Bop1 defines the old nucleolus, which is maintained during the SAC arrest. During exit from the mitotic arrest, the old nucleolus is outside the reforming nucleus defined by Nup49. As cells enter interphase a new nucleolus forms in the nucleus and for a period of time both the old nucleolus and new nucleolus are apparent (frames with an asterisk). The pixel intensity profiles highlight the disassembly of the old nucleolus (o) and reassembly of a new nucleolus (n) within the nucleus defined by Nup49. **(B)** Kymograph representation showing an extended time course of the same experiment. Scale bar, 5 µm.

Figure 4 Cyclin B is degraded in a temporally and spatially regulated normal manner during exit from SAC arrest without spindle function. (A) Cyclin B-GFP and Nup49-mCherry during transition through mitosis in benomyl at 90 s intervals. False colouring (Cyclin B*) depicts relative levels of Cyclin B in the different mitotic locales. Some Cyclin B is released from nuclei at entry into mitosis. The pool of Cyclin B concentrated in the SPB/kinetochore region remains constant during the mitotic arrest but is degraded as cells return to interphase. The inset at bottom shows images collected at 45 s intervals during mitotic exit for the same experiment. (B) Graph showing quantification of Cyclin B levels in the nucleus (green) and cytoplasm (grey) for the experiment shown in (A). The period of the SAC arrest is indicated by low Nup49 nuclear levels (red). Note that degradation of the nuclear pool of Cyclin B and initiation of Nup49 reassembly occur before the cytoplasmic pool of Cyclin B is degraded. An interphase bystander cell continued to accumulate Cyclin B during the same time course. (C) Cyclin B-GFP and the Ndc80-mCherry kinetochore marker during transition through mitosis in benomyl depicted in kymographs to highlight that the kinetochore/SPB focus of Cyclin B is maintained during the SAC arrest. Time-lapse images and quantification for this experiment are shown in Supplementary Figure S3. (D) Normalized nuclear Cyclin B levels during transit through a normal mitosis and during transit through SAC arrest in benomyl (error bars indicate $\pm$ s.d., $n = 4$). Each time series is aligned based on the half maximal intensity for Cyclin B-GFP during SIME. The decrease in nuclear levels of Cyclin B at the start of the time course marks mitotic entry when Cyclin B is partially released to the cytoplasm. Scale bar, 5 μ m.

extended but only for a defined period of time (37.4 ± 8.3 min, $n = 17$; Figure 7C and D). Although these cells remained arrested in mitosis, as indicated by the continued

mitotic dispersal of Nup49 and stabilization of Cyclin B, Mad1 remained dispersed from kinetochores (Figure 7; Supplementary Figure S9). Notably, Cyclin B continued to

Figure 5 SAC inactivation without spindle function. **(A)** In benomyl-treated cells, Mad2-GFP localizes to NPCs during interphase. Upon mitotic entry, Mad2-GFP is released from NPCs and associates with the Ndc80-mCherry kinetochore cluster, indicating that the SAC is active. During return of cells to interphase Mad2 returns to NPCs demonstrating the SAC is turned off. **(B)** Normalized total cellular levels of Mad2 in cells transiting SAC arrest in benomyl (error bars indicate $\pm$ s.d., $n = 5$). Each time series is normalized and aligned for mitotic entry at time = 0. The period of mitotic arrest for the cell in **(A)** is indicated in orange. **(C)** Time-lapse images and pixel intensity profiles showing that Mad1-GFP associated with kinetochores during SAC arrest disperses before Nup49-mCherry reassembly during SIME. Similarly to a normal mitotic exit (De Souza *et al*, 2009), Mad1 then accumulates in the nucleoplasm (arrowheads) before reassociating with NPCs at the nuclear periphery demonstrating the SAC is turned off. **(D)** As for **(B)** but showing total cellular levels of Mad1 (error bars indicate $\pm$ s.d., $n = 6$). Nuclear levels of Nup49 for a typical cell transiting SAC arrest in benomyl are shown in red. **(E)** Bub1-GFP localizes to kinetochores during the period of SAC arrest. **(F)** As for **(B)** but showing total cellular levels of Bub1 (error bars indicate $\pm$ s.d., $n = 4$). Nuclear levels of Nup49 for the experiment shown in **(E)** are indicated in red. Scale bar, 5 μ m.

accumulate in the SPB/kinetochore region even when Mad1 disappeared from kinetochores (Figure 7E and F). The SAC protein Bub1 also associated with kinetochores during a *bimE7^{APC1}* mitotic arrest without spindle function, before it was removed (Figure 7G and H). This occurred even though Bub1 is an APC/C substrate (Qi and Yu, 2007) which is likely stabilized in the absence of APC/C function, perhaps explaining why the duration of Bub1 kinetochore association (78 ± 27 min, $n = 10$) was longer than that of Mad1.

These experiments indicate that the recruitment of SAC proteins to kinetochores in the absence of spindle function is not permanent even when Cyclin B degradation and mitotic exit are prevented. This is consistent with SAC inactivation without normal spindle function being independent of either Cyclin B degradation or mitotic exit.

Protein synthesis is required for timely SAC inactivation without spindle function

Our data indicate that a mechanism exists which turns off the SAC under conditions when it cannot be satisfied

by bipolar spindle formation. One possibility is that in addition to the known levels of SAC regulation, the SAC could also be regulated by a mechanism which would inactivate the SAC after a period of mitotic arrest without correct spindle formation. To test if such a putative mechanism requires protein synthesis, we performed experiments including the translational inhibitor cycloheximide (Cheng *et al*, 2003). The addition of cycloheximide caused benomyl-treated cells to remain in mitosis for over twice as long as similarly treated cells without cycloheximide (Figure 8). Interestingly, the period of mitotic SAC arrest was similar whether cycloheximide was added to cells which had just entered SAC arrest or to cells which had already been arrested by the SAC for over 20 min (Figure 8C). This is consistent with protein synthesis being required towards the end of the normal period of mitotic SAC arrest in order for cells to exit the arrest in a timely manner. These data further support the hypothesis that turning off of the SAC without mitotic spindles is an active cell-cycle regulated process in *A. nidulans*.

Figure 6 Cell-cycle oscillations of SAC activation and inactivation without spindle function. (A) Kymographs following Nup49-GFP in the indicated strains and conditions. The periods of mitotic SAC arrest are indicated by vertical gold lines. (B) Bar graph showing the duration of interphase and mitotic SAC arrest in cells which undergo three consecutive cell cycles. (C) Kymographs showing Nup49-mCherry together with Cyclin B-GFP in a cell treated with benomyl which undergoes three rounds of SAC activation-inactivation. Time-lapse images for this experiment are shown in Supplementary Figure S6. (D) Western blot showing Cyclin B-HA levels following release from a *nimT23^{cdc25}* G2 arrest into benomyl. The bar graph shows the % cells with mitotically dispersed Nup49-mCherry from the same experiment. (E) Mad1-GFP together with Ndc80-mCherry in a cell undergoing two cell cycles without spindle function. Mad1 cycles from NPCs to kinetochores demonstrating that the SAC is active during each mitotic arrest (gold lines). Mad1 then cycles and back to NPCs showing that the SAC is inactive during each interphase. Imaging in this figure was carried out at 32°C. Scale bars, 5 µm.

Nuclear division can occur if microtubule function is returned following SIME

Our data indicate that cyclic activation and then inactivation of the SAC allows cells to continue through multiple cell cycles in a regulated manner without normal spindle function. To gain insight into why cells utilize such a mechanism, we examined if nuclei which had undergone SIME could divide if microtubules were allowed to re-polymerize. Cells were allowed to transit one mitosis in benomyl resulting in nuclei becoming diploid (Figure 9B). Following removal of benomyl, these nuclei transited interphase and entered another mitosis forming apparently normal spindles and frequently divided as diploid nuclei (Figure 9C). In other

benomyl wash out experiments, following several rounds of SIME, enlarged polyploid nuclei formed multiple spindles during mitosis often generating more than two nuclei from the parental polyploid nucleus. The presence of multiple spindles in these wash out experiments indicates that SPB duplication occurred during the cell cycles without microtubule function. These experiments also suggest that at some frequency the large polyploid nuclei which form following several rounds of SIME might be able to undergo a form of reductional mitosis to generate haploid nuclei. These data suggest a rationale, as described below, for why the SAC might be regulated at levels in addition to SAC fulfilment.

Figure 7 SAC inactivation can be uncoupled from mitotic exit. Imaging of *bimE7^{APC1}* strains at the non-permissive temperature of 42°C. Representative time-lapse images of cells entering a *bimE7^{APC1}* mitotic arrest (**A**, **C**, **E**, **G**) and graphs showing relative levels of the indicated proteins from the same experiments (**B**, **D**, **F**, **H**) are shown. Note that nuclear levels of Mad1 and Bub1 are only indicated for the period in which these proteins were located to kinetochores. Nup49-mCherry disassembles from NPCs during mitotic entry and remains dispersed during the *bimE7^{APC1}* mitotic arrest (see also Supplementary Figure S9). (**A**, **B**) In the absence of benomyl, Mad1-GFP is not maintained at kinetochore foci during a *bimE7^{APC1}* mitotic arrest. (**C**, **D**) When cells enter a *bimE7^{APC1}* mitotic arrest in the presence of benomyl Mad1 is maintained at kinetochore foci because spindle formation cannot occur and the SAC is kept active. However, after 37.4 ± 8.3 min ($n = 17$) Mad1 is no longer evident at kinetochore foci, indicating that the SAC has been inactivated even though cells remain in mitosis. (**E**, **F**) Cyclin B remains concentrated in the SPB/kinetochore region when Mad1 disappears from kinetochores, indicating that the SAC has been inactivated. (**G**, **H**) Bub1-GFP is maintained at mitotic kinetochores during a *bimE7^{APC1}* arrest in the presence of benomyl for 78 ± 27 min ($n = 10$). Scale bars, 5 μm.

Discussion

Collectively, our data demonstrate that the SAC is subject to regulation that is independent of bipolar microtubule attachments to kinetochores, the event which leads to SAC fulfilment and silencing during normal mitosis (Musacchio and Salmon, 2007). Most compellingly the hallmark of SAC activation, the kinetochore association of SAC proteins, is reversed without fulfilment of the SAC. Strikingly, however, this situation is not permanent as when these cells cycle through interphase and enter another round of mitosis, the SAC is again able to detect and respond to the lack of spindle

formation and mitotic arrest ensues. This can occur for at least three spindle-independent cell cycles in which each mitotic arrest corresponds with the localization of Mad1 and Mad2 to kinetochores and therefore SAC activation. At the end of each mitotic arrest, Mad1 and Mad2 return to their interphase localization at NPCs, demonstrating that the SAC is inactivated.

During normal mitosis bipolar spindle formation leads to satisfaction and silencing of the SAC, which then rapidly triggers the sequence of events required to reassemble nuclei and entry into interphase. This complex series of events includes the reassembly of NPCs, which facilitates nuclear

Figure 8 Protein synthesis is required for timely exit from SAC arrest. (A) Bar graph showing the average period of mitotic arrest as defined by Nup49–GFP dispersal in benomyl for experiments with or without 40 µg/ml cycloheximide (CHX) (error bars indicate $\pm$ s.d., $n \geq 4$). (B) Kymographs showing typical experiments in which benomyl-treated cells transited mitosis and underwent SIME with or without cycloheximide. (C) Graph showing the average length of mitotic arrest when cycloheximide was added to cells already arrested in mitosis by the SAC for either <10 min or >20 min (error bars indicate $\pm$ s.d., $n \geq 12$). Experiments in this figure were carried out at 32°C. Scale bar, 5 µm.

Figure 9 Following SIME, reversal of microtubule inhibition allows mitosis of a diploid nucleus. (A) A normal mitosis showing GFP–tubulin and Nup49–mCherry. (B) In the same strain, benomyl leads to SAC arrest maintaining the mitotic dispersal of Nup49 until the SAC is silenced and Nup49 returns to NPCs as cells reenter interphase. In the tubulin panel, the imaging and contrast are the same as in (A, C) but in the Tubulin* panels the contrast has been adjusted to visualize the depolymerized tubulin. (C) The same cell in (B) following wash out of benomyl. Microtubules re-polymerize and the cell transits interphase, forms a normal bipolar spindle and undergoes mitosis as a diploid. Experiments in this figure were carried out at 32°C. Scale bar, 5 µm.

transport and chromatin decondensation. In *A. nidulans*, these events are additionally coordinated with a mitotic exit cycle of nucleolar disassembly and reassembly (Ukil *et al*,

2009). We have previously shown that NPC reassembly and the mitotic nucleolar cycle can be uncoupled from nuclear segregation when SAC deficient cells transit mitosis without

spindle function, resulting in SIME (Ukil *et al*, 2009). In this scenario, there is no functional negative feedback informing cells of a problem with the spindle and the correct sequence of mitotic exit events are not delayed. The situation we demonstrate here is distinct in that the SAC is not defective and delays mitotic exit for over 10 times the length of a normal mitosis. SAC proteins are then removed from kinetochores and cells undergo a rapid transition in which the previously mitotically stable Cyclin B is degraded in a sequential-regulated manner similar to what occurs during normal mitosis. This is likely important for orchestrating the correct reassembly of nuclei. For example, similarly to a normal mitosis (De Souza *et al*, 2009), as Cyclin B disappears from the SPB/kinetochore region NPCs begin to reassemble even though Cyclin B is present elsewhere in the cell. Such highly regulated mitotic exit is clearly not simply a reversal of the mitotic state and it is hard to envisage how this could occur if the SAC was still engaged. Instead, together with the localization of SAC proteins, these findings indicate that the SAC is being inactivated without spindle function and that this allows normally regulated nuclear reassembly, albeit without generation of two daughter nuclei.

During progression through mitosis, SAC inactivation removes inhibition of the APC/C which leads to Cyclin B degradation and hence mitotic exit. As Cdk1/Cyclin B activity is important to maintain a mitotic SAC arrest (D'Angiolella *et al*, 2003; Brito and Rieder, 2006; Gavet and Pines, 2010b; Gong and Ferrell, 2010) inactivation of Cdk1/Cyclin B by Cyclin B degradation (Bruto and Rieder, 2006), accumulation of Cdk1 inhibitory proteins (Rieder and Maiato, 2004) or inhibitory Cdk1 tyrosine phosphorylation (Potapova *et al*, 2009) could potentially inactivate the SAC. However, our data indicate that SAC inactivation without spindle formation still occurs when the APC/C component BIME^{APC1} is genetically inactivated leading to a mitotic arrest in which Cyclin B is stabilized. Therefore, SAC inactivation without spindle formation can occur without Cyclin B degradation. In addition, as Cdk1/Cyclin B activity remains elevated when BIME^{APC1} is inactivated (Ye *et al*, 1998), these experiments strongly suggest that Cdk1/Cyclin B inactivation by accumulation of a Cdk1 inhibitory protein or by inhibitory Cdk1 tyrosine phosphorylation is not leading to SAC inactivation without spindle function. Further, cells containing a version of Cdk1 which cannot be tyrosine phosphorylated (Ye *et al*, 1996) undergo a benomyl-induced mitotic arrest of similar duration to wild-type cells before Cyclin B is degraded and cells return to interphase (Supplementary Figure S8). During both the APC/C inactivation and Cdk1F experiments, SAC inactivation occurred even though Cyclin B continued to accumulate in the SPB/kinetochore region during the mitotic arrest (Figure 7E and F; Supplementary Figure S8). Together, these experiments indicate that in *A. nidulans* SAC inactivation without spindle function occurs by a mechanism, which is likely independent of Cdk1/Cyclin B inactivation.

Our data demonstrate that a mechanism exists that regulates the duration of SAC arrest thereby allowing cells to exit mitosis and return to interphase without SAC satisfaction by bipolar spindle formation. Many mechanisms contribute to SAC silencing during mitosis (Hardwick and Shah, 2010) and these might also be involved in SAC silencing when spindle formation cannot occur. However, mechanisms

requiring biorientation (Vanoosthuysen and Hardwick, 2009) or involving translocation of SAC proteins along microtubules (Howell *et al*, 2001) are unlikely to contribute to SAC silencing in the absence of spindle function. Recently, the PP1 phosphatase was shown to contribute to SAC silencing by reversing phosphorylations mediated by SAC kinases (Pinsky *et al*, 2009; Vanoosthuysen and Hardwick, 2009). While dephosphorylation of mitotic substrates is likely important for the return of cells to interphase during SIME, it remains to be seen if PP1 contributes to inactivation of the SAC without spindle function.

Interestingly, we find that timely inactivation of the SAC requires protein synthesis. Although the full inventory of mitotically synthesized proteins is yet to be defined, several mitotic regulators are known to be synthesized during mitosis. In mammalian cells, the APC/C activator Cdc20 is synthesized during mitosis (Nilsson *et al*, 2008). As the SAC targets Cdc20 to inhibit APC/C activation, synthesis of Cdc20 during mitosis could eventually overwhelm the SAC, leading to APC/C activation and mitotic exit. Arguing against this is our finding that SAC inactivation can occur without APC/C function. This also suggests that SAC silencing mechanisms involving proteins which enhance APC/C-mediated ubiquitination (Reddy *et al*, 2007; Stegmeier *et al*, 2007; Garnett *et al*, 2009) are unlikely to contribute to SAC inactivation in *A. nidulans*. In addition to Cdc20, Cyclin B is also synthesized during mitosis in mammalian cells (Sciortino *et al*, 2001; Mena *et al*, 2010), although this synthesis is counterbalanced by APC/C activity (Malureanu *et al*, 2010). This is also likely the case in *A. nidulans* as Cyclin B levels increase during a mitotic arrest when the APC/C is inactivated. However, as Cyclin B does not accumulate during SAC arrest in wild-type cells, it is unlikely that mitotic synthesis of Cyclin B leads to SAC inactivation.

Inactivation of the SAC without spindle function leads to cell-cycle oscillations without mitotic division. In *A. nidulans*, similar oscillations have previously been uncovered upon rapid inactivation of the APC/C using the *bimA1*^{APC3} temperature-sensitive allele (Ye *et al*, 1998). It was hypothesized that the *bimA1*^{APC3} mutation rendered the APC/C less responsive to the accumulation of proteins which are both involved in APC/C activation and also APC/C substrates. Following rapid inactivation of *bimA1*^{APC3}, this then causes delay in a mitotic state while such proteins accumulate to a level that activates the mutant APC/C to allow mitotic exit and repetition of the process (Ye *et al*, 1998). A similar scenario could be in play regarding the cyclic SAC activation–inactivation reported here, but with an undefined protein(s) accumulating to a critical level during SAC arrest to trigger SAC inactivation and exit from mitosis into a new cell cycle. The extended SAC arrest observed when protein synthesis is perturbed is consistent with such a model.

Insights to how the SAC activation–inactivation cycle might be regulated and integrated with other cyclic cell-cycle regulatory systems come from several experimental systems in which cell-cycle oscillations can be uncoupled from cell-cycle progression (Haase *et al*, 2001; McClelland and O'Farrell, 2008; Orlando *et al*, 2008; Lu and Cross, 2010; Manzoni *et al*, 2010). These findings have led to the concept that Cdk1–Cyclin acts as a master oscillator which entrains a series of peripheral oscillators that control individual cell-cycle events (Lu and Cross, 2010). Thus, the SAC activation–inactivation cycle

might be a peripheral oscillator that is entrained to occur at the appropriate times by the Cdk1–Cyclin oscillator. Peripheral oscillators may also feedback on the Cdk1–Cyclin oscillator, resulting in mutual entrainment (Lu and Cross, 2010). This would be highly likely for a potential SAC regulatory oscillator because SAC inactivation leads to Cyclin B degradation allowing cells to transit interphase and reactivate the SAC when they enter the subsequent mitosis. Such a potential mechanism could explain how the cycles of SAC activation and inactivation that occur without spindle function become coupled with the cell cycle. Supporting this idea, preventing Cyclin B degradation and mitotic exit by inactivating the APC/C is not required to inactivate the SAC, but is required for reactivation of the SAC during a prolonged mitotic arrest without spindle function (Supplementary Figure S9). As we discuss below, such a system which allows cells to transit multiple cell cycles without normal spindle function might act as a survival mechanism in *A. nidulans*.

We have shown that deletion of the essential β -tubulin *benA* results in cell cycles with reversible mitotic arrests, essentially recapitulating the effects of benomyl treatment. Other mutations which prevent bipolar spindle formation, including loss of BIMC^{Kinesin5/Eg5} kinesin function (Enos and Morris, 1990) or γ -tubulin (*mipA*) function (Oakley *et al*, 1990), also do not cause a permanent mitotic arrest and result in the formation of polyploid nuclei. Therefore, it is likely that regulated inactivation of the SAC occurs whenever the checkpoint cannot be satisfied by the formation of a functional spindle. We reason that such a mechanism might provide an advantage to *A. nidulans* and other organisms, which undergo polarized growth and contain multiple nuclei within a syncytium. During cyclic activation and inactivation of the SAC, nuclei become polyploid while cells increase their biosynthetic capacity and continue polarized growth. Such polarized growth may allow cells to eventually escape from environmental conditions, which destabilize microtubules. As such cells periodically attempt mitosis, they may eventually be able to undergo successful mitosis when microtubule function returns. Indeed, our benomyl wash out experiments provide strong evidence that this is the case and suggest that for *A. nidulans* SAC inactivation without nuclear segregation is not a lethal event. Instead, SAC inactivation coupled with continued growth enables *A. nidulans* to periodically test its environment for compatibility with mitosis providing a rationale for why it may be advantageous to inactivate the SAC even when mitosis cannot be completed or the SAC fulfilled.

Our findings are of further interest in the light of the variability in the fate of different mammalian cell lines when they are unable to satisfy the SAC (Rieder and Maiato, 2004; Gascoigne and Taylor, 2008). In this regard, it is notable that in the presence of a microtubule poison HeLa cells can undergo several cell cycles in which cells arrest in mitosis but then return to interphase without nuclear division (Gascoigne and Taylor, 2008). These cell cycles of mitotic arrest are essentially identical to what occurs in *A. nidulans*. It will be interesting to define the status of the SAC during these spindle-independent HeLa cell cycles to determine if, as occurs in *A. nidulans*, the SAC is being cyclically activated and then inactivated in a manner distinct from which has hitherto been described for mitotic slippage.

Materials and methods

Strain generation

Strains used are listed in Supplementary Table S1 and were generated using standard techniques as described (Yang *et al*, 2004; Nayak *et al*, 2006; Szewczyk *et al*, 2006; De Souza *et al*, 2009). All GFP or mCherry tagged proteins represent the only version of the protein expressed from its endogenous promoter. Tagged versions of NPC proteins, Ndc80, Mad1, Mad2, Bop1 and Cyclin B used here have been described previously and are functional based on growth tests compared with the wild-type strains and the respective deleted alleles (Osmani *et al*, 2006a; De Souza *et al*, 2009; Ukil *et al*, 2009).

Heterokaryon rescue

To delete *benA* (Anid_01182.1) strain CDS999 (*pyrG89*; *wA3*; *nkua*^{Ku70} Δ ::*argB*; *Nup49-GFP::riboB*^{AF}; (*riboB2 fwA1 nirA14*)) was transformed with a *benA* Δ ::*pyrG*^{AF} targeting cassette and heterokaryons containing nuclei which were either wild-type *benA* and *pyrG89* or *benA* Δ ::*pyrG*^{AF}, identified as described (Osmani *et al*, 2006b). Uninucleate conidiospores were germinated from heterokaryons in media lacking uridine and uracil. Conidiospores which were *benA* Δ ::*pyrG*^{AF} were identified by their ability to undergo polarized growth in the absence of uridine and uracil. In contrast, conidiospores that were wild type for *benA* did not form germ tubes in the absence of uridine and uracil, as the *pyrG89* mutation was not complemented.

Live cell microscopy

For live cell imaging, conidiospores were germinated in minimal media containing 55 mM glucose and 10 mM urea in 35 mm glass-bottom microwell dishes (MatTech). Imaging was at room temperature for Figures 1, 2, 4 and 5. For other figures, imaging was carried out at 32°C except for Figure 7, which was at 42°C. Cells were maintained at 32 or 42°C using a Delta T4 culture dish controller and objective heater (Bioptechs). Imaging was with an Orca-ER camera (Hamamatsu) using a $\times 60$ 1.40 NA Plan Apochromatic objective (Nikon) on a TE300 inverted microscope (Nikon) configured with an Ultraview ER spinning disk confocal system (Perkin-Elmer) controlled by Ultraview software (Perkin-Elmer). For some experiments, an Orca-AG camera (Hamamatsu) and TE2000-U inverted microscope (Nikon) configured with an Ultraview ERS spinning disk confocal system (Perkin-Elmer) was used. Data are displayed as maximal intensity profiles. Benomyl (Sigma) was used at a concentration of 2.4 μ g/ml which is sufficient to depolymerize all microtubules (Horio and Oakley, 2005; De Souza *et al*, 2009).

Temperature shift experiments

Cells containing the temperature-sensitive *nimT23*^{cdc25} mutation (O'Connell *et al*, 1992; Ye *et al*, 1995; De Souza *et al*, 2000) were incubated at 42°C to facilitate G2 arrest and then released to permissive temperature to allow synchronous entry into mitosis. For Figure 1C, short germlings grown on coverslips were arrested at 42°C for 3 h and released to 32°C by exchange to media with or without 2.4 μ g/ml benomyl. Coverslips were fixed at each time point, DAPI stained for DNA (De Souza *et al*, 2009) and the chromosome mitotic index and mitotic dispersal of Nup188–GFP scored. For Figure 6D, 1500 ml cultures were inoculated at 2.5×10^6 conidiospores/ml, grown to a density of 0.2 ml/10 ml at 26°C, and then shifted to 42°C for 4 h to cause G2 arrest. Benomyl was added to a final concentration of 2.4 μ g/ml 15 min before release to 30°C. At each time point, mycelia were harvested and a small sample fixed to determine the mitotic index. Sample preparation and western blotting were as described (De Souza *et al*, 2000).

Strains carrying the *bimE7*^{APC1} mutation were germinated at room temperature, which is permissive for *bimE7*^{APC1}. Germlings were then shifted to the *bimE7*^{APC1} non-permissive temperature of 42°C for 1 h to inactivate the APC/C before imaging at 42°C.

Cycloheximide experiments

Cycloheximide (Sigma) was added to cultures containing 2.4 μ g/ml benomyl by exchange to media containing 40 μ g/ml cycloheximide (Cheng *et al*, 2003) and 2.4 μ g/ml benomyl. Live cell imaging was carried out before and after exchange to determine the effect of cycloheximide on cells which entered mitotic SAC arrest before

media exchange. For control experiments, the exchange was to media containing 2.4 µg/ml benomyl but no cycloheximide.

Quantification and image analysis

Image analysis, quantification, kymograph generation and pixel intensity profiles were carried out using ImageJ freeware (Rasband, WS, ImageJ, US National Institutes of Health, Bethesda, MD, USA, <http://rsb.info.nih.gov/ij/>, 1997–2008). Quantification of Cyclin B, Mad1, Mad2, Bub1, Nup49-GFP and NLS-DsRed was carried out essentially as previously described (Dultz *et al*, 2008). For a given region of interest, the average pixel intensity less the background was calculated and normalized as a percentage of the maximal intensity during the time course. To calculate the nuclear levels of Nup49 the average pixel intensity when Nup49 dispersed during mitosis was subtracted from the average pixel intensity at each time point.

Data points on graphs represent the mean $\pm$ s.d.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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