Introduction

The dynamic properties of the microtubule cytoskeleton underpin a range of cellular functions from cell migration to division. In a large number of eukaryotes, the challenge of generating a malleable and dynamic yet tightly regulated array is facilitated by restraining microtubule nucleation to discrete sites called microtubule-organizing centers (MTOCs). This localized microtubule nucleation from MTOCs can then be tightly controlled by regulating MTOC number and activity to modulate the architecture of the microtubule cytoskeleton. Therefore, understanding MTOC composition and control is central to the understanding of a spectrum of cell functions. The genetic tractability or lifestyle of several microbial systems has meant that many of the core principles of MTOC composition function have been established by the analysis of microbial model systems such as Chlamydomonas reinhardtii, Aspergillus nidulans, Tetrahymena thermophila, Saccharomyces cerevisiae, and the subject of this study, the fission yeast Schizosaccharomyces pombe (Oakley and Oakley, 1989; Hagan and Petersen, 2000; Dutcher, 2003; Jaspersen and Winey, 2004; Kilburn et al., 2007).

Mitotic commitment in S. pombe is accompanied by a dramatic change in the microtubule cytoskeleton, as cytoplasmic microtubules depolymerize and microtubules are nucleated by the two spindle pole bodies (SPBs; McCully and Robinow, 1971; Hagan and Hyams, 1988; Ding et al., 1993, 1997). The SPB is composed of a large cytoplasmic component that is connected to an ill-defined nuclear component by fine striations that run through the nuclear envelope that separates these two domains (Ding et al., 1997). SPB duplication in fission yeast is poorly understood. Genetic analyses and a recent EM study suggest that duplication occurred in G1 phase of the cell cycle (Vardy and Toda, 2000; Uzawa et al., 2004), whereas another EM study suggested that it was in G2 phase (Ding et al., 1997). The clear presence of a bridge structure between the cytoplasmic components of duplicated SPBs suggests that duplication in cut12.1 loss of function mutations. We show that the cut12.1 monopolar phenotype arises from a failure to activate and integrate the new SPB into the nuclear envelope. The activation of the old SPB was frequently delayed, and its integration into the nuclear envelope was defective, resulting in leakage of the nucleoplasm into the cytoplasm through large gaps in the nuclear envelope. We propose that these activation/integration defects arise from a local deficiency in mitosis-promoting factor activation at the new SPB.
fission yeast may well mimic that in budding yeast SPB, in which a half bridge extends from one side of the SPB to form a full bridge that is capped by a satellite structure from which a new SPB is assembled (McCully and Robinow, 1971; Ding et al., 1997; Adams and Kilmartin, 2000). Such conservative duplication is consistent with the ability to differentiate between old and new SPBs with a slow folding fluorescent protein in both budding and fission yeast (Pereira et al., 2001; Grallert et al., 2004).

The nuclear envelope that separates the nuclear and cytoplasmic components fragments upon commitment to mitosis to generate a fenestra in the nuclear membrane (Ding et al., 1997). This localized nuclear envelope breakdown is confined to the region within the SPBs. The two SPB domains then fuse to plug this hole and nucleate microtubules to form the spindle. During anaphase B, the nuclear envelope grows back once more between the two components to completely separate them in the next cycle (Ding et al., 1997).

The SPB components Cut11 and Sad1 contain regions that have the potential to integrate into the nuclear membrane (Hagan and Yanagida, 1995; West et al., 1998). Cut11 is the fission yeast equivalent of the conserved Ndc1 protein that was first identified because it was required for the insertion of the budding yeast SPB into the nuclear envelope (Winey et al., 1993; West et al., 1998; Stavru et al., 2006). Metazoan Ndc1 associates with nuclear pores throughout interphase, whereas budding yeast Ndc1 associates with both the SPBs and nuclear pores throughout the cell cycle (Chial et al., 1998; Stavru et al., 2006). Like other Ndc1 family members, cut11+ encodes six or seven regions that are predicted to constitute membrane-spanning domains (West et al., 1998; Stavru et al., 2006), and it associates with nuclear pores throughout the cell cycle and the SPB in mitosis. EM of a temperature-sensitive cut11 mutant revealed monopolar spindles emanating from a single SPB and a defect in SPB integration into the nuclear membrane. In extreme cases, SPBs failed to insert into the fenestra in the nuclear envelope and fell into the nucleoplasm (West et al., 1998). Sad1 possesses a single trans-membrane–spanning domain family of proteins that anchors centrosomes to nuclear membranes. In extreme cases, SPBs failed to nucleate microtubules and form the spindle. During anaphase B, the nuclear envelope grew back once more between the two components to completely separate them in the next cycle (Ding et al., 1997).

Sad1 is often preferentially enriched on the nonfunctional SPB (Bridge et al., 1998). To understand this phenotype at the level of EM and address the distinction between the functions of Cut11 in promoting the mitotic state of the SPB and Cut11 in physically inserting the SPB into the nuclear envelope. We show that the new SPB appears to be unable to be converted into a mitotic state and insert into the nuclear envelope in cut12.1. The old SPB is activated to nucleate microtubules; however, its integration into the nuclear envelope is defective, resulting in a gapped membrane deformation in the nuclear envelope through which nucleoplasm leaks into the cytoplasm. We discuss why we believe that this SPB insertion defect arises from defective MPF activation at the SPB.

**Results**

**One SPB fails to insert into the nuclear envelope of cut12.1 cells**

We previously established that one of the two SPBs fails to nucleate microtubules when cut12.1 cells are shifted from the permissive temperature of 25°C to the restrictive temperature of 36°C (Bridge et al., 1998). To understand this phenotype in greater detail, we processed cut12.1 cells for EM analysis 100 min after...
cells in a logarithmically growing culture have fluorescent SPBs; however, arresting cell cycle progression by nitrogen starvation to induce a G1 arrest provides sufficient time for the protein in the majority of the SPBs in the population to fold into an actively fluorescing state. The RFP moiety that is associated with SPBs that form after readdition of a nitrogen source to restore cell division does not generally have time to fold so that the old SPB fluoresces, whereas the new one does not (Pereira et al., 2001). In fission yeast, fusion of RFP to the pericentrin homologue Pcp1 (Flory et al., 2002) enables the differentiation of the old and new SPB (Grallert et al., 2004). We used this nitrogen starvation regimen to label the old SPBs in a cut12.1 pcp1.RFP strain before shifting the culture to 36°C. The inclusion of the nmt81.atb2GFP gene fusion in this strain enabled us to monitor microtubules. 3 h after shifting the cells to 36°C, the majority of the culture was processed for immunofluorescence microscopy and stained with antibodies against α-tubulin and Sad1 (Hagan and Yanagida, 1995). The remainder of the culture was observed by live cell microscopy at 36°C.

Immunofluorescence microscopy of cut12.1 cells established that 89% (n = 52) of mitotic cells had monopolar spindles. In 70% of these monopolar spindles, microtubules emanated from one of two distinct Sad1 SPB signals, whereas in the remaining

The old SPB is the active SPB in cut12.1 and cut11.1

SPB duplication is conservative (Grallert et al., 2004), raising the possibility that the failure of one of the SPBs to function at 36°C could arise because of an inherent difference between the old and the new SPB. SPB age in both budding and fission yeast can be determined by following the fluorescence of a fusion between the red fluorescent protein DsRed and a core SPB component. As the RFP moiety takes several hours to fold, very few
same culture emanated from a single red fluorescent Pcp1::RFP signal in all 17 cells examined (Fig. 2 C). We conclude that it is the new SPB of both cut12.1 and cut11.1 mutant cells that fails to insert into the nuclear envelope at 36°C (West et al., 1998). Nuclear/cytoplasmic partitioning persists in wild-type mitosis In addition to the SPB activation defect addressed in the previous sections, EM analysis revealed gapped membrane distortions in the nuclear envelope of cut12.1 cells at 36°C (Fig. 3, A–D). In many instances, the nucleoplasm spilled out through this gapped membrane distortion to mix with the cytoplasm (Fig. 3, A and D). When SPBs were discerned in a section with a hole in the nuclear envelope, the SPB was always adjacent to the hole (Fig. 3, A–C). Although we failed to observe such holes in or any distortion of the nuclear envelopes of wild-type cells (unpublished data), it is possible that the cut12.1 mutant cells had intact nuclear envelopes 30%, the microtubules extended from a single focus of SPB staining (Bridge et al., 1998; unpublished data). In contrast, live cell imaging of the same population indicated that in all cells in which a single focus of SPB staining was discerned, the microtubules were extending from the RFP fluorescent marker on the old SPB (n = 21; Fig. 2 A). We conclude that it is the new SPB that fails to activate and insert into the nuclear envelope in cut12.1 mutants.

Given the strong genetic interactions between cut12.1 and cut11.1 mutants (West et al., 1998), we asked whether there was a similar association between the competence of the SPB to nucleate microtubules and SPB age in cut11.1 mutants. Sad1/tubulin immunofluorescence analysis of the cut11.1 nmt81::GFPatb2 pcp1::RFP cultures used for live cell analysis established that 65.5% (n = 61) of the cells with monopolar spindles had two clear Sad1 foci, with the microtubule nucleating SPB often recruiting less Sad1 than the inactive one (Fig. 2 B). The monopolar GFP tubulin signal in live cell imaging of the same culture emanated from a single red fluorescent Pcp1::RFP signal in all 17 cells examined (Fig. 2 C). We conclude that it is the new SPB of both cut12.1 and cut11.1 mutant cells that fails to insert into the nuclear envelope at 36°C (West et al., 1998).

Nuclear/cytoplasmic partitioning persists in wild-type mitosis In addition to the SPB activation defect addressed in the previous sections, EM analysis revealed gapped membrane distortions in the nuclear envelope of cut12.1 cells at 36°C (Fig. 3, A–D). In many instances, the nucleoplasm spilled out through this gapped membrane distortion to mix with the cytoplasm (Fig. 3, A and D). When SPBs were discerned in a section with a hole in the nuclear envelope, the SPB was always adjacent to the hole (Fig. 3, A–C). Although we failed to observe such holes in or any distortion of the nuclear envelopes of wild-type cells (unpublished data), it is possible that the cut12.1 mutant cells had intact nuclear envelopes.
before fixation but that membrane disruption was induced during processing. Therefore, we decided to study the integrity of the nuclear envelope. We used a well-characterized nuclear marker, the NLS-GFP–β-galactosidase (β-Gal) fusion protein (Yoshida and Sazer, 2004). Live cell imaging of wild-type cells that expressed a red fluorescent tubulin fusion protein (pRep81Cherry-tubulin) confirmed that the fluorescent signal of this NLS-GFP–β-Gal remained constrained within the nuclear envelope throughout the cell cycle of wild-type cells (Fig. 4A and Video 1; Yoshida and Sazer, 2004).

### Nuclear integrity is maintained when a failure to interdigitate the two half spindles arrests mitotic progression

To determine whether NLS-GFP–β-Gal was retained within the nucleus during a prolonged mitotic arrest, we blocked mitotic progression by introduction of the cut7.24 mutation into the NLS-GFP–β-Gal pRep81Cherry-tubulin background. As cut7 encodes S. pombe kinesin 5, it is required for the interdigitation of the two half spindles (Hagan and Yanagida, 1990). Loss of Cut7 function activates the spindle assembly checkpoint to block mitotic progression (Kim et al., 1998). To confirm that the integrity of the nuclear envelope was not compromised by cut7.24 mutation, we processed cells that had been arrested at the restrictive temperature for 3 h for EM. Serial sections through the nuclei of 10 cells revealed that both SPBs had integrated into the nuclear envelope and that the two half spindles had indeed failed to interdigitate in every case (Fig. 5 and Fig. S3). Consistently, the NLS-GFP–β-Gal fusion protein was retained within the nucleus throughout the mitotic arrest until the cells leaked through the cell cycle arrest and the cytokinetic ring randomly cleaved the nucleus in two in a cut phenotype (Fig. 4B and Video 2).

### Nuclear integrity is compromised in cut12.1 and cut11.1 arrests

As the trans-membrane motif bearing protein Cut11 is required for the integration of the SPB into the nuclear envelope (West et al., 1998), we anticipated that the cut11.1 mutation would affect the integrity of the nuclear envelope and so would permit efflux of the NLS-GFP–β-Gal from the nucleus as they unsuccessfully attempted to integrate the SPB into their nuclear envelope. In line with this prediction, a burst of GFP fluorescence appeared throughout the cytoplasm when the red fluorescent interphase microtubules depolymerized to mark commitment to mitosis (Fig. 4C and Video 3). The reaccumulation of this marker within the nuclei after leakage is likely caused by the presence of
our ability to identify cells in which an active SPB had apparently lost its association with the membrane completely and fallen into the middle of the nucleus (Fig. 3 E) and the proximity of the SPB to these gaps in the membrane (Fig. 3, A–C).

The visualization of microtubules in the cut12.1, cut11.1, and cut7.24 backgrounds to monitor the timing of commitment to mitosis revealed an additional defect in SPB activation during mitotic commitment in cut11.1 and cut12.1 cells. The dissolution of cytoplasmic interphase microtubules is either coincident with or rapidly followed by (within 4 min) the nucleation of spindle microtubules from the mitotic SPBs when wild-type or the nuclear localization signal that will drive its reimport into nuclei once the nuclear envelope is resealed and a RAN (ras-related nuclear protein) GTP gradient is reestablished.

Having established that the NLS-GFP–β-Gal marker is retained within the nuclei of cells that maintain the integrity of the nuclear envelope during mitosis but not in those in which integrity is compromised, we monitored NLS-GFP–β-Gal fluorescence in cut12.1 cells at 36°C. 52 of the 54 cells observed formed a monopolar spindle with microtubules emanating from a single point within the cell. The formation of each of these monopolar spindles was accompanied by a brief efflux of GFP signal from the nuclei (Fig. 4 D and Video 4). Thus, the compromised Cut12 function of the cut12.1 allele disrupted the integrity of the nuclear envelope as cells attempted to form a spindle. This would explain our ability to identify cells in which an active SPB had apparently lost its association with the membrane completely and fallen into the middle of the nucleus (Fig. 3 E) and the proximity of the SPB to these gaps in the membrane (Fig. 3, A–C).

**cut12.1 and cut11.1 mutations delay SPB activation during commitment to mitosis**

The visualization of microtubules in the cut12.1, cut11.1, and cut7.24 backgrounds to monitor the timing of commitment to mitosis revealed an additional defect in SPB activation during mitotic commitment in cut11.1 and cut12.1 cells. The dissolution of cytoplasmic interphase microtubules is either coincident with or rapidly followed by (within 4 min) the nucleation of spindle microtubules from the mitotic SPBs when wild-type or
Levels of Cdc25 that suppress the spindle formation defect of cut12.1 do not suppress the nuclear integrity defect

Because the efflux of the NLS-GFP–β-Gal marker that accompanies the defective mitosis in cut12.1 and cut11.1 is an uncharacterized phenotype for these mutations, we asked whether the elevation of cdc25 levels suppressed the NLS-GFP–β-Gal efflux. To this end, we monitored wild-type, cut12.1, and cut11.1 cells harboring the cdc25.d1 NLS-GFP–β-Gal transgenes by live cell imaging at the restrictive temperature of 36°C (Fig. 7 C). There was no significant reduction in the frequency or duration of leakage of nucleoplasm from the nuclei of cut11.1 cells (Fig. 7, C–E). Surprisingly, in 50% (n = 79) of the cut12.1 cells that successfully completed mitosis, an efflux of the NLS-GFP–β-Gal marker accompanied mitotic commitment (Fig. 7, C and D). However, notably, this leakage was significantly briefer than in cut12.1 single mutant (Fig. 7 E). These data indicate that the level of Cdc25 activity required to suppress the SPB activation and insertion defect of the new SPB is below the threshold required to ensure a high fidelity of integration into the nuclear envelope. Thus, two distinct stages of nuclear remodeling can be differentiated by the cut12.1 mutation: SPB activation and correct integration of the activated SPBs within the nuclear envelope.

Discussion

We show that incubation of cells bearing the temperature-sensitive cut12.1 mutation at 36°C blocked both the activation and insertion of the new SPB into the nuclear envelope and compromised the fidelity with which the active old SPB inserted (Fig. 8).
The defective insertion of the old SPB was associated with leakage of nucleoplasm into the cytoplasm through gapped membrane distortions in the nuclear envelope. When an SPB was seen in the same electron microscopic section as a gap in the nuclear envelope, it was adjacent to this hole, suggesting that it was the defective attempt at SPB insertion that generated the breach in integrity. Consistently, we observed instances in which the active old SPB resided within the nucleoplasm, suggesting that it had fallen through a hole generated during the abortive attempt at spindle formation. The gap in the membrane seen in cut12.1

Figure 6. Activation of the old SPB is delayed in cut12.1 and cut11.1 mutants. (A–D) Wild-type (Wt) nmt81.atb2GFP (A; Video 1), cut7.24 nmt81.atb2GFP (B; Video 2), cut12.1 nmt81.atb2GFP (C; Video 3), or cut11.1 nmt81.atb2GFP (D; Video 4) cells were grown at 25°C and mounted for live imaging at 36°C. Images were captured every 4 min, and the time between the dissolution of the interphase microtubules and nucleation of mitotic microtubules was scored and plotted in E. (E) Numbers inside the circles correspond to the number of individual cells that spent this specific time interval (y axis) without microtubules (MT). For each strain, n = 15. Bar, 10 µm.
Figure 7. cut11.1 lethality is not rescued by boosting Cdc25. (A) Serial dilutions (1 in 5) of the strains indicated were plated at the permissive (left) and restrictive temperature (right). In contrast to the rescue of cut12.1 by stabilizing cdc25 mRNA (cut12.1 cdc25.d1), cut11.1 lethality at 36°C is not suppressed in the same conditions (cut11.1 cdc25.d1). (B) Frequency of cells that display a defective karyokinesis, including the cut phenotype and asymmetric or no segregation of the nucleus (n = 50). (C) Indicated strains bearing NLS-GFP-β-Gal marker were grown at 25°C and mounted at 36°C for live cell imaging. Stacks of images were taken every 4 min to monitor karyokinesis. Both cdc25.d1 control and cdc25.d1 cut12.1 double mutant are able to divide the nucleus successfully, whereas in the majority of cdc25.d1 cut11.1 cells, GFP signal leaks into the cytoplasm in mitosis. Note that the cells are very small because of the elevated levels of Cdc25. (D) Frequency of cells that show leakage at any point in the process of nuclear division (n = 50). (E) Mean duration of the nucleoplasm efflux in cells that were assessed in D (n = 40). Error bars show standard deviation. wt, wild type. Bar, 2 µm.
cells greatly exceeded that of the fenestra that normally forms in the nuclear envelope for SPB insertion, suggesting that the attempt to integrate is followed by a progressive disruption of membrane integrity.

These phenotypes are highly reminiscent of those arising from mutation of the cut11 gene. cut11 encodes a protein with six or seven predicted trans-membrane domains that associate with the nuclear pores in interphase and both the nuclear pores and SPBs of mitotic cells (West et al., 1998; Stavru et al., 2006). It is the functional homologue of the budding yeast Ndc1p protein (Winey et al., 1993; West et al., 1998). In cut11 cells, the old SPB nucleates microtubules, whereas the new SPB remains inactive. This old SPB can fail to associate with the nuclear envelope and, like the cut12 cells, can fall into the heart of the nucleoplasm (West et al., 1998). Sad1 often preferentially associated with the inactive SPB in both cut11 and cut12 mutants.

Despite these striking similarities between the cut11 and cut12 mutant phenotypes, the genetic relationship of cut11 with an integrated version of the cdc25.d1 allele that elevates the levels of Cdc25 to promote premature mitosis (Daga and Jimenez, 1999; Tallada et al., 2007) was different. Elevation of Cdc25 levels to boost MPF activity promoted the activation of the otherwise inactive new SPB in the majority of cut12 cells. In contrast, the presence of cdc25.d1 had no impact on the spindle formation or abnormal mitotic or temperature-sensitive lethality phenotypes of the cut11 mutation. Although this distinction is based on the analysis of a single allele, it would be consistent with the view that Cut11 is a physical component of the SPB that is required to generate an interface through which the proteinaceous SPB becomes an integral part of the nuclear envelope, whereas Cut12 is a regulatory protein that is required for SPB activation and the control of mitotic commitment.

Figure 8. A model of SPB insertion in wild-type, cut12.1, and cut11.1 cells. (A) A cartoon representing SPB integration into the nuclear envelope of wild-type cells. Note the modification that occurs during the maturation of the new SPB into an old SPB that accompanies transit through mitosis or from one cell cycle to the next (Grallert et al., 2004). (B) A cartoon representing the defective SPB integration of cut12.1 cells.
Because MPF is recruited to the SPBs of late G2 cells (Alfa et al., 1990; Decottignies et al., 2001), we propose that the compromised Cut12 function of cut12.1 cells reduced the activation of MPF on the new SPB below the critical threshold required for SPB integration into the membrane (Figs. 8 and 9). The elevation of global Cdc25 levels via the introduction of the cdc25.d1 allele then raised global MPF activity to drive the local level at the SPB back above the threshold for, and so restored, SPB integration. The morphology of the nuclear envelope underlying the cytoplasmic SPB component of the inactive SPB in cut12.1 mutants supports this view of defective MPF at the new SPB, as it retains the differentiated appearance that is the hallmark of an interphase SPB (Ding et al., 1997).

The restriction of the SPB activation/integration defect to one of the two SPBs suggests that Cut12 acts in cis in a local fashion on individual SPBs to promote MPF activation at individual SPBs, which drives integration of the SPB into the membrane (Fig. 9, red arrows), whereas the ability of the dominant cut12.s11 mutation to permit division of cdc25.Δ and cdc25.22 cells in a polo-dependent fashion (Hudson et al., 1990, 1991; Maclver et al., 2003) suggests that these events on the SPB are amplified and harnessed by different pathways to influence the global control of mitotic commitment (Fig. 9, green arrows). Such global control from a defined structure echoes the mechanism by which a single unattached kinetochore arrests cell cycle progression (Musacchio and Salmon, 2007). This proposed importance of SPB-associated events in regulating mitotic commitment in fission yeast is supported by the observation that constitutively active Plo1 kinase is only able to suppress cdc25.22 mutants when it is able to associate with the SPB (Petersen and Hagan, 2005).

Elevating Cdc25 levels suppressed the efflux of the NLS-GFP–β-Gal nuclear marker to a lesser degree than it suppressed the SPB activation and insertion defect. This suggests that SPB activation is regulated in a manner that is distinct from the controls that govern membrane insertion. A further striking feature of the efflux of the GFP marker was the speed with which the GFP nuclear marker reaccumulated in the nuclei after efflux in cut12.1 and cut11.1 cells. Current technologies have not allowed us to address the duration of the different phases of rupture, repair (assuming that there is repair), and reimport. It is possible that the breach of the nuclear envelope is very transient, after which it takes 40 min to reimport the entire population of NLS-GFP–β-Gal back into the nucleus, or that the rupture persists but that the RAN GTP system can establish a gradient that is sufficient to direct import even though membrane integrity is compromised at one point. Given the size of the breach to the nuclear envelope recorded in images such as that in Fig. 3 D, it is hard to imagine how the nuclear envelope could reseal. In this respect, it may be important to consider the distinctions between the point at which cut7 and cut12 mutant cells lose viability as they undergo a defective mitosis. The death of cut7.446 cells coincides with the inability to form the mitotic spindle, whereas the viability of cut12.1 cells has already fallen to 60% of its starting value by the time that the first spindle appears (Hagan and Yanagida, 1990, Bridge et al., 1998). This may indicate that although the NLS-GFP–β-Gal reaccumulates within the nuclei after efflux, there is lasting damage to nuclear integrity. This would favor the interpretation that the RAN GTP system can accommodate a moderate perturbation of nuclear cytoplasmic partitioning.

Sad1 often preferentially associates with the inactive new SPB in both cut11.1 and cut12.1 mutant cells (Fig. 2 B; Bridge et al., 1998). This asymmetry reflects the membrane association of the two SPBs. Like all SUN domain proteins that anchor MTOCs to the nuclear envelope (Tzur et al., 2006; Wilhelmsen et al., 2006), the trans-membrane domain of Sad1 gives it an intrinsic affinity for the nuclear envelope (Hagan and Yanagida, 1995). If this affinity were to be greater than its affinity for the SPB, Sad1 would preferentially partition with the inactive, membrane-associated rather than the active, membrane-free SPB. In contrast to cut12.1, a complete loss of Cut12 function arising from deletion of the cut12+ gene results in two equally staining Sad1 foci with microtubules emanating from another site within the heart of the nucleoplasm (Bridge et al., 1998). It is tempting to speculate that the complete absence of Cut12 protein from these germinating spores blocks the dissolution of the nuclear envelope within both SPBs, leaving the nuclear component to become active, nucleate microtubules, and drift away from the sites at which the cytoplasmic domains remain associated with the nuclear envelope. The asynchrony of germination of spores makes it impractical to address this phenotype by

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**Figure 9.** A model depicting the proposed distinctions between local and global MPF activation in wild-type and cut12.1 cells. The cartoons show the two types of signaling event that we believe the cut12.1 mutant phenotype and relationship between cut12+ and cdc25+ have revealed. (A) In addition to a global signaling event that commits the cell to mitosis (green), we propose that local activation of MPF (red) is required on individual SPBs to promote the insertion of these SPBs into the nuclear envelope (gray lines). (B) In cut12.1 mutants, the old (orange) but not the new SPB (gray) is competent to promote local activation. It is unclear at present whether the signal for global commitment in cut12.1 mutant cells comes from the old or new SPB, so this signal is omitted from this panel.
EM of germinating cut12.d1 spores; however, it maybe possible to address this in the future, should conditional mutants that mimic this phenotype arise in future analyses of Cut12 function.

Interpreting the significance of the appearance of monopolar spindles in the first cell division after G2 cells are shifted to the restrictive temperature relies on a concrete understanding of when SPB duplication occurs. If SPB duplication occurs in G1 phase, the data suggest that there is an intrinsic difference in the response of the two SPBs to the compromised Cut12 activity. However, if the SPB duplicates in G2 phase, it may simply be that the SPB that forms after the shift to the restrictive temperature failed to incorporate sufficient functional Cut12 and so was unable to function, whereas the molecular interactions within the older SPB enabled it to retain function. The fact that Cut11 only associates with the SPB upon mitotic commitment and yet cut11 mutants have a monopolar phenotype (West et al., 1998) argues that it arises from an intrinsic difference in the potential of the SPBs to cope with alterations in SPB function. This view is supported by our demonstration of an inherent functional distinction between the two anaphase B SPBs, as the septum initiation network promotes septation from the new, not the old, SPB (Sohrmann et al., 1998; Grallert et al., 2004). A recent study suggests that these distinctions are established by G2 phase, as the KASH (Klarsicht/ANC-1/Syne-1 homology) domain protein Kms2 associated with only one of the two Sad11-staining SPBs in imal1Δ mutants (King et al., 2008). Furthermore, Fin1 association with SPBs shows that, just like metazoan centrioles (Vorobjev and Chentsov, 1982), a novice S. pombe SPB actually takes more than one cell cycle to fully mature (Grallert et al., 2004). In the model, to account for this SPB maturation, we proposed that passage through mitosis or G1 modifies one of the two SPBs such that its behavior in the next cell cycle is altered (Grallert et al., 2004). In other words, exposure to a mitotic environment or transit through START could modify the SPB such that its threshold requirement for Cut12 function is lower than that of the neighboring new SPB that is yet to experience these modifications (Fig. 8 A). Whatever the explanation, it is clear that there are inherent differences between the two SPBs in each cell, and it appears that Cut12 function is sensitive to these distinctions.

Live cell imaging also revealed a variable delay between the dissolution of interphase microtubules and the formation of the mitotic spindle when the integrity of the nuclear envelope was compromised by either the cut11 or cut12 mutations. This delay may arise from altered partitioning of key cell cycle regulators between the cytoplasm and nucleoplasm or the leakage of critical spindle components, such as tubulin from the mitotic nuclei through the gapped membrane distortions that accompany the defective mitoses in these mutants. In conclusion, we propose that Cut12 acts in cis to promote the changes that drive the integration and activation of SPBs into the nuclear envelope at the start of mitosis and globally to control the rate at which cells execute the global decision to commit to mitosis.

Materials and methods

Cell growth

Growth and maintenance of strains were performed according to Moreno et al. (1991). The strains used in this study are listed in Table S1. Strains IH741 and IH5253 were gifts from T. Toda (Cancer Research UK London Research Institute, London, England, UK) and S. Sazer (Baylor College of Medicine, Houston, TX), respectively.

EM

Plunge freeze substitution fixation and subsequent preparation of cut7.24 cells for EM were performed according to Konbe et al. (1989). 3 h after shift of an early-log phase culture from 25°C to 36°C, cell suspensions were dotted onto filter paper and then sandwiched between two copper grids. Fixation was achieved by plunging into liquid propane. Subsequent substitution with anhydrous acetone containing 2% OsO4 and 0.05% uranyl acetate took 48 h at ~79°C. The temperature was then increased to ~20°C for 2 h followed by 1.5 h at 4°C before incubation at room temperature for 30 min. After four washes in anhydrous acetone, step-wise infiltration with Epon-Araldite led to a final infiltration of 100%. Samples were sandwiched between Teflon-coated glass and polymerized at 70°C for 48 h. Blocks were trimmed, and serial sections were prepared with a diamond knife and mounted on Formvar-coated single-slot grids for staining with uranyl acetate and lead citrate. Images were taken with an electron microscope (100 CX; JEOL) operated at 100 kW.

High pressure freeze substitution of cut12.1 cells was performed as described previously (Murray, 2008). cut12.1 cells were synchronized with respect to cell cycle progression by centrifugal elutriation according to Creenor and Mitchison (1979) using an elutriator rotor (JE-5.0; Beckman Coulter). After filtration onto 0.45-µm membrane filters (Millipore), cells were loaded into interlocking brass hats (Swiss Precision) and fixed by high pressure freezing with a liquid propane freezer (HPM100; Bal-Tec). Freeze substitution into 2% OsO4 + 0.1% uranyl acetate in anhydrous acetone was conducted using an automatic freeze substitution chamber unit (AFS; Leica) at 90°C for 72 h with a 5°C/h slope to raise the temperature to ~20°C, at which point cells were held for 2 h before a final increase to 4°C for 4 h at a rate of 5°C/h. After infiltration with Spurr’s resin, blocks were trimmed, and serial sections were prepared with a diamond knife, mounted on Formvar/carbon-coated single-slot grids, stained with Reynolds’s solution for 5 min, and imaged on a transmission electron microscope (model 1220; JEOL) at 80 kW.

Live cell microscopy

Live cell image capture and analysis were performed according to Grallert et al. (2006). Cells were grown in supplemented, filter-sterilized EM2 at 25°C before being mounted on an FC2 chamber (Biotechn) coated with soybean lectin (Sigma-Aldrich). The chamber was mounted onto a DeltaVision Spectris system (Applied Precision, LLC) that uses a microscope (IX71; Olympus). The Precision Control Weather Station heating chamber (Applied Precision, LLC) surrounding the stage and the FC2 chamber were set at 36°C as was the objective heating collar (Biotechn) on the 100× NA 1.45 objective (Carl Zeiss, Inc.) that was used to capture images. This led to a temperature shift of the cells from 25 to 36°C in 2 min after mounting on the microscope. Image capture started once the focus had stabilized (45 min). 20 0.3-µm consecutive slices were captured every 4 min with a camera (Cascade II 512b; Photometrix) using the SoftWoRx (Applied Precision, LLC) image capture program. The 2 series was then compressed to a maximal projection in Imaris software (Bitplane). Individual panels were extracted into Photoshop (Adobe) to generate the panels for the figures.

Immunofluorescence microscopy

Immunofluorescence microscopy was performed according to Hagan and Yanagida (1995), in which the TAT1 anti-tubulin monoclonal antibody (Woods et al., 1989) that was used at a dilution of 1 in 80 was detected with FITC-conjugated goat anti–mouse IgG (Sigma-Aldrich) and the cAP5 anti-Sad1 antibody that was used at a dilution of 1 in 25 was detected with CY3-conjugated goat anti–rabbit IgG antibody (Sigma-Aldrich). Samples were imaged and processed as for live imaging with the exception that cells were mounted on soybean lectin–coated, standard 18 x 18-mm 1.5 cover slips and no heating of the sample was used. pRep81Cherry-tubulin was a gift from K. Tanaka (University of Leicester, Leicester, England, UK).

Online supplemental material

Fig. S1 shows further detail of the cell shown in Fig. 1. Fig. S2 shows a second example of electron microscopic sections from a cut12.1 cell in which one SPB is active and nucleating microtubules, whereas the structure of the second SPB is highly reminiscent of the structure of a wild-type interphase SPB in which the cytoplasmic component associates with the outside of the nuclear envelope. Fig. S3 shows a second example of EM analysis of a cut7.24 cell to show the two active SPBs inserted into a continuous nuclear envelope. Videos 1 and 2 show the retention of the NLS-GFP–Gal nuclear integrity marker in wild-type and cut7.24 mutants, respectively.
Videos 3 and 4 show the transient efflux of this marker as cut11.1 and cut12.1 cells, respectively, transit mitosis. Table S1 lists the strains used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200812108/DC1.

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