Recruitment of NIMA kinase shows that maturation of the \textit{S. pombe} spindle-pole body occurs over consecutive cell cycles and reveals a role for NIMA in modulating SIN activity

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Mitotic exit in \textit{Saccharomyces cerevisiae} and septation in \textit{Schizosaccharomyces pombe} are regulated by a conserved signaling network called the mitotic exit and septum initiation networks (SIN), respectively. The network is active on one of the two anaphase B spindle-pole bodies (SPBs). Whereas the inherent asymmetry of growth by budding accounts for elements of the asymmetry in \textit{S. cerevisiae}, it has been unclear how, or why, the pathway is asymmetric in \textit{S. pombe}. We show that elements of SPB duplication in \textit{S. pombe} are conservative, and that the SIN is active on the new SPB. SIN association with the new SPB persists after transient depolymerization of microtubules. The localization of the NIMA-related kinase, Fin1, reveals further complexity in SPB inheritance. Fin1 associates with the SPB bearing the older components in all cells and with the “new” SPB in half of the population. Fin1 only binds the new SPB when this new SPB has arisen from the duplication of an SPB that is two or more cycles old. Thus, each of the four SPBs generated over two consecutive cell cycles are different, because they have distinct fates in the next cell cycle. Fin1 binds the SPB once the SIN is active and the association requires the SIN inhibitors Byr4 and Cdc16. Fin1 physically associates with Byr4. Compromising Fin1 function leads to SIN activation on both anaphase B SPBs and promotes septation, indicating that Fin1 restrains SIN activity on the old SPB.

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Commitment to mitosis is promoted by the activation of the Cdk1/cyclinB protein kinase complex called MPF [Nurse 1990]. MPF activation promotes a cascade of phosphorylation events that results in the formation of the mitotic spindle. Whereas some of these are due to direct phosphorylation of key substrates by MPF itself, for example, Blangy et al. [1995], the majority arise from the activity of kinases that are activated by MPF. These kinases include polo, aurora, and NIMA-related kinases [Nigg 2001].

Lower eukaryotes characterized to date contain a single NIMA-related kinase, whereas a family of NIMA-related kinases or Neks exist in higher eukaryotes [O’Connell et al. 2003]. \textit{Aspergillus nidulans} NIMA mutants arrest cell cycle progression in G2 with active MPF [Ye et al. 1995]. In contrast, deletion of the analogous kinase from either budding or fission yeast is not lethal [Jones and Rosamond 1990; Krien et al. 1998]. However, it does delay commitment to mitosis in fission yeast [Krien et al. 1998]. Despite the ability of cells to live without these genes, the phenotype of recessive conditional mutants in fission yeast suggests that fission yeast NIMA, Fin1, plays an important role in modulating spindle formation and spindle-pole maturation [Grallert and Hagan 2002]. The spindle formation phenotype of conditional \textit{fin1} mutants is reminiscent of the consequences of manipulation of one of the human and \textit{Xenopus} NIMA homologs, Nek2. Nek2 localizes to the centrosome and is required for the maturation of centrioles in \textit{Xenopus} egg extracts [Fry 2002]. Overproduction of Nek2 or injection of Nek2 antibodies affects the cohesion between centrioles. This is reminiscent of the requirement for the function of the \textit{Chlamydomonas} NIMA-related kinase, FA2, in breaking the connection between the basal bodies and the flagella [Mahjoub et al. 2002]. Mitotic functions, some at centrosomes, are now being ascribed to more Nek molecules [Belham et al. 2003].
2003; Swenson et al. 2003), emphasizing the important role played by NIMA kinases in cell cycle control and the function of microtubule organizing centers.

The association of NIMA kinases with the spindle poles in diverse systems resembles the behavior of the more intensively studied mitotic kinases, polo and aurora, which are also found on spindle poles [Nigg 2001]. Studies from fission yeast and humans suggest that the activation of polo kinase on the spindle pole is a critical step in regulating the autocalytic feedback loop that promotes full-scale activation of MPF to drive commitment to mitosis [Jackman et al. 2003; Madlver et al. 2003]. This amplification loop highlights the interplay between different mitotic kinases, as both the recruitment of polo kinase to the spindle pole and the activity of this feedback loop in fission yeast are modulated by the NIMA kinase Fin1 (Grallert and Hagan 2002).

Events at the spindle pole also play critical roles in controlling mitotic exit [for review of SIN and MEN, see Gard and Amon 2001; Simanis 2003]. Mitotic exit is controlled by the activity of a conserved regulatory network called the mitotic exit network [MEN] in budding yeast and the septum initiation network [SIN] in fission yeast. The critical events in modulating MEN/SIN activity occur on the spindle-pole body [SPB]. In budding yeast, they culminate in the release of the protein phosphatase Cdc14 from the nucleolus, which then dephosphorylates multiple targets, including those that were phosphorylated by Cdk1/cyclin B complexes at earlier stages of mitosis to promote mitotic exit. Although the precise details may differ, an analogous phosphatase exists in fission yeast, and its release from the nucleolus is also modulated by SIN activity (Bardin and Amon 2001; Cueille et al. 2001; Trautmann et al. 2001; Simanis 2003).

The SIN is anchored to the SPB via two scaffold proteins, Cdc11 and Sid4. SIN activity is determined by the status of a small G protein of the ras superfamily, Spg1. SPB-associated Spg1 is in a GDP-bound, inactive state throughout interphase. Upon mitotic commitment, there is a switch in the nucleotide status of the Spg1 associated with both SPBs, and it is in a GTP-bound state on both poles. This persists until early anaphase B, when the Spg1 on one SPB switches to a GDP-bound state. As this coincides with the formation of a GAP complex for Spg1 on this SPB, it is assumed that the GTP is hydrolyzed to create GDP Spg1. This effectively turns off the SIN on one of the two anaphase B SPBs. SIN activity persists on the other SPB until cytokinesis is complete. Active Spg1 binds the protein kinase Cdc7, and this activates the downstream components of the network. Analysis of both the MEN and the SIN highlight the importance of feedback loops in this conserved network. Components that are placed later in the network appear to feed back to either activate or inhibit components that map by genetics and cytology to earlier stages of the network [Pereira et al. 2002; Krapp et al. 2003; for review, see Simanis 2003].

The activation of the MEN/SIN on one of the two anaphase B SPBs highlights a distinction between two, apparently morphologically identical, SPBs. In budding yeast, the identity of the SPB that will host the active MEN is determined by the location of the SPB relative to the mother/bud axis within this highly asymmetric cell [Pereira et al. 2001]. The SPB that faces the daughter cell binds the MEN inhibitors Bub2 and Bfa1 [Pereira et al. 2001], whereas the downstream effector Cdc15 is only seen on the SPB in the mother cell prior to mitotic exit [Menssen et al. 2001]. The activation of the MEN on the SPB in the daughter cell appears to be modulated by the interaction of astral microtubules with the bud neck and cortex [for review, see Simanis 2003].

In contrast to budding yeast, fission yeast cells divide symmetrically, and yet the SIN is still located asymmetrically on one of the two SPBs. One obvious feature that could generate asymmetric markers within a fission yeast cell is the fact that one end was produced by cytokinesis at the end of the previous cell cycle, whereas the other persists from the previous cycle. As both ends of the cell have a different history, they could send different signals to the SPBs to direct asymmetric activation of the SIN. However, the SIN-active SPB faces these two ends with equal frequency [M. Sohrmann, A. Grallert, I. Hagan, and V. Simanis, unpubl.]. In this work, we show that the fission yeast SPB takes more than one cell cycle to mature, so that each of the four SPBs of two daughter cells has a distinct fate in the next cell cycle. This aspect of SPB behavior was revealed by the age dependence of the ability of the SPB to recruit the kinase Fin1. We also show that it is the new SPB that hosts the active SIN in anaphase B and that SIN inhibition on the old SPB is mediated, in part, by the recruitment of Fin1 to that SPB by the SIN GAP component Byr4.

Results

Fin1 associates with the mitotic apparatus in a stage-dependent manner

Two polyclonal antibodies were raised against the non-catalytic, C-terminal 441 amino acids of the Fin1. As affinity purification of antibodies from both sera gave the same patterns on Western blots and immunolocalization (data not shown), results with one, which we refer to as αFIN1, are described here. αFIN1 antibodies recognized a single 76-kD band in wild-type extracts [Fig. 1A, lane 1]. This band was absent from a fin1Δ strain from which the fin1Δ gene had been deleted [Fig. 1A, lane 2] and an extra band at 46 kD was seen in wild-type cells expressing the 441 C-terminal amino acids [Fig. 1A, lane 3].

Staining cells with αFIN1 and antibodies to the SPB marker Sad1 [Hagan and Yanagida 1995] showed that Fin1-associated with the SPBs of dividing, but not interphase cells [Fig. 1C], stained the region between the separating chromosome masses in early anaphase B [red arrows in Fig. 1D], and gave discrete, but variable, patterns between late anaphase B SPBs [yellow arrows in Fig. 1D']. To check that all of these staining patterns arose from recognition of Fin1 by αFIN1 antibodies, we mixed
Fin1 binds to the SPB in this arrest [data not shown]. A signal was only seen on the SPBs following αFIN1 staining in the
nda3.KM311-elongated, mononucleated, cells that had condensed chromatin [Fig. 1E, yellow arrows]. It was not
seen in the anaphase/G1 SPBs of fin1.Δ cells [Fig. 1E, red arrows]. This established that the patterns observed after
αFIN1 staining could be attributed to the recognition of Fin1. Furthermore, because the background fluorescence
was higher throughout the arrested nda3.KM311 cells,
this experiment suggests that αFIN1 antibodies can detect a cytoplasmic pool of Fin1. Fin1 associated with the SPB as soon as two distinct SPBs could be detected. In one-half of the population, SPB association persisted until septation was complete [Fig. 1D]. In the other, it was lost from one of the two SPBs midway through anaphase B [Fig. 1D’]. Whether or not Fin1 associated with one of both of the anaphase B/G1 SPBs, the intensity of the staining increased from the middle of anaphase B to peak just before septation.

We also generated strains in which Fin1 could be localized by alternative approaches. Three Pk epitopes were fused to the 3’ end a fin1+ ORF integrated at the leu1+ locus, whereas one and four copies of GFP were fused to the 3’ end of the endogenous fin1+ gene in separate strains [Fig. 1B, lanes 5,6]. With two exceptions, localizing the modified proteins with αFIN1 or Fin1.CPK with anti-Pk antibodies gave the same pattern as Figure 1D. These exceptions were the presence of several dots within the nucleus and staining of G2 SPBs [data not shown]. These data are consistent with a previous study that reported that both Fin1.HA and ectopically expressed Fin1.GFP associate with G2 SPBs [Krien et al. 2002]. It is unclear why the fusion of a tag to Fin1 should influence its localization in this way; however, the critical fact is that the staining between the anaphase B SPBs and the frequency with which Fin1 associated with one or two anaphase B/G1 SPBs was the same in these tagged strains as seen in wild-type cells with αFIN1.

We therefore analyzed fin1.4GFP cells by time-lapse fluorescence microscopy [Fig. 1C]. Expression of an α-tubulin–CFP fusion protein [Glynn et al. 2001] did not affect Fin1.4GFP distribution [Fig. 1F,G]. Deconvolution of stacks of Z series taken through such cells established that the Fin1.4GFP staining between the anaphase B SPBs arose from the association of Fin1 with the central spindle [Fig. 1F]. Like the αFIN1 staining of fixed cells, Fin1.4GFP fluorescence on these anaphase B spindles of living cells was rarely uniform and generally punctate [Fig. 1D,D’,G].

Fin1 leaves SPBs upon completion of septation

Rapidly growing Schizosaccharomyces pombe cells initiate and generally finish S phase before completion of cytokinesis [Nurse et al. 1976]. Loss of Fin1 staining from the SPB could therefore be linked to either the completion of S phase or the completion of cytokinesis. We therefore stained cells in which progression through START into S phase of the next cell cycle was blocked by mutation of the transcription factor Cdc10 [Nurse et al. 1976]. Fin1 was absent from these arrested cdc10.v50 cells [Marks et al. 1992; data not shown]. It was also absent from newly divided cig1.Δ cig2.Δ puc1.Δ cells [Martin-Castellanos et al. 2000], in which G1 phase extends through cytokinesis into the next cell cycle [data not shown]. Thus, Fin1 leaves the SPB as a consequence of the completion of septation rather than the transit of START or the initiation of S phase.

When Fin1 associates with one SPB, it always binds to the old SPB

The red fluorescent protein DsRed takes several hours to fold into an actively fluorescing molecule. This property has been exploited to great effect to differentiate between the old and new SPBs in budding yeast [Pereira et al. 2001; Tanaka et al. 2002]. In these studies, refeding starved, Spc42.RFP cells generated a dividing population in which the “old” SPB that persisted throughout the G1 arrest was fluorescent, whereas the “new” SPB that formed in the new cell cycle was not [Pereira et al. 2001]. To recapitulate this approach in fission yeast, we fused DsRed to the C terminus of the S. pombe SPB component Pcp1 [Flory et al. 2002] to generate Pcp1.RFP. Very few cells in a pcp1.RFP log-phase culture had fluorescent SPBs, whereas a starvation/refeed regime generated anaphase B/septating cells, in which one SPB was fluorescent and the other was not [Fig. 2A]. This asymmetry indicated that partition of actively fluorescing, aged Pcp1.RFP, was conservative. Imaging fin1.4GFP pcp1.RFP cells established that when Fin1 was confined to one of the two anaphase B SPBs, the green and red fluorescent signals were always on the same SPB [Fig. 2B,C, left]. Thus, Fin1 always bound the SPB that inherited the old Pcp1.RFP, and so, presumably, was the older of the two SPBs. In half of the cells, Fin1 was also on the RFP-negative SPB, whereas in the other half it was not.

**Figure 2.** SPB history determined Fin1 affinity for the SPB. [A] pcp1.RFP cells were starved of nitrogen by switching from supplemented EMM2 [containing ammonium chloride] to EMM – N [no ammonium chloride] for 16 h before resuspension in EMM2. At 8 h, images of the red fluorescence (left) and DIC (right) were taken. The single acentric spots in the largest cells highlight only one of the two anaphase/G1 SPBs. [B] fin1.4GFP pcp1.RFP cells were processed as in A, and the fluorescence signals captured as indicated. When Fin1 was on one of the two anaphase B SPBs, it was always on the same SPB as the Pcp1.RFP signal. (C) fin1.4GFP pcp1.RFP cells were grown on gas-permeable membranes under an EMM2 agarose pad, and cells in which the old SPB could be identified by the RFP signal were followed until both daughter cells had divided to determine the relationship between SPB inheritance and Fin1 recruitment to the SPB. Images were captured in 1-µm steps every 5 min, and each slice monitored to identify the SPB. The cartoon summarizes observations of 15 such pedigrees.

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Fin1 affinity for the new SPB is also determined by SPB age and history

We next asked whether the affinity of Fin1 for the new SPB in cells arising from a parent in which both SPBs had stained in the previous division was determined by the history of that SPB. We did this by starving \textit{fin1.4GFP pcp1.RFP} cells to differentially label the old SPB with fluorescent Pcp1.RFP. Refed cells were then placed underneath an agar pad on an oxygen-permeable membrane, and the SPB inheritance pattern was monitored with a widefield fluorescence microscope. As cells immediately form microcolonies under these conditions (Hagan et al. 1990), we assume that this provides a way of following the fate of cells that inherit particular SPBs without adversely affecting cell growth or division. We first addressed the fate of cells that produced by division of cells in which only one of the two SPBs was fluorescent. Both anaphase B SPBs bound Fin1.4GFP in the cell that had inherited the old SPB in the previous division, whereas only one of the anaphase B/G1 SPBs bound Fin1.4GFP if the cell had inherited the nonfluorescent new SPB from the previous cell division (Fig. 2C, left). We then followed the fate of cells produced by division of a parent in which both of the anaphase B SPBs had bound Fin1.4GFP. We saw exactly the same relationship between inheritance of the old and new SPB and recruitment of Fin1.4GFP in the next cell cycle (Fig. 2C, right). In other words, old poles always gave rise to cells in which both anaphase B/G1 SPBs recruited Fin1, whereas new poles always gave rise to cells in which Fin1 was recruited to only one SPB, irrespective of whether they had bound Fin1.4GFP in the previous division or not.

This inheritance pattern was unlikely to arise because of the inclusion of Pcp1.RFP in the SPBs, as it was recapitulated in pedigree analysis of \textit{fin1.4GFP} cells (Fig. 3). The green dots in the pedigrees in Figure 3B represent SPBs that bound Fin1, gray dots those which did not, and the numbers in Figure 3B refer to the respective cells in Figure 3A. Two distinct inheritance patterns were seen, depending upon whether Fin1 had associated with only one [Fig. 3B, orange block] or both [Fig. 3B, blue block] SPBs in the previous division. When Fin1 had been restricted to one SPB in the previous division [Fig. 3B, orange block], the cell that inherited the Fin1 positive, old, SPB always had Fin1 associated with both anaphase B/G1 SPBs in the subsequent division. In contrast, in cells that had inherited the Fin1 negative, new SPB, Fin1 only associated with one SPB. In the second pattern, progeny arising from a cell in which Fin1 had been associated with both anaphase B/G1 SPBs in the previous cell cycle gave rise to a population that showed a 1:1 ratio of cells with one or both SPBs staining during their divisions [Fig. 3B, blue block].

The division-dependent maturation of the SPB could occur through progressive accumulation of a marker or molecule until it reached a threshold level, after which it would dictate either that the SPB should bind Fin1, or that both it and its progeny should bind Fin1. Alternatively, the pattern could arise because of a change that is induced by transit of a specific cell cycle stage/s. Forcing cells to speed up transit through a cell cycle would differentiate between these two possibilities, because it would shorten the time between cell cycle events, while maintaining the order of these cell cycle transitions. We therefore scored the frequency with which Fin1 stained one or two anaphase B/G1 SPBs in \textit{wee1.50} cells, in which the size control over commitment to mitosis is removed by heat-induced inactivation of the MPF-inhib
iting kinase Wee1. Removal of this size control accelerates cells into mitosis (Nurse 1975). The ratio of cells with Fin1 on one or both anaphase B/G1 SPBs was monitored every 30 min for 3 h after shift to 36°C, and was unaffected by the acceleration into mitosis [data not shown]. This established that it is transit through the cell cycle rather than the precise age of the SPB that determined Fin1 affinity for the SPB.

We draw the following conclusions from this data. A new SPB that is produced by a SPB that was itself a new SPB in the previous division does not bind Fin1 in the cell cycle in which it is generated. It gains the competence to bind Fin1 during, or after, passage through mitosis into the next cell cycle. However, it cannot confer the ability to bind Fin1 upon its daughter SPB in this, the second cell cycle of its existence, but acquires this ability to dictate that its daughter SPB must bind Fin1 after passage into its third cell cycle.

**Mutation of SIN components blocks Fin1 association with the SPB**

The apparent complexity of the rules governing Fin1 association with the SPB suggested that understanding the molecular basis of this phenomenon would give greater insight into Fin1 function. We therefore asked which cell cycle regulators and SPB components were required for insight into Fin1 function. We therefore asked which cell cycle regulators and SPB components were required for the passage into its third cell cycle. However, it cannot confer the ability to bind Fin1 upon its daughter SPB in this, the second cell cycle of its existence, but acquires this ability to dictate that its daughter SPB must bind Fin1 after passage into its third cell cycle.

Fin1 binds to the SPB via association with the Spg1 GAP component Byr4

The dependence of Fin1 upon Sid4 and Cdc11 for recruitment to the SPB suggested that it either bound directly to these scaffold molecules or to the SIN components that they recruit to the SPB (Chang and Gould 2000; Krapp et al. 2001). We therefore screened Fin1 against all known SIN components in the budding yeast two-hybrid system with Fin1 as bait, and Spg1, Sid4, and Cdc11 as prey (data not shown). (B) Immunoprecipitation reactions were performed with α-FIN1 or α-Byr4 antibodies and blots of the immunocomplexes were probed with each antibody. The inclusion of the mob1Δ mutation attenuated the otherwise lethal hyperactivation of the SIN hyperactivity arising from deletion of byr4Δ, and so, enabled us to demonstrate the specificity of the Byr4 immunoprecipitation reaction with a strain that lacked Byr4, and yet divided normally. (D) Byr4 association with the SPB was unaffected by deletion or mutation of fin1Δ. Wild-type and fin1Δ cells were grown at 25°C, whereas fin1Δ len1Δ-fin1A5 cells were grown at 25°C before shift to 37°C for 3 h to compromise Fin1 function.

**Table 1. Fin1 staining in sin mutants: where conditional mutants are used, analysis was done at the restrictive temperature**

| Strain          | Fin1 on SPB | Fin1 on SPB |
|-----------------|-------------|-------------|
| cdc25.22        | −           | −           |
| cdc2.33         | −           | −           |
| cdc10.V50       | −           | −           |
| cut12.1         | +           | −           |
| nuc2.663        | +           | −           |
| cut7.24         | +           | −           |
| sad1.1          | +           | −           |
| nda3.KM311      | +           | −           |
| cdc11.136       | −           | −           |
| sid4.K12        | −           | −           |

**Figure 4.** Fin1 binds Byr4. (A) Fin1 failed to associate with SPBs when the SIN was either inactive or hyperactive. cdc16.116 and cdc7.A20 were shifted to 36°C for 60 min, and cdc11.136 for 180 min before being stained with α-FIN1 antibodies. The lack of SPB staining was not due to errors in processing, as the central spindle staining was clearly visible. (B) S. cerevisiae transformants containing two-hybrid plasmids transferred to Nitrocellulose and incubated on X-gal-containing filters. Bait and prey plasmids are indicated (the interaction between the cdc11 C terminus and full-length cdc11Δ is a positive control [Krapp et al. 2001]). Tests using fin1Δ as bait, and spg1Δ, cdc16Δ, sid1Δ, sid2Δ, mob1Δ, cdc11Δ, cdc14Δ, dma1Δ, zfs1Δ, and cdc7Δ as prey revealed no significant interaction. A weak interaction above background was noted with sid2Δ as prey (data not shown). (C) Immunoprecipitation reactions were performed with α-FIN1 or α-Byr4 antibodies and blots of the immunocomplexes were probed with each antibody. The inclusion of the mob1Δ mutation attenuated the otherwise lethal hyperactivation of the SIN hyperactivity arising from deletion of byr4Δ, and so, enabled us to demonstrate the specificity of the Byr4 immunoprecipitation reaction with a strain that lacked Byr4, and yet divided normally. (D) Byr4 association with the SPB was unaffected by deletion or mutation of fin1Δ. Wild-type and fin1Δ cells were grown at 25°C, whereas fin1Δ len1Δ-fin1A5 cells were grown at 25°C before shift to 37°C for 3 h to compromise Fin1 function.
assay. The only association we detected was between Fin1 and the component of the Spg1 GAP complex Byr4 [Fig. 4B]. Fin1 was found in isolated Byr4 immunocomplexes and Byr4 was found in isolated Fin1 immunocomplexes [Fig. 4C], suggesting that the two-hybrid interaction reflected a genuine association between these two molecules. Fin1 failed to bind to the SPB when byr4 was deleted (Table 1). The inability of Byr4 to bind to the SPB when its partner molecule, Cdc16, is defective [Li et al. 2000], was mimicked by Fin1, as it did not bind the SPBs of cdc16Δ strains [Fig. 4A]. To rule out the possibility that this failure to associate with cdc16Δ byr4Δ-defective SPBs was the consequence of a feedback loop arising from a hyperactive SIN, we utilized strains in which either cdc16 or byr4 had been deleted, and cells were kept alive by attenuation of the SIN through mutation of the downstream effector complex Sid2/Mob1 [Cueille et al. 2001; Fournier et al. 2001]. Fin1 still failed to localize in these strains (Table 1), suggesting that the inability to bind in cdc16Δ mutants was not due to the hyperactivation of Sid2 or effectors that function after it. The association of Byr4 with the SPB is independent of the activity of the SIN effectors, Cdc7, Sid2/Mob1, and Sid1/Cdc14 [Li et al. 2000]. However, the same was not true of Fin1, as it failed to associate with SPBs when these proteins were rendered inactive in conditional mutants [Table 1; Fig. 4A], suggesting that prior activation of SIN signaling is required for Fin1 to associate with SPBs. Whereas Fin1 required Byr4 to associate with the SPB, the converse was not true, as mutation, deletion, or over-expression of fin1Δ did not affect the affinity of Byr4 for the SPB [Fig. 4D; data not shown]. We draw two conclusions from these data; first, association of Fin1 with the SPB requires activation of the SIN; second, recruitment of Fin1 to the SPB requires the SIN inhibitor Byr4, with which it interacts.

The SIN is active on the new SPB

The association between Fin1 and a SIN inhibitor suggested that Fin1 may contribute to regulation of SIN activity. We therefore analyzed pcp1.RFP cdc7.GFP cells to ask whether the old or the new SPBs host the active SIN in anaphase B. This revealed that the GFP signal of the active SIN was always on the new SPB (the one that lacked any RFP signal, Fig. 5A). This conclusion was confirmed by indirect immunofluorescence microscopy of a cdc7.HA strain to localize Cdc7 and Fin1 in the same cell [Fig. 5B].

In an unperturbed budding-yeast cell cycle, the old SPB is inherited by the bud/daughter, whereas the new one stays in the mother. The SIN equivalent, the MEN, is inhibited on the old SPB that faces the bud and active on the new SPB in the mother cell [Pereira et al. 2001; Menssen et al. 2001]. Transient depolymerization of microtubules abolishes this bias, and the new SPB enters the bud as frequently as the old. Despite this randomization of SPB inheritance, the MEN inhibitors Bfa1 and Buh2 are still only found on the SPB that faces the bud tip [Pereira et al. 2001]. We therefore asked whether a transient depolymerization of microtubules would mimic the effect seen in budding yeast and lead to SIN activation on the old and new SPBs in equal measure. To do this, we differentiated between the SPB that hosted the active SIN from the one upon which the SIN was inactive by following the association of Cdc7.GFP with the SPB. The Cdc7.GFP fusion protein that marks the SPB that hosts the active SIN. 25 µg ml-1 CBZ was added to refed cdc7.GFP pcp1.RFP cells as they progressed through mitosis. At 15 min, the CBZ containing medium was replaced by drug-free medium, and the localization of the SIN relative to the old SPB was determined a further 10 and 15 min later. At these times, the mitotic cells that had reformed a spindle after the removal of the drug were in anaphase B. This revealed that the GFP signal of the old SPB maturation and NIMA kinase in SIN regulation
Sohrmann et al. (1998; Cerutti and Simanis 1999), suggested that Fin1 may regulate SIN activity. However, we were unable to address this possibility by simply inactivating Fin1 in a temperature-sensitive mutant and asking whether SIN activation remained asymmetric, because these mutants are unable to form a mitotic spindle after shift to the restrictive temperature (Grallert and Hagan 2002). The mutant cells would not, therefore, be able to get into the anaphase state where we could ask the question. To circumvent this problem, we asked whether fin1 mutants could undergo a normal anaphase if they had been allowed to enter mitosis at the permissive temperature? We exploited the exquisite reversibility of nda3.KM311/H9252-tubulin mutant. nda3.KM311 cells accumulate in prophase without microtubules at 20°C. Upon rewarming to 36°C, a spindle reforms, and within 10 min, most cells are in anaphase (Fig. 6A) (Uemura et al. 1987). If the fin1.A5 execution point were after that for nda3.KM311, then fin1.A5 nda3.KM311 mutants should also execute a normal anaphase after accumulating in prophase at 20°C. They did (Fig. 6B). We could therefore ask whether Fin1 function affected the symmetry of SIN activation in anaphase of a fin1.A5 nda3.KM311 cdc7.HA strain that had been subjected to the arrest/release regime. Whereas Cdc7.HA was asymmetric on the nda3.KM311 cdc7.HA anaphase B spindles, inactivation of Fin1 in the fin1.A5 nda3.KM311 cdc7.HA strain resulted in recruitment of Cdc7.HA to both anaphase B SPBs in 44% of cells [Fig. 6C,D] compared with 5.5% in the fin1+ control. This showed that Fin1 function was required to ensure the asymmetry of SIN activation on the SPBs.

**Loss of Fin1 function promotes septation**

Whereas the previous experiment established that Fin1 function contributes to the attenuation of Spg1 function on the old SPB, it did not establish whether this attenuation also influenced the strength of the positive signal that passed through the SIN network. We used two approaches to address this possibility.

Inactivation of Cdc16 in S-phase-arrested cells induces septation in a proportion of the cells in the culture (Cerutti and Simanis 1999). We examined the effects of this protocol on a fin1.A5 cdc16.116 mutant to assess whether the simultaneous loss of Fin1 and Cdc16 function were additive. If they were, it would indicate that Fin1 normally attenuated the flux through the SIN. Hydroxyurea was added to cultures of small G2 cells 1 h after elutriation at 25°C. After a normal mitosis, the block to replication led to an S-phase checkpoint-dependent arrest in the next cell cycle. Increasing the temperature to 37°C induced septation in 68% of cdc16.116 cells, but 91% in cdc16.116 fin1.A5 cells (Fig. 7A).

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**Figure 6.** Compromising Fin1 function derepresses Spg1 on old SPBs. (A,B) Fin1 is not required for spindle formation following release from a prophase arrest imposed by microtubule depolymerization through transient arrest with the cold-sensitive nda3.KM311 mutation (i.e., the fin1+ execution point is before that of nda3−). (A) nda3.KM311 cdc7.HA cells were incubated at 20°C for 8 h to inactivate the β tubulin product of the nda3 gene before being rewarmed to 37°C. Samples were processed to visualize the microtubules; SPBs and chromatin are as indicated. (B) Cells were devoid of microtubules at the restrictive temperature (0°C), but spindles formed within 5 min of return to the permissive temperature, and cells were in anaphase B 10 min after the shift to 37°C. Identical behavior was seen in nda3.KM311 cdc7.HA fin1.Δlea1::fin1.A5 cells. (C) The analysis was repeated and cells stained to show the distribution of Cdc7 with 12CA5 antibodies at 20 min after return to the permissive temperature. (D) The frequency with which Cdc7 was seen on both anaphase B/G1 SPBs was scored at the indicated time in the strains shown in A and in a nda3.KM311 cdc7.HA fin1.A strain that had been treated in the same way as the nda3.KM311 cdc7.HA fin1.Δlea1::fin1.A5 strain. Analogous results were obtained with strains in which cdc7+ was tagged with GFP rather than the 12CA5 epitope (data not shown).
We have labeled the fission yeast SPB with a protein that takes several generations to fold into a conformation that fluoresces. A starvation/refeed protocol resulted in a population of cells in which only one of the two anaphase B SPBs was fluorescent. This suggests that elements of the duplication of the SPB are conservative. Conservative segregation of SPB components is consistent with the defects seen in temperature-sensitive \( \text{alp6} \), \( \text{alp4} \), \( \text{cut12} \), \( \text{fin1} \), and \( \text{cut11} \) mutants, where one SPB works, while the other does not (Bridge et al. 1998; West et al. 1998; Vardy and Toda 2000; Grallert and Hagan 2002). It would seem logical that the functional SPB is one that assembled at the permissive temperature, whereas the nonfunctional one is likely to be composed of material that formed at the restrictive temperature when the defective proteins do not fold or function.

Analysis of a population of synchronized cells by electron microscopy (Ding et al. 1997) suggested that the SPB duplicates by fission of a large precursor into two smaller structures that then grow to the size of the parental SPB before mitosis. At first sight, our data might contradict this view; however, it could be possible that the entire population of folded, fluorescent Pcp1, and the anchor for Fin1/Byr4 are partitioned to one SPB during this fission event. Different regions of a single SPB may have different structures. The SPB component Cut12 can be detected along the entire surface of the SPB that abuts the outer face of the nuclear envelope, but an N-terminal epitope was only detected to one side of this face (Bridge et al. 1998). Alternatively, the fission yeast SPB may actually duplicate by a conservative mechanism in which a new SPB forms de novo each cycle, and that intermediates in SPB duplication have simply not been detected by...
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the electron microscopic studies conducted to date. Further developments in the field will doubtless resolve this. The mechanics of SPB duplication are, however, not pertinent to the findings we report here, as our main findings relate to the demonstration that the Pcp1.RFP and Fin1.GFP inheritance patterns establish distinct fates for specific SPBs.

The SIN is active on the new SPB in anaphase B/G1

Simultaneous imaging of the old SPB and a marker for the active SIN showed that the active SIN was on the new SPB. The controls that modulate the analogous pathway in budding yeast, the MEN, are more complex. In an unperturbed budding yeast mitosis, the old SPB migrates into the bud, whereas the new SPB stays in the mother. The MEN inhibitors, Bub2 and Bfa1, always associate with the bud-bound old SPB, whereas, until mitotic exit, the Cdc15 kinase that marks the active MEN is only seen on the SPB of the mother cell [Menssen et al. 2001; Pereira et al. 2001]. Transient microtubule depolymerization resets the inherent orientation in this system, and either SPB subsequently enters the bud with equal frequency. In these cells, the MEN inhibitors reside on the SPB that faces the bud tip, irrespective of its age [Pereira et al. 2001]. This shows that it is a combination of the specific architecture of the cell and the association between the astral microtubules and the bud cortex/neck/tip that dictates which SPB will host the active MEN, rather than the age of the SPB. Perhaps the distinction between the anchorage of the MEN and SIN arises from the use of an additional SPB anchor in fission yeast. The MEN is anchored to the budding yeast SPB via Nud1, whereas the fission yeast Nud1 homolog, Cdc11, cooperates with a second scaffold protein Sid4 to anchor SIN components to the \textit{S. pombe} SPB [Grunenberg et al. 2000; Krapp et al. 2001; Tomlin et al. 2002].

\textbf{The \textit{S. pombe} SPB takes at least one and a half cell cycles to mature}

We find the NIMA kinase Fin1 on the central spindle, the SPB, and the region between the separating anaphase chromatin masses. The affinity of these sites for Fin1 is stage dependent. The region between the chromatin masses only stains in early anaphase cells, whereas the central spindle stains toward the end of anaphase B. The SPB association shows that each of the four daughter cells in a population are distinct because they have a different fate in the subsequent cell cycle. Fin1 always bound the old SPB and associated with the new SPB in half of the cells in the population. The affinity of the new SPB for Fin1 was dependent upon the history of the parent SPB that gave rise to this new SPB. If the parent SPB that produced this new SPB had itself been a new SPB in the previous cell cycle (i.e., the parent had only ever experienced one mitosis/START), then the new SPB did not bind Fin1. If, on the other hand, the parent SPB had produced at least one daughter already and gone through two or more mitosis/START transitions, then the new SPB bound Fin1.

Various models can be proposed to account for this behavior. Perhaps the simplest is best illustrated by considering the fate of a totally naive SPB (i.e., a new SPB that arose from the duplication of a parent that had, itself, been produced as a new SPB in the previous cell cycle; Fig. 8). This naive SPB is unable to bind Fin1 in the cell cycle in which it is created. Upon passage through mitosis or START, it is modified so that it will now bind Fin1 in the next anaphase B. In addition to promoting the recruitment of Fin1 to the anaphase B SPB this change, or a modification that is conducted in parallel, primes the SPB to undergo a second change during passage through the next round of mitosis/START. This second change means that this mature SPB can dictate that the SPB it will now produce will be able to bind Fin1 in the subsequent anaphase [Fig. 8]. Were such a simple model to underlie the changes that we find, then phosphorylation of an SPB component by Cdk1/cyclin B or mitotic kinases, such as polo or aurora, may account for one or both of the SPB modifications. Whether this is the case or not, the modification that determines that a partner SPB will bind Fin1, it is not maintained through sporulation as only one of the two SPBs stained in germinating spores (data not shown).

\textbf{Fin1 as a new component of the SIN}

The association between Fin1 and the SIN inhibitor Byr4 is important for Fin1 association with the SPB, as it is unable to bind when Byr4 is missing from the SPB as a result of deletion of the gene encoding \textit{byr4} itself or its partner in the GAP complex \textit{cdc16} (Cerutti and Simanis 1999). However, as Fin1 associates with the new SPB that hosts the SIN in half of the cells in the population, and Byr4 does not bind this SPB [Cerutti and Simanis

![Figure 8](image)
Fin1 must associate with another SPB component after, or as a result of, its association with Byr4. In this context, it is important to note that both Byr4 and Fin1 associate with both SPBs early in mitosis, long before the asymmetry of SIN regulation. This may explain why the Fin1 signal was always brightest on the old SPB, as Fin1 may bind to both the unidentified SPB anchor and Byr4 on this SPB. The association between Fin1 and a SIN inhibitor appears to be biologically relevant, as compromising Fin1 function both enhances SIN signaling on the old SPB and promotes septation. Three points suggest that Fin1 is not a core SIN component; it was not identified in screens that exhaustively identified key SIN components (Fournier et al. 2001); cells can survive without it, and alteration of Fin1 function has only modest effects on SIN output. Fin1, therefore, appears to join molecules such as Dma1, Zîs1, and Amn1 as a regulator at the periphery of SIN/MEN control (Murone and Simanis 1996; Beltraminelli et al. 1999; Wang et al. 2003). Perhaps these molecules become critically important in growth states that have not been encountered in the controlled laboratory environment, such as stress and nutrient limitation. Removal of such elements might have only a moderate consequence upon flux through the pathway in standard growth conditions. Alternatively, the SPB may need to mature through distinct steps before it is fully competent to regulate the pathway. In this case, inhibition by molecules such as Fin1 or Amn1 could regulate the pathway on a subset of SPBs and ensure the maximum fidelity of mitotic controls. This view would be supported by the observations that the SIN is only activated on both SPBs in around half of the population following Fin1 inactivation, and that inactivating Cdc16 in S phase induces around half of the cells in a population to septate (Cerutti and Simanis 1999). Finally, the dependence of Fin1 recruitment upon the activity of SIN effectors could suggest that it is part of a feedback control that limits or dampens SIN activity on one or both SPBs once the network is active. As such, it would resemble the mitotic phosphorylation of the scaffold molecule Cdc11, which depends upon the activity of the kinase that it recruits to the SPB, Cdc7 (Krapp et al. 2003).

**Parallels between SPB maturation and centriole maturation**

The incremental maturation of the fission yeast SPB bears a striking resemblance to the changes in centriolar morphology that accompany maturation from newborn daughters into fully mature mother centrioles in pig epithelial cells (Vorobjev and Chentsov 1982). Mature mother centrioles have appendages that are not seen on their daughters. In the first stage of centrosome duplication, the single centriole pair splits to produce two independent centrioles that seed short procentrioles. These centrioles elongate during G2 to generate two prophase centrosomes that are similar in structure, except that only one of the pairs has a centriole that is equipped with appendages. This is the original mother centriole. The new mother that was produced by the G1 duplication fission event still lacks appendages. They do not appear on this “ex-daughter, now mother” until the transition from metaphase to anaphase B. At this time, this ex-daughter, now mother centriole acquires the appendages. The anaphase cell has two pairs of centrioles, and in each of these pairs, the oldest centriole has appendages. Vorobjev and Chentsov concluded that centriole maturation takes one and half-cell cycles in mammalian cells. The analysis of molecular markers of centriole maturation such as CDIB4 and ninein also reveal incremental changes in centriole structure [Lange and Gull 1995; Mogensen et al. 2000]. The fact that both centriole and SPB maturation may take similar times, raises the exciting possibility that the underlying mechanisms are related, and that studies of SPB maturation in yeasts may shed light upon the mechanism of centriole maturation in higher systems.

**What purpose could centriole/SPB maturation serve?**

There is increasing evidence from yeast and higher systems that the decision to commit to mitosis is taken at the centrosome/SPB [Jackman et al. 2003; Maclver et al. 2003]. Similarly, the role for yeast SPBs in regulating mitotic exit/septation is beyond question, and parallels are emerging in higher systems (Simanis 2003). The mother centriole plays a critical role in regulating the final stages of cytokinesis. It migrates to touch the midbody, and this contact then promotes cell fission [Piel et al. 2001]. It was also recently established that Centrinol, a component of centriole appendages, has a domain that is homologous to a domain found in both the yeast MEN/SIN scaffold proteins, Nud1/Cdc11 (Gromley et al. 2003). This raises the possibility that a control network analogous to the MEN/SIN associates with the appendages.

If a MEN/SIN-related network were to associate with the appendages, it may explain why appendages only form on daughter centrioles upon commitment to anaphase. The MEN modulates mitotic exit in budding yeast in response to interaction between astral microtubules and the cortex. Prior to anaphase, the spindle-assembly checkpoint can regulate mitotic progression, but once anaphase has started, the astral microtubule checkpoint, the MEN, becomes critical. As a MEN-equivalent network would only be important in anaphase B, there would only be a need for appendages at this time and not before. There may be a positive benefit to having only one site at which to coordinate cell cycle controls prior to anaphase, because two or more control points could generate conflicting signals and result in inappropriate cell proliferation. This would be particularly relevant for tumor cells, as many have multiple centrosomes [Nigg 2002]. Restoration of normal centriole maturation or the promotion of centrosome maturation when multiple centrosomes are present could be a powerful way to alter the proliferative capacity of such tumor cells, and so may offer novel avenues for therapeutic intervention.
Materials and methods

Strains, molecular biology, and antibody production

Strains are listed in Table 2. Standard fission yeast and molecular biology methods were used throughout (Moreno et al. 1991). PCR tagging used pSM822 and pSM1023 (Pereira et al. 2001; Maekawa et al. 2003) to generate pcp1.RFP and fin1.ΔGFP. An Ndel site was generated immediately before the STOP codon of fin1 ORF, a fragment encoding 3-Pk epitopes (Craven et al. 1998) inserted into this site and the 3.3-Kb fragment from −286 to +672 was inserted into pNNTA (Petersen and Hagan 2003) to direct disruption of the leu1′ locus with this modified fin1′ gene alongside ura4+. To generate pReplfin1.Δ282–722 amino acids, an Ndel site was inserted in pfin1.1 (Grallert and Hagan 2002) directly after nucleotide 1157 of the coding sequence (equivalent to residue 281/282) to generate pfin1.1Nde1int. The 1535-bp Ndel/XbaI fragment of pfin1.1Nde1int was inserted into pPREP41. To generate fusion protein for immunization, an Ndel site was introduced at the stop codon of pfin1.1Nde1int to generate pfin1.1Ndefrag. The 1323-bp Ndel fragment from pfin1.1Ndefrag was inserted into pAR3033 (Studier et al. 1990) to generate pfin1FP1, and this was used to generate fusion protein by standard procedures (Studier et al. 1990).

Table 2. Yeast strains used in this study

| Strain | Description |
|--------|-------------|
| IH163  | 972 h−      |
| IH592  | wee1.50 leu1.32 h− |
| IH3160 | leu1.32 ura4.D18 pRepl41 |
| IH3161 | leu1.32 ura4.D18 pRepl41 fin1.282-722aa |
| IH1824 | fin1::ura4 ura4.D18 his2 h+ |
| IH2772 | leu1::fin1::ura4 fin1::ura4 ura4.D18 |
| IH2282 | leu1::finpKc::ura4 fin1::ura4 ura4.D18 |
| IH2674 | fin1.GFP::kanR h− |
| IH2675 | fin1.RFP::kanR h− |
| IH2719 | pcp1.RFP::kanR fin1.4GFP::kanR |
| IH759  | cdc7.HA::ura4::ura4.D18 h− |
| IH1106 | cdc7.GFP::ura4::ura4.D18 h− |
| IH2721 | cdc7.GFP::ura4::pcp1.RFP::kanR ura4.D18 h− |
| IH635  | cdc25.22 ura4.D18 h− |
| IH164  | cdc2.33 h− |
| IH280  | cut2.1 leu1.32 h− |
| IH110  | mit2.663 leu1.32 h− |
| IH936  | cut7.24 leu1.32 h− |
| IH156  | sad1.1 leu1.32 h− |
| IH385  | cut11.1 leu1.32 h− |
| IH304  | nda3.KM311 leu1.32 h− |
| IH2333 | cdc11.123 leu1.32 h− |
| IH2332 | cdc11.136 ura4.D18 h− |
| IH2650 | sid4.K12 leu1.32 ura4.D18 h− |
| 1296   | cdc16.116 leu1.32 h− |
| IH3017 | cdc16::ura4::sid2.1 ade6.M210 leu1.32 ura4.D18 h− |
| IH2940 | cdc16::ura4::mob1.R4 ura4.D18 h− |
| IH3132 | byr4::ura4::byr4::ade6.M216/ade6.M210 leu1.32 leu1.32 ura4.D18/ura4.D18 h− |
| IH2992 | byr4::ura4::mob1.R4 leu1.32 ura4.D18 h− |
| IH1557 | spg1.B8 ura4.D18 leu1.32 his2 h− |
| IH738  | cdc7.A20 ura4.D18 h− |
| IH114  | cdc7.24 h− |
| IH2499 | cdc7::ura4/cdc7::ade6.M216/ade6.M210 leu1.32/leu1.32 ura4.D18/ura4.D18 h− |
| IH1469 | sid1.239 leu1.32 ura4.D18 h− |
| IH111  | cdc14.118 h− |
| IH1470 | sid2.250 leu1.32 ura4.D18 ade6.M216 h− |
| IH2939 | mob1.R4 ura4.D18 h− |
| IH1336 | spg1.HA::ura4 leu1.32 ura4.D18 |
| IH3162 | spg1.HA::ura4 leu1.32 ura4.D18 pRepl41fin1′ |
| IH2918 | cdc7.HA::ura4::nda3.KM311 ura4.D18 h− |
| IH2920 | cdc7.HA::ura4::nda3.KM311 fin1::ura4 ura4.D18 leu1.32 his2 h− |
| IH2942 | cdc7.HA::ura4::nda3.KM311 fin1::ura4 leu1::fin1.A5::ura4 ura4.D18 |
| IH2780 | cdc16.116 fin1::ura4 ura4.D18 leu1.32 |
| IH2762 | cdc16.116 fin1.ts1 leu1.32 |
| IH2774 | fin1::ura4 spg1.B8 ura4.D18 leu1.32 h− |
| IH3510 | spg1.B8 leu1.32 ura4.D18 pREP81 h− |
| IH3511 | spg1.B8 leu1.32 ura4.D18 pREP81fin1′ h− |
Cell biology
Standard Western blotting approaches following TCA precipitation and immunoprecipitation of cell extracts was performed as described previously (Mclver et al. 2003). Standard immunofluorescence procedures were used for tubulin staining with the anti- α tubulin antibody TAT11 (Wood et al. 1989), whereas Fin1 and Byr4 staining was performed following fixation with 1% formaldehyde for 5 min according to standard procedures (Hagan and Asycough 2000). For pedigree analysis, cells were mounted under agarose pads according to Hagan et al. (1990), with the exclusion that PVP was from the medium and use of EMM2 rather than YES. Images were captured on a Zeiss Axiovert 200M fitted with a PIFOC piezo objective driver and a Cool snap HQ camera (Roper Scientific). Images were captured in a set of 6–1-μm slices every 5 min, and processed with Metamorph software (Universal Imaging). Each slice was scored for the presence of SPBs. Images in Figure 1F were processed by the Metamorph nearest-neighbor algorithm. A Perkin Elmer spinning-disk confocal system was used to generate images in Figure 1F and G. Stacks were compressed with the maximum projection algorithm to generate the images for presentation using proprietary software.

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