The *Schizosaccharomyces pombe* septation initiation network (SIN) is required for spore formation in meiosis

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Summary

When nutrients are abundant, *S. pombe* cells grow as rods, dividing by fission after formation of a medially placed cell wall or division septum. Septum formation is triggered by a group of proteins, called the septation initiation network or SIN, that trigger contraction of the acto-myosin contractile ring at the end of mitosis. Ectopic activation of the SIN can uncouple septum formation from other cell-cycle events, whereas loss of SIN signalling gives rise to multinucleated cells due to the failure of cytokinesis. When starved, *S. pombe* cells of opposite mating types fuse to form a diploid zygote that undergoes meiosis and produces four spores. No septa or contractile rings are formed during meiosis. In this study, we have investigated the role of the SIN in meiosis. Our data show that, whereas the meiotic divisions appear normal, SIN mutants cannot form spores. Forespore membrane formation is initiated, but the nuclei are not encapsulated properly. The SIN proteins localise to the spindle pole body in meiosis. The protein kinases Sid1p and Cdc7p do not associate with the spindle pole body until meiosis II, when forespore membrane deposition begins. These data indicate a role for the SIN in regulating spore formation during meiosis.

Key words: Meiosis, Septation, Cell cycle, Fission yeast, Spore

Introduction

Gametogenesis is a developmental process during which diploid cells undergo meiosis to produce specialised germ cells. The equivalent event in *Schizosaccharomyces pombe* is sporulation, which is initiated when diploid cells are challenged by nutrient starvation, especially when nitrogen is limiting in the medium (reviewed by Yamamoto, 2004). Sporulation is preceded by one round of S phase and two meiotic divisions that generate four haploid nuclei, which are packaged into individual spores. This unique process involves the formation of membrane-bound haploid gametes (spores) within the cytoplasm of the mother cell (ascus). The four haploid nuclei produced by the meiotic nuclear divisions are packaged into double-layered membranes, termed forespores. During meiosis II, the spindle pole body (SPB) undergoes a morphological transformation from a compact dot into a multilayered expanded structure. Membrane vesicles are recruited to the vicinity of the modified SPBs to generate the forespore membrane. As the nucleus divides in anaphase II, the forespore membrane extends and encapsulates each of the four nuclei to form the prespores. The prespores then mature into spores by deposition of two layers of spore walls in the lumen between the inner and outer prespore membranes. Mature spores are finally liberated by autolysis of the ascus wall (reviewed by Shimoda, 2004; Shimoda and Nakamura, 2004).

To permit error-free segregation of chromosomes during the meiotic cell division, the internal compartmentalisation and meiotic nuclear divisions must be properly coordinated. A key structure linking these two events is the SPB. The SPB is required not only for meiotic spindle assembly, as the microtubule-organising center, but also for formation of the forespore membrane, because sporulation is totally abolished when SPB modification is blocked by a mutation of the SPB component Spo15p (Ikemoto et al., 2000). The SPB might therefore serve as a platform to coordinate nuclear division and prespore formation.

During the mitotic cycle, nuclear division must also be tightly linked to cytokinesis, the compartmentalisation event at the end of the cycle, which gives rise to two daughter cells. Cytokinesis in *S. pombe* is regulated by a signalling cascade termed the septation initiation network (SIN), which is required for the contraction of the actomyosin ring and formation of the septum (reviewed by Krapp et al., 2004; Wolfe and Gould, 2005). SIN components localise to the SPB to which they are anchored via the SIN scaffold complex formed of sid4p and cdc11p (Krapp et al., 2001; Tomlin et al., 2002). SIN signalling is governed by the nucleotide status of the small, Ras superfamily GTPase, Spg1p (Schmidt et al., 1997), which is kept inactive throughout interphase by the two-component GTPase activating protein (GAP) Cdc16p-Byr4p (Furge et al., 1998). Upon entry into mitosis, SPB-associated Spg1p accumulates in its GTP-bound form, which allows recruitment of the protein kinase Cdc7p (Sohrmann et al., 1998), followed by the Sid1p-Cdc14p complex to the new SPB (Guertin et al., 2000). These kinases have been proposed to activate and/or allow relocalisation of the Mob1p-Sid2p complex from the SPB to the medial ring, where this complex would trigger medial ring contraction and septation (Sparks

et al., 1999). The SIN has been conserved through evolution; its functional equivalent in budding yeast is called the mitotic exit network (MEN). In addition to cytokinesis, the MEN proteins also regulate mitotic exit (see Bardin and Amon, 2001; Simanis, 2003; Wolfe and Gould, 2005 for reviews of the MEN, including a comparison with the SIN).

Septum and spore formation are different compartmentalisation processes during the mitotic and meiotic cycle, respectively. However, both events require coordination with the nuclear cycle and involve recruitment of membrane and cell wall material. Since the SIN plays an essential role during the vegetative cycle in signalling initiation of septum formation, we asked whether the SIN plays a similar role during meiosis in signalling forespore membrane formation. In this study, we found that the SIN proteins localise to the SPB during meiosis and that the pathway is activated during the second meiotic division. Moreover, we have found that SIN signalling is essential in the proper forespore membrane formation around the haploid nuclei, suggesting that both compartmentalisation processes are regulated by the same pathway.

Results

The SIN is activated during the second meiotic division

SIN genes are transcribed during the meiotic cycle (Mata et al., 2002), when the cell does not form a contractile ring or a division septum. We therefore examined the localisation pattern of functional chromosomally GFP-tagged SIN proteins. Cells of opposite mating types were mated and visualised by fluorescence microscopy. The scaffold proteins Sid4p and Cdc11p, which are required to anchor the downstream SIN components to the SPB during vegetative growth, were detected on the SPB throughout meiosis, namely during the horsetail stage and the two meiotic divisions (Fig. 1A,B). Downstream SIN signalling molecules, such as Spg1p, Mob1p or Sid2p, showed the same localisation pattern as the scaffold molecules throughout meiosis, although the GFP signal was weak at the horsetail stage (Fig. 1C,G,H). Interestingly, the characteristic crescent shape of the modified SPB during the second meiotic division was not detected when using GFP-tagged SIN proteins (this is shown for Cdc11p-GFP and Mob1p-GFP; Fig. 1B and 1G, respectively), suggesting that the SIN proteins are not components of the meiotic outer plaque. During the mitotic cycle, the hallmarks of an active SIN are: displacement from the SPB of the two-component GAP Cdc16p-Byr4p, which allows activation of the Spg1p GTPase, and recruitment to the SPB of two kinases Cdc7p and Sid1p (Guertin et al., 2000; Sohrmann et al., 1998). Interestingly, the Sid1p and Cdc7p protein kinases were only

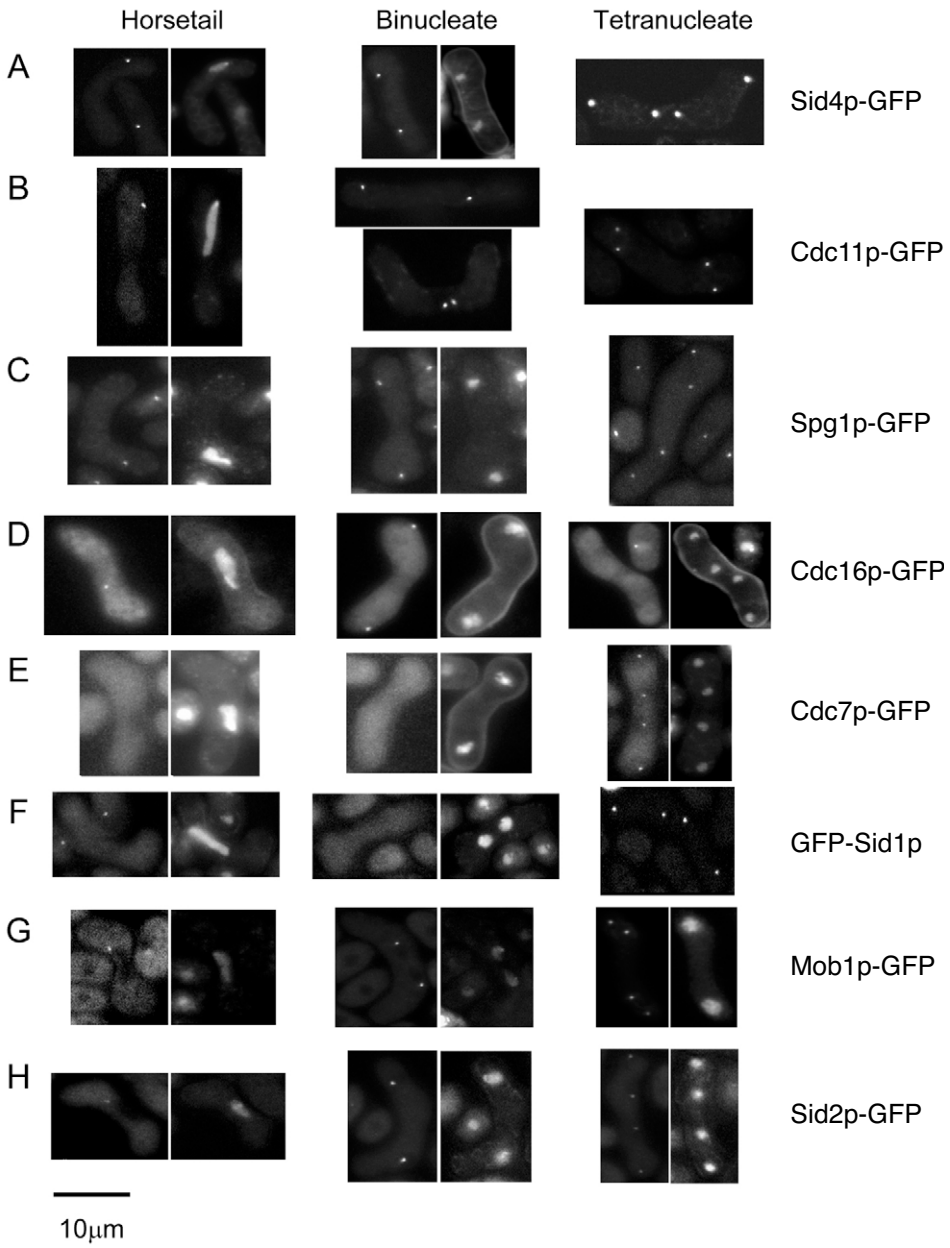

Fig. 1. Localisation of the SIN proteins during meiosis. Strains of opposite mating types expressing chromosomally GFP-tagged alleles of Sid4p (A), Cdc11p (B), Spg1p (C), Cdc16p (D), Cdc7p (E), Sid1p (F), Mob1p (G) or Sid2p (H) were mated on EMM-NH_4Cl plates at 25°C for 20 hours. Cells were then resuspended in EMM-NH_4Cl containing DAPI and visualised under a fluorescence microscope. The columns show cells in the horsetail stage, binucleate cells and tetranucleate cells. In cases where a DAPI image is shown, the DAPI is on the right, the GFP-fluorescence on the left. Bar, 10 μm.

detected on the SPBs during the second meiotic division (Fig. 1E,F), whereas the Cdc16p GAP component was found on the SPB only during the horsetail stage and the first meiotic division (Fig. 1D). These results suggest that the SIN is activated during the second meiotic division. However, this activation does not lead to the formation of a septum, suggesting that the SIN plays a different role during meiosis. None of the SIN proteins showed any discrete signal associated with mature spores (data not shown).

The SIN proteins are required for spore formation

The localisation data described above suggest that the SIN plays a role during meiosis. To investigate whether this is the case, sporulation was assessed in temperature-sensitive SIN mutants. Meiosis was induced by inactivation of Pat1p using the *pat1-114* mutation (Iino and Yamamoto, 1985; Nurse, 1985) in diploid SIN strains that are homozygous at the mat locus (Blanco et al., 2001; Murakami and Nurse, 1999; Perez-Hidalgo et al., 2003). Notice that the SIN mutant alleles used in this study are all inactivated at 33°C or less, a temperature that does not interfere with meiotic progression (Crandall et al., 1977). An isogenic SIN^+ strain was used as a control. Successful completion of meiosis was monitored by iodine staining of the spore cell wall. In contrast to cells carrying only the *pat1-114* mutation, spore walls were not formed in diploid cells homozygous for either the *sid4-SA1*, *cdc11-136* or *spg1-B8* mutation. As a control, cells returned to the permissive temperature (25°C) 3 hours after induction of meiosis were able to sporulate (Fig. 2A). These data indicate that the SIN plays an essential role during meiosis.

Asci derived from *cdc11-136* diploids were observed by phase-contrast microscopy and DAPI staining. Whereas mature ascospores were readily detectable in the *pat1-114* mutant 24 hours after induction of meiosis, no mature spores were observed in the *cdc11-136* mutant (Fig. 2B). However, asci derived from the *cdc11-136* diploids contained four nuclei, suggesting that these cells had progressed normally through the two meiotic divisions. The ability to form spore-containing asci was also monitored in *pat1-114/pat1-114* diploids containing other SIN-temperature-sensitive alleles (Fig. 2C). In all the cases, no spores were formed at the restrictive temperature, although tetranucleate cells were observed. By contrast, sporulation occurred normally when the cells were shifted back to the permissive temperature 3 hours after shift-up (Fig. 2C). These data show that SIN proteins are required for spore formation.

Meiotic divisions in the *cdc11-136* mutant are normal

To examine at which stage during meiosis SIN proteins are required, progression through meiosis was monitored in a diploid *pat1-114/pat1-114 cdc11-136/cdc11-136* mutant by determining the percentage of cells with one, two or four nuclei by DAPI staining. Fig. 3C shows that *cdc11-136* cells progressed normally through the two meiotic divisions. This indicates that the main meiotic defect in *cdc11-136* cells resides in spore formation.

To confirm that cdc11p is required only during the late stages of meiosis, diploid *pat1-114/pat1-114 cdc11-136/cdc11-136* cells were shifted to the restrictive temperature to induce meiosis and inactivate Cdc11p, and returned to the permissive temperature at intervals thereafter. Incubation times at the restrictive temperature, ranging from 90 to 210 minutes, allowed both control cells and *cdc11-136* cells to sporulate, although the percentage of asci that contain spores was low (Fig. 3A). Longer incubation times at the restrictive temperature progressively abolished sporulation in *cdc11-136* cells, whereas the percentage of asci-containing spores increased in the SIN^+ control cells (Fig. 3A). Assuming that cdc11p activity is restored after the shift to the permissive temperature, these data indicate that cdc11p activity is required around 4 hours after induction of meiosis, at the onset of the second meiotic division (Fig. 3B).

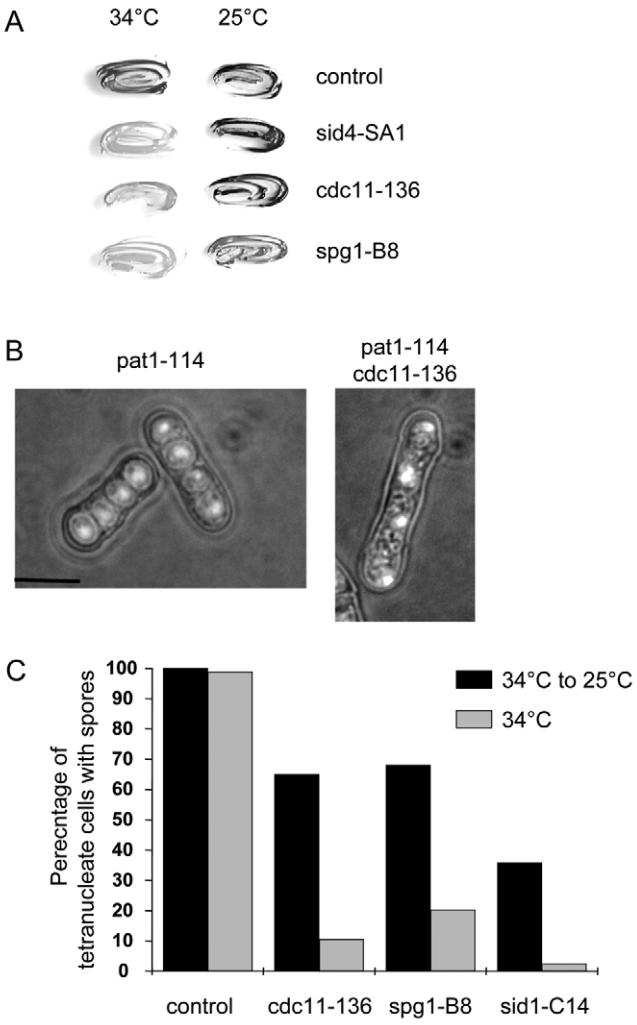

Fig. 2. The SIN proteins are required during meiosis. (A) *pat1-114/pat1-114* (control) and *pat1-114/pat1-114* homozygous for the indicated SIN temperature-sensitive mutation were replica-plated onto EMM-NH_4Cl, shifted to 34°C for 3 hours and then shifted back to 25°C (25°C), or kept at 34°C for 24 hours (34°C). Plates were then exposed to iodine vapour to stain spore walls. (B) *pat1-114/pat1-114* and *pat1-114/pat1-114 cdc11-136/cdc11-136* were induced to sporulate at 33°C during 24 hours, fixed and stained with DAPI. Bar, 10 μm. Notice the absence of spores in the *pat1-114 cdc11-136* mutant. (C) *pat1-114/pat1-114* (control) and *pat1-114/pat1-114* cells homozygous for the indicated SIN temperature-sensitive mutation were induced to sporulate at 34°C for 20 hours or for 3 hours and then shifted back to 25°C. Cells were then fixed, stained with DAPI and the percentage of tetranucleate cells containing spores was determined.

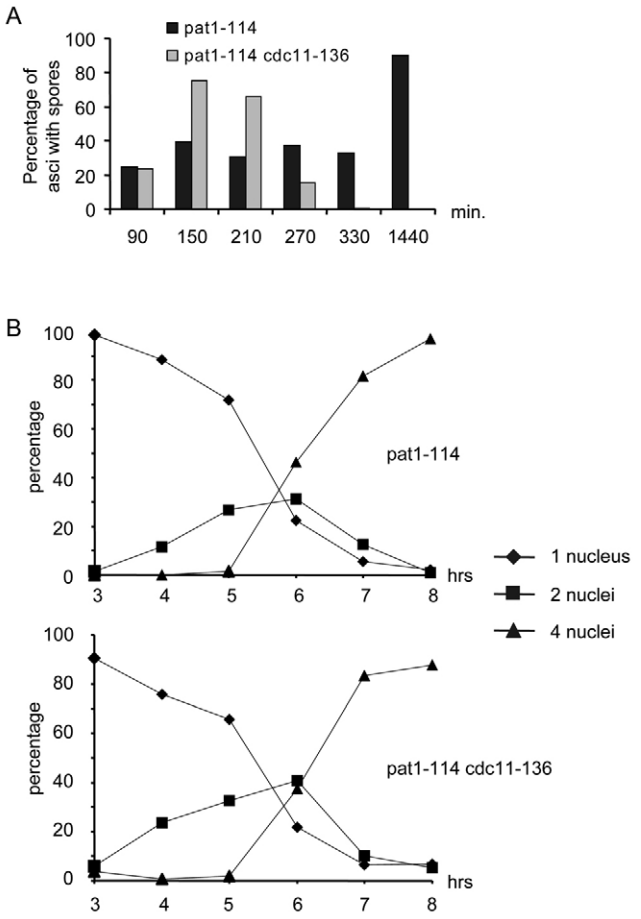

Fig. 3. *cdc11-136* is defective in spore formation. (A) Nitrogen-starved *pat1-114/pat1-114* and *pat1-114/pat1-114 cdc11-136/cdc11-136* cells were shifted to 34°C for the indicated time and then shifted back to 25°C. Cells were fixed and the percentage of asci that contained spores was determined by phase-contrast microscopy. (B) Kinetics of meiosis in *pat1-114/pat1-114* and *pat1-114/pat1-114 cdc11-136/cdc11-136* cells. Starved cells were induced to sporulate at 33°C, and a portion of the culture was fixed at the indicated time and stained with DAPI. Meiotic cells were classified according to the number of nuclei per cell. ◆, mononucleate cells; ■, binucleate cells; ▲, tetranucleate cells.

To investigate whether the spore formation defect results from a problem earlier in meiosis, we analysed DNA replication, horsetail movement, and the two meiotic divisions in the *cdc11* mutant. Premeiotic DNA replication was monitored in *pat1-114/pat1-114* and *pat1-114/pat1-114 cdc11-136/cdc11-136* cells by FACS analysis in a synchronous meiosis experiment (Fig. 4A). In both cases, the DNA content showed that a significant proportion of cells were arrested in G1 phase upon nitrogen starvation (2C peak) and then underwent a single round of pre-meiotic DNA replication after induction of meiosis (4C peak).

Using the GFP-tagged histone hht2p-GFP to visualise the nucleus (Wang et al., 2002), we observed that the characteristic nuclear shape adopted during the horsetail stage was normal in the diploid *cdc11-136* mutant (Fig. 4B). Examination of the behaviour of the SPB marker pcp1p-GFP revealed that the typical oscillatory movement of the nucleus occurred in the *cdc11-136* cells (Fig. 4E). Finally, cytoplasmic microtubules emanating from the SPB at the leading edge of the moving nucleus, formed normally in the cdc11-136 mutant, as visualised in cells expressing GFP-atb2p under the control of its own promoter (Fig. 4C).

During the first meiotic division, chromatin and SPB segregation appeared normal, as shown by visualisation of hht2p-GFP (Fig. 4B) and pcp1p-GFP (Fig. 4E), respectively. In addition, examination of GFP-atb2p revealed the presence of normal meiosis I spindles (Fig. 4D). Similar results were obtained upon examination of the second meiotic division. Nuclear segregation (assessed with Hht2p-GFP and the nuclear envelope marker Nup107p-GFP) appeared normal (Fig. 5B), as did the distribution of the kinetochore marker Nuf2p-GFP (Fig. 5B). Formation of spindles in meiosis II was examined with GFP-Atb2p (Fig. 5A) and also appeared unperturbed in *pat1-114/pat1-114* and *pat1-114/pat1-114 cdc11-136/cdc11-136* cells. Therefore, we conclude that Cdc11p is not essential for progression through the stages of meiosis prior to spore formation.

Abnormal forespore membrane formation in the cdc11-136 mutant

Since the *cdc11-136* mutant produces no mature spores, despite the fact that it undergoes meiosis with normal kinetics, we analysed the process of spore formation in more detail. Spore formation is initiated during the second meiotic division, when the SPB undergoes a drastic modification to form a complex structure called the meiotic plaque, which is essential for spore formation but not for the second meiotic division (Hirata and Shimoda, 1992; Ikemoto et al., 2000). The meiotic plaque is a multilayered extension of the SPB and many SPB components become incorporated into this structure (Bahler et al., 1993; Hirata and Shimoda, 1994; Shimoda et al., 1985). The protein Spo15p (Ikemoto et al., 2000) is located on the meiotic plaque and is required for its formation. Observation of the SPB protein Cut12p by using a chromosomally GFP-tagged allele (Bridge et al., 1998) or Spo15p-GFP expressed from a plasmid revealed the presence of the typical crescent-shaped SPB in both control and *cdc11-136* cells (Fig. 6A). This result suggests that cdc11p is not required for the meiosis-specific modification of the SPB.

The forespore membrane is assembled in the immediate vicinity of the meiotic plaque during anaphase II, and at the end of the second meiotic division, each haploid nucleus is engulfed by this membrane to form the pre-spore (Shimoda, 2004). Spo3p and Psy1p, a t-SNARE protein implicated in vesicle fusion (Nakamura et al., 2001), both localise to the developing forespore membrane (Fig. 6B) (Nakamura et al., 2001). In the *cdc11-136* mutant, the cup-like structure formed in the vicinity of the SPB initiated normally, but its later development was aberrant; the haploid nuclei were not encapsulated by the membrane and ectopic membrane-containing structures were observed in the cytoplasm (Fig. 6B). Co-staining of the nuclei with DAPI and Psy1p-GFP with an antibody against GFP confirmed that in most of the control cells, the forespore membrane encapsulated each haploid nucleus, whereas in the *cdc11-136* mutant Psy1p-GFP aggregates were observed near the nuclei (Fig. 6C). These results indicate that forespore membrane extension and/or nuclear encapsulation requires Cdc11p function, thereby

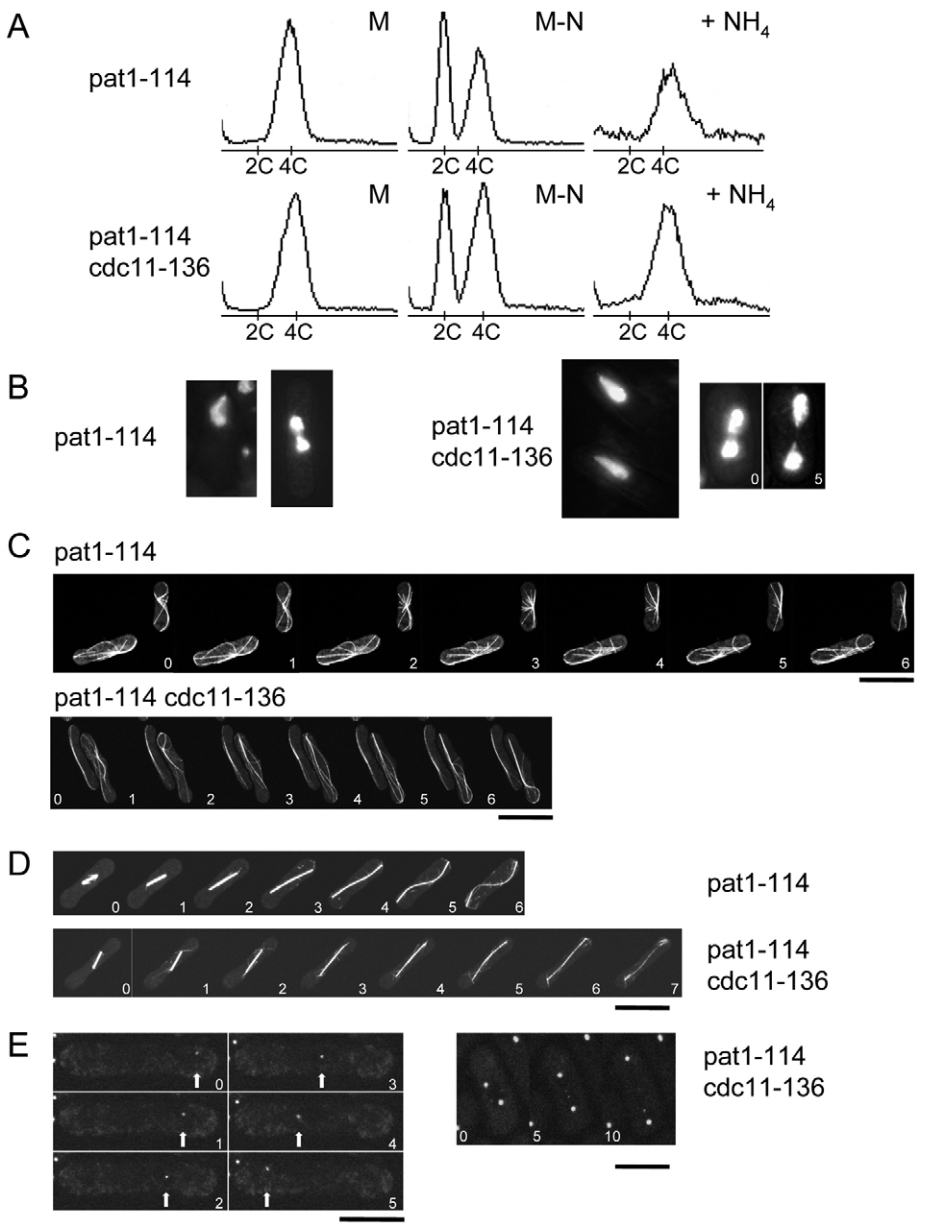

Fig. 4. *cdc11-136* diploid cells progress normally through the horsetail and the first meiotic division. (A) FACS analysis of diploid *pat1-114/pat1-114* and *pat1-114/pat1-114 cdc11-136/cdc11-136* cells growing vegetatively (M), starved in EMM-NH_4Cl during 16 hrs at 25°C (M-N) and induced to sporulate at 33°C for 7 hours (+ NH_4) were analysed for their DNA content as described in materials and methods. (B) Microscopy of living *pat1-114/pat1-114* and *pat1-114/pat1-114 cdc11-136/cdc11-136* cells expressing the chromosomally GFP-tagged histone *hht2* allele. In the left panel, two images of the same cell are shown, taken 5 minutes apart. (C,D) Observation of living *pat1-114/pat1-114* and *pat1-114/pat1-114 cdc11-136/cdc11-136* cells expressing GFP-Atb2p under the control of its own promoter from a plasmid, during the horsetail stage (C) or the first meiotic division (D). Images were recorded at 1-minute intervals. The time after the first images (*t*=0) is indicated. (E) Live cell microscopy of *pat1-114/pat1-114* and *pat1-114/pat1-114 cdc11-136/cdc11-136* cells expressing chromosomally GFP-tagged Pcp1p, to visualise the SPB. The left panel represents the horsetail stage. The position of the SPB is indicated by the white arrow. The right panel shows the first meiotic division. The time (in minutes) after the first image is indicated on each panel. Bars, 10 μm.

providing an explanation for the sporulation defect observed in the *cdc11-136* diploid.

SIN signalling is required for spore formation

The data above show that *cdc11* mutants are sporulation-defective because they fail to form a proper forespore membrane around the haploid nuclei. This meiotic role of Cdc11p could be owing to its function as a SIN signalling molecule or to a structural role as a SPB component. The former hypothesis is supported by the following observations. First, SIN proteins are required for spore formation (Fig. 2C) but not completion of the meiotic divisions (Fig. 7B). Second, diploid *pat1-114/pat1-114 cdc11-136/cdc11-136* cells containing GFP-tagged *cdc7*, *sid1* or *mob1* strains were constructed and localisation of these proteins was monitored after the second meiotic division. During the mitotic cycle, the mutant Cdc11-136 protein and Mob1p still localised to the SPB at the restrictive temperature (Krapp et al., 2001; Salimova et al., 2000), wheras Cdc7p and Sid1p were not recruited to the SPB (Krapp et al., 2001). Similar results were obtained during the meiotic cycle; whereas Sid1p-GFP and Cdc7p-GFP (Fig. 7A) localised to the SPB after the second meiotic division in the SIN^+ control cells, they were not detected in the diploid cells homozygous for *cdc11-136* (Fig. 7A). By contrast, Mob1p-GFP was readily detectable on the SPB in meiotic cells lacking a functional Cdc11p (data not shown). These results indicate that Cdc11p acts as a SIN scaffold during the meiotic cell cycle and vegetative growth.

Finally, in the *cdc2-N22* mutant, homozygous diploids undergo a single meiotic division to produce dyads rather than tetrads (Grallert and Sipiczki, 1990; MacNeill et al., 1991; Nakaseko et al., 1984). Thus, if SIN signalling is instrumental for spore formation, Cdc7p and Sid1p should be recruited to the SPB during the first meiotic division in this mutant. Consistent with this, in a homozygous *cdc2-N22* cross, Cdc7p-GFP (Fig. 7C) and Sid1-GFP (data not shown) localised to the

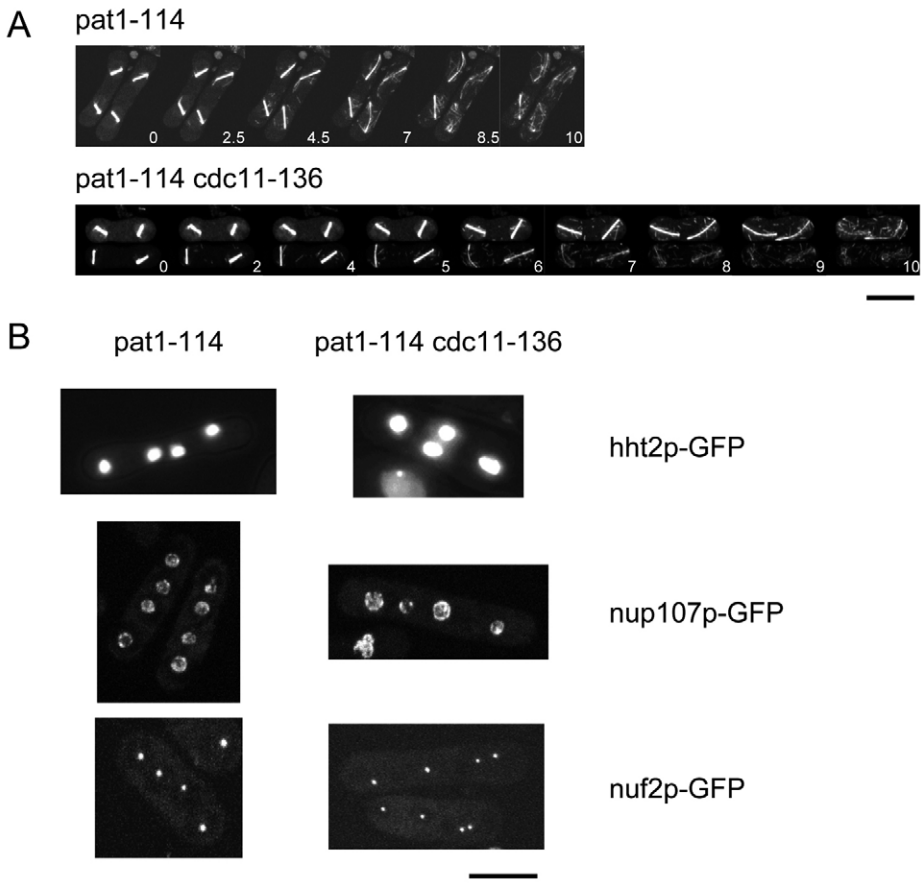

Fig. 5. *cdc11-136* diploid cells progress normally through the second meiotic division. (A) Observation of living *pat1-114/pat1-114* and *pat1-114/pat1-114 cdc11-136/cdc11-136* cells expressing GFP-Atb2p under the control of its own promoter from a plasmid after 6 hours at 34°C. The time in minutes after the first images is indicated on the panels. (B) Observation of living *pat1-114/pat1-114* and *pat1-114/pat1-114 cdc11-136/cdc11-136* cells expressing GFP-Hht2p, the nuclear envelope protein Nup107p-GFP, or the kinetochore Nuf2p-GFP. Cells were incubated for 6 hours at 34°C. Bars, 10 μm.

SPBs during the first meiotic division. Taken together, these results strongly suggest that the SIN signalling network is required for spore formation.

Ectopic activation of SIN during meiosis leads to septation

Localisation data of SIN proteins during meiosis suggest that the SIN is not fully activated before meiosis II, a stage at which it is implicated in the formation of forespore membrane. In growing vegetative cells, activation of the SIN during G2 phase by, for example overexpression of *spg1*, leads to ectopic septation (Schmidt et al., 1997). Similarly, precocious activation of the SIN during the early stages of meiosis might induce either septation or forespore membrane formation. To test this, the SIN was activated ectopically by overexpression of *spg1* in a diploid *pat1-114/pat1-114* strain and cells were induced to undergo meiosis at the restrictive temperature. As shown in Fig. 8A, overexpression of *spg1* during meiosis led to the formation of asci that contained septa as well as spores. Most of the mature asci contained four spores or nuclei, indicating that ectopic activation of the SIN does not induce spore formation during the early stages of meiosis. Time course analysis shows that the septation index increases while the cells are going through meiosis (Fig. 8B). Moreover, whereas 4 hours after induction of meiosis most septating cells were mononucleate, septa were found in tetranucleate cells 6 hours after induction of meiosis. These results indicate that, first, septation can occur during meiosis and, second, septation does not interfere with meiotic progression. Analysis of the position of the septa within the asci allowed us to distinguish at which time during meiosis septation took place. Cells containing all four nuclei on one side of the septum have septated from the horsetail stage onwards. By contrast, asci that bear two nuclei on each side of the septum must have septated at or after the first meiotic division. These two types of cells were readily detectable upon overexpression of *spg1* (Fig. 8C). Third, cells containing one and three nuclei on each side of the septum indicate that septation occurred after the second meiotic division. These types of cells were not detected upon overexpression of *spg1*, suggesting that septation cannot be induced after the second meiotic division. These results show that correct regulation of SIN activity is important to avoid septation during meiosis.

Discussion

Localisation of SIN proteins during meiosis

We have shown that, although septum formation does not occur in meiosis, SIN proteins are nonetheless present at the SPB. Three points are noteworthy. First, components of the SIN that constitutively localise to SPB during mitosis are present on the SPB throughout meiosis. By contrast, the protein kinases Cdc7p and Sid1p are present at the SPB only during meiosis II. Their localisation mirrors that of Cdc16p, the catalytic component of the GAP of Spg1p. The presence of Cdc7p and Sid1p at the SPB, as well as the absence of the two-component GAP is a hallmark of what is considered to be the active configuration of the SIN. This pattern of localisation therefore suggests that the SIN is not activated until the time of the

initiation of spore formation, consistent with the role we have uncovered for it in this study. During the mitotic cycle, a decrease of Cdc2p activity is required to permit association of Sid1p with the spindle pole body and activation of the SIN (Guertin et al., 2000). Assays of Cdc2p-associated kinase activity in synchronised meiotic cells have demonstrated that it decreases during meiosis II, prior to sporulation (Blanco et al., 2001; Izawa et al., 2005). Moreover, in the meiotic cycle, spore formation does not occur if cyclin Cdc13p is stabilised or cannot be degraded (Blanco et al., 2001), leading to the proposition that Cdc2p/cyclin-kinase activity prevents sporulation before the completion of meiosis II (Blanco et al., 2001). The timing of the appearance of Sid1p and Cdc7p at the spindle pole body during meiosis II correlates well with the previously observed decrease in Cdc2p activity. We therefore favour the idea that Cdc2p regulates the SIN negatively by controlling Sid1p/Cdc7p localisation during meiosis.

Second, during mitosis, the old and the new SPBs behave differently: Cdc7p and Sid1p localise to the newly generated SPB, which apparently functions as the active signalling centre for the initiation of septum formation (Grallert et al., 2004; Guertin et al., 2000; Sohrmann et al., 1998), whereas the two-component GAP is restricted to the old SPB. However, during meiosis the SPBs behave in a symmetrical fashion: SIN signalling proteins are either omnipresent or absent. This lack of asymmetry may result from a different mode of SPB duplication and/or maturation during meiosis, which would abrogate the difference between 'old' and 'new' SPBs. For example, during meiosis, the determinant controlling segregation of Cdc7p and Sid1p to only one pole during the vegetative cell cycle might segregate symmetrically. Future studies will address this point.

Finally, although many SPB antigens assume a characteristic crescent shape during the late stages of meiosis as the outer spindle plaque is remodelled to promote spore formation, the SIN proteins do not show this shape. Thus, either SIN proteins do not associate with the modified outer plaque or they do so only in a spatially limited manner.

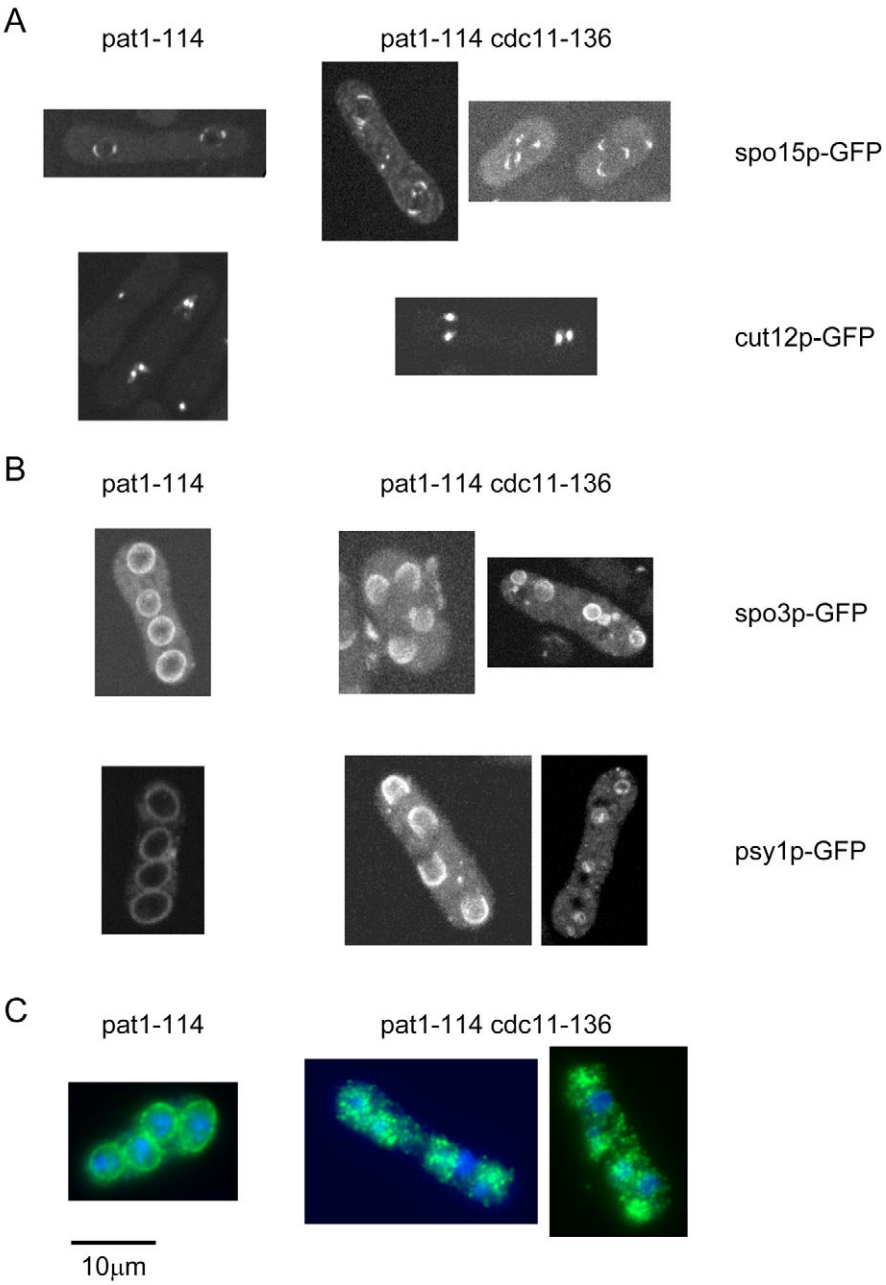

Fig. 6. Abnormal forespore membrane formation in the *cdc11-136* mutant. (A) Observation of living *pat1-114/pat1-114* and *pat1-114/pat1-114 cdc11-136/cdc11-136* cells expressing either spo15p-GFP from a plasmid, or chromosomally GFP-tagged alleles of *cut12*. Cells were incubated for 6 hours at 34°C. (B) Observation of living *pat1-114/pat1-114* and *pat1-114/pat1-114 cdc11-136/cdc11-136* cells expressing either spo3p-GFP or psy1p-GFP from a plasmid. Cells were incubated for 6 hours at 34°C. (C) *pat1-114/pat1-114* and *pat1-114/pat1-114 cdc11-136/cdc11-136* cells expressing Psy1p-GFP were fixed after 6 hours at 34°C as described in Materials and Methods. Forespore membranes and chromatin were examined by using anti-GFP antibody (green) and DAPI (blue), respectively. Bar, 10 μm.

The role of the SIN in meiosis

We have shown that, although progression through meiosis takes place normally, spore formation fails in the absence of SIN components and Cdc11p is required for the correct formation of the forespore membrane. This conclusion is not restricted to Cdc11p, because, first, spore formation – but not nuclear division – is affected in some other SIN mutants. Second, during meiosis Cdc11p acts as a SIN scaffold, as it

does during the vegetative cycle. Third, in the *cdc2-N22* mutant premature spore formation correlates with premature recruitment of Cdc7p and Sid1p to the SPB. However, currently available mutant alleles have not permitted all the known SIN components (particularly *sid2* and *mob1*) to be tested in the context of a *pat1-114*-induced meiosis. Nonetheless, we notice that a diploid heterozygous for a partial deletion of *mob1* has decreased spore viability (Salimova et al., 2000), suggesting that the truncated Mob1p has a dominant-negative effect upon meiotic events. The role of individual SIN proteins downstream of the scaffold and Spg1p in meiosis will be the subject of future studies.

Deletion of genes induced during meiosis has identified a number of proteins that are required for spore formation in meiosis (Martin-Castellanos et al., 2005). However, examination of the phenotype of these mutants suggests that, in most cases, the failure to form spores arises from defects earlier in meiosis. It will be interesting to determine whether any of these proteins are SIN substrates.

Forespore membrane formation starts with the modification of the SPB during which two or three layers are constructed outside the central plaque (Shimoda, 2004). Cdc11p seems not to be involved in the formation of this crescent-shaped extension of the SPB, because both Spo15p and Cut12p could be detected in these structures in the absence of functional Cdc11p. However, electron microscopy will be required to ascertain that the modified SPB does form as in wild-type cells. At present, we cannot rule out that specific components fail to be recruited to this structure in the Cdc11 mutant. In *S. cerevisiae*, Nud1p, the Cdc11p orthologue, is involved in the formation of the meiotic plaque. It binds to the meiosis-specific proteins Mpc54p and Mpc70p, and is thought to anchor them to the enlarged SPB structure during meiosis II (Knop and Strasser, 2000). However, these meiotic proteins have no orthologues in *S. pombe*, and this function of Nud1p is MEN-independent.

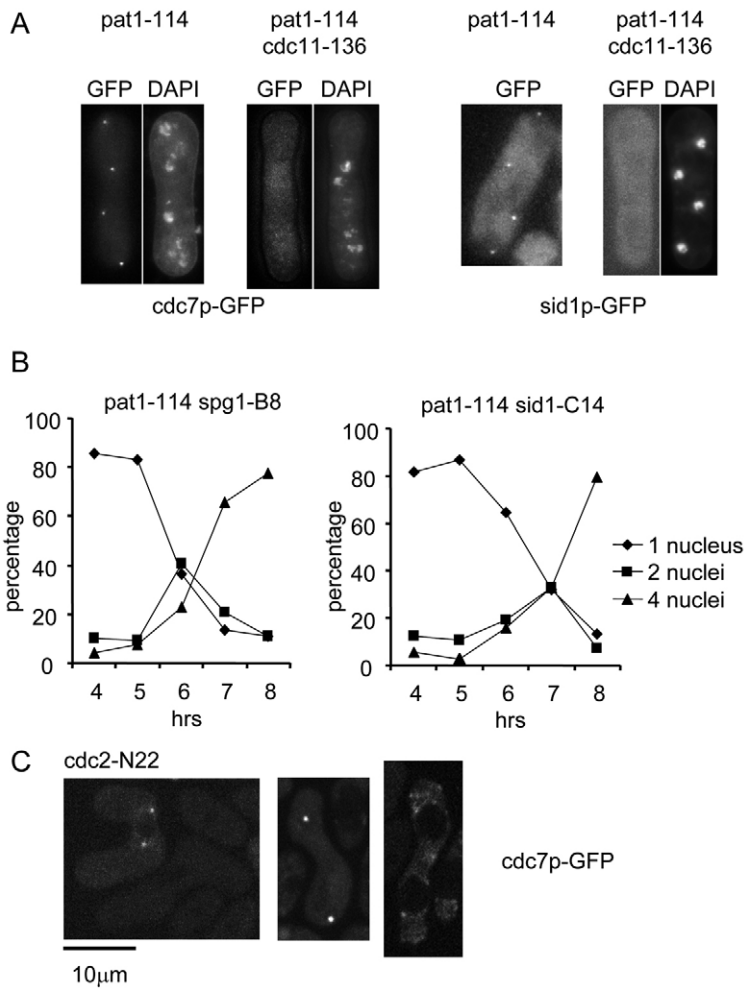

Fig. 7. The SIN is required for spore formation. (A) Microscopy of living *pat1-114/pat1-114* and *pat1-114/pat1-114 cdc11-136/cdc11-136* cells expressing chromosomally tagged Cdc7p-GFP (left) or Sid1p-GFP (right). Cells were induced to undergo meiosis by incubation for 6 hours at 34°C. (B) Analysis of the kinetics of meiosis in *pat1-114/pat1-114*, *pat1-114/pat1-114 spg1-B8/spg1-B8* and *pat1-114/pat1-114 sid1-C14/sid1-C14* cells. Starved cells were induced to sporulate at 34°C, and a portion of the culture was fixed at the indicated time and stained with DAPI. Meiotic cells were classified by the number of nuclei per cell. ◆, mononucleate cells; ■, binucleate cells; ▲, tetranucleate cells. (C) *cdc2-N22* cells of opposite mating types expressing Cdc7p-GFP were mated on EMM-NH_4Cl plates at 25°C for 24 hours. Cells were then resuspended in EMM-NH_4Cl and GFP fluorescence was examined. Bar, 10 μm.

In the absence of functional Cdc11p, the forespore membrane fails to encapsulate the haploid nuclei. This phenotype is reminiscent of that of Spo3p (Nakamura et al., 2001), Spo14p (Nakamura-Kubo et al., 2003), Spo20p (Nakase et al., 2001) or Sec9 (Nakamura et al., 2005) mutants. These gene products are part of the general secretory pathway or the vesicle fusion machinery, which are required for forespore membrane assembly. Interestingly, during vegetative growth, Psy1p (Nakamura et al., 2001), Spo3p and Spo20p localise to the division site, and a role in completion of the septum and cell separation has been assigned to Spo20p (Nakamura et al., 2001) and Sec9 (Nakamura et al., 2005). It is therefore tempting to speculate that the SIN might fulfil its function, at least in part, by controlling membrane and/or protein trafficking to the division sites both during mitosis and meiosis. In this respect it is interesting to notice that in higher eukaryotes, exocyst and SNARE protein localisation to the midbody is required for abscission and depends on centriolin, which shares homology with Cdc11p (Gromley et al., 2005).

A role in spore formation has also been reported for the MEN component Cdc15p, the orthologue of *S. pombe* Cdc7p. However, this role cannot be extended to the entire MEN pathway, since depletion of Tem1p, the Spg1p orthologue, does not affect meiosis in budding yeast (Kamieniecki et al., 2005).

Increased expression of *spg1* can induce septum formation from any point in the mitotic cell cycle (Schmidt et al., 1997) – even when mitotic Cdc2p activity is maximal, in cells arrested by the spindle assembly checkpoint (Guertin et al., 2002). Ring formation, however, does not require an active SIN (Wu et al., 2003) (for overviews see Krapp et al., 2004; Wolfe and Gould, 2005), yet paradoxically, *spg1* overexpression can trigger contractile ring and septum

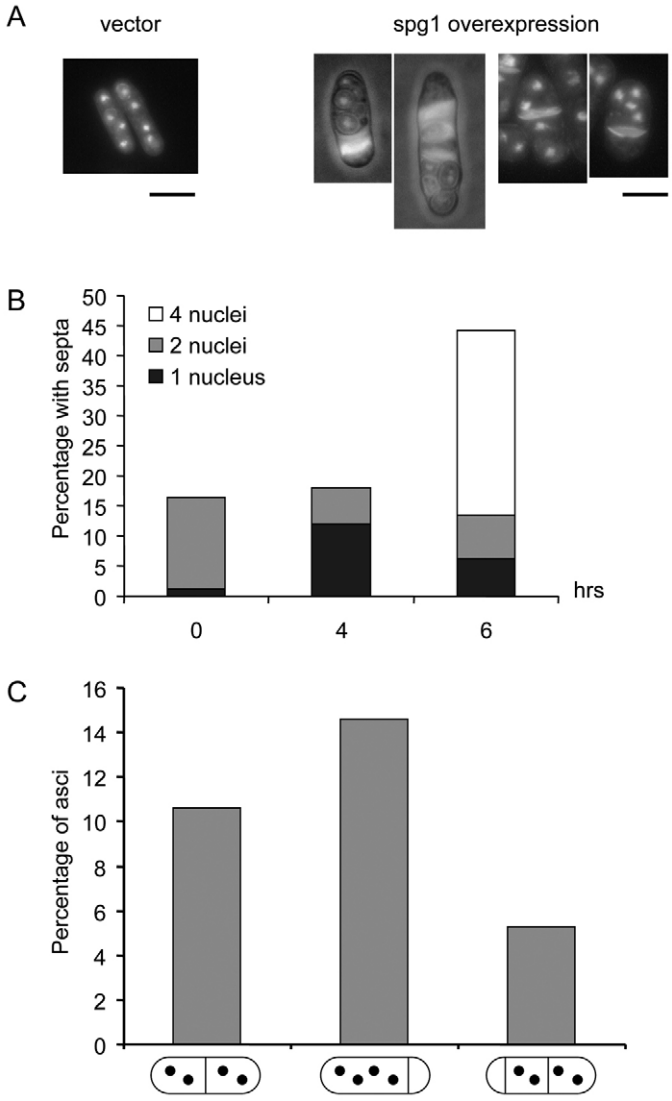

Fig. 8. Ectopic activation of the SIN leads to septation during meiosis. (A) *pat1-114/pat1-114* diploid cells carrying a plasmid expressing Spg1 under the control of the *nmt1* promoter were grown in minimal medium lacking thiamine for 4 hours at 25°C and then starved in EMM-NH_4Cl for 16 hrs at 25°C. Meiosis was induced by incubation at 34°C for 6 hours, when the cells were fixed and stained with DAPI and Calcofluor. The control was cells carrying an empty *nmt1* expression vector. (B) Cells were treated as in A, but samples were taken at different time points after induction of meiosis. The septation index in the total population was determined and subdivided into categories of cells containing one, two or four nuclei. (C) Cells were treated as in A. The septation index among the cells containing fournuclei was determined and subdivided into the following three categories: (1) asci containing two nuclei on each side of the division septum (septation occurred at or after MI); (2) asci containing four nuclei on the same side of the division septum (septation occurred before or after MI); (3) multiseptated asci. Bars, 10 μm.

formation (Schmidt et al., 1997). Ectopic activation of the SIN during meiosis results in formation of septa during the horsetail stage and meiosis I. However, septation can no longer be induced after the initiation of meiosis II. The reason for this is unclear, but it is possible that spores cannot be formed before formation of the meiotic plaque. Spg1p overexpression can probably not induce the formation of the meiotic plaque because we do not observe asci that containing two diploid spores.

This, in turn, raises the question of how formation of the contractile ring is suppressed during meiosis, because the required activity is clearly latent in the cell. The finding that septa cannot be induced after the initiation of meiosis II suggests that, once the SPB has been remodelled to form the meiotic plaque, either the targets for the induction of septum formation are inaccessible to the SIN or they have been eliminated. Future studies will address these questions.

Materials and Methods

Yeast strains, media and culture conditions

Fission yeast strains bearing GFP-tagged alleles of SIN genes used in this study have been described previously (Chang and Gould, 2000; Guertin et al., 2000; Krapp et al., 2001; Salimova et al., 2000; Schmidt et al., 1997; Sohrmann et al., 1998; Sparks et al., 1999). Standard techniques (Moreno et al., 1991) were used to create the strains described in the text. Yeast transformation was carried out by using the lithium acetate transformation protocol (Moreno et al., 1991). The GFP-Cdc16 strain was created by placing the *cdc16* promoter upstream of a GFP-Cdc16 fusion. This construction was then integrated at the *leu1* gene. The GFP-tagged *cdc16* gene inserted at *leu1* rescues a *cdc16* deletion. Integration of the GFP-Cdc16 fusion at *leu1* was necessary because the proximity (>150 bp) of the *cdc16* gene to the *rps13* gene precludes tagging at the native locus. For examination of GFP-Atb2, the *atb2* gene was cloned onto a multicopy plasmid expressed from its own promoter. The construction is not toxic to cells.

Diploid strains were obtained by mating on EMM-NH_4Cl plates (Blanco et al., 2001). Synchronous meiosis in *pat1-114/pat1-114* temperature-sensitive mutants was performed as follows. *h-/h-pat1-114/pat1-114* diploid cells were cultured in rich Y5 medium at 25°C for 1 day and transferred to EMM plus supplements (100 μg/ml) for another day. These cells were then washed and resuspended in EMM-NH_4Cl plus supplements (10 μg/ml) at a density of 2-3×10^6 cells/ml. After 16 hours at 25°C, most cells were arrested in G1 phase and the culture was shifted to 34°C in the presence of 0.5 g/l NH_4Cl and 10 μg/ml supplements to induce meiosis. The same protocol was followed for the diploid cells overexpressing Spg1p under the control of the *nmt1* promoter, with the exception that the cells were cultured in EMM containing 2 μM thiamine, transferred into thiamine-free EMM for 4 hours at 25°C and then resuspended in EMM-NH_4Cl.

Microscopy

DAPI staining (1 μg/ml) was performed on cells that had been fixed with 70% ethanol as described previously (Balasubramanian et al., 1997; Moreno et al., 1991). To estimate the proportion of cells in meiosis I, meiosis II or in sporulation, we determined the percentage of cells with one, two or four nuclei after DAPI (1 μg/ml) staining and the percentage of asci with mature spores with phase-contrast microscopy. Alternatively, DAPI was added to the culture medium of living cells at 0.2 μg/ml. The septation index was determined after fixation with 70% ethanol and staining with DAPI (1 μg/ml) and Calcofluor (10 μg/ml). Cells were fixed according to established protocols (Balasubramanian et al., 1997; Moreno et al., 1991). Psy1p-GFP was visualised either in living cells, or by indirect immunofluorescence microscopy using an antibody against GFP, followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes). Examination of GFP-tagged proteins in living cells was performed using a Zeiss axiovert 200 microscope equipped with a confocal scanner unit model CSU10 (Yokogawa Electric Corporation), a coolSNAP HQ camera (Photometrics), and 63× 1.4 NA plan-apo or 100× 1.4 NA plan-apo objective. Images were collected using Metamorph software (Universal Imaging, version 4.5). For examination of fixed specimens, TILLvisION software (v3.3; TILL Photonics GmBH) was used to analyse data captured with an IMAGO CCD camera mounted on an Olympus IX70 microscope. Images were assembled in Adobe Photoshop 7 or CS and Powerpoint 2003.

FACS analysis

Approximately 10^7 cells were collected by centrifugation, washed with water, fixed with 70% ethanol and processed for flow cytometry as described previously (Sazer and Sherwood, 1990). A Becton-Dickinson FACScan was used for flow cytometry.

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