

Role of the spindle pole body of yeast in mediating assembly of the prospore membrane during meiosis

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Spindle pole bodies (SPBs) are the centrosome equivalents in yeast, required for microtubule organization. In yeast, the SPB further serves as the attachment sites of the prospore membrane during meiosis. Here we report the identification of two new meiosis-specific components of the SPB, Mpc54p and Mpc70p, and the first protein specific for the prospore membrane, Don1p. Mpc54p and Mpc70p are not present in mitotic SPBs, and during meiosis II they are components of a meiosis-specific structural alteration of the outer plaque of the SPB. Both proteins are dispensable for the meiotic divisions but are essentially required for the formation of the prospore membrane. In the *mpc54* and *mpc70* mutants, the Don1p-containing precursors of the prospore membrane can still be found in the cytoplasm and associated with the SPB. Unexpectedly, however, the assembly of the precursors to a continuous membrane system is affected. Thus, the meiotic SPB is directly involved in the formation of a specialized membrane system, the membrane of the prospore.

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Introduction

Meiosis is a specialized form of cell division in which a diploid mother cell produces four haploid daughter cells within the mother cell. This process is accompanied by a single round of DNA replication followed by two rounds of DNA segregation. In the yeast *Saccharomyces cerevisiae* this process takes place within the boundaries of one undivided nucleus. Simultaneously with the onset of meiosis II, intracellular membrane systems are formed. These so-called prospore membranes are destined to form the enclosures of the future spores, the spore walls. They originate at the cytoplasmic side of the spindle pole bodies (SPBs). SPBs are multilayered proteinaceous structures that are always embedded in the nuclear envelope in this yeast. During meiosis II the prospore membranes grow and form domed structures that engulf lobes of the nucleus that contain the chromosomes. In addition, cytoplasmic material and organelles become enclosed. At the end of meiosis II, the nuclear lobes pinch off and the prospore membrane closes to form two membranes stacked on each other, the so-called prospore wall (Lynn and Magee, 1970; Moens and Rapport, 1971; Peterson *et al.*, 1972; Zickler

and Olson, 1975). Formation of the prospore membrane depends on *SEC* genes that act in late steps of secretion. Spo20p, a meiosis-specific yeast homologue of the human synaptonemal protein SNAP-25, is required for the proper completion of the prospore walls (Neiman, 1998).

The SPB is the functional equivalent of the centrosome in yeast and functions as the microtubule organizing center in a manner analogous to the pericentriolar material (PCM) in higher eukaryotes. A common feature of the SPB and the PCM of centrosomes is their content of coiled-coil proteins (Doxsey *et al.*, 1994; Fry *et al.*, 1998; Adams and Kilmartin, 1999). The SPB is comprised of different plaques. The outer and the inner plaque nucleate and organize the cytoplasmic and the nuclear microtubules, respectively. The central plaque spans the nuclear envelope.

Coiled-coil proteins are often found to build large oligomeric structures that undergo various interactions, for example, in the case of the SPB, with proteins required for microtubule nucleation (for review see Zimmerman *et al.*, 1999). Many coiled-coil proteins are also crucially important for the organization of various aspects of the cytoskeleton. For example, motor proteins, which exhibit large stalks formed by coiled-coil domains, provide motile functions essential for the organization of the microtubules in concert with ongoing mitotic processes (for a review see Vallee and Gee, 1998). Other coiled-coil proteins have been found to localize to the cytoplasm and to be associated peripherally with the cytoplasmic face of the organelles of the secretory pathway. A number of functions of these proteins have been proposed. These include roles of the proteins in the docking of donor vesicles to acceptor membranes, in homotypic membrane fusion and in the organization of the stacking of the Golgi apparatus (Sapperstein *et al.*, 1996; Nakamura *et al.*, 1997; Gournier *et al.*, 1998; Lowe *et al.*, 1998; Simonsen *et al.*, 1998; Sönnichsen *et al.*, 1998; VanRheenen *et al.*, 1998).

In this paper we describe the specific role of the yeast SPB during meiosis. We identified two novel coiled-coil proteins, Mpc54p and Mpc70p, which are components of a specific substructure of the SPB that is only present during meiosis. This unit, which we termed the meiotic plaque, forms the outmost cytoplasmic layer of the SPB during meiosis II. Mutant studies indicate that the meiotic plaque is not required for progression through meiosis, but apparently is required for prospore membrane formation. By utilizing a novel specific marker for the prospore membrane, Don1p, several subsequent steps in this assembly process can be distinguished. The process starts during meiosis I with the appearance of precursors of the prospore membrane in the cytoplasm. Subsequently, some of these precursors dock to the SPB in a process that does not require the meiotic plaque. The results, however, support the idea that formation of a continuous membrane

system, the prospore membrane, requires the meiotic plaque. Finally, growth, shaping and closure of the prospore membranes take place.

Results

Identification of meiosis-specific components of the SPB

A hallmark of all previously identified proteins of the SPB core particle (with the exception of Nud1p) is the presence of coiled-coil motifs (Wigge *et al.*, 1998). To identify proteins that are specific for SPBs of meiotic cells, we analyzed genes that are transcriptionally upregulated during meiosis (Chu *et al.*, 1998) for sequences predicted to code for coiled-coil domains (Lupas *et al.*, 1991). A number of candidate genes could be identified (18 genes in total). To determine whether these genes code for components of the meiotic SPB we constructed diploid strains that carried one copy of a C-terminal green fluorescent protein (GFP)-tagged variant of the respective gene by using a PCR-based one-step gene-tagging strategy (Wach *et al.*, 1994; Knop *et al.*, 1999). The strains constructed were induced to undergo synchronous sporulation and the expression and localization of the respective GFP-tagged proteins were then investigated by fluorescence microscopy. A few of these proteins could not be detected at all, while some were found to localize either to the nucleus or to spores. One protein, which we named Don1p, encoded by the open reading frame (ORF) YDR273w, seemed to localize specifically to the prospore membrane (see later sections of this article). Two other proteins appeared as four dots during meiosis II (Figure 1A), a localization that is characteristic for a component of the SPB. These two proteins, encoded by the ORFs YOR177c and YOL091w, were named Mpc54p and Mpc70p, respectively (Mpc = meiotic plaque component). Each of the proteins contains one central domain that is predicted to form coiled-coils (Figure 1B). Mpc54p and Mpc70p were detected at the ends of spindle microtubules in cells in meiosis II by immunofluorescence microscopy (data not shown), consistent with a localization of the two proteins at the SPB. Both proteins could not be detected in mitotic cells and in spores (for a summary see Figure 3A). Immunoblotting confirmed that these proteins were not present in mitotic cells (Figure 1C, time point 0 h). Maximal levels of Mpc54p and Mpc70p were reached towards the end of meiosis II (Figure 1C, 7.5 h). The proteins disappeared after the cells had passed meiosis II (Figure 1C, time points onwards of 8.5 h).

It has been described previously that during meiosis II the SPB grows in size and that the outmost cytoplasmic layer becomes more prominent (Moens and Rapport, 1971; Peterson *et al.*, 1972; Zickler and Olson, 1975). This layer appears first as an amorphous mass on top of the SPBs. During meiosis II it appears as a bi-layered highly electron-dense structure. Therefore, we speculated that Mpc54p and Mpc70p could be components of this meiosis-specific layer of the SPB. To address this question, we performed immunoelectron microscopy (immunoEM) (Figure 1D). The cells used for this experiment expressed GFP fusions of either Mpc54p or Mpc70p and the culture was prepared to have a large percentage of cells in meiosis II (40–60%, as judged by

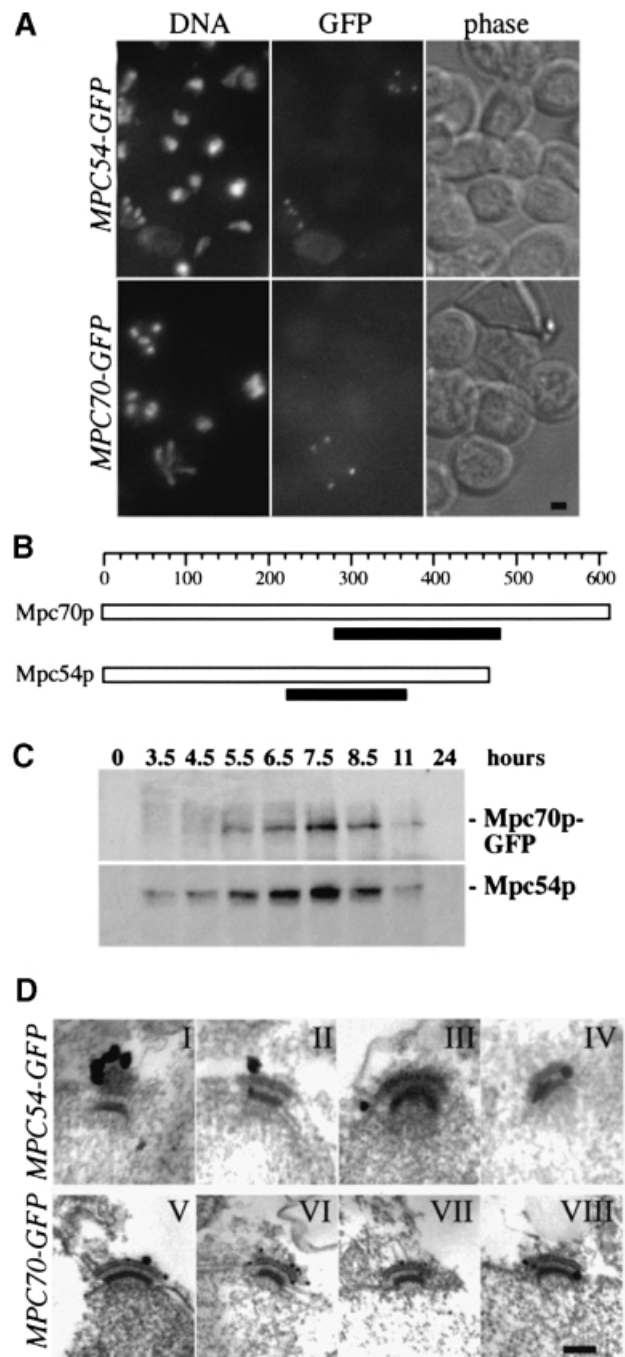


Fig. 1. Mpc54p and Mpc70p are meiosis-specific components of the SPB. (A) GFP fluorescence of Mpc54p-GFP and Mpc70p-GFP in sporulating cultures of strains YMK333-2 and YMK413-2. DNA was stained with DAPI. Phase, phase-contrast. Bar, 2 μ m. (B) Mpc54p and Mpc70p are coiled-coil proteins. The black bars indicate the regions of the proteins that are predicted to form coiled-coil with a probability of 1.0 (numbers: amino acids). (C) Levels of Mpc54p and Mpc70p-GFP in vegetatively growing cells (time point 0 h) and during synchronous sporulation (all other time points). Mpc54p was detected using specific polyclonal antibodies. Mpc70p-GFP was detected using anti-GFP antibodies. Cells used were from strain YMK413-2. Cells in meiosis II reached maximal levels at ~6.5 h, as judged by immunofluorescence. (D) ImmunoEM localization of Mpc54p-GFP and Mpc70p-GFP to an electron-dense plaque of the SPB. Primary rabbit anti-GFP antibodies were detected using 1.4 nm gold particles coupled to specific secondary Fab fragments (Nanoprobes). The gold particles were visualized by silver enhancement. Bar, 100 nm.

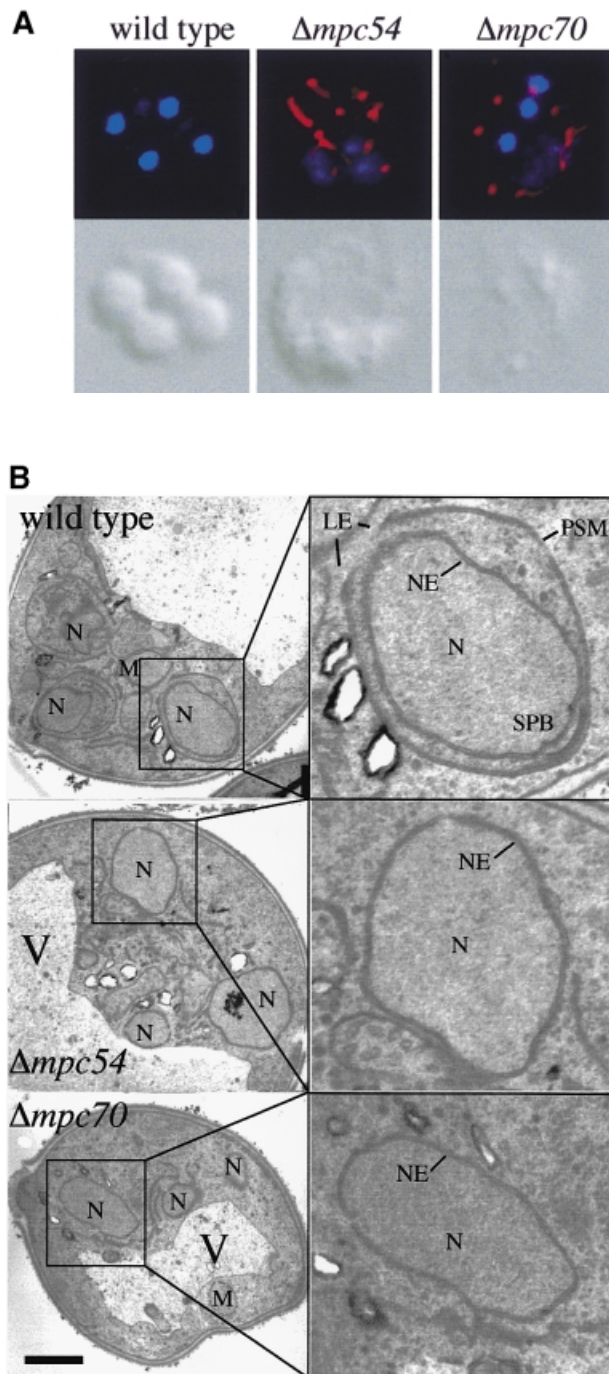


Fig. 2. *mpc54* and *mpc70* mutants are defective in the formation of the prospore membrane. (A) Immunofluorescence staining of DNA (blue) and tubulin (red) in wild-type, $\Delta mpc54$ and $\Delta mpc70$ cells. Cells from strains with the indicated genetic background (homozygous deletions) were synchronously sporulated and samples were taken and prepared for immunofluorescence. Pictures of single cells that have completed meiosis were imaged using deconvolution microscopy. (B) Electron microscopy of permanganate fixed cells. Cells from the above time course were harvested at a time point (7 h) where most cells have proceeded through meiosis II (>80%), as judged by immunofluorescence. Vacuoles (V), nuclei (N) and some mitochondria (M) are indicated. Permanganate fixation allows visualization of membranes; however, structures of the cytoskeleton such as microtubules are not preserved. Bar, 1 μ m. A section of each cell is shown at a higher magnification. SPB, spindle pole body; NE, nuclear envelope; PSM, prospore membrane; LE, leading edge of the prospore membrane.

immunofluorescence and visualization of tubulin and DNA). Mpc54p and Mpc70p were detected at SPBs that carried the meiosis-specific enlarged outer plaque (Figure 1D). The label could be detected on either face of this plaque. No label was found at SPBs that had no visibly enlarged outer plaques (data not shown). For Mpc54p we could also see a labeling of the amorphous structure (Figure 1D, I). Additionally, Mpc54p was sometimes detected at a filamentous extension of this electron-dense outer plaque (Figure 1D, III), which has not been described previously.

Taken together, the results indicate that Mpc54p and Mpc70p are components of a meiosis II-specific enlarged plaque at the cytoplasmic face of the SPB. Since both proteins are only detectable during meiosis, we named this unit of the meiotic SPB the meiotic plaque.

The meiotic plaque is not required for meiotic progression but for the formation of the spore wall

The composition of the mitotic SPB has been investigated in detail (Wigge *et al.*, 1998; Adams and Kilmartin, 1999). Genetic and biochemical analysis of the components revealed a function of the proteins in either SPB duplication and/or microtubule organization. When we analyzed $\Delta mpc54$ or $\Delta mpc70$ cells, which were fully viable, staining of microtubules and the DNA revealed no defects in progression through meiosis I or II (data not shown). Also, the fragments of microtubule bundles characteristic for the completion of meiosis II were visible. In contrast to the wild-type strain, no spores were formed in the mutants and the fragmented microtubules remained detectable (Figure 2A).

To analyze the phenotypes of the mutants in more detail, we prepared samples for electron microscopy from cells at various stages of meiosis. Wild-type cells in meiosis II showed the characteristic membrane structures that engulf the divided nucleus, indicative of the forming prospore membrane (Figure 2B, wild type). In contrast, in the mutants no prospore membranes surrounding the nuclei could be seen (Figure 2B). These findings indicate that the meiotic plaque is required for the formation of the prospore membrane.

SPB components in meiosis

Two-hybrid analysis of the interaction of components of the mitotic SPB, in conjunction with data obtained by immunoEM, allowed the construction of a model for the core of this particle (Adams and Kilmartin, 1999; Elliott *et al.*, 1999) (see Figure 8B for a schematic model of a mitotic SPB). To analyze the composition of meiotic SPBs with respect to known SPB components, we investigated the persistence of known SPB components in meiotic cells. For this purpose we constructed diploid strains that expressed GFP fusions of the set of proteins, which comprises the components of the cytoplasmic and central part of the SPB. These proteins are Spc42p, Cnm67p, Spc72p and Nud1p (Donaldson and Kilmartin, 1996; Brachat *et al.*, 1998; Knop and Schiebel, 1998; Wigge *et al.*, 1998). These strains were then analyzed by fluorescence microscopy for the presence of the respective SPB components during the stages of meiosis and sporulation (Figure 3A). All proteins investigated were

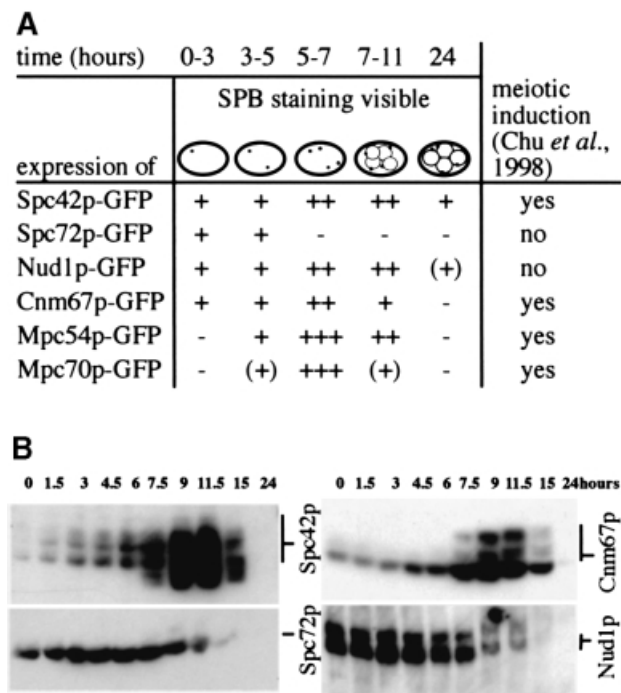


Fig. 3. Localization of mitotic SPB components to meiotic SPBs. (A) Strains expressing GFP fused C-terminally to the indicated genes were synchronously sporulated. The strains are listed in Table I. All GFP fusions are functional (Knop and Schiebel, 1998; Wigge *et al.*, 1998). Samples were taken at intervals of 1 h throughout the entire time course and inspected for the number of GFP dots visible per cell and for the appearance of visible spores. The various stages that are distinguishable are drawn schematically in the table. The intensity of the signal was always compared to vegetatively growing cells of the same strain. '+' denotes vegetative levels; '++' indicates an increased level of localized signal compared with vegetatively growing cells. In the cases of Mpc54p-GFP and Mpc70p-GFP (no signal in vegetative cells) the maximal signal visible is set arbitrarily to '+++'. Data about meiotic transcription upregulation of the indicated SPB components are from Chu *et al.* (1998). (B) Protein amounts of SPB components during sporulation. Samples from synchronously sporulating cells of strain YKS32 were taken at the indicated time points and the proteins indicated were detected using specific antisera (Knop and Schiebel, 1998; Elliott *et al.*, 1999). Multiple bands seen for Spc42p, Nud1p and Cnm67p result from phosphorylation of the proteins. Note that the sporulation conditions used for the experiments in this figure (2% potassium acetate) lead to a slower sporulation than the conditions used for all other experiments (Alani *et al.*, 1990) (see section Materials and methods). In this experiment, cells in meiosis II reached maximal levels ~9 h, as judged by immunofluorescence.

localized at the SPBs at the outset of meiosis. Cnm67p, Spc42p and Nud1p were found to be more abundant at the SPBs in meiosis II compared with meiosis I and mitotic diploid cells (Figure 3A). In contrast, Spc72p disappeared from the SPBs in late meiosis I and was no longer visible in meiosis II. This correlates with the observation that astral microtubules, which depend on the SPB outer plaque component Spc72p (Knop and Schiebel, 1998), are only detected until the end of meiosis I but not during subsequent steps of sporulation (Peterson *et al.*, 1972 and data not shown). For Spc72p, Spc42p and Cnm67p the observations correlated well with the respective changes of protein (Figure 3B) and mRNA levels (Chu *et al.*, 1998; summarized in Figure 3A). Similar to Spc72p, the Nud1p protein levels did not increase during meiosis II (Figure 3B) and no upregulation of the *NUDI* mRNA

was observed (Chu *et al.*, 1998). Therefore, the increased staining seen for Nud1p at the SPBs during meiosis II was most likely due to a recruitment of cytoplasmic pools of the protein to the SPBs. After completion of spore formation, the signal for Cnm67p disappeared and the signal for Nud1p became much fainter. Only the signal for Spc42p was found to be prominent in tetrads (Figure 3A). However, it could no longer be detected in tetrads by immunoblotting (24 h), which can be explained by the difficulty of extracting protein from fully differentiated spores (Figure 3B). We also investigated the localization of Mpc54p and Mpc70p during this time course (Figure 3A), which was performed under sporulation conditions that led to a delay in meiosis I (Alani *et al.*, 1990). We found that Mpc54p was already visible at the end of meiosis I at the SPBs, while for Mpc70p only rarely a faint SPB staining was detected at this stage. This finding correlates well with the observation that upregulation of *MPC54* mRNA occurs at an earlier time point than the upregulation of the *MPC70* mRNA (Chu *et al.*, 1998).

Taken together these data indicate that the meiotic SPB contains the central plaque and most of the outer plaque components. However, Spc72p seems to be replaced by the meiotic plaque, which contains Mpc54p and Mpc70p.

Protein-protein interactions in meiotic SPBs

We tested for two-hybrid interactions among Mpc70p, Mpc54p and the SPB components of the central and outer plaque. As shown in Figure 4A, we found that Mpc70p interacts strongly with itself, with full-length Mpc54p and with the C-terminal half of Nud1p. These interactions were independent of which domain was fused to the *lexA* DNA-binding domain and to the Gal4 activation domain. Mpc70p also showed interactions with Spc42p and the C-terminal domain of Cnm67p. These interactions, however, were observed only for one direction in the two-hybrid assay. For Mpc54p, self-interactions of the full-length proteins as well as the N-terminal and the coiled-coil domains were observed. Similar to Mpc70p, Mpc54p interacts with the C-terminal domain of Nud1p and with Spc42p. No interaction was observed with Cnm67p (Figure 4A). Moreover, Mpc54p and Mpc70p showed no interaction with a functionally unrelated coiled-coil protein (data not shown). The data obtained indicate an interaction of Mpc54p and Mpc70p with at least a subset of the mitotic SPB components of the outer plaque such as with Nud1p.

We looked for additional evidence for an interaction of Mpc54p or Mpc70p with components of the SPB. Therefore we prepared a cytosolic fraction and an SPB-enriched pellet fraction from meiotic cells that expressed protein A (ProA) fused to either Mpc54p or Mpc70p. Before immunoprecipitation, the SPB-enriched fraction was incubated under mild denaturing conditions to allow a fragmentation of the SPBs into soluble subcomplexes. For Mpc54p, we identified conditions (salt concentration, ion chelators) for the SPB fraction under which only a subset of SPB components, in this case Nud1p, co-precipitated (Figure 4B). None of the other SPB components tested (Cnm67p, Spc42p, Kar1p, Spc72p, Spc97p, Spc98p) co-precipitated under these conditions (not shown). This confirms the two-hybrid interaction of Mpc54p with Nud1p and points to a direct interaction of Mpc54p with

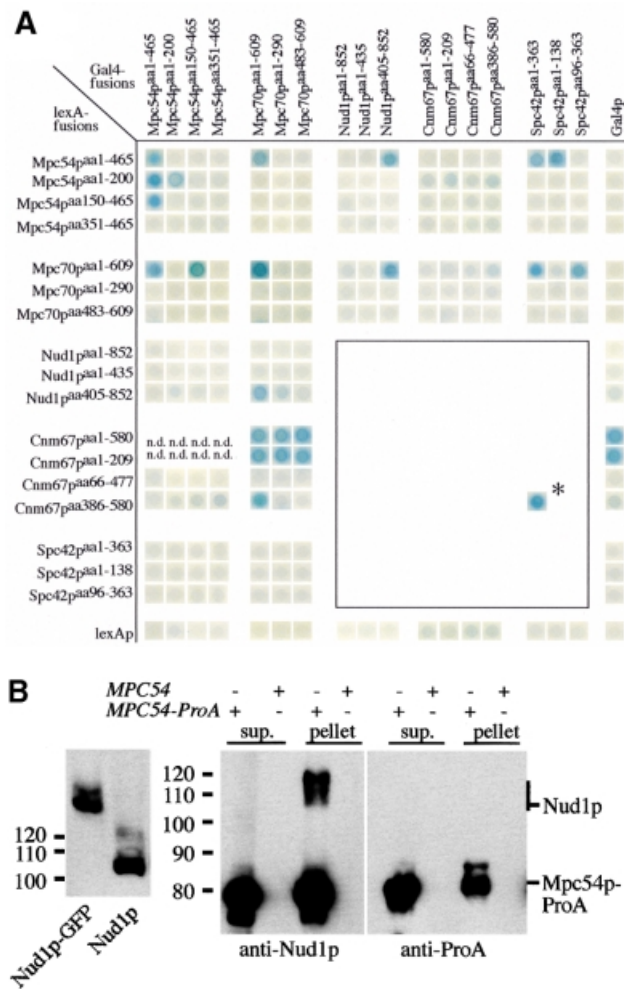


Fig. 4. Mpc54p and Mpc70p interact with components of the SPB outer plaque. (A) Two-hybrid interactions of Mpc70p and Mpc54p with components of the SPB outer plaque. Plasmids expressing the respective LexA or Gal4 fusions were tested for two-hybrid interaction using a β -gal overlay assay. The interactions that would be within the white square have been published previously (Adams and Kilmartin, 1999; Elliott *et al.*, 1999). The interaction shown within this field (*) was used as an internal standard in any test performed. n.d., not determined. (B) Immunoprecipitation of Mpc54p-ProA complexes from a soluble fraction and from high salt fragmented SPBs. Strains YMK363 (*MPC54-ProA*) and YMK367 (wild type) were used. The left panel shows the specificity of the anti-Nud1p antibody. The right panel shows the immunoprecipitates from the soluble (sup.) or the extracted SPB fraction (pellet) that were probed for detection of Nud1p (using anti-Nud1p antibodies) or Mpc54p-ProA (using non-specific antibodies).

Nud1p. Since Nud1p was found to form complexes with Cnm67p and Spc42p using different fragmentation conditions for the SPB (Elliott *et al.*, 1999), the observed two-hybrid interactions of the Mpc54p with Cnm67p and Spc42p might be mediated by Nud1p. For Mpc70p we failed to identify a fragmentation condition that allowed the co-precipitation of a subset of the SPB components. Either all components tested (Spc42p, Nud1p, Cnm67p and Mpc54p) co-precipitated, indicating insufficient fragmentation of the SPBs, or no co-precipitation at all was found (data not shown).

Taken together the data support the notion that the meiotic plaque is attached to proteins of the outer plaque of the SPB.

Mpc54p and Mpc70p are important for the formation of the meiotic plaque

The slight differences in the localization of Mpc70p and Mpc54p to the SPB during different stages of meiosis (Figure 3A) could suggest that the binding of Mpc70p to the SPB could depend on Mpc54p. This could indicate a subsequent assembly of layers of Mpc54p and Mpc70p. To investigate this, the localization of Mpc70p-GFP in Δ *mpc54* cells as well as of Mpc54p-GFP in Δ *mpc70* cells was investigated. In both cases, the respective proteins were still found to localize to the SPB, as indicated by the localization of Mpc70p and Mpc54p in living cells in meiosis (Figure 5A). Additionally, cells were observed that showed fainter dots in the cytoplasm, which could indicate the formation of aggregates. It was confirmed by immunofluorescence (Figure 5B) that the bright dots observed in Figure 5A corresponded to Mpc54p or Mpc70p, which were bound to the SPBs. To address further the effects caused by a deletion of either *MPC54* or *MPC70* or both genes simultaneously, we investigated the structure of the SPBs by electron microscopy. In wild-type cells, the meiotic plaque appeared as a very dense structure, and membranes from the prospore membrane were attached to it (Figure 5C, I). In comparison, in Δ *mpc54* and Δ *mpc70* cells a plaque was still detected, but it was less pronounced than the meiotic plaque (Figure 5C, II and III). Cells deleted for both genes were devoid of such a plaque (Figure 5C, IV). Only the material that connects the central plaque with the meiotic plaque in the wild type is still there. It most likely consists of Cnm67p and Nud1p. This confirms that both proteins can indeed specifically localize to the outer plaque of the SPBs in meiotic cells on their own and that both proteins have the ability to form a plaque. But both proteins have to be present before the meiotic plaque can assemble.

The prospore membrane is formed from specialized precursors

During the initial screen for the localization of meiotically induced coiled-coil proteins we identified a protein that showed a localization that strikingly resembled the pattern one would expect for the precursors of the prospore membrane and for the prospore membrane itself. This protein, which we named Don1p (encoded by the ORF YDR273w), is expressed exclusively during meiosis. Deletion of *DON1* led to no obvious defects in sporulation (data not shown). In Don1p-GFP-expressing cells, a dotted staining became visible when cells had reached early or middle stages of meiosis I (Figure 6A, I and B, I). Towards the end of meiosis I, Don1p localized also near the ends of the microtubules (Figure 6B, II). As soon as the cells had progressed to metaphase of meiosis II, all four ends of the two spindles showed Don1p staining. At the metaphase-to-anaphase transition the staining near the microtubule ends became more prominent and the Don1p dots disappeared from the cytoplasm (Figure 6B, III and IV). At later stages, four ring-like structures (donuts→Don1p) were visible, two of which surrounded each one bundle of microtubules throughout anaphase (Figure 6A, II and B, V and VI). These Don1p rings were detected adjacent to the septin Cdc3p (Figure 6C), which is found in filamentous and patchy structures along the prospore membrane (Fares *et al.*, 1996). Just after

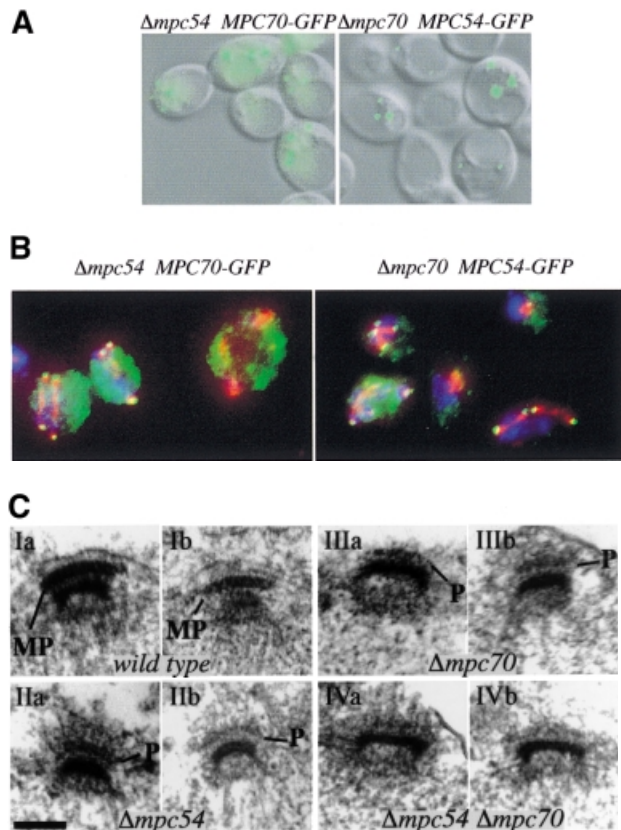


Fig. 5. Mpc54p and Mpc70p are essential for the assembly of the meiotic plaque. (A) Localization of Mpc70p-GFP in $\Delta mpc54$ cells (YMK467) and of Mpc54p-GFP in $\Delta mpc70$ cells (YMK470), as indicated in the figure. Cells were mostly in meiosis I or II. The GFP fluorescence is superimposed with the DIC picture. (B) Immunofluorescence localization of Mpc54p-GFP or Mpc70p-GFP in the strains in (A). Mpc54p-GFP or Mpc70p-GFP are detected using anti-GFP antibodies (green). Microtubules (red) and DNA (blue) are also shown. (C) Structure of the meiotic plaque in wild-type, $\Delta mpc54$, $\Delta mpc70$ and $\Delta mpc54 \Delta mpc70$ mutants, respectively. Two SPBs are shown for each strain. Cells mostly in meiosis II were used and prepared for electron microscopy. Strains used were (I) YKS53, (II) YMK467, (III) YMK470 and (IV) YKS65. The meiotic plaque (MP) and the plaques formed by Mpc54p and Mpc70p, respectively, are indicated (P). Bar, 100 nm.

meiosis II, indicated by fragmented microtubules, the Don1p rings were smaller in size and localized just adjacent to each other in the center of the cell (Figure 6B, VII). As soon as spores became visible, Don1p was found to localize to the prospore wall (Figure 6A, III). Immunofluorescence was performed to investigate in more detail the localization of Don1p in relation to visible structures. In cells that are in meiosis I or early stages of meiosis II (as indicated by the size and appearance of the SPBs), Don1p was found on circular structures above the meiotic plaque (Figure 6D, I and II). As soon as an assembled prospore membrane was visible, Don1p was detected next to the leading edge of this structure at sites where the membrane seems to be covered by a coat (Figure 6D, III–V, see also the enlargement in Figure 2B, wild type). We were not able to visualize the Don1p-containing cytoplasmic structures successfully, mainly because the method for immunofluorescence we had to use to detect Don1p at the prospore

membrane and at the SPB led to an extraction and fragmentation of cytoplasmic contents. We also investigated the localization of Don1p-GFP in a *spo20* mutant. *SPO20* codes for a meiotically expressed yeast homologue of the synaptonemal protein SNAP-25. Spo20p is not required for the formation of the prospore membrane, but is required for the formation of nuclei containing spores (Neiman, 1998). Consistent with this, deletion of *SPO20* did not abolish the formation of Don1p containing rings (Figure 6E, V and VI). This indicates that the assembly of the prospore membranes *per se* is not affected. However, the subsequent growth of the prospore membrane seemed to be delayed and the proper shaping of the membrane was impaired (compare Figure 6E with B).

These findings demonstrate that the prospore membrane is formed from specific precursors. Don1p is the first protein known to localize to these precursors and to the leading edge of the prospore membrane. This suggests that Don1p could be a unique marker to investigate the defects associated with the impaired function of the meiotic plaque in the *mpc*⁻ mutants.

Mpc54p and Mpc70p are required for the assembly of the prospore membrane at the SPB

The data obtained so far link the occurrence of the meiotic plaques at the SPBs with the ability of the cell to form the prospore membranes. However, Mpc54p and Mpc70p might have additional functions that are required for earlier steps in the biogenesis of the prospore membrane, e.g. during the formation of precursors of the prospore membrane. To address this possibility we investigated the localization of Don1p-GFP in $\Delta mpc54$ and $\Delta mpc70$ mutant cells (Figure 7A). In both cases, Don1p accumulated in the cytoplasm during meiosis in a dot-like fashion. However, we never observed cells where Don1p appeared as rings (compare Figure 7A and B with 6A and B). This indicates that the precursors of the prospore membrane can be formed, but their assembly into the prospore membranes is prevented. As shown by immunofluorescence, dots of Don1p were detected not only in the cytoplasm but also at the SPBs (Figure 7B). Using deconvolution microscopy and three-dimensional (3D) rendering of the cells we verified the localization of Don1p to the SPBs. (The panels in Figure 7 show planar projections of the 3D-rendered cells.) Thus, the meiotic plaque is not required for the localization of Don1p to the SPBs. It could suggest, however, that Mpc54p and Mpc70p, which can bind independently of each other to the SPBs, could also function independently in the localization of Don1p to the meiotic plaque. Therefore, the localization of Don1p was investigated in the $\Delta mpc54 \Delta mpc70$ double deletion strain. Also, in this case Don1p was able to bind to the SPB during meiosis (Figure 7B). This indicates that Mpc54p and Mpc70p are not required for the binding of Don1p to the SPB but for the subsequent formation of Don1p rings. Don1p was detected at round structures at the SPBs in cells that have not yet started to assemble the prospore membranes (Figure 6D, I and II). Similar round structures can also be seen at SPBs in the *mpc* mutants (Figure 5C, II–IV). We thus concluded that the meiotic plaque plays an essential function in the assembly of the precursors of the prospore membrane to one continuous membrane system.

Discussion

Molecular composition of the SPB in meiosis

SPBs are involved in the nucleation and organization of microtubules during cell division. Nuclear microtubules have essential functions in mitosis and meiosis. Cytoplasmic microtubules are required for nuclear movements during mitosis, but during meiosis, they are no longer detectable when cells have passed meiosis I (Peterson *et al.*, 1972 and data not shown). This indicates that cytoplasmic microtubules are no longer required for the completion of meiosis. Instead, the outer plaque, which organizes the cytoplasmic microtubules, seems to thicken during meiosis II and becomes involved in the binding of the prospore membrane to the nuclear envelope. In this paper we describe the identification of two components of the meiotic SPB. These proteins, Mpc54p and Mpc70p, are structural components of the characteristic thickened outer plaque of meiotic SPBs, which we termed the meiotic plaque. The meiotic plaque is the most distal structure of the cytoplasmic side of the SPB in meiosis II. Consistent with the observation that cytoplasmic microtubules are absent during meiosis II is the fact that deletion of *MPC54* or *MPC70* does not lead to a defect in the organization of microtubules by the SPB. We observed that all components of the SPB outer plaque, with the notable exception of Spc72p, are present at the SPBs in meiosis II. Interestingly, Spc72p is the receptor for the γ -tubulin complex of the outer plaque of the SPB, required for cytoplasmic microtubule nucleation and organization (Knop and Schiebel, 1998). Our two-hybrid assays indicate that Mpc54p and Mpc70p interact with components of the SPB. It is not clear whether all interactions that were observed resulted from direct protein interactions. Spc42p is a component of the central plaque of the SPB. This indicates that the observed interaction of Spc42p with Mpc54p and Mpc70p might be mediated by Nud1p and/or Cnm67p. However, the interaction of Mpc54p and possibly also of Mpc70p with Nud1p could be direct, because Mpc54p can be co-immunoprecipitated with Nud1p in the absence of precipitation of Cnm67p and Spc42p. This suggests that the meiotic plaque assembles with the SPB via Nud1p. The findings further suggest that the core of the SPB (Adams and Kilmartin, 1999) has the same composition in mitosis and meiosis (for a model see Figure 8B). Spc72p, which is not part of the core of the

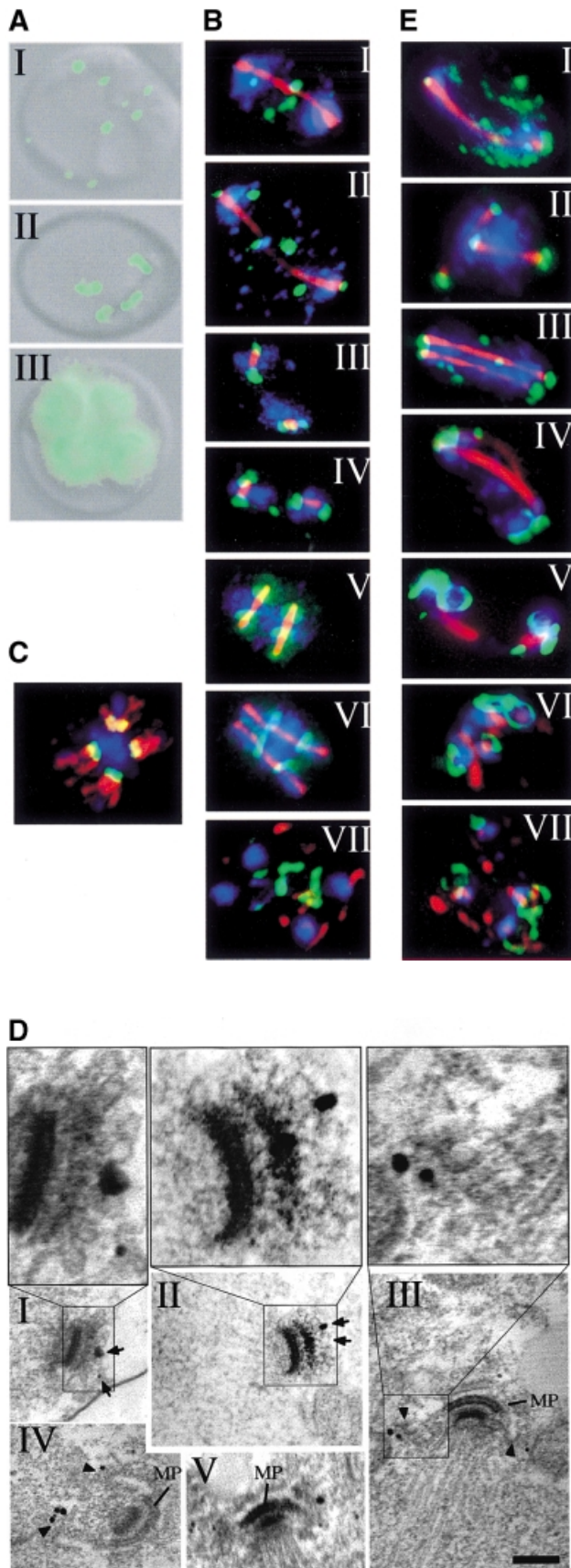


Fig. 6. Don1p is a specific marker for the prospore membrane.

(A) Localization of Don1p-GFP in cells at various stages of meiosis. Living cells were imaged by deconvolution microscopy and the fluorescence picture (green) was overlaid with the phase-contrast picture. (B) Immunofluorescence microscopy staining showing Don1p-GFP (green), tubulin (red) and DNA (blue) in cells of strain YKS53 (*DON1-GFP*). Single cells from different stages of meiosis are shown in the order of the meiotic events. (C) Localization of Cdc3p (red), Don1p-GFP (green) and DNA (blue) in a cell in meiosis II of strain YKS53. (D) Localization of Don1p-GFP by immunofluorescence. The experiment was performed as described in Figure 1B. Arrows point to round vesicular structures on top of an SPB where Don1p was localized. Arrowheads point to the leading edge of the prospore membrane. Three-fold enlargements of some of the SPBs (I, II) and of the leading edge of the prospore membrane (III) are shown. The meiotic plaque (MP) is also indicated. Bar, 125 nm. (E) As in (B) for a *spo20* mutant strain (YKS72).

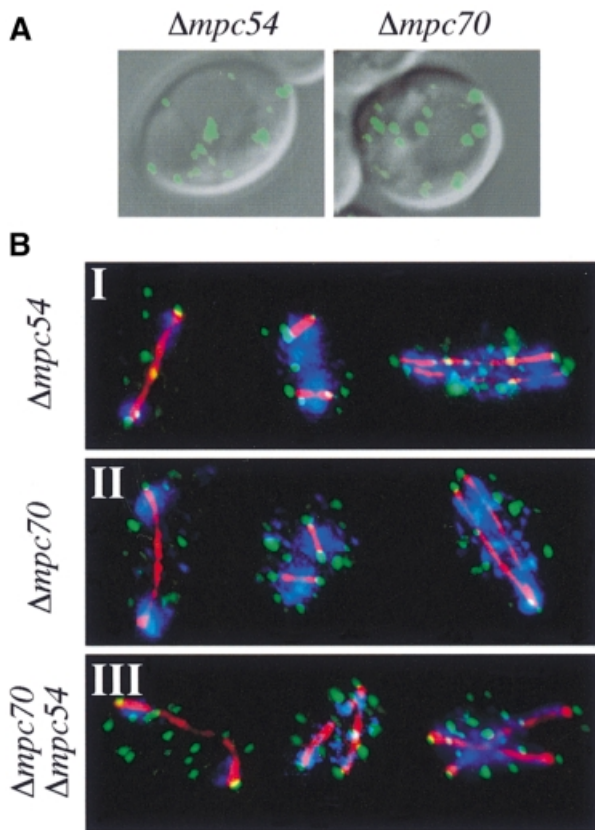


Fig. 7. Mpc54p and Mpc70p are required for the assembly of precursors of the prospore membrane at the SPB. (A) Imaging of living cells expressing *DON1-GFP* after progression through meiosis II. Strains used were YMK473 (Δ *mpc54* *DON1-GFP*) and YKS49-3 (Δ *mpc70* *DON1-GFP*). (B) Immunofluorescence localization of tubulin (red), DNA (blue) and Don1p-GFP (green) in cells of the strains in (A), or in cells of strain YKS65 (Δ *mpc54* Δ *mpc70* *DON1-GFP*). Three cells representative for meiosis I (left cells), early (middle) or late meiosis II (right cell) are shown.

SPB, is replaced by the meiotic plaque for the formation of the spore.

Prospore membrane assembly and the meiotic plaque

The morphological processes leading to the formation of the prospore membrane have been characterized previously using electron microscopy (Lynn and Magee, 1970; Moens and Rapport, 1971; Peterson *et al.*, 1972; Zickler and Olson, 1975). Precursors of the prospore membranes could not be identified, however, due to lack of characteristic structures or markers. We showed that Don1p, which we identified by the GFP fluorescence localization-based screening, might be such a marker. We showed that Don1p localized to cytoplasmic structures, visible as dots, which then became assembled to the prospore membrane. This strongly suggests the formation of specific precursors of the prospore membrane, which takes place during meiosis I. During growth of the prospore membrane, Don1p was detected at the leading edge of the membrane (a model is shown in Figure 8B). Finally, in tetrads Don1p localizes to the spore wall.

We were interested in identifying the structures to which Don1p localized. By immunoEM, we could localize

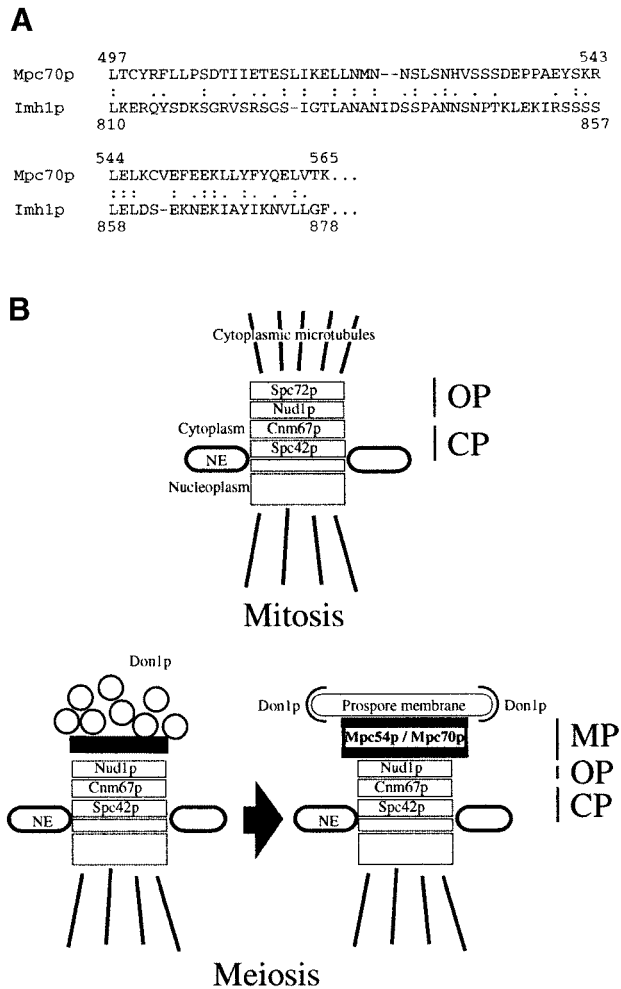


Fig. 8. Model for the assembly of the prospore membrane at the SPB. (A) Alignment of Imh1p and Mpc70p. The alignment shows a region of high homology, which lies outside of the regions of both proteins which were predicted to form coiled-coils. The alignment was generated using W.R.Person's SSEARCH program (version 2.0u). (B) The top scheme shows a mitotic SPB and the proposed localization of the proteins relevant to this work (Adams and Kilmartin, 1999; Elliott *et al.*, 1999). The scheme at the bottom shows a model for the proposed function of the meiotic plaque, as well as the localization of Mpc54p and Mpc70p. The position of the discussed substructures of the SPB is indicated (OP, outer plaque; MP, meiotic plaque; CP, central plaque). NE, nuclear envelope.

Don1p to two different types of structures. The first were round structures at the cytoplasmic face of SPBs that had no meiotic plaque. These round structures might be the membranes or the coats of vesicles that bind to the SPB at stages before the prospore membrane starts to assemble. This correlates with the finding, obtained by immunofluorescence microscopy, that Don1p can be found at the SPB at time points before the meiotic plaque is there. When the prospore membrane was detected in the electron microscope, which was always the case when a meiotic plaque was visible, Don1p localized to a coat-like structure covering at some distance the leading edge of this membrane. This localization explains the rings seen by immunofluorescence microscopy of cells at this stage. Finally, formation of a closed prospore membrane led to a dispersion of Don1p along the prospore wall. Taking these results together, Don1p appears to label specifically

Table I. Yeast strains and plasmids

| Name | Genotype construction | Source or reference |
|----------------------|---|---------------------------------|
| Yeast strains | | |
| NKY289 | <i>Mata ura3 lys2 ho::hisG</i> | Alani <i>et al.</i> (1987) |
| NKY292 | <i>Mata ura3 lys2 leu2::hisG ho::LYS2</i> | Alani <i>et al.</i> (1987) |
| YKS32 | diploid obtained by crossing NKY289 and NKY292 | this study |
| 1382-7C | <i>Mata his4-N met4 ura3 leu2 trp1 lys2 ho::LYS2</i> | Cold Spring Harbor yeast course |
| 1383-5C | <i>Mata his4-G met13 ura3 leu2 trp1 lys2 ho::LYS2</i> | Cold Spring Harbor yeast course |
| YMK333-2 | <i>MATA/α his4-N/his4-G met4/MET4 MET13/met13 ura3/ura3 leu2/leu2 trp1/trp1 lys2/lys2 ho::LYS2/ho::LYS2 ZIP1/Zip1-9Myc-kITRP1 MPC54/MPC54-eGFP-kanMX</i> | this study |
| YMK363 | <i>MATA/α his4-N/his4-G met4/MET4 MET13/met13 ura3/ura3 leu2/leu2 trp1/trp1 lys2/lys2 ho::LYS2/ho::LYS2 MPC54-TEV-ProA-7HIS-kanMX/MPC54-TEV-ProA-7HIS-kanMX</i> | this study |
| YMK367-2 | <i>MATA/α his4-N/his4-G met4/MET4 MET13/met13 ura3/ura3 leu2/leu2 trp1/trp1 lys2/lys2 ho::LYS2/ho::LYS2</i> | this study |
| YMK386 | <i>Mata/α his4-N/his4-G met4/met13 ura3/ura3 leu2/leu2 trp1/trp1 lys2/lys2 ho::LYS2/ho::LYS2 SPC42-eGFP-kanMX/SPC42-eGFP-kanMX</i> | this study |
| YMK402 | <i>Mata/α his4-N/his4-G met4/met13 ura3/ura3 leu2/leu2 trp1/trp1 lys2/lys2 ho::LYS2/ho::LYS2 SPC72-eGFP-kanMX/SPC72-eGFP-kanMX</i> | this study |
| YMK403 | <i>Mata/α his4-N/his4-G met4/met13 ura3/ura3 leu2/leu2 trp1/trp1 lys2/lys2 ho::LYS2/ho::LYS2 CNM67-eGFP-kanMX/CNM67-eGFP-kanMX</i> | this study |
| YMK404 | <i>Mata/α his4-N/his4-G met4/met13 ura3/ura3 leu2/leu2 trp1/trp1 lys2/lys2 ho::LYS2/ho::LYS2 NUD1-eGFP-kanMX/NUD1-eGFP-kanMX</i> | this study |
| YMK413-2 | <i>Mata/α his4-N/his4-G met4/met13 ura3/ura3 leu2/leu2 trp1/trp1 lys2/lys2 ho::LYS2/ho::LYS2 MPC70-eGFP-kanMX/MPC70-eGFP-kanMX</i> | this study |
| YMK467 | <i>Mata/α leu2::hisG/LEU2 lys2/lys2 ho::hisG/ho::LYS2 Δmpc54::kanMX/Δmpc54::kanMX MPC70-eGFP-kanMX/ MPC70-eGFP-kanMX</i> | this study |
| YMK470 | <i>Mata/α leu2::hisG/leu2::hisG lys2/lys2 ho::LYS2/ho::LYS2 Δmpc70::kanMX/Δ mpc70::kanMX MPC54-eGFP-kanMX/MPC54-eGFP-kanMX</i> | this study |
| YMK473 | <i>Mata/α leu2::hisG/LEU2 lys2/lys2 ho::hisG/ho::LYS2 Δmpc54::kanMX/Δmpc54::kanMX DON1-eGFP-kanMX/DON1-eGFP-kanMX</i> | this study |
| YKS49-3 | <i>Mata/α leu2::hisG/LEU2 lys2/lys2 ho::hisG/ho::LYS2 Δmpc70::kanMX/Δmpc70::kanMX DON1-eGFP-kanMX/DON1-eGFP-kanMX</i> | this study |
| YKS53 | <i>Mata/α ura3/ura3 LEU2/leu2::hisG lys2/lys2 ho::hisG/ho::hisG DON1-eGFP-kanMX/ DON1-eGFP-kanMX</i> | this study |
| YKS65-1 | <i>Mata/α LEU2/LEU2 ho::LYS2/ho::LYS2 lys2/lys2 Δmpc70-kanMX/Δmpc70-kanMX Δmpc54-kanMX/Δmpc54-kanMX DON1-eGFP-kanMX/DON1-eGFP-kanMX</i> | this study |
| YKS72 | <i>Mata/α leu2/LEU2 lys2/lys2 ho::LYS2/ho::hisGΔspo20::kanMX/Δspo20::kanMX DON1-eGFP-kanMX/DON1-eGFP-kanMX</i> | this study |
| YPH499 | <i>MATA ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1</i> | Sikorski and Hieter (1989) |
| Plasmids | | |
| pRS416 | CEN4, URA3 and ARS containing yeast <i>E.coli</i> shuttle vector | Sikorski and Hieter (1989) |
| pMM14 | pRS416 containing <i>MPC54</i> | this study |
| pKS1 | pRS416 containing <i>MPC70</i> | this study |
| pATH1 | bacterial expression plasmid with the trp promoter and most of trpE | Koerner <i>et al.</i> (1991) |
| pET28c(+) | <i>E.coli</i> expression vector | Novagen |
| pGEX-5X-1 | <i>E.coli</i> expression vector containing GST under control of the <i>lacZ</i> promoter | Pharmacia |
| pMM5 | p423-Gal1 carrying the the <i>lexA</i> DNA-binding domain and a Myc tag | Schramm <i>et al.</i> (2000) |
| pMM6 | p425-Gal1 carrying the the Gal4 activation domain and an HA tag | Schramm <i>et al.</i> (2000) |
| pMM15 | pATH1 containing codons 1–200 of <i>MPC54</i> | this study |
| pMM17 | pET28c(+) containing codons 1–200 of <i>MPC54</i> | this study |

precursor membranes and the prospore membranes during the entire course of formation of spores.

In cells where *MPC54* or *MPC70*, or both genes simultaneously, are deleted, the integrity of the meiotic plaque was affected. In this experiment the round structures at the SPB could still be seen (Figure 5C), which correlates with the ability of Don1p to localize to the SPBs also in the *mpc*⁻ mutants. It is likely that the round structures are vesicles that are precursors of the prospore membrane. Therefore, one could postulate the following model (Figure 8B). Specialized vesicles are formed and targeted via a specific protein(s) to the SPB outer plaque. Subsequent assembly of the meiotic plaque leads to the fusion of these vesicles to a continuous membrane, the prospore membrane. This would of course require that components of membrane fusion machinery become localized to the SPB, which could be one of the functions

of the meiotic plaque. To explain why the cytoplasmic pool of Don1p localized to dot-like structures, we favor a model in which the dots represent vesicles that are tethered together prior to their delivery to the SPBs.

When we analyzed the sequences of Mpc54p and Mpc70p, we found that Mpc70p shows homology to Imh1p, a protein involved in Golgi-associated protein transport together with Ypt6p, a member of the Rab family (Tsukada *et al.*, 1999). The homology of Mpc70p to Imh1p is not restricted to the coiled-coil regions of the proteins. Also, amino acids (aa) 810–878 of Imh1p show 28% identity and 51% similarity to aa 497–565 of Mpc70p (Figure 8A). A relationship between Imh1p and Mpc70p is further suggested by the localization of the respective genes on chromosome regions that could have arisen from an ancient chromosome duplication (Seoighe and Wolfe, 1999).

Previously, many other coiled-coil proteins have been shown to be involved in the organization of membranes. Examples are the function of the Rabaptins in endosome fusion (Gournier *et al.*, 1998), Uso1p in endoplasmic reticulum to Golgi trafficking (Sapperstein *et al.*, 1996), and the numerous proteins involved in the organization of Golgi membranes during mitosis (Lowe *et al.*, 1998). The Mpc⁻ proteins might fulfill similar functions in the process of prospore membrane biogenesis. The main difference to these systems, however, remains in the fact that the Mpc proteins are not cytosolic or solely membrane-associated factors, but that they fulfill their function as components of a large macromolecular structure that is a unit of the SPB. This may have several advantages. The number of prospore membranes is restricted to the number of SPBs and the orientation of the SPB ensures the proper orientation of the prospore membrane with respect to the structures that have to become encapsulated.

The SPB is further required for the anchorage of the prospore membrane to the nuclear envelope during the growth of the prospore membrane. The meiotic plaque must also fulfill this function, as it remains closely attached to the prospore membrane during this process. We found that mitotically overexpressed Mpc70p formed large aggregates that were always attached to the plasma membrane (data not shown). This indicates that Mpc70p can interact with lipids or a protein of the plasma membrane, and supports the hypothesis made by Neiman that the prospore membrane is related to the plasma membrane (Neiman, 1998).

The process of prospore membrane assembly in yeast could be analogous to membrane assembly processes observed in different higher eukaryotic cell systems. For example, cellularization of the multinucleated syncytial blastoderm during *Drosophila* embryogenesis requires the intracellular formation of plasma membranes (Loncar and Singer, 1995). In addition, gametophyte development and phragmoblast formation in plants involve intracellular synthesis and assembly of plasma membrane equivalent membranes (reviewed by McCormick, 1993; Staehlin *et al.*, 1996). Therefore, a molecular understanding of the formation and the shaping of the prospore membrane as well as of the underlying regulatory mechanisms may help understanding of similar processes in other systems in the future.

Materials and methods

Yeast strains, growth media and growth conditions

The genotypes of strains used in this work are listed in Table I. Basic yeast methods and growth media were as described previously (Guthrie and Fink, 1991). All yeast strains used are derivatives of SK-1 strains. Mating of haploid SK-1 cells was carried out on YPD plates for 6–8 h and diploid colonies were selected on YP-glycerol plates based on their specific colony shape. Chromosomal manipulations of yeast strains (gene deletions and C-terminal gene tagging) were performed using PCR-amplified cassettes as described previously (Wach *et al.*, 1994; Knop *et al.*, 1999). In all cases, the correct integration of the cassettes was verified by PCR. Out-crossed strains were verified by PCR for the presence of tags and/or disruptions and (in the case of homozygous deletions) for the absence of coding sequences of the deleted ORF(s) using chromosomal DNA of the respective strains and appropriate primer combinations. The C-terminal GFP or ProA fusions to *MPC54* or *MPC70* did not affect the functions of the proteins, as the homozygously tagged strains showed similar sporulation kinetics and frequencies to wild-type strains.

Synchronous sporulation was performed as described previously (Cao *et al.*, 1990). DAPI staining was performed routinely to monitor progression through meiosis. Sporulation media used were either 2% potassium acetate (experiments shown in Figure 3) or 0.3% potassium acetate and 0.02% raffinose.

Plasmid construction

Plasmids are listed in Table I. Construction was performed using standard procedures (Sambrook *et al.*, 1989). Cloned PCR products were either sequenced, or, in the case of the two-hybrid constructs, PCR products from two independent reactions were cloned and tested. The bacterial strains used were RR1 (for expression of TrpE fusions), DH5 α and SURE. *MPC70* and *MPC54* were cloned using chromosomal DNA of strain YPH499 (Sikorski and Hieter, 1989) with primers designed to amplify ~350 bp of flanking sequences. Potential PCR errors were eliminated by the 'gap repair' technique. Two-hybrid plasmids were constructed using primers designed to amplify the desired part of an ORF (as listed in Figure 4A). The primers contained either a *Bam*HI (5' primer) or a *Xho*I (3' primer) restriction site.

Antibody production

Antibodies specific for the N-terminal 200 aa of Mpc54p were raised against a TrpE fusion protein expressed from plasmid pMM15. The antiserum was affinity purified with purified His-tagged N-Mpc54p (aa 1–200, expressed from plasmid pMM17) that was coupled to BrCN–Sephacrose (Pharmacia).

Immunofluorescence, deconvolution, electron and immunoelectron microscopy

Immunofluorescence was performed as described previously (Knop and Schiebel, 1998). Fixation times for detection of Mpc54p–GFP and Mpc70p–GFP were 15 min; for all other experiments the cells were fixed for 105 min. Primary antibodies were mouse monoclonal anti- β -tubulin Wa3 (a gift from U. Euteneuer-Schliwa), rabbit anti-GFP (a gift from Pamela Silver), rabbit anti-Cdc3p (a gift from Tim Mitchison) or rabbit anti-N-Mpc54p (this study). Secondary antibodies were goat anti-rabbit ALEXA₄₈₈ (Molecular Probes) or donkey anti-mouse Cy5 (Jackson ImmunoResearch Laboratories).

For deconvolution microscopy, sections throughout the cells (spacing of 0.3 μ m) were collected using a z-axis focus drive and a 100 \times Plan-Apo objective (Leica). Deconvolution of the stacked images (McNally *et al.*, 1999) was performed using appropriate parameters for the individual chromophores and was carried out in a way that the result represented the picture seen by eyes, except that blurs from fluorescence out of the focal plane were removed. The staple of deconvolved images was then 3D rendered. The pictures shown in Figures 2A, 6 and 7 show planar projections of the 3D-rendered cells. All other immunofluorescence pictures are obtained by merging a staple of pictures without deconvolution.

For immunoelectron microscopy, the protocol of Adams and Kilmartin (1999) was followed (fixation times used for detection of Mpc54p–GFP, 13 min; for Mpc70p–GFP, 3 min; and for Don1p–GFP, 3 h), with the exception that the cells were treated with β -ME (Zickler and Olson, 1975) prior to digestion. Permanganate (K₂MnO₄) fixation (experiment in Figure 2B) was performed as described previously (Neiman, 1998), except that Agar 100 was used instead of Epon 812. For the experiment shown in Figure 5C, the cells were fixed and digested as described previously (Adams and Kilmartin, 1999) (fixation time 105 min). After digestion, the cells were postfixed with 2.5% glutaraldehyde in 50 mM KPO₄ pH 7 for 2 h and washed with water. Thereafter the protocol of Byers and Goetsch (1975) was followed, starting with the OsO₄ step.

Two-hybrid assay

Gene fusions with the DNA-binding domain of *lexA* were made using either plasmid pEG202 (Gyuris *et al.*, 1993) or plasmid pMM6 (Table I). Plasmids pGAD424 (Durfee *et al.*, 1993) and pMM5 (Table I) were chosen as activation domain (Gal4p) vectors. Cells harboring the listed gene fusions (Figure 3A) were assayed for two-hybrid interaction as described previously (Schramm *et al.*, 2000). The two-hybrid plasmids for Nud1p, Spc42p and Cnm67p have been described previously (Elliott *et al.*, 1999).

Cell lysis, immunoprecipitation and immunoblotting

Extraction of proteins from yeast cells was performed as described previously (Knop *et al.*, 1999). For immunoprecipitation, sporulating cells at the desired stage were incubated with phenylmethylsulfonyl fluoride (PMSF) (2 mM) for 5 min, harvested, frozen in liquid nitrogen

and stored at -80°C . Cell lysis and high salt extraction for immunoprecipitation of ProA-tagged Mpc54p (Mpc54p-ProA) were essentially carried out as described previously (Elliott *et al.*, 1999). Extraction buffer was 0.5 M NaCl, 50 mM Tris-HCl pH 7.5, 10 mM EDTA, 2 mM EGTA, 1% Triton X-100. Immunoprecipitation was performed using non-specific rabbit IgGs coupled covalently to Dynabeads M-280 (Dyna).

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