Phospholipase D and the SNARE Sso1p are necessary for vesicle fusion during sporulation in yeast

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Summary

Spore formation in *Saccharomyces cerevisiae* requires the de novo formation of prospore membranes. The coalescence of secretory vesicles into a membrane sheet occurs on the cytoplasmic surface of the spindle pole body. Spo14p, the major yeast phospholipase D, is necessary for prospore membrane formation; however, the specific function of Spo14p in this process has not been elucidated. We report that loss of Spo14p blocks vesicle fusion, leading to the accumulation of prospore membrane precursor vesicles docked on the spindle pole body. A similar phenotype was seen when the t-SNARE Sso1p, or the partially redundant t-SNAREs Sec9p and Spo20p were mutated. Although phosphatidic acid, the product of phospholipase D action, was necessary to recruit Spo20p to the precursor vesicles, independent targeting of Spo20p to the membrane was not sufficient to promote fusion in the absence of *SPO14*. These results demonstrate a role for phospholipase D in vesicle fusion and suggest that phospholipase D-generated phosphatidic acid plays multiple roles in the fusion process.

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Introduction

Phosphatidylcholine (PC)-specific phospholipase D (PLD) is a conserved enzyme that catalyzes the removal of the choline headgroup of PC to produce phosphatidic acid (PA) (Exton, 2000; McDermott et al., 2004). In mammalian cells, PLD has been proposed to function in a variety of cellular processes. The activity of mammalian PLD can be stimulated by Arf and Rho GTPases implicating PLD in such diverse events as vesicle trafficking, signal transduction, and regulation of the actin cytoskeleton (Cockcroft et al., 1994; Kahn et al., 1993; Malcolm et al., 1994; McDermott et al., 2004). In particular, in a number of different cell types PLD has been shown to have a role in vesicular trafficking events including transport from the Golgi (Chen et al., 1997; Freyberg et al., 2001), endocytosis (Du et al., 2004) and regulated exocytosis (Choi et al., 2002; Hughes et al., 2004; Vitale et al., 2001). In baker's yeast, the single PC-specific PLD isozyme is encoded by the *SPO14* gene (Rose et al., 1995). In vegetative cells Spo14p is localized throughout the cytoplasm and *spo14* mutants display no strong phenotypes (Rose et al., 1995). However, *spo14* mutants cannot sporulate (Rose et al., 1995).

Sporulation in yeast requires the creation of a new membrane compartment, the prospore membrane, which encapsulates the nuclei that arise from the meiotic divisions (Byers, 1981; Moens, 1971; Neiman, 1998). During meiosis II, a specialized structure termed the meiotic outer plaque is formed on the cytoplasmic face of each of the four spindle pole bodies (SPBs) (Byers, 1981; Moens and Rapport, 1971). These outer plaques serve as initiation sites for prospore membrane formation. Post-Golgi secretory vesicles are localized to each SPB and coalesce to form four distinct prospore membranes (Neiman, 1998). By continuing fusion of new vesicles, the prospore membranes expand, engulf, and finally enclose the daughter nuclei resulting in the generation of immature spores. As cells enter meiosis II, Spo14p relocalizes from the cytoplasm to the prospore membrane (Rudge et al., 1998). In *spo14* mutants, sporulation is blocked because of an absence of prospore membranes, suggesting a role for Spo14p in vesicular trafficking during sporulation (Rudge et al., 1998). Further, the catalytic activity of Spo14p is required for its function in sporulation, indicating that generation of PA is essential for assembly of prospore membranes (Rudge et al., 1998; Rudge et al., 2004).

In addition to *SPO14*, several other genes are necessary for prospore membrane formation. Many of these encode proteins required for vesicle fusion at the plasma membrane in vegetative cells (Neiman, 1998). However, a number of these are similar to *SPO14* in that they are uniquely required for assembly of the prospore membrane. For instance, the meiotic outer plaque components Mpc54p, Spo21p/Mpc70p and Spo74p form a complex at the initiation site for prospore membrane assembly (Bajgier et al., 2001; Knop and Strasser, 2000; Nickas et al., 2003). In the absence of any of these proteins, no prospore membranes are formed. In addition, specific SNAREs are

required to mediate the fusion of vesicles with the prospore membrane. The sporulation-specific SNARE Spo20p, a paralog of the plasma membrane SNARE Sec9p, is required for prospore membrane growth (Neiman, 1998). Spo20p and Sec9p display a partial redundancy in that *sec9* mutants have no sporulation phenotype, *spo20* mutants form defective prospore membranes and *spo20 sec9* double mutants lack prospore membranes entirely (Neiman, 1998). An additional difference between fusion of vesicles at the plasma membrane and prospore membrane is suggested by the observation that the t-SNARE Sso1p is required for spore formation, whereas Sso1p and Sso2p are redundant with respect to vegetative growth (Aalto et al., 1993; Jantti et al., 2002), though the precise nature of the sporulation defect in *sso1* cells has not been reported.

In this study, we report that *spo14* mutants accumulate precursor vesicles to the prospore membrane on and around the meiotic outer plaque, indicating that *SPO14* is required for vesicle fusion. Similar accumulations are seen in mutants lacking the t-SNARE Sso1p or both Sec9p and Spo20p SNAREs, and in cells expressing a catalytically inactive Spo14p. These studies reveal a role for PLD and its product, PA, in membrane fusion.

Results

spo14 mutants accumulate docked, unfused vesicles on the SPB during meiosis II

Cells lacking the PLD encoded by *SPO14* do not form prospore membranes (Rudge et al., 1998). As part of an ongoing study of the meiotic SPB, we examined the SPBs in *spo14* mutants in the transmission electron microscope (TEM) as a means of visualizing intact outer plaques lacking attached membranes. We found that the SPBs of *spo14* mutants were decorated with vesicles (Fig. 1A,B). Electron tomography was used to visualize the distribution of vesicles around the SPB in *spo14* cells (Fig. 1C-H; supplementary material Movie 1). The vesicle membranes appeared to be in direct contact with the surface of the outer plaque (Fig. 1E,F). Tomographic reconstructions demonstrated that these docked vesicles were arranged across the surface of the SPB in a regular pattern (Fig. 1G,H; supplementary material Movie 2). The vesicles were relatively uniform in size with an average diameter of 60 nm, consistent with previous measurements of post-Golgi secretory vesicles in *S. cerevisiae* (Mulholland et al., 1997). Reconstructions of several different *spo14* SPBs showed similar patterns. In each case the surface of the outer plaque was nearly fully occupied with vesicles. In addition to the vesicles on the surface of the SPB, additional vesicles appeared to be clustered in the vicinity of the spindle pole. The number of these additional vesicles varied between SPBs from a few vesicles to more than 40.

The presence of intact vesicles docked to the SPB in *spo14* cells suggests that the absence of prospore membranes in these cells is due to a defect in the fusion of vesicle precursors to the prospore membrane. Mutation of the t-SNARE *SSO1* blocks

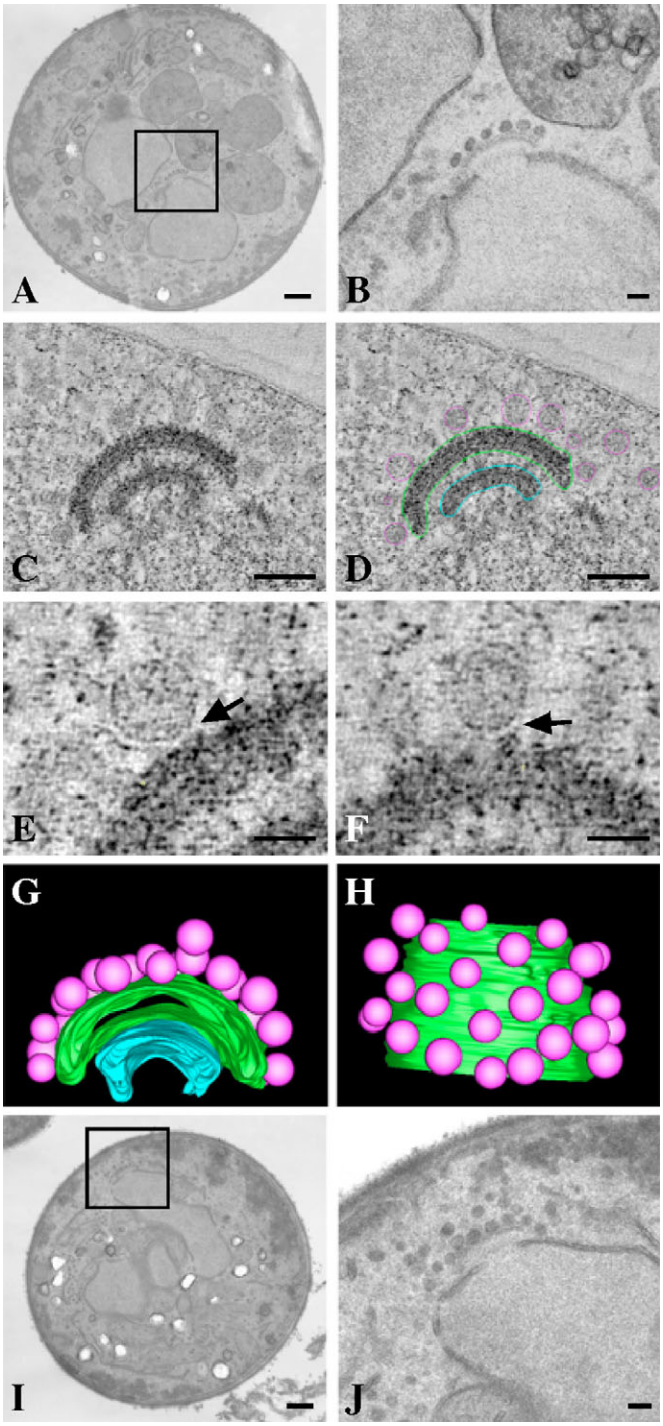


Fig. 1. Vesicles docked to the SPB accumulate in *spo14* and *sso1* mutants. (A) TEM image of a *spo14Δ /spo14Δ* (HI51) cell in meiosis II, SPB region is boxed. (B) Higher magnification view of the boxed region in A showing vesicles decorating the SPB. (C) A 1 nm *Z*-slice of a tomogram of a *spo14* SPB (for the complete tomogram see Movie 1 in supplementary material). (D) Outlines of vesicles, the outer plaque of the SPB and the central plaque of the SPB shown in purple, green, and light blue, respectively, in the same *Z*-slice. (E,F) Higher-magnification images from the tomogram of individual vesicles docked at the SPB. Arrows indicate sites of apparent contact between the outer plaque and the vesicular membrane. (G,H) Two views of a three-dimensional reconstruction of a *spo14* outer plaque modeled from the tomogram (see Movie 2 in supplementary material). Vesicles are in purple, the outer plaque surface is outlined in green, and the central plaque surface is outlined in light blue as in D. (I) TEM image of a *sso1* cell in meiosis II, SPB region is boxed. (J) Higher magnification view of the boxed region in I, showing vesicles decorating the SPB. Bars, 500 nm (A,I); 100 nm (B,C,D,J); 40 nm (E,F).

sporulation (Jantti et al., 2002). As *SSO1* is involved in vesicle fusion at the plasma membrane, we examined the *sso1* sporulation phenotype in the EM for comparison with *spo14* (Fig. 1J,I). Mutation of *sso1* caused a very similar phenotype to *spo14* both in the decoration of the SPB with docked vesicles and the clustering of extra-SPB vesicles in the spindle pole region. These cytological studies suggest that both *SPO14* and *SSO1* are required for the fusion of vesicles to initiate prospore membrane formation.

The vesicles accumulating at the SPB in the *spo14* mutant contain prospore membrane proteins

To determine if the vesicles accumulating on and around the SPB in the *spo14* mutant were prospore membrane precursors, we examined the localization of prospore membrane markers in the fluorescence microscope. A fusion of GFP to a catalytically inactive form of Spo14p (GFP-Spo14^{K1098H}p), was previously shown to localize to the spindle pole region in a *spo14* mutant (Rudge et al., 1998). Our data suggest that this localization might correspond to vesicles clustered at the SPB. To test this possibility, *GFP-SPO14*^{K1098H} was coexpressed in the *sso1* and *spo14* strains with an RFP fusion to the SPB component *MPC54*. In both the *spo14* mutant, as described previously (Rudge et al., 1998), and in the *sso1* mutant, GFP-Spo14^{K1098H}p was found in bright foci adjacent to each of the four SPBs indicating that it indeed localizes to the vesicle clusters (Fig. 2). TEM analysis of *spo14* cells expressing *GFP-SPO14*^{K1098H} demonstrated that unfused vesicles were present on the SPB, as in the *spo14* null strain (A.M.N., unpublished observations). Thus, the PLD catalytic activity of Spo14p is required to promote vesicle fusion.

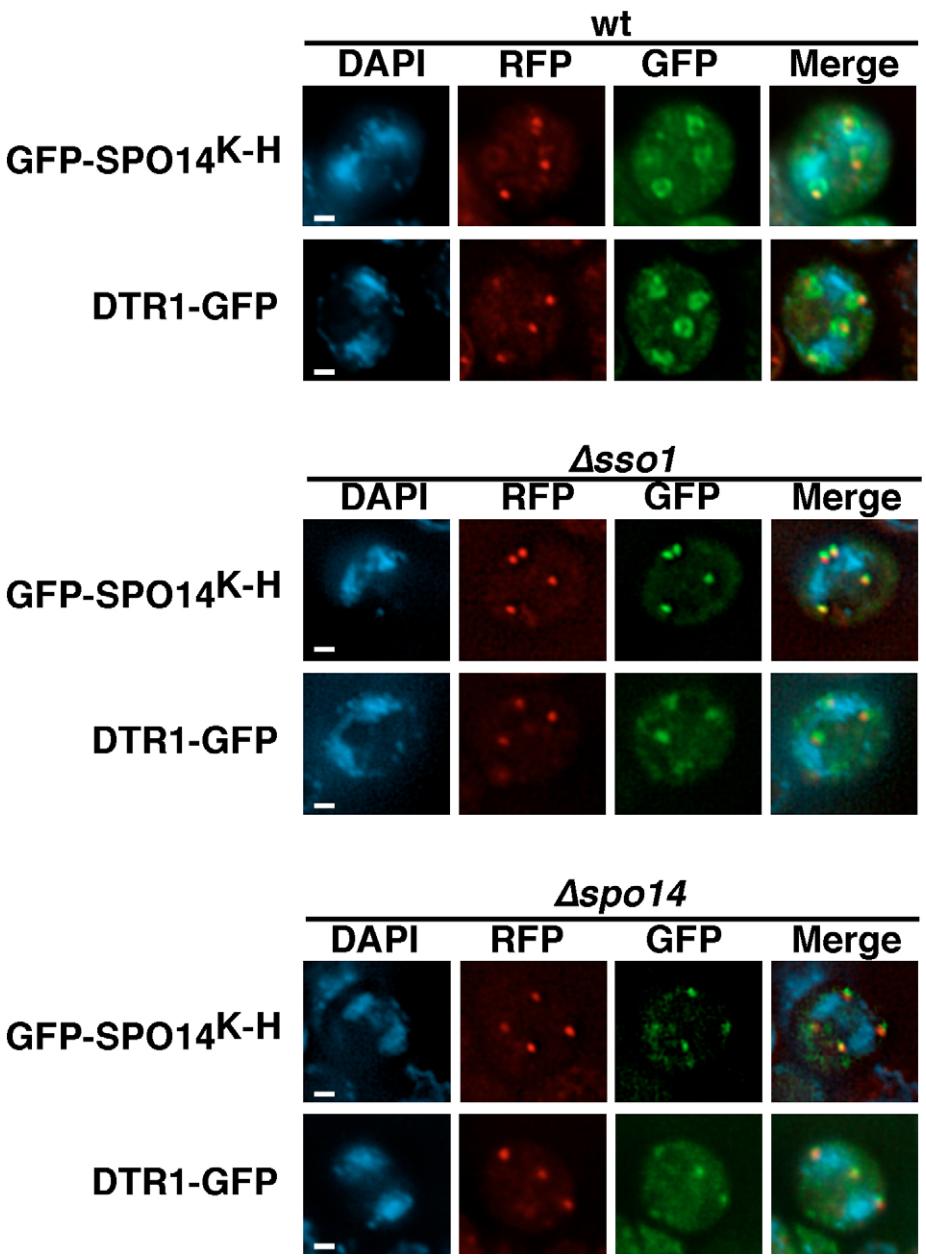


Fig. 2. Prospore membrane proteins accumulate at the SPB in *spo14* and *sso1* mutants. Wild-type (HI54), *sso1Δ*/*sso1Δ* (HI58) and *spo14Δ*/*spo14Δ* (HI51) cells expressing the prospore membrane markers GFP-Spo14^{K1098H} (from pRS424-GFP-SPO14^{K1098H}) or Dtr1-GFP (from pRS424-DTR1-GFP) were sporulated and examined by fluorescence microscopy. DNA was visualized using DAPI and SPBs were visualized using an RFP fusion to Mpc54p. Bars, 1 μm.

Spo14p is a cytoplasmic protein that is peripherally associated with membranes (Rudge et al., 1998). Therefore, it is possible that it localized to these vesicles without trafficking through the secretory pathway. To examine whether an integral membrane protein of the prospore membrane would also be found in these clusters, a fusion of GFP to the Dtr1p transporter was examined (Felder et al., 2002). In wild-type cells, Dtr1p-GFP was found exclusively on the prospore membrane, as reported previously (Felder et al., 2002). By contrast, in both the *sso1* and *spo14* cells Dtr1p-GFP was found in foci adjacent to the SPB, similar to GFP-Spo14^{K1098H}p. With both GFP fusions, the intensity of fluorescence from the SPB region was slightly less prominent in the *spo14* cells than in the *sso1* cells. Nonetheless, these data suggest that the vesicles that accumulate around the SPB in both *sso1* and *spo14* mutants are prospore membrane precursors.

Dtr1-GFP localizes to prospore membrane precursors in *spo14* and *sso1* cells

The cytological analyses demonstrated that *spo14Δ* and *sso1Δ* cells accumulate a population of vesicles that contain prospore membrane proteins. If these are precursor vesicles to the

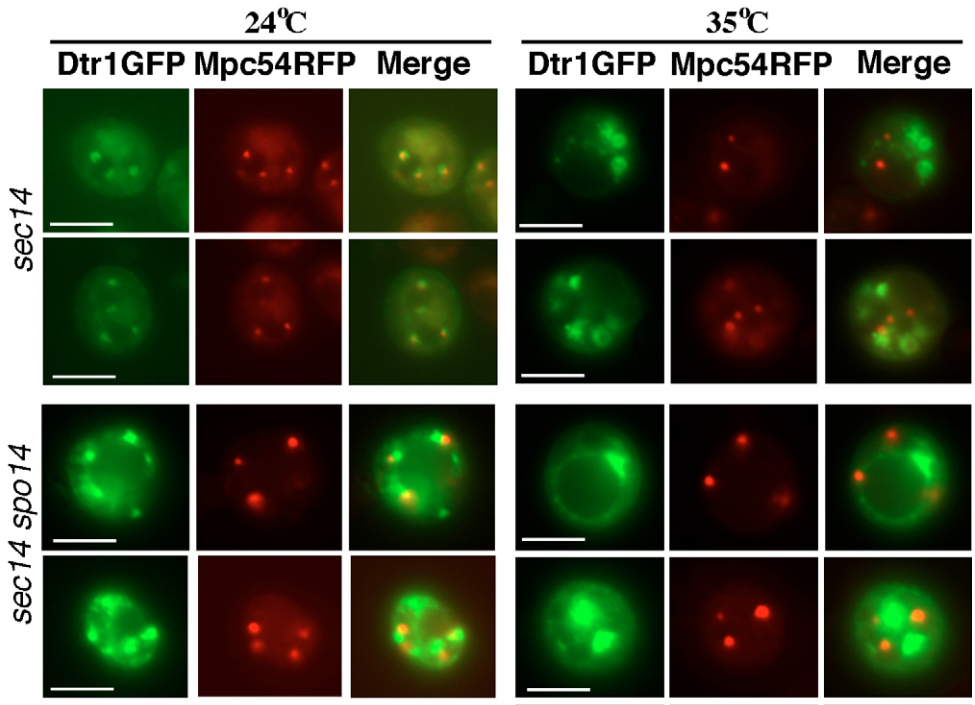


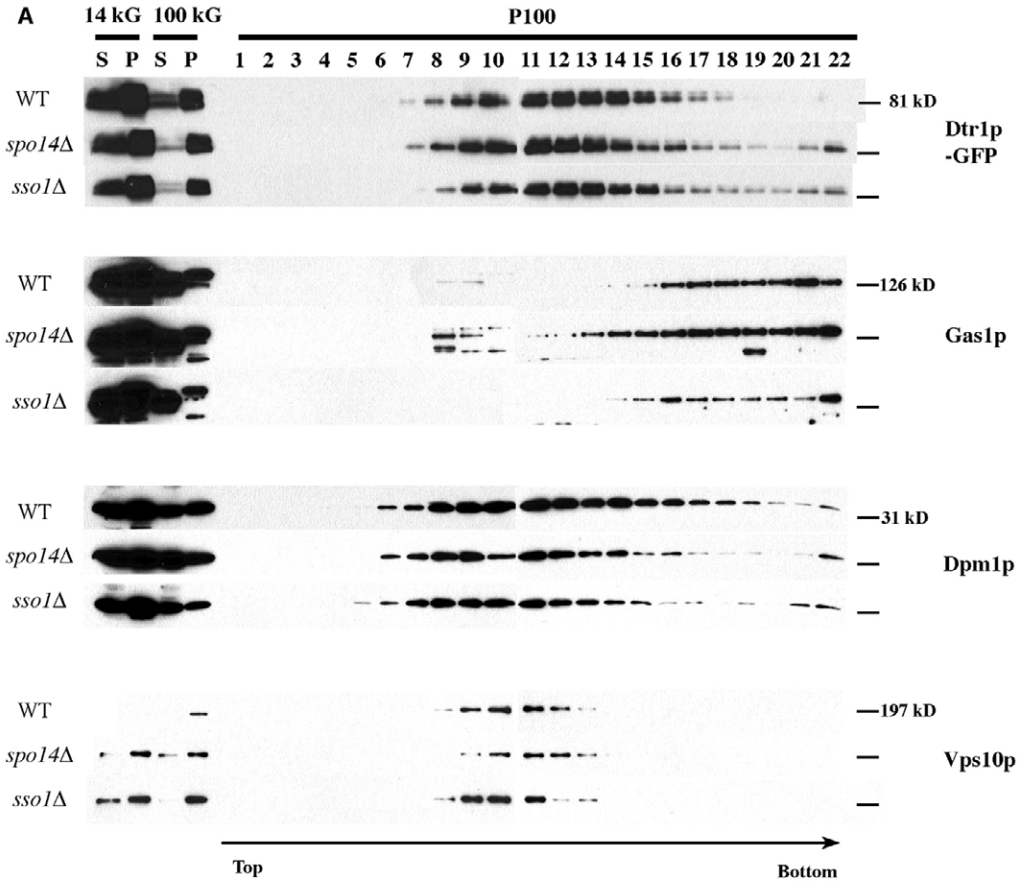
Fig. 3. Inactivation of *SEC14* blocks vesicle accumulation at the SPB in *spo14* mutants. *sec14-1/sec14-1* (Y5671) and *sec14-1/sec14-1 spo14Δ/spo14Δ* (Y5688) cells carry pRS424-Dtr1p-GFP were sporulated at either the permissive temperature (24°C) or the nonpermissive temperature (35°C) and examined by fluorescence microscopy. SPBs were visualized using an RFP fusion to Mpc54p. Bars, 5 μm.

prospore membrane, they should be derived from the Golgi (Neiman, 1998). This is consistent with the function of Sso1p in post-Golgi vesicle fusion in vegetative cells, but would represent a novel fusion function for Spo14p during sporulation.

Alternatively, rather than representing direct precursor vesicles, the accumulation of vesicles in *spo14* may be caused by a block earlier in the secretory pathway. To distinguish between these possibilities, localization of Dtr1p-GFP was examined in *spo14* cells in which exit from the Golgi was blocked by a temperature-sensitive mutation in the *SEC14* gene, which is required for the production of secretory vesicles from the Golgi (Bankaitis et al., 1989) and is essential for sporulation (Rudge et al., 2004). After induction of sporulation, *sec14-1* and *sec14-1 spo14* cells were either incubated at the permissive temperature or shifted to the nonpermissive temperature and Dtr1p-GFP fluorescence examined. At the permissive temperature, in meiosis II *sec14-1* and *sec14-1 spo14* cells, Dtr1p-GFP was found in bright foci adjacent to each of the four SPBs as expected. By contrast, in *sec14-1* and *sec14-1 spo14* cells shifted to the nonpermissive temperature, in meiosis II, the Dtr1p-GFP signal was random and was rarely found adjacent to the SPBs (Fig. 3). The same results were obtained for *sec14-1 sso1* (data not shown). Thus, the localization of Dtr1p-GFP-containing membranes to the SPBs is dependent on Golgi function.

As an additional test of the identity of the SPB-associated vesicles, cellular fractionation was performed to analyze the secretory pathway in these mutants. Cell lysates were subjected to differential centrifugation: a 14,000 ***g*** spin to pellet intact membrane organelles such as nuclei, mitochondria and vacuoles (P14) (Walworth et al., 1989; McCaffrey et al., 1991) followed by a high-speed spin (100,000 ***g***) to harvest the remaining membranes, vesicles and vesiculated organelles (P100). The resulting pellet was gently resuspended and loaded on a 20-60% sorbitol step gradient to separate different membranes compartments. Fractions were analyzed by western blot with antibodies against GFP to detect Dtr1p-GFP as a prospore membrane marker, Gas1p as a plasma membrane marker (Nuoffer et al., 1991), Dpm1p as an ER membrane marker (Orlean et al., 1988), or Vps10p as a marker for the late Golgi (Marcusson et al., 1994). Immunofluorescence studies show that Gas1p is found in both the plasma membrane and prospore membrane in sporulating cells (Neiman, 1998); however on these gradients the bulk of Gas1p was found in a heavier fraction, distinct from the peak of Dtr1-GFP (Fig. 4), suggesting that it fractionates predominantly with the plasma membrane. This is somewhat surprising, but is probably due to the harvesting of cells for the gradients during meiosis II, before newly synthesized Gas1p has a chance to accumulate in the prospore membrane and before there has been extensive turnover of plasma membrane localized Gas1p. The plasma membrane ATPase Pma1p behaved similarly to Gas1p on these gradients, indicating that the plasma membrane and prospore membrane indeed have distinct fractionation patterns (M.M. and J.E., unpublished observations). The fractionation of Dtr1-GFP was very similar in wild-type, *sso1*, and *spo14* cells indicating that the Dtr1p-GFP containing vesicles that accumulate in these mutants are similar in density to the prospore membrane.

Examination of the ER and Golgi membrane markers on the same gradients revealed that both markers peaked in a lighter fraction than Dtr1p-GFP (Fig. 4). Again, the fractionation pattern of these two markers was not changed by mutation of either *SSO1* or *SPO14*. Thus, the fractionation data indicate that in both *sso1* and *spo14* cells Dtr1p-GFP is found in a fraction of similar density to the prospore membrane that is overlapping with, but slightly more dense than, other intracellular membranes. Taken together with the cytological data, these results strongly imply that the same population of



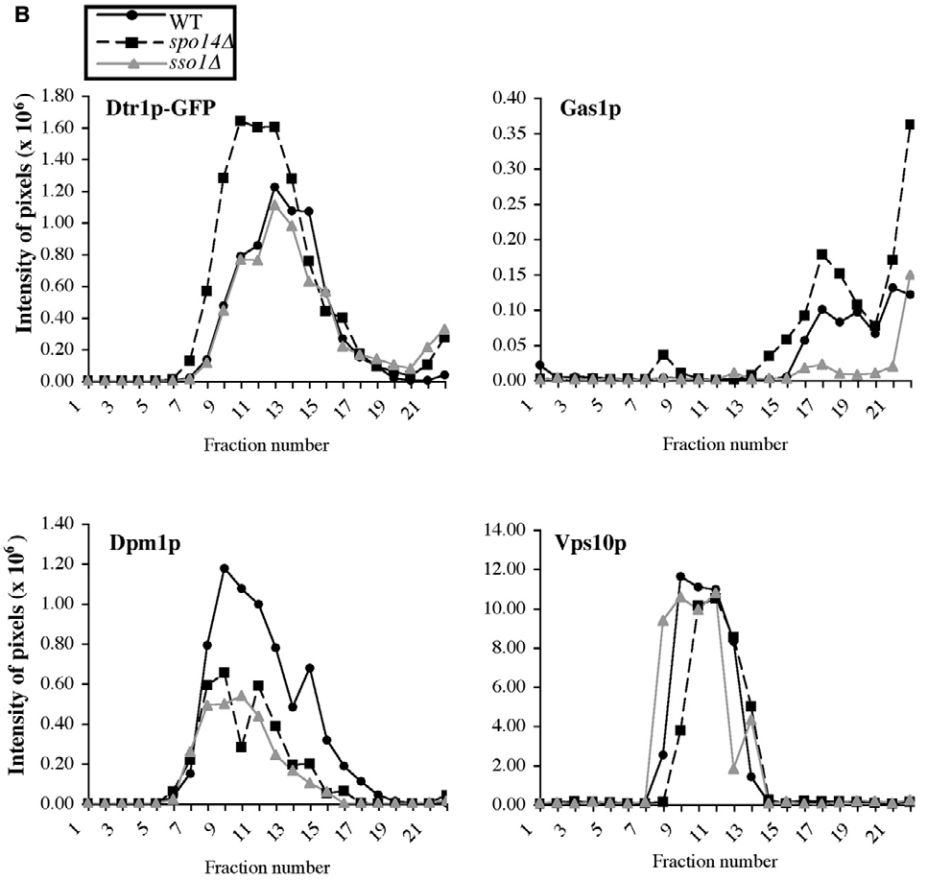


Fig. 4. Sorbitol density gradient fractionation of sporulating wild-type, *spo14Δ* and *sso1Δ* cells. (A) Immunoblots of fractionated extracts from wild-type (AN120), *spo14* (HI6), and *sso1* (HI3) cells expressing Dtr1-GFP. The 14,000 ***g*** supernatant (S14), pellet (P14), 100,000 ***g*** supernatant (S100), pellet (P100) and 20-60% sorbitol gradient fractions (1-22) were probed with anti-GFP to detect Dtr1p-GFP, anti-Gas1p and anti-Dpm1p, or anti-Vps10p (details in Materials and Methods). (B) Quantification of the pixel intensity of each band was performed using AlphaEase FC4.0 imager software. ●, WT; ■, *spo14*; ▲, *sso1*.

vesicles is accumulating in both mutants and that these vesicles are post-Golgi precursor vesicles for the prospore membrane.

The PA-binding region of Spo20p does not localize to prospore membrane precursors in *spo14* cells

An N-terminal region of the t-SNARE Spo20p binds to acidic phospholipids in vitro and the subcellular localization of a fusion of this region to GFP is specifically sensitive to perturbations of PA pools in vivo (Nakanishi et al., 2004). This fusion localizes to the prospore membrane during sporulation in wild-type cells (Nakanishi et al., 2004). Because PA is the product of the Spo14p enzyme and enzymatic activity is required to stimulate fusion of the precursor vesicles, we examined the localization of the GFP-Spo20$^{51\text{-}91}$p fusion in *sso1* and *spo14* cells. In *sso1* mutants GFP-Spo20$^{51\text{-}91}$p was localized in four bright punctae each located near the spindle pole as seen for the other prospore membrane markers (Fig. 5). However, in *spo14* cells no association of this fusion with the SPB was seen (Fig. 5). The failure of this specific marker to localize in *spo14* mutants suggests that the lack of PA in these vesicles may be the basis for the fusion defect in *spo14* mutants.

SPO20/*SEC9* and *SEC1* are also required for precursor vesicle fusion

We have reported previously that *spo20Δ* mutants display abnormal prospore membranes whereas a *spo20Δ sec9-ts* double mutant lacks prospore membranes (Neiman, 1998). Analysis of this *spo20Δ sec9-ts* double mutant expressing GFP-Spo20$^{51\text{-}91}$p revealed an accumulation of fluorescence adjacent to the spindle pole (Fig. 5), as in the *sso1* and *spo14* strains. Mutation of *SEC1*, encoding SNARE-interacting protein (Carr et al., 1999), also caused a similar accumulation (Fig. 6). By contrast, when a component of the meiotic outer plaque, *MPC54*, was mutated, no accumulation of precursor vesicles on the SPB was seen. Rather, plasma membrane fluorescence persisted longer in meiosis and numerous smaller dots of fluorescence, presumably small clusters of vesicles, were seen in the cytoplasm (Fig. 6). Thus, *SEC9*/*SPO20* and *SEC1* appear to be required for fusion of the vesicles, whereas an intact outer plaque is necessary for vesicles to accumulate at the spindle pole.

Independent targeting of Spo20p to the prospore membrane does not rescue the *spo14* phenotype

Our data indicate that *SPO14* is required for the recruitment of GFP-Spo20$^{51\text{-}91}$p to precursor vesicles and that *SPO20*/*SEC9* are required for vesicle fusion. One simple explanation for these observations is that the *spo14* phenotype results from a failure to recruit Spo20p and Sec9p to the precursor vesicles. To test this possibility, we targeted Spo20p to the prospore membrane independently of PA binding by fusing the region encoding amino acid residues 50-397 of Spo20p to the 3′ end of the *DTR1* gene. This fragment of Spo20p lacks the inhibitory domain and complements a *spo20Δ* mutant as well as full-length Spo20p (Neiman et al., 2000). The *DTR1-SPO20* fusion rescued the sporulation of a *spo20* mutant, indicating that this fusion gene encoded a functional SNARE (Table 1). However, when *spo14* cells expressing *DTR1-SPO20* were induced to sporulate, no spores were produced nor was an increase in vesicle fusion seen when cells were examined by fluorescence or in the electron microscope (Table 1; H.N. and A.M.N., unpublished observations). Similar results were obtained with a *DTR1-SEC9* fusion; the fusion partially rescued *spo20Δ*, but did not bypass *spo14Δ* either for ascus formation or for vesicle fusion (Table 1; H.N. and A.M.N., unpublished observations). The rescue of *spo20Δ* by *DTR1-SEC9* is noteworthy as wild-type *SEC9* cannot rescue *spo20Δ*, mainly because of the absence of efficient prospore membrane targeting provided by the Spo20p PA-binding domain (Neiman

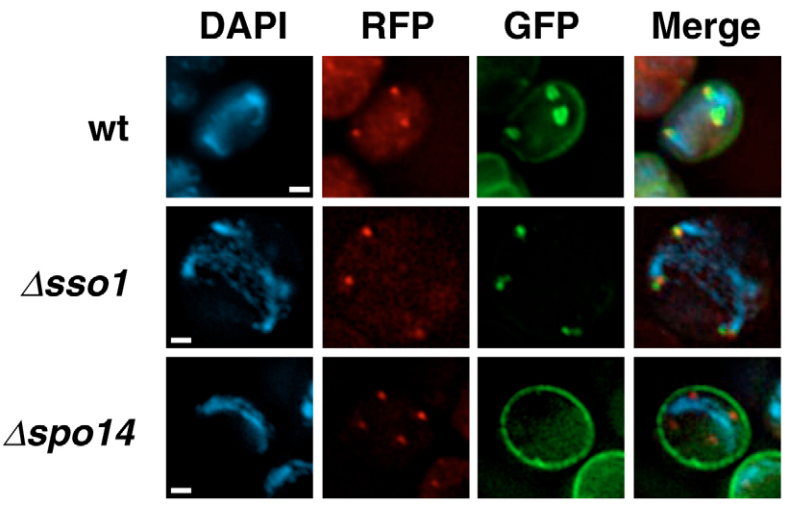


Fig. 5. The membrane-binding domain of Spo20p does not localize to prospore membrane precursor vesicles in the *spo14* mutant. Wild-type (HI54), *sso1Δ*/*sso1Δ* (HI58) and *spo14Δ*/*spo14Δ* (HI51) cells expressing GFP-Spo20p$^{51\text{-}91}$ (from pRS426-G20) were sporulated and examined by fluorescence microscopy. DNA was visualized using DAPI and SPBs were visualized using an RFP fusion to Mpc54p. Bars, 1 μm.

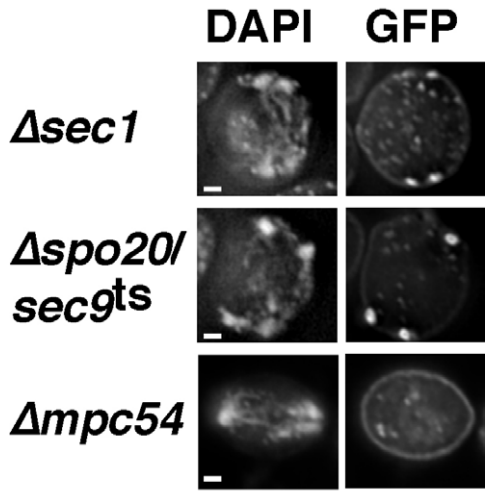


Fig. 6. Prospore membrane proteins accumulate at the SPB in *sec1* and *spo20 sec9* but not *mpc54* mutants. *sec1*^{ts}/*sec1*^{ts} (AN127), *sec9*^{ts}/*sec9*^{ts}, *spo20Δ*/*spo20Δ* (AN211) *mpc54Δ*/*mpc54Δ* (NY541) cells expressing GFP-Spo20p$^{51\text{-}91}$ (from pRS426-G20 or pRS424-G20) were sporulated and examined by fluorescence microscopy. DNA was visualized using DAPI. Bars, 1 μm.

Table 1. Membrane targeting of Spo20p or Sec9p does not suppress a *spo14Δ* mutant

Gene expressed	Number of asci (%)*	
	Δspo20	*Δspo14*
DTR1-GFP	0	0
DTR1-Δ3-51 SPO20	20	0
DTR1-Δ1-370 SEC9	10	0
Δ3-51 SPO20	39	0

*High-copy plasmids carrying the indixated genes were transformed into *spo20Δ/spo20Δ* (AN147) or *spo14Δ/spo14Δ* (HI6) mutants. Cultures were sporulated and then analyzed in the light microscope to determine the percentage sporulation. 500 cells were counted per culture.

et al., 2000). Rescue by this chimera is therefore consistent with the idea that fusion to Dtr1p targets these SNAREs to the prospore membrane. However this targeting is not sufficient to bypass the need for *SPO14*. In sum, although *SPO14* appears to be involved in recruitment of Spo20p to the membrane, these data indicate that recruitment of Spo20p is not the only role of Spo14p in promoting membrane fusion.

Discussion

This report describes a requirement for the phospholipase D, Spo14p, and the t-SNARE, Sso1p, in vesicle fusion during sporulation in *S. cerevisiae*. In mutants lacking either of these proteins, docked, unfused vesicles accumulate on the cytoplasmic face of the SPB during meiosis II. These vesicles contain prospore membrane markers and their accumulation is inhibited when exit from the Golgi is blocked, indicating that they are precursor vesicles to the prospore membrane. Consistent with this, the prospore membrane protein Dtr1-GFP fractionates differently from ER, Golgi, and plasma membrane markers in wild-type, *spo14Δ* and *sso1Δ* cells. The distinct fractionation of prospore membranes and plasma membranes was somewhat surprising and may reflect greater protein or lipid composition differences between these compartments than has previously been appreciated. Nonetheless, these results are consistent with a specific requirement for *SPO14* and *SSO1* in the fusion of prospore membrane precursor vesicles.

The defect in *spo14* cells is attributable, in part, to a failure to recruit the SNARE protein, Spo20p, to the precursor vesicles. However, targeting of Spo20p to the precursor vesicles independently of its PA binding domain does not suppress the membrane fusion defect of *spo14* mutants. Thus, the catalytic activity of Spo14p appears to be necessary for additional aspects of membrane growth. A recent, independent study of Spo14p and its sporulation-specific binding partner Sma1p reported the same accumulation of SPB-associated vesicles in *spo14* cells that we describe here (Riedel et al., 2005). Mutation of *SMA1*, however, resulted in formation of a membrane cap on the SPB, but a failure in continued expansion of the prospore membrane. Consistent with our results, the authors conclude that Spo14p has distinct functions during the initial formation and the subsequent expansion of the prospore membrane. As *spo20* mutants are defective in prospore membrane growth (Neiman, 1998), it may be that recruitment of Spo20p is essential for this latter role of Spo14p.

What is the function of PA during the initial coalescence of vesicles on the SPB? PA may be required for the recruitment of additional peripheral membrane proteins involved in fusion, such as Sec1p (Fig. 5). Alternatively, local generation of PA, or removal of phosphatidylcholine, may be directly required for efficient fusion of the membrane bilayers. Consistent with this latter possibility, a previous study demonstrated a role for Spo14p-generated PA in promoting Spo20p-mediated fusion independently of the Spo20p PA binding domain (Coluccio et al., 2004).

The vesicles that accumulate in *spo14* cells appear to be in direct contact with the outer plaque of the SPB (Fig. 1). As this structure is required for stable recruitment of vesicles to the SPB region (Fig. 5) and for their coalescence into a membrane sheet (Bajgier et al., 2001; Knop and Strasser, 2000), these observations reveal that the outer plaque functions as a docking complex prior to vesicle fusion. Correct docking of the vesicles on this surface may be required before fusion can proceed.

Mutation of the t-SNARE, *SSO1,* produced a similar phenotype to *spo14* in sporulating cells, an accumulation of docked precursor vesicles at the prospore membrane. Spo14p is present on the vesicles in an *sso1* mutant (Fig. 4) consistent with the possibility that Spo14p functions independently of Sso1p in the fusion process. In addition to *sso1*, we found that mutation of *SEC1*, or both *SEC9* and *SPO20*, causes a similar block to precursor vesicle fusion. Moreover, the Sec1p and Sso1p binding protein Mso1p has recently been reported to have a similar vesicle-accumulation phenotype (Knop et al., 2005). Therefore, the set of genes required for coalescence of precursor vesicles on the SPB includes the SNAREs *SSO1*, *SEC9* and *SPO20*, the SNARE regulators *SEC1* and *MSO1*, and *SPO14*. *SEC9* and *SEC1* are also required for the fusion of secretory vesicles with the plasma membrane during vegetative growth. However, *SPO14*, *MSO1* and *SPO20* are not required for fusion at the plasma membrane in vegetative cells and *SSO1* is redundant with *SSO2* for this function (Aalto et al., 1993; Neiman, 1998; Rose et al., 1995). Thus, fusion of secretory vesicles to form a prospore membrane has unique genetic requirements, suggesting that the SNARE complex acting at the SPB is specialized for this fusion event. This may be because the initial fusion events occurring on the SPB are homotypic fusions between vesicles rather than more standard heterotypic fusion between a vesicle and a stable membrane compartment. It should be noted that, except for *SPO20*, all of these genes are expressed in vegetative cells. It is possible, therefore, that this specialized fusion apparatus is present, but is not essential for growth, in vegetative cells.

In higher cells, the activity of the Pld1 isozyme is stimulated by ARF and has been linked to the regulated exocytosis of secretory granules in a variety of cell types including mast cells, pancreatic beta cells and neuroendocrine cells (Choi et al., 2002; Hughes et al., 2004; Kahn et al., 1993; Vitale et al., 2001). The fusion of GLUT4-containing vesicles with the plasma membrane in 3T3-L1 adipocytes requires Pld1 activity, and Pld1 has been implicated in the formation of fusion pores during release of dense-core vesicles in PC12 cells (Huang et al., 2005; Liu et al., 2005). Our data are thus consistent with a conserved role for phospholipase D in membrane fusion. Though these studies in higher cells indicate a requirement for PLD in release of secretory granule contents, the role of PLD in this process is unclear. The results presented here suggest that recruitment of PA-binding proteins required for membrane fusion is one probable function for PLD in these systems.

Table 2. Yeast strains used in this study

Name	Genotype	Source
AN127	*MAT***a**/*MATα ura3/ura3 LEU2/leu2 ARG4/arg4 sec1-1/sec1-1*	Neiman, 1998
AN147	*MAT***a**/*MATα ARG4/arg4-NspI his3ΔSK/his3ΔSK hoΔ::LYS2/hoΔ::LYS2 leu2/leu2 lys2/lys2 RME1/rme1Δ::LEU2 trp1::hisG/trp1::hisG ura3/ura3 spo20Δ::his5$^+$/spo20Δ::his5$^+$*	Neiman et al., 2000
AN211	*MAT***a**/*MATα his3/his3 ura3/ura3 trp1/ trp1 leu2/leu2 LYS2/lys2 ade2/ade2 sec9-4/sec9-4 spo20Δ::his5$^+$/ spo20Δ::his5$^+$*	Nakanishi et al., 2004
NY541	*MAT***a**/*MATα ARG4/arg4-NspI his3ΔSK/his3ΔSK hoΔ::LYS2/hoΔ::LYS2 leu2/leu2 lys2/lys2 RME1/rme1Δ::LEU2 trp1::hisG/trp1::hisG ura3/ura3 mpc54Δ::his5$^+$/mpc54Δ::his5$^+$*	Nickas et al., 2003
HI3	*MAT***a**/*MATα ARG4/arg4-NspI his3ΔSK/his3ΔSK hoΔ::LYS2/hoΔ::LYS2 leu2/leu2 lys2/lys2 RME1/rme1Δ::LEU2 trp1::hisG/trp1::hisG ura3/ura3 sso1Δ::his5$^+$/sso1Δ::his5$^+$*	This study
HI6	*MAT***a**/*MATα ARG4/arg4-NspI his3ΔSK/his3ΔSK hoΔ::LYS2/hoΔ::LYS2 leu2/leu2 lys2/lys2 RME1/rme1Δ::LEU2 trp1::hisG/trp1::hisG ura3/ura3 spo14Δ::ura3/spo14Δ::ura3*	This study
HI51	*MAT***a**/*MATα ARG4/arg4-NspI his3ΔSK/his3ΔSK hoΔ::LYS2/hoΔ::LYS2 leu2/leu2 lys2/lys2 RME1/rme1Δ::LEU2 trp1::hisG/trp1::hisG ura3/ura3 spo14Δ::ura3/spo14Δ::ura3* pRS303-MPC54-RFP	This study
HI54	*MAT***a**/*MATα ARG4/arg4-NspI his3ΔSK/his3ΔSK hoΔ::LYS2/hoΔ::LYS2 leu2/leu2 lys2/lys2 RME1/rme1Δ::LEU2 trp1::hisG/trp1::hisG ura3/ura3* pRS306-MPC54-RFP	This study
HI58	*MAT***a**/*MATα ARG4/arg4-NspI his3ΔSK/his3ΔSK hoΔ::LYS2/hoΔ::LYS2 leu2/leu2 lys2/lys2 RME1/rme1Δ::LEU2 trp1::hisG/trp1::hisG ura3/ura3 sso1Δ::his5$^+$/sso1Δ::his5$^+$* pRS306-MPC54-RFP	This study
NY541	*MAT***a**/*MATα ARG4/arg4-NspI his3ΔSK/his3ΔSK hoΔ::LYS2/hoΔ::LYS2 leu2/leu2 lys2/lys2 RME1/rme1Δ::LEU2 trp1::hisG/trp1::hisG ura3/ura3 mpc54Δ::his5$^+$/mpc54Δ::his5$^+$*	Nickas et al., 2003
K8409 *spo14*	*MAT***a**/*MATα HO/HO his3::hisG /his3::hisG ura3/ura3 trp1/trp1 P_{URA3}-tetR-GFPURA3:tetO224/P_{URA3}-tetR-GFPURA3:tetO224 REC8-HA3/REC8-HA3 spo14Δ::his5$^+$/spo14Δ::his5*	Rabitsch et al., 2001
Y3242	*MAT***a**/*MATα leu2/leu2 his4/HIS4 trp1::hisG/trp1::hisG ura3/ura3 lys2/ lys2 ho::LYS2/ho::LYS2 HIS3/his3Δ200 sec14-1/sec14-1*	Rudge et al., 2004
Y3753	*MAT***a**/*MATα leu2/leu2 his4/HIS4 trp1::hisG/trp1::hisG ura3/ura3 lys2/lys2 ho::LYS2/ho::LYS2 HIS3/his3Δ200 sec14-1/sec14-1spo14::LEU2/spo14::LEU2*	Rudge et al., 2004
Y5671	*MAT***a**/*MATα leu2/leu2 his4/HIS4 trp1::hisG/trp1::hisG ura3/ura3 lys2/lys2 ho::LYS2/ho::LYS2 HIS3/his3Δ200 sec14-1/sec14-1* pRS306-MPC54-RFP	This study
Y5688	*MAT***a**/*MATα leu2/leu2 his4/HIS4 trp1::hisG/trp1::hisG ura3/ura3 lys2/lys2 ho::LYS2/ho::LYS2 HIS3/his3Δ200 sec14-1/sec14-1* pRS306-MPC54-RFP	This study

Materials and Methods

Yeast strains and media

Unless otherwise noted, standard media and genetic methods were used (Rose and Fink, 1990). Cells were sporulated as described (Neiman, 1998). *S. cerevisiae* strains used in this study are listed in Table 2. Strain HI3 (*sso1Δ/sso1Δ*) was constructed as follows: a DNA fragment for targeted gene disruption was amplified by PCR using pFA6a-His3MX6 (Longtine et al., 1998) as a template, and HNO161 and HNO162 as primers. The PCR fragment was integrated into haploid wild-type cells, AN117-4B and AN117-16D (Neiman et al., 2000), and the resulting strains were mated. HI6 (*spo14Δ/spo14Δ*) was constructed in the same way except that an *Xba*I and *Cla*I fragment of pKR466 (Rose et al., 1995) was used for *SPO14* disruption. *Eco*RI-digested pRS303-MPC54-RFP and pRS306-MPC54-RFP (see Plasmids) were integrated into HI6 and HI3 to generate HI51 and HI58, respectively. HI54 was made by integration of *Eco*RI-digested pRS306-MPC54-RFP into the *MPC54* locus in strain AN120 (Neiman et al., 2000). To make HI51 and HI58, *Eco*RI-digested pRS303-MPC54-RFP and pRS306-MPC54-RFP were integrated into HI6 and HI3, respectively. Strain Y5671 (*sec14-1/sec14-1*) and Y5688 (*sec14/sec14 spo14/spo14*) were constructed by integrating *Eco*RI-digested pRS306-MPC54-RFP into the haploid derivatives of Y3242 and Y3753 (Rudge et al., 2004), respectively, and the resulting strains were mated. Plasmid RS424-DTR1-GFP was subsequently introduced by transformation.

Plasmids

Plasmids used in this study are listed in Table 3. Primers used are listed in Table 4. pRS303-MPC54-RFP and pRS306-MPC54-RFP were constructed as follows. First, the fast folding form of red fluorescent protein (RFP) (Bevis and Glick, 2002) was amplified by PCR using HNO581 and HNO582 as primers. This PCR fragment was digested with *Pac*I and *Asc*I, and cloned into similarly digested pFA6a-3HA-His3MX6 (Longtine et al., 1998) to make pFA6a-RFP-His3MX6. A C-terminal-tagging cassette for the chromosomal *MPC54* was generated by PCR using pFA6a-RFP-His3MX6 as a template and HT94 and HT77 as primers. The PCR fragment was transformed into AN117-4B. Using genomic DNA from the resulting

Table 3. Plasmids used in this study

Name	Yeast markers	Cloned gene	Source
pRS303-MPC54-RFP	*HIS3*, Integration	*RFP* fusion to *MPC54*	This study
pRS306-MPC54-RFP	*URA3*, Integration	*RFP* fusion to *MPC54*	This study
pRS424-DTR1-GFP	*TRP1*, 2μ	*GFP* fusion to *DTR1*	This study
pRS424-DTR1-Δ3-51SPO20	*TRP1*, 2μ	*DTR1* fusion to *SPO20*$^{\Delta 3\text{-}51}$	This study
pRS424-DTR1-Δ1-370SEC9	*TRP1*, 2μ	*DTR1* fusion to *SEC9*$^{\Delta 1\text{-}370}$	This study
pRS424-GFP-SPO14K→H	*TRP1*, 2μ	*GFP* fusion to *SPO14* K→H mutant	This study
pRS424-G20	*TRP1*, 2μ	*GFP* fusion to *SPO20* nucleotides 151-273	This study
pRS426-G20	*URA3*, 2μ	*GFP* fusion to *SPO20* nucleotides 151-273	Nakanishi et al., 2004
pRS426-SPO20pr-Δ3-51SPO20	*URA3*, 2μ	*SPO20*$^{\Delta 3\text{-}51}$	Nakanishi et al., 2004

Table 4. Primers used in this study

Name	Sequence
HNO161	5′-GAAAACCCTTTTACAATTAAAAAAGGCAATTAAAAATAGAAACAAATCAACGGATCCCCGGGTTAATTAA-3′
HNO162	5′-TATACAAAAGGGGAGTTCGGATAGAATAGAAATATAGAAAATAGTTGGAAGAATTCGAGCTCGTTTAAAC-3′
HNO501	5′-GTTCTTCTCGAGCTATCTGATACCTGCCAACC-3′
HNO531	5′-GTTCTTGGATCCTTAAGGGCTACTGTAACCTG-3′
HNO532	5′-GTTCTTGAATTCAAATTCTTTGGCAGTATATT-3′
HNO581	5′-GTTCTTTTAATTAACATGGCCTCCTCCGAGGACGT-3′
HNO582	5′-GTTCTTGGCGCGCCCTACAGGAACAGGTGGTGGC-3′
HNO731	5′-GTTCTTGAATTCTCAAGACAGCAGCAGCAGCA-3′
HT66	5′-GAAGAATTCAGATCTATATTACCCTGTTATCC-3′
HT77	5′-TCTTAAACATAATGTCTTTTTAATGTTTGTAGGTTAGGTTATAAACCTTAGAATTCGAGCTCGTTTAAAC-3′
HT94	5′-AGTATGTTACTGACTCACCAAGACTTAAAGAAAATGAACACGTTACAAATCGGATCCCCGGGTTAATTAA-3′
HT99	5′-GAAGAACTCGAGTACTTCGTTAACCGCGTCCGTCTC-3′

transformant as template and HT66 and HT99 as primers, PCR was used to amplify the C-terminal *RFP* fusion of *MPC54*. Finally, this PCR product was digested with *Bgl*II and *Xho*I and cloned into the *Bam*HI and *Xho*I sites of pRS303 or pRS306 (Sikorski and Hieter, 1989). Plasmids pRS424-DTR1-GFP, pRS424-DTR1-Δ3-51SPO20 and pRS424-DTR1- Δ1-370SEC9 were constructed as follows. First, the *DTR1* gene with 546 base pairs of the upstream sequence was amplified by PCR from yeast genomic DNA using HNO531 and HNO532 as primers. This PCR fragment was cloned between the *Bam*HI and *Eco*RI sites of pGFP-C-FUS (Niedenthal et al., 1996). The GFP fusion to *DTR1* was then cut out with *Bam*HI and *Xho*I, and cloned into similarly digested pRS424 (Christianson et al., 1992) to generate pRS424-DTR1-GFP. The *Eco*RI-*Xho*I fragment of YEp352GAP-Δ3-51SPO20 (Nakanishi et al., 2004) containing $SPO20^{\Delta 3\text{-}51}$ sequences was ligated into similarly digested pRS424-DTR1-GFP to generate pRS424-DTR1-Δ3-51SPO20. Similarly, a PCR fragment carrying the $SEC9^{\Delta 1\text{-}370}$ coding sequence was amplified using HNO501 and HNO731 and cloned into the *Eco*RI and *Xho*I sites of pRS424-DTR1-GFP to generate pRS424-DTR1- Δ1-370SEC9. Plasmid pRS424-GFP-$SPO14^{K1098H}$ was constructed as follows. The *Xba*I-*Hin*dIII and *Hin*dIII-*Hin*dIII fragments of pME1130 (Rudge et al., 1998) were sequentially cloned into *Xba*I and *Hin*dIII sites of pRS316. The resulting plasmid was digested by *Not*I and *Xho*I and the fragment carrying the GFP fusion to $spo14^{K1098H}$ was ligated into similarly digested pRS424. Plasmid pRS424-G20 was constructed by cloning the *Eco*RI-*Xho*I fragment from pRS426-G20 (Nakanishi et al., 2004) into similarly digested pRS424TEF (Mumberg et al., 1995).

Sporulation

For EM and fluorescence analysis, cells were sporulated essentially as described (Neiman, 1998). For cultures carrying plasmids, cells were incubated overnight in medium selective for the plasmid before dilution into YPAcetate medium.

Fluorescence microscopy

For direct detection of GFP and RFP fluorescence, cells were fixed with 3.7% formaldehyde for 10 minutes and then placed in mounting media containing DAPI. Images were acquired using a Zeiss Axioplan2 microscope (Carl Zeiss, Thornwood, NY) with a Zeiss mRM Axiocam and deconvolved using Zeiss Axiovision 3.1 software.

Temperature shift experiments

For inactivation of *sec14-1*, cultures were maintained at 24°C and induced to sporulate as described (Neiman, 1998). Sporulating cultures were shifted after 4 hours in sporulation medium. This allowed for meiotic progression and expression of Dtr1-GFP. Live cells were examined for GFP and RFP fluorescence. Images were acquired using a Zeiss Axioplan2 microscope (Carl Zeiss, Thornwood, NY).

Transmission electron microscopy and tomography

For permanganate staining, sporulating cells were fixed for 1 hour in 3% glutaraldehyde in cacodylate buffer (100 mM sodium cacodylate, 5 mM $CaCl_2$, pH 6.8), washed once in cacodylate buffer, resuspended in 4% $KMnO_4$ in distilled water, and incubated for 30 minutes at 23°C. Cells were then washed four times in dH_2O, resuspended in saturated uranyl acetate for 2 hours and then dehydrated by 10-minute incubations in a graded acetone series; two incubations each in 30%, 50%, 70% and 95% acetone and four incubations in 100% acetone. The dehydrated samples were embedded in Epon 812, sectioned, and images were collected on a JEOL 1200EX microscope at 80 kV. For tomography, strain K8409 *spo14* (Rabitsch et al., 2001) was sporulated and cells were then fast frozen using a high-pressure freezer (BAL-TEC HPM-010; Technotrade International, Manchester, NH) and prepared by freeze substitution as described (Ding et al., 1993; Winey et al., 1995). Thick sections (~250 nm) were cut and dual-axis-tilt series collected at ±60° with 1° increments on a Tecnai TF30 FEG TEM (FEI, Eindhoven, Netherlands). The IMOD software package (Mastronarde, 1997) was used to construct tomograms from the tilt series and to generate the models (O'Toole et al., 1999; O'Toole et al., 2002). Movies in the supplementary data were prepared from the tomographic and model data using Quicktime software.

Fractionation and western blot analysis

Approximately 5×10^9 cells (cultured in sporulation medium until ~50% of the cells were undergoing the second meiotic division; ~8 hours) were harvested by centrifugation. Cells were resuspended in spheroplast buffer I (100 mM Tris-HCl pH 9.4, 10 mM DTT) and incubated at RT for 5 minutes. Cells were collected by centrifugation, resuspended in spheroplast buffer II (1 M Sorbitol, 50 mM Tris-HCl pH 7.5, 2 mM $MgCl_2$, 0.14% β-mercaptoethanol), containing 0.21 μg (500 U)/10 ml lyticase (from *Arthrobacter luteus*, Sigma), and incubated at 30°C for 30 minutes for cell-wall digestion. Resultant spheroplasts were pelleted by centrifugation at low speed for 3 minutes, and washed once with and resuspended in ice-cold JR lysis buffer (100 mM Sorbitol, 50 mM potassium acetate, 2 mM EDTA, 20 mM HEPES pH 7.4, 1 mM DTT, 1 mM PMSF, 8 μg/ml leupeptin). Spheroplasts were burst by 20-stroke dounce homogenization on ice. Intact cells and cell debris were removed by centrifugation at 3000 ***g*** for 3 minutes at 4°C. The resultant supernatant (S3) was centrifuged at 14,000 ***g*** for 20 minutes at 4°C to obtain pellet (P14) and supernatant (S14) fractions. S14 fraction was further centrifuged at 100,000 ***g*** for 1 hour at 4°C (Optima™ TLX ultracentrifuge, TLA45 rotor) to obtain pellet (P100) and supernatant (S100). P100 fraction was resuspended in 1 ml ice-cold JR lysis buffer without sorbitol, overlaid onto 10 ml sorbitol gradient (20-60%) and centrifuged at 198,000 ***g*** for 40 hours at 4°C (Optima™ LE-80K ultracentrifuge, SW41 rotor). 500 μl fractions from the top to the bottom (1-22 fractions) were taken.

15 μl from each fraction (equivalent to about 45 μg of protein in S3) was loaded, separated on 15% PAGE and transferred to a nitrocellulose membrane. Dtr1p-GFP was blotted with anti-GFP antibody (Clontech/BD Biosciences, Palo Alto, CA) and horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Pierce, Rockford, IL) as secondary antibody and detected with West Femto substrate detection kit (Pierce). Other antibodies used for marker proteins in this experiment are as follows; primary antibodies, a rabbit polyclonal anti-Gas1p antibody for Gas1p (glycosylphophatidyl-inositol-anchored plasma membrane 1,3-β-glucanosyl-transferase) (a gift from H. Riezman, University of Geneva, Switzerland), and mouse antibodies for Dpm1p (an ER dolicholphosphate mannnosyl-transferase) and for Vps10p (a sorting receptor at late Golgi membrane) (Molecular Probes, Eugene, OR). Secondary antibodies used were HRP-conjugated anti-rabbit antibody (Pierce) and HRP-conjugated anti-mouse antibody (Pierce).

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