The exocytic Rabs Ypt3 and Ypt2 regulate the early step of biogenesis of the spore plasma membrane in fission yeast

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ABSTRACT During fission yeast sporulation, a membrane compartment called the forespore membrane (FSM) is newly formed on the spindle pole body (SPB). The FSM expands by membrane vesicle fusion, encapsulates the daughter nucleus resulting from meiosis, and eventually matures into the plasma membrane of the spore. Although many of the genes involved in FSM formation have been identified, its molecular mechanism is not fully understood. Here a genetic screen for sporulation-deficient mutations identified Ypt3, a Rab-family small GTPase known to function in the exocytic pathway. The ypt3-ki8 mutant showed defects in both the initiation of FSM biogenesis and FSM expansion. We also show that a mutation in Ypt2, another Rab protein that may function in the same pathway as Ypt3, compromises the initiation of FSM formation. As meiosis proceeds, both GFP-Ypt3 and GFP-Ypt2 are observed at the SPB and then relocalize to the FSM. Their localizations at the SPB precede FSM formation and depend on the meiotic SPB component Spo13, a putative GDP/GTP exchange factor for Ypt2. Given that Spo13 is essential for initiating FSM formation, these results suggest that two exocytic Rabs, Ypt3 and Ypt2, regulate the initiation of FSM formation on the SPB in concert with Spo13.

INTRODUCTION Sporulation in the fission yeast Schizosaccharomyces pombe is an intriguing cellular process that accompanies meiosis and also a cell specialization process that culminates in the formation of ascospores (Shimoda, 2004; Shimoda and Nakamura, 2004). A significant feature of sporulation is the de novo biogenesis of a double unit membrane called the forespore membrane (FSM) within the cytoplasm of the diploid mother cell (Yoo et al., 1973; Hirata and Tanaka, 1982; Tanaka and Hirata, 1982; Nakamura et al., 2001). The FSM expands by membrane vesicle fusion, which is followed by FSM closure, resulting in the formation of a prespore containing a haploid nucleus produced in meiosis (Hirata and Shimoda, 1994; Nakamura et al., 2008). After capture of the nucleus within the prespore, formation of the spore wall proceeds via deposition of spore wall materials between inner and outer layers of the FSM (Yoo et al., 1973; Hirata and Tanaka, 1982). The inner layer of the FSM becomes the plasma membrane of the nascent spore, and the outer layer eventually degrades during sporulation.

Assembly of the FSM initiates in the vicinity of the spindle pole body (SPB), which is equivalent to the centrosome of animal cells, during meiosis II. Before formation of the FSM, thick, multilayered, disk-shaped structures termed meiotic outer plaques form on the cytoplasmic side of the SPB (Hirata and Shimoda, 1994). This morphological alteration, referred to as SPB modification, is presumed to be indispensable for the initiation of FSM formation. Four SPB components essential for SPB modification have been identified—Spo2, Spo13, Spo15, and the calmodulin Cam1 (Takeda and Yamamoto, 1987; Takeda et al., 1989; Ikemoto et al., 2000; Nakamura et al., 2008; Itadani et al., 2010). During vegetative growth, the coiled-coil protein Spo15 is constitutively localized at the SPB in a Cam1-dependent manner (Itadani et al., 2010). At meiosis I, Spo2 and Spo13 are expressed and recruited to the SPB, dependent on the presence of Spo15 (Nakase et al., 2008). When meiosis II starts, another meiotic SPB component, Spo7, localizes to the SPB independently of Spo2, Spo13, Spo15, and Cam1 and is believed to...
coordinate formation of the leading edge of the FSM and the initiation of FSM assembly, thereby accomplishing accurate formation of this membrane (Nakamura-Kubo et al., 2011). An intriguing unresolved aspect of the initiation of FSM formation concerns which component of the SPB serves as the nucleation site for new membranes. Spo2, Spo13, Spo15, and Cam1 do not have domains associated with lipid-binding ability (Nakase et al., 2008), whereas Spo7 has a pleckstrin homology domain that can bind phospholipids within biological membranes. However, this domain is not essential for the initiation of FSM assembly (Nakamura-Kubo et al., 2011). Therefore unknown SPB components that anchor the FSM may be involved in meiosis-specific functions.

To ensure an orderly flow of membrane–vesicle traffic, vesicles must be highly selective in recognizing the appropriate target membrane with which to fuse. Ras-like guanine nucleotide-binding proteins, termed Ypts in yeast or Rabs in mammals, are highly conserved throughout evolution and play a central role in this process (Segev, 2001; Steinmark and Olkkonen, 2001; Takai et al., 2001). Each Rab protein has a characteristic distribution on cell membranes, and every organelle has at least one Rab protein on its cytosolic surface. Each Rab is essentially activated by a specific guanine nucleotide exchange factor (GEF), leading to its localization at a specific membrane (Blümer et al., 2013; Cabrera and Ungermann, 2013). Active GTP-bound Rab associates with the membrane via its geranylgeranylated C-terminus and recruits its effectors to the membrane, where they mediate various processes of vesicle transport (Pfeffer, 2001; Steinmark and Olkkonen, 2001). A regulatory mechanism termed the Rab cascade is involved in Saccharomyces cerevisiae exocytosis. In this cascade, the first Rab (ScYpt32) recruits the exchange factor (ScSec2) for its downstream Rab (ScSec4) to Golgi-derived secretory vesicles (Ortiz et al., 2002; Grosshans et al., 2006b; Medkova et al., 2006). The Rab cascade seems to be a general pathway conserved throughout evolution because similar cascades have been reported both in mammalian cells and at additional stages of membrane trafficking (Stenmark, 2009; Das and Guo, 2011; Hutagalung and Novick, 2011; Suda and Nakano, 2012; Pfeffer, 2013).

Components of the Rab cascade (Ypt3, Sec2, and Ypt2) are also highly conserved in S. pombe, and a mutation in either ypt3+ or ypt2+ severely inhibits post-Golgi membrane trafficking (Haurbruck et al., 1990; Craighead et al., 1993; Cheng et al., 2002). Of interest, Spo13 shares homology with the GEF domain of ScSec2 and can function as a GEF for Ypt2 (Yang and Neiman, 2010), implying that Spo13 shares homology with the GEF domain of ScSec2 and can associate with lipid-binding ability (Nakase et al., 2008), whereas Spo7 has a pleckstrin homology domain that can bind phospholipids within biological membranes. However, this domain is not essential for the initiation of FSM assembly (Nakamura-Kubo et al., 2011). Therefore unknown SPB components that anchor the FSM may be involved in meiosis-specific functions.

RESULTS

Ypt3 is required for the initiation of FSM formation and expansion of the FSM

Although studies of sporulation-deficient mutants (spo mutants) have led to the identification of many genes involved in sporulation, the molecular mechanism underlying sporulation is not completely understood. To gain further insight into the sporulation process, we isolated a novel spo mutant named spo26-ki8 (Figure 1A) from mutagenized Schizosaccharomyces pombe cells in which the FSM was visualized by green fluorescent protein (GFP)-tagged Psy1, an FSM-resident protein (Nakamura et al., 2001). The isolation and characterization of other spo mutants will be discussed elsewhere. The spo26 mutant also exhibited temperature-sensitive vegetative growth (Figure 1B), indicating that the spo26+ gene is required not only for sporulation, but also for vegetative growth. The spo26+...
gene was cloned via complementation of its temperature sensitivity and sporulation defect (see Materials and Methods). Nucleotide sequencing of the cloned DNA fragment and a complementation test with the ypt3-i5/its5-1 allele (Cheng et al., 2002) revealed that spo26 encodes the protein Ypt3, an orthologue of S. cerevisiae Ypt31/Ypt32 and mammalian Rab11 (Figure 1C; Miyake and Yamamoto, 1990; Urbe et al., 1993; Cheng et al., 2002). Ypt3 is known to regulate the exocytic pathway in fission yeast and is essential for cell viability (Cheng et al., 2002), but its role in sporulation is unclear.

To examine in detail how the ypt3-ki8 mutation impairs sporulation, we observed assembly of the FSM in the ypt3-ki8 mutant by using GFP-Psy1. Most wild-type cells displayed GFP-Psy1 fluorescence as circles of uniform brightness, representing FSMs in which a haploid nucleus was already enclosed (Figure 1A and Table 1). In contrast, ypt3-ki8 exhibited a severe defect in FSM formation. After incubation on sporulation medium at 25°C, about half of the ypt3-ki8 zygotes contained four small prespores (type I in Figure 1A and Table 1), possibly due to a defect in FSM expansion. The rest of the ypt3-ki8 zygotes exhibited a more severe phenotype in which a number of aggregates of GFP-Psy1 formed and no FSM was observed (type II in Figure 1A and Table 1). At higher temperature (28°C), the frequency of type II cells increased (Table 1).

Next we examined the initiation of FSM formation in ypt3-ki8 cells. In wild-type cells, the GFP-Psy1 signal was observed as two cup-like structures at each end of the spindle microtubules, which were visualized by mCherry-labeled Atb2, at metaphase II (Figure 1D and Supplemental Figure S1). In contrast, no GFP-Psy1 signal was observed at ~60% of the spindle poles in ypt3-ki8 cells (Figure 1D and Supplemental Figure S1). This phenotype is similar to that of mutants of meiotic SPB components such as Spo13 and Spo15 (Ikemoto et al., 2000; Nakase et al., 2008). Taken together, these data indicate that Ypt3 is essential for both the initiation of FSM formation and expansion of the FSM.

To determine the precise identity of the ypt3-ki8 allele, we isolated the mutant gene from genomic DNA by PCR amplification. Nucleotide sequencing revealed that ypt3-ki8 resulted from a single nucleotide change (C to T at position 221), which replaced alanine 74 with valine. Alanine 74 is located near the second effector region and is conserved among Rab11/Ypt3 proteins (Figure 1C).

Ypt3 localizes at the SPB and the FSM during sporulation

A comprehensive mRNA expression study previously revealed that transcription of the ypt3+ gene is up-regulated during meiosis (Mata et al., 2002), but Ypt3 protein levels during meiosis are unknown. To ascertain whether the levels of Ypt3 protein mirror those of ypt3 mRNA, we examined the abundance of GFP-Ypt3 protein during sporulation. A strain carrying the pat1-114 mutation and expressing GFP-Ypt3 was cultured at 34°C to induce synchronous meiosis. Unexpectedly, the abundance of GFP-Ypt3 remained essentially constant after the induction of sporulation (Supplemental Figure S2A).

In vegetative cells, Ypt3 localizes to cell tips and the medial region (Cheng et al., 2002). We next observed the behavior of GFP-Ypt3 during meiosis and sporulation. During prophase I, GFP-Ypt3 was observed as numerous dots dispersed throughout the cytoplasm (Figure 2A). The GFP-Ypt3 dots gradually accumulated around the nucleus before FSM formation (Figure 2, A and B, and Table 1).

![FIGURE 2: Localization of Ypt3 during sporulation. (A) Dual observation of Ypt3 and microtubules during sporulation. Homothallic haploid wild-type cells expressing GFP-Ypt3 and mCherry-Atb2 (K1114) were sporulated on ME at 28°C for 1 d. Chromosomal DNA was stained with Hoechst 33342 and analyzed by fluorescence microscopy. GFP-Ypt3 (green), mCherry-Atb2 (red), and Hoechst 33342 (blue) are overlaid in the merged images. Arrows indicate GFP-Ypt3 dots at the spindle poles. (B) Dual observation of Ypt3 and the FSM. Wild-type cells expressing GFP-Ypt3 and mCherry-Psy1 (K1118) were sporulated on ME at 28°C for 1 d. Chromosomal DNA was stained with Hoechst 33342 and analyzed by fluorescence microscopy. GFP-Ypt3 (green), mCherry-Psy1 (red), and Hoechst 33342 (blue) are overlaid in the merged images. (C) Dual observation of Ypt3 and Spo13. Wild-type cells expressing GFP-Ypt3 and Spo13-mCherry (K1132) were sporulated on ME at 28°C for 1 d and analyzed by fluorescence microscopy. GFP-Ypt3 (green) and Spo13-mCherry (red) are overlaid in the merged images. Bars, 10 μm.](image)
Sec2) and Type C, 2008), whereas the GFP-Ypt2

Thus these data indicate that Ypt3 localizes to the SPB before FSM formation and support the notion that Ypt3 is involved in the initiation of FSM formation.

Ypt2 is essential for FSM formation

In S. cerevisiae, ScYpt31 and ScYpt32 play important roles in the exocytotic pathway together with another Rab GTPase, ScSec4, which functions downstream of ScSec31 and ScSec32 (Haubruck et al., 1990). We therefore investigated whether Ypt2, an S. pombe orthologue of ScSec4, is involved in FSM formation. It has been shown that Ypt2 functions in a manner similar to ScSec4, because the ypt2-VN mutant shows defects in the late stage of the secretory pathway at the restrictive temperature (Craighead et al., 1993). Here GFP-Psy1 dots accumulated around SPBs to form cup-like structures in wild-type cells at metaphase II, but fewer of these structures were observed in ypt2-VN cells at a temperature permissive for proliferation (type A in Figure 3 and Table 2), indicating that the initiation of FSM formation is apparently defective or delayed in ypt2-VN.

To accurately determine the level of Ypt2 protein during sporulation, we cultured a strain carrying the pat1-I144 mutation and expressing GFP-Ypt2 at 34°C to induce synchronous meiosis. As in the case of Ypt3, the abundance of GFP-Ypt2 remained essentially constant after the induction of sporulation (Supplemental Figure S2B), although expression of the ypt2+ gene has been reported to be slightly up-regulated during synchronous meiosis (Mata et al., 2002).

In vegetative cells, GFP-Ypt2 was detected as dots around the plasma membrane (marked by mCherry-Psy1), especially at growing sites, cell tips, and septation sites (Figure 4A). At an early stage of meiosis (around prophase I), the GFP-Ypt2 signal was dispersed throughout the cytoplasm of the zygote (Figure 4B). At metaphase I, the GFP-Ypt2 signal was predominantly located at the spindle poles and overlapped with mCherry-labeled Spo15 during meiosis I (Figure 4, B and C), indicating that Ypt2 localizes to the SPB. When the cells proceeded to meiosis II, GFP-Ypt2 relocalized to the FSM (Figure 4D).

To determine whether localization of Ypt2 at the SPB is dependent on Spo13, we observed the localization of Ypt2 in spo13A cells. Of interest, GFP-Ypt2 fluorescence was not detected at the spindle poles in spo13A cells but was dispersed throughout the cytoplasm (Figure 5C and Supplemental Figure S4B). To examine whether this defect in Ypt2 localization at the SPB is a consequence of the defect in FSM formation, we observed the behavior of GFP-Ypt2 in spo7A cells. As shown in Figure 5C and Supplemental Figure S4B, GFP-Ypt2 dots at the SPB were observed in spo7A cells. Thus these data demonstrate that localization of Ypt2 is dependent on the meiotic SPB component Spo13. Conversely, localization of Spo13-GFP was not affected by the ypt2 mutation (Supplemental Figure S5A).

During meiosis II, cells were classified as follows: type A, containing four cup-like structures; type B, containing small FSM-like structures; type C, containing many dots of GFP-Psy1 (see Figure 3). Wild type, n = 84; ypt2-VN, n = 34.

TABLE 2: Classification of phenotypes of the FSM in meiosis II.

| Strain     | Type A | Type B | Type C |
|------------|--------|--------|--------|
| Wild type  | 88.1   | 7.1    | 4.8    |
| ypt2-VN    | 38.2   | 29.4   | 32.4   |

Localization of Ypt2 and Ypt3 depends on the meiotic SPB component Spo13

Generally, localization of Rab on the correct membrane is dependent on a specific guanine nucleotide exchange factor (GEF; Blümer et al., 2013; Cabrera and Ungermann, 2013). In S. pombe, two proteins, Sec2 (an orthologue of S. cerevisiae Sec2) and Spo13 (which has homology to the ScSec2 GEF domain), are believed to act as GEFs for Ypt2 (Figure 5A; Yang and Neiman, 2010). Because Spo13 is a meiosis-specific SPB component (Nakase et al., 2008), we hypothesized that Spo13 might recruit Ypt2 to the SPB. To confirm this, we observed the behavior of GFP-Ypt2 and Spo13-mCherry simultaneously. At an early stage of meiosis I, the Spo13-mCherry signal was detected on the SPB as dots (Figure 5B; Nakase et al., 2008), whereas the GFP-Ypt2 signal was diffused throughout the cytoplasm (Figure 5B). Subsequently, the GFP-Ypt2 signal was almost completely overlapped by Spo13-mCherry (Figure 5B), indicating that Spo13 is recruited to the SPB before Ypt2. This result was confirmed by live-cell imaging (Supplemental Figure S4A). During FSM formation, GFP-Ypt2 relocalized to the FSM, whereas Spo13-mCherry persisted on the SPB (Figure 5B).

To determine whether localization of Ypt2 at the SPB is dependent on Spo13, we observed the localization of Ypt2 in spo13A cells. Of interest, GFP-Ypt2 fluorescence was not detected at the spindle poles in spo13A cells but was dispersed throughout the cytoplasm (Figure 5C and Supplemental Figure S4B). To examine whether this defect in Ypt2 localization at the SPB is a consequence of the defect in FSM formation, we observed the behavior of GFP-Ypt2 in spo7A cells. As shown in Figure 5C and Supplemental Figure S4B, GFP-Ypt2 dots at the SPB were observed in spo7A cells. Thus these data demonstrate that localization of Ypt2 is dependent on the meiotic SPB component Spo13. Conversely, localization of Spo13-GFP was not affected by the ypt2 mutation (Supplemental Figure S5A).

Of note, Spo13 was also required for localization of Ypt3 at the SPB (Figure 6A and Supplemental Figure S3). The GFP-Ypt3 signal
The physical interaction of Ypt3 with Sec2 and Spo13 was investigated. In budding yeast, ScSec2, the GEF for ScSec4, is also an effector for ScYpt3 (Ortiz et al., 2002). Although Spo13 and Sec2 bound to inactive Ypt2 (Supplemental Figure S7) and are believed to act as Ypt2 GEFs in S. pombe (Yang and Neiman, 2010), they have not been identified as Ypt3 effectors. To evaluate the interaction of Ypt3 with Spo13 and/or Sec2 in more detail, we performed in vitro binding experiments with purified proteins. Recombinant GST-Ypt3 was immobilized on glutathione-Sepharose beads, which were then loaded with GDP or GTPγS, a GTP analogue. The beads were incubated with the lysate of cells expressing Spo13-3xGFP or Sec2-GFP and then collected and washed. Incubation of the beads with GST alone was used as a control for nonspecific binding. As shown in Figure 7A, Sec2 interacted preferentially with GST-Ypt3 bound to GTPγS. Typically, all GTPase effectors interact specifically with the GTP-bound form of a GTPase (Grosshans et al., 2006a,b; Hutagalung and Novick, 2011); thus the pull-down assay suggested that Sec2 is an effector of Ypt3 GTPase. In contrast, Spo13-3xGFP interacted with both forms of Ypt3 to a similar extent (Figure 7B). A similar pattern of interaction was detected when HA-Spo13 was used in the assay (Supplemental Figure S8). Therefore it was not possible to determine whether Spo13 acts as an effector of Ypt3, at least by in vitro binding assays.

**Localization of Sec2 during sporulation**

To investigate the role of Sec2 in FSM formation, we observed localization of Sec2-GFP during this process. Consistent with previous data (Matsuyama et al., 2006), Sec2-GFP was detected as dots at growing sites, cell tips, and the medial region during vegetative growth (Figure 8A). Similar to Ypt3 and Ypt2, Sec2-GFP was detected as numerous dots during meiosis I (Figure 8B). Some Sec2-GFP signals then accumulated on the spindle poles as meiosis proceeded, and an intense dot was observed at the center of each FSM, especially in anaphase II (Figure 8, B and C, and Supplemental Figure S9, A–C). After meiosis had completed, the concentrated Sec2-GFP dots were observed diffusely on the closed FSMs. In spo2, spo7, spo13, and spo15 mutant cells, concentrated Sec2-GFP signals were observed at the spindle poles (Supplemental Figure S9D).

In S. cerevisiae, active ScYpt3 recruits ScSec2 to its specific membrane, where ScSec2 activates ScSec4 to associate with the membrane (Mizuno-Yamashita et al., 2010). We therefore observed the behavior of Sec2-GFP in the ypt3-ki8 mutant. In vegetatively growing cells, the Sec2-GFP dots that localized at the growing sites appeared to be reduced in ypt3-ki8 cells, whereas their intensity in the cytoplasm increased (Supplemental Figure S10A). Similarly, Sec2-GFP dots at the spindle poles were significantly reduced in ypt3-ki8 cells during meiosis (Figure 9A). Taken together, these data indicate that recruitment of Sec2 to the SPB is dependent on Ypt3 but not on known meiotic SPB components such as Spo2, Spo7, Spo13, and Spo15.
The exocyst is also localized at the meiotic SPB and required for FSM formation

The exocyst, a highly conserved complex composed of eight subunits (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84), tethers secretory vesicles to the plasma membrane before exocytic membrane fusion (Heider and Munson, 2012). In budding yeast, components of the exocyst are recruited to secretory vesicles by GTP-bound ScSec4 (Guo et al., 1999; Boyd et al., 2004). To examine whether the exocyst is involved in formation of the FSM, we observed Sec8-GFP behavior during sporulation. During prophase I, the Sec8-GFP signal was observed as peripheral dots at the plasma membrane in wild-type cells. These dots then diffused and gradually appeared at the SBPs during anaphase I (Supplemental Figure S11A). The SPB localization of Sec8 was also detected in spo7Δ and spo13Δ cells (Supplemental Figure S11B), indicating that the exocyst is recruited to the SPB independently of Spo7 and Spo13.

Next we observed FSM formation in the sec8-1 mutant that shows defects in exocytosis (Wang et al., 2002). About one-third of the postmeiotic sec8-1 cells contained more than four small spore-like structures (Supplemental Figure S11, C and D), suggesting that Sec8 has a role in FSM formation. However, we did not observe a defect in the initiation of FSM formation in this mutant.

Ypt3 is required for SPB integrity during sporulation

Finally, we examined whether localization of Spo13 at the SPB is dependent on Ypt3. As shown in Figure 9B, Spo13-GFP dots were essentially detected at the spindle poles in ypt3-ki8 cells; however, a considerable fraction was localized at sites other than spindle poles. This defect in Spo13-GFP localization in ypt3-ki8 is reminiscent of that seen in a mutant of Spo20, a phosphatidylinositol/ phosphatidylinositol-transfer protein. In the spo20-H6 mutant, several SB-like structures called pseudo-SBs are separated from the spindle poles during the progression of meiosis. The pseudo-SBs contain various SB components, including essentially Spo13 and Spo15, but do not contain Alp4, a component of the γ-tubulin complex (Nakase et al., 2004). We therefore determined whether the extra Spo13-GFP dots observed in the ypt3-ki8 mutant are related to pseudo-SBs. As shown in Supplemental Figure S12, A–D, whereas Spo15-GFP was separated from the spindle poles, most Alp4 localized correctly at the spindle poles in the ypt3-ki8 mutant. These results suggest that, similar to Spo20, Ypt3 plays a role in maintaining the structural integrity of the meiotic SB but not in recruiting meiotic SB components to the SB.

DISCUSSION

Previous studies determined that various components of the membrane trafficking pathway play pivotal roles in FSM formation (Neiman, 1998, 2011; Nakase et al., 2001; Nakamura-Kubo et al., 2003; Nakamura et al., 2005). In this study, we isolated a novel spo mutant, spo26/ypt3, which exhibits a severe defect in FSM formation. Ypt3 encodes an orthologue of mammalian Rab11 GTPase, a master regulator of the post-Golgi secretory pathway. We also examined the role of Ypt2, another Rab protein involved in post-Golgi membrane vesicle transport, in the formation of the FSM. Our results demonstrate that both Rab GTPases are important in the initiation of FSM formation. The component of the SB that provides a key function as the nucleation site for the FSM has been unknown. We found that first Ypt2 and then Ypt3 are localized to the SB before formation of the FSM and are important in the initiation of FSM formation. Our results suggest that Ypt2 and
Ypt3 are potential proteins that anchor the membrane at the meiotic SPB.

During prophase I, GFP-Ypt2 signals were observed as many dots throughout the cytoplasm (Figure 4B), suggesting that Ypt2 is associated with membrane vesicles. At metaphase I, however, the GFP-Ypt2 signal was observed predominantly at the SPB, but some dots were dispersed homogeneously in the cytoplasm (Figure 4B). Furthermore, previous electron microscopy data showed that no obvious membrane- and vesicle-like structures are found on the SPB at this stage (Hirata and Tanaka, 1982; Hirata and Shimoda, 1994). Thus we presume that, during meiosis I, Ypt2 localizes to the SPB via Spo13 without associating with membrane vesicles. This is consistent with the results of the yeast two-hybrid assay, which showed that Spo13 preferentially interacts with Ypt2 in the GDP-bound form (Supplemental Figure S7). A GDP dissociation inhibitor (GDI) would bind to the Rab in GDP-bound form and inhibit the GDP/GTP exchange and the membrane association of the Rab protein. S. pombe is known to possess a single GDI, Gdi1. Of interest, Gdi1 interacts with Ypt2 physically and is up-regulated during meiosis (Mata et al., 2002; Ma et al., 2006). (A) The spindle and the merged images. Cells in meiosis II are shown in Supplemental Figure S3. (B) Localization of Ypt3 and Syb1 in S. pombe cells expressing GFP-Syb1 and mCherry-Atb2 were sporulated on ME at 28°C for 1 d. Chromosomal DNA was stained with Hoechst 33342 and analyzed by fluorescence microscopy. GFP-Ypt3 (green), mCherry-Atb2 (red), and Hoechst 33342 (blue) are overlaid in the merged images. Cells in meiosis II are shown in Supplemental Figure S5. (C) Localization of Syb1 in spo13Δ, spo7Δ, and spo7Δ spo13Δ cells expressing GFP-Syb1 and mCherry-Atb2 were sporulated on ME at 28°C for 1 d and analyzed by fluorescence microscopy. GFP-Syb1 (green) and mCherry-Atb2 (red) are overlaid in the merged images. Cells in meiosis II are shown in Supplemental Figure S6. Arrows indicate GFP dots at the spindle poles. The frequency of GFP dots at the spindle pole during late anaphase I is also indicated. Bars, 10 μm.

Figure 6: Secretory vesicles are recruited to the SPB in a Spo13-dependent manner. (A) Localization of Ypt3 in spo13Δ and spo7Δ in anaphase I. Wild-type (KI70), spo13Δ (KI99), and spo7Δ (KI98) cells expressing GFP-Ypt3 and mCherry-Atb2 were sporulated on ME at 28°C for 1 d. Chromosomal DNA was stained with Hoechst 33342 and analyzed by fluorescence microscopy. GFP-Ypt3 (green), mCherry-Atb2 (red), and Hoechst 33342 (blue) are overlaid in the merged images. Cells in meiosis II are shown in Supplemental Figure S3. (B) Localization of Syb1 in spo13Δ, spo7Δ, and spo7Δ spo13Δ cells expressing GFP-Syb1 and mCherry-Atb2 were sporulated on ME at 28°C for 1 d and analyzed by fluorescence microscopy. GFP-Syb1 (green) and mCherry-Atb2 (red) are overlaid in the merged images. Cells in meiosis II are shown in Supplemental Figure S6. Arrows indicate GFP dots at the spindle poles. The frequency of GFP dots at the spindle pole during late anaphase I is also indicated. Bars, 10 μm.

Figure 7: Physical interaction of Spo13 with Ypt3. GST or GST-Ypt3 was expressed in E. coli cells and immobilized on glutathioneSepharose beads. After preloading with either GDP or GTPγS, the beads were incubated with the lysate of yeast cells (TN104) overexpressing Sec2-GFP (A) or Spo13-3xGFP (B) from plasmid pKI202 or pKI201, respectively. The beads were precipitated by centrifugation, washed, and then subjected to immunoblotting with anti-GFP antibody (top). SDS-PAGE gels were stained with Coomassie brilliant blue (CBB) as a loading control (bottom).
The Rab and GEF proteins are recruited to the SPB at meiosis I, but FSM formation is initiated at meiosis II. How are the Rab and GEF proteins activated for the initiation of FSM formation? In mammalian cells, primary ciliogenesis—formation of the ciliary membrane—starts in the vicinity of the pericentrosome. The ciliary membrane also develops by membrane vesicle fusion. Rab11, Rabin8, and Rab8, corresponding to Ypt3, Sec2, and Ypt2, respectively, are involved in the formation of this membrane (Yoshimura et al., 2007; Knödler et al., 2010; Nachury et al., 2010; Feng et al., 2012). Under conditions of serum starvation, before formation of the ciliary membrane, Rabin8 is recruited to the membrane vesicle dependent on Rab11 and is transported to the centrosome (Westlake et al., 2011; Chiba et al., 2013). Ultimately, Rabin8 localizes to the centrosome in a Rabin8-dependent manner, facilitating the docking and fusion of vesicles and forming the ciliary membrane (Nachury et al., 2007; Westlake et al., 2011). These facts imply that the regulation of precursor vesicle fusion by Sec4 homologues on the SPB and centrosome has been conserved throughout evolution. The nuclear Dsk2-related (NDR) kinase 2 phosphorylates Rabin8, and this phosphorylation event is crucial for ciliogenesis (reviewed in Hergovich et al., 2006; Chiba et al., 2013). Of interest, S. pombe expresses a meiosis-specific NDR kinase, Mug27 (also known as Ppk35 and Slk1; Ohtaka et al., 2008; Pérez-Hidalgo et al., 2008; Westlake et al., 2011; Chiba et al., 2013). Although mug27 deletion reduces spore size, the mutant cells can initiate FSM formation. Therefore the regulation of Rabin8/Spo13/Sec2 may essentially differ between formation of the FSM and formation of the ciliary membrane. Further work is required to identify the molecules that regulate the Rab cascade in meiosis-specific functions.

FIGURE 8: Localization of Sec2. (A) Localization of Sec2 during vegetative growth. Wild-type (K129) cells expressing Sec2-GFP and mCherry-Psy1 were cultured on YE medium at 25°C for 1 d. Sec2-GFP (green) and mCherry-Psy1 (red) are overlaid in the merged images. (B) Dual observation of Sec2 and microtubules. Wild-type cells (K134) expressing Sec2-GFP from plasmid (pK133) and mCherry-Atb2 were sporulated on SSA including 15 μM thiamine at 28°C for 1 d and analyzed by fluorescence microscopy. Sec2-GFP (green) and mCherry-Atb2 (red) are overlaid in the merged images. Arrows indicate Sec2-GFP dots at the spindle poles. (C) Dual observation of Sec2 and the FSM. Wild-type cells (K153) expressing Sec2-GFP from plasmid (pK133) and mCherry-Psy1 were sporulated on SSA including 15 μM thiamine at 28°C for 1 d and analyzed by fluorescence microscopy. Sec2-GFP (green) and mCherry-Psy1 (red) are overlaid in the merged images. Arrows indicate Sec2-GFP dots on the FSM. Bars, 10 μm.

Therefore it is possible that the SPB localization of the exocyst and its potential binding partner Sec2 may depend on F-actin, which concentrates around the nucleus before FSM formation starts (Petersen et al., 1998; Toya et al., 2001). We previously showed that Psy1, the fission yeast orthologue of syntaxin 1, is selectively internalized by endocytosis and relocated to the nascent FSM (Kashiwazaki et al., 2011). Given that Syb1 is already localized to the SPB (Figure 6B and Supplemental Figure S6), one possibility is that the Rab cascade (Ypt3→Sec2→Ypt2 and/or Ypt3→Spo13→Ypt2) may regulate fusion between secretory vesicles, including Syb1 and endocytic vesicles containing Psy1 on the SPB. In support of this possibility, we identified psy1 and syb1 mutants in which FSM formation is completely inhibited (unpublished data). However, the exocyst mutant sec8-1 did not show a severe defect in the initiation of FSM formation (Supplemental Figure S11, C and D). We suggest that this is due to the weakness of the sec8-1 allele. Alternatively, membrane tethering by the exocyst might not be essential for the initiation of FSM formation as in the case of polarized growth in S. pombe (Wang et al., 2002).

MATERIALS AND METHODS

Yeast strains and culture conditions

The fission yeast strains used in this study are listed in Supplemental Table S1. Strains expressing GFP- or mCherry-tagged proteins were constructed by crossing two strains and/or by integration of the plasmid DNA listed in Supplemental Table S2. The tagged proteins were deemed functional on the basis of their ability to rescue the defects of the mutants (unpublished data). Vegetative cultures were deemed functional on the basis of their ability to rescue the defects of the mutants (unpublished data). Vegetative cultures were propagated in complete (yeast extract [YE]) or synthetic (synthetic defined [SD], modified minimal medium [MB], or minimal medium plus nitrogen [MM+N]) medium supplemented with nutrients essential for auxotrophic strains (Egel and Egel-Mitani, 1974; Moreno et al., 1990; Okazaki et al., 1990). For sporulation, cells precultured in YE, SD, or MM+N were incubated in malt extract medium (ME) or synthetic sporulation medium (SSA or MM+N), respectively. Synchronous meiosis was induced in diploid strains harboring the pat1-114 allele by a temperature shift as previously described (Iino et al., 1995).

Plasmids

The plasmids used in this study are listed in Supplemental Table S2. Genomic DNA encoding ypt3+, ypt2+, and sec2+ was amplified by PCR from L968 genomic DNA or the genomic library pTN-L1 (Nakamura et al., 2001) and used to construct plasmids carrying the respective genes. The cDNA clone of ypt3+, FYC239, was provided by the Yeast Genetic Resource Center (yeast.lab.nig.ac.jp/ig/index_en .html). The ypt3+ cDNA was amplified and cloned into pGEX-KG (Guan and Dixon, 1991) at a BamHI–EcoRI site to construct pK334, pGEX-KG (ypt3). A GTP- or GDP-locked mutation was introduced into ypt2+ (Q68L or S18V, respectively) by site-directed mutagenesis using the two-step PCR method, and the products were cloned into pGADT7 or pGBK77 (Clontech Laboratories, Mountain View, CA).
for 5 d, colonies with a defect in ascospore formation were detected by exposure to iodine vapor, which stains fission yeast colonies that have sporulated normally. Nonstained cells were observed under a fluorescence microscope to screen for spo mutants that completed mating and meiotic divisions but showed aberrant FSM formation.

**Cloning and identification of spo26** gene

The spo26 mutant K18 cells transformed with the S. pombe genomic library pTN-L1 (Nakamura et al., 2001) containing partial Sau3AI fragments constructed in the multi-copy plasmid pAL-KS (Tanaka et al., 2000) were spread on SD and incubated for 4 d at 37°C. Viable clones that recovered the temperature sensitivity, which is genetically linked to the sporulation defect, were screened. The isolated clones were then incubated on SSA for 2 d at 28°C, and suppression of the spo mutation was verified by microscopy.

**Fluorescence microscopy**

Proteins were visualized by fusion to GFP or mCherry. The nuclear chromatin region was stained with bisbenzimide H33342 fluorochrome trihydrochloride (Hoechst 33342). Living cells were observed under a fluorescence microscope (model BX51; Olympus, Tokyo, Japan), and images were obtained by using a cooled charge-coupled device camera (ORCA-R2; Hamamatsu Photonics, Hamamatsu, Japan) controlled by AQUA-COSMOS software (Hamamatsu Photonics). The time-lapse observation was performed as follows. Cells were incubated on ME at 28°C for 1 d. After conjugation, cells were inoculated to MM-N medium containing 15 μM thiamine on a cell culture dish with a glass bottom (Greiner Bio-One, Frickenhausen, Germany) and observed under a fluorescence microscope (model IX71; Olympus) as described in Nakamura et al. (2008). Images were processed with ImageJ (National Institutes of Health, Bethesda, MD).

**Western blotting**

Proteins were resolved by SDS–PAGE and then transferred to a polyvinylidene difluoride membrane (Immobilon-P; Merck Millipore, Darmstadt, Germany). Blots were probed with a rat anti-GFP antibody (a gift from S. Fujita, Mitsubishi Kagaku Institute of Life Sciences), the anti-α-tubulin antibody TAT-1 (Woods et al., 1989), or an anti-hemagglutinin antibody (3F10; Roche Diagnostics, Basel, Switzerland) at a dilution of 1:5000. Immunoreactive bands were revealed by ECL Select chemiluminescence (GE Healthcare, Little Chalfont, United Kingdom) with horseradish peroxidase–conjugated goat anti-rat immunoglobulin G (Biosource International, Camarillo, CA) or sheep anti-mouse IgG (GE Healthcare).

Further information on the plasmids constructed in this study is on the National BioResource Project Yeast website (yeast.lab.nig.ac.jp/nig.v2.1/index_en.html).

**Isolation of sporulation-deficient mutants**

Cells harboring GFP-Psy1, a fluorescent marker of the FSM, were mutagenized with DNA-alkylating agents as follows. TN414 cells were treated with N-methyl-N-nitro-N-nitrosoguanidine. The cells were spread on ME plates to induce sporulation. After incubation at 28°C for 5 d, colonies with a defect in ascospore formation were detected by exposure to iodine vapor, which stains fission yeast colonies that have sporulated normally. Nonstained cells were observed under a fluorescence microscope to screen for spo mutants that completed mating and meiotic divisions but showed aberrant FSM formation.

**Fluorescence microscopy**

Proteins were visualized by fusion to GFP or mCherry. The nuclear chromatin region was stained with bisbenzimide H33342 fluorochrome trihydrochloride (Hoechst 33342). Living cells were observed under a fluorescence microscope (model BX51; Olympus, Tokyo, Japan), and images were obtained by using a cooled charge-coupled device camera (ORCA-R2; Hamamatsu Photonics, Hamamatsu, Japan) controlled by AQUA-COSMOS software (Hamamatsu Photonics). The time-lapse observation was performed as follows. Cells were incubated on ME at 28°C for 1 d. After conjugation, cells were inoculated to MM-N medium containing 15 μM thiamine on a cell culture dish with a glass bottom (Greiner Bio-One, Frickenhausen, Germany) and observed under a fluorescence microscope (model IX71; Olympus) as described in Nakamura et al. (2008). Images were processed with ImageJ (National Institutes of Health, Bethesda, MD).

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Prophase I

Metaphase I

Anaphase I

Prophase II

Metaphase II

FIGURE 10: Working model of the initiation of FSM formation.

Pull-down assay
Recombinant glutathione S-transferase (GST) and GST-Ypt3 were purified as follows. Escherichia coli cells (BL21 Rosetta) harboring pTN174 or pKI34 were grown at 37°C overnight. The culture was diluted 1:1000 in 200 ml of Luria–Bertani broth (LB) and incubated at 37°C for 2 h. After incubation, expression was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactoside, and the culture was disrupted by grinding with a pestle and mortar in liquid nitrogen. The lysate was centrifuged at 18,000 × g for 15 min, and 50 μl of supernatant was incubated with the prepared beads at 4°C for 2 h in the presence of 0.2 mM GTPγS or GDP. The beads were washed with buffer C containing 10 μM GTPγS or GDP five or more times before analysis.

Yeast cells harboring pREP81 (sec2-GFP), pREP81 (spo13-3xGFp), or pREP41 (HA-spo13) were grown in MM+N for 20 h, harvested, and washed with water containing 1 mM PMSF. Next 3.0 × 10^10 cells were suspended in 1 ml of lysis buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2, 100 mM NaCl, 10 mM MgCl2, 1% Triton X-100, 1 mM DTT, 1 mM PMSF, and 1x PIC) before being rapidly frozen in liquid nitrogen. The cells were disrupted by grinding with a pestle and mortar in liquid nitrogen. The lysate was centrifuged at 18,000 × g for 15 min, and 50 μl of supernatant was incubated with the prepared beads at 4°C for 2 h in the presence of 0.2 mM GTPγS or GDP. The beads were washed with buffer C containing 10 μM GTPγS or GDP five or more times before analysis.

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