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Identification of sporulation-defective genes and a Rab-cascade model for the formation of the forespore membrane in *Schizosaccharomyces pombe*

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Abstract of the Dissertation

Identification of sporulation-defective genes and a Rab-cascade model for the formation of the forespore membrane in *Schizosaccharomyces pombe*

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In the fission yeast, *Schizosaccharomyces pombe,* environmental stress conditions such as nitrogen starvation induce mating of two haploid cells. The newly formed diploid cells immediately undergo meiosis and form spores, which are equivalent to gametes in higher organisms. The packaging of the haploid nuclei, which are formed after two rounds of meiosis, is achieved by the *de novo* formation of double membranes called forespore membranes, which form adjacent to the meiotic spindle pole bodies (SPBs). This work focuses on the initiation of the forespore membrane at the SPB and the genes that are required for the proper formation of the forespore membrane. To do this, I performed a systematic screen of a set of ~3,300 non-essential *S. pombe* gene deletion mutants to identify genes required for spore formation. By using high throughput genetic methods, I identified 34 genes whose deletion reduces sporulation. Fifteen genes are defective in forespore membrane morphogenesis. As a secondary project, I found that two Rab-GTPases in *S. pombe* that are involved in the late steps of the exocytic pathway, *ypt3* and *ypt2*, localize to the SPB and the forespore membrane. This suggests a Rab-cascade model for the initiation and the formation of the forespore membrane in *S. pombe* sporulation. Additionally, I found that a novel SPB-associated protein Mug79 is required for the localization of the class V myosin motor protein Myo51 to the SPB.

Dedication Page

All of my work is dedicated to my beloved husband Zafer who inspires, supports, protects, and believes in me; to my sons Omer and Ali whose love give me the strength to go on.

I couldn’t have it done without you.

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Chapter 1

Introduction

Yeast sporulation is a response to environmental stress conditions such as nitrogen starvation (Tomar *et al.*, 2013). It is a model for cellular differentiation and *de novo* membrane formation within a mother cell (Neiman, 2005). Starting with the mating of two haploid cells, the steps of the ascospore formation such as meiosis, spindle pole body modification, forespore membrane formation, successful closure of the membranes and finally the spore wall maturation constitute a highly regulated developmental process. During sporulation the mitotic machinery is remodeled in many ways to accommodate changing conditions as the cell differentiates from a vegetative cell into a spore (Neiman, 2005). The budding yeast *Saccharomyces cerevisiae* is the most studied model for the ascospore formation. Though the pathway of spore formation in *S. cerevisiae* and *Schizosaccharomyces pombe* is similar, the genes involved are not well conserved (Shimoda, 2004a). Comparison of sporulation in these two distantly related yeasts should provide greater insight into ascospore morphogenesis in ascomycetes in general.

The life cycle of Schizosaccharomyces pombe

The fission yeast *S. pombe* is a single-celled eukaryote and, similar to the budding yeast *S. cerevisiae,* it is commonly used as a model organism. These two yeasts diverged from a common ancestor about 1000 million ago (Forsburg, 2005). The complete genome of *S. pombe* was sequenced in 2002 and it has approximately 4800 genes (Wood *et al.*, 2002). The haploid *S. pombe* genome is 13.8 Mb that are organized into three chromosomes I (~5.7 Mb), II (~4.6 Mb) and III (~3.5 Mb) (Wixon, 2002). Unlike the budding yeast, the *S. pombe* genome shows no evidence for large scale gene duplication (Wood *et al.*, 2002). Nevertheless, similar to *S. cerevisiae*, *S. pombe* is a powerful tool for the identification of many biological processes that are active in humans (Bresch, 1968; Moreno *et al.*, 1991; Moreno, 2004).

An *S. pombe* cell has two distinct phases in its life cycle (Figure 1.1). The choice of the life cycle depends mostly on the availability of the nutrients. Under rich growth conditions, *S.*

pombe remains as a haploid (Hayles and Nurse, 1989), proliferates mitotically, grows exponentially and divides by medial fission to produce two equal-sized daughter cells (Moreno *et al.*, 1991; Wixon, 2002; Shimoda, 2004a; Shimoda, 2004b; Cooper, 2013) (Figure 1.1). This is called the vegetative state in the life cycle of *S. pombe*. In yeast, sporulation starts when a mitotically dividing cell decides to differentiate into spores. This change in the life cycle of a yeast cell is a form of a cell differentiation (Neiman, 2005). Cellular differentiation is a transition of a cell from one cell type into another specialized cell. Differentiation during development is achieved by a switch from one pattern of gene expression to another as the result of a gene regulatory network. Sporulation program is mostly regulated at the transcriptional level where the meiosis and sporulation specific genes are only expressed after nutrient starvation and otherwise silenced. There are master regulators in the transcription of these genes. For example, in *S. pombe ste11* is activated by nitrogen starvation and it induces the transcription of the genes that are required for mating and the early stages of the sporulation (van Werven and Amon, 2011). Thus, activation of a transcription factor initiates the differentiation pathway. Understanding the transcriptional regulation of genes that are silenced or activated during vegetative cells and spores will provide insight into the differentiation of stem cells into specialized cells in higher organisms. Because, similar to yeast spores, specialized cells share the exact same genome with the cells that they were originated; however, the expression pattern, which defines the role of these cells, is different.

In *S. pombe,* the decision to differentiate into spores depends on the lack of nitrogen and the mating type of the cell. When the cells encounter the lack of nitrogen source, they exit mitosis to differentiate into spores (Tanaka, 1982; Egel, 1989; Shimoda, 2004b) (Figure 1.1). In this stage two opposite mating-type cells undergo a pheromone-dependent arrest at the G1 phase of the cell cycle (Nurse and Bissett, 1981) and then mate and fuse to form a diploid zygote that enters immediately into meiosis and sporulation to form four haploid zygotes (Bresch, 1968; Yoo, 1973; Wixon, 2002; Moreno, 2004) (Figure 1.1).

In general, yeast ascospores are dormant cells that have a high degree of resistance to environmental stress factors including but not limited to heat, digestive enzymes and organic solvents like ethanol, ether and acetone (Neiman, 2005; Coluccio *et al.*, 2008; Smith, 2009;

Neiman, 2011). When the conditions improve, yeast spores can germinate and continue life by proliferating by mitosis.

Mating-type in S. pombe cells

In rich growth conditions, haploid *S. pombe* cells are sterile in that they do not express their mating-types and therefore they do not mate but grow by mitosis (Klar, 2007). When the cells are starved for nitrogen they start to express their cell types (Egel, 1989). The mating-type region of *S. pombe* is on Chromosome II and it is composed of three loci that are linked to each other: *mat1, mat2* and *mat3* (Matsuda et al., 2011) (Figure 1.2). The lab strains of *S. pombe* have two mating-types that are called *P*lus (*P* or h^+) and *M*inus (*M* or h^-). h^+ and h^- type cells may have one of these two different gene cassettes (*P* or *M*) on the *mat1* locus and this information on the *mat1* locus determines the mating-type of the cell (Yamada-Inagawa *et al.*, 2007; Matsuda *et al.*, 2011). h^+ cells contain the *mat1-P* genetic information at the mating-type locus whereas the h^- cells acquire the *mat1-M* allele (Klar, 2007; Yamada-Inagawa *et al.*, 2007). The two other *mat* loci which are located centromere-distal to the *mat1* locus on Chromosome II contain the information for plus and minus alleles, *mat2-P* and *mat3-M,* but they remain silenced and embedded in a heterochromatin domain (Matsuda *et al.*, 2011) (Figure 1.2). Wild-type homothallic strains, which are called h^{90}, have the ability to switch their mating types as a result of an epigenetic modification that is specific to switchable cells (Matsuda *et al.*, 2011). During cell division one of the silenced mating-type cassettes (*mat2-P* or *mat3-M*) serves as a donor for the daughter cells, giving rise to an opposite mating-type daughter cell and a parental mating-type daughter cell (Yamada-Inagawa *et al.*, 2007). By doing this, there are always h^+ and h^- cells available in a colony. This is important because meiosis and sporulation are induced only in diploid cells (Bresch, 1968). Thus, these strains do not need an opposite mating type strain but can mate within the same genetic background. Therefore mating of two genetically identical h^{90} cells yields to the formation of genetically identical homozygous diploids which in turn produces four haploid h^{90} spores (Bresch, 1968).

Meiosis produces spores and starts immediately after the mating of two haploid S. pombe cells

Mating of two haploid yeast cells produces strains with new genetic combinations or mutations that allow us to understand the effects of genetic interactions, and how different proteins partner together in biological processes, as in sporulation. (Wixon, 2002). When the nitrogen source is removed, two *S. pombe* cells with different mating types (h^+ and h^-) conjugate and the zygote undergoes karyogamy by fusing the two nuclei derived from the parental cells into one (Ohta *et al.*, 2012) (Figure 1.3). Nuclear fusion occurs by the fusion of two spindle pole bodies (SPBs) and the microtubule array anchored to the SPB causes the nucleus to oscillate back and forth in the cell (Chikashige *et al.*, 2007). This meiotic prophase specific SPB-driven nuclear oscillation is called horse-tail nuclear movement and is essential for promoting meiotic recombination of homologous chromosomes (Chikashige *et al.*, 2007; Ohta *et al.*, 2012).

During meiosis, pre-meiotic DNA replication and meiotic recombination are followed by two successive divisions (Meiosis I and Meiosis II) of a diploid nucleus that are necessary for sexual reproduction in eukaryotes (Alberts, 2002) (Figure 1.3). In *S. pombe*, similar to mitosis, the nuclear envelope remains closed during meiosis (Asakawa *et al.*, 2011). After Meiosis I, nuclei are separated into two discrete poles and in Meiosis II the chromosomes are segregated into four haploid nuclei which are then packaged into daughter cells (Shimoda, 2004a; Shimoda, 2004b) (Figure 1.3)

During Meiosis I and II, the SPB undergoes two rounds of duplication and the function of the cytoplasmic face of meiotic SPBs changes from microtubule nucleation to membrane initiation (Shimoda, 2004a) (Figure 1.3). The cytoplasmic face of the meiotic SPB is called the meiotic outer plaque (MOP) (Figure 1.4). MOPs are created by the recruitment of meiosis-specific proteins that triggers the initiation of forespore membrane formation and sporulation (Ikemoto *et al.*, 2000; Nakamura, 2001; Nakase *et al.*, 2008; Itadani *et al.*, 2010). Sporulation is completed with the deposition of spore wall material into the lumen of the forespore membrane after the expanding ends fuse to complete closure (Shimoda, 2004a; de Medina-Redondo *et al.*, 2008).

The spindle pole body serves as the initiation site of the forespore membrane

The SPB is the functional homolog of the centrosome in higher eukaryotes and serves as the microtubule-organizing center in yeast, where spindle microtubules initiate assembly during mitosis (Adams and Klimartin, 2000; Ohta *et al.*, 2012). Each SPB has a nucleoplasmic and a cytoplasmic face. In *S. pombe,* unlike *S. cerevisiae,* the SPB is positioned on the cytoplasmic side of the nuclear envelope during interphase (Ding *et al.*, 1997; Ohta *et al.*, 2012) and it is embedded in the nuclear envelope during mitosis. As cells enter M phase, the nuclear envelope opens a fenestra that allows the access of the SPB to the nucleus (Ding *et al.*, 1997; Adams and Klimartin, 2000).

The SPB plays an essential role both in mitosis and sexual reproduction and duplicates in mitosis and meiosis (Adams and Klimartin, 2000; Uzawa *et al.*, 2004). In mitosis and Meiosis I, the SPB structure is similar; however SPBs are remodeled at the beginning of Meiosis II (Shimoda, 2004a) (Figures 1.4 & 1.5). Ohta *et al.,* (2012) showed that when the horse-tail nuclear movement was observed, some SPB resident proteins (e.g. Plo1, Pcp1, Cut12 and Spo15) that are present during mitosis disappear from the SPB throughout meiotic prophase and reappear at the onset of Meiosis I. This dynamic movement of structural SPB proteins suggests a remodeling of the SPB in order to prepare for the subsequent steps of meiosis and sporulation.

The recovery of SPB components prior to Meiosis I presumably leads to the subsequent structural change of the SPBs from a dot-like structure to a crescent shape during Meiosis II (Hagan and Yanagida, 1995). The cytoplasmic face of SPBs change their protein composition and MOPs are formed, which causes the enlarged appearance of the SPB in the electron microscopy (Tanaka, 1982; Hirata and Shimoda, 1994; Shimoda, 2004a) (Figure 1.5). Nonetheless, this modification of the SPBs switches their function from microtubule nucleation to forespore membrane initiation (Ikemoto *et al.*, 2000; Shimoda, 2004a) (Figures 1.4 & 1.5). The components of the *S. pombe* MOP have not been completely identified. As mentioned earlier, the sporulation process in *S. cerevisiae* is similar to that in *S. pombe.* The MOP components of *S. cerevisiae* have been studied extensively. In budding yeast it is known that the MOP serves as a vesicle-tethering complex and regulates *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) -mediated membrane fusion (Yang and Neiman, 2010), in

which the SNARE proteins form bridges between the nascent forespore membrane and the newly arrived vesicles in order to promote vesicle docking and fusion to the newly synthesized membranes. Thus, SNARE-mediated membrane fusion is required for the formation of the forespore membrane (Shimoda, 2004a)

Forespore membrane formation

The forespore membrane initiates at the outer surface of the SPB during Meiosis II; strictly after the Meiosis I and the formation of the membrane is highly coordinated with the meiotic nuclear division (Tanaka, 1982; Hirata and Shimoda, 1994; Nakase, 2001; Shimoda, 2004b). Prior to Meiosis II, vesicles that are carrying forespore membrane proteins start to appear within the cytoplasm of the mother cell (Shimoda, 2004a). At the onset of Meiosis II, these vesicles gather around the MOP of the SPB and fuse to form the newly synthesized forespore membrane via SNARE-mediated membrane fusion (Figure 1.4) (Nakamura *et al.*, 2005; Nakase *et al.*, 2008; Maeda *et al.*, 2009; Yamaoka *et al.*, 2013) (Figure 1.4 & 1.5).

As in prospore membrane formation (equivalent to the forespore membrane) in *S. cerevisiae*, the origins of the forespore membrane are post-Golgi vesicles that are redirected towards the nascent SPBs during meiosis (Neiman, 1998b; Nakase, 2001; Nakamura-Kubo *et al.*, 2003; Neiman, 2005; Nakase *et al.*, 2008; Neiman, 2011) (Figure 1.4). *psy1* is a homolog of *S. cerevisiae SSO1* and mammalian STX1A (Yamaoka *et al.*, 2013) that encodes a SNARE of the plasma membrane docking and fusion complex. *psy1* is essential and it localizes to the plasma membrane during vegetative growth (Maeda *et al.*, 2009; Kashiwazaki *et al.*, 2011). During sporulation its transcription is enhanced, and, in fact, it relocates from the plasma membrane to the forespore membrane via an endocytic pathway (Kashiwazaki *et al.*, 2011).

Similar to *psy1*, *sec9* is the homolog of the mammalian SNARE SNAP-25 and is essential for growth (Nakamura *et al.*, 2005). *sec9* localizes to the plasma membrane during vegetative growth and to the forespore membrane during sporulation. *sec9* mutants have severe forespore membrane defects, thus *sec9* is indispensable for spore formation (Yamaoka *et al.*, 2013). Both Psy1 and Sec9 are t-SNAREs, meaning that they are the components of the membrane fusion complex localized to target membrane compartments (Nakamura *et al.*, 2005; Maeda *et al.*, 2009; Kashiwazaki *et al.*, 2011; Yamaoka *et al.*, 2013). Recently, another

component of the docking/fusion complex was found to be essential for the forespore membrane morphogenesis. The synaptobrevin ortholog *syb1* (*SNC1/SNC2* in *S. cerevisiae* and VAMP4 in human) is a v-SNARE that localizes to the vesicles (Yamaoka *et al.*, 2013) and is upregulated during sporulation (Mata *et al.*, 2002; Yamaoka *et al.*, 2013). Altogether, the important roles of these SNARE proteins during forespore membrane formation support the model that the precursors of the forespore membranes are the secretory vesicles. It also suggests roles for other secretory pathway components (e.g. RabGTPases and their GAPs and GEFs) during ascospore formation.

The spore wall

Sporulation ends when the expanding ends of the forespore membrane are closed and the spore wall material is deposited into the lumen of the double membrane. At this stage the inner layer of the forespore membrane becomes the plasma membrane of the haploid spore and the spore wall is built by the deposition of the wall material into the lumen of the forespore membrane (Yoo, 1973; Tanaka, 1982; Nakase, 2001). Finally, the mother cell collapses and the mature spores separate from each other (Nakase, 2001).

The vegetative *S. pombe* cell wall is composed of mannoproteins, α-glucan and β-glucan (de Medina-Redondo *et al.*, 2008) (Figure 1.6). There is also a small amount of glucosamine in the cell wall (Sietsma and Wessels, 1990; Matsuo *et al.*, 2004). Although all of the structural components are not well identified, it has been shown that the fission yeast spore wall has electron-transparent and electron-dense layers (Bush *et al.*, 1974; Horisberger and Rouvet-Vauthey, 1985) (Figure 1.6). The inner spore wall (electron-transparent) contains β-1,3-glucan, whereas the outer spore wall (electron-dense) is made of an amylose-like material that is stained dark brown color by iodine vapor (de Medina-Redondo *et al.*, 2008) (Figure 1.6). Alpha-glucans are shown to be more abundant and constitute ~46% of the total polysaccharide content in the ascospore cell wall whereas β-glucans are ~ 38% (Garcia *et al.*, 2006). Interestingly, β-1,3-glucan layer-defective mutants are not defective in iodine staining (Liu *et al.*, 2000). Although α-1,3-glucan linkages (synthesized by the α-glucan synthase subunits Mok12 and Mok13) are more abundant in the α-glucan layer, the minor α-1,4-glucan polymer (synthesized by Mok14) is the iodine-reactive material in the spore wall. The α-glucan synthases *mok12*, *mok13* and *mok14* are paralogs of the vegetative *mok1* gene (Hochstenbach *et al.*, 1998); however, these proteins are

synthesized only during sporulation (de Medina-Redondo *et al.*, 2008). The minor components of the spore wall (chitin and chitosan) form a matrix between the other polymers, hence giving overall support to the spore wall (Arellano *et al.*, 2000; Matsuo *et al.*, 2004; de Medina-Redondo *et al.*, 2008).

In this work, to gain more insight on the genes that are required for *S. pombe* sporulation we performed a genome-wide systematic screen of the *S. pombe* haploid deletion set. This screen identified 34 genes whose individual deletion diminishes the sporulation. Fifteen of these gene deletions are involved in the forespore membrane morphogenesis. Additionally, we analyzed the role of the late-secretory pathway genes during sporulation. We suggest that a Rab-cascade is essential for the recruitment and fusion of the secretory vesicles to the newly formed forespore membranes during sporulation.

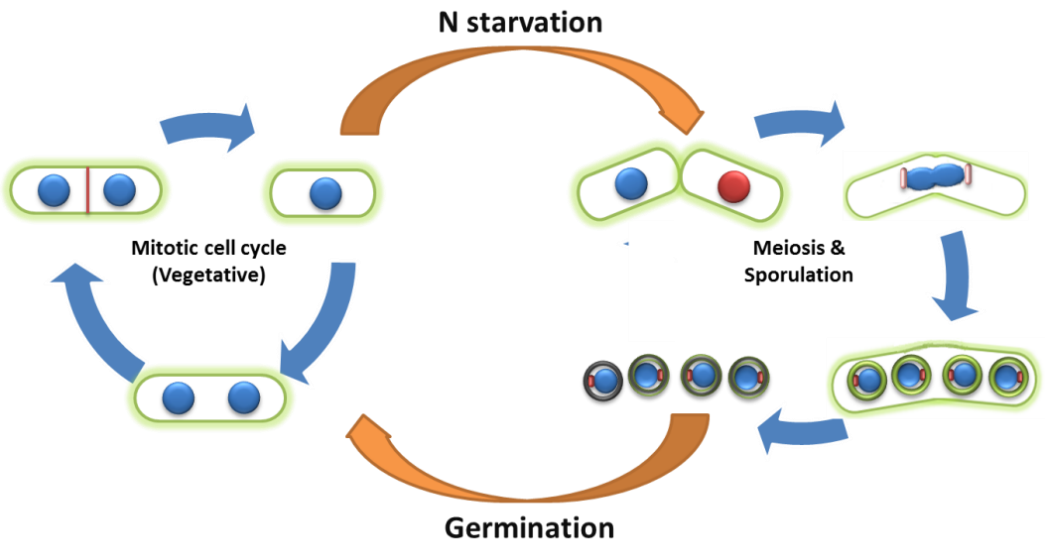


Figure 1.1 The life cycle of *S. pombe*

Mitotically proliferating *S. pombe* cells divide by medial fission under normal growth conditions. When the cells experience nitrogen depletion, they quit the mitotic cycle and sporulate.

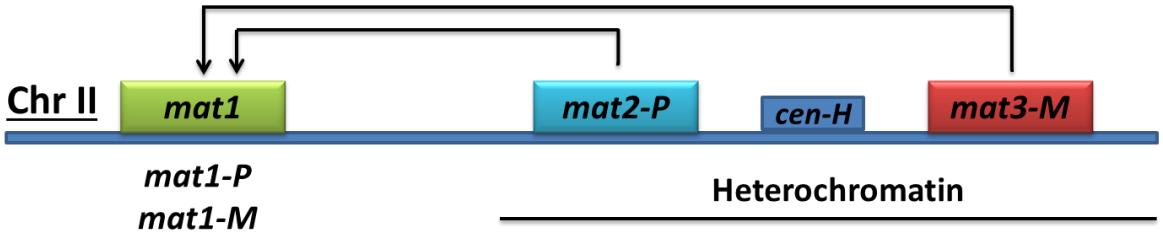


Figure 1.2 Mating-type locus of *S. pombe* on Chromosome II

mat1 (green box) is the active *mat* locus. Presence of *mat1-P* or *mat1-M* on this locus defines the mating-type of the cell. Inactive loci *mat2-P* (blue box) or *mat3-M* (red box) are embeded in heterochromation. In homothallic strains (h^{90}) that have the ability to switch their mating types, these two loci serve as the donors to the active *mat* locus for the daughter cells resulting in a mixture of *mat1-P* and *mat1-M* expressing cells.

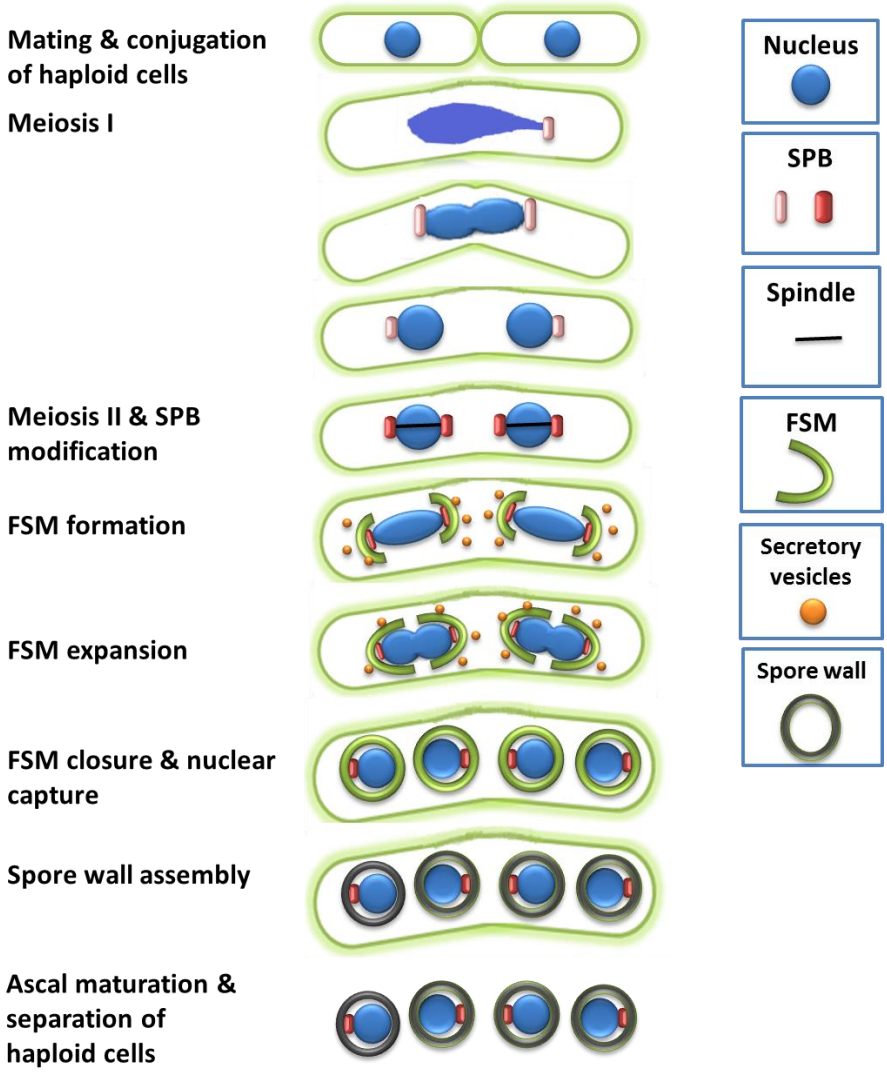


Figure 1.3 Steps of meiosis and sporulation in *S. pombe*

Under nitrogen starvation conditions two opposite mating type haploid *S. pombe* cells mate and directly undergo meiosis and sporulation. Blue circle indicates nucleus that goes through two rounds of meiosis and is packaged by a bilayer membrane. SPB (red) is duplicated twice during Meiosis I and Meiosis II. Post-Golgi vesicles (orange dots) form the forespore membrane (green).

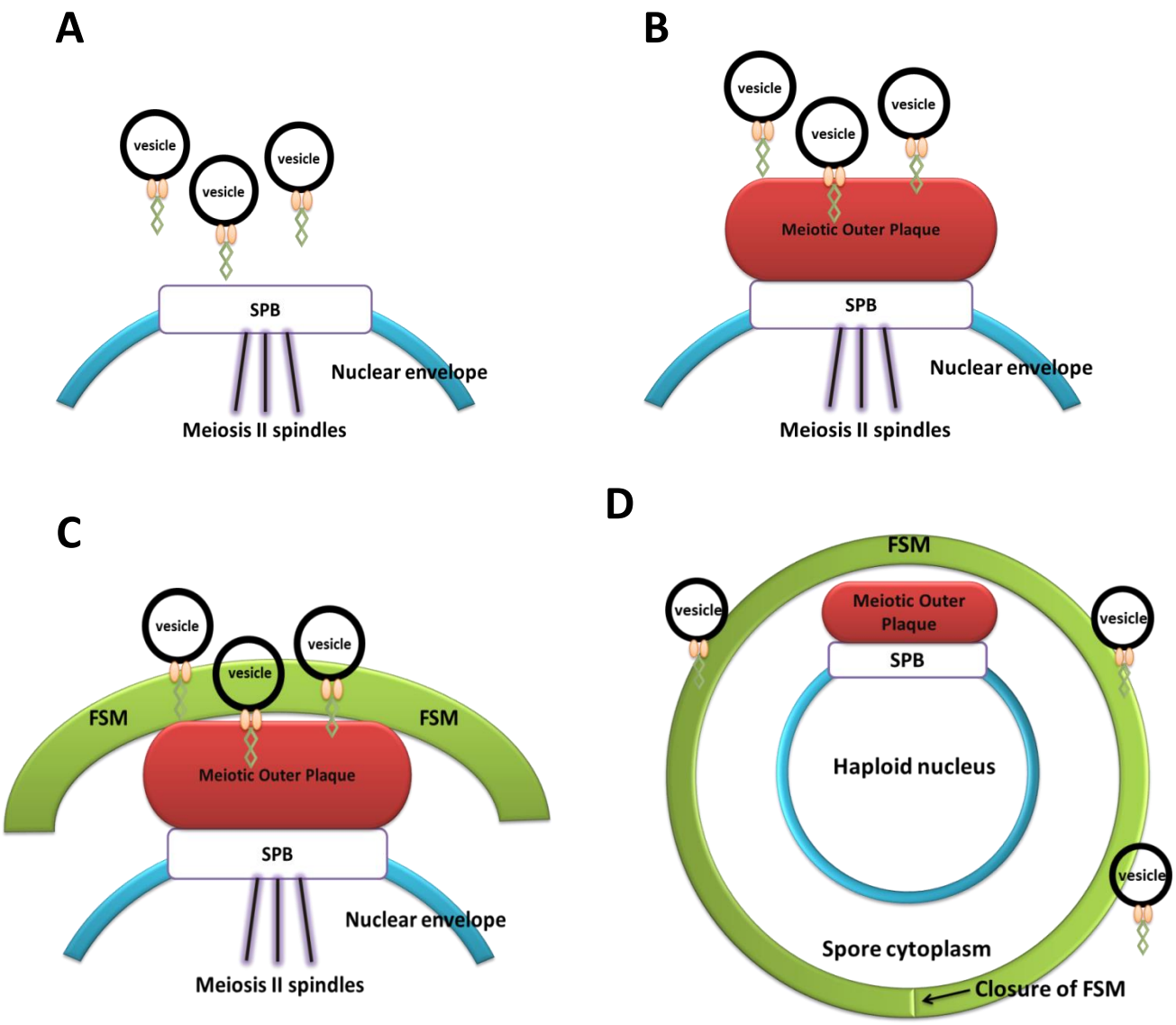


Figure 1.4 Spindle pole body modification and the vesicle transport

Schematic shows the steps of MOP formation, secretory vesicle recruitment and forespore membrane formation in *S. pombe*.

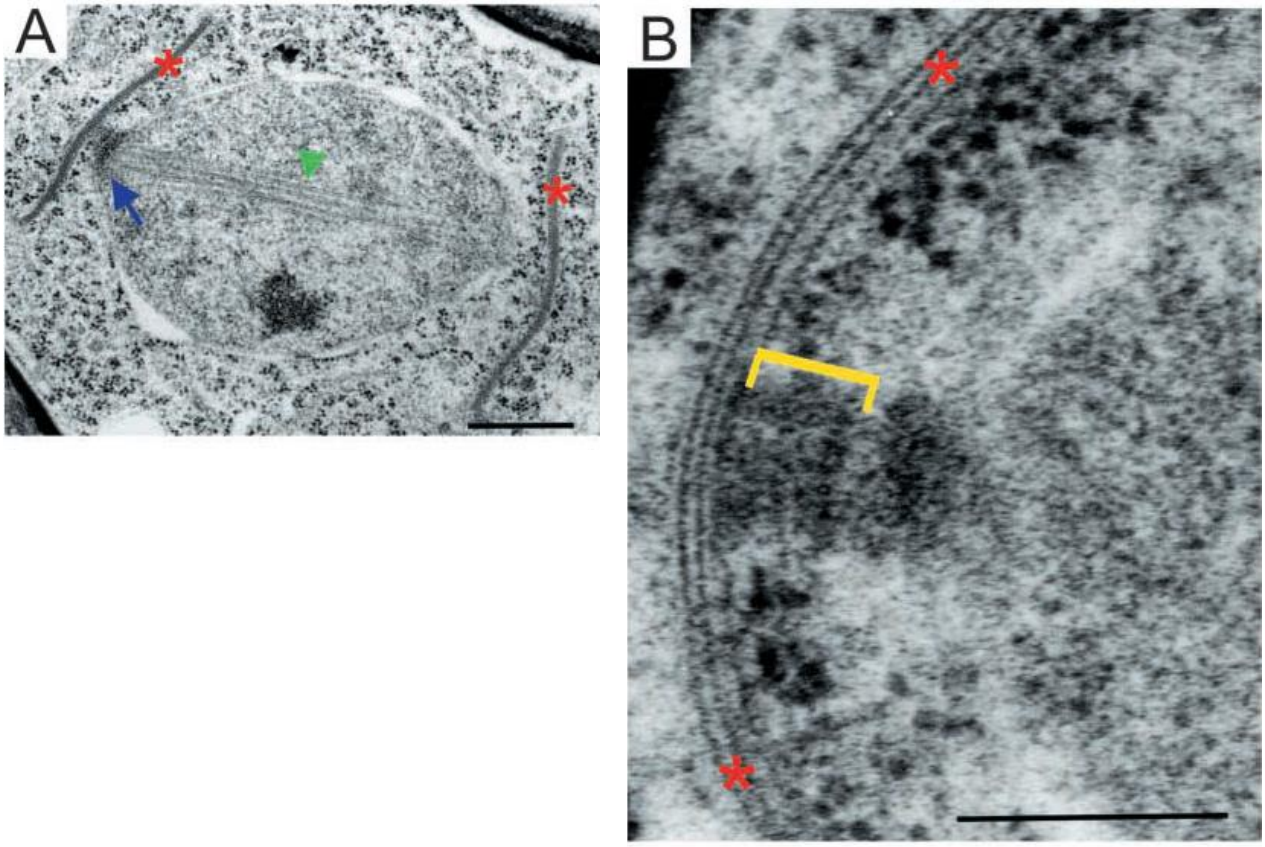
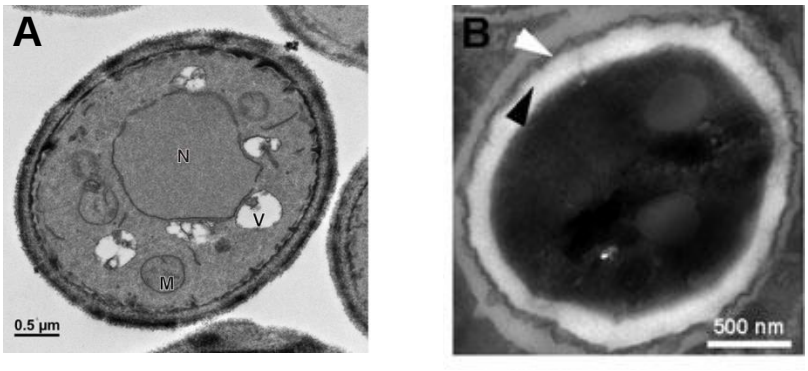


Figure 1.5 The spindle pole body and the forespore membrane formation

Electron microscopy images that show SPB and forespore membrane in wild-type *S. pombe* cells. (A) SPB (blue arrow), microtubules (green arrowhead) and the forespore membrane (red star) are shown, Scale bar = 0.5 microns. (B) expanding SPB is indicated by yellow line and the forespore membrane formation is shown with a red star, Scale bar = 0.5 microns (Shimoda, 2004a).



(Sun *et al.*, 2013) **(de Medina-Redondo *et al.*, 2008)**

Figure 1.6 Spore cell wall of the wild-type *S. pombe* strain

(A) TEM analysis of wild-type *S. pombe* spore. N, nucleus; M, mitochondrion; V, vacuole, (B) White arrowhead indicates the outermost α-glucan layer of the spore wall. Black arrowhead marks the inner β-glucan layer.

Chapter 2

A genome-wide screen for sporulation-defective mutants in *Schizosaccharomyces pombe*

(Reformatting of article *in press* in G3: Genes, Genomes, Genetics)

Yeast sporulation is a highly regulated developmental program by which diploid cells generate haploid gametes, termed spores. To better define the genetic pathways regulating sporulation, a systematic screen of the set of ~3,300 non-essential *Schizosaccharomyces pombe* gene deletion mutants was performed to identify genes required for spore formation. A high throughput genetic method was used to introduce each mutant into an h^{90} background and iodine staining was used to identify sporulation-defective mutants. The screen identified 34 genes whose deletion reduces sporulation, including 15 that are defective in forespore membrane morphogenesis. In *S. pombe*, the total number of sporulation-defective mutants is a significantly smaller fraction of coding genes than in *S. cerevisiae*, which reflects the different evolutionary histories and biology of the two yeasts.

2.1. Introduction

Ascospore formation in yeast is a response to nutrient deficiency (Tomar *et al.*, 2013). In *Schizosaccharomyces pombe*, cells exit mitosis to differentiate into spores when they encounter the lack of a nitrogen source (Tanaka, 1982; Egel, 1989; Shimoda, 2004b). First, haploid cells of opposite mating types fuse to form diploid zygotes. These diploids then immediately undergo meiosis to generate four haploid nuclei. During the course of meiosis, these nuclei become packaged into daughter cells, termed spores. Spores are created by a specialized form of cell division that occurs without cleavage of the mother cell (Shimoda, 2004a). Each of the four haploid nuclei produced by meiosis are packaged into daughter cells by envelopment within newly synthesized membranes called forespore membranes (Yoo, 1973; Shimoda, 2004b). Forespore membrane formation initiates on meiotic spindle pole bodies (SPBs) early in Meiosis II and as meiosis proceeds, each forespore membrane expands to engulf the associated nucleus (Shimoda, 2004a; Nakase *et al.*, 2008). Closure of the forespore membrane around a nucleus

completes cell division, and these cells then mature into spores by deposition of spore wall material (Yoo, 1973). All of these events occur within the cytoplasm of the original mother cell, which is referred to as the ascus.

Mutants defective in meiosis and sporulation have been identified in *S. pombe* in a number of different screens. Originally *spo* mutants were found by direct screening for sporulation defects (Bresch *et al.*, 1968; Kishida and Shimoda, 1986). More recently targeted mutagenesis of genes whose expression is sporulation-induced has identified additional genes involved in both processes (Gregan *et al.*, 2005; Martin-Castellanos *et al.*, 2005). While these screens have defined many genes involved in sporulation, these screens were not saturating and so additional genes likely remain to be identified.

The process of sporulation is similar in *S. pombe* and in the budding yeast *Saccharomyces cerevisiae*, though there appears to be only limited conservation of the specific genes involved in the process (Shimoda, 2004a). Systematic screening of the *S. cerevisiae* knockout collection has proven to be a valuable approach, identifying hundreds of genes required for sporulation (Rabitsch *et al.*, 2001; Deutschbauer *et al.*, 2002; Enyenihi and Saunders, 2003; Marston *et al.*, 2004; Neiman, 2005). Sporulation-defective mutants in *S. cerevisiae* can be divided into several broad categories: (1) genes required for aspects of cell physiology necessary to support sporulation, for example mitochondrial function or autophagy; (2) genes required for progression through meiotic prophase to the initiation of spore development; and (3) genes required for spore assembly, *per se*, for instance genes involved in growth of the prospore membrane (the *S. cerevisiae* equivalent of the forespore membrane) or for spore wall formation (Neiman, 2005).

To obtain a more comprehensive list of genes required for sporulation in *S. pombe*, we undertook a genome-wide systematic screen of the *S. pombe* haploid deletion set (~3300 strains in total). In *S. pombe*, nitrogen starvation induces haploid cells of opposite mating types (h^+ and h^-) to mate and then undergo meiosis and spore formation. Strains that carry the h^{90} allele at the *mat1* locus are homothallic, meaning the cells switch mating types during mitotic growth so that both the h^+ and h^- mating types are present in colonies originally derived from a single cell. Diploids generated by h^{90} strains are therefore completely homozygous, as they are a result of self-mating. This greatly simplifies the detection of meiotic and sporulation mutants, since

meiosis is normally induced only in diploid cells. The haploid deletion set was constructed in an h^+ mating type background. Therefore, it was necessary to introduce h^{90} into each deletion strain to enable the creation of homozygous mutant diploids. After these mutants were exposed to conditions that promote sporulation, iodine staining was used as an initial screen to determine whether spores were present (Garcia *et al.*, 2006). Secondary screens included direct observation of asci by phase contrast microscopy and examination of fluorescent markers for the forespore membrane and SPBs. Our screen identified > 90% of the previously known sporulation-defective mutants present in the collection, suggesting that the screen has identified the majority of non-essential genes required for spore formation. Among the novel sporulation genes are membrane trafficking proteins, signaling proteins, transcription factors, and metabolic enzymes. These results provide a wealth of information for future investigations.

2.2 Materials and Methods

Yeast strains and culture

Standard media and growth conditions were used unless otherwise noted (Forsburg and Rhind, 2006). For synthetic medium containing G418, pombe glutamate medium (PMG) was used (Sabatinos, 2010). Genotypes of the strains used in this study are listed in Table 2.1. Strain EAP20, which was used to introduce h^{90}, as well as genes encoding tagged versions of *psy1*$^+$ and *sid4*$^+$ (markers for the forespore membranes and SPBs, respectively) into the knockout collection, was constructed in several steps. First, a spontaneous cycloheximide resistant mutant of strain JLP18 (EAP3) was selected by plating cells on YES plates containing 10 mg/l of cycloheximide (Sigma-Aldrich Co. LLC). EAP11 was generated by transforming EAP3 with *Sph*I digested pEA4, which targets integration of the *S. cerevisiae URA3* gene adjacent to the *his5*$^+$ locus. *his5*$^+$ is tightly linked to *mat1*, which contains the h^{90} allele and the Ura$^+$ phenotype can then be used to follow the h^{90} allele in subsequent crosses. Next, an allele of the spindle pole body (SPB) gene *sid4*$^+$ fused to a gene encoding the fluorescent protein tdTomato (*sid4*$^+$-*tdTomato::hphMX6*) was introduced by crossing EAP11 with strain 843 (Doyle *et al.*, 2009) to generate EAP16. To introduce a marker for the forespore membrane, a strain [FY12295; (Nakase *et al.*, 2008)] carrying a green fluorescent protein (GFP) tagged allele of *psy1*$^+$ was crossed to EAP16 (Nakase *et al.*, 2008)(Nakase *et al.*, 2008)(Nakase *et al.*, 2008)(Nakase *et al.*, 2008)(Nakase *et al.*, 2008), generating EAP19. Finally, EAP19 was backcrossed to EAP16 to generate a segregant, EAP20, which carries the marked h^{90} locus, both fluorescent protein gene fusions, and cycloheximide resistance.

Plasmids

pEA4, which contains the *S. pombe his5*$^+$ gene in pRS306 (Sikorski and Hieter, 1989), was constructed by polymerase chain reaction (PCR) amplification of a 1.3kb fragment including *his5*$^+$ and its 5' and 3' regions from genomic DNA using EAO11 (5'-GTTCTTGGTACCGAGCGTGCTCAGTTTTCTATG-3') and HJO274 (5'-GTTGTTGAA TTCTTACAACACTCCCTTCGTGCTTGGG-3') oligonucleotides. The PCR product was engineered to contain *Kpn*I and *Eco*RI sites at its 5' and 3' ends, respectively, and was cloned into similarly digested pRS306.

pEA18, which expresses *wsc1*$^{+}$*-mTagBFP* under control of the *spo13* promoter was constructed in three steps. First, a yeast codon-optimized form of mTagBFP without a stop codon was PCR amplified from pRS426 *Spo20*$^{51–91}$*-mTagBFP* (Lin *et al.*, 2013) using EAO44 (GTTCTTCATATGGTTCTTGTTCCATGGATGTCTGAGGAGTTGATAAAGG) and EAO46 (GTTCTTGGATCCCTTGTTCTTGCGGCCGCGTTCAACTTGTGACCCAACTTTG) oligos and cloned as a *Nde*I/*Bam*HI fragment into similarly digested pREP42x (Forsburg, 1993) creating pEA13. Second, overlap PCR was used to construct a P*spo13-wsc1*$^{+}$ fusion. A ~500bp fragment of the *spo13* promoter region and the *wsc1*$^{+}$ open reading frame lacking the stop codon were amplified using the oligonucleotide pairs EAO47 (GTTCTTCTGCAGGGCACTCTGTAATTGTAAG) and EAO48 (GAGGAATTTAAAAAGACCATAGATCTTGTTTCAATTTTTTTTCCTTTCC), and EAO49 (GGAAAGGAAAAAAAATTGAAACAAGATCTATGGTCTTTTTAAATTCCTC) and EAO50 (GTTCTTCCATGGGTTCAAATTTGTGACACGC), respectively. These PCR products were mixed and used as template in a reaction with EAO47 and EAO50 to yield ~1.6 kb *spo13pr-wsc1*$^{+}$ fusion fragment. This product was digested with *Pst*I and *Nco*I and cloned into similarly digested pEA13 to replace the *nmt1* promoter of pREP42x in front of mTagBFP creating pEA17. Finally, pEA18 was created by amplifying mTagBFP with its stop codon from pRS426 *Spo20*$^{51–91}$*-mTagBFP* using EAO44 and EAO45 (GTTCTTGGATCCCTTGTTCTTGCGGCCGCTTAGTTCAACTTGTGACCCAACTTTG), digested with *Nco*I and *Not*I, and cloned into similarly digested pEA17.

Genetic screen

The haploid *S. pombe* deletion mutant library was purchased from Bioneer (South Korea). The knockouts are in an *h*$^{+}$ strain background (*h*$^{+}$ *ade6-M210 ura4-D18 leu1-32*). To examine sporulation, each mutant was crossed to strain EAP20 and a modified form of the synthetic gene array method was used to introduce the knockout alleles into an *h*^{90} background (Tong *et al.*, 2001; Baryshnikova *et al.*, 2010). The steps in this process are outlined in Figure 2.1. First, strains containing individual *geneX*Δ::*kanMX4* deletions were grown in liquid YES medium in microtiter dishes. To each well was then added 1/10th volume of a saturated culture of EAP20 grown in YES and the mixed cultures were pinned onto ME plates, allowing the cells to grow, mate, and sporulate (Forsburg and Rhind, 2006). Use of ME at this step produced higher

efficiency sporulation than other media (data not shown). The patches were then replica plated to YES plates supplemented with 200 mg/l Geneticin (G418; USBiological, New England, MA) and 10 mg/l cycloheximide. Combined, these drugs select for recombinant haploids from the cross. Geneticin selects for the knockout marker. Cycloheximide resistance is a recessive trait and cycloheximide therefore selects against both the original knockout strain and any diploid cells created by mating of EAP20 with the deletion strain. Inclusion of this step is essential to prevent a background of diploids heterozygous for the knockout allele from contaminating the patches (Baryshnikova *et al.* 2010). After three days incubation at 31°, patches were replica plated to EMM2 plates with 200 mg/l Hygromycin B (Calbiochem, Merck KGaA, Darmstadt, Germany). This medium selects for both uracil and leucine prototrophy, which are linked to h^{90} and the forespore membrane marker GFP-*psy1*$^{+}$, respectively and for the SPB marker *sid4*$^{+}$-*tdTomato::hphMX6*. In addition it also selects against the *ade6-M210* allele found in the deletion set. It is important that the resulting strain be *ade6*$^{+}$ as the red pigment created by the *ade6-M210* mutant complicates both the subsequent iodine staining and fluorescence analyses. We found that removal of the G418 selection at this step allowed the growth of cells lacking the knockout allele and so an additional replica plating to PMG plates with 200 mg/l G418 was performed. G418 selection is more efficient on PMG than EMM2 (Benko and Zhao, 2011). The resulting patches consist of h^{90} haploid deletion mutants harboring *sid4*$^{+}$*-tdTomato::hphMX6* and GFP-*psy1*$^{+}$. These patches were then replica plated to SPA plates to induce sporulation, incubated at 25° for three days and then inverted over a petri dish of iodine crystals for 2-3 min (Sabatinos, 2010). Staining of mature spore wall with iodine vapor produces a dark brown color (Meade and Gutz, 1975). Patches displaying absent or reduced staining with iodine were scored as sporulation-defective candidates.

Candidates were picked from the PMG plates to a fresh PMG plate with 200 mg/l G418, replica plated to SPA, and then retested for iodine staining. Cells from patches that failed to exhibit good iodine staining after the retest were then directly analyzed by light microscopy for the presence of visible spores and by fluorescence microscopy of the Sid4-tdTomato and GFP-Psy1 markers to evaluate progression through meiosis and forespore membrane formation.

Barcode sequencing

In construction of the knockout collection, each knockout incorporated "uptag" and "downtag" sequences that provide a unique barcode for each knockout (Kim *et al.*, 2010). To confirm the identity of the mutants identified in our screen, we amplified the uptag region for each one. This PCR product was then sequenced using EAO62 (GGGCGACAGTCACATCATGC) and the results were compared to the list of uptag sequences given by Kim *et al.* (2010). In addition, the *meu14* and *mfr1* deletions were also analyzed by PCR-amplification of the loci with flanking primers to distinguish the knockout and wild-type alleles.

Microscopy

Images were collected on a Zeiss Observer Z.1 microscope and processed using Zeiss Axiovision or Zen software.

Acetone resistance assays

Spore wall function was tested using an acetone resistance assay modified from (Smith, 2009). Wild-type spores are resistant to acetone, while unsporulated cells or cells with defective spore walls are killed. The wild type and the knockout strains were first incubated on PMG plates with 200 mg/l G418 at 31° for two days. Cells were then replica plated to SPA plates and incubated at 25° for three days to allow for mating and sporulation, and then replica-plated onto YES plates. An acetone-soaked filter paper (Whatman #3, 1003-090) was placed on a glass petri dish and inverted above the YES plate to expose the patches to acetone vapor for 15 min. These were then incubated at 31° for three days before being photographed.

2.3 Results and Discussion

Isolation of sporulation-defective mutants

Using a series of selective steps diagrammed in Figure 2.1, we constructed h^{90} homothallic derivatives of each deletion strain in the Bioneer *S. pombe* haploid deletion collection, at the same time introducing fluorescent markers for the SPBs and the forespore membrane. Because h^{90} strains are able to undergo mating type switching, h^{90} cells can be induced to self-mate and create homozygous diploids that then proceed through meiosis and sporulation. The ability of the strains in the deletion set to form spores was then assayed by exposure to iodine, which produces a dark brown stain in patches containing spores.

Eighty-five candidates passed the initial screen as well as a retest. In addition to sporulation-defective mutants, the assay of decreased iodine staining might also identify knockouts that cause h^-- specific mating defects, that is mutants that are unable to mate with h^+ haploids. The deletion strain background is h^+ and these cells are therefore able to mate with h^- cells present in the h^{90} background in the initial cross. However, these cells will be unable to self-mate once in the h^{90} background and so will not produce spores. Similarly, as the *URA3* marker is integrated approximately 10 cM from the *mat1* locus containing the h^{90}allele (Egel, 2004), recombination between *URA3* and the *mat* locus can produce *URA3* h^+ haploids that would slip through the selection procedure and these would also fail to sporulate. To test for such false positives, the 85 strains were assayed for their mating types by replica-plating to h^+ and h^- tester strains followed by iodine staining to examine whether diploids formed that could sporulate. Strains that are h^{90} are expected to mate to both h^- and h^+ cells. Three of the candidates mated only to the h^+ tester strains, and thirty-three mutants mated only to the h^- tester, demonstrating that a mating defect is indirectly responsible for the absence of spores. Of the strains that mated only to the h^- tester, two were deletions in *mam1* (M-factor transporter) and *mam2* (P-factor receptor), both of which are known to produce an h^- specific sterility (Kitamura and Shimoda, 1991; Christensen *et al.*, 1997). The remaining mutants we suspect were simply h^+ strains that leaked through the selection process. The strains with mating defects were not analyzed further.

The remaining candidates were sporulated and examined by phase contrast microscopy to determine the frequency of spore formation in the culture. Those strains in which no spores were detected were also examined by fluorescence microscopy of the Sid4-tdTomato and GFP-Psy1 markers to look at progression through meiosis and forespore membrane formation, respectively. Based on these microscopy assays the mutants can be divided into four classes: 1) reduced frequency of zygotes, suggesting that the sporulation defect is secondary to a mating defect; 2) near wild-type frequency of zygotes and spores, suggesting a defect in formation of the iodine-reactive layer of the spore wall; 3) no spores and no forespore membrane formation; 4) no spores with abnormal forespore membrane formation (Table 2.2).

To confirm the identity of the deleted gene in the knockout strains we used PCR to amplify the unique uptag region for many of the deletions (Kim *et al.*, 2010). These PCR products were then sequenced and compared with the published lists to confirm the identity of the knockouts. For 48 knockouts for which we obtained sequences, 32 matched the published barcodes. The knockouts that did not produce the expected barcode sequence are listed in Table 3. In three cases, the barcode sequence found corresponded to that of known sporulation-defective mutants, suggesting that the identification by the barcode sequence, rather than position in the collection, is correct. In all cases of misidentification, the expected knockout and the actual one are found in different plates within the collection. These errors are, therefore, unlikely to have been caused by cross-contamination during our handling of the collection as different plates were processed at different times. While this is a small sample, the surprisingly high error rate (33%) highlights the need for confirmation of knockout identity when using this collection.

To test the effectiveness of our screen, we culled from the literature a list of previously identified mutants that block spore formation. Several of the original *spo* mutants proved to be hypomorphic alleles of essential genes (Nakase, 2001; Nakamura-Kubo *et al.*, 2003) and so are not present in our deletion set; however we identified 13 known sporulation-defective mutants listed as present in the collection (Table 2.4). Amplification and barcode sequencing confirmed the presence of nine of these at the correct location in the collection and another two mutants were identified at different locations. Of these 11 mutants, 10 were identified in the screen. This yield suggests that the screen has identified ~90% of the sporulation-defective mutants present in the collection.

Classes of genes required for positive iodine staining phenotype

1. Genes required for zygote formation

For mutants that formed some level of visible spores, the frequency of zygote formation and of spore formation were examined by light microscopy (Table 2.5). Mutants that display bilateral mating defects, that is, are able to mate with the h^+ and h^- tester strains but are unable to self-mate to produce zygotes would pass the mating tests described above and show reduced sporulation. For seven mutants, zygote formation was reduced greater than three-fold from that seen in a wild-type h^{90} strain, indicative of a bilateral mating defect. Thus, the primary defect in these mutants is likely to be in the mating process or response to nitrogen starvation rather than in spore formation, *per se*. The two genes in this class with the strongest phenotype were *prm1*$^+$ and *cyp9*$^+$. Consistent with our interpretation, *prm1*$^+$ encodes an integral membrane protein recently shown to be necessary for conjugation (Sun *et al.*, 2013; Curto *et al.*, 2014). These results reveal a previously unknown role for *cyp9*$^+$ in the mating reaction.

2. Genes required for spores to be iodine-reactive

Mutants in seven additional genes formed zygotes at near normal frequency and displayed at most modestly reduced spore formation relative to wild-type. Because strains in this class form significant numbers of spores, their loss of staining may reflect defects in generation of the iodine reactive alpha-glucan component of the spore wall (Garcia *et al.*, 2006). It is noteworthy that a number of mutants known to disrupt assembly of the beta-glucan or chitosan layers of the spore wall were present in the collection but were not found in our screen, probably because those mutants that affect beta-glucan or chitosan do not alter iodine staining (Liu *et al.*, 2000).

Two of the genes in this class, *php3*$^+$ and *php5*$^+$, encode subunits of the CCAAT-binding transcription complex (McNabb *et al.*, 1997; Mercier *et al.*, 2006). While previous reports have implicated this complex in induction of transcription during nitrogen starvation and in the activity of meiotic recombination hotspots, no requirement for these genes in spore formation has been reported (Nakashima *et al.*, 2002; Steiner *et al.*, 2011). This work suggests that transcriptional induction by this complex of as yet unidentified genes is important for proper spore formation.

3. Genes required for entry into meiosis or for the initiation of forespore membrane assembly

The five genes identified in this class were previously known. Three of the genes are required for entry into meiosis. *mei2*$^{+}$ encodes an RNA-binding protein that is required for premeiotic DNA synthesis as well as progression into Meiosis I (Watanabe *et al.*, 1988; Watanabe and Yamamoto, 1994). *mei3*$^{+}$ is essential for the initiation of meiosis since it encodes a protein that binds and inhibits the meiosis-inhibitory protein kinase Pat1 during sporulation(McLeod and Beach, 1988). The transcription factor that is encoded by *mei4*$^{+}$ is a regulator necessary for the expression of many sporulation-induced genes (Horie *et al.*, 1998). The remaining two genes in this class, *mug79*$^{+}$/*spo7*$^{+}$ and *spo15*$^{+}$, both encode components of the meiotic SPB necessary for the SPB to catalyze the coalescence of secretory vesicles into a forespore membrane (Ikemoto *et al.*, 2000; Nakamura-Kubo *et al.*, 2011).

4. Genes that are essential for the proper formation and the maturation of the forespore membrane

Mutants in Class 4 genes progress through meiosis and initiate forespore membrane growth but the membranes display morphological defects and no spores are visible by light microscopy. There were 15 genes identified in this category of which five (*spo3*$^{+}$, *spo4*$^{+}$, *spo5*$^{+}$, *mes1*$^{+}$, *spn2*$^{+}$) were previously shown to be required for sporulation (Nakamura *et al.*, 2001; Nakamura *et al.*, 2002; Izawa *et al.*, 2005; Kasama *et al.*, 2006; Onishi *et al.*, 2010). Among the 10 genes in this class not previously associated with sporulation defects, two encode subunits of the COP9 signalosome (*csn1*$^{+}$ and *csn2*$^{+}$) and one encodes a reported interacting partner of the signalosome (*cdt2*$^{+}$) (Mundt *et al.*, 1999; Liu *et al.*, 2005). COP9 signalosome, also known as CSN, is a conserved protein complex that functions in the ubiquitin-proteosome pathway. It plays a key role in the regulation of cullin-based Ub-E3 ligases (Braus *et al.*, 2010). *S. pombe* has 6 subunits: *csn1, csn2, cns3, csn4, csn5, csn7 (Mundt et al., 1999).* Several other COP9 subunits are present in the collection but were not found to be iodine-negative in our screen. Thus, the Csn1 and Csn2 subunits of the signalosome may be specifically required for sporulation. A similar difference in function between Csn1/Csn2 and other COP9 subunits in sensitivity to DNA damage and a delayed progression through S-phase have been noted

previously (Mundt *et al.*, 2002; Braus *et al.*, 2010). Also in this class are *spe2*$^{+}$ and SPBC12C2.07c (*spe3*$^{+}$), genes that encode enzymes involved in consecutive steps in spermidine synthesis (Tabor and Tabor, 1985; Chattopadhyay *et al.*, 2002). This pathway has also been shown to be required for sporulation in *S. cerevisiae* (Cohn *et al.*, 1978), suggesting a conserved requirement for spermidine for spore formation in fungi.

Three of the mutants in this class have predicted functions within the secretory pathway. SPAC6C3.06c encodes a predicted phospholipid flippase orthologous to the *NEO1* gene of *S. cerevisiae*. Neo1 is localized to the endosome and to the Golgi and has been implicated in membrane trafficking (Hua and Graham, 2003; Wicky *et al.*, 2004). The *erp2*$^{+}$ and *erp5*$^{+}$ genes encode two *S. pombe* members of the p24 protein family. The p24 proteins are a highly conserved family of integral membrane proteins that act as cargo receptors and shuttle between the endoplasmic reticulum (ER) and the Golgi (Strating and Martens, 2009). In particular, they play an important role in cargo selection and packaging into COPII vesicles at ER exit sites (Strating and Martens, 2009). Consistent with the similar phenotypes of both *erp2* and *erp5* deletions, studies in *S. cerevisiae* suggest that the four family members function in a single complex (Hirata *et al.*, 2013). Knockouts of the other family members in *S. pombe*, *emp24*$^{+}$ (SPCC24B10.17.1) and *erv25*$^{+}$ (SPAC23H4.03c.1), were not present in the collection, though we predict mutants in these genes would display a similar sporulation defect. We predict that the p24 family is necessary for the exit of some protein(s) from the ER so that the cargo protein(s) can be transported through the secretory pathway to the forespore membrane and contribute to proper membrane growth.

The *erp2 and erp5 mutants do not cause a general block to ER exit.*

In *S. cerevisiae* a different class of ER cargo receptor, encoded by the *ERV14* and *ERV15* genes, is required for proper formation of the prospore membrane (the budding yeast equivalent of the forespore membrane) during sporulation (Powers and Barlowe, 1998; Nakanishi *et al.*, 2007). Though these genes are not essential for vegetative growth, their deletion creates a general block to ER exit of integral membrane proteins during sporulation (Nakanishi *et al.*, 2007). Since *ERV14* deletion mutants have small, abnormal forespore membranes similar to *erp2Δ* and *erp5Δ* mutants, we hypothesized that, parallel to the *S. cerevisiae* ER cargo receptors,

erp2$^+$ and *erp5*$^+$ might become essential for ER exit of integral membrane proteins in *S. pombe* sporulation. The GFP-Psy1 reporter is localized to the forespore membrane in *erp2*Δ and *erp5*Δ cells, however this does not provide a strong test of a role for *erp2*$^+$ and *erp5*$^+$ in ER exit as Psy1 is relocalized from the plasma membrane to the forespore membrane via the endosome (Kashiwazaki *et al.*, 2011). Therefore, to test a possible general role for *erp2*$^+$ and *erp5*$^+$ in ER exit, the strains were transformed with a plasmid carrying an integral plasma membrane protein, *wsc1*$^+$, fused with mTagBFP and placed under control of the sporulation-specific protein *spo13* promoter (Nakase *et al.*, 2008). When expressed in a wild-type strain, Wsc1-mTagBFP localized to the forespore membrane (Figure 2.2). In *erp2*Δ and *erp5*Δ mutants Wsc1-mTagBFP colocalized with GFP-Psy1 in the abnormal forespore membranes and no additional BFP fluorescence from the ER was seen, indicating that transport of Wsc1-mTagBFP is unaffected in the mutants (Figure 2.2). If loss of *erp2* or *erp5* cause forespore membrane defects indirectly by limiting the exit of some cargo from the ER, this is likely an effect on some specific cargo protein(s) and not due to a more general block in transport.

The lcf2$^+$ *and mcl1*$^+$ *gene products may contribute to spore wall function*

The spore wall provides the cell with resistance to environmental stresses such as acetone vapor (Egel, 1977). To examine spore wall function we tested mutants in Class 2 for resistance to acetone (Smith, 2009). Two of the mutants, *lcf2*Δ and *mcl1*Δ showed strong sensitivity to acetone exposure (Figure 2.3). This stress-sensitivity is striking as these mutants show near-normal levels of sporulation. This suggests a structural defect in the spore walls of these mutants, presumably in the alpha-glucan component of the spore wall. The *mcl1*$^+$ gene encodes a polymerase alpha accessory protein, so its effect on the spore wall is likely indirect (Williams and McIntosh, 2005). *lcf2*$^+$ encodes a predicted fatty-acyl CoA ligase, which could influence the composition of cellular membranes (Fujita *et al.*, 2007). The stress sensitivity and iodine staining defects in these cells may reflect an influence of *lcf2*$^+$ on the activity or delivery of the integral membrane Mok14 alpha-glucan synthase responsible for synthesis of the iodine-reactive polymer (Garcia *et al.*, 2006).

Sporulation genes in budding and fission yeast

In this screen we have produced the first survey of the non-essential knockout collection of *S. pombe* for sporulation defective mutants. One of the most striking results is the relatively small number of mutants that displayed a sporulation defect. In all, only ~1% of the *S. pombe* collection showed loss or reduction of spores. This low number is not due to poor recovery in our screen, as we identified known mutants with ~90% efficiency. By contrast, comparable screens of the knockout collection in *S. cerevisiae* found that over 10% of the knockouts produced a sporulation defect (Enyenihi and Saunders, 2003; Marston *et al.*, 2004). The reasons for this difference, in part, reflect the different biology of these two yeasts. Whole categories of genes essential for sporulation in *S. cerevisiae* are not found in our screen. For example, *S. cerevisiae* is a petite-positive yeast that can grow in glucose medium without functional mitochondria (Kominsky, 2000). However, sporulation is an obligatorily aerobic process. Therefore, any mutations that impair respiration are viable but sporulation defective. This accounts for over a quarter of the sporulation-defective mutants in *S. cerevisiae* (Neiman, 2005). In contrast, *S. pombe* cannot grow mitotically without mitochondrial function and so most of the orthologous genes should be essential in fission yeast (and therefore absent from the haploid deletion set). Another significant fraction of sporulation-defective genes in budding yeast are involved in autophagy, either directly or through effects on vacuolar function (Neiman, 2005). Though autophagy mutants were present in the *S. pombe* deletion set, only one, *atg12*, was iodine-negative and it displayed reduced zygote formation, indicating a role in mating, not sporulation (Table 2.2). This result is consistent with previous studies showing that many autophagy mutants display reduced mating, but if mutant cells succeed in mating they are capable of sporulation (Kohda *et al.*, 2007; Sun *et al.*, 2013). Finally, in *S. cerevisiae*, mutations that cause defects in meiotic recombination can lead to activation of a checkpoint that arrests cells in meiotic prophase thereby preventing them from producing spores (Roeder and Bailis, 2000). Though the orthologous genes and meiotic recombination checkpoint are present in *S. pombe*, failures in meiotic recombination in fission yeast lead only to a delay in meiotic progression (Shimada *et al.*, 2002; Perez-Hidalgo *et al.*, 2003). Thus, these mutants eventually do form spores and so were not found in our screen.

Even accounting for the absence of these three categories of mutants, there appear to be fewer non-essential genes required for spore assembly in *S. pombe* than in *S. cerevisiae*. This finding probably reflects the different evolutionary histories of the yeasts. The whole genome duplication that occurred during the evolution of *Saccharomyces* allowed for the emergence of distinct sporulation- and vegetative-specific isozymes (Wolfe and Shields, 1997). For example, the t-SNAREs *psy1*$^{+}$ and *sec9*$^{+}$ are both essential genes in *S. pombe* that are also essential for forespore membrane growth (Nakamura *et al.*, 2005; Maeda *et al.*, 2009). In *S. cerevisiae,* gene duplication has produced two versions of each gene, *SSO1*/*SSO2* for *psy1*$^{+}$ and *SEC9*/*SPO20* for *sec9*$^{+}$. In each case, one paralog is specifically required for sporulation (*SSO1* and *SPO20*) (Neiman, 1998a; Jantti *et al.*, 2002). In the absence of such extensive gene duplication, there has been less opportunity for sporulation-specific functions to evolve in *S. pombe* and as a result, we expect that more essential genes play "double duty" in both vegetative growth and sporulation.

In summary, we report the results of a systematic screen through the *S. pombe* haploid deletion set for mutants displaying spore formation defects. The genes identified provide multiple new avenues for investigation into spore differentiation. These include a role for the COP9 signalosome in forespore membrane formation, sporulation-specific requirements for the p24 family of ER export cargo receptors, and the possible function of fatty acid metabolism in regulation of spore wall assembly.

Table 2.1 Strains used in this study

Name	Genotype	Source
JLP18	*h^{90} ura4-D18 leu1-32 his3-127*	This study
EAP3	*h^{90} ura4-D18 leu1-32 his3-127 cyh^{R}*	This study
EAP11	*h^{90} his5::URA3::his5^{+} ura4-D18 leu1-32 his3-127 cyh^{R}*	This study
843	*h^{90}myo51^{+}-GFP::kanMX6 sid4^{+}-tdTomato::hphMX6 ura4-D18 leu1-32*	(Doyle *et al.*, 2009)
EAP16	*h^{90} his5::URA3::his5^{+} sid4^{+}-tdTomato::hphMX6 ura4-D18 leu1-32 his3-127 cyh^{R}*	This study
FY12295	*h^{90} spo15::ura4^{+} ura4-D18 leu1^{+}::GFP-psy1^{+}*	(Nakase *et al.*, 2008)
EAP19	*h^{90} leu1::GFP-psy1^{+}-LEU2 sid4^{+}-tdTomato::hphMX6 ura4-D18 leu1-32*	This study
EAP20	*h^{90} his5::URA3::his5^{+} leu1^{+}::GFP-psy1^{+} sid4^{+}-tdTomato::hphMX6 ura4-D18 leu1-32 cyh^{R}*	This study
Bioneer deletion set	*h^{+} ade6-M210 ura4-D18 leu1-32 geneXΔ::kanMX4*	(Kim *et al.*, 2010)
deletion mutants after outcrosses	*h^{90} his5::URA3::his5^{+} leu1^{+}::GFP-psy1^{+} sid4^{+}-tdTomato::hphMX6 ura4-D18 leu1-32 cyh^{R} geneXΔ::kanMX4*	This study

Table 2.2 Genes identified in the sporulation-defective screen

Class 1. Genes required for zygote formation (*n*=7)

Gene	Gene ID	Comments[a]
atg12^{+}	SPAC1783.06c	Autophagy associated ubiquitin-like modifier
cyp9^{+}	SPCC553.04	Predicted cyclophilin family peptidyl-prolyl cis-trans isomerase
mmd1^{+}	SPAC30C2.02	Predicted deoxyhypusine hydroxylase
prm1^{+}	SPAP7G5.03	Integral membrane protein important for cell-cell fusion
	SPBC1711.12	Predicted oxidised protein hydrolase
	SPBC18E5.08	Predicted *N*-acetyltransferase
	SPBC146.02	Sequence orphan

Class 2. Genes required for spores to be iodine-reactive (*n*=7)

Gene	Gene ID	Comments
fsc1^{+}	SPAC22H12.05c	Fasciclin domain protein
lcf2^{+}	SPBP4H10.11c	Long-chain-fatty-acid-CoA ligase
mam3^{+}	SPAP11E10.02c	Cell agglutination protein
mcl1^{+}	SPAPB1E7.02c	DNA polymerase alpha accessory factor
php3^{+}	SPAC23C11.08	CCAAT-binding factor complex subunit
php5^{+b}	SPBC3B8.02	CCAAT-binding factor complex subunit
rik1^{+}	SPCC11E10.08	Silencing protein

Class 3. Genes required for entry into meiosis or for the initiation of forespore membrane assembly (*n*=5)

Gene	Gene ID	Comments
mei2^{+}	SPAC27D7.03c	RNA-binding protein required for meiosis
mei3^{+}	SPBC119.04	Required for the initiation of meiosis
mei4^{+}	SPBC32H8.11	Transcription factor regulating meiotic gene expression
mug79^{+} (*spo7*$^{+}$)	SPAC6G9.04	Meiotic spindle pole body component
spo15	SPAC1F3.06c	Meiotic spindle pole body component

Class 4. Genes that are essential for the proper formation and the maturation of the forespore membrane (*n*=15)

Gene	Gene ID	Comments
csn1^{+}	SPBC215.03c	COP9/signalosome complex subunit
csn2^{+}	SPAPB17E12.04c	COP9/signalosome complex subunit
cdt2^{+}	SPAC17H9.19c	COP9/signalosome associated factor
erp2^{+}	SPAC17A5.08	ER exit receptor for secretory cargo
erp5^{+}	SPBC16E9.09c	ER exit receptor for secretory cargo
mes1^{+}	SPAC5D6.08c	Meiotic APC/C regulator
spe2^{+}	SPBP4H10.05c	S-adenosylmethionine decarboxylase proenzyme
(spe3^{+}*)*	SPBC12C2.07c	Predicted spermidine synthase
spn2^{+}	SPAC821.06	Septin
spo3^{+}	SPAC607.10	Required for spore formation
spo4^{+}	SPBC21C3.18	Kinase required for spore formation
spo5^{+}	SPBC29A10.02	Meiotic RNA-binding protein
tpp1^{+}	SPAC19G12.15c	Trehalose-6-phosphate phosphatase
	SPAC6C3.06c	Predicted P-type phospholipid flippase
	SPCC1739.04c	Sequence orphan

[a]Descriptions are based on PomBase (Wood *et al.*, 2012b) (www.pombase.org)

[b]knockout not confirmed by barcode sequencing

Table 2.3 Gene deletions that do not have the correct barcode

Gene	Gene ID	Barcode information
atg15^{+}	SPAC23C4.16c	matches with *spo5*
atp10^{+}	SPAC4G8.11c	no match
atp14^{+}	SPBC29A3.10c	no match
ctf1^{+}	SPBC3B9.11c	no match
lsk1^{+}	SPAC2F3.15	matches with *mei4*
mei4^{+}	SPBC32H8.11	no match
mfr1^{+}	SPBC1198.12	matches with SPAC17H9.14c *mfr1* knockout is not present as determined by PCR with flanking primers,
scd1^{+}	SPAC16E8.09	matches with *mei4*
spo5^{+}	SPBC29A10.02	no match
spo6^{+}	SPBC1778.04	no match
	SPBC15C4.06c	no match
apq12^{+}	SPBC428.04	matches with *cyp9*
	SPBC21H7.06c	matches with *cyp9*
	SPAC139.01c	matches with *nrd1*
	SPBC23G7.06c	matches with *nrd1*
	SPBC1711.08	matches with *nrd1*

Table 2.4 Known sporulation-defective genes listed in the *S. pombe* haploid deletion set

Gene	Comment	Phenotype in our study
spo3^{+}	confirmed by barcode sequence	sporulation defect
spo4^{+}	confirmed by barcode sequence	sporulation defect
spo5^{+}	knockout found at different position in the collection[a]	sporulation defect
spo6^{+}	not present[a]	n.d.[b]
mug79^{+}/*spo7*$^{+}$	confirmed by barcode sequence	sporulation defect
spo15^{+}	confirmed by barcode sequence	sporulation defect
mei2^{+}	confirmed by barcode sequence	sporulation defect
mei3^{+}	confirmed by barcode sequence	sporulation defect
mei4^{+}	knockout found at different position in the collection[a]	sporulation defect
mes1^{+}	confirmed by barcode sequence	sporulation defect
mfr1^{+}	not present[a]	n.d.[b]
meu14^{+}	knockout is present as determined both by PCR with flanking primers and by barcode sequence	normal sporulation
cdt2^{+}	confirmed by barcode sequence	sporulation defect

[a] see Table 3

[b] not determined

Table 2.5 Mating and sporulation efficiency of different mutants

Gene	Gene ID	Class [a]	% of zygotes[b] (s.d.[c])	% of sporulation[e] (s.d.[c])
WT			67.0 (4.0)	76.7 (5.5)
*cyp9*Δ	SPCC553.04	1	<0.5	n.d.[d]
*prm1*Δ	SPAP7G5.03	1	<0.5	n.d.[d]
	SPBC1711.12	1	9.0 (2.2)	21.3 (1.2)
	SPBC18E5.08	1	9.8 (2.4)	87.7 (2.5)
	SPBC146.02	1	14.8 (5.0)	3.3 (0.6)
*atg12*Δ	SPAC1783.06c	1	19.0 (8.6)	47.7 (6.8)
*mmd1*Δ	SPAC30C2.02	1	21.5 (11.2)	71.0 (8.9)
*fsc1*Δ	SPAC22H12.05c	2	27.3 (12.6)	52.0 (4.4)
*mcl1*Δ	SPAPB1E7.02c	2	38.5 (8.3)	56.3 (12.5)
*php3*Δ	SPAC23C11.08	2	38.5 (6.6)	43.0 (6.6)
*mam3*Δ	SPAP11E10.02c	2	39.5 (4.8)	76.3 (6.7)
*lcf2*Δ	SPBP4H10.11c	2	43.8 (11.5)	58.5 (6.1)
*rik1*Δ	SPCC11E10.08	2	57.8 (7.1)	85.3 (3.9)

[a]Class 1 = Genes required for zygote formation; Class 2 = Genes required for spores to be iodine-reactive.

[b]The average of at least three experiments. At least 100 cells were counted in each experiment.

[c]standard deviation.

[d]not determined.

[e]The average of at least three experiments. At least 100 asci were counted in each experiment.

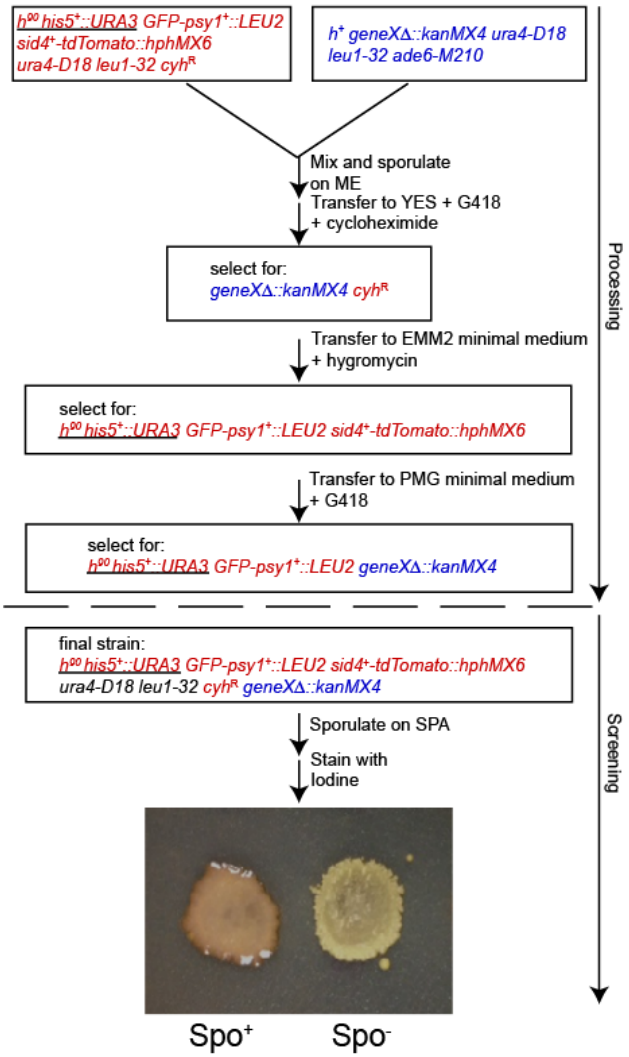


Figure 2.1 Outline of generation of homothallic mutant strains and the screen for sporulation defective mutants.

Genotypes of cells at each stage are boxed. Blue indicates genes from the deletion set strains; red indicates genes derived from EAP20. These two strains were first mixed in liquid and then spotted onto ME plates to allow mating and sporulation. Cells were then replica plated to plates containing G418 and cycloheximide to select for recombinant progeny containing both the *geneX*Δ::*kanMX4* and the *cyh*^{R} alleles. These haploids were transferred to minimal medium containing hygromycin to select for those segregants that also carry the h^{90} mating type (linked to *URA3*, indicated by the underline) and harbor markers for the forespore membrane (*GFP-psy1*$^{+}$) and the SPB (*sid4*$^{+}$*-tdTomato::hphMX6*). A final transfer to minimal medium containing G418 ensures that the deletion alleles are still present. Meiosis and sporulation were induced by replica plating the patches to SPA medium and spore formation was assayed by exposure of the cells to iodine vapor, which causes spores to exhibit a red colony color. An example of sporulation proficient and defective patches is shown.

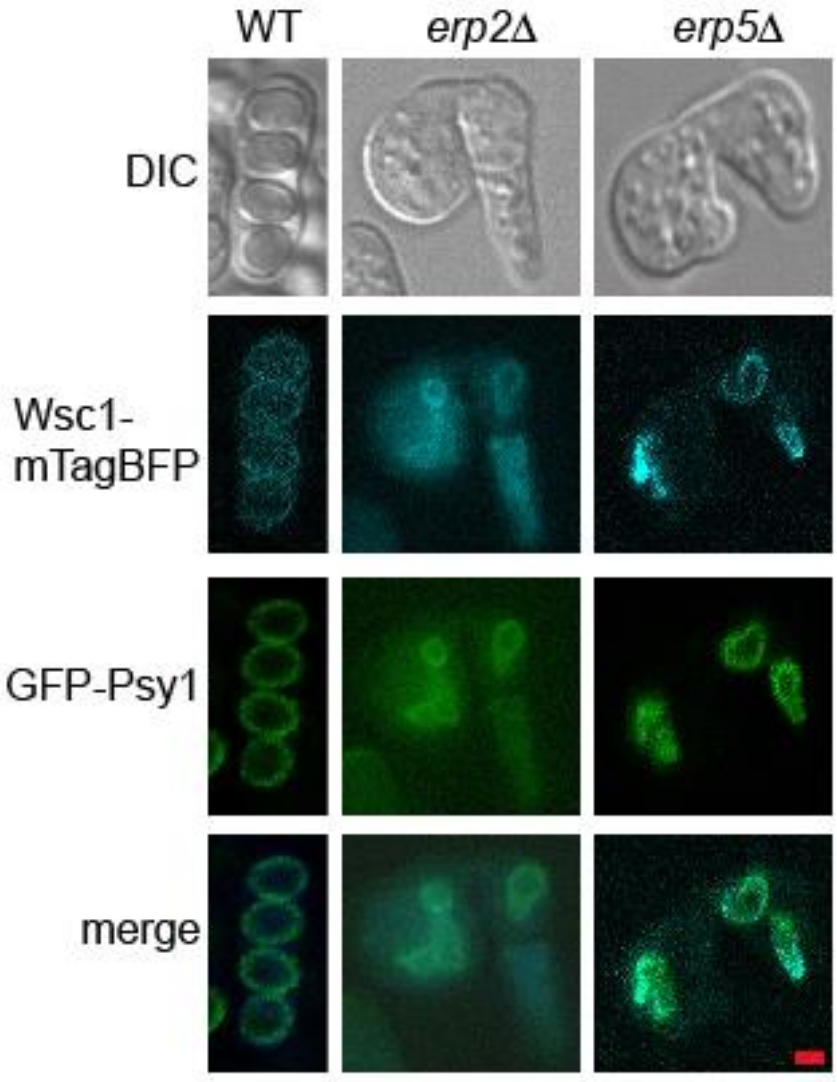


Figure 2.2 Wsc1-mTagBFP localization in *erp2* and *erp5* mutants.

Wild type, *erp2*Δ or *erp5*Δ cells expressing *Pspo13-wsc1*$^{+}$*-mTagBFP* and *GFP-psy1*$^{+}$ were imaged after 24 hr incubation on SPA plates. Scale bar = 2 microns.

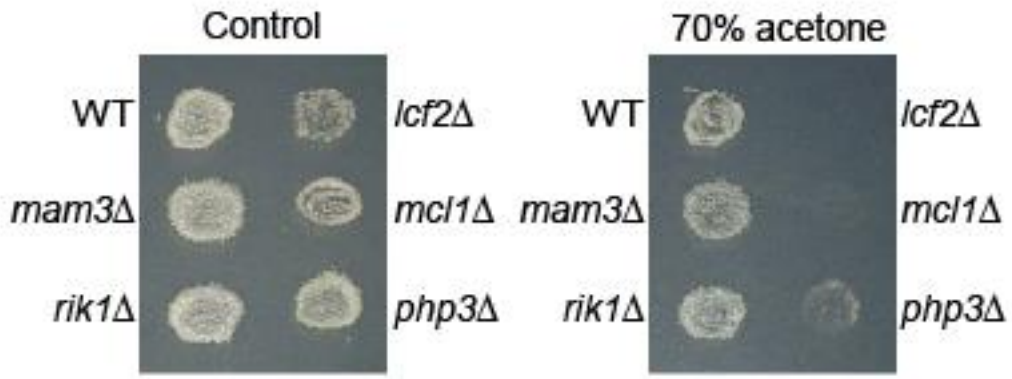


Figure 2.3 Acetone resistance assay.

Indicated mutants were sporulated on SPA plates and then replicated to YES plates. Left panel: growth without exposure to acetone. Right panel: strains were exposed to 70% acetone for 15 min before incubation at 31° for 3 days.

Chapter 3

A Rab-cascade model for the formation of forespore membrane during *Schizosaccharomyces pombe* sporulation

3.1 Introduction

Yeast sporulation, equivalent to gametogenesis in higher organisms, is a differentiation process that is induced by starvation (Neiman, 2005; Piekarska *et al.*, 2010; Neiman, 2011; Tomar *et al.*, 2013). Two complex processes, meiosis and spore morphogenesis, form the developmental program of sporulation (Neiman, 2005; Neiman, 2011). Spindle pole body (SPB) modification during Meiosis II initiates the formation of a membrane called the forespore membrane (Shimoda, 2004b; Neiman, 2011). Each haploid nucleus that was formed after the two rounds of meiotic nuclear division is encapsulated by a forespore membrane. Eventually, the inner bilayer of the forespore membrane becomes the plasma membrane of the spore. Forespore membrane formation during yeast sporulation is a model for *de novo* membrane formation. In the budding yeast *Saccharomyces cerevisiae,* it has been shown that the precursors of this membrane are post-Golgi vesicles (Neiman, 1998b). Like many other cellular processes, the secretory pathway is altered during sporulation so that post-Golgi vesicles normally destined for the plasma membrane are redirected towards the SPBs where they fuse to form forespore membrane. The underlying molecular mechanisms for *S. pombe* SPB modification and forespore membrane formation are not known.

The spindle pole body proteins

SPB modification is the signal that links forespore membrane formation to meiosis. Meiotic outer plaques (MOPs) are formed during Meiosis II on the cytoplasmic faces of the four SPBs (Shimoda, 2004a). These morphological changes, which are referred as SPB modification, can be observed by fluorescence immunostaining as a change in the shape of the SPB from a dot to a crescent during Meiosis II (Hagan and Yanagida, 1995). Similar to their human counterparts (centrosomes) SPBs contain coiled-coil proteins (Ikemoto *et al.*, 2000). Three proteins; Spo15,

Spo2 and Spo13 localize to the MOP in a hierarchic order during Meiosis II and are indispensable for forespore membrane formation and sporulation (Ikemoto *et al.*, 2000; Nakase *et al.*, 2008) (Figure 3.1). Spo15, a 220 kDa (1957aa) coiled-coil protein, is a constitutive component of the SPB (Ikemoto *et al.*, 2000). Spo2, a 15 kDa (133aa) protein with no apparent motifs, and Spo13, a 16 kDa (138aa) coiled-coil protein, are synthesized *de novo* during meiosis (Nakase *et al.*, 2008). Our lab previously discovered that Spo13 has a guanine nucleotide exchange factor (GEF) activity and small Rab-GTPase Ypt2 is its substrate (Yang and Neiman, 2010). Deletion mutants of *spo15, spo2, and spo13* fail to assemble the MOP and to form forespore membranes (Ikemoto *et al.*, 2000; Nakase *et al.*, 2008).

In addition to these three *spo* genes, recent studies revealed novel proteins that localize in the vicinity of the SPBs or to the forespore membrane during Meiosis I and Meiosis II; which are a Rab-GTPase Ypt3, a class V myosin motor protein Myo51, and a coiled-coil protein Mug79. Rabs collaborate with class V myosins to move and position their secretory vesicles to the plasma membrane (Pruyne *et al.*, 1998; Pruyne and Bretscher, 2000). It is possible that a similar collaboration regulates the formation of the forespore membranes during sporulation. Here, I describe the properties and the interactions of these proteins in detail.

Rab proteins regulate membrane fusion

The vesicular traffic process is divided into distinct steps: cargo selection in the donor compartment, vesicle formation, vesicle transport, and finally the tethering and fusion of the vesicles to their target membranes (Ortiz *et al.*, 2002; Novick *et al.*, 2006; Rivera-Molina and Novick, 2009). Transport of the secretory vesicles between membrane compartments is under the control of different Rab (Ypt) proteins (Figure 3.2). The Rab GTPases are molecular switches that regulate vesicle traffic and define the specificity of the membrane fusion (Pfeffer, 2001; Grosshans *et al.*, 2006; Del Conte-Zerial *et al.*, 2008). They are activated by GEFs, which mediate GTP loading and are inactivated by GTPase activating proteins (GAPs) (Stenmark and Olkkonen, 2001; Zerial and McBride, 2001). Generally the GTP-bound form of a Rab GTPase associates with its effector molecules, membrane of an organelle or a transport vesicle (Del Conte-Zerial *et al.*, 2008). After the fusion of the vesicle to its target membrane, a Rab protein hydrolyzes its bound GTP and the inactive GDP-bound protein returns to the cytosol and remains there until reactivated. The upstream GTP-bound Rab on the vesicles recruits a GEF which in

turn recruits and activates the subsequent Rab GTPase. This functional cycling of one GTPase that is coupled to another one is called a Rab-cascade (Ortiz *et al.*, 2002; Del Conte-Zerial *et al.*, 2008). This coordination of the small GTPases of the Rab family regulates the transport of vesicles from their donor compartments to their final destinations where they dock and fuse with the acceptor membranes (Zerial and McBride, 2001; Rink *et al.*, 2005; Grosshans *et al.*, 2006; Del Conte-Zerial *et al.*, 2008; Rivera-Molina and Novick, 2009). In *S. cerevisiae* the prospore membrane (equivalent of forespore membrane) is derived from the fusion of the post-Golgi vesicles around the haploid nucleus (Neiman, 1998b; Neiman, 2005; Neiman, 2011). The *S. pombe* forespore membrane is likely to be formed by post-Golgi vesicles because the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins involved in plasma membrane fusion are required for forespore membrane formation (Nakase, 2001; Nakamura-Kubo *et al.*, 2003; Nakamura *et al.*, 2005). Since the Rab-GTPases regulate the different steps of the exocytic pathway, the may also have a role in the forespore membrane formation.

Rab effectors are a wide range of molecules including coiled-coil proteins that are associated with membrane tethering and docking, enzymes, and cytoskeleton-associated proteins (Stenmark and Olkkonen, 2001). Since it can interact with several types of effectors, a Rab GTPase is probably capable of regulating more than one event at a specific membrane location. For example, the human endosomal Rab GTPase Rab5a mediates endocytic vesicle tethering and fusion (Gorvel *et al.*, 1991) while it regulates the vesicle formation at the plasma membrane and the microtubule-dependent motility of the endocytic organelles (McLauchlan *et al.*, 1998; Nielsen *et al.*, 1999). The function of a Rab is restricted to specific membrane domains and this is achieved by the receptor molecules that also work on these membranes. Therefore, it is important to know which receptor molecules work together with a Rab and its effectors. Since there is a plethora of evidence suggesting that the eukaryotic membrane traffic requires the participation of specific Rab proteins, Rab GTPases are also likely to be important for the forespore membrane formation in yeast.

S. pombe has conserved Rab (Ypt) family proteins that are involved in the regulation of different intracellular trafficking events where the individual members localize to specific subcompartments of the endomembrane system (Newman *et al.*, 1992). Ypt3 and Ypt2 are two

Rab-GTPases that are essential for growth (Haubruck *et al.*, 1990; Miyake and Yamamoto, 1990). The *S. pombe ypt3* gene encodes a 23.86 kDa (214aa) small GTPase of a Rab family that is homologous to mammalian Rab11 (Urbe *et al.*, 1993) and *S. cerevisiae* Ypt31/32 (Miyake and Yamamoto, 1990; Benli *et al.*, 1996; Jedd *et al.*, 1997; Cheng *et al.*, 2002) (Table 3.1). Ypt3 functions in multiple steps of the exocytic pathway in *S. pombe.* Cheng *et al.*, (2002) reported that temperature-sensitive *ypt3-i5* mutation (R29H) causes cell wall integrity, vacuole fusion and cytokinesis defects. Additionally temperature-sensitive *ypt3-ki8* mutants display severe defects in forespore membrane formation (Imada *et al.*, 2011).

The *ypt2* gene of *S. pombe* encodes a 22.75 kDa (200aa) Rab GTPase that is homologous to mammalian Rab8 and *S. cerevisiae* Sec4 (Haubruck *et al.*, 1990; Craighead *et al.*, 1993) (Table 3.1). Rab8, Sec4, and Ypt2 are known to act at the last stages of the exocytic pathway, which is the delivery of the secretory vesicles to the plasma membrane (Craighead *et al.*, 1993; Walch-Solimena *et al.*, 1997; Donaldson and Segev, 2000; Ortiz *et al.*, 2002). *S. cerevisiae* Sec4 localizes to the secretory vesicles and promotes fusion of the vesicles to their target membranes when it is activated by its GEF Sec2 (Novick *et al.*, 2006). In *S. cerevisiae* both the Rab-GTPase Sec4 and the GEF Sec2 concentrate on surface of the secretory vesicles as the vesicles approach to the exocytic sites (Novick *et al.*, 2006). The previous step of Sec4, which is the transport of vesicle from the Golgi to the target membrane, is under the control of redundant Ypt31/32 proteins (Ortiz *et al.*, 2002; Novick *et al.*, 2006; Rivera-Molina and Novick, 2009). In its GTP-bound active form; the GEF Sec2 binds to the upstream Rab-GTPase Ypt31/32 and this interaction recruits Sec4 (Ortiz *et al.*, 2002; Novick *et al.*, 2006). Thus, a GEF undertakes a dual action by acting as an effector for a Rab while promoting the activation of another Rab.

Rab-cascades are also found in mammalian cells. Human Rab11 and Rab8 interaction is also essential for cilia formation where Rab11 activates the Rab8 GEF, Rabin 8 (*S. cerevisiae* Sec2), which then activates Rab8 and *de novo* membrane formation during ciliogenesis (Knodler *et al.*, 2010; Westlake *et al.*, 2011; Hsiao *et al.*, 2012). Interestingly, Yang and Neiman (2010) reported that the sporulation-specific SPB component Spo13 has a functional GEF domain that can only activate Ypt2, but not Ypt3 *in vitro*. Thus, the GEF is not mobile in the cytoplasm and recruited to the secretory vesicles but instead it is immobilized within a structural component. During sporulation, the forespore membrane forms and expands by the fusion of vesicles while

the membrane curves and encapsulates a haploid nucleus (Yoo, 1973; Nakase *et al.*, 2008). A Rab-cascade probably regulates the formation of the forespore membrane during sporulation and Ypt2 is the last Rab GTPase of this cascade. Ypt2 is recruited to the SPB where it is activated by Spo13. However, the upstream Rab is not known. We believe that Ypt3 and Ypt2 function in a Rab-cascade that brings the post-Golgi vesicles to the SPB during the initiation of forespore membrane formation. Therefore the localization of Ypt3 and Ypt2 during sporulation as well as the upstream events need to be clarified.

Interactions between Class V Myosin motor proteins and the Rab-GTPases regulate intracellular trafficking pathways

The class V myosin motor protein Myo51 localizes to the SPB during sporulation (Doyle *et al.*, 2009). Rab GTPases and myosin class V motor protein interactions are essential for secretory vesicle trafficking during *S. pombe* growth as well as in the mammalian and the budding yeast systems. In wild-type mitotically growing *S. pombe* cells GFP-tagged Ypt3 co-localizes with filamentous actin at the cell tips and the septum (Cheng *et al.*, 2002), and also co-localizes with the class V motor protein Myo52 at the cell tips forming a punctate appearance (Lo Presti and Martin, 2011). However, this punctate structure of Ypt3 is not related directly to the Golgi but represents the endosomal compartments in the cell (Cheng *et al.*, 2002). Thus, Ypt3 and Myo52 are involved in many steps of the intracellular trafficking; possibly by loading onto the transport vesicles. All these data led us to ask the role of the interaction between the myosin motors and the Rab-GTPases in *S. pombe* spore morphogenesis.

Myosins are evolutionarily conserved motor proteins that engage with the actin-cytoskeleton. Class V myosins are responsible for cargo (e.g. secretory vesicles) delivery to discrete locations in the cell (East and Mulvihill, 2011). The N-terminal ATP-binding motor domain binds to actin. The α-helical neck domain connects the highly variable C-terminal globular tail domain to the head. The class-specific globular tail domain binds to its cargo directly or via an adaptor molecule and walks it along the actin cables (Hammer and Sellers, 2012). There are two class V myosin motor proteins in *S. pombe*: Myo51 and Myo52. Both Myo51 and Myo52 have discrete localizations and roles in vegetative and sporulating cells (Win *et al.*, 2000). Myo52 is found abundantly where the cortical actin is accumulated; e.g. underneath

the tips of the growing ends during cell growth, septum formed between the two cells during cytokinesis, cell fusion site during mating, and the vicinity of nucleus during meiosis.

Myo51 and Myo52 are both shown to localize to the conjugation site while two haploid cells are mating (Doyle *et al.*, 2009). Unlike Myo52, Myo51 is not highly active in vegetative cells; however, its expression increases at the onset of Meiosis I (Mata *et al.*, 2002; Wood *et al.*, 2012a) and Myo51 is the only fungal myosin that has been reported to associate with SPBs during sporulation (Doyle *et al.*, 2009; East and Mulvihill, 2011). Doyle *et al.*, (2009) showed that Myo51 localizes to the outer face of the SPB at the end of prophase I. They have also shown that the maintenance of Myo51 on the SPB is not dependent on F-actin but on the microtubule cytoskeleton. More importantly, the localizations of Myo51 and the SPB resident protein Spo15 are independent from each other. Thus, *myo51* is not required for the SPB modification and its localization is independent of the MOP alteration. Finally, Myo51 can still localize to the SPB in *myo52* deletion cells (Doyle *et al.*, 2009).

Mug79 localizes to the SPB and it is essential for the forespore membrane formation

mug79 encodes a 150.9 kDA (1318aa) protein, which is a meiosis-specific component of the SPB (Nakamura-Kubo *et al.*, 2011). *mug79* deletion mutants are viable and able to complete meiosis, but they display a severe sporulation deficiency (Nakamura-Kubo *et al.*, 2011). Mug79-GFP signal is positioned outside Spo15 but overlaps with Spo2 and Spo13 (Nakamura-Kubo *et al.*, 2011). However, recruitment of Mug79 to the SPB is independent of *spo15*, *spo2*, and *spo13* (Nakamura-Kubo *et al.*, 2011). Reciprocally, the localizations of these *spo* proteins do not require *mug79*. It has been shown that Spo13 is recruited to the SPB prior to Mug79 (Nakamura-Kubo *et al.*, 2011). Similar to Myo51, Mug79 is not essential for SPB modification but is indispensable for forespore membrane formation; however, its specific role is not clear.

Mug79 has an N-terminal coiled-coil domain that is similar to Spo15 (Nakamura-Kubo *et al.*, 2011). Since this N-terminus is essential for sporulation (Nakamura-Kubo *et al.*, 2011), we suspect that this N-terminus facilitates its localization to the SPB. The protein also harbors a C-terminal pleckstrin homology (PH) domain (Nakamura-Kubo *et al.*, 2011). Proteins that are related to membrane trafficking and lipid modification contain the PH domain, which regulates

binding to phospholipids (Yu *et al.*, 2004). Nakamura-Kubo *et al.*, (2011) showed that the deletion of the PH domain resulted in the aberrant formation of forespore membranes. Our BLAST analysis also revealed the existence of a possible ARF-GEF domain; however deletion of this domain does not result in a phenotype.

In this study, I tried to identify the localization, function and the possible partners of novel SPB proteins Ypt3, Myo51 and Mug79 during ascospore formation. During the course of this study, I used genetic methods and fluorescence microscopy to tag these proteins and visualize them, respectively, during sporulation.

3.2 Materials and Methods

Yeast Strains and media

Standard *S. pombe* genetics methods**,** media and growth conditions were used unless otherwise noted (Forsburg and Rhind, 2006). The strains and the primers used in this study are listed in Table 3.2 and Table 3.3, respectively.

The strains of this study have SPB marker *sid4*-tdTomato and the forespore membrane marker GFP-*psy1* when needed. *sid4* is an essential gene and its product is a constitutive resident of SPBs (Tomlin *et al.*, 2002). Syntaxin 1 homolog Psy1 is a t-SNARE and it is required for the proper formation of the forespore membranes (Maeda *et al.*, 2009). Psy1 localizes to the PM during growth and it is selectively internalized by endocytosis and recruited to the forespore membranes during sporulation (Kashiwazaki *et al.*, 2011). GFP-*psy1* is a powerful tool to visualize the different stages of the forespore membrane formation (Figure 3.3). By simply tracking the localization of GFP-Psy1 protein I could easily identify the mating and conjugation of two haploid cells, transport of Psy1-bound vesicles in the cytoplasm, initiation and expansion of the forespore membranes, and defects in the forespore membranes (Figure 3.3).

Random spore analysis was used for the construction of EAP strains (Sabatinos, 2010). First, a spontaneous cycloheximide resistant mutant of strain JLP18 (EAP3) was selected by plating cells on YES plates containing 10 mg/l of cycloheximide (Sigma-Aldrich Co. LLC). Second, an allele of the spindle pole body (SPB) gene *sid4* fused to a gene encoding the fluorescent protein tdTomato (*sid4*$^{+}$*-tdTomato::hphMX6*) and an allele of class V myosin motor protein *myo51* fused to a gene encoding the fluorescent protein GFP (*myo51*$^{+}$*-GFP::kanMX6*) were introduced by crossing EAP3 with strain 843 (Doyle *et al.*, 2009) to generate EAP7. EAP3 was backcrossed to EAP7 to generate strain EAP15, which has an allele of *sid4*-tdTomato and is *myo51*$^{+\cdot}$ EAP7 and 426 (Doyle *et al.*, 2009) were crossed to combine *myo51Δ::ura4*$^{+}$ as well as *sid4*$^{+}$*-tdTomato::hphMX6* and the segregant was named as EAP12. EAP12 was crossed with HJP2 (a gift from Hui-Yang) to obtain an allele of *mug79* fused to GFP in *myo51Δ* (EAP13; *myo51Δ::ura4*$^{+}$ *mug79*$^{+}$*-GFP::kanMX6 sid4*$^{+}$*-tdTomato::hphMX6*) as well as in *myo51*$^{+}$ cells (EAP14; *mug79*$^{+}$*-GFP::kanMX6 sid4*$^{+}$*-tdTomato::hphMX6*). Strain EAP18 (*mug79Δ-GFP::kanMX6 sid4*$^{+}$*-tdTomato::hphMX6*) is a segregant of a cross of EAP15 and HJP3 (a gift

from Hui-Yang). The forespore membrane marker *psy1* fused to GFP was introduced by crossing EAP15 to FY12295 (Nakase *et al.*, 2008), generating EAP19. EAP12 was crossed to YSM106 (Lo Presti and Martin, 2011) to generate two segregants, EAP24 and EAP25, which carry both *myo52^{+}-GFP::kanMX6 sid4^{+}-tdTomato::hphMX6* in combination with *myo51^{+}* or *myo51Δ::ura4^{+}*.

EAP27, a segregant of a cross of EAP15 and FY12476 (Yang and Neiman, 2010), and EAP12 were plated on EMM2 plates with 0.1% 5-FOA to select uracil auxotrophs and the strain isolated from EAP27 was named EAP30 (*spo13Δ::ura4^{-} sid4^{+}-tdTomato::hphMX6*) and the strain isolated from EAP12 was named as EAP31 (*myo51Δ::ura4^{-} sid4^{+}-tdTomato::hphMX6*).

EAP7 and EAP31 were used for a polymerase chain reaction (PCR)-based gene replacement (Davidson *et al.*, 2002) to construct EAP10 (*mug79Δ::LEU2 myo51^{+}-GFP::kanMX6 sid4^{+}-tdTomato::hphMX6*) and EAP37 (*myo51Δ::ura4^{-} myo52Δ::ura4^{+} sid4^{+}-tdTomato::hphMX6*), respectively. For the generation of the PCR overlap piece to target the allele of *mug79*, in the first round, ~450bp of 5' UTR of *mug79* with primers EAO13 and EAO14, and ~600bp 3' UTR of *mug79* with primers EAO17 and EAO18 from gDNA, and *S. cerevisiae LEU2* with primers EAO15 and EAO16 from FY532 were amplified. PCR products were purified with PCR-purification kit (Qiagen). In the second round, EAO13 and EAO18 oligos were used to overlap the three products to yield ~2.3kb *mug79Δ::LEU2* disruption allele. Integration was confirmed by using EAO20 and EAO23 oligos. Likewise, to construct the PCR overlap fusion to delete the allele of *myo52* in EAP31 strain ~230bp 5' UTR of *myo52* with EAO51 and EAO52 primers, and ~270bp 3' UTR of *myo52* with EAO55 and EAO56 by using gDNA, and *ura4* cassette from pREP42x with EAO53 and EAO54 primers were amplified. The three pieces were fused by using EAO51 and EAO56. ~2.3kb disruption allele was integrated to generate *myo52Δ::ura4^{+}* deletion. Integration was confirmed by using EAO51 and EAO59 oligos in EAP37. Additionally, *myo51Δ::ura4^{-}* was confirmed by using EAO59, EAO60 and EAO61 oligos together in EAP37.

Plasmids

The plasmids used in this study are listed in Table 3.4. To make plasmid pEA13, which contains mTag-BFP without the stop codon in pREP42x, a yeast codon-optimized form of

mTagBFP was PCR amplified from *pRS426 Spo20*$^{51–91}$*-mTagBFP* (Lin *et al.*, 2013) using EAO44 and EAO46 (without the stop codon) primers. The PCR product is engineered to contain both *Nde*I and *Nco*I sites at the 5' ends and *Not*I and *BamH*I at the 3' ends. It was digested with *Nde*I and *BamH*I and cloned into similarly digested pREP42x. The integration of the mTagBFP into pREP42x was confirmed by PCR analysis by using EAO29 and EAO43 primers.

pEA15 and pEA16, which contained *ypt2* and *ypt3* respectively, were constructed by polymerase chain reaction (PCR) amplification of the full-length genes from the genomic DNA of a wild-type *S. pombe* strain by using EAO38 and EAO39 primers for the amplification of *ypt2* and EAO40 and EAO41 primers for the amplification of *ypt3*. PCR products were digested with *Not*I and *BamH*I and cloned into similarly digested pEA13 to the C-terminus of the mTagBFP that did not have a stop codon. The oligo pairs of EAO29- EAO38 and EAO29-EAO40 were used to confirm the integration of *ypt2* and *ypt3,* respectively.

Fluorescence Microscopy

Freshly grown *S. pombe* cells were incubated on SPA sporulation plates for 1-2 days before imaging. Images were collected on a Zeiss Observer Z.1 microscope and processed using Zeiss Axiovision or Zen software.

3.3 Results

Rab-GTPases localize to the SPB and the forespore membrane during S. pombe sporulation

Rab GTPases are regulators of intracellular trafficking pathways (Zerial and McBride, 2001). *S. pombe* has two conserved Rab-GTPases, *ypt3* and *ypt2*, which are essential for growth. However, their roles during sporulation are not defined. Imada *et al.*, (2011) showed that Ypt3 localizes to the nascent forespore membrane during *S. pombe* sporulation and the forespore membrane formation is severely inhibited in *ypt3-ki8* mutants. They also suggest that localization to the forespore membrane is independent of the SPB resident protein Spo15. However, this is not possible because *spo15Δ* mutants are defective in forespore membrane formation (Ikemoto *et al.*, 2000), therefore there will be no forespore membrane to observe in the absence of Spo15, which means the GFP signal that they observed cannot be on the forespore membrane. The protein is probably accumulated around the nucleus. This leaves us only with the observation that Ypt3 localizes to the forespore membrane from the early stages of the membrane formation. Forespore membrane formation is initiated at the SPBs therefore it is likely thatYpt3 localizes to the SPB as well.

To test the localization of Ypt3, I first examined the GFP-tagged Ypt3 in the EAP15 strain. I identified the localization of GFP-Ypt3 around the nucleus during Meiosis II (data not shown); however, it was not possible to clarify the localization because the EAP15 strain does not have a forespore membrane marker and the forespore membrane marker *psy1* is also tagged with GFP. In need of a third color to test the localization of the Ypt3 on the forespore membrane, I constructed a plasmid (pEA16) by tagging *ypt3* with BFP. *mTagBFP-ypt3* (pEA16) expressed in strain EAP19 localizes to the vicinity of the SPB and then colocalizes with the forespore membrane marker GFP-Psy1 from the early stages of the forespore membrane formation (Figure 3.4). Thus, Ypt3 localizes to the SPB and, consistent with the previous finding, to the forespore membrane in wild-type cells during *S. pombe* sporulation.

ypt3 is the homolog of *S. cerevisiae YPT31/32* and mammalian Rab11, where both Ypt32 and Rab11 localization to a membrane compartment recruits the subsequent Rab-GTPases Sec4 and Rab8, respectively. If Ypt3 localizes to the SPB, it may recruit Ypt2 as well. Therefore, I

analyzed the localization of Ypt2 during *S. pombe* sporulation by expressing *mTagBFP- ypt2* (pEA15) in EAP19 strain. In these cells *sid4*-tdTomato and GFP-*psy1* are the SPB and the forespore membrane markers. The co-localization of the BFP signal with the markers showed that, similar to Ypt3, mTagBFP-Ypt2 localizes to the SPB during Meiosis II and then to the forespore membrane starting from the early stages of forespore membrane formation (Figure 3.5).

Ypt3 and Ypt2 co-localize on the forespore membrane

During my fluorescent microscopy analyses, I observed that Ypt2 arrives at the forespore membrane while it expands (Figure 3.6) and both Rab-GTPases Ypt3 and Ypt2 localize to the SPB and to the nascent forespore membrane. The timing of recruitment of these two proteins to the SPB and forespore membrane is important to understand if there is any hierarchal order for these Rab-GTPases or if they arrive in a random manner. I co-expressed *GFP-ypt3* and *mTagBFP-ypt2* in EAP15 cells to examine their behavior for the SPB localization (Figure 3.7). In a different experiment, I co-expressed *tdTomato-ypt3* and *mTagBFP-ypt2* in EAP19 cells for their localization to the forespore membrane (Figure 3.8) To do the co-localization analysis, I incubated cells on sporulation plates and took pictures of cells at random times. According to the images, it is likely that Ypt3 arrives at the SPB prior to Ypt2; however, it is not enough to conclude without the observation of live cells (Figure 3.7). It seems that once the forespore membrane starts to form and expand, both Ypt3 and Ypt2 co-localize homogenously on the forespore membrane (Figure 3.8). On the other hand, a clear understanding of the timing and the order of Ypt arrival at the SPB and the forespore membrane will only be available by live imaging.

Localization of Ypt3 and Ypt2 to the SPB and the forespore membrane confirms the involvement of Rab-GTPases in the formation of the forespore membrane during sporulation. Rab-GTPases interact with effector molecules and their GEFs/GAPs. Next I tried to identify the partners/effectors of Ypt3 and Ypt2 around the SPB and the forespore membrane.

Spindle pole body modification is required for the recruitment of Rab-GTPases to the SPB

Three SPB proteins Spo15, Spo2, and Spo13 form the *S. pombe* MOP, thus provide the SPB modification for the initiation of the forespore membrane formation (Nakase *et al.*, 2008) (Figure 3.1). Localization of GFP-fused proteins in *spo15*, *spo2* and *spo13* deletion mutants showed that recruitment of these proteins to SPB is in the order Spo15, Spo2, Spo13, and that Spo13 resides at the membrane-proximal side of the MOP and it is essential for the forespore membrane formation (Nakase *et al.*, 2008). Without any of these three proteins the SPB modification is not completed. Next, I asked whether SPB modification is required for the localization of Ypt3 and Ypt2 to the SPB. To do this, I expressed *mTagBFP-ypt3* (pEA16) and *mTagBFP-ypt2* (pEA15) in *spo13Δ* (EAP30) strains. Neither Rab localized to the SPB in the absence of the MOP protein Spo13 (Figures 3.9 and 3.10). It seems that recruitment of Ypts to the SPB is dependent on the successful formation of the MOP.

Mug79 is not required for Ypt localization to the SPB

Mug79 localizes to the SPB during Meiosis II and its localization is independent of Spo15 (and Spo2 and Spo13, since they are recruited to the SPB only in the presence of Spo15). Reciprocally, Mug79 is not required for Spo15. This means that Mug79 is not essential for SPB modification but is indispensable for forespore membrane formation (Nakamura-Kubo *et al.*, 2011).

I wanted to repeat testing of Ypt localization to the SPB in the absence of *mug79.* To do that, I expressed *mTagBFP-ypt3* (pEA16) or *mTagBFP-ypt2* (pEA15) in a mug79*Δ* strain EAP18. Interestingly, Ypt3 and Ypt2 localization to the SPB is independent of *mug79* (Figure 3.9 and 3.10). Even though the *mug79* deletion mutant has no forespore membrane formation at all, Ypt3 and Ypt2 vesicles are recruited to the SPB. It seems that a protein that has no effect on the SPB modification is also not required for the localization of Ypts.

Class V myosin motor protein Myo51 localizes to the SPB and is not required for forespore membrane formation

S. pombe has two class V myosin motor proteins: Myo51 and Myo52. Myo52 is more active during growth however Myo51 is upregulated during meiosis. Myo51 localizes to the SPB during Meiosis I however *myo51Δ* does not affect spore formation (Doyle *et al.*, 2009). Localization of Myo51 to the SPB is independent of Spo15, Myo52 and F-actin (Doyle *et al.*, 2009). Thus, the exact role of Myo51 on the SPB is not defined. I tested forespore membrane formation in a *myo51Δ* strain EAP37. GFP-*psy1* was the marker to visualize the forespore membranes. The forespore membrane formation was similar to that seen in wild-type cells in the absence of *myo51* (Figure 3.11). Consistent with the previous report, even though Myo51 had a dynamic localization to the SPB from the early stages of meiosis, absence of *myo51* does not cause a phenotype (Figure 3.11).

Myo52 relocalizes in the absence of Myo51

myo51Δ cells can make spores that are similar to wild-type (Figure 3.11). Since there are two class V myosin motor proteins in *S. pombe* we asked whether the second class V myosin motor protein Myo52 can take over the role of Myo51 when it is absent. I analyzed GFP-tagged Myo52 in *myo51*$^{+}$ (EAP24) and *myo51Δ* (EAP25) cells. In both strains *sid4*-tdTomato was used as the SPB marker. In *myo51*$^{+}$ cells, GFP-Myo52 signal appeared in the vicinity of the SPB and around the nucleus (Figure 3.12). Unfortunately, EAP24 *myo51*$^{+}$ strain does not have a forespore membrane marker because the marker *psy1* is also tagged with GFP. Therefore, it is not possible to conclude whether Myo52 localizes to the forespore membrane or not. On the other hand, in *myo51Δ* cells, GFP-Myo52 co-localized with the SPB marker Sid4-tdTomato (Figure 3.12). It is clear that Myo52 localization changes in the absence of Myo51 and may therefore substitute for Myo51. The ability of *myo51*Δ cells to make spores may reflect this substitution by Myo52.

Class V myosin motor protein double deletion mutants show severe defect in mating and fusion

Myo51 localize to the SPB from the early stages of meiosis, yet, *myo51Δ* cells do not have a sporulation defect (Figure 3.11) and that is probably because Myo52 can substitute in the

absence of *myo51*$^{+}$ (Figure 3.11). We wanted to investigate whether post-Golgi vesicles can still be recruited to the SPB in the absence of both motor proteins. To do this, I constructed strain EAP37 *myo51Δ::ura4*$^{-}$ *myo52Δ::ura4*$^{+}$, where both motor proteins were deleted. However, this mutant is sick and has a severe mating defect (data not shown). The mutant was not analyzed further.

Mug79 localizes to the SPB and is required for Myo51 localization to the SPB

Published results and my data show that *mug79* deletion mutants can complete meiosis but display a severe sporulation deficiency (Figure 3.11). It is interesting that *mug79* deletion does not affect Spo15, Spo2, Spo13, Ypt3, and Ypt2 localization; however, it may be important for Myo51 localization. To clarify this, I investigated whether the localization of Myo51 and Mug79 are dependent on each other. Comparison of Myo51-GFP in *mug79*$^{+}$ and *mug79Δ* cells showed that *mug79* is essential for the localization of Myo51-GFP to the SPB during sporulation (Figure 3.13 and Table 3.5). On the other hand, even though Myo51 localizes to the SPB during Meiosis I, before Mug79 (Doyle *et al.*, 2009) Mug79 localization is independent of Myo51 (Figure 3.14 and Table 3.5). Unfortunately, redundancy of Myo51 with Myo52 makes the Mug79 localization result difficult to interpret.

Fluorescence recovery after photobleaching analysis showed that Myo51 rapidly recovers at the SPB after photobleaching during Meiosis I, however, the signal does not fully recover after photobleaching in Meiosis II (Doyle *et al.*, 2009). Thus, Mug79 may be stabilizing Myo51 on the SPB; however, for a better understanding, we also need to test the localization of Myo52 in *mug79Δ* strains. Besides, *mug79Δ* strains have severe forespore membrane defect. It may be that Mug79 regulates the initiation of the forespore membrane, which indirectly controls the localization of Myo51 to the SPB. Further work is necessary to clarify the interaction between Myo51 and Mug79.

3.4. Discussion

Forespore membrane formation is initiated at the SPB during meiosis. Secretory vesicles are recruited to the SPB where they fuse and form a double layer membrane. Initiation of the forespore membrane formation is under the control of proteins that are recruited to the SPB. Therefore, identification of the SPB proteins is crucial in understanding the spore morphogenesis. The MOP proteins Spo15, Spo2 and Spo13 generate the modification of the SPB so that forespore membrane formation can begin. In this work, I have shown that Rab-GTPases Ypt3 and Ypt2 localize to the SPB and the forespore membrane. Recruitment of Ypts to the SPB requires Spo13 but is independent of Mug79. This finding suggests that there are two independent modules happening around the SPB that are both required for the forespore membrane formation. It is likely that SPB modification that is carried out by the hierarchal recruitment of Spo15, Spo2 and Spo13 also brings the Ypt3-bound vesicles to the SPB, which in turn recruits Ypt2. On the other hand, Mug79 and myosin motor proteins Myo51 and maybe Myo52 support forespore membrane formation via a separate mechanism. For a better understanding, we need to test whether functional Ypts are required for the localization of Mug79 to the SPB. Since *ypt3* and *ypt2* are essential for growth, conditional mutants need to be constructed to analyze their effect during only sporulation.

Sporulation-specific MOP component Spo13 has a GEF domain that can activate Ypt2 (Yang and Neiman, 2010). As mentioned earlier, this is a novel Rab-GEF interaction because the GEF Spo13 is a structural member of the SPB. It is likely that Ypt3-bound vesicles are transported to the SPB and then Ypt2 is recruited and activated by Spo13. In the absence of *spo13* neither of these two Rab-GTPases localize to the SPB. So it is likely that even though Ypt3 is not a substrate of Spo13, the successful modification of the meiotic outer plaque initiates the recruitment of post-Golgi vesicles to the SPB during forespore membrane formation.

In *S. cerevisiae* GTP-bound active Ypt31/32 (Ypt3) recruits the GEF Sec2 (Spo13) to the vesicles. Sec2 first recruits Sec4 (Ypt2) and then its effector Sec15 (Novick *et al.*, 2006). Sec15 is a component of the exocyst, which is an octameric protein complex that docks the secretory vesicles to the exocytic sites on the yeast plasma membrane (TerBush *et al.*, 1996). Sec15 associates with the vesicles by binding to the GTP-bound Sec4 (Ypt2) (Guo *et al.*, 1999). The exocyst complex protein Sec15 and Ypt32 (Ypt3) compete with each other to bind to Sec2 and

Sec15-Sec2 interaction is 3-fold higher affinity than Ypt32-Sec2 (Medkova *et al.*, 2006). Once Sec15 is bound to Sec2, it dissociates Ypt32 from the vesicle. Dissociation of Ypt32 promotes the activation of Sec4. When the vesicle is tethered to the plasma membrane, Sec2 is released from the vesicle and it recycles back to the cytosol for another vesicle. This model suggests that two successive Rab-GTPases co-localize on a vesicle for a very short period of time and this timing is regulated by a GEF and an effector molecule. However, I observed that Ypt3 and Ypt2 co-localize to the forespore membrane while both ends of the membrane expands (Figure 3.8). Although the exact mechanism is not clear, it is likely that both GTPases remain on the vesicles simultaneously during the active docking and fusion of the vesicles to the nascent forespore membrane.

It is interesting that Spo13 remains on the SPB while its substrate Ypt2 can be observed on the growing forespore membrane. My current data cannot explain if there is any interaction between Spo13 and Ypt2 that is on the nascent forespore membrane and this needs to be further investigated. It is noteworthy that there is a predicted *sec2* gene in the *S. pombe* genome (Wood *et al.*, 2012a). However, there is no published study in the literature about the function of *sec2.* It may be that Spo13 recruits the Rab-GTPases Ypt3 and Ypt2 for the initiation of the forespore membrane formation on the SPB and Sec2 acts as another GEF for Ypt2 for the addition of vesicles to the growing forespore membrane.

Class V myosin motor protein Myo52 expression does not significantly change during meiosis and sporulation. However, it is highly active in a variety of different cellular processes during mitosis. It was originally identified for its role in directing the localization of the alpha-glucan synthase Mok1 to promote appropriate cell wall synthesis (Win *et al.*, 2000). This is interesting because *S. pombe* has three sporulation-specific paralogues (*mok12, mok13, and mok14*) of *mok1* that carry out the alpha-glucan synthesis in the spore wall (Hochstenbach *et al.*, 1998; Garcia *et al.*, 2006). *mok14* is specifically required for synthesis of the amylose-like spore wall polymer (Garcia *et al.*, 2006). Additionally, Myo52 delivers the β-1,3-glucan synthase Bgs1 to the cell division sites (Mulvihill *et al.*, 2006). It has been shown that a sporulation-specific β-1,3-glucan synthase homolog *bgs2* is required for the proper formation of the spore wall (Liu *et al.*, 2000; Martin *et al.*, 2000). Myo52 has also been implicated at the transport of SNARE protein synaptobrevin Syb1 to the medial region of the cell (Edamatsu and Toyoshima, 2003).

This is very striking because recently Yamaoka *et. al.*, (2013) showed that *syb1* is essential for the forespore membrane formation and specifically for the maturation of the spore wall (Yamaoka *et al.*, 2013). Taken together, although a specific role is not attributed to Myo52 during sporulation, this class V myosin motor protein may have an essential role on the transport of the spore wall proteins.

Here, I propose a Rab-maturation model where consecutive Rab proteins and their binding partners regulate the movement, recruitment, and fusion of secretory vesicles to the SPB and to the forespore membrane during *S. pombe* sporulation (Figure 3.1). Sporulation specific SPB proteins Spo15, Spo2, and Spo13 localize to the SPB in a hierarchal order (Nakase *et al.*, 2008) (Figure 3.1, part A). The class V motor protein Myo51 localizes to the SPB starting from Meiosis I, however its dynamic behavior changes during Meiosis II (Doyle *et al.*, 2009). This may be because Mug79 localizes to the SPB during Meiosis II (Nakamura-Kubo *et al.*, 2011) and it is required for the stabilization of Myo51 on the SPB (Figure 3.1, part B). During fluorescence microscopy analysis, I observed that both Ypt3 and Ypt2 localize to the SPB and then to the forespore membrane in wild-type cells. Once the SPB modification is completed by the recruitment of Spo13, Ypt-3 bound vesicles flush towards the SPB and Ypt2 is also recruited at this time (Figure 3.1, part C). The Rab-cascade composed of Ypt3 and Ypt2 aids the formation of the forespore membrane by post-Golgi vesicles (Figure 3.1, part D). It seems that on the SPB Ypt2 is activated by Spo13; however its activation on the forespore membrane needs to be clarified.

Table 3.1 *S. pombe*, *S. cerevisiae*, and human Rab-GTPase and GEF homologs discussed

	S. pombe	***S. cerevisiae***	**Mammalian**
Rab-GTPase	Ypt3	Ypt31/32	Rab11
Rab-GTPase	Ypt2	Sec4	Rab8
GEF[a]	Spo13	Sec2	Rabin8

[a]GEFs that binds and activates only Ypt2, Sec4 or Rab8

Table 3.2 Strains used in this study

Name	Genotype	Source
EAP3	*h^{90} ura4-D18 leu1-32 his3-127 cyh^{R}*	This study
EAP7	*h^{90} myo51^{+}-GFP::kanMX6 sid4^{+}-tdTomato::hphMX6 ura4-D18 leu1-32 cyh^{R}*	This study
EAP10	*h^{90} mug79Δ::LEU2 myo51^{+}-GFP::kanMX6 sid4^{+}-tdTomato::hphMX6 ura4-D18 leu1-32 cyh^{R}*	This study
EAP12	*h^{90} myo51Δ::ura4^{+} sid4^{+}-tdTomato::hphMX6 ura4-D18 leu1-32 cyh^{R}*	This study
EAP13	*h^{90} myo51Δ::ura4^{+} mug79^{+}-GFP::kanMX6 sid4^{+}-tdTomato::hphMX6 ura4-D18 leu1-32 cyh^{R}*	This study
EAP14	*h^{90} mug79^{+}-GFP::kanMX6 sid4^{+}-tdTomato::hphMX6 ura4-D18 leu1-32 cyh^{R}*	This study
EAP15	*h^{90} sid4^{+}-tdTomato::hphMX6 ura4-D18 leu1-32 his3-127 cyh^{R}*	This study
EAP18	*h^{90} mug79Δ-GFP::kanMX6 sid4^{+}-tdTomato::hphMX6 ura4-D18 leu1-3 his3-127 cyh^{R}*	This study
EAP19	*h^{90} leu1::GFP-psy1^{+}-LEU2 sid4^{+}-tdTomato::hphMX6 ura4-D18 leu1-32*	This study
EAP23	*h^{90} myo52^{+}-GFP::kanMX6 ade6 ura4-D18 leu1-32 cyh^{R}*	This study
EAP24	*h^{90} myo52^{+}-GFP::kanMX6 sid4^{+}-tdTomato::hphMX6 ura4-D18 leu1-32*	This study
EAP25	*h^{90} myo51Δ::ura4^{+} myo52^{+}-GFP::kanMX6 sid4^{+}-tdTomato::hphMX6 ura4-D18 leu1-32*	This study
EAP27	*h^{90} spo13Δ::ura4^{+} sid4^{+}-tdTomato::hphMX6 ura4-D18 leu1-32 his3-127*	This study
EAP30	*h^{90} spo13Δ::ura4^{-} sid4^{+}-tdTomato::hphMX6 ura4-D18 leu1-32 his3-127*	This study
EAP31	*h^{90} myo51Δ::ura4^{-} sid4^{+}-tdTomato::hphMX6 ura4-D18 leu1-32*	This study
EAP37	*h^{90} myo51Δ::ura4^{-} myo52Δ::ura4^{+} sid4^{+}-tdTomato::hphMX6 ura4-D18 leu1-32*	This study
JLP18	*h^{90} ura4-D18 leu1-32 his3-127*	This study
HJP2-G1	*h^{90} mug79^{+}-GFP::kanMX6 ura4-D18 leu1-32 his3-127*	This study
HJP3	*h^{90} mug79Δ-GFP::kanMX6 ura4-D18 leu1-32 his3-127*	This study
426	*h^{90} myo51Δ::ura4^{+} ura4-D18 leu1-32*	(Doyle *et al.*, 2009)

843	*h^{90}myo51^{+}-GFP::kanMX6 sid4^{+}-tdTomato::hphMX6 ura4-D18 leu1-32*	(Doyle *et al.*, 2009)
FY12295	*h^{90} spo15Δ::ura4^{+} ura4-D18 leu1::GFP-psy1^{+}-LEU2*	(Nakase *et al.*, 2008)
FY12476	*h^{90} spo13Δ::ura4^{+} ura4-D18 sid4^{+}-GFP::kanMX6*	(Yang and Neiman, 2010)
YSM106	*h^{-} myo52^{+}-GFP::kanMX ade6 leu1-32 ura4-D18*	(Lo Presti and Martin, 2011)

Table 3.3 Primers used in this study

Name	Sequence	Relevant function
EAO13	GGAAGGTCAGATTGTTGCTC	5' UTR *mug79*$^+$
EAO14	CGTAGTCGATCGACAAGCTTCCTTTTTCGTGGATAATTATAC	5' UTR *mug79*$^+$
EAO15	GTATAATTATCCACGAAAAAGGAAGCTTGTCGATCGACTACG	*LEU2* from FY532
EAO16	CCTAATGTCTTTTCATCACAAGCTTGTCGAGGAGAACTTC	*LEU2* from FY532
EAO17	GAAGTTCTCCTCGACAAGCTTGTGATGAAAAGACATTAGG	3' UTR *mug79*$^+$
EAO18	CTCAGGTTATGTTAACCGTTAGC	3' UTR *mug79*$^+$
EAO20	GTATATGCGTCAGGCGACCTCTG	Confirmation of *mug79Δ::LEU2*
EAO23	CCAGGCGTTGGAATGTAGAAC	Confirmation of *mug79Δ::LEU2*
EAO29	GTACTCGTTGTCGGAGATCAAG	Confirmation of *ypt2*$^+$ and *ypt3*$^+$ integration
EAO38	GTTCTTGCGGCCGCGTTCATGTCTACAAAATCCTACGATTATTTAATC	Introduces *Not*I to *ypt2*$^+$
EAO39	GTTCTTGGATCCCTAACAACACCTCTTCACCG	Introduces *BamH*I to *ypt2*$^+$
EAO40	GTTCTTGCGGCCGCGTTCATGTGTCAAGAGGACGAATACG	Introduces *Not*I to *ypt3*$^+$
EAO41	GTTCTTGGATCCCTAACAACATTGGGAAGAAGAC	Introduces *Not*I to *ypt3*$^+$
EAO43	GGGTTTCACATGGGAGAGGG	Confirmation of mTagBFP integration into pREP42x
EAO44	GTTCTTCATATGGTTCTTGTTCCATGGATGTCTGAGGAGTTGATAAAGG	Introduces *Nde*I and *Nco*I to mTagBFP amplification
EAO46	GTTCTTGGATCCCTTGTTCTTGCGGCCGCGTTCAACTTGTGACCCAACTTTG	Introduces *Not*I and *BamH*I mTagBFP (without stop codon)
EAO51	GCGAAGTCATCTCCATATTAACATCAG	5' UTR *myo52*$^+$
EAO52	CAGTGGGATTTGTAGCTAAGCTTGACCGGTAGAAATTACAAGACTC	5' UTR *myo52*$^+$
EAO53	GAGTCTTGTAATTTCTACCGGTCAAGCTTAGCTACAAATCCCACTG	Amplification of *ura4*$^+$ from pREP42x

EAO54	CAGAGCACCTTGAAAAATAACTAGATATGTGATATTGAC GAAACATCTAATTTATTC	Amplification of *ura4*$^+$ from pREP42x
EAO55	GAATAAATTAGATGTTTCGTCAATATCACATATCTAGTTA TTTTTCAAGGTGCTCTG	3’ UTR *myo52*$^+$
EAO56	CTGAAACCAATGCGTACTCCGC	3’ UTR *myo52*$^+$
EAO59	CTTGATAACACAGACATAGGGTC	Confirmation of *myo52Δ::ura4*$^+$
EAO60	GCACATCGAAACTCAAGTTACC	Confirmation of *myo51Δ::ura4*$^+$
EAO61	CAAGGGTGCTTGAAAGTTCTCC	Confirmation of *myo51Δ::ura4*$^+$

Table 3.4 Plasmids used in this study

Name	Relevant function	Source
pREP42x	*ura4*$^{+}$	(Forsburg, 1993)
FYP532	*GFP-psy1*$^{+}$*::LEU2*	(Nakamura, 2001)
pSM893	*GFP-ypt3*$^{+}$*::LEU2*	(Lo Presti and Martin, 2011)
pSM928	*tdTomato-ypt3*$^{+}$*::LEU2*	(Lo Presti and Martin, 2011)
	Spo20$^{51–91}$*-mTagBFP::URA3*	(Lin *et al.*, 2013)
pEA13	*mTAG-BFP::ura4*$^{+}$ (without stop codon)	This study
pEA15	*mTAG-BFP-ypt2*$^{+}$*::ura4*$^{+}$	This study
pEA16	*mTAG-BFP-ypt3*$^{+}$*::ura4*$^{+}$	This study

Table 3.5 Myo51 and Mug79 localization on the SPB in wild-type and mutant cells

Name		on SPB	Total number of SPB	%
Myo51-GFP	**Wild-type**	19	24	79.2
	mug79Δ	3	106	2.8
Mug79-GFP	**Wild-type**	35	52	67.3
	myo51Δ	16	28	57.1

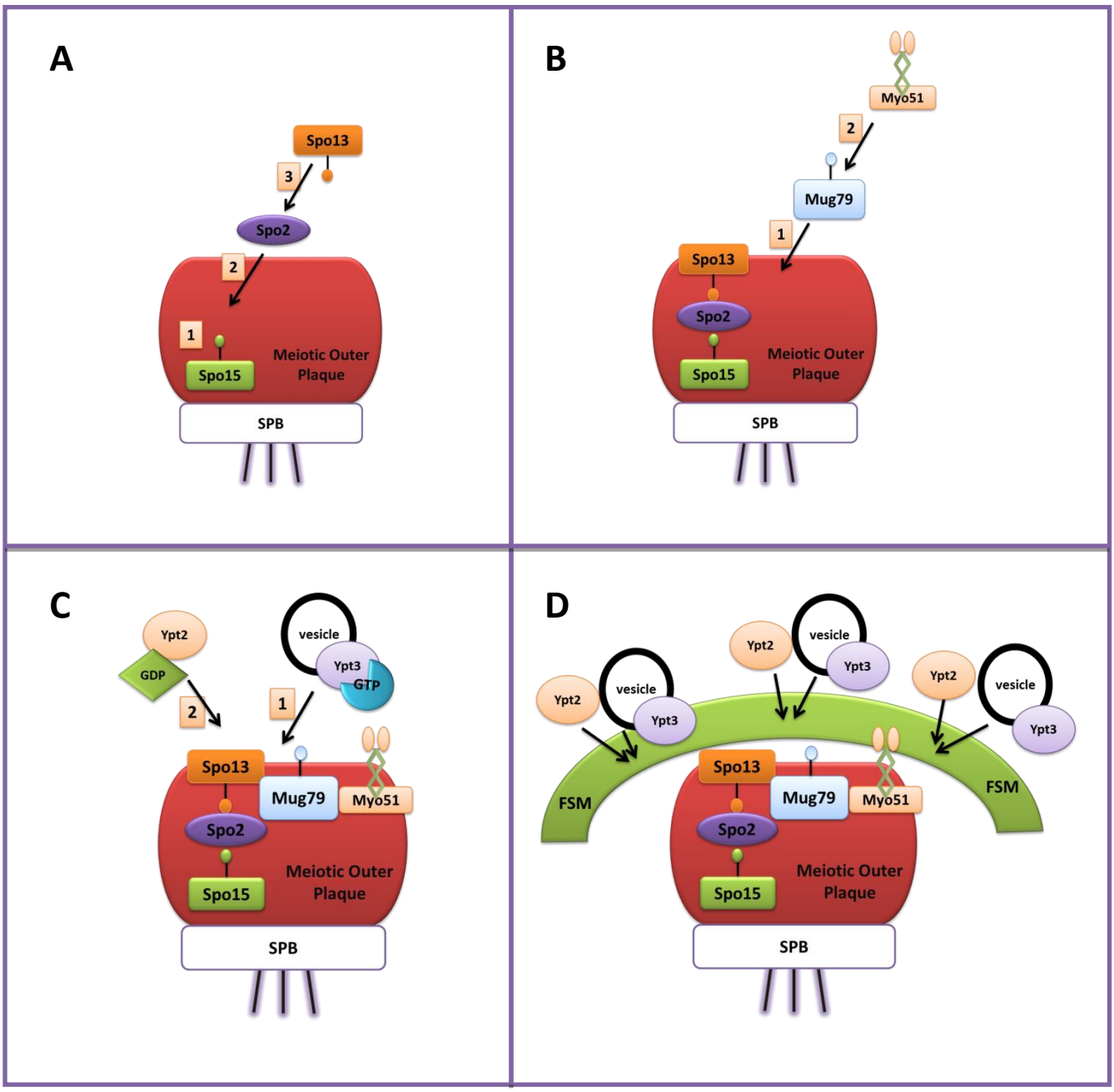


Figure 3.1 Rab-cascade model for the formation of forespore membrane.

A) Sporulation-induced Spo15 recruits Spo2, which in turn recruits Spo13 to the SPB. **B)** Mug79 localizes and stabilizes Myo51 on the SPB. **C)** Ypt3-bound vesicles and Ypt2 localize to the SPB, Ypt2 is activated by Spo13. **D)** Ypt3 and Ypt2 regulate the recruitment and fusion of the vesicles to the *de novo* synthesized forespore membrane as it forms and expands.

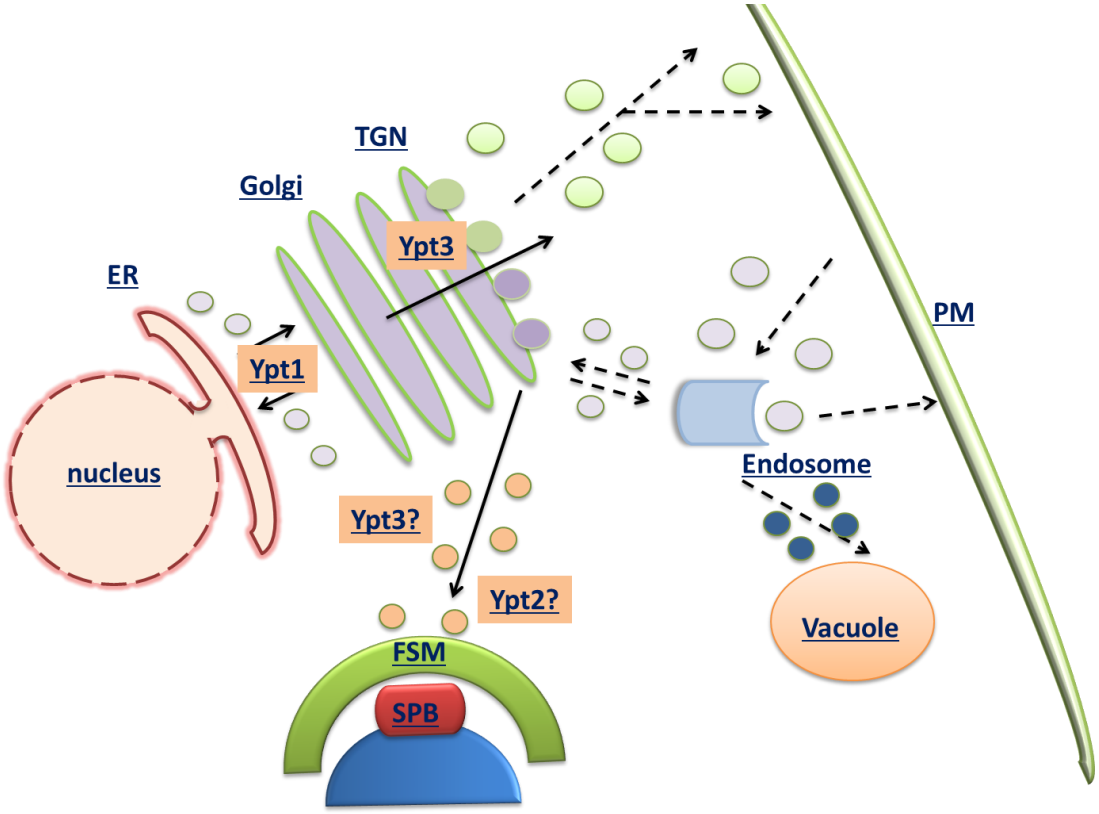


Figure 3.2 Schematic of the secretory pathway in *S. pombe*

Specific Ypt proteins that regulate vesicle transport between membrane compartments are shown. Post-Golgi vesicles that are transported to the PM in vegetative cells are redirected towards nascent forespore membrane during sporulation.

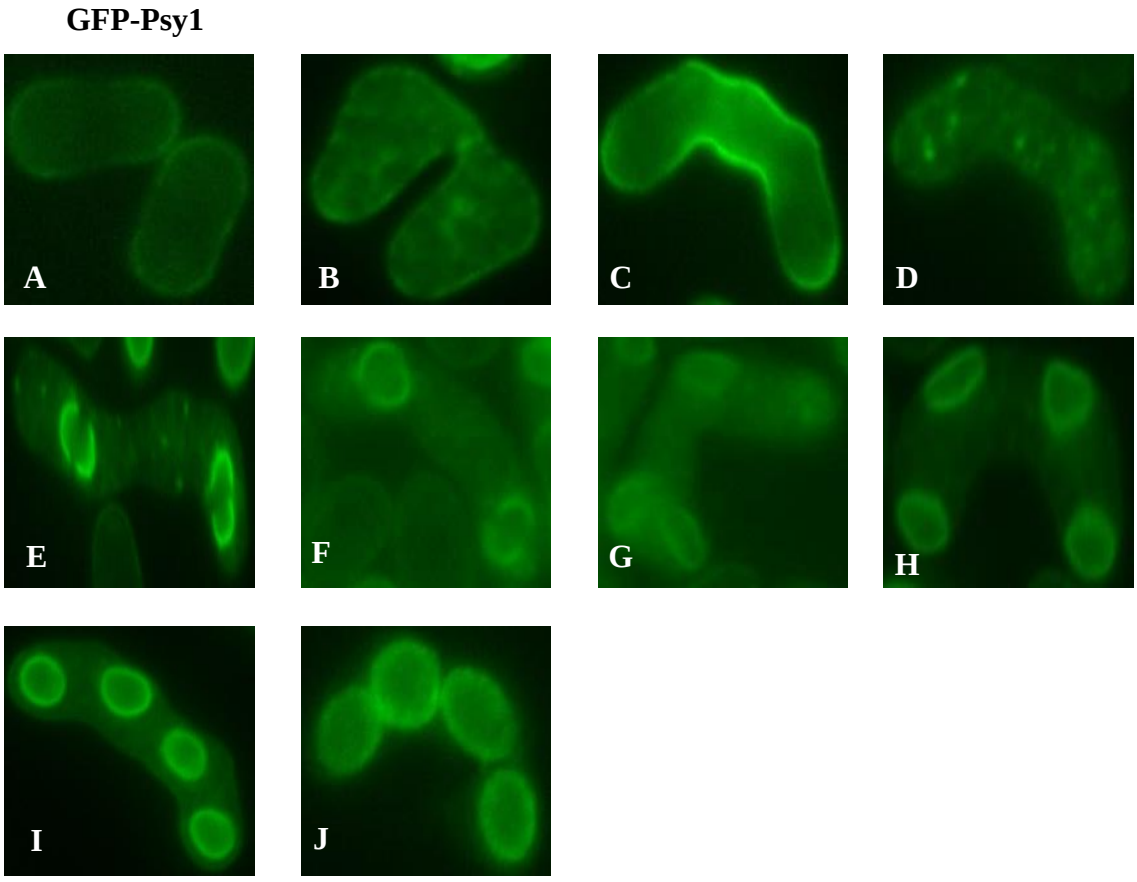


Figure 3.3 Tracking GFP-Psy1 from the plasma membrane to the forespore membrane

GFP-tagged Psy1 (green) is used as the forespore membrane marker during the course of the study. Cells that harbor GFP-*psy1* were imaged after 24 hr incubation on SPA plates. **(A-C)** GFP-Psy1 localizes to the plasma membrane in vegetative cells and in asci before Meiosis I **D)** at the onset of Meiosis I GFP-Psy1 is internalized by endocytosis from the plasma membrane (**E-J)** GFP-Psy1 localizes to the forespore membrane.

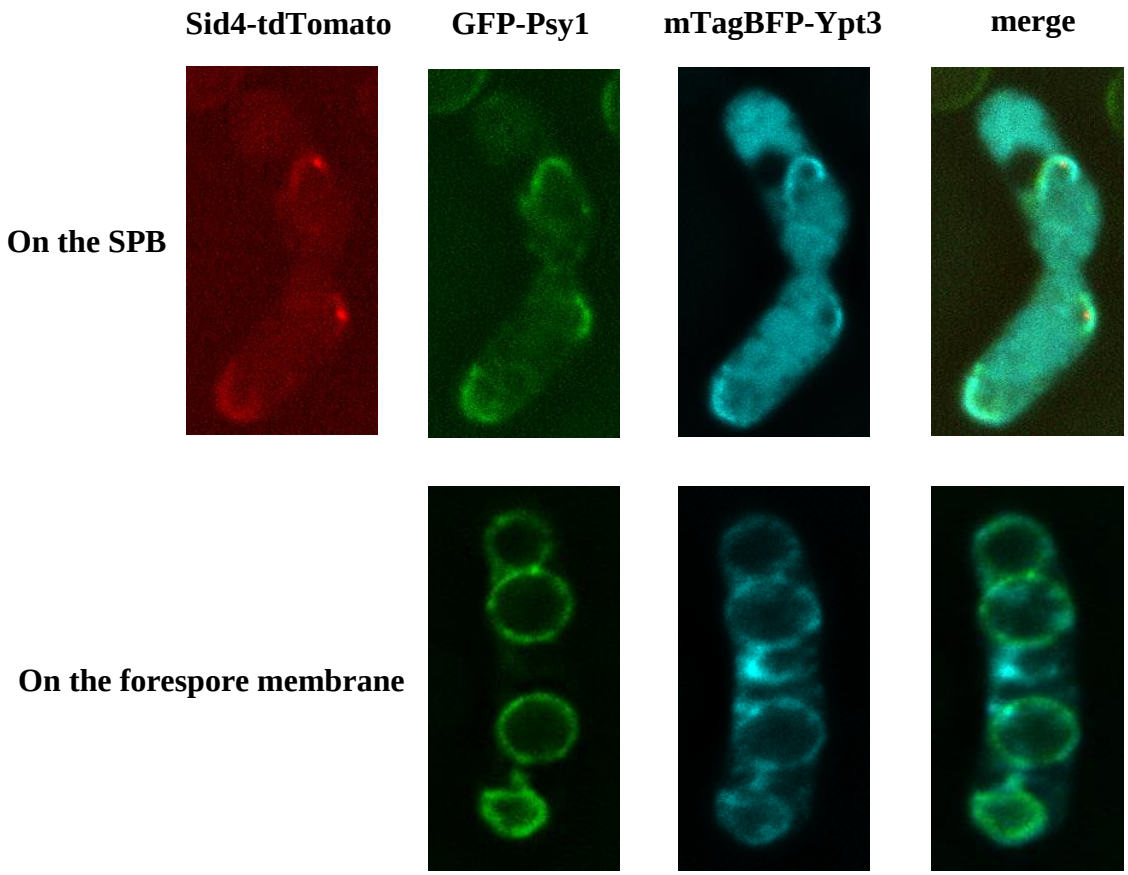


Figure 3.4 mTagBFP-Ypt3 localization on the SPB and the forespore membrane

Wild type cells expressing *PmTagBFP-ypt3* (blue), *GFP-psy1* (green) and *sid4-tdTomato* (red) were imaged after 24 hr incubation on SPA plates.

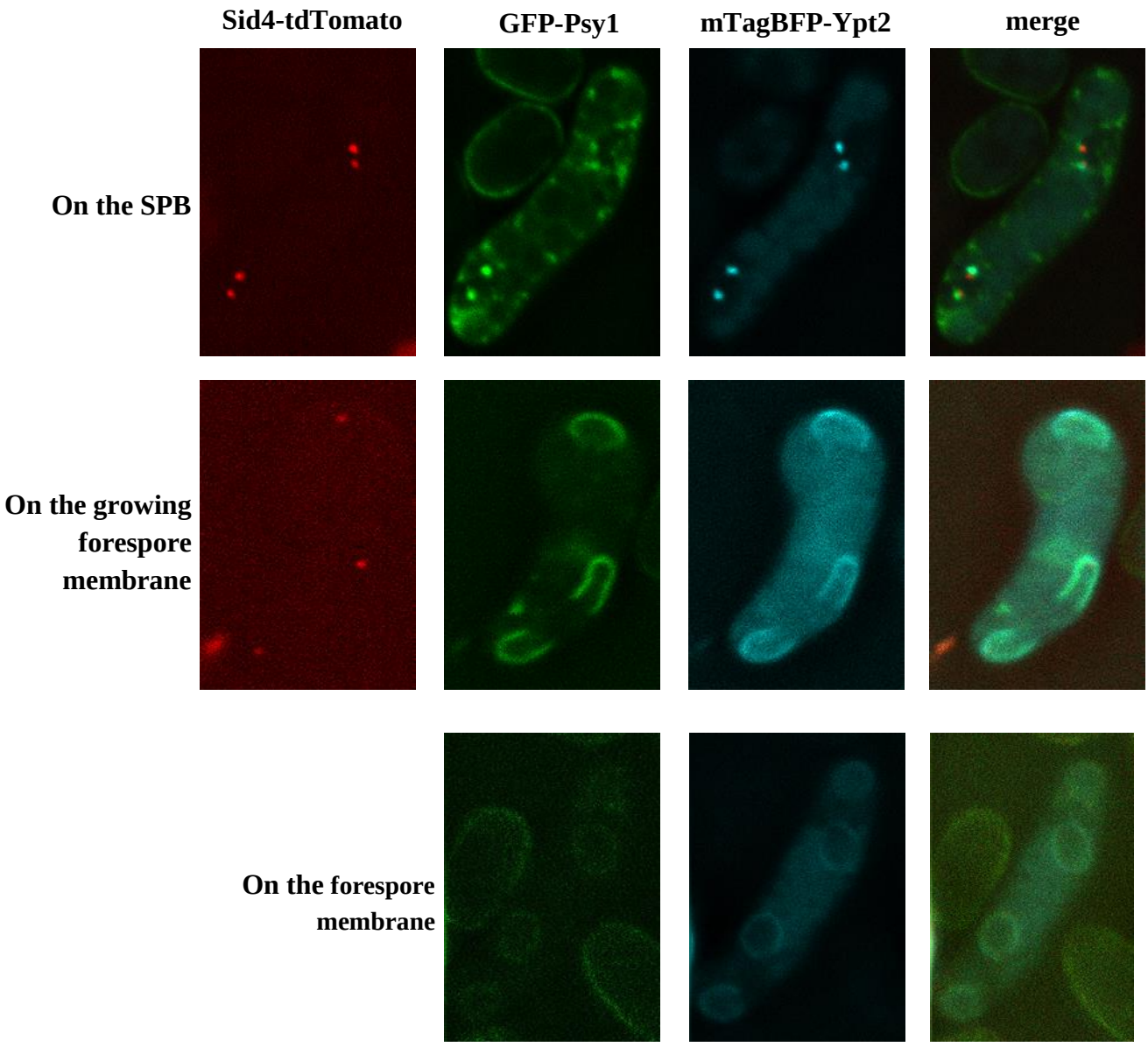


Figure 3.5 mTagBFP-Ypt2 localization on the SPB and the forespore membrane

Wild type cells expressing *PmTagBFP-ypt2* (blue), *GFP-psy1* (green) and *sid4-tdTomato* (red) were imaged after 24 hr incubation on SPA plates.

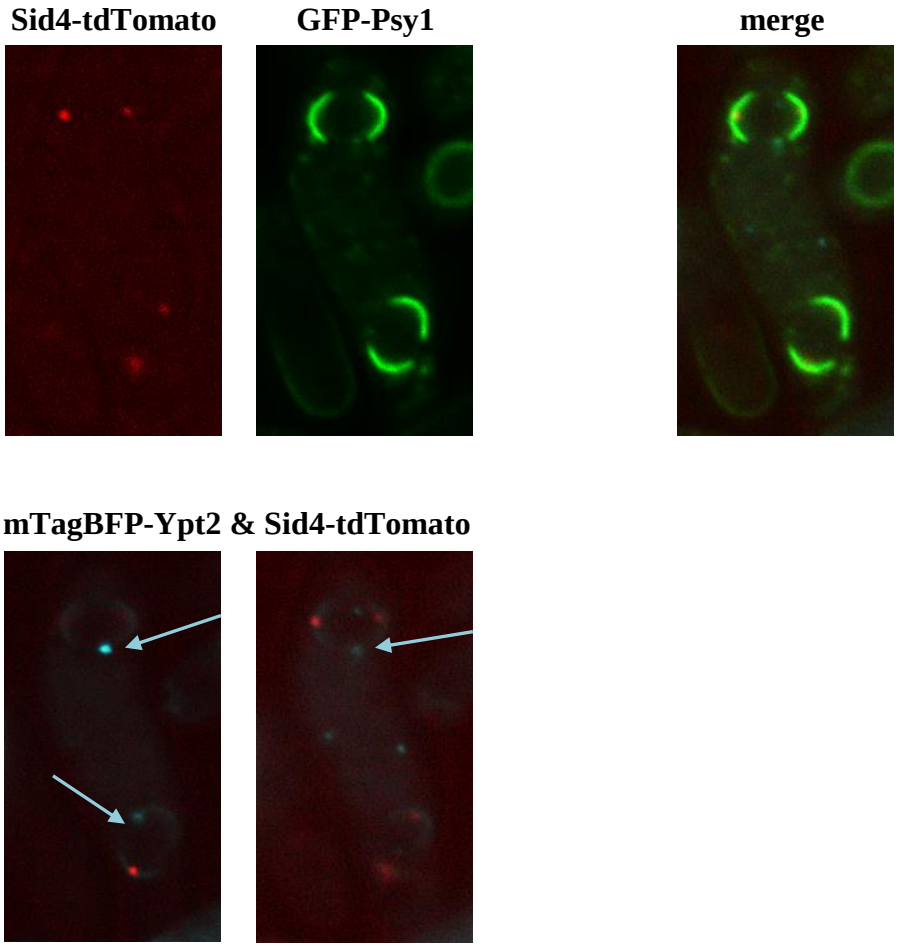


Figure 3.6 Ypt2 is recruited to the forespore membrane during the forespore membrane expansion

Wild type cells expressing *PmTagBFP-ypt2* (blue), *GFP-psy1* (green) and *sid4-tdTomato* (red) were imaged after 24 hr incubation on SPA plates. Arrows on the below panel show mTagBFP-Ypt2 vesicles that localize to the vicinity of the forespore membrane.

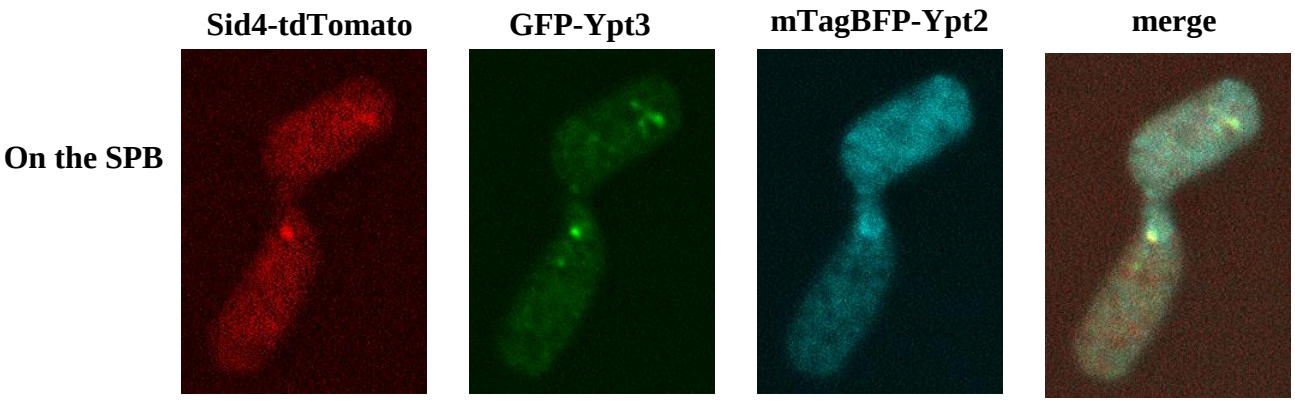


Figure 3.7 GFP-Ypt3 appears on the SPB before mTagBFP-Ypt2 signal is apparent

Wild type cells expressing *PGFP-ypt3* (green), *PmTagBFP-ypt2* (blue), and *sid4-tdTomato* (red) were imaged after 24 hr incubation on SPA plates.

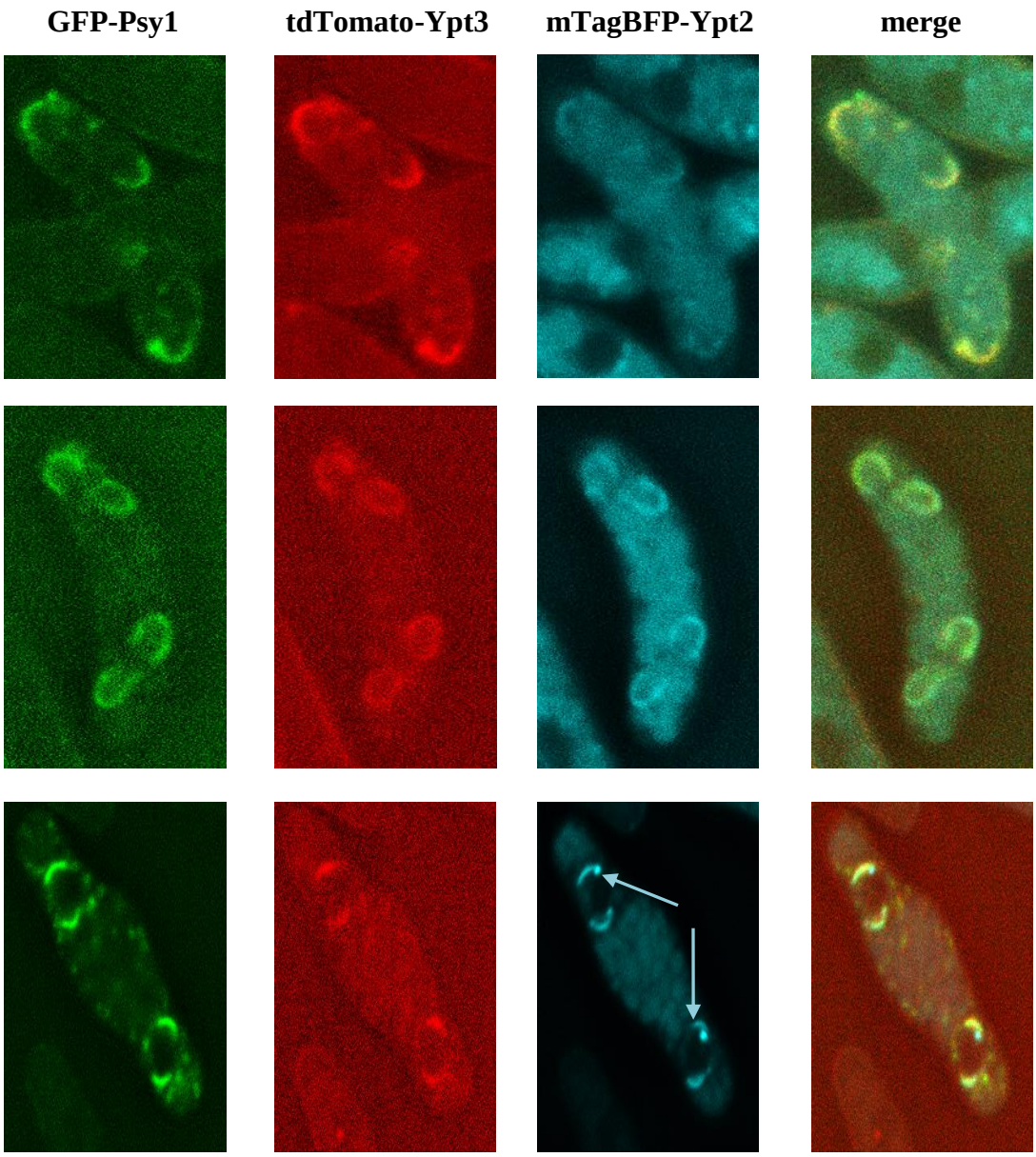


Figure 3.8 tdTomato-Ypt3 and mTagBFP-Ypt2 localize to the vicinity of the expanding forespore membrane

Wild type cells expressing *GFP-psy1* (green), *PtdTomato-ypt3* (red) and *PmTagBFP-ypt2* (blue) were imaged after 24 hr incubation on SPA plates. In three different cells tdTomato-Ypt3 (red) and mTagBFP-Ypt2 (blue) localize close to the forespore membrane region. On the below panel, arrows shows mTagBFP-Ypt2 vesicles that are in the vicinity of the forespore membrane.

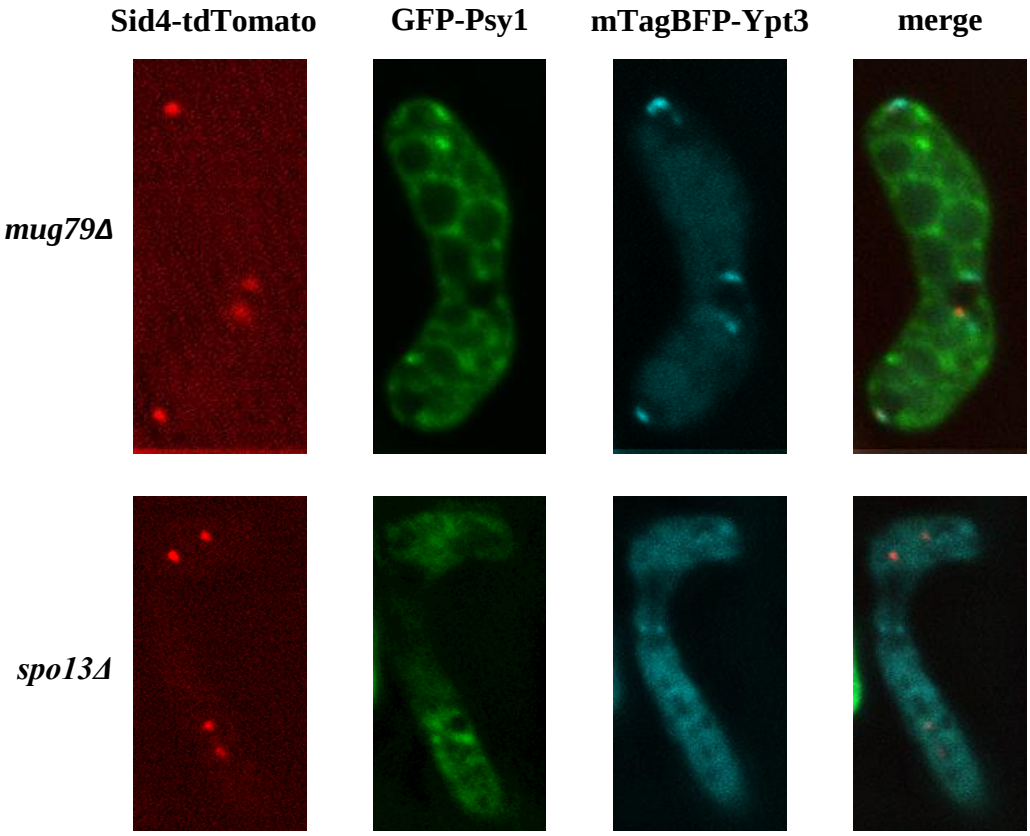


Figure 3.9 mTagBFP-Ypt3 localization in *mug79Δ* and *spo13Δ* mutants

mug79Δ (upper panel) and *spo13Δ* (lower panel) cells expressing *sid4-tdTomato* (red), *GFP-psy1* (green) and *PmTag-BFP-ypt3* (blue) were imaged after 24 hr incubation on SPA plates.

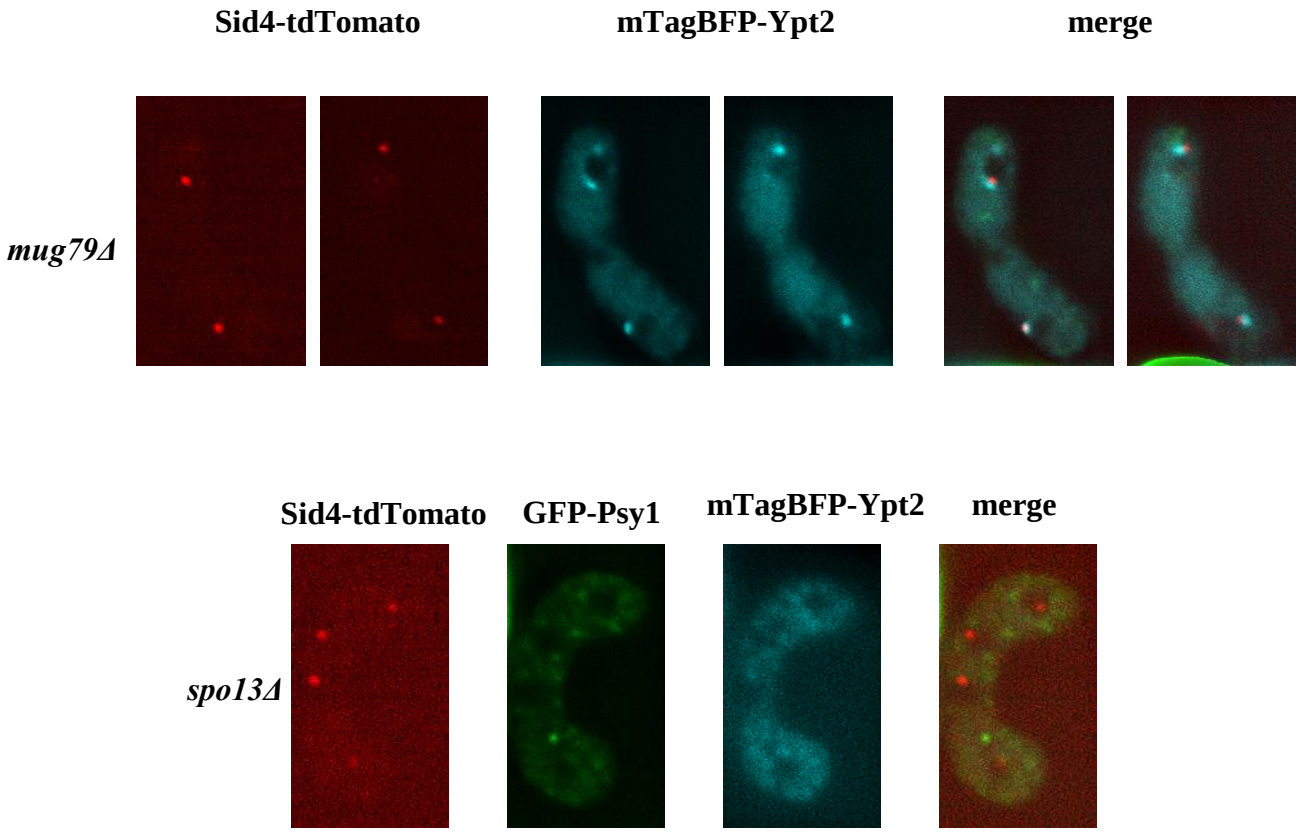


Figure 3.10 mTagBFP-Ypt2 localization in *mug79Δ* and *spo13Δ* mutants

mug79Δ (upper panel) and *spo13Δ* (lower panel) cells expressing *sid4-tdTomato* (red), *GFP-psy1* (green) and *PmTag-BFP-ypt3* (blue) were imaged after 24 hr incubation on SPA plates. GFP-Psy1 data is not shown in *mug79Δ* cells.

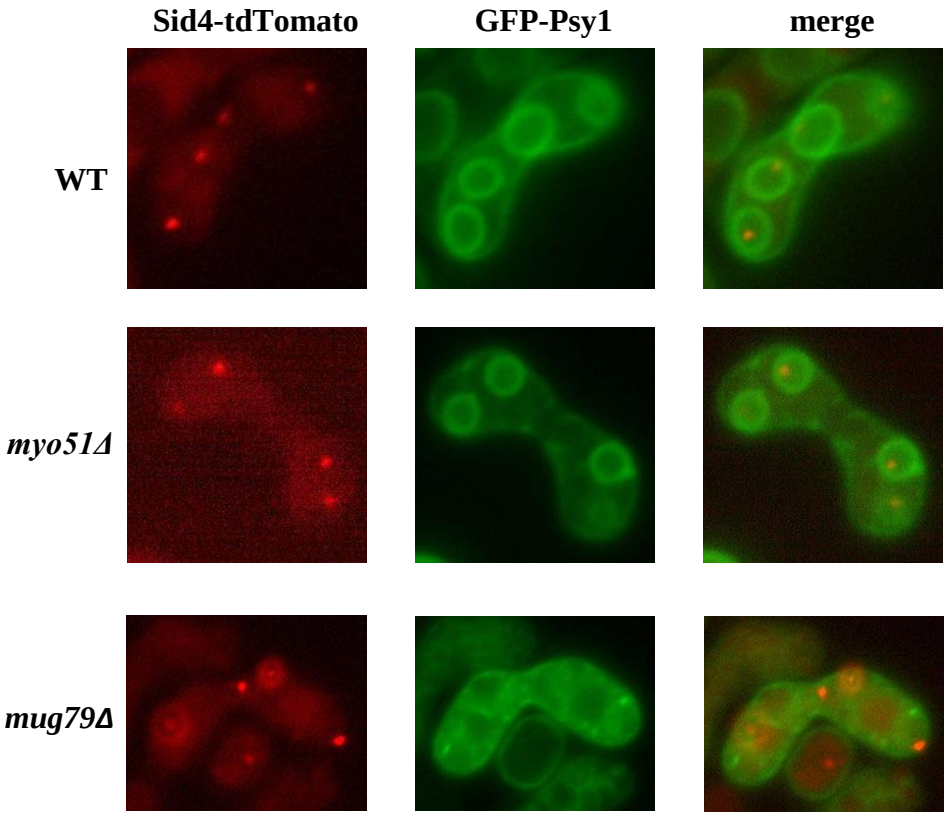


Figure 3.11 Forespore membrane formation in wild-type, *myo51Δ* and *mug79Δ* mutants

Wild type, *myo51Δ* and *mug79Δ* cells expressing *sid4-tdTomato* (red) and *GFP-psy1* (green) were imaged after 24 hr incubation on SPA plates.

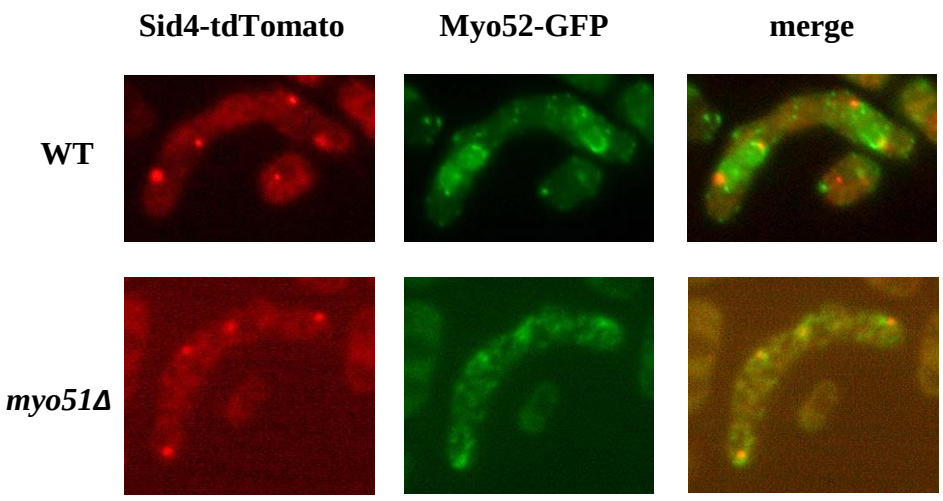


Figure 3.12 Myo52-GFP localization in wild-type and *myo51Δ* mutants

Wild type and *myo51Δ* expressing *sid4-tdTomato* (red) and *myo52-GFP* (green) were imaged after 24 hr incubation on SPA plates.

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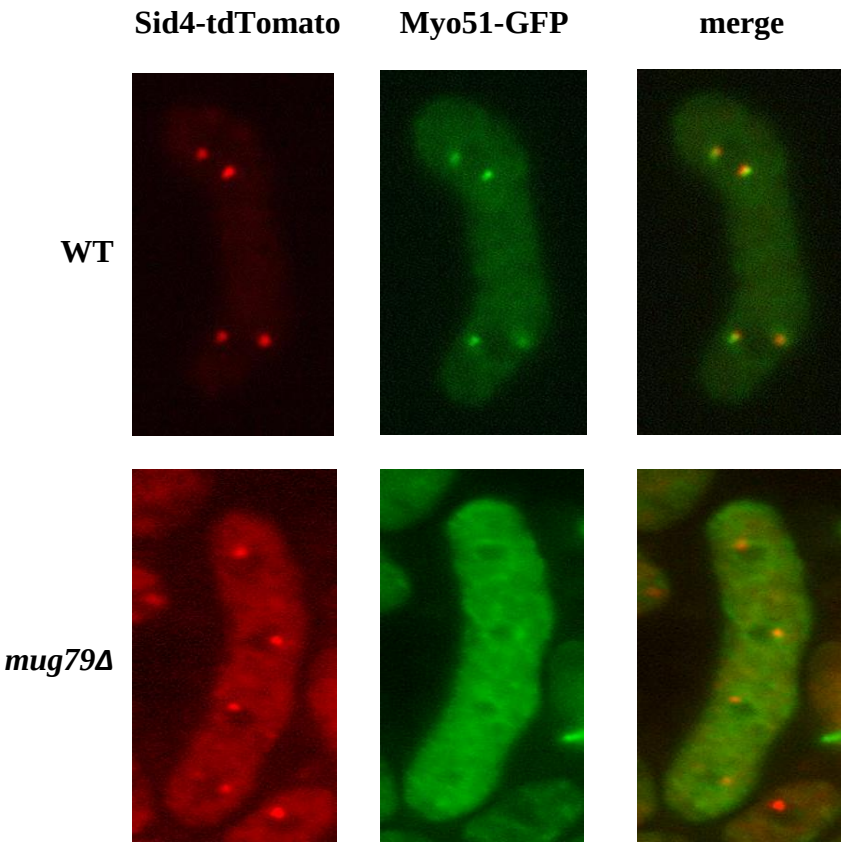

Figure 3.13 Myo51-GFP localization in wild-type and *mug79Δ* mutants

Wild type and *mug79Δ* cells expressing *sid4-tdTomato* (red) and *myo51-GFP* (green) were imaged after 24 hr incubation on SPA plates.

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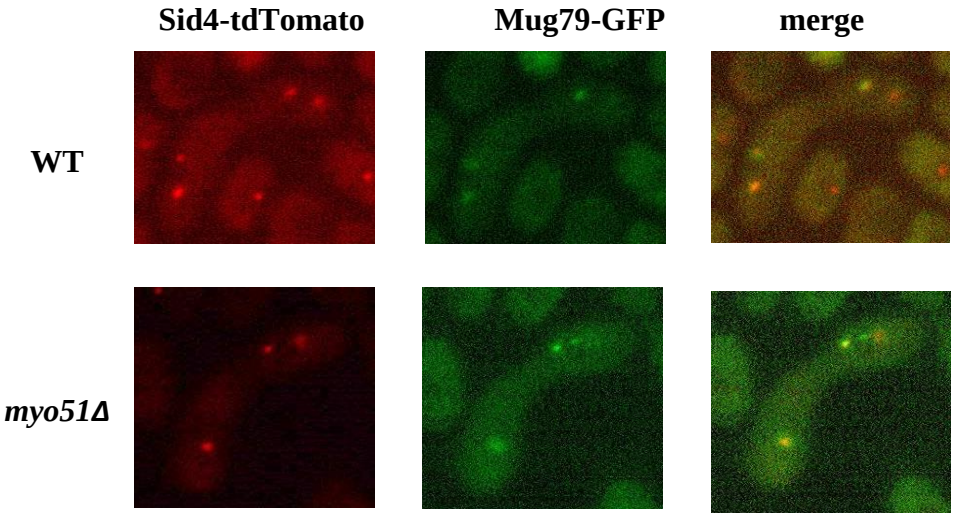

Figure 3.14 Mug79-GFP localization in wild-type and *myo51Δ* mutants

Wild type and *myo51Δ* cells expressing *sid4-tdTomato* (red) and *mug79-GFP* (green) were imaged after 24 hr incubation on SPA plates.

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Chapter 4

Discussions and Perspectives

Yeast sporulation is the gametogenesis phase of the life cycle that consists of meiosis of diploid cells followed by the differentiation of the meiotic products into gametes called spores. Haploid spores are packaged inside the ascus, which is the differentiated form of the mother cell within which sporulation occurs. Membrane vesicles are delivered to the modified MOP to fuse and form the forespore membrane, which is a double layer sheath that initiates at the SPB during Meiosis II and eventually becomes the spore plasma membrane. *De novo* synthesis of the forespore membrane is a model for membrane morphogenesis. Proteins that are recruited to the SPB and to the nascent forespore membrane regulate membrane biogenesis. Even though *S. pombe* sporulation is similar to the other well-studied fungal system *S. cerevisiae,* the proteins involved are not conserved (Shimoda, 2004a). In this work, I tried to identify novel sporulation-defective genes by screening a collection of ~3,300 non-essential *S. pombe* gene deletion mutants. This screen identified 34 gene deletions that reduce the sporulation. Among them, 15 gene deletions cause defects in forespore membrane formation. A part of this work also focuses on novel SPB-associated proteins, the RabGTPase Ypt3, the class V myosin motor protein Myo5, and a coiled-coil protein, Mug79.

Forespore membrane assembly resembles ciliary membrane formation

Cilia are microtubule-based sensory/motile projections found on the surface of many eukaryotic cells. There are two types of cilia, motile and non-motile. The non-motile cilium (the primary cilium) is found nearly on all cell types in humans (Singla and Reiter, 2006; Eggenschwiler and Anderson, 2007). On polarized epithelial cells, the primary cilium is positioned at the apical surface and extends towards the extracellular region where it functions as an antenna for the cell to communicate with its surrounding environment (Reiter and Mostov, 2006; Singla and Reiter, 2006; Goetz and Anderson, 2010). The primary cilium body, the axoneme, is made up of nine microtubule doublets nucleated from a modified centrosome at its base, known as the basal body (Lancaster and Gleeson, 2009; Pearson and Winey, 2009) (Figure 4.1). The axoneme is ensheated by a membrane that is connected to the plasma membrane; however the ciliary membrane contains receptors and proteins that are involved in signaling (Veland *et al.*, 2009). Deregulation or dysfunction of cilia causes a group of human diseases known as ciliopathies, which include Bardet-Biedle syndrome, Joubert syndrome, Meckel-Gruber syndrome, nephronophthisis, polycystic kidney disease, retinitis pigmentosa, and Sensenbrenner syndrome (Goetz and Anderson, 2010; Hildebrandt *et al.*, 2011; Hsiao *et al.*, 2012).

In the core of the centrosome there is a pair of cylindrical microtubule-based, self-organizing organelles called centrioles (Marshall, 2009). Identification of genes that are required for the centriole assembly suggests that centriole-like structures found in divergent eukaryotes are derived from a common ancestor (Azimzadeh and Marshall, 2010). The microtubule and membrane-nucleating activities of the functional core unit of the centrosome are highly conserved (Marshall, 2009). Through its microtubule-nucleation function, centrosomes control cell division, migration, polarity, nuclear positioning, and primary cilia formation in higher organisms (Azimzadeh *et al.*, 2012). The association of centrioles/basal bodies with cytoskeletal elements is a highly conserved mechanism that ensures cell polarization to form the sensory/motile structure of the organism as well as the generation of cell geometry during cell division (Azimzadeh and Marshall, 2010). For example, in *Chlamydomonas*, a set of microtubule bundles anchor to the basal bodies and determine the site of cell division. Also in *Paramecium*,

Trichomonas, *Chitrids*, and choanoflagellates basal bodies associate with the microtubule cytoskeleton directly or through a microtubule-organizing center (Carvalho-Santos *et al.*, 2011).

Similar mechanisms in two microtubule-organizing centers, centrioles and SPBs, suggest that the membrane nucleation capability of the microtubule anchored structures is not a coincidence but a highly conserved mechanism. Centrioles have evolved probably for the formation of cilia (and flagella; similar in organization to the cilia) because proteins involved in cilia formation are recruited to the centrioles, which become the basal bodies (Deane *et al.*, 2001), and gene defects in centriolar proteins cause ciliopathies (Azimzadeh and Marshall, 2010). Centriole duplication occurs at the G1/S transition when the pre-existing mother centriole gives rise to a daughter centriole at right angle next to it. In quiescent cells (G1/G0 stage), the modified mother-centriole migrates to and docks at the cell cortex to form the basal body and serve as the membrane nucleation site (Marshall, 2009; Steere *et al.*, 2012). Electron microscopy analysis has shown that only the mother centriole has a pinwheel-like structure at the distal end, which is called the distal appendage (Anderson, 1972). At the first step of the ciliogenesis, these distal appendages determine the site for centriole-to-membrane docking, therefore they are essential for ciliogenesis (Tanos *et al.*, 2013). Similar to the initiation of the forespore membrane at the outer layer of the SPBs during yeast sporulation, the ciliary membrane formation nucleates at the distal end of the basal body during ciliogenesis (Veland *et al.*, 2009). The interaction between the distal ends of the centrioles and the membrane vesicles is followed by the outgrowth of the ciliary microtubules from the centrioles to form the axoneme (Tanos *et al.*, 2013).

The membrane nucleation function of the microtubule-organizing centers is evolutionarily conserved among species and based on the phylogenetic tree we propose that the outer plaque of the SPB is derived from the centriole. In the Kingdom Fungi, the flagellum is still present in the phyla Chytridiomycota and Blastocladiomycota, where centrioles are still functional (Stajich *et al.*, 2009) (Figure 4.2). On the other hand, in some phyla of the Fungi, e.g. Ascomycota and Mucoromycotina, the microtubule-organizing centers are no longer centrioles, but the morphological and the functional equivalent the SPB (Stajich *et al.*, 2009) (Figure 4.2). In different Fungi, the assembly of the SPB has significantly diverged (Celio *et al.*, 2006). For example in Ascomycota the SPBs can be found as a quadrilateral plaque, an unlayered or two-layered disk with an intact nuclear envelope, or a layered disk with small polar fenestra in the

nuclear envelope (e.g. *S. pombe*) (Celio *et al.*, 2006) (Figure 4.2). Regardless of their shape, all SPBs are attached to the microtubules, in contact with the nuclear envelope and have the electron-dense appearance which may reflect their ability of membrane nucleation site (Celio *et al.*, 2006).

Ciliary and the forespore membrane formation both depend on the trafficking and fusion of Golgi-derived vesicles to the distal end of the basal body or to the outer face of the SPB, respectively (Rohatgi and Snell, 2010). Proper formation and the function of the primary cilium is coupled to the polarized vesicle trafficking because the components of cilia are synthesized in the cytoplasm and transported to the cilium (Eggenschwiler and Anderson, 2007). The transport of the ciliary proteins is under the control of Rab GTPases (Hsiao *et al.*, 2012). Disruption of the localization or the function of RabGTPases at the primary cilia causes ciliary diseases due to the loss of the ciliary function (Yoshimura *et al.*, 2007). The Rab GTPase Rab8 (*S. pombe* Ypt2) has a central role in vesicle targeting as well as the entrance of the proteins to the primary cilium (Hsiao *et al.*, 2009; Hsiao *et al.*, 2012) (Figure 4.1). The GTP-bound form of the upstream Rab GTPase, Rab11-GTP (*S. pombe* Ypt3) accumulates at the basal body of the primary cilium and activates Rabin8 (*S. pombe* Spo13), which is the GEF for Rab 8 (Knodler *et al.*, 2010; Westlake *et al.*, 2011; Hsiao *et al.*, 2012; Chiba *et al.*, 2013). Binding of Rabin8 to GTP-bound Rab11 is required for Rab8 activation and this process is similar to that seen in the *S. cerevisiae* Rab-cascade where GEF Sec2 binds to GTP-bound Ypt31/32, which in turn recruits the Rab GTPase Sec4 (Ortiz *et al.*, 2002; Novick *et al.*, 2006; Chiba *et al.*, 2013).

I have shown that during *S. pombe* forespore membrane formation Rab-GTPases Ypt3 and Ypt2 co-localize on SPB and forespore membrane. Interestingly, in human cells Rabin8 and Rab11-GTP are observed only on the basal body whereas Rab8 first localizes to the basal body and then is distributed along the cilia (Nachury *et al.*, 2007; Yoshimura *et al.*, 2007; Knodler *et al.*, 2010). Rabin8 does not localize to the post-Golgi vesicles but accumulates around the basal body during ciliogenesis (Westlake *et al.*, 2011). This behavior of Rabin8 is very similar to Spo13 which is recruited to become a structural component of the MOP (Yang and Neiman, 2010). It is likely that both Rabin8 and Spo13 can initiate the first vesicle fusion events that are required for *de novo* synthesis of a membrane. In this context, mammalian Rab-GTPases and

GEFs are conserved in similar mechanisms; therefore proteins that are found in fungi serve as a model for higher organisms.

Interactions between Rab GTPases and class V myosin motor protein are essential for secretory vesicle trafficking in the mammalian and the budding yeast systems. The mammalian class V myosin motor, Myo5B directly interacts with Rab11 and Rab8 and this interaction is required in multiple trafficking pathways (Roland *et al.*, 2007; Roland *et al.*, 2009; Roland *et al.*, 2011). Proper localization of Myo5B to the subapical endosomes is provided by Rab11 binding, which subsequently triggers the interaction with Rab8. Since Rab11 and Rab8 interaction is essential for cilia formation, myosins may also be involved in ciliogenesis. Although it is not clear yet, there may be a mammalian homolog of Mug79 and it will be interesting to examine whether it is involved in ciliogenesis. Since defects in cilia-related genes cause diseases, and both systems have parallel mechanisms, identification of mammalian homologs of sporulation-defective genes will provide more insight into the ciliary diseases.

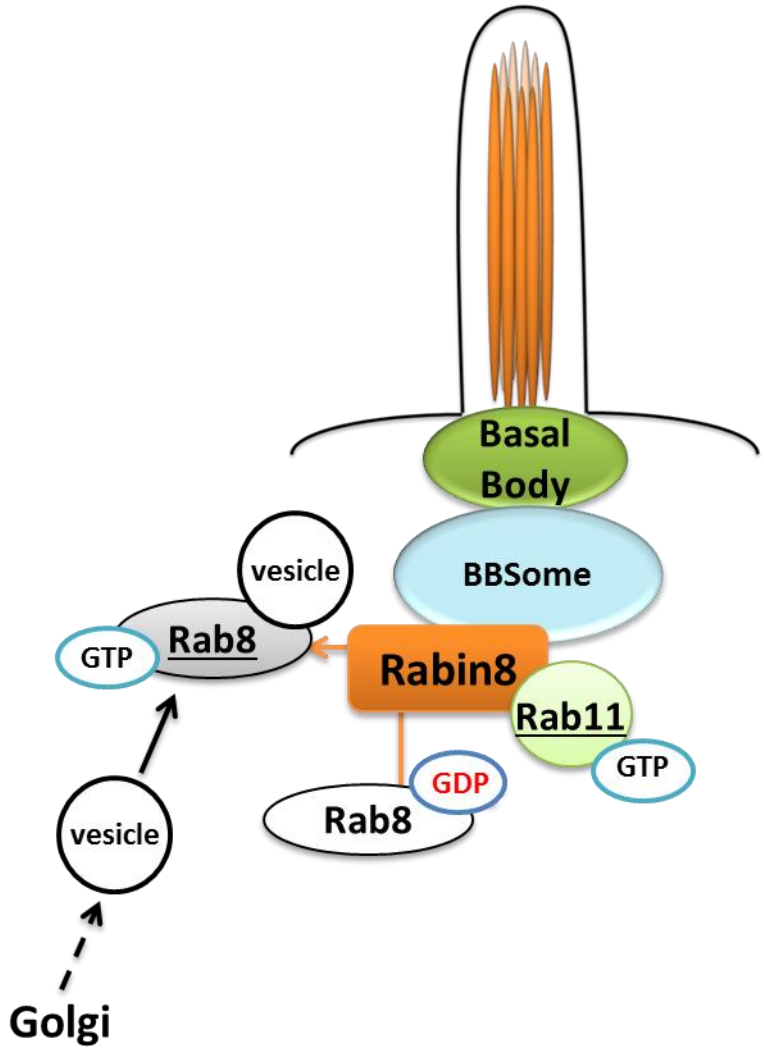

Figure 4.1 Primary cilia initiation is dependent on Rab11-GTP dependent trafficking of Rabin8

Primary cilia formation and function is regulated by polarized vesicle trafficking (Modified from Hsiao *et al*, 2012). GEF Rabin8 interacts with BBSome protein BBS1 (BBSome: a ciliary complex that consists of seven disease-related proteins: BBS1, BBS2, BBS4, BBS5, BBS7, BBS8, BBS9, BBIP10). Rab11-GTP binds to the same region of Rabin8 that binds to BBS1 and facilitates binding of Rabin8 and BBS1. Rabin8 activates Rab8 by loading GTP. Rab8-GTP regulates membrane trafficking to the cilia base.

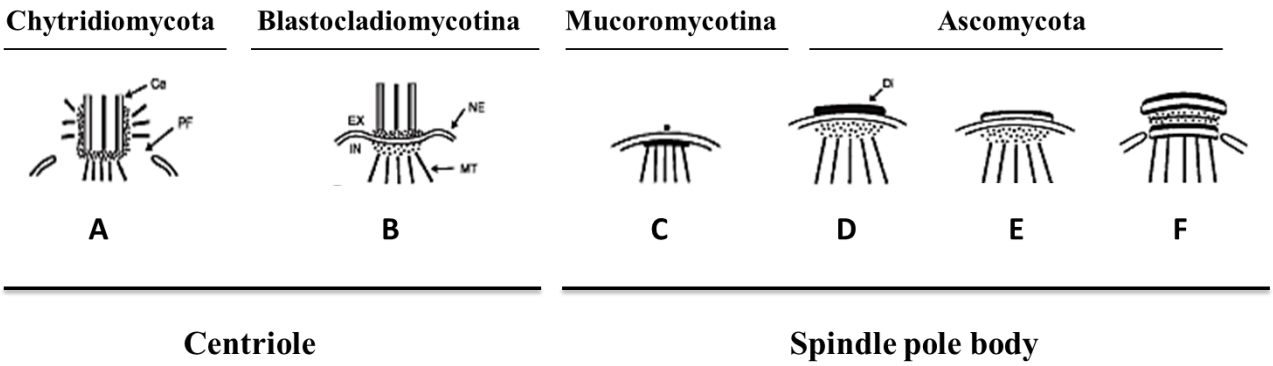

Figure 4.2 Centriole and SPB in different fungi

Centriole is replaced by SPB in some fungi. (A) Nuclear envelope has loose polar fenestra in Chytridiomycota, whereas it is (B) intact in Blastocladiomycotina, and (C) Mucoromyotina, and (D-F) in some ascomycetes (modified from Celio *et al.*, 2006). The layered disk shape with small polar fenestra in nuclear envelope in Ascomycota structure shown in (F) corresponds to the SPB structure observed in *S. pombe.* Ce, centriole; Di, unlayered disk; EX, extranuclear area; IN, intranuclear area; MT, microtubules; NE, nuclear envelope; PF, polar fenestra.

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