Sporulation in the Budding Yeast *Saccharomyces cerevisiae*

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ABSTRACT In response to nitrogen starvation in the presence of a poor carbon source, diploid cells of the yeast *Saccharomyces cerevisiae* undergo meiosis and package the haploid nuclei produced in meiosis into spores. The formation of spores requires an unusual cell division event in which daughter cells are formed within the cytoplasm of the mother cell. This process involves the *de novo* generation of two different cellular structures: novel membrane compartments within the cell cytoplasm that give rise to the spore plasma membrane and an extensive spore wall that protects the spore from environmental insults. This article summarizes what is known about the molecular mechanisms controlling spore assembly with particular attention to how constitutive cellular functions are modified to create novel behaviors during this developmental process. Key regulatory points on the sporulation pathway are also discussed as well as the possible role of sporulation in the natural ecology of *S. cerevisiae*.

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doi: 10.1534/genetics.111.127126
Manuscript received January 22, 2011; accepted for publication April 18, 2011
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SACCHAROMYCES cerevisiae cells that are heterozygous for the mating type locus can respond to changes in the nutrient status of the environment in a variety of ways. Some nutritional limitations can cause cells to enter stationary phase (Hartwell 1974) or to alter their morphology to a filamentous form (Gimeno *et al.* 1992). Alternatively, the absence of a nitrogen source combined with the presence of a nonfermentable carbon source leads cells to enter the developmental pathway of meiosis and sporulation (Freese *et al.* 1982).

The formation of spores involves a form of cell division that is radically different from the budding process in mitotic cells (Byers 1981; Esposito and Klapholz 1981; Kupiec *et al.* 1997; Neiman 2005). Rather than dividing the chromosomes through mitosis and the mother and daughter cells by cytokinesis at the bud neck, in sporulation the chromosomes are segregated by meiosis, resulting in the production of four haploid nuclei. Each of these nuclei is then enveloped within *de novo*-formed plasma membranes within the cytoplasm of the mother cell to form immature spores. After the spores have fully formed, the anucleate but still intact mother cell becomes the ascus encasing the four spores of the tetrad.

These morphogenetic events involve the alteration of the vegetative machinery for a variety of cellular processes including RNA processing, chromosome segregation, the cell cycle, the secretory pathway, and organellar segregation. In some instances sporulation-specific functions replace those in use in vegetative cells, while in other cases sporulation-specific modifications repurpose vegetative functions for this developmental pathway. This review describes the regulatory cascade controlling these alterations in the cell as well as our current understanding of the cytoplasmic events that create the spore.

Overview of Sporulation

Sporulation occurs in three major phases. The early phase begins when cells make the decision to differentiate into spores, on the basis of multiple factors including lack of nitrogen, lack of glucose, and mating type (Mitchell 1994). This leads to exit from the mitotic cycle in G1 and entry into premeiotic S phase. After DNA replication, the events of meiotic prophase including homolog recombination and pairing occur. Completion of the early phase of sporulation requires both changes in the cell cycle machinery and alterations in RNA processing (Shuster and Byers 1989; Clancy *et al.* 2002).

The middle phase includes the major cytological events of sporulation, in which the meiotic divisions give rise to four haploid nuclei that are then packaged into daughter cells (Figure 1A). This packaging requires a host of changes in the cell cytoplasm. Initially, the four spindle pole bodies (SPBs) present in meiosis II are modified so that they become the sites of formation for new membrane compartments, termed prospore membranes (Moens 1971; Byers 1981; Neiman 1998). Prospore membrane formation also requires changes in the late stages of the secretory pathway so that post-Golgi secretory vesicles are redirected from the plasma membrane to the prospore membranes to generate and expand these compartments (Moens 1971; Byers 1981; Neiman 1998). The prospore membranes grow so that each one engulfs the forming haploid nucleus adjacent to it (Figure 1A). Also during this phase, mitochondria and other organelles enter the cytoplasmic space between the nuclear envelope and the prospore membrane (Byers 1981). After karyokinesis gives rise to the daughter nuclei, each prospore membrane completes the engulfment of a nucleus. Fusion of the ends of the

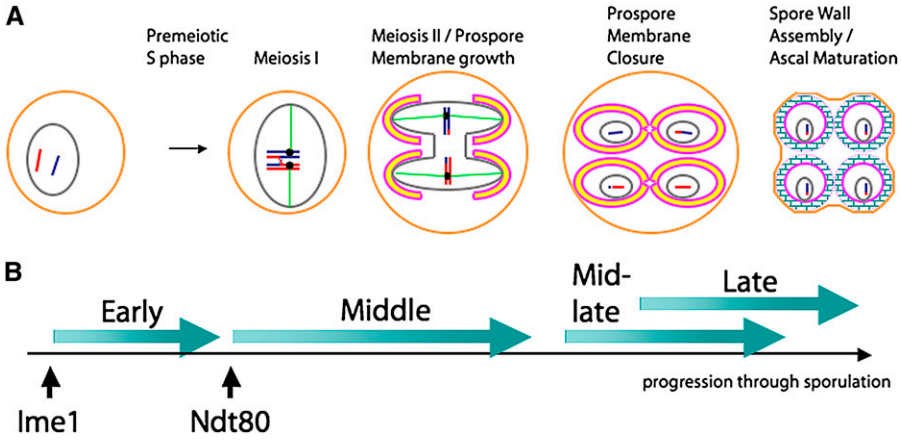

Figure 1 The morphogenetic events of spore formation are driven by an underlying transcriptional cascade. (A) The landmark events of meiosis and sporulation are shown in temporal order. Orange lines indicate the mother cell plasma membrane (which becomes the ascal membrane). Gray lines indicate the nuclear envelope. Blue and red lines represent homologous chromosomes. Green lines represent spindle microtubules. Prospore membranes are indicated by pink lines and the lumen of the prospore membrane is highlighted in yellow. After membrane closure, the prospore membrane is separated into two distinct membranes. The one closest to the nucleus serves as the plasma membrane of the spore, while the outer membrane, indicated by thin, dashed pink line, breaks down during spore wall assembly. Blue hatching represents the spore wall. (B) The shaded arrows indicate the relative timing of the different transcriptional classes with respect to the events in A. The black arrows indicate the points at which the transcription factors Ime1 and Ndt80 become active.

prospore membrane to enclose a nucleus is a cytokinetic event as it separates that nucleus from the cytoplasm of the surrounding mother cell (now referred to as the ascus) (Figure 1A).

The late phase of spore formation occurs after the closure of the prospore membrane. Assembly of a thick coat, or spore wall, around each spore begins only after membrane closure and is critical for the maturation of the spore (Briza *et al.* 1990a; Coluccio *et al.* 2004a) (Figure 1A). In addition, compaction of the chromatin in the spore nucleus as well as regeneration of certain organelles occurs after closure (Roeder and Shaw 1996; Krishnamoorthy *et al.* 2006; Suda *et al.* 2007). All of these events occur within the cytoplasm of the ascus. After spore wall assembly is complete, the original mother cell collapses around the spore to give rise to the tetrahedral mature ascus.

A Regulatory Cascade Controls the Events of Sporulation

The successive phases of sporulation are promoted by an underlying transcriptional regulatory cascade that orchestrates both meiosis and spore formation (Smith and Mitchell 1989; Mitchell 1994; Chu and Herskowitz 1998; Kassir *et al.* 2003) (Figure 1B). The differentiation process is triggered by the expression of the Ime1 transcription factor. Ime1 acts as a master regulator of the sporulation process; ectopic expression of Ime1 is sufficient to induce sporulation of vegetative diploid cells (Kassir *et al.* 1988; Smith *et al.* 1990). Thus, the decision to express *IME1* defines a choice of cell fate. Expression of *IME1* is regulated at transcriptional, post-transcriptional, and post-translational levels by a variety of different factors including mating type, nitrogen source, carbon source, storage carbohydrate, and extracellular pH (Kassir *et al.* 1988; Smith *et al.* 1990; Su and Mitchell 1993; De Silva-Udawatta and Cannon 2001).

Activation of Ime1 leads to the induction of the first transcriptional wave, or "early" genes (Mitchell 1994). These early genes have a common regulatory element, the URS1 site, in their promoters (Buckingham *et al.* 1990; Vershon *et al.* 1992; Bowdish and Mitchell 1993). This element is bound by the Ume6 protein, which acts to repress transcription of these genes during vegetative growth (Park *et al.* 1992; Strich *et al.* 1994; Steber and Esposito 1995). Binding of Ime1 to Ume6 is thought to disrupt the interaction of Ume6 with a repressive histone deacetylase complex and allow for transcriptional activation of the early genes (Washburn and Esposito 2001). The mechanism by which Ime1 interaction causes activation is unsettled as both activation by the Ime1/Ume6 complex and Ime1-dependent proteolysis of Ume6 have been proposed (Washburn and Esposito 2001; Mallory *et al.* 2007).

The early gene set includes genes required for entry into premeiotic S phase, for the chromosome recombination and pairing events of meiotic prophase (Primig *et al.* 2000), and for the subsequent induction of the middle genes. In addition to promoting Clb–Cdc28 activation (Dirick *et al.* 1998), the Ime2 kinase collaborates with Cdc28 in the control of different cell cycle changes that prime the cell for entry into the meiotic divisions (Guttmann-Raviv *et al.* 2001). One critical example of their collaboration is the expression of *NDT80*, which encodes the transcription factor that regulates the middle wave of gene expression and, therefore, entry into the middle phase of spore formation (Shin *et al.* 2010).

Expression of *NDT80* initiates entry of the cells into the meiotic divisions and, therefore, as with *IME1*, *NDT80* expression is tightly controlled at the transcriptional level (Pak and Segall 2002a). The *NDT80* promoter contains a URS1 element bound by Ime1/Ume6, as do early genes. In addition, the promoter contains a "middle sporulation element" or MSE, which is the binding site for Ndt80, indicating that Ndt80 promotes its own expression in a positive feedback loop (Pak and Segall 2002a). MSE elements are found upstream of most Ndt80-regulated genes (Hepworth *et al.* 1995; Ozsarac *et al.* 1997; Chu *et al.* 1998). However, despite the

presence of Ime1/Ume6 at the *NDT80* promoter, *NDT80* is not expressed with other early genes. This is due to the presence of a repressor protein, Sum1, which has overlapping binding specificity to Ndt80 and also recognizes the MSE element (Xie *et al.* 1999). Sum1 bound at the *NDT80* promoter recruits the histone deacetylase Hst1, which leads to repression of *NDT80* expression, even when Ume6 is converted to an activator by binding of Ime1 (Xie *et al.* 1999). The Sum1–Hst1 complex is also bound to MSE elements at many other Ndt80-regulated genes where it is responsible for their repression in vegetative cells (McCord *et al.* 2003).

Induction of *NDT80* expression requires both activation of Ume6 by Ime1 binding and a weakening of Sum1 repression (Pak and Segall 2002a). This weakening is achieved by phosphorylation of Sum1 by multiple kinases including Ime2 (Shin *et al.* 2010). These phosphorylations disrupt the interaction between Sum1 and Hst1, allowing Ume6/Ime1-mediated transcriptional activation. The requirement for Ime2, an Ime1/Ume6-induced gene, means that the induction of *NDT80* occurs after the broader induction of early genes and it is therefore referred to as a pre-middle gene (Hepworth *et al.* 1998). A few other genes with similar early-middle timing have been reported and, in at least one instance, the *SMK1* gene, it appears that a combination of URS1 and MSE elements may regulate expression (Hepworth *et al.* 1998; Pierce *et al.* 1998).

Weakening of Sum1 repression leads to an initial low-level expression of *NDT80*. Full expression requires positive feedback in which the newly synthesized Ndt80 protein displaces Sum1 from the *NDT80* promoter and leads to even higher levels of *NDT80* expression (Pak and Segall 2002a; Pierce *et al.* 2003). Similarly at other Ndt80-regulated genes, a combination of competition from Ndt80 and phosphorylation by Ime2 is thought to displace Sum1 from the MSE elements and activate transcription (Pierce *et al.* 2003; Ahmed *et al.* 2009). Whether or not this displacement occurs at all Ndt80-regulated genes is not yet clear. Sum1 and Ndt80 bind to overlapping but not identical DNA sequences and so the relative affinity of each protein for specific MSE elements will be different depending on the precise sequence of the elements (Wang *et al.* 2005). Studies using chromatin immunoprecipitation of Sum1 or Ndt80 from sporulating cells followed by microarray hybridization suggest a complicated pattern (Klutstein *et al.* 2010). Sum1 or Ndt80 were found alone on some promoters, while on others Sum1 was present even in the presence of Ndt80. The picture is further clouded by the observation that restoration of Sum1 binding may be important for turning off middle gene expression during the later stages of sporulation (Klutstein *et al.* 2010). The apparent cooccupancy of Sum1 and Ndt80 may, therefore, result from asynchrony of individual cells in the population. Thus, the underlying basis for the wave of middle gene expression may be the displacement of a repressor protein (Sum1) by an activator (Ndt80) followed by the subsequent displacement of the activator by the repressor (Klutstein *et al.* 2010).

Induction of the middle genes defines the onset of the meiotic divisions. In the next wave of gene expression, the mid-late genes are induced at the end of meiosis, probably only after closure of the prospore membrane (Briza *et al.* 1990a; Primig *et al.* 2000). As compared to middle genes (~300) there are relatively few mid-late genes (Primig *et al.* 2000). The best studied of these, *DIT1* and *DIT2*, are involved in spore wall assembly. The control of the timing of mid-late gene expression is not as well understood as for early or middle genes. In the *DIT1* promoter, *cis*-acting DNA sites required for proper expression have been defined, though their *trans*-acting binding factors have not been fully identified (Friesen *et al.* 1997). Nrg1 and Rim101 bind to one of these sites and act together to repress *DIT1* in vegetative cells, but it is unclear whether either is required for transcriptional induction during sporulation (Rothfels *et al.* 2005). The identified regulatory site includes an MSE, suggesting that Ndt80 and/or Sum1 might play a role in controlling *DIT1* expression (Friesen *et al.* 1997). Indeed, overexpression of *NDT80* in vegetative cells leads to *DIT1* expression (Chu *et al.* 1998) and chromatin immunoprecipitation from sporulating cells indicates that Sum1 is present at the *DIT1* promoter (Klutstein *et al.* 2010); however, a direct role for either gene in control of mid-late gene expression has not been established. Finally, the Gis1 transcription factor is required for induction of *DIT1* and at least one other mid-late gene (Coluccio *et al.* 2004a; Yu *et al.* 2010). Though direct binding of Gis1 to the *DIT1* promoter has not been demonstrated, the promoter does contain multiple matches to the consensus Gis1 binding site (Yu *et al.* 2010).

Subsequent to the induction of the mid-late genes, the late genes are induced (Law and Segall 1988; Primig *et al.* 2000). How, or whether, these genes act in spore assembly or maturation is not clear, as no common theme emerges from their known functions. One of the canonical late genes, *SPS101/CTT1*, is also induced by various stresses during vegetative growth (Law and Segall 1988; Marchler *et al.* 1993). Several other late genes are induced by stress in vegetative cells (Primig *et al.* 2000), suggesting that some late gene expression might be a stress response. No transcription factors directly responsible for late gene expression have been identified, though Gis1 is required for induction of the late gene *SPS100*, and the promoter of this gene harbors a consensus Gis1 binding site (Law and Segall 1988; Yu *et al.* 2010).

Key Events in the Phases of Sporulation

The early phase: alterations in the cell cycle and RNA processing machinery

In the early phase of sporulation, cells replicate their DNA in premeiotic S phase and then enter meiotic prophase. These nuclear events require modifications to the cell cycle machinery that alter the genetic requirements for passage into and through S phase from those in mitotic cells (Schild

and Byers 1978; Shuster and Byers 1989; Hollingsworth and Sclafani 1993; Dirick *et al.* 1998; Benjamin *et al.* 2003). For example, the early gene *IME2* encodes a protein kinase that inactivates the cyclin-dependent kinase inhibitor Sic1 (Dirick *et al.* 1998; Sedgwick *et al.* 2006). This inactivation bypasses the usual mitotic control of Clb5,6–Cdc28 and allows cells to enter premeiotic S phase without passing through the canonical START control point of the G1/S transition (Dirick *et al.* 1998). These changes in cell cycle control, as well as the chromosomal biology leading to and during meiosis, will be discussed in detail in a subsequent review in this series.

The early phase also includes alterations in the modification and processing of mRNAs that are important for proper expression of the early gene set. Ime4, which was originally identified as required for efficient expression of *IME1* (Shah and Clancy 1992), is homologous to mRNA N6-adenosine methyltransferase in higher cells. During sporulation, Ime4 mediates N6-adenosine methylation of bulk mRNA, including the *IME1* and *IME2* transcripts (Clancy *et al.* 2002; Bodi *et al.* 2010). These observations imply that methylation of *IME1* (and *IME2*) transcripts may control their expression, though the responsible mechanism is not yet clear.

Meiosis-specific splicing of certain messages also contributes to the control of gene expression during sporulation. Roughly 20 sporulation-induced transcripts contain introns (Juneau *et al.* 2007; Munding *et al.* 2010). Strikingly, most of these transcripts are spliced efficiently only in sporulating cells (Juneau *et al.* 2007). The best-studied case is the *MER1*-regulon, where splicing is controlled by the general splicing factor Nam8 in conjunction with the sporulation-specific Mer1 protein (Engebrecht *et al.* 1991; Spingola and Ares 2000). *MER1* is an early gene that encodes a splicing enhancer protein (Engebrecht and Roeder 1990; Engebrecht *et al.* 1991). The Mer1 protein binds directly to an element found in the regulated introns of target genes and in the absence of *MER1* these genes are not spliced (Nandabalan *et al.* 1993; Spingola and Ares 2000). Four direct targets of Mer1 have been identified: *MER2*, *MER3*, *SPO22*, and *AMA1* (Engebrecht *et al.* 1991; Nakagawa and Ogawa 1999; Cooper *et al.* 2000; Davis *et al.* 2000; Spingola and Ares 2000). *SPO22* and *MER3* are both early genes induced by Ume6/Ime1, while *MER2* is constitutively transcribed, but unspliced, in vegetative cells (Engebrecht *et al.* 1991; Munding *et al.* 2010). As *MER3* and *SPO22* are cotranscriptionally regulated with their splicing enhancer, full expression of these proteins must be delayed until the Mer1 protein has had time to accumulate (Munding *et al.* 2010). The *MER2*, *MER3*, and *SPO22* genes are all involved in the pairing and recombination of homologous chromosomes required for meiotic prophase (Engebrecht *et al.* 1990; Nakagawa and Ogawa 1999; Tsubouchi *et al.* 2006). The absence of any of these gene products leads to recombination defects that trigger a checkpoint that interferes with the activity of the Ndt80 transcription factor and, therefore, the induction of middle genes (see below). Thus, the delay in expression imposed by *MER1*-dependent splicing has been proposed to play a role in controlling the timing of middle gene induction with respect to early genes (Munding *et al.* 2010).

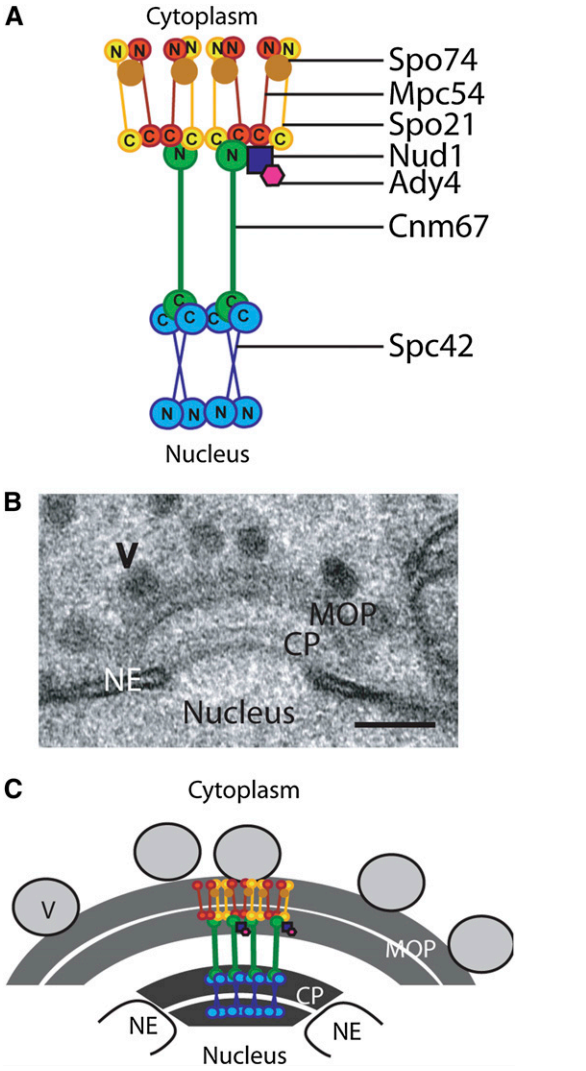

Figure 2 Organization of meiosis II outer plaque. (A) Diagram of the arrangement of meiosis II outer plaque subunits within the complex. The coiled-coil proteins Mpc54, Spo21, Cnm67, and Spc42 are depicted as dumbbells with their N- and C termini indicated. The likely positions of Spo74, Nud1, and Ady4 are also shown. (B) Electron micrograph of a meiosis II SPB prior to prospore membrane formation. V, prospore membrane precursor vesicle; CP, central plaque; MOP, meiosis II outer plaque; NE, nuclear envelope. Bar, 100 nm. (C) Cartoon of image in B overlaid with the schematic from A to show the positions of proteins within the structure. This figure is adapted from Mathieson *et al.* (2010b).

The middle phase: building a membrane and forming a cell

Modification of the spindle pole body: The SPB is the sole microtubule-organizing center in *S. cerevisiae* cells. It is arranged as a cylinder composed of several stacked "plaques" that appear as alternating light and dark layers in the electron microscope (Byers 1981; Muller *et al.* 2005). The SPB

is embedded in the nuclear envelope, similar to a nuclear pore, so that the cylinder has distinct cytoplasmic and nucleoplasmic faces. During mitosis, the nuclear face is the site of nucleation for the spindle microtubules and the cytoplasmic face is the source of astral microtubules (Palmer *et al.* 1992).

In meiosis, the SPBs duplicate twice: first at the beginning of meiosis I, and then again at the transition to meiosis II to generate the four SPBs necessary for the second division. In meiosis I, the two SPBs appear similar to those in mitotic cells. However, during meiosis II, the cytoplasmic faces of the four SPBs change their composition and switch their function from microtubule nucleation to membrane nucleation (Moens and Rapport 1971).

Microtubule nucleation by the cytoplasmic face of the SPB requires Spc72, which acts as a receptor for the γ-tubulin complex (Chen *et al.* 1998; Knop and Schiebel 1998; Soues and Adams 1998). At meiosis II, Spc72 disappears (presumably by proteolysis) and several sporulation-specific proteins are recruited to form a greatly expanded cytoplasmic face termed the meiosis II outer plaque (MOP) (Moens and Rapport 1971; Knop and Strasser 2000) (Figure 2). The major MOP proteins are Spo21/Mpc70, Mpc54, and Spo74 (Knop and Strasser 2000; Bajgier *et al.* 2001; Nickas *et al.* 2003). The constitutive SPB proteins Cnm67 and Nud1 are also present in the MOP, as is Ady4, a minor component important for MOP complex stability (Knop and Strasser 2000; Nickas *et al.* 2003; Mathieson *et al.* 2010a).

The cylinder of the SPB is created by vertically arranged layers of coiled-coil proteins, with the globular heads and tails of the proteins and the central coiled-coil regions likely giving rise to the alternating electron-dense and electron-lucent layers seen in the TEM, respectively (Schaerer *et al.* 2001). Similarly, the MOP proteins Spo21 and Mpc54 are also predicted coiled-coil proteins and fluorescence resonance energy transfer studies suggest that they are arranged with their N termini out toward the cytoplasm and their C termini inward (Mathieson *et al.* 2010b) (Figure 2A). The C termini are located near the N terminus of Cnm67, which links the MOP to the central domain of the SPB (Schaerer *et al.* 2001) (Figure 2). The positions of Nud1 and Spo74 within the complex have not been clearly defined, but on the basis of protein interactions, Nud1 is likely found near the Cnm67/Spo21/Mpc54 interface, while Spo74 is an integral component of the MOP (Nickas *et al.* 2003).

MOP-mediated membrane assembly is essential for spore formation. In mutants lacking Mpc54, Spo21, or Spo74, an organized MOP does not assemble on the SPB and hence no prospore membranes are formed (Knop and Strasser 2000; Bajgier *et al.* 2001; Nickas *et al.* 2003). That the MOP specifies where prospore membranes form is shown by experiments in *cnm67*Δ mutant cells (Bajgier *et al.* 2001), which lose the link between the MOP and the SPB. As a result, MOP complexes assemble at ectopic sites in the cytoplasm and generate prospore membranes that fail to capture daughter nuclei.

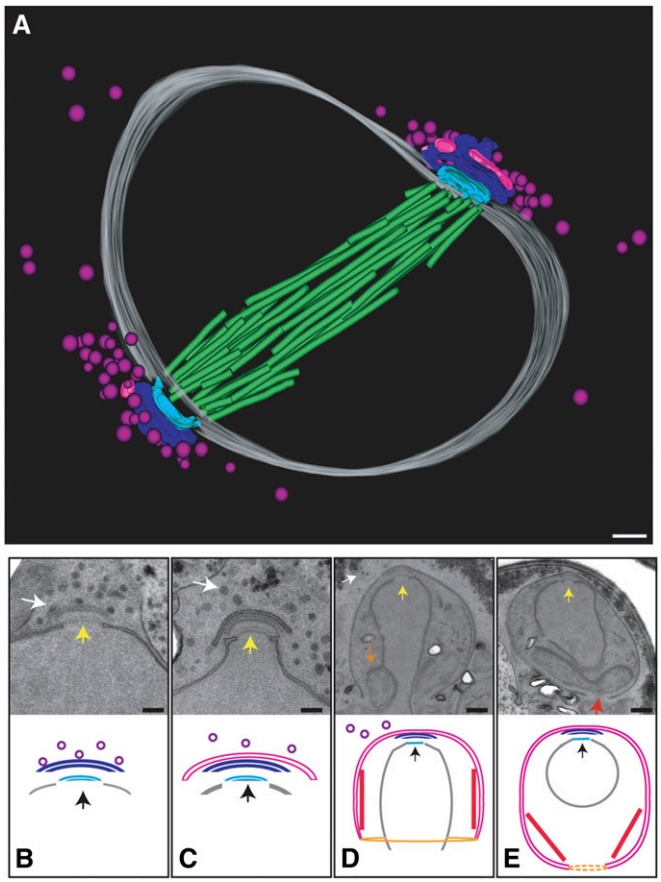

Figure 3 Stages of prospore membrane growth. (A) Model of a meiosis II spindle at the time prospore membrane formation initiates on the basis of a 3D EM tomographic reconstruction. Green cylinders indicate the position of spindle microtubules and the gray lines the location of the nuclear envelope. Dark blue structures are the MOP, while light blue indicates the central plaque of the SPB. Purple spheres are vesicles while bright pink shows prospore membranes beginning to form on the MOP surface. Bar, 100 nm. (B–E) (Upper) Electron micrographs of prospore membranes at different stages of growth. (Lower) Cartoons corresponding to the EM images. (B) Docking of vesicles to the MOP prior to fusion. Yellow arrows are within the nucleus and point to the position of the SPB. White arrow indicates precursor vesicles. Bar, 100 nm. (C) Initial fusion of vesicles creates a prospore membrane "cap" on the MOP. Labels are as in B. (D) Expansion of the prospore membrane, the lobe of the nucleus. White and yellow arrows are as in B. Orange arrow indicates an extension of nuclear envelope wrapping around a mitochondrion. Bar, 200 nm. (E) Just prior to closure, the prospore membrane has engulfed a divided nucleus. Yellow arrow is as in B. Red arrow indicates the site where the prospore membrane is closing. Bar, 400 nm. In the cartoons, structures are colored as in A. In addition, the red bars and orange rings in D and E indicate the positions of the septins and the leading edge complex, respectively, though these structures are not visible in the EM images. Stippling of the orange ring in E indicates that the leading edge complex is removed from the membrane prior to closure (see text).

The MOP structure acts as a vesicle docking complex (Riedel *et al.* 2005; Nakanishi *et al.* 2006). Secretory vesicles come in to the spindle pole region and dock onto the MOP surface (Figure 3, A and B). After docking, the vesicles fuse to form a small membrane cap (Moens and Rapport 1971) (Figure 3C). Fusion of additional vesicles then expands the

prospore membrane beyond the MOP (Figure 3D). Mutations in conserved residues in the N-terminal (membrane-proximal) domain of Mpc54 cause a defect in which vesicles associate with the MOP but do not dock stably onto its surface (Mathieson *et al.* 2010b). These undocked, MOP-associated vesicles do not fuse with each other. Thus, docking of the vesicles to the MOP is an essential prerequisite to their fusion. Furthermore, the Rab family GTPase Sec4, as well as several components of the exocyst complex, are present on vesicles when they are docked to the MOP but absent from the MOP-associated vesicles in the *mpc54* point mutants (Mathieson *et al.* 2010b). These observations suggest that the MOP controls the formation of prospore membranes in two ways. First, it provides positional information to ensure that membranes are initiated and held at the correct place. Second, by recruiting key regulators of the membrane fusion process, the MOP promotes the fusion of vesicles at that location.

Prospore membrane initiation: Docking of vesicles onto the MOP is a necessary prerequisite for prospore membrane formation, but the fusion of vesicles also requires additional factors such as a SNARE complex that acts specifically at the prospore membrane (Neiman 1998; Jantti *et al.* 2002; Yang *et al.* 2008). SNAREs act as fusogens in intracellular membrane transport events and different combinations of SNAREs mediate fusion at different organelles (Pelham 1999, 2001). The prospore membrane SNARE illustrates how the sporulation program, by modestly modifying a constitutive function, can dramatically alter cellular behavior.

In vegetative cells, the fusion of post-Golgi secretory vesicles with the plasma membrane requires a SNARE complex formed by three components: (i) Sso1 or Sso2 (a redundant pair), (ii) Sec9, and (iii) Snc1 or Snc2 (another redundant pair) (Gerst *et al.* 1992; Aalto *et al.* 1993; Brennwald *et al.* 1994). Sso1/2 and Sec9 form a binary complex on the plasma membrane that interacts with Snc1/2 arriving with the vesicle to create the active fusogen (Rossi *et al.* 1997; McNew *et al.* 2000). At the prospore membrane, vesicle fusion requires Sso1, Snc1/2, and a sporulation-specific Sec9 paralog called Spo20, whereas Sec9 and Sso2 are not required (Neiman 1998; Jantti *et al.* 2002; Yang *et al.* 2008). It is the presence of Sec9 *vs.* Spo20 that determines at which membranes the complex will function (Neiman *et al.* 2000); even when their expression patterns are reversed, neither protein can substitute for the other (Neiman 1998).

Many proteins that function upstream of SNAREs for plasma membrane fusion in vegetative growth also play that role in sporulating cells. For example, the SM family protein, Sec1, the exocyst tethering complex, and the Rab GTPase Sec4 are all required for fusion at the prospore membrane (Neiman 1998). Thus, the alteration of the SNARE machinery by introduction of Spo20 seems to be the major basis for diverting secretory vesicles to fuse at the prospore membrane instead of the plasma membrane. In addition, two other changes from the vegetative pathway are known. Mso1, a Sec1 binding protein, plays a minor role in vegetative secretion but is strongly defective in prospore membrane assembly (Knop *et al.* 2005) and, Spo14, a constitutively expressed phospholipase D, is dispensable for secretory pathway function during vegetative growth but is absolutely required for prospore membrane assembly (Rose *et al.* 1995; Rudge *et al.* 1998; Nakanishi *et al.* 2006).

The requirements for Spo14 and Spo20 are likely interrelated. Spo14 localizes to the prospore membrane and its precursor vesicles (Rudge *et al.* 1998), and its role in sporulation requires its ability to hydrolyze phosphatidylcholine to phosphatidic acid (Rudge *et al.* 1998). In turn, the N terminus of Spo20 contains a membrane-binding motif that has *in vivo* selectivity for phosphatidic acid (Nakanishi *et al.* 2004) and is required for its localization to the prospore membrane. In contrast, Sec9 lacks this motif and does not efficiently localize to the prospore membrane (Neiman *et al.* 2000), but targeting Sec9 to the prospore membrane allows it to rescue a *spo20*Δ mutant (Nakanishi *et al.* 2006). Phosphatidic acid may also have a separate effect on fusion by Spo20 SNARE complexes. Evidence both *in vivo* and *in vitro* suggests that SNARE complexes containing Spo20 are less efficient fusogens than those containing Sec9, and that Spo14 activity or phosphatidic acid can specifically enhance the activity of the Spo20 complexes (Coluccio *et al.* 2004b; Liu *et al.* 2007). Thus, Spo14 activity plays at least two roles in promoting fusion at the SPB: (1) recruitment of the Spo20 SNARE and (2) enhancement of the fusogenic properties of the formed SNARE complexes. Spo14 must have at least one additional role, as targeting Sec9 (which does not require phosphatidic acid for fusogenicity) to the prospore membrane independently of phosphatidic acid does not rescue the fusion defect of a *spo14*Δ mutant (Nakanishi *et al.* 2006).

Finally, whereas Sso1 and Sso2 function interchangeably in vegetative cells, Sso1 is specifically required during sporulation. The basis for this preference is incompletely understood, but current evidence suggests that modest differences in expression level and the ability to bind to cofactor lipids combine to separate the functionality of Sso1 and Sso2 at the prospore membrane (Oyen *et al.* 2004; Mendonsa and Engebrecht 2009).

Membrane expansion: Once formed on the MOP, the prospore membrane rapidly expands beyond the spindle pole (Figure 3, C–E). In this expansion phase, the membrane remains attached to the MOP, which serves to anchor it adjacent to the nuclear envelope. However, the continuing delivery and fusion of vesicles with the membrane must be independent of the MOP because fusion appears to occur at sites distant from the MOP structure (*e.g.*, see Figure 3, C and D). As the membrane grows, it does so with a characteristic curvature and in the appropriate direction so that it can engulf the forming daughter nucleus (Figure 3, D and E). Videomicroscopy studies demonstrate that the membranes initially appear as small horseshoes that become small

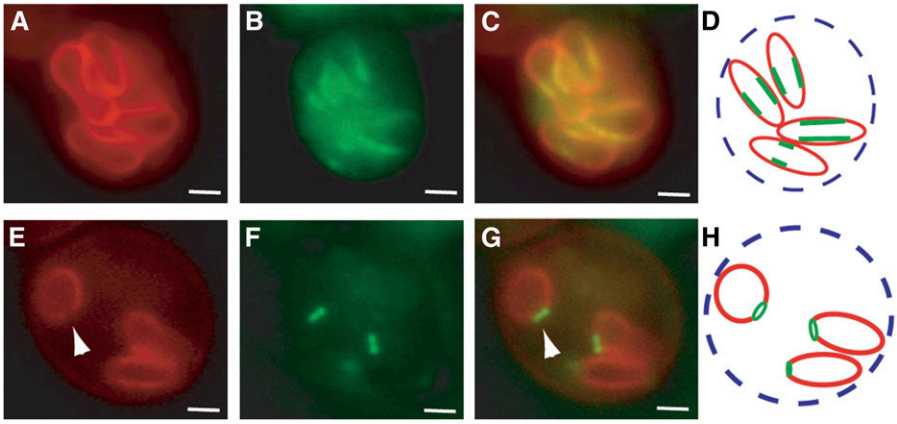

Figure 4 Prospore membrane associated cytoskeletal elements. (A) Prospore membranes are indicated by Spo20$^{51-91}$–RFP. (B) Septins are shown by Spr28–GFP. (C) Merge of the images in A and B. (D) Representation of the fluorescence image in C. Dashed line indicates the outline of the cell, red lines the prospore membranes, and green the position of the septins. (E) Prospore membranes are indicated by Spo20$^{51-91}$–RFP. (F) Leading edge complex is visualized by Don1–GFP. (G) Merge of images in D and E. (H) Representation of the fluorescence image in G. Dashed line indicates the outline of the cell, red lines the prospore membrane, and green the position of the leading edge complex. The arrowheads in E and G indicate the mouth of one prospore membrane. Bars, 1 μm.

circles before abruptly expanding into long cylindrical tubes (Diamond *et al.* 2008). This transition may correspond to the lengthening of the meiosis II spindle during anaphase. These tubes then round into ovals before returning to a spherical shape coincident with membrane closure (Diamond *et al.* 2008). Both membrane-associated cytoskeletal elements and components of the membrane itself are required to control this stereotyped growth pattern of the membrane.

Membrane–cytoskeletal interactions: Though the actin cytoskeleton is intimately associated with the plasma membrane in yeast, there is no obvious association of actin with the growing prospore membrane nor does disruption of the actin cytoskeleton have significant effects on prospore membrane growth (Taxis *et al.* 2006). Similarly, no direct role for microtubules in growth of the prospore membrane has been reported. Rather two different cytoskeletal systems associate with the growing membrane: septins and a ring structure at the lip of the membrane termed the leading edge complex (Figure 3, D and E).

Septins: Septins are a conserved family of filament-forming proteins (Oh and Bi 2010). In vegetative cells, septins form a ring at the bud neck. This ring creates a diffusion barrier between mother and daughter (Barral *et al.* 2000), and it also helps localize several proteins involved in cytokinesis and signaling (Demarini *et al.* 1997; Lippincott and Li 1998; Longtine *et al.* 2000). The septin ring is composed of five proteins: Cdc3, Cdc10, Cdc11, Cdc12, and Sep7/Shs1. The building block of the septin filament is a linear octamer composed of two head-to-head tetramers [Cdc11-Cdc12-Cdc3-Cdc10]-[Cdc10-Cdc3-Cdc12-Cdc11] (Bertin *et al.* 2008).

As with SNARE proteins, septins are changed during sporulation by replacement of two of the vegetative components with sporulation-specific paralogs. *SPR3* and *SPR28* encode sporulation-specific septins most closely related to *CDC12* and *CDC11*, respectively, that are induced as middle genes (Holaway *et al.* 1987; Ozsarac *et al.* 1995; De Virgilio *et al.* 1996; Fares *et al.* 1996). Interestingly, the vegetative septins *CDC3* and *CDC10* are also transcriptionally upregulated during sporulation, while *CDC12*, *CDC11*, and *SHS1* are not (Kaback and Feldberg 1985; Chu *et al.* 1998). Thus, Spr3 and Spr28 likely replace Cdc12 and Cdc11 in the octamer (*i.e.*, [Spr28-Spr3-Cdc3-Cdc10]-[Cdc10-Cdc3-Spr3-Spr28]), though Cdc11 still shows some localization to septin structures during sporulation (Fares *et al.* 1996; Pablo-Hernando *et al.* 2008). *In vivo* fluorescent pulse labeling indicates that during sporulation, the septin filaments are composed of mixtures of newly synthesized and old septins. Consistent with the patterns of transcriptional regulation, preexisting Cdc10 protein is incorporated into septin bars in sporulating cells but Cdc12 is replaced by Spr3 (McMurray and Thorner 2008).

This change in composition results in a change in behavior. Rather than a static ring, the septins localize in a dynamic pattern on the prospore membrane (Fares *et al.* 1996). When membranes are small, corresponding to the horseshoe shape described above, the septins appear as a ring near the MOP. However, as the membranes expand into cylinders, this ring resolves into bars or sheets that run down the nuclear-proximal side of the prospore membrane and are absent from the region near the MOP (Figure 4). The septins continue to follow the leading edge of the membrane so as the membrane rounds up, the bars form a "V" with the vertex near the site of closure. After membrane closure, this tight organization falls apart and the septins become uniformly distributed around the periphery of the spore (Fares *et al.* 1996).

This dynamic behavior of the septins requires both of the sporulation-specific subunits. Loss of Spr28, which is predicted to sit at the ends of the octamer, disrupts the bar-like organization and the remaining septins distribute uniformly around the prospore membrane as it expands (Pablo-Hernando *et al.* 2008). Deleting *SPR3* causes loss of the bar structure plus greatly reduced association of the remaining septins with the prospore membrane (Fares *et al.* 1996; Pablo-Hernando *et al.* 2008). The higher order organization

of the septin filaments on the prospore membrane is not known. When ectopically expressed in vegetative cells, Spr3 is not incorporated into septin filaments at the bud neck; reciprocally, Cdc12 is present during sporulation but does not enter the septin structures on the prospore membrane (Fares *et al.* 1996; McMurray and Thorner 2008; Pablo-Hernando *et al.* 2008). These observations, along with the dynamic rather than static behavior of the septins, suggest that the organization of septins at the prospore membrane is different from that at the bud neck.

Proper organization of septins on the prospore membrane also requires the protein phosphatase Glc7 and its sporulation-specific regulatory protein, Gip1 (Tachikawa *et al.* 2001). The Gip1–Glc7 complex colocalizes with the septins along the membrane and deletion of *GIP1* or an allele of *GLC7* that interferes with Gip1 binding results in loss of septins from the membrane, similar to an *spr3*Δ mutant (Tachikawa *et al.* 2001). In vegetative cells, septins are both phosphorylated and sumoylated (Johnson and Blobel 1999; Tang and Reed 2002; Dobbelaere *et al.* 2003), but at present no direct effect of Gip1–Glc7 on the modification state of any of the septins has been reported. In an *spr3*Δ mutant, where the septins are delocalized, Gip1 remains associated with the prospore membrane (though uniformly distributed, not in a bar-like pattern), which raises the possibility that Gip1–Glc7 acts structurally as an anchor rather than catalytically as a modifier to recruit septins to the membrane (H. Tachikawa, personal communication).

Despite the striking behavior of septins during sporulation, their functional role at the prospore membrane remains unclear. Deletion of *SPR3* or *SPR28* produces, at most, only a modest defect in sporulation (De Virgilio *et al.* 1996; Fares *et al.* 1996). By contrast, *gip1*Δ mutants show defects in spore wall formation that are not seen in *spr3*Δ mutants, despite similar septin localization defects, suggesting a septin-independent function for *GIP1* (see below). The Gip1-binding protein Ysw1 also colocalizes with septin complexes (Ishihara *et al.* 2009). Mutation of *YSW1* results again in only a modest defect in spore formation; however, prospore membrane morphology is more strongly affected (Ishihara *et al.* 2009).

Leading edge complex: Like the septins, the leading edge complex is associated with the growing prospore membrane. This complex consists of at least three components: Ssp1, Ady3, and Don1 (Knop and Strasser 2000; Moreno-Borchart *et al.* 2001; Nickas and Neiman 2002). The organization of the proteins within the complex is not known, but the localization of Don1 depends on Ady3 and the localization of both Ady3 and Don1 depends on Ssp1 (Moreno-Borchart *et al.* 2001). Consistent with these relationships, Ssp1 binds directly to inositol phospholipids, suggesting that it is the membrane-proximal component of the complex (Maier *et al.* 2007). Prior to membrane formation, Ssp1 and Don1 localize diffusely in the cytoplasm, perhaps associated with precursor vesicles (Moreno-Borchart *et al.* 2001). Ady3, by contrast, is found at the SPB (Moreno-Borchart *et al.* 2001; Nickas and Neiman 2002). As the membrane forms, these three proteins form a ring structure that is localized to the leading edge of the membrane (Moreno-Borchart *et al.* 2001) (Figure 4). This ring remains associated with the leading edge as the prospore membrane grows.

Despite their colocalization, Don1, Ady3, and Ssp1 have distinct functions. Deletion of *DON1* produces no obvious phenotype (Knop and Strasser 2000). Deletion of *ADY3* results in a modest reduction in sporulation efficiency and a large increase in the proportion of asci that contain fewer than four spores (Moreno-Borchart *et al.* 2001; Nickas and Neiman 2002). This mutant displays no obvious defect in prospore membrane formation or growth. Instead, some of the prospores fail to elaborate spore walls (Moreno-Borchart *et al.* 2001; Nickas and Neiman 2002), which is caused by a failure to efficiently segregate mitochondria into the spores (see below). In contrast to *don1*Δ and *ady3*Δ, *ssp1*Δ mutants show a severe sporulation defect, in which prospore membrane growth is abnormal and the membranes collapse onto the nuclear envelope (Moreno-Borchart *et al.* 2001). Membrane closure is also abnormal and, as a result, no spores are formed. Thus, the Ssp1 ring at the prospore membrane lip is essential for proper membrane growth.

Membrane curvature: While the prospore membranes in *ssp1*Δ mutant cells collapse onto the nuclear envelope, in other mutants such as *erv14*Δ or *sma2*Δ, the prospore membranes are often abnormally wide or "boomerang" shaped (Nakanishi *et al.* 2007). Sma2 is an integral membrane protein localized to the prospore membrane (Nakanishi *et al.* 2007; Maier *et al.* 2008). In *sma2*Δ cells, not only is prospore membrane shape abnormal, but the leading edge complex is abnormally expanded in shape and spore formation is blocked (Rabitsch *et al.* 2001). Erv14 is an ER-localized cargo receptor necessary for the export of some integral membrane proteins from the ER (Powers and Barlowe 2002). The defect in the *erv14*Δ mutant cells is likely due to effects on export of Sma2 (Nakanishi *et al.* 2007).

The function of Sma2 is connected to two other proteins: Spo1 and Spo19. Spo1 is a secreted putative phospholipase B required for spore formation (Tevzadze *et al.* 1996, 2000), whose loss of function phenotypes resemble *sma2*Δ mutants (Maier *et al.* 2008). *SPO19* was identified as a high-copy suppressor of both *sma2*Δ and *spo1*Δ mutants (Tevzadze *et al.* 2007; Maier *et al.* 2008). The suppression phenotypes suggest that Sma2 and Spo1 act together upstream of Spo19 in a pathway controlling membrane shape (Maier *et al.* 2008).

Spo19 is a spore wall-localized protein that is predicted to be GPI anchored (Tevzadze *et al.* 2007; Maier *et al.* 2008). Suppression of *sma2*Δ or *spo1*Δ was also seen using other highly expressed GPI-anchored proteins (Tevzadze *et al.* 2007; Maier *et al.* 2008). Moreover, deletion analyses indicated that it is the GPI lipid moiety rather than the protein that is essential for rescue (Tevzadze *et al.* 2007). By immuno-EM, Sma2 was found only in the bilayer of the

prospore membrane closest to the nucleus—*i.e.*, the portion that will become the spore plasma membrane after closure (Maier *et al.* 2008). While *sma2*Δ cells display abnormally wide open prospore membranes and in *ssp1*Δ cells the membranes collapse onto the nuclear envelope, in the *sma2*Δ *ssp1*Δ double mutant, some balance seems to be restored and the membranes have a more wild-type appearance (Maier *et al.* 2008). Taken together, these observations suggest a model in which Sma2 and Spo1 organize GPI-anchored proteins into the luminal leaflet of the inner prospore membrane bilayer (Maier *et al.* 2008), which may create a force that promotes curvature of the entire compartment. Opposing this force is the leading edge complex that helps to hold the membrane open and the balance of these two activities controls the overall shape of the membrane (Maier *et al.* 2008).

Membrane closure: The closure of the prospore membrane is a cytokinetic event that separates the spore cytoplasm from the ascal cytoplasm; concomitantly, membrane fusion separates the single, continuous bilayer of the prospore membrane into separate inner and outer bilayers. In contrast to mitotic cells, where cytokinesis involves both a constrictive actomyosin ring and synthesis of an extracellular septal wall (Tolliday *et al.* 2003), prospore membrane closure occurs without any obvious role for actin or spore wall material, which is not synthesized until after closure (Coluccio *et al.* 2004a; Taxis *et al.* 2006). Thus, cytokinesis in sporulation is likely to be mechanistically distinct from that in mitotic growth.

The leading edge complex is present throughout prospore membrane growth, and then it breaks down just prior to membrane closure due to proteolysis of Ssp1 (Maier *et al.* 2007; Diamond *et al.* 2008). In addition to its positive role in proper membrane growth, Ssp1 plays a negative role in regulating the closure of the prospore membrane (Maier *et al.* 2007). A C-terminal truncation of Ssp1 stabilizes the leading edge complex and blocks spore formation, apparently by interfering with closure of the prospore membrane.

Cytokinesis must be coordinated with meiosis so that closure occurs only after nuclear division is complete. Premature closure could cause either complete failure to capture nuclei or nuclear fragmentation. Membrane closure is coordinated with meiotic exit through the action of the anaphase promoting complex (APC) and its regulatory subunit Ama1 (Diamond *et al.* 2008). The APC is an E3 ubiquitin ligase that is directed to specific substrates through regulators of the Cdc20 family (Vodermaier 2001). In the mitotic cycle, there are two such proteins, Cdc20 and Cdh1 (Schwab *et al.* 1997; Visintin *et al.* 1997). Meiotic divisions require Cdc20 but not Cdh1 (Salah and Nasmyth 2000; Lee and Amon 2003; Tan *et al.* 2010). *AMA1* encodes a sporulation-specific member of the Cdc20 family (Cooper *et al.* 2000). Though expressed as an early-middle gene, its activity is repressed early in meiosis by CDK phosphorylation and by another APC subunit, Mnd2, which leaves the APC during meiosis II (Oelschlaegel *et al.* 2005; Penkner *et al.* 2005). If this regulation is disrupted (*e.g.*, in *mnd2*Δ mutants), premature activation of APC–Ama1 leads to defects in chromosome segregation (Oelschlaegel *et al.* 2005; Penkner *et al.* 2005). Though there is some evidence for an Ama1 function during meiosis, the combined action of Mnd2 and CDK likely limits the primary functions of APC–Ama1 to the end of meiosis II (Cooper *et al.* 2000; Oelschlaegel *et al.* 2005; Tan *et al.* 2010).

Deletion of *AMA1* blocks spore formation (Cooper *et al.* 2000; Coluccio *et al.* 2004a). Though *ama1*Δ cells complete the meiotic divisions (as judged by the appearance of four distinct DAPI staining nuclei) and form prospore membranes, no spores are formed and markers for spore wall formation are absent (Coluccio *et al.* 2004a). A fluorescence loss in photobleaching assay revealed that in postmeiotic *ama1* mutants the presumptive spore and ascal cytoplasms remain connected (Diamond *et al.* 2008); *i.e.*, the prospore membranes fail to close. This phenotype is suppressed by a temperature-sensitive *ssp1* allele, implying that the defect reflects stabilization of Ssp1 (Diamond *et al.* 2008). Thus, APC–Ama1 coordinates meiosis and cytokinesis by linking meiotic exit to Ssp1 degradation.

Organellar segregation: The closure of the prospore membrane around a daughter nucleus ensures inheritance of a complete haploid set of chromosomes by the spore. As in any cell division, the daughter cells must also inherit sufficient cytoplasm and organelles to be viable. In the inheritance of organelles, sporulation is distinctly different from mitotic divisions. During mitosis, polarized actin cables and myosin motors (Pruyne *et al.* 2004) are used to transport into the bud multiple organelles including vacuolar precursors, cortical ER elements, some Golgi elements, peroxisomes, and mitochondria (Hill *et al.* 1996; Simon *et al.* 1997; Rossanese *et al.* 2001; Fehrenbacher *et al.* 2002; Estrada *et al.* 2003; Fagarasanu *et al.* 2006). Because sporulation produces four daughter cells simultaneously rather than a single bud, mechanisms besides vectoral transport of organelles are required.

Sporulation also differs from mitotic divisions in that not all of the cellular contents are packaged into the progeny cells. In vegetative growth, the cytoplasm and its contents are divided between the mother and daughter. In sporulation, however, the contents are divided between the four spores and the ascus. Estimates based on serial reconstructions in electron micrographs suggest that only ~30% of the mother cell volume ends up in the spores (Brewer and Fangman 1980). Thus, most cellular organelles remain in the ascus and are not inherited.

This five-way division of material includes the nucleus itself. When the four haploid nuclei pinch off from the original nucleus, a remnant nuclear body remains, which is left behind in the ascus (Moens and Rapport 1971; Fuchs and Loidl 2004). This body contains no chromosomal DNA, but it includes the contents of the nucleolus (excluding the

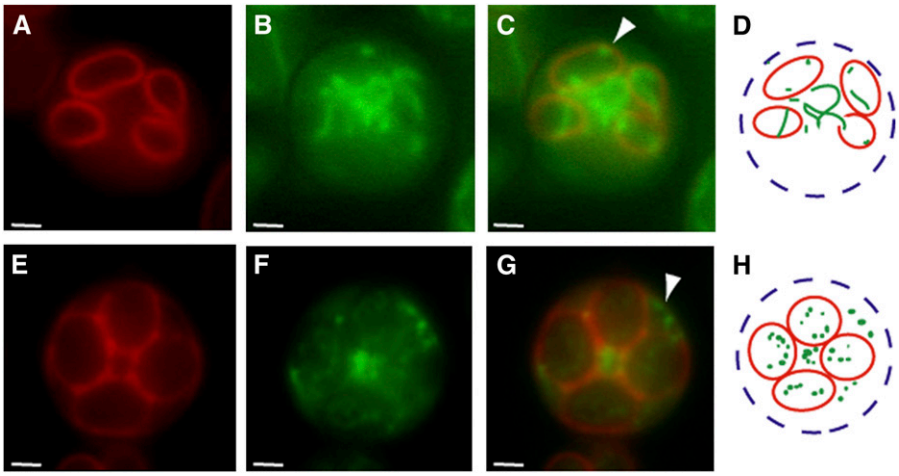

Figure 5 Segregation of mitochondria in the spore. (A) Spo20$^{51-91}$–RFP indicating the prospore membranes in a cell in meiosis II. (B) GFP-tagged *MRPS17*. (C) Merge of images in A and B. Arrowhead indicates mitochondrial material located within the prospore membrane. (D) Representation of the fluorescence image in C. Dashed line indicates the outline of the cell, red lines the prospore membrane, and green speckles the mitochondrial protein. (E) Spo20$^{51-91}$–RFP in mature spores. (F) Mrps17–GFP. (G) Merge of images in D and E. Arrowhead indicates mitochondria that have remained in the ascus. (H) Representation of the fluorescence image in G. Dashed line indicates the outline of the cell, red lines the prospore membrane, and green speckles the mitochondrial protein. Bars, 1 μm.

rDNA) and the majority of nuclear pore complexes (Fuchs and Loidl 2004). Nucleolar antigens are absent from the nuclei of newly formed spores, but the nucleolus subsequently regenerates (Fuchs and Loidl 2004). Thus rather than inherit old nucleoli, spores build new ones.

A similar pattern of regeneration rather than inheritance is also seen for some cytoplasmic organelles. For example, fluorescent markers for both the vacuolar lumen and the vacuolar membrane remain behind in the ascus when spores are formed (Roeder and Shaw 1996). New vacuoles appear within spores about 12 hr after closure (Suda *et al.* 2007). Thus, like nucleoli, spores regenerate vacuoles rather than inherit them.

The behavior of other organelles also suggests a regeneration process. Cortical ER, which is actively segregated in vegetative growth (Fehrenbacher *et al.* 2002; Estrada *et al.* 2003), disappears during meiosis (Suda *et al.* 2007). Marker proteins for the cortical ER relocalize to the nuclear envelope and segregate into the spore with the nucleus and then reappear beneath the spore plasma membrane after prospore membrane closure (Suda *et al.* 2007). The reabsorption of the cortical ER into the nuclear envelope during meiosis may help provide enough membrane to accommodate the expansion of surface area created by extension of the two meiosis II spindles. It also ensures entry of cortical ER proteins into the spore. In contrast to the vacuole and cortical ER, Golgi elements appear within the presumptive spore cytoplasm as the prospore membrane is expanding (Suda *et al.* 2007), though it is not known whether preexisting Golgi migrate into the spore or whether newly derived Golgi become "trapped" within the prospore membrane.

An exception to this pattern of organellar regeneration is the mitochondrion, which cannot be formed *de novo* and hence must be inherited. Early in sporulation, the mitochondria fuse to form an extended branched tubular structure at the cell periphery (Stevens 1981; Miyakawa *et al.* 1984). When cells enter meiosis, the bulk of the mitochondria migrate inward and become associated with the nuclei, with the mitochondrial outer membranes often closely apposed to the nuclear envelope (Stevens 1981) (Figure 3D). Because of this association with the nuclear envelope, at meiosis II the mitochondria form a dense cluster near the middle of the two spindles (Miyakawa *et al.* 1984). Tendrils of mitochondria extend out from this cluster and into the presumptive spore cytoplasm underneath the prospore membrane (Suda *et al.* 2007) (Figure 5). Closure of the membrane severs these tendrils from the greater mitochondrial mass and thus captures mitochondria within the spore, though most of the mass remains in the ascus (Brewer and Fangman 1980; Miyakawa *et al.* 1984; Gorsich and Shaw 2004) (Figure 5).

The actin-based pathways for mitochondrial inheritance in vegetative cells (Frederick *et al.* 2008) are not operative during sporulation. Instead, segregation of mitochondria into the spore relies in part on the leading edge complex protein Ady3 (Suda *et al.* 2007). In *ady3*Δ mutants only ~50% of the prospores inherit mitochondria and only those prospores that inherit mitochondria go on to form mature spores (Suda *et al.* 2007). Yet because 50% still receive mitochondria, other factors must contribute to segregation as well.

The leading edge proteins are situated at the interface between the presumptive ascal and spore cytoplasms. As such, they are well positioned to control transit between the two compartments, analogous to the way the septin ring at the bud neck functions in vegetative growth (Barral *et al.* 2000). However, Ady3 serves not to exclude mitochondria from the spore but to enhance their entry. Because of the association between the mitochondria and the nuclear envelope, nuclear division could provide the motive force to pull mitochondria into the spores as the spindle extends. Ady3 might assist the passage of the mitochondria through the mouth of the prospore membrane.

Why is so much of the cellular content left behind in the ascus? Two explanations have been proposed (Zubenko and Jones 1981; Fuchs and Loidl 2004). First, these components

may have functions in the ascus that help foster spore maturation. Indeed, vacuolar protease function within the ascus is required for it to properly collapse around the spores at the end of the process (Zubenko and Jones 1981). Moreover, some mRNAs are specifically enriched in the ascus (Kurtz and Lindquist 1986). Retention of cytoplasmic functions would allow expression of these mRNAs even though the ascus lacks nuclear DNA. A second suggestion is that disposal of old organelles may be important for resetting the aging process (Fuchs and Loidl 2004). One basis for aging is thought to be the accumulation of cellular damage over time, including modified proteins and extrachromosomal DNAs (Sinclair and Guarente 1997; Lai *et al.* 2002; Aguilaniu *et al.* 2003). During mitotic division, most of this abnormal material remains in the mother cell, allowing an "old" mother cell to give rise to a "young" daughter. During sporulation, all four progeny are young daughters. Thus, shedding cellular contents into the ascus and regenerating organelles may help spores reset the aging clock by ridding themselves of damaged components.

The late phase: settling down inside a protective coat

The closure of the prospore membrane marks the transition to the late phase of spore formation during which the prospores develop into mature spores. The major event of this phase is the assembly of the spore wall, which is the distinguishing feature of the spore and provides protection against a variety of different environmental stresses (Smits *et al.* 2001). In addition to spore wall assembly, the late phase includes changes to the nucleus, where altered histone modifications affect packing of the chromatin, and to the spore cytoplasm, where the secretory pathway returns to a more vegetative-like arrangement.

Chromatin changes: As cells progress through meiosis, different histone modifications appear (Ahn *et al.* 2005; Borde *et al.* 2009; Govin *et al.* 2010a). While some occur early and are likely linked to the chromosome pairing events of meiotic prophase, other modifications accumulate as cells undergo the meiotic divisions. Phosphorylation of histone H4 on serine 1 (Ser1) is undetectable in vegetative cells but begins to appear as cells enter meiosis and accumulates to high levels in postmeiotic cells (Krishnamoorthy *et al.* 2006). This modification is broadly distributed on nucleosomes across the genome (Govin *et al.* 2010b) and requires the protein kinase Sps1, though it is not known whether Sps1 directly phosphorylates H4 (Krishnamoorthy *et al.* 2006). *SPS1* is an *NDT80*-regulated gene (Chu *et al.* 1998), so the accumulation of this modification on histones may parallel the expression of the kinase. In addition to Ser1 phosphorylation, histone H4 becomes acetylated late in sporulation on lysines 5, 8, and 12 (Govin *et al.* 2010a).

What is the result of these changes in histone modification? Measurements of nuclear volume, based on DAPI staining, indicate that chromatin may be more compact in spores than in vegetative haploids (Krishnamoorthy *et al.* 2006). Moreover, blocking Ser1 phosphorylation on histone H4 increases the DAPI-stained volume, and this effect is exacerbated in cells that cannot acetylate the H4 lysines (Krishnamoorthy *et al.* 2006; Govin *et al.* 2010a). Thus, these modifications may lead to increased compaction of the chromatin late in sporulation. However, the function of this condensation is not yet clear. Mutation of H4 Ser1 causes only modest effects on spore formation or viability (Krishnamoorthy *et al.* 2006). Mutating the acetylated lysines to nonacetylated arginines causes reduced sporulation and inviable spores (Govin *et al.* 2010a), but given the potential for pleiotropic effects, it remains to be determined whether these sporulation phenotypes are caused by the chromatin condensation defect.

Restoration of vegetative cytoplasmic organization: Upon closure, many of the rearrangements of the cytoplasm and endomembrane system that drive prospore membrane growth are reversed, so that the maturing spore displays an organization similar to vegetative cells. For example, as noted earlier, just prior to closure the Ssp1 protein is degraded and the leading edge complex disassembles (Maier *et al.* 2007). Similarly, after closure, the septins lose their bar-like organization and redistribute uniformly around the spore, while the Glc7 phosphatase returns to the nucleus (Tachikawa *et al.* 2001). As with the leading edge complex, the MOP complex disappears at the time of closure (Knop and Strasser 2000), though it is not known whether this is due to protein degradation or disassembly.

Other aspects of the secretory pathway also return to a more vegetative cell-like state. The cortical ER reforms and, strikingly, actin rapidly becomes important for vesicle trafficking at the spore plasma membrane (Taxis *et al.* 2006; Suda *et al.* 2007). While there is evidence for endocytic trafficking of proteins from the mother cell plasma membrane to the prospore membrane (Morishita *et al.* 2007), recycling of material from the growing prospore membrane has not been reported. Mutations in endocytosis genes (*e.g.*, *ARP2* and *END3*) do not disrupt prospore membrane growth but do cause spore wall defects (Morishita and Engebrecht 2005; Taxis *et al.* 2006), implying that endosomal trafficking within the spore facilitates wall assembly. In summary, the cytoplasmic rearrangements seen during meiosis are rapidly reversed after cytokinesis.

Formation of the spore wall: The major event of the late phase is the assembly of the spore wall. Given the restoration of vegetative-like organization of the spore cytoplasm, the spore wall is the main feature that distinguishes spores from stationary phase haploid cells. The spore wall differs from the vegetative cell wall in two important respects: it contains additional components, and it must be assembled *de novo*. Newly formed vegetative cell walls, such as those surrounding a bud, can be formed by extension of the existing cell wall to cover newly inserted plasma membrane. By contrast, for the spore wall there is

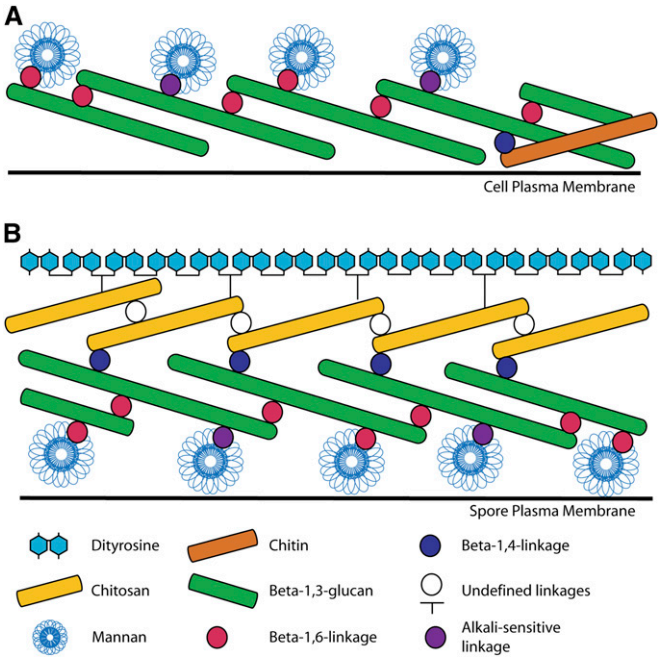

Figure 6 Model of spore wall organization. (A) Model for the vegetative cell wall showing the relationsip of three major components to the plasma membrane. (B) Model for the layered organization of the spore wall. The linkages between the mannan, β-1,3-glucan, and chitosan layers are based on work on the structure of the vegetative cell wall. The chemical linkages between chitosan chains, between dityrosine monomers, and linking the chitosan and dityrosine are unknown.

no preexisting structure available to act as a template, and so its assembly presents a unique challenge to the yeast cell.

The vegetative cell wall consists of two major components. First is a layer composed of long β-1,3 linked glucan chains, which lie relatively close to the plasma membrane (Figure 6A). Outside of these β-glucans is a thicker layer of mannoproteins (or mannan), which consists of a variety of different secreted proteins that are heavily mannosylated through asparagine (N-linked) or serine/threonine (O-linked) residues (Klis *et al.* 2002). In addition to these major components, the cell wall contains a lesser amount of chitin, a β-1,4–linked N-acetyl glucosamine polymer concentrated in the septum and at the bud neck (Klis *et al.* 2002; Lesage and Bussey 2006) (Figure 6A). These different layers are cross-linked to themselves and each other through a variety of linkages. In particular, short chains of β-1,6–linked glucoses are used as cross-linkers so that the cell wall as a whole can be thought of as a mesh of different sugar polymers (Kollar *et al.* 1997; Lesage and Bussey 2006).

Like the cell wall, the spore wall contains both mannan and β-1,3-glucan layers as major components (Smits *et al.* 2001). However, they are reversed in order with respect to the spore plasma membrane so that the mannan is inside of the β-glucans (Kreger-Van Rij 1978) (Figure 6B). Presumably, these layers are linked by β-1,6-glucans as in the vegetative wall, though this has not been demonstrated.

In addition to mannan and β-glucans, the spore wall incorporates two unique components, chitosan and dityrosine (Briza *et al.* 1988, 1990b) (Figure 6B). Chitosan, a β-1,4–linked glucosamine polymer, forms a distinct layer on the outside of the β-glucan layer (Briza *et al.* 1988). On the outer surface of the chitosan is a fourth layer of the spore wall, which is enriched in the cross-linked amino acid dityrosine. While the structure of this polymer is not known, it is distinct from the other spore wall layers in that it is not composed primarily of polysaccharides (Briza *et al.* 1990b). These spore-specific layers of chitosan and dityrosine provide the spore wall with many of its distinctive properties (see below).

Order of assembly: Assembly of the spore wall begins in the luminal space between the two bilayers (the spore plasma membrane and the outer membrane) created by closure of the prospore membrane (Lynn and Magee 1970). As the prospore membrane grows, the width of the lumen remains uniform until membrane closure. This luminal space expands after closure, presumably driven by the deposition of spore wall components (Coluccio *et al.* 2004a). Cells lacking *AMA1*, which have a closure defect, fail to initiate spore wall assembly (Coluccio *et al.* 2004a; Diamond *et al.* 2008). Thus, closure of the prospore membrane may generate a signal that initiates the spore wall assembly process.

A time course analysis using fluorescent markers for the different spore wall layers revealed that the different layers are deposited in a specific temporal order that matches their order within the final wall: mannan, β-1,3-glucan, chitosan, dityrosine (Tachikawa *et al.* 2001). Thus, the wall is built outward from the first layer. In these experiments, it is important to note that the different layers are identified using reagents that detect the presence of the components and do not require their assembly into a structured layer. Therefore, the fact that chitosan staining is not seen until well after β-glucan staining indicates that chitosan synthesis itself is delayed relative to β-glucan synthesis. These observations suggest the existence of monitoring systems that trigger the synthesis of each layer only after the preceding one is complete.

Mannan layer: After closure, there is a large increase in mannoproteins present in the lumen, which can be seen in the EM as an expansion of the luminal space (Coluccio *et al.* 2004a). Secretory vesicle carriers must mediate delivery of these mannoproteins, though whether they come solely from within the spore or also from the ascal cytoplasm has yet to be determined.

This early stage of spore wall formation is blocked in strains lacking Gip1 (Tachikawa *et al.* 2001), which promotes spore wall assembly in a manner distinct from its role in septin organization, as mentioned earlier. In principle, the spore wall block in *gip1*Δ mutants could be a secondary consequence of a cytokinesis defect, as with *ama1*Δ mutants (Coluccio *et al.* 2004a; Diamond *et al.* 2008). However, a fluorescence loss in photobleaching assay indicates that

*gip1*Δ mutants complete prospore membrane closure (J. S. Park, personal communication). Therefore, Gip1 may function in a signaling pathway that allows the spore to sense membrane closure and initiate wall formation.

β-*Glucan layer:* β-1,3-glucan synthase is an integral membrane protein localized at the plasma membrane of vegetative cells, where it binds the nucleotide sugar UDP-glucose in the cytoplasm, couples the glucose moieties together (with release of the nucleotide), and extrudes glucan chains into the extracellular space (Shematek *et al.* 1980). Three genes encode predicted β-glucan catalytic subunits in *S. cerevisiae*: *FKS1*, *FKS2/GSC2*, and *FKS3*. *FKS1* is the predominant form in vegetative cells, though in *fks1*Δ mutants, *FKS2* becomes upregulated (Mazur *et al.* 1995). Simultaneous deletion of *FKS1* and *FKS2* is lethal (Inoue *et al.* 1995; Mazur *et al.* 1995). During sporulation, Fks2 is primarily responsible for synthesis of the β-glucan layer, due largely to its higher expression (Mazur *et al.* 1995; Ishihara *et al.* 2007).

Fks3, also plays a role in spore wall assembly, as *fks3*Δ mutants display spore wall defects (Ishihara *et al.* 2007; Suda *et al.* 2009). But the function of *FKS3* may not be directly related to that of *FKS1* and *FKS2*, as overexpression of neither *FKS1* nor *FKS2* can rescue the *fks3*Δ defects (Ishihara *et al.* 2007). Expression of *FKS3* in vegetative cells can rescue an *fks1* temperature-sensitive allele, but it does so by influencing upstream regulators of *FKS1* rather than by providing glucan synthase activity (Ishihara *et al.* 2007). Thus, the precise role of Fks3 in assembly of the β-glucan layer remains to be determined.

In vegetative cells, glucan synthase activity is regulated by interaction of the catalytic subunit with the small GTPase Rho1 (Qadota *et al.* 1996). Whether similar regulation occurs during sporulation has not yet been examined, but two other forms of regulation have been reported (Huang *et al.* 2005; Iwamoto *et al.* 2005). The first involves regulated delivery of Fks2 to the prospore membrane by the sporulation-specific kinase Sps1. Cells lacking Sps1 display heterogeneous defects in spore wall assembly, including differing severities for spores in the same ascus (Friesen *et al.* 1994), consistent with an inability to coordinate the different assembly stages. Sps1 can bind negatively charged phospholipids *in vitro* (Zhu *et al.* 2001; Moravcevic *et al.* 2010), suggesting a membrane association *in vivo*, and Sps1–GFP colocalizes with endosomal markers in sporulating cells or when ectopically expressed in vegetative cells (Iwamoto *et al.* 2005). In *sps1*Δ mutants Fks2–GFP is retained in an intracellular compartment and fails to reach the prospore membrane (Iwamoto *et al.* 2005), implicating Sps1 in the delivery of the β-glucan synthase to the prospore membrane.

The second level of regulation involves interaction of Fks2 with another sporulation-specific kinase, Smk1. Like *sps1*Δ cells, *smk1*Δ cells display heterogeneous spore wall defects (Krisak *et al.* 1994). Hypomorphic alleles of *smk1* display more uniform and distinct assembly defects, suggesting that different levels of kinase activity are required at different transition points in the assembly process (Wagner *et al.* 1999). Fks2 binds Smk1 in sporulating cells and β-glucan synthase activity is elevated in the *smk1*Δ mutant, indicating that Smk1 may inhibit Fks2 (Huang *et al.* 2005). In *smk1*Δ mutants, chitosan synthesis is delayed or absent (Huang *et al.* 2005), but in *smk1*Δ *fks2*Δ double mutants, chitosan staining is restored. This suggests that elevated glucan synthase activity in the *smk1*Δ mutant inhibits the activation of chitosan synthesis (Huang *et al.* 2005). Therefore, negative regulation of Fks2 by Smk1 may be required for the transition to synthesis of the next spore wall layer. Conceivably, the presence of properly assembled β-glucans could trigger Smk1-mediated inactivation of Fks2, which in turn allows for activation of the chitosan synthesis.

Assembly of an intact β-glucan layer requires extracellular enzymes to extend and cross-link the β-glucan chains to themselves and to other wall components (Lesage and Bussey 2006). Several enzymes that produce these linkages are known from studies of the vegetative cell wall (Goldman *et al.* 1995; Carotti *et al.* 2004; Cabib *et al.* 2008), but in the spore wall these functions are often performed by sporulation-specific paralogs. For example, the five *GAS* genes encode β-1,3-glucosyltransferases (Ragni *et al.* 2007b), which link the β-1,3 chains extruded by glucan synthase into extended polymers found in the mature wall (Carotti *et al.* 2004). In vegetative cells, Gas1 and Gas5 are the predominant forms (Ragni *et al.* 2007b), whereas *GAS2* and *GAS4* are specifically induced during meiosis as part of the Ndt80 regulon (Chu *et al.* 1998; Ragni *et al.* 2007a). *gas2*Δ *gas4*Δ double mutants show severe defects in spore wall formation (Ragni *et al.* 2007a), including a weakened connection at the interface between the β-glucan and chitosan layers (Ragni *et al.* 2007a). The vegetative protein Gas1 also localizes to the spore wall (Neiman 1998), so it is not clear why the sporulation-specific isoforms are required. Interestingly, while Gas1, Gas2, and Gas4 all catalyze the same reaction *in vitro* (Ragni *et al.* 2007b), *in vivo* experiments suggest different pH optima for these enzymes (Ragni *et al.* 2007a); namely, *GAS2* and *GAS4* rescued *gas1*Δ when the growth medium was buffered near neutral pH, but not at the acidic pH of unbuffered medium. This is noteworthy because sporulation is optimum at neutral pH and because this stage of spore wall assembly occurs in the lumen of the prospore membrane (Ohkuni *et al.* 1998; Coluccio *et al.* 2004a). Thus, the presence of sporulation-specific paralogs of different cell wall assembly enzymes may reflect the need to function at a more alkaline pH than the acidic milieu in which the cell wall is assembled (Ragni *et al.* 2007a). Several other vegetative/sporulation paralogs exist, including *ECM33/SPS2* (or *SPS22*), *CRH1/CRR1*, and *EXG1/SPR1* (Nebreda *et al.* 1986; Muthukumar *et al.* 1993; San Segundo *et al.* 1993; Terashima *et al.* 2003; Coluccio *et al.* 2004a; Gomez-Esquer *et al.* 2004; Cabib *et al.* 2008). Whether these paralogs similarly differ in their pH optima has not been reported.

In addition to the assembly enzymes, additional sporulation-specific factors influencing the assembly of the

β-glucan layer have been identified including Spo73, Spo77, and Ssp2 (Sarkar *et al.* 2002; Coluccio *et al.* 2004a; Li *et al.* 2007). Though no molecular function has been ascribed to any of these proteins, each localizes to the cytoplasmic side of the prospore membrane, suggesting that they affect assembly indirectly (*e.g.*, as regulators of the synthase).

Chitosan synthesis: The chitosan of the spore wall is derived from chitin. Similar to the β-1,3 glucan chains of the β-glucan layer, the β-1,4–linked glucosamine chains of the chitosan layer are synthesized by an integral membrane enzyme that binds nucleotide sugars in the cytoplasm and extrudes the polymer to the extracellular space (Orlean 1997). In vegetative cells, cell wall chitin is produced by three different chitin synthases: Chs1, Chs2, and Chs3 (Silverman *et al.* 1988; Cabib *et al.* 1989; Silverman 1989; Shaw *et al.* 1991; Valdivieso *et al.* 1991). During sporulation, chitosan synthesis is mediated solely by Chs3 (Pammer *et al.* 1992). The immediate product of Chs3 enzymatic activity is chitin (a β1,4–N-acetylglucosamine polymer), whereas synthesis of chitosan requires both Chs3 and two sporulation-specific deacetylases, Cda1 and Cda2 (Christodoulidou *et al.* 1996, 1999; Mishra *et al.* 1997). These secreted proteins interact with the chitin extruded by Chs3 and remove the acetyl groups to produce chitosan (Christodoulidou *et al.* 1996, 1999; Mishra *et al.* 1997). This conversion is required for proper spore wall assembly. In *cda1*Δ *cda2*Δ mutants, Chs3 is active but the spore wall lacks chitosan and contains only chitin, which does not form a distinct layer on the outside of the β-glucan, and hence the dityrosine layer never forms (Christodoulidou *et al.* 1996, 1999).

In vegetative cells, Chs3 is regulated by a regulatory subunit, Chs4 (Chuang and Schekman 1996; Ono *et al.* 2000). Chs4 regulates chitin synthase activity both by increasing enzyme activity and by controlling its intracellular localization (Demarini *et al.* 1997; Trilla *et al.* 1997; Ono *et al.* 2000). Chs4 can bind to the Bni4 protein, which in turn binds to septins and the Chs4–Bni4 interaction allows recruitment of Chs3 to the bud neck for synthesis of the bud scar (Demarini *et al.* 1997).

The regulation of Chs3 is modified during sporulation. Chs4 is replaced by a sporulation-specific activator, Shc1 (Sanz *et al.* 2002). *SHC1* expression in vegetative cells can rescue the enzyme activity defect in a *chs4*Δ mutant but not the localization defect, suggesting that Shc1 cannot interact with Bni4 to link the enzyme to the septins (Sanz *et al.* 2002). Moreover, unique to sporulating cells and similar to the glucan synthase, in *sps1*Δ mutants, Chs3p is retained in an intracellular compartment and does not reach the prospore membrane (Iwamoto *et al.* 2005). Thus Sps1 is required for transport of both polysaccharide synthases.

Both the presence of chitosan and its assembly into a distinct layer of the wall distinguishes spore walls from cell walls. A number of mutants have been identified in which the chitin synthase is clearly active but the chitosan layer does not form, suggesting that these gene products could be involved in assembly of the chitosan layer. In addition to the *cda1*Δ *cda2*Δ mutant mentioned above, mutation of the transcription factor *GIS1* or of the *OSW1* or *MUM3* genes produces a similar phenotype (Coluccio *et al.* 2004a). While the effect of the *gis1*Δ mutation is likely indirect, the Osw1 protein localizes to the spore wall and so may be directly involved in assembly of the chitosan layer (Coluccio *et al.* 2004a; Li *et al.* 2007). The Mum3 protein has not been localized but has homology to acyltransferases (Neuwald 1997), suggesting that it has an enzymatic activity that could play a role in assembly of this spore wall layer as well.

Outer membrane breakdown: In addition to the start of chitosan synthesis, another change that coincides with the completion of the β-glucan layer is the disruption of the outer membrane (Coluccio *et al.* 2004a). While assembly of the mannan and β-glucan layers occurs in the lumen between the spore plasma membrane and the outer membrane, the chitosan and dityrosine layers are exposed directly to the ascal cytoplasm as they are built. Nothing is known about how the disruption of the outer membrane occurs or how it is achieved without damaging the spore or ascal plasma membranes. It is also unclear whether disruption is necessary for assembly of the outer spore wall, but membrane lysis could allow assembly factors to gain access to the forming wall. For example, the Osw1 protein is localized to the spore wall but lacks an obvious signal sequence for secretion to the prospore membrane lumen. Moreover, it is localized in the cytoplasm earlier in sporulation (Coluccio *et al.* 2004a; Li *et al.* 2007). Thus, Osw1 might remain in the ascal cytoplasm and only enter the spore wall after outer membrane dissolution.

Dityrosine layer: The most unique aspect of the spore wall is the outermost dityrosine layer, as it is constituted of neither protein nor polysaccharide (Briza *et al.* 1990b). Instead, the major constituent is the modified, cross-linked diamino acid N-N-bisformyl-dityrosine (hereafter, dityrosine) (Briza *et al.* 1990b, 1996). Dityrosine is synthesized in the spore cytoplasm in a two-step biosynthetic pathway catalyzed by Dit1 and Dit2 (Briza *et al.* 1994). Dit1 is an N-formyl transferase, which formylates free L-tyrosine, and Dit2 is a cytochrome P450 family enzyme that covalently crosslinks two molecules of N-formyl–L-tyrosine into dityrosine (Briza *et al.* 1986).

The dityrosine is exported from the cytosol by the action of a dedicated MDR family transporter, Dtr1 (Felder *et al.* 2002), and then polymerized into a much larger structure that assembles on the surface of the chitosan (Briza *et al.* 1990b). Incorporation into the polymer leads to the isomerization of ~50% of the dityrosine molecules from the L,L stereoisomer to the D,L form (Briza *et al.* 1990b). Additionally, assembly must take place on the surface of the chitosan layer because defects in chitosan synthesis or assembly block the formation of the dityrosine layer (Pammer *et al.* 1992). Beyond this, however, the structure of the polymer and its assembly pathway are unknown.

Regulators of assembly: The two best-studied regulators of spore wall assembly are the Smk1 and Sps1 kinases

described above, though additional candidates are implied by other mutants with spore wall defects similar to those in *sps1*Δ and *smk1*Δ strains (Wagner *et al.* 1997; Ufano *et al.* 1999; Straight *et al.* 2000; Coluccio *et al.* 2004a). Smk1 is a member of the MAP kinase family and, like other members of this group, is activated by phosphorylation of tyrosine and threonine residues in the activation loop (Krisak *et al.* 1994; Schaber *et al.* 2002). Unlike other yeast MAP kinases, however, there is no obvious MAP kinase kinase to activate Smk1. Instead, this activation may involve two essential kinases, Mps1 and Cak1, as hypomorphic forms of each kinase cause spore wall defects reminiscent of *smk1*Δ mutants (Wagner *et al.* 1997; Straight *et al.* 2000). Cak1 is known to activate several kinases by phosphorylation of activation loop threonines, and indeed Smk1 is not phosphorylated in the *cak1* mutant, and so Cak1 likely functions as a direct activator of Smk1 (Espinoza *et al.* 1996, 1998; Kaldis *et al.* 1996; Schaber *et al.* 2002; Yao and Prelich 2002; Ostapenko and Solomon 2005). It is not known whether Mps1 directly phosphorylates Smk1. Addtionally, mutations in the APC subunit Swm1 cause a spore wall defect similar to *smk1*Δ mutants (Ufano *et al.* 1999; Hall *et al.* 2003). This may reflect the requirement for the APC activator Ama1 for Smk1 activation (McDonald *et al.* 2005).

SPO75 encodes an integral membrane protein and *spo75*Δ cells display heterogeneous wall phenotypes ranging from an early block in formation to the assembly of wild-type spore walls (Coluccio *et al.* 2004a). Interestingly, a proteomic screen identified a physical interaction between Spo75 and Sps1 (Krogan *et al.* 2006). Thus, Spo75 might function with Sps1 in regulating the delivery of the polysaccharide synthases to the prospore membrane.

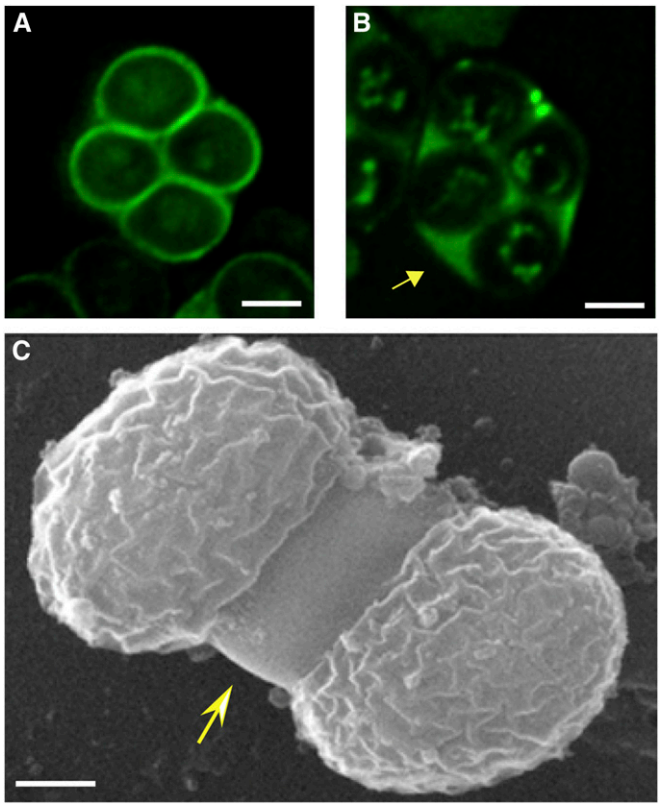

Figure 7 Features of the spore wall. (A) Localization of a secreted GFP molecule to the spore wall of wild-type spores. Bar, 2 μm. (B) Localization of the same secreted GFP in spores lacking a dityrosine layer. The arrow indicates localization of the GFP fusion to the ascal cytoplasm. Bar, 2 μm. (C) Scanning electron micrograph of a pair of spores. The arrow indicates the interspore bridge that links the two spores together. Bar, 1 μm.

Properties of the assembled spore wall: The mature spore is a quiescent cell that is resistant to multiple forms of stress, including organic solvents, heat, and digestive enzymes (Kupiec *et al.* 1997). The spore wall, and in particular its chitosan and dityrosine layers, is primarily responsible for this stress resistance (Briza *et al.* 1990a; Pammer *et al.* 1992). While the basis for resistance to ether vapor or heat shock is unclear, some insight has been gained into how the dityrosine layer protects against digestive enzymes. A secreted form of GFP expressed during sporulation initially accumulates in the prospore membrane lumen (Suda *et al.* 2009). Yet after lysis of the outer membrane, this fluorescent protein remains in the spore wall (Suda *et al.* 2009) (Figure 7A), implying the presence of a barrier to its diffusion out of the periplasmic space. By contrast, in *dit1*Δ or *chs3*Δ mutants this same protein leaks out from the wall into the ascal cytoplasm within a few hours of the appearance of mature spores (Suda *et al.* 2009) (Figure 7B), indicating that the dityrosine layer is responsible for forming this diffusion barrier (Suda *et al.* 2009). If we imagine the polysaccharide layers of the spore wall as a mesh of glycan fibers, then the dityrosine can be thought of as filling the outermost pores of that mesh. Presumably, this barrier would also block the diffusion of protein-sized molecules into the wall, perhaps explaining the dityrosine-based resistance to lytic enzymes.

Scanning EM analysis revealed that the outer chitosan and dityrosine layers not only surround each individual spore but they also form bridges that link adjacent spores of the tetrad together (Coluccio and Neiman 2004) (Figure 7C). These bridges help the spores remain associated even when the surrounding ascus is removed. Their formation provides another possible rationale for why the outer membrane breaks down before chitosan synthesis—so that different spore walls can be connected. The function of these bridges is unclear, though it has been speculated that they could help promote mating between sister spores after sporulation (Coluccio and Neiman 2004).

Maturation of the ascus: The final event of sporulation is the collapse of the surrounding mother cell around the mature spores to form an ascus. Very little is known about this process, though it must involve some remodeling of the cell wall around the ascus so that it can shrink. Similarly, there must be some degradation of the contents of the ascal cytoplasm to allow collapse. This latter process may involve

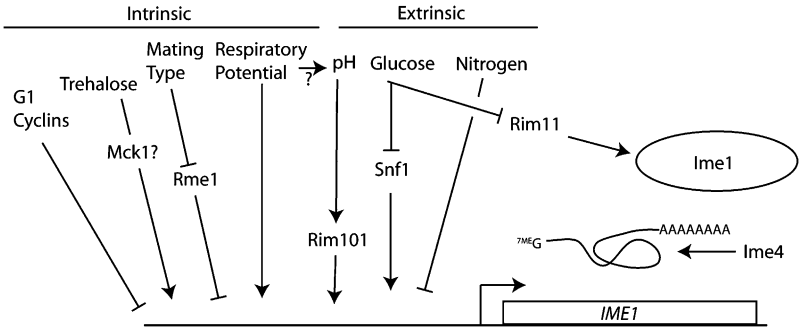

Figure 8 Factors controlling expression and activity of Ime1. Expression of *IME1* is the key event in triggering sporulation. A variety of intracellular and extracellular signals are integrated at the level of the *IME1* promoter to control gene expression and developmental choice. In addition, Ime1 activity is also controlled at the post-transcriptional and post-translational levels.

vacuoles in the ascus, as loss of the vacuolar protease Prb1 interferes with ascal collapse (Zubenko and Jones 1981). Finally, it seems likely that the timing of ascal maturation is coordinated with spore wall assembly to prevent premature collapse of the ascus.

Integrating the Phases of Sporulation: Key Control Points

During sporulation there are three major control points where information is integrated to ensure that the process proceeds properly. These occur at the start of each of the phases just described: the decision to begin sporulation, entry into the meiotic divisions, and exit from meiosis. These decision points were outlined above and the inputs and outputs of these regulatory nodes are examined in more detail below.

Entry into sporulation: control of Ime1 activity

Expression of the master regulator Ime1 serves as a control point for the cell to take inputs from various intracellular (and extracellular factors and integrate these into the decision to differentiate (Figure 8). The majority of these stimuli control *IME1* transcription, but there is also evidence for post-transcriptional and post-translational control. The best-studied inputs are mating type, glucose, and nitrogen. Mating-type regulation is mediated by the Rme1 repressor (Mitchell and Herskowitz 1986), which is expressed in haploid cells and represses *IME1* transcription. *RME1* is repressed in *MAT***a**/*MAT*α diploids, thereby relieving one brake to *IME1* expression (Mitchell and Herskowitz 1986).

The *IME1* upstream regulatory region is unusually large, reflecting the diverse factors affecting expression (Sagee *et al.* 1998). This region contains a multiplicity of positive and negative elements that respond to glucose, acetate, nitrogen, or mating type (Sagee *et al.* 1998). However, besides Rme1, only a few other transcriptional regulators, such as Msn2/Msn4 and Yhp1, have been shown to bind directly at the upstream region (Sagee *et al.* 1998; Kunoh *et al.* 2000). Thus, much remains to be learned about how environmental conditions directly influence *IME1* promoter activity.

Ime1 is inhibited by glucose in at least two ways. First, glucose inhibits *IME1* transcription (Kassir *et al.* 1988). In particular, glucose inhibits the Snf1 kinase, whose activity is required for *IME1* transcription (Honigberg and Lee 1998). Second, glucose controls Ime1 activity at the post-translational level through a pathway involving Ras and the kinase Rim11 (Bowdish *et al.* 1994; Malathi *et al.* 1999; Rubin-Bejerano *et al.* 2004). Here, glucose stimulates Ras activity, which in turn inhibits Rim11 (Rubin-Bejerano *et al.* 2004). When active, Rim11 phosphorylates both Ime1 and its binding partner Ume6, which promotes Ime1–Ume6 binding and the transcription of early genes (Malathi *et al.* 1999). Thus, through both pathways the absence of glucose activates Ime1 by relieving its repression.

Ime1 activity is also responsive to the presence or absence of a nitrogen source in the medium. Though less well understood than glucose regulation, the response to nitrogen is at least partially mediated at the transcriptional level (Kassir *et al.* 1988). In addition, the nitrogen-responsive TOR signaling pathway acts post-translationally to control the nuclear localization of Ime1 (Colomina *et al.* 2003).

In addition to these classical regulators of *IME1*, other regulatory factors include the respiration potential of the cell, the storage carbohydrate trehalose, the G1 cyclins, and extracellular pH (Colomina *et al.* 1999; De Silva-Udawatta and Cannon 2001; Jambhekar and Amon 2008). Trehalose promotes Ime1 expression, possibly via the kinase Mck1, while G1 cyclins repress its expression (Colomina *et al.* 1999; De Silva-Udawatta and Cannon 2001). This latter control may help ensure that cells enter the sporulation pathway from early in G1, before G1 cyclins accumulate.

Expression of *IME1* is also regulated by the Rim signaling pathway. *RIM* genes were identified in a screen for mutants defective in *IME2* induction and many of them proved to be components of a single signaling pathway that responds to extracellular pH (Su and Mitchell 1993; Li and Mitchell 1997). The Rim pathway consists of the transmembrane protein Rim21 as well as the protease Rim13, the transcription factor Rim101, and several additional components, including subunits of the ESCRT complex (Su and Mitchell 1993; Boysen and Mitchell 2006; Herrador *et al.* 2010). These cytoplasmic components assemble onto the endosome (Boysen and Mitchell 2006). In response to increases in the pH of the medium, Rim13 becomes activated and cleaves the C-terminal tail of Rim101 (Li and Mitchell 1997; Futai *et al.* 1999). The truncated Rim101 then translocates to the

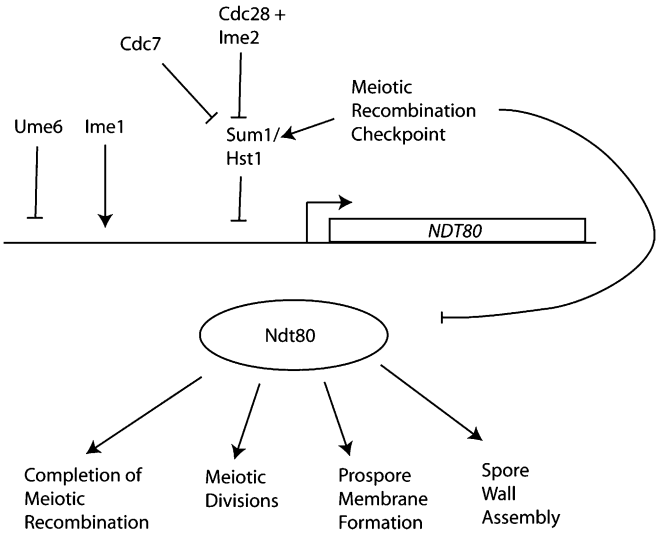

Figure 9 Inputs and outputs to Ndt80 activity. Ndt80 controls entry into the meiotic divisions. Expression is subject to nutritional, cell-cycle, and checkpoint control. Once active, Ndt80 induces multiple, independent downstream pathways.

nucleus to regulate the expression of responsive genes (Li and Mitchell 1997).

The requirement for the Rim pathway may contribute to the concentration dependence of sporulation in liquid medium. Optimal sporulation occurs at a cell density of $\sim 2 \times 10^7$ cells/ml (Fowell 1967). At higher or lower cell concentrations, sporulation efficiency drops off significantly. The basis for this dependence is that cells, prior to initiating sporulation, alkalinize the medium (Hayashi *et al.* 1998; Ohkuni *et al.* 1998). At optimal cell density, the pH of the medium reaches 7 to 8, whereas at lower or higher cell concentrations, the pH remains too acidic or becomes too alkaline. Buffering of the medium at pH 7 bypasses the effects of cell density (Ohkuni *et al.* 1998). Presumably, the RIM pathway is required to monitor pH and translate this information into the regulation of *IME1* expression.

The alkalinization of the medium is caused by the excretion of bicarbonate, which has been shown to be a byproduct of the tricarboxylic acid (TCA) cycle (Ohkuni *et al.* 1998). Thus, increase in extracellular pH is a byproduct of the need for respiration in sporulation medium (which lacks a fermentable carbon source). This pH effect may also help explain the observation that the transcription of *IME1* is regulated by the "respiratory potential" of the cell, though comparison to *rim101Δ* strains suggest that the effect of respiration defective mutants on sporulation is not solely mediated via pH of the medium (Jambhekar and Amon 2008).

IME1 expression controls entry into the sporulation pathway. After transfer to sporulation medium, different cells within a yeast culture vary greatly in the length of time it takes them to sporulate (Deutschbauer and Davis 2005). This cell-to-cell variability results from differences in the time from transfer to the induction of *IME1*, rather than differences in the rate of meiosis or spore formation (Nachman *et al.* 2007). The variation in *IME1* timing likely reflects the diversity of factors that influence its expression.

Transition to meiotic division: control of NDT80

The expression and regulation of *NDT80* constitute the second major control point in the sporulation process (Figure 9). As with *IME1*, induction of *NDT80* requires integration of multiple input signals. As described above, the initial expression of *NDT80* involves both *IME1*-mediated activation and relief of *SUM1*-mediated repression. Relief of *SUM1* repression provides the basis for some controls on *NDT80* expression. For instance, the cell cycle kinases Cdc28 and Ime2 redundantly regulate *NDT80* induction by phosphorylating Sum1 (Ahmed *et al.* 2009; Shin *et al.* 2010). Mutating phosphorylation sites for either kinase has no phenotype, but mutation of both sets of phosphorylation sites on Sum1 blocks the expression of middle genes (Shin *et al.* 2010). In addition, activity of the cell cycle kinase Cdc7 also promotes expression of *NDT80* by relief of Sum1 repression (Lo *et al.* 2008; N. Hollingsworth, personal communication). Multiple cell cycle functions thus impinge on *NDT80* expression.

NDT80 is also subject to nutritional regulation in at least two ways. Its initial induction requires activation by Ime1/Ume6 and so is affected by nutritional controls acting on Ime1 (Pak and Segall 2002a). In addition, Ime2 is also subject to direct regulation by glucose (Purnapatre *et al.* 2005; Gray *et al.* 2008). In the presence of glucose, Ime2 is rapidly degraded via the SCF ubiquitin ligase Grr1 and degradation signals in the Ime2 C terminus (Purnapatre *et al.* 2005; Sari *et al.* 2008). Thus, reintroduction of glucose early in sporulation can block further progression down this developmental pathway, at least in part, by inactivating Ime2.

Regulation of Ndt80 is also the ultimate target of the meiotic recombination checkpoint. Induction of Ndt80 is required for cells to exit from meiotic prophase (Xu *et al.* 1995). Many of the chromosomal events of meiosis I, including introduction of double strand breaks, formation of recombination intermediates, and pairing of homologous chromosomes by the synaptonemal complex occur prior to *NDT80* expression. However, resolution of recombination intermediates and dissolution of the synaptonemal complex require Ndt80-mediated transcription of the *CDC5* kinase (Clyne *et al.* 2003; Sourirajan and Lichten 2008). The checkpoint monitors the progress of meiotic recombination and inhibits the activity of Ndt80 if incomplete recombination products are present (Roeder and Bailis 2000). The mechanism by which Ndt80 is inhibited is not yet well understood but the checkpoint may act at both the transcriptional level through Sum1 as well as at the post-translational level through phosphorylation and inactivation of Ndt80 (Tung *et al.* 2000; Pak and Segall 2002b; Shubassi *et al.* 2003). Thus, cell cycle, nutritional, and checkpoint signals all converge on Ndt80 to control the transition into the middle phase of sporulation.

NDT80 outputs: Expression of *NDT80* leads to multiple independent outputs by the induction of different downstream transcriptional targets (Chu and Herskowitz 1998). These include the completion of meiotic prophase, progression into the meiosis I and II nuclear divisions, and assembly of the prospore membrane and spore wall. For the completion of meiotic prophase, the key regulatory target is *CDC5* (Sourirajan and Lichten 2008). Expression of *CDC5* from an inducible promoter is sufficient to trigger the completion of recombination and breakdown of the synaptonemal complex in an *ndt80Δ* strain, though these cells do not progress into the nuclear divisions of meiosis (Sourirajan and Lichten 2008). The B-type cyclins, in particular Clb1 and Clb4, are necessary for the meiotic divisions and multiple *CLB* genes are induced by *NDT80*, indicating that *NDT80* likely controls meiotic progression through control of cyclin expression (Grandin and Reed 1993; Dahmann and Futcher 1995; Chu and Herskowitz 1998). Interestingly, the activity of Cdc28 in complex with the different B-type cyclins is differentially regulated in meiosis I and meiosis II so that Clb1 plays a more important role in the first division and Clb3 in the second division (Carlile and Amon 2008). Moreover, the major mitotic B-type cyclin, Clb2, is not expressed during meiosis (Grandin and Reed 1993). Thus, some of the B-type cyclins in *S. cerevisiae* have acquired specialized meiotic functions. Many of the gene products required for formation of the prospore membrane are regulated by Ndt80, including the MOP proteins, the proteins of the leading edge complex, and multiple septin genes (Chu *et al.* 1998; Primig *et al.* 2000). Similarly, many of the sporulation-specific genes involved in spore wall formation are part of the *NDT80* regulon and are therefore expressed well before their functions are required (Chu *et al.* 1998; Primig *et al.* 2000). Thus, activation of *NDT80* puts into motion multiple different aspects of the differentiation pathway.

Commitment to sporulation and NDT80: If cells are placed in sporulation medium for a short period of time and then nutrients are reintroduced, they will cease differentiation into spores and return to mitotic growth. After a sufficient time, however, they will become insensitive to the nutrients and complete sporulation. In this case the cells are said to be "committed" to meiosis and sporulation (Ganesan *et al.* 1958; Simchen *et al.* 1972). The molecular event specifying the commitment point has not been defined, but the timing suggests that it is an event driven by *NDT80* or the induction of *NDT80* itself (Horesh *et al.* 1979; Friedlander *et al.* 2006). That is, once *NDT80* has set in motion the multiple events described above, then the cell must complete sporulation before returning to mitotic growth. Interestingly, even though committed cells appear insensitive to nutrients in that they complete meiosis and sporulation, microarray studies reveal that at the transcriptional level they respond as if preparing to enter the mitotic cycle, including the upregulation of ribosomal proteins and the downregulation of both *NDT80* and most *NDT80*-regulated genes (Friedlander *et al.* 2006). How this disconnection between cellular behavior and the transcriptional response to nutrients is achieved remains to be determined.

Commitment to sporulation means not only that cells will complete the process if nutrients are reintroduced, but also if the remaining nutrients in the medium are removed (Davidow *et al.* 1980; Srivastava *et al.* 1983). For example, cells transferred to water 2 hr after being placed in sporulation medium will simply arrest, whereas cells transferred at later time points complete sporulation even though no nutrients are present (Srivastava *et al.* 1983). Under these conditions the cells do not form tetrads (asci with four spores), but rather form dyads (asci with only two haploid spores) (Davidow *et al.* 1980; Srivastava *et al.* 1983). Thus, cells respond to depletion of the carbon source in the sporulation medium not by arresting but by limiting the number of spores that are formed, perhaps to ensure that enough biosynthetic capacity is available to complete the process.

How does the cell regulate the number of spores that are formed? It does so by controlling the spore formation process at its initial step, the assembly of the MOP complex on the SPB (Davidow *et al.* 1980; Nickas *et al.* 2004). In carbon-depleted cells, only two of the four spindle poles form MOPs. As a result, only two prospore membranes and two spores are formed. Analysis of centromere-linked markers revealed that one nucleus from each of the two meiosis II spindles is packaged into the dyad spores (Davidow *et al.* 1980), so that they contain homologous rather than sister chromosomes. For this reason the phenomenon is referred to as nonsister dyad formation.

How does the cell sense carbon depletion? The response is based on the abundance of intermediates in carbon metabolism (Nickas *et al.* 2004). The usual carbon source in sporulation medium is acetate, and depletion of acetate can trigger nonsister dyad formation (Davidow *et al.* 1980). Metabolism of acetate involves both its oxidation in the TCA cycle for energy and its conversion into gluconeogenic precursors for biosynthesis by the glyoxylate cycle. This latter pathway is critical for the cell to monitor carbon availability (Nickas *et al.* 2004). Mutation of genes encoding glyoxylate pathway enzymes results in nonsister dyad formation. Thus, the decision to form dyads represents an attempt by the cell to respond to its biosynthetic potential.

Formation of nonsister dyads involves two separable processes: the reduction in spore number and the choice of which SPBs to modify. The choice of SPB is based on its age. SPBs duplicate prior to spindle formation and their duplication is conservative, so that each spindle has an older (mother) and a younger (daughter) pole (Byers and Goetsch 1974). In response to carbon depletion, the cells preferentially assembly MOPs on the daughter poles (Nickas *et al.* 2004; Taxis *et al.* 2005). Titration of the acetate in the medium can cause formation of monads and triads in addition to dyads (Taxis *et al.* 2005); here, the younger SPBs are still preferred. For example in triad asci, it is the oldest SPB, the one present before cells entered meiosis I, that is avoided (Taxis *et al.* 2005).

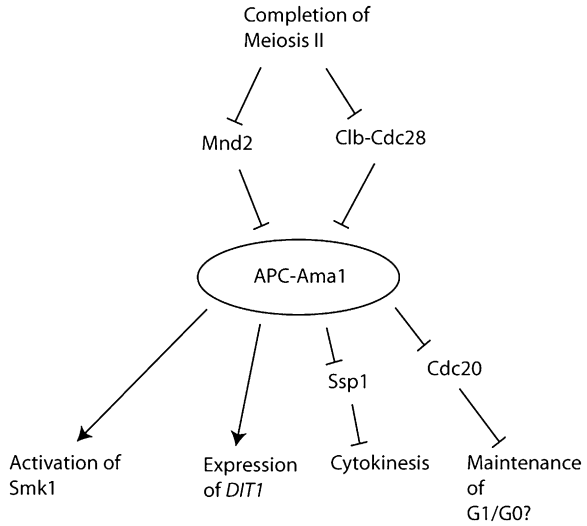

Figure 10 Coordination of meiotic exit with downstream events by APC–Ama1. The completion of meiosis leads to the upregulation of the APC–Ama1 ubiquitin ligase. This complex then triggers downstream events such as cytokinesis, spore wall assembly, and possibly entry into G1 by targeting specific substrates for degradation. Ssp1 and Cdc20 are established targets of APC–Ama1 but the substrates leading to Smk1 activation and *DIT1* expression have yet to be established.

That the choice of SPB is distinct from the reduction in spore number is revealed by mutants of the constitutive outer plaque component, Nud1 (Gordon *et al.* 2006). In *nud1-1* mutants sporulated in carbon-depleted conditions, dyads still form but the ability of the cell to distinguish old and new SPBs is lost and hence the assembly of MOPs becomes random. Thus, even though the cell cannot choose the SPBs properly, it still reduces the spore number. It is not known how the reduction in spore number is achieved. But it is noteworthy that strains heterozygous for deletion of any of the major MOP component genes (*MPC54*, *SPO21*, or *SPO74*) display increased nonsister dyad formation in normal sporulation conditions, suggesting that reduced expression of one or all of these genes could underlie the response (Bajgier *et al.* 2001; Wesp *et al.* 2001; Nickas *et al.* 2003). Indeed, sporulation in limited acetate leads to reductions in the levels of the MOP proteins plus the leading edge proteins Ady3 and Ssp1 (Taxis *et al.* 2005). These are all *NDT80*-regulated gene products, raising the possibility that carbon depletion may trigger a general reduction in expression of the *NDT80* regulon.

Integration of nuclear and cytoplasmic events at the end of meiosis

Induction of *NDT80* sets in motion multiple downstream pathways, including both the nuclear divisions of meiosis and the cytoplasmic events of prospore membrane formation. Surprisingly, once begun there is no apparent feedback control between meiotic events and prospore membrane growth. For example, mutants defective in membrane assembly nonetheless progress through the meiotic divisions with normal kinetics (Nag *et al.* 1997; Bajgier *et al.* 2001). Similarly, the arrest or delay of meiotic events does not induce a corresponding change in membrane growth (Schild and Byers 1980). It is important, therefore, to bring these events back into register before cytokinesis to ensure the proper segregation of nuclei into the spore. The APC and its targeting subunit Ama1 provide this integration (Figure 10).

Though *AMA1* is induced as a pre-middle gene, the activity of APC–Ama1 is restricted by the action of the APC subunit Mnd2 and by Clb–CDK phosphorylation, so that it does not become fully active until late in meiosis II (Oelschlaegel *et al.* 2005; Penkner *et al.* 2005). As described earlier, once APC–Ama1 is active, it leads to degradation of the leading edge protein Ssp1 (though direct Ama1-dependent ubiquitylation of Ssp1 has not been demonstrated) and this serves to link membrane closure to the end of meiosis (Diamond *et al.* 2008). In addition, APC–Ama1 regulates the onset of spore wall synthesis. Induction of the mid-late gene *DIT1* is blocked in *ama1*Δ cells, and this is not a consequence of the failure to degrade Ssp1 as *DIT1* induction is not affected in cells expressing the nondegradable form of Ssp1 (Coluccio *et al.* 2004a; J. S. Park, personal communication). Additionally, *AMA1* is required for the activation of the Smk1 kinase that regulates spore wall assembly (McDonald *et al.* 2005). Again, this effect on activation is independent of Ssp1 degradation (E. Winter, personal communication). Whether the effects on *DIT1* expression and Smk1 activation are linked will require identification of the relevant APC–Ama1 substrate, but these results indicate that Ama1 also links spore wall assembly to meiotic exit separately from cytokinesis.

The other demonstrated *in vivo* target of APC–Ama1 is a second APC activator, Cdc20 (Tan *et al.* 2010). Cdc20 is necessary for meiosis, but at the end of meiosis it is degraded in an Ama1-dependent fashion (Tan *et al.* 2010). Nevertheless, sporulation is normal when Cdc20 is stabilized by mutation of two consensus degradation motifs, indicating that turnover is not necessary for meiotic progression (Tan *et al.* 2010). In vegetative cells, Cdc20 degradation in late mitosis and early G1 is important for maintaining the order of cell cycle events (Huang *et al.* 2001). Thus, APC–Ama1-mediated degradation of Cdc20 at meiotic exit might help the spore enter or maintain a G0 or early G1 state. Ama1 thus acts to coordinate the completion of meiotic divisions with turnover of meiosis-specific proteins, cytokinesis, induction of spore wall synthesis, and entry into a quiescent cell cycle stage.

Functions of the Spore: Dispersal to New Environments

Sporulation is a starvation response. In a similar environment, haploid *S. cerevisiae* simply cease division, whereas diploid cells not only package themselves into a specialized form but link this process to meiosis. The evolutionary advantage of this elaborate response is not immediately

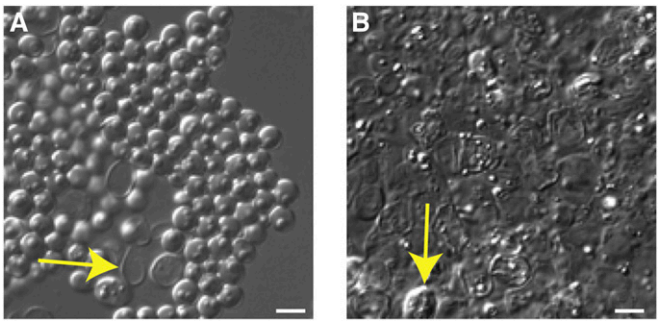

Figure 11 Spores survive passage through the insect gut. (A) Spores in the frass of *Drosophila melanogaster*. Arrow indicates a lysed vegetative cell among the spores. Bar, 4 μm. (B) Vegetative cells in the frass of *D. melanogaster*. Arrow indicates a rare intact vegetative cell among the lysed cells. Bar, 4 μm.

apparent. Despite our rich understanding of the cell biology of *S. cerevisiae*, there is relatively little information on its ecology. *S. cerevisiae* has been cultured from a variety of plants, such as grapes and oak tree exudates (Naumov *et al.* 1998; Mortimer and Polsinelli 1999). In these environments it presumably must interact with a variety of insects. In particular, yeasts are a favorite food of *Drosophilid* species and *S. cerevisiae* has been cultured from the crops of *Drosophila* captured in the wild (Phaff *et al.* 1956; Begon 1986).

Given that the spore wall is the major unique feature of the spore, what is its function? Although the spore wall confers resistance to a variety of insults, common laboratory treatments such as exposure to ether vapor or brief incubation at 55° seem unlikely to reflect real environmental conditions (Dawes and Hardie 1974; Briza *et al.* 1990a). Furthermore, for most treatments designed to mimic natural environmental extremes, such as repeated freeze–thaw cycles or dessication, spores are not more resistant than stationary phase vegetative cells (Coluccio *et al.* 2008). Notably, however, in addition to ether and heat, spores are significantly more resistant to treatments with mild base or acid as well as degradative enzymes (Coluccio *et al.* 2008). These results suggest that yeast spores may be adept at surviving predation by insects, as they are likely to encounter both digestive enzymes and altered pH in the insect gut (House 1974; Dow 1992). Indeed, spores are roughly 10 times more likely than vegetative cells to survive passage through the gut of *Drosophila melanogaster* (Reuter *et al.* 2007; Coluccio *et al.* 2008) (Figure 11). Importantly, this increased survival is absolutely dependent on the chitosan and dityrosine layers of the spore wall (Coluccio *et al.* 2008).

These findings provide a rationale for formation of the spore wall. Upon starvation, yeast cells differentiate into a specialized cell type (a spore) that will allow them to move into a new environment by being consumed and then deposited elsewhere by an insect vector. Dispersal of yeasts by *Drosophila* has been seen in ecological studies and is directly analogous to the manner in which some plant seeds are dispersed by avian vectors (Gilbert 1980; Howe 1986). In this view, the function of the yeast spore is not survival in adverse environments *per se*, but rather dispersal from adverse environments.

While this view can explain why the spore wall is built under starvation conditions, it leaves open the question of why sporulation is linked to meiosis. Why not simply assemble a more robust coat around the cell without meiosis? One possible answer is the increased genetic diversity provided by meiotic recombination and independent assortment. From the viewpoint of the population, increasing genetic diversity prior to dispersal increases the chance that one or more of the cells will have a high fitness in the newly encountered environment (Lenormand and Otto 2000). Thus, linking meiosis to dispersal may provide a selective advantage to the species as cells move to new environments.

Maintaining genetic diversity in the population is a particular issue for *S. cerevisiae* because they are homothallic; *i.e.*, haploid cells can switch mating type and mate with their own progeny to produce diploids that are homozygous at every locus (except *MAT*) (Herskowitz and Jensen 1991). As a result, the heterozygosity and genetic diversity of the parental diploid is lost. Perhaps to counter this effect, spores display high levels of outbreeding (mating between spores from different asci) after passage through *Drosophila* (Reuter *et al.* 2007), and a related tendency even without passage through insects suggests additional mechanisms may promote outbreeding (Murphy and Zeyl 2010). The drive to maintain genetic diversity also provides a rationale for the formation of nonsister dyads. By capturing each set of homologous chromosomes rather than sister chromatids, these asci maintain the maximum genetic diversity within their two spores (Taxis *et al.* 2005). While speculative, these notions highlight the important role that more information on the natural history and ecology of *S. cerevisiae* can play in interpreting the cell biology and behavior of the organism.

Perspectives

Though much has been learned in the last 15 years about the cell biology of spore formation, many important issues remain to be explored in all aspects of the process. In membrane growth, how assembly of the MOP is regulated by metabolic signals and, in particular, how the cell distinguishes the age of the different SPBs are open questions. The answers may have implications for higher cells where differentiation between mother and daughter centrioles is important in processes such as ciliogenesis and asymmetric cell division. Additionally, understanding how the closure of the membrane is achieved should provide broader insight into mechanisms of cytokinesis.

With respect to the spore wall there is a great deal to learn about the regulatory pathways that coordinate construction. While a rudimentary outline has begun to emerge, understanding the details should reveal novel MAPK and Ste20 kinase regulated-signal transduction pathways.

Finally, the process of ascal maturation is unusual for yeast in that it is a nearly unexplored morphogenetic event. As with other aspects of yeast biology, it is likely to prove a complex and interesting process.

Acknowledgments

I thank Nancy Hollingsworth, Peter Pryciak, and members of the Neiman laboratory for comments on the manuscript and for helpful discussions. I am deeply grateful to Cindi Schwartz for her help with the tomography shown in Figure 3. I am indebted to Erin Mathieson, Susan Van Horn, and Alison Coluccio for the EM images used and to Jae-Sook Park, Hiroyuki Tachikawa, Nancy Hollingsworth, and Ed Winter for communicating results prior to publication. Work in the Neiman laboratory is supported by National Institutes of Health grants R01GM072540 and P01GM088297.

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Communicating editor: P. Pryciak