The fission yeast spore is coated by a proteinaceous surface layer comprising mainly Isp3

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ABSTRACT **The spore is a dormant cell that is resistant to various environmental stresses. As compared with the vegetative cell wall, the spore wall has a more extensive structure that confers resistance on spores. In the fission yeast *Schizosaccharomyces pombe*, the polysaccharides glucan and chitosan are major components of the spore wall; however, the structure of the spore surface remains unknown. We identify the spore coat protein Isp3/Meu4. The *isp3* disruptant is viable and executes meiotic nuclear divisions as efficiently as the wild type, but *isp3Δ* spores show decreased tolerance to heat, digestive enzymes, and ethanol. Electron microscopy shows that an electron-dense layer is formed at the outermost region of the wild-type spore wall. This layer is not observed in *isp3Δ* spores. Furthermore, Isp3 is abundantly detected in this layer by immunoelectron microscopy. Thus Isp3 constitutes the spore coat, thereby conferring resistance to various environmental stresses.**

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INTRODUCTION

The spores of yeasts are quiescent cells that are highly resistant to various stresses, such as heat, digestive enzymes, and organic solvents (Egel, 1977; Kupelic *et al.*, 1997). This stress resistance of spores is thought to be attributable to the spore wall, which is more extensive than the vegetative cell wall (Yoo *et al.*, 1973; Kreger-Van Rij, 1978). The fission yeast *Schizosaccharomyces pombe* initiates a sporulation program when challenged with nutrient starvation (Yoo *et al.*, 1973; Shimoda and Nakamura, 2004). Sporulation consists of two overlapping processes—meiosis and spore morphogenesis. In the latter process, double-layered intracellular membranes called forespore membranes (FSMs), which become the spore plasma membrane, are newly formed during meiosis II (Yoo *et al.*, 1973). FSMs expand by the fusion of membrane vesicles and eventually encapsulate a haploid nucleus generated by two rounds of meiotic nuclear division, producing the membrane-bound precursor of the spore, the prespore (Yoo *et al.*, 1973; Hirata and Tanaka, 1982; Nakamura *et al.*, 2001, 2008). After capture of nuclei within FSMs, formation of the spore wall proceeds by deposition of the spore wall materials in the space between the inner and outer membranes of the FSM (Yoo *et al.*, 1973; Hirata and Tanaka, 1982). Finally, the inner layer of the FSM becomes the spore plasma membrane, while the outer layer of the membrane autolyzes. Mature spores are then liberated from an ascus upon autolysis of the ascus walls (Tanaka and Hirata, 1982).

The budding yeast *Saccharomyces cerevisiae* essentially sporulates in a way similar to that of *S. pombe* (reviewed by Neiman, 2011). In *S. cerevisiae*, formation of the spore wall has been well characterized. The spore wall of *S. cerevisiae* is composed of four layers of different polymers (Smits *et al.*, 2001). The two inner layers consist mainly of mannoproteins and glucans, and their chemical composition is very similar to that of the vegetative cell wall (Kreger-Van Rij, 1978). The third and fourth layers are specific to the spore and comprise chitosan and a dityrosine-containing polymer, respectively (Briza *et al.*, 1986, 1988). These two outer wall layers primarily provide the enhanced resistance of the spore to various environmental stresses (Briza *et al.*, 1986, 1990; Coluccio *et al.*, 2008). About half of the mass of the dityrosine layer comprises the nonpeptide *N,N'*-bisformyldityrosine, a highly fluorescent dimer of tyrosine (Briza *et al.*, 1994). Dityrosine molecules are synthesized in the cytoplasm of the maturating spore by the action of the *DIT1* and *DIT2* genes (Briza *et al.* 1994), which encode a formyltransferase and a member of the cytochrome P450 family protein, respectively

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Abbreviations used: CBB, Coomassie brilliant blue; FSM, forespore membranes; GFP, green fluorescent protein; IgG, immunoglobulin G; SEM, scanning electron microscope.

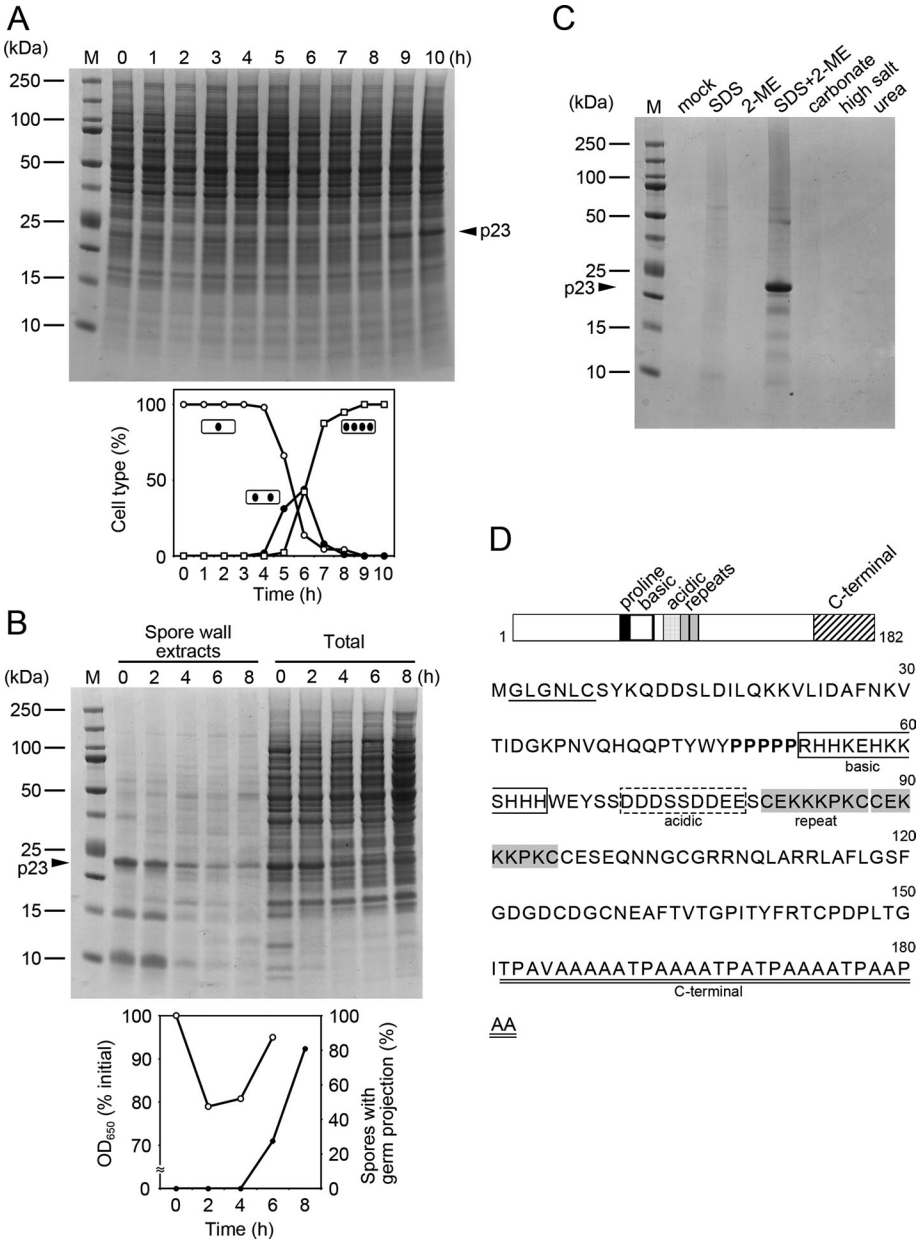


FIGURE 1: p23/Isp3 is a major spore wall protein. (A) Changes in the abundance of p23 during sporulation. Diploid cells harboring *pat1* (JZ670) were allowed to proceed through synchronous meiosis. Aliquots were removed at hourly intervals, the protein extracts were analyzed by SDS–PAGE, and the gel was stained with CBB. The progression of meiosis was monitored by 4′,6-diamidino-2-phenylindole staining. One, two, and four dots in rectangles represent mononucleate, binucleate, and tetranucleate cells, respectively. (B) Changes in the abundance of p23 during germination. Gradient-purified spores (L968) were inoculated into YE medium at a cell density of OD_{650} = 0.5 and then incubated at 28°C. The progression of germination was monitored by changes in the optical density of the cell suspension at 650 nm and morphological changes during spore germination (Nishi *et al.*, 1978). Symbols: open circles, optical density of the cell suspension; closed circles, spores with a germ projection. (C) Extraction of p23 from spores. Isolated spores were extracted with either 0.1 M Tris-HCl (pH 7.5) (mock), 1% SDS, 5% β-mercaptoethanol (2-ME), 1% SDS + 5% β-mercaptoethanol (SDS + 2-ME), 0.1 M sodium carbonate (pH 11.5) (carbonate) and 0.5 M NaCl (high salt), or 4 M urea. The extracts were analyzed by SDS–PAGE, and the gel was stained with CBB. (D) Schematic diagram and deduced amino acid sequence of Isp3. The residues identified by Edman degradation are underlined. The location of the proline cluster is shown in bold. The regions rich in basic and acidic residues are boxed with a solid line and a dashed line, respectively. Two repeat sequences are shaded. The C-terminal alanine-rich region is double underlined.

(Briza *et al.*, 1994). Dityrosine is exported from the cytosol by the action of a dedicated multidrug resistance 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.*, 1990). Although the genes directly involved in dityrosine polymerization and incorporation into the spore wall have not been identified, a recent study using a genetic approach revealed a network of genes that function redundantly to control synthesis of the dityrosine layer (Lin *et al.*, 2013).

As compared with *S. cerevisiae*, little is known about the chemical composition of spore walls in *S. pombe*. Immunoelectron microscopy has shown that 1,3-β-glucan is present at the inner layer of the spore wall (Klis, 1994; Tougan *et al.*, 2002). Mature spores are not formed in *mok12* and *mok13* (encoding α-glucan synthases), *bgs2* (β-glucan synthase), *chs1* (chitin synthase), or *cda1* (chitin deacetylase) mutants (Arellano *et al.*, 2000; Liu *et al.*, 2000; Martin *et al.*, 2000; Matsuo *et al.*, 2005; Garcia *et al.*, 2006). These data indicate that, as in *S. cerevisiae*, glucan and chitosan are major components of the spore wall in *S. pombe*. In contrast, dityrosine is not detected in sporulated cultures from *S. pombe* (Prillinger *et al.*, 1997). Furthermore, homologues of genes responsible for synthesis of the dityrosine layer (i.e., *DIT1*, *DIT2*, and *DTR1*) are not conserved in *S. pombe*. Therefore the structure of the spore surface seems to essentially differ between these two yeasts. However, the composition of the outermost layer of the spore wall of *S. pombe* remains unknown.

The 23K protein (hereafter referred to as p23) was previously found to be the most abundant polypeptide in the spore wall fraction (Shimoda, 1983), suggesting that it plays an important role in spore wall formation. The aim of this study was to characterize the p23 polypeptide and to investigate its function within the fission yeast spore by a series of genetic and microscopic approaches.

RESULTS

p23 is encoded by *isp3*$^+$

To determine exactly when the rise in p23 level occurs during sporulation, we used the *pat1-114* temperature-sensitive strain, which enters meiosis in a highly synchronous manner when it is shifted to its restrictive temperature (Iino *et al.*, 1995). The amount of p23 began to increase ~9 h after induction and became most abundant at 10 h, when the cells had completed meiosis (Figure 1A). We also examined the abundance of p23

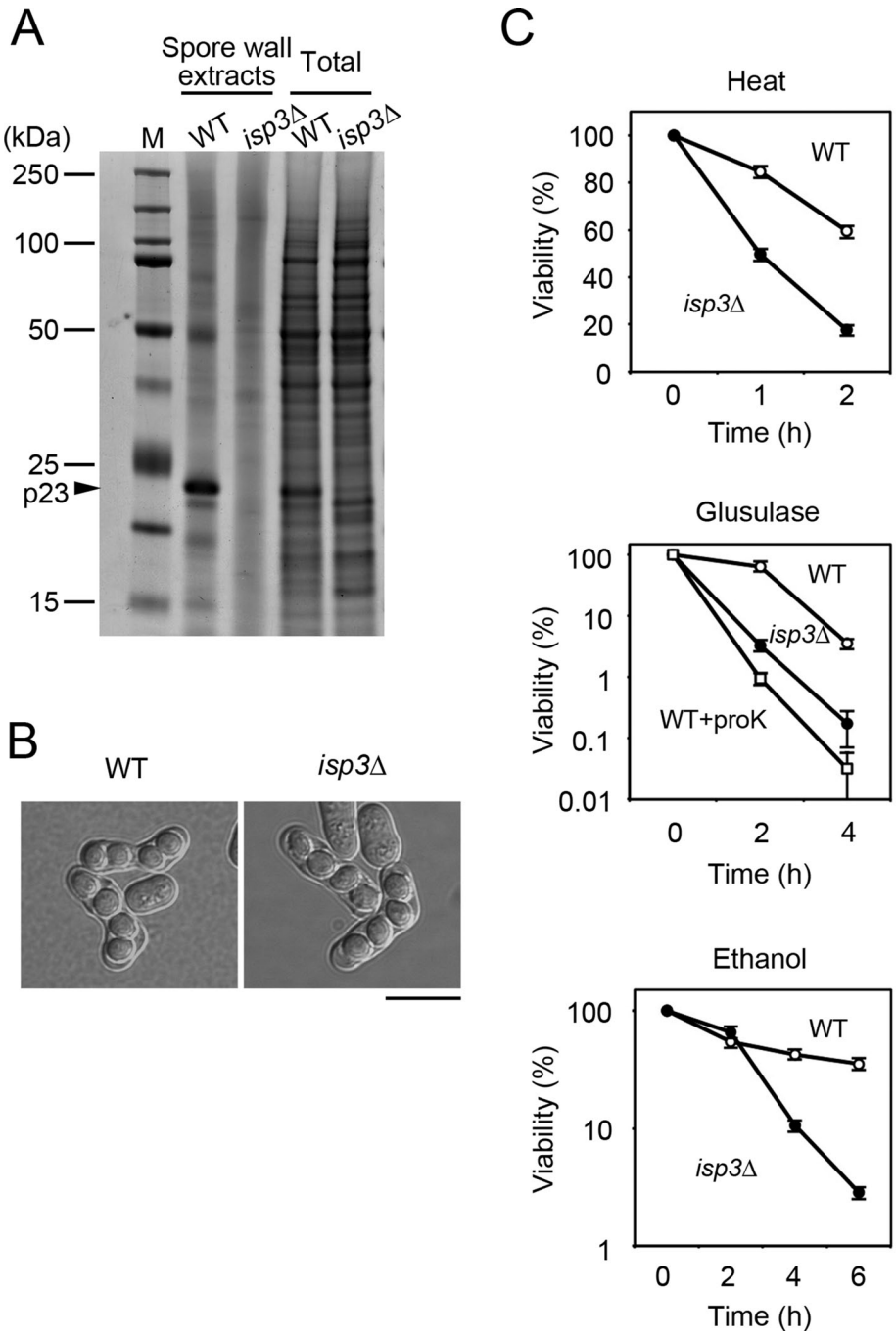


FIGURE 2: Phenotype of *isp3Δ* mutants (A) SDS–PAGE pattern of proteins from isolated spores of wild-type (L968) and *isp3Δ* (KN3). Spore wall extracts and whole-spore homogenates were subjected to SDS–PAGE, and the gel was stained with CBB. (B) Differential interference contrast microscopic images of wild-type and *isp3Δ* asci. Wild-type (L968) and *isp3Δ* (KN3) were sporulated on SSA medium at 28°C for 2 d. Scale bar: 10 μm. (C) Sensitivity to various stresses of wild-type (L968) and *isp3Δ* (KN3) spores. Spores purified by Urografin gradient were assayed by plating onto YEA medium after exposure to heat (55°C), 6% (vol/vol) glusulase (with or without pretreatment with 0.5 mg/ml of proteinase K for 2 h), or 30% ethanol for the indicated times.

during germination. The amount of p23 immediately decreased when spores were incubated in complete medium (YE; Figure 1B), supporting the notion that p23 is a spore-specific protein. A previous study showed that p23 is extracted from the spore wall by treatment with SDS and β-mercaptoethanol (Shimoda, 1983). We found, however, that p23 was not extracted by either SDS or β-mercaptoethanol alone. Moreover, alkaline, high-salt, and urea treatment did not solubilize p23 (Figure 1C). Taken together, these data indicate that p23 is firmly associated with the spore wall.

For determination of the gene that encodes p23, the spore wall fraction was separated by SDS–PAGE, and the 23K band from the gel was partially digested with V8 protease and fractionated by electrophoresis according to a previously reported method (Cleveland *et al.*, 1977). The six N-terminal amino acid residues of the peptide digests were sequenced by Edman degradation. The amino acid sequence obtained, GLGNLC, was searched against the *S. pombe* genome sequence database, which demonstrated that p23 is encoded by *isp3*$^{+}$*/meu4*$^{+}$. Although *isp3*$^{+}$*/meu4*$^{+}$ was originally identified as a meiosis–up-regulated gene by subtractive screening (Sato *et al.*, 1994; Watanabe *et al.*, 2001), its detailed function in sporulation remains unclear.

The predicted Isp3 protein is composed of 182 amino acids and has a molecular mass and isoelectric point of 19.7 kDa and 7.1, respectively. The Isp3 protein has a number of characteristic features, such as a five-proline cluster, a basic amino acid–rich region, an acidic amino acid–rich region, and two repeat sequences rich in basic amino acids (Figure 1D). Furthermore, the C-terminal region of Isp3 is rich in alanine and has five threonine–proline–alanine repeats. However, Isp3 exhibits no similarity to other known proteins.

Isp3 is important for the formation of functional spores

For investigation of the biological function of *isp3*$^{+}$, a null mutant was created by conventional gene disruption. The 23K band disappeared in *isp3Δ* spores (Figure 2A), indicating that *isp3*$^{+}$ indeed encodes p23. A previous study reported that *isp3Δ* cells show decreased spore formation ability (Sato *et al.*, 1994). Unexpectedly, we could not find any defects in the progression of meiotic nuclear division (Supplemental Figure S1) or spore formation rate (Supplemental Table S1). We presume that this discrepancy may be due to differences in strain background or experimental details. Furthermore, the *isp3Δ* mutation did not affect the shape, size, or germination rate of spores (Figure 2B and Table S1). Mature *S. pombe* spores are refractile under a phase-contrast microscope (Shimoda, 1980), and this refractility is suggested to be dependent largely on a major component of the spore. However, *isp3Δ* spores did not show altered refractility.

Normal mature spores are resistant to various stresses, including heat, digestive enzymes, and organic solvents (Egel, 1977; Shimoda,

1980). To determine whether *isp3Δ* mutants form functional spores, we examined their sensitivity to 55°C, 6% (vol/vol) glusulase, and 30% ethanol. Wild-type spores showed very high levels of tolerance (Figure 2C). By contrast, the viability of *isp3Δ* spores was reduced by at least threefold after 2 h of incubation at 55°C. Moreover, the resistance of *isp3Δ* spores to glusulase and ethanol was approximately an order of magnitude lower than that of wild-type spores (Figure 2C). Thus Isp3 is important for the formation of functional spores.

The outermost layer is not formed in *isp3Δ* spores

Given that *isp3Δ* spores showed altered sensitivity to various stresses, we considered that Isp3 may be involved in spore wall formation. Therefore we examined the fine structure of the spore wall of *isp3Δ* in more detail. The *S. pombe* spore has a characteristic surface structure from which many spikes project outward (Nakamura *et al.*, 2004). The surface appearance of *isp3Δ* spores did not differ from that of wild-type spores under a scanning electron microscope (SEM; Figure 3A). However, the *isp3Δ* spores seemed to be abnormally adhesive: that is, debris, probably derived from ascus wall or medium, was often attached to the *isp3Δ* spores (Figure 3A), suggesting that Isp3 might confer physiochemical properties on the spore surface in addition to stress resistance.

Both a previous study and genomic data predict that *S. pombe* lacks a dityrosine layer (Prillinger *et al.*, 1997). Because the dityrosine layer of *S. cerevisiae* has a strong bluish fluorescence (Briza *et al.*, 1986; Figure 3B), we directly observed *S. pombe* spores by fluorescence microscopy. As predicted, the bluish fluorescence was not detected in *S. pombe* spores (Figure 3B). These data indicate that the composition of the outermost layer of *S. pombe* is considerably different from that of *S. cerevisiae*. We therefore examined the *S. pombe* spores by transmission electron microscopy. In wild-type spores, the outermost surface of the spore wall, which appeared as a thin and electron-dense layer, surrounded an underlying more diffuse layer, probably composed of polysaccharides (Figure 3C). Electron-dense structures were initially detected on the surface of the forming wild-type spores; they then seemed to expand and eventually fuse with each other to form a layer (Figure 3D). In contrast, electron-dense layers were not observed on the surface of *isp3Δ* spores (Figure 3C). Taken together, these data indicate that Isp3 plays an important role in formation of the outermost layer of the spore wall.

A previous study has shown that 1,3-β-glucan is present in the inner part of the spore wall (Klis, 1994). Immunoelectron microscopy using an anti–1,3-β-glucan antibody showed that there was almost no difference between wild-type and *isp3Δ* spore walls (Figure 3E). This observation indicates that Isp3 is not required for proper localization of 1,3-β-glucan in the spore wall and also supports the notion that Isp3 contributes to formation of the outermost layer.

Isp3 is a major component of the outermost layer of the spore wall

Because it is present abundantly in the spore wall, we predicted that Isp3 is a major component of the outermost layer. First, we examined the sensitivity of *S. pombe* spores to protease treatment. As shown in Figure 2C, the sensitivity of spores to glusulase was dramatically enhanced by pretreatment with proteinase K, supporting the notion that *S. pombe* spores are coated by a proteinaceous layer. Next we constructed strains in which green fluorescent protein (GFP)-tagged *isp3*$^{+}$ was chromosomally integrated and driven by its own promoter. The KN7 strain expressing Isp3-GFP was incubated in MM–N medium to induce sporulation. Western blot analysis showed that the Isp3-GFP protein was the expected size and was highly induced during sporulation (Figure 4A), consistent with the p23 results (Figure 1A) and previous transcriptional analyses (Watanabe *et al.*, 2001; Mata *et al.*, 2002). Using this fusion gene, we observed localization of Isp3-GFP in asci. As shown in Figure 4B, Isp3-GFP was observed at the spore rim with a bright signal.

Next we used immunoelectron microscopy to determine the precise localization of Isp3 in the spore wall. Thin sections of cells (KN7) undergoing sporulation were labeled with anti-GFP antibody and detected by immunogold-conjugated secondary antibody. Consistent with the results of fluorescence microscopy, the label was confined to an area on the outermost layer (Figure 4C). Thus these data indicate that Isp3 is a major component of the outermost layer of the spore wall. Hereafter, we refer to this layer as the "Isp3 layer." If the Isp3 layer is exposed on the surface of the spore, it should be accessible to antibodies. We therefore carried out an immunofluorescence microscopic analysis in which spores expressing Isp3-GFP were incubated with anti-GFP and Cy3-conjugated secondary antibodies without fixation and permeabilization. As shown in Figure 4D, a strong fluorescence signal of Cy3 surrounding the Isp3-GFP spores was observed; by contrast, no signal was detected on wild-type spores, which did not express the fusion protein, indicating that Isp3 is indeed localized on the spore surface.

In *S. cerevisiae*, formation of the dityrosine layer is dependent on the second outer layer composed of chitosan, which is produced from chitin by chitin deacetylase (Pammer *et al.*, 1992). *S. pombe* has two chitin synthases, Chs1 and Chs2, and a chitin deacetylase, Cda1. These genes are essential for proper spore formation (Arellano *et al.*, 2000; Matsuo *et al.*, 2004, 2005). We therefore examined the abundance of p23 in mutants of these genes. As shown in Figure 5A, the amount of p23 decreased in both *chs1Δchs2Δ* and *cda1Δ* spores. Although four rings of Isp3-GFP were observed, the signal intensity differed among the spores within the *chs1Δchs2Δ* and *cda1Δ* asci (Figure 5B), suggesting that chitosan is important for proper assembly of the Isp3 layer.

Behavior of Isp3 during sporulation

To determine the way by which Isp3 is assembled on the spore surface, we examined the localization of Isp3-GFP over time. To monitor the progression of sporulation, we tagged the *psy1*$^{+}$ gene, which encodes an FSM-resident protein, with mCherry and performed two-color imaging (Figure 6 and Supplemental Movie S1). No Isp3-GFP signal was observed before the start of meiosis II. During metaphase II, when the mCherry-Psy1 fluorescence signal was visible as a pair of semicircles, Isp3-GFP appeared within the regions surrounded by FSMs. The Isp3-GFP signal persisted there during meiosis II. After the FSMs closed to form a sac (39 ± 1.3 min), Isp3-GFP was observed as rings that overlapped with the FSMs and became brighter. A magnified view of the spore shows that the Isp3-GFP signal appeared outside the mCherry-Psy1 (Figure 6B). Relocalization of Isp3 to the spore rim was coincident with the appearance of spores as viewed by differential interference contrast microscopy (Figure 4B). Thus these data indicate that Isp3 is exported to the surface from the cytoplasm of maturing spores.

DISCUSSION

In the present study, we have demonstrated that *S. pombe* spores are coated by a layer mainly comprising the Isp3 protein. Treatment of spores with various reagents, including SDS and alkali, did not elute Isp3. Furthermore, Isp3 was present abundantly and was detected as an electron-dense structure by electron microscopy. Similar to what is seen with the removal of the dityrosine layer of *S. cerevisiae*, *S. pombe* spores lacking the Isp3 layer showed sensitivity to various stresses. Taken together, these facts indicate that Isp3 forms

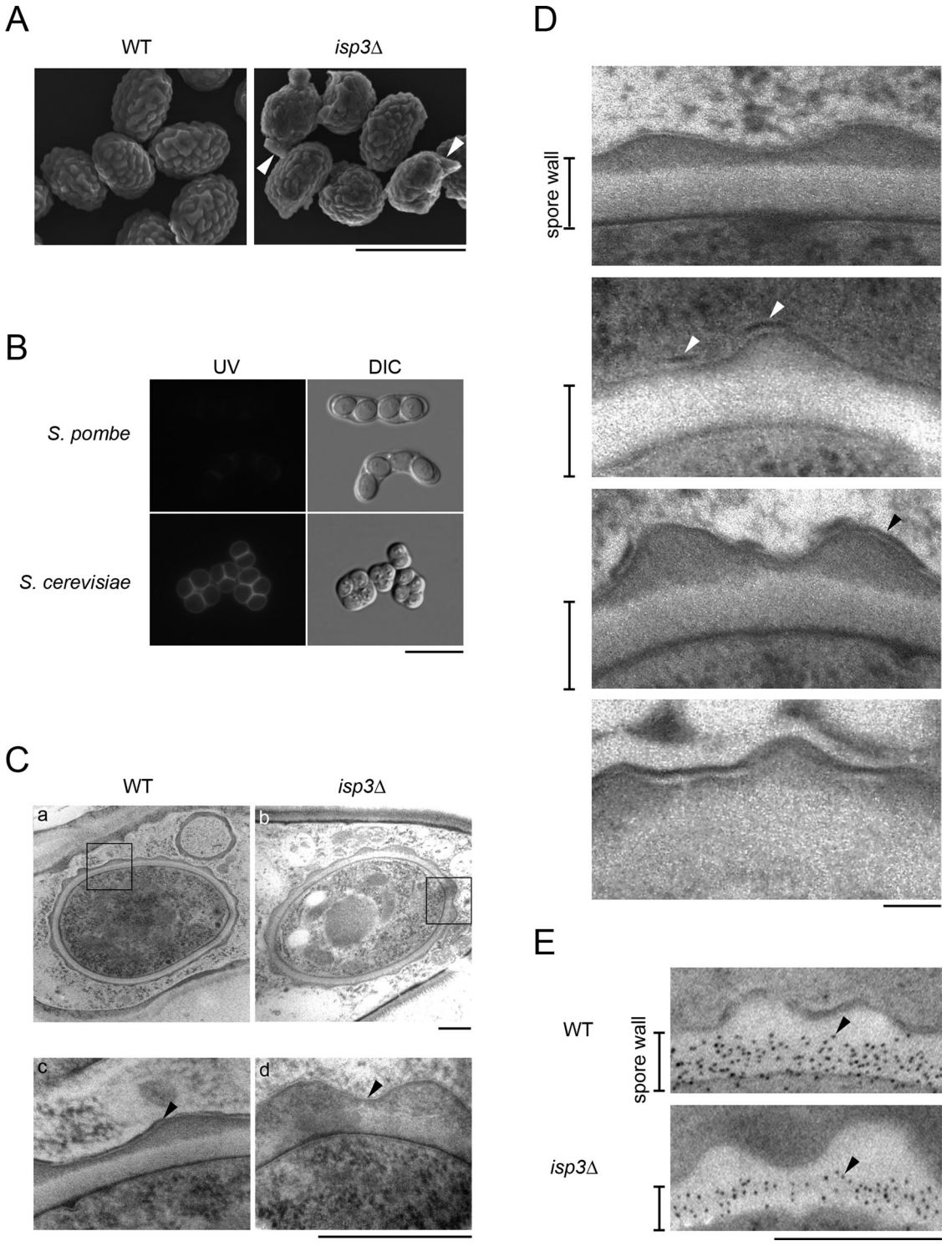


FIGURE 3: The outermost layer is defective in *isp3Δ* spores. (A) SEM images of wild-type (L968) and *isp3Δ* (KN1) spores. Arrowheads indicate debris probably derived from the ascus wall or medium. Scale bar: 5 μm. (B) Natural fluorescence of spores from *S. pombe* and *S. cerevisiae*. *S. pombe* (L968) and *S. cerevisiae* (KYC800) were sporulated and observed by fluorescence microscopy as described previously (Briza *et al.*, 1986). Scale bar: 10 μm. (C) Electron micrographs of representative examples of wild-type (L968) and *isp3Δ* (KN3) spores. (c) and (d) are magnified images of the boxed regions in (a) and (b), respectively. Arrowheads indicate the surface of the spore. Scale bars: 500 nm. (D) Development of the electron-dense layer of wild-type (L968) spores (shown by arrowheads). Scale bars: 100 nm. (E) Distribution of 1,3-β-glucan in wild-type (L968) and *isp3Δ* (KN3) spores imaged by immunoelectron microscopy using an antibody against 1,3-β-glucan and a gold-labeled secondary antibody. Arrowheads indicate one of the gold particles labeling the 1,3-β-glucan for each panel, respectively. Scale bar: 500 nm.

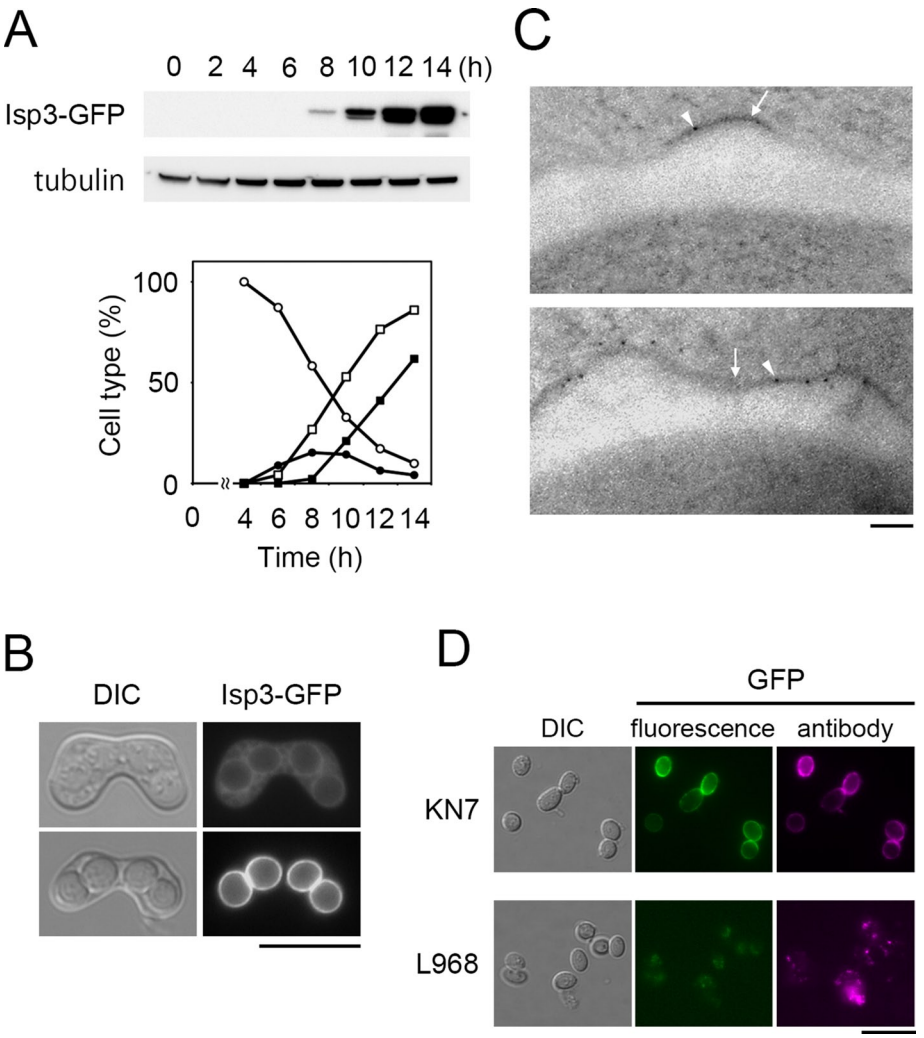


FIGURE 4: Isp3 is a component of the outermost layer of the spore wall. (A) Changes in the abundance of Isp3-GFP during sporulation. KN7 cells expressing Isp3-GFP were precultured overnight in MM+N medium and then transferred to MM-N sporulation medium. Aliquots were removed at every 2 h, and the protein extracts were subjected to Western blot analysis with mouse anti-GFP antibody. α-Tubulin was used as a loading control. Zygotes were classified based on the number of nuclei per cell. For each sample, ~200 zygotes were counted. The percentage of asci is also shown. Open circles, mononucleate zygotes; closed circles, binucleate zygotes; open squares, tri- or tetranucleate zygotes; closed squares, asci. (B) Localization of Isp3-GFP. Wild-type cells (KN14) expressing Isp3-GFP were sporulated on SSA at 28°C for 1 d. Scale bar: 10 μm. (C) Immunoelectron localization of Isp3-GFP to an electron-dense layer of the spore wall. The primary mouse anti-GFP antibody was detected by using 10-nm gold particles coupled to a specific secondary anti-mouse IgG. Arrowheads and arrows indicate a gold particle labeling Isp3-GFP and the electron-dense layer, respectively. Scale bar: 100 nm. (D) Isp3 is a spore surface protein. L968 and KN7 cells expressing Isp3-GFP were sporulated on MEA at 28°C for 1 d. Cells were processed for immunofluorescence microscopy with anti-GFP antibody followed by Cy3-conjugated anti-mouse IgG antibody. Scale bar: 10 μm.

a robust and insoluble structure on the spore surface, thereby conferring resistance to various stresses.

The surface morphology of *S. pombe* spores differs considerably from that of *S. cerevisiae*. Under an SEM, many spikes are observed on the surface of *S. pombe* spores (Figure 3A; Nakamura *et al.*, 2004), whereas *S. cerevisiae* spores have many wrinkles (Coluccio *et al.*, 2004). Because these wrinkles are not observed in *dit1* mutants, the dityrosine layer may be strongly related to formation of the wrinkles (Coluccio *et al.*, 2004). In contrast, the spikes on *S. pombe* spores formed before the Isp3 layer (Figure 3D) and were observed in *isp3Δ* spores (Figure 3A). Therefore these spikes may form independently of the Isp3 layer. We previously reported that an ADAM family metalloprotease Mde10 is involved in spike formation (Nakamura *et al.*, 2004). At present, however, the molecular function of Mde10 in regulating the spike formation remains unknown.

The *S. cerevisiae* dityrosine layer consists of neither protein nor polysaccharide (Briza *et al.*, 1990). In contrast, our results show that *S. pombe* spores are covered by a proteinaceous surface layer. Spore surface proteins have been well characterized in the bacterium *Bacillus subtilis*. Similar to yeasts, *Bacillus* spores show high resistance to various stresses. They have three distinct structures: the core, cortex, and coat. The *Bacillus* spore coat is a complex structure composed of several layers containing more than 50 proteins and is important in spore resistance to certain chemicals and lytic enzymes but has little or no role in resistance to heat, radiation, and other chemicals (reviewed by Setlow, 2006). In contrast, the *S. pombe* Isp3 layer might comprise a single or few proteins and is important for resistance to various stresses (lytic enzymes, heat, and ethanol). Furthermore, Isp3 shares no significant sequence similarity with any bacterial spore coat proteins.

The behavior of Isp3 is reminiscent of the fungal proteins hydrophobins. Hydrophobins are abundant cell-surface proteins that are ubiquitously expressed in fungi and can spontaneously form amphiphilic layers on hydrophilic/hydrophobic interfaces. Via this unique ability, hydrophobin mediates fungal attachment to hydrophobic surfaces and thus plays multiple roles in various cellular processes, including formation of the fruiting body and aerial hyphae (reviewed by Wessels, 1997; Wosten and de Vocht, 2000; Linder *et al.*, 2005). Interestingly, some hydrophobins are major components of the rodlet, a hydrophobic sheath that covers fungal spores, and are essential for its formation (Bell-Pedersen *et al.*, 1992; Stringer and Timberlake, 1995). These notions suggest that Isp3 functions in a manner similar to the spore coat hydrophobins. However, Isp3 shares no similarity with any known hydrophobins. Moreover, in an assay of microbial adhesion to hydrocarbons (Holder *et al.*, 2007) the *isp3Δ* mutation did not affect hydrophobicity of the spore surface (Fukunishi and Nakamura, unpublished data). Although there are few data about involvement of spore coat hydrophobins in stress resistance, in the case of entomopathogenic fungus, spores lacking hydrophobin are not sensitive to but are more resistant to heat as compared with wild-type spores (Zhang *et al.*, 2011). Thus we presume that Isp3 and spore hydrophobins have distinct roles: the primary role of hydrophobins may be to render the spore surface hydrophobic and water-resistant, thereby facilitating

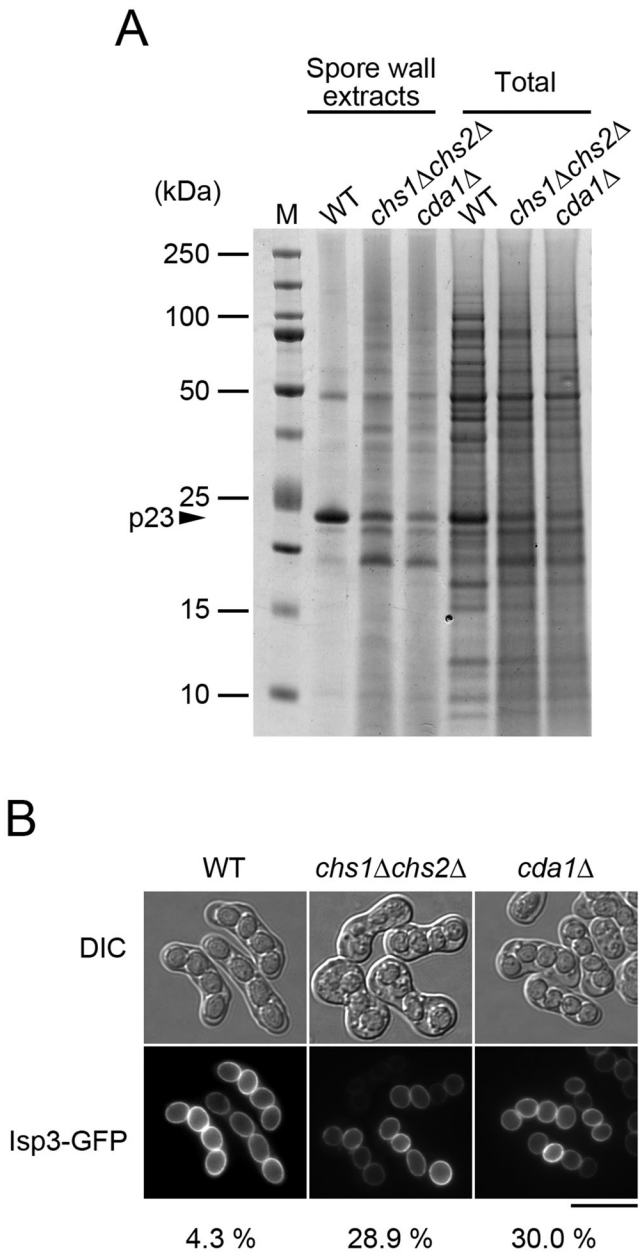


FIGURE 5: Chitosan is important for proper assembly of the Isp3 layer. (A) SDS–PAGE pattern of proteins from isolated spores of wild-type (L968), *chs1Δchs2Δ* (YM576), and *cda1Δ* (YM802) cells. Spore wall extracts and whole-spore homogenates were subjected to SDS–PAGE, and the gel was stained with CBB. (B) Localization of Isp3-GFP in wild-type (KN7), *chs1Δchs2Δ* (KN26), and *cda1Δ* (KN27) asci. The percentage of asci in which the signal intensity of the four Isp3-GFP rings differed among the spores is also indicated. Scale bar: 10 μm.

spore dispersal in the air, whereas that of Isp3 is to form a protective spore coat against various stresses.

On the basis of results from the present and previous studies, we propose a model in which the Isp3 layer forms during sporulation (Figure 7). During the middle stage of sporulation (around meiosis II), *isp3*$^+$ is expressed dependent on the primary meiotic transcription factor Mei4 (Horie *et al.*, 1998; Watanabe *et al.*, 2001). Isp3 proteins first accumulate in the cytoplasm of the prespore, from which they are exported to the surface. The apparent export of Isp3 from the spore cytosol to the spore wall makes an interesting parallel to dityrosine in *S. cerevisiae*, which is also exported from the spore cytoplasm to the spore wall, where it polymerizes on the surface via the action of the spore plasma membrane transporter Dtr1 (Felder *et al.*, 2002). As is not the case for *S. cerevisiae*, the mechanism by which Isp3 is exported from maturing spores remains to be elucidated for *S. pombe*. The predicted Isp3 protein has no potential signal sequence, and Isp3 isolated from spores indeed contained the N-terminal amino acids of the mature protein (Figure 1D), suggesting that Isp3 is not exported by the general secretory pathway. Recently Isp3 has been reported to be heavily palmitoylated in sporulation cells (Zhang *et al.*, 2013). Palmitoylation is a fatty acid modification that increases the hydrophobicity of proteins and is known to control the membrane trafficking and function of many proteins. Although the function of the palmitoylation of Isp3 is not clear, one possibility is that palmitoylation of Isp3 is important for export from the cytoplasm. Once exported, Isp3 proteins self-assemble on the surface to form a layer that is partly dependent on the underlying chitosan layer. Careful transmission electron microscopy analysis in *cda1* and *chs1chs2* mutants will be needed to address the mechanism underlying Isp3 layer assembly on the chitosan layer. Solubilization of Isp3 by SDS requires β-mercaptoethanol, suggesting that Isp3 proteins might be interlinked by disulfide bonds.

In nature, budding yeasts are dispersed by insects, in particular fruit flies, which feed on yeast (Begon, 1986). It has been reported that spores display enhanced survival relative to vegetative cells during passage through the gut of *Drosophila melanogaster* and that the dityrosine layer is important for survival. Therefore *S. cerevisiae* spores may be a cell type specialized for dispersal in the environment via *Drosophila* vectors (Coluccio *et al.*, 2008). By contrast, little is known about where *S. pombe* lives and how it disperses in nature. Interestingly, as in *S. cerevisiae*, vegetative cells are sensitive to digestion by *Drosophila*, whereas spores display increased survival in *S. pombe* (although, in both forms, *S. pombe* is more sensitive than *S. cerevisiae* to digestion), raising the possibility that *S. pombe* is also dispersed by insect vectors (Coluccio *et al.*, 2008). We attribute the difference in sensitivity between the two yeasts to the outermost layer of the spore: the dityrosine layer may be more resistant to digestion by *Drosophila* than the Isp3 layer. The spores of all species of *Saccharomyces* and *Kluyveromyces* contain dityrosine, whereas those of *Nadsonia fulvescens*, *Debaryomyces hansenii*, and some *Pichia farinosa* species do not (Prillinger *et al.*, 1997). Therefore the surface structure of yeast spores might have been evolved as a strategy for surviving drastic changes in the environment and/or adaptation for insect vectors. Further characterization of Isp3 will advance our understanding of not only the spore function of fission yeast but also its ecology and evolution.

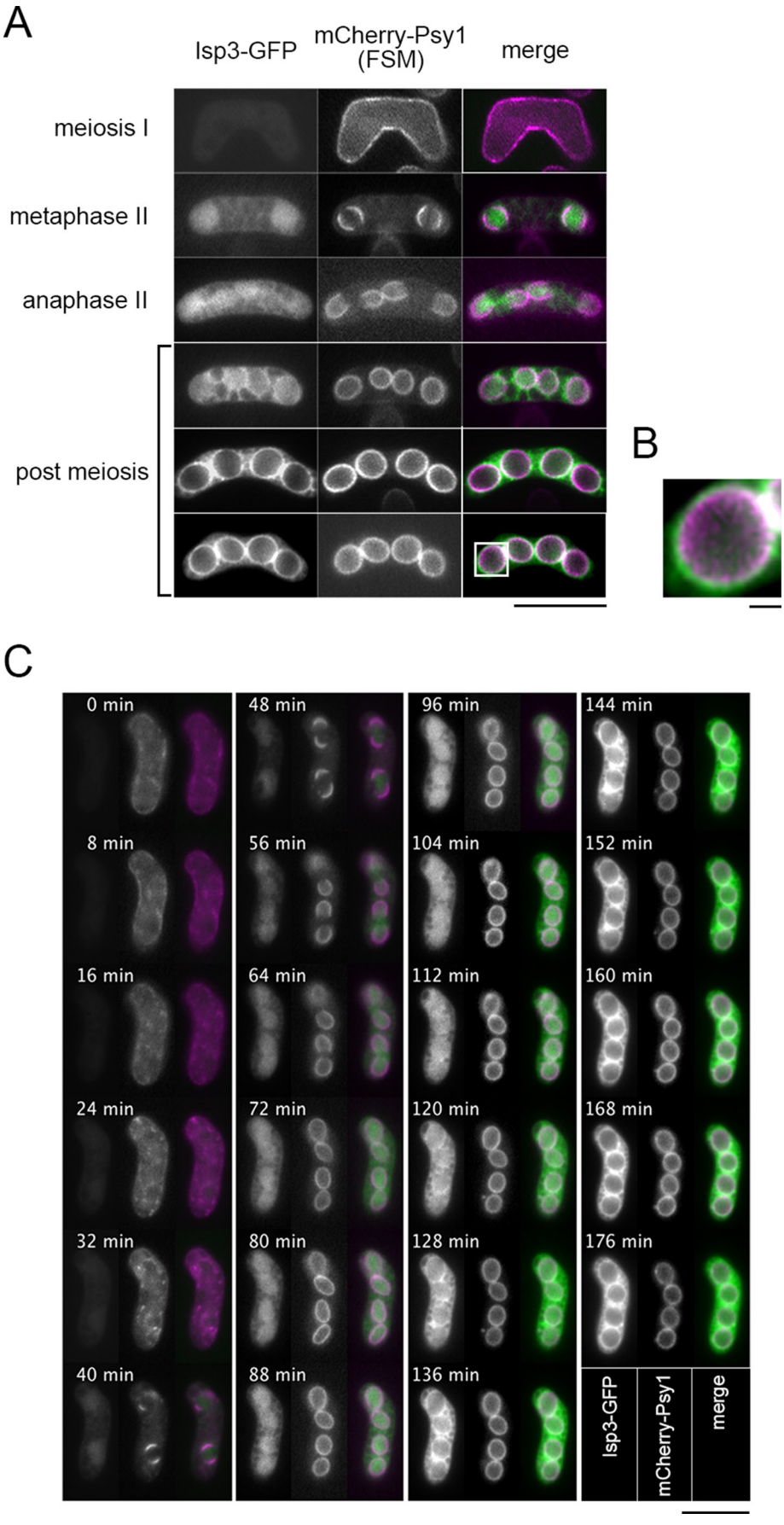
MATERIALS AND METHODS

Yeast strains, media, and plasmids

The *S. pombe* strains used in this study are listed in Table 1. Complete medium (YE) and synthetic medium (MM+N) were used for growth, and malt extract medium (MEA) and synthetic sporulation media (SSA, SSL-N, and MM-N) were used for mating and sporulation (Egel and Egel-Mitani, 1974). Synchronous meiosis was induced in strains carrying the *pat1-114* allele by a temperature shift as described (Iino *et al.*, 1995).

Disruption of the *isp3*$^+$ gene

The *isp3*$^+$ gene was disrupted with *ura4*$^+$ as follows. PCR primers 5′-GAGTAGGGCCCATCTTAGTAACATATAAGA-3′ and 5′-GACCAGAGCTCCTGGTACTTAATATCAAGT-3′ were used to amplify a



2.5-kb fragment encompassing the entire *isp3*$^+$ gene. The resulting *Apa*I–*Sac*I fragment was then subcloned into pBluescript II-KS$^+$ (Stratagene, La Jolla, CA) to generate pBS(isp3). After digestion with *Bst*XI and *Pst*I, pBS(isp3) was filled in and ligated to a *Bam*HI linker. The resulting plasmid was digested with *Bam*HI, and a 1.7-kb *ura4*$^+$ fragment was inserted at the *Bam*HI site. The resulting plasmid was digested with *Afl*II and *Hpa*I, and used to transform strain TN29. Disruption was confirmed by genomic Southern hybridization (unpublished data).

Construction of a strain expressing GFP-tagged Isp3

An Isp3 protein fused to GFP at the C-terminus was made as follows. PCR primers 5′-GAGTAGGGCCCATCTTAGTAACATA-TAAGA-3′ and 5′-GATTAGCGGCCGCAT-GCGGCAGGAGCAGCAGG-3′ were used to amplify a 2.5-kb fragment encompassing the promoter region and the open reading frame of the *isp3*$^+$ gene, and the resulting *Apa*I–*Not*I fragment was subcloned into pBluescript II-KS$^+$ to generate pBS(isp3)KN1. A 1.0-kb *Not*I–*Sac*I fragment of pSLF273 (Forsburg and Sherman, 1997) was ligated into the corresponding site of pBS(isp3)KN1, yielding pBS(isp3)KN2. A 0.8-kb *Not*I–*Sma*I fragment of pTN197 (Nakamura *et al.*, 2001) including the GFP gene was ligated into the corresponding site of pBS(isp3)KN2, yielding pBS(isp3)KN3. PCR primers 5′-GTCGACCCGGGTAGAG-CAAAAATGCCCTTT-3′ and 5′-GACCA-GAGCTCCTGGTACTTAATATCAAGTT-3′ were used to amplify a 2.5-kb fragment encompassing the terminator region and the *isp3*$^+$ gene, and the resulting *Sma*I–*Sac*I fragment was subcloned into the corresponding site of pBS(isp3)KN3 to generate pBS(isp3-GFP-isp3t). A 3.5-kb *Afl*II–SacI fragment of pBS(isp3-GFP-isp3t) containing Isp3-GFP was introduced into the *isp3Δ* strain, KN1. Uracil-negative transformants

FIGURE 6: Behavior of Isp3-GFP during sporulation. (A) Fluorescence signals from Isp3-GFP and mCherry-Psy1 were observed at multiple stages of sporulation after induction of sporulation by nitrogen starvation in KN14 cells. The merged images show Isp3-GFP (green) and mCherry-Psy1 (magenta). Scale bar: 10 μm. (B) Magnified image of the boxed region in (A). Scale bar: 1 μm. (C) Selected frames from time-lapse observations of Isp3-GFP and mCherry-Psy1 in a living cell (KN14). Scale bar: 10 μm.

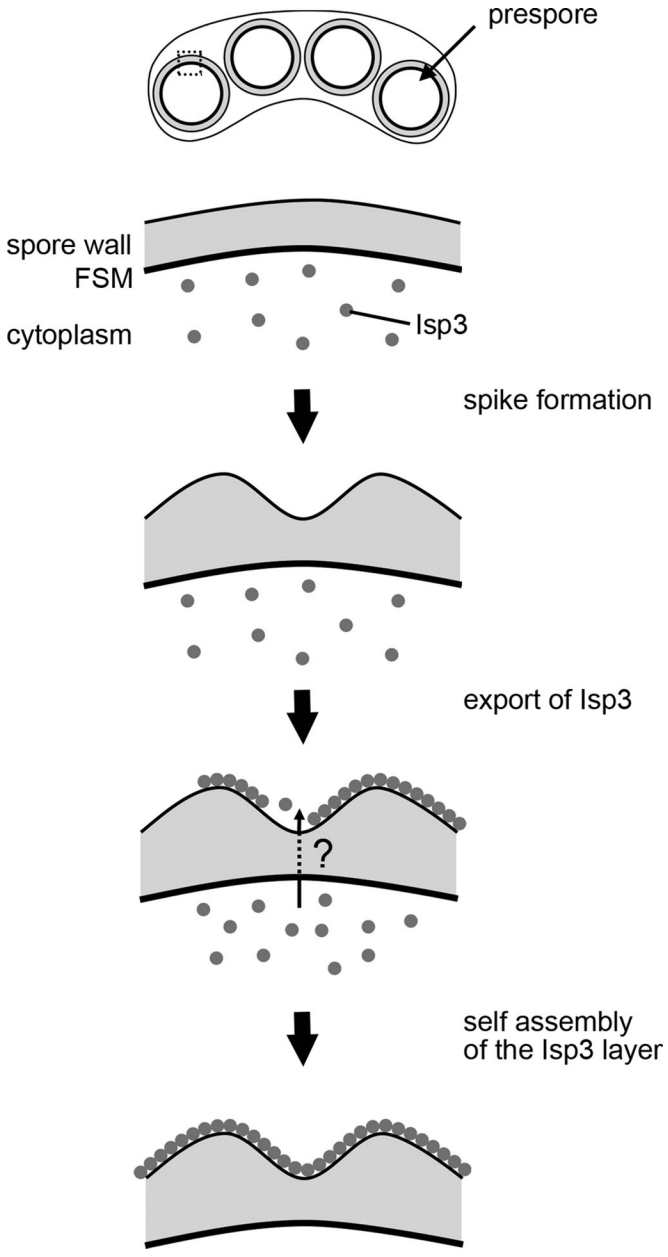


FIGURE 7: Model of the assembly of the Isp3 layer.

were obtained by screening for clones that survived in medium containing 5-fluoroorotic acid. The tagged proteins appeared to be functional on the basis of their ability to rescue the defects of *isp3Δ* (unpublished data).

Isolation of spores by Urografin gradient centrifugation

The homothallic haploid strains were grown in ME liquid medium for 7 d at 28°C. Ascal walls were spontaneously dissolved, and single spores were liberated. Spores were isolated by linear 25–55% Urografin (Bayer, Leverkusen, Germany) density-gradient centrifugation as described previously (Nishi *et al.*, 1978).

Identification of the p23 protein

Spores isolated by Urografin gradient centrifugation were treated with 2% SDS and 5% β-mercaptoethanol at 100°C for 4 min. After centrifugation, the supernatant was fractionated by electrophoresis in SDS on 12% acrylamide gels, which were then stained with Coomassie brilliant blue (CBB). The bands in each well were overlaid with a solution containing V8 protease. p23 was partially digested and separated on a gel according to the Cleveland method (Cleveland *et al.*, 1977), transferred to an Immobilon-PSQ

Strain (accession no.)[a]	Genotype[b]	Source
	S. pombe	
L968 (FY7520)	h^{90}	Leupold
KN1 (FY17852)	h^{90} *isp3::ura4⁺ leu1-32 ura4-D18*	This study
KN2 (FY17853)	h^{90} *isp3-gfp leu1-32 ura4-D18*	This study
KN3 (FY17854)	h^{90} *isp3::ura4⁺ ura4-D18*	This study
KN7 (FY17858)	h^{90} *isp3-gfp*	This study
KN14 (FY17862)	h^{90} *isp3-gfp leu1<<mCherry-psy1*	This study
KN18	h^{90}/h^{90} *isp3::ura4⁺/ isp3::ura4⁺ ade6-M210/ ade6-M216 ura4-D18/ura4-D18*	This study
KN19	h^{90}/h^{90} *ade6-M210/ade6-M216*	This study
KN26 (FY17873)	h^{90} *isp3-gfp chs1::ura4⁺ chs2::LEU2 leu1-32 ura4-D18*	This study
KN27 (FY17875)	h^{90} *isp3-gfp cda1::ura4⁺ leu1-32 ura4-D18*	This study
YM576 (FY14016)	h^{90} *chs1::ura4⁺ chs2:LEU2 ade6-M210 leu1-32 ura4-D18*	Matsuo *et al.*, 2004
YM802 (FY17731)	h^{90} *cda1::ura4⁺ ade6-M210 leu1-32 ura4-D18*	Matsuo *et al.*, 2005
MKW5 (FY7456)	h^{90}	Nakamura-Kubo *et al.*, 2003
TN29 (FY7816)	h^{90} *leu1-32 ura4-D18*	Ikemoto *et al.*, 2000
JZ670 (FY7051)	h^{-}/h^{-} *pat1-114/pat1-114 ade6-M210/ade6-M216 leu1-32/leu1-32*	Yamamoto
	S. cerevisiae	
KYC800 (BY19600)	*MATa/MATα ho::LYS2/ ho::LYS2 ura3/ura3 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2/ lys2*	YGRC

[a]Accession numbers are from the Yeast Genetic Resource Center of Japan, supported by the National BioResource Project (YGRC/NBRP; http:/yeast.lab.nig.ac.jp/nig). The *S. pombe* strains constructed in this study have been deposited with the YGRC/NBRP under the accession numbers shown here.
[b]*x* << *y* means that gene *y* is integrated at gene *x*.

TABLE 1: Strains used in this study.

membrane (Millipore, Billerica, MA), and stained with amido black. Edman degradation of the bands revealed N-terminal amino acid sequences of eight residues, GLGNLC.

Immunofluorescence microscopy

Isp3-GFP on the spore surface was detected as follows. The homothallic haploid strains L968 and KN7 were sporulated on MEA for 1 d at 28°C. Cells were collected by centrifugation and resuspended in PEMBAL (100 mM PIPES-KOH, pH 6.9, 1 mM ethylene glycol tetraacetic acid, 1 mM $MgSO_4$, 1% bovine serum albumin, 0.1% NaN_3, 100 mM lysine hydrochloride). After incubation at room temperature for 30 min, cells were incubated with a monoclonal anti-GFP antibody (Roche Diagnostics, Indianapolis, IN) at a 1:100 dilution in PEMBAL. Cells were then washed twice with PEMBAL and incubated with Cy3-conjugated goat anti-mouse immunoglobulin G (IgG; Sigma-Aldrich, St. Louis, MO) at a 1:1000 dilution in PEMBAL. After being washed in PEMBAL, cells were suspended in phosphate-buffered saline and analyzed by fluorescence microscopy.

Western blotting

KN7 cells were precultured overnight in MM+N medium and then transferred to MM–N sporulation medium. At intervals, portions of the culture were collected, and crude cell extracts were prepared as described by (Masai *et al.*, 1995). Polypeptides were resolved by SDS–PAGE on a 12% gel and then transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore). Filters were probed with a mouse anti-GFP antibody (Roche Diagnostics, Indianapolis, IN) at a 1:10,000 dilution. Blots were also probed with the anti–α-tubulin antibody TAT-1 (Woods *et al.*, 1989) to ensure that approximately equal amounts of protein were loaded. Immunoreactive bands were revealed by ECL select chemiluminescence (GE Healthcare, Little Chalfont, Buckinghamshire, UK) with horseradish peroxidase–conjugated goat anti-mouse IgG (GE Healthcare).

Scanning electron microscopy

Spores isolated by Urografin gradient centrifugation were fixed with 2.5% glutaraldehyde, postfixed with 1% OsO_4, and then dehydrated by 15-min incubations in the following graded ethanol series: two incubations each in 50, 70, 80, 90, and 95% ethanol; and two incubations in 99% ethanol. The samples were critical point dried from *t*-butanol and sputter-coated with a 7-nm-thick layer of osmium with an ion sputter (model OPC-80T; Filgen, Nagoya, Japan). Images were collected by using an SEM (model S-3500N; Hitachi, Tokyo, Japan) at 5 kV with an in-lens detector.

Transmission electron microscopy and immunoelectron microscopic analysis

Cells were mounted on a copper grid to form a thin layer and immersed in liquid propane (–187°C) cooled with liquid nitrogen in a Leica EM CPC cryo-workstation (Leica Microsystems, Wetzlar, Germany). The frozen cells were transferred to anhydrous acetone containing 2% OsO_4 at –80°C in a Leica EM AFS automatic freeze substitution apparatus, held at –80°C for 78 h, warmed gradually to 0°C over 11.4 h, held at 0°C for 1.5 h, warmed gradually to 23°C over 3.9 h, and then held at 23°C for 2 h. After three washes with anhydrous acetone, the samples were infiltrated with increasing concentrations of Spurr's resin in anhydrous acetone, and finally with 100% Spurr's resin. After polymerization (5 h at 50°C followed by 60°C for 2–3 d) in capsules, ultrathin sections were cut on a Leica Ultracut UCT microtome and stained with uranyl acetate and lead citrate.

The sections were immunostained with mouse anti-GFP (Roche) or 1,3-β-glucan antibody (Biosupplies, Bundoora, Australia) and immunogold conjugate (10-nm gold) EM goat anti-mouse IgG (BB International, Cardiff, UK) and then stained with 3% uranyl acetate for only 2 h. The sections were then viewed under a Hitachi H-7600 electron microscope (TEM) at 100 kV.

Time-lapse analysis

Time-lapse observation was performed as previously described (Nakamura *et al.*, 2008; Yamaoka *et al.*, 2013). For the colocalization study, cells expressing only Isp3-GFP or mCherry-Psy1 were also observed to rule out the possibility of channel bleed-through. Digital images were processed with Image J and Adobe Photoshop CS6 (Adobe, San Jose, CA).

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