**SPO71 Mediates Prospore Membrane Size and Maturation in Saccharomyces cerevisiae**

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The mechanisms that control the size and shape of membranes are not well understood, despite the importance of these structures in determining organelle and cell morphology. The prospore membrane, a double lipid bilayer that is synthesized de novo during sporulation in *S. cerevisiae*, grows to surround the four meiotic products. This membrane determines the shape of the newly formed spores and serves as the template for spore wall deposition. Ultimately, the inner leaflet of the prospore membrane will become the new plasma membrane of the cell upon germination. Here we show that Spo71, a pleckstrin homology domain protein whose expression is induced during sporulation, is critical for the appropriate growth of the prospore membrane. Without *SPO71*, prospore membranes surround the nuclei but are abnormally small, and spore wall deposition is disrupted. Sporulating *spo71*Δ cells have prospore membranes that properly localize components to their growing leading edges yet cannot properly localize septin structures. We also found that *SPO71* genetically interacts with *SPO1*, a gene with homology to the phospholipase B gene that has been previously implicated in determining the shape of the prospore membrane. Together, these results show that *SPO71* plays a critical role in prospore membrane development.

The membrane is an important determinant of the shape of biological structures (34). As both organelles and cells are bounded by lipid bilayers, membranes are instrumental in their morphology. However, the mechanisms that underlie the control of the size and shape of these limiting membranes are not fully understood.

Diploid *Saccharomyces cerevisiae* cells undergo sporulation in response to a lack of nitrogen and fermentable carbon sources (reviewed in reference 26). During this process, the cell undergoes meiosis and remodels its interior as it packages the meiotic products into spores, the equivalent of its gametes. Four spores are formed within the mother cell, which becomes known as the ascus. Upon reintroduction of nutrients into the environment, these spores can either grow vegetatively as haploid cells or mate with cells of the opposite mating type to create diploid cells.

The shape of these spores is determined by the prospore membrane (PSM), a double membrane that is synthesized de novo during sporulation by post-Golgi vesicle fusion at the spindle pole body. The PSM grows to surround the meiotic nuclei and undergoes a cytokinetic event to encapsulate each nucleus. The growth of the PSM must be regulated such that it grows to properly encapsulate nuclei and cytoplasmic material and matures into a spherical configuration (9, 40). Following completion of PSM development, the lumen of the double membrane expands and serves as the site of spore wall deposition. The spore wall, comprised of mannoprotein, β-glucan, chitosan, and dityrosine layers, differs from the vegetative cell wall in its composition and offers increased protection to environmental stresses (7). Midway through spore morphogenesis, the outer prospore membrane is lysed and removed, with the inner leaflet becoming the plasma membrane of the new cells (6).

Two structures have been associated with the growing PSM. First, a protein complex known as the leading-edge protein (LEP) complex localizes to the lip of the growing PSM (15, 23, 27). This complex includes Don1, a coiled-coil protein. Second, septins, which are filament-forming proteins that have been implicated in cellular morphology in multiple organisms (22, 28, 46), are also localized to the PSM. During sporulation, some components of the vegetative septin complex are replaced by the sporulation-specific septins Spr3 and Spr28 (8, 29). The localization of septins is dynamic during PSM growth, as the septins form circular structures during early PSM development which transition into sheets or bars as the PSM expands and ultimately grow to surround each spore (13, 30). The exact function of the septins during sporulation is not well understood.

Genes important for the proper curvature of the PSM have been identified (20, 25) and include *SPO1*, which encodes a putative phospholipase B (42, 43). Cells lacking *SPO1* can have abnormally wide PSMs (20), and *SPO1* was proposed to be involved in promoting the proper curvature of the PSM.

In this work, we show that proper PSM size is also dependent on *SPO71*, a pleckstrin homology (PH) domain-encoding gene previously identified as necessary for sporulation (5, 12, 33, 49). PH domains have been previously shown to bind to specific phospholipids found in membranes (18). Our analysis of *spo71* mutant alleles revealed that loss of *SPO71* reduces the size of PSMs. *spo71*Δ cells properly localize the leading edge component Don1 but do not properly localize the Spr28 septin and do not properly deposit spore wall materials. Additionally, we observed that Spo71 can localize to the plasma membrane when ectopically expressed in vegetatively growing cells. Finally, we found that *SPO71* genetically interacts with *SPO1*, such that loss of *SPO71* partially rescues...
### TABLE 1  S. cerevisiae strains used in this study

| Strain | Genotype | Reference |
|--------|-----------|-----------|
| LH177  | MATa/MA Tα h:hisG/h:hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA | 14 |
| LH185  | MATa/MA Tα h:hisG/h:hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA smk1::TRP1C::smk1::TRP1C:: | 14 |
| LH900  | MATa/MA Tα h:hisG/h:hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA spo71::TRP1C::spo71::TRP1C:: | This study |
| LH901  | MATa/MA Tα h:hisG/h:hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA SPO71-13 × MYC-TRP1/ SPO71-13 × MYC-TRP1 | This study |
| LH902  | MATa/MA Tα h:hisG/h:hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA HTB2-mCherry-TRP1C::HTB2-mCherry-TRP1C:: | This study |
| LH903  | MATa/MA Tα h:hisG/h:hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA HTB2-mCherry-URA3K::HTB2-mCherry-URA3K:: | This study |
| LH904  | MATa/MA Tα h:hisG/h:hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA spo71::TRP1C::spo71::TRP1C:: HTB2-mCherry-TRP1C::HTB2-mCherry-TRP1C:: | This study |
| LH905  | MATa/MA Tα h:hisG/h:hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA SPO71-13 × URA3K::SPO71-13 × URA3K:: | This study |
| LH906  | MATa/MA Tα h:hisG/h:hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA spo71(1–1030)-zz-URA3K::spo71(1–1030)-zz-URA3K:: | This study |
| LH907  | MATa/MA Tα h:hisG/h:hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA spo71(1–758)-zz-URA3K::spo71(1–758)-zz-URA3K:: | This study |
| LH908  | MATa/MA Tα h:hisG/h:hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA spo71(1–1030)-zz-URA3K::spo71(1–1030)-zz-URA3K:: /HTB2-mCherry-URA3K::HTB2-mCherry-URA3K:: | This study |
| LH909  | MATa/MA Tα h:hisG/h:hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA spo71(1–758)-zz-URA3K::HTB2-mCherry-URA3K::HTB2-mCherry-URA3K:: | This study |
| LH910  | MATa/MA Tα h:hisG/h:hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA DON1-GFP-HIS3MX6/DON1-GFP-HIS3MX6 | This study |
| LH911  | MATa/MA Tα h:hisG/h:hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA DON1-GFP-HIS3MX6/ DON1-GFP-HIS3MX6 spo71::TRP1C::spo71::TRP1C:: /HTB2-mCherry-TRP1C::HTB2-mCherry-TRP1C:: | This study |
| LH912  | MATa/MA Tα h:hisG/h:hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA HTB2-mCherry-TRP1C::HTB2-mCherry-TRP1C:: / HTB2-mCherry-TRP1C::HTB2-mCherry-TRP1C:: spo71::TRP1C::spo71::TRP1C:: | This study |
| LH913  | MATa/MA Tα h:hisG/h:hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA HTB2-mCherry-TRP1C::HTB2-mCherry-TRP1C:: / HTB2-mCherry-TRP1C::HTB2-mCherry-TRP1C:: spo71::TRP1C::spo71::TRP1C:: | This study |
| LH914  | MATa/MA Tα h:hisG/h:hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA HTB2-mCherry-TRP1C::HTB2-mCherry-TRP1C:: spo71::TRP1C::spo71::TRP1C:: | This study |
| LH915  | MATa/MA Tα h:hisG/h:hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA HTB2-mCherry-TRP1C::HTB2-mCherry-TRP1C:: | This study |
| LH916  | MATa/MA Tα h:hisG/h:hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA HTB2-mCherry-TRP1C::HTB2-mCherry-TRP1C:: | This study |
| LH917  | LH902 plus pRS426-G20 | This study |
| LH918  | LH903 plus pRS424-G20 | This study |
| LH919  | LH904 plus pRS426-G20 | This study |
| LH920  | LH908 plus pRS424-G20 | This study |
| LH921  | LH909 plus pRS424-G20 | This study |
| LH922  | LH970 plus pRS426-M20 | This study |
| LH923  | LH910 plus pRS426-M20 | This study |
| LH924  | LH904 plus pRS426-G71(1–1245) | This study |
| LH925  | LH904 plus pRS426-G71(1–1037) | This study |
| LH926  | LH904 plus pRS426-G71(1–758) | This study |
| LH927  | LH904 plus pRS426-G71(753–1037) | This study |
| LH928  | LH904 plus pRS426-G71(963–1245) | This study |
| LH929  | LH904 plus pRS426-G71(753–1245) | This study |
| LH930  | LH915 plus pRS426-G20 | This study |
| LH932  | LH916 plus pRS426-G20 | This study |

a C.g., Candida glabrata; K.l., Kluyveromyces lactis.
spo1Δ’s PSM defect, suggesting that SPO71 and SPO1 exert antagonistic effects on the developing PSM.

MATERIALS AND METHODS

Strains used in this study. Strains used in this study are listed in Table 1. All strains were derived from the SK1 background. Genomic alterations (tagging and deletions) were performed as previously described (19, 32). Strains were constructed using the primers and plasmids in Table S1 in the supplemental material. Primer sequences are located in Table S2.

Plasmids. Plasmids used in this study are listed in Table S3 in the supplemental material. The pTEF2-driven GFP-spo71 alleles were constructed as follows. First, the full-length SPO71 open reading frame (ORF) was amplified by PCR from SK1 genomic DNA with primers OLH1155 and OLH1127. psRS426-G20 (a gift from A. Neiman) was amplified by PCR with primers OLH1122 and OLH1123, which excised the spo2051-91 fragment and inserted HindIII and XhoI sites onto the vector. The SPO71 ORF was then ligated into the green fluorescent protein (GFP) vector, creating an N-terminally tagged SPO71. Truncation alleles were constructed using similar methods, by amplifying only the desired SPO71 regions using the following primers: for spo21-1037, OLH1155 and OLH1126; for spo21-758, OLH1155 and OLH1158; for spo71-1037, OLH1124 and OLH1126; for spo71-753-1245, OLH1125 and OLH127; and for spo71-753-1245, OLH1124 and OLH1127. All amplified regions were sequenced.

psRS426-M20 was constructed by replacement of the GFP gene in psRS426-G20 with mCherry. psRS426-G20 was amplified using OLH929 and OLH990, which excised the GFP gene and inserted a BamHI site before the SPO20 fragment. mCherry was amplified from pSET-B mCherry (gift from A. Veraksa) using OLH932 and OLH933, which created flanking EcoRI and BamHI sites. The amplified mCherry-containing fragment was then inserted into the psRS426-spo20(S1-91) backbone.

Sporulation. Sporulation was performed as previously described (14). Briefly, cells were grown to saturation in YPD (2% peptone, 1% yeast extract, 2% dextrose), and transferred to presporulation medium (YPA [2% peptone, 1% yeast extract, 1% potassium acetate]). Cells were grown in presporulation medium overnight and then shifted to sporulation medium (2% potassium acetate). When cells contained plasmids, selective medium (0.67% yeast nitrogen base without amino acids, 2% dextrose, and 0.6 M NaCl) was used instead of YPD prior to sporulation induction. All sporulation steps following this were the same as described above.

Bioinformatics. The SPO71 sequence was obtained from www.yeastgenome.org. Sequences of fungal homologs were obtained using BLAST at NCBI. PH domains were defined using SMART (36). Sequences of fungal homologs were obtained using blast at NCBI (www.ncbi.nlm.nih.gov). Sequences of fungal homologs were obtained using blast at NCBI (www.ncbi.nlm.nih.gov). The percentage of PSMs that captured nuclei was determined by examining whether a formed PSM properly surrounded a meiotic nucleus. The number of PSMs per ascus was quantified by counting the number of PSMs made in each ascus. We considered a structure a PSM if it appeared circular or oval and not as punctate clusters. As with nucleus capture, only meiotic cells were quantified.

Protein immunoblotting. Protein lysates for immunoblotting were prepared by trichloroacetic acid (TCA) denaturation, as previously described (48). TCA-precipitated proteins were resuspended in sample buffer and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were blotted onto polyvinylidene fluoride and probed with rabbit preimmune antisera to detect the α-satellite spore protein. We found increased expression levels of Spo71 protein at 6 h into sporulation, at around meiosis II (Fig. 1A). This increase in expression is consistent with the time of RNA induction seen in microarray studies. Comparisons of wild-type and spo71Δ strains were performed using a two-tailed Fisher’s exact test (GraphPad).
the more closely related species, including the *Saccharomyces sensu stricto* clade and *Kluyveromyces lactis*. The following B region is more broadly conserved, appearing across much of the fungal kingdom, including the more distantly related phylum *Basidiomycota*. At the C terminus, we detect two PH domains. Like the B region, the PH domains are found in the Spo71 protein throughout the fungal kingdom, including the phylum *Basidiomycota*.

**SPO71 is required for the proper size of the prospore membranes.** To determine the specific sporulation defect in *spo71Δ* cells, we examined the development of the prospore membrane (PSM) and saw that PSMs in *spo71Δ* cells were smaller than those in wild-type cells (Fig. 2A). PSMs were visualized using pRS426-G20, which contains amino acids 51 to 91 from Spo20, shown to be sufficient for PSM localization, fused to green fluorescent protein (GFP) (24). Quantification of PSM sizes in postmeiotic cells revealed that *spo71Δ* cells display significantly smaller PSMs than wild-type cells (Fig. 2B) (Tukey-Kramer HSD, α = 0.01, which shows that all mutants are significantly distinct from the wild type). To determine if the phenotypes of the truncation alleles were reflective of a difference in protein levels as opposed to a difference due to missing protein domains, we performed Western blotting on the truncation alleles and were able to detect protein at levels comparable to those in wild-type Spo71 (see Fig. S2 in the supplemental material). Thus, both PH domains appear to be required for Spo71 activity.

Previous studies have demonstrated that as PSMs develop, they take on recognizable shapes indicative of particular stages (9). PSMs begin as dots that grow into small half-circles, then change to elongated tubes followed by ovals, and finally mature to form spheres (Fig. 3). When we examine the PSM during its development in *spo71Δ* cells, we see shapes corresponding to many of the stages seen in wild-type cells. Interestingly, even though *spo71Δ* cells make smaller terminal PSMs, they form the elongated tubes observed during PSM development in wild-type cells (Fig. 3, column iv). We did not readily find the oval PSMs (Fig. 3, column v). Whether this is due to a defect in elongation or whether the lack of oval shape is due to the small PSMs not having enough membrane to form the elongated shape is unclear. Thus, beyond the clear

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**FIG 1** *SPO71* encodes a double pleckstrin homology domain protein essential for sporulation. (A) Immunoblot probed with anti-Myc antibody, showing Spo71-myc [LH901] expression. LH901 was sporulated, and samples were taken at the indicated times after transfer to SPM. (B) *SPO71* [LH902] and *spo71Δ* [LH904] cells 24 h after induction of sporulation. The histone gene HTB2 was tagged with mCherry for visualization of meiotic progression. (C) Schematic of Spo71 displaying sequence conservation across fungi. Spo71 contains four conserved regions. Asterisks denote pathogenic fungi. Tree topology was adapted from the work of Dujon (11).

**FIG 2** *spo71* mutants form small PSMs. (A) Representative images of PSMs in postmeiotic wild type and *spo71* mutants harboring the PSM marker, pRS426-GFP-spo2051–91. The schematics show the wild type (LH917 and the *spo71Δ* (LH918), *spo71Δ*-1030 (LH920), and *spo71Δ*-758 (LH921) (*spo71* mutants are truncation alleles with one or both PH domains truncated). Images shown are from live samples; no difference in morphology was found when samples were fixed prior to imaging. (B) Quantification of PSM sizes in the strains used for panel A. The PSMs were quantitated in strains transformed with *GFP*-spo2051–91, as follows: wild type [LH902 and LH177], 321; *spo71Δ* [LH900 and LH903], 257; *spo71Δ*-1030 [LH906], 144; *spo71Δ*-758 [LH907], 148. Bars show 95% confidence intervals.
reduction in PSM size, we did not detect other obvious PSM defects.

Loss of SPO71 affects Spr28 but not Don1. To determine whether, despite its small size, the spo71 mutant PSM behaves normally, we examined the localization of the leading-edge protein complex and the septins by assessing Don1 and Spr28 localization, respectively. Don1 was properly localized at the leading edge of the PSM in spo71/H9004 cells (Fig. 4A).

In contrast, localization of the sporulation-specific septin Spr28 was aberrant in spo71Δ cells. The sporulation specific septins Spr3 and Spr28 were previously shown to localize in a dynamic fashion, first localizing in a circular fashion during early PSM development, transitioning into bar- or sheet-like structures, and eventually returning to a more circular pattern surrounding the meiotic nuclei (13, 30). Loss of SPO71 resulted in aberrant Spr28 localization (Fig. 4B), such that the elongated-bar pattern seen in wild-type cells was absent in spo71Δ cells. Instead, Spr28 localized in a circular structure, both during meiosis II and postmeiotically. Furthermore, the Spr28 circles do not always surround the nuclei. Thus, although the leading edge appears normal in spo71Δ cells, septins are expressed but mislocalized.

SPO71 is necessary for proper spore wall deposition. As a major role of the PSM is to facilitate spore wall deposition, we sought to determine if SPO71 was necessary for spore wall formation. The outermost layer of the mature spore wall, dityrosine, is readily detected using UV fluorescence. Sporulated wild-type cells produce the dityrosine layer, as evidenced by the fluorescence of the patch of yeast cells (Fig. 5A). Cells lacking SPO71 did not fluoresce, indicating that spo71Δ cells do not properly synthesize the dityrosine layer.

To determine the ability of spo71Δ cells to form the first three spore wall layers, we examined these layers by indirect immunofluorescent detection (41). In wild-type cells, the mannan, β-glucan, and chitosan layers appear as circular structures surrounding the spore nuclei. In contrast, spo71Δ cells display an improper localization of spore wall structures. Unlike the dityrosine layer, spo71Δ cells apparently synthesize mannan, β-glucan, and chitosan, yet the materials are inappropriately deposited. The spore wall layers appear as clumps, with no apparent encapsulation of the meiotic nuclei, unlike the encapsulation seen in wild-type cells (Fig. 5B).

Spo71 can localize to the plasma membrane. To assess the subcellular localization of Spo71, we created N- and C-terminally tagged versions of Spo71. Unfortunately, we were unable to detect Spo71 expression at native levels during sporulation. Thus, we expressed GFP-Spo71 under the control of the strong TEF2 promoter on a high-copy-number plasmid, pRS426 (4). The TEF2 promoter has been shown to drive high levels of expression during both sporulating and vegetatively growing cells (5). While Spo71 is not normally expressed during vegetative growth, GFP-Spo71 localizes to the plasma membrane when expressed under these conditions (Fig. 6). During sporulation, the fluorescent signal becomes undetectable, despite the fact that expression of GFP-Spo71 is detectable using immunoblot analysis throughout spo-
rulation (see Fig. S3 in the supplemental material). While the mechanism behind our inability to visualize GFP-Spo71 in sporo-

lating cells is unclear, it could reflect localization of the protein to an environment incompatible with GFP fluorescence or a de-

crease in protein levels below the level of detection for epifluores-

cence microscopy (16, 47). Although we could not detect GFP-

Spo71 in the microscope during the time Spo71 is normally

induced, the pTEF2-GFP-Spo71 construct complemented spo71Δ

cells, as assayed by the formation of refractile spores (see Fig. S3).

Given the ability of Spo71 to localize to the plasma membrane in vegetatively growing cells, we used this localization to assess which regions of the protein are necessary for such membrane localization. We fused different domains of Spo71 to GFP (Fig. 6) and examined their localization patterns. The PH domains alone (GFP-Spo711753-1037 or GFP-Spo71963-1245) and in tandem (GFP-Spo71753-1245) were insufficient to confer the plasma membrane localization seen with the full-length construct. We then tested other combinations of the Spo71 domains (GFP-Spo711-1037 and GFP-Spo711-758) and found that none of these alleles localized to the plasma membrane.

We tested the ability of these alleles to complement the spo71Δ

phenotype and found that unlike the full-length construct, none could rescue the sporulation defect. We checked whether these GFP-tagged alleles were expressed by immunoblotting and found that all were expressed in vegetatively growing cells (see Fig. S4 in the supplemental material). Taken together, these results suggest that a single domain of Spo71 is unlikely to be sufficient for its localization to the membrane.

SPO71 and SPO1 genetically interact. SPO1 was previously shown to be important for the shape of the PSM, as spo1Δ cells displayed aberrant, wide prospore membranes with wide leading edges (20). We examined spo1Δ cells during sporulation and found that while wide PSMs can occur, the majority of sporulating spo1Δ cells are unable to form PSMs, with GFP-Spo2051–91 label-

ing clusters aggregating aberrantly throughout the mother cell (Fig. 7A). These clusters are likely aggregates of phosphatidic acid (PA)-containing membranes, as Spo2051–91 can bind to PAs (24). We classified the PSM phenotypes that occur in spo1Δ mutants into two groups. Cells were counted as a class I phenotype if they made no discernible PSM and displayed inappropriately aggregated membrane clusters. Cells were counted as a class II phenotype if they did not display inappropriate membrane aggregation.
and made a minimum of one PSM per mother cell. We also examined other phenotypes of spo1Δ cells and found that when PSMs are made, they sometimes do not capture the nuclei, and that the spo1Δ PSMs are smaller than wild-type PSMs (Fig. 7B).

Interestingly, spo71 partially suppresses the PSM defect caused by spo1. The spo1Δ spo71Δ double mutant shifts the distribution of cells significantly toward the less aberrant class II phenotype in which PSMs are made (Fig. 7A) (Fisher’s exact test, \( P = 0.0004 \)). We found that the spo1Δ spo71Δ double mutant showed significant improvement in the frequency of PSM production compared to spo1 mutants (Fig. 7B) (Tukey-Kramer HSD, \( \alpha = 0.01 \)). However, when we assayed the ability of the PSM to capture nuclei and measured PSM perimeter, we found that there was no significant improvement in spo1 mutants when SPO71 was removed (Fig. 7C and D) (Tukey-Kramer HSD, \( \alpha = 0.01 \), which shows that the spo1 spo71 and spo1 mutants are in the same class, distinct from the wild type, with regard to PSM capturing nuclei; Tukey-Kramer HSD, \( \alpha = 0.01 \), which shows that the spo71, spo1 spo71, and spo1 mutants are in the same class, distinct from the wild type, with regard to PSM perimeter).

Finally, we examined how the loss of SPO1 impacts spore wall deposition in the spo71Δ background. spo1Δ mutants have mannan and chitosan located throughout the mother cell, as opposed to the inappropriate clustering of spore wall layers seen in the spo71Δ mutant (Fig. 8). spo1 appears to be epistatic to spo71 for this defect, as the spo1Δ spo71Δ double mutant cells also show that mannan and chitosan localized throughout the mother cell.

FIG 6 SPO71 can localize to the vegetative plasma membrane. Different domains of Spo71 were fused to GFP and transformed into spo71Δ yeast as plasmids. Localization of spo711–1245 (LH924), spo711–1037 (LH925), spo711–758 (LH926), spo71753–1037 (LH927), spo71753–1245 (LH928), and spo71753–1245 (LH929) is shown. A diagram of GFP-spo71 alleles is shown on the left. Complementation was assayed by examining sporulation efficiency and comparing it to wild type sporulation efficiency under similar conditions.

FIG 7 SPO71 genetically interacts with SPO1. (A) Representative images of postmeiotic PSMs in wild-type (LH917), spo71Δ (LH919), spo1Δ (LH931), and spo71Δ spo1Δ (LH932) cells. Class phenotypes are described in the text. (B) Quantification of the strains used for panel A for the number of PSMs formed per ascus. The numbers of asci examined were as follows, wild-type (LH917), 48; spo71Δ (LH919), 50; spo1Δ (LH931), 47; and spo71Δ spo1Δ (LH932), 59. (C) Quantification of the strains used for panel A for the percentage of PSMs made that captured nuclei. The number of PSMs examined were as follows: wild-type (LH917), 175; spo71Δ (LH919), 162; spo1Δ (LH931), 63, and spo71Δ spo1Δ (LH932), 141. (D) Quantification of PSM perimeters. The numbers of PSMs measured were as follows, wild type (LH917, LH918, and LH177 transformed with GFP-Spo2051–91), 321; spo71Δ (LH919 and LH903 transformed with GFP-Spo2051–91), 257; spo1Δ (LH931), 46; and spo71Δ spo1Δ (LH932), 141. Note that the data for the wild type and the spo71Δ mutant are the same as those used in Fig. 1. Fewer spo1Δ PSMs were measured because PSMs are formed less frequently in spo1Δ cells. Bars show 95% confidence intervals.
The morphology of the PSM is important for the size and shape of the spores; it serves as the template for spore wall deposition, and its inner leaflet will become the plasma membrane as the spore matures. Here, we show that SPO71 is required for the proper size of the PSM, and the two PH domains of Spo71 are important for this activity. Despite the small size of spo71 PSMs, the leading-edge protein Don1 is appropriately localized, although the sporulation-specific septin Spr28 is not. Furthermore, SPO71 is needed for the proper targeting of spore wall materials to the PSM. Spo71 can associate with membranes, although neither PH domain is sufficient for this localization. SPO71 genetically interacts with SPO1, another gene implicated in PSM shape.

**Role of SPO71 during sporulation.** Our work shows that SPO71 is important for proper PSM development: the size of the PSMs, the localization of the septins to the PSM, and the ability of the PSM to act as a template for spore wall deposition are all disrupted in cells lacking spo71. How might SPO71 act to affect PSM development? The mislocalization of septins and spore wall materials suggests a role for Spo71 in directing appropriate trafficking of materials to the PSM. However, it is also possible that without the proper development of the PSM, the localization of the septins and spore wall materials is an indirect consequence of the lack of PSM maturation. Although PSMs in spo71 cells are smaller than normal, the terminal shape for the spo71 mutant PSM is spherical, as in the wild type. Whether spo71 PSMs are spherical because of a completed cytokinetic event (9) or because of other factors, such as membrane energetics favoring the formation of this spherical shape (44), remains to be determined.

We were intrigued by the ability of ectopically expressed Spo71 to localize to the plasma membranes of vegetatively growing cells, since a simple model for Spo71 function could involve Spo71 localization via its PH domains to the PSM. However, although PH domains can mediate membrane localization (18), and although previous studies demonstrated that the PH domains of Spo71 can bind the phosphoinositide phosphatidylinositol 3-phosphate (PI3) promiscuously and with weak affinity (49), neither PH domain of Spo71 was sufficient to mediate membrane localization in vegetatively growing cells. It is important to note that our experiments showing that the PH domains are not sufficient for membrane localization do not rule out a relationship between SPO71’s PH domains and phosphatidylinositol phosphates (PIPs), as other PH domain proteins have been shown to require regions outside the PH domain for the PH domain to correctly bind PIPs (17, 39).

We were also intrigued by the localization of Spo71 to the plasma membranes of vegetatively growing cells because the PA binding domain of Spo20 (Spo2051-91) (24) localizes to the plasma membrane during vegetative growth and the PSM during sporulation. Furthermore, the membrane phosphoinositide phosphatidylinositol 4,5-bisphosphate [PI(4,5)] has been demonstrated to localize to the PSM and the vegetative plasma membrane (35). During sporulation, some of the PI(4,5) is likely further metabolized to the PA that Spo20 binds by the phospholipase D Spo14 (37, 38). Unfortunately, we were unable to determine the localization of Spo71 during sporulation, either as a genomically integrated GFP-tagged allele or when overproduced on a high-copy-number plasmid. While it is possible that this lack of detectable localization in sporulation is due to technical limitations, it is also possible that Spo71 can associate with the plasma membrane but not the PSM because the compositions of the two membranes differ, such that the component with which Spo71 interacts on the plasma membrane is not present on the PSM.

**SPO71 and SPO1 genetic interaction.** Our data suggest that the relationship between SPO71 and SPO1 is complex. For PSM formation, spo71 can partially suppress the spo1 defects, suggesting an antagonistic relationship between the two genes. However, for spore wall deposition, spo1 appears to be epistatic to spo71. The spo1 spo71 double mutant appears to have diffuse spore wall component localization, like that seen in the spo1 mutant, despite the PSMs being more normal in this double mutant than in spo1 single mutants. This difference in genetic interaction may reflect a difference in the roles of SPO1 and SPO71 in spore wall deposition versus PSM development.

**SPO71 is important to fungi.** Although we did not find orthologs of SPO71 beyond fungi, we can identify orthologs in many fungal species, including the distantly related *Schizosaccharomyces*
pombe (estimated to have diverged from S. cerevisiae 350 to 1000 million years ago [2]) and the even more distantly related species within the phylum Basidiomycota. All orthologs have maintained a B domain that lies N terminal to two tandem PH domains. Conservation of the A domain is seen within the Saccharomyces sensu stricto clade, which is estimated to have diverged from other fungi about 20 million years ago; at this distance, the protein sequence diversity within this clade is considered comparable to that of the protein sequence diversity found between mammals and birds (10). Conservation within the A domain is also found in K. lactis, suggesting the A domain came to be before the genome-wide duplication event found in the Saccharomyces sensu stricto clade that did not occur in K. lactis (11). Interestingly, the S. pombe ortholog mrg56 (SPAC26H5.11) is induced during sporulation (21), consistent with a conserved role in sporulation. This evolutionary conservation suggests an important role for SPO71 in fungi, including pathogenic fungi with varying degrees of evolutionary relatedness to S. cerevisiae.

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