Protein Phosphatase Type 1-Interacting Protein Ysw1 Is Involved in Proper Septin Organization and Prospore Membrane Formation during Sporulation\textsuperscript{\textcopyright}

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Sporellation of \textit{Saccharomyces cerevisiae} is a developmental process in which four haploid spores are generated inside a diploid cell. Gip1, a sporulation-specific targeting subunit of protein phosphatase type 1, together with its catalytic subunit, Glc7, colocalizes with septins along the extending prospore membrane and is required for septin organization and spore wall formation. However, the mechanism by which Gip1-Glc7 phosphatase promotes these events is unclear. We show here that Ysw1, a sporulation-specific coiled-coil protein, has a functional relationship to Gip1-Glc7 phosphatase. Overexpression of \textit{YSW1} partially suppresses the sporulation defect of a temperature-sensitive allele of \textit{gip1}. Ysw1 interacts with Gip1 in a two-hybrid assay, and this interaction is required for suppression. Ysw1 tagged with green fluorescent protein colocalizes with septins and Gip1 along the extending prospore membrane during spore formation. Sporulation is partially defective in \textit{ysw1}\textsuperscript{Δ} mutant, and cytological analysis revealed that septin structures are perturbed and prospore membrane extension is aberrant in \textit{ysw1}\textsuperscript{Δ} cells. These results suggest that Ysw1 functions with the Gip1-Glc7 phosphatase to promote proper septin organization and prospore membrane formation.

Diploid cells of \textit{Saccharomyces cerevisiae} subjected to nitrogen limitation in the presence of a nonfermentable carbon source undergo the developmental process of sporulation (14, 23, 35). Four nuclei produced by two rounds of nuclear division, meiosis I and II, are encapsulated by newly formed double-membrane structures, called prospore membranes, and are finally packaged into spores covered with layered spore walls (35).

In this process, prospore membrane formation is one of the most dynamic events. Early in meiosis II, the cytoplasmic surface of the meiotic spindle pole body (SPB) is modified by the recruitment of septin complexes that act as a site of vesicle recruitment (2, 22, 39). Post-Golgi secretory vesicles dock to the surface of the SPBs and fuse with each other, generating prospore membranes (33, 34). The prospore membranes then grow to engulf daughter nuclei through a series of stages that are categorized by the membranes’ appearance in the fluorescence microscope (12). Initially, the membranes appear as small horseshoes that enlarge to become small round membrane structures. The prospore membranes then extend into a tube-like shape, engulfing the nucleus, as well as some cytosol and organelles (12). After this extension, prospore membranes undergo a rapid change to a mature round form. This rounding of the membrane is coordinated with membrane closure (12). Spore wall materials are then deposited into the luminal space created by closure of the prospore membrane (9).

In addition to the meiotic plaque of the SPB, two protein complexes are associated with the prospore membrane as it forms. One is the leading edge protein complex, which exists at the lip of the prospore membranes and consists of three components: Ssp1, Ady3, and Don1 (27, 30, 38). Ssp1 is the most important of the three and is required for proper extension of the prospore membrane (30). The second complex is a sporulation-specific septin structure. The septins are a family of cytoskeletal proteins, which form filaments (18, 50). Septins are conserved from yeast to mammals. They were originally found and have been extensively studied in \textit{S. cerevisiae}. In vegetatively growing \textit{S. cerevisiae} cells, five septin proteins—Cdc3, Cdc10, Cdc11, Cdc12, and Shs1—form a ring at the bud neck that serves as a scaffold for many additional proteins, as well as a barrier to diffusion of proteins between the mother and the bud (19, 29, 50). In sporulating cells, the set of septin proteins is changed. Cdc3 and Cdc10, along with two sporulation-specific septins, Spr3 and Spr28, form a pair of parallel bars or sheets associated with each prospore membrane (11, 15, 29). Although deletion of sporulation-specific septins has
only modest effects on sporulation (11, 15), their specific localization suggests that they have some function during pros︑ pore membrane formation. Septin organization in vegetatively growing cells is regulated by phosphorylation and dephospho︑ ylation of septin components and septin-associated proteins (29). In sporulating cells, a sporulation-specific protein phos︑ phatase type 1 (PP1) complex Gip1-Glc7 is required for the formation of septin structures (46), although whether this phos︑ phatase acts directly on the septin proteins is unknown. The PP1 catalytic subunit is highly conserved in eukaryotes and is involved in a variety of cellular processes (8, 44). In S. cerevisiae it is encoded by an essential gene, GLC7, and func︑ tions in glycyogen synthesis, glucose repression, chromosome segregation, cell wall organization, endocytosis, mating, and sporulation (3, 17, 24, 42, 44, 47, 53). The specificity of this enzyme is determined by targeting subunits. GIP1 was origi︑ nally isolated in a two-hybrid screen by using GLC7 as a bait, and this interaction was confirmed by cimmunoprecipitation of the two proteins (48). GIP1 is a sporulation-specific gene required for sporulation. Further analysis revealed that Gip1 and Glc7 colocalize with septins during sporulation and are required for both septin organization and spore wall formation (46). The specific targets or cofactors of this PP1 complex are unknown.

To elucidate the role of Gip1-Glc7 phosphatase, we screened for high-copy suppressors of a temperature-sensitive allele of gip1 and isolated YSW1. Ysw1 interacts with Gip1 and colocalizes with septins similar to Gip1. Furthermore, a ysw1Δ mutant displays aberrant septin structures and pros︑ pore membrane extension. These results suggest that Ysw1 may function with Gip1-Glc7 to regulate proper septin organization and pros︑ pore membrane formation.

### MATERIALS AND METHODS

**Strains and growth media.** Unless otherwise noted, standard media and ge︑ netic techniques were used (1, 20). The strains used in the present study are listed in Table 1. All strains are in the fast-sporulating SK-1 background except for AH109 (Clontech, Mountain View, CA) used for two-hybrid analysis. The primers used in the present study are listed in Table 2. To construct the YSW1-GFP haploid, NY19, HT21, and HT22 were used as primers to amplify the green fluorescent protein (GFP)-tagging cassette in pFA6a-yEGFP-HIS3MX6 (38), and the product was used to transform strain AN117-4B (36). The YSW1-ΔHA haploid, NY21, was created in the same way using pFA6a-HT22-GFP as a template. The SPR28-ΔFP diploid strain, TC522, was constructed by transfection of AN117-4B and AN117-16D (36) with a red fluorescent protein (ΔFP)-tagging cassette, amplified with oligonucleotides HT322 and HT323 from Escherichia coli DH5α, and isolated by self-ligation to delete part of the ΔFP sequence the plasmid. Transformation was performed with pRS304 (43) digested with EcoRI, pRS304-ΔFP with NotI digestion of pRS304, followed by mating of resulting haploids. NY703 was constructed by PCR-mediated knockout of SPR28 in AN117-4B and AN117-16D using pFA6a-HT22-GFP as a template.

**Plasmids.** The plasmids used in the present study are listed in Table 3. To construct pRS306-GIP1, the GIP1 gene was amplified from genomic DNA of AN120 (36) with MAC1 and HT306 as primers and cloned into Asp718 and SacII sites of pRS306 (43). pRS803ΔGpa1 was constructed by PstI digestion of pRS803, followed by self-ligation to delete part of OR34 coding sequence. To recover and sequence the gpa1-7 allele, PstI digests of genomic DNA were first prepared from the gpa1-7 mutant, MY101. These digests were self-ligated and used to transform Escherichia coli DH5α to obtain an ampicillin-resistant clone, pRS803-

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**TABLE 1. S. cerevisiae strains used in this study**

| Strain       | Genotype                                      | Source or reference |
|--------------|-----------------------------------------------|---------------------|
| AN117-4B     | MA TA αara3 leu2 trp1 his3ΔSK arg4-4Npl1 his2α ho::LYS2 rme1::LEU2 | 36                   |
| AN117-16D    | MA TA αara3 leu2 trp1 his3ΔSK arg4-4Npl1 his2α ho::LYS2 rme1::LEU2 | 36                   |
| NY19         | MA TA αara3 leu2 trp1 his3ΔSK arg4-4Npl1 his2α ho::LYS2 rme1::LEU2 YSW1-GFP::his5α | This study           |
| NY21         | MA TA αara3 leu2 trp1 his3ΔSK arg4-4Npl1 his2α ho::LYS2 rme1::LEU2 YSW1-ΔHA::his5α | This study           |
| NY528        | MA TA αara3 leu2 trp1 his3ΔSK arg4-4Npl1 his2α ho::LYS2 rme1::LEU2 YSW1::his5α | This study           |
| NY703        | MA TA αara3 leu2 trp1 his3ΔSK arg4-4Npl1 his2α ho::LYS2 rme1::LEU2 YSW1::his5α | This study           |
| TC522        | MA TA αara3 leu2 trp1 his3ΔSK arg4-4Npl1 his2α ho::LYS2 rme1::LEU2 YSW1::his5α | This study           |
| TC7          | MA TA αara3 leu2 trp1 his3ΔSK arg4-4Npl1 his2α ho::LYS2 rme1::LEU2 YSW1::his5α | This study           |
| TC8          | MA TA αara3 leu2 trp1 his3ΔSK arg4-4Npl1 his2α ho::LYS2 rme1::LEU2 YSW1::his5α | This study           |
| TC504        | MA TA αara3 leu2 trp1 his3ΔSK arg4-4Npl1 his2α ho::LYS2 rme1::LEU2 YSW1::his5α | This study           |
| TC551        | MA TA αara3 leu2 trp1 his3ΔSK arg4-4Npl1 his2α ho::LYS2 rme1::LEU2 YSW1::his5α | This study           |
| TC552        | MA TA αara3 leu2 trp1 his3ΔSK arg4-4Npl1 his2α ho::LYS2 rme1::LEU2 YSW1::his5α | This study           |
| TC553        | MA TA αara3 leu2 trp1 his3ΔSK arg4-4Npl1 his2α ho::LYS2 rme1::LEU2 YSW1::his5α | This study           |
| MIY101       | MA TA αara3 leu2 trp1 his3ΔSK arg4-4Npl1 his2α ho::LYS2 rme1::LEU2 YSW1::his5α | This study           |
| AH109        | MA TA αara3 leu2 trp1 his3ΔSK arg4-4Npl1 his2α ho::LYS2 rme1::LEU2 YSW1::his5α | Clontech            |
gip1-7. pRS424-YSW1 and pRS304-YSW1 were constructed by cloning the
BamHI-Asp718 fragment from one of the suppressor candidate clones into
pRS424 (7) and pRS304. pRS424-YSW1 and pRS304-YSW1 were constructed by cloning the
YSO319...........................GAAGAACTCGAGGATGGAAACTATTTTGCAGCCAAAGGCTAGA
YSO191...........................GAAGAAGGATCCATGGCATTTTAGAACTGAACGT
YSO178...........................GTGTTTGGTGTCTGAGCCACAGCTTTGGCAGGGCCTTCAAGAACATTC
YSO177...........................GAATGTTCTTGAAGGCCCTGCCAAAGCTGTGGCTCAGACACCAAACAC
YSO171...........................AGCAGCCTCGAGTTTCAATGAGTTAATTTCCT
MAC22............................GAAGAACTCGAGGCTCAATTGGTCGAGCTCTTC
MAC21............................GAAGAACTCGAGAGGACCCATATTGGATTTGGT
MAC20............................GAAGAACTCGAGCTGGTCGATAGTATATCTATT
MAC16............................GAAGAACTCGAGTTAAGATGTTAAGGTGGAGG
MAC7..............................GAAGAATTCACCCAGGAAATGGCATTACTC
MAC6..............................GAAGAACTCGAGCTGGCGTCTTAACAAGAGATTA
MAC1..............................GAAGAAGGTACCCTGCTGACATAGAAAGTAGAAA
HT319 .............................TATTGCACCGGTGTATTAACATATATAAGGATACGTACGAACATAACATCCGGATCCCCGGGTTAATTAA
HT315 .............................GAAGAACTCGAGTCAAAAAACATCCTCATCAAGC
HT306 .............................GAAGAACCGCGGTGATAGGCAAAAAATTGCACAG
HT259 .............................AAGAGAAACGAGAAACAATG
HT66K ............................GAAGAAGGTACCAGATCTATATTACCCTGTTATCC
HT66 ...............................GAAGAATTCAGATCTATATTACCCTGTTATCC
HT22 ...............................AATAAACGAGTTTTTAAGCGATCTATAATATTTTTATTGAGTGATAGTTAGAATTCGAGCTCGTTTAAAC
HT21 ...............................GTACAAAGTTGATAAAAGACAGCAAGAATGGCGCCTCCACCTTAACATCTCGGATCCCCGGGTTAATTAA

TABLE 2. Primers used in this study

| Name                  | Sequence (5’-3’) |
|-----------------------|-----------------|
| HT21                  | GTACAAGTGTGAAAAGACAGCAAGAATGCGGCTCCACTTAATATTTTTTATGAGTGATAGTTAGAATTCGAGCTCGTTTAAAC |
| HT22                  | ATATAACGAGTTTTTAAGCGATCTATAATATTTTTATTGAGTGATAGTTAGAATTCGAGCTCGTTTAAAC |
| HT32                  | GAAGAAGGTACCCTGCTGACATAGAAAGTAGAAA |
| HT39                  | CGATCACTTAAGACCTAAGCTAGTATTAAAAATATCAACAGGATCCCAGGTTAATTAACATGAGTTAGAATTCGAGCTCGTTTAAAC |
| HT40                  | ATATTTATTCTTTTGAGTTGACCAAGGCTAGTATTATTTTATGAGTGATAGTTAGAATTCGAGCTCGTTTAAAC |
| HT66                  | GAAGAAGGTACCCTGCTGACATAGAAAGTAGAAA |
| HT66K                 | GAAGAAGGTACCCTGCTGACATAGAAAGTAGAAA |
| pRS242-YSW1-GFP       | YSW1-GFP        |
| pRS304-YSW1-GFP       | YSW1-GFP        |
| pRS314-YSW1-GFP       | YSW1-GFP        |
| pGBD-GIP1             | GIP1            |
| pGAD-YSW1 series      | See Fig. 4      |
| pSB5                  | HA-GIP1         |
| pSB6                  | HA-GIP1         |
| pSB7                  | SPR28-GFP       |
| 424-G20               | GFP-SPO251-91   |

TABLE 3. Plasmids used in this study

| Plasmid     | Description               | Source or reference |
|-------------|---------------------------|---------------------|
| pRS304      | Integration vector        | 43                  |
| pRS306      | Integration vector        | 43                  |
| pRS314      | Low-copy vector          | 43                  |
| pRS424      | Multicopy vector          | 7                   |
| pRS306-GIP1 | GIP1                      | This study          |
| pRS306aPst  | pRS306 with a part of     | This study          |
|             | URA3 deleted              |                     |
| pRS306-gip1-7 | gip1-7                   | This study          |
| pRS424-YSW1 | YSW1                      | This study          |
| pRS304-YSW1 | YSW1                      | This study          |
| pRS424-YBR147w | YBR147w                 | This study          |
| pRS424-YSW1-GFP | YSW1-GFP                | This study          |
| pRS314-YSW1-GFP | YSW1-GFP                 | This study          |
| pRS304-YSW1*** | ysw1***                  | This study          |
| pRS424-YSW1-HA | YSW1-HA                  | This study          |
| pRS424-YSW1***-HA | ysw1***-HA               | This study          |
| pGBD-GIP1   | GIP1                      | This study          |
| pGAD-YSW1   | GIP1                      | This study          |
| pGBD-GIP1   | GIP1                      | This study          |
| pGAD-YSW1   | GIP1                      | This study          |
| pSB5        | HA-GIP1                   | 46                  |
| pSB6        | HA-GIP1                   | 46                  |
| pSB7        | SPR28-GFP                 | 46                  |
| 424-G20     | GFP-SPO251-91             | 32                  |
NSY01 (46). Colonies on each plate were collected in bulk by scraping of the plate, sporulated at 34°C, and subjected to ethanol treatment. For this treatment, ~10⁸ cells from each plate were incubated in 540 μl of 28% ethanol for 40 min, and plated on synthetic dextrose minimal (SD) plates. Colonies formed on each plate were collected, and plasmids were isolated. These plasmids were used to transform the gip1-7 cells and subjected to another round of sporulation and ethanol treatment. Plasmids were isolated from each of the surviving colonies, and suppression of gip1-7 was confirmed by retransformation and sporulation, followed by observation under microscope.

**Sporulation assays.** Cells were sporulated in liquid medium as described previously (34). Briefly, strains were grown at 30°C overnight in YPD or in SD medium when required. Cells were then preincubated at 30°C overnight in yeast extract-peptone-acetate (YP) medium. Cells were collected and resuspended in sporulation medium (2% potassium acetate) at an optical density of 1.5 at 600 nm, and these cultures were incubated at 30°C. For spore number analysis, more than 200 cells were counted.

**Two-hybrid analysis.** Two-hybrid analysis was performed as described by the manufacturers (Clontech). AH109 was transformed with pGBD-GIP1 and pGAD-YSW1 fusions. Transformants were grown overnight on SD medium without tryptophan, leucine, and histidine, and 10-fold dilutions were spotted onto SD plates without tryptophan, leucine, adenine, and histidine.

**Immunoblotting.** For the Western blot analysis of Ysw1-1HA, cells were induced for sporulation for 7.5 h, and total protein was prepared by using glass beads as described previously (1). Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Western blotting with anti-HA antibody (12CA5). Bands were visualized by horseradish peroxidase-conjugated goat antirabbit immunoglobulin G antibody (Biosource International, Camarillo, CA).

**Microscopy.** Differential interference contrast (DIC) images were obtained by using a BX70 microscope and DP Controller soft ware (Olympus, Tokyo, Japan). Fluorescence and immunofluorescence microscopy was performed as described previously (46) using a BX70 or a Zeiss Axioplan2 microscope (Carl Zeiss, Thornwood, NY) with a Zeiss mRM Axiocam and processed using Zeiss Axiosvision 4.7 software. Cells were fixed with 3.7% formaldehyde when required. Fluorescence and immunofluorescence microscopy was performed as described previously (46). Briefly, strains were grown at 30°C overnight in YPD or in SD plates without tryptophan, leucine, adenine, and histidine.

**RESULTS**

**Isolation of a temperature-sensitive gip1 mutant.** Gip1 is a sporulation-specific targeting subunit of Gic7, the yeast PP1 catalytic subunit, and is required for sporulation (46). However, targets and cofactors of this Gip1-Gic7 phosphatase complex are not known. To isolate genes that have a functional relationship to GIP1, we took a genetic approach. Temperature-sensitive sporulation-defective mutants of gip1 were isolated by introduction of randomly mutagenized GIP1 gene into the gip1 deletion mutant, followed by screening of transformants for those that formed ether-resistant spores at 22°C and not at 30°C. Of 1,000 colonies tested, four colonies showed temperature sensitivity. Among those, gip1-7 showed a complete sporulation defect at 30°C (Fig. 1A and B), which was ideal for suppressor screening. The gip1-7 cells subjected to sporulation at 22°C sporulated well, although the asci formed are predominantly monads and dyads (Fig. 1A and B). In contrast, at 30°C, the gip1-7 cells were indistinguishable to those in gip1Δ cells (Fig. 1A). In addition, spores formed at 22°C were aberrant in their shape; oval or football-like spores were observed (Fig. 1A). DNA sequence analysis of gip1-7 revealed that it has three mutations that affect the amino acid sequence of the encoded protein (K399R, D481G, and L500P).

The interaction of gip1-7 with GIP1 was assessed by two-hybrid analysis and was comparable to that of wild-type GIP1 (data not shown).

**Isolation of YSW1 as a multicopy suppressor of a temperature-sensitive gip1 mutant.** A screen was performed to isolate multicopy suppressor genes of the gip1-7 temperature-sensitive sporulation defect. The gip1-7 strain was transformed with a genomic library constructed using gip1Δ genomic DNA, sporulated at 33°C, and clones that formed ethanol-resistant spores were isolated. A total of 2 × 10⁶ colonies were subjected to screening and four plasmids were isolated as multicopy suppressors. All four plasmids contained a genomic region, which encompasses YBR147w and YSW1 (YBR148w). Therefore, YBR147w and YSW1 were cloned separately into multicopy vectors, introduced into the gip1-7 mutant, and sporulation was examined at restrictive temperature. YSW1, not YBR147w, restored sporulation, although sporulation efficiency was low and the ascii formed were mostly monads and dyads (Fig. 1C and D). Expression of Ysw1 from low copy vector suppressed gip1-7 at a level comparable to that of Ysw1 expression from multicopy plasmid (data not shown). Suppression was not observed when YSW1 was introduced into gip1Δ cells (data not shown). These results indicate that overexpression of YSW1 partially suppresses the temperature-sensitive sporulation-defective phenotype of the gip1-7 mutant and that YSW1 has a genetic interaction with GIP1.

A conserved region of Ysw1 is required for interaction with Gip1 and suppression of gip1-7. YSW1 encodes a predicted protein of 609 amino acids (aa) that contains two coiled-coil domains. It was originally isolated in a large-scale analysis of gene expression and protein localization as a protein induced during meiosis and sporulation (4). A truncated form of Ysw1 fused to lacZ localized to the prospore membrane. The genetic interaction between YSW1 and GIP1 led us to examine whether Ysw1 can physically interact with Gip1. A two-hybrid analysis was performed, and Ysw1 was found to interact with Gip1 (Fig. 2A). To identify the region of Ysw1 involved in the interaction with Gip1, we constructed deletion series of Ysw1 and tested for interaction with full-length Gip1. Surprisingly, we found that two separate regions of Ysw1 interact with Gip1 (Fig. 2A). The first region includes aa 201 to 297, and the second region spans aa 401 to 473, which encompasses the second coiled-coil domain of Ysw1.

Comparative genomic studies have revealed that a whole-genome duplication occurred in the evolution of hemiascomycetes, *S. cerevisiae* being one of the postduplication species (52). Duplicated genes are referred to as ohnologs. It is reported that YSW1 is the ohnolog of SPO21, which encodes a meiosis-specific component of the SPB, although they have only 13% identity and do not hit each other in the basic local alignment tool for protein sequences (BLASTP) (51). Based on this report, we compared the YSW1 sequence with the SPO21/YSW1 genes of preduplication yeast species and found a small region (aa 230 to 240) of Ysw1, amino terminal to the first coiled-coil domain, that is conserved at the analogous position in the Spo21/Ysw1 orthologs of *Kluyveromyces lactis*, *Ashbya gossypii*, and *Kluyveromyces waltii* (Fig. 2B). This region is not conserved in *S. cerevisiae* Spo21. The motif resides in the first Gip1-interacting region of Ysw1 defined in our deletion studies. Therefore, to examine whether the motif was imper-
tant for the interaction, we introduced the three point mutations Y233A, F235A, and F237A into conserved residues of Ysw1 (hereafter referred to as Ysw1***; Fig. 2A). Neither the full-length Ysw1*** nor aa 201 to 400 of Ysw1*** alone interacted with Gip1 in two-hybrid assay, indicating the importance of this conserved motif for binding of Ysw1 to Gip1. To examine whether Ysw1*** is functional or not, we expressed Ysw1***-HA in the gip1-7 mutant. The gip1-7 cells expressing Ysw1-HA sporulated to ca. 20% at a nonpermissive temperature (Fig. 2C). However, at 30°C the gip1-7 cells expressing Ysw1***-HA were completely defective in sporulation, as was the case with gip1-7 cells harboring vector alone (Fig. 2C). A Western blot with anti-HA antibody confirmed that Ysw1***-HA is expressed at a level similar to that of Ysw1-HA during sporulation (Fig. 2D). These results strongly indicate that interaction of Ysw1 with Gip1 through this conserved region is required for suppression of gip1-7 by YSW1.

Ysw1 colocalizes with septins and Gip1 along the extending prospore membrane. To investigate the localization of full-length Ysw1 protein during sporulation, we tagged the Ysw1 protein with GFP at its C terminus and examined the protein by fluorescence microscopy. Ysw1-GFP was at least partially functional, because overexpression of YSW1-GFP allowed the gip1-7 strain to sporulate at only a slightly lower level compared to the same strain overexpressing YSW1 (Fig. 1D). Ysw1-GFP was seen as four small rings or four pairs of short bars around the nucleus in early meiosis II, and four pairs of long bars along prospore membrane in late meiosis II (Fig. 3A). The Ysw1-GFP signal disappeared in postmeiotic cells, and no specific localization pattern was observed (data not shown). The localization pattern of Ysw1-GFP during meiosis II was similar to that reported for septins and Gip1 (46), although the absence of Ysw1 in postmeiotic cells was quite different from the spore periphery pattern seen for septins and Gip1 at this later stage (15, 46).

To confirm that Ysw1 colocalizes with septins and Gip1 in meiosis II, a YSW1-GFP plasmid was introduced into strains expressing Spr28-RFP and HA-Gip1, respectively. Colocalization of Ysw1-GFP with Spr28-RFP and HA-Gip1 was observed in meiosis II cells (Fig. 3B and C). These results suggest that...
Ysw1, Gip1, and Glc7 colocalize on septin structure along the extending prospore membranes.

Given that septins form filaments and function as a scaffold for many proteins at the bud neck in vegetatively growing cells, we reasoned that the Ysw1 localization pattern would be septin dependent. Therefore, we analyzed Ysw1-GFP localization in septin mutants. Deletion of either of the sporulation-specific septins SPR3 or SPR28 disrupts the organization of the remaining septin proteins (41). Loss of SPR28 causes the remaining septins to localize uniformly around the prospore membrane rather than in bars, while the loss of SPR3 leads to a failure of the remaining septins to associate with the prospore membrane.
In the case of Ysw1-GFP, deletion of either SPR3 or SPR28 caused the same phenotype, an even distribution around the prospore membrane rather than a bar-like pattern (Fig. 3D). These observations indicate that localization of Ysw1 into bars, although not its association with the prospore membrane, is septin dependent.

Septins structures are aberrant in the ysw1Δ mutant. To examine the role of Ysw1 during sporulation, a ysw1Δ mutant was constructed and analyzed for sporulation. As reported in genomewide analysis (28), the ysw1Δ mutant asci had a reduced number of spores, and many dyads and triads were observed (Fig. 4A and B). Using DAPI staining, the meiotic progression of the ysw1Δ mutant was monitored and found to be wild type (data not shown). These observations suggest that Ysw1 functions in spore formation rather than in meiosis. Expression of Ysw1** did not rescue the sporulation defect of the ysw1Δ mutant (Fig. 4B), suggesting that interaction between Ysw1 and Gip1 is required for the function of Ysw1.

The involvement of Ysw1 in spore formation, together with its relationship to septins and Gip1, prompted us to examine the localization of septins and Gip1 in the ysw1Δ mutant. Spr28-GFP and HA-Gip1 were expressed in the mutant, and their localization during sporulation was determined by fluorescence microscopy. Septin structures were formed during sporulation in the ysw1Δ mutant (Fig. 4C). Both bars in meiosis II cells and distribution of proteins around the spore periphery in postmeiotic cells were observed. However, the bars in the ysw1Δ cells were abnormal and appeared excessively bent or twisted. A similar localization defect of Gip1 was observed in ysw1Δ cells (Fig. 4D), indicating that HA-Gip1 can localize to the septin structure in the ysw1Δ cells. These results suggest that Ysw1 is required for proper septin organization.

Ysw1 is required for proper prospore membrane formation. The bar-like structures containing Gip1, Ysw1, and septins form along the prospore membrane (15, 46). Therefore, we examined whether prospore membranes are properly formed in the ysw1Δ mutant. A fragment of the Spo20 protein fused to GFP (32) was used to visualize prospore membranes. In wild-type cells, as the cells go through meiosis II, prospore membranes progressively display horseshoe-like, tubular, and round morphologies (12) (Fig. 5A). In addition, all four prospore membranes forming within a wild-type cell appear to grow at similar rates. In the ysw1Δ cells, prospore membranes were also formed but appeared somewhat uncoordinated in their size (Fig. 5A). Strikingly, counting of prospore membranes in early- and post-meiosis II cells revealed that number of mature round prospore membrane per ascus was reduced, about half of the asci contained only two prospore membranes (Fig. 5B). This correlated well with distribution of spore number in mature asci. Very small prospore membranes and/or remnants of them were frequently observed in the ysw1Δ asci. These results suggest that the ysw1Δ cells are defective in prospore membrane growth.

To further analyze the defect of ysw1Δ cells in prospore membrane formation, we performed time-lapse imaging. In wild-type cells, the extension of the four prospore membranes appeared coordinated (Fig. 5C and see Movie S1 in the supplemental material). In contrast, prospore membrane extension was slower and looked less coordinated in ysw1Δ cells (Fig. 5C and see Movies S2 to S5 in the supplemental material). Fewer than four prospore membranes extended in many asci, with the remaining prospore membranes stopped at the small round phase or earlier. These results indicate that Ysw1 is required for proper prospore membrane growth.
FIG. 4. Sporulation is aberrant in the ysw1Δ mutant. (A) TC504 (ysw1Δ) was sporulated and analyzed by DIC microscopy. (B) AN120 (wild-type), TC551 (ysw1Δ pRS304), TC552 (ysw1Δ, pRS304-YSW1), and TC553 (ysw1Δ, pRS304-ysw1***)) were sporulated, and the distribution of the spore number is represented. (C) AN120 (wild-type) and TC504 (ysw1Δ) harboring pSB7 (SPR28-GFP) were analyzed by fluorescence microscopy. (D) AN120 (wild-type) and TC504 (ysw1Δ) harboring pSB5 (HA-GIP1) were analyzed by immunofluorescence with anti-HA antibody. Arrowheads in panels C and D indicate cells showing aberrant pattern. Scale bars (A, C, and D), 5 μm.
In *Saccharomyces cerevisiae*, the sporulation-specific PP1 complex Gip1-Glc7 is essential for sporulation (46). However, its cofactors or downstream targets are unknown. In the present study, we identified Ysw1 as a new factor that functions with Gip1-Glc7 during sporulation. Ysw1 genetically and physically interacts with Gip1 and colocalizes with Gip1 and septins during meiosis II. Deletion of the *YSW1* gene affects septin organization and prospore membrane formation. Thus, Ysw1 may work with the Gip1-Glc7 phosphatase to control proper septin assembly and prospore membrane growth.

Overexpression of *YSW1* partially suppressed *gip1Δ*, but not *gip1Δ*, indicating that Ysw1 requires partially functional Gip1 for suppression. Taken together with the physical interaction of Ysw1 with Gip1 and the requirement of this interaction for suppression, Ysw1 does not substitute for, but may function in the same pathway as, Gip1. Because the sporulation defect of *ysw1Δ* is not as severe as that of *gip1Δ*,

**FIG. 5.** Prospore membrane growth is defective in *ysw1Δ* mutant. (A) AN120 (wild-type) and TC504 (*ysw1Δ*) were transformed with 424-G20 (*GFP-SPO2051-91*), and prospore membranes were visualized during sporulation. (B) The numbers of small prospore membranes per cell (left) and mature prospore membranes per cell (middle) or spores per ascus (right) were counted. These categories correspond to cells in early meiosis II, late meiosis II, and postmeiosis, respectively. (C) AN120 (wild-type) and TC504 (*ysw1Δ*) carrying 424-G20 (*GFP-SPO2051-91*) were sporulated and analyzed by time-lapse fluorescence microscopy. Each image is a projection through a deconvolved image stack. The numbers indicate the time elapsed, in minutes. Scale bars (A and C), 5 µm.
we favor the idea that Ysw1 is a cofactor of Gip1-Glc7. Western blot analysis of Ysw1-HA expressed in wild-type and the gip1Δ mutant cells showed no obvious mobility difference (data not shown), although we cannot rule out the possibility that Ysw1 is also a target of the Gip1-Glc7 phosphatase.

Ysw1 localizes with septins along the extending prospore membrane in wild-type cells, but it localizes evenly on the prospore membrane in both spr3Δ and spr28Δ cells, indicating that Ysw1 can localize to the prospore membrane in the absence of septin structures. Because Spr3 is required for the association of other septins with the prospore membrane (41), we suggest that Ysw1 localization to prospore membrane is not dependent on interaction with septins but rather with Gip1 or some other protein(s) on prospore membrane.

Septins form hetero-oligomeric complexes and filaments are formed by polymerization of the complexes (29, 50). In vegetatively growing cells, they are organized into higher-order structures that appear as patches, collars, or rings coordinated with cell cycle (16). Septin subunits in these cells are phosphorylated and dephosphorylated coordinated with cell cycle, so it is likely that this phosphorylation and dephosphorylation is involved in the regulation of septin organization. Septin-associated kinases and phosphatases are responsible for the modification of septin subunits (13, 21, 25, 31, 49). In sporulating cells, the formation of the sporulation-specific structure of septins requires Gip1-Glc7 phosphatase (46), suggesting the existence of similar regulatory mechanisms. In the ysw1Δ mutant, septant structures are perturbed during prospore membrane formation. Considering that Ysw1 interacts with Gip1-Glc7 phosphatase, it is possible that the absence of Ysw1 may cause subtle changes in Gip1-Glc7 phosphatase localization or activity that may, in turn, affect septin organization. The Gip1-Glc7 phosphatase may dephosphorylate septins and/or septin-associated proteins, and Ysw1 may help regulate septin organization through interaction with Gip1. Further analysis of the organization and modification of septins during sporulation is required to elucidate the mechanism by which Gip1-Glc7 and Ysw1 function.

In addition to the defect in septin organization, the ysw1Δ mutant displays defects in prospore membrane extension, indicating that Ysw1 is involved in proper prospore membrane growth. Our recent analysis of the gip1Δ mutant revealed that prospore membranes formed in this mutant are smaller than those in wild-type cells (I. Inoue and H. Tachikawa, unpublished observations). Thus, Ysw1 may function in proper prospore membrane formation through interaction with Gip1.

There are many reports of sporulation-defective mutants that form predominantly dyads. Most of these mutants have defects in SPB modification or stability, leading to the generation of less than four prospore membranes (2, 10, 22, 37, 39). The ady1Δ mutant defines a different class in which there is a defect in spore wall formation but no defect in prospore membrane formation (38, 45). The ysw1Δ mutant also forms dyads; however, it cannot be placed in either of these classes. It initially forms four prospore membranes, but coordination of the extension is defective, resulting in the formation of fewer than four mature prospore membranes and mature spores. Thus, the ysw1Δ mutant represents a new type of dyad-forming mutant that has defects in coordinated prospore membrane extension.

Ysw1 and a meiosis-specific component of the SPB, Spo21, are ohnologs, that is, a paralogous S. cerevisiae gene pair formed by gene duplication (51). It is noteworthy that Ady3, which is a component of leading edge complex is also the ohnolog of Cnm67, another component of SPB (5). Therefore, it is tempting to speculate that genome duplication in ascomycetes may have produced prospore membrane-associated proteins from SPB components. Spo21 interacts with Cnm67 (30); thus, from an evolutionary point of view, it would be interesting to test whether Ysw1 interacts with Ady3.

Glc7, Gip1, septins, and Ysw1 colocalize in bars during prospore membrane extension. After closure, however, the proteins appear to dissociate. While septins and Gip1 remain at the spore periphery, Glc7 relocates to the nucleus and Ysw1 disappears (15, 46). Closure of the prospore membrane is coordinated with the end of meiosis by the anaphase-promoting complex-dependent removal of the leading edge component Sp1 (12). The disappearance of Ysw1 also appears to correlate well with the time of prospore membrane closure. It may be that Ysw1 is also a target of anaphase-promoting complex-mediated removal or degradation at the end of meiosis II, and the turnover of Ysw1 may contribute to the dissociation of the septin structures after cytokinesis.

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