# A Gip1p–Glc7p phosphatase complex regulates septin organization and spore wall formation

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Sporulation of *Saccharomyces cerevisiae* is a developmental process in which a single cell is converted into four haploid spores. *GIP1*, encoding a developmentally regulated protein phosphatase 1 interacting protein, is required for spore formation. Here we show that *GIP1* and the protein phosphatase 1 encoded by *GLC7* play essential roles in spore development. The *gip1Δ* mutant undergoes meiosis and prospore membrane formation normally, but is specifically defective in spore wall synthesis. We demonstrate that in wild-type cells, distinct layers of the spore wall are deposited in a specific temporal order, and that *gip1Δ* cells

display a discrete arrest at the onset of spore wall deposition. Localization studies revealed that Gip1p and Glc7p colocalize with the septins in structures underlying the growing prospore membranes. Interestingly, in the *gip1Δ* mutant, not only is Glc7p localization altered, but septins are also delocalized. Similar phenotypes were observed in a *glc7–136* mutant, which expresses a Glc7p defective in interacting with Gip1p. These results indicate that a Gip1p–Glc7p phosphatase complex is required for proper septin organization and initiation of spore wall formation during sporulation.

## Introduction

Sporulation of *Saccharomyces cerevisiae* is a developmental process in which a single diploid cell is converted into an ascus containing four haploid spores. Diploid cells subjected to nitrogen starvation in the presence of a nonfermentable carbon source undergo two meiotic nuclear divisions, meiosis I and II, and packaging of the four resulting haploid nuclei into spores. Spore formation occurs in a series of well-defined steps (Lynn and Magee, 1970; Moens, 1971; Moens and Rapport, 1971; Neiman, 1998; Kupiec et al., 1997). Late in meiosis I, the spindle pole body (SPB)* undergoes a structural change and its outer plaque becomes enlarged. As cells enter meiosis II, secretory vesicles coalesce on the cytoplasmic surface of each outer plaque to form a novel intracellular membrane, termed the prospore membrane. The prospore membranes extend out as double membranes from the SPBs and engulf the adjacent nuclear lobes. After the

completion of meiosis II, nuclear division gives rise to four haploid nuclei. At the same time, the ends of each double membrane meet and fuse with themselves, encapsulating the haploid nuclei to form prospores. Membrane closure triggers the final step of spore morphogenesis, spore wall formation. Spore wall materials are deposited in the luminal space between the two membranes derived from the prospore membrane.

The spore wall consists of four layers. The two inner layers are composed of glucan and mannan, components of the vegetative cell wall (Klis, 1994). The third layer is composed largely of chitosan, a glucosamine polymer, produced by the combined action of the chitin synthetase, Chs3p, and chitin deacetylases (Briza et al., 1988; Pammer et al., 1992; Christodoulidou et al., 1996; Mishra et al., 1997). The outermost layer consists largely of cross-linked dityrosine molecules (Briza et al., 1986). Dityrosine precursors are synthesized in the spore cytosol by the enzymes encoded by *DIT1* and *DIT2* (Briza et al., 1990, 1994). These genes are induced specifically around the time of prospore membrane closure. The chitosan and dityrosine layers are specific to spores, and provide much of the spore's resistance to environmental stress. Several mutants and genes related to spore wall formation have been isolated and characterized. Among these are genes coding for protein kinases, *SPS1*, *SMK1*, *CAK1*, and *MPS1*, and a gene encoding a nuclear protein,

*SWM1*, raising the possibility that signal transduction pathways might regulate the spore wall formation (Friesen et al., 1994; Krisak et al., 1994; Wagner et al., 1997; Ufano et al., 1999; Straight et al., 2000).

Septins are a conserved family of proteins characterized by a central core domain and P-loop nucleotide-binding motif (Longtine et al., 1996; Kartmann and Roth, 2001). Most of them also contain a coiled-coil domain that could be involved in their assembly into filaments. In vegetatively growing yeast cells, septins localize to the bud neck and function as a scaffold interacting with a variety of proteins (DeMarini et al., 1997; Longtine et al., 2000). There are seven septin genes in *S. cerevisiae*, four of which are transcriptionally upregulated during sporulation: *SPR3* and *SPR28* are sporulation specific, and *CDC3* and *CDC10* are induced more than tenfold (Kaback and Feldberg, 1985; Chu et al., 1998; De Virgilio et al., 1996; Fares et al., 1996; Primig et al., 2000). During spore development, septin localization is different than that seen in vegetative cells (Fares et al., 1996). No septin localization to the cell periphery is seen. Rather, the septins appear in early meiosis II as four ring-like structures around each SPB. As the prospore membrane extends, septins disappear from the SPB region and display an extended band-like pattern underlying the prospore membrane. After closure of the prospore membrane, the septins become diffusely localized in the spore periphery (Fares et al., 1996). Surprisingly, disruption studies have revealed only modest sporulation defects in septin mutants; *spr3Δ spr28Δ* homozygous diploids and *cdc10Δ* diploids sporulate well (De Virgilio et al., 1996; Fares et al., 1996). Although no strong sporulation phenotype has been observed in septin mutants, their expression profile and specific localization suggest that they have some function in sporulation.

Protein phosphatase type 1 (PP1) is a highly conserved phosphoserine-/phosphothreonine-specific protein phosphatase that plays important roles in a variety of cellular processes including cell cycle progression, glycogen metabolism, glucose repression, and sporulation (Mumby and Walter, 1993; Depaoli-Roach et al., 1994; Shenolikar, 1994). In *S. cerevisiae*, PP1 is encoded by the essential gene *GLC7* (Feng et al., 1991). Consistent with the multiple functions of Glc7p, the localization of this protein is dynamic throughout the cell cycle. In addition to a predominant nuclear localization, it is also observed at the SPB, bud neck, and actomyosin ring at distinct times in the cell cycle (Bloecher and Tatchell, 2000). The localization and substrate specificity of Glc7p are thought to be regulated by interaction with different targeting or regulatory subunits. For instance, bud neck localization of Glc7p requires the septin-binding protein Bni4p (unpublished data), and Gac1p is required to localize Glc7p to glycogen particles (Francois et al., 1992; Stuart et al., 1994). As in vegetative cells, there are multiple points at which *GLC7* is required during sporulation, including premeiotic DNA synthesis and passage through meiosis I (Ramaswamy et al., 1998; Bailis and Roeder, 2000).

*GIP1* encodes a potential developmentally regulated targeting subunit of Glc7p. *GIP1* was isolated as a *GLC7* interacting gene in a two-hybrid screen (Tu et al., 1996). This interaction was confirmed by coimmunoprecipitation (Tu et al., 1996). *GIP1* is a sporulation-specific gene and diploid strains homozygous for a deletion of this gene are blocked in sporulation and fail to induce *SPS100*, one of the very late sporulation genes (Tu et al., 1996). Additionally, several *glc7* mutant alleles defective in sporulation have been isolated, and in most instances sporulation efficiency correlates with the strength of the two-hybrid interaction between the *glc7* mutant protein and Gip1p (Baker et al., 1997; Ramaswamy et al., 1998). These previous reports raise the possibility that *GIP1* dependent regulation of Glc7p might be important for sporulation.

In this study, we examine the roles of *GIP1* and *GLC7* in sporulation. Cytological analyses of mutant strains revealed that *GIP1* and *GLC7* are required for spore wall formation.

Table I. **Strains used in this study**

| Strain | Genotype | Source |
| --- | --- | --- |
| NH144 | *MATa/MATα ura3/ura3 leu2Δ::hisG/leu2-k his4-x/HIS4 arg4-NspI/ARG4 lys2/lys2 hoΔ::LYS2/hoΔ::LYS2* | Neiman, 1998 |
| AN103 | *MATa/MATα his3ΔSK/his3ΔSK ura3/ura3 leu2Δ::hisG/leu2Δ::hisG trp1::hisG/trp1::hisG arg6/ARG6 lys2/lys2 hoΔ::LYS2/hoΔ::LYS2 rme1::LEU2/rme1::LEU2 gip1::HIS3/gip1::HIS3* | This study |
| SB247 | *MATa/MATα ura3-52/ura3-52 trp1-1/trp1-1 leu2/leu2 glc7::LEU2/glc7::LEU2 [pNC160-glc7-136 (TRP1)]* | Baker et al., 1997 |
| AN117-4B | *MATα his3 ura3 trp1::hisG leu2 arg4-NspI lys2 hoΔ::LYS2 rme1::LEU2* | Neiman et al., 2000 |
| AN117-16D | *MATa his3 ura3 trp1::hisG leu2 lys2 hoΔ::LYS2* | Neiman et al., 2000 |
| AN120 | *MATa/MATα his3/his3 ura3/ura3 trp1::hisG/trp1::hisG leu2/leu2 arg4-NspI/ARG4 lys2/lys2 hoΔ::LYS2/hoΔ::LYS2 rme1::LEU2/RME1* | Neiman et al., 2000 |
| NY1 | *MATα his3 ura3 trp1::hisG leu2 arg4-NspI lys2 hoΔ::LYS2 rme1::LEU2 gip1::HIS3* | This study |
| NY2 | *MATa his3 ura3 trp1::hisG leu2 lys2 hoΔ::LYS2 gip1::HIS3* | This study |
| NY501 | *MATa/MATα his3/his3 ura3/ura3 trp1::hisG/trp1::hisG leu2/leu2 arg4-NspI/ARG4 lys2/lys2 hoΔ::LYS2/hoΔ::LYS2 rme1::LEU2/RME1 gip1::HIS3/gip1::HIS3* | NY1 × NY2 |
| NY13 | *MATα his3 ura3 trp1::hisG leu2 arg4-NspI lys2 hoΔ::LYS2 rme1::LEU2 gip1::his5⁺-spo20pr-HA-GIP1* | This study |
| NY14 | *MATa his3 ura3 trp1::hisG leu2 lys2 hoΔ::LYS2 gip1::his5⁺-spo20pr-HA-GIP1* | This study |
| NY509 | *MATa/MATα his3/his3 ura3/ura3 trp1::hisG/trp1::hisG leu2/leu2 arg4-NspI/ARG4 lys2/lys2 hoΔ::LYS2/hoΔ::LYS2 rme1::LEU2/RME1 gip1::his5⁺-spo20pr-HA-GIP1 / gip1::his5⁺-spo20pr-HA-GIP1* | NY13 × NY14 |
| NY523 | *MATa/MATα his3/his3 ura3/ura3 trp1::hisG/trp1::hisG leu2/leu2 arg4-NspI/ARG4 lys2/lys2 hoΔ::LYS2/hoΔ::LYS2 rme1::LEU2/RME1 glc7::his5⁺/GLC7* | This study |
| NY525 | *MATa/MATα his3/his3 ura3:glc7-136:URA3/ura3 trp1::hisG/trp1::hisG leu2/leu2 arg4-NspI/ARG4 lys2/lys2 hoΔ::LYS2/hoΔ::LYS2 rme1::LEU2/RME1 glc7::his5⁺/GLC7* | This study |
| NY527 | *MATa/MATα his3/his3 ura3:glc7-136:URA3/ura3:glc7-136:URA3 trp1::hisG/trp1::hisG leu2/leu2 arg4-NspI/ARG4 lys2/lys2 hoΔ::LYS2/hoΔ::LYS2 rme1::LEU2/RME1 glc7::his5⁺/glc7::his5⁺* | This study |

Both proteins are found to colocalize with septins and are required for septin assembly during sporulation. These results suggest that *GIP1* and *GLC7*, and perhaps the septins, participate in a signal transduction pathway necessary to monitor prospore membrane growth and initiate the synthesis of the spore wall.

## Results

### Spore wall formation is blocked in *gip1Δ* mutant cells

To investigate the essential role of *GIP1* during sporulation, we examined where in the process of sporulation *gip1Δ* cells are defective. First, a homozygous *gip1Δ* mutant strain, NY501 (Table I), was constructed in the rapidly sporulating SK1 background (Kane and Roth, 1974), and the progression of meiosis was monitored using the DNA-binding dye DAPI. In wild-type and *gip1Δ* mutant cells, the number of nuclei seen in each cell increased from one to two, then to four, with nearly identical kinetics (Fig. 1). This indicates that meiosis I and II proceed normally in the *gip1Δ* mutant, and suggests that the sporulation defect in *gip1Δ* mutants must be due to a failure in some aspect of spore packaging.

To examine this possibility, EM analysis of sporulating wild-type and the *gip1Δ* mutant cells was performed. In wild-type cells, prospore membranes capturing each nucleus were observed (Fig. 2 A), and at later stages, spore walls were seen between each of the inner and outer membrane surrounding the spore nucleus (Fig. 2 B). In *gip1Δ* cells, prospore membranes resembling those in wild-type cells were also observed capturing nuclei (Fig. 2, C and D). Yet strikingly, no spore walls were observed in the mutant cells. The inner and outer membranes derived from each prospore membrane remained closely apposed, suggesting that no spore wall material was deposited. The *gip1Δ* cells also displayed a proliferation of darkly staining material around the ascal surface (*, Fig. 2, C and D). Accumulation of similar material has been observed in some spore wall defective mutants (Christodoulidou et al., 1999; Wagner et al., 1999). These data suggest that *GIP1* is required to initiate spore wall formation.

### *DIT1* expression is blocked in *gip1Δ* mutants

Although the EM study indicated a lack of spore wall material in the *gip1* mutant, we could not rule out the possibility that there are some components or layers of spore wall deposited that are not clearly visible by EM. Dityrosine-containing macromolecules can be observed by their natural fluorescence (Briza et al., 1990). Thus, to examine whether the dityrosine layer is formed in *gip1Δ* mutant, we assayed the sporulated *gip1Δ* mutant for dityrosine fluorescence. Dityrosine fluorescence was absent in the *gip1Δ* mutant (unpublished data), indicating that no dityrosine is produced in this mutant.

The *DIT1* gene is required for the synthesis of dityrosine and expression of *DIT1* is induced in sporulation before *SPS100*, but after middle genes such as *GIP1* or *SPR3* (Briza et al., 1990; Chu et al., 1998; Primig et al., 2000). *GIP1* is required for expression of the late gene *SPS100* (Tu et al., 1996). This previously described effect of *gip1Δ* mutation on *SPS100* expression, as well as the lack of dityrosine fluorescence, led us to examine *DIT1* expression in the *gip1Δ* mutant. A *DIT1–lacZ* fusion gene was introduced into wild-type and *gip1Δ* mutant strains. As a control, a *lacZ* fusion to *SPR3* was used. Whereas *SPR3–lacZ* was induced with the same kinetics in the isogenic wild-type and *gip1Δ* diploids, no induction of *DIT1–lacZ* was observed when *GIP1* was absent (Fig. 3). Thus, the absence of dityrosine staining in the *gip1Δ* mutant can be explained by the failure to transcribe *DIT1*.

### Fluorescence studies reveal the temporal order of spore wall deposition

To examine the formation of spore wall layers other than the dityrosine layer, a series of fluorescent markers was used. The mannan layer was visualized using the mannose-binding lectin concanavalin A coupled to FITC. A monoclonal antibody directed against β1–3Glucan was used to identify the glucan layer, and the chitin-binding dye Calcofluor white was used to visualize the chitosan layer. The use of secondary antibodies conjugated to a red fluor to localize the glucan antibodies allowed all three markers to be used simultaneously. As shown in Fig. 4, the staining patterns of wild-type asci varied accord-
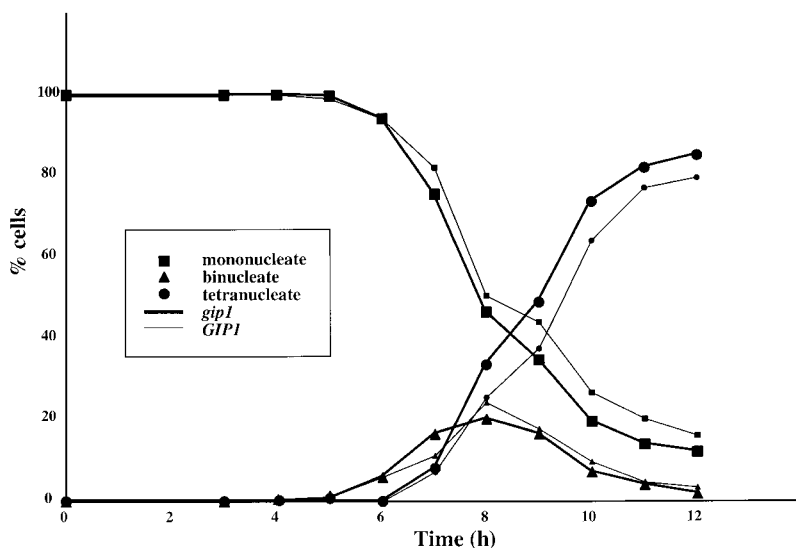


Figure 1. **Meiotic progression is normal in the *gip1Δ* mutant.** At each time point, aliquots of strains AN120 (*GIP1/GIP1*, thin line) and NY501 (*gip1Δ/gip1Δ*, thick line) were removed from sporulation medium, stained with DAPI, and analyzed for meiotic progression. Percentage of mononucleate (■), binucleate (▲), and tetranucleate (●) cells are shown. At least 200 cells were counted at each time point.

Figure 2.    **The *gip1Δ* mutant fails to form spore walls.** EM analysis of *GIP1* (NH144, A and B) and homozygous *gip1Δ* mutant (AN103, C and D) cells at a late stage of sporulation. Prospore membranes (PrM); spore walls (SW); and nuclei (N) are indicated. *, Darkly staining material formed in the ascal cytoplasm of *gip1* mutants. Bar, 500 nm.
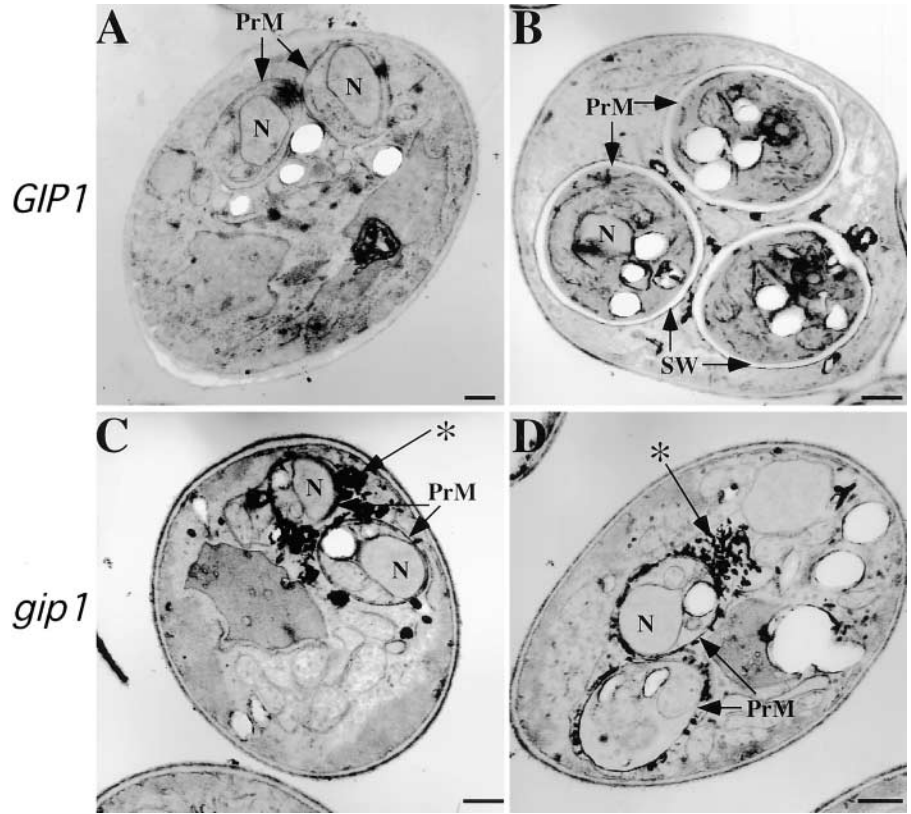


ing to the stage of spore development. After counting and classifying these asci, we ordered the patterns as follows (Table II): when cells are in the stage of the prospore membrane formation and extension, those membranes are stained only with FITC-ConA (Class 2). Approximately 30% of cells showed



Figure 3.    ***DIT1–lacZ* is not induced in *gip1Δ* cells.** Sporulating cultures of AN120 (*GIP1/GIP1*, △) and NY501 (*gip1Δ/gip1Δ*, □) carrying either (A) *SPR3–lacZ* or (B) *DIT1–lacZ* were analyzed at various time points for β-galactosidase activity. β-galactosidase activity is given in Miller Units.

this pattern at the seven hour time point. Subsequently, a population of cells appears that is positive for both FITC-ConA and anti-β1–3Glucan (Class 3). At the 7- and 8-h time points, ∼20% of the cells showed staining with anti-β1–3Glucan in the spore precursors. After a transient period of triply positive staining (Class 4), the ascus loses anti-β1–3Glucan staining (Class 5). At this point, the FITC-conA staining pattern changes to stain only the spore periphery. Finally, Calcofluor white no longer stains the spore wall (Class 6). Therefore, these markers allow all the major spore wall polysaccharides to be visualized during the course of spore wall formation and suggest that these components are deposited in a specific sequence: mannan before glucan before chitosan.
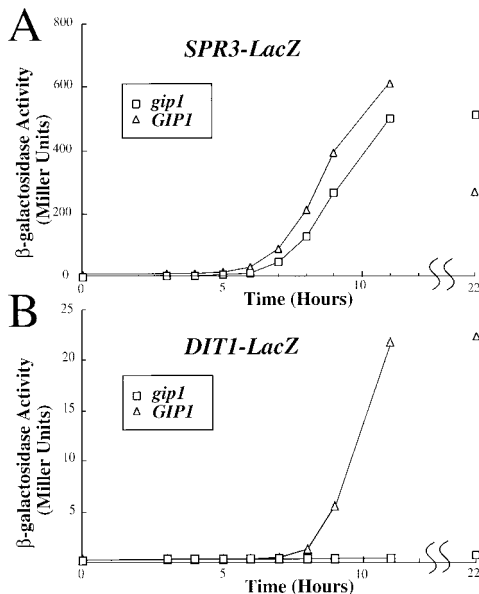
### The *gip1Δ* mutant fails to form the inner layers of spore wall

To examine the spore wall defect of *gip1Δ*, we applied this method to *gip1Δ* mutant cells. As in wild-type cells, the developing prospore membranes stained with FITC-ConA (Fig. 4, P–R). However, the *gip1Δ* mutant cells never progressed to show anti-β1–3Glucan or Calcofluor white staining. Taken together with the lack of the dityrosine layer, these results indicate that the prospore wall in *gip1Δ* mutants contains only those mannoproteins present during prospore membrane formation. No additional spore wall material is deposited at the completion of prospore membrane synthesis.

### Gip1p colocalizes with Spr28p during spore formation

To examine where in the cell Gip1p functions, the Gip1 protein was localized by immunofluorescence analysis. COOH-terminal–tagged versions of Gip1p were nonfunctional (unpublished data). Therefore, targeted integration was used to
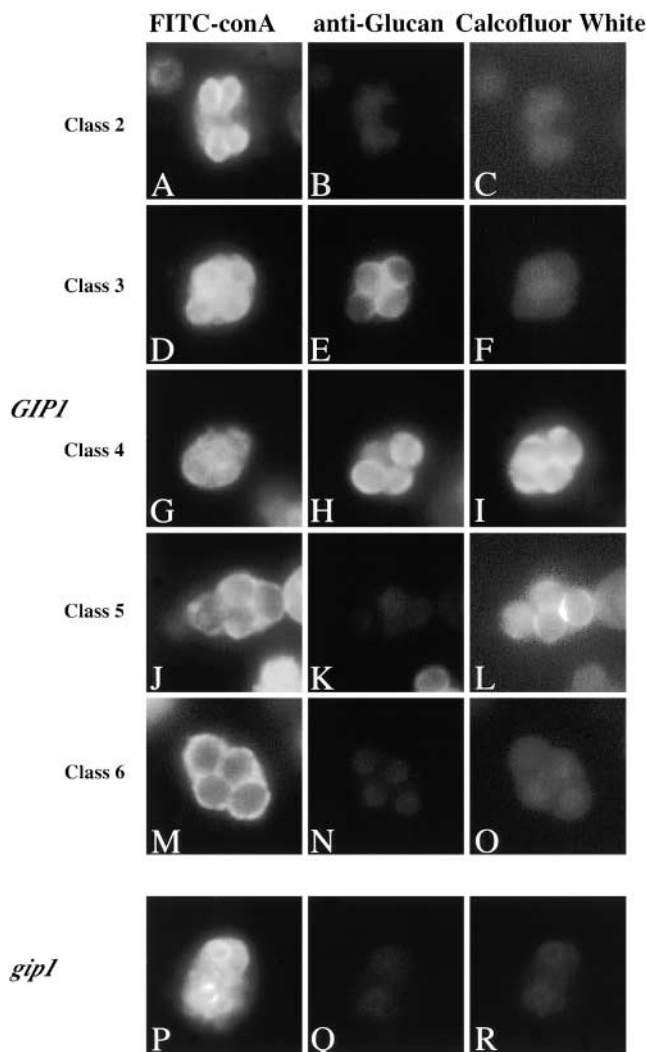
Figure 4. **Analysis of spore wall formation in wild type and *gip1Δ* mutant.** (A–O) Samples were removed from sporulating culture of AN120 (*GIP1/GIP1*) at 6-, 7-, 8-, and 9-h time points, subjected to the immunofluorescence analysis of spore walls as described in Materials and methods. Classification of the cells are described in Results. (A, D, G, J, and M) FITC-ConA staining; (B, E, H, K, and N) Anti-β-glucan staining; (C, F, I, L, and O) Calcofluor white staining. (P–R) NY501 (*gip1Δ / gip1Δ*) was sporulated and subjected to the immunofluorescence analysis of spore walls. (P) FITC-ConA staining; (Q) Anti–β-glucan staining; (R) Calcofluor white staining.

Table II. **Analysis of spore wall deposition**

| | Staining for | | | GIP1 | | | | gip1 |
|---|---|---|---|---|---|---|---|---|
| Pattern | Mannan | Glucan | Chitosan | 6 h | 7 h | 8 h | 9 h | 9 h |
| Class 1 | − | − | − | 95.7 | 66.3 | 31.8 | 13 | 52 |
| Class 2 | + | − | − | 4.3 | 22.8 | 17.8 | 4 | 48 |
| Class 3 | + | + | F | 0 | 9.4 | 16.8 | 7.5 | 0 |
| Class 4 | + | + | + | 0 | 0 | 1.4 | 0.5 | 0 |
| Class 5 | + | − | + | 0 | 0.5 | 0.9 | 3.5 | 0 |
| Class 6 | + | − | F | 0 | 1 | 31.3 | 71.5 | 0 |

At each time point, cells were removed from sporulating AN120 (*GIP1/ GIP1*) or NY501 (*gip1Δ/gip1Δ*) cultures and processed as described in Materials and methods. Cells were placed into six classes based on their staining pattern. +; strong staining, −, no staining; F, faint staining. The percentage of cells in each class is shown. At least 200 cells were counted at each time point.
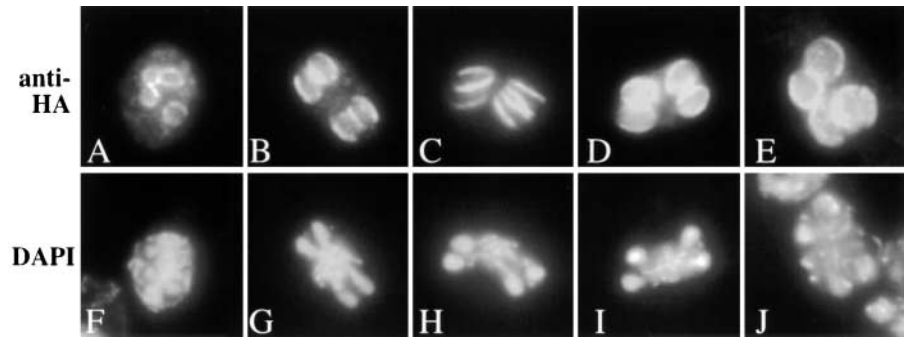
Gip1p localization during sporulation was examined by immunofluorescence analysis using anti-HA antibodies. HA–Gip1p appears initially in sporulating cells as four rings during meiosis II, and then localizes to the region of extending prospore membranes following the leading edge (Fig. 5). At this stage, parallel bar-like structures were observed. After completion of meiosis II, round structures surrounding the four nuclei were seen. Identical results were obtained when the integrated *HA–GIP1* was examined, indicating that the localization is not a consequence of overexpression (unpublished data). This localization pattern of Gip1p resembles the reported localization of septins during sporulation. Therefore, we examined whether Gip1p colocalizes with septins. Gip1p–HA and Spr28p–green fluorescent protein (GFP) were expressed in wild type cells and their localization during sporulation was compared. The distribution of the two proteins overlapped extensively with each other (Fig. 6, A–D). These results indicate that Gip1p colocalizes with septins during spore formation.

To determine if the bar-like pattern of Gip1p represents a section through a more complicated structure, image stacks were collected and three dimensional renderings made. Quicktime movies displaying the resulting images are available at http://www.jcb.org/cgi/content/full/jcb.200107008/ DC1. The results indicate that the Gip1p-containing structures are actually pairs of parallel sheets that appear as bars when optically sectioned.

## Glc7p shows a dynamic localization pattern during sporulation and colocalizes with Gip1p in meiosis II

Because *GIP1* encodes a potential Glc7p targeting protein, we examined Glc7p localization in sporulating cells. *GFP–GLC7* was introduced into the wild-type strain and the localization of the fusion protein was observed by GFP fluorescence (Fig. 7). Early in sporulation, cells displayed a predominant nuclear staining with a bright nucleolar dot as observed in vegetative cells (Bloecher and Tatchell, 2000). In early meiosis II, in addition to the nuclear staining, four dots were observed near the nuclear periphery suggesting some Glc7p is localized to the SPB. At later stages of meiosis II, Glc7p clearly displayed a bar-like pattern that resembles those of Spr28p and Gip1p. Finally, in postmeiotic cells, Glc7p was again found predominantly in the spore nucleus. These observations indicate that the localization of Glc7p is

fuse the hemagglutinin (*HA*) epitope to the 5′ end of the *GIP1* gene. To preserve the sporulation-specific expression of *GIP1*, the fusion gene was under the control of *SPO20* promoter. *SPO20* is a sporulation specific gene (Neiman, 1998) with a similar expression profile to *GIP1* (Chu et al., 1998). When NY509 (in which both copies of the genomic *GIP1* genes are replaced with the fusion gene) was sporulated, the efficiency of sporulation was similar to wild-type cells (82% asci). However, only 26% of the asci were tetrads, with the remainder predominantly dyads and triads, suggesting that the fusion gene is not completely functional. When the fusion gene was cloned into a multicopy vector and introduced into the homozygous *gip1* deletion strain, NY501, NY501 produced tetrads at a comparable level (>60%) to the wild-type strain. This strain carrying multicopy *HA-GIP1* was therefore used for examination of Gip1p localization.

Figure 5. **Localization of HA-Gip1p in sporulating cells.** NY501 (*gip1Δ / gip1Δ*) carrying 2 μ *HA-GIP1* was sporulated and analyzed by immunofluorescence. (A–E) Anti-HA antibody staining is shown. (F–J) Staining of the same cells with DAPI.



dynamic during sporulation, and that Glc7p displays a similar localization pattern to Gip1p and Spr28p during meiosis II.

To confirm this last point, colocalization of Gip1p and Glc7p was examined directly. *GIP1–HA* and *GFP-GLC7* were expressed in AN120 and the localization of the proteins was analyzed. The distribution of the two proteins showed extensive overlap (Fig. 6, E–H), indicating that Glc7p colocalizes with Gip1p. Taken together with the immunofluorescence analysis of Gip1p and Spr28p, these results demonstrate that Gip1p and Glc7p colocalize with septins during meiosis II.

## Glc7p and Septins are not properly localized in the *gip1Δ* mutant

By analogy to other Glc7p targeting proteins, Gip1p might be a targeting subunit that recruits Glc7p to the septins at the prospore membrane. To test this hypothesis, we examined the localization of Glc7p in the *gip1Δ* mutant. In this strain, Glc7p did not show a septin-like staining pattern in meiosis II. Instead, it often showed cytosolic staining with four discrete dots, which may indicate SPB localization (Fig. 8 C). Thus, localization of Glc7p to the prospore membrane is dependent on *GIP1*.

Similar to their function at the bud neck of vegetative cells, the septins may serve as a scaffold to which Gip1p recruits Glc7p. Alternatively, recruitment of Glc7p might be important for the organization of the septins themselves. To

address these possibilities, we examined Spr28p localization in the *gip1Δ* mutant. We found that Spr28p is delocalized in many of the mutant cells (Fig. 8, G and I). In postmeiotic cells, ~20% of the mutant cells showed a ring-like pattern surrounding four nuclei, indicating localization to the periphery of the prospore. The majority of cells showed only a uniform cytosolic staining pattern. Importantly, in cells in meiosis II, a pattern of bars like those in wild-type cells was only rarely observed (<1%). Similar results were obtained using antibodies to a second sporulation-specific septin Spr3p (unpublished data). Thus, Gip1p does not serve simply as a targeting subunit to connect Glc7p to the septins; rather, *GIP1* is required for proper septin organization and the failure of Glc7p to localize to the prospore membrane in *gip1Δ* cells may reflect the absence of septin structures.

## The Don1p ring is not affected by *gip1Δ*

*DON1* encodes a coiled-coil protein that localizes to a ring-like structure at the leading edge of the prospore membrane, and this structure is distinct from the septin bars that underlie the prospore membrane (Knop and Strasser, 2000). To examine whether the effect of *gip1* deletion is specific to septins or is perhaps a more general effect on prospore membrane–associated structures, we examined the localization of Don1p in the *gip1Δ* mutant. *DON1-GFP* was introduced into wild type and *gip1Δ* cells, and the localization of the fusion protein was observed using anti-GFP antibodies. In both wild-type and

Figure 6. **HA-Gip1p colocalizes with septins and GFP-Glc7p.** (A to D) AN120 carrying both pCEN-*SPR28-GFP* and 2μ *HA-GIP1* was sporulated for 7 h and analyzed by staining with anti-HA antibodies. (A) GFP fluorescence. (B) Anti-HA staining. (C) Merge of A and B. (D) DAPI staining of the same cell. (E to H) AN120 carrying both *GFP-GLC7* and 2 μ *HA-GIP1* was sporulated for 7 h and analyzed by staining with anti-HA antibodies. (E) GFP fluorescence; (F) Anti-HA staining; (G) Merge of E and F; (H) DAPI staining of the same cell.
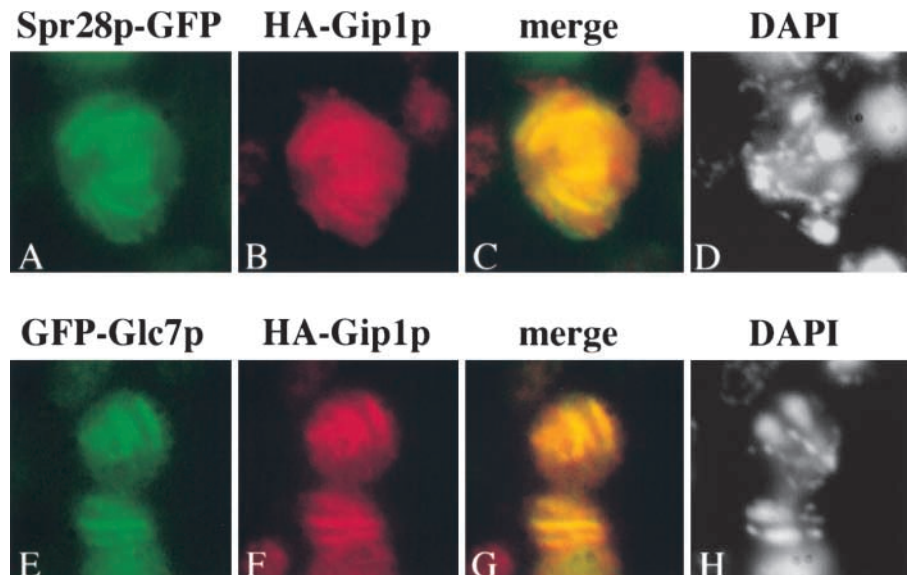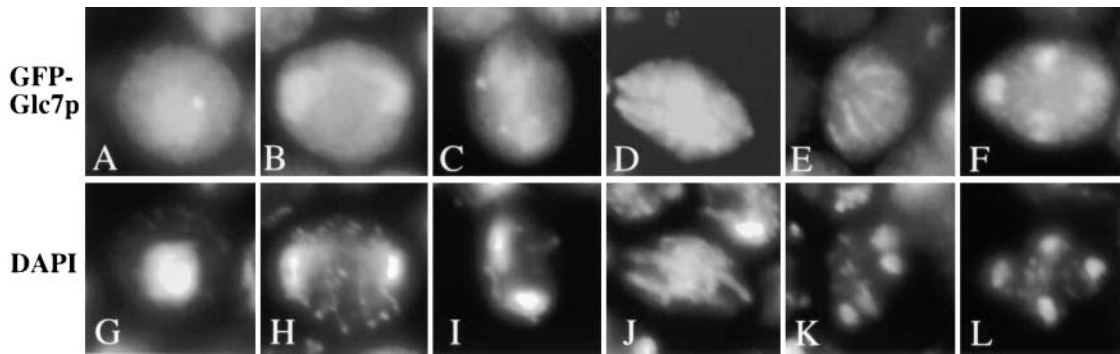
Figure 7.   **Localization of GFP-Glc7p during sporulation.** AN120 carrying *GFP-GLC7* was sporulated and GFP fluorescence was observed. (A–F) GFP fluorescence; (G–L) DAPI staining of corresponding cells in A–F.

*gip1Δ* cells, ring-like structures were observed at the lip of the prospore membranes (Fig. 9). Thus, assembly of the Don1p ring is not affected by *gip1* deletion, suggesting that the effects of *gip1Δ* are specific to assembly of the septin complex.

## The *glc7–136* mutant shows similar defect to the *gip1Δ* mutant

To determine whether the phenotypes of the *gip1Δ* mutant are caused by a failure to properly localize Glc7p or a second function of the Gip1 protein, we examined the sporulation phenotype of a strain carrying the *glc7–136* allele. The *glc7–136* mutant causes a sporulation defect (Baker et al., 1997), and the protein is defective in interaction with Gip1p (unpublished data). Thus, if the sporulation defect of the *gip1Δ* mutant is due to altered localization of Glc7p, the *glc7–136* mutant should display similar phenotypes. EM analysis of SB247, a *glc7–136* mutant in the JC482 background (Baker et al., 1997), was performed. The cytological phenotype of *glc7–136* was more heterogeneous than *gip1Δ*, probably due to the leaky sporulation defect of the mutant (2% asci). Nonetheless, a significant fraction of the cells (50%) displayed prospores with no apparent deposition of spore wall material (Fig. 10 A), similar to the *gip1Δ* mutant (Fig. 2). Thus, *glc7–136* mutant cells display a block to spore wall deposition similar to that seen in the *gip1* deletion strain.

To examine the localization of Spr28p and Gip1p in the *glc7–136* strain, the *glc7–136* mutation was introduced into the same SK1 strains used for the *gip1* analysis. As in the JC482 background, the *glc7–136* mutant is leaky in SK1, giving 5% asci. *SPR28-GFP* and *HA–GIP1* were introduced into NY527, and the proteins were localized by immunofluorescence. Most of the cells expressing *SPR28-GFP* displayed uniform cytosolic staining. In ∼30% of the cells, staining of the spore periphery was observed, as is the case with septin localization in the *gip1Δ* mutant (Fig. 10, B and D). HA–Gip1p displayed similar localization defects to Spr28p-GFP in the *glc7–136* strain (Fig. 10, F and H*)*. Thus, as with the spore wall, the *glc7–136* mutant shows similar septin organization defects to the *gip1Δ* mutant. Further, Gip1p and Glc7p are interdependent for localization; interaction of the two proteins is required for either to localize to the prospore membrane. Taken together, these data indicate that Gip1p and Glc7p function together to promote both organization of the septins and spore wall formation.

## Discussion
### *GIP1* and *GLC7* are required to signal the onset of spore wall formation
Strains deleted for *GIP1* display two clear phenotypes, a block to spore wall formation and delocalization of the septins from
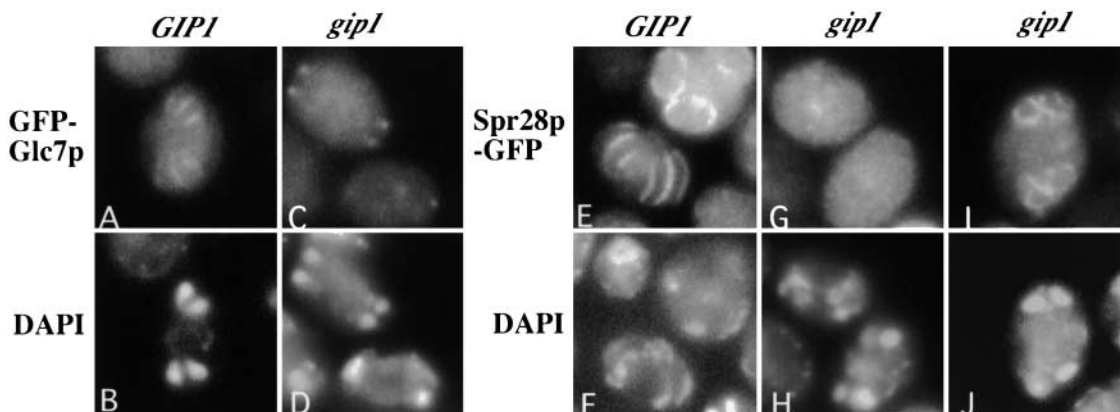


Figure 8.   **Localization pattern of GFP-Glc7p and Spr28p-GFP are altered in the *gip1* mutant.** AN120 (*GIP1/GIP1*) and NY501 (*gip1Δ / gip1Δ*) carrying either *GFP-GLC7* or pCEN-*SPR28-GFP* were sporulated and analyzed by fluorescence microscopy. (A) GFP-Glc7p localization in AN120. (B) DAPI staining of cell in A. (C) GFP-Glc7p localization in NY501. (D) DAPI staining of cells in C. (E) Spr28p-GFP localization in AN120. (F) DAPI staining of cells in E. (G and I) Spr28p-GFP localization in NY501 (H and J) DAPI staining of cells in G and I, respectively.
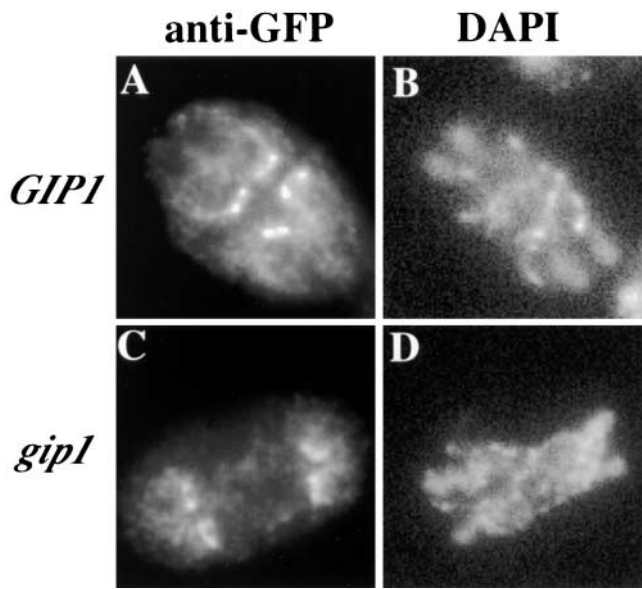
**Figure 9.  Don1p ring is formed normally in the *gip1Δ* mutant.** AN120 (*GIP1/GIP1*) and NY501 (*gip1Δ / gip1Δ*) carrying *DON1-GFP* were sporulated and analyzed by immunofluorescence using anti-GFP antibodies. (A) Don1p-GFP localization in AN120. (B) DAPI staining of cell in A. (C) Don1p-GFP localization in NY501. (D) DAPI staining of cell in C.

the forming prospore membrane. The spore wall phenotype of *GIP1* mutants is a discrete arrest at the initiation of spore wall formation. In wild-type cells, deposition of spore wall material is never seen until after the closure of the prospore membrane (Lynn and Magee, 1970). Consistent with this cytological observation, expression of the *DIT1* gene appears to occur after prospore closure (Briza et al., 1990). These observations suggest that the cell must have some mechanism to trigger spore wall formation in response to prospore membrane closure. Given their colocalization with septins, the Gip1p–Glc7p holoenzyme would be ideally located to participate in this process. Though we favor the idea that this phosphatase complex acts as a monitor to signal the closure of the prospore membrane, we cannot at present rule out the possibility that the Gip1p–Glc7p phosphatase is required for membrane closure itself. A defect in either membrane closure or the ability to sense that the membrane has closed would result in a failure to initiate spore wall formation.

*CHS3* encodes the chitin synthase responsible for the synthesis of the chitosan layer. In contrast to *DIT1*, the *CHS3* gene is not transcriptionally induced during sporulation (Pammer et al., 1992). Therefore, the failure of *gip1Δ* mutants to produce chitosan suggests that *GIP1* may be required for posttranslational regulation of this enzyme. In vegetative cells, Chs3p activity is controlled in part by regulated recycling through an endosomal compartment (Ziman et al., 1998). It will be of interest to examine the localization of Chs3p in sporulating wild-type and *gip1Δ* cells.

### Spore wall formation is an ordered process

Previous studies have shown that Calcofluor white transiently stains the chitosan layer of the developing spore (Briza et al., 1990). The loss of Calcoflour white staining in mature asci is due to maturation of the overlying dityrosine layer blocking access of the dye to its ligand (Briza et al., 1988, 1990). We interpret the transient staining of spores with the anti–β-glucan antibody in the same way. That is, spores are positive for staining until the overlying chitosan layer forms and obscures access of the antibody to the glucan layer. Surprisingly, staining with FITC-ConA is not lost as spores mature, even though the inner mannan layer should also be obscured as the outer layers form. However, at the same time that β-glucan staining is lost, the appearance of the FITC-ConA staining changes markedly. This change probably reflects a loss of access to the mannan layer of the inner spore wall, but residual staining of mannoproteins present in the outer prospore membrane or ascal cytoplasm condensed around the spores. Thus, our immunofluorescence assay of spore wall formation suggests a definitive order of spore wall assembly. Mannoproteins are deposited as the prospore membrane forms. After prospore membrane closure, the glucan layer forms, followed by the chitosan layer and finally the dityrosine layer.
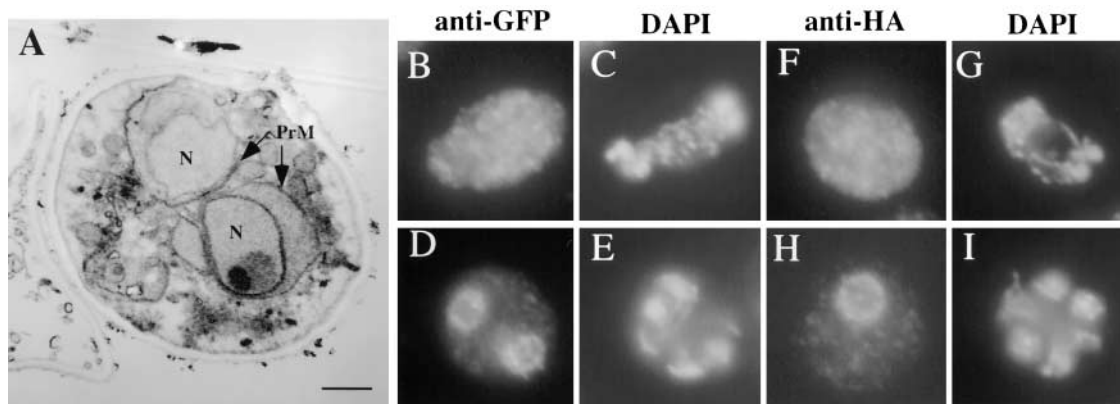


**Figure 10.  *glc7–136* mutants show a similar phenotype to the *gip1Δ* mutant.** (A) SB247 (*glc7–136/glc7–136*) at a late stage of sporulation was analyzed by transmission EM. Prospore membranes (PrM) and nucleus (N) are indicated. (B–I) NY527 (*glc7–136/glc7–136*) harboring either pCEN-*SPR28-GFP* or 2 μ *HA-GIP1* was sporulated and analyzed by fluorescence microscopy. (B and D) anti-GFP staining; (C and E) DAPI staining of cells in B and D, respectively; (F and H) Anti-HA staining; (G and I) DAPI staining of cells in F and H, respectively. Bar, 500 nm.

Surprisingly, the layers are deposited from innermost to outermost, with respect to the spore cytoplasm. This suggests that precursors to the chitosan and dityrosine layer must be able to pass through the glucan and mannan layers before assembling. Alternatively, some of the spore wall layers may be constructed from precursors introduced from the ascal cytoplasm rather than the spore cytosol. This possibility has been suggested previously based on specific localization of some sporulation-induced transcripts to the ascal cytoplasm (Kurtz and Lindquist, 1986), and is also consistent with the accumulation of darkly staining vesicles in the region of the ascal cytoplasm surrounding the prospores that is seen in *gip1Δ* (Fig. 2) and other spore wall–defective mutants (Christodoulidou et al., 1999; Wagner et al., 1999).

**Relationship of *GIP1* to other spore wall synthesis genes.**
Our data indicate that not only is spore wall deposition coordinated with respect to prospore membrane closure, but also a temporal order of deposition, glucan before chitosan before dityrosine, must be maintained. Previously reported spore wall–defective mutants fall broadly into one of two categories: those such as *dit1* or *chs3* that remove specific spore wall layers (Briza et al., 1990; Pammer et al., 1992), or those such as *smk1* and *swm1* in which individual spores within an ascus display heterogeneous wall defects (Krisak et al., 1994; Ufano et al., 1999). The heterogeneous spore wall defects seen in these latter mutants could result from an inability to coordinate the timing of deposition within individual spores.

*SMK1* is particularly interesting in this regard. Though deletion of *SMK1* causes a heterogeneous spore wall phenotype, point mutations produce more homogeneous arrests (Wagner et al., 1999). In the most severe allele, *smk1–4*, this arrest appears similar to *gip1Δ*. Further, it has been reported that formation of the glucan, chitosan, and dityrosine layers requires increasing levels of Smk1p activity, respectively (Wagner et al., 1999). As this matches the temporal order of deposition of these layers, *SMK1* activity might provide a mechanism to control the timing of synthesis of the different layers. Gip1p–Glc7p phosphatase could control the initiation of spore wall synthesis, at least in part, by regulating the activation of Smk1p.

**Gip1p–Glc7p phosphatase regulates septin organization**
The Gip1p–Glc7p complex colocalizes with the septins during prospore membrane growth and the first evident phenotype of the *gip1Δ* mutation is not the spore wall defect, but a failure to organize the septins onto the prospore membrane. However, at times after prospore closure, in a fraction of the *gip1Δ* mutant cells the septins can be found in the spore periphery as in wild-type cells. These results indicate a specific requirement for the Gip1p–Glc7p phosphatase to organize the septins into structures associated with the growing prospore membrane. Whether this effect is mediated by direct dephosphorylation of the septin proteins or some other modifier of septin organization is not yet known. In vegetative cells, mutation of several different protein kinases has effects on organization of the septin ring (Bouquin et al., 2000; Longtine et al., 2000). For instance, mutation of *GIN4* leads to the arrangement of the septins as a series of parallel bars (Longtine et al., 1998a). It has

not yet been reported whether any of the septin proteins are direct substrates of any of these kinases. Nonetheless, taken together with our results, it seems likely that phosphorylation and dephosphorylation regulate septin architecture.

The fact that septins are lost from the prospore membrane in the *gip1Δ* mutant but that the prospore membrane nonetheless forms, indicates that the septins are not required for membrane assembly or growth. One possibility is that the septins function to keep Gip1p–Glc7p associated with the prospore membrane. In this instance, mutation of the septin genes might cause a similar spore wall phenotype to the *gip1Δ* mutation. However, no significant sporulation phenotype has been reported in septin mutants (De Virgilio et al., 1996; Fares et al., 1996). Therefore, it remains to be determined whether the spore wall and septin phenotypes of the *gip1Δ* mutant are linked, or whether they represent two independent aspects of Gip1p–Glc7p function.

In higher cells, as in yeast, septins are found at sites of cytokinesis (Neufeld and Rubin, 1994; Kinoshita et al., 1997). However, in contrast to mitotic yeast cells, the septins of higher cells exhibit dynamic localization and are often found in association with regions of active membrane growth (Fares et al., 1995; Kinoshita et al., 1997; Hsu et al., 1998). Thus, the behavior of septins during sporulation is similar to that in higher cells. Our results indicate that the Gip1p–Glc7p complex is required to organize the septins onto the prospore membrane. Given the high degree of conservation of PP1, it will be of interest to learn if septin organization is also regulated by this enzyme in higher cells.

Bailis and Roeder (2000) have reported that *GLC7*-dependent dephosphorylation of the synaptonemal complex component Red1p is required for progression through the meiotic pachytene checkpoint. In contrast to our findings, this study reported that *gip1Δ* mutants arrest in pachytene. Further, *GIP1* was required for the localization of Glc7p to meiotic chromosomes and overexpression of *GLC7* suppressed the *gip1Δ* sporulation defect. From these results it was suggested that the function of Gip1p is to target Glc7p to meiotic chromosomes. We found no evidence of a meiotic arrest in *gip1Δ* in two different strain backgrounds, SK1 (Fig. 1) and JC482. Additionally, overexpression of *GLC7* does not relieve the spore formation defect of *gip1Δ* mutants in our strains (unpublished data), as was observed in the BR strain background (Bailis and Roeder, 2000). The reason for the differing phenotypes of *gip1Δ* in different strain backgrounds remains unclear; however, our results clearly demonstrate roles for *GIP1* and *GLC7* in postmeiotic events.

In sum, our data suggest a model in which Gip1p is induced during sporulation, binds to Glc7p, and the Gip1p–Glc7p complex then organizes the septins onto the prospore membrane. The Gip1p–Glc7p complex remains associated with the septins during meiosis II, and this places the phosphatase in a position to act as a monitor of prospore membrane growth. Upon closure of the prospore membrane, we propose that Gip1p and Glc7p initiate a signaling cascade that triggers events leading to the synthesis of the spore wall. This includes the induction of *DIT1* as well as the activation of chitin and β-glucan synthases. Further work will be necessary to elucidate the nature of this intracellular signaling pathway.

# Materials and methods

## Yeast strains and media

*S. cerevisiae* strains used in this work are listed in Table I. AN103 was created by selection of 5-fluoroorotic acid–resistant derivatives of MCY3259 (Tu et al., 1996). To construct NY501, AN117–4B and AN117–16D (Neiman et al., 2000) were transformed with *Pvu*II-digested pJT26-HIS3 (Tu et al., 1996) to produce NY1 and NY2, respectively. For this and all other integrations, proper arrangement of the genomic locus was confirmed by PCR. These strains are mated to create diploid NY501. To create a strain carrying NH₂ terminally HA-tagged *GIP1* under control of the *SPO20* promoter, pFA6a-His3MX6-prSPO20-HA was subjected to PCR with HT1 (5′-CTATTTTCTTCCT-CCTTGCCTATTCGTTTGGTGGCTTTCTTTATAATTCGGAATTCGAGCTC-GTTTAAAC-3′) and HT2 (5′-CTTTTCAAAGACTCAAATGGTCTAGCCTTTG-GCTGCAAAATAGTTTCCATTTTGTATAGTTCATCCATGC-3′), and the product was used to transform AN117–4B and AN117–16D to obtain NY13 and NY14, respectively. These strains were mated to create diploid NY509. To create the *DON1*–GFP fusion gene in yeast, pFA6a-GFP (S65T)-His3MX6 (Longtine et al., 1998b) was amplified with HT15 (5′-AAGATGAAAAAAA-GCAGGTTCATCCATCTAGCAAGAATTAAGTTTTACGCGGATCCCCGGGT-TAATTAA-3′) and HT16 (5′-AAAGGCAAATAAGCGCAGAAGAAGCAA-TGGTGGTGTCTGTTCATTGCGAGGAATTCGAGCTCGTTTAAAC-3′), and the product was used to transform AN117–4B to create NY17. To construct NY527, the *his5⁺* gene of *Schizosaccharomyces pombe* was amplified from pFA6a-His3MX6 (Longtine et al., 1998b) with HT28 (5′-AACTGGGG-AGAGGAAAGCAGATTACCACAATATACATTCAAATTAAAGAACGGAT-CCCCGGGTTAATTAA-3′) and HT29 (5′-TAATAAGTATTTTCCTTTTTAAA-CTTTGATTTAGGACGTGAATCTATTTAGAATTCGAGCTCGTTTAAAC-3′), and the product was used to transform AN120 to create the *glc7* heterozygous deletion strain, NY523. NY523 was then transformed with pSB14 to create NY525, which has *glc7–136* integrated at the *ura3* locus. NY525 was sporulated and dissected. Progeny that had both the *glc7* deletion and the *glc7–136* integration were identified and mated to produce NY527. Unless otherwise noted, standard media and growth conditions were used (Rose et al., 1990).

## Plasmids

To create pFA6a-His3MX6-prSPO20-HA, the promoter sequence of the *SPO20* gene was amplified directly from yeast genome using ANO195 (5′-CCTTGAGATCTAAGTCTAGGCGCTTTCAAC-3′) and ANO201 (5′-CCTTGTTAATTAAAGACATTATATATCTAAAAATGGC-3′). The PCR product was then digested with *Pac*I and *Bgl*II and used to replace *GAL1* promoter region of pFA6a-His3MX6-PGAL1-3HA (Longtine et al., 1998b). To clone the *prSPO20*-HA-*GIP1* fusion gene, genomic DNA from NY13 was subjected to PCR with ANO195 and HT6 (5′-CAGTTGCCTACCAAT-GTTTC-3′). The product was cloned into *Sma*I sites of pRS424 and pRS426 (Christianson et al., 1992) to create pSB5 and pSB6, respectively. pNC160-GFP-*GLC7* (Bloecher and Tatchell, 2000) was used to express GFP-tagged version of Glc7p. pSB7 was created by excising the *SPR28*–GFP fusion gene from YEplac181-SPR28-GFP (De Virgilio et al., 1996) with *Eco*RI and *Bam*HI and cloning it into pRS314 (Sikorski and Hieter, 1989). To clone *DON1*–GFP into plasmid, genomic DNA prepared from NY17 was subjected to PCR using HT10 (5′-AAACAGATCTATATTAC-CCTG-3′) and HT19 (5′-GAAGAATTCGATATAGCTCTGAACAATTC-3′). The product was digested with *Eco*RI and *Bgl*II and ligated into similarly digested pRS314 to create pSB8. To integrate *glc7–136* at the *ura3* locus, the *glc7–136* gene was cut out from pSB56 (Baker et al., 1997) with *Hin*dIII and *Sac*I, and cloned into pRS306, creating pSB14. pSB14 was linearized with *Pst*I before transformation to target integration to the *ura3* locus. The plasmids pHindIII-DIT1-lacZ, a gift of J. Segall (University of Toronto, Toronto, Canada), and pGK16 (Holaway et al., 1987) carrying the *DIT1*–lacZ and *SPR3*–lacZ reporters, respectively, were used to monitor gene induction.

## Microscopy

For analysis of meiotic progression, cells were sporulated as described (Neiman, 1998). At intervals, aliquots were removed and fixed with 3.7% formaldehyde at 4°C overnight. Cells were collected and mounted in medium containing DAPI (Molecular Probes). EM was performed as described (Neiman, 1998). Samples were analyzed using a JEOL 1200EX transmission electron microscope at the Stony Brook University Microscopy Imaging Center (Stony Brook, NY).

For immunofluorescence analysis, cells were sporulated and fixed with 3.7% formaldehyde for 2 h, collected, resuspended in SHA buffer (1 M Sorbitol, 0.1 M Hepes, pH 7.5, 5 mM NaN₃) and stored at 4°C. Immunofluorescence analysis of proteins was performed as described previously (Gao and Dean, 2000). Monoclonal anti-HA antibodies (12CA5)

were provided by N. Dean (SUNY Stony Brook, Stony Brook, NY), and affinity-purified anti-GFP antibodies, a gift of N. Davis (Louisiana State University Medical Center, Shreveport, LA), were used at a 1:10 and 1:200 dilution, respectively. Anti-Spr3p antibodies (Fares et al., 1996) were used at a 1:10 dilution. Alexa Fluor 488 goat anti–mouse IgG conjugate, Alexa Fluor 488 goat anti–rabbit IgG conjugate, Alexa Fluor 546 goat anti–rabbit IgG conjugate, and Texas red goat anti–rabbit IgG conjugate (Molecular Probes) were used as secondary antibodies at a 1:400 dilution.

Immunofluorescence analysis of spore walls was performed as follows. Fixed sporulating cells were suspended in Zymolyase solution (27 μg/ml Zymolyase and 0.2% β-mercaptoethanol in SHA buffer) and incubated at 30° for 1 h. Cells were then treated 5 min with SHA buffer containing 0.1% Triton X-100, and 10 min with SHA buffer containing 0.2 mg/ml Calcofluor white (Sigma-Aldrich), washed twice with SHA buffer, and adhered to wells of a polylysine-coated slide. Cells on the slide were treated with methanol for 6 min, followed by acetone for 30 s, and then dried. After blocking with WT buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Milk, 5 mg/ml bovine serum albumin), cells were incubated with a monoclonal anti–β-1,3 glucan antibody (Biosupplies) (Humbel et al., 2001) at a 1:300 dilution in WT buffer overnight. Wells were washed with PBS-BSA (7 mM Na₂HPO₄, 3 mM NaH₂PO₄, 130 mM NaCl, 5 mg/ml bovine serum albumin) and incubated with PBS-BSA containing 1:400 diluted Alexa Fluor 546 goat anti–mouse IgG and 0.1 mg/ml FITC-ConA (Sigma-Aldrich) for 2 h. Finally, wells were washed with PBS-BSA and covered with mounting media. For detection of native GFP fluorescence, cells were fixed with 3.7% formaldehyde for 10 min, washed once with PBS, and mounted with mounting media containing DAPI or processed for immunofluorescence.

Immunofluorescence images were acquired using a Zeiss Axioskop with attached SPOT camera (Diagnostic Instruments) and Adobe Photoshop 5.0, or on a Zeiss Axioplan 2 microscope with a Zeiss Axiocam and Zeiss Axiovision 3.0.6 software. Figures were prepared using Adobe Photoshop 6.0 and Canvas 5.0 (Deneba Software).

## β-Galactosidase and dityrosine assays

Natural fluorescence of the dityrosine molecules was observed as described previously (Briza et al., 1990). *DIT1–lacZ* and *SPR3–lacZ* were separately introduced into both AN120 and NY501. The resulting cells were sporulated and aliquots were collected at each time point and subjected to β-galactosidase assay as described (Stern et al., 1984).

## Online supplemental material

To create three-dimensional projections of Gip1 immunofluorescence, cells carrying *HA–GIP1* were prepared after 7 h in sporulation medium, and stacks of images were collected with a Bio-Rad Radiance 2000 confocal microscroscope (25mW Arlaser, Nikon TE300, 100 X Plan Apo objective 1.4 NA). Images were collected at 0.2-μm intervals in the Z-axis at 1.5% laser power, pinhole aperture at 2.6, Kalman filtration of four images. Three-dimensional projections were created using Bio-Rad Lasersharp2000 software. Projected images were tilted at angles of −45°–45° at 1°-intervals.

To create three-dimensional projects of HA-Gip1p and DAPI fluorescence, stacks of images were collected through an Olympus UPlanFl 100 × 1.3 NA objective with a Roper Coolmax HQ CCD Camera using Scanalytics IPlab Spectrum software. Images were collected at 0.2-μm intervals in the Z-axis, and three-dimensional projections were created using Iplab software. Projected images were tilted at angles of −45°–45° at 5°-intervals. Both projections were saved as Apple QuickTime movies, available at http://www.jcb.org/cgi/content/full/jcb.200107008/DC1

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