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The Arf-GTPase-Activating Protein Gcs1p Is Essential for Sporulation and Regulates the Phospholipase D Spo14p

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***SPO14*, encoding the major *Saccharomyces cerevisiae* phospholipase D (PLD), is essential for sporulation and mediates synthesis of the new membrane that encompasses the haploid nuclei that arise through meiotic divisions. PLD catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid (PA) and choline. PA stimulates Arf-GTPase-activating proteins (Arf-GAPs), which are involved in membrane trafficking events and actin cytoskeletal function. To determine if Spo14p-generated PA mediates its biological response through Arf-GAPs, we analyzed the sporulation efficiencies of cells deleted for each of the five known and potential yeast Arf-GAPs. Only *gcs1*Δ mutants display a sporulation defect similar to that of *spo14* mutants: cells deleted for *GCS1* initiate the sporulation program but are defective in synthesis of the prospore membrane. Endosome-to-vacuole transport is also impaired in *gcs1*Δ cells during sporulation. Furthermore, Arf-GAP catalytic activity, but not the pleckstrin homology domain, is required for both prospore membrane formation and endosome-to-vacuole trafficking. An examination of Gcs1p-green fluorescent protein revealed that it is a soluble protein. Interestingly, cells deleted for *GCS1* have reduced levels of Spo14p-generated PA. Taken together, these results indicate that *GCS1* is essential for sporulation and suggest that *GCS1* positively regulates *SPO14*.**

Saccharomyces cerevisiae undergoes sporulation upon transfer to conditions where diploid cells are starved for nitrogen in the presence of a nonfermentable carbon source (35). The developmentally regulated pathway of sporulation consists of meiosis and spore morphogenesis. Meiosis involves a single round of DNA replication, followed by two nuclear divisions, taking place within a single nuclear envelope (35). The nuclei that arise from the meiotic divisions are packaged into four spores.

Spore formation requires de novo synthesis of a double-layered membrane known as the prospore membrane (7). Analyses of secretory mutants with defects in sporulation suggest that the vesicles that fuse to form this prospore membrane are derived from the Golgi (47). The prospore membrane originates on the cytoplasmic face of the four spindle pole bodies (SPBs) as an extension of the outer plaque (7). As meiosis II (MII) proceeds, each prospore membrane extends around the segregated nucleus, thus encapsulating the four haploid nuclei (42). The membrane closest to the daughter nucleus now serves as the plasma membrane of the spore. Exactly how this pathway of membrane formation is regulated and how it differs from other membrane trafficking events are not precisely known.

The closure of each prospore membrane triggers the final step of spore morphogenesis, spore wall formation. Spore wall components are laid down within the luminal space between the two membranes. Once the layers of the spore wall are deposited, the resulting spore is resistant to environmental stress (70).

The *SPO14* gene encodes the major yeast phospholipase D (PLD) and is essential for sporulation (15, 25, 61, 79). Spo14p catalyzes the hydrolysis of phosphatidylcholine (PC) to generate phosphatidic acid (PA) and choline; PA is the likely mediator of Spo14p function (67). Analyses of *spo14*Δ diploids during sporulation revealed that they are defective in completion of the meiotic divisions and are unable to form prospore membranes (25, 61, 65). Furthermore, Spo14p relocalizes from a detergent-resistant cytoplasmic pool to prospore membranes during meiosis (65). One probable target of Spo14p-generated PA is Spo20p, a sporulation-specific t-SNARE (t-soluble NEM-sensitive factor receptor) that mediates the fusion of vesicles with the prospore membrane (45, 47). Spo20p contains an amphipathic helix that mediates membrane binding in vivo and binds acidic phospholipids in vitro (45). However, it is likely that there are additional targets of Spo14p-generated PA.

ADP-ribosylation factors (Arfs) are 21-kDa proteins of the Ras superfamily of GTP-binding proteins. Arfs have been shown to have a number of biochemical activities, including recruitment of coat proteins to membranes, activation of PLD in mammalian cells, and stimulation of phosphatidylinositol 4-phosphate [PI(4)P] kinase (44, 49, 58). These activities contribute to the maintenance of Golgi morphology and the formation of vesicles involved in both secretory and endocytic pathways (8, 18, 34, 80). Arf proteins are therefore involved in a plethora of activities required for membrane trafficking within the cell.

There are three *ARF* genes in yeast, namely, *ARF1*, *ARF2*, and *ARF3*. *ARF1* and *ARF2* are 96% identical, functional ho-

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mologues and together are required for viability. *ARF1* produces 90% of the Arf protein in the cell, and thus the deletion of *ARF1* significantly impairs Arf function (71). Consistent with a role in membrane trafficking, *arf1*Δ mutants display altered glycosylation of secreted proteins (72) and also grow slowly, are cold sensitive, and are supersensitive to fluoride (71). *ARF3* is most similar to mammalian *ARF6* and is not essential for viability (36). Arf3p has been found to play roles in cell polarity (27) and, more recently, in the endocytic process in yeast (12).

Arf proteins have been shown to directly activate mammalian phosphatidylinositol-(4,5)-bisphosphate (PIP_2)-dependent PLD enzymes (21, 22). Changes in the lipid composition of membranes mediated through PLD are thought to promote the assembly as well as the release of clathrin-coated vesicles at the *trans*-Golgi network and the plasma membrane (8, 80). Although Arf1p has been shown to be a potent activator of mammalian PLD1 (21, 22, 50), Arf1p does not influence the catalytic activity of Spo14p in yeast (64). An analysis of a particular allele, *arf1-myc*, in an *arf2*Δ mutant showed that this allele supported vegetative growth but that homozygous diploids failed to sporulate (64). Furthermore, visualization of green fluorescent protein (GFP)-Spo14p in *arf1-myc arf2*Δ cells revealed an improper formation of spore compartments (64). Although Arf1p activity does not appear to directly act through Spo14p, it does play an important role in sporulation. In contrast, deletion of *ARF3* has no effect on sporulation efficiency (17).

Arf's activity is dependent on cycling between its GTP- and GDP-bound forms. Arf proteins contain little intrinsic GTPase activity and therefore rely on GTPase-activating proteins (31). Arf-GTPase-activating proteins (Arf-GAPs) are lipid-activated enzymes and are considered key regulators of membrane trafficking events (73). For some Arf-GAPs, the lipids most potent in activation include PA, a direct product of PLD catalytic activity, and diacylglycerol, which can be formed as a result of the dephosphorylation of PA (57, 82). It has been proposed that Arf-GAPs mediate vesicle formation, cargo selection, and uncoating of transport vesicles (37, 74, 82). In addition, Arf-GAPs have Arf-independent roles in cellular functions (58).

Five genes have been identified in yeast which encode polypeptides that share significant sequence similarity to Arf-GAPs: these are *AGE2*, *GCS1*, *GLO3*, *SAT1*, and *SPS18*. Age2p, Gcs1p, Glo3p, and Sat1p are known Arf-GAPs (52, 54, 55, 83), while Sps18p, a sporulation-specific protein thought to function during sporulation (11), has not, to our knowledge, been tested for Arf-GAP activity.

Genetic studies have shown that the four known yeast Arf-GAPs have overlapping functions (83). For instance, Glo3p and Gcs1p provide overlapping essential functions for retrograde vesicular transport from the Golgi to the endoplasmic reticulum (53). On the other hand, Gcs1p and Age2p form an essential pair that provides overlapping functions for *trans*-Golgi network transport (54). Furthermore, these Arf-GAPs have been shown together to be required for both Sec14p-dependent and -independent Golgi secretory function (82). Sec14p is the major yeast phosphatidylinositol transfer protein, which maintains a lipid environment essential for protein transport from the Golgi complex (3, 4, 10). In the absence of Sec14p, Spo14p contributes to the lipid milieu for the maintenance of Golgi function, also through the action of Arf-GAPs (82).

TABLE 1. Genotypes of yeast strains

Strain	Genotype
BY4743	*MAT***a**/α *his3/his3 leu2/leu2 met15/MET15 LYS2/lys2 ura3/ura3*
32008[a]	BY4743 but homozygous for *sps18*::*kanMX*
33924[a]	BY4743 but homozygous for *gcs1*::*kanMX*
36121[a]	BY4743 but homozygous for *glo3*::*kanMX*
31437[a]	BY4743 but homozygous for *age2*::*kanMX*
34358[a]	BY4743 but homozygous for *sat1*::*kanMX*
Y304	*MAT***a** *leu2-x his4-x trp1 ura3-1 thr1 lys2 CYH*
Y305	*MAT*α *leu2-y his4-y trp1 ura3-1 ade2-1 lys2 CYH*
Y315	*MAT***a**/α *leu2-x/leu2-y his4-x/his4-y trp1/trp1 ura3-1/ura3-1 thr1/+ lys2/lys2 CYH/CYH +/ade2-1*
Y4403	Y315 but homozygous for *gcs1*::*kanMX*
Y4690	Y315 but homozygous for *GCS1-GFP*
Y4804	Y315 but homozygous for $GCS1^{PH\Delta}$-*GFP*
Y5408	Y315 but homozygous for *gcs1R54K*
Y5397	Y315 but homozygous for *gcs1R54K-GFP*
Y5328	Y315 but heterozygous for *gcs1R54K-GFP*
Y5609	Y315 but heterozygous for *SNF7-GFP*
Y5617	Y4403 but homozygous for *SNF7-GFP*
Y5450	Y315 but homozygous for *SPC42-dsRED*
Y5466	Y4403 but homozygous for *SPC42-dsRED*
Y5502	Y5450 plus pME1096 (*GFP-SPO14* 2μm *LEU2*)
Y5503	Y5466 plus pME1096 (*GFP-SPO14* 2μm *LEU2*)
Y5040	Y315 but homozygous for *spo14*::*LEU2*
Y5492	Y315 but homozygous for *gcs1*::*kanMX* and *spo14*::*LEU2*
Y4995	Y315 but homozygous for *ndt80*::*kanMX*
Y5664	Y315 but homozygous for *arl1*::*kanMX*
Y5204	Y315 plus pUN105 (*CEN LEU2*)
Y5205	Y315 plus YEp351 (2μm *LEU2*)
Y5206	Y315 plus pME2206 (*GCS1 CEN LEU2*)
Y5207	Y315 plus pME2306 (*GCS1* 2μm *LEU2*)
Y5209	Y4403 plus pUN105 (*CEN LEU2*)
Y5210	Y4403 plus YEp351 (2μm *LEU2*)
Y5211	Y4403 plus pME2206 (*GCS1 CEN LEU2*)
Y5212	Y4403 plus pME2306 (*GCS1* 2μm *LEU2*)
Y5545	Y4403 plus pME2399 (*GCS1-GFP* 2μm *TRP1*)
Y4496	Y4403 plus pME1096 (*GFP-SPO14* 2μm *LEU2*)
Y5707	Y5040 plus pME2399 (*GCS1-GFP* 2μm *TRP1*)
Y5703	Y315 plus pME2377 (*DTR1-GFP* 2μm *TRP1*)
Y5697	Y4403 plus pME2377 (*DTR1-GFP* 2μm *TRP1*)
Y5700	Y5040 plus pME2377 (*DTR1-GFP* 2μm *TRP1*)
Y5701	Y5492 plus pME2377 (*DTR1-GFP* 2μm *TRP1*)

[a] Record number from Research Genetics homozygous deletion project.

To determine if Arf-GAPs are downstream effectors of Spo14p-generated PA during sporulation, we examined a homozygous mutant of each of the five known or potential Arf-GAPs. Only *gcs1*Δ diploid cells displayed a similar sporulation defect to that of the *spo14*Δ mutant. Our findings indicate that Gcs1p plays an important role in prospore membrane formation in budding yeast, in part through the regulation of Spo14p.

MATERIALS AND METHODS

Yeast strains and media. Routine growth and manipulation of *S. cerevisiae* strains were performed as described previously (62). The yeast strains used in this study are all derived from the S288C strain background, unless otherwise noted (Table 1). The BY4743 deletions obtained from Research Genetics were confirmed by PCR. DNA-mediated transformations of yeast cells were done by the lithium acetate procedure (29). Gene replacement and disruptions (see below) were performed by the one-step method (63). *gcs1*::*kanMX* and *ndt80*::*kanMX*, which replace the entire open reading frames with the marker *kanMX4* (78), were generated by amplifying a region ~300 bp upstream and downstream of the disrupted open reading frames from a previously constructed knockout strain obtained from Research Genetics.

GFP and dsRED (tdimer2 variant of red fluorescent protein) chromosomal fusions were constructed using the reagents described by Longtine et al. (39) and Sheff and Thorn (69), respectively. All integrants were confirmed using PCR and

the appropriate synthetic oligonucleotide primers. Gcs1p$^{PH\Delta}$-GFP was created by generating primers that placed the GFP-Kan tag 227 amino acids into the open reading frame. When the corresponding PCR product was transformed into haploid strains, a deletion of 125 amino acid residues at the C terminus of Gcs1p was created.

Plasmids. Plasmid ME2206 contains the *GCS1* complementing region in an ~2.2-kb SacI-BamHI (PCR or primer generated) fragment at the corresponding sites of pUN105 (16), a low-copy-number (*CEN*) *LEU2* plasmid. The 2.2-kb fragment was cloned into the SacI-BamHI sites of the high-copy-number (2μm) plasmid YEp351 (23) to generate pME2306 (*LEU2*). Plasmid ME913 (*spo14*::*LEU2*, which removes 4,187 bp of the open reading frame) was used in generating the *spo14*Δ and *gcs1*Δ *spo14*Δ chromosomal deletions/disruptions as described by Rudge et al. (65). Plasmid ME913 was targeted for integration in yeast with XhoI-NotI.

Site-directed mutagenesis was performed on pME2207 (*GCS1* 2μm *LEU2*) to generate *gcs1R54A* (pME2221) and *gcs1R54K* (pME2355), using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as recommended by the manufacturer. A mutagenic upstream primer (5′-CTTGAATGTGCCGGTATC CAT**G**CAGGGCTTGGTGTGCATATATC-3′; the bold residue indicates the introduced mutation) and downstream primer (5′-GATATATGCACACCAAG CCC**TGC**ATGGATACCGGCACATTCAAG-3′) were used to generate the desired R54A point mutation. A different mutagenic upstream primer (5′CTTGA ATGTGCCGGTATCCAT**AAA**GGGCTTGGTGTGCATATATC-3′) and downstream primer (5′GATATATGCACACCAAGCCC**TTT**ATGGATACCGGCA CATTCAAG-3′) were used to generate the desired R54K point mutation. The SacI-XbaI N-terminal fragments of pME2221 (containing *gcs1R54A*) and pME2355 (containing *gcs1R54K*) and the XbaI-BamHI fragment containing the C-terminal unmutagenized fragment of pME2206 were moved into the SacI and BamHI sites of pUN105 and pUN15 (16) to generate pME2325 (*gcs1R54A CEN LEU2*) and pME2358 (*gcs1R54K CEN TRP1*), respectively, which maintained the Spo$^-$ phenotype and contained only the R54A or R54K mutation, respectively, as verified by sequencing. The EcoRI-BamHI fragment containing *gcs1R54K* from pME2358 was moved into the corresponding sites of YIp5, a yeast integrating plasmid, to generate pME2363 and was confirmed by sequencing to only contain the R54K mutation. Plasmid ME2363 was targeted for integration into wild-type haploid strains with XbaI. The *MAT*α *gcs1R54K* haploid strain was crossed with a strain of the opposite mating type to generate the *gcs1R54K* homozygous mutant. The original *MAT*α *gcs1R54K* haploid strain was additionally chromosomally tagged with GFP by the method of Longtine et al. (39). The homozygous GFP-tagged diploid strain was generated as described above, but haploid *MAT***a** segregants were isolated by G418 resistance conferred by the *kanMX* marker.

GFP-SPO14 was expressed from a high-copy-number (2μm) *LEU2* plasmid (pME1096) and has been previously described (65). *DTR1-GFP* (a generous gift from Aaron Neiman, State University of New York at Stony Brook, Stony Brook, NY) was expressed from a high-copy-number (2μm) *TRP1* plasmid. *HOP1-lacZ*, *NDT80-lacZ*, and *DIT1-lacZ* (generous gifts from Andrew Vershon, Waksman Institute of Microbiology, Rutgers State University of New Jersey, Piscataway, NJ) were expressed from high-copy-number (2μm) *LEU2* plasmids.

To generate *GCS1-GFP* expressed from a plasmid, the *GCS1-GFP-kan* cassette was amplified from Y4690 using an upstream primer that binds ~1,000 bp upstream of the *GCS1* open reading frame and a downstream primer containing a KpnI restriction site in its sequence. A SacI-KpnI fragment containing *GCS1-GFP-kan* was originally moved into the corresponding sites of pUN15, a low-copy-number (*CEN*) *TRP1* plasmid, to generate pME2369, which complemented the *gcs1*Δ mutant. The ~4.5-kb SacI-KpnI fragment containing *GCS1-GFP-kan* was cloned into corresponding sites of pME856, a high-copy-number (2μm) *TRP1* plasmid, to generate pME2399, which additionally complements the *gcs1*Δ mutant.

Analysis of sporulation. Cells were grown on YPAD (yeast extract-peptone-dextrose plus 10 mM adenine) or a medium lacking appropriate amino acids to maintain the selection of plasmids and were incubated overnight. Cells were replica plated onto sporulation medium and incubated at 30°C for 3 days. Sporulation was monitored by differential interference contrast (DIC) microscopy. For liquid cultures, 2 ml of YPAD or medium lacking appropriate amino acids was inoculated, and cells were grown for 24 h at 30°C. Approximately 1×10^7 to 2×10^7 cells were then transferred to 4 ml YP-acetate medium and incubated at 30°C for 24 h. The cells were collected by centrifugation, washed one time with double-distilled water (ddH_2O), and resuspended in 2 ml 2% potassium acetate (KAc) to induce sporulation. For the BY4743 strain background, cells were sporulated in 10 ml SPM medium (0.3% KAc, 0.02% raffinose). In some experiments, the meiotic divisions were analyzed after fixation by staining with 4′,6′-diamidino-2-phenylindole (DAPI) as described previously (40). Final sporulation counts were taken after 3 days.

Analysis of GFP and dsRED fusion proteins. Strains expressing *GFP-SPO14* (65), *DTR1-GFP*, *SPC42-dsRED*, *GCS1-GFP*, *gcs1R54K-GFP*, and *GCS1$^{PH\Delta}$-GFP* (Table 1) were grown and sporulated as described above. At various times after induction, live cells were examined by fluorescein isothiocyanate or rhodamine optics on a Zeiss Axioskop 2 fluorescence microscope.

Analysis of FM4-64 uptake. Cells were induced to sporulate in liquid as described above (sporulating cells), or log-phase cultures grown in YPAD (vegetative cells) were analyzed. FM4-64 [*N*-(3-thiethylammoniumpropyl)-4-(*p*-diethylaminophenylhexatrienyl) pyridinium dibromide; Molecular Probes, Inc., Eugene, OR] (77) was added to 1 ml of culture at a final concentration of 2 μg/ml (from a 1-mg/ml stock solution in dimethyl sulfoxide) at 14 h and incubated on ice for 5 min in the dark. The cells were collected by centrifugation, washed once, and inoculated into fresh 2% KAc. The cells were observed 0, 10, 20, 30, and 60 min after incubation with shaking at 30°C.

In vivo BODIPY-PC analysis. BODIPY-PC (4 μM final concentration; Molecular Probes, Eugene, OR) was added directly to cultures ($\sim 2 \times 10^8$ cells) 12 h after induction in sporulation medium. Cells were harvested 3 h later, and lipids were extracted and analyzed by thin-layer chromatography as previously described (66). All assays were performed in triplicate. The percentage of conversion of BODIPY-PC to BODIPY-PA was determined from the pixel intensities obtained from an image of the thin-layer chromatography plate, using AlphaEase FC4.0 imager software (Alpha Innotech).

Transmission electron microscopy (TEM). Cells were sporulated as described above. Ten milliliters of cells was fixed by the addition of glutaraldehyde to the culture medium at a final concentration of 2.5% and incubation for 1 h at room temperature. Cells were washed two times with ddH_2O, resuspended in 1 ml 4% potassium permanganate ($KMnO_4$) (in water), and incubated at room temperature for 30 min. Cells were then washed with ddH_2O until the supernatant appeared clear, followed by resuspension in 1 ml saturated uranyl acetate, and were incubated at room temperature for 30 min. Samples were finally dehydrated through serial washes in acetone (five times for 15 min each). The dehydrated samples were embedded in Mollenhauer medium (41), and thin sections were obtained. The images were collected on a Philips CM120 microscope at 80 kV.

Preparation of particulate and cytosolic fractions. Extracts were prepared from cells ($\sim 8 \times 10^8$) undergoing meiotic divisions as described above (sporulating cells) or from log-phase cultures grown in YPAD (vegetative cells). Cells were harvested and resuspended in 1 to 3 ml lysis buffer (20 mM triethanolamine, 300 mM sorbitol, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). One milliliter of the resuspension was added to 100 μl acid-treated glass beads on ice. Cells were vortexed for 40 seconds and placed on ice for 30 seconds, and this was repeated five times. Lysed cells were subjected to a prespin at $1,000 \times g$ for 3 min to remove unlysed cells and cell debris. Whole-cell extracts were taken at this point. The supernatant was then spun at $14,000 \times g$ for 20 min. The supernatant obtained at this speed (14K supernatant) was transferred to a new tube, and the 14K pellet was resuspended in an equivalent volume of lysis buffer. The 14K fractions were isolated at this point. The remaining supernatant was then spun at $100,000 \times g$ for 1 h. The 100K supernatant was transferred to a new tube, and the 100K pellet was resuspended in an equivalent volume of lysis buffer. The above procedure was performed on ice, and centrifugation was done at 4°C. The isolated fractions were combined with an equal volume of 2× sample buffer, boiled at 95°C for 5 min, placed on ice for 5 min, and spun at maximum speed for 1 min. Cell equivalents were used for immunoblot analysis.

Immunoblot analysis. Cell extracts were prepared as described above and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 8% or 10% SDS-polyacrylamide gels. Proteins were electrophoretically transferred onto nitrocellulose membranes (0.45-μm pore size; Bio-Rad Technologies) for 2 h. Blots were blocked by incubation with 3% nonfat dry milk for 1 h at room temperature. Blots were then probed with an anti-GFP monoclonal antibody (Ab) (Clontech/BD Biosciences, Palo Alto, CA) diluted 1:1,000 in 3% nonfat dry milk in TBS-T (Tris-buffered saline with 0.1% Tween 20 [vol/vol]) or a rabbit polyclonal anti-Gas1p antibody (1:25,000 dilution in 3% nonfat dry milk in TBS-T) to detect the glycosylphosphatidylinositol-anchored plasma membrane 1,3-β-glucanosyl transferase (a gift from H. Riezman) and incubated at room temperature for 1 h. After three washes with TBS-T, the anti-GFP blots were incubated with a horseradish peroxidase-conjugated anti-mouse secondary Ab (Pierce, Rockford, IL) diluted 1:3,000 in 3% nonfat dry milk in TBS-T, the anti-Gas1p blots were incubated with a horseradish peroxidase-conjugated anti-rabbit secondary Ab (Pierce, Rockford, IL) diluted 1:3,000 in 3% nonfat dry milk in TBS-T, and both types of blot were incubated at room temperature for 1 h.

TABLE 2. Sporulation of single gene deletion mutants in BY4743 strain background

Strain	Relevant genotype	% Spore formation[a]
BY4743		27.7 ± 4.9
32008	*sps18*Δ	21.2 ± 13.8
33924	*gcs1*Δ	1.2 ± 0.9*
36121	*glo3*Δ	6.2 ± 1.6*
31437	*age2*Δ	19.8 ± 4.0
34358	*sat1*Δ	30.5 ± 9.2

[a] A minimum of 500 cells were counted per liquid culture over a minimum of three independent experiments. The final % sporulation includes cells that contained two, three, or four mature spores. Values are means ± standard deviations (SD) from three independent experiments. *, % sporulation was statistically significantly different for *gcs1*Δ and *glo3*Δ, with *P* values of <0.001 and 0.002, respectively.

After three final washes with TBS-T, proteins were visualized by enhanced chemiluminescence detection (Pierce, Rockford, IL) on preflashed film.

RESULTS

Genetic analysis of Arf-GAPs during sporulation. To analyze the role of Arf-GAPs in sporulation, we examined a Research Genetics deletion collection strain (BY4743) harboring individual homozygous deletions of all five known or predicted Arf-GAPs. The gene deletions analyzed included *age2*Δ, *gcs1*Δ, *glo3*Δ, *sat1*Δ, and *sps18*Δ. A genomic screen previously reported that only a *gcs1*Δ mutant had a sporulation phenotype; however *glo3*Δ was not analyzed (17). As shown in Table 2, we have confirmed that *gcs1*Δ mutants largely fail to form spores when induced in sporulation medium, while *age2*Δ, *sat1*Δ, and *sps18*Δ mutants sporulate similarly to the wild type. In addition, we found that *glo3*Δ mutants also displayed a sporulation defect (Table 2). The finding that strains deleted for the sporulation-specific *SPS18* gene showed no significant sporulation defect led us to investigate this gene further. Since Arf-GAPs have both overlapping and distinct roles in cell function in vegetative cells, we examined the consequence of deleting *SPS18* in conjunction with the other Arf-GAP mutants and saw no enhancement of phenotype (data not shown). Thus, the role of *SPS18* remains enigmatic.

Cells deleted for *GCS1* and *GLO3* were examined further to determine where in the sporulation program these mutants stalled. First, we analyzed the ability of the mutants to undergo meiotic divisions. At various times after transfer to sporulation medium, *glo3*Δ mutants were fixed and stained with DAPI, which revealed that most of the cells failed to undergo either meiotic division (data not shown). To determine if meiosis was initiated in *glo3*Δ cells, we monitored the expression of *HOP1*, a sporulation-specific gene that is expressed early in meiosis (24, 76). β-Galactosidase activity was measured for cells harboring a *HOP1*::*lacZ* fusion induced in sporulation medium (76). The results of the assay showed that *HOP1* was not expressed in *glo3*Δ cells, indicating that *glo3*Δ mutants cannot initiate the sporulation program (data not shown). The role of this gene was not investigated further.

In contrast to the case for *glo3*Δ mutants, cells deleted for *GCS1* induced the meiotic program, as *HOP1*::*lacZ* was expressed and some cells underwent meiotic divisions, as monitored by DAPI staining (data not shown). This led to increased interest in the *gcs1*Δ mutant; phenotypic characterization of the *gcs1*Δ mutant is presented below.

TABLE 3. Sporulation in S288C strain background

Strain	Relevant genotype	% Spore formation[a]
Y315	*GCS1*	69.3 ± 4.0
Y4403	*gcs1*Δ	<0.1
Y5408	*gcs1R54K*	<0.1
Y5040	*spo14*Δ	<0.1
Y5492	*gcs1*Δ *spo14*Δ	<0.1
Y5664	*arl1*Δ	46.3 ± 9.8
Y4690	*GCS1-GFP*	41.7 ± 3.8
Y4804	$GCS1^{PH\Delta}$-*GFP*	62.7 ± 8.4
Y5396	*gcs1R54K-GFP*	<0.1
Y5328	*gcs1R54K-GFP* × *GCS1*	70.7 ± 5.4
Y5609	*GCS1 SNF7-GFP*	75.6 ± 8.6
Y5617	*gcs1*Δ *SNF7-GFP*	<0.1

[a] A minimum of 300 cells were counted per liquid culture over a minimum of three independent experiments. The final % sporulation includes cells that contained two, three, or four mature spores. Values are means ± SD from three independent experiments.

***GCS1* plays an essential role in sporulation.** Due to the difficulty in inducing sporulation in BY4743, we moved the *gcs1*::*kanMX* deletion cassette into an S288C-like strain background, as in previous studies performed in our lab (13).

As shown in Table 3, the S288C *gcs1*Δ mutant sporulated very poorly (<0.1% of *gcs1*Δ cells versus 69.3% ± 4.0% of *GCS1*-expressing cells). Analysis of the meiotic divisions by DAPI staining revealed that some *gcs1*Δ cells underwent mei-

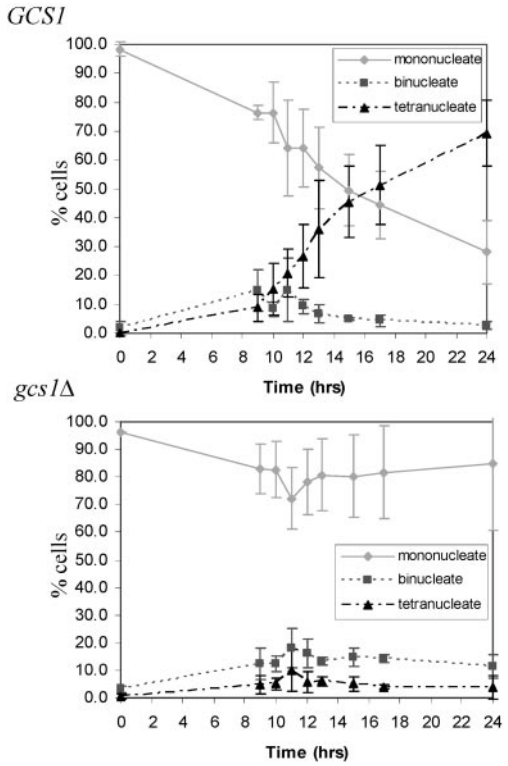

FIG. 1. *gcs1*Δ cells undergo meiotic division with reduced efficiency. Nuclear divisions were analyzed in *GCS1*-expressing and *gcs1*Δ cells by staining with the DNA-specific dye DAPI. A minimum of 300 cells were counted over three independent experiments.

TABLE 4. Complementation and suppression analysis of *gcs1*Δ mutant

Strain	Relevant genotype	% Spore formation
Y5204	*GCS1* + *CEN*	30.3 ± 0.7
Y5205	*GCS1* + 2μm	30.4 ± 2.9
Y5206	*GCS1* + *GCS1 CEN*	35.1 ± 1.7
Y5207	*GCS1* + *GCS1* 2μm	28.6 ± 3.3
Y5209	*gcs1*Δ + *CEN*	0.2 ± 0.4
Y5210	*gcs1*Δ + 2μm	0.1 ± 0.2
Y5211	*gcs1*Δ + *GCS1 CEN*	34.6 ± 6.8
Y5212	*gcs1*Δ + *GCS1* 2μm	28.9 ± 0.8
Y5545	*gcs1*Δ + *GCS1-GFP*-2μm	20.9 ± 6.6
Y4496	*gcs1*Δ + *GFP-SPO14*-2μm	0.1 ± 0.2
Y5707	*spo14*Δ + *GCS1-GFP*-2μm	<0.1

[a] A minimum of 300 cells were counted over three independent experiments performed on plates. The final % sporulation includes cells that contained two, three, or four mature spores. Values are means ± SD from three independent experiments.

osis I (MI) but that only about 5% of the cells progressed through MII (Fig. 1). Meiotic progression was also analyzed by examining the expression of sporulation-specific genes, which are regulated temporally and can be grouped into early, middle, mid-late, and late genes (9). We monitored the expression of early (*HOP1*) (24, 76), middle (*NDT80*) (9), and mid-late (*DIT1*) (6) genes by using *lacZ* fusions. Consistent with the analysis of the meiotic divisions in the *gcs1*Δ mutant, β-galactosidase assays with strains harboring these fusions revealed that *HOP1* and *NDT80* were expressed at similar levels to those of the wild type, while *DIT1* was expressed at a lower level in cells deleted for *GCS1* (data not shown). Therefore, the sporulation program is initiated in *gcs1*Δ mutants, but the mutants fail to efficiently progress through the meiotic divisions and form mature spores. To ensure that the sporulation defect was due to the deletion of *GCS1*, complementation analysis was performed, using low-copy-number (*CEN*) and high-copy-number (2μm) plasmids containing *GCS1*. Sporulation analyzed on plates was rescued to levels of 34.6% ± 6.8% (*CEN*) and 28.9% ± 0.8% (2μm), indicating that the deletion of *GCS1* is solely responsible for the sporulation phenotype. These same plasmids transformed into wild-type cells had no additional effect on sporulation (Table 4).

***gcs1*Δ mutant is defective in prospore membrane formation.** During the second meiotic division, the secretory system is modified to direct the synthesis of a distinct double-layered internal membrane termed the prospore membrane (40). To determine whether *gcs1*Δ synthesized the prospore membrane, we expressed *GFP-SPO14*, which is essential for and whose gene product localizes to the prospore membrane (65). Additionally, these strains were marked with *SPC42-dsRED*. Spc42p is a central plaque component of the SPB (14); thus, the dsRED marker can be used to monitor the meiotic divisions in live cells (48). In the wild type, GFP-Spo14p moves to the SPB and then marks the prospore membrane as it grows to surround the haploid nuclei (Fig. 2A) (30, 43, 64, 65). Visualization of GFP-Spo14p revealed that most cells deleted for *GCS1* that formed an MII spindle displayed a punctate pattern of GFP fluorescence (Fig. 2B) and that a small population displayed localization of GFP-Spo14p to the four SPBs (Fig. 2C),

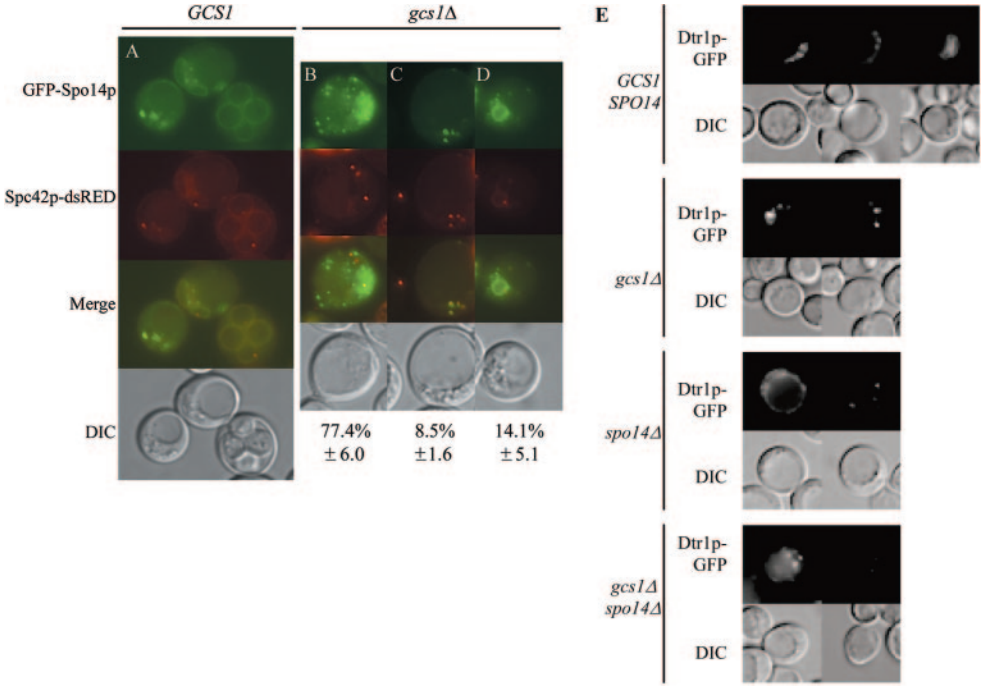

FIG. 2. *gcs1*Δ mutants are defective in prospore membrane formation. Fluorescence and DIC images of the same cells are shown. (A) Wild-type cells analyzed at ~12 h in sporulation medium. (B to D) Localization of GFP-Spo14p to punctate structures (B), the four SPBs (C), and immature prospore membranes (D) in *gcs1*Δ cells tagged with *SPC42-dsRED* to indicate the location of the SPBs under sporulation conditions. (E) Dtr1p-GFP localizes similarly to GFP-Spo14p in wild-type and *gcs1*Δ cells, with the majority of *gcs1*Δ cells displaying the patterns shown and a small percentage forming immature prospores. Additionally, Dtr1p-GFP localizes to punctate structures or the four SPBs in *spo14*Δ and *gcs1*Δ *spo14*Δ cells. This experiment was repeated a minimum of three times, with similar results.

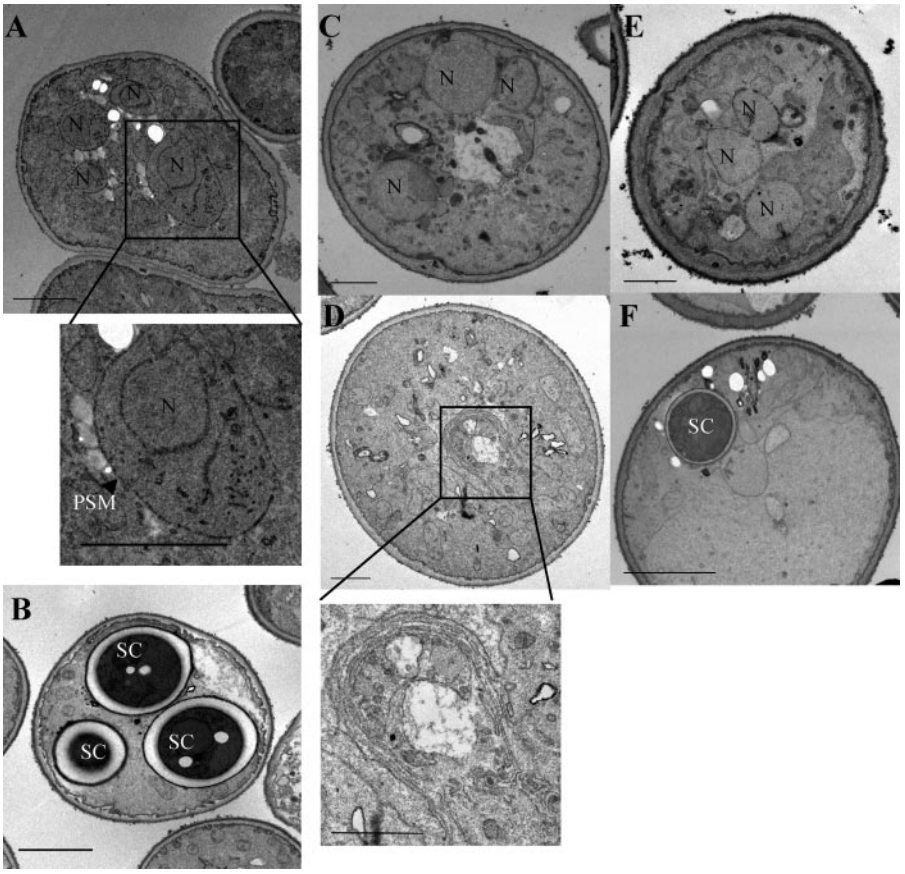

FIG. 3. TEM of *GCS1*-expressing and *gcs1* mutant cells during sporulation. (A and B) Wild-type cells show the presence of four nuclei and the corresponding prospore membranes (PSMs), and some cells have reached compartmentalization. (C) *gcs1*Δ cells largely fail to synthesize the prospore membrane and display abnormal morphology. (D) Some *gcs1*Δ cells contain aberrant structures reminiscent of "class E compartments." (E and F) *gcs1R54K*-expressing cells display less severe morphological defects overall, and one cell with a spore compartment can be observed. N, nuclei; SC, spore compartments; arrowhead, prospore membrane. Bars, 2 μm.

similar to what is observed with the *spo14*Δ mutant (30, 65). In addition, rare cells contained internal membranes; however, these membranes were smaller than those of the wild type, and cells usually contained fewer than four membrane compartments (Fig. 2D). Another prospore membrane marker, Dtr1p-GFP (19), gave a similar pattern of localization in the *gcs1*Δ mutant (Fig. 2E).

To confirm that *gcs1*Δ mutants were defective in prospore membrane formation, we examined thin sections of yeast cells induced to sporulate by TEM. In wild-type cells, the presence of prospore membranes encapsulating nuclei after MII, as well as cells containing spore wall compartments, was readily observed (Fig. 3A and B). In contrast, in the majority of cells deleted for *GCS1*, no prospore membrane or spore structures were seen (Fig. 3C). In addition, many *gcs1*Δ cells had abnormal morphologies and an abundance of membranous structures. The abnormal membrane structures were reminiscent of what is seen in class E mutants defective in endosome-to-vacuole trafficking (Fig. 3D) (1). Thus, *gcs1*Δ mutants are defective in prospore membrane formation and display additional defects in cellular membrane compartments.

Deletion of *GCS1* impairs endosome-to-vacuole trafficking during sporulation. The aberrant membrane structures observed by TEM and the previous report that Gcs1p interacts with the actin cytoskeleton (5) led us to examine FM4-64 uptake by the *gcs1*Δ mutant. FM4-64 is a lipophilic dye that is internalized by endocytosis at the plasma membrane and is transported through the endosome to the vacuolar membrane (77). In vegetative cells, the kinetics of uptake were similar for wild-type and *gcs1*Δ mutant cells: FM4-64 was primarily observed in punctate structures immediately after removal from the dye and 10 min later was found at the vacuolar membrane (Fig. 4A).

Endocytosis plays an important role in spore morphogenesis (43), and thus we monitored the uptake of FM4-64 during sporulation by the *gcs1*Δ mutant (Fig. 4B). Cells undergoing meiotic divisions were stained with FM4-64, the dye was removed, and the cells were examined at various times. Wild-type cells rapidly took up FM4-64 into punctate structures and then the vacuolar membrane; >80% of the cells showed vacuolar membrane staining by 60 min after removal from the dye (Table 5). Many cells deleted for *GCS1* displayed a very bright signal and the appearance of aberrant vacuoles immediately after removal from the dye. As time progressed, most of the cells showed FM4-64 in punctate or bar-like structures surrounding the vacuole. Some cells showed vacuolar membranes

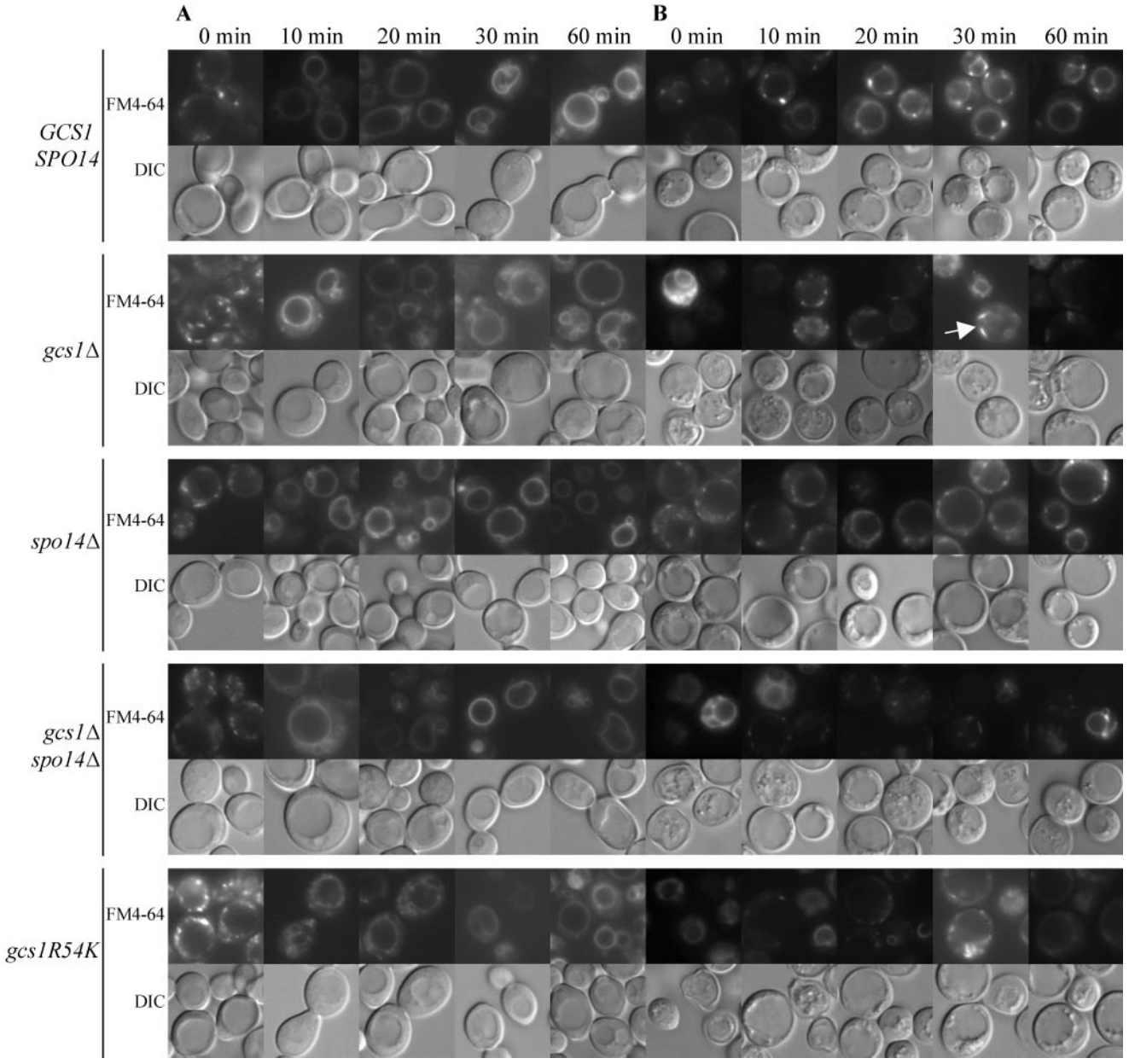

FIG. 4. FM4-64 staining in vegetative and sporulating cells. Fluorescence and DIC images of the same cells are shown. (A) FM4-64 uptake by vegetative cells. (B) Cells induced during meiosis for 14 h. An analysis of FM4-64 uptake was performed 0, 10, 20, 30, and 60 min after removal of the cells from the dye. Arrow, bar-like structures. (C) Localization analysis of FM4-64 and the endosomal marker Snf7p-GFP in wild-type (60 min after removal of the dye) and *gcs1*Δ (30 min [top panel] and 60 min [bottom panel] after removal of the dye) cells. Arrowhead, "class E compartment"; arrow, bar-like structures. These experiments were repeated a minimum of three times, with similar results.

stained with FM4-64 that appeared to be convoluted or fragmented, as previously seen by Zhang et al. (83). Only ~30% of *gcs1*Δ cells showed vacuolar membrane staining by 60 min, while ~33% of the cells showed punctate and bar-like structures (Table 5).

Colocalization of FM4-64 and Snf7p-GFP, an endosomal marker (2), was analyzed in wild-type and *gcs1*Δ cells to determine the nature of the punctate and bar-like structures (Fig. 4C). Significant colocalization of FM4-64 and Snf7p-GFP was not observed, although in many cells the signals appeared adjacent to one another. Thus, the punctate and bar-like structures (Fig. 4C, arrow) visualized in *gcs1*Δ cells were not Snf7p-marked endosomal structures. Additionally, *gcs1*Δ mutants contained a small population of cells (~1%) that showed a small ring-like structure next to the vacuole that was labeled with both FM4-64 and Snf7p-GFP (Fig. 4C, arrowhead). This structure most likely represents the "class E compartment," corresponding to a late endosomal intermediate unable to form intraluminal vesicles (2, 33, 56).

Gcs1p-GFP is a soluble protein that becomes concentrated at spore peripheries during maturation. Strains expressing Gcs1p-GFP, either chromosomally or from a high-copy-number plasmid, were constructed, and localization was examined by GFP fluorescence in living cells. The Gcs1p-GFP fusion was

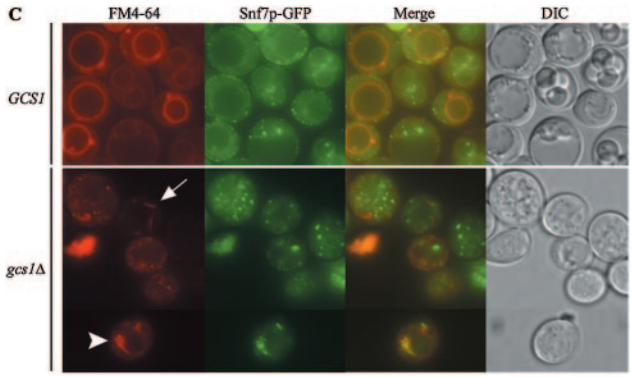

FIG. 4—*Continued.*

largely functional, as assessed by complementation of the sporulation phenotype (Tables 3 and 4). In vegetative cells and early during the sporulation program, Gcs1p-GFP was seen throughout the cytoplasm (Fig. 5A). As sporulation progressed, the GFP signal became concentrated in the developing spore, and during spore maturation, the GFP signal was primarily seen at the spore periphery (Fig. 5A).

Cell fractionation was performed to determine whether Gcs1p was membrane associated. The predicted molecular mass of Gcs1p is 39 kDa and that of GFP is 26 kDa. Gcs1p-GFP was specifically detected with anti-GFP antibody as an ~75-kDa protein that was absent from extracts made from cells lacking the tagged protein (Fig. 5B, *GCS1* WCE). Gcs1p-GFP was found exclusively in the soluble fraction after both a 14,000 × *g* spin and a 100,000 × *g* spin of extracts from both vegetative and sporulating cells. In contrast, the membrane-anchored Gas1p protein was found predominately in the pellets after spinning at 14,000 × *g* and 100,000 × *g*. Therefore, Gcs1p-GFP is a largely soluble protein. In addition, Gcs1p-GFP isolated from sporulating cells migrated slower by SDS-polyacrylamide gel electrophoresis than did Gcs1p-GFP isolated from vegetative cells (Fig. 5B). This shift in molecular mass suggests that the protein undergoes posttranslational modification during sporulation.

Arf-GAP activity, but not the pleckstrin homology (PH) domain, is essential for Gcs1p function in sporulation. All Arf-GAPs described thus far contain one invariant arginine residue, which corresponds to position 54 in Gcs1p. This residue, when mutated to alanine or lysine in Gcs1p, has been shown to abolish enzymatic activity in vitro (82). Cells expressing *gcs1R54K* or *gcs1R54A* as their only source of *GCS1* failed to sporulate (Table 3 and data not shown). Thus, *GCS1* Arf-GAP activity is crucial for in vivo function during sporulation.

We analyzed strains expressing *gcs1R54K* for prospore membrane formation and FM4-64 uptake as described above for the null mutant. Like *gcs1*Δ cells, *gcs1R54K*-expressing cells largely failed to make prospore membranes, although rarely a cell containing a single spore compartment was observed (Fig. 3E and F; data not shown), and *gcs1R54K*-expressing cells were additionally defective in FM4-64 trafficking during sporulation (Fig. 4B). Thus, the phenotype of *gcs1*Δ mutants during sporulation is largely attributable to the absence of GAP activity.

gcs1R54K was chromosomally tagged with GFP to analyze the expression and localization of this mutant. Gcs1p^{R54K}-GFP behaved similarly to Gcs1p-GFP in sporulating *GCS1*-expressing cells, exhibiting a diffuse staining pattern, that is, concentrated to spore peripheries in maturing wild-type spores, and was expressed at similar levels (Fig. 5D).

TABLE 5. Analysis of endocytosis by FM4-64 in sporulating cells

Strain and time of analysis (min)	Relevant genotype	% FM4-64 signal[a]		
		Punctate	Vacuole[b]	No signal
Y315	*GCS1 SPO14*			
0		59.4	3.1	37.5
10		31.0	41.0	28.0
20		4.4	80.6	15.0
30		1.1	76.6	22.3
60		0.0	82.9	17.1
Y4403	*gcs1*Δ			
0		27.5	34.5	38.0
10		32.1	10.2	57.7
20		46.6	21.9	31.5
30		25.3	22.8	51.9
60		32.5	29.6	37.9
Y5040	*spo14*Δ			
0		83.2	3.4	13.4
10		94.5	1.6	3.9
20		46.1	30.9	23.0
30		1.1	95.2	3.7
60		0.0	97.7	2.3
Y5492	*gcs1*Δ *spo14*Δ			
0		8.6	38.9	52.5
10		23.8	41.4	34.8
20		12.7	27.6	59.7
30		8.6	26.8	64.6
60		9.1	38.8	52.1
Y5408	*gcs1R54K*			
0		8.4	33.7	57.9
10		26.8	25.6	47.6
20		32.0	23.2	44.8
30		30.7	23.2	46.1
60		28.6	27.8	43.6

[a] Localization of FM4-64 to punctate or vacuolar structures within cells. This experiment was performed multiple times revealing similar results. A minimum of 300 cells was counted in each experiment. These numbers reflect one individual experiment.
[b] Cells showing localization of FM4-64 to normal or fragmented vacuoles.

In addition to the Arf-GAP region of the protein, Gcs1p contains a PH domain that consists of the C-terminal 125 amino acid residues. PH domains have been implicated in phosphoinositide binding in a variety of proteins (20, 32). Phosphoinositides, such as PIP_2, play important roles in meiosis and have been shown to activate Spo14p (67). The PH domain of Gcs1p is dispensable for vegetative growth (82). The same deletion in sporulating cells appears to have no effect on Gcs1p function (Table 3).

Gcs1p does not act through Arl1p during sporulation. Recently, Gcs1p has been shown to exhibit GAP activity towards Arl1p (Arf-like) as well as Arf1p (38). Thus, it was possible that the unique role Gcs1p plays in sporulation is mediated through Arl1p. To test this hypothesis, we deleted *ARL1* in the S288C strain background and examined sporulation in the homozygous mutant. The *arl1*Δ mutant sporulated with a

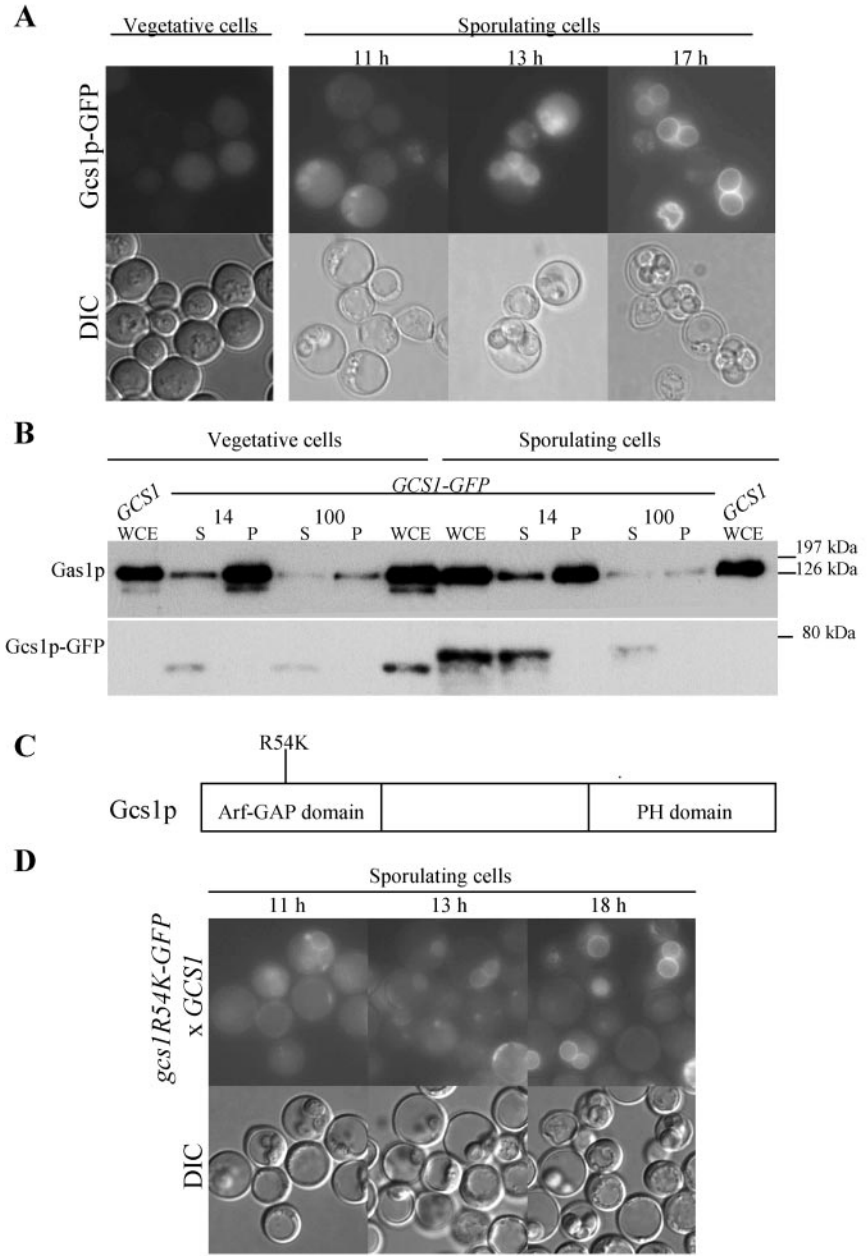

FIG. 5. Gcs1p-GFP is a soluble protein that eventually localizes to mature spore peripheries. (A) Localization of Gcs1p-GFP expressed from a 2μm plasmid in vegetative and sporulating cells. Fluorescence and DIC images of the same cells are shown. (B) Autoradiograph of Western blot analysis of proteins extracted from cells induced during meiosis (14 h) and from vegetative cells probed with anti-GFP to detect Gcs1p-GFP and anti-Gas1p to detect the membrane protein Gas1p. (C) Gcs1p contains 352 amino acids and consists of Arf-GAP and PH domains. A mutation at position 54 (R54K) abolishes Arf-GAP activity. (D) Gcs1p^{R54K}-GFP localizes similarly to the wild-type protein in *GCS1*-expressing cells. These experiments were repeated a minimum of three times, with similar results.

close-to-wild-type efficiency, indicating that Gcs1p does not act exclusively through Arl1p during sporulation (Table 3).

Relationship between Gcs1p and Spo14p during sporulation. To determine the relationship between Gcs1p and Spo14p during sporulation, we constructed the *gcs1*Δ *spo14*Δ double mutant. Like the case for the *spo14*Δ mutant, no prospore membranes were formed in the *gcs1*Δ *spo14*Δ mutant (Fig. 2E). However, the double mutant behaved similarly to the *gcs1*Δ mutant with respect to FM4-64 uptake (Fig. 4). Thus, the *spo14*Δ mutant is epistatic to the *gcs1*Δ mutant for prospore membrane formation, but Gcs1p plays additional roles independent of Spo14p during sporulation (e.g., endosome-to-vacuole trafficking).

The effect of overexpressing *GCS1* in a *spo14*Δ strain and in a strain with a temperature-sensitive hypomorphic allele of *SPO14* was analyzed. High levels of *GCS1* were unable to rescue the sporulation defects associated with these *spo14* mutants. Likewise, overexpression of *SPO14* had no effect on *gcs1*Δ mutants (Table 4; data not shown).

***gcs1*Δ has reduced levels of Spo14p-generated PA.** To determine whether the deletion of *GCS1* had any effect on

TABLE 6. Spo14p-catalyzed hydrolysis of internalized BODIPY-PC

Strain	Relevant genotype	% BODIPY-PA[a]
Y5040	*spo14*Δ	0.1 ± 0.1
Y315	*GCS1 SPO14*	3.0 ± 0.8
Y4403	*gcs1*Δ	0.6 ± 0.3*
Y5492	*gcs1*Δ *spo14*Δ	0.1 ± 0.02
Y5408	*gcs1R54K*	0.5 ± 0.2*
Y4995	*ndt80*Δ	2.5 ± 0.9

[a] The percentage of conversion of intracellular BODIPY-PC to BODIPY-PA was determined in cells sporulated at 30°C as described in Materials and Methods. Values are means ± SD from three independent experiments. *, % BODIPY-PA is statistically significantly different for *gcs1*Δ and *gcs1R54K*, with *P* values of 0.043 and 0.035, respectively.

SPO14 function in sporulating cells, we monitored Spo14p PLD activity by measuring the conversion of internalized BODIPY-PC to BODIPY-PA. BODIPY-PC is a fluorescently labeled substrate that is readily internalized by cells. The hydrolysis of BODIPY-PC occurs almost exclusively through the action of Spo14p, as cells deleted for *SPO14* do not generate any appreciable levels of BODIPY-PA (43, 66, 67). The conversion rate of BODIPY-PC to BODIPY-PA in *gcs1*Δ cells was markedly reduced compared to that in the wild type (0.6% ± 0.3% and 3.0% ± 0.8%, respectively), indicating that the deletion of *GCS1* had a significant effect on Spo14p catalytic activity (Table 6). Additionally, *gcs1R54K*-expressing cells showed a similar reduction in the generation of BODIPY-PA, mimicking the effect seen in *gcs1*Δ cells. The BODIPY-PA generated in the *gcs1*Δ mutant is due to the activity of Spo14p, as the *gcs1*Δ *spo14*Δ double mutant failed to produce any appreciable BODIPY-PA. Furthermore, similar levels of BODIPY-PC were internalized in all strains analyzed in this experiment (data not shown).

A reduction in PA might be expected for cells that are not efficiently progressing through meiosis and generating prospore membranes. To ensure that the reduced levels of PA in *gcs1*Δ and *gcs1R54K*-expressing cells were not due to a failure to efficiently progress through meiosis and sporulation, Spo14p activity was monitored in *ndt80*Δ mutants. Cells deleted for *NDT80* arrest prior to the MI division (81). In this assay, *ndt80*Δ cells generated wild-type levels of Spo14p-generated PA (Table 6). Thus, the reduction in Spo14p-generated PA in *gcs1* mutants was not due to a failure to internalize the substrate or efficiently progress through sporulation, but rather the data suggest that Gcs1p regulates the activity of Spo14p.

DISCUSSION

Gcs1p has a unique function during sporulation and regulates Spo14p. Arf-GAPs have been widely studied in cycling cells, where they play roles in many aspects of membrane trafficking. Sporulation not only needs a functional secretory system, as in vegetative cells (67), but it also requires unique components for the generation of the prospore membrane (47). Furthermore, Spo14p-generated PA plays a central role in the formation of the prospore membrane (67), and given the lipid responsiveness of Arf-GAPs, it seemed likely that a subset, either singly or in combination, functions in this process. We found that deletion of either *GCS1* or *GLO3* alone impaired sporulation to a significant extent, while deletion of the other Arf-GAPs did not have an effect on sporulation. *glo3*Δ mutants grow slowly (53), and we found that they do not initiate the sporulation program, suggesting a general defect in cell function. On the other hand, *gcs1*Δ mutants induce the sporulation program but largely fail to synthesize the prospore membrane. Thus, in contrast to the situation in mitotically dividing cells, where different pairs of Arf-GAPs function redundantly, Gcs1p alone is essential for sporulation. However, it is likely that, although not essential, the other Arf-GAPs function during sporulation and may contribute to the phenotype of the *gcs1*Δ mutant.

In vegetative cells, in addition to its central role in vesicular transport (53, 54, 83), Gcs1p has been implicated in the maintenance of mitochondrial morphology and actin cytoskeletal organization (5, 26). Furthermore, *GCS1* was originally identified as a gene conditionally required for the reentry of cells into the cell cycle after stationary-phase growth (28). Similar to the case in cycling cells, it appears that Gcs1p functions in many processes during sporulation, including meiotic progression, prospore membrane formation, and endosome-to-vacuole transport. Thus, the inactivation of *GCS1* impinges on a number of different cellular processes in both vegetative and sporulating cells.

With respect to prospore membrane formation, the phenotype of *gcs1* mutants is reminiscent of that of the *spo14*Δ mutant in that many cells display a punctate pattern of prospore membrane markers within the cytoplasm, and in some cells these markers accumulate at the SPB (30, 65). However, unlike *spo14*Δ cells, a minor proportion of *gcs1*Δ cells generate small prospore membranes, and, rarely, even a mature spore is produced. *gcs1*Δ *spo14*Δ cells do not form these structures. The presence of small prospore membranes in some cells may be explained by the low but detectable level of Spo14p PLD activity in *gcs1*Δ mutants.

PA has been shown to directly stimulate Gcs1p Arf-GAP activity in vitro and appears to function downstream of Spo14p during Sec14p-independent growth (82). In contrast, we found a reduction in Spo14p-generated PA levels in *gcs1*Δ mutants, suggesting that Gcs1p positively regulates Spo14p during sporulation. How Gcs1p influences Spo14p activity is currently unknown, but it is likely to be indirect. Gcs1p converts both $Arf1p^{GTP}$ and $Arl1p^{GTP}$ to their GDP-bound forms (38), and an analysis of *gcs1R54K*-expressing cells showed that they closely mimicked *gcs1*Δ cells in all assays, indicating that Arf-GAP activity is specifically required for proper functioning of Gcs1p during sporulation. Given that the deletion of *ARL1* has no significant effect on sporulation, Arl1p is unlikely to influence Spo14p. Furthermore, there is no direct stimulation or interaction between Arf1p and Spo14p (64). Taken together, these results indicate that the Arf cycle is important for sporulation and suggest that this cycle impinges on Spo14p, but not directly, as observed in mammalian cells (21, 22, 50). Perhaps the Arf cycle influences Spo14p activity by recruitment of other proteins or by modulating the lipid environment.

Recent studies indicated that Spo14p is specifically involved in vesicle fusion for prospore membrane formation (46, 60). The results reported here indicate that Gcs1p also plays a role in prospore membrane formation and suggest that there is a feedback loop between these signaling molecules (Fig. 6). Spo14p generates PA, which has been shown to stimulate

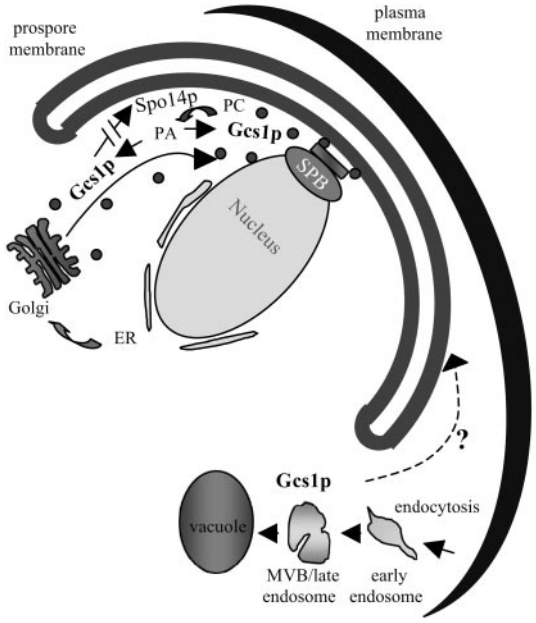

FIG. 6. Potential functions of Gcs1p during sporulation. Gcs1p is involved in multiple steps of membrane trafficking during sporulation. Gcs1p may aid in the transport of vesicles from the Golgi and/or in vesicle fusion at the site of prospore membrane synthesis. The interrupted arrow indicates that Gcs1p stimulation of Spo14p is most likely indirect. Additionally, Gcs1p functions in endosome-to-vacuole trafficking. Whether this pathway contributes to the sporulation process is uncertain, as indicated by the dashed arrow and question mark.

Gcs1p (82), while in turn Gcs1p converts $Arf1p^{GTP}$ to $Arf1p^{GDP}$ and indirectly influences Spo14p activity. Transport vesicles require coat removal prior to vesicle fusion, which is mediated by hydrolysis of Arf-bound GTP (75). Therefore, the hydrolysis of $Arf1p^{GTP}$ to $Arf1p^{GDP}$ by Gcs1p may be involved in shedding the protein coat and subsequently stimulating the fusion of prospore membrane transport vesicles, which additionally requires Spo14p-generated PA. Therefore, Spo14p, Gcs1p, and the Arf cycle are important and intermingled to facilitate prospore membrane formation.

Localization and modification of Gcs1p during sporulation. Previous studies revealed that, depending on growth conditions, the majority of Gcs1p is cytoplasmic (26). Consistent with this, we found that in vegetative cells, Gcs1p-GFP fluorescence was seen throughout the cytoplasm, and in extracts, the protein remained in the soluble fraction after centrifugation. During sporulation, Gcs1p-GFP was present in the cytoplasm of both the mother cell and the developing spore. At a late stage of spore maturation, Gcs1p-GFP became concentrated at the spore peripheries. However, Gcs1p-GFP remained soluble during fractionation. Therefore, the localization of Gcs1p-GFP to the spore peripheries as sporulation progresses perhaps occurs through association with a secondary protein.

Immunoblot analysis of protein extracts from cells expressing *GCS1-GFP* revealed that Gcs1p-GFP changes its electrophoretic mobility during meiosis, as was seen for Spo14p (65). The change in electrophoretic mobility of Spo14p isolated from sporulating cells was shown to be due to phosphorylation (65). Furthermore, genetic analysis suggested that this modification is specifically important for sporulation (68). Although we cannot rule out the possibility that the change in mobility of Gcs1p-GFP is due to proteolytic breakdown or other modifications, we favor the idea that like Spo14p, Gcs1p is modified by phosphorylation. This suggests that proteins that function during both vegetative growth and sporulation are modified posttranslationally to carry out sporulation-specific functions. The distinct nature of this posttranslational modification and its generality in modifying proteins for sporulation await future investigation.

Role of endosome-to-vacuole transport during sporulation. In addition to defects in prospore membrane synthesis, we found that Gcs1p facilitates endosome-to-vacuole transport during sporulation. Furthermore, TEM indicated that there are abnormal membrane structures in *gcs1*Δ sporulating cells. The role of Gcs1p in endosome-to-vacuole transport during vegetative growth has previously been reported (83); however, other studies have not found any defect in this transport step (26). The difference may be due to the strain background or growth conditions. Nonetheless, our studies suggest that sporulation is more sensitive to a loss of Gcs1p with respect to endosome-to-vacuole trafficking. Recently, Morishita and Engebrecht (43) reported that endocytosis is important for spore wall formation and suggested that trafficking between the plasma membrane and prospore membranes is important during the early stages of sporulation. This raises the possibility that Gcs1p may play a role in this trafficking event as well.

While this defect in endosome-to-vacuole trafficking was apparent in *gcs1*Δ cells, we found that the involvement of Gcs1p in prospore membrane formation is likely the major contribution of this protein during sporulation. TEM revealed aberrant structures in *gcs1*Δ mutants indicative of "class E compartments" usually seen in cells lacking any of the 17 class E *VPS* genes (51, 56, 59). Additional analyses of these mutants during sporulation revealed that sporulation was not impaired in most of the mutants in this class (17). Thus, this endosome-to-vacuole trafficking step does not appear to be required for efficient sporulation, and therefore is unlikely to be the main function of Gcs1p in sporulation.

In conclusion, our findings that Spo14p-generated PA is reduced in the absence of *GCS1* and that Gcs1p is stimulated by PA (82) suggest that Gcs1p and Spo14p function together in the developmentally regulated transport step that mediates prospore membrane formation.

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