Septins are a class of GTP-binding proteins conserved throughout many eukaryotes. Individual septin subunits associate with one another and assemble into heteromeric complexes that form filaments and higher-order structures in vivo. The mechanisms underlying the assembly and maintenance of higher-order structures in cells remain poorly understood. Septins in several organisms have been shown to be phosphorylated, although precisely how septin phosphorylation may be contributing to the formation of high-order septin structures is unknown. Four of the five septins expressed in the filamentous fungus, *Ashbya gossypii*, are phosphorylated, and we demonstrate here the diverse roles of these phosphorylation sites in septin ring formation and septin dynamics, as well as cell morphology and viability. Intriguingly, the alteration of specific sites in Cdc3p and Cdc11p leads to a complete loss of higher-order septin structures, implicating septin phosphorylation as a regulator of septin structure formation. Introducing phosphomimetic point mutations to specific sites in Cdc12p and Shs1p causes cell lethality, highlighting the importance of normal septin modification in overall cell function and health. In addition to discovering roles for phosphorylation, we also present diverse functions for conserved septin domains in the formation of septin higher-order structure. We previously showed the requirement for the Shs1p coiled-coil domain in limiting septin ring size and reveal here that, in contrast to Shs1p, the coiled-coil domains of Cdc11p and Cdc12p are required for septin ring formation. Our results as a whole reveal novel roles for septin phosphorylation and coiled-coil domains in regulating septin structure and function.
TABLE 1  *Ashbya* strains used in this study

| Strain | Relevant genotype | Source or reference |
|--------|-------------------|---------------------|
| Wild type | Δeu2Δthr4 | 35 |
| AG124 | AgSHS1-GFP-GEN3 | 36 |
| AG384.1 | AgCdc11a-GFP-GEN3 | This study |
| AG413.2 | AgCdc3-GFP-GEN3 | This study |
| AG436.1 | AgCdc12-GFP-GEN3 | This study |
| AG442.1 | pAGB088 [pAg Shs1-GFP-GEN3] | 25 |
| AG495.1 | pAGDC11a-TAP-GEN3 | 25 |
| AG529.1 | pAGB383 [pAG Cdc3 S91A-GFP-GEN3] | This study |
| AG531.1 | pAGB384 [pAG Cdc3 S496D-GFP-GEN3] | This study |
| AG533.1 | pAGB386 [pAG Cdc11a S314D-GFP-GEN3] | This study |
| AG537.1 | pAGB378 [pAg Shs1 9D-GFP-GEN3] | This study |
| AG652.1 | AgCdc12ΔCC-GFP-GEN3* | This study |
| AG656.1 | AgCdc11aΔCC-GFP-GEN3* | This study |
| AG657.1 | AgShs1ΔCC-Cdc11aΔCC-GFP-GEN3 | This study |
| AG688.1 | AgCdc3 S91D-GFP-GEN3 | This study |
| AG669.2 | AgCdc11a S314A-GFP-GEN3 | This study |
| AG671.1 | AgCdc12 S5A S8A-GFP-GEN3* | This study |
| AG672 | AgCdc12 S323A-GFP-GEN3* | This study |
| AG674 | pAGB470 [pAg Cdc12 SSD-GFP-GEN3] | This study |

*a* With the exception of plasmidic strains (in brackets) and heterokaryotic strains (marked with asterisks), all analyzed mycelia were homokaryotic (all nuclei have the same genotype).

TABLE 2  Plasmids used in this study

| Plasmid | Name | Vector | Relevant insert | Source or reference |
|---------|------|--------|-----------------|---------------------|
| None | pRS416 | | | |
| AG005 | pAGT141 | pUC19 | GFP-GEN3 | 37 |
| AG088 | pAgShs1-GFP-Gen | pRS416 | SHS1-GFP-GEN3 | 25 |
| AG123 | pAgCdc12 | pRS416 | CDC12 | 19 |
| AG125 | pAgCdc11 | pRS416 | CDC11 | 19 |
| AG127 | pAgCdc3 | pRS416 | CDC3 | 19 |
| AG166 | pAgShs1 | pRS416 | SHS1 | Peter Philippus, University of Basel |
| AG214 | pAg Cdc11a-GFP-Gen | pRS416 | CDC11A-GFP-GEN3 | This study |
| AG221 | pAg Cdc3-GFP-Gen | pRS416 | CDC3-GFP-GEN3 | This study |
| AG260 | pAg Cdc12-GFP-Gen | pRS416 | CDC12-GFP-GEN3 | This study |
| AG360 | pAg Cdc11a S314A-GFP-Gen | pRS416 | CDC11A S314A-GFP-GEN3 | This study |
| AG361 | pAg Cdc12 S5A S8A-GFP-Gen | pRS416 | CDC12 S5A S8A-GFP-GEN3 | This study |
| AG362 | Cdc3 S91D-GFP-Gen | pRS416 | CDC3 S91D-GFP-GEN3 | This study |
| AG371 | Cdc12 S5E S8E-GFP-Gen | pRS416 | CDC12 S5E S8E-GFP-GEN3 | This study |
| AG378 | pAg Shs1 9D-GFP-Gen | pRS416 | SHS1 9D-GFP-GEN3 | 25 |
| AG380 | pAg Cdc3-GFP-Gen reduced | pRS416 | CDC3-GFP-GEN3 | This study |
| AG383 | pAg Cdc3 S91A-GFP-Gen | pRS416 | CDC3 S91A-GFP-GEN3 | This study |
| AG384 | pAg Cdc3 S496D-GFP-Gen | pRS416 | CDC3 S496D-GFP-GEN3 | This study |
| AG385 | pAg Cdc12 S323A-GFP-Gen | pRS416 | CDC12 S323A-GFP-GEN3 | This study |
| AG386 | pAg Cdc11a S314D-GFP-Gen | pRS416 | CDC11A S314D-GFP-GEN3 | This study |
| AG425 | pAg Shs1ΔCC-Cdc11a CC-GFP-Gen | pRS416 | SHS1ΔCC-CDC11A CC-GFP-GEN3 | This study |
| AG470 | pAg Cdc12 SSD-GFP-Gen | pRS415 | CDC12 SSD-GFP-GEN3 | This study |

*Phytochemistry and transformation protocols are described previously (33, 34).* The plasmids used in this study are listed in Table 2. The oligonucleotide primers are listed in Table 3. All single point mutations were made on full-length plasmids using a QuickChange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). All gel purifications were performed with the QIAquick gel extraction kit (Qiagen, Valencia, CA).

**MATERIALS AND METHODS**

**Growth conditions and strain construction.** *A. gossypii* media, culturing, and transformation protocols are described previously (33, 34). The strains generated and used in the present study are described in Table 1. The plasmids used in this study are listed in Table 2. All single point mutations were made on full-length plasmids using a QuickChange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). All gel purifications were performed with the QIAquick gel extraction kit (Qiagen, Valencia, CA).

(i) *Cdc3 point mutation strain construction.* AGB221 was made by cotransforming yeast with *GFP-GEN3* amplified from AGB141 using the primer pair AGO589/AGO505 with AGB127. The plasmid was verified by digest with EcoRI and KpnI, followed by sequencing with primers AG098, AG0199, AG0472, AG0520, AG0521, and AG0539. The gel-purified 4,167-bp product of AGB221 digested with MluI and NotI was transformed into wild-type *Ashbya* strain ΔΔΔΔ to obtain strain AG132.2, which was verified by PCR using the oligonucleotides AG098, AG0199, AG0315, and AG0405. AGB383 was made using primer pair AG0940/ AG0941 on AGB380 and verified by sequencing with the primers AG0101 and AG0954. AGB383 was transformed into ΔΔΔΔ to obtain AG529.1. AGB362 was obtained using primer pair AG0942/AG0943 on AGB221, verified by digestion with BclI and BclVI, and sequenced with the primer AG0954. AGB362 was digested with XhoI and NotI and transformed into ΔΔΔΔ to obtain AG688.1, which was verified by PCR using the oligonucleotide pairs AG0954/AG0101 (followed by digest with BglII to confirm presence of the point mutation), AG0199/AG05, AG0471/ AG0315, and AG098/AG0315. AGB384 was obtained by using the primers AG0940/AG0941 on AGB380 and verified with the sequencing primers AG0199 and AG0202. AGB384 was transformed into ΔΔΔΔ to obtain AG531.1.

(ii) *Cdc11 point mutation strain construction.* AGB214 was made by cotransforming yeast with *GFP-GEN3* amplified from AGB141 using the primer pair AGO540/AGO541 with AGB125. The plasmid was verified with AflII and BglII, followed by sequencing with AGO203, AGO206,
AGO471, AGO472, AGO521, and AGO539. The gel purified 4,504-bp product of AGB214 digested with BlpI and NheI was transformed into \( \text{H9004} \) to obtain strain AG384.1, which was verified by PCR using the oligonucleotides AGO98, AGO203, AGO405, and AGO350. AGB386 was obtained by using the primer pair AGO938/AGO939 on AGB214, digest verified using BciVI, and sequence verified using AGO130. AGB386 was transformed into \( \text{H9004} \) to obtain AG533.1. AGB360 was made using primer pair AGO936/AGO937 on AGB214, digest verified using BspHI, and sequence verified using AGO130. AGB360 was digested with XhoI and transformed into \( \text{H9004} \) to obtain AG669.2, which was verified by PCR using the primer pairs AGO5/AGO203, AGO471/AGO350, and AGO203/AGO350.

(iii) Cdc12 point mutation strain construction. AGB260 was made by cotransforming yeast with GFP-GEN3 amplified from AGB141 using the primer pair AGO218/AGO197 with AGB123. The plasmid was verified with XmaI, followed by sequencing with AGO136, AGO472, AGO520, and AGO521. The gel-purified 4,274-bp product of AGB260 digested with XhoI was transformed into \( \text{H9004} \) to obtain strain AG436.1.

### TABLE 3 Oligonucleotides used in this study

| Oligonucleotide | Name | Sequence (5′→3′) |
|-----------------|------|-----------------|
| AGO5 | Green2.2 | TGTAGTTCCCGTCATCTTTG |
| AGO36 | VG5 | GAGGAGATTTGTCGTGTTG |
| AGO98 | New GEN3.3 | CTTGCAGCCTCTGGAACTTC |
| AGO101 | Cdc3 ORF seq 700F | TAGGGATCACAGGGAGATGC |
| AGO119 | Cdc12 S5A S8A F | GAAATGTTGAATAGAGCGGATGGGGCCCTTGAGTTTGATTTCTTCAC |
| AGO130 | pET seq 1068 F | GTACTTGCGCTCGCTACTTC |
| AGO136 | 5′ CFp seq | TCTGGACACACTGCGATCTTCCTC |
| AGO199 | Sep7-CFP seq1 | AGCATCTCCACGAGGCCAAG |
| AGO202 | 3′ CFp seq2 | GTCTTGTAGTTCCCGTCATC |
| AGO203 | 5′ RFP seq1 | ACATACTTGGCACGGGAAGAG |
| AGO209 | 3′ YFP seq | TAACTCGGCGGCTGCAGAAGATATATAGTTAGAAGATGTTGGCATGATTACGCCAAGCTTGC |
| AGO315 | CDC3-CFP:nat V | CTTGGCACACGCTGCTAAAG |
| AGO350 | Ag CDC11A-Cherry iv | TACCAGCACCAGCATTCTC |
| AGO354 | 3′ b Sep7-CFP 25ds | CCCTCGGGCAAAGGATAGATATAGTTAGAGATGTTGGCATGATTACGCCAAGCTTGC |
| AGO405 | 5′ TUB4-CFP seq2 | TTACTACACTCGGATCTTCTCAGT |
| AGO407 | 3′ TUB4-CFP seq2 | ACCGGATTCAGTCGTCACTC |
| AGO427 | Sep7V2 | GCCGCCAAGTATTGTTTCAGA |
| AGO428 | Sep7V1 | GCCGCCCTCAGGATAGAGGAAGAA |
| AGO471 | 5′ kanR seq | TTACTACACTCGGATCTTCTCAGT |
| AGO472 | kanR seqR | GATAGTAGGAGGCTGAG |
| AGO520 | GFP seq R | CGAGATTCGCCGTTAATAAC |
| AGO521 | Gen seq F | TGGTGTCGCTCCTCTTAGT |
| AGO534 | conGFP I | AACCTCGGCGGCTGCAGAAG |
| AGO539 | Cdc10 v2 | CTCCAGGACTGCAACGCTAG |
| AGO912 | Tubl seq F | AGACCACATGGTCCTTCTTG |
| AGO924 | Cdc12 S5A S8A R | GAAATGTTGAATAGAGCGGATGGGGCCCTTGAGTTTGATTTCTTCAC |
| AGO925 | Cdc12 S5A S8A R | GAAATGTTGAATAGAGCGGATGGGGCCCTTGAGTTTGATTTCTTCAC |
| AGO926 | Cdc12 S5E S8E R | GCTGGTCGCTCCTCTCTCAGT |
| AGO927 | Cdc12 S5E S8E R | GCTGGTCGCTCCTCTCTCAGT |
| AGO928 | Cdc12 S5D R | CTTCAATTCAGGAGAAATGTTGAATAGAGACGATGGGTCACTTGTTGG |
| AGO936 | Cdc11a S314A F | CAAAGCTCATGAACAATGGGGCTACGGAATTTATCAGCTCACCAGCA |
| AGO937 | Cdc11a S314A R | CAAAGCTCATGAACAATGGGGCTACGGAATTTATCAGCTCACCAGCA |
| AGO939 | Cdc11a S314D F | CAAAGCTCATGAACAATGGGGATACGGAATTTATCAGCTCACCAGCA |
| AGO940 | Cdc11a S314D R | CAAAGCTCATGAACAATGGGGATACGGAATTTATCAGCTCACCAGCA |
| AGO942 | Cdc11a S314D R | CAAAGCTCATGAACAATGGGGATACGGAATTTATCAGCTCACCAGCA |
| AGO943 | Cdc11a S314D R | CAAAGCTCATGAACAATGGGGATACGGAATTTATCAGCTCACCAGCA |
| AGO944 | Cdc11a S314D R | CAAAGCTCATGAACAATGGGGATACGGAATTTATCAGCTCACCAGCA |
| AGO946 | Cdc3 S91A F | CATCCACCGCAAGATCGCAGGATACGTGGGGTTCGCAAACC |
| AGO947 | Cdc3 S91A R | GGTTTGCGAACCCCACGTATCCTCGGATCTGGGATGTG |
| AGO949 | Cdc3 S91D F | CCACCGCAAGATCGACGGATACGTGGGGTTCGCAAACC |
| AGO950 | Cdc3 S91D R | GGTTTGCGAACCCCACGTATCCTCGGATCTGGGATGTG |
| AGO954 | Cdc3 upstream seq F | ACATCCACCGCAAGATCGCAGGATACGTGGGGTTCGCAAACC |
| AGO966 | Cdc3 upstream seq F | ACATCCACCGCAAGATCGCAGGATACGTGGGGTTCGCAAACC |
| AGO982 | Cdc12 S323A F | CTTCCTTCTGCTGTCGAGTACGTGGGGTTCGCAAACC |
| AGO983 | Cdc12 S323A R | CTTCCTTCTGCTGTCGAGTACGTGGGGTTCGCAAACC |
| AGO1065 | Cdc11a ACC F | CTCGCGCAGCAAGGGAAGACGCTAGCTCACAATCCAAAATTTAAGGAGG |
| AGO1066 | Cdc11a ACC R | CTCGCGCAGCAAGGGAAGACGCTAGCTCACAATCCAAAATTTAAGGAGG |
| AGO1067 | Cdc12 ACC F | CTCGCGCAGCAAGGGAAGACGCTAGCTCACAATCCAAAATTTAAGGAGG |
| AGO1068 | Cdc12 ACC R | CTCGCGCAGCAAGGGAAGACGCTAGCTCACAATCCAAAATTTAAGGAGG |
| AGO1146 | Cdc12 VS | CTTCCTTCTGCTGTCGAGTACGTGGGGTTCGCAAACC |
| AGO1167 | Shs1 seq F | GTCAACCAATCGGATATCACC |

AGO471, AGO472, AGO521, and AGO539. The gel purified 4,504-bp product of AGB214 digested with BpiI and NheI was transformed into \( \Delta \Delta \Delta t \) to obtain strain AG384.1, which was verified by PCR using the oligonucleotides AGO98, AGO203, AGO471/AGO350, and AGO203/AGO350.

(iii) Cdc12 point mutation strain construction. AGB260 was made by cotransforming yeast with GFP-GEN3 amplified from AGB141 by using the primer pair AGO218/AGO197 with AGB123. The plasmid was verified with XmaI, followed by sequencing with AGO136, AGO472, AGO520, and AGO539. The gel-purified 4,274-bp product of AGB260 digested with XhoI was transformed into \( \Delta \Delta \Delta t \) to obtain strain AG436.1,
which was verified by PCR using the oligonucleotides AGO119, AGO202, AGO209, and AGO407. AGB361 was made by using the primer pair AGO924/AGO925 on AGB260, digest verified using Apal, and sequence verified using AGO955. AGB361 was digested with BciVI and PacI and transformed into ΔΔΔ to obtain AG671,1, which was verified by PCR with primer pairs AGO955/AGO101 (followed by digestion with Apal to verify point mutation), AGO203/AGO5, AGO471/AGO119, and AGO209/AGO119. AGB385 was obtained by using the primer pairs AGO982/AGO983 on AGB260, digest verified using Nhel, and sequence verified using AGO5 and AGO954. AGB385 was digested using Apal and BciVI and transformed into ΔΔΔ to obtain AG672, which was verified using primer pairs AGO203/AGO5, AGO471/AGO119, and AGO209/AGO119. AGB470 was obtained using the primer pairs AGO928/929 on AGB260, digest verified using ApaI, and sequence verified using AGO5 and AGO954. AGB470 was transformed into ΔΔΔ to obtain strain AG674. AGB371 was obtained using the primer pairs AGO926/AGO927, digest verified using SpeI, and sequence verified by using AGO5 and AGO955.

AG537.1 was obtained by transforming AGB378 into ΔΔΔ. AG652.1 was made by transforming ΔΔΔ with a gel-purified 2,901-bp product amplified off BglII/PciI-digested AGH05 template DNA by using the primer pair AGO1067/AGO1068. Heterokaryons were verified by using the primers AGO136, AGO405, AGO471, and AGO1146. AGH656.1 was obtained by transforming ΔΔΔ with a gel-purified 2,901-bp product amplified off BglII/PciI-digested AGH05 template DNA by using the primer pair AGO1065/AGO1066. Heterokaryons were verified by using the primers AGO130, AGO350, AGO405, and AGO407. Strain construction is described previously (40).

Microscope setup and image processing. A Zeiss Axiophoto-M1 upright light microscope (Carl Zeiss, Jena, Germany) equipped with a Plan-Apochromat ×63/1.4NA objective lens was used for wide-field fluorescence microscopy and a Plan-Neofluar ×100/1.3 NA objective lens was used for phase-contrast imaging of spores. The green fluorescent protein (GFP) signal was visualized with a Zeiss 38HE filter set and Alexa Fluor 568 signal was visualized with Chroma filter set 41002B. An Exfo X-Cite 120 lamp was used as the fluorescent light source. Images were acquired on an Orca-AG charge-coupled device camera (C4742-80-12AG; Hamamatsu, Bridgewater, NJ) driven by Volocity 5 (Perkin-Elmer, Waltham, MA). Live and immunofluorescence images were captured using a Hamamatsu ImagEM C9100-13 EM-CCD camera. GFP was imaged using 30% laser power (491 nm laser) exposed for 250 ms/slice. Seventeen-slice z-stacks (0.5-μm steps) through the entire hypha were imaged at 30-s intervals.

Live Ashbya cells were imaged on 2% agarose gel pads made with ×2 low-fluorescence minimal media, covered with a glass coverslip, and sealed with VALAP. All images presented here are maximum projections of three-dimensional volumes.

Fluorescence intensity and FRAP analysis. Fluorescence intensity measurements were taken in Velocitoy 4 and FRAP (fluorescence recovery after photobleaching) measurements were taken in Velocitoy 5. Mean fluorescence intensity measurements for septin rings and septin clouds at hyphal tips were measured using a region of interest of the same area for each structure type. Cytosolic background was measured by subtracting an average of five background mean fluorescence measurements in the same cell as the measured ring from the mean fluorescence intensity of the ring.

To measure the intensity of a given ring over the course of a FRAP movie, a region of interest was drawn around the entire ring, and the mean fluorescence value per pixel of the selected area was recorded. An adjacent region of cytosol was measured, and this mean fluorescence value was subtracted from the mean fluorescence of the ring to yield the mean fluorescence of the ring minus the background. Photobleaching was corrected for using the mean fluorescence intensity of a ring of the same type in the same field. The regions of interest around the rings were shifted if necessary to compensate for cell drift during the movie. The mobile fractions for each ring were calculated by dividing the percent recovery by the percent bleach, and mobile fractions were averaged to obtain the average mobile fractions and standard deviations listed in Table 4. The t1/2 for each ring was calculated using the formula $t_{1/2} = \frac{\log\left(0.5\right)}{\log\left(1/\text{mobile fraction}\right)}$, where $t_{\text{max}}$ is the time point where recovery plateaus. The average $t_{1/2}$ and standard deviation values are listed in Table 4. The statistical significance of the mobile fraction and $t_{1/2}$ measurements were determined by two-tailed t tests.

Immunofluorescence. Immunofluorescence was performed as described previously (40). Rabbit anti-ScCdc11p (Santa Cruz Biotechnology, Santa Cruz, CA) or Rabbit anti-GFP (Invitrogen) was diluted in 1× phosphate-buffered saline plus 1 mg of bovine serum albumin/ml and used at a 1/100 dilution. Cells were incubated with primary antibody overnight at 4°C. Primary antibody was detected and visualized using Alexa Fluor 488-labeled anti-rabbit antibody (Invitrogen) at a 1/200 dilution.

Protein extraction and purification. Cells were grown in Ashbya full media (AFM) for 16 h and harvested by vacuum filtration. For lysis, 500 mg of cells was resuspended in 1 ml of ice-cold lysis buffer (50 mM HEPES-KOH [pH 7.6], 1 M KCl, 1 mM MgCl$_2$, 1 mM EGTA, 5% glycerol, 0.45% Tween 20, 2× protease inhibitor cocktail). For lysates used to analyze the phosphorylation status of the septin complex by mass spectrometry, phosphatase inhibitors (50 mM NaF and 100 mM β-glycerophosphate) were added to the lysis buffer. Cells were lysed by bead beating with 0.3-mm zirconia/silica beads in a MiniBeadbeater-8 (BioSpec Products, Bartlesville, OK) at top speed for five intervals of 90 s with 30-s rest. Cell lysates were clarified by centrifugation for 10 min at 16,100 × g. Protein concentrations were determined by using the Bradford assay.

Septin complexes to be analyzed by mass spectrometry were purified from AG495.1 (Cdc11-TAP) lysates using rabbit IgG (Milipore, Billerica,

| Strain | n | Mobile fraction (%) | t_{1/2} (s) |
|--------|---|---------------------|-------------|
| Shs1-GFP (AG442.1) | 7 | 25.3 ± 4.9 | 54.7 ± 8.5 |
| Shs1-GFP 9D (AG537.1) | 7 | 37.8 ± 11.1* | 38.8 ± 13.1* |

*P < 0.05 (two-tailed t test).
RESULTS

**Ashbya septins are phosphorylated.** We hypothesized that septins in *Ashbya* are phosphorylated, based on previous data revealing that the kinases Elm1p and Gin4p lead to a loss of septin structure formation (19). To identify phosphorylation sites, we first immunoprecipitated TAP-tagged Cdc11p in order to pull out the entire septin complex and assessed the coprecipitating proteins by SDS-PAGE (Fig. 1A). The five constituents of the septin complex resolve as four bands, due to the comigration of high-molecular-weight Shs1 with Cdc11-TAP and low-molecular-weight Shs1 with Cdc3 (Fig. 1A). All four bands were excised from the gel and submitted for mass spectrometry analysis. Although we analyzed the proteins for several posttranslational modifications—phosphorylation, acetylation, and SUMOylation—the only modification identified on any of the septins was phosphorylation. We previously reported Shs1p is multiphosphorylated at nine sites (25) and show here that three of the remaining four septins are also phosphorylated (Fig. 1B and Table 5). The majority of these sites are novel, that is, they are not conserved on the primary sequence level with phosphorylation sites previously identified in the septins of *S. cerevisiae* and *C. albicans* (see Fig. S1 in the supplemental material) (23). Many of the *Ashbya* septin phosphorylation sites are found in proximity to septin polybasic and coiled-coil domains, which are thought to be important in septin interactions with membranes and with one another. We set out to examine whether phosphorylation of these sites influences the assembly of septins into higher-order structures.

**Mutation of septin phosphorylation sites affects cell and septin morphology.** In order to assess the function of septin phosphorylation, we made phosphomimetic (site changed to aspartic acid or glutamic acid) and nonphosphorylatable (site changed to alanine) point mutations to the identified phosphorylation sites of GFP-tagged septins. These mutants were tested for their effects on cell and septin morphology. Some of the mutations caused no obvious cell or septin phenotypes (Table 6). Representative images of normal-looking septin structures at the interregion, branch sites, and at hyphal tips are shown for nonphosphorylatable Cdc12 S5A S8A and Cdc12 S323A mutants in Fig. 2A. The normal cell growth and septin structure formation in these mutants indicate that a subset of septin phosphorylation sites are dispensable for normal cell growth and assembly of higher-order septin structures. Other alterations to phosphorylation sites did lead to obvious cell and septin phenotypes. The nonphosphorylatable mutant Cdc11 S314A and the phosphomimetic mutant Cdc3 S91D led to production of abnormally shaped ascospores. Wild-type ascospores are straight and needle-like, however both the Cdc11 S314A and Cdc3 S91D mutations resulted in spores that were curved and crescent-shaped (Fig. 2B). Despite their unusual shape, the crescent-shaped ascospores were not detectably defective in germination. This alteration in spore shape also did not lead to any significant change in cell growth, based on radial growth of cells on agar plates over the course of 10 days (Fig. 2C), indicating that spore shape does not impact mycelial growth rates.

Intriguingly, when Cdc11 S314A and Cdc3 S91D mutant cells were assessed for their ability to form higher-order septin structures, we found that the mutant septins were not localizing to any

### TABLE 5 Septin phosphorylation sites identified by mass spectrometry

| Septin | Phosphorylation site(s) |
|--------|-------------------------|
| Cdc11  | S314                    |
| Cdc12  | S5, S8, S323            |
| Cdc3   | S91, S496               |
| Cdc10  | No sites identified     |
| Shs1   | S359, S362, S380, T394, S396, S408, S431, T555, S558 |

**Western blotting.** Western blotting was performed by standard methods as previously described (25). Membranes were probed with rabbit polyclonal anti-GFP antibody (ab290; Abcam, Cambridge, MA) and rat monoclonal anti-α-tubulin antibody (AbD Serotec, Raleigh, NC), both at a 1:1,000 dilution. Densitometry analysis to determine relative abundance of proteins was done in ImageJ (National Institutes of Health, Bethesda, MD) using the gel analysis tools.
higher-order structure (Fig. 2D). Remarkably, when the opposite mutant (i.e., Cdc11 S314D and Cdc3 S91A) for each of these was tested, we found that neither had any impact on cell or septin morphology (Table 6). This indicates that specific changes in phosphorylation status of individual septin phosphorylation sites can have severe functional consequences.

**Phosphomimetic mutant Cdc12 SSE S8E is dominant lethal.** Although the majority of mutants we tested did not affect cell health, some mutants had extremely deleterious effects on cell viability. The phosphomimetic mutant Cdc12 SSE S8E was lethal to *Ashbya* cells even when introduced on a plasmid in the presence of the wild-type genomic Cdc12 (Table 6). Attempts to heterologously express this mutant on a plasmid in the budding yeast *Saccharomyces cerevisiae* also resulted in a lethal phenotype. Due to its dominant lethal effects, we were unable to assess the precise role of Cdc12 SSE S8E in septin organization. The single phosphomimetic mutant Cdc12 SSD expressed on a plasmid in *Ashbya* displayed normal cell and septin morphology (Table 6), indicating that the deleterious effects of Cdc12 SSE S8E are due either to the double mutant or S8E alone. Thus, we have identified Cdc12 SSE S8E, as a novel dominant-negative septin allele.

**Phosphomimetic Shs1p has increased dynamics at IR rings.** The Shs1p coiled-coil domain leads to a greatly increased mobile fraction of Shs1 at IR rings without affecting the dynamics of other septins (25). We tested this possibility by performing FRAP on Shs1p-GFP or Shs1p-9D-GFP at the IR rings (n = 7 rings each). These FRAP experiments revealed that Shs1p-GFP has a significantly increased mobile fraction and statistically significantly decreased t_{1/2} at the IR rings compared to wild-type Shs1p-GFP (Fig. 3C and Table 4, P < 0.05 for both mobile fraction and t_{1/2}). Thus, although the steady-state fluorescence intensities are similar for the mutant compared to the wild type, Shs1p-9D-GFP is more dynamic, i.e., a greater percentage of Shs1p-9D-GFP can recover more quickly than the wild type. It is unclear whether this change in dynamics could affect cell viability in the absence of any wild-type Shs1p, or if some other unidentified property of the phosphomimetic mutant is the culprit for the lethal phenotype.

**Shs1p requires its specific coiled coil to incorporate into septin rings.** Increased dynamics in mutant Shs1p is not without precedent. We demonstrated in previous work that deletion of the Shs1p coiled-coil domain leads to a greatly increased mobile fraction of Shs1 at IR rings without affecting the dynamics of other septins (25). The Shs1p coiled-coil deletion mutant also exhibits greatly expanded branch rings that extend beyond the boundaries of normal branch rings. We sought to determine whether these dynamics- and ring size-limiting properties were specific to the Shs1p coiled coil. To test this, we truncated Shs1p after residue 358, which phenocopies the deletion of the Shs1p coiled-coil domain alone for all features tested (25), replaced the deleted region with the predicted coiled-coil domain of Cdc11 (residues 371 to 411, Fig. 4A), and observed the effects on septin structure. Strikingly, the chimeric Shs1p (residues 1 to 358) with the Cdc11 coiled-coil domain (Shs1p-Cdc11 CC-GFP) does not localize to any septin structures but is instead distributed throughout the cytoplasm of the cell (Fig. 4B). We hypothesized that the loss of Shs1p at the rings would lead to an inability of septin rings to form at all and were surprised to find that when Cdc11 is visualized via immunofluorescence in the Shs1p-Cdc11 CC-GFP cells, we see it localizing to expanded branch ring structures that phenocopy those found in the Shs1p deleted deletion cells (Fig. 4C). To exclude the possibility that the Shs1-Cdc11 CC-GFP construct is defective in GFP fluorescence and thus not able to be visualized in the septin assemblies, we confirmed its cytoplasmic localization using anti-GFP immunofluorescence (Fig. 4D). The expression levels of Shs1p-Cdc11 CC-GFP were comparable to the wild type, as assessed by Western blotting (Fig. 4E), so the localization defect is not simply a result of misexpression of the chimeric protein. These data indicate that septin rings in *Ashbya* can form in the absence of Shs1p in the higher-order structure and that the introduction of the Cdc11 coiled coil to Shs1p inhibits the incorporation of Shs1p into higher-order septin structures. Furthermore, the expanded

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**TABLE 6 Summary of phosphorylation site mutation phenotypes**

| Phosphorylation site mutation | Ashbya strain no. | Phenotype |
|------------------------------|-----------------|-----------|
| Cdc11 S314A                  | AG669.2         | Sickle-shaped spores |
| Cdc11 S314D*                | AG533.1         | None observed |
| Cdc12 SSA S8A                | AG671.1         | None observed |
| Cdc12 SSE S8E*              | —               | Dominant lethal |
| Cdc12 SSD*                  | AG674           | None observed |
| Cdc12 S323A                  | AG672           | None observed |
| Cdc3 S91A*                  | AG529.1         | None observed |
| Cdc3 S91D                   | AG668.1         | Sickle-shaped spores |
| Cdc3 S496D*                 | AG531.1         | None observed |
| Shs1 9A                      | AG643.1         | No immediately obvious cell, spore, or septin ring morphological defects. |
| Shs1 9D*                     | AG537.1         | Increased dynamics at IR rings |

*All mutants were scored for cell, spore, and septin ring morphological defects. Mutations indicated by asterisks (*) were introduced on a plasmid, while the remainder were integrated at the endogenous locus. All were tagged with GFP to observe localization to septin structures.*

*a Mesereau et al. (25).*

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**Interpretation:**

1. The Shs1p coiled-coil domain is required for proper septin ring formation.
2. The phosphomimetic mutant Cdc12 SSE S8E is dominant lethal.
3. Shs1p-9D-GFP has increased dynamics at IR rings compared to wild-type Shs1p-GFP.
4. The chimeric Shs1p-Cdc11 CC-GFP does not localize to septin structures but is distributed throughout the cytoplasm.
5. The loss of Shs1p at the rings leads to the inability of septin rings to form.

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**Legend:**

- **Shs1p** is a septin protein.
- **Cdc11** and **Cdc3** are septin-interacting proteins.
- **IR rings** are interregion rings that form during cell division.
- **FRAP** is a fluorescence recovery after photobleaching technique used to study protein dynamics.
- **GFP** is a green fluorescent protein used for marking proteins.

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**Figure Legends:**

- **Fig. 2D** shows the higher-order structure of septin rings.
- **Fig. 3C** illustrates the increased mobile fraction of Shs1p-9D-GFP at IR rings.
- **Fig. 4A** displays the coiled-coil domain of Cdc11.
- **Fig. 4B** shows the distribution of Shs1p-Cdc11 CC-GFP in the cytoplasm.
- **Fig. 4C** demonstrates the localization of Shs1p-Cdc11 CC-GFP to expanded branch ring structures.
- **Fig. 4D** confirms the cytoplasmic localization of Shs1p-Cdc11 CC-GFP.
- **Fig. 4E** assesses the expression levels of Shs1p-Cdc11 CC-GFP.

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**Conclusion:**

The phosphorylation of septins has been shown to be important in cell division and septin organization. The study demonstrates that the phosphomimetic mutant Cdc12 SSE S8E is dominant lethal, and the phosphomimetic mutant Shs1p has increased dynamics at IR rings, indicating the importance of septin phosphorylation in septin dynamics and cell division.
Septin structures observed in the mutant strain suggest that any loss of the Shs1p coiled coil, even if replaced with another septin’s coiled coil, leads to aberrantly large septin rings. We previously showed that loss of the Shs1 C terminus leads to more frequent lateral branches (25). In the Shs1-Cdc11 CC-GFP chimera, despite the loss of Shs1p at the ring, the distance between lateral branches is not significantly altered in the chimeric mutant compared to the wild type (19.8 ± 11.8 μm for wild type versus 19.1 ± 11.2 μm for the chimera, n > 95, P > 0.6 by two-tailed t test). These results suggest that the normal position and frequency of lateral branches is not dependent on the localization of Shs1p to the ring. Finally, this also suggests that the increased branching frequency previously observed (25) does depend on the presence of the mutant Shs1 at the ring and is not simply the consequence of expanded ring zones.

Cdc11p and Cdc12p coiled coils are required for normal septin ring morphology. Based on the remarkable septin organization phenotypes we observed upon swapping the Shs1p coiled coil for that of Cdc11p, we hypothesized that the coiled-coil domains of other septins also impact septin ring morphology. We tested this by separately deleting the coiled coils of Cdc11p (residues 371 to 411) and Cdc12p (residues 352 to 390) (Fig. 5A). The loss of either coiled-coil domain led to major defects in septin higher-order structures. Cdc11p-GFP was observed to localize diffusely to the cytoplasm (Fig. 5B); however, the GFP signal is very dim, and when Cdc11p-GFP expression levels were assessed...
by Western blotting, they appear to be much lower than wild-type Cdc11-GFP levels (Fig. 5C). Loss of the Cdc12p coiled coil leads to several cellular effects. Spore germination is greatly reduced in these cells; thus, obtaining a quantity of fully grown cells for this strain is challenging. Those that do grow demonstrate diverse septin phenotypes. In the majority of Cdc12\textsuperscript{+}/H9004 cells (78%, \(n = 27\) cells total), Cdc12\textsuperscript{+}/H9004-GFP localizes diffusely to the cytoplasm (Fig. 5B, Cdc12\textsuperscript{+}/H9004-GFP left-hand panel); however, in smaller percentages of cells, the protein localizes to cytoplasmic circles and crescents (15%, Fig. 5B, Cdc12\textsuperscript{+}/H9004-GFP, middle panel) or septin rings (7%, Fig. 5B, Cdc12\textsuperscript{+}/H9004-GFP, right-hand panel). Thus, in 93% of the cells observed, Cdc12\textsuperscript{+}/H9004-GFP is unable to localize to septin rings. These data suggest that septin coiled-coil domains are able to perform different roles: the Shs1p coiled coil limits septin ring size, although the Cdc12p, and perhaps Cdc11p, coiled coils are required for septin higher-order structures to form at all.

**DISCUSSION**

We demonstrate in this study the novel and various effects of septin phosphorylation and coiled-coil domain mutations in *Ashbya*. Alteration to the status of septin phosphorylation sites leads to diverse phenotypes. Mutation of some phosphorylation sites have no apparent impact on cell or septin morphology, while others led to defects in cell and septin structure shape. The phosphomimetic mutants Cdc12 S5E S8E and Shs1 9D are so deleterious as to cause cell lethality. Similarly, we show here that septin coiled-coil domains also perform diverse functions. The Shs1p coiled-coil domain acts to limit septin ring size, while the Cdc12p and Cdc11p coiled-coil domains are required for the formation of septin rings. The variety of effects of septin phosphorylation sites and coiled-coil domains suggests that septins can be regulated in many different ways to achieve the functions they perform in the cell. Importantly, phosphorylation does not provide a generic
switch but rather can promote or limit organization depending on the subunit context.

The variability in the impact of Ashbya septin phosphorylation is intriguing. Studies focusing on the roles of septin phosphorylation in other organisms such as the budding yeast *Saccharomyces cerevisiae* and the fungus *Candida albicans* have revealed that prevention of septin phosphorylation affects cell and septin morphology (22, 28). Mutations made to *S. cerevisiae* Shs1p phosphorylation sites result in mutant cells that are larger and have aberrant cell morphology (22). *C. albicans* Cdc11p phosphorylation is required for cells to transition to hyphal growth but did not impact septin localization (28). Loss of the Gin4p kinase, which has been shown to direct phosphorylate Shs1p in *S. cerevisiae* and *C. albicans*, impairs septin ring formation and dynamics (24, 27, 28, 43, 44). Although we have yet to identify whether any septins are the target of the Gin4p and Elm1p kinases in Ashbya, deletion of either of these kinases leads to an inability of IR rings to assemble (19).

We show here that mutations to individual sites in the septins Cdc3p and Cdc11p causes Ashbya to produce misshapen ascospores and prevents either of the mutant proteins from localizing to septin structures. It is interesting that the mutation to Cdc3 is phosphomimetic (S91D), whereas the Cdc11 mutation is non-phosphorylatable (S314A). This suggests that, depending upon the context, inhibition of phosphorylation and dephosphorylation...
tion of the septins can have similar effects. Remarkably, if the opposite mutation is made to these phosphorylation sites (i.e., Cdc3 S91A or Cdc11 S314D), we see no apparent cell or septin phenotypes. While it is unclear why these particular sites may be important, we speculate that the nearness of the Cdc3 S91 site to this septin’s polybasic region (Fig. 1B) may enable phosphorylation to regulate the exposure of this region and help direct its interaction with the membrane. Although S314 is not particularly near to any of Cdc11’s conserved domains, it was the only site we identified for Cdc11, and its phosphorylation clearly plays some important functional role in cell morphology and septin structure formation. Future work with these mutants will be required to parse out the mechanism of these sites’ functionalities.

In addition to the cell and septin phenotypes we observed with the aforementioned Cdc11 and Cdc3 mutants, we identified some septin phosphorylation sites to be even more crucial to cell health. The phosphomimetic double point mutant Cdc12 S5E S8E was dominant lethal and phosphomimetic mutations to all nine Shs1p phosphorylation sites led to cell lethality if expressed as the only copy of \textit{SHS1} in the cell. The severity of these phosphorylation mutant phenotypes is unprecedented in the septin field, and we aim to uncover a mechanism for the lethality in the future.

The effects of Cdc12 S5E S8E may also be explained by the nearness of these sites to the polybasic domain of Cdc12. It is possible that the negative charge carried by both of these sites in the double phosphomimetic mutant interferes with the functions of the polybasic domain and alters the interaction of septins with the membrane in such a way that it causes the membrane to become unstable. If the membrane cannot be remodeled properly, the cell cannot grow. Another possibility for the dominant lethal effect is that the double point mutant interferes with the septins’ ability to scaffold a necessary signaling protein to the proper place during cell development. Because \textit{Ashbya} Cdc12 S5E S8E has the same lethal effect when introduced to \textit{S. cerevisiae}, the mislocalized protein would likely be a conserved factor. When only one of the sites is made phosphomimetic (Cdc12 S8D), the dominant lethal effect observed for the double point mutation is not phenotype by the single (Table 6). This suggests that both sites must be negatively charged for lethality to occur, although it is also possible that Cdc12 S8D alone would be sufficient for the lethal phenotype. Remarkably, the opposite double mutant, Cdc12 S5A S8A, has no apparent cell or septin phenotypes, reinforcing the idea that specific charges on septin phosphorylation sites can make a dramatic difference in the functionality of the protein.
Another phosphomimetic mutant, Shs1 9D, is also lethal if it is expressed as the only copy of SHS1 in the cell. Although this is not as extreme as the dominant lethality observed for Cdc12 S5E S8E, it is still quite astonishing. Because it is recessive lethal, we were able to observe the effects of the mutant protein expressed on a plasmid in cells where a wild-type copy of SHS1 is present. Cells were healthy and septin rings appeared normal, however, we observed that the Shs1 9D-GFP at IR rings was more dynamic than wildtype. Changes in septin dynamics correlated with the presence or absence of kinases or phosphatases have been previously reported in other systems (24, 31, 41); however, none of these studies directly assessed the affects of septin phosphorylation status on dynamics at the septin ring. We previously showed that the non-phosphorylatable mutant Shs1 9A-GFP does not have significantly altered dynamics at either IR or branch rings in Ashbya (25), so again we found that specific changes to septin phosphorylation sites direct septin functionality. It is unlikely that the increased dynamics of Shs1 9D alone are responsible for the mutant’s lethal effects; however, it is possible that either decreased stability of septin structures as a result of increased turnover or changes in septin-septin or septin-membrane interactions caused by the mutation contribute to the lethality.

It is curious that the phosphomimetic SHS1 allele leads to cell lethality, while the SHS1-null mutant is viable, although the null mutant is sick and does not assemble septin rings (19). Why would the complete absence of Shs1 be less deleterious than forcing nine sites on the protein to be phosphomimetic? One possibility is that the loss of Shs1p and septin rings leads to a decrease in proteins that are normally scaffolded by septins to their proper location. Perhaps some of the proteins can still arrive where they need to be stochastically, so the cells can survive, but they are not as healthy as they would be with an intact septin cytoskeleton. On the other hand, phosphomimetic Shs1p may be interacting too tightly with factors that wild-type Shs1p only interacts with transiently, leading to an accumulation of mislocalized protein, which could have a toxic effect. It will be interesting use the Shs1 9D protein expressed off a plasmid in Ashbya to assess whether it can stably bind to proteins that its wild-type counterpart does not.

We were surprised to discover that swapping the coiled-coil domain of Shs1p with that of Cdc11p resulted in the inability of the Shs1 chimera to incorporate into septin structures. Given that Shs1p with its coiled coil completely deleted is able to localize to the expanded ring structures characteristic of that mutant (25), it was puzzling that the Shs1-Cdc11 CC hybrid localized only to the cytoplasm. Intriguingly, Cdc11 readily localizes to septin structures in cells expressing the Shs1 chimeric protein, and these structures phenoxy the expanded branch rings and ectopic fibers witnessed in the Shs1p coiled-coil deletion mutants. A possible explanation for the exclusion of the chimeric protein at septin structures is that the Cdc11 coiled coil may be preventing lateral associations normally made by Shs1 to form rings (45). Evidently, the absence of the Shs1p coiled coil, even if replaced with another coiled coil, is sufficient to expand the septin branch structures beyond their normal boundaries. To put it another way, the Shs1p coiled coil specifically is required to limit septin ring size. It has been shown in S. cerevisiae that other septins localize to the ring at the mother-bud neck in shs1Δ cells, although the ring organization is abnormal (45, 46). It is possible that some similar mechanism is at work in Ashbya. The size or shape of the Cdc11p coiled coil appended to Shs1p may be prohibitive for the chimeric protein to associate with other septins or may prevent complexes that have the chimera from further assembling into longer polymers. In this case, septin complexes comprised of the other four septins would be the constituents of the septin rings, and the absence of the Shs1p coiled coil leads to the expanded septin structures.

In contrast to large assemblies forming, loss of the coiled coils of Cdc11p or Cdc12p eliminates nearly all higher-order structures. These phenotypes are very different from the Shs1p coiled-coil deletion and are even somewhat different from one another. Clearly, the Cdc11p and Cdc12p coiled coils do not act to limit septin ring size like that of Shs1p. The loss of the Cdc11p coiled coil results in a complete lack of septin structures. While Cdc12p coiled-coil deletion cells can infrequently assemble rings (7%), the remainder of these mutant cells display only cytoplasmic localization of Cdc12ΔCC-GFP, either diffusely or in small circles and crescents. Both of these mutants’ phenotypes are consistent with the theory that the septin coiled coils are required for septin-septin interactions. Studies in yeast have revealed that coiled coils of Cdc11p, Cdc12p, and Cdc3p facilitate septin-septin interactions, and in the absence of these coiled coils, yeast cells have aberrant, elongated morphology and often fail to form normal septin rings (47, 48). Furthermore, one of the most widely used conditional mutants used in S. cerevisiae, cdc12-6, contains a C-terminal truncation (49). It will be interesting to dissect the differences between the septin coiled coils and how precisely they perform their apparently different cellular functions in future studies.

Overall, the data we present here delineate novel roles for septin phosphorylation and coiled-coil domains in regulating septin structure formation, as well as cell morphology and other functions. The specific mechanisms underlying the mutant phenotypes have yet to be described. It is possible that phosphorylation or lack thereof is important for masking or revealing certain parts of a given septin protein, allowing the septin to interact with another protein or with the membrane. Similarly, coiled coils may be influencing septin-septin or septin-membrane interaction. This study provides a first step toward understanding diverse effects of individual septin modifications and conserved domains and how these components may influence septin structure and function.

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