The Transcription Factor SomA Synchronously Regulates Biofilm Formation and Cell Wall Homeostasis in *Aspergillus fumigatus*

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**ABSTRACT** Polysaccharides are key components of both the fungal cell wall and biofilm matrix. Despite having distinct assembly and regulation pathways, matrix exopolysaccharide and cell wall polysaccharides share common substrates and intermediates in their biosynthetic pathways. It is not clear, however, if the biosynthetic pathways governing the production of these polysaccharides are cooperatively regulated. Here, we demonstrate that cell wall stress promotes production of the exopolysaccharide galactosaminogalactan (GAG)-dependent biofilm formation in the major fungal pathogen of humans *Aspergillus fumigatus* and that the transcription factor SomA plays a crucial role in mediating this process. A core set of SomA target genes were identified by transcriptome sequencing and chromatin immunoprecipitation coupled to sequencing (ChIP-Seq). We identified a novel SomA-binding site in the promoter regions of GAG biosynthetic genes *agd3* and *ega3*, as well as its regulators *medA* and *stuA*. Strikingly, this SomA-binding site was also found in the upstream regions of genes encoding the cell wall stress sensors, chitin synthases, and β-1,3-glucan synthase. Thus, SomA plays a direct regulation of both GAG and cell wall polysaccharide biosynthesis. Consistent with these findings, SomA is required for the maintenance of normal cell wall architecture and compositions in addition to its function in biofilm development. Moreover, SomA was found to globally regulate glucose uptake and utilization, as well as amino sugar and nucleotide sugar metabolism, which provides precursors for polysaccharide synthesis. Collectively, our work provides insight into fungal adaptive mechanisms in response to cell wall stress where biofilm formation and cell wall homeostasis were synchronously regulated.

**IMPORTANCE** The cell wall is essential for fungal viability and is absent from human hosts; thus, drugs disrupting cell wall biosynthesis have gained more attention. Caspofungin is a member of a new class of clinically approved echinocandin drugs to treat invasive aspergillosis by blocking β-1,3-glucan synthase, thus damaging the fungal cell wall. Here, we demonstrate that caspofungin and other cell wall stressors can induce galactosaminogalactan (GAG)-dependent biofilm formation in the major fungal pathogen *Aspergillus fumigatus*. We further identified SomA as a master transcription factor playing a dual role in both biofilm formation and cell wall homeostasis. SomA plays this dual role by direct binding to a conserved motif upstream of GAG biosynthetic genes and genes involved in cell wall stress sensors, chitin synthases, and β-1,3-glucan synthase. Collectively, these findings reveal a transcriptional control pathway that integrates biofilm formation and cell wall homeostasis and suggest SomA as an attractive target for antifungal drug development.
Biofilms are organized communities of surface-associated microorganisms embedded in a polymeric extracellular matrix. They are common microbial growth forms in nature and during human infection (1, 2). Emerging evidence suggests that pathogenic fungi produce biofilms during infection, where they play a crucial role in mediating adherence to both host tissues and biomedical devices and provide protection from host immune defenses and antifungal therapy (3).

*Aspergillus fumigatus* is a common opportunistic mold that causes invasive infections in immunosuppressed patients (4). One strategy used by *A. fumigatus* to establish and maintain infection is the production of biofilms within pulmonary tissues (5, 6). Recent studies have established a key role for exopolysaccharide galactosaminogalactan (GAG) in biofilm formation of *A. fumigatus*. GAG is a cationic linear heteropolymer composed of \( \alpha \)-1,4-linked galactose, \( N \)-acetylgalactosamine (GalNAc) and galactosamine (GalN) (7–9). GAG binds to the surface of hyphae via charge-charge interactions, resulting in a polysaccharide sheath that covers the hyphae. GAG is also secreted and is an important component of the biofilm extracellular matrix in *Aspergillus* species (10). Degrading GAG within *A. fumigatus* biofilms with the GAG-specific hydrolase Sph3 significantly enhances the activity of antifungal agents, highlighting the importance of GAG in antifungal resistance (11).

A cluster of five genes on chromosome 3 is predicted to encode the enzymes required for the synthesis of GAG (10). These genes encode a glucose 4-epimerase (*uge3*) (8, 9), a secreted polysaccharide deacetylase (*agd3*) (10, 12), a putative transmembrane glycosyltransferase (*gtb3*), and two glycoside hydrolases (*ega3* and *sph3*) (13, 14). The fungal developmental regulators MedA and StuA and the transcription factor SomA positively regulate expression of the *uge3* gene within the GAG biosynthetic cluster (8, 15), while the Lim-domain binding protein PtaB regulates expression of both *uge3* and *agd3* expression (16). SomA forms a complex with PtaB to regulate the expression of *medA* and *stua*, suggesting the SomA/PtaB complex acts upstream of *medA* and *stua* (17). However, the mechanisms underlying this regulation are not fully defined. Factors governing expression of other genes within the GAG biosynthetic cluster also remain unknown.

In addition to their role in biofilm matrix, polysaccharides are also the main components of the fungal cell wall. The cell wall of *A. fumigatus* consists of linear and branched polysaccharides, including \( \alpha \)-glucans, \( \beta \)-glucans, chitin, and galactomannans (18–20). GAG shares some common substrates and intermediates with the synthesis of these cell wall polysaccharides. The synthesis of GAG involves the nucleotide sugars UDP-galactose and UDP-GalNAc as the substrates. UDP-galactose can also be converted to UDP-galactofuranose (UDP-Galf) by UDP-galactofuranose mutase (Ugm1). UDP-Galf is a key substrate for the synthesis of the cell wall polysaccharide galactomannan (21). Deletion of *ugm1* results in increased production of GAG, suggesting a link between these two pathways (8). Moreover, UDP-GlcNAc, required for chitin synthesis, is converted to UDP-GalNAc by Uge3 for the synthesis of GAG (9). An increase in the GlcNAc content of the cell wall of the *uge3* deletion mutant was observed, suggesting that the increased availability of UDP-GlcNAc leads to increased chitin synthesis in this mutant (8). Collectively, these observations suggest a link between GAG and other cell wall polysaccharide biosynthetic pathways. However, the signaling pathways that underlie these connections remain unknown.

The cell wall stress response has been shown to link cell wall polysaccharide biosynthetic pathways in many fungi (22). Moreover, the studies in the *Candida albicans* revealed a partial link between cell wall integrity pathway and biofilm matrix production (23). We therefore hypothesized that studying the effects of cell wall stress on biofilm formation would reveal the links between this process and cell wall homeostasis. In this study, we demonstrate that cell wall stress promotes GAG-mediated biofilm
production via transcription factor SomA. Expression profiling and full genome chromatin immunoprecipitation (ChIP) revealed that SomA regulates the expression of genes encoding GAG biosynthesis and cell wall homeostasis via distinct pathways. Our work provides insight into fungal adaptive mechanisms in response to cell wall stress and sheds light on a regulatory circuit that couples biofilm formation and cell wall homeostasis.

RESULTS

Cell wall stress promotes GAG-mediated biofilm formation. To test the impact of cell wall stress on the A. fumigatus biofilm formation, wild-type (WT) hyphae were grown in the presence of cell wall stressors, including the chitin-binding agents calcofluor white (CFW) and Congo red (CR), the ionic detergent sodium dodecyl sulfate (SDS), and the β-1,3-glucan synthase inhibitor caspofungin (CAS). At high concentrations, all four agents inhibited the growth of A. fumigatus (see Fig. S1A to D in the supplemental material). However, at lower concentrations, CFW, CR, and CAS exposure resulted in an increase in biofilm formation (Fig. 1A to D). This effect was most marked following CFW exposure, with a 2-fold increase in adherent biofilm biomass when hyphae were grown in the presence of 12.8 μg/ml CFW. Cell wall stress-induced biofilm formation was further validated using quantitative analysis of biofilm biomass (Fig. 1E), scanning electron microscopy (SEM) of hyphae (Fig. 1F), and confocal microscopy using a GAG-specific fluorescein-tagged soybean agglutinin lectin (SBA-FITC) (Fig. 1G and H). The data are presented as the percentages of the mean fluorescence intensity (MFI) of A. fumigatus WT hyphae grown under the same conditions described for panel G. The data are presented as the percentages of the MFI of the WT strain grown in MM, and the standard deviations represent averages from four independent biological samples, each with five hyphal sections measured (***, P < 0.0001).
formation was observed reproducibly among *A. fumigatus* isolates, including the common laboratory strain AF293 and two clinical isolates, AFc06 and AFc08, as well as the nonpathogenic species *Aspergillus nidulans* (Fig. 1E), a species which is relatively biofilm deficient due to the low expression of *uge3* (24).

Given the key role of GAG in *A. fumigatus* biofilm formation, we hypothesized that CFW-dependent augmentation of biofilm production might reflect an increase in GAG production. Consistent with this hypothesis, scanning electron microscopy (SEM) of the hyphal surface revealed that CFW exposure resulted in a significant increase in hyphal surface decorations and intercellular matrix, findings that have been associated with GAG production (Fig. 1F). These findings were also confirmed by GAG-specific fluorescein-tagged soybean agglutinin lectin (SBA-FITC) staining; the mean fluorescence intensity (MFI) on the hyphae was significantly increased by 5-fold when exposed to CFW (Fig. 1G and H). CFW exposure failed to enhance biofilm formation by the Δ*uge3* mutant strain (see Fig. S2). Moreover, CFW exposure had no effect on biofilm formation by the pathogenic yeasts *Candida albicans* and *Cryptococcus neoformans* (Fig. 1E), which lack the GAG biosynthetic gene cluster (10). Collectively, these data indicate that the cell wall stress induces GAG-mediated biofilm formation in *Aspergillus* species.

**SomA mediates cell wall stress-induced biofilm formation.** Previous work has demonstrated that GAG biosynthesis is regulated by the transcription factor SomA, which forms a complex with the Lim-binding domain protein PtaB to regulate the expression of the developmental regulatory proteins MedA and StuA (8, 16, 17). We therefore sought to determine whether stress-induced GAG-mediated biofilm formation was dependent on elements of this regulatory pathway. Since the deletion of somA was incapable of production of conidia, a Tet-*somA* strain was constructed by replacing its promoter region with the inducible Tet-On system (17, 25), which could conditionally express the *somA* gene upon addition of doxycycline to the medium.

Consistent with previous reports, the Δ*medA*, Δ*stuA*, Δ*ptaB*, and Tet-*somA* (OFF) strains all exhibited a severe defect in the biofilm formation under normal condition (Fig. 2A and B). Exposure to 12.8 μg/ml CFW dramatically enhanced biofilm production by the Δ*stuA* and Δ*ptaB* mutants (Fig. 2A) but not by the Δ*medA* and Tet-*somA* (OFF) mutants (Fig. 2A and B). These findings suggest that cell wall stress-induced biofilm formation is dependent on SomA and MedA. Since SomA acts as upstream of MedA, we focused on SomA in further study. Consistent with these findings, SEM demonstrated significantly reduced surface decoration and intercellular matrix in the Tet-*somA* (OFF) mutant, compared to both wild-type and Tet-*somA* (ON) strains under normal conditions (Fig. 2C). Strikingly, the CFW-mediated increase in GAG production that appeared in wild-type and Tet-*somA* (ON) strains was abolished in the Tet-*somA* (OFF) mutant (Fig. 2C; see also Fig. S3).

To confirm these findings, the effects of *somA* on the expression of GAG biosynthetic genes were assessed under both normal and cell wall stress conditions. Under normal conditions, the expression of three of five genes on the GAG biosynthetic cluster (*uge3, agd3*, and *ega3*) was dependent on SomA. When exposed to the cell wall stressor CFW, four of five genes on the GAG biosynthetic cluster (*uge3, agd3, sph3*, and *ega3*) were upregulated from 3- to 6-fold (Fig. 2D) in the Tet-*somA* (ON) strain. In contrast, only a minimal increase in *ega3* and *sph3* expression was observed in response to CFW exposure in the Tet-*somA* (OFF) mutant (Fig. 2D). Taken together, these data suggest that SomA is a key regulator of GAG production under both normal and cell wall stress conditions.

**SomA globally regulates glucose uptake, utilization, and amino sugar and nucleotide sugar metabolism.** Given the importance of SomA in regulation of *A. fumigatus* biofilm formation and GAG biosynthesis, we carried out transcriptomic analysis (RNA-seq) of the Tet-*somA* strain under normal growth and cell wall stress conditions in the presence or absence of doxycycline. During growth in minimal media, 873 genes were upregulated (fold change, >2; *P* < 0.05) and 1,432 genes were downregulated in the Tet-*somA* (OFF) strain. In the presence of CFW, 921 genes were
upregulated and 1,679 genes were downregulated in the Tet-somA (OFF) strain (see Table S1 in the supplemental material). To identify potential roles of SomA-dependent genes in specific fungal processes, we subjected these genes to pathway analysis using KEGG (see Table S2). Strikingly, transcripts whose abundance was directly linked to SomA expression were most significantly enriched in genes with functions in amino sugar and nucleotide sugar metabolism under both normal growth and cell wall stress conditions (Fig. 3A and C). In contrast, transcripts that were less abundant during conditions of SomA expression included tyrosine metabolism and glycolysis/gluconeogenesis (Fig. 3B and D). Overall, these findings suggest that SomA plays a key role in the control of carbon flux and the production of precursors for polysaccharide synthesis.

To identify genes under direct transcriptional control of SomA, ChIP coupled to sequencing (ChIP-seq) was performed. The two biological ChIP-seq replicates identified 581 and 586 SomA-binding peak regions, respectively, sharing 476 common targets (see Table S3) corresponding to 426 genes (see Table S4) ($q < 0.001$, fold enrichment $> 2$). We further investigated the correlation between SomA occupancy and mRNA levels by comparing the ChIP-seq and the RNA-seq data sets. Of the 426 genes with associated SomA DNA binding, the transcripts of 182 genes (42.7%) and 161 genes (37.8%) were identified as SomA dependent by RNA-seq under normal and cell wall stress conditions, respectively (Fig. 3E and F). These results suggest that many of these SomA-dependent genes are likely indirectly regulated by SomA.

Among the 426 direct targets of SomA were two genes have potential roles in governing glucose uptake and utilization in A. fumigatus, including snf3 (AFUB_030220) and hox2 (AFUB_089570) (Fig. 3G). The orthologue of snf3 in S. cerevisiae encoding a
The major facilitator superfamily monosaccharide transporter responsible for glucose uptake (26). The orthologue of hxxk2 in S. cerevisiae encoding a putative hexokinase plays a role in glucose phosphorylation (27). Hexo/glucokinase-mediated glucose phosphorylation during the first step of glycolysis is crucial for fungal cell wall construction (28).

**FIG 3** ChIP-seq and transcript analysis identify SomA target genes. (A and B) KEGG enrichment tables of downregulated (A) and upregulated (B) genes in the Tet-somA (OFF) versus Tet-somA (ON) mutant in the absence of CFW. (C and D) KEGG enrichment of downregulated (C) and upregulated (D) genes in the Tet-somA (OFF) mutant compared to the Tet-somA (ON) strain in the presence of CFW. False discovery rate values and gene numbers are represented using a gradient of color and bubble size, respectively. (E and F) Venn diagram of intersecting genes observed being differentially expressed (fold change > 2 and P < 0.05) in RNA-seq and genes identified as directly bound by SomA in ChIP-seq analyses in the absence (E) or in the presence (F) of CFW. (G) Genome browser images depicting the relative enrichment and transcript levels of two indicated glucose uptake and utilization genes based on ChIP-seq (blue) and RNA-seq (gray). ChIP1 and ChIP2 are two independent repetitions. Scale bar, 1 kb.
The expression of *snf3* and *hxt2* were significantly decreased in the Tet-somA (OFF) strain compared to the Tet-somA (ON) strain both under normal and cell wall stress conditions (Fig. 3G). Collectively, these data suggest that SomA globally regulates glucose uptake, utilization, and amino sugar and nucleotide sugar metabolism.

SomA binds to the promoters of genes related to GAG biosynthesis. The SomA-bound DNA motifs were identified using multiple expectation maximum for motif elicitation (MEME) of the identified peaks. The SomA DNA binding motif with the highest E value (5.6E–63) and frequency (119/476) is an 11-bp “GTACTCCGTAC” binding region (Fig. 4A).

Next, SomA binding sites that were identified in proximity to the GAG biosynthetic cluster genes and their known transcriptional regulators *medA* and *stuA* were examined in greater detail. Five SomA-binding peaks were identified in proximity to genes within the GAG biosynthetic cluster (Fig. 4B). Four of the five SomA-binding peaks regions contained the identified SomA-binding motif (Fig. 4C and D). The peak intensity map shows that SomA binding was significantly enriched at the region between *agd3* (1,048 bp upstream of the translational start site) and *gtb3* (1,299 bp upstream of the translational start site) which are in opposite orientations on the chromosome (Fig. 4B).
Given that the RNA-seq studies demonstrated higher transcriptional levels of agd3 but not gtb3 in the Tet-somA (ON) strain, these data suggest that SomA directly regulates the expression of agd3. Two SomA-binding sites were found on the upstream regions of ega3 (983 and 2,506 bp upstream of translational start site, respectively) (Fig. 4B). As with agd3, expression of ega3 was positively regulated by SomA by RNA-seq, suggesting that SomA plays a direct role in regulating ega3 expression. Two SomA-binding sites were found on the open reading frame (ORF) region and 3′ untranslated region (UTR) of sph3 (Fig. 4B and C), respectively. However, the expression of sph3 was not significantly different between the Tet-somA (ON) and (OFF) strains (Fig. 4B). Surprisingly, although the expression of uge3 was dramatically reduced in the Tet-somA (OFF) strain, no SomA-binding sites were found in the promoter, the ORF region, or the UTRs of uge3 (Fig. 4B and C), suggesting that SomA indirectly regulates uge3 expression. Electrophoretic mobility shift assays (EMSAs) further confirmed the in vitro binding of SomA to GTACTCCGTAC motif-containing promoter fragments of agd3 and ega3 (Fig. 4E). Excess unlabeled DNA or mutation of the GTACTCCGTAT motif to GggtgCCGTAT in the agd3 fragment blocked the interaction of SomA with the promoter fragments (Fig. 4F), highlighting the specificity of this protein-DNA interaction.

Previous studies have demonstrated a role for StuA and MedA in the regulation of GAG biosynthetic genes (8). RNA-seq and RT-qPCR demonstrated a reduced abundance of both medA and stuA mRNA in the Tet-somA (OFF) strain (see Fig. S4A and B in the supplemental material). Consistent with these findings, multiple SomA occupancy sites were found in the upstream of medA and stuA (see Fig. S4B), suggesting direct regulation of these factors by SomA. Collectively, these findings identify a role for both direct and indirect regulation of GAG biosynthetic genes by SomA.

**SomA positively regulates cell wall-related genes.** Gene Ontology (GO) analysis of the ChIP-seq data revealed that, in addition to the genes involved in GAG synthesis, SomA occupancy was observed to be associated with genes which encode proteins involved in chitin biosynthesis (P < 0.00001), cell wall organization (P = 0.00026), and cell adhesion (P = 0.00142) (see Table S4). These genes included midA and wsc3, which encode transmembrane sensors that respond to cell wall perturbations (29); fks1, encoding a 1,3-β-glucan synthase catalytic subunit (30); and genes involved in chitin synthesis and remodeling: chsE, chsF, chs3, and chs7 (31, 32) (see Table S4). Analysis of the sequences upstream of each of these genes revealed the presence of a conserved GTACTCCGTAC motif (Fig. 5A). Consistent with these findings, RT-qPCR analysis revealed increased mRNA accumulation (FC > 2) of these genes following exposure of the wild type to 100 μg/ml wall-perturbing agent CFW for 0.5 to 2 h (Fig. 5B), suggesting that these genes play a role in the compensatory response to cell wall stress. Similar findings were observed with exposure of the Tet-somA strain (ON) to CFW (Fig. 5B). Exposure of the Tet-somA strain (OFF) to CFW revealed three patterns of gene expression (Fig. 5B). The expression of fks1 and chs7 were downregulated (FC > 2) in the Tet-somA (OFF) strain compared to the Tet-somA (ON) and wild-type strains under both normal growth and in the presence of CFW. In comparison, the expression of chsC was downregulated (FC > 2) in the Tet-somA (OFF) strain compared to the Tet-somA (ON) and wild-type strains under normal culture conditions but exhibited a similar level of upregulation in response to CFW exposure. Finally, chsA, chsB, chsE, chs3, and wsc3 exhibited levels of basal expression in the Tet-somA strain (OFF) similar to those observed in the Tet-somA (ON) and wild-type strains but reduced expression levels (FC > 2) in response to CFW exposure. These results indicate that SomA is likely part of a complex regulatory network that governs expression of cell wall-related genes under both normal growth and cell wall stress conditions.

To confirm the role of SomA in the regulation of cell wall stress responses, the susceptibilities of the Tet-somA mutant to multiple cell wall stressors were determined in the presence or absence of doxycycline. As predicted by our ChIP-seq and gene expression studies, the Tet-somA strain (OFF) was hypersensitive to the cell wall-perturbing agents CR, CFW, and CAS (Fig. 5C). Normal resistance to these agents was
restored in the Tet-somA strain (ON) (Fig. 5C). Taken together, our data suggest that SomA is a global regulator of genes encoding cell wall polysaccharide biosynthesis.

**SomA regulates cell wall architecture and composition.** To explore the effects of SomA on cell wall architecture, the hyphal cell wall was inspected by transmission electron microscopy (TEM) (Fig. 6A). Strikingly, the thickness of cell wall in the Tet-somA strain (OFF) was found to be 2-fold thicker than that in the Tet-somA (ON) strain (Fig. 6B), indicating that SomA plays a potential role in cell wall architecture.

The cell wall monosaccharide compositions in Tet-somA and wild-type strains were further analyzed by gas chromatography. Overall, the total amount of cell wall sugars in the Tet-somA strain (OFF) was dramatically decreased compared to that in the Tet-somA (ON) and in the wild type. Among these strains, the amount of cell wall glucose in the Tet-somA strain (OFF) decreased to 50% compared to that in the Tet-somA strain (ON) (Fig. 6C), suggesting a decreased glucan content in the cell wall of the Tet-somA strain (OFF). Consistent with these findings, the total β-1,3-glucan content, as assessed by aniline blue, was dramatically decreased in the Tet-somA strain (OFF) (Fig. 6D). In comparison, only a minor decrease in the GlcNAc and galactose content of the cell wall in the Tet-somA strain (OFF) was observed (Fig. 6C), and CFW staining of chitin revealed only minor differences between the wild-type, Tet-somA
and Tet-somA (ON) strains (Fig. 6E). Taken together, these results demonstrate that SomA plays a crucial role in the maintenance of cell wall composition and architecture.

**DISCUSSION**

Given their absence from human hosts and their key role in fungal viability, fungal cell wall biosynthetic enzymes are promising targets for antifungal development. The success of the echinocandins, which target the fungal cell wall by blocking β-1,3-glucan synthase, highlights the potential of this antifungal development strategy (33). Use of these agents, however, has revealed several fungal adaptive mechanisms that can reduce the activity of echinocandins. These include target mutations in the regions of (OFF), and Tet-somA (ON) strains (Fig. 6E). Taken together, these results demonstrate that SomA plays a crucial role in the maintenance of cell wall composition and architecture.

**DISCUSSION**

Given their absence from human hosts and their key role in fungal viability, fungal cell wall biosynthetic enzymes are promising targets for antifungal development. The success of the echinocandins, which target the fungal cell wall by blocking β-1,3-glucan synthase, highlights the potential of this antifungal development strategy (33). Use of these agents, however, has revealed several fungal adaptive mechanisms that can reduce the activity of echinocandins. These include target mutations in the regions of

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**FIG 6** SomA regulates cell wall architecture and compositions. (A) Representative TEM images of hyphae of WT, Tet-somA (ON), and Tet-somA (OFF) strains cultured on MM. Scale bar, 100 nm. (B) Quantification of the mean cell wall thickness of WT, Tet-somA (ON), and Tet-somA (OFF) strains as in panel A. The data are presented as the means and standard deviations of three biological samples, with 10 sections were measured for each. (***, P < 0.001). (C) Absolute monosaccharide composition of WT, Tet-somA (ON) and Tet-somA (OFF) mutants mycelial cell walls. The data are presented as the means and standard deviations of three biological replicates. (*, P < 0.05; ***, P < 0.001); (D and E) Staining of the WT, Tet-somA (ON), and Tet-somA (OFF) strains for β-1,3-glucan with aniline blue (D) and chitin with CFW (E). Scale bar, 10 μm.
target enzyme, Fks (34, 35), and activation of cell wall stress pathways leading to compensatory effects on cell wall composition, such as increased chitin content (36, 37). This phenomenon may underlie the paradoxical effect, wherein echinocandins are observed to be less effective in vitro at high concentrations (38). Here, our data indicate that caspofungin and other cell wall stressors can also induce GAG-mediated biofilm formation. These results are consistent with the observation that deletion of the β-1,3-glucan synthase-encoding gene fks1 in A. fumigatus resulted in a compensatory increase of both chitin and GAG (30). Considering the important roles of GAG in modulating the immune response during invasive infection and enhancing antifungal resistance, the long-term use of an antifungal drug which causes cell wall stress may increase the risk of biofilm overproduction and subsequent multiple drug resistance and GAG-mediated suppression host inflammatory responses to facilitate fungal survival in vivo.

In the present study, we demonstrate that SomA plays a central role in the signaling pathway that integrates biofilm formation and cell wall homeostasis (Fig. 7). Multiple lines of evidence implicate the transcription factor SomA in the regulation of both the GAG-mediated biofilm formation and cell wall homeostasis: (i) cell wall stress induced biofilm formation in a SomA-dependent manner; (ii) SomA regulated cell wall architecture and compositions under both normal and cell wall stress conditions; (iii) the downregulation of somA resulted in a severe biofilm formation defect and hypersensitivity to cell wall stressors; (iv) SomA governed the expression of GAG biosynthetic genes and cell wall-related genes under both normal and cell wall stress conditions; and (v) ChIP-seq analysis demonstrated SomA-binding sites proximal to both GAG biosynthetic genes and cell wall-related genes encoding chitin biosynthesis and glucan biosynthesis.

SomA orthologues play a conserved role in the regulation of adherence and biofilm formation in both S. cerevisiae (39) and A. fumigatus (17). In the nonpathogenic yeast S. cerevisiae, Flo8 governs aggregation and biofilm formation by direct regulation of the expression of flo11, a gene encoding cell surface-bound protein containing serine threonine-rich conserved repeats. A. fumigatus lacks Flo11 homologous adhesins, and adherence and biofilm formation of this filamentous fungus are mediated by the production of the exopolysaccharide GAG, which is absent in yeast (10). In A. fumigatus, SomA directly regulates the expression of the GAG biosynthetic genes agd3 and ega3, demonstrating that these fungi utilize a conserved regulator of adhesion and biofilm formation despite marked divergence of the downstream effectors of these pathways.
Our findings further reveal that SomA regulates GAG production and biofilm formation through distinct pathways depending on the conditions. Under normal growth, the SomA/PtaB complex controls GAG production through regulation of the expression of the GAG biosynthetic genes *uge3*, *agd3*, and *sph3*, as well as the GAG biosynthetic regulators *medA* and *stuA*. Under cell wall stress conditions, the Lim domain protein PtaB and the GAG biosynthetic regulator StuA were not involved in increased biofilm formation. SomA may form complexes or otherwise interact with new partners to regulate the expression of GAG biosynthetic genes *uge3*, *agd3*, *ega3*, and *sph3*. Targeting regulators that are crucial for stress responses may provide a powerful strategy for antifungal drug development. One strategy is to combine inhibitors of the stress response with conventional antifungals for treatment of fungal infections (40). Since SomA lacks an identifiable ortholog in humans but is required for stress responses, biofilm formation, and cell wall homeostasis in *A. fumigatus*, it may prove an attractive target for antifungal drug development.

**MATERIALS AND METHODS**

**Strains, media, and culture conditions.** All strains used in this study are listed in Table S5 in the supplemental material. *A. fumigatus* A1160 (∆ku80 pyrG) was purchased from the Fungal Genetics Stock Center; its complemented strain, A1160C (A1160 pyr4) (41), was used as the parental wild type (WT). All *Aspergillus* strains were grown on minimal medium (MM) containing 1% glucose as carbon sources, 70 mM NaNO₃ as nitrogen sources, and trace elements at 37°C, except as noted. To induce the expression of *somA* in the ΔsomA mutant, the medium was supplemented with 1 μg/ml doxycycline.

For routine culture, *Candida albicans* and *Cryptococcus neoformans* strains were incubated in liquid YPD (1% yeast extract, 2% dextrose, and 2% peptone) at 30°C. For biofilm formation assays, *C. albicans* and *C. neoformans* strains were grown in RPMI 1640 without sodium bicarbonate and phenol red at 37°C.

**Construction of genetic mutant strains.** To generate the indicated mutant strains, the fusion PCR method was used, as previously described (42). For ΔsomA mutant construction, the endogenous promoter of somA was replaced with a conditional doxycycline-inducible Tet-On promoter (17, 25). Briefly, the pyrithiamine resistance cassette and the Tet system from pCH008 were amplified with the primer pair TetT/TetR. Approximately 1 kb of the upstream and downstream flankning sequences of the *somA* promoter regions at positions −802 and +1 were amplified with the primer pairs Tet-somAP1/Tet-somAP3 and Tet-somAP4/Tet-somAP6, respectively. The three purified PCR products were then used as a template to generate the Tet-somA cassette with the primers Tet-somAP2/Tet-somAP5. The resulting fusion product was cloned into the pEASY-Blunt Zero cloning kit (TransGen Biotech) and used to transform the WT recipient strain. Transformants were grown on media supplemented with 0.1 μg/ml pyrithiamine (Sigma) and verified by diagnostic PCR using the primer pairs Tet-somASF/SR, Tet-somAP1/Tet-ptrA down, and Tet-somAP6/Tet-ptrA up.

To construct the deletion strain of *medA*, the ORF of *medA* was replaced with a selective marker *pyr4*. The selective marker *pyr4* was amplified from the pAL5 plasmid using the primer pair Pyr4F/4R. Approximately 1 kb of the upstream and downstream flankning sequences of the *medA* ORF were used as template to generate the ΔmedA knockout cassette with the primers MedAP1/P3 and MedAP4/P6, respectively. These three PCR products were used as a template to generate the ΔmedA deletion cassette with the primers MedAP2/P5. The resulting fusion product was cloned into the pEASY-Blunt Zero cloning kit (TransGen Biotech) and used to transform the recipient strain A1160. The transformants were grown on MM and verified by diagnostic PCR using primers MedASF/SR, MedAP1/Cpyr4R, and MedAP6/Cpyr4F, respectively. A similar strategy was used to construct the ΔstuA mutant.

To generate the FLAG-tagged SomA strain, the *flag* and *hph* fragment were amplified with primer pairs FlagSF/Flag-hphSR and Hph-FLAGH/hphSR, respectively. The two purified PCR products were then used as template to generate the *flag-hph* fragment using the primers FlagSF/HphSR. Approximately 1 kb of the upstream and downstream flankning sequences of the SomA ORF were amplified with the primer pairs SomAflagP1/P3 and SomAflagP4/P6, respectively. The upstream, downstream, and *flag-hph* fragments were used as templates to generate somA-FLAG cassette with the primers SomAflagP2/P5, and the resulting fusion products were sequenced verified and then used to transform the WT recipient strain. Transformants were grown on media supplemented with 200 μg/ml hygromycin B and verified by diagnostic PCR and Western blotting. All primers used in this study are listed in Table S6 in the supplemental material.

**Biofilm formation assay.** *Aspergillus* biofilm visualization and quantification were performed as previously described (15) with minor modifications. Briefly, 96-well non-tissue-culture-treated plates (Corning) were inoculated with 150 μl of MM per well containing 2×10⁵/ml conidia, followed by incubation at 37°C. After the indicated incubation period, the biofilms were washed twice with 200 μl of distilled water. Adherent biofilms were stained with 100 μl of 0.1% (wt/vol) crystal violet for 10 min at room temperature. The excess crystal violet solution was removed, and the stained biofilms were washed twice with 200 μl of distilled water. The biofilms were then destained by adding 125 μl of ethanol to each well for 10 min. The quantification of fungal biofilm by determining the absorbance of 75 μl of destain solution at 600 nm.
C. albicans and C. neoformans biofilm visualization and quantification were performed as previously described (43–45) with minor modifications. Yeast strains were precultured in YPD at 30°C overnight and then diluted to an optical density at 600 nm (OD_{600}) of 0.5 in RPMI 1640 medium. The 96-well microtiter plates were inoculated with 150 μl of fungal suspension, followed by incubation for 48 h at 37°C. Biofilm-containing wells were washed once time with 200 μl of distilled water and air dried for 10 min. The wells were stained with 100 μl of 0.2% (wt/vol) crystal violet for 10 min and then washed. Biofilms were destained with 200 μl of 100% ethanol for 10 min. The quantification of fungal biofilm by determining the absorbance of 75 μl of destain solution at 600 nm.

Scanning electron microscopy analysis of the cell surface. For hyphal surface characterization, SEM was performed as previously described (24) with minor modifications. Briefly, WT and Tet-somA (ON) strains were grown statically in MM with or without 12.8 μg/ml calcofluor white (CFW) for 24 h. To compensate for their reduced growth rate, the Tet-somA (OFF) strain was grown for 36 h in MM with or without 12.8 μg/ml CFW. Mycelia were washed with phosphate-buffered saline (PBS), fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at room temperature for 2 h, and then sequentially dehydrated in 30, 50, 70, and 80% ethanol for 15 min each. Samples were then dehydrated twice in 90% ethanol for 20 min, followed by 100% ethanol. The samples were dried at a critical point, followed by sputter coating with Au-Pd (Quorum Q150T ES plus), and then imaged with a field-emission scanning electron microscope (Zeiss Gemini SEM500).

Galactosaminogalactan characterization. To characterize the galactosaminogalactan (GAG) on the surface of mycelium, an immunofluorescence assay was performed as previously described (8). Briefly, fungi were grown in MM with or without CFW on glass coverslips. After 8 h of growth, the samples were washed twice with PBS and subsequently stained with fluorescein-conjugated soybean agglutinin (Vector Labs) in a dark chamber. The mycelia were then washed twice and imaged using a microscope (Zeiss).

RNA isolation and RT-qPCR. To analyze the relative expression levels of genes within the GAG cluster under normal growth conditions, WT and Tet-somA strains were incubated in MM for 24 h at 37°C. To analyze the relative gene expression levels of the GAG cluster and chitin synthase genes under conditions of CFW stress, WT and Tet-somA strains were incubated in MM for 22 h, and then the samples were supplemented with 100 μg/ml CFW for 0.5, 1, or 2 h. To induce the expression of somA in the Tet-somA mutant, the medium was supplemented with 1 μg/ml doxycycline. The samples were collected and subsequently frozen using liquid nitrogen. Total RNA was isolated using UNIQ-10 column total RNA purification kit (Shanghai Sangon Biotech) according to the manufacturer’s instructions. For gDNA digestion and cDNA synthesis, the HIScript q RT SuperMix for qPCR (+gDNA wiper) kit (Vazyme) were used according to manufacturer’s instructions. To analyze the relative expression of the interest genes, the resulting cDNAs were used for quantitative PCR, performed with an ABI one-step fast thermocycler (Applied Biosystems) and AceQ qPCR SYBR green master mix (Vazyme). The results were then normalized to tubA, and expression levels were calculated using the ΔΔ_{ct} method (46).

RNA-seq. For RNA sequencing, the Tet-somA strain was grown in MM with or without 1 μg/ml doxycycline for 22 h and then exposed or not exposed to 100 μg/ml CFW for 2 h. The samples were collected and subsequently frozen using liquid nitrogen. After mRNA purification and library construction, the samples were sequenced by next-generation sequencing (NGS) based on the Illumina sequencing platform. The threshold value of differentially expressed genes were a fold change of >2 and a P value of <0.05. RNA isolation, mRNA purification, and cDNA synthesis and sequencing were performed by Shanghai Personal Biotechnology (China). All the samples were evaluated using three biological repetitions.

ChIP-seq and MEME analysis. The SomA-FLAG strain was incubated in MM for 24 h and then cross-linked by 1% formaldehyde for 10 min at 37°C. Cross-linking was stopped by supplementation with 0.125 M glycine and incubation for 5 min at room temperature. The samples were then washed twice with ice-cold PBS and frozen with liquid nitrogen. Immunoprecipitation of DNA was performed as previously described (47, 48). Briefly, after cell lysis, the samples were sheared by sonication (Diagenode Bioruptor Pico) to approximately 500- to 1,000-bp fragments. Immunoprecipitation was performed using Dynabeads-protein G (Thermo Fisher) and anti-FLAG M2 monoclonal antibody (Sigma). ChIP-seq libraries were constructed according to the manufacturer’s instructions for Illumina ChIP-seq library preparation. The output data were processed with a cutoff q-value of 0.001 and a fold enrichment of >2. Immuno precipitation and sequencing were performed by Bio-Tech & Consult (Shanghai).

To identify conserved SomA binding sequences, the output target sequences of SomA were analyzed by using the MEME suite (http://meme-suite.org/tools/meme) with the class mode for motif discovery and a site distribution of zero or one occurrence per sequence ("zoops"), and the minimum and maximum widths of the motif were set at 6 and 12, respectively.

SomA protein expression, purification, and electrophoretic mobility shift assay. For SomA protein recombination studies, the full-length cDNA sequence of SomA was amplified with the primer pair pet30-SomAF/R, and the resulting product was cloned into the NdeI and HindIII site of pET-30a (+). The constructs were then transformed into Escherichia coli DE3 (TransGen Biotech). SomA was purified using His tag purification resin (Beyotime).

For DNA probe preparation, the probe was labeled with Cy5 using two-step PCR. Briefly, ~200 bp of the target sequence-containing motifs were amplified with the primer pair EMSAF/R (e.g., agd3EMSAF/R), which included probe primer oligonucleotide. The resulting products were then used as the template and amplified with a probe primer labeled with Cy5 (Cy5labeled) to generate Cy5-labeled probe DNA.

To generate the agd3 mutant probe, a fusion PCR method was used. Briefly, ~1,000 bp of the upstream and downstream flanking sequences of the conserved motif were amplified with the primer...
pairs $agd3mutF1$/$R1$ and $F2$/$R2$, respectively. For site-directed mutagenesis, the complementary primers $agd3mutR1$ and $agd3mutF2$ harboring the desired mutation in the centromeric position were designed and synthesized. The two purified products were then used as a template to generate a mutant sequence using the primer pair $agd3fusionF/R$. The sequenced mutant sequence was then used as a template to generate Cy5-labeled $agd3$ mutant probe DNA according to the method described above.

The EMSA was performed as described previously (49) with minor modifications. For EMSA, 1 µg of salmon sperm DNA was used as a nonspecific competitor, and 20-fold nonlabeled DNA was used as a competitive cold probe. The reaction mixtures consisted of 30 µl of 1 × EMSA binding buffer containing nonspecific competitor, 100 ng of probe DNA, and 1 or 1.5 µg of recombination protein. The samples were incubated at 37°C for 30 min and then separated on a 5% polyacrylamide gel in 0.5 Tris-borate EDTA buffer. After electrophoresis, the Cy5-labeled probes were detected with an Odyssey machine (LI-COR).

Plate assays. To test the sensibility of WT and Tet-somA strains to cell wall-perturbing agents, minimal medium was supplemented with 50 µg/ml CFW, 20 µg/ml Congo red, or 1.25 µg/ml caspofungin. Then, 2-µl portions of conidial suspensions ($1 \times 10^5$, $1 \times 10^6$, or $1 \times 10^7$ conidia/ml) of the indicated strains were spotted onto the relevant media plates with or without doxycycline, grown at 37°C for 48 h, and observed and imaged.

Transmission electron microscopy analysis of the cell wall. The cell walls of WT and Tet-somA strains were examined by TEM, as previously described (50). After the indicated incubation period, the mycelia were fixed overnight in 0.1 M sodium phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde at 4°C. The samples were embedded in 1% agar, fixed in 0.1 M sodium phosphate buffer (pH 7.4) containing 1% OsO4 for 2 h, and sequentially dehydrated in 50, 70, 80, 90, 95, and 100% ethanol and 100% acetone for 15 min each. Samples were embeded in 812 epoxy resin monomer (SPI), sliced into 60- to 80-nm ultrathin sections using an ultrathin microtome (Leica UC7), stained with uranyl acetate and lead citrate, and imaged at 80 kV using a transmission electron microscope (Hitachi HT7700).

Cell wall monosaccharide analysis. WT and Tet-somA strains were incubated in MM for 24 h at 37°C. After incubation, fungal balls were collected by Miracloth filtration and washed in 70% ethanol. The resulting biomass was crushed in a glass cell homogenizer in 70% ethanol, Pellets were washed five times with 70% ethanol at 70°C and then in a solution of methanol and chloroform (1:1 [vol/vol]) for 24 h and acetone for 24 h. Pellets were dried, and 1 mg of the resulting preparation was analyzed by gas chromatography-mass spectrometry. Samples were hydrolyzed with either 2 M trifluoroacetic acid for 2 h at 110°C or 6 M hydrochloric acid (HCl) for 4 h at 100°C. After drying, samples were derivatized and analyzed as previously described (13). Briefly, samples were converted in methyl glycosides by heating in 1 M methanol-HCl (Supelco) for 16 h at 80°C. Samples were dried and washed twice with methanol prior re-N-acetylating hexosamine residues. Re-N-acetylation was performed by incubation with a mix of methanol, pyridine, anhydride acetic acid (10:2:3) for 1 h at room temperature. Samples were then treated with hexamethyldisilazane-trimethylchlorosilane-pyridine solution (3:1:9; Supelco) for 20 min at 80°C. The resulting TMS methyl glycosides were dried, resuspended in 1 ml of cyclohexane, and injected in the Trace1300 GC-MS system equipped with a CP-Sil5-CB capillary column (Agilent Technologies). Elution was performed using the following temperature gradient: 120 to 160°C at a rate of 10°C/min, 160 to 220°C at a rate of 1.5°C/min, and 220 to 280°C at a rate of 20°C/min. Identification and quantification of each monosaccharide was carried out using standards and response factors determined for each monosaccharide.

Calcofluor white and aniline blue staining. Mycelium were stained with CFW and aniline blue as previously described (51). For CFW staining, hyphae were washed with PBS and stained with 10 mg/ml CFW for ~2 min. For aniline blue staining, samples were stained with a freshly prepared 0.05% (wt/vol) aniline blue solution for 60 min. These samples were then washed with PBS and immediately imaged by fluorescence microscopy.

Data analysis. All statistical analyses were performed using GraphPad Prism 6 software. Multiple comparisons were analyzed by one-way analysis of variance. A P value of <0.05 was considered statistically significant.

Data availability. The RNA-seq and ChIP-seq data have been deposited in the NCBI Sequence Read Archive under accession numbers PRJNA647130 and PRJNA647621, respectively. Other relevant data supporting the findings of this study are available in this article and its associated supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 0.5 MB.
FIG S2, TIF file, 0.1 MB.
FIG S3, TIF file, 0.5 MB.
FIG S4, TIF file, 0.5 MB.
TABLE S1, XLS file, 1.8 MB.
TABLE S2, XLSX file, 0.05 MB.
TABLE S3, DOC file, 0.2 MB.
TABLE S4, XLSX file, 0.1 MB.
TABLE S5, DOC file, 0.1 MB.
TABLE S6, DOCX file, 0.02 MB.
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Y.C., L.L., and S.Z. designed the experiments. Y.C., F.L.M., Y.W., and R.L. performed the experiments. Y.C. and F.L.M. analyzed the data. Y.C., D.C.S., and S.Z. wrote the manuscript.

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