Cryptococcus neoformans Mediator Protein Ssn8 Negatively Regulates Diverse Physiological Processes and Is Required for Virulence

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Abstract

Cryptococcus neoformans is a ubiquitousity distributed human pathogen. It is also a model system for studying fungal virulence, physiology and differentiation. Light is known to inhibit sexual development via the evolutionarily conserved white collar proteins in C. neoformans. To dissect molecular mechanisms regulating this process, we have identified the SSN8 gene whose mutation suppresses the light-dependent CWC1 overexpression phenotype. Characterization of sex-related phenotypes revealed that Ssn8 functions as a negative regulator in both heterothallic α-α mating and same-sex mating processes. In addition, Ssn8 is involved in the suppression of other physiological processes including invasive growth, and production of capsule and melanin. Interestingly, Ssn8 is also required for the maintenance of cell wall integrity and virulence. Our gene expression studies confirmed that deletion of SSN8 results in de-repression of genes involved in sexual development and melanization. Epistatic and yeast two hybrid studies suggest that C. neoformans Ssn8 plays critical roles downstream of the Cpk1 MAPK cascade and Ste12 and possibly resides at one of the major branches downstream of the Cwc complex in the light-mediated sexual development pathway. Taken together, our studies demonstrate that the conserved Mediator protein Ssn8 functions as a global regulator which negatively regulates diverse physiological and developmental processes and is required for virulence in C. neoformans.

Introduction

Transcription is an essential process for gene expression in both prokaryotic and eukaryotic organisms, which requires RNA polymerase to decode the DNA sequences. Transcription in eukaryotic cells is a complex process which requires the interactions of the RNA polymerase II (RNAPII) general transcriptional machinery with a whole array of DNA-binding transcriptional factors [1]. Activation and repression of RNAPII activity by the positive and negative regulatory proteins is fundamental to the regulation. An important group of highly conserved proteins forms the Mediator complex that functions in conveying the regulatory signals from these transcription factors to RNAPII [2–4]. The Mediator complex is comprised of 24 subunits in Saccharomyces cerevisiae. The number of subunits varies in different species, but overall, the Mediator is well conserved from yeast to human in its protein structure [5–7].

The Mediator subunits are organized structurally into 4 major parts: head, middle, tail and a free module [8]. The former three parts form the core Mediator which functions together with RNAPII as the holoenzyme [4]. Ssn8, also known as Srb11, a C-type Cyclin, and its specific Cyclin-dependent kinase (CDK), Ssn3/Srb10, are members of the free module in the Mediator complex. Therefore, this free module has a kinase role. The free module is recruited by repressors to phosphorylate Ser2 and/or Ser5 in the heptapeptide repeats of CTD [9,10]. This phosphorylation of RNAPII complex, that prevents its interaction with the core Mediator, results in the repression of gene expression. On the other hand, transcription is activated when activators recruit the core Mediator complex without the free module to associate with RNAPII complex at the promoter region [2,11,12]. Although most studies indicate that the free module serves in a negative role, some have demonstrated its activation function. In S. cerevisiae, the free module is required for the induction of the GAL genes by phosphorylating the Gal4 activator which may prevent the repressor, Gal80, from binding to the promoter region [13,14].

Ssn8 is involved in the regulation of carbon utilization in S. cerevisiae. It was first identified as a suppressor of the snf1 mutation [15]. Ssn8 was later rediscovered as a suppressor in the snf1mg1 double mutant screening for gluconeogenic growth [16]. Snf1 and Mgl1 in yeast are two key regulators involved in carbon catabolite repression [17]. Ssn8, also named Um35, plays critical roles in meiosis [18]. Destruction of Ssn8 is required for induction and execution of meiotic development; deletion of Ssn8 results in unscheduled meiotic gene expression [19]. Genome-wide studies in S. cerevisiae revealed that 173 genes (approximately 3% of the genome) are negatively regulated by the Srb11 containing free module, of these genes 75 genes are involved in nutrient scavenging.
been identified and characterized. Deletion of the negatively regulating filamentation in sexual development, Ssn8 was conducted under various conditions. The results indicate that filamentation pathway. An ssn8 mutant shows slow growth, reduced conidiation, increased pigmentation, female sterility and sensitivity to stress conditions. In addition, it also reduces the production of DON (deoxynivalenol), a protein synthesis inhibitor which causes toxicity, and fails to infect corn plants [27]. These reports describe the global regulatory roles of Ssn8 homologues including fungal pathogens.

Cryptococcus neoformans is a globally distributed human fungal pathogen that exists in different ecological niches [28]. To adapt different environments and to adjust to the transition and challenges upon entering the host, various sensing mechanisms to co-opt the external cues or stimuli have been developed [29]. \( C. \) neoforms grows vegetatively as the yeast form; filamentation is primarily associated with two sexual processes, heterothallic \( a-\alpha \) mating and \( a-\alpha \) same-sex mating [30,31]. Blue light is known to inhibit the production of sexual filaments in \( C. \) neoforms and two evolutionally conserved blue light regulators, Cwc1 and Cwc2, play critical roles in these processes [32,33]. Mutants carrying a deletion in either \( CWC1 \) or \( CWC2 \) are blind to the inhibitory effect of light on mating and are sensitive to ultraviolet light. Interestingly, they also show a reduction of virulence in a murine model [32]. Elevating transcript level of \( CWC1 \) or \( CWC2 \) with synthetic constructs causes inhibition of mating filamentation in the light [33].

In order to dissect the molecular mechanisms of blue light-inhibited sexual development in \( C. \) neoforms, a genome-wide mutagenesis was conducted under the \( CWC1 \) overexpression background to screen for mutants that restored filamentous growth in the light [34]. A T-DNA insertion into the Mediator Ssn8 gene not only suppresses the light-dependent \( CWC1 \) overexpression mating phenotype but also shows dramatic de-repression of same-sex mating [34]. In this study, we aimed to characterize the roles of Ssn8 in \( C. \) neoforms and its relationship to the light-mediated filamentation pathway. An \( ssn8 \) mutation was introduced into different strain backgrounds and their phenotypic characterization was conducted under various conditions. The results indicate that \( C. \) neoforms Ssn8 functions as a global negative regulator involved in diverse physiological and developmental processes. In addition to negatively regulating filamentation in sexual development, Ssn8 also suppresses melanization, capsule formation and invasive growth, and is also required for the maintenance of cell wall integrity and virulence.

Results

C. neoforms Ssn8 Is a Mediator Protein Containing the Conserved Cyclin Box and PEST Domain

Our previous studies showed that \( C. \) neoforms Cwc1 and Cwc2 are two central regulators which coordinately mediate blue light-inhibited sexual filamentation [32,33]. To understand how blue light inhibits filamentation, we set up a genome-wide mutagenesis screen and identified mutants suppressing the \( CWC1 \) light-dependent overexpression phenotype [34]. One of the suppressors, \( AY18 \), restored mating filamentation and also showed dramatic de-repression of monokaryotic fruiting. Further characterization confirmed that a mutation of \( C. \) neoforms Ssn8 Mediator homologue is responsible for its phenotypes [34].

Sequence analysis revealed that \( C. \) neoforms Ssn8, encoding a 439 amino-acid protein with two introns, is a putative Cyclin-like component of the RNA polymerase II holoenzyme. The conserved Cyclin box (46–122 amino acid) and CCL1 domain (244–280 amino acid) are identified. \( C. \) neoforms Ssn8 also contains a predicted PEST-rich region (298–315 amino acid) which has been known to be responsible for the degradation of Ssn8 in \( S. \) cerevisiae [23]. Homologues of the Ssn8 protein are widely present among diverse organisms. Overall, Ssn8 is low among these proteins and high in the conserved domain regions. Unlike other ascomycetous homologues, no predicted destruction box (RxxL) is found in the \( C. \) neoforms Ssn8 protein [23,25,27]. The homologue most closely related to \( C. \) neoforms Ssn8 is the umb06212 protein of \( U. \) maydis; they shares 28% overall identity and 52% identity in the Cyclin box. \( C. \) neoforms Ssn8 protein also shares 18% and 19% overall identities respectively with \( S. \) cerevisiae Ssn8 and \( F. \) verticillioides Fcc1, and 39% and 44% in the Cyclin box. Phylogenetic tree based on the whole protein sequence was generated (Fig. S1A) and amino acid sequence alignment of the Cyclin domains among the Ssn8 homologues is shown (Fig. S1B). \( C. \) neoforms Ssn8 also shows similar close relationship with the basidiomycetous homologues.

To confirm \( C. \) neoforms Ssn8 gene is a Mediator homolog, functional complementation was conducted in \( S. \) cerevisiae. \( S. \) cerevisiae \( ssn8 \) mutant exhibits a flocculation phenotype when cultured in liquid medium. The \( C. \) neoforms Ssn8 gene was expressed under control of the \( GAL1 \) promoter in pYES2 and transformed into the \( S. \) cerevisiae \( ssn8 \) mutant and the transformants recovered the flocculation phenotype when grown in SD medium containing 2% galactose (Fig. S2). These results indicated that \( C. \) neoforms Ssn8 gene, encoding a functional mediator protein, is able to complement the phenotype of \( S. \) cerevisiae \( ssn8 \) mutant.

Deletion of Ssn8 Does Not Affect General Growth Capability, But Influences Galactose Utilization in C. neoforms

To determine the functions of Ssn8 in \( C. \) neoforms, \( ssn8 \) null mutants and overexpression strains were generated. Southern blot analysis was carried out to confirm the strains (Fig. S3A and S3B). Real-time PCR analysis was also conducted to verify the levels of Ssn8 transcript. The results indicated that Ssn8 expression was not detected in the mutants and highly elevated Ssn8 transcript levels, at least 40 fold greater than the wild type, were detected in the overexpression strains (Fig. S3C).

To further verify if deletion of Ssn8 affects the general growth capability of \( C. \) neoforms, we carried out cell growth assays on different media (Materials and Methods S1). When serially diluted yeast cells were spotted onto YPD rich and FA starvation media, the \( ssn8 \) mutants and reconstituted strains all grew to the same extent as the wild-type strains (Fig. 1A). The Ssn8 overexpression strains also showed normal growth (Fig. 1A). We also examined the growth of mutants at 37°C and 39°C. No difference in growth rate was detected between the \( ssn8 \) mutant and wild-type strain under these high temperature conditions (Fig. S3D). Therefore, deletion of the Ssn8 gene does not affect the general growth ability of \( C. \) neoforms.
Since *S. cerevisiae* Ssn8 is known to involve in the regulation of carbon utilization, we also determined whether *SSN8* deletion affects carbon utilization. The wild-type strains, *ssn8* mutants and *SSN8* overexpression strains were grown on YNB medium supplemented with different carbon sources, including glucose, sucrose, glycerol, ethanol, galactose, and sodium acetate. The *ssn8* mutants showed no growth defect on different carbon sources except for galactose-containing medium (Fig. 1B; data not shown).

In addition, we also examined the expressions of *HXT* (α hexose transporter, CNB02680) and *SNF1* in the *C. neoformans MATα* strains including three *ssn8* mutants in YPD liquid medium. Elevated transcript levels of these genes were detected in the mutants (Table S1). These results suggest that *C. neoformans* Ssn8 protein is possibly involved in the regulation of carbon, specifically galactose utilization.

**Heterothallic α–α Mating Is Negatively Regulated by *SSN8***

In our previous study, *SSN8* was identified as a gene whose mutation restored mating filamentation in the *CWCI* light-dependent overexpression phenotype [34]. We postulated that *C. neoformans* Ssn8 may play a negative role in sexual differentiation. Hence, mating assays were conducted to determine the role of *SSN8* in the heterothallic α–α mating process. On V8 plate mating, both the *ssn8* unilateral and bilateral mutant crosses showed early formation of mating filaments at 8 h post incubation (Fig. S4A). However, upon further incubation filamentation around the edges of mutant mating spots was less pronounced when compared to the wild-type spot (Fig. S4B).

To more precisely examine the effect of *SSN8* deletion on mating, we further conducted a slide mating assay by diluting and spreading mating cells onto V8 agar film on microscope slides. In the unilateral (Fig. 2d and f) and bilateral mutant (Fig. 2h) crosses, more filaments were consistently observed in the diluted middle parts of mating cultures throughout the process when compared to the wild-type cross (Fig. 2b). More filaments at the edges of mutant mating spreads were also observed at early stages of the mating process. However, similar filamentation levels were seen in the mutant and wild-type crosses at later times (Fig. 2a, c, e, and g). Bilateral crosses between the *MATα* and *MATα* *SSN8* reconstituted strains (Fig. 2o and p) showed a filamentation pattern similar to the wild-type. In contrast, unilateral and bilateral crosses involving the *MATα* and *MATα* *SSN8* overexpression strains consistently exhibited less filamentation than those of the wild-type crosses when observed in the plate mating assay (Fig. 2i–n). Although filamentation levels were altered in crosses of mutant and overexpression strains, the timing for initiation of basidia formation in these crosses occurred around 36 h post incubation, which was similar to that in the wild-type cross. Fruiting structures produced by these crosses appeared normal (insets in the photos of Fig. 2). Based on our mating assays, we have demonstrated that deletion of the *SSN8* gene results in enhanced mating filamentation, whereas artificially elevating the *SSN8* transcript level reduces mating filamentation, suggesting that *C. neoformans* Ssn8 plays a negative role in heterothallic α–α mating process.

**Same-Sex Mating Is Dramatically De-repressed in the *MATα* and *MATα* *ssn8* Mutants**

Same-sex mating, also called monokaryotic fruiting, occurs in same mating type cell, predominantly in the *C. neoformans MATα* strains [30]. A mutation of the *SSN8* gene by T-DNA integration, or by homologous replacement, in the *CWCI* overexpression strain surprisingly showed de-repression of same-sex mating [34]. We examined the effect of *SSN8* mutation on this process in the *MATα* and *MATα* wild-type strain background. Same-sex mating is a slow differentiation process in *C. neoformans*, and the serotype D *MATα* wild-type strain JEC21 started to produce filaments on FA medium after 2 days and filamentation was clearly seen 5 days post incubation (Fig. 3Aa). In contrast, the *MATα* wild-type strain JEC20 failed to undergo same-sex mating and no filament was observed after 5 days (Fig. 3Ac) and even after a prolonged incubation. As expected, same-sex mating was significantly de-repressed not only in the *MATα ssn8* mutants but also in the *MATα* *ssn8* mutants. Filamentation took place earlier in the *ssn8* mutant strains and more filaments were observed 2 days post incubation when compared to the wild-type strains. After 5 days, both *MATα* and *MATα* *ssn8* mutants showed profuse monokaryotic filaments, although more pronounced in the *MATα* *ssn8* mutants (Fig. 3Bb). In the mutant strains, *MATα* and *MATα* *SSN8* reconstitution strains (Fig. 3Ad and Ah). On the other hand, same-sex mating process was completely blocked by *SSN8* overexpression. Both *MATα* and *MATα* *SSN8* overexpression strains failed to undergo this fruiting process after 5 days (Fig. 3Ac and Ag). No filament was developed even after 2 weeks incubation (data not shown).

We further conducted the same-sex mating assay, with diluted *C. neoformans* cells, on microscope slides coated with FA agar. The results showed that no filament was found in the wild-type strain at 12 h, and even up to 60 h post incubation, whereas, monokaryotic filaments were easily observed in the *ssn8* mutants (Fig. 3Bb). In these mutants, filamentous structures were observed as early as 8 h post incubation. Interestingly, most filaments arose from cells of larger size than regular yeast cells and no cell-cell conjugation occurs. Therefore, *SSN8* deletion can hasten the fruiting process of *C. neoformans*.
event was found (Fig. 3C). These results suggest that *C. neoformans* Ssn8 negatively regulates the same-sex mating event in both MATα and MATα cells and plays a critical role in the control of this unusual sexual development.

Mating Related Genes Are De-repressed in the ssn8 Mutants

Observations of enhanced filamentation phenotypes of the *ssn8* mutants in the heterothallic α-α mating and also in the same-sex mating prompted us to further examine the expression of genes involved in these processes. Bilateral crosses of the wild-types, *ssn8* mutants, and *SSN8* overexpression strains were conducted on V8 agar media. Cells were collected at different time points and subjected to RNA extraction. The relative transcript levels of MFα and GPA2 during α-α mating were measured by quantitative real time PCR analyses. MFα pheromone gene is known to be induced when strains of opposite mating type co-culture under nutritional starvation conditions [35]. MFα expression in the wild-type cross was low at 0 h and apparently induced to 18-fold at 3 h, and reached a peak of 178-fold at 12 h. Thereafter, the MFα transcript stayed high, but slightly declined to 158-fold at 24 h (Fig. 4Aa). In the *ssn8* bilateral mutant cross, the transcript level of MFα was 24-fold higher than that of the wild-type cross at 0 h. Mating induction of the pheromone gene in the mutant cross was more dramatic and its level was continually elevated and reached up to 238-fold at 9 h. At 12 h post incubation, the MFα level decreased in the *ssn8* mutant cross and showed similar expression level, 180-fold, to that in the wild-type cross. At 24 h, the MFα transcript level was significantly down-regulated and dropped to the basal level, 13-fold (Fig. 4Aa). In the bilateral cross of *SSN8* overexpression strains, the induction trend of MFα was generally similar over 24 hour period. However, the transcript level was significantly attenuated (Fig. 4Aa) when compared to the other two crosses.

*C. neoformans* G protein α subunit Gpa2 is a member of heterotrimeric G protein complex functioning upstream of the Cpk1 MAPK pheromone response pathway and its level is highly induced during mating process [36]. We also examined the GPA2 transcript levels in the wild-type and *ssn8* mutant crosses and found that the overall GPA2 expression patterns of these crosses were quite

Figure 2. Mutation of the *SSN8* gene enhances heterothallic α-α mating responses. The MATα and MATα wild-type, *ssn8* mutant, and reconstituted strains were crossed as indicated. Mating was conducted on microscope slides coated with V8 agar at 26 °C under light conditions and photos were taken from different areas of mating culture 44 h post incubation at 100× magnification under a microscope. Mating filaments around the edges of mating colonies were shown in upper panels (a, c, e, g, i, k, m, and o) and filamentation in the middle of mating culture was shown in lower panels (b, d, f, h, j, l, n, and p) at magnifications of 100× and 200× respectively. Insets showed chains of basidiospores and basidia at 400× magnification.

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similar, and they both showed two peaks of GPA2 transcript level at 6 and 24 h time points. However, higher induction levels were seen in the ssn8 bilateral mutant cross throughout the 24 h period (Fig. 4Ab). In the bilateral cross involving the SSN8 overexpression strains, the GPA2 levels were lower than the wild-type cross at all time points except at its peak level at the 9 h time point (Fig. 4Ab).

Genes involved in heterothallic a–a mating also play roles in same-sex mating [30,35]. Deletion of SSN8 showed hyper-filamentation phenotype. To determine the MFα transcripts level, C. neoformans strains were grown on FA agar and cells were harvested at 0 and 48 h for RNA analysis by Northern blot hybridization. MFα was barely detectable in the wild-type strain. In contrast, the ssn8 mutants exhibited high levels of MFα pheromone expression at 0 and 48 h (Fig. 4Ba and Bb). As in the wild-type strain, MFα transcripts were also extremely low in the SSN8 overexpression and reconstituted strains (Fig. 4Ba and Bb).

Our expression studies revealed that mating-related genes such as MFα and GPA2 were highly elevated and still responsive during mating in the ssn8 mutant cross. In addition, high pheromone transcript levels were also detected in the ssn8 mutants during same-sex mating process. We speculated that Ssn8 may function as a general regulator repressing sexual-related genes during vegetative growth and therefore their expressions in YPD rich medium were further examined. C. neoformans MATα strains including three ssn8 mutants were grown in YPD liquid medium for 22 hours. RNA was extracted and subjected to real time PCR analyses. The transcript levels of MFα, GPA2, GPB1, SXI1α, CPR2, and other mating related genes among the ssn8 mutants were significantly higher than those in the wild-type strain (Fig. 4C and Table S1). No significant differences were observed in the SSN8 overexpression and reconstituted strains. In summary, our gene expression analyses have demonstrated that genes involved in sexual development are de-repressed in the ssn8 mutants under non-mating and mating conditions. The ssn8 mutants maintain the responsiveness to mating inducing signals, but the intensity and timing of gene expression pattern are changed.

C. neoformans Ssn8 Plays Critical Roles Downstream of the Cpk1 MAPK Cascade and Ste12 in the Mating Signaling Pathway

As demonstrated earlier, C. neoformans SSN8 negatively regulates heterothallic a–a mating and same-sex mating processes and deletion of SSN8 causes de-repression of sex-related genes (Fig. 2, 3 and 4). We therefore set out to determine the relationship between Ssn8 and components of the Cpk1 MAPK pheromone signaling pathway. We first conducted epistatic studies and generated double mutants of interest, including gb1ssn8, ste20ssn8, cpk1ssn8 and ste12ssn8. Their phenotypes were examined and compared with respective single mutants. As gb1 and cpk1 mutants are sterile, the gb1ssn8 and cpk1ssn8 double mutants produced profuse mating filaments, but slightly less than the ssn8 mutant (Fig. 5A). The filamentation level in the ste20ssn8 double mutant cross was comparable to that in the ste20 mutant cross but slightly less than that in the ssn8 mutant cross (Fig. 5A). In contrast, the ste12ssn8 double mutant cross produced profuse filaments similar to the ssn8 mutant but more than the ste12 cross (Fig. 5A).

It is known that GPB1 is required and STE20, CPK1, and STE12 are all essential for the same-sex mating process [37]. Yet, we found that all the double mutants exhibited enhanced filamentation similar to the ssn8 mutant, although to different extents (Fig. 5B). STE12, located in the mating type locus, is a major

Figure 3. Same-sex mating is dramatically de-repressed in the MATα and MATα ssn8 mutants. (A) The MATα and MATα wild-type, ssn8 mutants, reconstituted strains, and SSN8 overexpression strains were spotted on filament agar plate and incubated at 26 C under light conditions. Photos were taken after 5 days at 100× magnification. (B) The wild-type (a) and ssn8 (b) cells were grown on microscope slides coated with FA agar film. Monokaryotic filaments were produced from the ssn8 mutant cells. Photo was taken at 12 h post incubation at 100× magnifications. (C) Development of monokaryotic filament from a single enlarged ssn8 mutant cell was monitored on microscope slide coated with FA agar at 12 h (a), 24 h (b), 36 h (c), 50 h (d) and 60 h (e) post incubation at 200 × magnification.

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transcription regulator of same-sex mating. The ste12ssn8 double mutant exhibited profuse monokaryotic filaments much the same as the ssn8 mutant, which was in stark contrast to the sterile phenotype of the ste12 mutant (Fig. 5B).

To support our phenotypic observation, we further examined the expression levels of MFα pheromone gene in the cpk1 mutant background. The cpk1 and ssn8 single mutants, and three cpk1ssn8 double mutants were grown in YPD liquid medium. RNA was extracted from these samples and subjected to quantitative real time PCR analysis. The results demonstrated that pheromone gene was de-repressed in the ssn8 mutant, 3-fold higher than the wild-type strain; whereas, the MFα gene stayed at the basal level in the cpk1 mutant. The expression levels of pheromone gene in three cpk1ssn8 double mutants were all elevated and similar to that in the ssn8 mutant (Fig. 5C). Taken together, our epistatic and gene expression studies suggest that Ssn8 plays critical roles downstream of the Cpk1 MAPK cascade and Ste12 and negatively regulates heterothallic α-α mating and same-sex mating processes.

Disruption of SSN8 Alters Cell Wall Structure and Integrity

While growing the ssn8 mutants in YPD liquid medium, we noticed some abnormally large aggregates of cells floating at the

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Figure 4. Mutation of the SSN8 gene causes de-repression of genes involved in sexual development. (A) C. neoformans α-α bilateral crosses of the wild-type, ssn8-1 mutants and SSN8 overexpressed strains were conducted on V8 agar medium in the dark and cells were collected at indicated time for RNA preparation. The abundance of the MFα (a) and GPA2 (b) transcripts were measured by quantitative real time PCR analysis. (B) C. neoformans strains were grown on FA medium and cells were harvested at 0 (a) and 48 (b) h for RNA extraction. The expression of MFα was detected by northern blot analysis and RNA loading for each sample was shown by rRNA staining. Samples in lane 1 and 2 (MATα wild-type), lane 3 and 4 (MATα ssn8-1), lane 5 and 6 (MATα ssn8-2), lane 7 and 8 (SSN8 overexpression strain), lane 9 and 10 (SSN8 reconstituted strain) are as indicated.

(C) C. neoformans MATα wild-type, ssn8 mutants, reconstituted strain, and SSN8 overexpression strain were grown in YPD liquid medium for 22 h. Cells were harvested and subjected to RNA extraction. Expression of the GPB1, GPA2, MFα and Sxi1α genes was measured by quantitative real time PCR analysis.

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surface of the culture after 2 days of cultivation. Interestingly, upon microscopic observation, we found some of the ssn8 mutant cells displayed an elongated and deformed morphology instead of the spherical yeast cells normally seen in the wild-type (Fig. 6Aa and Ac). Some of the elongated cells appeared to be septated but failed to separate. Under the same growth conditions, the reconstitution
and overexpression strains both showed normal yeast cell morphology (data not shown). Quantification revealed that more than 17% of the ssn8 mutant cells exhibited this atypical morphology (Table 1). To examine if cell wall structure was altered in C. neoformans ssn8 mutants, we stained the cells with Eosin Y to visualize chitosan distribution in the cell wall [38]. In contrast to the uniform staining in the wild-type yeast cells (Fig. 6Ab), some of the ssn8 mutant cells exhibited irregular patches by Eosin Y staining, suggesting the cell wall structure of the ssn8 mutants was altered (Fig. 6Ad and Ae).

Figure 6. Cell wall structure and integrity are changed in the ssn8 mutant strains. (A) The C. neoformans wild-type strain showed normal yeast cells (a, b), but the ssn8 mutant displayed high percentage of morphological deformation (c, d, e) when grown in YPD liquid medium for 2 days. Fluorescence images of the same cells by Eosin Y staining are shown (b, d, e). Photos were taken at a magnification of 400× under a confocal microscope. (B) Equal number of the MATa wild-type, ssn8 mutants, SSN8 overexpression and reconstituted cells were serially diluted and spotted on YPD medium and YPD supplemented with Congo red, calcofluor white (CFW) and caffeine. Plates were incubated at 30°C for 5 days under dark condition. (C) The wild-type and ssn8 mutant strains were cultured in YPD liquid medium for 4 days. Cells were collected and stained with anti-β-1,3-glucan antibody and Cy3-labeled goat-anti-mouse secondary antibody for immunofluorescent observation. The DIC (a, d), fluorescent (b, e) and merged images (c, f) of the wild-type and ssn8 mutant strains were shown. The projections of a series of z-axis fluorescent images were shown in the insets (b, e). Arrows indicate staining spots in the wild-type and ssn8 mutant cells. Scale bar indicates 20 μm. (D) Transmission electron microscopy illustrates the wild-type (a, b) and the ssn8 mutant (c, d) cells. Higher magnification images of the cells (b, d) were focused on the cell wall structure. Arrows indicate lipid droplets (c) and arrowhead indicates glycogen-like structure (d) in the ssn8 mutant cells.

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Table 1. Cell deformation rate of the ssn8 mutants in the YPD liquid medium.

| Strain               | Deformation Rate | Strain               | Deformation Rate |
|----------------------|------------------|----------------------|------------------|
| JEC20                | 0                | JEC21                | 0.12%±0.24%      |
| MATa ssn8-1          | 43.53%±7.7%      | MATa ssn8-1          | 22.26%±4.42%     |
| MATa ssn8-2          | 26.53%±4.12%     | MATa ssn8-2          | 17.85%±3.44%     |
| MATa pGPD1::SSN8     | 0                | MATa pGPD1::SSN8     | 0                |
| MATa ssn8+ SSN8      | 0                | MATa ssn8+ SSN8      | 0                |

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exhibited round shape with a ragged cell surface (Fig. S5). When further stained with trypan blue and cosin Y, most of the ragged mutant cells showed strong staining with irregular patches, whereas the smooth and rounded wild-type cells showed uniform staining (Fig. S5).

Since mutation of SSN8 caused abnormal morphology, we further examined if the ssn8 mutants showed sensitivity to stress reagents. Yeast cells were serially diluted and spotted onto YPD medium containing different stress reagents, including Congo red, sodium dodecyl sulfate (SDS), caffeine, calcofluor white, and other osmotic, oxidative and nitrosative reagents. Their growths on these media were monitored. The results showed that the ssn8 mutants were not sensitive to most of the stress agents, only showing sensitivity to Congo red (Fig. 6B; Fig. S6). Congo red is known to interact with the important cell wall component β-1,3-glucan, and strains with cell wall defects will show sensitivity to this chemical [39].

Since ssn8 mutants showed sensitivity to Congo red, we further examined the distribution of β-1,3-glucan by immunofluorescent staining. C. neoformans wild-type yeast cells showed relatively weak staining by anti-β-1,3-glucan antibody and only a small portion of cells showed fluorescent spots in the cell wall (Fig. 6Cb and Cc). In stark contrast, deformed yeast cells or filament structures of the ssn8 mutant exhibited bright fluorescent staining in the cell wall (Fig. 6Ce and Cf). These observations confirm that the organization or distribution of β-1,3-glucan in the ssn8 mutant cell wall is changed.

To reveal the detailed structural changes caused by the mutation of SSN8, we conducted transmission electron microscopy. In the C. neoformans wild-type strain, yeast cells overall displayed uniform cellular structures and compact cell wall organization (Fig. 6Da and Db). In contrast, the elongated and deformed ssn8 mutant cells showed uneven cellular organization. Most mutant cells showed relatively light or uneven staining, and disordered structures or fractured organelles were found. Some mutant cells contained large vacuoles and round-shaped lipid droplets and glycogen (Fig. 6Dc and Dd), indicating that the mutant cells might be old or in different physiological stage due to increased storage components. Furthermore, the mutant cell wall appeared thicker and less condensed than the wild-type, consistent with the view that the cell wall structure and organization is changed in the ssn8 mutant. Taken together, our findings indicate that a mutation of the SSN8 gene affects cell wall structure and integrity in C. neoformans.

The ssn8 Mutants Display Invasive Growth on Rich Medium

C. neoformans ssn8 mutants produced more abundant hyphae during sexual development processes. The ssn8 mutants also showed elongated filament-like structures in liquid rich medium. We further examined if mutation of the SSN8 gene caused any abnormal growth phenomenon on solid rich medium. To assess the agar invasion phenotype, the wild-type, ssn8 mutant, reconstituted, and overexpression strains were incubated on YPD medium (Materials and Methods S1). After 10 days of incubation, yeast colonies were repeatedly washed from the agar surface with sterile water. Interestingly, all the strains including the SSN8 overexpression strains were readily removed from the agar surface except for the ssn8 mutants (Fig. 7A). The remaining cells of the ssn8 mutants were observed under a microscope and a mixture of hyphae and deformed yeast cells was found (Fig. 7B and C). We observed similar phenotypes when the ssn8 mutants were grown on V8 or FA medium and hyphal invasion was clearly seen on these media (Fig. S7). Therefore, we conclude that Ssn8 also plays a negative role in regulating invasive hyphal growth in C. neoformans.

Production of Melanin and Capsule Is Negatively Regulated by the SSN8 Gene in C. neoformans

To determine if mutation of SSN8 affects the virulence traits of C. neoformans, we examined in vitro production of melanin and capsule in the ssn8 mutants. In the melanin production assay, C. neoformans ssn8 mutants started to accumulate melanin earlier than the wild-type strain. By 6 days, melanin accumulation was apparent in the mutant strains, which was clearly different from the wild-type strain. No dramatic difference was observed among the wild-type, reconstitution, and SSN8 overexpression strains (Fig. 8A). To confirm melanin accumulation in the ssn8 mutants, we examined the expression levels of LAC1 among these strains. We first checked the SSN8 transcript level in the ssn8 mutants and SSN8 overexpression strains under induced conditions. No SSN8 mRNA was detected in the ssn8 mutants and high level of SSN8 expression in the SSN8 overexpression strain were confirmed (Fig. 8B). When laccase gene expression was examined, high levels of LAC1 were detected in the ssn8 mutants. Quantitative measurement by real-time PCR analysis revealed 120- and 75-fold higher levels than the wild-type in two independent ssn8 mutants. The reconstitution and SSN8 overexpression strains exhibited LAC1 level similar to the wild-type strain (Fig. 8B).

To examine the formation of capsule in the ssn8 mutants, strains were negatively stained by India ink after 4 days of induction and thirty cells from each strain were measured. The capsule sizes of the ssn8 mutants were significantly larger than those of the other strains tested. The SSN8 reconstitution and wild-type strains exhibited normal capsule sizes, whereas the overexpression strain displayed smaller capsule (Fig. 8C). These results indicate that C. neoformans Ssn8 also plays negative roles in the regulation of melanin and capsule production.

C. neoformans Ssn8 Is Required for Virulence

Mutation of SSN8 resulted in increased accumulation of melanin and capsule, two well-known virulence factors of C. neoformans; however, the ssn8 mutants also displayed altered phenotypes in other cellular and physiological processes. We next asked the question whether C. neoformans Ssn8 contributes to virulence by conducting animal survival, tissue burden, and cerebrospinal fluid analyses in a murine animal model. The MATa wild-type, ssn8 mutant, and reconstituted strains were each injected intravenously via the lateral tail vein into ten C57LB/6 mice each, and host health and survival were recorded daily. Initial death of mice infected with the wild-type or reconstituted strains were observed between 27 and 35 day, and subsequently all individuals of these two groups died within 70 days after inoculation (Fig. 9A). In contrast, mice infected with the ssn8 mutant stayed healthy except for one death when close to day 70 since the inoculation (Fig. 9A).

To monitor the survival of C. neoformans strains inside the host body, we examined the yeast cell loads in different organs and cerebrospinal fluid after infection. Brain and lung were analyzed because they are the major targets of cryptococcal infection. In addition, cerebrospinal fluid was examined because it is the predisposed site of cryptococcosis in clinical presentations. We also analyzed liver because this organ is characterized by the presence of Kupffer cells; and Kupffer cells have been proven important for the clearance of circulating pathogens by extracellular or/and intracellular killing [40,41]. On the day of the experiment, we injected 106 yeast cells each of the wild-type, ssn8 mutant, and reconstituted strains intravenously into the lateral tail vein of three C57LB/6 mice, individually. After 24 hours, the yeast cell loads in the brain, lung, liver and cerebrospinal fluid were examined. The CFUs of the wild-type strain showed highest infection in all four
samples, whereas the ssn8 mutant exhibited the lowest cell counts in the brain, liver and cerebrospinal fluid (Fig. 9B). The reconstituted strain partially complemented the mutant defect and restored infection and cell loads in the brain, liver and cerebrospinal fluid. Interestingly, no apparent difference in the cell numbers found in lung tissue was observed one day post-inoculation. However, there was a significant decrease in brain infection (Fig. 9B). These in vivo animal studies demonstrated that the ssn8 mutant is defective in virulence and Ssn8 contributes to the virulence of C. neoformans.

C. neoformans Ssn8 Does Not Directly Interact with the Cwc1 or Cwc2 Protein and Possibly Functions Downstream of the Cwc Complex

To identify components in the C. neoformans blue light mediated filamentation process, we conducted a genome wide mutagenesis and identified suppressor genes including SSN8 in the screen [34]. These candidate genes may function either together with or downstream of the Cwc complex, or in an independent pathway. To determine whether Ssn8 physically interacts with Cwc1 or Cwc2, we conducted a yeast two-hybrid assay. Positive interactions were detected in the strains expressing AD-Cwc1 and BD-Cwc2, or AD-Cwc2 and BD-Cwc1 (Fig. S8B), as observed previously [32]. In contrast, no physical interaction was detected between Ssn8 and Cwc1/Cwc2 as no growth was observed in the strains containing AD/BD-Ssn8 and AD/BD-Cwc1/Cwc2 in combination (Fig. S8B). Control strains containing one C. neoformans gene and one empty vector also failed to grow. The yeast two hybrid analyses indicated that Ssn8 does not directly interact with Cwc1 or Cwc2.

We further determine the relationship between Ssn8 and Cwc1 by epistatic analysis. Strains containing SSN8 and CWC1 deletion, their respective overexpression, as well as their combined double mutants in the MATa and MATa background were tested in the slide mating assay. The timing and level of filamentation in the middle parts of diluted mating mixtures were recorded and compared. At 18 h post incubation, the bilateral mutant crosses of ssn8 (Fig. 10d) and cwc1 (Fig. 10h) showed more profuse filaments than the wild-type cross (Fig. 10b). At 9 h post incubation, however, filaments appeared in the ssn8 unilateral or bilateral mutant cross (Fig. 10c; data not shown), while few or no filaments were produced in the wild-type (Fig. 10a) and cwc1 mutant crosses (Fig. 10g). The bilateral cross of the SSN8 overexpression strain showed few filaments (Fig. 10f), whereas no filaments (one filament occasionally observed in one field) were seen in the unilateral cross of the MATa CWC1 overexpression ssn8 mutant (Fig. 10i). Cross between the MATa CWC1 overexpression ssn8 mutant and MATa ssn8 mutant (Fig. 10f) exhibited less filamentation than the ssn8 bilateral mutant cross (Fig. 10d). These results confirmed that mutation of SSN8 suppresses the light-dependent CWC1 overexpression phenotype and Ssn8 does not function upstream of Cwc1.

Three additional pieces of evidence suggested that Ssn8 functions in one major branch downstream of the Cwc complex.
First, the bilateral cross of the cwc1 SSN8 overexpression strains (Fig. 10n) showed slightly more filaments than that of the SSN8 overexpression bilateral cross (Fig. 10f). Second, filamentation level in the cross between the MATa cwc1 ssn8 mutant and MATa cwc1 mutant (Fig. 10p) was also slightly lower than that of the cwc1 bilateral mutant cross (Fig. 10h). Third, the ssn8 mutants were still responsive to light and their mating filaments were inhibited by light (data not shown). Based on the results from yeast two-hybrid and epistasis studies, we conclude that Ssn8 does not interact with the Cwc1 or Cwc2 protein and, instead, Ssn8 possibly functions in one major filamentation pathway downstream of the Cwc complex.

### Discussion

The Mediator complex is highly conserved among diverse organisms and is required for transcriptional regulation in eukaryotic cells [2,4,5,42]. We studied light-regulated sexual development in C. neoformans and identified SSN8 as a gene for a C-type cyclin Mediator whose mutation suppresses the CWC1 light-dependent overexpression phenotypes [34]. In this study, we further characterized the roles of Ssn8 in details and found that this Mediator protein is important for diverse physiology in C. neoformans. Deletion of SSN8 leads to pleiotropic phenotypes, including enhanced heterothallic a–a mating and same-sex mating responses, increased production of melanin and capsule, invasive growth, modified cell wall structure and integrity, and compromised virulence toward its mammalian host. Expression studies also reveal that genes involved in these processes are regulated by Ssn8. These results suggest that Ssn8 serves predominantly as a negative regulator in different physiological and differentiation processes in C. neoformans.

C. neoformans can undergo two types of sexual differentiation, heterothallic a–α mating and same-sex mating, and they have many features in common. They occur under similar conditions and share common signaling components including Cpk1 MAPK-mediated pheromone signaling pathway [30,37]. It is also known that blue light inhibits both mating filamentation processes [32,33]. Based on characterization of mutant phenotypes and the studies of gene expression in related strains under different conditions, we suggest that SSN8 functions as a negative regulator in both processes. As for the mating phenotypes, the ssn8 mutant strains exhibit enhanced mating responses including early onset and profuse production of dikaryotic filaments. In contrast, reduced mating filamentation is seen in the SSN8 overexpression strains (Fig. 2). The ssn8 mutant phenotypes correlate well with derepression of sex-related genes under mating conditions (Fig. 4Aa and b). The absence of Ssn8 in the mating cells causes early derepression of mating genes and consequently early appearance of filaments and better heterothallic a–a mating response are seen on V8 medium. Therefore, Ssn8 negatively regulates a–α opposite sex mating process. Furthermore, Ssn8 plays a dominant role in repressing same-sex mating process. The MATa and MATα ssn8 mutants both show dramatic same-sex mating, whereas overexpression of SSN8 blocks this process (Fig. 3A). Genes related to this same-sex mating process, especially the constitutively active
In YPD rich medium, genes involved in sexual development are normally repressed in the wild-type cells. Mutation of the **SSN8** gene releases repression, and sex-related genes including **GPB1**, **GPB2**, **MFα** and **SXI1** are expressed (Fig. 4C; Table S1). However, successful mating between *ssn8* mutants of opposite mating types cannot occur under nutritional rich conditions (data not shown), suggesting additional signals or regulatory mechanisms are required for proper sexual development. In this study, we also examined the utilization of different carbon sources and found that *C. neoformans ssn8* mutants show growth defect on galactose medium, suggesting Ssn8 plays a positive role in galactose utilization. This is also in accord with the finding in *S. cerevisiae*, in which **SXX0** positively regulates the transcription of **Gal** genes [13,14]. Furthermore, we found the transcript levels of two carbon utilization related genes were also elevated in the *ssn8* mutants under nutritionally rich condition (Table S1). Based on these results, we believe that *C. neoformans* Ssn8 is involved in the regulation of carbon utilization; nutritional repression of sexual development may at least partly be mediated by Ssn8 and its levels in mating cells may also play critical roles for proper sexual response.

**C. neoformans** SSN8 gene was identified in our suppressor screen for possible components downstream of the Cwc complex in the blue light-inhibited sexual process [34]. We also determined the relationship between Ssn8 and Cwc complex as well as other known components in the pheromone signaling pathway by epistasis and gene expression studies (Fig. 5 and 10). We found that a mutation of **SSN8** in the *gpb1* and *cpk1* mutant background suppresses their sterile mating phenotypes and that the *ssn8*mut*ste12* double mutant also produces filaments similar to the wild-type strain, but more than the *ste12* mutant alone (Fig. 5A). Similar observations were also found in same-sex mating (Fig. 5B). Because Ssn8 has been shown to function as a global regulator, we cannot exclude the possibility that Ssn8 may regulate sexual development at multiple steps in the pheromone signaling pathway in *C. neoformans*. Our findings strongly suggest that Ssn8 plays critical roles downstream of the Cpk1 MAPK cascade and of the transcription factor Ste12. De-repression of sex-related genes in the *ssn8* mutants may be caused by first de-repressing a transcription factor downstream of Cpk1 or Ste12. Furthermore, direct physical interaction between Ssn8 and Cwc1/Cwc2 has not been detected by yeast two hybrid assay (Fig. 5A). Similar observations were also found in same-sex mating (Fig. 5B). Phenotypic comparison of strains with *CWC1/SSN8* backgrounds indicated that Ssn8 also possibly regulates sexual development at one of major branches downstream of the Cwc complex (Fig. 10).

In *U. maydis*, Prf1 is a key transcription factor that binds specific pheromone response element in the promoter region of mating related genes. Prf1 is regulated by the MAPK and cAMP-PKA pathways both at the transcriptional and post-translational levels [45,46]. A recent study has indicated that the *U. maydis* Ssn8 homologue functions downstream of Prf1, but is regulated independently by kpp2/juc7, which are *C. neoformans* CPK1/STE7 homologues [47]. In other words, *U. maydis* Ssn8 can be induced by Prf1, which is activated through an unknown kinase rather than by Kpp2 or other activation mechanisms. *C. neoformans* and *U. maydis* are evolutionarily related basidiomycetes. However, no role in sexual development has been revealed by the disruption of the *C. neoformans* **PRF1** homologue [48]. Instead of **PRF1**, a recent study has identified two transcription factors, Mat2 and Znf2, which function downstream of the Cpk1 MAPK cascade and regulate the pheromone response and hyphal development [48]. Furthermore, the expression of **CPR2** also depends on **MAT2** [43].

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**Figure 9. The ssn8 mutant is attenuated for virulence.** (A) Groups of ten C57/LB/6 mice were infected with the MATα wild-type, ssn8 mutant, or the reconstituted strain and the survival of mice was monitored daily. The wild-type and reconstituted strains caused total morbidity in less than 70 days, whereas only one mouse was dead in the ssn8 mutant group. (B) Groups of three C57/LB/6 mice were infected with 10⁶ yeast cells of the MATα wild-type, ssn8 mutant, or the reconstituted strain via lateral tail vein injection for tissue burden studies. The tissues of brain, liver, lung and CSF were recovered after 24-hour infection. Quantitative fungal burden was performed by plating the tissue homogenates or CSF on YPD medium. The culture data from each sample was averaged and analyzed with a paired Student’s t test. Data expressed as CFU were means of triplicate of the inoculated plates ± SD from two experiments (P<0.025). Notice that they are in different y-scales.

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pheromone receptor-like gene **CPR2**, are also de-repressed in the *ssn8* mutants (Table S2) [43]. Same-sex mating is a slow differentiation process which requires diploidization in fruiting cells of the same mating type [30]. This unusual mating event has been suggested as being responsible for disease outbreak in Canada [44]. In our study, detailed monitoring of the *ssn8* mutants on microscope slides revealed that monokaryotic filaments are mostly originated from cells with larger cell size (Fig. 3C). Characterization of the ploidy and other features of these large cells may reveal additional details of this differentiation process. Therefore, the *ssn8* mutants may serve as a platform to gain further insights into the nature of the same-sex mating process.
It will be of interest to determine the relationship between Ssn8 and these two regulators.

Invasive growth is thought to be a differentiation event for scavenging nutrients in response to nutritional starvation [49]. *C. neoformans ssn8* mutants show the invasive growth phenotype on rich medium (Fig. 7 and Fig. S7), suggesting that SSN8 regulates the stress responses. In *S. cerevisiae*, nutrition deprivation leads to invasive growth in haploid cells or pseudohyphal growth in diploid cells, both of which allow cells to scavenge limited nutrients [50,51]. In *S. cerevisiae*, STE12, a transcription factor downstream the FUS3/KSS1 MAP kinase cascade, is required for invasive growth [52,53]. *S. cerevisiae ras2* mutants also display reduced invasive growth [54]. Mutation of the *S. cerevisiae SSN8* or *SSN3* genes induces invasive growth in haploid strains [55]. Epistasis analysis suggests that *SSN8* and *SSN3* function downstream of *RAS2* to regulate invasive growth in *S. cerevisiae* [56]. Moreover, *S. cerevisiae* Ssn3 has been found to inhibit filamentous growth by phosphorylating Ste12 when cells are growing in rich medium [57], suggesting that nutritional signals may act through the C-type cyclin/CDK pair to regulate filamentous growth. In *C. neoformans*, the *ssn8* mutants display invasive growth on nutritional rich medium and have more severe invasion phenotypes in nutrition limited conditions (Fig. 7 and Fig. S7). The dominant active *RAS1* strain also exhibits invasive growth [58]. Deletion of *C. neoformans STE12* displays a defect in filamentous growth during the monokaryotic fruiting process and no role has been previously noted regarding invasive growth [59,60]. Characterization of the *ssn8ste12* double mutant suggests that *SSN8* functions downstream of *STE12* in the mating processes. We are currently constructing the *ras1ssn8* double mutants and the *SSN8* overexpression mutant in the dominant active *RAS1* background and will use them to determine the relationship between Ssn8, Ras1 and Ste12 in the invasive growth pathway of *C. neoformans*. These results also suggest that Ssn8 is an important switch which connects nutritional cues to developmental programs in *C. neoformans*.

*C. neoformans* ssn8 mutants accumulate more melanin than the wild-type strain (Fig. 8A). Deletion of *SSN8* also results in dramatic de-repression of *C. neoformans LAC1* gene, the major gene responsible for melanin biosynthesis (Fig. 8B). Fungal Ssn8 homologues appear to be evolutionally conserved in regulating

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**Figure 10. C. neoformans Ssn8 possibly functions at one of the major branches downstream of the Cwc complex.** Epistasis analysis of the CWC1 and SSN8 genes was conducted by observing the mating phenotype on strains indicated on slide cultures under light condition. Photos were taken in the middle of the mating mixtures at 9 h (a, c, e, i, k, m, and o) and 18 h (b, d, f, h, j, l, n, and p) post incubation. Photos were taken at 100× magnification under a microscope. doi:10.1371/journal.pone.0019162.g010
secondary metabolism including pigmentation and toxin production. *F. verticillioides* FCC1 and *F. graminearum* CID1 are both required for mycotoxin production, fumonisin and DON toxin respectively [25,27]. Deletions of both genes also results in the accumulation of pigments and the gene expression in related processes is also altered. *F. verticillioides* Fck1, the Cyclin-dependent kinase partner of Fcc1 and *S. cerevisiae* Sfn3 homologue, has also been shown to coordinately regulate these processes with Fcc1 [26]. How Sfn8 is involved in the regulation of secondary metabolism, via a global or specific mechanism, requires further investigation.

In addition to melanin, the production of polysaccharide capsule is also negatively regulated by *SSN8*. The *C. neoformans* ssn8 mutants show a larger capsule size than the wild-type strain (Fig. 8C). However, the ssn8 mutant shows attenuated virulence in a murine model (Fig. 9A). Similar findings have been described in an *in vivo* screen of signature-tagged mutants [61]. In this study, several *C. neoformans* genes including *SSN8* are predicted to be required for virulence due to their reduced STM scores. The question of how Sfn8 contributes to the virulence is still an unresolved issue. One possible explanation for the virulence defect is that ssn8 mutant cells may have difficulty in crossing the blood brain barrier due to their elongated cell morphology, or may be defective in putative brain predilection factor(s), which is yet to be demonstrated. Interestingly, it has been found that *C. neoformans* pseudohyphal structure can be induced by co-culturing with soil amoebae and the pseudohyphal strains are also attenuated in virulence [62]. Antigenic modifications in these cells have been suggested to evoke a stronger immune attack from the host [63]. Our studies have shown that ssn8 mutants display abnormal cell morphology and sensitivity to Congo red medium (Fig. 6). The deformed ssn8 mutant cells resemble pseudohyphal structures. This unusual cell morphology has also been described in the *C. neoformans* rom2 deletion strain [64]. *ROM2* has been identified as a gene that is required for virulence in a nematode-based screen [65]. *ROM2*, the guanyl nucleotide exchange factor (GEF) of Rho1, is a regulatory component in the PKC-cell wall pathway. Rom2 can activate Pkc1 by turning Rho1 into the GTP-binding activated form that mediates the formation of cell wall structure. The rom2 mutant displays normal virulence traits but has sensitivity to Congo red, and its avirulent phenotype has been confirmed in a murine model [65,66]. These authors suggest that cell wall defect may be linked to the avirulence phenotype. Interestingly, Sfn8 has been shown to be regulated by Ste2 in the conserved PKC pathway in *S. cerevisiae* [22,23]. Chitosan and β-1,3-glucan are both present in *C. neoformans* cell wall [38,67]. Our studies using TEM and fluorescent microscopy have revealed that the cell wall organization and the distribution of chitosan and β-1,3-glucan are altered in the cell wall of ssn8 mutant (Fig. 6). A study from another fungal pathogen, *Candida albicans*, suggests that a *SSN8* mutation leads to increased exposure of β-D-glucan, which is normally protected by an outer layer of mannanproteins. Such exposure elicits increased production of proinflammatory cytokines from primary macrophages that leads to enhanced immunity of the host and consequently, the ssn8 mutant may become challenged and quickly lose the battle [68]. Our observations of immunofluorescent staining by anti-β-1,3-glucan antibody have revealed that β-1,3-glucan in the cell wall is also masked and not stained in the *C. neoformans* wild-type cells. In contrast, the ssn8 mutant cells are heavily stained, suggesting that increased exposure of the β-1,3-glucan may also occur. Based on these results, we suggest that a mutation of *SSN8* causes modified cell wall structure and integrity that the mutant becomes increasingly immunity challenged and reduced fitness and consequently the ssn8 mutant shows the attenuated virulence.

In summary, we have demonstrated that Sfn8 is an important negative regulator in both heterothallic a·α mating and same-sex mating processes. Our studies indicated that Sfn8 possibly functions at one of the major branches downstream of the Cwc complex and also plays critical roles downstream of the Cpk1- MAPK and Ste12 in the light-mediated sexual response pathway. Interestingly, Sfn8 acts as a global regulator in other physiological processes including virulence of this human pathogen. Our findings have also provided evidence that the member of the free module in the Mediator complex plays crucial roles in *C. neoformans*.

### Materials and Methods

#### Strains and Growth Conditions

*Cryptococcus neoformans* congeneric serotype D strains JEC20 (*MATa*), and JEC21 (*MATa*) were used as the wild-type strains [69]. The auxotrophic derivative strains such as JEC34 (*MATa ura3*), JEC43 (*MATa ura3*), and others were also used in this study [70]. All the strains utilized and generated in this study are listed in Table 2. *C. neoformans* strains were routinely cultured on YPD (1% yeast extract, 2% peptone and 2% dextrose) medium at 30°C. Synthetic media (SD) lacking specific nutrient supplement were utilized for genotype verification and selection of transformants [71]. V8 agar medium was used for heterothallic a·α mating assay [72]. Filament agar (FA, pH 5.0) was used for same-sex mating assay [73].

#### Sequence Alignment of the Ssn8 Homologues and Generation of the Phylogenetic Tree

The Sfn8 homologues were identified using the BLASTP program against the NCBI GenBank database. The amino acids sequences were downloaded and subjected to sequence analyses. Sequence alignments and comparisons were conducted by ClustalX program [74]. Phylogenetic tree based on the maximum likelihood method was constructed by submitting to the on-line program provided by the Tree-Puzzle website (http://mobyle.pasteur.fr/).

#### Complementation of the *S. cerevisiae* ssn8 mutant

The cDNA of *C. neoformans* SSN8 gene was subcloned into pYES2 yeast expression vector to generate pYES2::CaSSN8, which *SSN8* was expressed under control of the *GAL1* promoter. The pYES2::CaSSN8 and pYES2 constructs were delivered into YNL025C by biolistic transformation. Transformants were selected on SD medium lacking uracil and screened by PCR.

To examine the complementation phenotype, yeast cells were incubated in liquid SD medium lacking uracil and screened by PCR.

#### Disruption and Reintroduction of the *C. neoformans* SSN8 Gene

To disrupt the *C. neoformans* SSN8 gene, an *ssn8::NAT* mutant allele previously constructed was used for transformation [34]. The disruption construct was delivered into the wild-type and other strains by biolistic transformation [75]. Transformants were selected on YPD medium with 100 µg/ml nourseothricin and verified by PCR and Southern blot analysis.

To generate the SSN8 reconstitution construct, a 5810 bp genomic fragment containing the SSN8 open reading frame and...
Table 2. Fungal strains used in this study.

| Strain   | Description | Reference |
|----------|-------------|-----------|
| Cryptococcus neoformans | MATa WT | [69] |
| JEC20    | MATa WT    | [69] |
| JEC21    | MATa ura5  | [70] |
| JEC34    | MATa ura5  | [70] |
| WSC129   | MATa gpd1::URA5 ura5 | [35] |
| RDC23    | MATa ste12::URA5 ura5 | [37] |
| RDC5     | MATa cpl1::ADE2 ade2 | [37] |
| CSB7     | MATa ste20::URA5 ura5 | [82] |
| YKC7     | MATa cwc1::URA5 ura5 | [33] |
| YKC9     | MATa cwc1::URA5 ura5 | [33] |
| YKC38    | MATa pGPD1::CWC1-URAS ura5  | [34] |
| YSC1     | MATa ssn8::NAT + pGPD1::CWC1-URAS ura5 | [34] |
| YSC2-1   | MATa ssn8::NAT (MATa ssn8-1) | This study |
| LIC1     | MATa ssn8::NAT #157 (MATa ssn8-2) | This study |
| YSC2-2   | MATa ssn8::NAT (MATa ssn8-3) | This study |
| LIC2     | MATa ssn8::NAT #9 | This study |
| YSC3-1   | MATa ssn8::NAT (MATa ssn8-1) | This study |
| LIC3     | MATa ssn8::NAT #99 (MATa ssn8-2) | This study |
| YSC3-2   | MATa ssn8::NAT | This study |
| LIC4     | MATa ssn8::NAT #19 | This study |
| YSC9     | MATa pGPD1::SSN8::URA5 ura5 | This study |
| YSC10    | MATa pGPD1::SSN8::URA5 ura5 | This study |
| YSC11    | MATa ssn8::NAT + SSN8::HYG | This study |
| YSC12    | MATa ssn8::NAT + SSN8::HYG | This study |
| YSC7-1   | MATa cwc1::URA5 ura5 ssn8::NAT | This study |
| LIC5     | MATa pGPD1::CWC1-URAS ura5 ssn8::NAT | This study |
| LIC6     | MATa pGPD1::CWC1-URAS ura5 ssn8::NAT | This study |
| LIC7     | MATa gpd1::URA5 ura5 ssn8::NAT #7 | This study |
| LIC8     | MATa gpd1::URA5 ura5 ssn8::NAT #8 | This study |
| LIC9     | MATa ste20::URA5 ura5 ssn8::NAT #10 | This study |
| LIC10    | MATa ste20::URA5 ura5 ssn8::NAT #20 | This study |
| LIC11    | MATa ste12::URA5 ura5 ssn8::NAT #57 | This study |
| LIC12    | MATa ste12::URA5 ura5 ssn8::NAT #21 | This study |
| LIC13    | MATa cpl1::ADE2 ade2 ssn8::NAT #7 | This study |
| LIC14    | MATa cpl1::ADE2 ade2 ssn8::NAT #8 | This study |
| LIC15    | MATa cpl1::ADE2 ade2 ssn8::NAT #9 | This study |
| LIC16    | MATa pGPD1::CWC1-URAS ura5 ssn8::NAT + SSN8::HYG | This study |
| LIC17    | MATa pGPD1::CWC1-URAS ura5 ssn8::NAT + SSN8::HYG | This study |

Examination for Cell Morphology

To examine cell morphology, C. neoformans strains were cultured in 50 ml YPD liquid medium at 30°C for 2 days. Ten microliters of cell suspension was taken out, spotted onto haemacytometer, and examined under a microscope (Olympus BX41). Deformed cells were counted and the deformation rate was expressed as the number of deformed cells divided by the total cell number.

To observe the distribution of chitosan in the cell wall, a described staining method was followed [38]. Briefly, the tested strains were grown in different liquid media at 30°C for 2 days, and 0.5 ml of cell suspension was centrifuged, washed once with sterile water, and resuspended in 0.5 ml McIlvaine’s buffer (pH 6.0). Thirty microliters of eosin Y solution (5 mg/ml, Sigma) was added into cell suspension, gently shaken at 30°C for 30 min, and 0.5 ml trypan blue solution (0.4%, Sigma) was then added and incubated for another 10 min. Finally, the stained cells were washed and resuspended in 0.3 ml McIlvaine’s buffer. Trypan blue and eosin Y staining were respectively examined by brightfield and fluorescence illumination under a confocal microscope (A1R, Nikon).

Heterothallic α-α mating and Same-Sex Mating Assays

Two types of mating assays, plate and slide mating, were conducted. Strains subjected to mating assays were first streaked on YPD agar and grown at 30°C for 2 days. Single yeast colonies were picked and resuspended in sterile water. Strains of opposite mating type were mixed in equal amount for the heterothallic α-α mating assay, otherwise, cell suspensions of single mating type flanking regions was amplified by primers WC530 and WC469. The PCR product was purified, digested by XbaI and XhoI, and cloned into pJAF15 [76], which contains the hygromycin selectable marker. The SSN8 reconstitution plasmid was biolistically transformed into the ssn8 mutant strains in both MATa and MATα mating types. Transformants were selected on YPD with 200 µg/ml hygromycin, re-confirmed the nourseothricin resistance and finally verified by PCR and Southern blot analysis. Primers used in this study are listed (Table S2).

Overexpression of the C. neoformans SSN8 Gene

To generate the C. neoformans SSN8 overexpression construct, a 2.0 kb fragment containing the SSN8 open reading frame and 3’-flanking terminator sequences was amplified by primers WC415 and WC446. The fragment was digested with BamHI and Smal, and subcloned into the pYKL8, which contains the C. neoformans GPD1 promoter and URA5 selectable marker [33]. The plasmid was sequence-verified and transformed into JEC34 (MATa ura5), JEC43 (MATα ura5), and other strains by biolistic transformation. Uracl prototrophic transformants were picked from SD plate lacking uracil, screened by PCR amplification, and finally verified by Northern blot and real-time PCR analyses.

Northern Blot and Real-Time PCR Analyses

Total RNA was extracted from the C. neoformans samples using TRIzol total RNA isolation reagent (Invitrogen). Protocols for Northern blot hybridization were conducted as previously described [77]. Northern hybridization probe for the pheromone genes was amplified by PCR primers WC572 and WC573.

Real-time PCR analysis was conducted according to the manufacturer’s instructions (Applied Biosystems). Relative gene expression level was normalized with the constitutively expressed C. neoformans GPD1 gene. Primers used for real-time PCR analyses are listed (Table S2).

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strain were directly conducted for the same-sex mating assay. Cells were cultured at 26°C in the light and dark. For plate mating assay, cell suspension was spotted onto V8 agar/FA plate. For slide mating assay, thin film of V8 agar/FA was made on microscope slides. Cell suspension was added at one side of the slide and the slide was then tilted to make the mixture spread across the V8 agar/FA film. Mating filamentation and fruiting structures were observed and photographed under a microscope (Olympus BX41).

Melanin Production Assay

Strains subjected to melanin assay were first cultured in 5 ml YPD liquid medium and grown at 30°C for overnight. Cells were harvested by centrifugation, washed twice with sterile water, and finally resuspended in sterile water. Five microliters of cell suspension from each tested strain was spotted on asparagine salt agar with L-DOPA (100 mg/l) and kept in the dark at 26°C [78]. Melanization of the colony was checked after 2–5 days and recorded by a digital camera (Sony DSC-S85). Strains subjected to the study of LAC1 gene expression were initially grown overnight in 5 ml YPD liquid medium. Cells were collected, washed twice with sterile water, and transferred to asparagine broth medium (0.1% glucose). Cultures were further incubated at 30°C until mid-log phase. Cells were then harvested, repeatedly washed, transferred to asparagine broth medium without glucose, and grown for additional 2 hours to induce LAC1 gene expression [79].

Phenotypic and Quantitative Analyses of Capsule Formation

To examine capsule formation, fresh single yeast colony of tested strains from YPD plate was inoculated in 5 ml 0.1× Sabouraud medium buffered with 50 mM MOPS for capsule induction [80]. Cultures were grown at 30°C for 3–4 days and the formation of capsule was examined by negative staining with India ink under a microscope (Olympus BX41). To conduct the quantitative analysis of capsule formation, same growth procedure was followed. The thickness of capsule layer around individual cell was manually measured. The relative ratio of capsule size was expressed as the difference in diameter between the whole cell and cell body without capsule divided by the diameter of the whole cell. Thirty randomly selected cells were measured for each tested strain and statistical analysis was performed by Fisher's LSD method.

Sample Preparations for Gene Expression Analyses

Strains subjected to gene expression analyses were first grown on YPD agar for 2 days. For YPD culture, single colony was inoculated in 5 ml YPD liquid medium and incubated overnight at 30°C. Then 1.5 ml culture was inoculated into 13.5 ml YPD liquid medium and grown at 30°C for additional 22 hours. Cells were harvested and immediately frozen in liquid nitrogen for RNA extraction. For same-sex mating samples from filament agar, single colony was inoculated in 5 ml YPD liquid medium and incubated overnight at 30°C. The whole culture was transferred into 45 ml YPD liquid medium and grown with agitation at 30°C for an additional 22 hours. Cells were harvested, washed once with sterile water, and resuspended in 12.5 ml sterile water. Cell density was counted and adjusted to 10^8 cells/ml. Then 20 μl of cell suspension was spotted onto FA medium and a total of 10 spots were added on each plate. Plates were incubated at 26°C in the dark for up to 24 hours. Cells were harvested from the plates of the plates at 3, 6, 9, 12 and 24 hours post incubation and immediately frozen in liquid nitrogen for RNA extraction.

Yeast Two Hybrid Assay

Yeast two hybrid assay was followed as described [81]. The cDNAs of SSN8, CWC1, and CWC2 were amplified and cloned into pGAD-C1 and pGBDU-C1 respectively. All the inserted sequences were confirmed by sequencing. The respective prey and bait constructs with a different insert as well as the empty vectors were co-transformed into the yeast strain, PJ69-4A, to detect the interaction. Transformants were selected on SD medium lacking uracil and leucine to confirm the successful transformation of the two constructs. Yeast two hybrid assay was conducted on SD medium lacking uracil, leucine and histidine medium but supplemented with 50 mM 3-Amino-1,2,4-triazole (3-AT) to detect positive interaction.

Generation of the Double Mutant Strains for Epistasis Analysis

The snn8 disruption construct with nourseothricin selection marker was biolistically transformed into the cwc1 and gpb1 mutant strains, as well as into the CWC1 overexpression strain. Transformants were selected on YPD medium with 100 μg/ml nourseothricin and verified by PCR. Other double mutants, including ste12snn8, ste20snn8, cwk1snn8, and SSN8 overexpression in the cwc1 mutant background, were generated by crossing the strains with appropriate genotypes. Progeny with expected genotypes were screened by PCR and verified by growing on selective media.

Immunofluorescent Study

The strains subjected to immunofluorescent observation were cultured in YPD liquid medium for 4 days at 30°C. Five hundred microliters of cells were collected and washed twice with PBS/0.1% BSA, and followed by staining with 20 μg/ml of anti-β-1,3-glucan antibody (Biosupplies Inc., Parkville, Australia) at 25°C for 3 hours. Cells were then briefly washed with PBS/0.1% BSA three times and further incubated with 10 μg/ml Cy3-labeled goat-anti-mouse secondary antibody at 25°C for 2 hours. After washing with PBS/0.1% BSA, stained samples were observed under DeltaVision Core deconvolution microscope (Applied Precision).

Transmission Electron Microscopy

The C. neoformans strains subjected to TEM examination were first cultured in YPD liquid medium for overnight. Cells were collected by centrifugation at 10,000 g for 30 min and fixed overnight in 2.5% glutaraldehyde at 4°C. Cells were then washed with 0.1 M phosphate buffered saline (PBS) three times, and further subjected to postfixation in 1% osmium tetroxide for 80 min. After being washed in 0.1 M PBS three times, cell samples were dehydrated in a graded series of ethanol and finally washed in 100% ethanol three times. Cells were embedded in resin, and 70 nm thin sections were prepared. Samples were examined and photographed in JOEL JEM-1400 electron microscope.
Animal Virulence Assays

Mouse survival measurements were performed with 6-week old C57LB/6 mice. Three groups of 10 mice were infected with a total of 10^6 yeast cells of the MATa wild-type, ssn8 mutant, and reconstituted strain via lateral tail vein injection, individually. Survival was monitored daily, and moribund mice or those in pain were sacrificed by CO2 inhalation. The survival of groups of 10 infected mice was determined for the infected mice.

For the analyses of tissue burden and cell load in the cerebrospinal fluid, three groups of three 6-week old C57LB/6 mice were infected with a total of 10^5 yeast cells of the MATa wild-type, ssn8 mutant, and reconstituted strain via lateral tail vein injection. After 24 hours infection, mice were euthanized which was conducted by anesthetizing i.p. by ketamine and xylazine (100 mg per body weight, respectively) and exanguinating by cardiac puncture. The cerebrospinal fluid was drawn, and the brain, lung and liver tissues were harvested. Organ tissues were resuspended in PBS and homogenized. Quantitative cultures were performed by plating dilutions of the tissue homogenates and cerebrospinal fluid on YPD plates.

Supporting Information

Figure S1 Phylogenetic analysis and sequence alignment of the C. neoformans Ssn8 and related homologues. (A) Phylogenetic tree was constructed by the maximum likelihood method based on the protein sequences of the Ssn8 homologues. The names of the organisms were abbreviated and the gene name or locus number for each homologue was indicated. Af, Aspergillus fumigatus; An, Aspergillus nidulans; Ca, Candida albicans; Cc, Coprinopsis cinerea; Ce, Caenohabditis elegans; Cn, Cryptococcus neoformans; Dm, Droshila melanogaster; Gm, Gibberella moniliformis; Hs, Homo sapiens; Kf, Kluyveromyces lactis; Lb, Lodderomyces elongisporus; Le, Laccaria bicolor; Mg, Magnaporthe grisea; Ml, Malassezia globosa; Nc, Neurospora crassa; Sc, Saccharomyces cerevisiae; Um, Usitaga maydis; Xl, Xylopus laevis. Numbers above each branch are bootstrap values based on 1000 replications. (B) Amino acid sequence alignment of the cyclin domain among the Ssn8 homologues. (TIF)

Figure S2 The C. neoformans SSN8 gene complements the florocation phenotype of the S. cerevisiae ssn8 mutant. Yeast strains were grown in SD medium containing 2% glucose (Lane 1–2) and SD medium lacking uracil containing 2% glucose (Lanes 4 and 6) or 2% galactose (Lane 3 and 5) at 30°C overnight. The cultures were mixed and photographed after 90 min. Yeast strains: 1, wild-type (BY4742); 2, ssn8 mutant (YNL025C); 3, 4, ssn8 mutant+YPE2; 5, 6, ssn8 mutant+pYES2::CaSSN8. (TIF)

Figure S3 Deletion of the C. neoformans SSN8 gene in the wild-type strains, and C. neoformans ssn8 mutant strains display no growth defect under high temperature conditions. (A) An ssn8::NAT disruption allele was generated by replacing the SSN8 coding region with the NAT selectable marker [34]. (B) Southern hybridization was conducted to verify the ssn8 mutants and reconstituted strains. Genomic DNA from the MATa wild-type (Lane 1), ssn8 mutants (Lane 2–4), and reconstituted strain (Lane 5) and the MATa wild-type (Lane 6), ssn8 mutants (Lane 7–9), ssn8 mutant with ectopic integration (Lane 10), and reconstituted strain (Lane 11) was digested with HindIII and hybridized with the probe as indicated in (A). (C) The relative expression levels of Ssn8 in the MATa strains as indicated were detected by quantitative real-time PCR and normalized with the C. neoformans GPD1 gene. (D) The 10-fold diluted cells were spotted on YPD medium. Plates were kept at high temperature as indicated. (TIF)

Figure S4 Early formation of dikaryotic filaments is observed in the bilateral ssn8 mutant cross. (A) The ssn8 bilateral mutant cross displayed early formation of mating filaments compared to the wild-type cross. Mating assay was conducted on V8 plate under light condition. Photos were taken at different time points. (B) The mating reactions of strains as indicated were conducted on V8 plates and photos were taken at 18 h post incubation under light condition. (TIF)

Figure S5 Deletion of SSN8 results in modified cell wall structure. The ssn8 mutant cells exhibited rugged surface (d) compared to smooth surface of the wild-type cells (a) in YNB medium. High proportion of the ssn8 mutant cells (c) were heavily stained by trypan blue, but only few in the wild-type cells (b). Uniform staining by eosin Y was found in the wild-type cells (c); whereas, irregular patches of eosin Y staining was observed in the ssn8 mutant cells (f). All photos were taken at 400x magnification under a confocal microscope. (TIF)

Figure S6 C. neoformans ssn8 mutant strains exhibit no growth defect under different stress conditions. The 10-fold diluted cells were spotted on YPD medium or YPD media containing different stress reagents, including 3 mM NaNO2 (A), 1 M KCl, 0.03% SDS, and 3 mM H2O2 (B). (TIF)

Figure S7 Deletion of SSN8 induces invasive growth on different media. (A) The ssn8 mutant strains displayed severe invasive growth on V8 medium at 26°C under light condition after 24 h. Colonies were washed off by sterile water and photos were taken. (B) C. neoformans strains as indicated were incubated on filament agar at 26°C for 9 days. The margins of the ssn8 mutant colonies showed more abundant hyphal like than those of the wild-type, reconstituted and overexpression strains. (C) The 9-day fruiting colonies on filament agar were washed by sterile water and photos were taken. The ssn8 mutants showed more severe invasive growth than the wild-type strains. (TIF)

Figure S8 C. neoformans Ssn8 does not directly interact with the Cwc1 or Cwc2 protein. The coding sequences of SSV8, CWC1 and CWC2 were cloned into pGAD-C1 or pGBDU-C1 and cotransformed into S. cerevisiae strain PJ69-4A to test physical interaction. Transformants were streaked on SD-ura-leu medium (A) and SD-ura-leu-his50 mM 3-AT medium (B) and positive interaction was detected only between Cwc1 and Cwc2. (TIF)

Materials and Methods S1

Table S1 The relative expression levels of C. neoformans genes in the wild-type and SSN8-related strains in YPD liquid medium. (DOC)

Table S2 Primers used in this study. (DOC)

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Author Contributions

Conceived and designed the experiments: LIW YSL AJJ WCS. Performed the experiments: LIW YSL KHL AJJ. Analyzed the data: LIW YSL KHL AJJ WCS. Contributed reagents/materials/analysis tools: AJJ WCS. Wrote the paper: LIW AJJ WCS.
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