Abstract

Qsp1 is a secreted quorum sensing peptide required for virulence of the fungal meningitis pathogen Cryptococcus neoformans. Qsp1 functions to control cell wall integrity in vegetatively growing cells and also functions in mating. Rather than acting on a cell surface receptor, Qsp1 is imported to act intracellularly via the predicted oligopeptide transporter Opt1. Here, we identify a transcription factor network as a target of Qsp1. Using whole-genome chromatin immunoprecipitation, we find Qsp1 controls the genomic associations of three transcription factors to genes whose outputs are regulated by Qsp1. One of these transcription factors, Cqs2, is also required for the action of Qsp1 during mating, indicating that it might be a shared proximal target of Qsp1. Consistent with this hypothesis, deletion of CQS2 impacts the binding of other network transcription factors specifically to Qsp1-regulated genes. These genetic and genomic studies illuminate mechanisms by which an imported peptide acts to modulate eukaryotic gene expression.

Author summary

For many fungal pathogens, the ability to adapt to changing and diverse environments forms the basis for their ability to infect and survive inside macrophages and other niches in the human body. These changes are accomplished by transcription factors. Many pathogenic microbes coordinate their gene expression as a function of cell density in a process known as quorum sensing. Here, in the human fungal meningitis pathogen Cryptococcus neoformans, we find that an imported eukaryotic quorum sensing peptide that promotes virulence, Qsp1, controls the binding of three different transcription factors to promoters, thereby modulating the expression of Qsp1-regulated genes. This discovery reveals the mechanism for how an imported peptide affects gene expression.
Introduction

The opportunistic basidiomycete yeast *Cryptococcus neoformans* is the most common cause of fungal meningitis, causing over 200,000 deaths annually [1]. The unique features of this organism that drive its virulence are incompletely understood. In many bacterial pathogens, quorum sensing plays a key role in the regulation of group behaviors and virulence [2,3]. In previous work, we described a peptide-based quorum sensing system in *Cryptococcus neoformans*, the first described in a eukaryote [4]. This system is mediated by an 11 residue peptide dubbed Qsp1, first purified because it complements a low-density phenotype produced by *C. neoformans* lacking a transcriptional co-repressor, Tup1 [5]. Our analysis revealed that Qsp1 is secreted as a pro-peptide that is matured extracellularly by the cell wall-associated serine protease, Pqp1 into a biologically active form [4]. The action of Qsp1 requires an oligopeptide transporter, Opt1 [4]. As cytosolic expression of the mature form of Qsp1 complements the *qsp1Δ* knockout phenotype (a dry colony phenotype), we infer that Qsp1 acts intracellularly after import [4].

In this prior work, we demonstrated that a WOPR domain transcription factor, Liv3, which is related to key regulatory proteins *C. albicans* Wor1 and *H. capsulatum* Ryp1, acts downstream of Qsp1 [4,6–10]. Like cells lacking other components of the Qsp1 system, cells lacking Liv3 display a rough colony morphology phenotype. Cells that lack Qsp1 or Liv3 are also attenuated for virulence in an intranasal mouse model of infection [4,11]. Others have discovered that Qsp1 also regulates unisexual and bisexual mating in *C. neoformans* as well as mating-induced transcription [12]. This function also requires Opt1 and a previously uncharacterized transcription factor, Cqs2, which has also been called Zfc3 [12,13]. The relationships between the roles of Qsp1 in colony morphology, virulence, and mating are not well-understood.

In this paper, we demonstrate that mutants of two transcription factors in addition to Liv3 display a rough colony phenotype when deleted, Nrg1 and Cqs2. By performing a series of transcriptomics experiments, we show that these transcription factors and Qsp1 regulate a common set of target genes. Whole-genome chromatin immunoprecipitation demonstrates that these transcription factors generally bind together to a common set of target genes, forming a highly connected transcription factor network. Significantly, the presence of Qsp1 impacts the binding of all three transcription factors to a subset of target genes which are highly enriched for genes whose expression is controlled by Qsp1. Cqs2 is particularly sensitive to the presence of Qsp1 for its genomic binding. Cqs2 is strongly required for the binding of Nrg1 and Liv3 to Qsp1-regulated genes, suggesting it may be an upstream factor in the pathways. Furthermore, while Qsp1 seems to negatively regulate protein levels of Nrg1 and Liv3, the association of these factors with promoters is still greatly decreased in the *qsp1Δ* mutant. These experiments illuminate the mechanism by which an imported quorum-sensing peptide impacts gene expression.

Results

Phenotypic identification of predicted transcription factors that act downstream of Qsp1

Wild-type yeast form glossy colonies, whereas cells lacking the *QSP1* gene (*qsp1Δ*) exhibit a wrinkled colony morphology phenotype at either 25°C or 30°C [4]. We previously published that the transcription factor Liv3 mediates a large portion of the Qsp1 response in rich media, and that a *liv3Δ* knockout strain forms dry, wrinkled colonies at 30°C. We hypothesized that the deletion of genes encoding factors involved in the response to Qsp1 signaling would also exhibit a wrinkled colony morphology. Therefore, we screened strains in a *C. neoformans*
knockout collection generated in our laboratory for genetic candidates. We discovered two additional strains that exhibited a qsp1Δ-like colony morphology that is also temperature-dependent, corresponding to genes encoding the transcription factors Cqs2 and Nrg1. Cqs2 was recently reported as a regulator of the Qsp1 response for unisexual filamentation [12]. Nrg1 is a transcriptional regulator that plays a role in several cellular processes, including carbohydrate acquisition, metabolism, and virulence [14].

In contrast to the qsp1Δ mutant, each transcription factor deletion strain exhibits this phenotype at a more restricted range of temperatures (Fig 1A). Colonies formed by nrg1Δ cells display their strongest phenotype at room temperature, and liv3Δ and cqs2Δ colonies show their strongest phenotype at 30˚C. This dry and wrinkled colony morphology is not caused by an inability of these transcription factor deletion strains to synthesize Qsp1 peptide, as they are still able to secrete wild-type levels of Qsp1 peptide (Fig 1B). Additionally, each transcription factor deletion strain is able to complement a qsp1Δ strain when patched nearby on a plate, due to Qsp1 peptide diffusing through the agar (Fig 1C). In contrast, the colony morphology phenotype of the transcription factor deletion strains could not be complemented by the peptide produced by a wild-type strain (Fig 1C). Therefore, while each transcription factor knockout strain is able to produce Qsp1 peptide, none are complemented by the peptide. This supports the idea that all three of these transcription factors act downstream of Qsp1 production to promote wild-type colony morphology.

Saturated cultures of the qsp1Δ mutant are sensitive to the cell wall stressor SDS, a phenotype that can be rescued by prior growth of the cells in the presence of synthetic Qsp1 peptide [4]. To determine whether these three transcription factors could be involved in Qsp1 signaling in this context, we tested the sensitivity of the corresponding deletion mutants to SDS. We grew each strain to saturation in rich media, then incubated the cells in different concentrations of SDS (Fig 1D). The cells were then plated on YPAD agar to assay for viability following SDS treatment. The liv3Δ strain is not sensitive to SDS treatment, but nrg1Δ and cqs2Δ strains display sensitivity (Fig 1E). Thus, Nrg1 and Cqs2 function to promote resistance to cell wall stress, while Liv3 is dispensable for this phenotype.

To test whether Nrg1, Cqs2, or Liv3 were downstream of Qsp1, we created double knockouts of each transcription factor gene and QSP1 and grew these strains with or without an excess of synthetic Qsp1 peptide for 48 hours (Fig 1F). The qsp1Δnrg1Δ double mutant appears to not be complemented (compare dilutions for SDS sensitivity vs. dilutions for culture titer), and the qsp1Δcqs2Δ double mutant is unable to respond to peptide. The SDS sensitivity of the qsp1Δliv3Δ mutant could be rescued by prior growth in synthetic Qsp1, indicating that Liv3 is not required for the ability of Qsp1 peptide to promote SDS resistance (as expected from the lack of SDS sensitivity in the liv3Δ strain). These data are consistent with a model in which Cqs2 and Nrg1 function downstream of Qsp1 to promote resistance to a cell wall stress.

RNA-seq analysis reveals shared roles for Qsp1 and the three transcription factors

In previous work, we found that loss of Liv3 significantly impacts the response of cells to Qsp1 [4]. These experiments were performed at a single time point in rich media. To test more broadly media and culture density conditions for subsequent analysis, we collected RNA from either wild type or qsp1Δ mutant cultures grown in either rich media (YPAD) or minimal media (YNB) at an optical density at 600 nm (OD600) of 1, 5 and 10 (Fig 2A). We then performed RNA-seq analysis to identify differentially expressed genes. Over 400 genes were significantly affected by the loss of the QSP1 gene in minimal media at an OD600 of 1 (OD1) or OD600 of 5 (OD5), more genes than in rich media at any culture density (Fig 2B). Therefore,
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A) Temperature effects on colony morphology

B) Western blot analysis of Qsp1

C) Complementation assay results

D) SDS treatment protocol

E) SDS concentration vs. cell input

F) Synthetic Qsp1 addition results
we chose to proceed with OD1 and OD5 conditions in minimal media for the subsequent experiment.

To assess whether Liv3, Nrg1, and Cqs2 were required for the expression of genes involved in the Qsp1 response, we performed RNA-seq analysis on RNA extracted from wild type, qsp1Δ, liv3Δ, cqs2Δ, and nrg1Δ cultures grown to OD1 or OD5 in minimal media. We compared differentially expressed genes from the qsp1Δ mutant and the three transcription factor deletion strains relative to wild type at both timepoints (Fig 2C). The \( P \)-value for the overlap in differentially expressed genes between targets of pairs of transcription factors or Qsp1 was significant under at least one condition, indicating that the targets of these transcription factors overlap with each other, and with Qsp1 targets, more than would be expected due to random chance (Fig 2C). While the Qsp1-dependent gene set consistently overlapped with those dependent on Cqs2 or Nrg1, this was not the case for the Liv3-dependent set (Fig 2C). The latter only significantly overlapped the Qsp1-dependent set for genes derepressed in the mutants at OD1 (Fig 2C). These data reveal strong similarities between the transcript signatures of the qsp1Δ mutant and those of cqs2Δ and nrg1Δ mutants, with only weak similarity to the liv3Δ mutant signature.

To further examine the involvement of these three transcription factors in Qsp1 signaling, each transcription factor was tagged with a FLAG epitope tag and expressed from their native promoters in either a wild type or qsp1Δ mutant background. To confirm that the tags did not affect protein function, we tested that each tagged strain displayed the expected colony morphology (not shown). Additionally, we confirmed that Qsp1 biology was not affected in these strains by verifying that qsp1Δ mutant strains harboring tagged C-terminally tagged Cqs2 and Liv3 and N-terminally tagged Nrg1 were sensitive to SDS in a manner that could be rescued by growing these strains in an excess of synthetic Qsp1 peptide (S1 Fig).

Quantitative immunoblots show a slight reduction in Cqs2 levels between wild type cells and cells lacking the QSP1 gene at OD1 in minimal media, though this difference was not significant (Fig 3A and S1 Table). However, cells lacking QSP1 expressed significantly more Nrg1 and Liv3 protein (Fig 3A and S1 Table). Due to the strong overlaps between genes controlled by Qsp1 and these three factors at OD1 in minimal media, we conducted ChIP-seq under this condition (Fig 3B and 3C, S2 Fig). To quantify transcription factor binding, a ChIP score for each gene was calculated as the sum of the read depth over a 1 kb region upstream of each transcription start site, normalized to the untagged strain. We employed a k-means clustering approach to divide the genes into groups whose promoters were significantly bound or not-bound (See Methods). We found that in wild type, these transcription factors generally bind to the same promoters (Fig 3C and 3D and S2 Table). The majority of the genes bound by any transcription factor are also bound by one or two others, with 274 genes bound by all three transcription factors (Fig 3D). The overlaps between the sets of promoters that are bound by any two of these three transcription factors are highly significant, further supporting the conclusion that these transcription factors are part of a network (Fig 3E and S2 Table).
Fig 2. Cqs2, Nrg1, and Liv3 are part of a transcription factor network that shares targets with Qsp1. A) Schematic of how cultures were grown and harvested for RNA-seq. B) Number of significantly differentially expressed genes in qsp1Δ vs. wild type as determined by DE-seq2 analysis. C) Comparisons of significantly changed, increased, and decreased genes (mut/WT).
Qsp1 affects the binding of all three transcription factors to a common set of promoters

To test whether Qsp1 could influence the binding of these transcription factors, we constructed tagged transcription factor strains that also harbor a knockout of QSP1, and conducted ChIP-seq. In specific regions, binding of the transcription factors is abolished or diminished in the absence of QSP1, indicating that Qsp1 peptide is required for binding of these factors to these promoters (Fig 3C). Our analysis revealed that Qsp1 affects the binding of Cqs2, Liv3, and Nrg1 to a large fraction of bound promoters (Fig 3F, S2 Fig, S3l Fig and S1 Table). We confirmed this by quantifying the amount of binding of each factor to two of the promoters that showed the highest differences by ChIP-seq using ChIP-qPCR (S4 Fig). In the majority of cases, Qsp1 promotes rather than inhibits the binding of a transcription factor to its targets. Overall, we observed a shift to lower levels of binding in the qsp1Δ mutant by Cqs2, with only 7 genes exhibiting an increase in ChIP signal compared to nearly 200 genes exhibiting a decrease in binding. Nrg1 and Liv3 binding increased for the majority of promoters and decreased for others (Fig 3G, S3 Fig, and S2 Table). These data indicate that Qsp1 promotes the binding of Cqs2, and modulates the binding of Nrg1 and Liv3, to upstream regions.

We next sought to understand whether Qsp1 influences the binding of Cqs2, Nrg1, and Liv3 to the same sets of promoters, and whether this influence was positive or negative. We examined the degree of overlap between the sets of promoters that displayed a mean fold-change greater than 1.5-fold in either direction that were called as bound in either wild type or qsp1Δ mutants and tested whether the overlaps between these sets were significant. We observed highly significant ($P < 1x10^{-100}$) overlaps between promoters that displayed lower levels of binding by any two transcription factors in the qsp1Δ mutant, more so than between groups of promoters that are more bound in the qsp1Δ mutant (Fig 3G and 3H, and S2 Table). This indicates that Qsp1 functions to promote the binding of all three transcription factors upstream of a subset of genes. The overlaps between genes that are differentially bound by any two of these three transcription factors in the qsp1Δ mutant are also highly significant (Fig 3F and S2 Table), but most of the significance comes from genes that are less bound in the mutant (Fig 3G and S2 Table).

Cqs2, Nrg1, and Liv3 are transcription factors that bind to DNA and influence gene expression. Therefore, we tested whether the binding of each transcription factor upstream of a gene impacts the expression of that gene. We compared genes bound by a tagged transcription factor in wild type to genes whose expression was affected by loss of the corresponding transcription factor (Fig 4A). Genes that are bound by a transcription factor, but whose expression is not affected by deletion of a transcription factor, are very often the majority, as they are for Nrg1, Liv3, and Cqs2 (Fig 4A). The expression of these genes may be regulated by other signaling inputs that override the presence of Nrg1, Liv3, or Cqs2 at these promoters, such as a repressor, or required in addition to these factors to promote expression. Transcriptional activators are often bound to their target promoters, even in non-inducing conditions, indicating that this is a general feature of transcriptional regulation [15]. Nonetheless, the overlaps between these two sets are significant in all comparisons, supporting the conclusion that these three transcription factors influence gene expression via binding to target genes (Fig 4A and S1 Table).
Genomic binding regulation by imported quorum sensing peptide

A) Protein Levels

B) DAY 1

OD1 → OD5

Inoculate to <0.01 OD

Harvest 50 OD600 units of cells

C) Cqs2-FLAG

D) Nrg1

E) significantly bound by Nrg1

F) differentially bound by Nrg1

G) p = 0.037

H) less bound by Nrg1 in qsp1Δ

less bound by Liv3 in qsp1Δ

less bound by Cqs2 in qsp1Δ

less bound by Cqs2 in qsp1Δ

less bound by Nrg1 in qsp1Δ

42

63

99

p < 1 e-100

PLOS Genetics | https://doi.org/10.1371/journal.pgen.1008744 September 21, 2020 8 / 24
Qsp1 promotes the binding of Cqs2, Nrg1, and Liv3 to promoters, which activates expression of these genes

To test whether the Qsp1-dependent binding events had functional consequences, we next investigated whether the genes whose promoters were differentially bound in a qsp1Δ mutant by each transcription factor were also differentially expressed in a qsp1Δ mutant under a particular condition for all three transcription factors. For all three transcription factors, we observed significant overlaps between genes differentially bound by a transcription factor and genes that were differentially expressed in a qsp1Δ mutant compared to wild type, indicating that the influence of Qsp1 on binding of Cqs2, Nrg1, and Liv3 is important for gene expression (Fig 4B and S2 Table).

To test if there was a relationship between the combination of transcription factors bound and gene expression, we created a heatmap displaying data for genes whose promoters are differentially bound in a qsp1Δ mutant by any transcription factor at OD1, with their corresponding change in expression in a qsp1Δ mutant compared to wild type (S5 Fig and S4 Table). We observed that the largest impact on gene expression occurs for genes whose promoters were much less occupied by all three transcription factors together in the qsp1Δ mutant (Fig 4C, S5 Fig and S2 and S4 Tables). This decrease in occupancy of all three transcription factors in a qsp1Δ mutant compared to wild type corresponds to a significant decrease in expression of about a third of these genes (Fig 4C–4E and S2, S3 and S4 Tables).

From our conservative analysis, there are fourteen genes where Qsp1 promotion of Cqs2, Nrg1, and Liv3 binding correlates with a significant change in expression in the qsp1Δ mutant compared to wild type (Table 1). Five encode predicted transporters of sugars, amino acids, or other types of nutrients. Interestingly, one of the genes encodes Ral2, which is essential for mating in Schizosaccharomyces pombe [16]. Ral2 activates Ras1, a GTPase that is also activated by Ste6, the alpha mating factor transporter and exchange factor for Ras1 [16,17]. Another of these genes encodes Agn1, a putative α-glucanase, and could be related to the cell wall phenotype of qsp1Δ and cqs2Δ mutants [18].

Together, these data indicate that Qsp1 regulates the binding of Cqs2, Nrg1, and Liv3 together to a subset of Qsp1-regulated genes, and the loss of binding of all three of these factors results in altered expression of a set of genes predicted to be involved in nutrient sensing, signaling, and acquisition as well as cell wall remodeling.

Loss of NRG1 or CQS2 affects Liv3, Nrg1, and Cqs2 binding to promoters

To test whether Cqs2, Nrg1, and Liv3 impacted each other’s binding, we attempted to delete the genes encoding the other two transcription factors in the tagged strains described above. We conducted ChIP-seq on FLAG-tagged Liv3, Nrg1, or Cqs2 strains harboring deletions of NRG1 or CQS2 (we were unable to obtain deletions of LIV3) grown to OD1 in minimal media. Immunoblotting demonstrated that no significant difference in the levels of each of these tagged transcription factors in the mutant background compared to wild type (Fig 5A and S5 Table). An example of the binding pattern of Liv3, Nrg1, and Cqs2 in these backgrounds across...
Fig 4. Qsp1 regulation of binding of Cqs2, Nrg1, and Liv3 to promoters correlates with a change in expression. A) Overlap between promoters bound by each transcription factor in wild type and genes that are differentially expressed in the corresponding transcription factor mutant, at OD1 in minimal media. B) Overlap.
part of chromosome 1 is shown (Fig 5B). We calculated a ChIP score for transcription factor binding for each promoter in each strain and plotted these for each gene in each transcription factor mutant versus wild type (S5 Fig and S6 Table). Strikingly, deletion of CQS2 results in reduced Liv3 binding to promoters. 357 genes exhibited a >1.5-fold decrease, and only 3 genes exhibited a >1.5-fold increase in binding (Fig 5C, S6 Fig, S2 and S6 Tables). In contrast, deletion of CQS2 both reduced and increased Nrg1 binding, depending on the promoter. Deletion of NRG1 also dramatically impacted Liv3 binding to targets, again primarily reducing binding. Finally, deletion of NRG1 increased Cqs2 binding to more targets than it decreased.

We sought to understand further the transcription factor network in the context of Qsp1 signaling. To accomplish this, we examined how the loss of NRG1 or CQS2 compared with loss of QSP1 on altering binding of Cqs2, Liv3, and Nrg1 to promoters, by comparing the sets of promoters that were affected by each deletion. Strikingly, almost all of the Qsp1-dependent promoters also exhibit a Cqs2-dependence for binding of Nrg1 and Liv3, and in the same direction (Fig 5D, S7 Fig, and S2 and S6 Tables). In other words, the same group of promoters that exhibit altered Nrg1 and Liv3 binding in a qsp1Δ knockout is also impacted in the same direction in a cqs2Δ knockout. Liv3 and Cqs2 binding to Qsp1-dependent promoters is also significantly regulated by Nrg1, but to a lesser extent (Fig 5D, S7 Fig and S2 and S6 Tables).

<table>
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<tr>
<th>Gene</th>
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<th>Cqs2 binding difference, qspΔ / WT (fold change)</th>
<th>Liv3 binding difference, qspΔ / WT (fold change)</th>
<th>Nrg1 binding difference, qspΔ / WT (fold change)</th>
<th>Expression difference, qspΔ / WT (log2fold change)</th>
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Table 1. Thirteen genes are differentially bound by Cqs2, Liv3, and Nrg1 and differentially expressed in minimal media at OD1.

https://doi.org/10.1371/journal.pgen.1008744.g004

https://doi.org/10.1371/journal.pgen.1008744.t001

https://doi.org/10.1371/journal.pgen.1008744.s007
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Thus, there is a notable overlap between promoters whose transcription factor binding is promoted by Qsp1 and those that display transcription factor interdependencies for binding.

**Discussion**

Single-celled organisms often cooperate in a type of community-oriented signaling called quorum sensing, mediated by the accumulation of secreted autoregulatory molecules. Quorum sensing coordinates cellular adaptations that allow the cells to survive in response to environmental cues, such as in mating, biofilm formation, and host infection. Quorum sensing has been reported in many different microbes to regulate mating and competence [19–23], starvation and mating [24], regulation of sporulation in response to starvation [25], nutrient acquisition and virulence [3]. Our experiments have uncovered that Qsp1 regulates gene expression by influencing three transcription factors that play roles in mating, virulence, and nutrient acquisition, providing insight into the mechanism by which a eukaryotic quorum sensing molecule can influence gene expression and clues to the role that quorum sensing plays in the biology of *C. neoformans*.

Our previous work in *C. neoformans* demonstrated that Qsp1 is secreted as a precursor that is cleaved outside the cell [4]. The mature peptide accumulates in the culture supernatant, then appears to be imported back into the cell by Opt1, where it induces a transcriptional response [4]. This is remarkably similar to some gram-positive bacteria, which also secrete quorum sensing peptides that are imported into the cell via oligopeptide permeases. Once imported, these small peptides interact with phosphatases [26,27] or transcriptional regulators [28–30] to influence gene expression. In *C. neoformans*, the two transcription factors Liv3 and Cqs2 have been identified as regulators of the Qsp1 response [4,12]. In this study, we uncovered Nrg1 as a third Qsp1-regulated transcription factor. We showed that the response to Qsp1 signaling is mediated by a network formed by these three transcription factors (Fig 2), which were identified on the basis of a temperature-regulated rough colony morphology that is also exhibited by a *qpqLA* knockout (Fig 1). Surprisingly, we found that Qsp1 seems to promote the binding of Cqs2 to promoters and alter the binding of Nrg1 and Liv3 (Fig 3). These Qsp1-dependent promoters are shared between all three transcription factors, with the largest and most significant overlaps occurring between promoters to which Qsp1 promotes transcription factor binding (Fig 3). This decrease in transcription factor binding to Qsp1-dependent promoters correlates with a decrease in gene expression in cells lacking *QSP1* compared to wild type (Fig 4). We observed this correlation with reduced binding leading to reduced expression in spite of higher levels of Cqs2 and Nrg1 protein in *qpqLA* mutants in this condition (Fig 3). Furthermore, Cqs2 promotes Liv3 binding to promoters (Fig 5). Additionally, Cqs2 impacts Nrg1 and Liv3 binding on almost all Qsp1-dependent promoters, given the strong overlap between promoters affected for binding of these transcription factors by loss of *QSP1* and loss of *CQS2* (Fig 5). Nrg1 also impacts Cqs2 and Liv3 binding (Fig 5). Qsp1 may influence on Nrg1 and Liv3 binding by promoting Cqs2’s affinity for promoter sites, since Cqs2 appears to be the principal factor through which Qsp1 acts under these conditions (Fig 6). Together, these data support the model that Qsp1 influences gene expression by controlling a network of transcription factors’ ability to bind to DNA (Fig 6). It is unclear if Qsp1 directly binds to Cqs2, or if there is another
unidentified Qsp1-regulated signaling factor upstream of Cqs2 that regulates the affinity of Cqs2 for target promoters. Binding of Qsp1 to a transcription factor could promote binding through a conformational change. Prokaryotic quorum sensing peptides directly bind to transcription factors via the tetratricopeptide repeat (TPR) domain of the regulator, causing conformational changes that alter the activity of the transcription factor, in some cases [26,28–30]. However, Nrg1, Liv3, and Cqs2 do not contain a TPR motif or similar repeats, so if Qsp1 directly binds these transcription factors, it would do so through an as-yet unidentified motif.

Fig 6. Model for how Qsp1 triggers changes in gene expression in *Cryptococcus neoformans*. Following import into the cytoplasm, Qsp1 alters the binding of Nrg1 and Liv3 by modulating the ability of Cqs2 to bind promoters, thereby causing changes to gene expression. Dotted lines indicate functional rather than physical interactions.

https://doi.org/10.1371/journal.pgen.1008744.g006
that has evolved for a similar purpose. One appealing hypothesis is that Qsp1 directly promotes cooperativity in DNA binding of Cqs2, Nrg1 and Liv3 to a subset of promoters that display a particular pattern of binding site spacing, sequences and orientations. It is possible that Qsp1 disrupts interactions in other contexts. Testing these hypotheses will require reconstitution of such binding in vitro using purified proteins.

Cqs2, Nrg1, and Liv3 are transcription factors that play roles in mating, nutrient acquisition and virulence in *C. neoformans*. Qsp1 has recently been shown to signal through Cqs2 to regulate unisexual reproduction and filamentation [12]. Liv3 is required for proliferation in the lung [11]. Liv3 is also a homolog of Wor1, the master regulator of white-opaque switching in *C. albicans*, a functional and morphological switch in phenotype that can be triggered by various environmental cues and determines which area of the body the fungus is best equipped to colonize [6,8,9]. Nrg1 is a transcriptional regulator that promotes bisexual mating and virulence, and plays a role in several cellular processes, including carbohydrate acquisition, metabolism, and capsule formation [14]. Homologs of Nrg1 in other fungi play roles in filamentation, nutrient sensing, and metabolism in response to environmental cues and are also regulated by quorum sensing [31–37]. Here, we show that Nrg1 and Liv3 protein levels are repressed by the *QSP1* gene in minimal media (Fig 3A). We also found that Qsp1 promotes the binding of Cqs2 to promoters and influences the binding of Nrg1 and Liv3, and that these transcription factors influence each other’s binding (Fig 3). These experiments provide a mechanistic basis for quorum sensing control of these factors and further evidence for the implication of quorum sensing in mating and pathogenesis of *C. neoformans*.

It is unclear why Cqs2, Nrg1, and Liv3 transcription factors have been integrated into a quorum sensing system. One possibility is that quorum sensing enables cells to anticipate and prepare for future starvation and associated stresses, which could be critical in particular host niches or when deciding to mate. In prokaryotes, starvation and quorum sensing signaling pathways regulate each other [38]. In *Saccharomyces cerevisiae*, the production of autoregulatory aromatic alcohols is coupled to both culture density and nitrogen starvation, and serves as a species-specific trigger for transformation into a filamentous form [39]. In both bacteria and yeast, it is thought that entry into stationary phase once nutrients are exhausted provides benefits to the cell such as thickening of the cell wall, accumulation of reserve nutrients, and an increased resistance to environmental stressors, allowing the cells to survive long term [38,40]. Integration of quorum sensing and starvation signaling could explain why Qsp1 signaling increases as culture density increases in rich media as nutrients run out, but has the opposite trend in minimal media, where cells are starved immediately (Fig 2). In addition, we found that Qsp1 promotion of resistance of stationary-phase cells to cell wall stress requires Nrg1 and Cqs2 (Fig 1), further solidifying the relationship between quorum sensing and starvation responses.

In line with this idea, one of the promoters that exhibited a very dramatic dependence on Qsp1 for binding of all three transcription factors was the *LAC1* gene (CNAG_03465) (S3 Fig), which encodes the melanization factor laccase [40–42]. Melanization is known to be a key virulence trait for *C. neoformans* infection [40,43]. In our previous work, we found that cells lacking Qsp1 display altered capacities to produce melanin when plated on media containing the substrate for melanin synthesis [4]. Although our conditions did not reveal laccase transcript regulation by Qsp1, it may be that these three transcription factors bind to the laccase promoter in the presence of Qsp1 in order to prime the cell to activate transcription as soon as the correct signals are received.

In conclusion, it seems that these three transcription factors are at the core of a gene regulatory network that integrates Qsp1 signaling with starvation or other unknown signaling inputs to determine which genes to express in different contexts (such as in the host or in different media), ultimately influencing the mating and virulence of this organism.
Methods

Cryptococcal strain construction

Gene deletions were generated using nourseothricin (NAT) resistance, neomycin (NEO) resistance, or hygromycin (HYG) resistance cassettes. Proteins were tagged with 2x-FLAG or 3x-FLAG epitope tags using one of these three resistance cassettes as previously described [41]. Constructs were made via homologous recombination using fragments amplified with the primers in Table 2. Strains constructed in this study are listed in Table 3.

All strains are derived from the KN99alpha (CM26) parent.

Cell wall stress assay

Part of a colony was cultured in 5 ml of YPAD (1% yeast extract, 2% Bacto-peptone, 2% glucose, 0.015% L-tryptophan, 0.004% adenine) for 48 hours, when all cultures were fully saturated. 1 uM pure synthetic Qsp1 peptide (LifeTein) was added to indicated cultures at the time of inoculation. 50 ul of saturated culture was mixed with 150ul of either water or SDS in a series of 1:2 dilutions (starting at 10%) and incubated for 3 hours at room temperature without shaking. The supernatant of settled incubations was partially replaced with water following SDS incubation and prior to cell resuspension to minimize the amount of SDS transferred to the recipient plate, and 3 ul were spotted on YPAD plates containing no SDS to assay survival. Cells incubated in water were then serially diluted 1:6 to provide a measure of the titer of the input culture. Plates were allowed to grow for 4 days at room temperature.

Immunoblots

Two biological replicates from each strain were cultured for immunoblot. 2 OD₆₀₀ units of cells per sample were fixed with 10% TCA, then 100% acetone, then lysed by two 1.5 minute rounds of bead-beating in sample buffer. Samples were then boiled for 5 min and cell debris was spun down. For supernatant analysis, 2 mL of conditioned media were snap frozen and lyophilized overnight, then resuspended in 150 μl of 1x Laemmli Sample Buffer. 5–10 μl of each sample was loaded on 4–12% Bis-Tris gels (Thermofisher).

RNA-Seq and ChIP-Seq cultures

Each strain of C. neoformans was inoculated in YPAD or YNB at 30˚C. The next day, larger cultures were started from the starter cultures at an OD < 0.01. On the following day, as each culture grew in density 50 OD₆₀₀ units of cells were harvested sequentially from the same culture at the indicated ODs.

50 OD₆₀₀’s of cells at each optical density (OD 1, 5, and 10) for each replicate for each strain were harvested sequentially as the cultures grew. For ChIP-seq samples, 50 OD₆₀₀’s of cells were crosslinked in a 50 mL volume of conditioned media from the same culture, harvested at the same time as the cells.

RNA-Seq

Total RNA was isolated from 50 OD₆₀₀’s of cells as previously described [42] and libraries prepared as previously described [43]. In brief, cell pellets were lyophilized overnight and then RNA was isolated using TRizol (Invitrogen) as previously described [42] and DNase treated as previously described [44]. 0.5 µg RNA was then prepared for sequencing using the QuantSeq 3’-mRNA-Seq Library Prep Kit FWD (Lexogen) according to the manufacturer’s instructions. Input RNA quality and mRNA purity were verified by Bioanalyzer Pico RNA chips (Agilent). Libraries were sequenced on the HiSeq 4000 platform (Illumina).
Table 2. Primers used to create genetic constructs to create strains used in this study.

<table>
<thead>
<tr>
<th>qsp1Δneo in pRS316</th>
<th>nrg1Δhyg in pRS316</th>
</tr>
</thead>
<tbody>
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<td>W1_Qsp1KO in DKS strains_95F</td>
<td>c_Nrg1Δ_95F</td>
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<td>c_Nrg1Δ_96R</td>
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https://doi.org/10.1371/journal.pgen.1008744.t002
RNA-Seq analysis

Expression analysis for each transcription factor mutant was performed by counting the number of reads aligned by STAR for each transcript [45]. DEseq2 was used to determine genes differentially expressed between mutant and wild type conditions. Noncoding genes were not included in the analysis. See GEO accession #GSE147378 for raw data.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously [4], with the following changes: 50 OD_{600} units of cells were crosslinked in 50 mL total of conditioned media. Lyophilized pellets were resuspended in 600 ul ChIP lysis buffer with protease inhibitors for bead beating until >95% of cells were lysed. The chromatin pellet was resuspended in 350 ul ChIP lysis buffer for sonication. After sonication and removal of cell debris via centrifugation, the supernatant was brought to 3 ml in ChIP lysis buffer. Immunoprecipitation was performed at 4°C overnight with nutation in 1 ml chromatin aliquots with 3 ul of anti-FLAG M2 antibody (F3165, Sigma) and 20 ul of Protein G Dynabeads (Invitrogen). See GEO accession #GSE147378 for raw data. ChIP-qPCR primers are shown in Table 4.

Table 4. ChIP-qPCR primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Locus</th>
<th>Sequence</th>
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<td>CNAG_03465 promoter (chr8:1,020,374–1,020,622)</td>
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<td>c397F_ChIPqPCR:CNAG_00758_F1 (DKS)</td>
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<td>c8371 qPCR (CEN-Hpy99I)</td>
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</table>

https://doi.org/10.1371/journal.pgen.1008744.1004
ChIP-seq library construction

ChIP-seq library construction was performed as described previously [4] with the following changes: For each genotype, libraries for two biological replicates were prepared. Adaptors were selected out using Sera-Mag SpeedBead Carboxylate-Modified Magnetic Particles (Hydrophobic) and products between 200-500bp were selected by gel extraction. Library quality and concentration were determined by High Sensitivity DNA Bioanalyzer analysis (Agilent) and Qubit (Thermofisher), respectively.

ChIP-Seq analysis

ChIP-seq reads were trimmed with CutAdapt and aligned using Bowtie1 [46]. Up to two mismatches within the seed sequence. If a read could align to multiple loci, it was aligned at random. Indexed, sorted bam files were created for each dataset using SAMtools [47]. Bedgraph files were created using BEDtools [48,49], and normalized to the untagged sample by subtraction. The ChIP signal for each gene in each replicate was calculated as the sum of the read depth over the promoter region, defined as the 1kb upstream of the annotated transcription start site. Using the ChIP signal, genes were clustered into bound or unbound via k-means analysis. There was a high degree of overlap between genes called as bound in either replicate (S7 Table), but genes were only considered as bound in our analysis if it was called as bound in both replicates by k-means analysis (S6 Table). The average of the ChIP signal from both replicates was taken for subsequent analysis. Bedgraphs were plotted using Integrative Genomics Viewer 2.0.30 (Broad Institute). Genes were determined as differentially bound if the fold change in ChIP signal between both conditions was greater than 1.5-fold in either direction (based on [50]).

Statistical analysis

Immunoblot quantification was performed with ImageJ analysis on two biological replicates, and significance was determined using the student’s \( t \)-test. \( P \)-values <0.05 were considered significant. All other \( P \)-values were adjusted (\( P \)-adj) for multiple testing using the Bonferroni correction (S2 Table).

Supporting information

S1 Fig. Tagging Cqs2, Liv3, and Nrg1 does not affect protein function or the ability of \( qsp1\Delta \) mutants to respond to Qsp1. Each genotype shown was tested for their ability to survive increasing concentrations of the cell wall stressor SDS. 1 uM synthetic Qsp1 peptide was added to the indicated cultures (+) from the time of inoculation, or not (-). Water dilutions of each culture are shown to the right as a measure of cell input. Plates were allowed to grow up at room temperature for 4 days.

S2 Fig. Closeups of binding patterns across all genotypes in this study for four individual promoters. ChIP-seq data visualized using the Integrative Genomics Viewer software. Scale bars measuring 1kb are shown at the top of each screenshot, along with chromosomal location. Gene transcripts are shown in black boxes with white arrows showing directionality.

S3 Fig. Deletion of \( QSP1 \) affects the binding of Cqs2, Liv3, and Nrg1 to promoters. ChIP score for each gene was calculated as the read depth in the 1 kb region upstream of the transcription start site, normalized to the untagged control. Only promoters that are called as
bound in either genotype by k-means analysis are shown, with promoters that are more or less bound (>1.5-fold changed) by each factor in the qsp1Δ mutant highlighted in orange or light blue, respectively. The number of promoters in either of these groups is labeled with the corresponding color.

(TIF)

S4 Fig. ChIP-qPCR validation of ChIP-seq results. ChIP was performed on tagged strains followed by qPCR to quantify binding of CNAG_00758 and CNAG_03465 by tagged Nrg1, Liv3, and Cqs2 in wild type or qsp1Δ knockout using the primers in Table 4.

(TIF)

S5 Fig. A heatmap of genes that are differentially bound by either Cqs2, Liv3, or Nrg1, and their respective log2-fold expression difference in qsp1Δ compared to wild type. Non-significant differences are colored in white, significant decreases in mutant are shown in blue, and significant increases in qsp1Δ over wild type are shown in yellow.

(TIF)

S6 Fig. CQS2 or NRG1 deletion affects the binding of Cqs2, Liv3, and Nrg1 to their promoters. The ChIP score for each gene was calculated as the read depth in the 1 kb region upstream of the transcription start site, normalized to the untagged control. Only genes that are called as bound in either genotype by k-means analysis are shown, with genes that are differentially bound by each factor in mutant compared to wild type (greater than 1.5-fold changed) highlighted in light blue.

(TIF)

S7 Fig. Promoters that are differentially bound by transcription factors in a qsp1Δ mutant are also differentially bound by these transcription factors in an nrg1Δ or cqs2Δ mutant compared to wild type. The ChIP score for each gene was calculated as the read depth in the 1 kb region upstream of the transcription start site, normalized to the untagged control. Only genes that are called as bound in either genotype by k-means analysis are shown, with genes that are differentially bound (>1.5-fold changed) by each transcription factor in the qsp1Δ mutant highlighted (light blue and yellow). Promoters that are differentially bound by each transcription factor in the transcription factor mutant are highlighted in yellow.

(TIF)

S1 Table. Data used to generate Fig 3A. Quantification of bands in fluorescent immunoblots was performed using the ImageJ gel analysis tool. A student’s T-test was performed to determine significance of the difference in averages of transcription factor expression between wild type and qsp1Δ mutant conditions.

(XLSX)

S2 Table. Analysis of the degree and significance of overlaps between different sets of genes either bound by Nrg1 (N), Liv3 (L), or Cqs2 (C) in each genotype, differentially bound by these three transcription factors in qsp1Δ vs. wild type, or differentially expressed in qsp1Δ vs. wild type. The Fig generated from the data is indicated. Analysis of differentially bound (diff), decreased in binding level (less bound), or increased in binding levels (more bound) of these transcription factors to sets of genes in different mutants compared to wild type is included.

(XLSX)

S3 Table. Values used to create S3 Fig. Set of genes that are differentially bound by either Cqs2, Liv3, or Nrg1, and their respective log2-fold expression difference in qsp1Δ compared to
wild type. Non-significant differences are given a value of "NA", significant decreases in mutant are shown in blue, and significant increases in \textit{qsp1Δ} over wild type are shown in yellow.

\textbf{(XLSX)}

\textbf{S4 Table. Numbers used to generate the heatmap in Fig 4C & 4D.} Genes that are differentially bound by all three transcription factors are shown, with their fold change in Cqs2, Nrg1, or Liv3 binding in \textit{qsp1Δ} mutants vs wild type, and the gene's respective log2fold expression change in \textit{qsp1Δ} mutant vs wild type.

\textbf{(XLSX)}

\textbf{S5 Table. Data used to generate Fig 5A.} Quantification of bands in fluorescent immunoblots was performed using the ImageJ gel analysis tool. A student’s T-test was performed to determine significance of the difference in averages of transcription factor expression between wild type and mutant conditions.

\textbf{(XLSX)}

\textbf{S6 Table. ChIP score values.} Values of ChIP scores for binding of each transcription factor in each genotype for each promoter, whether the promoter was called as bound by k-means analysis ("1") or not ("0"), values for fold change in binding levels in differential binding analysis, and log2fold-change for expression of the gene downstream of the promoter in \textit{qsp1Δ} vs. wild type by RNA-seq followed by DE-seq analysis.

\textbf{(XLSX)}

\textbf{S7 Table. Comparison of the number of promoters called as bound in either replicate.} Bound genes in each sample were determined by k-means analysis.

\textbf{(XLSX)}

\textbf{Acknowledgments}

We thank members of the Madhani lab for helpful discussions and review of the paper, especially Sandra Catania, Jordan Burke, and Daniele Canzio. We thank Nguyen Nguyen for media preparation. DKS and HDM designed the study. DKS and BR performed the experiments, DKS and DSP performed the bioinformatics analysis, and HDM supervised the work.

\textbf{Author Contributions}

**Conceptualization:** Diana K. Summers, Hiten D. Madhani.

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**Funding acquisition:** Hiten D. Madhani.

**Investigation:** Diana K. Summers, Beiduo Rao.

**Project administration:** Hiten D. Madhani.

**Supervision:** Hiten D. Madhani.

**Writing – original draft:** Diana K. Summers.

**Writing – review & editing:** Diana K. Summers, Hiten D. Madhani.

\textbf{References}


