Title
Coordinate genomic association of transcription factors controlled by an imported quorum sensing peptide in Cryptococcus neoformans

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Coordinate genomic association of transcription factors controlled by an imported quorum sensing peptide in Cryptococcus neoformans

by
Diana Summers

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Acknowledgements: A Quorum at Work

I could not have completed the work presented here without help from people in my community.

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Coordinate genomic association of transcription factors controlled by an imported quorum sensing peptide in *Cryptococcus neoformans*

by

DIANA K. SUMMERS

Abstract

Qsp1 is 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. We found that rather than acting on a cell surface receptor, Qsp1 is imported to act intracellularly via the predicted oligopeptide transporter Opt1. We also demonstrate that a transcription factor network is a target of Qsp1. 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 Qsp1's action in 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.

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Chapter 1: Introduction

1.1 Sensing and Signaling

Cells survive by integrating signals that alter gene expression through regulation of transcription factors

The ability to sense and respond to signals is an integral part of cell physiology. All cells have evolved to sense their surroundings and respond to a variety of environmental cues and stimuli. Cells that grow into multicellular organisms depend on external signals such as contact with neighboring cells, the extracellular matrix, and local concentrations of diffusible signaling molecules to determine what genes are expressed, what type of cell they become, and when to die or divide. Single-celled organisms move towards nutrients and light and must quickly adapt their gene expression, and thus their behavior, in order to survive in rapidly changing environments. In addition, single-celled microbes have evolved the ability to form communities that are regulated by the secretion of one or several autoregulatory molecules that signal the population to work together.

Comparative genome analysis has revealed that gene number is not strictly correlated with organismal complexity (1). There are many ways in which a small number of genes could be utilized to generate organismal complexity over evolutionary time, such as alternative splicing of transcribed messenger RNA (which allows for several versions of a protein from one gene) (2), or increases in number of protein domains and combinations of domains (3) . However, it has been argued that the number of gene expression programs over an organism's life cycle is what contributes the most to organismal complexity (1).

The reception of a signal is coupled to the expression of different combinations of genes to meet a variety of functional needs. Cells rely on transcription factors that bind to gene regulatory sequences in DNA for control of gene expression. Transcription factors are regulated in response to various signals and can also influence each other. The expression level of a gene depends on

the interactions of combinations of transcription factors at its regulatory sequences (4). This type of combinatorial control allows cells to adapt quickly and robustly to changing and challenging environments, such as survival of a pathogenic microbe in the environment of the host.

1.2 Quorum Sensing in Bacteria and Fungi

Individual bacterium secrete basal levels of quorum sensing molecules into their local environments and are receptive to the local concentration of these molecules (Figure 1A). As the cells divide and grow, the concentration of this molecule increases, triggering a signaling cascade that results in changes in gene expression in all cells that have receptors for the molecule. Individual cells can also experience an increase in concentration of the signaling molecule and react to it when trapped in an enclosed space, such as inside a macrophage (5,6). Thus, integration of other contextual clues via activation of other signaling cascades in addition to quorum sensing are often important for determining the proper combination of genes to be expressed.

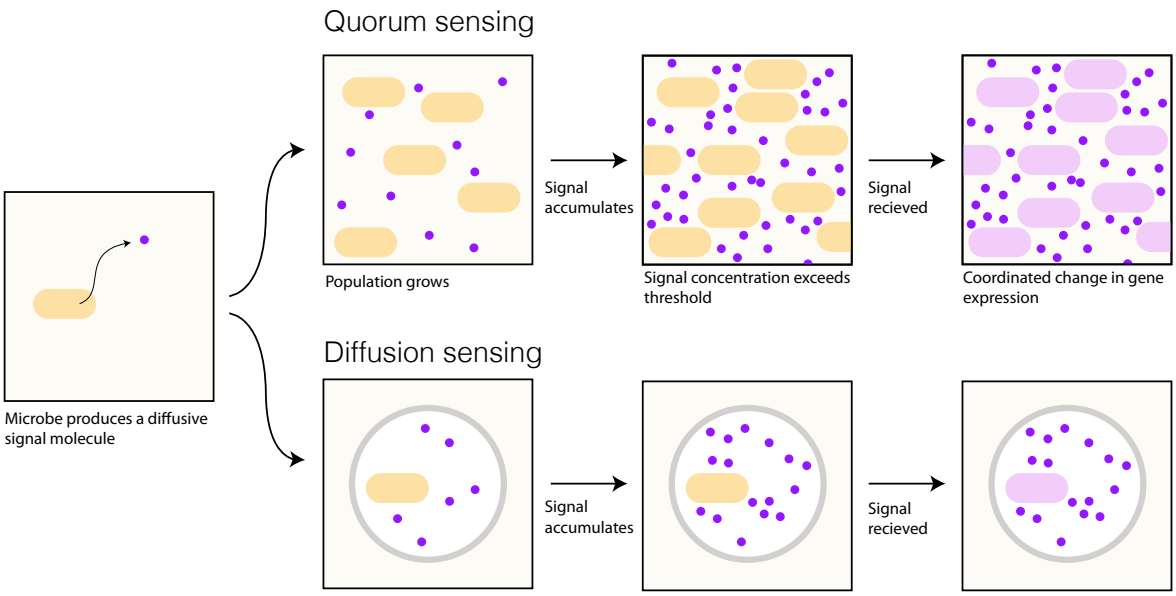

Figure 1.1. Quorum Sensing vs Diffusion Sensing. A single-celled microbe produces a signaling molecule at a constant rate. As the population grows (quorum sensing), or if the microbe is trapped in an enclosed space (diffusion sensing), the local concentration of the signal increases until a certain threshold is reached, triggering a change in gene expression.

Quorum sensing circuits were first discovered in regulation of bioluminescence in the marine bacterium Vibrio fisheri (7–10). Since then, quorum sensing pathways have been discovered in

many different bacterial species, controlling conjugation (11–13), induction of genetic competence (14,15) and DNA transfer, biofilm formation (16,17), exoprotease production (18), and other factors that impact virulence (17,19). Control of these processes are likely coordinated since they provide the most benefit to the individual cell if all of the members of the population cooperate by acting together.

Gram-negative bacteria primarily utilize acylated homoserine lactones (AHLs) for quorum sensing, while gram-positive bacteria typically utilize small oligopeptides (Figure 1B). The peptide is often secreted as a pro-peptide that must be cleaved to become active, providing another level of regulation. Some of these peptides are sensed via their cognate histidine kinase on the plasma membrane (20), while others are imported into the cell via an oligopeptide permease, where they bind to a cytoplasmic receptor such as a phosphatase that dephosphorylates a response regulator (21), or in other cases, to the response regulator itself (22–24).

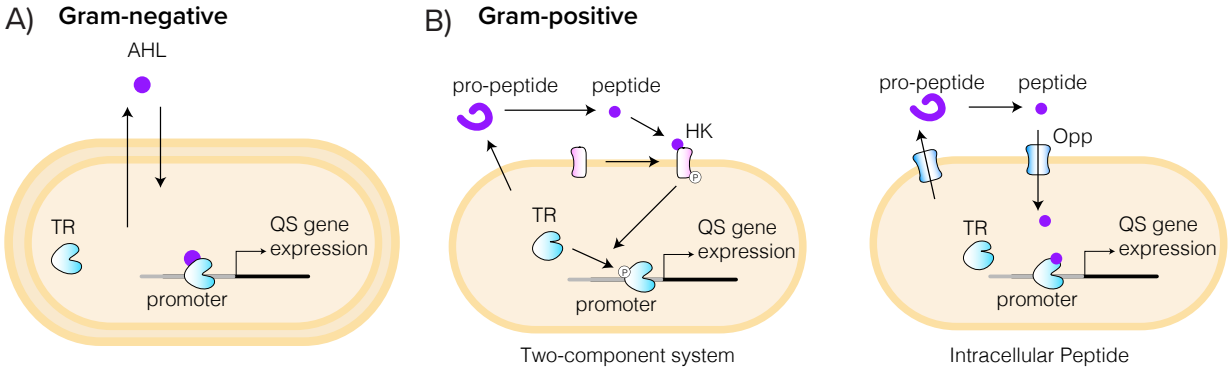

Figure 1.2. Prokaryotic Quorum Sensing Mechanisms. A) Gram-negative bacteria primarily utilize acyl homoserine lactones (AHLs), which freely diffuse between the cytoplasm and extracellular space. **B,C)** Gram-positive bacteria typically use peptides are processed to become active via protease cleavage and are exported. Once outside the cell, the peptide then either **B)** binds their cognate histidine kinase receptor on the plasma membrane, causing phosphorylation of the downstream transcription factor (TF), or **C)** is imported into the cell by an oligopeptide permease (Opp), where it binds to a downstream regulator such as a phosphotase or TF. In all three cases, the signaling molecule causes a change in the activity of the transcription factor, altering its behavior and inducing a change in gene expression.

Until recently, it was thought that quorum sensing was restricted to prokaryotes, but it is clear that eukaryotes can also regulate certain phenotypes based on inoculum size (25). Indeed, many fungi have been shown to demonstrate signs of quorum sensing-like behavior, but the identity of the signaling molecule remains unknown in most cases (26). The first quorum sensing molecules

to be discovered in fungi were the alcohols farnesol and tyrosol in *Candida albicans*. Farnesol inhibits biofilm formation and the yeast-to-hypha cell morphological transition in dense cultures (27,28), while tyrosol promotes biofilm formation (29). *Saccharomyces cerevisiae* also participates in quorum sensing mediated by the aromatic alcohols 1-phenylethanol and tryptophol, which also regulate morphogenesis in response to nitrogen starvation (30). In these cases, even though the identity of the signaling molecule is known, the signaling pathway remains unclear.

In previous work, we described a peptide-based quorum sensing system in *Cryptococcus neoformans*, the first to be described in a eukaryote (31). This system is mediated by an 11 residue peptide dubbed Qsp1, purified because it complements a low-density phenotype produced by C. neoformans lacking a transcriptional co-repressor, Tup1 (32). This system presents the unique opportunity to study how a quorum sensing molecule functions in a eukaryotic organism.

1.3 Qsp1 Signaling in *Cryptococcus neoformans*

The opportunistic basidiomycete yeast *Cryptococcus neoformans* is the most common cause of fungal meningitis, causing over 200,000 deaths annually (31). The unique features of this organism that drive its virulence are incompletely understood. Previous work in our laboratory established that cells lacking the *QSP1* gene exhibit a defect in virulence, a wrinkled colony morphology, and cells in saturated cultures become sensitive to cell wall stress (29). We also discovered that Qsp1 influences gene expression under saturating conditions in rich media (29). By screening our knockout collection for knockouts that form *qsp1Δ*-like wrinkled colonies, we uncovered genes that may act either downstream or in parallel to Qsp1 (Table 1). 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 (Figure 1C) (29). However,

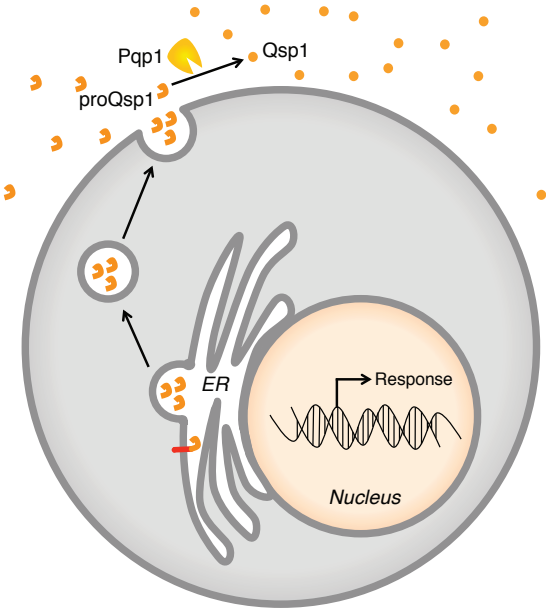

Figure 1.3 Qsp1 biogenesis in *C. neoformans*

it was still unclear what the role of the other genetic candidates was in Qsp1 signaling and how Qsp1 acts to elicit changes in gene expression.

This thesis describes some aspects of the mechanism by which Qsp1 signals. In Chapter 2, I show that intracellular expression of the mature form of Qsp1 is sufficient to complement all *qsp1Δ* phenotypes and is able to bypass the need for the oligotransporter Opt1. In Chapter 3, I uncover the transcription factor Nrg1 as a novel participant in Qsp1 signaling network together with the transcription factors Liv3 and Cqs2. I then show these three factors are part of a transcription factor network that is regulated by Qsp1. We also probe the network via deletion of either of the TFs to tease apart potential hierarchy within the Qsp1 signaling pathway. In Chapter 4, I attempt to determine whether Cqs2, Liv3, or Nrg1 serve as the intracellular receptor for Qsp1 and provide future directions for discovering the intracellular receptor for Qsp1.

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Chapter 2: The Oligopeptide Transporter of Qsp1

In which we discover that intracellularly expressed Qsp1 is functional in cells lacking Opt1.

2.1 Summary

Qsp1 is a secreted autoregulatory peptide that is important for virulence. Sensing of the peptide seems to require the putative oligopeptide transporter Opt1, as cells lacking the *OPT1* gene (*opt1Δ)* share all *qsp1Δ* phenotypes, yet are not able to be complemented by extracellular addition of synthetic Qsp1. However, the function of Opt1 in Qsp1 signaling was unknown. Here, we distinguish between models where Opt1 acts as a transporter of Qsp1, the cell surface receptor for Qsp1, or acting in some other way in the Qsp1 signaling pathway by testing the ability of intracellularly expressed Qsp1 (iQsp1) to rescue various phenotypes shared by *qsp1Δ* and *opt1Δ* mutants. We found that cytoplasmic expression of iQsp1 was capable of rescuing the *qsp1Δ* knockout for all phenotypes tested, indicating that Qsp1 was likely imported into the cell to function. Surprisingly, iQsp1 was also able to rescue two of the three phenotypes of *opt1Δ* mutant phenotypes, indicating that Opt1 acts to import Qsp1. However, iQsp1 expression could only partially rescue the dry colony morphology phenotype of Opt1 mutants. This result indicates that Opt1 may also serve an additional function required for full complementation of the colony morphology phenotype by Qsp1, such as the import of other peptides.

2.2 Introduction

The *QSP1* gene encodes a peptide that contains a putative secretory signal upstream of a precursor peptide of 24 amino acids, which directs the peptide into the secretory pathway for release outside the cell. Pqp1, a cell-associated extracellular protease, then cleaves it into the biologically active Qsp1 peptide of 11 amino acids we call Qsp1 (1)(Figure 2.1). Yeast lacking the *QSP1* gene (*qsp1Δ* mutants) form dry, wrinkled colonies on a plate at room temperature or 30°C, but are smooth and glossy at 37°C. In addition, saturated cultures are sensitive to SDS treatment, and the cells have a thicker capsule when grown in capsule-inducing conditions at room temperature. These *qsp1Δ* mutant phenotypes are able to be complemented by extracellular supplementation of synthetic Qsp1 peptide to the cultures or to the media (Figure 2.3A).

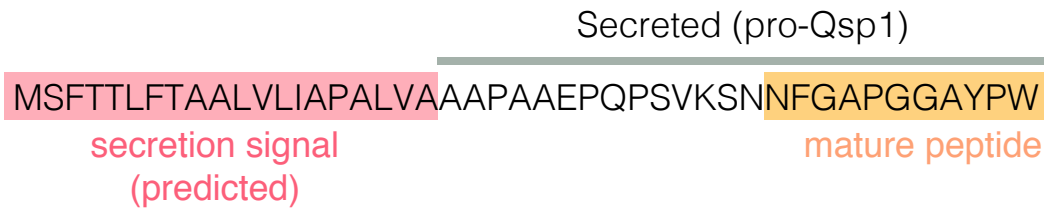

Figure 2.1. Amino acid sequence of Qsp1. The QSP1 gene encodes a 24 amino acid peptide with an N-terminal predicted secretion signal. This 24-residue peptide is cleaved outside the cell to produce the 11-residue mature Qsp1 peptide.

We hypothesized that strains lacking other factors required for Qsp1 signaling would also exhibit a temperature-dependent dry colony morphology. We screened strains in our gene deletion in *Cryptococcus neoformans* for potential candidates and found that yeast lacking the *OPT1* gene also exhibit a *qsp1Δ*-like colony morphology. Cells lacking *OPT1* are indistinguishable from *qsp1Δ* cells in colony morphology, sensitivity to SDS treatment, and capsule thickness, but are unable to be rescued by the supplementation of extracellular synthetic Qsp1 peptide (Figure 2.3). This inability of *opt1Δ* mutants to respond to Qsp1 peptide indicated that Opt1 may acts downstream of Qsp1 in the Qsp1 signaling pathway. As the *OPT1* gene encodes a putative oligopeptide transporter, we hypothesized that Opt1 likely acts as a transporter of Qsp1. Alternatively, it could also function as a cell surface receptor for Qsp1 or act downstream of extracellular Qsp1 sensing in the Qsp1 signaling pathway in another way.

Here, we test this hypothesis by investigating the ability of intracellular Qsp1 expression to rescue colony morphology, cell wall stress, and capsule thickness phenotypes of *opt1Δ* and *qsp1Δ* mutants and illuminate the role of Opt1 in Qsp1 signaling.

2.3 Results

To distinguish between possibilities for Opt1 function, we expressed Qsp1 peptide in the cytoplasm to determine if intracellular Qsp1 (i-Qsp1) could functionally complement cells (Figure 2B). We replaced the endogenous Qsp1 promoter and gene with the construct shown in Figure 2C using homologous recombination. The endogenous Qsp1 promoter was replaced by a copper-repressible promoter, which allows control of expression by growing the cells in media that contains either copper sulfate (repressed) or the copper chelator BCS (expressed). Since adding a methionine to the N-terminus of synthetic Qsp1 peptide inhibits its activity (not shown), we required expression of mature Qsp1 without the initiator methionine for this experiment. To do this, we took advantage of a ubiquitin cleavage pathway. We expressed the 11 amino acids of Qsp1 as a ubiquitin fusion protein with ubiquitin at the N-terminus. The ubiquitin is then cleaved off of the mature peptide by a ubiquitin ligase to release the mature peptide into the cytoplasm (2,3).

We tested the ability of induced i-Qsp1 to rescue each of the phenotypes exhibited by the *qsp1Δ*

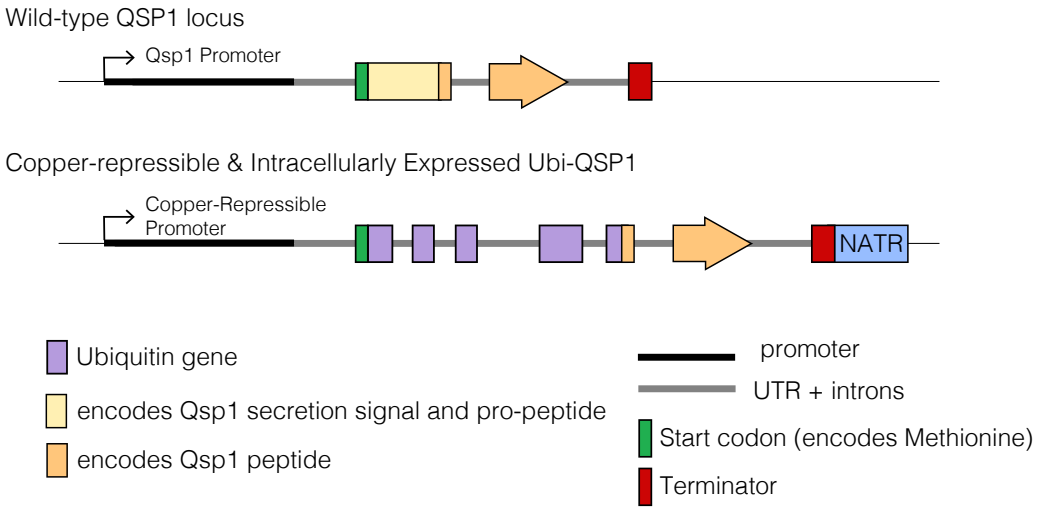

Figure 2.2. Gene structure of inducible expression of cytosolic Qsp1. The endogenous QSP1 gene and promoter were replaced by a copper-repressible promoter driving the expression of a fusion of Ubiquitin to mature Qsp1.

mutant: colony morphology, cell wall stress, and capsule size. On media containing copper, the i-Qsp1 strain is rough and looks much like the *qsp1Δ* mutant, as expected (Figure 2.3B). Strikingly, on media containing the copper chelator BCS, the i-Qsp1 strain forms smooth and glossy colonies that look indistinguishable from wild type colonies (Figure 2.3B).

To test the sensitivity of each strain to cell wall stress, each was grown to saturation for 48 hours in rich media, then incubated in serial dilutions of the cell wall stressor SDS for 3 hours without shaking, or serially diluted in water to assay cell input (Figure 2.3C). At the end of the incubation period, cells were spotted on plain YPAD to assay survival. Saturated cultures of the i-Qsp1 strain grown under repressive conditions were just as sensitive as *qsp1Δ* mutants to SDS, but when i-Qsp1 expression was induced by growth in BCS, the i-Qsp1 strain was able to survive higher concentrations of SDS during the incubation period, similar to saturated cultures of wild-type cells (Figure 2.3C).

To determine capsule size, cells were cultured in a capsule-inducing medium at room temperature at low density, stained with india ink, and cell and capsule diameter of 100 cells was measured (Figure 2.3D). i-Qsp1 cells cultured with BCS in the media had similar capsule to cell size ratio as wild type cells, and i-Qsp1 cells cultured with Cu in the media had enlarged capsules that were much closer in thickness to that of *qsp1Δ* mutant cells (Figure 2.3D). The ability of intracellularly expressed Qsp1 peptide to rescue all three phenotypes indicates that Qsp1 functions intracellularly for the phenotypes examined.

We next wanted to know if Opt1 is responsible for importing Qsp1 peptide into the cytoplasm. To answer this question, we decided to express i-Qsp1 in an *opt1Δ* knockout, to determine if intracellularly expressed Qsp1 bypasses the requirement for Opt1. Since the *OPT1* gene lies tangential to *QSP1* in the genome, we replaced the endogenous *QSP1* gene and the endogenous OPT1 gene with the i-Qsp1 construct but with a homology arm that containing the sequence upstream of the *OPT1* gene such that the *OPT1* gene would be recombined out at the same time as the endogenous *QSP1* gene is replaced.
The resulting i-Qsp1; *opt1Δ* strain was tested for its ability to rescue the colony morphology,

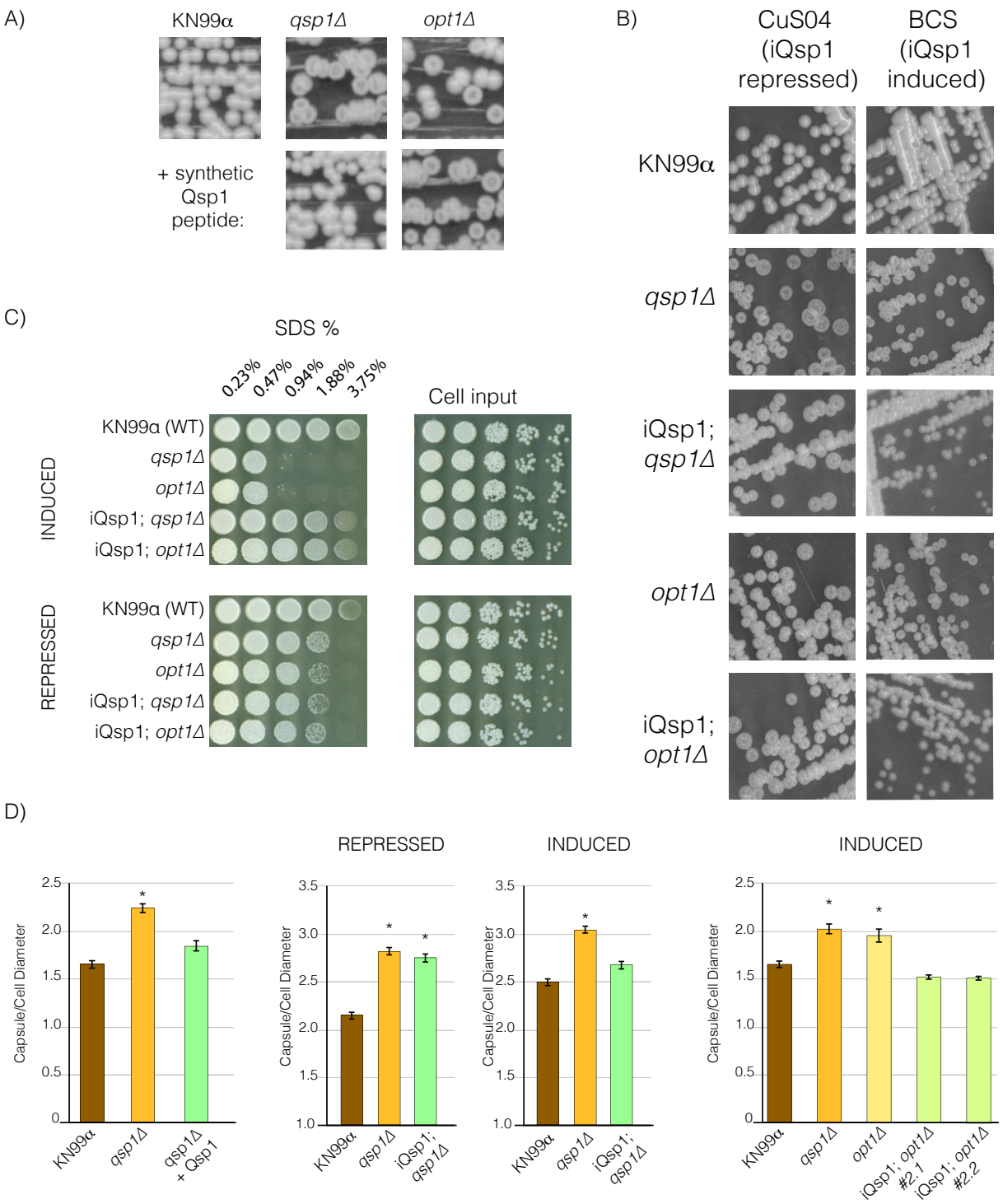

Figure 2.3 Intracellular expression of Qsp1 complements phenotypes of *qsp1Δ* and *qsp1Δopt1Δ* mutants. A) Colony Morphology B) SDS sensitivity, and C) capsule thickness of strains in cells lacking *QSP1* or *OPT1* induced or repressed for expression of intracellular Qsp1

sensitivity to cell wall stress, and capsule size when expression was induced by growth in copper chelating conditions (Figure 2.3B-D). Interestingly, though the colony morphology of the i-Qsp1; *opt1Δ* strain was more wrinkled and dry on plates where the expression of i-Qsp1 was repressed, it still formed dry and wrinkled colonies under inducing conditions (Figure 2.3B). Thus, intracellular expression of Qsp1 was only partially able to rescue the colony morphology phenotype.

In comparison to wild type cells, the *opt1Δ* mutant has a thicker capsule, much like the *qsp1Δ* mutant, at room temperature. To test if intracellular expression could change the capsule thickness of *opt1Δ* cells to be more thin like wild-type cells, the i-Qsp1; *opt1Δ* strain was grown with BCS or in copper containing capsule-inducing media at room temperature (Figure 2.3D). The result that intracellular expression of Qsp1 in an *opt1Δ* mutant is sufficient to rescue the *opt1Δ* phenotype supports the hypothesis that Opt1 is the oligopeptide transporter responsible for import of Qsp1, .

We next looked to see if i-Qsp1 expression in the *opt1Δ* mutant could rescue the sensitivity to cell wall stress exhibited by the *opt1Δ* mutant. As expected, when expression was repressed, the i-Qsp1; *opt1Δ* strain was just as sensitive to SDS as the *opt1Δ* strain. When expression was induced, the i-Qsp1; *opt1Δ* strain was no longer as sensitive to cell wall stress, and behaved like wild-type cells in this assay (FIgure 2.3C). This indicates that Opt1 is no longer necessary for cells to strengthen their cell wall when Qsp1 is expressed intracellularly. Taken together, these data indicate that Qsp1 functions intracellularly, and Opt1 is not required for intracellular function.

2.4 Discussion

Our previous work in the eukaryote *Cryptococcus neoformans* has shown that Qsp1 is secreted as a pro-peptide that is cleaved outside the cell (1) . Mature peptide accumulates in the culture supernatant, then appears to be imported back into the cell by Opt1, where it induces a transcriptional response. However, not all phenotypes of cells lacking Opt1 are able to be rescued by internal expression of the Qsp1 peptide, indicating that Opt1 may also serve an additional

function required for full complementation Qsp1 phenotypos other than import of Qsp1. It is possible that Opt1 could import other peptides that are required in addition to Qsp1 to promote a wild type colony morphology. It is possible that Opt1 serves as a scaffold for other factors that influence Qsp1 signaling downstream, such as an enzyme that modifies Qsp1. In addition, Opt1 contains a mysterious and rather large folded domain at its N-terminus that is not present in other oligopeptide transporters encoded in the family, the function of which is unknown.

While there are many examples of extracellular signaling peptides in eukaryotes, all of them signal by binding to cell surface receptors and are not imported. For example, one of the most highly studied peptide signaling molecules in yeast are the mating pheromones, which are short peptides that act on surface-bound receptors to signal. Plants utilize extracellular signaling peptides that are matured in the secretory pathway, secreted, then sensed by receptors on the cell surface. Even small intracellular signaling peptides generated by proteolysis of larger proteins have been discovered in higher eukaryotes, though not much is known about their mechanism of action (4,5). This mechanism of signaling via import of an extracellularly matured peptide is paralleled in the quorum sensing systems of gram-positive bacteria, which also secrete quorum sensing peptides that are matured extracellularly, then imported into the cell via oligopeptide permeases. However, the discovery that the autoregulatory peptide Qsp1 is a must be imported to function is unprecedented in eukaryotes.

None of the components of the QS system in *C. neoformans* has a proximal ancestor in bacteria, indicating that this mechanism of signaling has convergently evolved in these two branches of life (1). The discovery that *C. neoformans* utilizes an imported peptide for signaling is unprecedented. Generally, imported peptides are often associated in eukaryotes as a nitrogen source that are hydrolyzed into signal amino acids for use. Most oligopeptide transporters are utilized for the import of short di- and tri- residue peptides for nutrition. For example, the oligopeptide transporters encoded by the *C. albicans* genome are mainly utilized to import peptides as a nitrogen source and have different preferences for peptide size (6). It is not known whether members of the OPT family of transporters is capable of importing an 11-residue peptide, but some oligopeptide transporters have been shown to import larger peptides, up to 35 amino acids

in length (7).

Once inside the cell, peptides are generally digested by various peptidases that digest peptides. It is likely that quorum sensing peptides such as Qsp1 and bacterial QS peptides are also subject to degradation by intracellular peptidases. Therefore, the affinity of the peptide for its intracellular receptor must be high enough to allow for the signal to be received. Interestingly, we have never been able to detect internalized Qsp1 via immunoblotting of protein extracts made from cell pellets, even in those made from strains expressing an excess of cytoplasmic iQsp1 (not shown). It is possible that after import, Qsp1 is either degraded quickly or modified such that the antibody no longer recognizes the peptide. A modification could also protect Qsp1 peptide from peptidases.

2.5 References

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Chapter 3: The Qsp1-regulated transcription factor network

In which we discover that Qsp1 influences gene expression by altering the binding of three transcription factors to DNA

3.1 Summary

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.

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, and 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 is important for 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.

3.2 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* Ryp, acts downstream of Qsp1 (4,6–10). 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 expression 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 an imported quorum-sensing peptide impacts gene expression.

3.3 Results

3.2.1 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 (4). 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 (Figure 3.1). 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 (Figure 3.2A). 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 (Figure 3.2B). 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 (Figure 3.2B). Therefore, while each transcription factor knockout strain is able

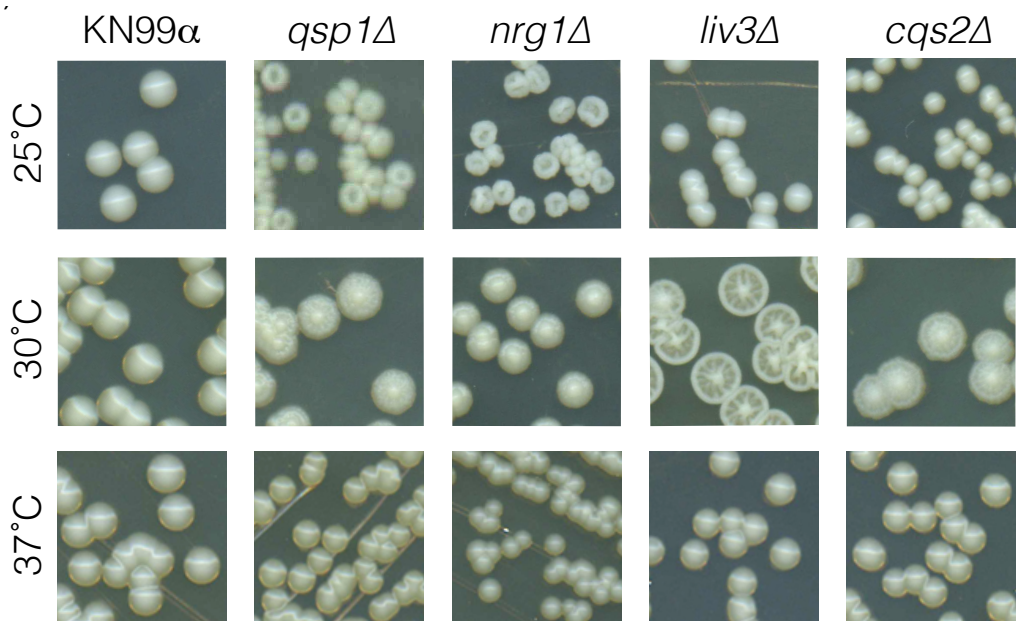

Figure 3.1. Knockouts of *NRG1, LIV3,* and *CQS2* form rough colonies at a narrow range of temperatures. Colony morphology of QSP1 and transcription factor knockout strains streaked on YPAD agar at 25°C, 30°C,

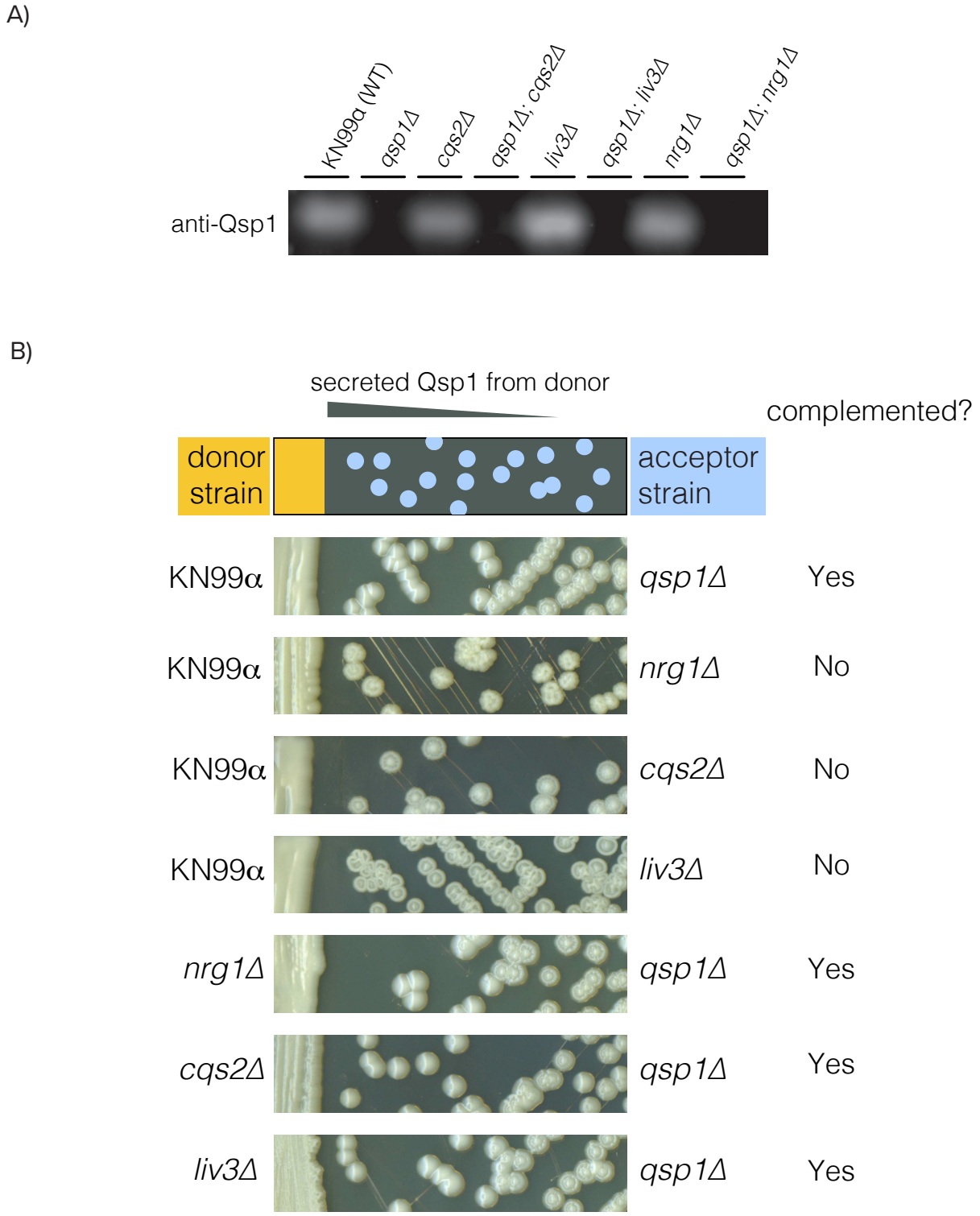

Figure 3.2. Transcription factor mutants still express Qsp1, but are unable to be rescued by Qsp1 peptide. A) Anti-Qsp1 immunoblot of Qsp1 peptide in supernatant of knockout strains cultured at 30°C to an optical density at 600nm of 12. B) A Qsp1-secreting donor strain is patched on the left, and colonies from a wrinkled acceptor strain streaked to the right are tested for its ability to be complemented by Qsp1 from the donor strain.

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

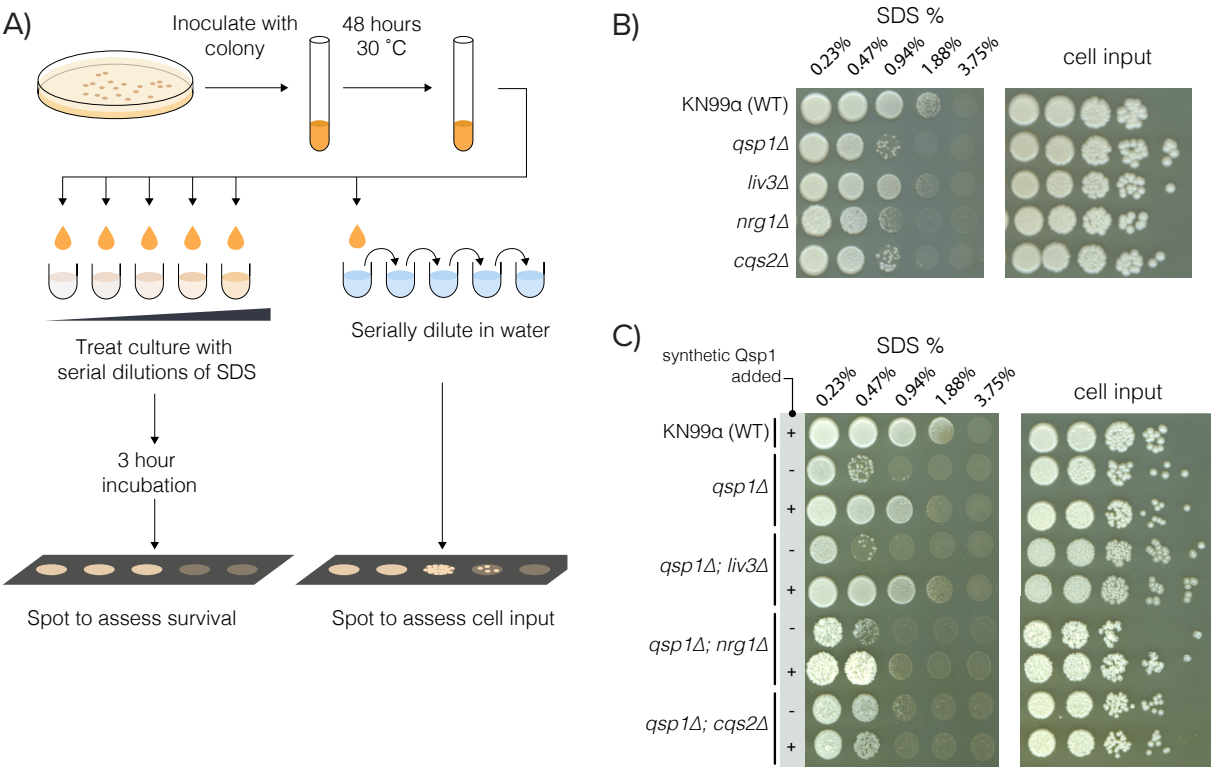

Figure 3.3. Nrg1 and Cqs2 promote cell wall stress, and mutants lacking these factors are unable to be rescued by Qsp1 peptide. A) Schematic for how strains were tested for their ability to survive different concentrations of the cell wall stressor SDS. B) Single mutants for each of the genes shown were tested for their ability to survive increasing concentrations of the cell wall stressor SDS. Water dilutions are shown to the right. Plates were allowed to grow up at room temperature. C) 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.

SDS (Figure 3.3A). 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 displayed sensitivity (Figure 3.3B). 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 (Figure 3.3C). The *qsp1Δnrg1Δ* double mutant is only modestly complemented, and the *qsp1Δcqs2Δ* double mutant is completely 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 involved in responding to Qsp1 peptide in cell wall stress. These data are consistent with a model in which Cqs2 and Nrg1 function downstream of Qsp1 to promote resistance to a cell wall stress.

3.2.2 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 stationary phase 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 (OD_{600}) of 1, 5 and 10 (Figure 3.4). We then performed RNA-seq analysis to

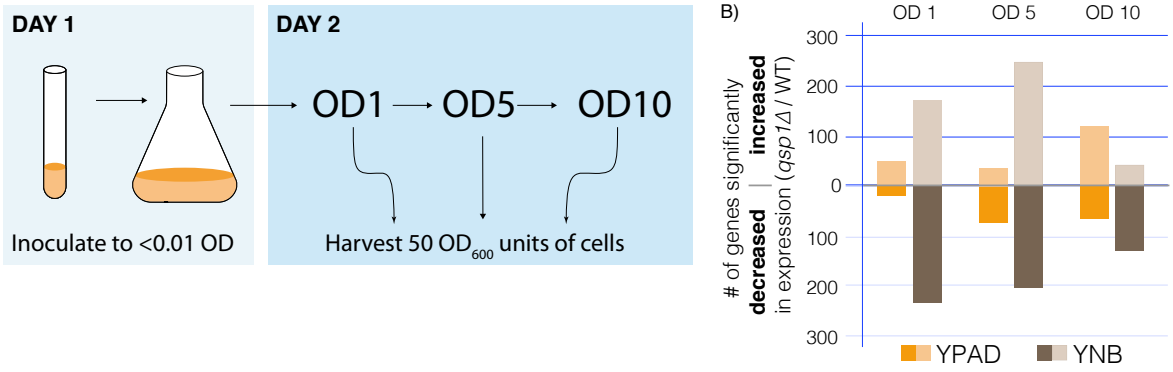

Figure 3.4. Differential gene expression in *qsp1Δ* cells compared to wild type in rich and mininal media. A) Schematic for how RNA-Seq cultures were harvested. B) Number of significantly changed genes in qsp1Δ over wild-type as determined by DE-seq2 analysis.

identify differentially expressed genes. Over 400 genes were significantly affected by the loss of the *QSP1* gene in minimal media at an OD_{600} of 1 (OD1) or OD_{600} of 5 (OD5), more genes than in rich media at any culture density (Figure 3.4B). Therefore, 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 (Figure 3.5). There were significant overlaps between

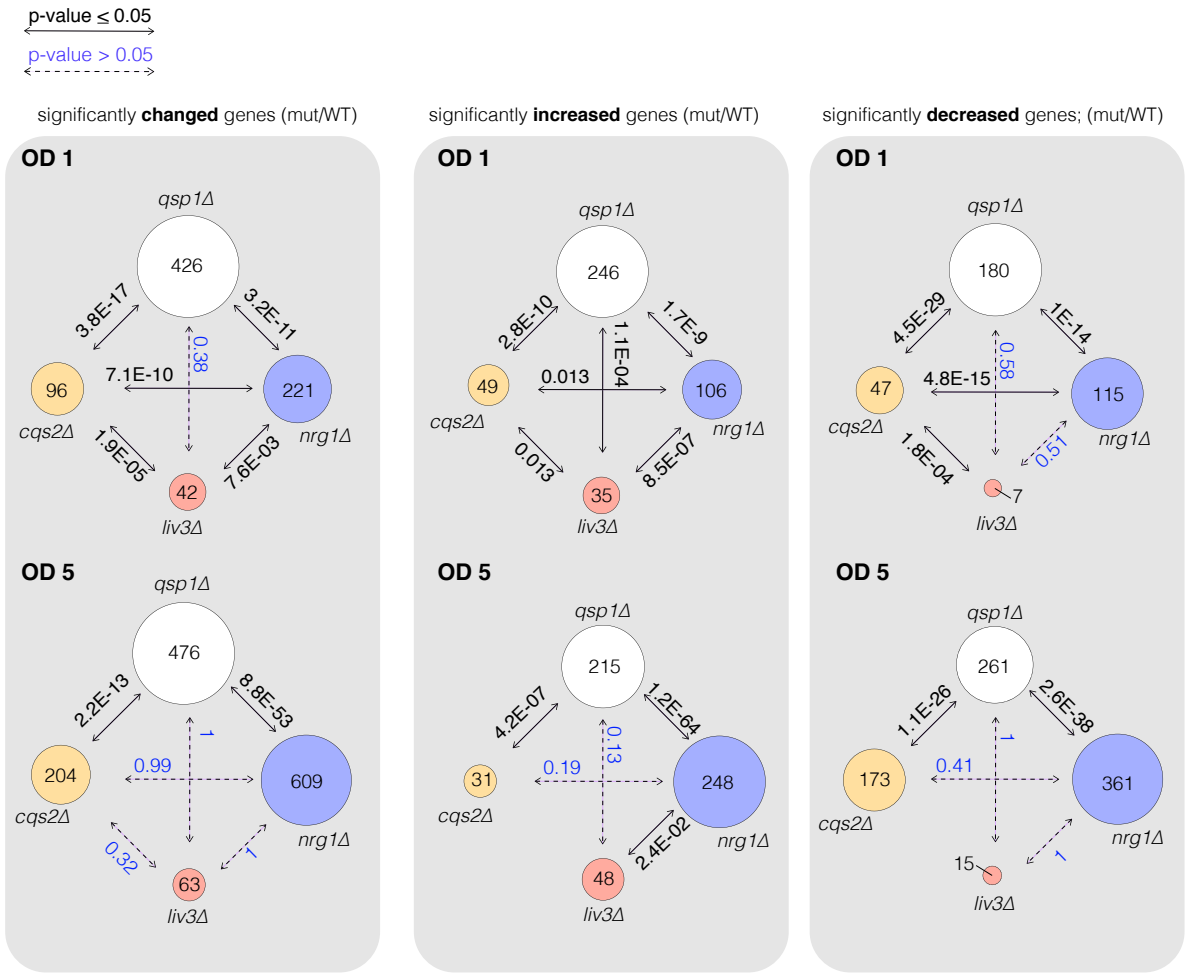

Figure 3.5. Cqs2, Nrg1, and Liv3 are part of a transcription factor network that shares targets with *qsp1Δ*. Comparisons of significantly changed genes from DEseq2 analysis of each mutant compared to wild type (WT) and their *P*-values shown above the arrows. Solid lines and bold text indicate that the overlap is significant (p<0.05), dotted lines and blue text indicate a non-significant *P*-value (p>0.05).

groups of differentially expressed genes under at least one condition (Figure 3.5). 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 (Figure 3.5). The latter only significantly overlapped the Qsp1-dependent set for genes derepressed in the mutants at OD1 (Figure 3.5). These data reveal strong similarities between the transcript signatures of *qsp1Δ* mutant and those of *cqs2Δ* and *nrg1Δ* mutants, with only weak similarity to the *liv3Δ* mutant signature.

3.2.3 ChIP-seq reveals that Cqs2, Nrg1, and Liv3 bind to a common set of promoters.

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. Nrg1 was tagged on its N-terminus, as a C-terminal tag rendered the *qsp1Δ* knockout unable to respond to synthetic Qsp1 peptide in the cell wall stress assay.

Quantitative immunoblots show a slight reduction in Cqs2 levels in cells lacking the *QSP1* gene at OD1 in minimal media, though this difference was not significant (Figure 3.6). However, cells lacking *QSP1* expressed significantly more Nrg1 and Liv3 protein (Figure 3.6).

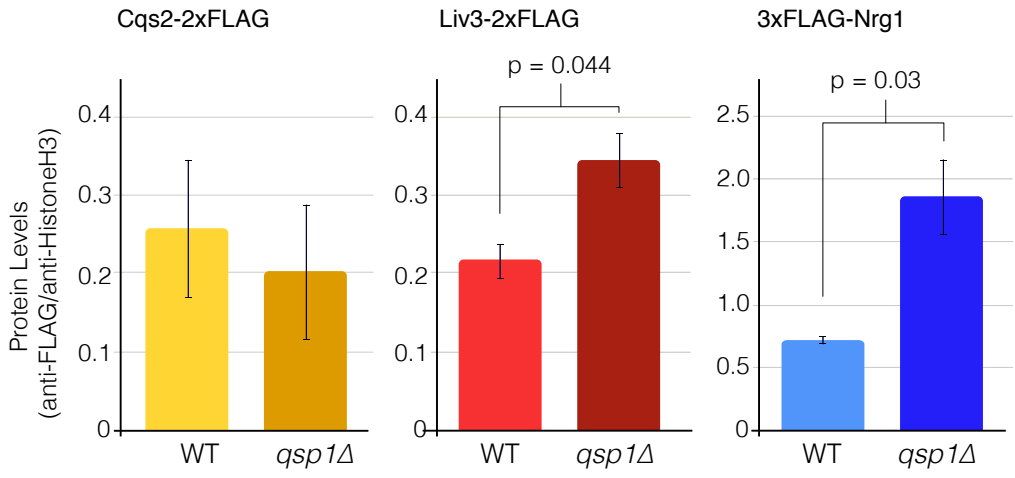

Figure 3.6 *QSP1* represses Liv3 and Nrg1 protein levels. Expression of Cqs2, Liv3, and Nrg1 protein levels in wild type (WT) or *qsp1Δ* mutants at OD1 in YNB. The average of two biological replicates is shown for each condition, along with the *P*-value if the difference between WT and mutant is significant.

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 (Figure 3.7, 3.8A, 3.8B). We found that in wild type, these transcription factors generally bind to the same promoters (Figure 3.8A, 3.8B). 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 (Figure 3.8B). 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 (Figure 3.8C).

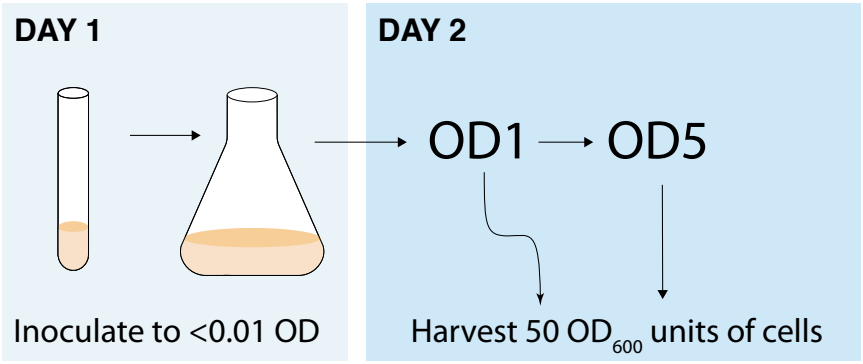

Figure 3.7. Schematic for how cultures were grown for ChIP-seq. Cultures were grown in minimal media then harvested at OD1 and OD5 for ChIP-seq or ChIP qPCR.

3.2.4 Qsp1 affects the binding of all 3 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 (Figure 3.8A). To quantify this, the ChIP score of transcription factor binding 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). This analysis revealed that Qsp1 affects the binding of Cqs2, Liv3, and Nrg1 to a large fraction of bound promoters (Figure 3.9 and Figure 3.8C). We confirmed this by quantifying the amount of

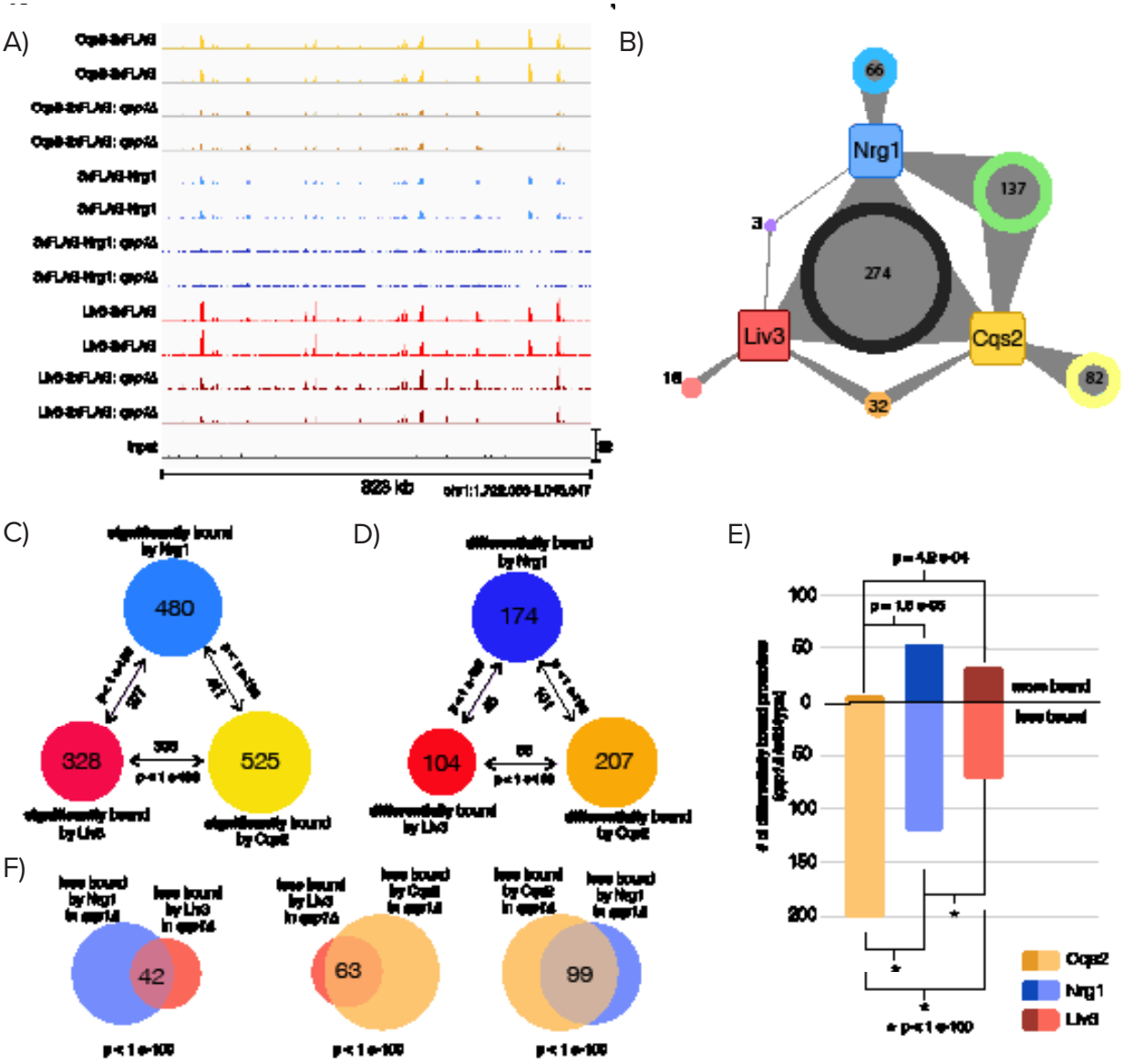

Figure 3.8. Qsp1 affects Cqs2, Nrg1, and Liv3 binding to a common set of promoters. A) ChIP-Seq data was visualized using the Integrative Genomics Viewer software. Binding across part of chromosome 1 is shown. **B)** Network diagram of promoters bound by Nrg1, Liv3, and Cqs2 in wild type cells in YNB at OD1. **C)** Overlaps between promoter sets significantly enriched for Liv3, Cqs2, or Nrg1 binding and their significance. **D)** Overlaps between promoter sets differentially bound (>1.5-fold) by Liv3, Cqs2, or Nrg1 in the *qsp1Δ* mutant compared to wild type. **E)** Breakdown of promoters that are bound more or less by a given transcription factor in *qsp1Δ* compared to wild type, filtered by promoters significantly bound in either genotype. Significant overlaps between groups are noted with the *P*-value. **F)** Overlaps between promoter sets that are >1.5-fold less bound by Liv3, Cqs2, or Nrg1 in a *qsp1Δ* mutant compared to wild type, and their significance. Only promoters that are called as bound in either genotype by k-means analysis are shown.

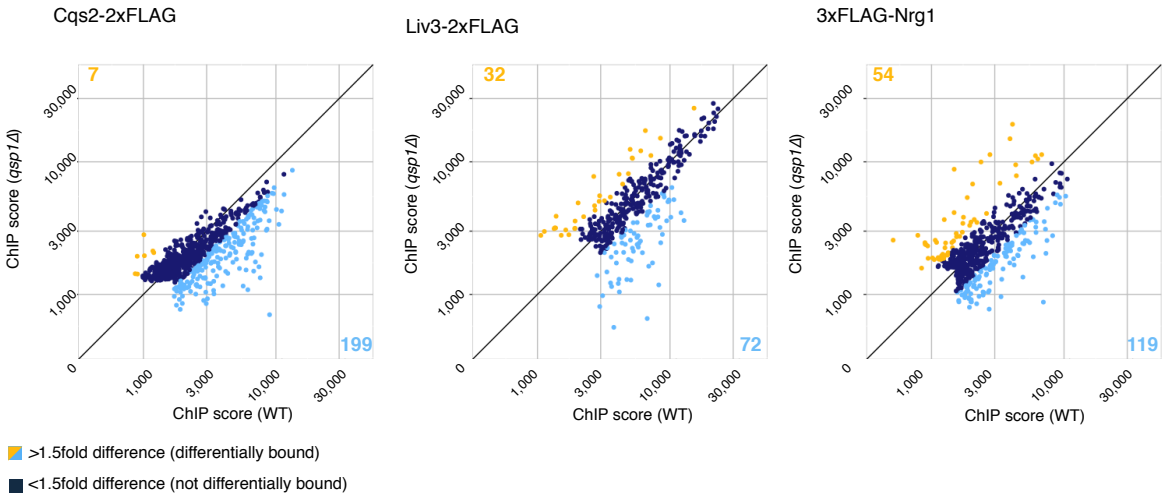

Figure 3.9 Deletion of *QSP1* affects the binding of Cqs2, Liv3, and Nrg1 to promoters. ChIP score for each gene was calculated as the read depth in the 1 kb region upstream of the transcription start site, normalized to the untagged control. Only promoters that are called as bound in either genotype by k-means analysis are shown, with genes 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 genes in either of these groups is labeled with the corresponding color.

binding of each factor to two of the promoters that showed the highest differences by ChIP-seq using ChIP-qPCR (Figure 3.10). Overall, we observed a shift to lower levels of binding in the *qsp1Δ* mutant by Cqs2, whereas Nrg1 and Liv3 binding increased for some promoters and decreased for others (Figure 3.8E and Figure 3.9). In the majority of cases, Qsp1 promotes rather than inhibits the binding of a transcription factor to their targets. This indicates that Qsp1 promotes the binding of Cqs2 upstream of genes and affects the binding of Nrg1 and Liv3.

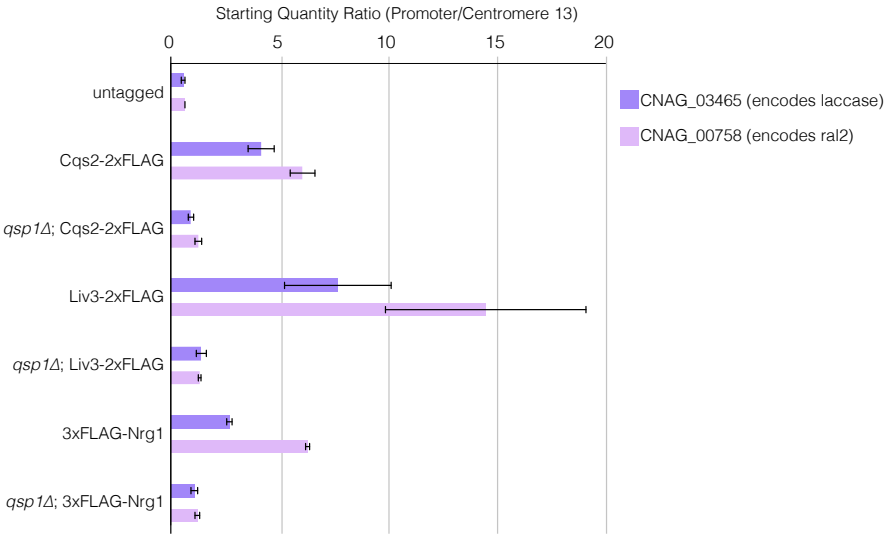

Figure 3.10. ChIP-qPCR validation of ChIP-seq results. ChIP was performed on tagged strains followed by qPCR to quantify binding of CNAG_00758 and CNAG_03465 by tagged Nrg1, Liv3, and Cqs2 in wild type or *qsp1Δ* knockout. Averages of 2 biological replicates wvith 2 technical replicates are shown.

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 were greater than 1.5-fold changed in either direction that were called as bound in either wild-type or *qsp1Δ* mutants, and 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 (Figure 3.8E, 3.8F). 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 (Figure 3.8D), but most of the significance comes from genes that are less bound in the mutant (Figure 3.8E).

3.2.4 Cqs2, Nrg1, and Liv3 are transcription factors that bind to DNA and influence gene expression

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. The overlaps between these two sets were significant in all comparisons, supporting the conclusion that these three transcription factors influence gene expression via binding to target genes under the conditions tested (Figure 3.11). Non-overlapping genes may be regulated indirectly or via other inputs.

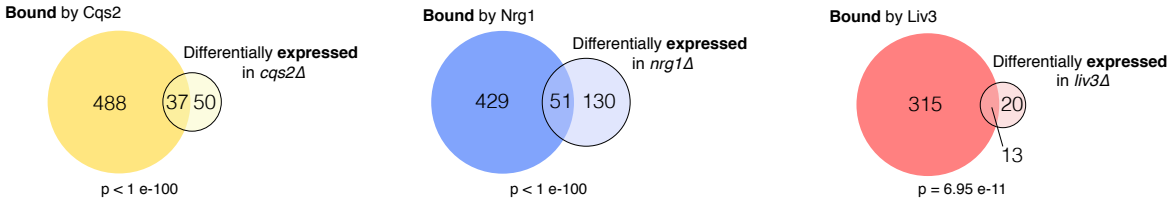

Figure 3.11. Cqs2, Nrg1, and Liv3 are transcription factors that bind to DNA and influence gene expression. Overlap between promoters bound by each transcription factor in wild type and genes that are differentially expressed in the corresponding transcription factor mutant compared to wild type, at OD1 in minimal media.

3.2.5 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 promoters that 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 (Figure 3.12).

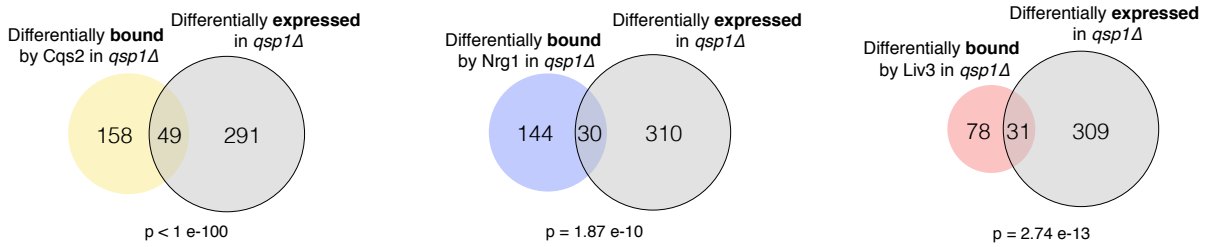

Figure 3.12. A Qsp1-dependence on binding levels of Cqs2, Nrg1, and Liv3 to promoters correlates with a change in expression in *qsp1Δ* mutants compared to wild type. Overlap between promoters differentially bound by each transcription factor and genes that are differentially expressed in a *qsp1Δ* mutant relative to wild type, at OD1 in minimal media.

To test if there was a relationship between the combination of transcription factors bound and gene expression, we created a heatmap displaying 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 (Figure 3.13A). We observed that the largest impact on gene expression happened for genes whose promoters were much less occupied by all three transcription factors together in the *qsp1Δ* mutant (Figure 3.13B). This decrease in occupancy of all three transcription factors in a *qsp1Δ* mutant compared to wild type corresponds with a significant decrease in expression of about a third of these genes (Figure 3.13B, C, & D).

From our conservative analysis, there are thirteen genes where Qsp1 promotion of Cqs2, Nrg1, and Liv3 binding correlates with a significant change in expression in the *qsp1Δ* mutant compared

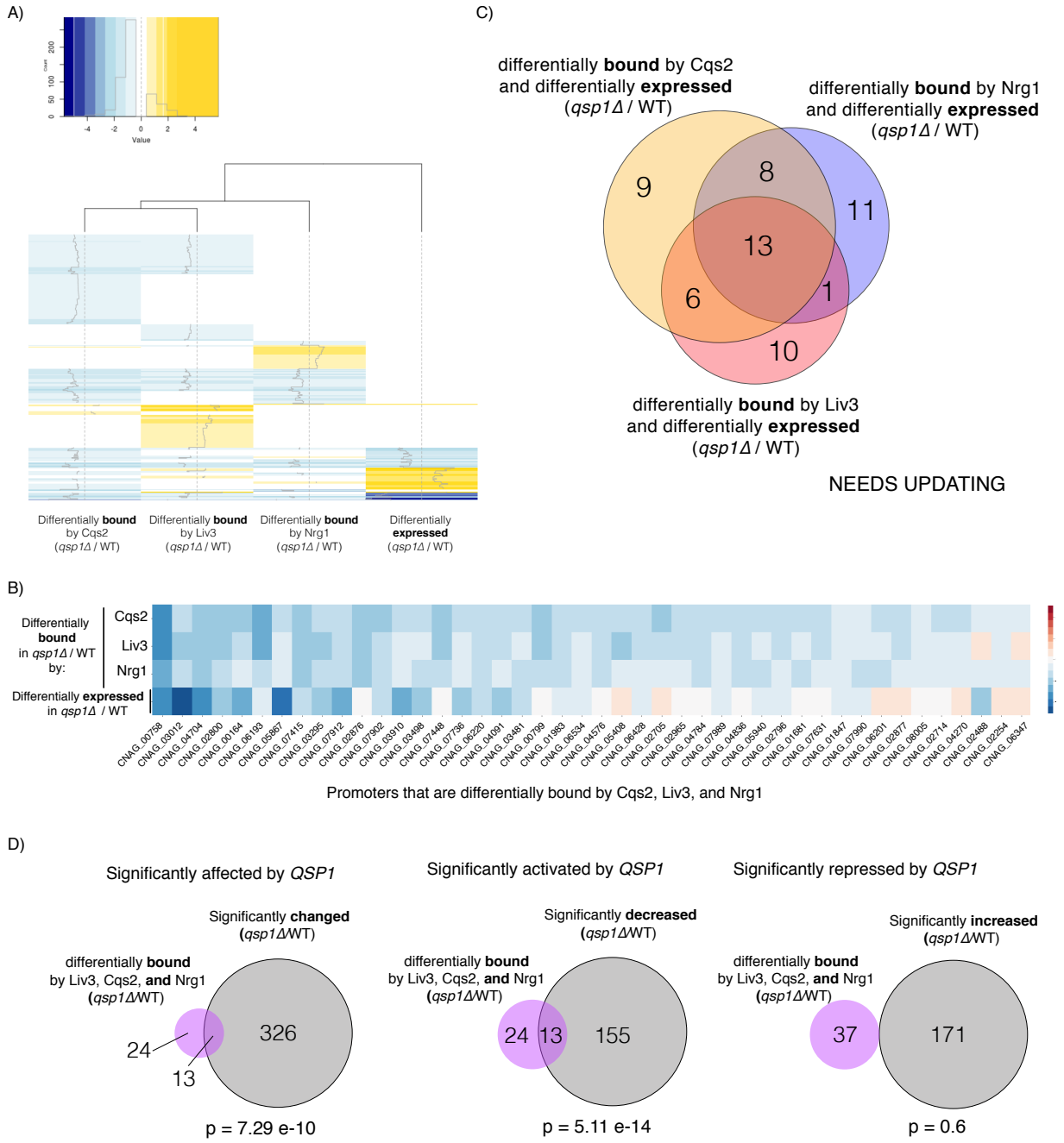

Figure 3.13. Differential binding of three Qsp1-regulated transcription factors to promoters is correlated with differential expression in a *qsp1Δ* knockout. Heatmap of promoters that are differentially bound by **A)** at least one transcription factor or **B)** all three transcription factors, and the respective log2-fold expression difference for the downstream gene in *qsp1Δ* mutants 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 or red. **C)** The amount of overlap between genes that are differentially bound by a transcription factor and genes that are differentially expressed in a *qsp1Δ* mutant relative to wild type, and genes that are differentially bound by another transcription factor and differentially expressed in *qsp1Δ* mutants. **D)** Overlap between genes that are differentially bound by all three transcription factors (Liv3, Cqs2, and Nrg1) in the *qsp1Δ* compared to wild type and genes that are significantly changed, decreased, or increased in the *qsp1Δ* mutant over wild type.

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* (15). Ral2 activates Ras1, a GTPase that is also activated by Ste6, the alpha mating factor transporter and exchange factor for Ras1 (15,16). Another of these genes encodes Agn1, a putative α-glucanase, and could be related to the cell wall phenotype of *qsp1Δ* and *cqs2Δ* mutants (17).

Table 3.1. Thirteen genes are differentially bound by Cqs2, Liv3, and Nrg1 and differentially expressed in minimal media at OD1. Predictions are based on the FungiDB database (www.fungidb.org/fungidb).

Gene	**FungiDB prediction**	Cqs2 **binding difference**, *qsp1*Δ / WT (fold change)	Liv3 **binding difference**, *qsp1*Δ / WT (fold change)	Nrg1 **binding difference**, *qsp1*Δ / WT (fold change)	**Expression difference**, *qsp1*Δ / WT (log2fold change)
CNAG_04704	MFS transporter, SHS family, lactate transporter	-2.146984984	-2.086963676	-1.812106251	-3.353711453
CNAG_00164	hypothetical protein	-1.723124352	-1.570017835	-1.102078907	-3.02915977
CNAG_00758	Ral2	-3.661595619	-3.42453112	-3.065837112	-3.134524317
CNAG_05867	L-fucose transporter	-1.10169001	-0.8190109623	-1.038811792	-4.054070329
CNAG_06220	allergen	-1.537145061	-0.9763878669	-1.339831079	-1.169683049
CNAG_03481	ribonuclease P protein component	-1.502722947	-0.9396796768	-1.324953767	-1.241050662
CNAG_07902	AAT family amino acid transporter	-2.055690063	-1.300864527	-1.427461922	-1.243178786
CNAG_07415	hypothetical protein	-1.864334723	-2.060466898	-1.916548414	-1.055923056
CNAG_02800	hypothetical protein	-2.215638285	-1.729995297	-1.317722549	-2.231716326
CNAG_03295	hypothetical protein	-1.537740823	-2.041700727	-1.317847643	-1.853757787
CNAG_07736	glucan endo-1,3-alpha-glucosidase agn1	-1.097461985	-0.7092281975	-0.999094169	-2.268387208
CNAG_04091	hypothetical protein	-1.210716649	-0.900720007	-0.8430093613	-1.942559812
CNAG_03910	myo-inositol transporter, putative	-1.516107887	-0.8657361164	-0.814114154	-2.415593154

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 genes predicted to be involved in nutrient sensing, signaling, and acquisition as well as cell wall remodeling.

3.2.6 Loss *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 difference in expression of each of these tagged

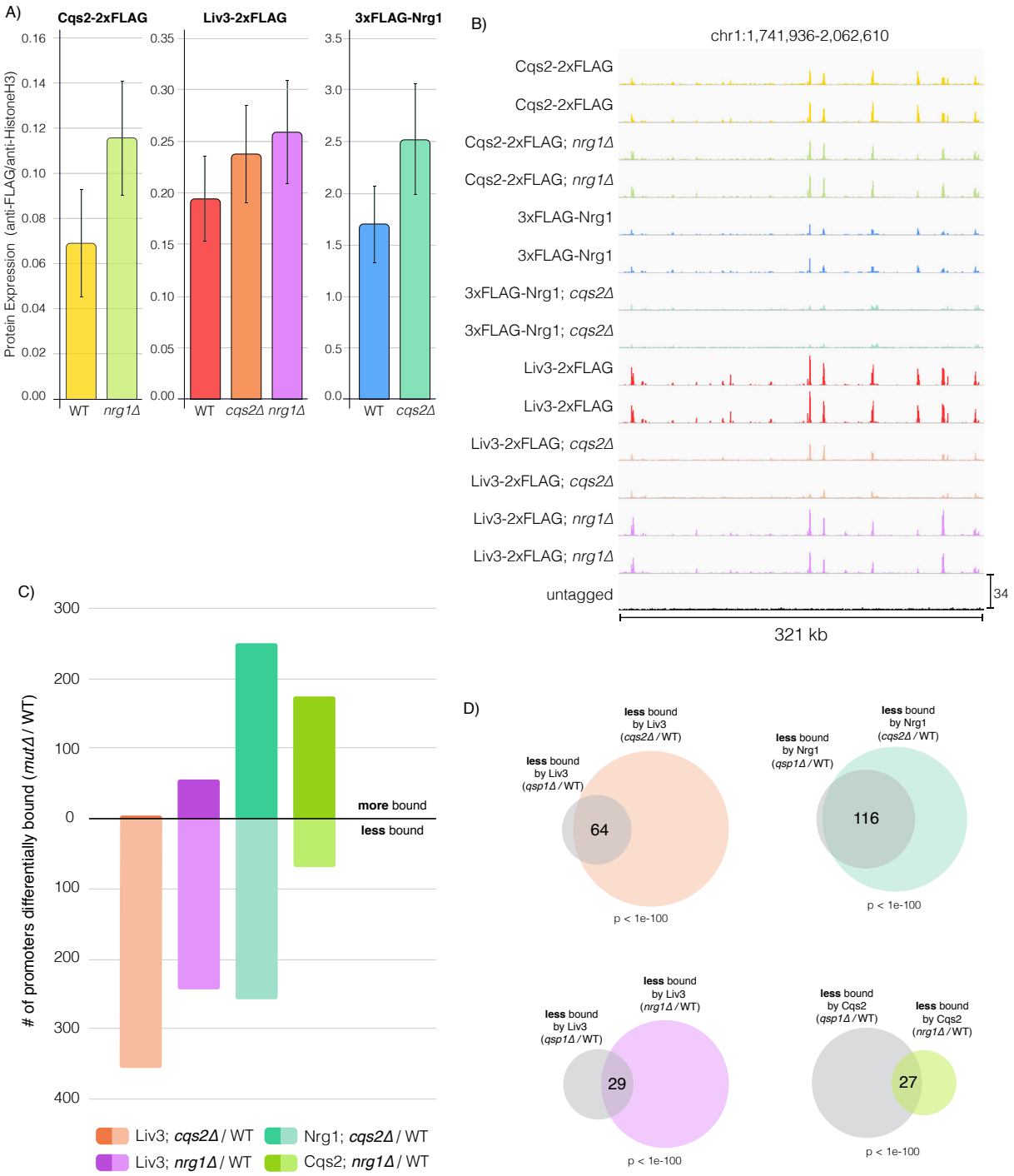

Figure 3.14. Qsp1 acts through Cqs2 to affect transcription factor binding to promoters. A) Protein levels of FLAG-tagged Cqs2, Liv3, and Nrg1 in each transcription factor deletion strain background. Average of two biological replicates is shown. **B)** ChIP-Seq data was visualized using the Integrative Genomics Viewer software. Binding across part of chromosome 1 is shown. **C)** Breakdown of genes that are bound more or less by a given transcription factor in *qsp1Δ* compared to wild type, filtered by promoters significantly bound in either genotype. **D)** Overlaps between gene sets that are less bound by each transcription factor in the *qsp1Δ* mutant and genes that are less bound by each transcription factor in the *nrg1Δ* or *cqs2Δ* mutants.

transcription factors in the mutant background compared to wild type (Figure 3.14A). An example of the binding pattern of Liv3, Nrg1, and Cqs2 in these backgrounds across part of chromosome 1 is shown (Figure 3.14B). 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 (Figure 3.15). 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 (Figure 3.14, 3.15). In contrast, deletion of *CQS2* both reduced and increased

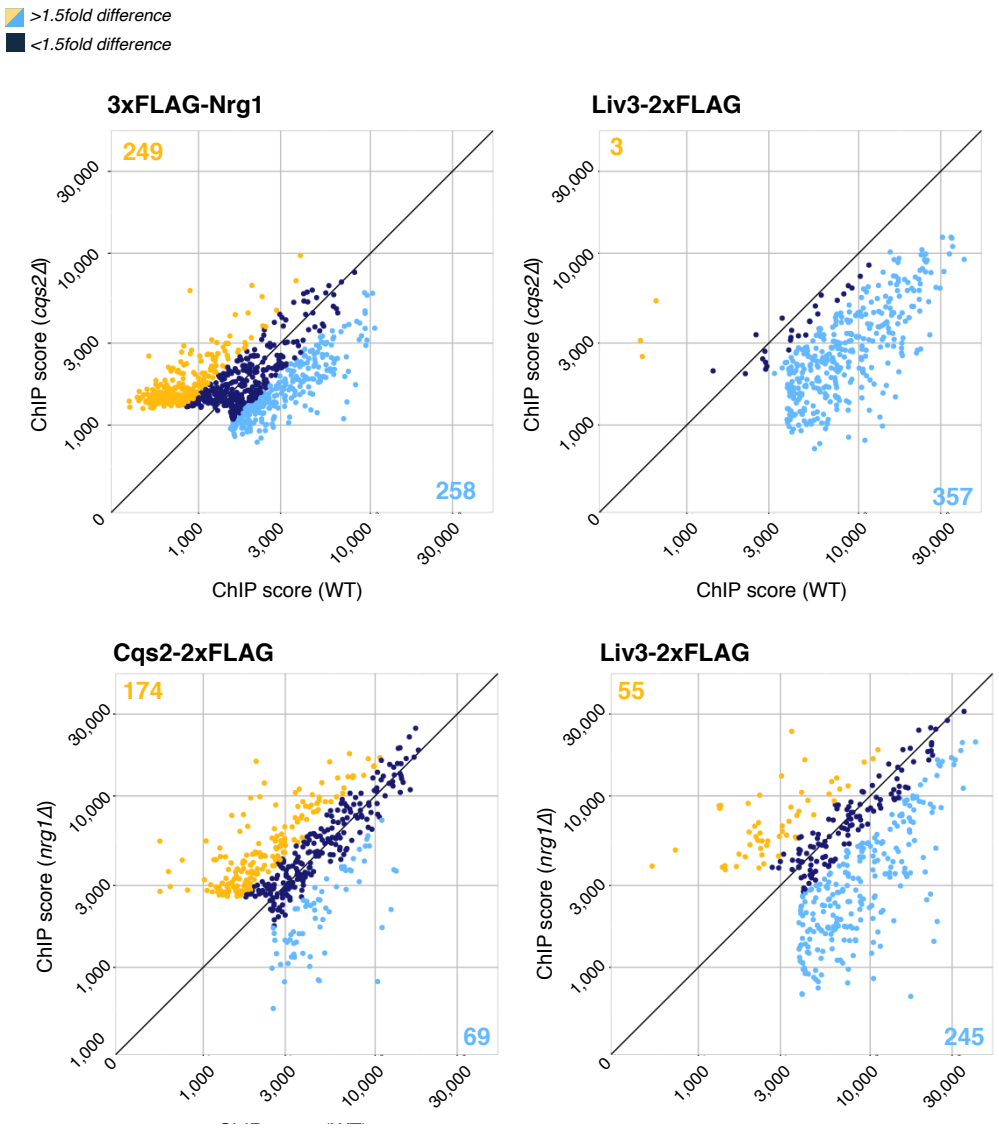

Figure 3.15. *CQS2* or *NRG1* deletion affects the binding of Cqs2, Liv3, and Nrg1 to their promoters. The ChIP score for each gene was calculated as the read depth in the 1 kb region upstream of the transcription start site, normalized to the untagged control. Only genes that are called as bound in 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 yellow or light blue.

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 (Figure 3.14D and Table 3.2). 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 (Figure 3.14D, Figure 3.15, and Table 3.2). 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.

Together, these data support the model that Qsp1 influences gene expression by affecting a network of transcription factors' ability to bind to DNA, and that Cqs2 appears to be the main factor through which Qsp1 acts under these conditions.

3.4. 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 starvation, mating, biofilm formation, and host infection. Quorum sensing has been reported in many different microbes to regulate competence (18–22), starvation and mating (23), regulation of sporulation in response to starvation (24), nutrient acquisition and virulence (25). Our experiments have uncovered that Qsp1 regulates gene expression by influencing three transcription factors that play roles in mating, virulence, and nutrient acquisition, providing

Table 3.2. Analysis of the degree and significance of overlaps between different sets of genes. Promoters 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 are compared. The figure generated from the data is indicated.

Comparison of promoters bound by each TF in WT or *qsp1Δ*	SET 1	SET 2	SET 1 (# of genes)	SET 2 (# of genes)	Overlap (# of genes)	p value	p adjusted	Figure
	Bound by C	Bound by N	525	480	411	0	0	Figure 3.8C
	Bound by N	Bound by L	480	328	277	0	0	Figure 3.8C
	Bound by L	Bound by C	328	525	306	0	0	Figure 3.8C
	Bound by C	Bound by C in *qsp1Δ* vs. WT	525	709	475	0	0	
	Bound by N	Bound by N in *qsp1Δ* vs. WT	480	426	349	0	0	
	Bound by L	Bound by L in *qsp1Δ* vs. WT	328	324	269	0	0	

Comparison of promoters that are differentially bound by each TF in qsp1Δ vs. WT	SET 1	SET 2	SET 1 (# of genes)	SET 2 (# of genes)	Overlap (# of genes)	p value	p adjusted	Figure
	Diff bound by C in *qsp1Δ* vs. WT	Diff bound by N in *qsp1Δ* vs. WT	207	174	101	0	0	Figure 3.8D
	Diff bound by N in *qsp1Δ* vs. WT	Diff bound by L in *qsp1Δ* vs. WT	174	104	45	0	0	Figure 3.8D
	Diff bound by L in *qsp1Δ* vs. WT	Diff bound by C in *qsp1Δ* vs. WT	104	207	66	0	0	Figure 3.8D
	Less bound by C in *qsp1Δ* vs. WT	Less bound by N in *qsp1Δ* vs. WT	200	120	99	0	0	Figure 3.8E, 3.8F
	Less bound by N in *qsp1Δ* vs. WT	Less bound by L in *qsp1Δ* vs. WT	120	72	42	0	0	Figure 3.8E, 3.8F
	Less bound by L in *qsp1Δ* vs. WT	Less bound by C in *qsp1Δ* vs. WT	72	200	63	0	0	Figure 3.8E, 3.8F
	Less bound by C in *qsp1Δ* vs. WT	More bound by N in *qsp1Δ* vs. WT	200	54	0	0.79	1	
	Less bound by N in *qsp1Δ* vs. WT	More bound by L in *qsp1Δ* vs. WT	120	32	3	2.05E-03	0.182	
	Less bound by L in *qsp1Δ* vs. WT	More bound by C in *qsp1Δ* vs. WT	72	**7**	0	0.07	1	
	More bound by C in *qsp1Δ* vs. WT	Less bound by N in *qsp1Δ* vs. WT	7	120	0	0.11	1	
	More bound by N in *qsp1Δ* vs. WT	Less bound by L in *qsp1Δ* vs. WT	54	72	0	0.43	1	
	More bound by L in *qsp1Δ* vs. WT	Less bound by C in *qsp1Δ* vs. WT	32	200	2	0.06	1	
	More bound by C in *qsp1Δ* vs. WT	More bound by N in *qsp1Δ* vs. WT	7	54	2	1.49E-05	1.33E-03	Figure 3.8E
	More bound by N in *qsp1Δ* vs. WT	More bound by L in *qsp1Δ* vs. WT	54	32	0	0.22	1	Figure 3.8E
	More bound by L in *qsp1Δ* vs. WT	More bound by C in *qsp1Δ* vs. WT	32	7	1	4.20E-04	3.74E-02	Figure 3.8E

ChIP seq vs RNA-seq (for each TF, in wild-type)	SET 1 (ChIP seq)	SET 2 (RNA-seq)	SET 1 (# of genes)	SET 2 (# of genes)	Overlap (# of genes)	p-value	p adjusted	Figure
sig vs sig	Bound by C	*cqs2Δ* vs. WT (sig changed)	525	87	37	0	0	Figure 3.11
sig vs sig	Bound by N	*nrg1Δ* vs. WT (sig changed)	480	181	51	0	0	Figure 3.11
sig vs sig	Bound by L	*liv3Δ* vs. WT (sig changed)	328	33	13	0	6.19E-09	Figure 3.11
sig vs up	Bound by C	*cqs2Δ* vs. WT (sig increased)	525	41	11	2.98E-05	2.65E-03	
sig vs up	Bound by N	*nrg1Δ* vs. WT (sig increased)	480	84	23	9.14E-10	8.13E-08	
sig vs up	Bound by L	*liv3Δ* vs. WT (sig increased)	328	26	8	1.52E-06	1.35E-04	
sig vs down	Bound by C	*cqs2Δ* vs. WT (sig decreased)	525	46	26	0	0	
sig vs down	Bound by N	*nrg1Δ* vs. WT (sig decreased)	480	97	28	0	3.93E-10	
sig vs down	Bound by L	*liv3Δ* vs. WT (sig decreased)	328	7	5	6.87E-08	6.11E-06	

Comparison of promoters differentially bound by each TF to genes differentially expressed in qsp1Δ vs. WT	SET 1 (genes >1.5x diff bound and called as bound in either genotype)	SET 2 (RNA-seq)	SET 1 (# of genes)	SET 2 (# of genes)	Overlap (# of genes)	p-value	p adjusted	Figure
sig vs sig	diff bound by C in *qsp1Δ* vs. WT	*qsp1Δ* vs WT (sig changed)	207	340	49	0	0	Figure 3.12
sig vs sig	diff bound by N in *qsp1Δ* vs. WT	*qsp1Δ* vs WT (sig changed)	174	340	30	1.87E-10	1.66E-08	Figure 3.12
sig vs sig	diff bound by L in *qsp1Δ* vs. WT	*qsp1Δ* vs WT (sig changed)	104	340	26	0	0	Figure 3.12
down vs up	less bound by C in *qsp1Δ* vs. WT	*qsp1Δ* vs WT (sig increased)	200	171	17	1.58E-06	1.41E-04	
down vs up	less bound by N in *qsp1Δ* vs. WT	*qsp1Δ* vs WT (sig increased)	72	171	4	3.10E-02	1	
down vs up	less bound by L in *qsp1Δ* vs. WT	*qsp1Δ* vs WT (sig increased)	120	171	2	0.57	1	
down vs down	less bound by C in *qsp1Δ* vs. WT	*qsp1Δ* vs WT (sig decreased)	200	169	32	0	0	
down vs down	less bound by N in *qsp1Δ* vs. WT	*qsp1Δ* vs WT (sig decreased)	72	169	18	0	0	
down vs down	less bound by L in *qsp1Δ* vs. WT	*qsp1Δ* vs WT (sig decreased)	120	169	21	0	0	
up vs up	more bound by C in *qsp1Δ* vs. WT	*qsp1Δ* vs WT (sig increased)	7	171	0	0.16	1	
up vs up	more bound by N in *qsp1Δ* vs. WT	*qsp1Δ* vs WT (sig increased)	32	171	3	7.29E-03	0.648	
up vs up	more bound by L in *qsp1Δ* vs. WT	*qsp1Δ* vs WT (sig increased)	54	171	7	4.34E-05	3.86E-03	
up vs down	more bound by C in *qsp1Δ* vs. WT	*qsp1Δ* vs WT (sig decreased)	7	169	0	0.16	1	
up vs down	more bound by N in *qsp1Δ* vs. WT	*qsp1Δ* vs WT (sig decreased)	32	169	1	0.18	1	
up vs down	more bound by L in *qsp1Δ* vs. WT	*qsp1Δ* vs WT (sig decreased)	54	169	0	7.30E-01	1	

Promoters differentially bound by all 3 TFs compared to differentially expressed genes in *qsp1*Δ vs. WT	**SET 1**	**SET 2**	**SET 1 (# of genes)**	**SET 2 (# of genes)**	**Overlap (# of genes)**	**p-value**	**p adjusted**	**Figure**
	diff bound by C,N, & L	*qsp1*Δ vs WT (sig changed)	37	339	13	6.76E-10	7.40E-09	Figure 3.13
	diff bound by C,N, & L	*qsp1*Δ vs WT (sig increased)	37	171	0	0.6	1	Figure 3.13
	diff bound by C,N, & L	*qsp1*Δ vs WT (sig decreased)	37	168	13	0	0	Figure 3.13
	less bound by C, N & L	*qsp1*Δ vs WT (sig changed)	36	339	13	4.39E-10	3.94E-08	
	less bound by C, N & L	*qsp1*Δ vs WT (sig increased)	36	171	0	0.59	1	
	less bound by C, N & L	*qsp1*Δ vs WT (sig decreased)	36	168	13	0	0	
	more bound by C, N & L	*qsp1*Δ vs WT (sig changed)	0	339	0	0	0	
	more bound by C, N & L	*qsp1*Δ vs WT (sig increased)	0	171	0	0	0	
	more bound by C, N & L	*qsp1*Δ vs WT (sig decreased)	0	168	0	0	0	

Comparison between sets of genes bound by each TF in each *tf*Δ mutant vs. WT	**SET 1**	**SET 2**	**SET 1 (# of genes)**	**SET 2 (# of genes)**	**Overlap (# of genes)**	**p-value**	**p adjusted**	**Figure**
	Bound by C	Bound by C in *nrg1*Δ	337	401	270	0	0	
	Bound by L	Bound by L in *nrg1*Δ	371	249	199	0	0	
	Bound by N	Bound by N in *cqs2*Δ	480	709	357	0	0	
	Bound by L	Bound by L in *cqs2*Δ	371	236	221	0	0	

Comparison of promoters differentially bound by each TF in each mutant	**SET 1**	**SET 2**	**SET 1 (# of genes)**	**SET 2 (# of genes)**	**Overlap (# of genes)**	**p-value**	**p adjusted**	**Figure**
sig vs sig	diff bound by N in *qsp1*Δ	diff bound by N in *cqs2*Δ	174	507	142	0	0	
sig vs sig	diff bound by C in *qsp1*Δ	diff bound by C in *nrg1*Δ	207	243	96	0	0	
sig vs sig	diff bound by L in *qsp1*Δ	diff bound by L in *cqs2*Δ	104	360	78	0	0	
sig vs sig	diff bound by L in *qsp1*Δ	diff bound by L in *nrg1*Δ	104	300	53	0	0	
sig vs sig	diff bound by L in *cqs2*Δ	diff bound by L in *nrg1*Δ	360	300	245	0	0	
down vs down	less bound by N in *qsp1*Δ	less bound by N in *cqs2*Δ	120	258	116	0	0	Figure 3.14C, 3,14D, 3.15
down vs down	less bound by C in *qsp1*Δ	less bound by C in *nrg1*Δ	200	69	27	0	0	Figure 3.14C, 3,14D, 3.15
down vs down	less bound by L in *qsp1*Δ	less bound by L in *cqs2*Δ	72	357	64	0	0	Figure 3.14C, 3,14D, 3.15
down vs down	less bound by L in *qsp1*Δ	less bound by L in *nrg1*Δ	72	245	29	0	0	Figure 3.14C, 3,14D, 3.15
down vs down	less bound by L in *cqs2*Δ	less bound by L in *nrg1*Δ	357	245	231	0	0	
down vs up	less bound by N in *qsp1*Δ	more bound by N in *cqs2*Δ	120	249	0	0.99	1	
down vs up	less bound by C in *qsp1*Δ	more bound by C in *nrg1*Δ	200	174	67	0	0	
down vs up	less bound by L in *qsp1*Δ	more bound by L in *cqs2*Δ	72	3	1	3.12E-04	2.78E-02	
down vs up	less bound by L in *qsp1*Δ	more bound by L in *nrg1*Δ	72	55	10	0	4.66E-10	
down vs up	less bound by L in *cqs2*Δ	more bound by L in *nrg1*Δ	357	55	13	4.10E-07	3.65E-05	
up vs down	more bound by N in *qsp1*Δ	less bound by N in *cqs2*Δ	54	258	0	0.87	1	
up vs down	more bound by C in *qsp1*Δ	less bound by C in *nrg1*Δ	7	69	2	3.13E-05	2.79E-03	
up vs down	more bound by L in *qsp1*Δ	less bound by L in *cqs2*Δ	32	357	13	1.30E-10	1.16E-08	
up vs down	more bound by L in *qsp1*Δ	less bound by L in *nrg1*Δ	32	245	11	3.19E-10	2.84E-08	
up vs down	more bound by L in *cqs2*Δ	less bound by L in *nrg1*Δ	3	245	0	0.1	1	
up vs up	more bound by N in *qsp1*Δ	more bound by N in *cqs2*Δ	54	249	26	0	0	Figure 3.14C, 3,14D, 3.15
up vs up	more bound by C in *qsp1*Δ	more bound by C in *nrg1*Δ	7	174	0	0.16	1	Figure 3.14C, 3,14D, 3.15
up vs up	more bound by L in *qsp1*Δ	more bound by L in *cqs2*Δ	32	3	0	0.0137	1	Figure 3.14C, 3,14D, 3.15
up vs up	more bound by L in *qsp1*Δ	more bound by L in *nrg1*Δ	32	55	3	1.05E-04	9.35E-03	Figure 3.14C, 3,14D, 3.15
up vs up	more bound by L in *cqs2*Δ	more bound by L in *nrg1*Δ	3	55	1	1.81E-04	1.61E-02	

insight into the mechanism by which a eukaryotic quorum sensing molecule can influence gene expression and clues to the role 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). 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 inside the cell, these small peptides interact with phosphatases (26,27). 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 (Figure 3.5), which were identified on the basis of a temperature-regulated rough colony morphology that is also exhibited by a *qsp1Δ* knockout (Figure 3.1). Surprisingly, we found that Qsp1 seems to promote the binding of Cqs2 to promoters and alter the binding of Nrg1 and Liv3 (Figure 3.8, Figure 3.9). 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 (Figure 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 (Figure 3.9). We observed this correlation with reduced binding by leading to reduced expression in spite of higher levels of Cqs2 and Nrg1 protein in *qsp1Δ* mutants in this condition (Figure 3.6). Furthermore, Cqs2 promotes Liv3 binding to promoters (Figures 3.14, 3.15). 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* (Figure 3.14). Nrg1 also impacts Cqs2 and Liv3 binding (Figure 3.14, 3.15). 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 (Figure 3.14). Together, these data support the model that Qsp1 influences gene expression by affecting a network of transcription factors' ability to bind to DNA (Figure 3.16). However, 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.

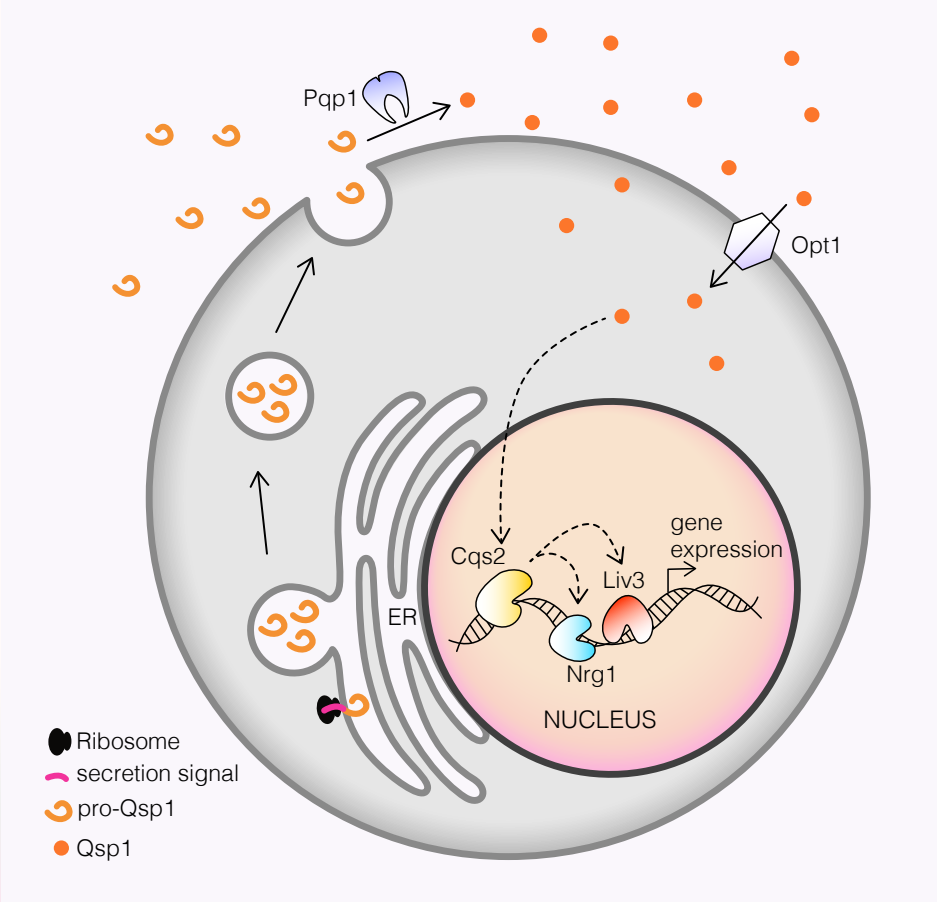

Figure 3.16. Model for how Qsp1 triggers changes in gene expression in Cryptococcus neoformans. Following import into the cytoplasm, Qsp1 alters the binding of Nrg1 and Liv3 by modulating the ability of Cqs2 to bind promoters, thereby causing changes in gene expression.

Cqs2, Liv3, and Nrg1 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 (31–34). In *C. albicans* and *S. japonicus*, Nrg1 is also regulated by quorum sensing (35–37). Here, we show that Nrg1 and Liv3 protein levels are repressed by the *QSP1* gene in minimal media (Figure 3.6). We also found that Qsp1 promotes the

binding of Cqs2 to promoters and influences the binding of Nrg1 and Liv3 (Figure 3.8, 3.9), and that these transcription factors influence each other's binding (Figure 3.15). 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 a nutrients are exhausted provide 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 (Figure 3.4). In addition, we found that Qsp1 promotion of resistance of stationary phase cells to cell wall stress requires Nrg1 and Cqs2 (Figure 3.3), 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) (Figure 3.10), which encodes the melanization factor laccase (41–43). Melanization is known to be a key virulence trait for C. neoformans infection (41,44). In our previous publication, we cound that cells lacking Qsp1 display altered capacities to produce melanin when plated on plates containing the substrate for melanin production (4). Though our conditions were not sufficient to promote laccase expression, it likely that these three transcription factors bind to the laccase promoter in the presence of Qsp1 in order to prime the cell for transcription as soon as the proper signal for laccase expression is 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 medai), ultimately influencing the mating and virulence of this organism.

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Chapter 4: The search for the intracellular receptor of Qsp1

In which I test the hypothesis that Qsp1 acts either directly on Nrg1, Liv3, and Cqs2, and present future directions.

4.1 Summary

Qsp1 is a secreted quorum sensing peptide required for virulence of the fungal meningitis pathogen Cryptococcus neoformans. Qsp1 controls the genomic associations of three transcription factors, Cqs2, Liv3, and Nrg1, to genes whose expression is regulated by Qsp1. Remarkably, these three transcription factors form a network and bind to a common set of promoters. Deletion of *CQS2* and *NRG1* affects binding levels of other two transcription factors in the network to promoters *in vivo*. However, it is not known whether Qsp1 directly influences these factors, or if these factors operate cooperatively to bind promoters together. Here, we were unable to find evidence that the binding of these factors to radiolabeled DNA is enhanced by Qsp1 *in vitro* using recombinantly expressed and purified Cqs2, Liv3, and Nrg1. Furthermore, we investigate the potential role of Pka2, a protein kinase A homolog, in Qsp1 signaling via phosphorylation of a PKA consensus motif on Nrg1. These biochemical and genetic studies further explore mechanisms by which an imported peptide acts to modulate eukaryotic gene expression.

4.2 Introduction

The cryptococcal quorum sensing peptide Qsp1 is secreted as a precursor that is processed extracellularly by Pqp1, then presumably imported by the oligopeptide transporter Opt1, where it is active intracellularly (1). Qsp1 influences gene expression by controlling the genomic association of the three transcription factors Cqs2, Nrg1, and Liv3 to promoters (Chapter 3). We found that these transcription factors generally bind together to a common set of target genes, forming a highly connected transcription factor network (Chapter 3). 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 (Chapter 3).

A similar quorum sensing paradigm exists in gram-positive bacteria, where an exported precursor peptide is processed, then imported by an oligopeptide permease. Once inside the cell, the peptide then binds to an intracellular receptor such as a transcriptional regulator or a phosphatase to influence gene expression.

While it is known that Qsp1 is active intracellularly and effects transcription factors, how it is able to act is unknown. We hypothesized that Qsp1 may be binding to Cqs2, Nrg1, or Liv3 to directly influence the ability of one or these factors to bind DNA, a mechanism that if true would be unprecedented in eukaryotes. In this chapter, I test the ability of synthetic Qsp1 peptide to alter the binding affinity of recombinantly expressed and purified Cqs2, Nrg1, or Liv3. I also investigate the ability of Cqs2, Nrg1, and Liv3 to act cooperatively to bind DNA.

Furthermore, cells lacking components of the Qsp1 signaling pathway display a rough colony morphology phenotype. To determine if there could be another factor upstream of Nrg1, Liv3, and Cqs2, we screened strains in our gene deletion in *Cryptococcus neoformans* for this phenotype. We found that a strain harboring a deletion of a gene encoding the catalytic subunit of protein kinase A, *PKA2,* formed rough colonies on a plate at room temperature. These catalytic subunits together with the regulatory subunit Pkr1, form the PKA complex. An intracellular increase in cyclic AMP (cAMP) produced by adenylyl cyclase (Cac1) leads to a conformational

change in the regulatory subunits, freeing the catalytic subunits for activation of downstream regulators (2). The G-protein-regulated cyclic AMP (cAMP) pathway has been studied in *C. neoformans* as one of the signaling pathways regulating virulence and morphology (2–4). *C. neoformans* has two protein kinase A subunits, Pka1 and Pka2, as a result of a gene duplication event (5). Pka1 regulates mating and expression of virulence factors in serotype A (6,7)failed to produce melanin or capsule, and were avirulent. The PKR1 gene encoding the protein kinase A (PKA. However, it is unknown what role Pka2 plays. Intriguingly, Nrg1 contains a conserved PKA phosphorylation motif. We hypothesized that Pka2 phosphorylation of this motif could be an avenue of Qsp1-dependent regulation.

4.3 Results

4.3.1 Qsp1 does not influence binding of recombinantly expressed and purified transcription factors to radiolabeled promoters.

To investigate the hypothesis that Qsp1's influence on this transcription factor network was direct, and that these transcription factors bind DNA in a cooperative manner, we purified full length Nrg1, Cqs2, and Liv3 and tested their ability to bind DNA, both individually (Figure 4.1A) and with each other or with synthetic Qsp1 peptide (Figure 4.1B, 4.1C). We chose to radiolabel 200bp fragments of promoters (except CNAG_02711, which we tested as a 400bp fragment) corresponding to promoters where we saw the largest binding peaks in transcription factor binding in wild type cells and largest decreases in binding in the in the *qsp1Δ* mutant *in vivo*. Tagged Nrg1 and Liv3 proteins appeared to bind DNA, as increasing the concentration of each protein in the reaction resulted in shifting of more free probe (0 nM lanes) higher in the gel, indicating formation of a protein:DNA complex that migrates slower (Figure 4.1A). Cqs2-FLAG only faintly bound DNA under these conditions (Figure 4.1A).

We next tested if addition of an excess of synthetic Qsp1 peptide to the binding reaction could affect the affinity of any of these factors for binding of DNA. There was no difference in the proportion of free probe to bound probe at each concentration of tagged Nrg1 or Liv3 or Cqs2, in conditions with Qsp1 peptide compared to conditions without (Figure 4.1B). Lastly, we tested

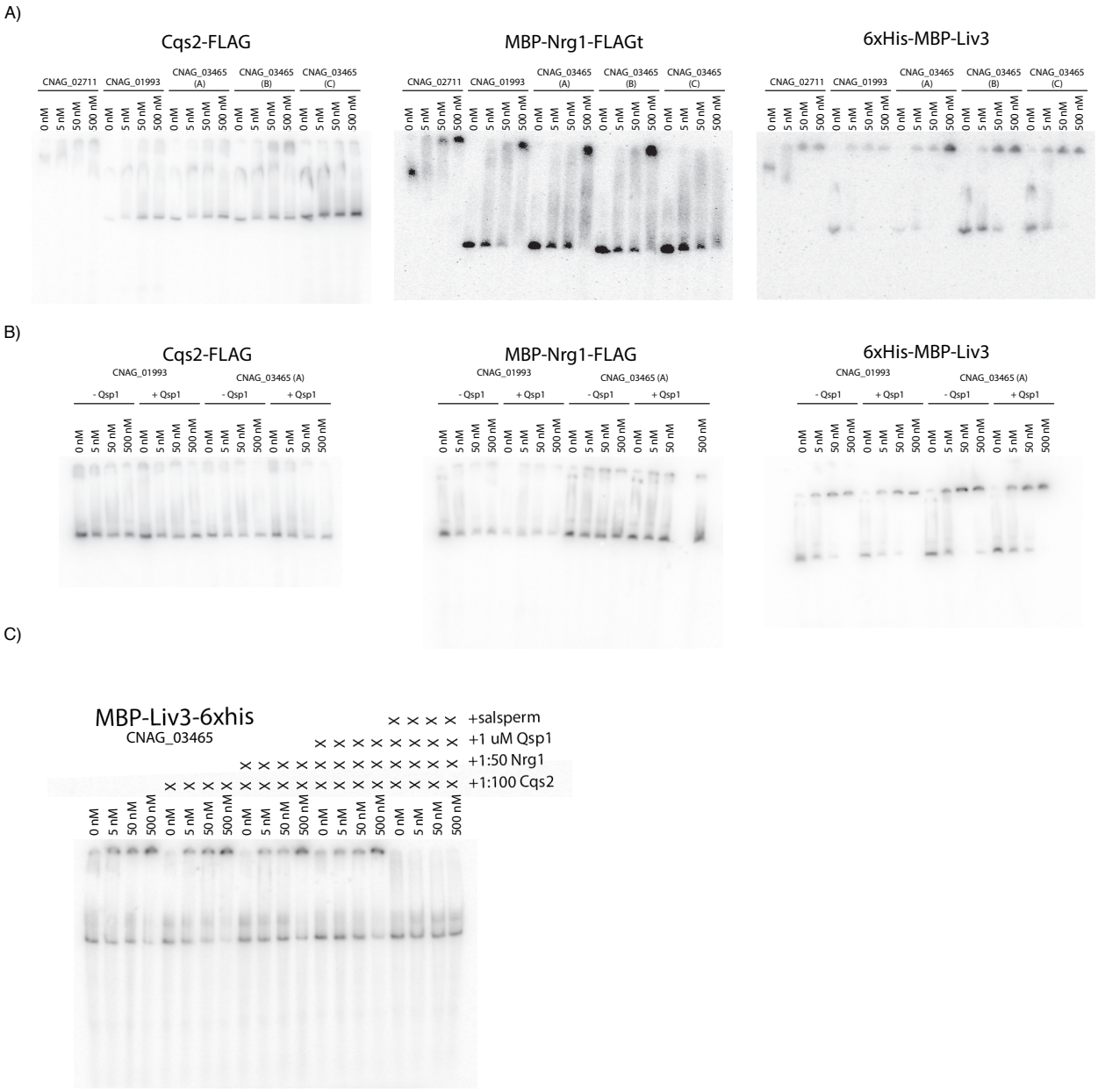

Figure 4.1. Qsp1 is not sufficient to effect binding of purified transcription factors to promoters *in vitro*. Tagged and purified transcription factors were tested for their ability to bind radiolabeled promoter fragments that exhibited Qsp1-dependence for binding of these factors by chromatin immunoprecipitation, **A)** by themselves, **B)** with and without 1uM Qsp1 peptide, or **C)** in combination with each other and Qsp1 via electrophoresis mobility shift assays.

adding Cqs2, Cqs2 and Nrg1, or Cqs2 and Nrg1 and Qsp1 peptide to Liv3 binding reactions to determine if adding these factors together to DNA could affect the formation of protein:DNA complexes in the binding reaction. None of these combinations affected the amount of free probe, indeed, all of these binding reactions looked very similar to each other (Figure 4.1C). Additionally, it looked as if the binding exhibited by tagged Nrg1 and Liv3 was nonspecific, as adding unlabeled salmon sperm DNA to the reaction quenched the formation of the complex (Figure 4.1C and not shown). Testing the ability of Nrg1 and Cqs2 to bind to probes containing either their own binding sequence or the other factors binding motif supported this conclusion, as both factors were able to bind to both motifs similarly (not shown).

In conclusion, we were not able to find evidence that Qsp1 directly affects the ability of these transcription factors to bind DNA *in vitro*. It is possible that we were not able to achieve the proper conditions for specific binding in this experiment, and if we were to find these conditions, we might be able to see modulation of that activity by Qsp1 or the addition of other transcription factors in the network to the binding reaction. However, it is likely that these factors require other yet unidentified factors to bind DNA in a sequence specific and/or Qsp1-dependent manner.

4.3.2 Phenotypic identification of other potential candidates involved in Qsp1 signaling

It is possible that another factor exists in Qsp1 signaling upstream of transcription factor binding, through which Qsp1 acts to modulate transcription factor affinity for promoters and thus gene expression. Since all known Qsp1 signaling pathway components have been identified on the basis of a rough colony morphology exhibited by genetic deletions of these factors, we chose to screen our gene deletion collection for other promising candidates. We found that a strain harboring a deletion of *PKA2* formed rough colonies on a plate at room temperature (Figure 4.2A).

4.3.3 Involvement of components of PKA signaling in colony morphology and sensitivity to SDS.

Pka2Δ mutants still produce Qsp1 peptide but are not able to be complemented by the peptide, indicating that Pka2 may act downstream of Qsp1 (not shown). However, none of the other

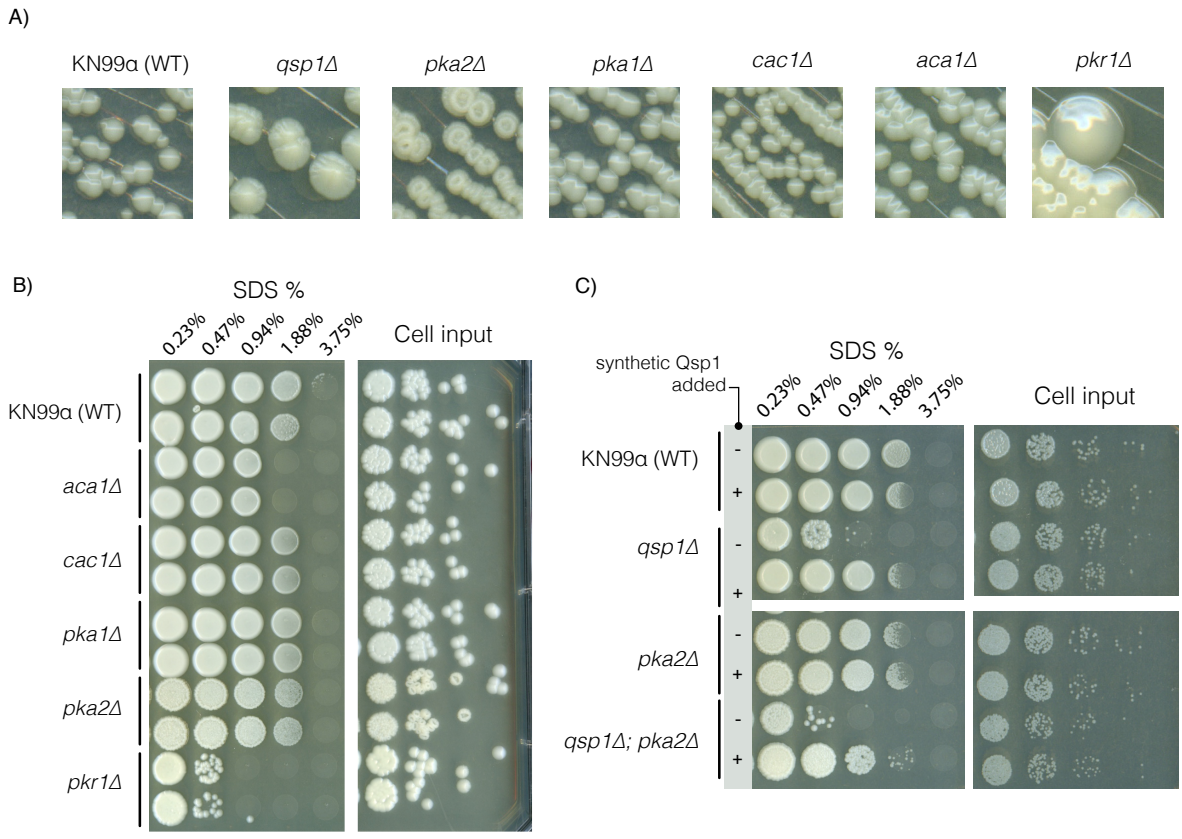

Figure 4.2. Deletions of genes encoding PKA2 and other components of PKA signaling share some phenotypes with Qsp1 signaling pathway mutants. A) Colony morphology of wild type cells or cells lacking *QSP1* or components of the PKA signaling pathway. B, C) Saturated cultures were tested for their ability to survive increasing concentrations of SDS. Water dilutions of the input cell culture are shown to the right as a measure of cell input prior to incubation in SDS. 1 uM synthetic Qsp1 peptide was added to the indicated cultures (+) from the time of inoculation, or not (-).

components involved in cAMP signaling seem to be involved in this colony phenotype, since deletions of genes encoding the adenylyl cyclase that produces cAMP (*cac1Δ*), the activator of Aca1 (*aca1Δ*), the regulatory subunits of PKA (*pkr1Δ*), or the other homolog of the catalytic subunit of PKA (*pka1Δ*) did not yield rough colonies (Figure 4.2A). It is possible that a specific target of Pka2 (that is not targeted by Pka1 or intracellular cAMP) is required for a wild type colony morphology under these conditions.

Cells lacking Qsp1, Nrg1, and Cqs2 exhibit a decrease in their ability to resist high concentrations of the cell wall stressor SDS under saturating culture conditions (Chapter 3, Figure 3.3). Thus, we tested Pka2 and other components of PKA signaling for SDS sensitivity (Figure 4.2B). The *pka2Δ* mutant was indistinguishable from wild type (Figure 4.2B). In addition, a double qsp1Δpka2Δ

mutant exhibits a weakness to SDS that is rescued by growth of the cells in the presence of synthetic Qsp1 peptide (Figure 4.2C). This indicates that Pka2 is dispensable for Qsp1's effect on cell wall strength. However, cells lacking *PKR1* were severely sensitive, and cells lacking *ACA1* were modestly sensitive (Figure 4.2B). Therefore, while Pka2 may not be directly involved in cell wall stress or in Qsp1 signaling for this phenotype, it appears that intracellular cAMP signaling could be either downstream, or also be another input for cell wall strength. Furthermore, since the *C. neoformans* genome contains another catalytic subunit of PKA, Pka1, we wanted to check whether functional redundancy between Pka1 and Pka2 could be rescuing cells harboring only a single knockout of either gene. However, a double *pka1Δpka2Δ* knockout strain did not form rough colonies or exhibit SDS sensitivity (not shown). Further research is required to understand how Qsp1 signaling and cAMP signaling intertwine to promote resistance to SDS.

4.3.4 Phosphorylation state of Nrg1 impacts cell wall sensitivity, but does not impact the ability of cells to respond to Qsp1 peptide.

Intriguingly, Nrg1 contains a conserved PKA phosphorylation motif that is not present in homologs of Nrg1 in *C. albicans* or *S. cerevisiae* (Figure 4.3)(8). We hypothesized that Pka2 could function upstream of Nrg1 in Qsp1 signaling through phosphorylation of this motif. We mutated the endogenous Nrg1 sequence by replacing the predicted phosphorylated serine with a residue that mimics the charge of a phosphorylated residue or by a single amino acid substitution in the sequence that is predicted to be recognized by PKA, rendering the site unphosphorylatable by a PKA (Figure 4.3).

Cells lacking Qsp1 and Nrg1 exhibit a decrease in their ability to resist high concentrations of the cell wall stressor SDS under saturating culture conditions (Chapter 3, Figure 3.3). Since cells lacking *PKA1* or *PKA2* (Figure 4.2B) or both (not shown) did not exhibit SDS sensitivity, it is unlikely that activity from either catalytic subunit is necessary for cell wall stress. As expected, cells expressing the phosphomimic or unphosphorylatable versions of Nrg1 proteins did not exhibit SDS sensitivity (Figure 4.4). However, we did observe that cells lacking *QSP1* that expressed Phosphomimic-Nrg1 were moderately more resistant to SDS treatment (Figure 4.4A). However, cells expressing unphosphorylatable Nrg1 did not appear to be less resistant to SDS

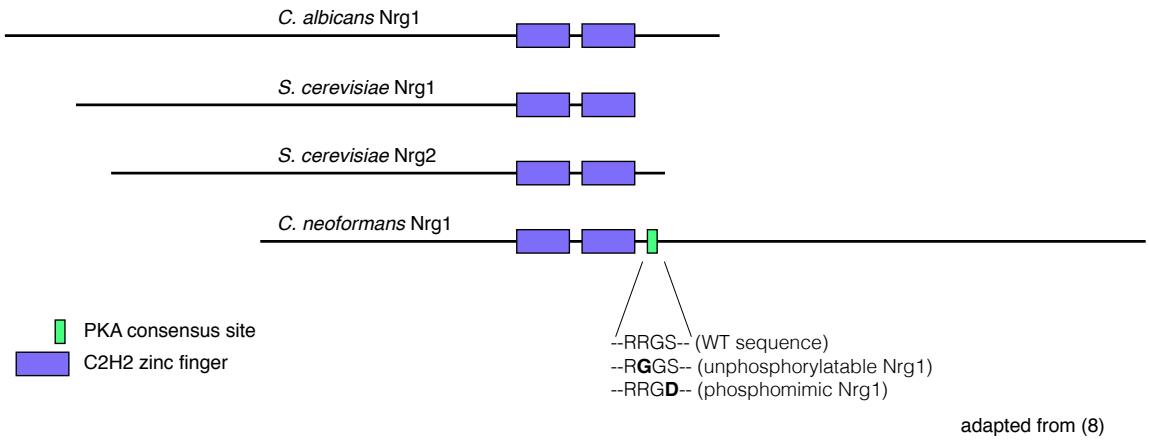

Figure 4.3. *C. neoformans* Nrg1 contains a PKA consensus motif. Nrg1 contains two C2H2 DNA-binding domains and in *C. neoformans* also contains a motif for phosphorylation by Protein Kinase A. Modifications in the consensus sequence used in this study are shown.

treatment (Figure 4.4B). Growth of these strains with Qsp1 peptide resulted in rescue of the *qsp1Δ* phenotype to levels of cell wall strength exhibited wild type cells (Figure 4.4). Therefore, the phosphorylation state of the PKA consensus motif in Nrg1 does not seem to interfere with Qsp1 signaling to promote cell wall stress. In addition to cell wall stress, we also examined the colony morphology of these strains, but did not see any effect of Nrg1 phosphorylation state on the smooth colony morphology of wild type cells or the rough colony morphology of *qsp1Δ* mutant cells, nor did we see evidence for interference with rescue of the *qsp1Δ* mutant phenotype with Qsp1 peptide (not shown). In conclusion, it does not seem that phosphorylation of Nrg1 via PKA is necessary for Qsp1 signaling in these contexts.

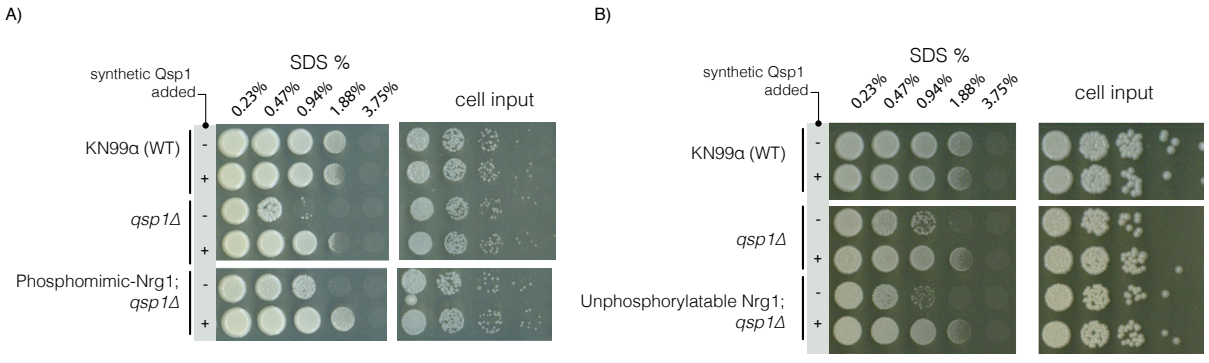

Figure 4.4. Effect of mimics of Nrg1 phosphorylation state on cell wall stress. Saturated cultures were tested for their ability to survive increasing concentrations of SDS. Water dilutions of the input cell culture are shown to the right as a measure of cell input prior to incubation in SDS. 1 uM synthetic Qsp1 peptide was added to the indicated cultures (+) from the time of inoculation, or not (-).

4.4 Discussion

We did not find any evidence that Qsp1 is sufficient for regulation of the binding of three transcription factors to promoters *in vitro*. It is possible that the proteins purified were not completely functional following purification, even if they remained soluble following cleavage of MBP (not shown). It is also possible that the tags interfered with binding (in the case of Cqs2) or interaction of these proteins with Qsp1, or the conditions for binding did not favor binding specificity. Re-purification of these factors could be useful for testing this hypothesis in the future. However, it is likely that Qsp1 influences these transcription factors through another unidentified factor.

We investigated the possibility that Qsp1 influences these transcription factors through Pka2. Cells lacking *PKA2* exhibit a similar colony morphology to cells lacking other components of Qsp1 signaling, but saturated cultures are not sensitive to cell wall stress (Figure 4.2A, 4.2B). In addition, both *qsp1Δ* and *pka2Δ* mutants exhibit a rough colony morphology that is not rescued by the addition of Qsp1 peptide, but *qsp1Δpka2Δ* double mutants are able to be rescued by Qsp1 peptide for cell wall stress (Figure 4.2B). Together, these results indicate that Pka2 may function downstream of Qsp1 for colony morphology but is not involved in promoting cell wall strength or transduction of the Qsp1 signal to rescue the defect in cell wall strength.

Though the hypothesis that Pka2 phosphorylates Nrg1 to regulate Qsp1 phenotypes is tantalizing, we did not find any evidence linking phosphorylation state of the consensus PKA motif of Nrg1 to phenotypes exhibited by cells lacking *QSP1* or *NRG1*, other than a very mild increase in cell wall strength in the *qsp1Δ;*Phosphomimic-Nrg1 strain. This lack of a dramatic effect is not surprising, since Pka2 seems dispensable for SDS sensitivity in *C. neoformans* (Figure 4.2B). However, neither mutation of the PKA consensus motif in Nrg1 had an effect on colony morphology, though *pka2Δ* and *nrg1Δ* mutants do display a rough colony morphology. These data indicate that the influence Pka2 has on colony morphology is not through phosphorylation of Nrg1. It is also possible that Pka2 phosphorylates Cqs2 or another unidentified protein that is required for Qsp1 signaling, or that these mutations were not sufficient to mimic the phosphorylation state of Nrg1.

Further experiments, some of which are described in the next section, would be necessary to understand whether Pka2 is involved in any way in Qsp1 signaling and whether this site on Nrg1 is indeed phosphorylated in wild type cells and affected by Qsp1.

4.5 Future Directions

The rough colony morphology exhibited by deletions of components of Qsp1 signaling was extremely fruitful for identifying these components as involved in Qsp1 function in previous chapters and in previous research from our lab (Chapter 2, 3 and reference 1). Therefore, the fact that deletion of *PKA2* leads to a rough colony morphology thus strongly hints at involvement in Qsp1 signaling, though it seems cAMP signaling and Qsp1 signaling both influence colony morphology independently of each other. However, the experiments I performed were not sufficient to prove that Pka2 is not involved in Qsp1 signaling. Additionally, colony morphology and cell wall stress may not be the best way to investigate Pka2 involvement in the context of Qsp1 signaling.

Some additional experiments to investigate the role of Pka2 and PKA dependent phosphorylation of factors involved in Qsp1 signaling include:

1. Examination of the phosphorylation state of Nrg1 in wild type, *qsp1Δ*, *pka1Δ*, *qsp1Δpka1Δ, pka2Δ, qsp1Δpka2Δ, pka1Δpka2Δ,* and *qsp1Δpka1Δpka2Δ* mutants. This could be accomplished via separation of phosphorylated from unphosphorylated Nrg1 (and potentially Cqs2 or Liv3) on a phos-tag gel. Alternatively, these factors could be affinity purified and phosphorylation to identified by mass spectrometry.

2. Determine whether if *pka1Δpka2Δ* double mutants look transcriptionally similar to *qsp1Δ* mutants. We did not observe a difference in RNA-seq performed on *pka1Δ* and *pka2Δ* mutants at saturation (48 hours) in rich media, but this could have been due to redundancy between the *PKA1* and *PKA2* gene products. A culture density of OD1 in minimal media could also be a better condition in which to look at this, based on experiments done in Chapter 3.

3. For a more quantitative and more globally descriptive phenotype than colony morphology and SDS sensitivity, ChIP-seq could be performed to look at how binding of Phospho-mimic Nrg1 or unphosphorylatable Nrg1 compares to wild type. It would also be interesting to look at how binding of Nrg1, Liv3, and Cqs2 are altered in cells that lack *PKA1*, *PKA2,* or both. Are there differences, and if so, are those changes only affecting promoters that exhibit Qsp1-dependent binding by these factors?

Direct interaction of Qsp1 peptide with purified Nrg1, Cqs2, and Liv3 could be further explored in fluorescence anisotropy or label-free binding experiments. Preliminary experiments involving Cqs2-flag and Qsp1 peptide labeled N-terminally with bodipy-FL were inconclusive, but further studies are required to determine whether Qsp1 is capable of binding these three transcription factors directly. A scrambled peptide would serve as an excellent control for these experiments.

It is also likely that the intracellular receptor for Qsp1 is not these three transcription factors, or Pka2, but another yet unidentified factor. While the screening our gene deletion has been fruitful in identifying factors that promote Qsp1 signaling, we are missing factors that may inhibit Qsp1 signaling. Additionally, since it is still not fully understood why or how a rough colony morphology and sensitivity to cell wall stress phenotypes are exhibited by Qsp1 components, and how well these phenotypes fully represent Qsp1 signaling, it is possible that we are missing factors that are involved that may not influence these phenotypes. Our gene deletion collection could be screened for mutants that display sensitivity to SDS, although there would likely be a lot of genes that contribute to this phenotype independent of Qsp1 signaling or downstream of the factors involved and may not be the most effective use of our efforts.

Instead, I propose utilizing an unbiased approach for identification of the Qsp1 receptor. I have identified a synthetic modified Qsp1 peptide that is biotinylated and modified to be cross-linkable that is able to partially the colony morphology of *qsp1Δ* mutant cells streaked on a late.could identify the intracellular receptor in an unbiased manner. If the biotin and PEG linker do not affect Qsp1's ability to bind its intracellular receptor, this approach would allow us to physically crosslink Qsp1 to any intracellular binding partners, including transient interactors, and purify

these complexes using streptavidin beads. A scrambled control peptide would control for non-specific interactions. I have identified and sourced a protocol for this, and the reagents needed to perform the experiment are stored in the laboratory.

4.6 References

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Chapter 5: Methods

5.1 Materials and Methods

5.1.1 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 (1). Constructs in Table 5.1 were made via homologous recombination. Strains constructed in this study are listed in Table 5.2. All strains are derived from the KN99alpha (CM26) parent.

5.1.2 Cell Wall Stress Assay

Part of a colony was cultured in 5 ml of YPAD (1% yeast extract, 2% Bacto-peptone, 2% glucose,

Table 5.1. Bacterial stocks of plasmids used to make these strains. BHM #'s for frozen stocks of DH5alpha strains containing plasmids

Bacterial stock #	Name	Description	Backbone	Linearization
BHM 2349	*qsp1*Δ(neo)	KO *QSP1* with Neomycin resistance cassette	pRS316	PmeI and AccI
BHM 2350	*nrg1*Δ(hyg)	KO *NRG1* gene with Hygromycin resistance cassette	pRS316	PmeI
BHM 2351	*liv3*Δ(neo)	KO *LIV3* gene with Neomycin resistance cassette	pRS316	PmeI
BHM 2352	*pka2*Δ(neo)	KO *PKA2* gene with Neomycin resistance cassette	pRS316	PmeI
BHM 2353	*pka1*Δ(hyg)	KO *PKA1* with Hygromycin resistance cassette	pRS316	PmeI
BHM 2354	*gat201*Δ(hyg)	KO *GAT201* with Hygromycin resistance cassette	pRS316	PmeI
BHM 2355	3xFLAG-Nrg1(hyg)	Nterminally tagged Nrg1 with 3xFLAG, replaces endogenous NRG1 gene. Hygromycin resistance cassette is downstream	pRS316	PmeI
BHM 2356	Cqs2-cbp-2xFLAG(hyg)	Cterminally tagged Cqs2 with CBP-2xFLAG, replaces endogenous CQS2 gene. Hygromycin resistance cassette is located downstream.	pRS316	PmeI
BHM 2357	Phosphomimic-Nrg1(hyg)	Serine in putative PKA consensus replaced with Aspartic Acid to create "constitutively phosphorylated" Nrg1. Replaces endogenous NRG1 gene. Hygromycin resistance cassette is located downstream.	pRS316	PmeI
BHM 2358	UnPhosphorylatable-Nrg1(hyg)	Mutation of putative PKA consensus site (RRGS) in Nrg1 to RGGS to prevent phophorylation. Replaces endogenous NRG1 gene. Hygromycin resistance cassette is located downstream.	pRS316	PmeI
BHM 2359	Ctr4p:iQsp1(nat) (aka Q1C22)	Replaces endogenous QSP1 gene and promoter with copper-repressible Ctr4 promoter followed by Ubiquitin fused to mature Qsp1 peptide. Nourseothricin resistance cassette is located downstream.	pRS316	PmeI and AccI
BHM 2360	Ctr4p:iQsp1;*opt1*Δ(nat) (aka Q1C23)	Replaces endogenous OPT1 gene & QSP1 gene and promoter with copper-repressible Ctr4 promoter followed by Ubiquitin fused to mature Qsp1 peptide. Nourseothricin resistance cassette is located downstream.	pRS316	PmeI and AccI

Table 5.2. Strains used in this study and strains not mentioned that may be useful for future studies. CM (Madhani) or CK (Crypto knockout collection) #'s are shown for each strain, along with the resistance cassette associated with each genotype (nat = nourseothricin, neo = neomycin, hyg = hygromycin).

Strains Used in this Study:

CM or CK #	Genotype
CM026	KN99α
CK0286	*qsp1*Δ(nat)
CK0326	*opt1*Δ(nat)
CM1686	iQsp1; *qsp1*Δ (nat)
CM1687	iQsp1; *opt1*Δ (nat)
CK0080	*liv3*Δ(nat)
CK0252	*nrg1*Δ(nat)
CK0624	*cqs2*Δ(nat)
CM1711	*qsp1*Δ(nat), *liv3*Δ(neo)
CM2028	*qsp1*Δ(nat), *nrg1*Δ(hyg) #2.3
CM2030	*qsp1*Δ(neo), *cqs2*Δ(nat) #1
CM1937	3xFLAG-Nrg1(hyg)
CM1938	*qsp1*Δ(nat); 3xFLAG-Nrg1(hyg)
CM1939	Liv3-2xFLAG(neo)
CM1940	*qsp1*Δ(nat); Liv3-2xFLAG (neo)
CM1941	Cqs2-2xFLAG (hyg)
CM1942	*qsp1*Δ(nat); Cqs2-2xFLAG (hyg)
CM2003	Liv3-2xFLAG (neo); *nrg1*Δ(nat)
CM2004	Liv3-2xFLAG (neo); *cqs2*Δ(nat)
CM2005	3xFLAG-Nrg1(hyg); *cqs2*Δ(nat)
CM2006	Cqs2-2xFLAG (hyg); *nrg1*Δ(nat)
CK1541	*pka2*Δ(nat)
CK2914	*pka1*Δ(nat)
CK3200	*pkr1*Δ(nat)
CK5586	*aca1*Δ(nat)
CK1948	*cac1*Δ(nat)
CM2030	*pka1*Δ(nat); *pka2*Δ(neo)
CM2031	*qsp1*Δ(nat); *pka2*Δ(neo)
CM2032	*qsp1*Δ(nat); *pka2*Δ(neo); *pka1*Δ(hyg)
CM2033	Phosphomimic-Nrg1(hyg)
CM2034	*qsp1*Δ; Phosphomimic-Nrg1(hyg)
CM2035	unphosphorylatable-Nrg1(hyg)
CM2036	*qsp1*Δnat; unphosphorylatable-Nrg1(hyg)

Strains with potential use for future studies:

CM#	Genotype
CM1832	iQsp1(nat); *liv3*Δ(neo)
CM1833	iQsp1(nat); *pka2*Δ(neo)#1.2
CM1834	iQsp1(nat); *pka2*Δ(neo)#2.1
CM2029	*qsp1*Δ(nat), *nrg1*Δ(hyg) #2.5
CM2031	*qsp1*Δ(neo), *cqs2*Δ(nat) #9
CM2037	*liv3*Δ(nat); *nrg1*Δ(hyg) #2
CM2038	*nrg1*Δ(nat); *liv3*Δ(neo) #1
CM2039	*qsp1*Δ(nat); *nrg1*Δ(hyg), *liv3*Δ(neo) #8
CM2040	*qsp1*Δ(nat); *nrg1*Δ(hyg), *liv3*Δ(neo) #4
CM2041	*gat201*Δ(hyg)
CM2042	*qsp1*Δ(nat); *gat201*Δ(hyg)
CM2043	*nrg1*Δ(nat); *gat201*Δ(hyg)
CM2044	*liv3*Δ(nat); *gat201*Δ(hyg) #1
CM2045	*liv3*Δ(nat); *gat201*Δ(hyg) #3
CM2046	*qsp1*Δ(nat); *nrg1*Δ(hyg), *gat201*Δ(neo) #5.1

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.

5.1.3 Immunoblots

Cultures were grown as indicated. 2 OD_{600} units of cells per sample were fixed with 10% TCA, then 100% acetone, then lysed by two 1.5min 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 ul of 1x Laemmli Sample Buffer. 5-10 ul of each sample was loaded on 4-12% Bis-Tris gels (Thermofisher).

5.1.4 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 OD_{600} units of cells were harvested sequentially from the same culture at the indicated ODs. 50 OD_{600}'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 OD_{600}'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.

5.1.5 RNA-Seq

Total RNA was isolated from 50 OD_{600}'s of cells as previously described (2) and libraries prepared as previously described (3). In brief, cell pellets were lyophilized overnight and then RNA was isolated using TRIzol (Invitrogen) as previously described (2) and DNase treated as previously described (4). 0.5 ug 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).

5.1.6 RNA-Seq Analysis

Expression analysis for each transcription factor mutant was performed by counting the number of reads aligned by STAR for each transcript (5). DEseq2 was used to determine genes differentially expressed between mutant and wild type conditions.

5.1.7 Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously (6), 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). Immunoprecipitated DNA was used for either ChIP-qPCR using the primers listed in Table 5.3, or for ChIP-seq.

Table 5.3. Primers used for ChIP-qPCR. Primers were designed to span the largest peak visualized in the bedgraph files, as all three transcription factors had similar ChIP profiles on these genes. Centromere 13 was used as a negative control.

Primer	Locus	Sequence
c391F_ChIPqPCR_laccase_F1 (DKS)	CNAG_03465 promoter (chr8: 1,020,374-1,020,622)	CCAACCTTCTCAGGGTACTCGCAC
c392R_ChIPqPCR_laccase_R1 (DKS)		GGACGGAAACGCTAAGACGTTGG
c397F_ChIPqPCR:CNAG_00758_F1 (DKS)	CNAG_00758 promoter (chr1: 1,990,291-1,990,508)	AGGAAGGGCAATTCGATTACATTAGAAAGG
c398R_ChIPqPCR:CNAG_00758_R1 (DKS)		GAAGCGTCTCACCGTGTCAGG
c8371 qPCR (CEN-Hpy99I)	CEN 13 (chr13:603,981-604,091)	TGTCCCCAGTCTCTTAGAG
c8372 qPCR (CEN-Hpy99I)		CTTGGTGAGTGAAGTAATG

5.1.8 ChIP-seq library construction

ChIP-seq library construction was performed as described previously (6) 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.

5.1.9 ChIP-Seq analysis

ChIP-seq reads were trimmed with CutAdapt and aligned using Bowtie1 (7), 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 kmeans analysis. There was a high degree of overlap between genes called as bound in either replicate, but genes were only considered as bound in our analysis if it was called as bound in both replicates by kmeans analysis. 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 (11)).

5.1.10 Statistical Analysis

Immunoblot quantification was performed with ImageJ analysis, and significance was determined using the student's T-test. *P*-values <0.05 were considered significant. All other *P*-values were computed using the hypergeometric package in R using the phypher() command configured for enrichment analysis using a significance threshold of < .05.

5.1.11. Constructs for Recombinant Protein Expression

Transcription factor cDNA reverse-transcribed from RNA purified from wild type cells was placed into the constructs using the primers listed below. Each of the three transcription factors was tagged with a N-terminal MBP (plus a linker) to enhance solubility of the purified protein. Nrg1 and Cqs2 were tagged with C-terminal 1xFLAG tags, and Liv3 was tagged with an N-terminal 6x-Histidine tag.

5.1.12 Recombinant Protein Expression

BL21 *E. coli* cells were transformed with constructs expressing tagged Nrg1, Cqs2, or Liv3. A mixture of colonies was used to inoculate overnight cultures in Terrific Broth. The

following morning, the overnight culture was used to inoculate 4-6 L Terrific Broth at 37°C at OD 0.2 containing 25 uM $ZnCl_2$. When the culture density reached OD2, cultures were shifted to 18°C and 1M IPTG was added to induce expression for 3 hours.

5.1.13 Recombinant Protein Purification

BL21 cells were then spun down and the pellet was frozen at -80C. A cryogrinder (SPEX Sample Prep) was used to lyse the cells in liquid nitrogen for 10 cycles of 2 min @ 12 cps, with a 5 min rest in between cycles. 10 mL of cold lysis buffer containing benzonaze was added per gram of grindate, and the resuspension was allowed to stir at 4°C until the consistency of the lysate thinned. Cell debris was spun out for 15min at 25,000 rpm for 15 minutes. MBP-Nrg1-FLAG and MBP-Cqs2-FLAG were purified from the supernatant using Anti-Flag M2 magnetic beads (Sigma), eluted with 3xFLAG peptide (Sigma), and then size selected using size exchange chromatography. 6xHis-MBP-Liv3 was purified using agarose NiNTA beads followed by size exchange chromatography. Fractions containing the purified proteins were concentrated to 1uM.

5.1.14 Electrophoresis Mobility Shift Assays

Radiolabeled probes were made by taking PCR products and radiolabeling them with P32. Reaction conditions were as follows: 20 mM Tris pH 7.5, 5% glycerol, 75 mM NaCl, 0.5 mM KCl, 0.5 mM TCEP, 1 mM DTT, 0.1% NP-40, 1 ug/ul BSA*, 5 mM MgCl, 2 mM $ZnCl_2$, 5 nM radiolabeled DNA. Proteins were allowed to incubate with radiolabeled DNA for an hour on ice, then the reaction was mixed with 6x Ficoll loading dye (without dye for reactions containing protein). Complexes were separated from free DNA by electrophoresis in 6% acrylamide gels in the cold, then dried in a gel dryer before imaging with a Typhoon scanner.

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