Title
Identifying Functional Target Genes of the Candida albicans Biofilm Network

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Publication Date
2021

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA, MERCED

Identifying Functional Target Genes of the *Candida albicans* Biofilm Network

A

dissertation

submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

in

Quantitative and Systems Biology

by

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Merced, California

2021

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Dedication

This thesis is dedicated to God for providing me all the tools to complete my degree despite many setbacks and moments of self-doubt throughout this journey. This work is also dedicated to my parents, Virginia Rodriguez and Leonardo Rodriguez, and to my siblings, for their emotional, moral, and financial support when I needed it most.

Table of Contents

List of Tables

List of Figures

List of Supplemental Files

Dataset S2.1. Compilation and analysis of regulator binding interactions and target gene expression.

Videos S3.1-S3.36. BioFlux microfluidics biofilm assay videos.

Dataset S3.1. Priority list of target genes.

Data Set S3.2. Optical density screen.

Acknowledgements

I thank my graduate advisor, Dr. Clarissa Nobile, for all the academic support she provided me. I am grateful for the substantial feedback and guidance from the members of my dissertation committee: Dr. Aaron Hernday, Dr. Mike Cleary, and Dr. Katrina Hoyer. Special thanks to my mentors – Dr. Laura Beaster-Jones, Dr. Jesus Romo, Dr. Rudy Ortiz, and Dr. Ruby Gonzalez – whose generous advice helped me to make consistent progress in my PhD. Thank you to my fellow lab mates in the Nobile lab, who made my experience enjoyable and to the members of the Hernday lab, for allowing me to borrow material for my experiments whenever our lab was out. Thank you to the National Science Foundation (NSF) and the University of California Institute for Mexico and the United States (UC MEXUS) for supporting my PhD program with the GRFP NSF and the UC MEXUS: CONACYT fellowships; their financial support was crucial in completing this work.

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Graduate Student Researcher Aug 2015-Present
The University of California at Merced, Merced, CA
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- Studying the genetic regulation of biofilm formation in *Candida albicans*
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Microbiology Laboratory Volunteer Sep 2014 - Aug 2015
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Mentor: Dr. Clarissa Nobile

- Constructed gene deletion mutants for candidate genes involved in biofilm formation in *C. albicans* using traditional fusion PCR and CRISPR methodologies, extracted genomic DNA, and prepared different culture media

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Team Leader Aug 2013 - May 2014
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Publications

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- Gulati, M., Lohse, M.B., Ennis, C.L., Gonzalez, R.E., Perry, A.M., Bapat, P., Valle-Arevalo, A., **Rodriguez, D.L.**, Nobile, C.J. (2018) *In Vitro* Culturing and Screening of *Candida albicans* Biofilms. *Current Protocols in Microbiology* 50 (1): e60.
- Gulati, M., Ennis, C.L., **Rodriguez, D.L.**, & Nobile, C.J. (2017). Visualization of Biofilm Formation in *Candida albicans* Using an Automated Microfluidic Device. *Journal of visualized experiments* 130: e56743.

Publications in Progress

- **Rodriguez, D.L.**, Hartooni, N., Fox, E.P., Sanchez, H., Andes, D.R., Nobile, C.J. Identifying Functional Target Genes of the *Candida albicans* Biofilm Network. In Preparation.

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- **Rodríguez, D.L.** and Nobile, C.J. Identifying Functional Target Genes of *Candida albicans* Biofilm Formation. *The University of Texas Health Science Center at Houston, Seminar.* Mar. 17, 2021. Houston, TX.
- **Rodríguez, D.L.** and Nobile, C.J. Identifying Functional Target Genes of *Candida albicans* Biofilm Formation. *University of California Santa Barbara STEM Camp.* Jul. 11, 2019. Santa Barbara, CA.

Oral Conference Presentations

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- **Rodríguez, D.L**. and Ortiz, R.M. Antagonism of Angiotensin, but not the Mineralocorticoid Receptor, Ameliorates ANG II-dependent Hypertension through Reduction of Sodium Reabsorption. *Undergraduate Summer Research Symposium*. Aug 8, 2014. Merced, CA.

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- **Rodríguez, D.L.** and Nobile, C.J. Identifying Functional Target Genes of Candida albicans Biofilm Formation. *Candida and Candidiasis*. Mar. 23, 2021. https://microbiologysociety.org/event/full-events-listing/candida-and-candidiasis-2021.html.

- **Rodríguez, D.L.** and Nobile, C.J. Identifying Functional Target Genes of Candida albicans Biofilm Formation. *20th Microbiology Student Symposium.* Apr. 27, 2019. Berkeley, CA.

- **Rodríguez, D.L.** and Nobile, C.J. Identifying Functional Target Genes of Candida albicans Biofilm Formation. *14th ASM Conference on Candida and Candidiasis*. Apr. 16, 2018. Providence, RI.

- **Rodríguez, D.L.** and Nobile, C.J. Identifying Functional Target Genes of Candida albicans Biofilm Formation. *Northern California American Society of Microbiology, 35th Annual Spring Meeting*. Mar. 2, 2018. Pleasanton, CA.

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Teaching Experience

Teaching Assistant — Spring 2017
Upper Division Course: BIO122, Microbial Pathogenesis
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- Led discussion sessions (20 students)
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- Designed review sessions and distributed study material such as worksheets

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- Interacted with students during weekly office hours
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Teaching Assistant — Spring 2016
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- Led three weekly discussion sessions with 25+ students each
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Abstract of the Dissertation

Identifying Functional Target Genes of the *Candida albicans* Biofilm Network

by
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Doctor of Philosophy in Quantitative and Systems Biology
University of California, Merced, 2021
Advisor: Dr. Clarissa J. Nobile

The objective of this dissertation is to provide a framework to discover biofilm-specific antifungal drug targets of the opportunistic human fungal pathogen *Candida albicans* by answering two main questions: (1) which genes are functionally important for biofilm formation? (2) what are the primary roles of these genes during biofilm formation? In chapter one, I provide fundamental background information about *C. albicans*. In chapter two, I summarize the current understanding of the transcriptional regulatory circuits that govern three major developmental processes in *C. albicans*: biofilm formation, the white-opaque cell type switch, and the commensal to pathogen switch. In chapter three, I describe the findings of my main thesis project and highlight a set of genes that are part of the *C. albicans* biofilm regulatory network that are required for biofilm formation and play important roles in the different stages of biofilm development. Lastly, I conclude by summarizing the main points from each of the previous chapters and discussing their overall significance.

Chapter 1. Introduction

1.1 *Candida albicans* as an Opportunistic Pathogen

C. albicans is a normal colonizer of human mucosal surfaces such as the gastrointestinal and genitourinary tracts of healthy individuals, and it is estimated to be present in ~80% of the population in the United States (Ghannoum and Rice, 1999; Perlroth et al., 2007). While it is mostly harmless in healthy individuals, disturbances in the microbiota or the host immune system can result in superficial or systemic and sometimes life-threatening infections. *C. albicans* infections are most common in individuals who have been treated with antibiotics and/or in individuals with compromised immune systems, such as patients undergoing cancer treatment and organ transplantation (Perlroth et al., 2007; Sudbery, 2011). The major risk factors to develop invasive *Candida* infections include prolonged hospitalizations, and the presence of implanted medical devices (Perlroth et al., 2007). On the other hand, less severe infections that occur with higher incidence rates are seen in individuals who undergo multiple rounds of antibiotics, where administration of antibiotics perturbs the host microbiota and allows for overgrowth of *C. albicans* (Maraki et al., 2001; Mavromanolakis et al., 2001).

1.2 Role of Cell Morphology in Virulence

The transition of *C. albicans* from an asymptomatic colonizer (i.e., a commensal) to a pathogen depends on multiple factors, including *C. albicans* cell morphology and the host environmental conditions. One important characteristic of *C. albicans* cells is their ability to grow in different morphological forms and to switch between them. *C. albicans* exist in two prominent morphological forms: yeast and hyphae (Sudbery et al., 2004). Ellipsoidal yeast cells grow by budding, are usually found on epithelial surfaces, and play an important role for dissemination in the blood (Pope and Cole, 1982). On the other hand, the elongated cell form, hyphae, is referred to as the invasive form since it is strongly correlated with increased virulence and promotes tissue penetration in organs such as the kidneys (Phan et al., 2000; Saville et al., 2003; MacCallum and Odds, 2005). Moreover, the switch between these two cell morphologies can be induced under specific *in vitro* conditions, such as human physiological temperature (37°C) and the addition of serum or phosphate (Hornby et al., 2004; Whiteway and Oberholzer, 2004). These morphological changes do not go unnoticed by the host's innate immune system, where innate immune cells play critical roles in surveying the fungal burden and discriminating between non-pathogenic and pathogenic *C. albicans* cells. In this regard, previous studies have shown differential recognition of yeast and hyphal cells by the immune system (D'Ostiani et al., 2000; van der Graaf et al., 2005; Bi et al., 2010). For example, *C. albicans* yeast cells induce the production of interleukin-12 (IL-12) from dendritic cells whereas hyphal cells induce interleukin-4 (IL-4) (D'Ostiani et al., 2000). Differences in recognition by the immune system have been attributed to changes in the composition of the *C. albicans* cell wall, particularly differences in mannan levels (Netea et al., 2006). Thus, surveillance by the host immune system as well as intrinsic cell attributes, such as the morphological state of the *C. albicans* cells, are crucial in determining whether *C. albicans* exists as a harmless commensal or an invasive pathogen.

1.3 Biofilm Formation by *C. albicans*

A major virulence factor contributing to pathogenesis of *C. albicans* is its ability to form biofilms, where yeast and hyphae constitute the main cell types and provide architectural support to the biofilm structure. In general, biofilms are defined as microbial communities that are enclosed in an extracellular matrix and are formed at semi-liquid interfaces, or on surfaces (Donlan and Costerton, 2002). Although the vast majority of studies have focused on the planktonic growth state, biofilms are now recognized as the preferred mode of growth of most microorganisms in their natural settings (López et al., 2010). *C. albicans* biofilm formation has been extensively studied both *in vitro* and *in vivo* (Jabra-Rizk et al., 2004; Nett and Andes, 2006; Finkel and Mitchell, 2011; Tournu and van Dijck, 2012; Mathé and van Dijck, 2013). The *C. albicans* biofilm life cycle occurs in four distinct stages: adherence, proliferation, maturation, and dispersal (Hawser and Douglas, 1994; Baillie and Douglas, 1999; Chandra et al., 2001; Douglas, 2003; Nobile and Mitchell, 2006; Uppuluri et al., 2010a). Initiation of biofilm formation begins when free floating yeast cells encounter a surface and adhere to it, serving as an anchor of the biofilm to the surface. Biofilm and Cell Wall Regulator-1 (Bcr1), Enhanced Filamentous Growth-1 (Efg1), Regulatory Factor X-2 (Rfx2), and Transposon Enhancement Control-1 (Tec1) are all important transcription factors regulating adherence during biofilm formation (Li and Palecek, 2003; Nobile and Mitchell, 2005; Hao et al., 2009; Sahni et al., 2010). Through regulation by the transcription factors Biofilm Regulator-1 (Brg1), Efg1, Flocculation-8 (Flo8), Non-DiTyrosine-80 (Ndt80), Regulatory Factor X-2 (Rfx2), Regulator of Biofilm (Rob1), and Tec1, the cells proliferate, and hyphal formation takes place, causing cells to aggregate while also providing structural support to the biofilm (Schweizer et al., 2000; Ramage et al., 2002b; Sahni et al., 2010; Nobile et al., 2012; Fox et al., 2015). After cell proliferation and hyphal formation, biofilm cells enter the maturation stage, where the cells continue to filament and produce an extracellular matrix (ECM) that is composed of glycoproteins (55%), carbohydrates (β-1,6-glucans and β-1,3-glucans) (25%), lipids (15%) and extracellular DNA (5%) (Zarnowski et al., 2014). ECM production is governed by the transcription factors Resistance to Lethality of MKK1P386-1 (Rlm1), Zinc-responsive Activator Protein-1 (Zap1), and Carbon Catabolite Repression-4 (Ccr4) (Nobile et al., 2009; Verma-Gaur and Traven, 2016). In the final stage of the biofilm life cycle, yeast cells and/or fractions of the biofilm are released into the environment seeding new sites of infection, and the cycle begins again. Negative Regulator of Glucose-controlled Genes-1 (Nrg1) and Unscheduled Meiotic Gene Expression-6 (Ume6) have been identified as important transcription factors regulating dispersal during the biofilm life cycle (Uppuluri et al., 2010a, 2010b). A *C. albicans* biofilm is considered mature after 24-48 h of growth under most nutrient-rich conditions (Andes et al., 2004; Řičicová et al., 2010; Kaneko et al., 2013).

1.4 Medical Relevance of *C. albicans* Biofilms

The National Institutes of Health (NIH) estimates that ~80% of all infections are caused by biofilms (Penesyan et al., 2019), and more than half of all nosocomial infections are caused by *C. albicans*, with an attributable mortality rate of up to 40%, even after antifungal therapy (Fraser et al., 1992; Blau and Fauser, 2000; Richards et al., 2000; Mora-Duarte et al., 2002; Kullberg et al., 2005). Although multiple species from the *Candida* clade (e.g., *Candida parapsilosis* and *Candida dubliniensis*) also form biofilms, *C. albicans* appears to form the most robust biofilms under the conditions studied (Chandra et al., 2001; Shin et al., 2002; Mancera et al., 2021). Additionally, *C. albicans* is able to form biofilms on a wide range of biotic and abiotic surfaces and causes a wide range of

infections, with significant clinical implications. For example, *C. albicans* biofilms are the source of infection in central venous catheters and many implanted medical devices, including indwelling catheters, cardiac implants, and hip replacements (Crump and Collignon, 2000; Phelan et al., 2002; Hauser et al., 2003). In the United States, more than ten million device-associated infections are attributed to biofilm formation on indwelling medical devices annually, and these device-associated infections are also correlated with the development of systemic infections (Donlan and Costerton, 2002; Kojic and Darouiche, 2004). Overall, there is a strong unmet medical need to effectively prevent and treat biofilm associated infections caused by *C. albicans* and other biofilm forming species.

1.5 Drug Resistant Properties of *C. albicans* Biofilms

Cells within biofilms possess distinct metabolic properties from cells growing in planktonic cultures (Kolter and Greenberg, 2006; Kolter, 2010; López et al., 2010). Due to their structural complexity, biofilms act as a protective barrier against environmental stressors, thus providing an increased competitive fitness to the cells growing within them; this in turn makes recognition by the immune system more challenging (Meiller et al., 2009; Mathé and van Dijck, 2013). A striking difference between *C. albicans* biofilms and planktonic cells is sensitivity to antifungals, with biofilms being up to 4000-fold more resistant than planktonic cells to antifungal drugs such as fluconazole, amphotericin B and caspofungin (Hawser and Douglas, 1995; Ramage et al., 2001; Miceli et al., 2009). Numerous studies have demonstrated multiple mechanisms for increased antifungal resistance in *C. albicans* biofilms (Chandra et al., 2001; Mukherjee et al., 2003; Taff et al., 2013; Desai et al., 2014). These mechanisms include the overexpression of drug efflux pumps (Nobile and Johnson, 2015), the presence of the extracellular matrix (Mukherjee et al., 2003), and changes in the copy number of genes and mutations in genes that encode for drug targets (Berman and Krysan, 2020). Deletion mutant strains for genes encoding the major facilitator transporters such as Multidrug Resistance-1 (Mdr1*)*, *Candida* Drug Resistance-1 (Cdr1*),* and *Candida* Drug Resistance-2 (Cdr2) were highly susceptible to fluconazole (Ramage et al., 2002a). These same strains showed an increased resistance when grown under biofilm inducing conditions, highlighting the contributions of the biofilm structure to drug resistance (Ramage et al., 2002a). Likewise, mutations in the gene encoding Ergosterol Biosynthesis-3 (Erg3*)* and Ergosterol Biosynthesis-11 (Erg11), which play roles in cell wall synthesis through the ergosterol pathway, confer resistance to fluconazole (Akins and Sobel, 2017). More recently, aneuploidy has been implicated as a player in increasing antifungal resistance during biofilm formation (Yang et al., 2019).

1.6 Current Therapeutics

When compared to antibiotics for bacterial infections, the availability of antifungal drug therapeutics to treat fungal infections is much more limited. This is because fungi, like humans, are also eukaryotes; thus, the development of antifungal agents requires careful examination to prevent toxicity towards the host. Furthermore, given the recalcitrant nature of biofilms, the availability of therapeutic agents to treat biofilm associated infections is even more limited (Tsui et al., 2016). While azoles, including fluconazole, itraconazole, and voriconazole, are commonly used in treating superficial *Candida* infections, echinocandins and amphotericin B are often used to treat biofilm-related infections (Bachmann et al., 2002; Kuhn et al., 2002; Mukherjee and Chandra, 2004; Kucharíková et al., 2010; Mathé and van Dijck, 2013; Ghannoum et al., 2015). The azoles generally have a fungistatic effect on *C. albicans* by inhibiting the production of

ergosterol within the fungal cell membrane (Ghannoum and Rice, 1999; Williams and Lewis, 2011; Mathé and van Dijck, 2013), but their indiscriminate use has resulted in the evolution of drug resistant clinical isolates (Mathé and van Dijck, 2013). Only antifungals with a fungicidal activity, such as the echinocandin class (e.g., caspofungin and micafungin), when administered intravenously, have shown efficacy against recalcitrant biofilm-associated and systemic infections (Walsh, 2002; Williams and Lewis, 2011). Echinocandins work by blocking the synthesis of β-1,3-glucan, an essential cell wall component of *C. albicans* cells (Denning, 2003).

Clearly, there is a need for the development of targeted antifungal therapeutics and a comprehensive approach to treat and prevent biofilm associated infections, especially those caused by *C. albicans*. The following chapters provide a summary of the current knowledge on the regulatory circuits controlling various developmental processes in *C. albicans*, while highlighting the regulation of biofilm formation (Chapter 2). I also present the results from a reverse genetic approach used to evaluate biofilm development capabilities *in vitro* and *in vivo* of a subset of target genes that are part of the *C. albicans* biofilm regulatory network (Chapter 3). The results reveal new target genes required for biofilm formation, some of which may be useful in the development of novel antifungal drugs that specifically target the biofilm mode of growth.

1.7 References

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Chapter 2. Transcriptional Circuits Regulating Developmental Processes in *Candida albicans*

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Frontiers in Cellular and Infection Microbiology, 2020

2.1 Abstract

Candida albicans is a commensal member of the human microbiota that colonizes multiple niches in the body including the skin, oral cavity, and gastrointestinal and genitourinary tracts of healthy individuals. It is also the most common human fungal pathogen isolated from patients in clinical settings. *C. albicans* can cause a number of superficial and invasive infections, especially in immunocompromised individuals. The ability of *C. albicans* to succeed as both a commensal and a pathogen, and to thrive in a wide range of environmental niches within the host, requires sophisticated transcriptional regulatory programs that can integrate and respond to host specific environmental signals. Identifying and characterizing the transcriptional regulatory networks that control important developmental processes in *C. albicans* will shed new light on the strategies used by *C. albicans* to colonize and infect its host. Here, we discuss the transcriptional regulatory circuits controlling three major developmental processes in *C. albicans*: biofilm formation, the white-opaque phenotypic switch, and the commensal-pathogen transition. Each of these three circuits are tightly knit and, through our analyses, we show that they are integrated together by extensive regulatory crosstalk between the core regulators that comprise each circuit.

2.2 Introduction

C. albicans is a common human commensal that asymptomatically colonizes the skin, oral cavity, and gastrointestinal and genitourinary tracts of healthy individuals (Kennedy and Volz, 1985; Kumamoto, 2002, 2011; Achkar and Fries, 2010; Spiliopoulou et al., 2010; Nobile and Johnson, 2015; Kan et al., 2020). It is also an opportunistic pathogen that is capable of causing superficial mucosal and life-threatening disseminated infections, especially in immunocompromised individuals (Wenzel, 1995; Calderone and Fonzi, 2001; Hube, 2004; Pappas et al., 2004; Mayer et al., 2013), such as in AIDS, chemotherapy and organ transplant patients, as well as in individuals with implanted medical devices (Wenzel, 1995; Nobile and Johnson, 2015). Multiple regulatory pathways controlling important *C. albicans* developmental processes allow this opportunistic fungal pathogen to adapt to and proliferate in distinct environmental niches in the host. In this review, we discuss the “core” transcriptional circuits controlling three major developmental processes in *C. albicans*: biofilm formation, the white-opaque phenotypic switch, and the commensal-pathogen transition. The core circuitry is defined as the direct physical interactions between transcriptional regulators that control these developmental processes and their respective upstream intergenic regions, where at least one direct binding interaction with other members of the circuit has been experimentally observed. These three circuits were chosen because they regulate persistent phenotypic changes in *C. albicans* that have been characterized using genome-wide transcriptional profiling (RNA-sequencing and/or microarray) and binding (chromatin immunoprecipitation) approaches. In our discussion of these circuits, we focus largely on transcription factors (TFs) that bind to DNA in a sequence-specific manner; however, we also include some discussion of important cofactors for which genome-wide transcriptional profiling and binding data are available. In addition, we include information on “auxiliary” transcriptional regulators of these three developmental

processes that we define as those that are known to regulate these processes, but that lack direct binding interactions with the core transcriptional regulators or binding data is not available for these transcriptional regulators under the growth condition of interest.

2.3 Regulation of Biofilm Formation

Biofilms are communities of adherent microbial cells encased in protective extracellular matrices (Kolter and Greenberg, 2006; Nobile and Johnson, 2015; Gulati and Nobile, 2016). Biofilms are ubiquitous in nature and are typically associated with interfaces, such as solid-liquid, liquid-gas, and liquid-liquid interfaces (Davey and O'toole, 2000; Kolter and Greenberg, 2006; Wilking et al., 2011; Desai and Ardekani, 2020). They are problematic when they form in industrial settings, such as in water distribution systems and on food preparation settings, and even more so when they form inside a host on tissues and on implanted medical devices. *C. albicans* biofilms are composed of several cell types, including round budding yeast-form cells, oval pseudohyphal cells, and elongated hyphal cells, encased in a protective extracellular matrix (Chandra et al., 2001; Desai and Mitchell, 2015). *C. albicans* biofilm formation occurs in four basic temporal stages: (i) adherence of yeast-form cells to a surface; (ii) growth and proliferation of yeast-form cells forming a basal layer of anchoring cells; (iii) differentiation of a proportion of yeast-form cells into hyphal cells and production of the extracellular matrix; and (iv) dispersion of yeast-form cells out of the biofilm to cause bloodstream infections or to colonize new sites for biofilm formation (**Figure 2.1.**) (Desai and Mitchell, 2015; Nobile and Johnson, 2015; Gulati and Nobile, 2016). Indeed, *C. albicans* is a common cause of bloodstream infections worldwide, which often originate from biofilms (Edmond et al., 1999; Richards et al., 1999; Pfaller and Diekema, 2007). Given that cells within *C. albicans* biofilms are inherently resistant and tolerant to most antifungal drug treatments compared to planktonic (free-floating) cells, biofilm infections are particularly challenging to treat in the clinic. Understanding the genetic regulatory mechanisms that control *C. albicans* biofilm formation could lead to the development of novel therapeutic strategies effective in treating biofilm infections.

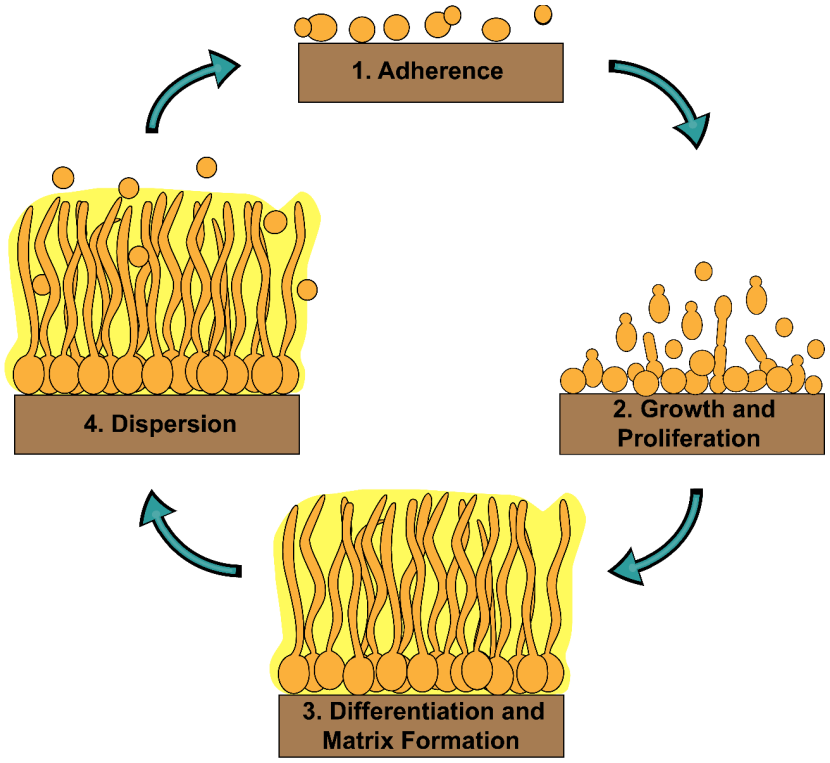

Figure 2.1. Stages of *C. albicans* biofilm formation. *C. albicans* biofilm formation occurs in four basic temporal stages: (1) adherence of yeast-form cells to a surface; (2) growth and proliferation of yeast-form cells forming a basal layer of anchoring cells; (3) differentiation of a proportion of yeast-form cells into hyphal cells and production of the extracellular matrix; and (4) dispersion of yeast-form cells out of the biofilm to cause bloodstream infections or to colonize new sites for biofilm formation.

The *C. albicans* transcriptional network controlling biofilm formation was first described eight years ago (Nobile et al., 2012). Six "master" biofilm transcriptional regulators (Bcr1, Tec1, Efg1, Ndt80, Rob1, and Brg1) were identified by screening a library of 165 transcription factor (TF) mutant strains (Homann et al., 2009) for defects in biofilm formation under standard *in vitro* biofilm growth conditions (Nobile et al., 2012). Here, we define a master biofilm transcriptional regulator as one whose deletion impairs biofilm formation throughout a 48-hour period of biofilm growth under these standard conditions. All six TF mutant strains identified additionally had clear defects in biofilm formation in at least one of two *in vivo* animal models for biofilm formation (Nobile et al., 2012). Using genome-wide transcriptional profiling and chromatin immunoprecipitation techniques to study mature 48-hour biofilms, a complex interconnected transcriptional network was discovered consisting of those six master transcriptional regulators, along with 1,061 downstream "target" genes (Nobile et al., 2012). These six master transcriptional regulators directly bound to the upstream intergenic regions and positively regulated the expression of each other, forming a tightly knit core biofilm circuit (Fox and Nobile, 2012; Nobile et al., 2012). Additionally, with the exception of Tec1, all of the six master biofilm transcriptional regulators acted as both repressors and activators of their directly bound biofilm target genes; Tec1, on the other hand, primarily acted as an activator (Nobile et al., 2012). Each of the six master biofilm transcriptional regulators controlled target genes

that were in common with the other core transcriptional regulators in the circuit, as well as target genes that were unique to each transcriptional regulator. These findings suggest that each master biofilm transcriptional regulator in the circuit controls certain elements of biofilm formation independently, but that they also work together to coordinate concerted efforts important for biofilm formation. For example, Ndt80 regulates the expression of drug transporters independent of the other master biofilm transcriptional regulators in the circuit (such as, *CDR4*), and some in common with several of the other master biofilm transcriptional regulators in the circuit (such as, *CDR3*) (Nobile et al., 2012). Additionally, each master biofilm transcriptional regulator likely responds to unique environmental inputs, such as oxygen and nutrient availability, pH, temperature, and waste products. How different environmental inputs influence the biofilm transcriptional circuit is an intriguing area of future research. For example, we know that the six master biofilm transcriptional regulators discovered using *in vitro* biofilm assays are still required for *in vivo* biofilm formation in at least one of two *in vivo* biofilm models (Nobile et al., 2012). The majority (four) of the master biofilm transcriptional regulators discovered in this study were essential for biofilm formation in both *in vivo* biofilm models used; however, two of the master biofilm transcriptional regulators played different roles depending on the *in vivo* biofilm model (Nobile et al., 2012). Specifically, Bcr1 was essential for biofilm formation in a rat catheter biofilm model but was dispensable in a rat denture biofilm model (Nobile et al., 2012). Similarly, Brg1 was essential for biofilm formation in a rat denture biofilm model but was dispensable in a rat catheter biofilm model (Nobile et al., 2012). Future work on these master transcriptional regulators will determine their unique influences on biofilm formation dependent on the environmental inputs present.

In a subsequent study, three additional transcriptional regulators, Gal4, Rfx2, and Flo8, were added to the core biofilm transcriptional circuit (Fox et al., 2015). Gal4, Rfx2, and Flo8 were found to directly bind to the upstream intergenic regions of one or more of the previously identified six master biofilm transcriptional regulators and vice versa during biofilm development (Nobile et al., 2012; Fox et al., 2015). Gal4, Rfx2, and Flo8 were identified (in addition to the six previously identified transcriptional regulators) by screening a TF mutant library containing 192 TF mutant strains (Fox et al., 2015). This TF library contained the same 165 TF mutants (Homann et al., 2009) from the Nobile et al. 2012 study (Nobile et al., 2012) plus 27 additional newly constructed TF mutant strains. The TF mutants in this larger library were screened for their abilities to form biofilms over time at 90 minutes, 8 hours, 24 hours, and 48 hours of biofilm growth (Fox et al., 2015). Flo8, like the other six previously identified master biofilm transcriptional regulators, was required for biofilm formation throughout a 48-hour course of biofilm growth, and thus was deemed to be a master biofilm transcriptional regulator; Gal4 and Rfx2 were only required for normal biofilm formation at specific intermediate time points (Fox et al., 2015). Given that the initial biofilm circuit consisting of six master transcriptional regulators was discovered by assessing biofilm formation at a single mature time point (48 hours) (Nobile et al., 2012), performing the genetic screen as a biofilm develops over time, with the additional TF mutant strains, contributed to the expansion of the core biofilm circuit (Fox et al., 2015). Genome-wide binding data was not performed for Gal4, Rfx2, and Flo8 as part of this study; however, directed chromatin immunoprecipitation followed by quantitative PCR was performed to determine that these three new transcriptional regulators are integrated into the core biofilm circuit, which now consists of nine core transcriptional regulators, seven of which are considered to be master biofilm transcriptional regulators (**Figure 2.2.**) (Nobile et al., 2012; Fox et al., 2015). We note that although genome-wide binding experiments have been performed for Gal4 and Flo8 (Askew et al., 2009; Polvi et al.,

2019), these experiments were not performed under biofilm conditions and thus the resulting data cannot be integrated into the biofilm transcriptional circuit. Overall, although the logic of the biofilm transcriptional circuit (defined as how each transcriptional regulator contributes to the regulatory dynamics of the circuit) has yet to be fully elucidated, the high degree of interconnectivity between the core biofilm transcriptional regulators likely contributes to the robustness, yet reversibility, of the biofilm state.

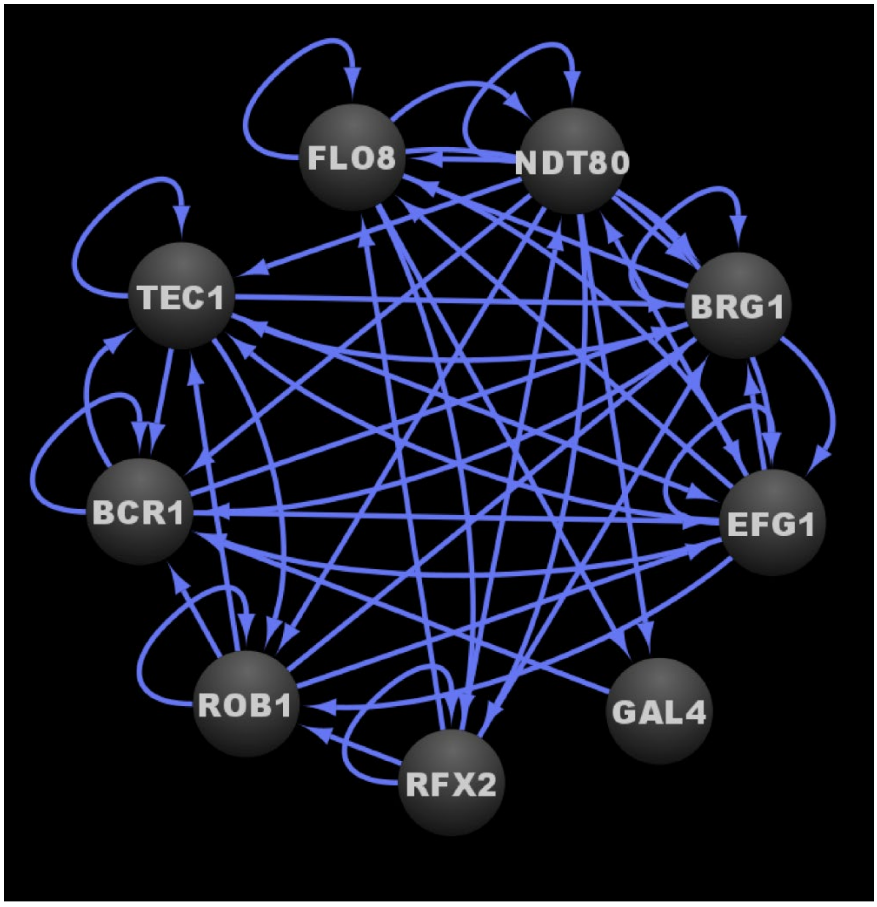

Figure 2.2. Transcriptional circuit controlling *C. albicans* biofilm formation. Ovals indicate each of the core biofilm transcriptional regulators with their respective names. Arrows indicate direct binding events. See **Dataset S2.1., Tab4** for binding interactions. Data were derived from (Nobile et al., 2012; Fox et al., 2015). Figure was generated using Cytoscape (Shannon et al., 2003).

Although the nine core biofilm transcriptional regulators are known to be important for biofilm formation, how each one specifically contributes to biofilm processes (e.g. adhesion, filamentation, antifungal drug resistance, etc.), through detailed analyses of their mutant strains, has not been systematically determined. **Table 2.1.** summarizes the current knowledge of the roles of all known transcriptional regulators in known biofilm-related processes. Eight of the nine core biofilm transcriptional regulators (Bcr1, Brg1, Efg1, Flo8, Ndt80, Rfx2, Rob1, and Tec1) have been implicated in regulating filamentation (Schweizer et al., 2000; Bockmüh and Ernst, 2001; Cao et al., 2006; Elson et al., 2009; Hao et al., 2009; Sellam et al., 2010; Vandeputte et al., 2011; Du et al., 2012b; Nobile et

al., 2012), which is a critical process necessary for maintaining the architectural stability of the biofilm structure. Four of the nine core biofilm transcriptional regulators (Bcr1, Efg1, Rfx2, and Tec1) have been implicated in regulating adhesion (Dieterich et al., 2002; Hao et al., 2009; Sahni et al., 2010; Finkel et al., 2012), including both cell-cell and cell-substrate adhesion, which is an essential process for both the initiation of biofilm formation as well as for the maintenance of a mature biofilm. Three of the nine core biofilm transcriptional regulators (Bcr1, Efg1 and Ndt80) are known to be involved in the regulation of antifungal drug resistance and/or tolerance (Chen et al., 2004; Sellam et al., 2009; Prasad et al., 2010; Desai et al., 2013), an important feature that contributes to the overall recalcitrance of established biofilms to antimicrobial compounds. Of the nine core biofilm transcriptional regulators, we know the least about the biofilm specific roles of Gal4, and only that it contributes to the structure of a biofilm at intermediate stages of biofilm development (Fox et al., 2015). In the future, additional roles of the nine core biofilm transcriptional regulators during biofilm formation will certainly be elucidated. For example, it seems likely that some of the core biofilm transcriptional regulators would be involved in the formation of the extracellular matrix; however, this role has not been examined to date in the mutant strains of the core biofilm transcriptional regulators. In addition, the ability of cells within biofilms to communicate with one another, called quorum sensing, is an important process for coordinating biofilm formation of many microorganisms; however, this role has yet to be examined in the mutant strains of the core biofilm transcriptional regulators. In fact, little is known in general on the regulation of quorum sensing during *C. albicans* biofilm development.

In addition to these nine transcriptional regulators that make up the core biofilm circuit, there are 50 “auxiliary” transcriptional regulators that have been implicated in biofilm formation (**Table 2.1.**). The majority of these auxiliary biofilm transcriptional regulators are also bound in their upstream intergenic regions by at least one of the initial six master biofilm transcriptional regulators (Bcr1, Tec1, Efg1, Ndt80, Rob1, or Brg1; note that of the nine core biofilm transcriptional regulators, there is not genome-wide chromatin immunoprecipitation data available for Gal4, Rfx2, and Flo8, and thus we do not know whether they bind to the auxiliary biofilm transcriptional regulators) (**Table 2.1.**) (Nobile et al., 2012). As such, several of the 50 auxiliary transcriptional regulators are integrated into the larger biofilm network that includes the core nine transcriptional regulators and all of their directly bound target genes (Nobile et al., 2012). Based on existing phenotypic analyses of the mutant strains of the auxiliary biofilm transcriptional regulators, the majority (48) are implicated in the regulation of adhesion and/or filamentation (Brown et al., 1999; Kadosh and Johnson, 2001; Cheng et al., 2003; Uhl et al., 2003; García-Sánchez et al., 2004; Kelly et al., 2004; Mulhern et al., 2006; Kim et al., 2008b; Shen et al., 2008; Wheeler et al., 2008; Homann et al., 2009; Nobile et al., 2009; Pukkila-Worley et al., 2009; Uppuluri et al., 2010a, 2010b; Askew et al., 2011; Bonhomme et al., 2011; Ganguly et al., 2011; Finkel et al., 2012; Kamthan et al., 2012; Langford et al., 2013; Delgado-Silva et al., 2014; Tsai et al., 2014; Chen and Lan, 2015; Fox et al., 2015; Ghosh et al., 2015; Kakade et al., 2016, 2019; Böttcher et al., 2020; Lagree et al., 2020; Omran et al., 2020; Wang et al., 2020); sixteen are implicated in drug resistance and/or tolerance (Bruno et al., 2006; Cornet et al., 2006; Mulhern et al., 2006; Xu et al., 2007; Dunkel et al., 2008; Wheeler et al., 2008; Homann et al., 2009; Prasad et al., 2010; Nett et al., 2011; Vandeputte et al., 2012; Langford et al., 2013; Vasicek et al., 2014); two are implicated in the production of the extracellular matrix (Finkel et al., 2012; Delgado-Silva et al., 2014); and two are implicated in dispersion (Uppuluri et al., 2010b, 2010a). Similar to the core biofilm transcriptional regulators, detailed analyses of the mutant strains of the auxiliary

biofilm transcriptional regulators have not been systemically studied for known biofilm processes. Rather, most of their roles in biofilm processes have been determined through large-scale genetic screens. Of the auxiliary biofilm transcriptional regulators, we understand the least about the biofilm specific roles of Bpr1/Orf19.6874, which is only known to contribute to biofilm biomass throughout biofilm development (Fox et al., 2015). Future detailed phenotypic analyses of the auxiliary transcriptional regulator mutant strains in biofilm specific processes will certainly reveal new and additional roles for these transcriptional regulators in biofilm development.

Table 2.1. Known transcriptional regulators with roles in *C. albicans* biofilm formation.

Core Biofilm Transcriptional Regulators				
Orf19#	**Name**	**Known biofilm-related process affected in mutant strain**	**Gene upstream intergenic region bound by one or more of the core biofilm regulators?**	**References**
Orf19.723	Bcr1	Adhesion, Filamentation, Drug Resistance/Tolerance	Yes	(Nobile and Mitchell, 2005; Elson et al., 2009; Homann et al., 2009; Fanning et al., 2012; Finkel et al., 2012; Desai et al., 2013)
Orf19.4056	Brg1	Filamentation	Yes	(Du et al., 2012b; Nobile et al., 2012)
Orf19.610	Efg1	Adhesion, Filamentation, Drug Resistance/Tolerance	Yes	(Bockmüh and Ernst, 2001; Dieterich et al., 2002; Ramage et al., 2002; Li and Palecek, 2003; Prasad et al., 2010; Nobile et al., 2012)
Orf19.1093	Flo8	Filamentation	Yes	(Cao et al., 2006; Fox et al., 2015)
Orf19.5338	Gal4	Unknown	Yes	(Fox et al., 2015)
Orf19.2119	Ndt80	Filamentation, Drug Resistance	Yes	(Chen et al., 2004; Sellam et al., 2009; Sellam et al., 2010; Nobile et al., 2012)
Orf19.4590	Rfx2	Adhesion, Filamentation	Yes	(Hao et al., 2009; Fox et al., 2015)
Orf19.4998	Rob1	Filamentation	Yes	(Vandeputte et al., 2011)
Orf19.5908	Tec1	Adhesion, Filamentation	Yes	(Schweizer et al., 2000; Staib et al., 2004; Nobile and Mitchell, 2005; Sahni et al., 2010)

Auxiliary Biofilm Transcriptional Regulators				
Orf19#	**Name**	**Known biofilm-related process affected in mutant strain**	**Gene upstream intergenic region bound by one or more of the core biofilm regulators?**	**References**
Orf19.6124	Ace2	Adhesion, Filamentation, Drug Resistance/Tolerance	No	(Kelly et al., 2004; Mulhern et al., 2006; Finkel et al., 2012)
Orf19.2331	Ada2	Adhesion, Filamentation, Drug Resistance/Tolerance	No	(Bruno et al., 2006; Pukkila-Worley et al., 2009; Finkel et al., 2012)
Orf19.7381	Ahr1	Adhesion, Filamentation, Drug Resistance/Tolerance	Yes	(Homann et al., 2009; Askew et al., 2011)
Orf19.4766	Arg81	Adhesion, Filamentation, Drug Resistance/Tolerance	No	(Homann et al., 2009; Finkel et al., 2012)
Orf19.6874	Bpr1	Unknown	Yes	(Fox et al., 2015)
Orf19.4670	Cas5	Adhesion, Drug Resistance/Tolerance	Yes	(Finkel et al., 2012; Vasicek et al., 2014)
Orf19.2356	Crz2	Adhesion, Drug Resistance/Tolerance	Yes	(Homann et al., 2009; Finkel et al., 2012)
Orf19.3127	Czf1	Adhesion, Filamentation, Drug Resistance/Tolerance	Yes	(Brown et al., 1999; Finkel et al., 2012; Langford et al., 2013)
Orf19.3252	Dal81	Adhesion	No	(Finkel et al., 2012)
Orf19.3193	Fcr3	Adhesion	Yes	(Finkel et al., 2012)
Orf19.6680	Fgr27	Adhesion, Filamentation	No	(Uhl et al., 2003; Finkel et al., 2012)
Orf19.1358	Gcn4	Filamentation	Yes	(García-Sánchez et al., 2004; Kamthan et al., 2012)
Orf19.4000	Grf10	Adhesion, Filamentation	Yes	(Ghosh et al., 2015)
Orf19.2842	Gzf3	Adhesion, Drug Resistance/Tolerance	Yes	(Homann et al., 2009; Fox et al., 2015)
Orf19.4225	Leu3	Adhesion	No	(Finkel et al., 2012)
Orf19.5312	Met4	Adhesion	No	(Finkel et al., 2012)
Orf19.4318	Mig1	Filamentation, Drug Resistance/Tolerance	Yes	(Homann et al., 2009; Lagree et al., 2020)
Orf19.5326	Mig2	Filamentation	No	(Lagree et al., 2020)
Orf19.6309	Mss11	Adhesion, Filamentation	Yes	(Tsai et al., 2014)
Orf19.2012	Not3	Adhesion, Filamentation	No	(Cheng et al., 2003; Finkel et al., 2012)

Orf19.7150	Nrg1	Filamentation, Drug Resistance/Tolerance, Dispersion	Yes	(Wheeler et al., 2008; Uppuluri et al., 2010b)
Orf19.4093	Pes1	Filamentation, Drug Resistance/Tolerance, Dispersion	No	(Xu et al., 2007; Shen et al., 2008; Uppuluri et al., 2010a)
Orf19.2823	Rfg1	Adhesion, Filamentation	Yes	(Kadosh and Johnson, 2001; Fox et al., 2015)
Orf19.1604	Rha1	Filamentation	Yes	(Omran et al., 2020)
Orf19.7247	Rim101	Adhesion, Filamentation, Drug Resistance/Tolerance	Yes	(Cornet et al., 2006; Fox et al., 2015)
Orf19.4662	Rlm1	Drug Resistance/Tolerance, Extracellular Matrix Production	No	(Nett et al., 2011; Delgado-Silva et al., 2014)
Orf19.5953	Sfp1	Adhesion	Yes	(Chen and Lan, 2015)
Orf19.5871	Snf5	Adhesion	No	(Finkel et al., 2012)
Orf19.4961	Stp2	Adhesion, Filamentation	Yes	(Böttcher et al., 2020)
Orf19.7319	Suc1	Adhesion	No	(Finkel et al., 2012)
Orf19.798	Taf14	Adhesion, Filamentation	No	(Finkel et al., 2012; Wang et al., 2020)
Orf19.4062	Try2	Adhesion	No	(Finkel et al., 2012)
Orf19.1971	Try3	Adhesion	No	(Finkel et al., 2012)
Orf19.5975	Try4	Adhesion	Yes	(Finkel et al., 2012)
Orf19.3434	Try5	Adhesion	Yes	(Finkel et al., 2012)
Orf19.6824	Try6	Adhesion	Yes	(Finkel et al., 2012)
Orf19.4941	Tye7	Filamentation	Yes	(Bonhomme et al., 2011)
Orf19.7317	Uga33	Adhesion	No	(Finkel et al., 2012)
Orf19.1822	Ume6	Filamentation, Dispersion	Yes	(Uppuluri et al., 2010a, 2010b)
Orf19.391	Upc2	Adhesion, Drug Resistance/Tolerance	No	(Silver et al., 2004; Dunkel et al., 2008; Kakade et al., 2019)
Orf19.1035	War1	Adhesion	No	(Finkel et al., 2012)
Orf19.3794	Zap1	Filamentation, Extracellular Matrix Production	Yes	(Kim et al., 2008b; Nobile et al., 2009; Ganguly et al., 2011; Finkel et al., 2012)
Orf19.1718	Zcf8	Adhesion	Yes	(Finkel et al., 2012)
Orf19.4767	Zcf28	Adhesion	No	(Finkel et al., 2012)
Orf19.5924	Zcf31	Adhesion	Yes	(Finkel et al., 2012)
Orf19.5940	Zcf32	Adhesion, Filamentation	No	(Kakade et al., 2016, 2019)

Orf19.6182	Zcf34	Adhesion, Drug Resistance/Tolerance	No	(Homann et al., 2009; Oh et al., 2010; Finkel et al., 2012)
Orf19.7583	Zcf39	Adhesion	No	(Finkel et al., 2012)
Orf19.6781	Zfu2	Adhesion, Drug Resistance/Tolerance	No	(Finkel et al., 2012; Vandeputte et al., 2012)
Orf19.3187	Znc1	Adhesion	No	(Finkel et al., 2012)

2.4 Regulation of the White-Opaque Phenotypic Switch

The white-opaque switch in *C. albicans* is a form of phenotypic switching that gives rise to two distinct cell types called “white” and “opaque” that display distinct phenotypic characteristics at the single cell and colony levels (Anderson and Soll, 1987; Slutsky et al., 1987; Rikkerink et al., 1988; Bergen et al., 1990; Soll, 1992; Soll et al., 1993). White cells represent the standard budding yeast form of *C. albicans,* forming shiny, white, dome-shaped colonies on solid media plates, while opaque cells are larger and more elongated than white cells and form dull, off-white, flattened colonies on solid media plates (Slutsky et al., 1987; Soll et al., 1993; Lohse and Johnson, 2009; Noble et al., 2017). White and opaque cells differ in their virulence characteristics, metabolic preferences, mating competencies, interactions with the host innate immune system, and responses to environmental stimuli (Kolotila and Diamond, 1990; Lan et al., 2002; Lockhart et al., 2002; Miller and Johnson, 2002; Bennett et al., 2003; Geiger et al., 2004; Dumitru et al., 2007; Lohse and Johnson, 2008; Ramírez-Zavala et al., 2008; Huang et al., 2009, 2010; Lohse et al., 2013, 2016a; Xie et al., 2013; Du and Huang, 2016; Ene et al., 2016; Dalal et al., 2019). In total, nearly 20% of the transcriptome is differentially expressed, by at least twofold, between the two cell types, highlighting that the white-opaque switch involves major transcriptional rewiring (Tuch et al., 2010; Hernday et al., 2013). Under standard switch permissive growth conditions, switching between the white cell type, considered the "ground” state, and the opaque cell type, considered the “excited” state, occurs stochastically at a frequency of roughly one switch event per 1,000-10,000 cell divisions (Rikkerink et al., 1988; Bergen et al., 1990; Ramírez-Zavala et al., 2008; Alby and Bennett, 2009b). Each cell type is heritably maintained without any change to the primary sequence of the genome, thus fitting the classic definition of an epigenetic switch (Slutsky et al., 1987; Soll et al., 1993; Zordan et al., 2006, 2007). The switch is responsive to the combined effects of environmental signals, such as carbon source, pH, CO_2 levels, and temperature, which can differentially bias the cell population towards one of the two cell types (Dumitru et al., 2007; Ramírez-Zavala et al., 2008; Alby and Bennett, 2009a; Huang et al., 2009; Huang, 2012; Lohse et al., 2013; Du and Huang, 2016; Ene et al., 2016; Dalal et al., 2019). Mating type can also influence the ability of the cells to undergo white-opaque switching, where *MTL* heterozygous (**a**/α) cells are typically “locked” in the white state, while *MTL* hemizygous (**a**/Δ, α/Δ), homozygous (**a**/**a**, or α/α), and haploid (**a** or α) cells are capable of undergoing stochastic white-opaque switching (Hull and Johnson, 1999; Lockhart et al., 2002; Miller and Johnson, 2002). This mating type dependency, however, is not exclusive to all strains; in fact, a significant fraction of *MTL* heterozygous clinical isolates can be induced to form opaque cells under specific growth conditions that promote white to opaque switching in *MTL* hemizygous, homozygous, or haploid cells (Xie et al., 2013).

Through a combination of forward and reverse genetic approaches, a total of 112 transcriptional regulators and one protein binding cofactor (Ssn6) have been identified

which, when deleted, significantly impact the frequency of white-opaque switching (**Table 2.2.**) (Huang et al., 2006; Srikantha et al., 2006; Zordan et al., 2006, 2007; Hernday et al., 2013, 2016; Lohse et al., 2013, 2016a; Du et al., 2015; Lohse and Johnson, 2016). Of these 113 switch regulating proteins, eight (Wor1, Wor2, Wor3, Wor4, Czf1, Efg1, Ahr1, and Ssn6) are considered to be core switch regulators, and have been extensively characterized by genome-wide transcriptional profiling and chromatin immunoprecipitation approaches in white and opaque cell types; the remaining 105 switch regulating proteins are considered to be auxiliary switch regulators (**Table 2.2.**) (Sonneborn et al., 1999; Srikantha et al., 2000; Huang et al., 2006; Zordan et al., 2006, 2007; Vinces and Kumamoto, 2007; Lohse and Johnson, 2010, 2016; Wang et al., 2011; Hernday et al., 2013, 2016; Lohse et al., 2013). Together, these eight core switch regulators form complex cell type specific networks, with 203 bound target genes in white cells and 756 bound target genes in opaque cells (Hernday et al., 2013, 2016; Lohse et al., 2013; Lohse and Johnson, 2016). At the center of the white and opaque specific regulatory networks are two distinct transcriptional circuits (see **Figure 2.3A.** for the white circuit, **Figure 2.3B.** for the opaque circuit, and **Figure 2.3C.** for the combined white and opaque overlayed circuits) that consist of interconnected positive and negative feedback loops that govern the cell fate and heritable maintenance of the white and opaque cell types (Vinces et al., 2006; Vinces and Kumamoto, 2007; Zordan et al., 2007; Hernday et al., 2013, 2016; Lohse and Johnson, 2016). Although several groups have identified kinases, chromatin modifiers, and other proteins that also affect white-opaque switching (Hnisz et al., 2009; Noble et al., 2017; Rai et al., 2018), here we focus on the eight core switch regulators (TFs: Wor1, Wor2, Wor3, Wor4, Czf1, Efg1, Ahr1; and cofactor: Ssn6) for which genome-wide transcriptional profiling and chromatin immunoprecipitation data are available.

Table 2.2. Known transcriptional regulators and a protein cofactor with roles in the *C. albicans* white-opaque switch‡.

Core White-Opaque Transcriptional Regulators and a Protein Cofactor				
Orf19#	Name	**Known effect on white-opaque switch in mutant strain***		**Gene upstream intergenic bound by one or more of the core white-opaque regulators?**
		White to Opaque	**Opaque to White**	
Orf19.7381	Ahr1	2.0	-7.8	Yes
Orf19.3127	Czf1	-21.9	-16.8	Yes
Orf19.610	Efg1	24.0	-62.7	Yes
Orf19.6798	Ssn6	N/A	N/A	Yes
Orf19.4884	Wor1	-20.8	N/A	Yes
Orf19.5992	Wor2	-32.9	N/A	Yes
Orf19.467	Wor3	-2.4	-3.9	Yes
Orf19.6713	Wor4	-13.3	N/A	Yes
Auxiliary White-Opaque Transcriptional Regulators				

Orf19#	Name	Known effect on white-opaque switch in mutant strain*		Gene upstream intergenic bound by one or more of the core white-opaque regulators?
		White to Opaque	Opaque to White	
Orf19.7436	Aaf1	-1.1	-2.7	Yes
Orf19.2272	Aft2	-2.8	-1.7	Yes
Orf19.4766	Arg81	1.8	-2.3	Yes
Orf19.166	Asg1	-21.6	-22.1	Yes
Orf19.5343	Ash1	-1.2	-26.9	Yes
Orf19.6874	Bas1	-1.5	2.5	Yes
Orf19.723	Bcr1	2.2	N/A	Yes
Orf19.4056	Brg1	1.9	-1.5	Yes
Orf19.1623	Cap1	-1.5	-4.4	Yes
Orf19.4670	Cas5	1.4	-2.3	Yes
Orf19.4433	Cph1	-2.2	-2.6	Yes
Orf19.1187	Cph2	-2.3	-1.4	No
Orf19.7359	Crz1	1.9	-5.6	Yes
Orf19.3794	Csr1	1.0	2.6	Yes
Orf19.7374	Cta4	-1.1	-5.9	Yes
Orf19.4288	Cta7	2.4	-2.1	Yes
Orf19.5001	Cup2	-1.2	-1.6	Yes
Orf19.6514	Cup9	4.7	-15.4	Yes
Orf19.3252	Dal81	-6.1	-1.8	Yes
Orf19.2088	Dpb4	-3.1	-2.4	Yes
Orf19.2623	Ecm22	1.3	-2.2	Yes
Orf19.5498	Efh1	1.7	-1.6	Yes
Orf19.6817	Fcr1	-1.9	-1.6	Yes
Orf19.2054	Fgr15	-17.7	4.8	Yes
Orf19.1093	Flo8	-27.8	N/A	No
Orf19.5338	Gal4	-23.9	-1.3	Yes
Orf19.3182	Gis2	-1.2	-9.4	Yes
Orf19.4000	Grf10	1.4	-6.9	Yes
Orf19.2842	Gzf3	-12.3	1.7	Yes
Orf19.1228	Hap2	-28.6	-1.6	No

Orf19.4647	Hap3	-3.0	1.3	Yes
Orf19.517	Hap31	-22.7	-1.3	Yes
Orf19.740	Hap41	-9.6	1.1	Yes
Orf19.1481	Hap42	-2.0	-1.9	No
Orf19.1973	Hap5	-6.7	-3.0	Yes
Orf19.4853	Hcm1	18.3	-3.7	Yes
Orf19.3063	Hfl1	-21.0	2.1	Yes
Orf19.7539	Ino2	-23.5	-3.6	Yes
Orf19.837.1	Ino4	-3.0	-1.2	Yes
Orf19.7401	Isw2	3.4	2.9	Yes
Orf19.3736	Kar4	-2.0	1.2	Yes
Orf19.4776	Lys143	7.3	-1.1	Yes
Orf19.5380	Lys144	1.3	-2.4	Yes
Orf19.7068	Mac1	-19.4	-1.6	Yes
Orf19.4318	Mig1	-29.7	1.4	Yes
Orf19.5326	Mig2	1.6	-1.6	Yes
Orf19.4752	Msn4	1.9	-4.7	Yes
Orf19.2119	Ndt80	-10.1	1.9	Yes
Orf19.5910	Nto1	2.8	-1.5	Yes
Orf19.1543	Opi1	4.0	-2.4	Yes
Orf19.4231	Pth2	3.0	-4.1	Yes
Orf19.1773	Rap1	16.0	-1.6	Yes
Orf19.5558	Rbf1	N/A	-32.4	Yes
Orf19.6102	Rca1	-9.1	-1.9	Yes
Orf19.7521	Rep1	-1.5	2.4	Yes
Orf19.2823	Rfg1	1.1	2.1	Yes
Orf19.3865	Rfx1	1.8	1.7	Yes
Orf19.4590	Rfx2	1.5	-1.7	Yes
Orf19.1604	Rha1	1.0	-2.7	Yes
Orf19.4438	Rme1	2.1	-1.8	Yes
Orf19.513	Ron1	-1.2	-1.7	Yes
Orf19.1069	Rpn4	18.2	-1.5	No
Orf19.4722	Rtg1	-2.1	-2.9	Yes
Orf19.2315	Rtg3	-2.8	-2.2	Yes
Orf19.1926	Sef2	1.1	-3.3	Yes
Orf19.454	Sfl1	-1.1	2.0	Yes
Orf19.971	Skn7	1.1	-1.5	Yes
Orf19.1032	Sko1	-1.8	-2.4	No
Orf19.4961	Stp2	9.2	-6.9	Yes
Orf19.909	Stp4	3.3	-2.2	Yes
Orf19.4545	Swi4	-4.5	1.0	Yes

Orf19.4941	Tye7	2.0	-1.0	Yes
Orf19.7317	Uga33	-1.0	-1.7	Yes
Orf19.1822	Ume6	-1.6	2.0	Yes
Orf19.2745	Ume7	-2.0	1.4	Yes
Orf19.391	Upc2	-1.1	3.1	Yes
Orf19.1035	War1	-3.2	-1.1	No
Orf19.5210	Xbp1	-6.4	-1.2	Yes
Orf19.2808	Zcf16	1.5	1.2	Yes
Orf19.3305	Zcf17	1.3	2.2	Yes
Orf19.431	Zcf2	-1.7	-2.8	Yes
Orf19.4145	Zcf20	-1.5	-2.2	Yes
Orf19.4166	Zcf21	-4.1	-40.4	Yes
Orf19.4251	Zcf22	1.8	-1.1	Yes
Orf19.4524	Zcf24	-1.0	-3.2	Yes
Orf19.4568	Zcf25	8.5	-2.9	Yes
Orf19.4649	Zcf27	-1.9	1.5	Yes
Orf19.5251	Zcf30	1.1	-1.7	Yes
Orf19.5924	Zcf31	-2.4	3.2	Yes
Orf19.6182	Zcf34	-2.9	-4.6	Yes
Orf19.1685	Zcf7	4.7	-2.7	Yes
Orf19.1718	Zcf8	-2.1	-2.2	Yes
Orf19.6781	Zfu2	-1.9	2.3	Yes
Orf19.6888	Zfu3	-5.0	-16.2	Yes
Orf19.5026	Zms1	-2.8	-1.2	Yes
Orf19.1150		1.2	-1.3	No
Orf19.1274		-1.4	1.2	No
Orf19.1577		-1.1	-1.5	No
Orf19.1757		1.0	-1.6	Yes
Orf19.217		-1.7	-1.7	Yes
Orf19.2476		1.9	2.5	Yes
Orf19.2612		2.4	1.4	Yes
Orf19.2961		7.0	2.0	Yes
Orf19.3928		5.7	-4.4	Yes
Orf19.7098		7.8	1.1	Yes

‡Data derived from (Zordan et al., 2007; Hernday et al., 2013, 2016; Lohse et al., 2013, 2016a; Lohse and Johnson, 2016). *Fold change in switch frequency is relative to a wildtype reference strain.

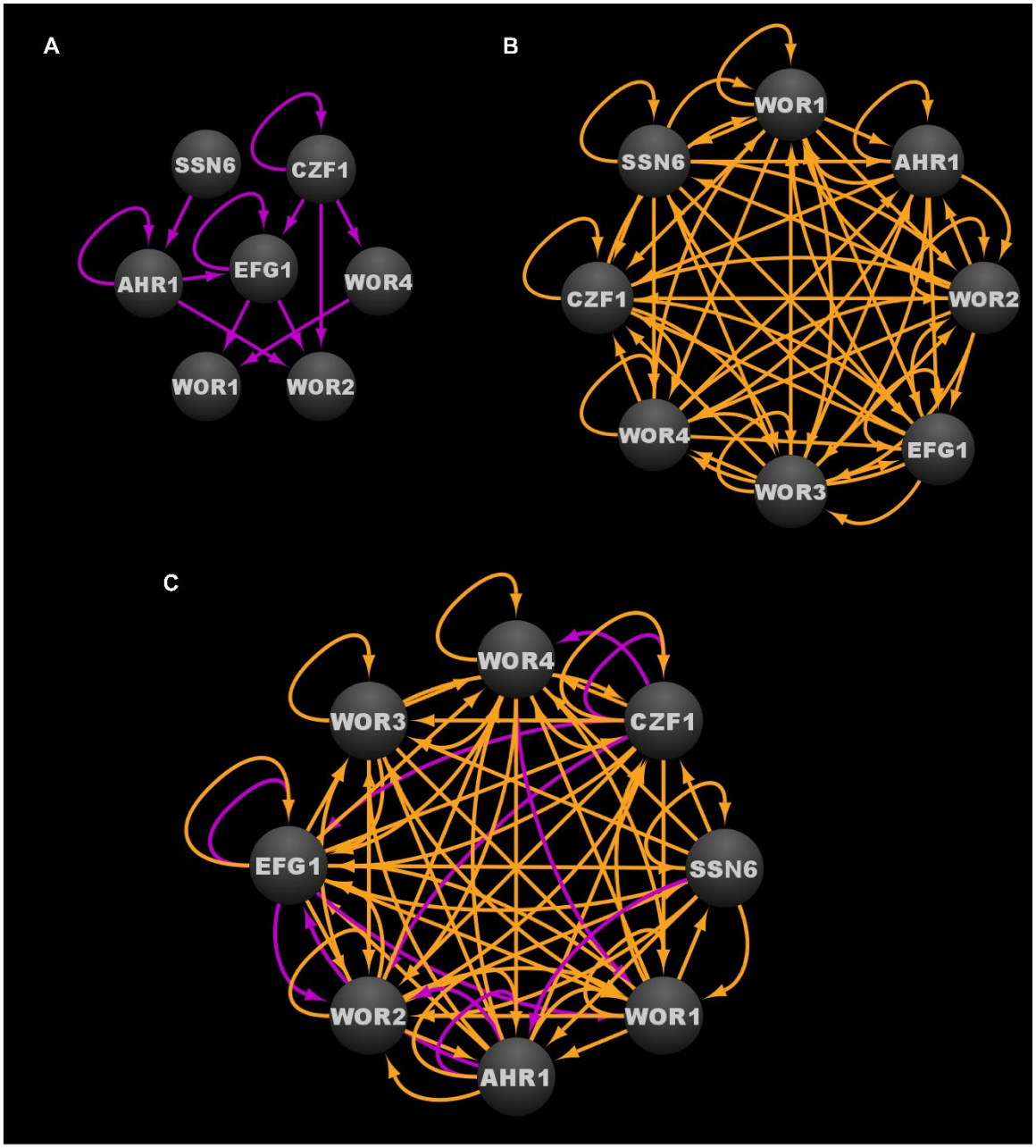

Figure 2.3. Transcriptional circuit controlling the *C. albicans* white-opaque phenotypic switch. (A) Transcriptional circuit of the white state. (B) Transcriptional circuit of the opaque state. (C) Overlayed transcriptional circuits regulating the white and opaque states. Ovals indicate each of the core regulators with their respective names. Arrows indicate direct binding events. See **Dataset S2.1., Tab4** for binding interactions. Data were derived from (Zordan et al., 2007; Hernday et al., 2013, 2016; Lohse et al., 2013; Lohse and Johnson, 2016). Figure was generated using Cytoscape (Shannon et al., 2003).

Wor1 is considered to be the master regulator of the white-opaque switch, as it is the only switch regulator that is known to be required for both the transition to, and

heritable maintenance of, the opaque cell type (Huang et al., 2006; Srikantha et al., 2006; Zordan et al., 2006, 2007; Hernday et al., 2013, 2016; Lohse et al., 2013; Lohse and Johnson, 2016). Furthermore, ectopic *WOR1* expression can rescue opaque cell formation in all known mutant backgrounds that fail to spontaneously switch to the opaque cell type (Zordan et al., 2007; Du et al., 2012a; Lohse and Johnson, 2016). *WOR1* expression is repressed in white cells, where Wor1 protein levels have been found to be nearly undetectable (Huang et al., 2006; Srikantha et al., 2006; Zordan et al., 2006, 2007; Lohse and Johnson, 2010). In opaque cells, *WOR1* is highly transcribed, and Wor1 protein levels have been found to accumulate to elevated levels (Huang et al., 2006; Srikantha et al., 2006; Zordan et al., 2006, 2007; Lohse and Johnson, 2010). Stochastic white to opaque switching is thought to be the result of transcriptional noise within the white cell circuit that occasionally allows Wor1 levels to surpass a critical threshold necessary to induce the transition to the opaque state (Srikantha et al., 2006; Hernday et al., 2010; Lohse and Johnson, 2010, 2016; Nobile et al., 2012; Guan and Liu, 2015; Horwitz et al., 2015; Lohse et al., 2016a; Tandonnet and Torres, 2017). Once established, the excited opaque cell circuit is stably maintained by a series of nested feedback loops, including a positive autoregulatory feedback loop generated by Wor1 binding to the upstream intergenic region of *WOR1* (Zordan et al., 2007; Hernday et al., 2013). This Wor1-induced positive feedback loop, along with other opaque specific binding interactions between the white and opaque regulators and their respective upstream intergenic regions, is proposed to be a central mechanism that mediates the epigenetic heritability of the opaque cell type (Huang et al., 2006; Srikantha et al., 2006; Zordan et al., 2006, 2007; Lohse and Johnson, 2010, 2016; Wang et al., 2011; Hernday et al., 2013, 2016; Lohse et al., 2013). Stochastic opaque to white switching is believed to occur when transcriptional noise causes Wor1 levels to drop below a critical threshold, thus leading to a collapse of the excited opaque cell transcriptional program and a return to the ground white cell transcriptional program (Srikantha et al., 2006; Zordan et al., 2006; Lohse and Johnson, 2010).

The core transcriptional circuit in white cells consists of a series of feed-forward loops that ultimately repress the expression of *WOR1* and *WOR2*, both of which are key players in the establishment and/or maintenance of the opaque cell type (Zordan et al., 2007). Efg1, Ahr1, and Ssn6 all contribute to the stability of the white cell circuit and are believed to directly or indirectly repress the expression of *WOR1* and *WOR2* (Zordan et al., 2007; Tuch et al., 2010; Hernday et al., 2013, 2016). Deletion of *EFG1*, *AHR1*, or *SSN6* destabilizes the white cell circuit such that most, if not all, of the cells in the population transition to the opaque state (Sonneborn et al., 1999; Srikantha et al., 2000; Vinces et al., 2006; Vinces and Kumamoto, 2007; Zordan et al., 2007; Wang et al., 2011; Hernday et al., 2016). Czf1, Wor3, and Wor4 are capable of destabilizing the white cell circuit, and induced expression of *CZF1*, *WOR3*, or *WOR4* in white cells can promote white to opaque switching in a Wor1 dependent manner (Zordan et al., 2007; Hernday et al., 2013; Lohse et al., 2013; Lohse and Johnson, 2016). Interestingly, neither Czf1 nor Wor3 is required for the heritable maintenance of the opaque state once switching has occurred (Zordan et al., 2007; Lohse et al., 2013). Based on these results and the structure of the white cell regulatory circuit (**Figure 2.3A.**)**,** Czf1 and Wor4 are thought to destabilize the white cell type by directly and indirectly antagonizing the white cell stabilizing activities of Ssn6, Ahr1, and Efg1, and by inducing opaque promoting factors such as *WOR3*, thus introducing the transcriptional noise that leads to the stochastic activation of the *WOR1* positive feedback loop and the transition to the opaque state. In addition to repression of *WOR1* and *WOR2*, the white cell transcriptional program results in repression of opaque enriched transcripts (e.g. *WOR3* and *CZF1*) as well as the activation of white enriched

transcripts (e.g. *EFG1*), thus creating a series of feed-forward loops that act to stabilize the white cell circuit and prevent activation of the opaque state (Zordan et al., 2007; Hernday et al., 2013; Lohse et al., 2013).

In contrast to the core transcriptional circuit of the white cell type (**Figure 2.3A.**), the core transcriptional circuit of the opaque cell type is extensively intertwined (**Figure 2.3B.**). All of the core switch regulators are active in opaque cells, and they are each found to bind to their own upstream intergenic regions, along with the upstream intergenic regions of most, if not all, of the other core switch regulators (**Figure 2.3B.**) (Huang et al., 2006; Srikantha et al., 2006; Zordan et al., 2006, 2007; Wang et al., 2011; Hernday et al., 2013, 2016; Lohse et al., 2013; Lohse and Johnson, 2016). To highlight this point, 58 of the 64 possible binding interactions between the core switch regulators and their respective upstream intergenic regions are observed in opaque cells (**Dataset S2.1., Tab1**). Although the logic of the opaque transcriptional circuit has yet to be fully elucidated, the high degree of interconnectivity between the core opaque regulators likely contributes to the robustness, yet reversibility, of the opaque cell state. Similar to the white cell circuit, Wor1 is a critical player in the opaque cell circuit; however, it is the sustained high levels of *WOR1* expression, rather than its repression, that is required for the formation and stable maintenance of the opaque cell type (Huang et al., 2006; Srikantha et al., 2006; Zordan et al., 2006). Although not strictly required for the formation of an opaque cell, Wor2 and Wor4 also play important roles in the heritable maintenance of the opaque transcriptional program (Zordan et al., 2007; Lohse and Johnson, 2016). Strains lacking *WOR2* or *WOR4* are locked in the white cell type and fail to undergo spontaneous white to opaque switching, yet can be induced to form opaque cells by ectopic expression of *WOR1* (Frazer et al., 2020). These induced opaque cells, however, are unstable, and quickly revert to the white cell type when ectopic *WOR1* expression is repressed, indicating that Wor2 and Wor4 play essential roles in the heritability of opaque cells (Zordan et al., 2007). Interestingly, with the exception of Ahr1, all switch regulators discovered to date have been found to contain prion-like domains that enable liquid-liquid demixing and the formation of phase-separated condensates (Frazer et al., 2020). Several of the switch regulators, including Wor1 and Wor4, have been shown to undergo phase separation *in vitro*, and to form condensates at genomic loci *in vivo*, in a manner similar to the formation of mammalian super-enhancers (Frazer et al., 2020). Combined with the observation that many of the target genes bound by the switch regulators are flanked by unusually large upstream intergenic regions (Zordan et al., 2007; Hernday et al., 2013), and the discovery that specific residues within the Wor1 prion-like domain are required for condensate formation and white to opaque switching, it seems likely that these phase-separated condensates formed by the core switch regulators in opaque cells are critical factors that contribute to the formation and heritable maintenance of the opaque cell type.

2.5 Regulation of the Commensal-Pathogen Transition

C. albicans typically exists as a commensal member of the healthy human microbiota. It can also transition into a pathogen in response to specific host environmental cues. In its pathogenic state, *C. albicans* can cause a wide range of infections, from acute to chronic superficial mucosal infections to severe and life-threatening disseminated bloodstream infections (Wenzel, 1995; Hube, 2004; Pappas et al., 2004). Although immunocompetent individuals with healthy and balanced microbiota are typically not adversely affected by *C. albicans*, immunocompromised individuals can suffer severe infections with significant morbidity and mortality (Wenzel, 1995; Nobile and Johnson, 2015). Understanding the genetic regulatory mechanisms that control the *C. albicans*

commensal-pathogen transition has the potential to lead to the development of targeted therapeutic strategies against *C. albicans* in its pathogenic state, without affecting its commensal state and the delicate balance of the microbiota.

Two distinct *C. albicans* transcriptional networks controlling the commensal-pathogen transition were described in 2011 and 2013, one governing iron homeostasis, and the other governing proliferation in the host, respectively (see **Figure 2.4A.** for the iron homeostasis circuit, **Figure 2.4B.** for the proliferation in the host circuit, and **Figure 2.4C.** for the combined commensal-pathogen overlayed circuits) (Chen et al., 2011; Pérez et al., 2013). As a commensal of the gastrointestinal (GI) tract, *C. albicans* is exposed to varying and often abundant levels of iron from food, and thus a tightly regulated transcriptional response is important for *C. albicans* to control iron assimilation and to avoid iron toxicity in the GI tract (McCance and Widdowson, 1938; Martin et al., 1987; Miret et al., 2003). On the other hand, when *C. albicans* causes a disseminated bloodstream infection, iron is extremely limiting, and to survive, *C. albicans* must conserve and scavenge iron from the bloodstream. Three transcriptional regulators, Sef1, Sfu1, and Hap43, were found to form a tightly knit transcriptional network, encompassing 214 downstream target genes (Chen et al., 2011). These three transcriptional regulators control iron homeostasis and were found to be essential for *C. albicans* to survive as both a commensal and as a pathogen within the mammalian host (Chen et al., 2011). Iron homeostasis in many other fungi (such as in other ascomycetes and the basidiomycete, *Cryptococcus neoformans*) is commonly regulated by a bipartite regulatory circuit composed of orthologs of Sfu1 and Hap43, where Sfu1 orthologs repress iron acquisition genes and *HAP43* orthologs, while Hap43 orthologs repress nonessential iron utilization genes and *SFU1* orthologs. This mutually repressive regulatory interaction between orthologs of Sfu1 and Hap43 in other fungi is significantly altered in *C. albicans* by the intercalation of Sef1 as a third player within this circuit (**Figure 2.4A.**) (Chen et al., 2011). In *C. albicans*, Sfu1 directly represses *SEF1* and iron acquisition genes under iron replete conditions (Chen et al., 2011). In response to iron limitation, Sef1 serves to directly activate *HAP43* and iron uptake genes, while Hap43 directly represses *SFU1* and iron utilization genes (Chen et al., 2011). Although the roles for Hap43 in *C. albicans* are similar to those of other fungi, the reciprocal interaction between Sfu1 and *HAP43* is altered in *C. albicans* by the inclusion of Sef1, which serves as an intermediary between Sfu1 and *HAP43*. *C. albicans SEF1* and *SFU1* are differentially expressed between growth in the GI tract versus growth in the bloodstream (Chen et al., 2011), thus providing dual inputs into the circuit controlling iron acquisition and utilization. While both Sef1 and Sfu1 serve to promote commensalism in a mouse GI commensal model, only Sef1 is required for virulence in a mouse disseminated infection model (Chen et al., 2011). Interestingly, deletion of *SFU1* conferred a significant competitive advantage over wildtype cells in the disseminated infection model (Chen et al., 2011), indicating that Sfu1 serves not only to promote commensalism in the GI tract, but also to attenuate virulence in the bloodstream. (See **Table 2.3.** for information on these three core transcriptional regulators in the commensal-pathogen transition.) Ultimately the *C. albicans* iron homeostasis circuit produces a well conserved transcriptional output consisting of increased iron uptake and reduced iron utilization in iron limited environments, and decreased iron uptake and increased iron utilization in iron replete conditions. Despite being well conserved in its transcriptional output, the iron homeostasis circuit appears to be uniquely evolved in *C. albicans* to control the delicate balance between its commensal and pathogenic growth states.

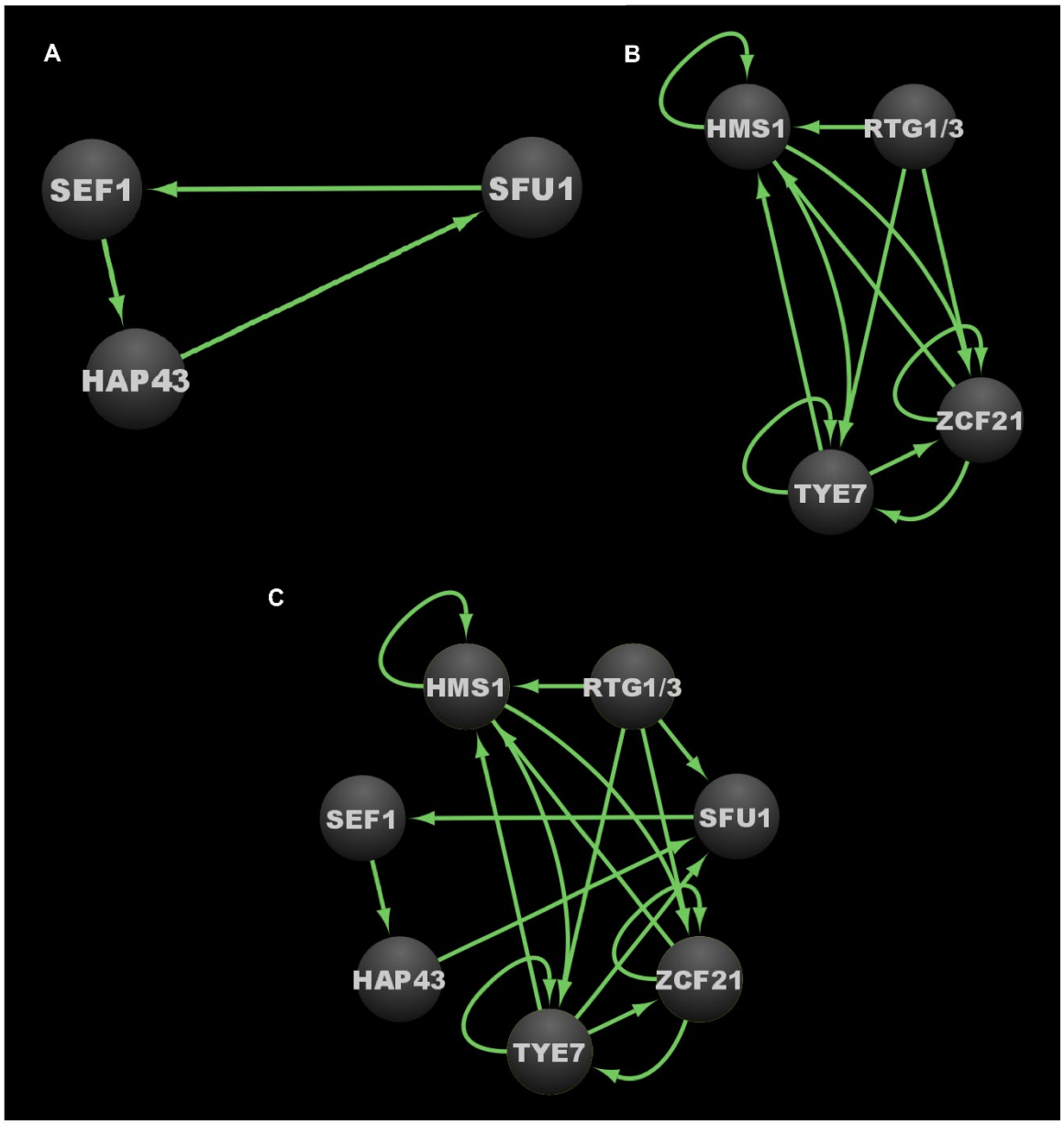

Figure 2.4. Transcriptional circuits controlling the *C. albicans* commensal-pathogen transition. (A) Transcriptional circuit controlling iron homeostasis. (B) Transcriptional circuit controlling proliferation in the host. (C) Overlayed transcriptional circuits controlling the commensal-pathogen transition. Ovals indicate each of the core regulators with their respective names. Arrows indicate direct binding events. Note that since Rtg1 and Rtg3 function as a heterodimer, and do not appear to bind DNA independently, they are represented as a single node in regulatory circuit diagrams. See **Dataset S2.1., Tab4** for binding interactions. Data were derived from (Chen et al., 2011; Pérez et al., 2013). Figure was generated using Cytoscape (Shannon et al., 2003).

Table 2.3. Known transcriptional regulators with roles in the *C. albicans* commensal-pathogen transition.

Core Iron Homeostasis Transcriptional Regulators				
Orf19#	**Name**	**Known commensal-pathogen-related process affected in mutant strain**	**Gene upstream intergenic region bound by one or more of the core regulators?**	**References**
Orf19.681	Hap43	Iron Utilization	Yes	(Baek et al., 2008; Chen et al., 2011; Hsu et al., 2011)
Orf19.3753	Sef1	Iron Uptake	Yes	(Chen et al., 2011)
Orf19.4869	Sfu1	Iron Acquisition	Yes	(Lan et al., 2004; Chen et al., 2011)
Core Host Proliferation Transcriptional Regulators				
Orf19#	**Name**	**Known commensal-pathogen-related process affected in mutant strain**	**Gene upstream intergenic region bound by one or more of the core biofilm regulators?**	**References**
Orf19.921	Hms1	GI Colonization, Disseminated Infection	Yes	(Shapiro et al., 2012; Pérez et al., 2013)
Orf19.4722	Rtg1	GI Colonization, Disseminated Infection	Yes	(Jia et al., 1997; Pérez et al., 2013)
Orf19.2315	Rtg3	GI Colonization, Disseminated Infection	Yes	(Jia et al., 1997; Pérez et al., 2013)
Orf19.4941	Tye7	GI Colonization	Yes	(Pérez et al., 2013)
Orf19.4166	Zcf21	Disseminated Infection	Yes	(Pérez et al., 2013)

A subsequent study identified eight transcriptional regulators (Tye7, Orf19.3625, Lys144, Zcf21, Lys14, Hsm1, Rtg1 and Rtg3) that influence *C. albicans* proliferation in the commensal and/or pathogenic growth states (Pérez et al., 2013). These regulators were identified by screening a subset of the commonly used *C. albicans* TF mutant library (Homann et al., 2009) for defects in a commensal (GI colonization) mouse model and a pathogenic (disseminated infection) mouse model. This subset of the TF mutant library consisted of those mutant strains that revealed no phenotypes in a diverse panel of *in vitro*

growth conditions, and was screened to identify transcriptional regulators that were specifically required for normal (wildtype) levels of growth in either of the two mouse models (Homann et al., 2009; Pérez et al., 2013). Of the eight regulators that were identified, six (Rtg1, Rtg3, Tye7, Hms1, Orf19.3625, and Lys144) were required for GI colonization, while five (Rtg1, Rtg3, Hms1, Lys14, and Zcf21) were required for robust growth in the disseminated infection model (Pérez et al., 2013). Overall, Tye7, Orf19.3625, and Lys144 were found to be specific to commensal colonization of the GI tract; Zcf21 and Lys14 were found to be specific to disseminated infections; and Rtg1, Rtg3, and Hms1 were found to be associated generally with growth in the host (Pérez et al., 2013). Based on genome-wide transcriptional profiling and chromatin immunoprecipitation data, seven of these regulators (Tye7, Lys144, Zcf21, Lys14, Hsm1, Rtg1 and Rtg3) were found to form a transcriptional network consisting of 808 directly bound target genes. Significant overlap was observed between the bound target genes of this network and those genes that were upregulated in the mouse GI model compared to growth *in vitro*. Orf19.3625 was excluded from this analysis as it is a predicted subunit of a histone remodeling complex, and thus was not considered to be a specific regulator within the commensal-pathogen network. In contrast to the transcriptional network defined by Sef1, Sfu1, and Hap43, which is primarily responsible for regulating genes involved in iron homeostasis (Chen et al., 2011), the transcriptional network defined by Tye7, Lys144, Zcf21, Lys14, Hsm1, Rtg1 and Rtg3 appears to primarily regulate genes involved in the acquisition and metabolism of carbon and nitrogen, as well as genes that encode transporters and cell surface proteins (Pérez et al., 2013). The binding profiles for Rtg1 and Rtg3 were observed to be identical, and thus they likely function as a heterodimer to bind DNA (Pérez et al., 2013), which is consistent with their orthologs in *Saccharomyces cerevisiae* (Liu and Butow, 2006). Of the 153 direct target genes in this network that are upregulated during GI colonization and disseminated infection, 108 of them are bound by the Rtg1/3 heterodimer (Pérez et al., 2013), highlighting the central role that Rtg1/3 plays in this network. We note that a subsequent study by the same group identified five transcriptional regulators that influence fitness in an oropharyngeal candidiasis model (Cup9, Zcf8, Zcf21, Zcf27, and Orf19.217), and identified a set of genes that are differentially regulated in response to deletion of *CUP9* (Meir et al., 2018). We did not include this data in our analyses since binding experiments that would be necessary to integrate these additional regulators into the commensal-pathogen transcriptional circuit have not been performed.

At the core of this commensal-pathogen transcriptional network lies a tightly interwoven regulatory circuit defined by the binding interactions between five of these transcriptional regulators (Hms1, Zcf21, Tye7, Rtg1 and Rtg3) and their respective upstream intergenic regions (**Figure 2.4B.**). While Lys14 and Lys144 are clearly important for pathogenic and commensal growth, respectively, they are not integrated into the core transcriptional circuit and instead appear to function as auxiliary regulators. Interestingly, *RTG1* and *RTG3* are not regulated at the transcriptional level in response to growth in the GI tract and are not direct targets of any of the members of this commensal-pathogen transcriptional circuit (Pérez et al., 2013). Instead, Rtg1/3 seems to function as a major regulatory input into, rather than target of, this commensal-pathogen circuit. In *S. cerevisiae*, the Rtg1/3 heterodimer is known to be post-translationally modified and translocated into the nucleus in response to growth on poor nitrogen sources or mitochondrial dysfunction, suggesting that nitrogen assimilation and metabolic adaptation could be critical factors for the proliferation of *C. albicans* in the host (Liao and Butow, 1993; Jia et al., 1997; Liu and Butow, 2006). Hms1, which is also required for both commensal and pathogenic growth in the host, is known to be activated in response to

elevated temperatures (Shapiro et al., 2012), indicating that temperature, along with nitrogen source(s), represent two critical environmental signals that influence the commensal and pathogenic growth programs of *C. albicans*. Zcf21 represses a variety of genes that encode key virulence factors, and plays a major role in pathogenesis by balancing the positive effects of these virulence factors during disseminated infection against the increased susceptibility to host immune system recognition and clearance that is correlated with their expression (Böhm et al., 2016). Finally, Tye7 has been implicated in the metabolism of carbohydrates, such as oligosaccharides and polysaccharides, as well as in the regulation of hyphal growth and biofilm formation (Askew et al., 2009; Bonhomme et al., 2011). (See **Table 2.3.** for information on these five core transcriptional regulators in the commensal-pathogen transition.) Although both the iron homeostasis and the host proliferation transcriptional networks are critical to the ability of *C. albicans* to grow as a commensal and as a pathogen, there is limited interconnectivity between these networks at the level of the core regulators of each circuit (**Figure 2.4C.**). *SFU1* serves as the sole point of integration between the two circuits, being bound by Rtg1/3 and Tye7. There are no binding interactions observed between the iron homeostasis regulators (Sef1, Sfu1, and Hap43) and the genes encoding the host proliferation regulators, suggesting that under certain growth conditions which alter the binding of Rtg1/3 and/or Tye7, the iron homeostasis circuit may function as a sub-circuit of the host proliferation circuit. Together, the transcriptional regulators involved in iron homeostasis and acquisition, and host proliferation, confer *C. albicans* with the ability to proliferate in different niches of the host as well as to transition between commensal and pathogenic states in response to changes in the host environment.

2.6 Integration of Networks

In total, the three larger regulatory networks, consisting of the core regulators and all of their directly bound target genes involved in biofilm formation, the white-opaque phenotypic switch, and the commensal-pathogen transition in *C. albicans* encompass at least 1657 directly bound individual target genes, making up a little over 25% of genes in the entire genome (note that Flo8, Gal4, and Rfx2 were excluded from this analysis since there is not genome-wide chromatin immunoprecipitation data available for them) (**Dataset S2.1., Tab2**) (Zordan et al., 2007; Chen et al., 2011; Nobile et al., 2012; Hernday et al., 2013, 2016; Lohse et al., 2013; Pérez et al., 2013; Fox et al., 2015; Lohse and Johnson, 2016). These three networks are highly intertwined, with 40% (667/1657) of the target genes shared between at least two of the networks, and 11% (188/1657) of the target genes shared between all three networks (**Dataset S2.1., Tab2**). This high degree of interconnectivity is even more pronounced at the level of the core transcriptional circuits that control these three networks, as is evident by the extensive binding interactions present between the core regulators themselves (**Figure 2.5.** and **Dataset S2.1., Tab1**). Together, the twenty transcriptional regulators for which we have genome-wide chromatin immunoprecipitation data available form a total of 225 binding interactions within and between their core circuits, distributed roughly evenly between intra-circuit (49%) and inter-circuit (51%) interactions (note that the Rtg1/3 heterodimer is counted as a single regulator since neither subunit is known to bind independently) (**Dataset S2.1., Tab3**). The commensal-pathogen circuit and the biofilm circuit are highly intertwined with the regulators in the other circuits, with 66% and 59% inter-circuit interactions, respectively, while the opaque cell circuit appears to be much more isolated, with the majority (64%) of its interactions being intra-circuit (**Dataset S2.1., Tab3**). Perhaps the most striking example of integration between the circuits is exemplified by Ndt80 in the biofilm circuit,

which binds to the upstream intergenic regions of 22 out of 24 of the core regulators (all but the upstream intergenic regions of *RTG1* and *RTG3*) (**Dataset S2.1., Tab1**). The percentage of inter-circuit binding events is highest for Tye7 (79%), Zcf21 (75%), Bcr1 (71%), Brg1 (67%), and Rtg1/3 (67%), accounting for at least two out of three binding events for each of these regulators within the three core circuits (**Dataset S2.1., Tab3**). At the opposite end of the spectrum, Hap43, Hms1 and Sfu1 are exclusive to the commensal-pathogen circuit. In addition, at least two thirds of the binding events observed for Wor3 (88%), Czf1 (75%), Rob1 (71%), Ahr1 (70%) and Wor4 (70%) within the three core circuits occur within their respective core circuits (**Dataset S2.1., Tab3**). Interestingly, the degree of Efg1 inter-circuit interaction is unique to the circuit within which it lies, where 61% inter-circuit interactions are observed for Efg1 in the biofilm circuit, while only 42% inter-circuit interactions are observed for Efg1 in the white-opaque circuit (**Dataset S2.1., Tab3**). *BRG1* is the most highly integrated target within the three circuits, where it is bound by seventeen of the twenty core regulators evaluated (leaving out Gal4, Rfx2, and Flo8, and considering Rtg1 and Rtg3 as a single regulator) (**Dataset S2.1., Tab1**). Overall, more than half (thirteen out of twenty-four) of the regulators that make up the three core circuits are bound by at least half (eleven or more) of the twenty core regulators evaluated (**Dataset S2.1., Tab1**). These rather striking numbers highlight the degree to which these circuits are intertwined, and these numbers are only likely to increase as additional core regulators are identified and incorporated into the three transcriptional circuits.

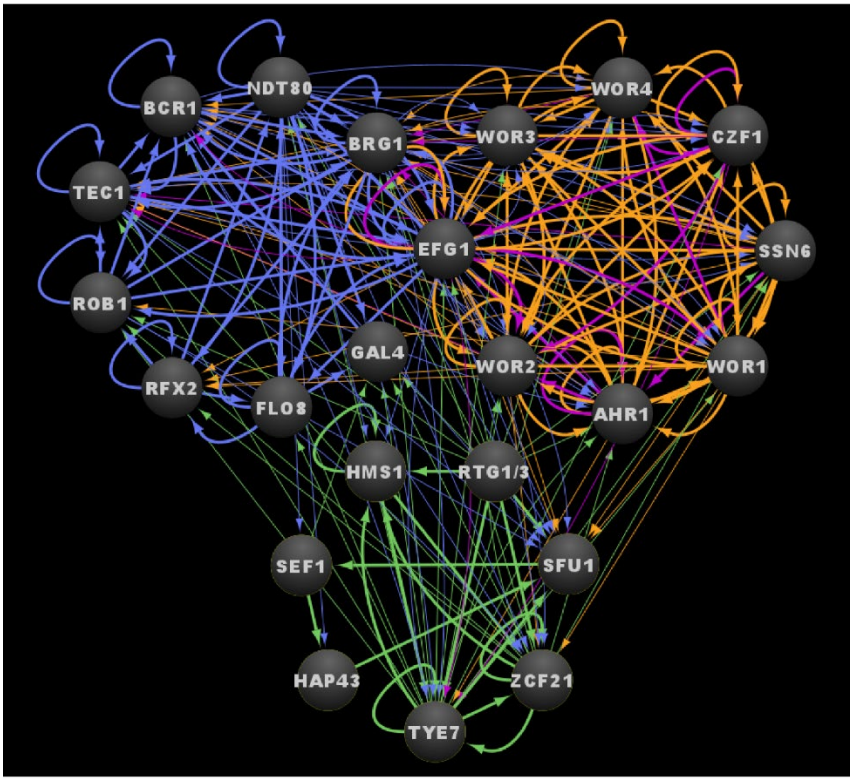

Figure 2.5. Integrated transcriptional circuits of *C. albicans* biofilm formation, the white-opaque switch and the commensal-pathogen transition. Ovals indicate each of the core regulators with their respective names. Arrows indicate direct binding events. See **Dataset S2.1., Tab4** for binding interactions. Data were derived from (Nobile et al., 2012;

Fox et al., 2015; Zordan et al., 2007; Hernday et al., 2013, 2016; Lohse et al., 2013; Lohse and Johnson, 2016; Chen et al., 2011; Pérez et al., 2013). Figure was generated using Cytoscape (Shannon et al., 2003).

The extensive integration between these core transcriptional circuits appears to have significant functional relevance. For example, fourteen of the twenty-four regulator genes discussed (*AHR1*, *BCR1*, *BRG1*, *CZF1*, *GAL4*, *HAP43*, *HMS1*, *RFX2*, *SEF1*, *SFU1*, *TEC1*, *WOR1*, *WOR3*, *ZCF21*) are differentially expressed by at least twofold between planktonic and biofilm growth conditions; of these fourteen genes, all but *GAL4* are upregulated in biofilms (**Dataset S2.1., Tab1**) (Nobile et al., 2012). A similar trend is observed during white-opaque switching, where eleven of the twenty-four regulator genes (*BRG1*, *CZF1*, *EFG1*, *GAL4*, *HMS1*, *RFX2*, *ROB1*, *TYE7*, *WOR1*, *WOR2*, *WOR3*) are differentially expressed by at least twofold between white and opaque cell types (**Dataset S2.1., Tab1**) (Tuch et al., 2010). The interactions between the biofilm circuit and the white-opaque circuit are particularly striking. All eight of the core white-opaque regulator genes are bound by at least four of the six core biofilm regulators, and six of the eight white-opaque regulator genes (all but *EFG1* and *WOR4*) are differentially expressed by twofold or more between planktonic and biofilm conditions (*WOR1*, *AHR1*, *CZF1* and *WOR3* are upregulated by threefold, fivefold, eightfold, and 32-fold, respectively, while *WOR2* and *SSN6* are both downregulated by twofold) (**Dataset S2.1., Tab1**). Conversely, five of the nine core biofilm regulator genes are bound by at least four of the eight white-opaque regulators in opaque cells (*EFG1*, *BRG1*, *BCR1*, *TEC1*, and *RFX2* are bound by eight, eight, five, five, and four white-opaque regulators, respectively), and five of the nine biofilm regulator genes are differentially expressed by at least twofold between white and opaque cells (*BRG1* and *RFX2* are upregulated in opaque cells, while *EFG1*, *GAL4*, and *ROB1* are upregulated in white cells) (**Dataset S2.1., Tab1**). The commensal-pathogen circuit regulators are closely intertwined with the biofilm circuit; however, there is relatively little overlap between the overlayed white-opaque circuit and the overlayed commensal-pathogen circuit. Six of the eight commensal-pathogen regulator genes (all but *RTG1* and *RTG3*) are bound by at least one biofilm core regulator, half of which (*SFU1*, *TYE7*, and *ZCF21*) are bound by at least four of the biofilm regulators (**Dataset S2.1., Tab1**). All six of the commensal-pathogen regulator genes that are bound by biofilm regulators are differentially expressed by twofold or more between planktonic and biofilm conditions, with all but *TYE7* being upregulated in biofilms (**Dataset S2.1., Tab1**). In contrast to the high degree of functional interaction between the biofilm circuit and the overlayed commensal-pathogen circuit, only three of the eight commensal-pathogen regulator genes (*SFU1*, *TYE7*, and *ZCF21*) are bound by any of the white-opaque regulators, and of the three target genes, only *TYE7* is differentially expressed between white and opaque cells (upregulated twentyfold in opaque cells). The effect of growth under conditions relevant to the overlayed commensal-pathogen circuit (i.e., low iron or growth in the GI tract) is relatively limited when compared to the effects of biofilm formation and white-opaque switching. Upon growth in low iron, only the three regulator genes involved in iron homeostasis (*HAP43*, *SEF1*, *SFU1*) are differentially expressed (**Dataset S2.1., Tab1**) (Chen et al., 2011). While growth in the GI tract does affect the expression of core regulator genes in the other circuits, the impact of this expression is relatively limited, where *AHR1* and *TEC1* are upregulated and *ROB1* is downregulated in the GI tract versus growth *in vitro* (Rosenbach et al., 2010).

2.7 Perspectives

The *C. albicans* transcriptional regulatory circuits controlling the developmental processes of biofilm formation, the white-opaque phenotypic switch, and the commensal-pathogen transition are individually tightly knit, and we show that they are integrated together by extensive regulatory crosstalk between the core regulators that comprise each circuit. If we take into consideration all of the target genes in each of the larger transcriptional networks, each regulator controls individual subsets of target genes regulating distinct functions as well as subsets of target genes with functions in common with the other core regulators in each network. Strikingly, these three major transcriptional networks, together, encompass a little over 25% of genes in the entire genome, indicating that there is a high degree of functional redundancy across the networks. The complexity and functional redundancy of these network structures often make dissecting the logic of each network extremely challenging. The networks we discuss here in this review are overall structurally very similar to networks controlling complex transcriptional developmental processes in higher eukaryotes, such as the mammalian embryonic stem cell state (pluripotency) network (Boyer et al., 2005; Kim et al., 2008a). Given that mammals and *C. albicans* diverged from a common ancestor around 1.5 million years ago (Wang et al., 1999), it is notable that the structures of these independently evolved transcriptional networks are so similar. There are a couple hypotheses as to how these transcriptional networks could appear so structurally similar (Sorrells and Johnson, 2015). The first hypothesis is that these complex transcriptional networks represent the optimal solutions for organizing the biological processes they control (François and Hakim, 2004; Prill et al., 2005). The second hypothesis is that these transcriptional networks are not optimal solutions but are rather non-adaptive structures that have been retained over evolutionary time scales by purifying selection and are thus the result of high-probability evolutionary trajectories (Sorrells and Johnson, 2015). As we begin to discover and deconvolute complex transcriptional networks, we will begin to test these hypotheses and shed new light on the logic of these complex network structures.

2.8 Contributions to the Field

Candida albicans is a common member of the human microbiota as well as an opportunistic human fungal pathogen. The ability of *C. albicans* to be successful within the host requires sophisticated transcriptional regulatory programs. Here, we discuss the transcriptional regulatory circuits controlling three major developmental processes in *C. albicans*: biofilm formation, the white-opaque phenotypic switch, and the commensal-pathogen transition. Each of these three circuits are tightly knit and, through our analyses, we show that they are integrated together by extensive regulatory crosstalk between the core regulators that comprise each circuit.

2.9 Supplementary Material

Supplemental Dataset Sheet 2.1 (Dataset S2.1) can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.605711/full#supplementary-material

Dataset S2.1. Compilation and analysis of regulator binding interactions and target gene expression. Tab1 labeled “Tab1_Combined Core Circuits” contains compiled genome-wide chromatin immunoprecipitation (ChIP-seq or ChIP-chip) and expression profiling (RNA-seq or microarray) data for the core circuit regulators and their respective

target genes. ChIP data and RNA-seq data values are in $\log_2$ format. Biofilm regulators ChIP data and differential gene expression data were derived from (Nobile et al., 2012). White-opaque regulators ChIP data were derived from (Zordan et al., 2007; Hernday et al., 2013, 2016; Lohse et al., 2013; Lohse and Johnson, 2016). White-opaque differential gene expression data were derived from (Tuch et al., 2010). Iron homeostasis regulators ChIP data and differential gene expression data were derived from (Chen et al., 2011). Host proliferation ChIP data were derived from (Pérez et al., 2013). Host proliferation differential gene expression data were derived from (Rosenbach et al., 2010). **Tab2** labeled "Tab2_Combined Networks" contains compiled genome-wide chromatin immunoprecipitation (ChIP-seq or ChIP-chip) and expression profiling (RNAseq or microarray) data for the core circuit regulators and all possible target genes in the *C. albicans* genome. ChIP data and RNA-seq data values are in $\log_2$ format. Biofilm regulators ChIP data and differential gene expression data were derived from (Nobile et al., 2012). White-opaque regulators ChIP data were derived from (Zordan et al., 2007; Hernday et al., 2013, 2016; Lohse et al., 2013; Lohse and Johnson, 2016). White-opaque differential gene expression data were derived from (Tuch et al., 2010). Iron homeostasis regulators ChIP data and differential gene expression data were derived from (Chen et al., 2011). Host proliferation ChIP data were derived from (Pérez et al., 2013). Host proliferation differential gene expression data were derived from (Rosenbach et al., 2010). **Tab3** labeled "Tab3_Inter- vs Intra-circuit" contains an analysis of the genome-wide ChIP data from **Tab1**, tabulating the total number of bound targets for each of the regulators for which genome-wide binding data is available, and calculating the percentage of binding events that represent inter- versus intra-circuit binding interactions. **Tab4** labeled "Tab4_Cytoscape Interactions" contains a representation of the genome-wide ChIP data from **Tab1** in an interaction table format for visualization in Cytoscape (Shannon et al., 2003). This dataset was used to generate all of the circuit diagrams shown in **Figures 2.2-2.5**.

2.10 References

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Chapter 3: Functional Target Genes of Biofilm Formation Identified from the *Candida albicans* Biofilm Transcriptional Network

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3.1 Abstract

The majority of new *Candida albicans* infections arise from the presence of *C. albicans* cells existing in biofilms – resilient, surface-associated, communities of microorganisms. Biofilm infections are highly correlated with implanted medical devices, and current antifungal agents are largely ineffective at treating these recalcitrant infections. We previously discovered the transcriptional network governing *C. albicans* biofilm formation, which consists of six "master" transcriptional regulators and ~1,000 downstream target genes. Here, we analyzed the target genes of this network by constructing, screening, and characterizing a priority set of 208 target gene mutants. We screened this mutant library to assess biofilm formation using standard *in vitro* biofilm assays and an *in vivo* intravenous rat catheter infection model. Our results revealed that *DEF1, RHA1, KEX2, ECE1, SOK1, ORF19.7214, CAN3, SAP7,* and *ATO1* were found to be required for biofilm formation *in vitro* and *DEF1, KEX2*, and *ATO1* were found to be required for normal biofilm formation both *in vitro* and *in vivo.* Additionally, *DEF1, KEX2, and SOK1,* were found to be required for filamentation and *ORF19.7214* and *CAN3* were found to play roles in adherence during biofilm formation.

3.2 Author Summary

Biofilms are the preferred mode of growth for most microorganisms in their natural settings. *Candida albicans* biofilm associated infections originating from implanted medical devices are a leading cause of systemic infections in humans. Given that cells in biofilms can be much more resistant than planktonic cells, the treatment of biofilm associated infections is particularly challenging. Moreover, our limited antifungal drug arsenal suggests that there is an urgent unmet medical need for the development of novel antifungal drugs, especially for those with efficacy against biofilms. We previously identified the transcriptional network controlling *C. albicans* biofilm formation. Based on our analysis of this network, here we identified a high priority set of 209 target genes and assessed their functional importance in biofilm formation. We systematically analyzed this set of target gene mutant strains for biofilm formation and identified several genes that are important for normal biofilm formation, some of which may encode for novel antifungal drug targets.

3.3 Introduction

Biofilms are defined as communities of microbial cells that are encased in an extracellular matrix and are tightly adhered to each other and to a surface (Costerton et al., 1995; Donlan, 2002). Biofilms are the preferred mode of growth of microbes in most natural settings and their properties differ from those of free-floating (planktonic) cells (Kolter and Greenberg, 2006; López et al., 2010). Microbes growing as biofilms are much more resistant to the host innate immune system and to physical and chemical

perturbations than their planktonic counterparts (Flemming et al., 2000; López et al., 2010). For example, studies have shown that biofilms can be up to 4000 times more resistant to antifungals such as fluconazole than planktonic cells (Hawser and Douglas, 1995a; Ramage et al., 2001a, 2001b). Due to their recalcitrance, biofilms present a significant health risk, especially when they form on indwelling medical devices, such as catheters and heart valves, as a biofilm can act as a reservoir for systemic and life-threatening infections (Ramage et al., 2006; Saldanha Dominic et al., 2007).

Candida albicans, a common fungal member of the human microbiota and an opportunistic pathogen, is a leading cause of biofilm associated infections originating from indwelling medical devices, including dentures, heart valves, catheters, and prostheses (Wenzel, 1995; Ramage et al., 2006; Pfaller and Diekema, 2007; Saldanha Dominic et al., 2007). Device-associated infections have a high risk of becoming systemic and life-threatening (Wenzel, 1995; Zander and Becker, 2018). Although certain antifungal drugs are effective against *C. albicans* infections, these drugs are largely effective against the planktonic cell state and are less effective or completely ineffective at targeting its biofilm form (Hawser and Douglas, 1995b; Chandra et al., 2001; Ramage et al., 2001c, 2002a; Kuhn et al., 2002). Oftentimes, the only method to effectively treat biofilm-associated infections is through removal of the infected medical device. Therefore, biofilm-associated infections represent a major problem in clinical settings, and their effective treatment requires the development of targeted (i.e., biofilm specific) antifungal therapeutics and a better understanding of the underlying molecular mechanisms that lead to biofilm formation.

C. albicans biofilm development occurs in four distinct stages: adherence, proliferation, maturation, and dispersal (Hawser and Douglas, 1994; Baillie and Douglas, 1999; Chandra et al., 2001; Douglas, 2003; Nobile and Mitchell, 2006; Uppuluri et al., 2010a). Biofilm formation begins when free-floating yeast cells adhere to a surface. Next, the adhered-yeast cells replicate and differentiate, forming pseudohyphae and hyphae. This proliferation stage is followed by maturation, where an extracellular matrix, primarily composed of proteins, DNA, and carbohydrates is produced. Lastly, in the dispersal stage, yeast cells are released from the biofilm and disperse out to the environment to colonize new sites within the host, and the cycle repeats.

Previous studies identified nine core transcriptional regulators (Flo8, Ndt80, Brg1, Efg1, Gal4, Rfx2, Rob1, Bcr1, and Tec1) that control biofilm formation in *C. albicans* (Nobile et al., 2012; Fox et al., 2015). In the context of biofilm formation, Bcr1, Efg1, Rfx2, and Tec1 regulate adhesion (Dieterich et al., 2002; Hao et al., 2009; Sahni et al., 2010; Finkel et al., 2012); Bcr1, Brg1, Efg1, Flo8, Ndt80, Rfx2, Rob1, and Tec1 regulate filamentation (Schweizer et al., 2000; Bockmüh and Ernst, 2001; Cao et al., 2006; Elson et al., 2009; Hao et al., 2009; Sellam et al., 2010; Vandeputte et al., 2011; Du et al., 2012; Nobile et al., 2012); Bcr1, Efg1 and Ndt80 regulate antifungal drug resistance (Chen et al., 2004; Prasad et al., 2010; Desai et al., 2013); and Gal4 regulates the architectural stability of biofilms (Fox et al., 2015). For six of the nine core biofilm transcriptional regulators (Ndt80, Brg1, Efg1, Rob1, Bcr1, and Tec1), we also know their downstream target genes (over 1000 genes) (Nobile et al., 2012). In this study, we prioritized and selected 245 out of 1000 downstream target genes to investigate for their contributions to biofilm formation in *C. albicans*. We generated a homozygous gene deletion library of 208 of these downstream target genes (35 are putatively essential) and screened this library *in vitro* and *in vivo* to identify functionally relevant target genes of biofilm formation.

3.4 Results

3.4.1 Selection Criteria for Target Genes of Interest

We previously discovered the transcriptional network controlling biofilm formation in *C. albicans* consisting of six master biofilm regulators (Bcr1, Tec1, Efg1, Ndt80, Rob1 and Brg1) and all their downstream target genes (~1000 genes) (Nobile et al., 2012). From these 1000 target genes, we selected 245 genes that we deemed as high priority based on their interconnectedness in the network, enrichment levels for binding by the master regulators, differential expression under biofilm compared to planktonic conditions, and differential expression under biofilm conditions upon deletion of the master regulators (**Data Set S3.1. Tab1**).

3.4.2 Identifying Downstream Target Genes Important for Biofilm Formation *In Vitro*

Of the 245 selected high priority target genes, twenty were already known to play roles in biofilm formation (Staab, 1999; Nantel et al., 2002; Ramage et al., 2002b; Nobile and Mitchell, 2005; Nobile et al., 2006a, 2006b, 2008, 2009, 2012, 2014; Norice et al., 2007; Sellam et al., 2009; Ganguly et al., 2011; Vylkova et al., 2011; Finkel et al., 2012; Ghosh et al., 2015; Crawford et al., 2018; Kurakado et al., 2018; Lagree et al., 2020), and thus we did not construct deletion mutants for those target genes. Of the remaining 225 high priority target genes, we successfully created homozygous gene deletion strains for 208 genes (**Dataset S3.2.** and **Table S3.1.**), which we refer to as our high priority target gene mutant library; we were unable to construct deletion mutant strains for seventeen target genes, which we deem as putatively essential. A standard optical density (OD) *in vitro* biofilm assay in 96-well polystyrene plates (Lohse et al., 2017; Gulati et al., 2018) was used to screen our high priority target gene mutant library for biofilm formation. From this screen, 35 mutant strains were identified to form defective biofilms (p-value $\leq$ 0.05) relative to the wildtype reference strain (**Table 3.1.**, **Fig. S23.1 (A-E)**, **Data Set S3.2.**). These 35 strains were then screened for biofilm formation in an *in vitro* microfluidics biofilm assay that mimics a catheter environment as a secondary assay. From this screen, 20 mutant strains formed significantly defective biofilms (p-value $\leq$ 0.05) compared to the wildtype reference strain (**Fig. 3.1.**, **Fig. S3.2.** and **Supplemental Videos S3.1.- S3.36.**). Next, these 20 strains, which were "hits" in both the OD biofilm assay and in the microfluidics biofilm assay, were examined using confocal scanning laser microscopy (CSLM) to assess their cellular morphologies compared to the wildtype reference strain (**Fig. 3.2.** and **Fig. S3.3.**). Visual examination by CSLM revealed nine mutant strains (*def1*Δ/Δ, *rha1*Δ/Δ, *kex2*Δ/Δ, *ece1*Δ/Δ, *sok1*Δ/Δ, *rf19.7214*Δ/Δ, *can3*Δ/Δ, *sap7*Δ/Δ, and *ato1*Δ/Δ) that formed severely visibly defective biofilms compared to the wildtype reference strain (**Fig. 3.2** and **Fig. S3.3.**). Adding the wildtype alleles back into these ten target gene mutant strains restored wild-type levels of biofilm formation to the mutant strains (**Fig. S3.4.**). Overall, our results from the OD, microfluidics, and CSLM biofilm assays indicated that nine out of the 208 mutants screened were severely defective at forming biofilms under *in vitro* conditions (**Table 3.1.**, **Fig 3.1.**, **Fig 3.2.**, **Fig. S3.1.(A-E)**, **Fig. S3.2.**, **Fig. S3.3.,** and **Supplemental Videos S3.1.- S3.36.**).

3.4.3 Identifying Downstream Target Genes Important for Biofilm Formation *In Vivo*

To determine whether Def1, Rha1, Kex2, Ece1, Sok1, Orf19.7214, Can3, Sap7, and Ato1 are important for biofilm formation *in vivo*, we tested their mutant strains in an *in*

vivo rat catheter biofilm infection model. Scanning electron microscopy (SEM) images from the inner lumen of the catheters collected 24 hours post-infection showed that the *def1*Δ/Δ, *kex2*Δ/Δ, and *ato1*Δ/Δ target gene mutant strains displayed severe defects in biofilm formation *in vivo* (**Fig. 3.3.**). The *sok1*Δ/Δ mutant strain, which appeared thinner and was made up of only yeast cells, displayed a moderate defect in biofilm formation *in vivo* relative to the wildtype strain (**Fig. 3.3.**). The e*ce1*Δ/Δ and *can3*Δ/Δ mutant strains displayed minor defects in biofilm formation *in vivo* (**Fig. 3.3.**) and appeared to contain fewer hyphae than the wildtype strain (**Fig. 3.3.**). The *rha1*Δ/Δ, *orf19.7214*Δ/Δ, and *sap7*Δ/Δ mutant strains formed relatively normal biofilms *in vivo* (**Fig. 3.3.**). Interestingly, the *orf19.7214*Δ/Δ mutant strain formed a biofilm that primarily consisted of yeast cells (**Fig. 3.3.**).

Table 3.1. Mutant strains with significant biofilm defects in the *in vitro* optical density biofilm assay.

Gene ID (ORF#)[a]	Strain [b]	Biofilm Effect (%)[c]	Standard Deviation (%)[d]
	WT		2.9
ORF19.7561	*def1*Δ/Δ	-61.3	1.2
ORF19.7561	*rbd1*Δ/Δ	-47.2	1.8
ORF19.2158	*nag3*Δ/Δ	-45.0	3.7
ORF19.4459	*orf19.4459*Δ/Δ	-38.7	1.5
ORF19.5843	*srr1*Δ/Δ	-37.5	2.0
ORF19.5447	*hgt19* Δ/Δ	-36.4	2.1
ORF19.6874	*orf19.6874*Δ/Δ	-36.0	1.8
ORF19.2823	*rfg1*Δ/Δ	-34.1	2.0
ORF19.1604	*rha1*Δ/Δ	-32.2	9.9
ORF19.4755	*kex2*Δ/Δ	-31.8	1.6
ORF19.3839	*sap10*Δ/Δ	-31.8	2.0
ORF19.265	*orf19.265*Δ/Δ	-31.5	1.9
ORF19.3374	*ece1*Δ/Δ	-31.2	4.8
ORF19.451	*sok1*Δ/Δ	-31.1	2.5
ORF19.5585	*sap5*Δ/Δ	-27.4	2.9
ORF19.5302	*pga31*Δ/Δ	-27.1	0.7
ORF19.721	*orf19.7214*Δ/Δ	-27.1	2.8
ORF19.815	*dck1*Δ/Δ	-27.1	4.0
ORF19.4761	*hst1*Δ/Δ	-24.7	3.3
ORF19.1350	*orf19.1350*Δ/Δ	-24.1	1.5
ORF19.6720	*orf19.6720*Δ/Δ	-23.3	1.9
ORF19.84	*can3*Δ/Δ	-23.1	2.1
ORF19.852	*sap98*Δ/Δ	-22.4	2.4
ORF19.756	*sap7*Δ/Δ	-21.8	3.3
ORF19.4433	*cph1*Δ/Δ	-21.8	2.2
ORF19.6734	*tcc1*Δ/Δ	-21.6	3.1
ORF19.1362	*smm1*Δ/Δ	-21.1	1.6
ORF19.7196	*orf19.7196*Δ/Δ	-20.1	0.5

ORF19.6169	*ato1Δ/Δ*	-19.8	1.4
ORF19.6577	*flu1Δ/Δ*	-19.0	2.8
ORF19.1285	*orf19.1285Δ/Δ*	-18.6	2.2
ORF19.4654	*orf19.4654Δ/Δ*	-16.7	1.5
ORF19.6420	*pga13Δ/Δ*	-16.5	3.2
ORF19.1490	*msb2Δ/Δ*	-15.6	1.2
ORF19.7247	*rim101Δ/Δ*	-15.5	2.0

Footnotes:
[a]This column includes each homozygous gene deletion strain that had a significantly (*p-value* ≤ 0.05) reduced optical density (OD_{600}) relative to the wildtype reference strain (SN250). Statistical significance was calculated using a Student's two-tailed paired t-test using six technical replicates per mutant and wildtype strains, from two independent experiments.
[b]This column lists the percent difference in OD for each mutant strain compared to the wildtype reference strain. Negative values represent decreased biofilm formation relative to the wildtype reference strain and are ordered from smallest to largest.
[c]This column shows the percent difference standard deviation across six technical replicates for each mutant strain compared to the reference strain, from two independent experiments.
See Figure **S3.1.** for the complete set of strains screened.

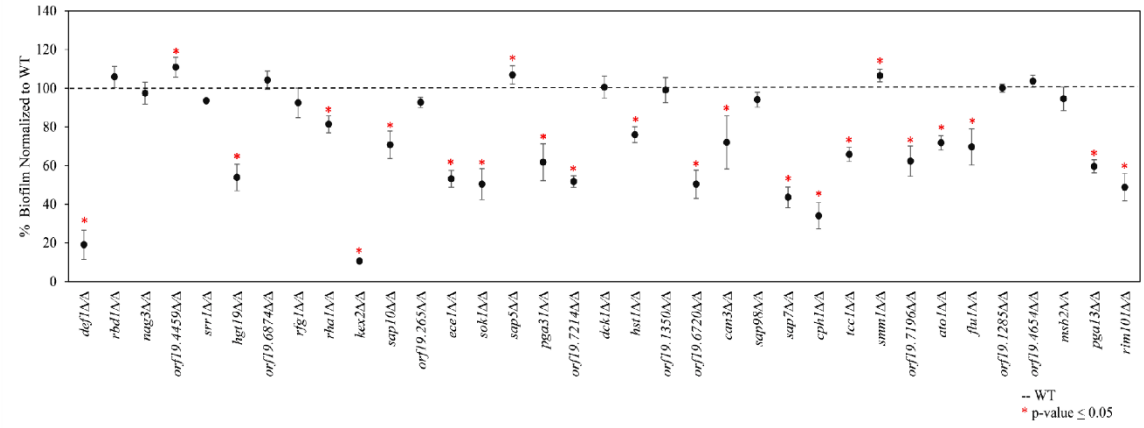


Figure 3.1. *In vitro* biofilm secondary screen of hits in a BioFlux microfluidics device. Percent area of biofilm formed under flow conditions. 35 strains with significantly (*p-value* ≤ 0.05) reduced optical density values were grown in Spider medium under constant flow in a BioFlux microfluidics device. The average percent area covered by the biofilm was quantified and normalized to the wildtype reference strain (SN250). Biofilms of two replicates per mutant strain at three different locations within the microchannel plate were grown, visualized and quantified. Significance (*p-value* ≤ 0.05) was calculated using a two-tailed Student's t-test and is indicated by red asterisks in the plot. Error bars represent standard deviation.

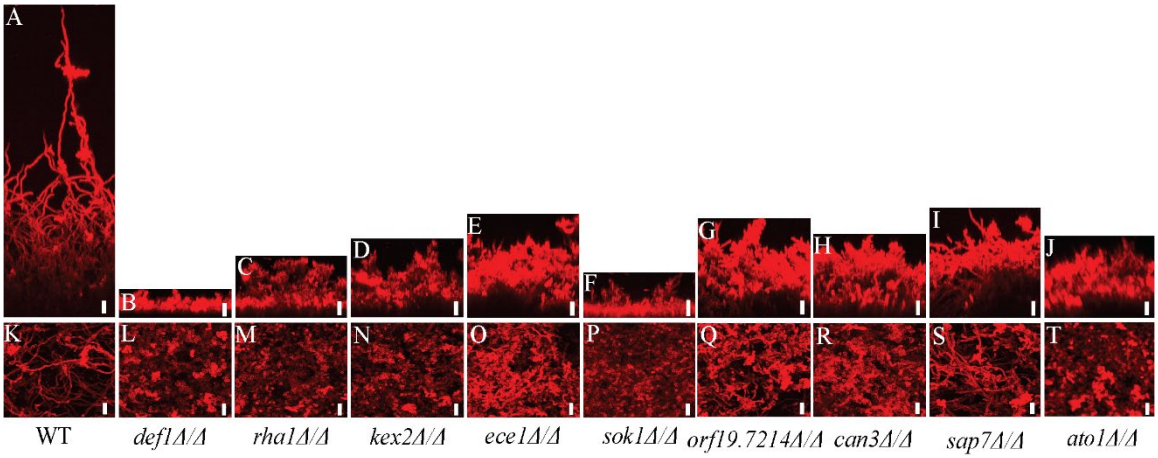


Figure 3.2. Nine target gene mutant strains have severe defects in biofilm formation *in vitro* by CSLM. The wildtype reference strain (SN250), *ece1ΔΔ*, *sap7ΔΔ*, *kex2*, *rha1ΔΔ*, *can3ΔΔ*, *orf19.7214ΔΔ*, *def1ΔΔ*, *sok1ΔΔ*, and *ato1ΔΔ* mutant strains were grown in Spider medium for 24 h on silicone squares pretreated overnight with bovine serum. Biofilms were stained with concanavalin A-Alexa 594 dye. (A-J) Confocal Scanning Laser Microscopy (CSLM) side view images of the wildtype reference strain and nine homozygous gene deletion strains defective in biofilm formation. Scale bars represent 20μm. (K-T) CSLM top view images of the wildtype reference strain and nine mutant strains defective in biofilm formation. Scale bars represent 20μm.

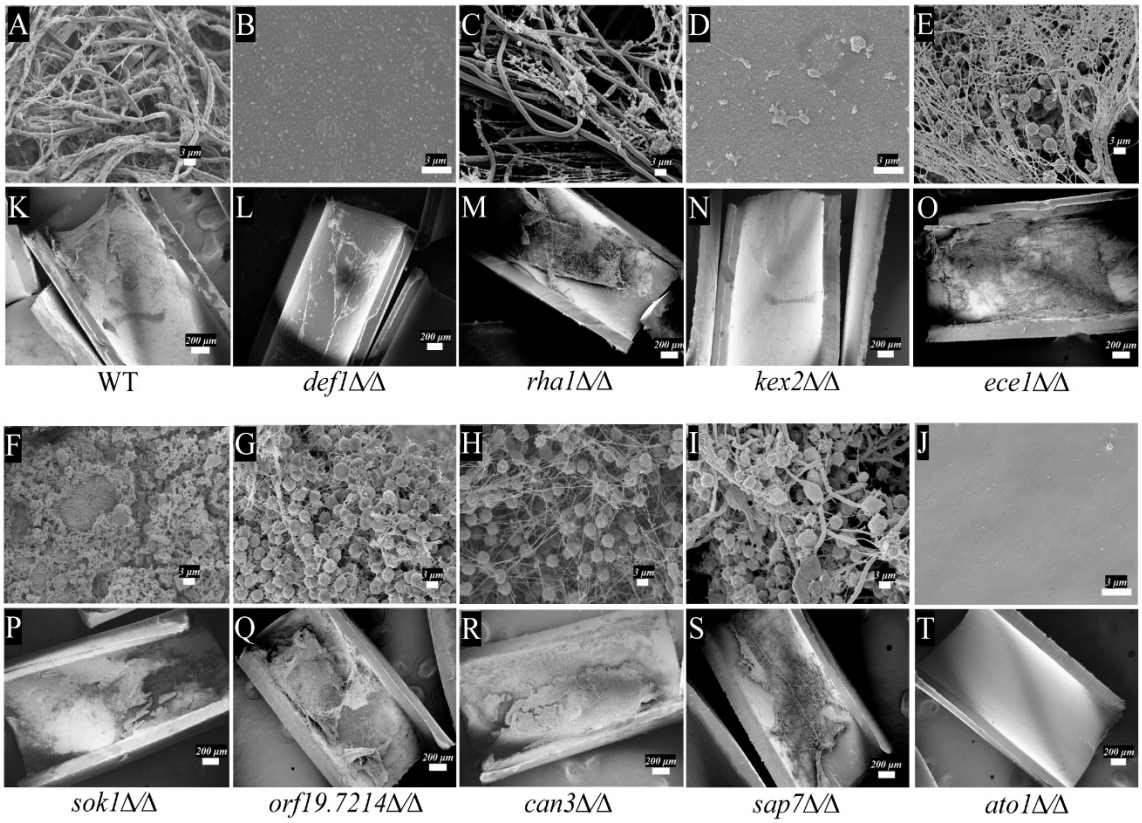


Figure 3.3. Biofilm formation in an *in vivo* rat catheter model. The wildtype reference strain (SN250) and nine homozygous gene deletion strains defective in biofilm formation in both *in vitro* biofilm assays were inoculated intravenously in an *in vivo* rat catheter infection model. Scanning electron microscopy (SEM) images of biofilms formed in the lumen of the catheters were obtained at magnifications of 100x and 5000x after 24 hour of growth. (A-J) SEM catheter images of the wildtype reference strain and nine mutant strains at magnifications of 5000X. Scale bars represent 3μm. (K-T) SEM catheter images of the reference strain and nine mutant strains at magnifications of 100X. Scale bars represent 200μm.

3.4.4 Functional Roles of *ATO1*, *SOK1*, *RHA1*, *CAN3*, *KEX2*, *ECE1*, *SAP7*, *ORF19.7214*, and *DEF1* in Biofilm Formation

To ensure that the target genes we identified have specific roles in biofilm formation, and are not simply due to growth defects, we first assessed whether any of these target gene mutant strains had growth defects under planktonic conditions. When grown in YPD medium at 30°C, *def1Δ/Δ*, *rha1Δ/Δ*, *ece1Δ/Δ, sok1Δ/Δ, orf19.7214Δ/Δ*, *sap7Δ/Δ*, and *kex2Δ/Δ* mutant strains showed no major growth differences relative to the wildtype strain (**Fig. 3.4.**). On the other hand, *can3Δ/Δ* and *ato1Δ/Δ* mutant strains displayed moderate growth defects relative to the wildtype strain at either stationary phase (*can3Δ/Δ*) or logarithmic phase (*ato1Δ/Δ*) of growth (**Fig. 3.4.**). Based on these findings, the *can3Δ/Δ* and *ato1Δ/Δ* mutant strains may have biofilm defects that could, at least in part, be due to growth defects.

To assess whether the target gene mutant strains were involved in important known processes for biofilm formation, we next assessed whether the strains were

involved in adherence and filamentation. To assess adherence, we performed a standard *in vitro* adhesion assay (Krom and Willems, 2016; Lohse et al., 2017; Gulati et al., 2018). We found that the *orf19.7214*Δ/Δ and *can3*Δ/Δ mutant strains had the most noticeable adherence defects compared to the wildtype strain in all medium used (**Fig. 3.5.**). For the remaining mutant strains, their adherence defects were less obvious and varied depending on the medium used; some mutant strains even displayed significantly enhanced adherence compared to the wildtype reference strain (**Fig. 3.5.**). Based on these findings, the *orf19.7214*Δ/Δ and *can3*Δ/Δ mutant strains likely have biofilm defects that could, at least in part, be due to these clear adherence defects. To assess filamentation, we performed standard *in vitro* filamentation assays in RPMI medium (Nadeem et al., 2013). We found that the *def1*Δ/Δ, *kex2*Δ/Δ, *sok1*Δ/Δ, and *rha1*Δ/Δ mutant strains had clear filamentation defects (**Fig. 3.6.**). The *def1*Δ/Δ and *sok1*Δ/Δ mutant strains were the most defective in filamentation, where no hyphae were observed, followed by the *kex2*Δ/Δ mutant strain, and then the *rha1*Δ/Δ mutant strain, whose filamentation defects were more moderate (**Fig. 3.6.**). Based on these findings, the *def1*Δ/Δ, *kex2*Δ/Δ, *sok1*Δ/Δ, and *rha1*Δ/Δ mutant strains likely have biofilm defects that could, at least in part, be due to defects in filamentation.

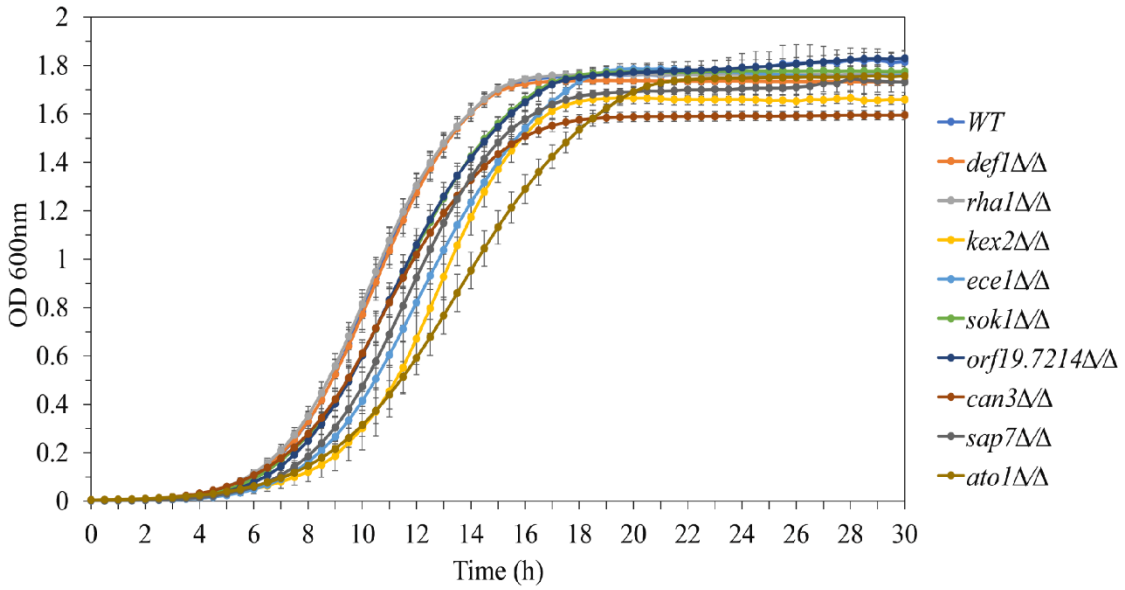


Figure 3.4. Growth curves of biofilm defective mutant strains. Growth curves of the wildtype reference strain and nine mutant strains defective in biofilm formation in all *in vitro* biofilm assays. Growth was assessed in YPD at 30°C for 30 h. The mean optical density (OD_{600}) of six replicates per strain in three separate experiments was calculated and plotted. OD_{600} was recorded every 30 mins.

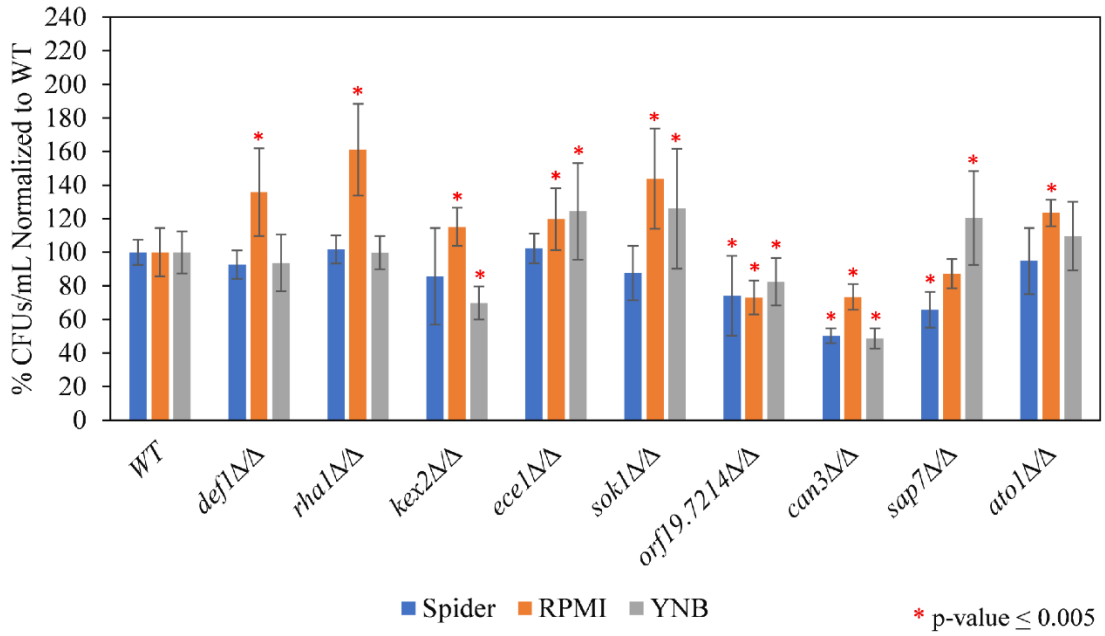


Figure 3.5. Adhesion assay of biofilm defective mutant strains. Adhesion in Spider, RPMI, and YNB media. Overnight cultures of the wildtype reference strain, *rha1Δ/Δ*, *ece1Δ/Δ*, *sap7Δ/Δ*, *ato1Δ/Δ*, *can3Δ/Δ, orf19.7214Δ/Δ, kex2Δ/Δ*, *sok1Δ/Δ* and *def1Δ/Δ* mutant strains were diluted to OD=0.5 (approximately $1X10^7$ cells) and grown in Spider, RPMI, or YNB media for 90 mins. Unadhered cells were removed by washing with PBS as previously published (Lohse et al., 2017). Four tenfold serial dilutions were made and were plated on YPD agar plates. Each bar represents the mean number of colonies in four technical replicates of three experiments. Significance was calculated using a paired Student's t-test (p-value $\leq$ 0.005) and is indicated by red asterisks. Error bars represent the standard deviation.

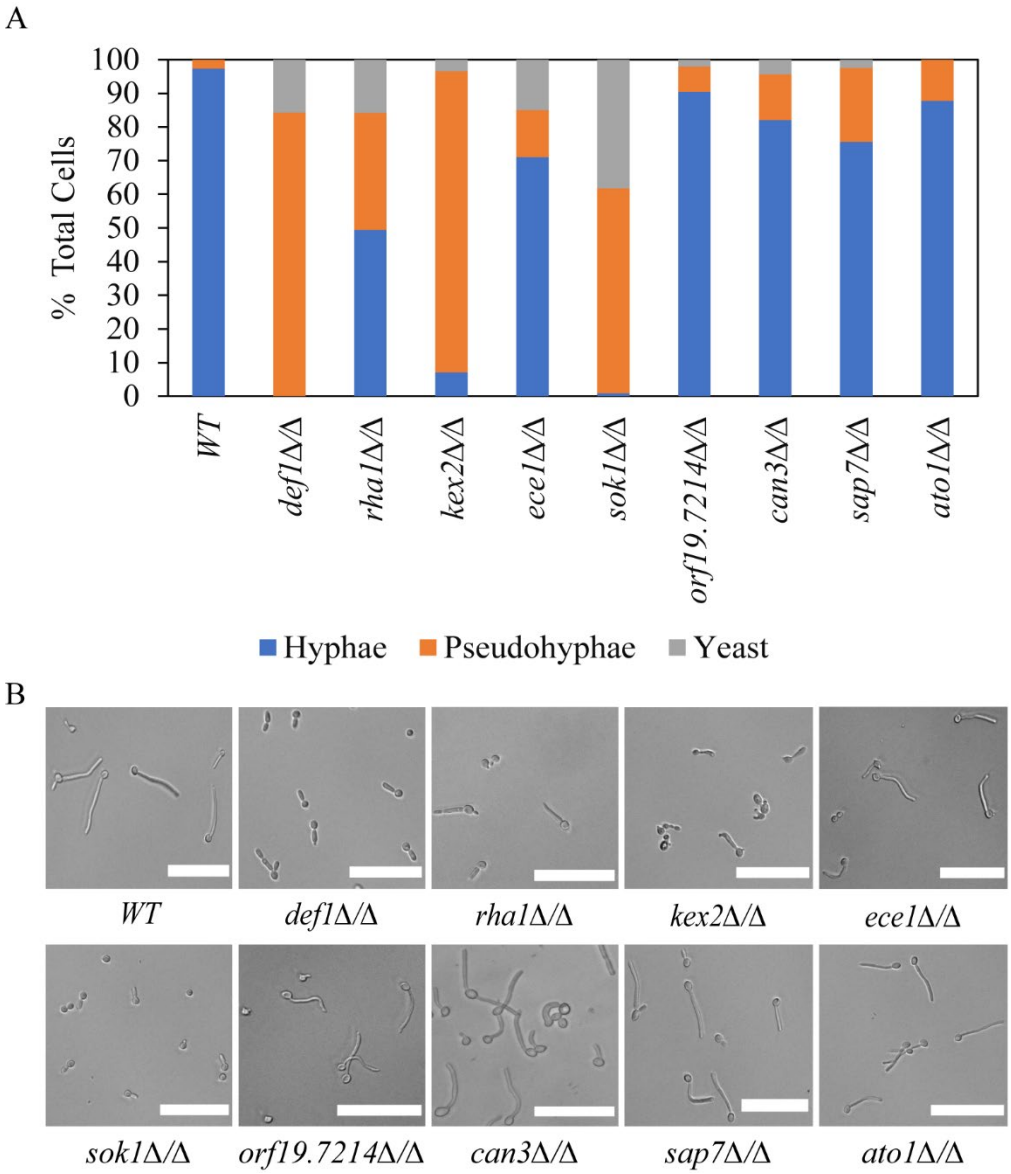


Figure 3.6. Filamentation assay of biofilm defective mutant strains. (A) The bars represent the percentage of cells in a total of ten images per mutant strain relative to the wildtype reference strain. Overnight cultures of *rha1ΔΔ*, *ece1ΔΔ*, *sap7ΔΔ*, *ato1ΔΔ*, *can3ΔΔ, orf19.7214ΔΔ, kex2ΔΔ*, *sok1ΔΔ* and *def1ΔΔ* mutant strains were diluted to OD=0.5 ($1X10^7$) in 2 mL of RPMI medium and incubated for 2h at 30℃. (B) Representative images of cells of the mutant strains and the wildtype strain. Scale bars represent 100μm.

3.5 Discussion

In this study, we constructed and screened for biofilm formation a high priority target gene mutant library consisting of 208 *C. albicans* mutants that were identified and prioritized from the biofilm transcriptional network (Nobile et al., 2012) that we previously discovered. These target genes were previously shown to be highly differentially regulated

under biofilm compared to planktonic conditions and were found to be regulated by one or more of the master *C. albicans* biofilm transcriptional regulators (Nobile et al., 2012).

Overall, from the different *in vitro* biofilm assays performed, nine target gene mutant strains (*def1ΔΔ, rha1ΔΔ*, *kex2ΔΔ*, *ece1ΔΔ*, *sok1ΔΔ*, *orf19.7214ΔΔ*, *can3ΔΔ*, *sap7ΔΔ*, and *ato1ΔΔ*) were severely defective for biofilm formation, and their role in biofilm development, prior to this work, had not been reported. Ece1, Kex2, Sap7, and Def1 have other verified functions in *C. albicans* (Birse et al., 1993; Newport and Agabian, 1997; Naglik et al., 1999; Zakikhany et al., 2007; Martin et al., 2011; Aoki et al., 2012; Moyes et al., 2016; Richardson et al., 2018), but here we show that they are important for biofilm development. The functions of Ato1, Sok1, Rha1, Can3, and Orf19.7214 are unknown, and here we show that they play important roles in biofilm development. Ece1 is a hypha-specific cytolytic toxin and is crucial for mucosal infection (Birse et al., 1993; Moyes et al., 2016; Richardson et al., 2018). Sap7 and Kex2 are classified as proteases (Newport and Agabian, 1997; Naglik et al., 1999; Aoki et al., 2012; Richardson et al., 2018). While Sap7 is a self-processing enzyme expressed in human oral infection (Naglik et al., 1999; Aoki et al., 2012), Kex2 is necessary for hyphal growth, for maturation of Ece1, and it is known to process other Saps (Newport and Agabian, 1997; Richardson et al., 2018). Def1 is structurally similar to Def1 in *S. cerevisiae*, where it functions as an RNA polymerase II regulator (Woudstra et al., 2002; Somesh et al., 2005; Zakikhany et al., 2007; Martin et al., 2011). In *C. albicans*, Def1 has known roles in filamentation and adhesion, which is confirmed by our results, and it is also important in *C. albicans* dissemination and host epithelial cell escape (Zakikhany et al., 2007; Martin et al., 2011). Additionally, our screen also revealed two predicted transmembrane proteins (Ato1 and Can3), of which Ato1 is fungal specific (Lan et al., 2002; Vylkova et al., 2011), a predicted kinase (Sok1) (Lu et al., 2014b), a glucan 1,3-beta-glucosidase (Orf19.7214), and a predicted Gal4-like DNA binding transcription factor (Rha1) (Skrzypek et al., 2017) with roles in biofilm development. Additionally, we show that Can3 and Orf19.7214 are also involved in adherence during biofilm formation, suggesting that Can3 may be a cell surface protein with roles in cell-cell and cell-substrate adherence rather than a transporter. The functions of Orf19.7214 are completely uncharacterized and our results demonstrate its involvement in adherence during biofilm formation.

Our results also indicate a close relationship between filamentation and biofilm formation as four of the nine mutant stains *def1ΔΔ, rha1ΔΔ*, *kex2ΔΔ*, and *sok1ΔΔ* were defective in filamentation. Kex2, Sok1, and Def1 have been previously implicated in filamentation under planktonic conditions (Newport and Agabian, 1997; Zakikhany et al., 2007; Martin et al., 2011; Lu et al., 2014b, 2014a; Richardson et al., 2018). For example, Sok1 plays a role in the hyphal induction program through the activation of the cAMP-PKA pathway (Lu et al., 2014a, 2014b), and it is necessary for degradation of Nrg1, which is a known repressor of hyphal genes (Uppuluri et al., 2010b). Like Sok1, Def1 and Kex2 also play roles in filamentation, where Def1 is a member of the same signaling cascade as Sok1 (Newport and Agabian, 1997; Kadosh and Johnson, 2005; Noble et al., 2010; Martin et al., 2011). Although other groups have previously reported roles for Def1 in adherence and filamentation (Kadosh and Johnson, 2005; Martin et al., 2011), we only observed a role for Def1 in filamentation. These inconsistencies between our findings with those of others are likely due to differences in assay conditions.

Overall, our findings highlight that adherence and filamentation are the main contributors to normal biofilm formation in *C. albicans*. Thus, targeting some of the proteins required for either adherence or filamentation that we describe here could represent promising antifungal drug targets.

3.6 Methods

3.6.1 Media

C. albicans strains were cultured at 30℃ in yeast extract-peptone-dextrose (YPD) (2% Bacto peptone, 2% dextrose, 1% yeast extract, pH 6.8). Heterozygous and homozygous gene mutants were selected on synthetic defined (SD) media (6.7% YNB with ammonium sulfate, 2% dextrose, and without histidine and or leucine). Nourseothricin-resistant mutants were selected on YPD + NAT200 (2% Bacto peptone, 2% dextrose, 1% yeast extract, and 200 µg/ml nourseothricin (GoldBio, N-500-5)) and later grown on SD media without leucine to remove the NAT marker and the Cas9 and gRNA cassettes. Nourseothricin-sensitive isolates were patched on YPD and YPD + NAT400 plates (2% Bacto peptone, 2% dextrose, 1% yeast extract, supplemented with 400 µg/ml nourseothricin) as previously reported (Noble and Johnson, 2005; Nguyen et al., 2017). Biofilms were grown in YPD medium, in Spider medium (10 g/l nutrient broth (Difco), 10 g/l mannitol, 4 g/l K2HPO4, pH 7.2) or in RPMI (RPMI-1640 with L-Glutamine, without sodium bicarbonate (MP Biomedicals, 0910601) supplemented with MOPS (Sigma, M3183), pH 7.0).

3.6.2 Prioritization of Target Genes

To identify the functionally relevant target genes of biofilm formation, we prioritized the 1,007 target genes previously discovered from the *C. albicans* biofilm network (Nobile et al., 2012) based on genome-wide gene expression data and chromatin immunoprecipitation data. From the 1,007 target genes, we selected 245 “high priority” genes of interest (**Data Set S3.1.**) based on three criteria: (i) the gene is highly upregulated under biofilm compared to planktonic conditions, (ii) the gene is bound by one or more of the six master regulators under biofilm conditions, and (iii) the gene is positively controlled by one or more of the master regulators under biofilm conditions.

3.6.3 Construction of C. albicans Deletion Mutants

Of the 245 high priority genes of interest, we constructed 209 deletion mutants using either the traditional gene deletion or recently developed CRISPR methods, as described previously (Noble and Johnson, 2005; Nguyen et al., 2017). In brief, for the standard traditional gene deletion method (Noble and Johnson, 2005), we used the isogenic reference strain SN152 (HIS- LEU- ARG-) as an auxotrophic base strain and performed two rounds of transformation. In the first round of transformation, we inserted a *HIS1* cassette, which replaced one of the alleles of the desired gene through homologous recombination. The cell suspension from the first transformation was then plated on selective media (synthetic dextrose (SD HIS-)). In the second round, a *LEU2* cassette replaced the second allele of the target gene, and homozygous deletion mutants were selected by plating on SD HIS- LEU-. Gene deletion was confirmed through colony polymerase chain reaction (cPCR) using three primer pairs. Each of two primer pairs were used to confirm the insertion of the HIS and LEU cassettes, which replaced the target gene. A third pair of primers was used to confirm the absence of the gene of interest. For the CRISPR/Cas9 method (Nguyen et al., 2017), which was newly developed at the time and significantly increased the rate of mutant strain construction, we used a *C. albicans LEU2* heterozygous base strain disrupted by the integration of a fragment that contains the Cas9 expression cassette, a nourseothricin-resistance marker (*NAT*), and a gRNA construct. Transformants were selected on Yeast Peptone Dextrose (YPD) plates

containing nourseothricin, and the CRISPR components were removed by streaking the transformed colonies on minimal media containing LEU as indicated in Nguyen et al., 2017 (Nguyen et al., 2017). Target gene deletions were verified by colony PCR using a pair of primers to confirm the integration of the donor DNA in place of the target gene and another pair of primers to confirm the absence of the gene of interest. Gel electrophoresis was performed to verify the size of the amplicons. All primers are listed in **Table S3.2**. Twenty mutants included in our high priority target gene set have previously known roles in biofilm formation (**Dataset S3.1.**). We were unable to create mutants for seventeen high priority target genes after several attempts, and therefore, deemed these mutants as putatively essential (**Data set S3.1.**). Complemented strains for *ATO1, SOK1, RHA1, CAN3, KEX2, ECE1, SAP7, ORF19.7214,* and *DEF1* were constructed using the CRIPSR/Cas9 method published by Nguyen et al., 2017 (Nguyen et al., 2017).

3.6.4 Optical Density Biofilm Assay

The optical density biofilm assay was performed as previously described (Lohse et al., 2017; Gulati et al., 2018). Briefly, each well of a 96-well plate was inoculated with $1x10^7$ cells (OD_{600} = 0.5) of an overnight culture grown in YPD to a final volume of 200 µL in Spider, YPD, or RPMI media. Cells were allowed to adhere for 90 minutes at 37ºC with shaking at 250 rpm. The wells were then washed once with 200µL of 1X phosphate-buffered saline (PBS). After washing, 200µL of Spider medium were added to each well and the plate was incubated for 24 h at 37ºC, with shaking at 250 rpm. After 24 h, the media and unadhered cells were removed, and the OD_{600} was measured using a BioTek Epoch 2 plate reader (BioTek).

3.6.5 BioFlux Microfluidics Biofilm Assay

The BioFlux microfluidics biofilm assay was performed as described previously (Winter et al., 2016; Gulati et al., 2017). Three technical replicates of each strain were grown on the bottoms of 48-well BioFlux plates (FLUXION, 910-0047), in Spider medium for 12 h, at 37ºC, with a constant shear flow of 0.5 dyn/cm^2. Biofilm formation was quantified using the BioFlux Montage software (Fluxion, Version 7.8.4.0), which measures the area covered by cells within the viewing field. For specific information on experiment setup and software settings on this assay, see Gulati et al., 2017 (Gulati et al., 2017).

3.6.6 Confocal Microscopy Biofilm Assay

The confocal scanning laser microscopy (CSLM) biofilm assay was performed as previously described (Nobile and Mitchell, 2005; Nobile et al., 2012). Briefly, silicone squares (Cadiovascular Instrument Corp, PR72034-04N) were placed in a 12-well polystyrene plate that was pretreated overnight with Bovine serum (Corning, MT35010CV), and washed with 2 mL of 1XPBS. Two wells per strain were inoculated at an OD_{600} = 0.5 (approx. $1x10^7$ cells/mL) in 2 mL Spider medium. The plate was incubated for 90 min, at 37ºC, with shaking at 200 rpm. Unadhered cells were removed by gently aspirating the medium from the wells and washing with 2 mL of 1XPBS. Fresh Spider medium (2 mL) was then added to each well. The plate was incubated for 24 h at 37ºC with shaking at 200 rpms. Concanavalin A Alexa Flour 594 conjugate (conA-594) (Thermo Fisher, C11253) (50µg/ml) was used to dye the biofilms for 1 h in the dark at 37ºC under gentle shaking. The biofilms were visualized with a 555 nm diode laser and a 40x/0.8W objective using a Zeiss LSM 700 confocal microscope located at the UC Merced microscopy facility. Side and depth images were assembled using ImageJ (ImageJ bundled with 64-bit Java 1.8.0_112).

3.6.7 In vivo Rat Catheter Biofilm Model

The *in vivo* rat central venous catheter biofilm assay was performed as previously described (Andes et al., 2004). Briefly, pathogen-free Sprague-Dawley rats were inserted a polyethylene catheter (inner diameter, 0.76 mm; outer diameter, 1.52 mm) into the external jugular vein, 24 h prior to infection. The proximal end of the catheter was externalized through the skin, and the rats were infected via intraluminal instillation with 10^6 cells/mL of *C. albicans* cells. The cells were adhered for 4 h and then removed by flushing the catheter with heparinized NaCl. The catheter containing the biofilm was collected after 24 h, and the biofilm was analyzed by scanning electron microscopy (SEM).

3.6.8 Growth Curves

Overnight cultures were diluted to an OD_{600} of 0.05 in YPD (200µL final volume) in each of 6 wells per strain, in a 96-well polystyrene plate. An additional 10-fold dilution was done in YPD (200µL final volume). Strains were incubated for 30 h and 60 h at 30℃ and 225 rpms, and absorbance was recorded every 30 min.

3.6.9 Filamentation Assay

Cell cultures were added to RPMI (2mL final volume) at a starting OD_{600} of 0.5 and were grown for 2 h at 37℃ and 225 rpms. 10µL of culture was obtained and added onto a slide to visualize under the microscope. 10 images per strain were captured using an EVOS (Life Technologies, FL Cell Imaging System) inverted microscope and a 40x objective.

3.6.10 Adhesion Assay

The biofilm adhesion assay was performed as previously described (Gulati et al., 2018). Strains were grown on the bottom of 96-well plates at a starting OD_{600} of 0.5 (approximately equivalent to $1X10^7$ cells) in Spider, RPMI, or YNB (Yeast Nitrogen Base) media, for 90 mins, at 37℃ and 250 rpms. Unadhered cells were removed by washing the wells twice with 200 µL 1XPBS. After the second wash, the PBS was aspirated out and the cells were thoroughly resuspended in 200 µL of fresh 1XPBS then serially diluted to 1:1,000 in 1XPBS. 100 µL of the final dilution were plated on YPD plates and were incubated for 48 h at 30℃. CFU counts were recorded for six plates per strain. This assay was done three times in each Spider, RPMI, and YNB media.

3.7 Author Contributions

Conceptualization, D.L.R. and C.J.N.; Formal Analysis, D.L.R. and C.J.N.; Investigation, D.L.R., N. H., E. F.., H. S., D. R. A., and C.J.N.; Resources, D. R. A. and C.J.N.; Data Curation, D.L.R. and C.J.N.; Writing – Original Draft Preparation, D.L.R. and C.J.N.; Writing – Review & Editing, D.L.R. and C.J.N.; Visualization, D.L.R. and C.J.N.; Supervision, C.J.N.; Project Administration, C.J.N.; Funding Acquisition, C.J.N.

3.8 Funding

This work was supported by the National Institutes of Health (NIH) National Institute of General Medical Sciences (NIGMS) award R35GM124594, by a Pew Biomedical Scholar Award from the Pew Charitable Trusts, and by the Kamangar family in the form of an endowed chair to C.J.N. D.L.R. was supported by the National Science Foundation (NSF) Graduate Research Fellowship Program (GRFP) award 1744620. The confocal microscopy data in this work was collected with a confocal microscope acquired

through the National Science Foundation MRI Award Number DMR-1625733. The funders had no role in the study design, data collection and interpretation, or the decision to submit the work for publication.

3.9 Conflict of Interest

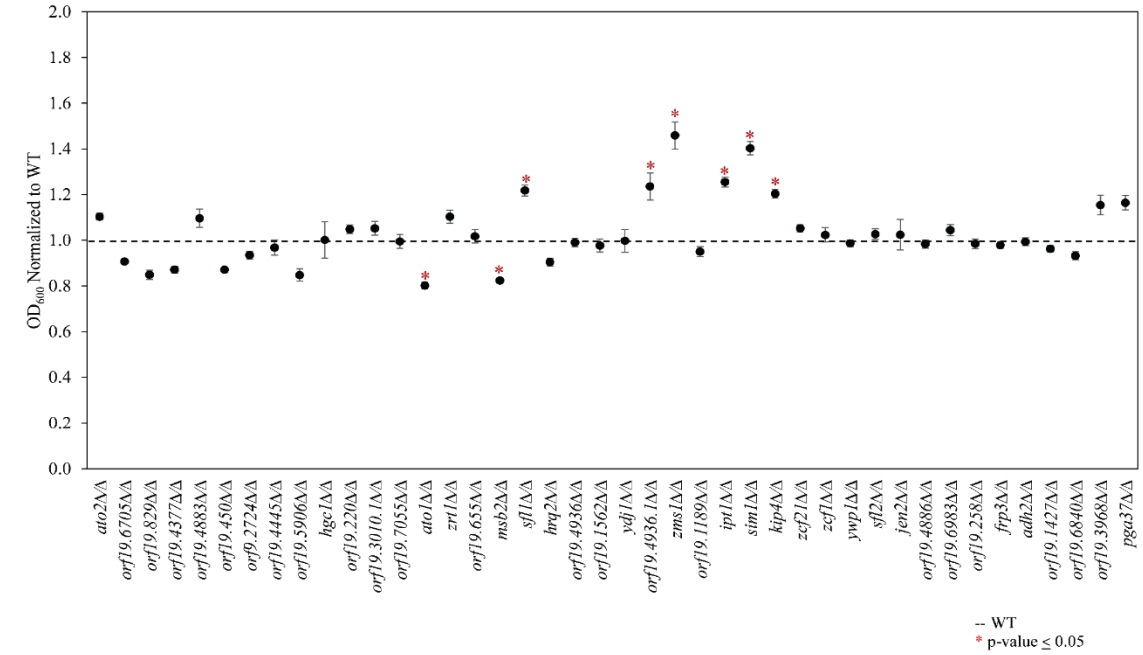
Clarissa J. Nobile is a cofounder of BioSynesis, Inc., a company developing inhibitors and diagnostics of biofilms.

3.10 Acknowledgements

We thank Aaron Hernday, Morgan Quail, and Namkha Nguyen for advice on strain construction. We are grateful to Anand Subramaniam, Joseph Pazzi, Alexander Li, and Vaishnavi Girish for technical assistance with the confocal microscope. We also thank all members of the Nobile and Hernday labs for insightful discussions on the topic of this manuscript.

3.11 Supplemental Figures

A



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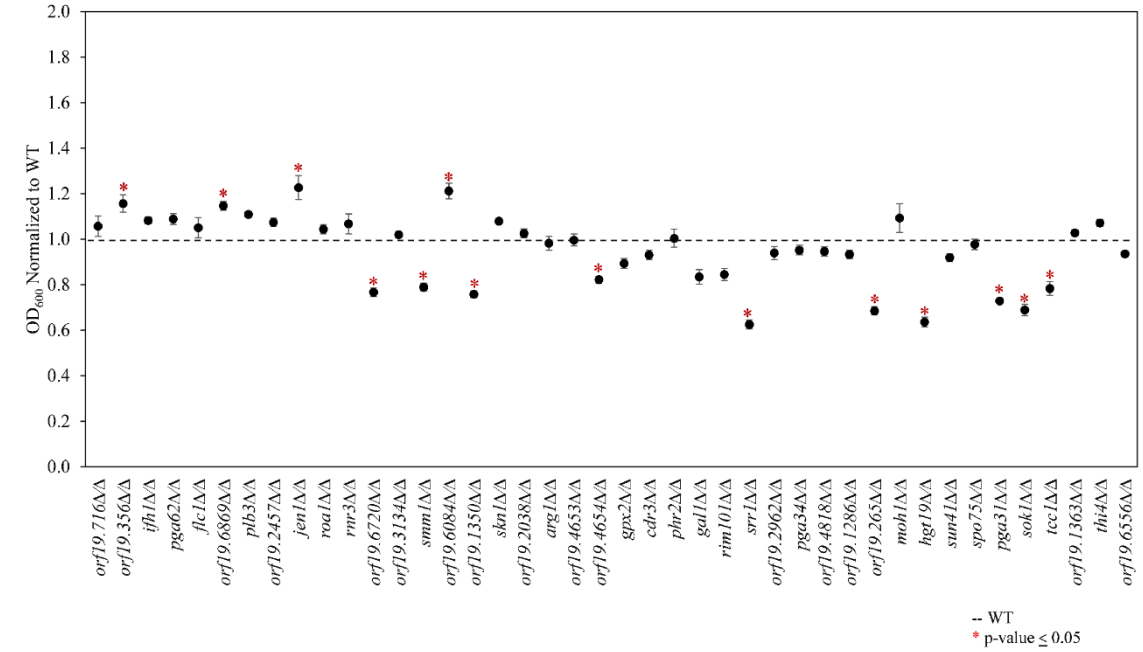
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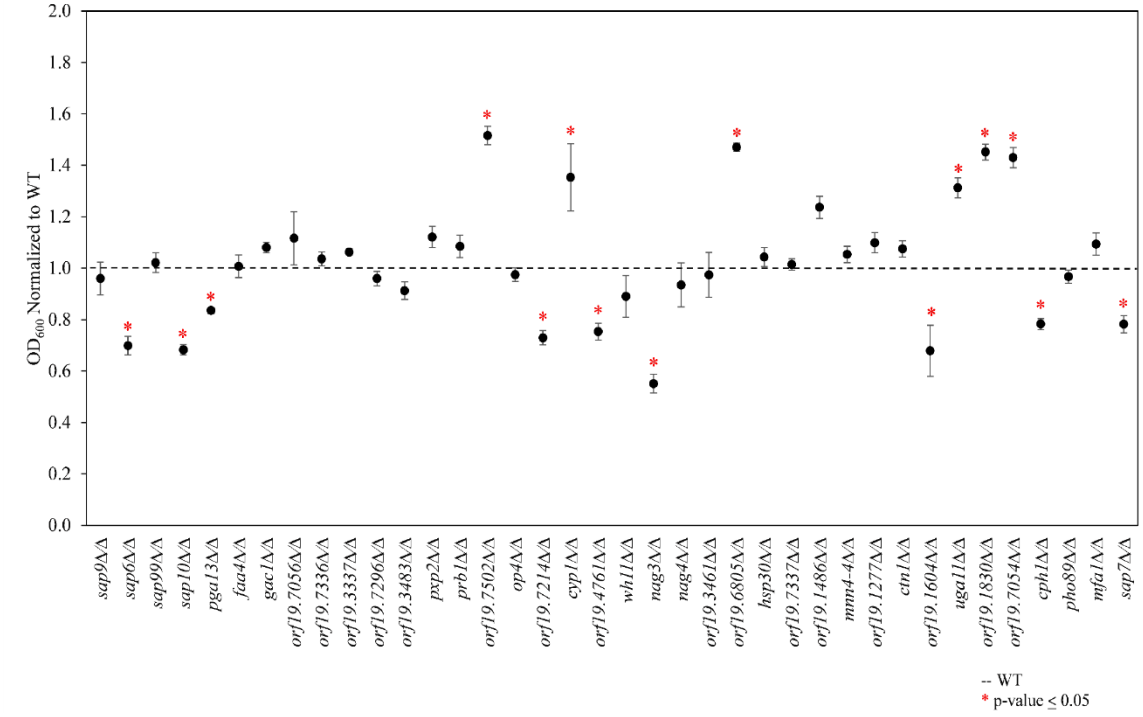
C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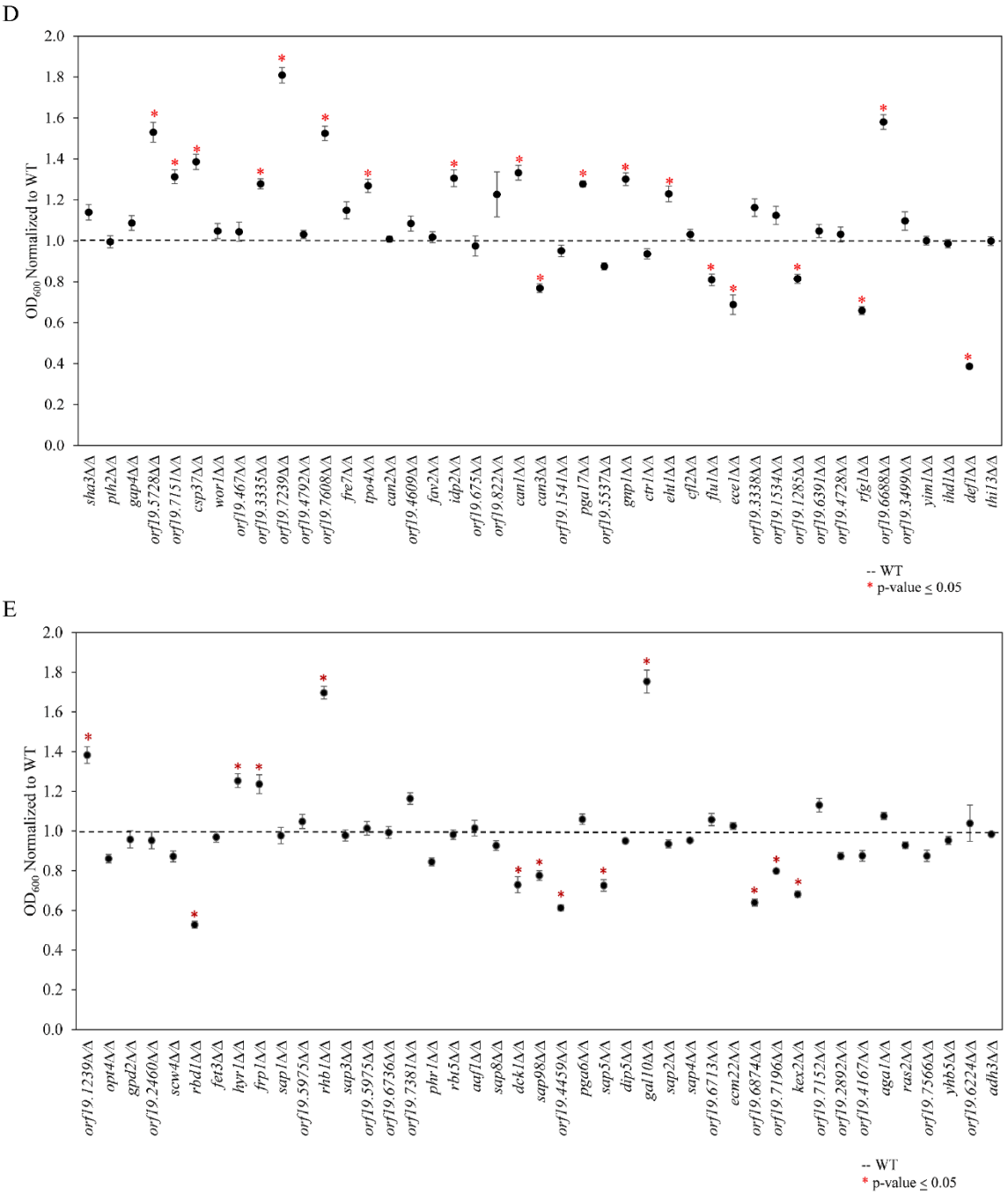

Figure S3.1. Optical density screen of *C. albicans* homozygous gene deletion strains (A-E), Related to Table 3.1. Optical density (OD_{600}) was determined for a set of 209 homozygous gene deletion strains. The average OD and standard deviation of each strain was calculated from four experimental replicates. A two-tailed Student's t-test was used to calculate statistical significance (p-value) and is represented by red asterisks for strains with OD values significantly (*p-value* ≤ 0.05) different from the wildtype reference strain (SN250). Error bars represent the standard deviation.

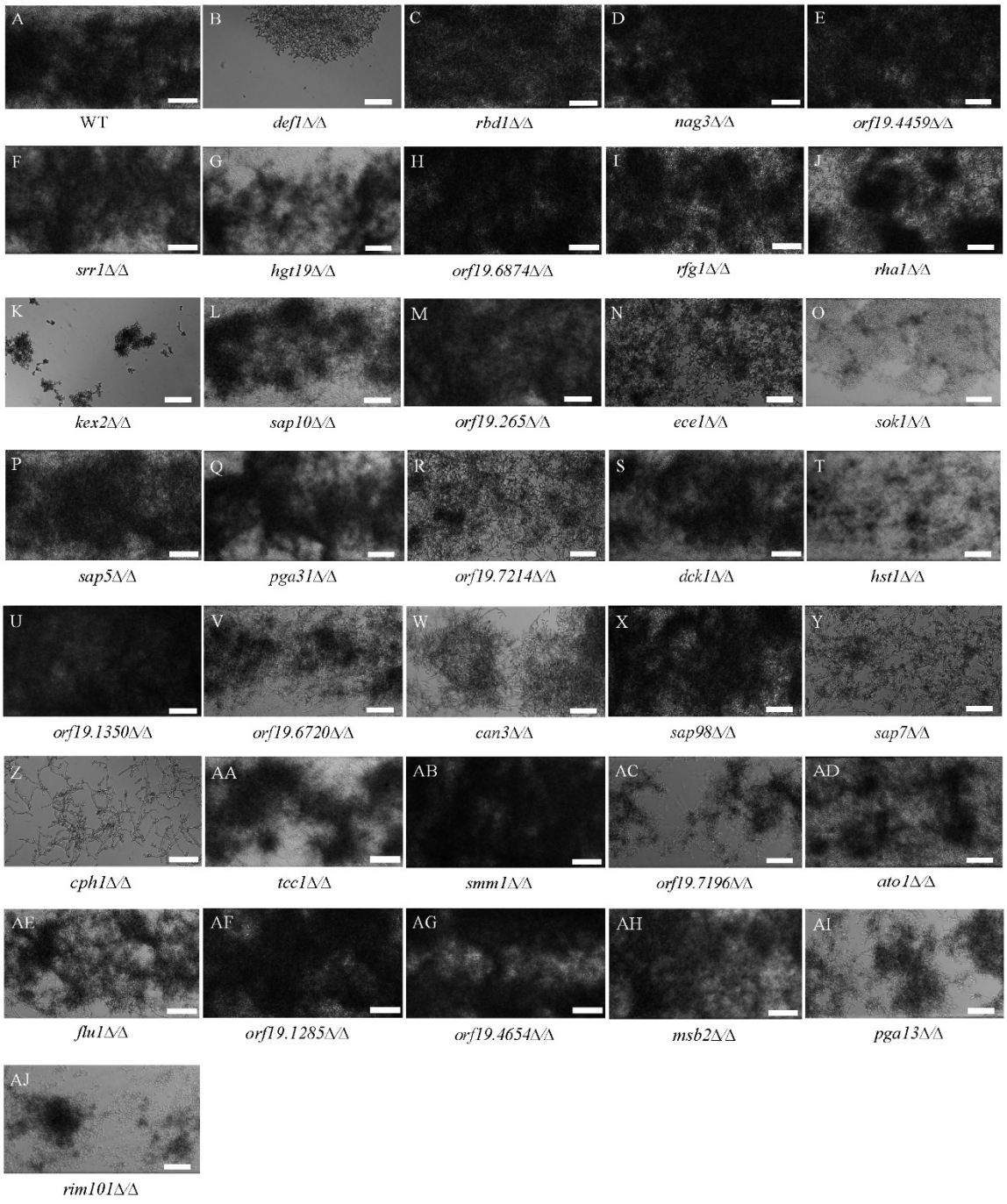

Figure S3.2. BioFlux microfluidics biofilm assay still images. Representative images of biofilms formed in Spider medium by the wildtype reference strain (A) and 35 mutant strains (B-AK) taken at 12 h post adherence under constant flow (0.5 dyne/cm^2). Scale bars represent 100μm in each panel. See Supplemental Videos S1-S37 for corresponding videos.

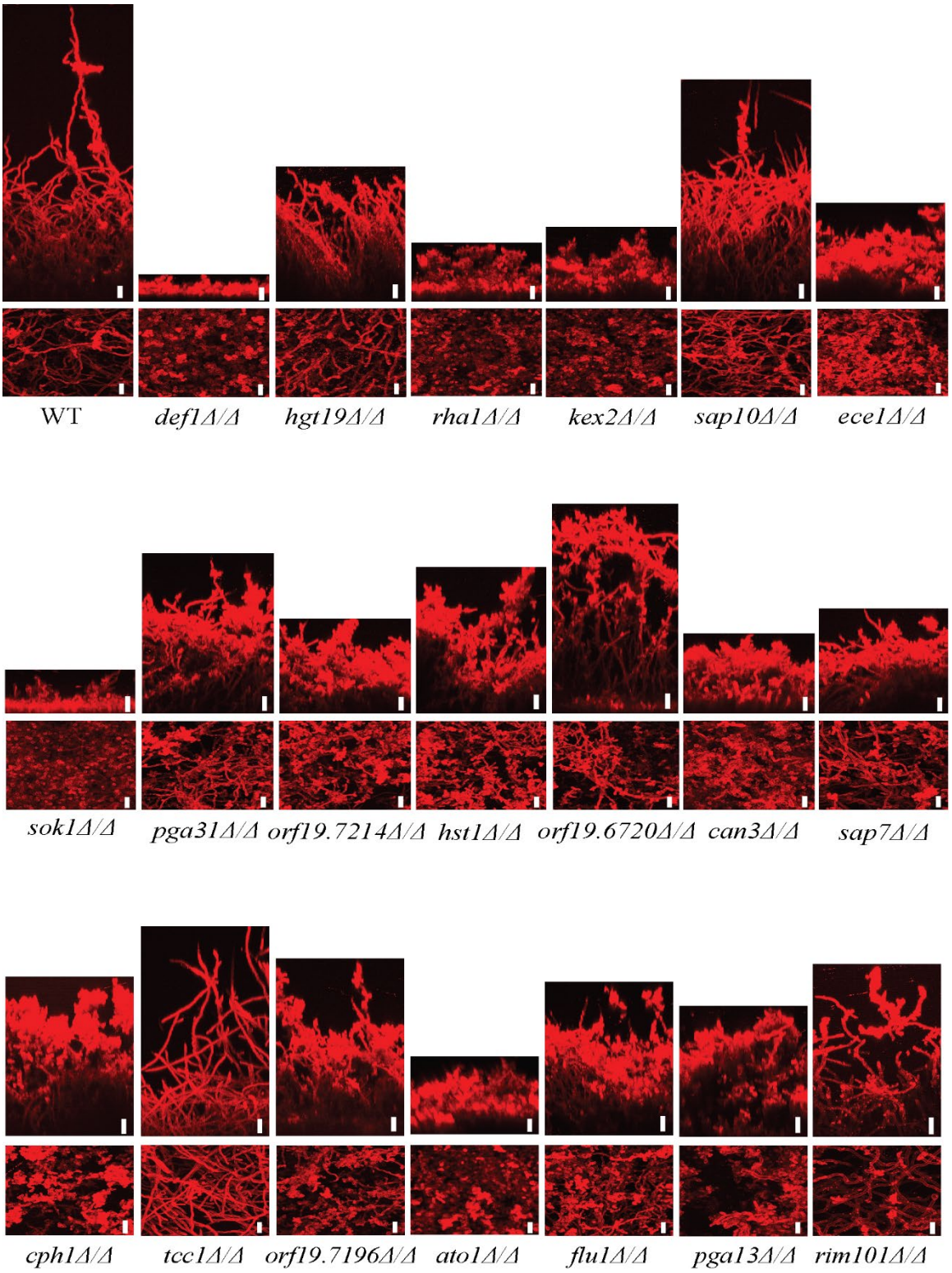

Figure S3.3. Confocal microscopy screen of *C. albicans* homozygous gene deletion strains. The wildtype reference strain (SN250) and 20 mutant strains were grown in Spider medium for 24 h on silicone squares pretreated overnight with bovine serum.

Biofilms were stained for one hour with concanavalin A-Alexa 594 dye. Images were obtained using Confocal Scanning Laser Microscopy (CSLM) and assembling side and top view images of the reference strain and the homozygous gene deletion strains. Scale bars represent 20μm.

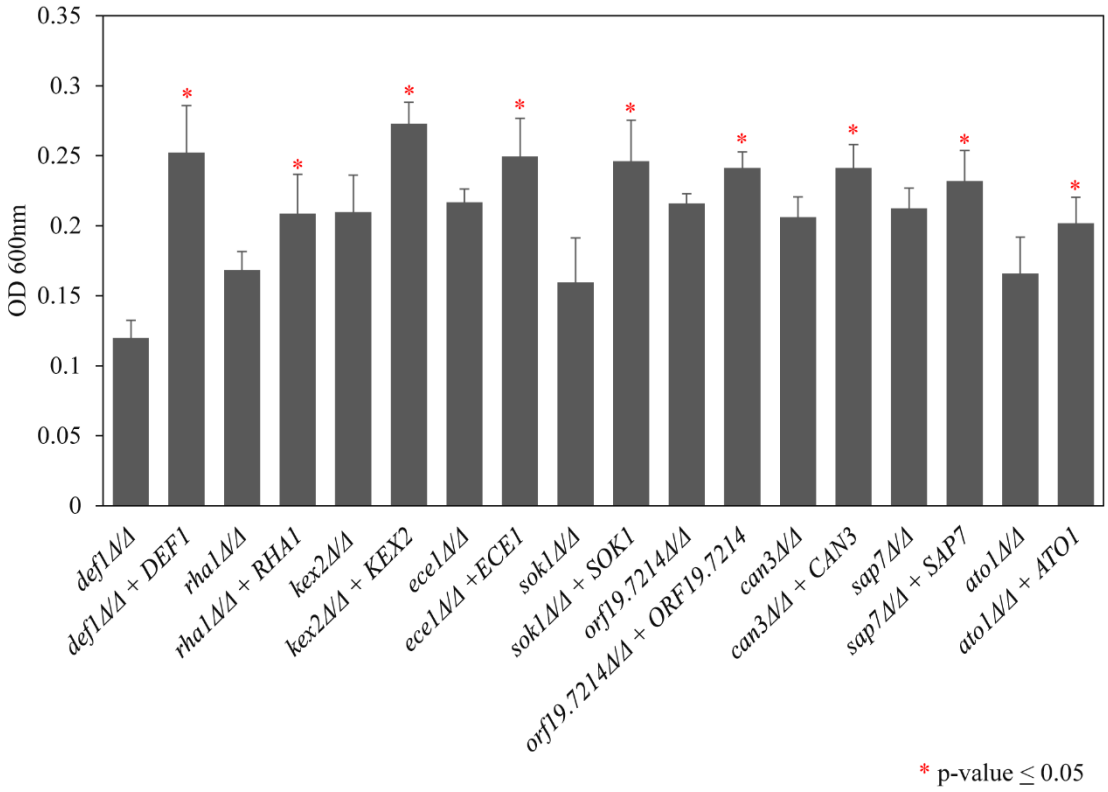

Figure S3.4. Biofilm formation by homozygous gene deletion and complemented strains. Homozygous gene deletion strains and their complemented strains were grown for 24 h in spider medium at 37°C and 250 rpms. The average optical density (OD_{600}) and standard deviation of each strain was calculated from four technical replicates and three independent experiments. A two-tailed Student's t-test was used to calculate statistical significance (p-value) and is represented by red asterisks for strains with OD values significantly (*p-value* ≤ 0.05) different from the wildtype reference strain (SN250). Error bars represent the standard deviation.

3.12 Supplemental Information

Videos (S3.1-S3.36). BioFlux microfluidics biofilm assay videos. Time lapse videos of biofilm formation in Spider medium grown for 12 h post adhesion under constant flow (0.5 dyne/cm^2) in a BioFlux 1000Z microfluidics device. Videos were assembled for all 36 mutant strains that showed significantly reduced optical density (OD) values relative to the wildtype strain (see **Data Set S3.2.**). **Videos S3.1-S3.36** can be found at: https://github.com/anaid16/Biofilm-Formation-Manuscript.git

Table S3.1. Strains Used in This Study. Complete list of *C. albicans* strains used in this study. Table S3.1. can be found at: https://github.com/anaid16/Biofilm-Formation-Manuscript.git

Table S3.2. Primers Used in This Study. Sequences of oligonucleotides used in this study. Table S3.2. can be found at: https://github.com/anaid16/Biofilm-Formation-Manuscript.git

Data Set S3.1. Priority List of Target Genes. Complete list of all downstream target genes of the biofilm regulatory network selected for this study. Data Set S3.1. can be found at: https://github.com/anaid16/Biofilm-Formation-Manuscript.git

Data Set S3.2. Optical Density Screen. Tab1. Complete list of all downstream target genes of the biofilm regulatory network selected for this study. **Tab2**. Genes with known effect in biofilm formation. **Tab3**. Putatively essential genes. Data Set S3.2. can be found at: https://github.com/anaid16/Biofilm-Formation-Manuscript.git

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Chapter 4. Conclusions and future directions

4.1 Conclusions

The opportunistic fungal pathogen, *C. albicans*, is highly relevant for human health, and determining the molecular mechanisms by which it forms and maintains its biofilms will be very valuable for improving the treatment of *Candida* infections in the clinic. This work will provide a framework for the development of targeted antifungal therapeutics against *C. albicans* biofilm associated infections. It will also advance our understanding on the behavior of fungal cells in a community. Treatment of severe device-associated infections currently involves the use of antifungal drugs with high toxicity to humans, or the complete removal of infected medical devices. Current antifungal drugs are highly inefficient against biofilms, and in many cases, they are completely ineffective. Thus, much work is ahead of us in the prevention and control of biofilm-based infections. This dissertation sheds new light on the genes that govern *C. albicans* biofilm formation that would be future drug targets.

In chapter one, we reviewed the properties of *C. albicans* as an opportunistic fungal pathogen, the unique characteristics of its biofilms, and relevant statistics on the medical implications of *Candida* infections. We discussed the roles that different cell morphologies play in biofilm structure and pathogenicity, particularly the switch from the yeast to hyphal form, and outlined the current knowledge on each of the stages of biofilm formation and their key regulators. We also discussed some of the main mechanisms of antifungal resistance in biofilms, which are absent or diminished in planktonic cells. We explained some of the current therapeutic options to treat biofilm-associated infections and other interventions to clear biofilm infections.

Chapter two is a compilation of our current knowledge on the regulatory circuits that control biofilm formation, the switch between white to opaque cell states, and the transition from a commensal to pathogenic state in *C. albicans*. As discussed, the transcriptional regulatory circuit of biofilm formation is composed of nine core regulators (Bcr1, Tec1, Efg1, Ndt80, Rob1, Brg1, Gal4, Rfx2, and Flo8) and 50 auxiliary transcriptional factors, each with distinct roles in biofilm development. Some transcription factors play roles in a single step of biofilm development, while others play multiple roles. In the case of the white-opaque switch, this epigenetic switch is controlled by the core regulatory factors Wor1, Wor2, Wor3, Wor4, Czf1, Efg1, Ahr1, and Ssn6, and 105 auxiliary switch regulating proteins. Among the core TFs, Wor1 is recognized as the master regulator as it is essential in the transition of the white to opaque state and maintenance of the opaque cell state. The third cell process is the transition from commensal to pathogen states, governed by two transcriptional regulatory circuits: iron homeostasis and host proliferation. Hms1, Zcf21, Tye7, Rtg1, and Rtg3 are important regulators of pathogenic and commensal growth, and Sef1, Sfu1, and Hap43 control iron homeostasis. Together these circuits regulate the ability of *C. albicans* to grow as a commensal and as a pathogen in the context of the human host.

In chapter three, we presented the results from a systematic analysis of 245 downstream target genes that are part of the *C. albicans* biofilm regulatory network and utilized different biofilm developmental assays *in vitro* and *in vivo*. The results indicate that Rha1, Ece1, Sok1, Orf19.7214, Can3, and Sap7 are important for biofilm formation *in vitro* and Ato1, Kex2, and Def1 are required for normal biofilm formation *in vitro* and *in vivo.* Overall, the findings from this study suggest that adhesion and filamentation are critical

for initiation and maintenance of biofilm formation and suggest that Def1, Kex2, Orf19.7214, and Can3 could be potential targets for novel antifungal drugs against *C. albicans* biofilms.

Together, the current knowledge on the molecular and cellular plasticity of *C. albicans*, its ability to form biofilms, and the close interactions between multiple cell processes, in combination with the results from *in vitro* and *in vivo* experiments we present here, reflect the complexity of *C. albicans* as a microorganism and highlights the need to incorporate multiple approaches in the future to mitigate the clinical impact of *C. albicans* biofilms. Such approaches should include forward and reverse genetic screens, large scale screens of small molecules to find novel antifungal drugs, and the implementation of strategies to prevent the colonization of embedded medical devices, such as antimicrobial coatings.

4.2 Future directions

In this dissertation, I analyzed known *C. albicans* transcriptional networks for biofilm formation, the white-opaque switch, and the commensal to pathogen transition (Chapter 2); and identified nine downstream target genes from the *C. albicans* biofilm network whose protein products have important roles in biofilm formation (Chapter 3). As additional transcriptional networks are discovered, future studies will uncover new relationships between the transcriptional networks that we discussed here and other newly discovered networks.

Based on the overall findings presented in Chapter 3, future studies will uncover the roles of Def1, Kex2, Ece1, Sap7, Orf19.7214, Can3, Rha1, Sok1 and Ato1 in other biofilm forming *Candida* species, such as *Candida dubliniensis* and *Candida parapsilosis* (Silva et al., 2011; Henriques et al., 2016). These proteins, with the exception of Ece1, have homologs in *C. dubliniensis* and *C. parapsilosis* (Skrzypek et al., 2017), but they have not been characterized in other *Candida* species in the context of biofilm formation. We hypothesize that some of these proteins will have conserved functionality within the *Candida* clade species. To determine their roles in biofilm formation, *in vitro* and *in vivo* functional characterization of these proteins in biofilm formation in other *Candida* species will be performed.

Currently, we do not yet know all of the signaling cascades that trigger biofilm formation in *C. albicans*. I hypothesize that the signaling pathways regulating filamentation, quorum sensing, and biofilm formation are interconnected. Consistent with this idea, my results showed that several gene deletion strains that were impaired in filamentation were also defective in biofilm formation, linking these two signaling pathways. Additionally, a known *C. albicans* quorum sensing molecule, farnesol, inhibits both filamentation and biofilm formation (Ramage et al., 2002). It seems likely that quorum sensing pathways (which were not assessed in my studies) will also be linked with filamentation and biofilm formation. In fact, a recent study found that *C. albicans* Def1 plays major roles in both filamentation and quorum sensing (Polke et al., 2017). Thus, future studies are needed to explore the transcriptional relationships between filamentation, quorum sensing, and biofilm formation.

Finally, some of the genes identified in Chapter 3 (e.g., *KEX2* and *SAP7*) encode for enzymes, including proteases, kinases, and glucosidases. Based on these findings, the inhibition of biofilm formation through enzyme inhibition could be a promising avenue

for discovering novel biofilm specific drug targets. Thus, important next steps are to determine the relevance of enzyme activity in biofilm formation and to explore enzyme inhibitors as novel drug targets. The findings from these experiments could be valuable in the future development of new antifungal drugs.

4.3 References

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