UNIVERSITY OF CALIFORNIA, MERCED

Identifying the regulatory network controlling *Candida albicans* interactions with host mucins

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Quantitative and Systems Biology

by

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2021

The dissertation of Ashley Valle Arevalo, titled, “Identifying the regulatory network controlling *Candida albicans* interactions with host mucins”, is approved, and is acceptable in quality and form for publication on microfilm and electronically:

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2021

Dedications

This work is dedicated to my family. I am certain this journey would have been 10x harder without the support system that has been with me since the first day I started this program. I thank my beautiful mother, Ada, for always providing guidance and support, for being the best mother in the world. Without her, I know I would not be earning my doctorate degree. I thank my caring sister, Alexis, for being the one person who understands me better than anyone else in this world and for everything I continue to learn from her. You have always been a role model to me. I thank my brother-in-law, Daniel, for being a one and only B.I.L. and the love you've brought to our family. It's always a great time spent with you. I thank my grandma, Mamá Lidia, for always being my number one fan and being so proud of your "doctora científica". Although it hurts you are no longer here, I know you are watching over me and will always cherish the times spent with you.

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Table of Contents

I. List of Abbreviations vi
II. List of Figures vii
III. Acknowledgments iviii
IV. Vita for Ashley Valle Arevalo ix
V. Dissertation Abstract xiv
Chapter 1: Interactions of microorganisms with host mucins: a focus on *Candida albicans* 1
Chapter 2: Identifying the regulatory network controlling *C. albicans* interactions with host mucins 22
Chapter 3: Epithelial infection with *Candida albicans* elicits a multi-system response in planarians 43
Chapter 4: Conclusions and future directions 76

I. List of Abbreviations

TF	Transcription factor
TFKO	Transcription factor knockout
MUC	Mucin
WT	Wildtype
hpi	Hours post infection

II. List of Figures

Figure 1.1 Mucosal membranes covering epithelial cell layers of the human body.

Figure 1.2 Schematic drawing of a mucin monomer.

Figure 1.3 Summary of known and hypothesized interactions between *C. albicans* and mucins.

Figure 2.1 Transcription factor deletion mutant library screen.

Figure 2.2 Genes involved in adherence and filamentation are differentially regulated in the presence of mucin.

Figure 2.3 Mucin differentially regulates target genes of the classical biofilm network.

Figure 2.4 The tec1Δ/Δ strain in the presence of mucins has reversed expression of many target genes of the tec1Δ/Δ strain in the classical biofilm network.

Figure 2.5 Functional enrichment reveals important enriched gene sets.

Figure 2.6 The complete transcriptional network controlling *C. albicans* interactions with mucin.

Figure 2.7 Core circuitry of transcriptional interactions among biofilm regulators.

Figure 2.8 Newly identified *C. albicans* motifs.

Figure 3.1 *Candida albicans* infection leads to an early wound and mitotic response in planarians.

Figure 3.2 *Candida albicans* Als3 and Bcr1 are important for virulence in planarians.

Figure 3.3 The planarian mitotic response decreases in infections with less virulent *C. albicans als3* and *bcr1* mutant strains.

Figure 3.4 The Dectin signaling pathway plays a role in the host response to and clearance of *C. albicans* infection.

Figure 3.5 Expression of planarian neoblast subclasses throughout infection with *C. albicans.*

Figure 3.6 Neurons, protonephridia, and specific mesenchymal neoblast markers are upregulated in planarians during infection with *C. albicans*.

Figure 3.7 Model of the cascade of host–pathogen interactions that occur following an epithelial infection of planarians with *C. albicans*.

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Thank you all for the impact you have made in my life.

V. Vita for Ashley Valle Arevalo

Education

Doctoral Candidate, Quantitative and Systems Biology 2015 – 2021
University of California, Merced, CA

Master of Science, Quantitative and Systems Biology 2015 – 2018
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Research Experience

Graduate Student Researcher*, University of California, Merced.* 2015 – 2021
PI: Professor Clarissa Nobile
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Collaborative planaria project: Investigating host-microbe interactions between Schmidtea mediterranea and pathogenic Candida species
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International Research Assistant, *Universidad de El Salvador* June – Aug. 2014
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Project: Travelled to El Salvador to teach Dr. Joyce's co-advised graduate student at UES how to perform DNA extractions and AFLPs.
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Teaching Experience

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Valle Arevalo, A., Qasim, M.N., Gunasekaran, D., Paropkari, A.D., Hughes, S., Cooper, K., Hernday, A.D., Nobile, C.J., (2021). Identifying the regulatory network controlling *C. albicans* interactions with host mucins: In prep.

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Takagi, J., Aoki, K., Turner, B.S., Lamont, S., Lehoux, S., Kavanaugh, N., Gulati, M., **Valle Arevalo, A.**, Lawrence, T.J., Kim, C.Y., Bakshi, B., Nobile, C.J., Cummings, R.D., Wozniak, D., Tiemeyer, M., Hevey, R., and Ribbeck, K. (2021). Mucin O-glycans are

natural inhibitors of *C. albicans* filamentation, biofilm formation, and dual-species competition. *Nature Chemical Biology*: In revisions.

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Qasim, M.N., **Valle Arevalo, A.**, Hernday, A.D., Nobile, C.J., (2021). The roles of chromatin accessibility in regulating the *Candida albicans* white-opaque phenotypic switch. *Journal of Fungi.*

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Valle Arevalo, A., Nobile, C. J. (2020). Interactions of microorganisms with host mucins: a focus on *Candida albicans*. *FEMS Microbiology Reviews.*

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Lohse, M. B., Gulati, M., **Valle Arevalo, A**., Fishburn, A., Johnson, A. D., & Nobile, C. J. (2017). Assessment and optimizations of *Candida albicans in vitro* biofilm assays. *Antimicrobial Agents and Chemotherapy*.

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Valle Arevalo, A., Nobile, C.J. (2019, March). Identifying the regulatory network controlling *Candida albicans* interactions with mucin. Poster presented at the *30th Fungal Genetics*, Pacific Grove, CA.

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Committee on Academic Planning and Resource Allocation
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VI. Dissertation Abstract

Identifying the regulatory network controlling *Candida albicans* interactions with host mucins

Ashley Valle Arevalo

Doctor of Philosophy

University of California, Merced

2021

Supervisor: Professor Clarissa J. Nobile

Candida albicans is a commensal member of the human microbiota that is also capable of causing superficial and disseminated infections and is known to be the predominant fungal pathogen of humans in clinical settings. Mucosal linings are critical for protecting the host against infections, and it is the biological and viscoelastic properties of mucus, predominantly attributed to glycoproteins called mucins, that provide this protection. Chapter 1 reviews the interactions by which mucins are involved in maintaining a healthy microbial balance on mucosal linings when directly interacting with microbes. Oftentimes invading microbes colonize mucosal surfaces by forming microbial communities called biofilms. Biofilms can have detrimental impacts on the host because, once formed, they are highly resistant to chemical and mechanical perturbations. Chapter 2 aims to determine the regulators that mediate the ability of *C. albicans* to form biofilms on mucin-coated surfaces. We screened a library of 211 homozygous transcription factor deletion mutants using *in vitro* biofilm assays in the presence of mucin to identify the regulators involved in controlling the interactions with mucin. A total of 12 “master” regulators were found to be defective or enhanced in their biofilm formation abilities in the presence of mucins. Using a combination of genetic and genome-wide approaches, we mapped the regulatory connections of each transcription factor to one another and to downstream target genes to determine the regulatory network controlling the ability of *C. albicans* to interact with mucin. Understanding how *C. albicans* modulates its interactions with mucin will shed new light on the regulatory control that evolved to mediate this important interaction with the host. In Chapter 3, we explore host-pathogen interactions between *C. albicans* and the planarian *Schmidtea mediterranea.* We studied early infection with *C. albicans* and identified that there are key players that mediate epithelial infection of planaria and elicit an immune response of the planarian host to effectively clear the infection. Chapter 4 summarizes the work from the first three chapters and explores potential future directions.

Chapter 1

Interactions of microorganisms with host mucins: a focus on *Candida albicans*

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Keywords: mucus, mucins, mucin monomer, *Candida albicans*, innate immunity, biofilms, host-pathogen interactions, mucosal surface, epithelial cell layer, viscoelasticity

Summary

This review describes the current knowledge on the interactions between microorganisms and host mucins, with a focus on the opportunistic human fungal pathogen and commensal *Candida albicans*.

Abstract

Mucus is an important host innate defense factor that lines most epithelial cell layers of the body and provides crucial physical and biological protection against pathogenic microorganisms. Mucins are the main glycoproteins of mucus that are responsible for interacting with microorganisms and are critical for the antimicrobial properties of mucus. The mechanisms by which microorganisms interact with mucins are poorly understood, especially in terms of fungi, and these interactions are continually evolving. Work in bacterial pathogens has shown that mucins inhibit bacterial virulence traits, including quorum sensing, toxin secretion, and biofilm formation. Among the fungal clade, the common opportunistic human fungal pathogen and commensal *Candida albicans* engages in constant battle with the host innate immune system. This battle creates strong selective pressures for *C. albicans* to evolve in response to the host. Recent work in *C. albicans* found that mucins inhibit specific virulence traits, such as surface adherence, filamentation, biofilm formation, and the production of secreted proteases. Here we review the current knowledge of microbial interactions with mucins, with a special emphasis on the interactions between *C. albicans* and mucins.

Introduction

The healthy human microbiota is composed of hundreds of trillions of diverse microorganisms, including bacteria, fungi, and archaea, that share and compete for nutrients and environmental niches in the body (Ursell *et al.* 2013; Wang *et al.* 2017). Over the course of millions of years of evolution, these microorganisms have coevolved

in a mutually symbiotic relationship with the host, where they contribute to host physiological processes; the host, in turn, provides a hospitable environment for these microorganisms to reside (Relman 2008; Chow *et al.* 2010; Pickard *et al.* 2017). In addition to providing physiological benefits to the host (e.g. through nutrient acquisition and synthesis), these microorganisms also protect the host from invading pathogens that may enter and colonize the host from the outside environment (Chow *et al.* 2010; Buffie and Pamer 2013; Sassone-Corsi and Raffatellu 2015; Chiu *et al.* 2017). Although the majority of members of the microbiota typically behave as mutualists or commensals, some of these microorganisms can have pathogenic potential under certain circumstances (Casadevall and Pirofski 2001; Casadevall 2017; Libertucci and Young 2019). Most research to date has focused on studying bacterial members of the microbiota, but fungal members have been found to play increasingly important roles in interacting with the host and in shaping the functions of the microbiota (Underhill and Iliev 2014; Lukeš *et al.* 2015; Kumamoto 2016). Both members of the microbiota and invading pathogens from the environment typically make contact with and often reside on mucosal membranes covering epithelial cell layers found in the body, such as the oral cavity, eyes, nose, ears, respiratory, gastrointestinal and reproductive tracts (**Figure 1**) (Linden *et al.* 2008; Hansson 2012; Frenkel and Ribbeck 2015). Regardless of where they originated from (the microbiota or the outside environment), those microorganisms that can breach the mucosal barrier have the potential to cause systemic infections in the host (Linden *et al.* 2008; Belkaid and Hand 2014). Consistently, defects in mucus production, mucus physiochemical properties, or in the expression of mucins can lead to several disease states in humans, such as cystic fibrosis, inflammatory bowel disease, ulcerative colitis, Sjogren's syndrome, cancer, and preterm birth, which are all associated with microbial dysbiosis and often the presence of an infection (Henke *et al.* 2004, 2007; Kim and Ho 2010; Williams, Ranjendran and Ramage 2016; Wagner, Wheeler and Ribbeck 2018; Schroeder 2019). In this review, we focus on the interactions between mucins, the major components of mucus responsible for mediating microbial interactions with the host, and microorganisms with pathogenic potential, with an emphasis on *Candida albicans*, a common opportunistic fungal pathogen and commensal of humans.

It is estimated that fungal diseases cost the United States approximately $7.2 billion annually, where *Candida* infections account for ~20% of these costs (Benedict *et al.* 2019). *C. albicans*, the most commonly isolated fungal pathogen from clinical settings, typically resides as a commensal fungus in the microbiota of the skin, vagina, gastrointestinal and urogenital tracts of humans (Kennedy and Volz 1985; Kumamoto 2002, 2011; Achkar and Fries 2010; Ganguly and Mitchell 2011). When alterations to the host microbiota occur, such as by changes in pH or residing microorganisms, *C. albicans* can overgrow, become invasive, and cause a wide range of infections (Odds 1987; Kim and Sudbery 2011; Nobile and Johnson 2015). These infections, collectively referred to as candidiasis, can range from superficial skin infections to severe bloodstream infections, the latter of which typically occur in immunocompromised individuals and can be life-threating (Haynes 2001; Kullberg and Oude 2002; Kim and Sudbery 2011). *C. albicans* possesses numerous virulence traits that contribute to its pathogenicity, such as the production of host recognition molecules, the ability to undergo morphological transitions, and the release of secreted aspartyl proteases and phospholipases that can damage host cells (Calderone and Fonzi 2001). In addition, the ability to form biofilms, recalcitrant communities of cells encased in extracellular

matrices, is another important virulence trait of *C. albicans* that enhances its survival in the host (Kumamoto 2002; Douglas 2003; Ganguly and Mitchell 2011; Nobile and Johnson 2015; Gulati and Nobile 2016; Lohse *et al.* 2018).

C. albicans biofilm formation in a host setting begins when *C. albicans* cells colonize a mucosal surface covering a layer of epithelial cells or an implanted medical device. The *C. albicans* biofilm life cycle consists of four basic stages (Chandra *et al.* 2001; Douglas 2003; Gulati and Nobile 2016). In the first stage, round yeast form cells adhere to a solid surface (e.g. the intestinal mucosa or an implanted central venous catheter) (Kennedy *et al.* 1987; Hawser and Douglas 1994; Baillie and Douglas 1999; Nobile and Johnson 2015). This is followed by proliferation of the adhered cells and early stage filamentation (Baillie and Douglas 1999; Nobile and Johnson 2015). As the biofilm matures, extensive filamentation takes place along with the production of the extracellular matrix, which is comprised of proteins, polysaccharides, nucleic acids and lipids (Baillie and Douglas 1999; Zarnowski *et al.* 2014; Pierce *et al.* 2017). As a result, the mature biofilm architecture is such that it provides structural protection to the cells within the biofilm from both chemical and mechanical insults (Mitchell, Zarnowski and Andes 2016). In addition to this structural protection, cells within the biofilm upregulate drug efflux pumps, further enhancing the resistance and tolerance of biofilms to inhibitory compounds. In the final stage of the *C. albicans* biofilm life cycle, round yeast form cells disperse from the biofilm to colonize new sites (Uppuluri *et al.* 2010; Nobile and Johnson 2015). Taken together, *C. albicans* biofilms can not only be highly resistant and tolerant to chemical and mechanical perturbations but can also act as reservoirs to seed new sites of infection. Interestingly, recent work in *C. albicans* found that specific fungal virulence traits, such as surface adherence, filamentation, and biofilm formation, are compromised in the presence of mucins (Kavanaugh *et al.* 2014).

In the following sections of this review, we begin by discussing the properties, functions, and structures of mucins. We then review the types of mucins present in the human body and examine their production and biosynthesis. Lastly, we review known interactions between microorganisms and mucins, with a focus on the interactions between *C. albicans* and mucins.

Mucus and mucins – an overview

Mucus is a viscoelastic hydrogel that is comprised of 95% water, 3% mucin glycoproteins, and 2% other small molecules, including immunoglobulin A (IgA), lipids and antimicrobial peptides (Celli *et al.* 2005). Mucus provides lubrication and hydration to epithelial linings and is a critical innate defense factor that protects the host against infection; this protection is largely attributed to large glycoproteins called mucins, which can be secreted or membrane-bound (Gendler 1995; Liévin-le Moal and Servin 2017; Petrou and Crouzier 2018). The unique physiochemical properties of mucus are such that mucus limits microbial penetration through the epithelial cell layer, while at the same time permits the passage of water and gases (Cone 2009; Bakshani *et al.* 2018). In addition to these properties, mucosal surfaces continuously regenerate, allowing for the efficient removal of contaminants, and preventing them from reaching the underlying epithelial cell layer (Cone 2009). Mucins within mucus are known to mediate physical interactions with microorganisms, serve as receptor binding sites for the adhesion of molecules, act as nutrient sources for microorganisms, serve as biochemical signals,

and support gaseous exchange and nutrient absorption between host cells (Wagner, Wheeler and Ribbeck 2018). Some of these roles depend on where the mucins are produced and localized in the body. For example, a major role of lung mucins is to support gaseous exchange between host cells, while for gut mucins, it is to support nutrient absorption (Corfield 2015). Lastly, it is known that mucins can inhibit virulence traits, such as biofilm formation, motility, and cellular morphology changes in opportunistic pathogens, thus maintaining them in a commensal state (Ogasawara *et al.* 2007; Celli *et al.* 2009; Caldara *et al.* 2012; Kavanaugh *et al.* 2014; Co *et al.* 2018).

Mucin monomer molecular weights range from 0.5-20 MDa (Bansil and Turner 2006; Balabushevich *et al.* 2018), where ~80% of the molecular weight of a mucin monomer comes from polysaccharides that are attached to the protein core, including N-acetylgalactosamine, N-acetylglucosamine, sialic acid, fucose, and galactose (Bansil and Turner 2006; Brockhauser, Schachter and Stanley 2009). The protein core consisting of proline, threonine, and serine (called the PTS domain) makes up the remaining 20% of the molecular weight of a mucin monomer, and is the main glycosylated region of the protein (**Figure 2**) (Bansil and Turner 2006; Brockhauser, Schachter and Stanley 2009).

Human mucin glycoproteins belong to the MUC protein family, which is currently known to consist of 21 known secreted and membrane-bound mucins (Corfield 2015, 2018). The five major secreted gel-forming mucins in the human body, which are important contributors to the viscoelasticity of mucus are MUC2, MUC5AC, MUC5B, MUC6, and MUC19 (Thornton, Rousseau and McGuckin 2008). MUC5AC and MUC5B are structurally similar proteins but are found in different niches of the host. For example, MUC5AC is found in mucus of the gastrointestinal and respiratory tracts, and MUC5B is found in salivary and cervical mucus. Currently, there are three known secreted but non-gel forming MUC proteins, MUC7, MUC8, and MUC9 (Corfield 2018). Unlike the gel-forming secreted mucins, MUC7 and MUC8 are not implicated in mediating viscoelasticity, however they have been shown to exhibit microbe-binding and anti-inflammatory properties (Xu *et al.* 2016; Cha and Song 2018). MUC7 specifically has been shown to possess fungicidal activity via a histatin-like domain found at its N-terminal region (Gururaja *et al.* 1999; Puri and Edgerton 2014). The remainder of the MUC protein family is made up of large membrane-bound mucins (also called tethered or cell surface-associated mucins) that form the glycocalyx mucus barrier and are known to mediate adherence to mucosal surfaces and to limit access of microorganisms to epithelial cell layers (Linden *et al.* 2008; Roy *et al.* 2014). These membrane-bound mucins include MUC1, MUC3A/B, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17, MUC20, MUC21, and MUC22 (Linden *et al.* 2008; Pelaseyed and Hansson 2020).

Production of mucus and biosynthesis of mucins

Mucus is produced by mucus cells found in the surface epithelium, such as in goblet epithelial cells, in mucus glands, and in mixed glands containing mucus and serous cells (Lillehoj, Kato and Wenju Lu 2013; Pelaseyed *et al.* 2014). From there, mucus is secreted onto the epithelial cell layer forming the mucosal surface (Linden *et al.* 2008; Pelaseyed *et al.* 2014; Corfield 2015).

Polymerization and secretion of gel-forming secreted mucins into the mucosa is critical for creating the viscoelastic properties of mucus, while localization of membrane-bound mucins to the cell membrane is critical for forming the glycocalyx mucus barrier. For secreted mucins, oligomerization occurs by rapid dimerization of mucin monomers in the endoplasmic reticulum (ER) (Linden *et al.* 2008; Corfield 2015). This is followed by O-glycosylation in the Golgi apparatus (Linden *et al.* 2008; Corfield 2015). For membrane-bound mucins, which are monomeric, proper synthesis in the ER is dependent on cleavage of an SEA domain into two subunits by autoproteolysis concurrent with N-glycosylation (Macao *et al.* 2006). For membrane-bound mucins, similar to secreted mucins, O-glycosylation also takes place in the Golgi apparatus, but for membrane-bound mucins, the newly synthesized mucin monomer is then tethered to the cell membrane (Linden *et al.* 2008; Corfield 2015).

Microbial interactions with mucins

Mucins in mucus are critical in the host's defense against invading microorganisms. Consistent with this concept, host genes encoding mucins have been found to be upregulated in the presence of invading microorganisms (Wagner, Wheeler and Ribbeck 2018). In one example, exposure of ear epithelial cells to the otitis media causing bacteria *Streptococcus pneumoniae*, *Haemophilus influenza*, and *Moraxella catarrhalis* led to an upregulation of host genes encoding MUC2, MUC5AC, and MUC5B (Kerschner *et al.* 2014; Wagner, Wheeler and Ribbeck 2018). In another example, exposure of lung epithelial cells to *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis,* and *Streptococcus pyogenes* led to an upregulation of host genes encoding MUC5AC and MUC2 (Dohrman *et al.* 1998; Wagner, Wheeler and Ribbeck 2018). From the pathogen perspective, in response to host mucins, invading microorganisms can defend themselves in a number of different ways. They can, for example, secrete hydrolytic enzymes, such as proteases and glycosidases that degrade mucins and promote microbial invasion into the underlying host epithelial cell layer (Derrien *et al.* 2010). For example, in response to mucins, *E. coli* secretes the metalloprotease SslE, *Vibrio cholerae* secretes the metalloprotease TagA, and *C. albicans* secretes the aspartyl protease Sap2, which can all degrade mucins, potentially allowing for microbial penetration of the mucosal barrier (Colina *et al.* 1996; Szabady *et al.* 2011; Luo *et al.* 2014; Valeri *et al.* 2015).

Interestingly, other than invading microorganisms, some symbiotic members of the microbiota can also degrade mucins and are important in maintaining healthy host metabolic processes (Derrien *et al.* 2010; Tailford *et al.* 2015). For example, *Akkermansia muciniphila*, a bacterial colonizer of the mucosal layer of the intestinal tract that can degrade mucins is depleted in individuals with metabolic disorders, such as in diabetic and obese individuals (Collado *et al.* 2007; Cani and de Vos 2017; Shin *et al.* 2019; Xu *et al.* 2020). *A. muciniphila* is known to degrade and utilize mucins as a nutrient source by producing sialidases, fucosidases, N-acetyl-β-glucosaminidases, and GlcNAc-sulfatases, which breakdown mucin monomers (Derrien *et al.* 2004; Ottman *et al.* 2016; Geerlings *et al.* 2018; Xu *et al.* 2020). Using mucins as a nutrient source is particularly useful in the colon, where carbon sources are extremely limited (Derrien *et al.* 2008). Interestingly, *A. muciniphila* colonizes the mucosal layer, but does not invade it, which is an intriguing distinction from most of the mucin degrading microorganisms

that have pathogenic potential. In the case of *A. muciniphila*, the host may support its colonization of the mucosal layer in exchange for the benefits it provides to the host in preventing metabolic disorders.

In addition to these mucin degrading enzymes, microorganisms can also directly bind to mucins, an interaction that is continuously evolving between the host and pathogen. Many commensal and pathogenic bacterial species, such as *Helicobacter pylori, Yersinia enterocolitica, P. aeruginosa,* and *E. coli*, to name a few, are known to physically bind to mucins (Sajjan and Forstner 1990; Wanke *et al.* 1990; Mantle and Husar 1994; Lindén *et al.* 2009; Dhar and McAuley 2019; Wheeler *et al.* 2019; Hoffman, Lalsiamthara and Aballay 2020). From the host perspective, microbial binding to mucins can be beneficial by allowing for the removal of pathogens through mucus flow and excretion, and even by "mucin shedding" (Linden *et al.* 2008; Van Putten and Strijbis 2017). In an example of the latter concept, the human opportunistic bacterial pathogen, *H. pylori*, which typically colonizes the digestive tract, has been found to bind via its BabA and SabA adhesins to Lewis^{b}, sialyl Lewis^{a}, and sialyl Lewis^{x} extracellular domain antigens of the carbohydrate portion of the membrane-bound mucin MUC1 (Lindén *et al.* 2009; Dhar and McAuley 2019). This binding event induces shedding of the microbe-bound MUC1 from the gastric epithelial cell layer, thereby preventing *H. pylori* from adhering to host epithelial cells (Lindén *et al.* 2009; Dhar and McAuley 2019). This is followed by excretion of the microbe bound MUC1 into the stomach, where it is digested by stomach acids. Mucin shedding can significantly limit disease progression by pathogens, which in the case of *H. pylori,* is the development of chronic peptic ulcers in the host.

In another example of microbe-mucin binding, the opportunistic bacterial pathogen, *P. aeruginosa,* commonly found in the respiratory tract of humans is known to physically bind via multiple strain dependent surface adhesins, including flagellins, to the sialyl Lewis^{x} extracellular domain antigens of the carbohydrate portion of airway mucins (Carnoy *et al.* 1994; Scharfman *et al.* 1999; Lillehoj, Kim and Kim 2002). From the host perspective, this binding interaction can lead to attenuation of *P. aeruginosa* virulence by inducing a downregulation of numerous bacterial virulence genes, including genes involved in quorum sensing (e.g. *lasR*), toxin secretion (e.g. *pcrV*), and siderophore biosynthesis (e.g. *pvdA*), as well as by inducing active biofilm dispersion (Caldara *et al.* 2012; Wheeler *et al.* 2019). From the pathogen perspective, on the other hand, *P. aeruginosa* can use mucin binding to its advantage to cause disease by enhancing its ability to adhere to and colonize the mucosal surface, and by aiding its penetration to the underlying epithelial cell layer (Derrien *et al.* 2010; Hoffman, Lalsiamthara and Aballay 2020). When this occurs, *P. aeruginosa* binds mucins primarily at the N-acetylgalactosamine and N-acetylglucosamine polysaccharide portions of mucin monomers, leading to their degradation, thus allowing *P. aeruginosa* to access the underlying epithelial cell layer (Hoffman, Lalsiamthara and Aballay 2020).

In terms of pathogenic fungi, there are many unanswered questions on how fungi interact with mucins, but the most mechanistic information is known for *C. albicans* (**Figure 3**). One study determined that the gel-forming mucins MUC5AC, MUC5B, and MUC2 prevent *C. albicans* from transitioning from the round yeast cell state to the elongated hyphal cell state that is critical for this fungus to invade the host epithelial cell layer and is an important structural feature of its biofilms (Kavanaugh *et al.* 2014; Basmaciyan *et al.* 2019). In addition, when hyphal *C. albicans* cells were exposed to

mucins under hyphal inducing conditions, newly budded cells from these hyphae were in the round yeast form rather than the elongated hyphal form. In contrast, in the absence of mucins, newly budded cells under these conditions were always in the hyphal form. In addition, methylcellulose, a viscosity control used in this study to mimic the viscosity of mucins did not affect filamentation, suggesting that the biological properties of mucins, rather than their physical properties, are important in their ability to suppress *C. albicans* filamentation. Taken together, these results indicate that mucins suppress the development of hyphae from yeast cells and also suppress the development of new hyphae from existing hyphal cells. Not surprisingly in this study, several *C. albicans* genes involved in filamentation were downregulated in the presence of mucins, including *EFG1,* which encodes a major transcriptional regulator of filamentation, as well as *ALS3, ECE1,* and *HWP1* (Kavanaugh *et al.* 2014). Interestingly, mucins appear to induce *C. albicans* cells to transition into a novel yeast morphology that phenotypically resembles the oval mating competent opaque cell type of this fungus, but that is functionally distinct since the morphology induced in the presence of mucins is not mating competent (Kavanaugh *et al.* 2014). This morphology also bears some resemblance to the *C. albicans* gastrointestinally induced transition (GUT) cell type identified as a commensal cell state that occurs when *C. albicans* cells are passaged through the gastrointestinal tract of a mouse (Pande, Chen and Noble 2013; Noble, Gianetti and Witchley 2017). It is possible that this novel mucin induced morphology may occur uniquely and specifically in response to mucins.

Another study found that *C. albicans* filamentation was inhibited by salivary mucins in a dose dependent manner (Ogasawara *et al.* 2007). In this study, *C. albicans* cells were grown for 24 hours under hyphal inducing conditions in the absence and presence of different concentrations of salivary mucins ranging from 125 – 1000 µg/mL (Ogasawara *et al.* 2007). In the absence of mucins, *C. albicans* cells formed long and numerous hyphae, while in the presence of mucins, there was a clear dose dependent reduction in hyphal formation as higher concentrations of mucins were used, and at 1000 µg/mL of mucins, no hyphae were observed whatsoever in the culture (Ogasawara *et al.* 2007). No differences in growth rates were observed in the absence versus the presence of mucins at concentrations up to 1000 µg/mL of mucins (Ogasawara *et al.* 2007). The expression of *RAS1*, which encodes the Ras1 GTPase that regulates the cAMP and MAP kinase pathways involved in the induction of hyphal formation, was also measured in this study (Feng *et al.* 1999; Leberer *et al.* 2001; Ogasawara *et al.* 2007). In the absence of mucins under hyphal inducing conditions, *RAS1* expression levels were increased throughout the course of the experiment, while in the presence of mucins under the same conditions, *RAS1* expression levels were significantly repressed (Ogasawara *et al.* 2007). This repression of *RAS1* also correlated with a decrease in the expression of *EFG1.* Taken together, these results indicate that salivary mucins suppress the development of hyphae from yeast cells.

Other than filamentation, it has been shown that mucins, specifically MUC5AC, inhibit adherence of *C. albicans* cells to abiotic (polystyrene) and biotic (human epithelial cell) surfaces (Kavanaugh *et al.* 2014). The adherence inhibitory effects of mucins on these surfaces was observable after 30 minutes and increased significantly over the course of a 1-hour adhesion assay. Interestingly, the methylcellulose viscosity control also inhibited surface adhesion, suggesting that the physical properties of mucins contribute to their anti-adherence properties. Consistent with the finding that mucins inhibit adherence, a number of *C. albicans* genes involved in adherence were

downregulated in the presence of mucins, such as the adhesins *ALS1* and *ALS3* (Kavanaugh *et al.* 2014). Therefore, by preventing *C. albicans* cells from adhering to surfaces, mucins impede the ability of *C. albicans* to achieve the first step necessary in the process of breaching the epithelial cell layer.

Since filamentation and adherence are important processes during *C. albicans* biofilm formation, it follows that biofilm formation would also be inhibited in the presence of mucins. Indeed, over the course of a 48-hour biofilm experiment in the presence of mucins, biofilm formation was severely constrained, where few hyphae were observed throughout the rudimentary (~60 µm thick) biofilm formed (Kavanaugh *et al.* 2014). This is in contrast to the robust (~500 µm thick) biofilm formed in the absence of mucins, containing long and extensive hyphae. The methylcellulose viscosity control in this experiment also inhibited biofilm formation, suggesting that the physical properties of mucins contribute to their antibiofilm properties. Consistent with the finding that mucins inhibit biofilm development, the genes *BCR1*, *EFG1*, and *NDT80*, encoding three of the six core *C. albicans* biofilm master regulators, were downregulated in the presence of mucins (Nobile *et al.* 2012; Kavanaugh *et al.* 2014).

Other than inhibiting filamentation, adherence, and biofilm formation in *C. albicans*, mucins also suppress the expression of *C. albicans* secreted aspartyl protease encoding genes, such as *SAP5* and *SAP2* (Kavanaugh *et al.* 2014). By suppressing the expression of these hydrolytic enzymes, which are known *C. albicans* virulence factors that are similar to those produced by bacterial pathogens, mucins protect themselves from degradation and limit the ability of *C. albicans* to colonize the mucosal surface and invade the underlying epithelial cell layer (Colina *et al.* 1996; Naglik, Challacombe and Hube 2003; Nikou *et al.* 2019).

Although the molecular mechanisms for *C. albicans* direct binding to mucins are unknown, the adhesin Als1 is known to bind via its N-terminal region to fucose-containing glycans (Donohue *et al.* 2011). Based on these findings and the fact that mucins are heavily comprised of fucose glycans, it seems feasible that mucins could directly bind to Als1 as well as to the structurally similar protein Als3. Additionally, the hyphal specific cell surface protein Hwp1 is another candidate for mucin binding that is already known to interact with the host. Hwp1, which has a domain that resembles mammalian transglutaminase substrates, can bind to and form stable bonds of attachment to mammalian transglutaminases on the surfaces of host buccal epithelial cells (Staab *et al.* 1999). Hwp1 is also known to have complementary surface adhesion functions with Als1 and Als3 (Nobile *et al.* 2008). Taken together, it is plausible that Hwp1 could also directly bind to mucins. From the pathogen perspective, binding of *C. albicans* surface adhesins to mucins could increase adherence to the mucosal surface, allowing for *C. albicans* to penetrate to the epithelial cell layer. From the host perspective, binding of *C. albicans* surface adhesins to mucins could allow for mucin shedding to occur, where the *C. albicans* cells bound to mucins could be excreted in mucus flow, thereby reducing the number of *C. albicans* cells available to invade the host epithelial cell layer.

Another study assessing the abilities of several *Candida* species to bind to small intestinal mucins observed a hierarchy of mucin binding capabilities that appears to correlate with the abilities of the different species to cause disease in mammals, suggesting that direct mucin binding is an important virulence factor in the *Candida* clade

(De Repentigny *et al.* 2000; Hirayama *et al.* 2020). The authors found that *C. albicans*, *Candida dubliniensis* and *Candida tropicalis* strongly adhered to mucins; *Candida parapsilosis* and *Candida lusitaniae* moderately adhered to mucins; and *Candida krusei* and *Candida glabrata* weakly adhered to mucins (De Repentigny *et al.* 2000). *S. cerevisiae*, which was used as a non-pathogenic outlier species, adhered to mucins the weakest relative to the *Candida* species tested. The binding of *Candida* species to mucins in this study appeared to be, in part, dependent on the *C. albicans* secreted aspartyl protease Sap2 (De Repentigny *et al.* 2000). The authors suggest that the C-terminal glycosylated region of small intestinal mucins may be involved in the direct binding of *Candida* adhesins to mucins, and that this region of the mucin monomer is a substrate specifically for Sap2 (De Repentigny *et al.* 2000). Taken together, although the mucosal surface acts as a barrier to *Candida* species from accessing the epithelial cell layer, mucins are likely substrates for several *Candida* secreted proteases that can degrade mucins, allowing *Candida* cells to access the underlying epithelial cell layer (De Repentigny *et al.* 2000).

Finally, it has been postulated that *C. albicans* cell type heterogeneity in the gastrointestinal tract, which is lined with mucus, can be modulated by the immune status of the host (Kumamoto and Pierce 2011; Pierce and Kumamoto 2012). This model predicts that *C. albicans* produces phenotypic variants with two distinct functions: one optimized for persistence as a commensal in the host and one optimized for pathogenic interactions with the host (Kumamoto and Pierce 2011; Koh 2013). When alterations in the host's immune status occur, the levels of these phenotypic variants are postulated to shift, changing the pathogenic potential of the population (Kumamoto and Pierce 2011). This model is supported by studies showing that variability in the levels of the transcriptional regulators Efg1 and Efh1 in mouse infection models can shift the *C. albicans* population between the commensal and pathogenic states (Pierce and Kumamoto 2012). Specifically, if a change in the host status selects for *C. albicans* cells with low Efg1 activity, then the *C. albicans* cell population shifts to become pathogenic, while if this change selects for cells with low Efh1 activity, then the population shifts to become commensal, and vice versa (White *et al.* 2007; Pierce and Kumamoto 2012). In terms of heterogeneity in cell morphology, another study showed that the *C. albicans* yeast form over other morphological forms is the commensal morphological form in the gastrointestinal tract in a monocolonized gnotobiotic mouse model (Böhm *et al.* 2017). This finding is logical given that the filamentous form is the morphological form that can breach the mucosal barrier (Basmaciyan *et al.* 2019). Interestingly, when the mice were treated with antibiotics, a morphologically heterogeneous population of cell types containing yeast and filamentous forms was formed, thereby increasing the pathogenic potential of the population (Böhm *et al.* 2017). This study also identified three transcriptional regulators, Zcf8, Zfu2 and Try4, that are required for maintaining this yeast form morphology and thus the commensal state of *C. albicans* in the mouse gastrointestinal tract (Böhm *et al.* 2017). Interestingly, these regulators also promote the adherence of *C. albicans* to mucin coated surfaces as well as to mucus producing intestinal epithelial cells (Böhm *et al.* 2017).

Much less is known about the interactions of the non-*Candida* fungal pathogens with mucins. *Aspergillus fumigatus*, an opportunistic human fungal pathogen that can colonize the respiratory tract and cause aspergillosis, is known to degrade mucins using hydrolytic enzymes, including proteases and glycosidases (St. Leger and Screen 2000; Oguma *et al.* 2011; Cowley *et al.* 2017). One study found that approximately 75% of the

protein portions and 40% of the polysaccharide portions of mucins were degraded by *A. fumigatus* secreted proteases and glycosidases, respectively, that were produced under *in vitro* growth conditions in the presence of mucins (St. Leger and Screen 2000). Consistent with this finding, another study found that the *A. fumigatus* secreted serine protease Alp1 was highly upregulated at both the protein and transcript level in the presence of mucins (Farnell *et al.* 2012). From the pathogen perspective, degrading mucins using secreted proteases and glycosidases can allow *A. fumigatus* cells to access the underlying epithelial cell layer. In addition, studies have suggested that *A. fumigatus* likely uses mucins as a nutrient source (St. Leger and Screen 2000; Oguma *et al.* 2011; Cowley *et al.* 2017). From the host perspective, in response to *A. fumigatus* secreted proteases, the host compensates by upregulating the expression of MUC5AC in airway epithelial cells, which can be a double-edged sword (Cowley *et al.* 2017). The upregulation of MUC5AC could be protective against infection by inhibiting fungal colonization of the mucosal layer, but if MUC5AC becomes highly upregulated or upregulated for too long, this can lead to diseases related to mucus hypersecretion, such as allergic bronchopulmonary aspergillosis, which typically occurs in individuals with asthma or cystic fibrosis (Oguma *et al.* 2011; Gao *et al.* 2012).

Concluding remarks

Mucus is an important host innate defense factor that lines most epithelial cell layers of the body and provides crucial physical and biological protection against pathogenic microorganisms. Mucins are the main glycoproteins of mucus that are responsible for interacting with microorganisms and are critical for the antimicrobial properties of mucus. The physiochemical properties of mucins can suppress key virulence traits in microorganisms, maintaining them in a commensal state. In the opportunistic human fungal pathogen *C. albicans*, adherence, filamentation, biofilm formation, and the production of secreted proteases are suppressed by mucins, although the molecular mechanisms behind this suppression are unknown. In general, further work is needed to elucidate the molecular mechanisms involved in microbe-mucin interactions, which will be essential for our understanding of how a healthy mucosal barrier is maintained. In order to carry out such studies, there is a need for tractable mucus model systems that can be used to study microbe-mucin interactions. Mucosal model systems would also be helpful in the development of novel therapeutics to treat mucosal diseases, such as cystic fibrosis, inflammatory bowel disease, and ulcerative colitis. Finally, given that mucins are such critical players against infection, mechanistically understanding their biological and physical properties will be useful in the development of novel therapeutic strategies against pathogenic microorganisms.

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Conflicts of interest

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

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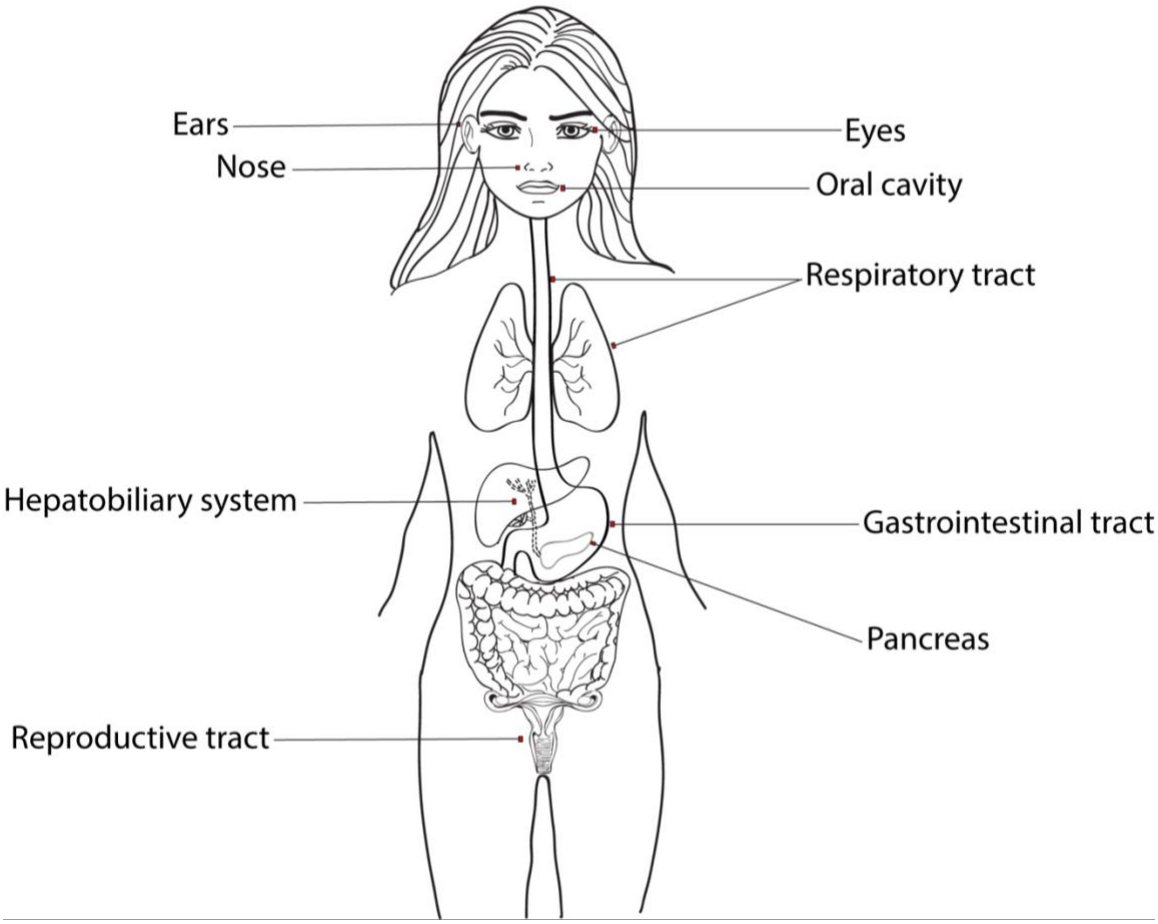

Figure 1. Mucosal membranes covering epithelial cell layers of the human body. Microorganisms are typically associated with mucosal membranes of the eyes, nose, oral cavity, respiratory, gastrointestinal and reproductive tracts. These microorganisms can include both commensals and pathogens. Figure adapted from (Frenkel and Ribbeck2015)

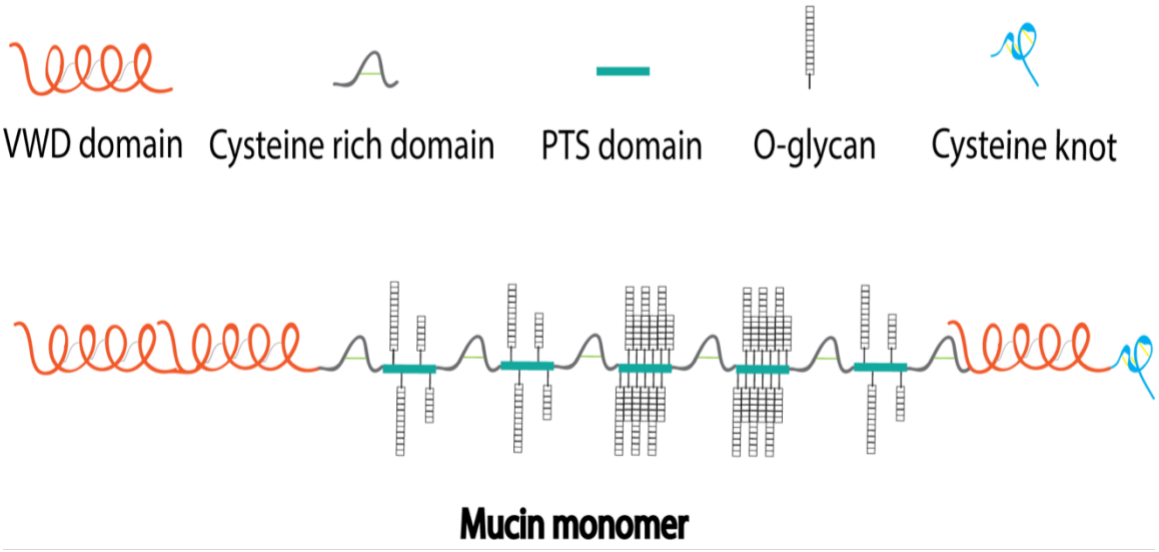

Figure 2. Schematic drawing of a mucin monomer. Each mucin monomer consists of a protein core called the PTS domain that is comprised primarily of proline, threonine and serine. O-glycosylation of polysaccharides occurs at PTS domains between cysteine rich domains. Typically, the C-terminus of the protein backbone contains a cysteine knot and the N-terminus contains several von Willebrand D (VWD) domains.

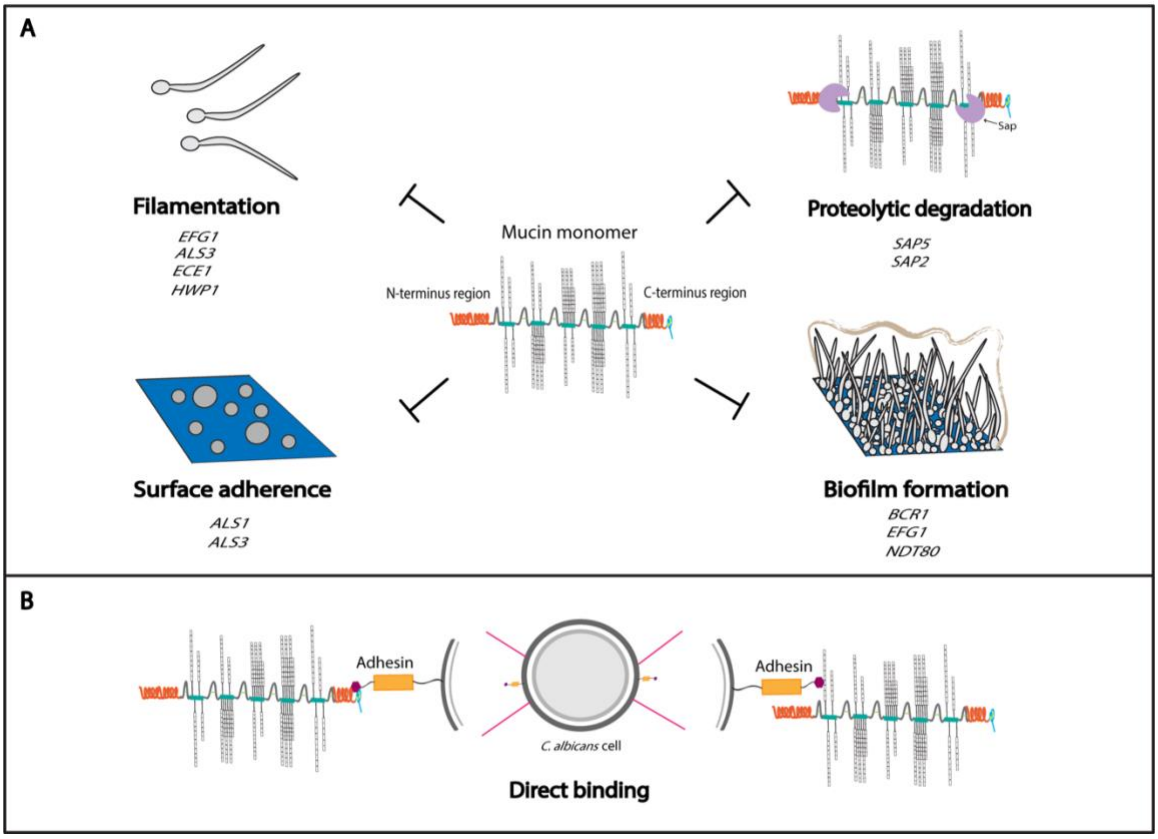

Figure 3. Summary of known and hypothesized interactions between *C. albicans* and mucins. (A) Known interactions. These include the suppression of *C. albicans* adherence, filamentation, biofilm formation and secreted protease production in the presence of mucins. Several *C. albicans* genes encoding important virulence processes are known to be downregulated in the presence of mucins, including *ALS1* and *ALS3* (adherence); *EFG1, ALS3, ECE1* and *HWP1* (filamentation); *BCR1, EFG1* and *NDT80* (biofilm formation); and *SAP5* and *SAP2* (proteolytic degradation).(B) Hypothesized interactions. *C. albicans* adhesins may directly bind to a mucin monomer at the C-terminus of the PTS domain and/or at a glycan monosaccharide.

Chapter 2

Identifying the regulatory network controlling *Candida albicans* interactions with host mucins

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Abstract

Candida albicans is a common human commensal fungus that can become pathogenic when advantageous situations arise, such as when the host is immunocompromised, and due to fluctuations in temperature, pH, and nutrients. One common *C. albicans* virulence factor is its ability to form biofilms, resilient groups of adhered cells composed of different fungal morphologies, protected by an extracellular matrix. In the host, *C. albicans* is in constant interaction with mucosal linings of the body, which protect surrounding tissues from potentially pathogenic invading microorganisms, including *C. albicans*. Mucins, the primary glycoproteins found in mucus, are the main biologically active components of mucus that protects the host from infection and provide the physical viscoelastic properties to mucus. Here we discover and analyze the transcriptional network controlling the interactions of *C. albicans* with mucins using a combination of genetic and genome-wide approaches. The network is composed of eight "master" transcriptional regulators and ~3,000 downstream "target" genes, and provides new insights into how *C. albicans* interacts with the host and forms biofilms on host mucosal surfaces.

Keywords

Mucins, mucus, *Candida albicans,* transcriptional regulation, transcriptional networks, host-pathogen interactions, biofilms, filamentation, adherence

Introduction

Hundreds of trillions of diverse microorganisms inhabit the healthy human microbiota, which include bacteria, fungi, and archaea. These commensal microorganisms coexist, sometimes by sharing nutrients and environmental niches, and other times by competing for the same resources (Ursell et al., 2013; Wang et al., 2017).

These commensal microorganisms have mutually beneficial relationships with the host, where the microorganisms gain a hospitable, nutrient-rich environment to live, while the host gains certain physiological advantages (Chow et al., 2010; Pickard et al., 2017; Relman, 2008). In addition to providing physiological advantages to the host, these microorganisms also provide protection against invading pathogens that can cause disease (Buffie & Pamer, 2013; Chiu et al., 2017; Sassone-Corsi & Raffatellu, 2015).

Candida albicans is a common opportunistic fungal pathogen that is generally found as a commensal of the skin, gut, and genitourinary tract of humans (Kennedy & Volz, 1985; Kumamoto, 2011; Kumamoto & Pierce, 2011). When the normal microbiota changes, *C. albicans* can take advantage of the situation and overgrow, which can ultimately lead to an infection. When this occurs *C. albicans* becomes invasive and can cause superficial skin infections to life-threating bloodstream infections. One major *C. albicans* virulence trait is its ability to form biofilms, communities of tightly adhered cells consisting of different cellular morphologies (Gulati & Nobile, 2016; Lohse et al., 2018; Nobile & Johnson, 2015). *C. albicans* biofilms are highly resistant to chemical and mechanical perturbations (Douglas, 2003; Gulati & Nobile, 2016; Kumamoto, 2002; Lohse et al., 2018; Nobile & Johnson, 2015). *C. albicans* biofilms have been found to grow on implanted medical devices and mucosal surfaces, resulting in superficial and life-threating systemic infections. The *C. albicans* biofilm life cycle occurs in four distinct stages: (i) yeast cells adhere to a surface, (ii) cells grow and proliferate forming a basal cell layer, (iii) pseudohyphae and hyphae develop, and a protective extracellular matrix encases all the cells, and (iv) yeast cells disperse from the biofilm to colonize new sites (Chandra et al., 2001; Douglas, 2003; Gulati & Nobile, 2016).

The transcriptional network governing *C. albicans* biofilm development was originally identified in Nobile et al., 2012. By screening a library of 165 sequence-specific DNA binding transcription factor (TF) deletion mutants (Homann et al., 2009), six “master” biofilm transcriptional regulators (Bcr1, Tec1, Efg1, Ndt80, Rob1, and Brg1) were identified (Nobile et al., 2012). The author’s defined a master biofilm transcriptional regulator, also referred to as a transcription factor (TF), as one whose deletion impairs mature biofilm formation under standard *in vitro* conditions. These master regulators were found to control 1,061 downstream target genes. This TF mutant library containing 165 TF deletion mutant strains was later expanded in Fox et al., 2015 to now include an additional 27 newly constructed TF mutant strains, which were subsequently screened again for biofilm formation (Fox et al., 2015). From screening the expanded 192 TF mutant strain library, the biofilm transcriptional network was further expanded to include three additional transcriptional regulators (Gal4, Rfx2, and Flo8) controlling *C. albicans* biofilm formation (Fox et al., 2015).

Since *C. albicans* commonly resides as a biofilm on mucosal surfaces that line epithelial layers in the human host, it is often in contact with mucus. Mucus is predominantly composed of water, mucin glycoproteins, and other small molecules including lipids and peptides (Celli et al., 2005). It is the large mucin glycoproteins that primarily provide the viscoelastic properties of mucus and protect the host against pathogen invasion (Petrou & Crouzier, 2018). Prior work has shown that mucins suppress several key virulence traits in *C. albicans*, including filamentation, adherence, and biofilm formation (Kavanaugh et al., 2014). Therefore, we aimed to identify the regulatory network controlling *C. albicans* interactions with host mucins. By combining high-throughput *in vitro* biofilm assays, genetic, and genome-wide approaches, including

RNA-seq and CUT&RUN assays, along with CRISPR genetic engineering technologies, we identified the complete transcriptional network governing *C. albicans* interactions with host mucins. Overall, we discovered eight master transcriptional regulators along with ~3,000 downstream target genes involved in interacting with mucins.

Results

Identification of the master transcriptional regulators mediating *C. albicans* interactions with mucins.

Using a *C. albicans* TF deletion mutant library consisting of 211 mutant strains (Fox et al., 2015; Homann et al., 2009), we screened these mutants for their abilities to form biofilms in the presence of mucins in the standard developmental inhibition biofilm assay (Gulati et al., 2018; Lohse et al., 2017). *C. albicans* cells were inoculated on the bottom of polystyrene plates in the presence and absence of mucins. The cells were allowed to grow into a mature biofilm for 24 hours, media was removed, and optical density (OD) readings at 600nm were taken, which has been shown to correspond to the relative thickness of the biofilm (Gulati et al., 2018; Lohse et al., 2017). From this screen, we identified three mutant strains that were defective in forming a mature biofilm in the presence of mucins, and nine mutants that were enhanced in forming biofilms in the presence of mucins. The three TF mutant strains defective in forming biofilms in the presence of mucins were *tup1Δ/Δ, ssn6Δ/Δ* and *fcr1Δ/Δ*; and the nine TF mutant strains that formed enhanced biofilms in the presence of mucins were *tec1Δ/Δ, nto1Δ/Δ, fgr27Δ/Δ, zfu2Δ/Δ, zfu3Δ/Δ, swi4Δ/Δ, uga3Δ/Δ, zcf39Δ/Δ,* and *upc2Δ/Δ* (Fig. 1). All 12 TF mutant strains exhibited a statistically significant difference in their abilities to form biofilms compared to the wildtype (P<0.0001).

Of the 12 mutants identified from this genetic screen, *tec1Δ/Δ* was previously known to be a master regulator of *C. albicans* biofilm formation (Nobile et al., 2012). These results indicate that when *C. albicans* is exposed to mucins during biofilm formation, it regulates interactions with mucins in the context of a biofilm through a transcriptional network that is largely distinct (at least in terms of its master regulators) from the transcriptional network known to regulate classical biofilm formation (i.e., in the absence of mucins).

Identification of the transcriptome of the master transcriptional regulators mediating *C. albicans* interactions with mucins.

Identifying the relationships between transcription factors and their target genes is essential to understanding their functions in many biological and developmental processes. Here, we performed RNA-seq on the TF mutants treated with mucins to identify the target genes differentially expressed in the mutants when mucins are present. Using the *in vitro* developmental inhibition biofilm assay (Gulati et al., 2018; Lohse et al., 2017), as was performed for the initial genetic screen, we grew the 12 TF mutant strains in the presence and absence of mucins. Total RNA was extracted from biofilm harvested cells after 24-hours of biofilm growth. Sequencing ready libraries were then prepared and sequenced on the Illumina Hi-seq platform. The resulting sequencing data was then analyzed using a DESeq2 data analysis pipeline developed in our lab.

Using this strategy, we identified many downstream target genes regulated by the master TFs identified in our genetic screen.

Our RNA-seq results indicate that there are thousands of genes differentially expressed in the presence of mucins. On average, over 2,000 genes are differentially expressed in each of the biofilm defective mutant strains (*tup1Δ/Δ, ssn6Δ/Δ* and *fcr1Δ/Δ*) in the presence of mucins. Roughly 60% of these genes are upregulated and the remaining 40% are downregulated in the presence of mucins (padj < 0.05, threshold of log2 fold change of < -0.58 and >0.58). Similarly, for the enhanced biofilm forming mutant strains (*tec1Δ/Δ, nto1Δ/Δ, fgr27Δ/Δ, zfu2Δ/Δ, zfu3Δ/Δ, swi4Δ/Δ, uga3Δ/Δ, zcf39Δ/Δ,* and *upc2Δ/Δ*) in the presence of mucins, ~1,300 to ~3,000 genes are differentially regulated.

Adherence and filamentation are two of the developmental stages known to be critical for classical biofilm formation. Therefore, we hypothesized that the transcriptional regulators identified in our screen will include target genes previously known to play roles in regulating these processes. Remarkably, all three of the regulators identified as defective biofilm formers in the presence of mucins (*tup1Δ/Δ, ssn6Δ/Δ* and *fcr1Δ/Δ*) upregulated six other TF genes (*WOR1*, *EFH1*, *UME6*, *ACE2*, *TRY6*, and *ZAP1*). Interestingly, these genes are known to encode proteins with known roles in filamentation and adherence (Fig. 2A). We note that Zap1 is also a known regulator of biofilm matrix production (Nobile et al., 2009).

The transcriptional program controlling filamentation in *C. albicans* includes many known TFs that either activate or repress downstream genes that regulate filamentation. We next analyzed a set of functionally relevant genes encoding TFs (n=86) with a primary function in *C. albicans* filamentation (Villa et al., 2020) (Fig. 2B). We found a subset of genes that were highly upregulated in all nine of the enhanced biofilm former mutants in the presence of mucins. Interestingly, of those genes, *ZAP1* was upregulated in all regulator mutants, both in defective biofilm formers and enhanced biofilm formers, suggesting that it is a major player in regulating filamentation and biofilm matrix formation throughout biofilm development in both the presence and absence of mucins (Ganguly & Mitchell, 2011; Nobile et al., 2009).

Lastly, we asked whether the regulators, when in the presence of mucins, are differentially regulating target genes in common with the classical biofilm network (i.e., in the absence of mucins). Of the 1,061 target genes that are differentially regulated in the classical biofilm network, we focused on target genes that were either upregulated or downregulated by at least twofold (Fig. 3). Interestingly, roughly half of the genes that were upregulated in the classical biofilm network (n=150) were downregulated in the presence of mucins (Fig. 3A). The downregulated genes of the classical biofilm network (n=260), on the other hand, were upregulated in the presence of mucins (Fig. 3B). In other words, interestingly, about 40% of the target genes of the classical biofilm network showed expression patterns that had reversed in direction when *C. albicans* cells encountered mucins.

Tec1 is one of the master regulators identified in our mucins screen, and it is the only master regulator that also overlaps with the master regulators of the classical biofilm network. In the classical biofilm network, Tec1 binds to the upstream intergenic regions of all six of the other master biofilm regulators, and a *tec1Δ/Δ* strain is defective in classical biofilm formation. On the other hand, in the presence of mucins, the *tec1Δ/Δ*

strain is enhanced in biofilm formation. These observations suggest that Tec1 may be a key regulator responsible for the reversal in the expression of many downstream target genes between the classical biofilm network and the interaction with mucins network. We, therefore, analyzed the transcriptional profiles for the *tec1Δ/Δ* strain in the presence of mucins compared that of the *tec1Δ/Δ* strain in the absence of mucins from Nobile et al., 2012. Intriguingly, we found that ~50% of the *tec1Δ/Δ* target genes show flipped gene expression profiles in the presence of mucins (Fig. 4). The *tec1Δ/Δ* cells for the classical biofilm network differentially express 454 target genes by at least 1.5-fold, the majority of which appear to be reversed in direction in the presence of mucins. Remarkably, Tec1 directly binds to the upstream intergenic regions of four of the classical master biofilm TFs *EFG1, ROB1, BCR1,* and *BRG1* in the presence of mucins, but the expression of all four of these TFs are reversed in expression in the mucin network compared to the classical biofilm network. Taken together these results suggest that Tec1 is an integrated player regulating biofilm formation in the presence and absence of mucins, and may be responsible for rewiring the classical biofilm network in the presence of mucins.

Mucins regulate genes involved in processes like filamentation, adherence, and biofilm matrix formation.

The differentially expressed target genes discussed above were analyzed for enriched functions using Gene Ontology (GO) to determine groups of genes that are involved in the response to mucins. As expected, several biofilm processes, such as filamentation, adherence, and extracellular matrix, are significantly enriched in the transcriptional regulator mutants in the presence of mucins. The terms that showed significant enrichment in our functional enrichment analysis include hyphal cell wall, yeast-form cell wall, and fungal biofilm matrix (Fig. 5). These groups of genes were upregulated in the presence of mucins, and more specifically, were upregulated in the enhanced biofilm former mutants, which make up most of the identified mucin regulators.

Target genes of interest that stood out for further follow-up experiments include *ORF19.251*, *ENO1*, *TDH3*, *PGK1*, *IPP1*, *PGI1*, and *GRP2* (Fig. 5). These genes were chosen for downstream study because they were i) highly upregulated in the presence of mucins and ii) mutually identified in both gene sets pertaining to hyphal cell wall and biofilm matrix functions. In terms of the defective biofilm formers in the presence of mucins, we noticed that enrichment for filamentation, adherence, and biofilm formation was enriched, but differentially downregulated.

Identification of the directly bound target genes of the master mucin transcriptional regulators.

To determine the regulatory relationships between the master mucin transcriptional regulators and their downstream target genes, we performed Cleavage Under Targets and Release Using Nuclease (CUT&RUN) assays (Skene et al., 2018) on GFP-tagged *C. albicans* cells of the mucin regulators grown in the presence and absence of mucins. CUT&RUN is a popular alternative to ChIP-seq, and both methods determine the genome-wide binding locations for a protein of interest. Briefly, *C. albicans*

biofilms grown for 24 hours in the presence and absence of mucins were harvested. Intact nuclei were isolated from the cells and were bound to magnetic beads. Subsequently, the bead-bound nuclei were incubated with a primary anti-GFP antibody followed by the addition of pAG-MNase fusion protein. The pAG-MNase was activated, and limited chromatin digestion was carried out before stopping the reaction. The DNA-protein complex was then allowed to diffuse out of the cell. DNA collected from the supernatant was cleaned and sequencing ready libraries were prepared for sequencing on the Illumina sequencing platform, as described previously (Skene et al., 2018).

Currently, we have CUT&RUN data for eight of the twelve regulators; we are awaiting CUT&RUN data for Fgr27, Zfu2, Uga3, and Ssn6. Thus far, we calculated the following number of peaks at intergenic regions for each regulator in the absence (-) and presence (+) of mucins: 1,327 (-) and 18 (+) for Nto1, 309 (-) and 12 (+) for Zfu3, 221 (-) and 2 (+) for Fcr1, 1,218 (-) and 8 (+) for Swi4, 251 (-) and 4 (+) for Tec1, 2,029 (-) and 46 (+) for Tup1, 31 (-) and 42 (+) for Upc2, and 559 (-) and 8 (+) for Zcf39.

Mucins mediate biofilm formation by disrupting a significant number of DNA-binding events.

Our CUT&RUN results indicate that the identified regulators bind to hundreds of loci throughout the genome when the biofilms are not treated with mucins. Interestingly, when biofilms are treated with mucins, most of the binding events are disrupted and the transcription factors are bound to significantly fewer regions compared to the untreated condition. In other words, each of the regulators bind to the intergenic regulatory region of numerous target genes only in the absence of mucins. These results indicate that mucins mediate biofilm formation by disrupting the DNA-binding ability of the identified transcription factors (Fig. 6).

Determining transcriptional relationships among master mucin regulators.

Coupling the differential expression analysis from the RNA-seq experiments with the direct binding interactions from the CUT&RUN experiments, we discovered the complete transcriptional network controlling *C. albicans* interactions with mucins. Our analysis suggests that the network is currently composed of 3,186 target genes that are bound in their promoter regions by at least one of the eight regulators (for which we currently have CUT&RUN data for) (Fig. 7). Of the 3,186 genes, 412 are downregulated (shown in yellow circles), 214 are upregulated (shown in blue circles), and 2,560 are not differentially expressed (shown in grey circles) in the presence compared to absence of mucins. Furthermore, a total of 17 target genes are bound by all eight of the master mucin transcriptional regulators. Interestingly, only genes encoding the master regulators Fcr1 and Zcf39 are downregulated in the network in the presence compared to absence of mucins, while the rest of the mucin regulators show no differential expression. Master regulators Nto1 and Tup1 control the largest individual sets of target genes and share a group of genes not controlled by any of the other regulators. Since Tup1 is a known corepressor with Ssn6 (Keleher et al., 1992), we anticipate that once we have Ssn6 CUT&RUN data, it will show regulation of the same gene sets as Tup1. Thus far, the transcription network controlling *C. albicans* interactions with mucins

resembles a highly interconnected network that shows both dependent and independent relationships among the regulators and their downstream target genes.

A closer look at the binding of the master mucin transcriptional regulators to one another, reveals a core network that is composed of six master mucin regulators controlling each other's expression (Fig. 8). Nto1 binds to the upstream intergenic regions of *SWI4*, *ZCF39*, *FCR1*, and *TEC1*; Swi4 binds to and regulates *FCR1* and *TEC1*; and binds to its own intergenic regulatory region and autoregulates itself. Tup1 binds to and regulates *SWI4* and *FCR1*, but interestingly, it itself is not bound by any of the other regulators. Zcf39 only regulates *SWI4*, and Tec1 only regulates *FCR1*. Fcr1 does not regulate any of the other regulators but is regulated directly by three regulators and indirectly by two. Since Zfu3 and Upc2 have no direct binding interactions with the other regulators thus far, they are currently excluded from the core circuitry.

De novo motif discovery for the transcriptional regulators.

Based on several hundred significant binding events from our CUT&RUN data, we were able to identify statistically significant motifs *de novo* for all eight of the master mucin regulators. This motif compilation was based exclusively on our CUT&RUN data. We were able to determine statistically significant motifs using MEME software (Bailey et al., 2015) for all eight regulators (Fig. 8). Interestingly, we note that none of the mucin master regulators have known unambiguous orthologs in *Saccharomyces cerevisiae*, and thus motif comparison to *S. cerevisiae*, which is often standardly performed for *de novo* motifs identified in *C. albicans*, was not possible.

Discussion

The master transcriptional network controlling *C. albicans* interactions with mucins.

C. albicans biofilms are communities of various cell types including yeast, pseudohyphae, and hyphae, protected by an extracellular matrix. Generally, in a human host, *C. albicans* is in contact with mucins, the main glycoproteins found in the mucosal layer lining the organs where *C. albicans* is known to inhabit. Here, we have described a transcriptional network composed of eight master regulators bound to numerous downstream target genes that control *C. albicans* interactions with mucins in the context of a biofilm. Our network includes regulators and target genes functionally involved in filamentation, adhesion, and biofilm formation. These eight master transcriptional regulators form an interconnected transcriptional network, where each regulator binds to other regulators in the network and the majority of the differentially expressed target genes in the network are controlled by more than one regulator. Six out of the eight master regulators (except for Zfu3 and Upc2) have direct binding interactions with each other. Taking into consideration all the target genes of the eight regulators, the network controlling *C. albicans* interactions with mucins is comprised of 3,186 genes, roughly 50% of the genes found in the *C. albicans* genome.

Of the 12 regulators comprising this mucin mediated transcriptional network, only Tec1, a known master regulator of classical biofilm formation (Nobile et al., 2012), was identified in our screen as one of the master mucin regulators. Thus, the mucin mediated transcriptional network is controlled by a set of regulators largely distinct from those controlling the classical biofilm network. Interestingly, the classical biofilm regulators and their target genes are nevertheless involved in this mucin mediated transcriptional network since the majority of the classical biofilm master regulators are target genes of the mucin master regulators. This, along with other similarities shared by the classical biofilm network and the mucin mediated transcriptional network, suggests that the classical biofilm network is significantly rewired when mucin is in contact with *C. albicans*. Our results with Tec1 provide support for this "rewiring" event. Tec1controls hundreds of target genes in the classical biofilm network. In the presence of mucins, however, Tec1 plays a significantly different regulatory role in the following two ways. First, a ***tec1Δ/Δ*** strain forms enhanced biofilms in the presence of mucins, as opposed to when mucins are absent, where ***tec1Δ/Δ*** strains are highly defective in biofilm formation. Second, the expression levels of many Tec1 target genes reverse in direction when mucins are present, including the expression of the classical biofilm regulators *EFG1, ROB1, BCR1*, and *BRG1.* In short, Tec1 is a key regulator in the mucin mediated transcriptional network that may be responsible for rewiring the transcriptional network when *C. albicans* cells encounter mucins.

Mucins may be controlling biofilm formation in *C. albicans* by repressing genes involved in activating biofilm formation, which is consistent with our results and depicted by the changes in binding events that occur for each mucin regulator in the presence versus absence of mucins. Known interactions between *C. albicans* and mucins include suppression of adherence, filamentation, biofilm formation, and proteolytic degradation (Kavanaugh et al., 2014; Valle Arevalo & Nobile, 2020). One possible mechanism for *C. albicans* to physically interact with mucins is through *C. albicans* adhesins, which are proteins involved in adherence, filamentation and biofilm formation. These fungal adhesins may directly bind to mucins at their C-terminal PTS domain and/or at the glycan monosaccharide (Valle Arevalo & Nobile, 2020). Further analysis of this idea will need to be explored in the future to confirm that this is in fact the way that mucin modulates physical interactions with *C. albicans.*

Materials and Methods

Fungal strains and growth conditions. *C. albicans* SN250 was used as WT strain. The TF mutant library strains were obtained from Homann et al., 2008 and Fox et al., 2015. *C. albicans* strains were grown at 30°C in YPD agar plates for 48 hours, and overnight cultures were grown at 30°C in YPD broth with shaking for 12-16 hours.

Mucin purification. Mucin protein powder (Sigma Aldrich Cat No. 84082-64-4) was weighed out to make a 0.5% solution in 1X PBS. The solution was left on a nutator shaking at 4°C overnight to completely homogenize into solution. Afterwards, the solution was transferred into a 70mL gamma-irradiated dialysis cassette and dialyzed in 1X PBS. After dialysis, the solution was collected in 15mL conical tubes and lyophilized using a Labconco FreeZone 4.5 Liter -105°C freeze dryer. After freeze drying for 24

hours, lyophilized protein powder was reconstituted into solution with RPMI-1640 medium for use with biofilm assay experiments.

***In vitro* developmental inhibition biofilm assays.** *In vitro* biofilm assays were carried out following the standard Lohse et al., 2017 developmental biofilm assay protocol. Briefly, 96-well microtiter plates were inoculated with *C. albicans* cells in RPMI-1640 medium (Sigma Aldrich Cat No. R6504-10X1L), incubated with shaking for an initial 90 mins at 37°C, washed with 1X PBS, and added to either fresh RPMI-1640 medium or RPMI with mucin medium and incubated for 24 hours. Optical density readings were taken after 24 hours of biofilm growth.

RNA sample preparation and extraction. Biofilms for RNA-seq analysis were grown in RPMI-1640 medium at 37°C in 12-well polystyrene plates following the Lohse et al., 2017 developmental inhibition biofilm assay protocol. RNA extraction was performed using the Invitrogen RiboPure RNA Purification Kit (Cat No. 850013SA). Samples were then library prepped using Lexogen's QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina (Cat No. 015x96) and sequenced on an Illumina HiSeq4000.

CUT&RUN sample preparation and sequencing. Samples were prepared for CUT&RUN following (Skene et al., 2018). Briefly, each transcriptional regulator was tagged with GFP tag at the C- terminal end of the protein in the SN250 wildtype strain background. GFP-tagged regulators were grown using the developmental inhibition biofilm assay in 12-well microtiter plates in the presence and absence of 0.5% mucins. The biofilms were then harvested, and nuclei were obtained from spheroplasts of native cells. Polyclonal anti-GFP antibody (Takara Bio, Cat No. 632592) was used to bind to the ConA-bound nuclei, pA-MNase fusion protein was added to bind to the GFP antibody, and cleavage of the DNA-protein target complex is initiated with the addition of $CaCl_2$. The reaction was stopped, and the DNA-protein target complex diffused out of the cell. Final DNA clean-up was performed using bead-based size selection with SPRIselect (Beckman Coulter, Cat No. B23317). Illumina paired-end sequencing was performed to obtain 3-5 million reads of target DNA.

Differential gene expression analysis of RNA-seq data.

Analysis of the raw.fastq files after sequencing was done using an RNA-seq pipeline available in the lab that utilizes DESeq2 (Love et al., 2014).

Motif analysis.

Motif analysis was performed using MEME software (Bailey et al., 2015).

Statistical analysis. Identification of target genes was performed using an R-source code with a two-tailed Student's *t*-test; a p-value ≤ 1e-04, normalized to the wildtype, was considered significant.

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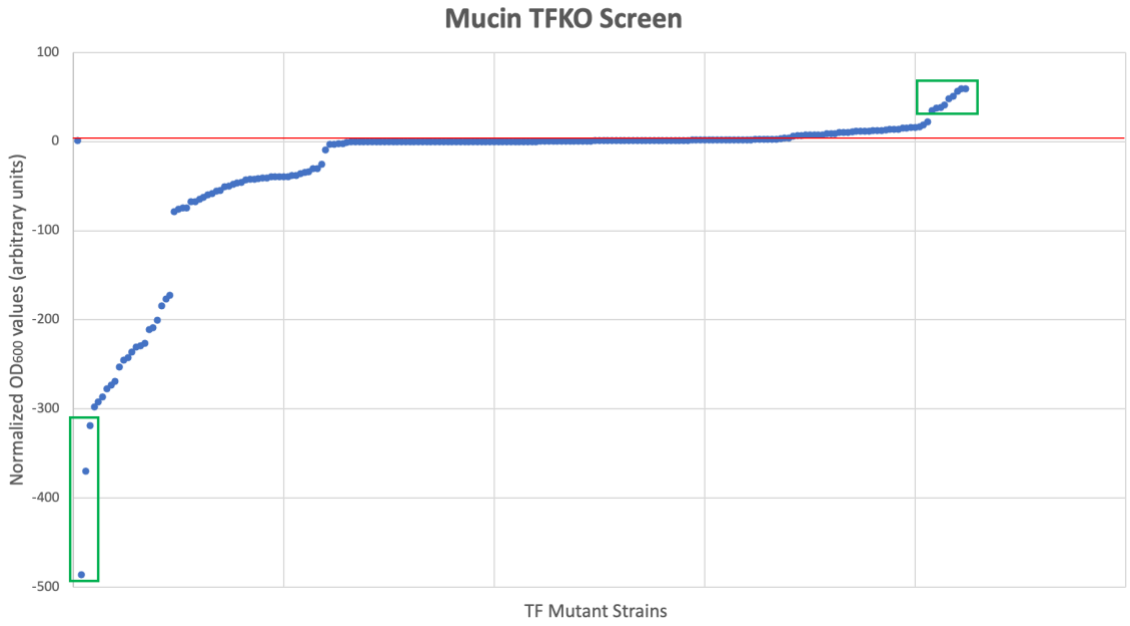

Figure 2.1 Transcription factor deletion mutant library screen. Mutants and wildtype were grown in the presence and absence of mucin. All 211 TFKO mutants are plotted (blue dots) and wildtype was set to equal 1 (red line). Statistical significance was determined using a two-tailed Student's t-test with a p-value ≤ 1e-04, normalized to the wildtype. The lower green box represents the top defective biofilm formers: *tup1Δ/Δ, ssn6Δ/Δ* and *fcr1Δ/Δ*; the upper green box represents the top enhanced biofilm formers: *tec1Δ/Δ, nto1Δ/Δ, fgr27Δ/Δ, zfu2Δ/Δ, zfu3Δ/Δ, swi4Δ/Δ, uga3Δ/Δ, zcf39Δ/Δ,* and *upc2Δ/Δ.*

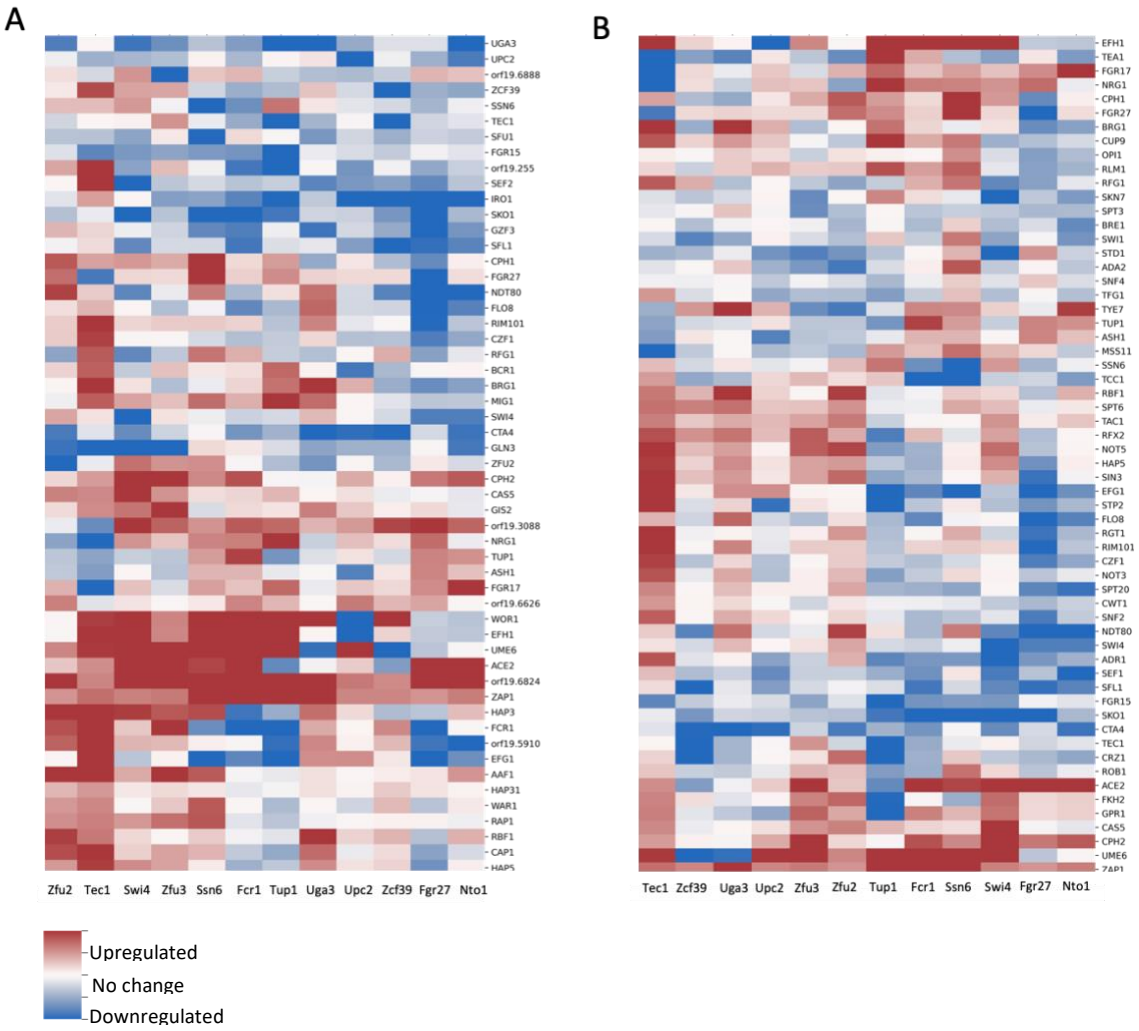

Figure 2.2 Genes involved in adherence and filamentation are differentially regulated in the presence of mucin. A) Heatmap representing 54 known genes involved in adherence and filamentation. The defective biofilm formers in the presence of mucin (*tup1Δ/Δ, ssn6Δ/Δ* and *fcr1Δ/Δ)* highly upregulated genes, such as *WOR1*, *EFH1*, *UME6*, *ACE2*, *TRY6*, and *ZAP1.* B) Heatmap representing genes with functions exclusively in filamentation.

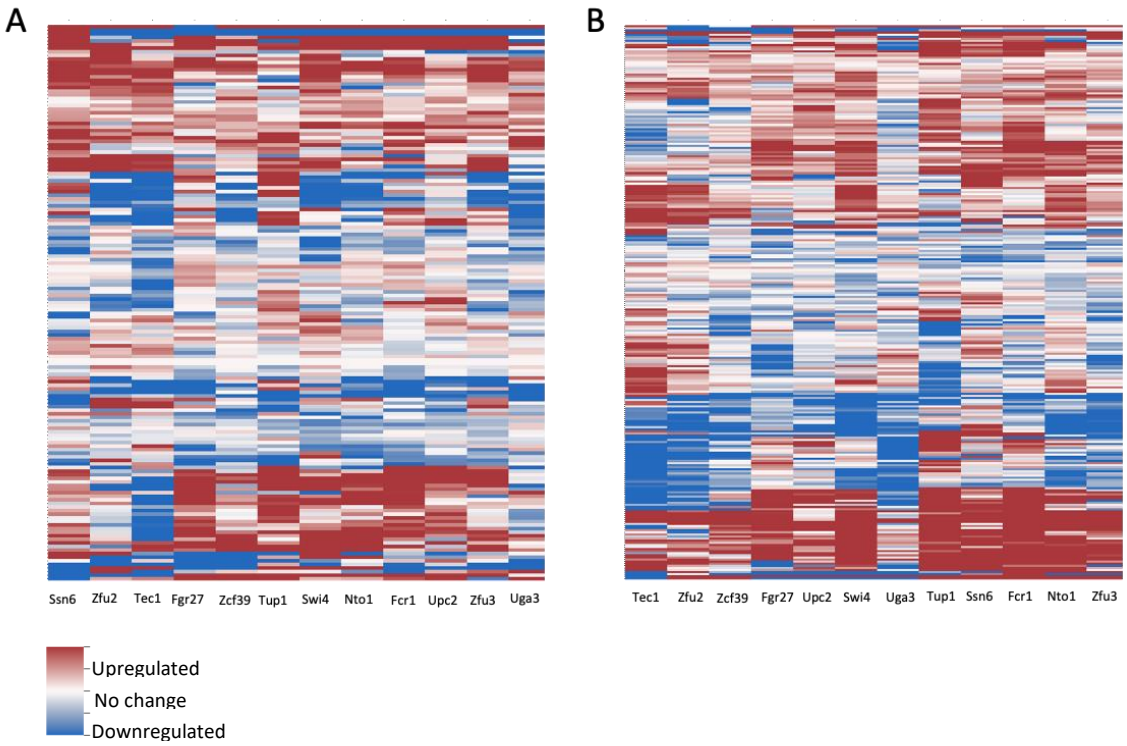

Figure 2.3 Mucin differentially regulates target genes of the classical biofilm network. A) Upregulated biofilm target genes, > 2-fold change B) Downregulated biofilm target genes, < -2-fold change.

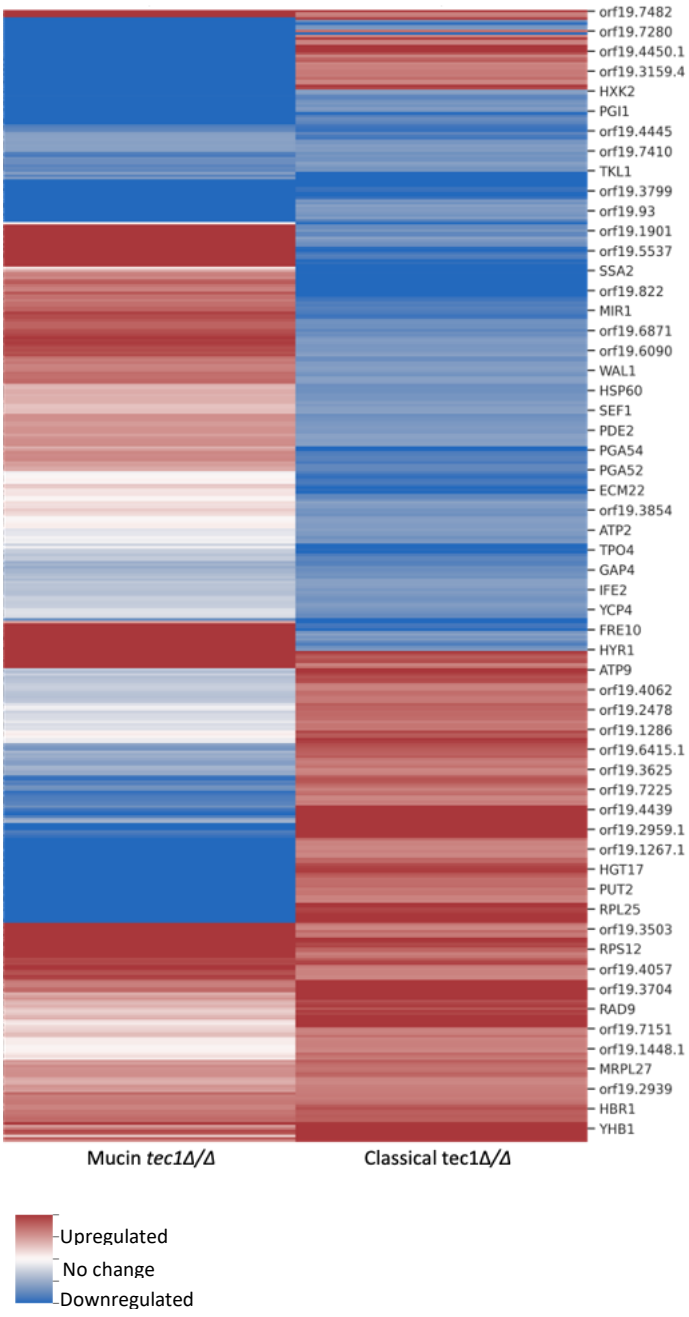

Figure 2.4 The *tec1Δ/Δ* strain in the presence of mucins has reversed expression of many target genes of the *tec1Δ/Δ* strain in the classical biofilm network. Heatmap showing *tec1Δ/Δ* in the presence of mucins (left) and *tec1Δ/Δ* from the classical biofilm network (right).

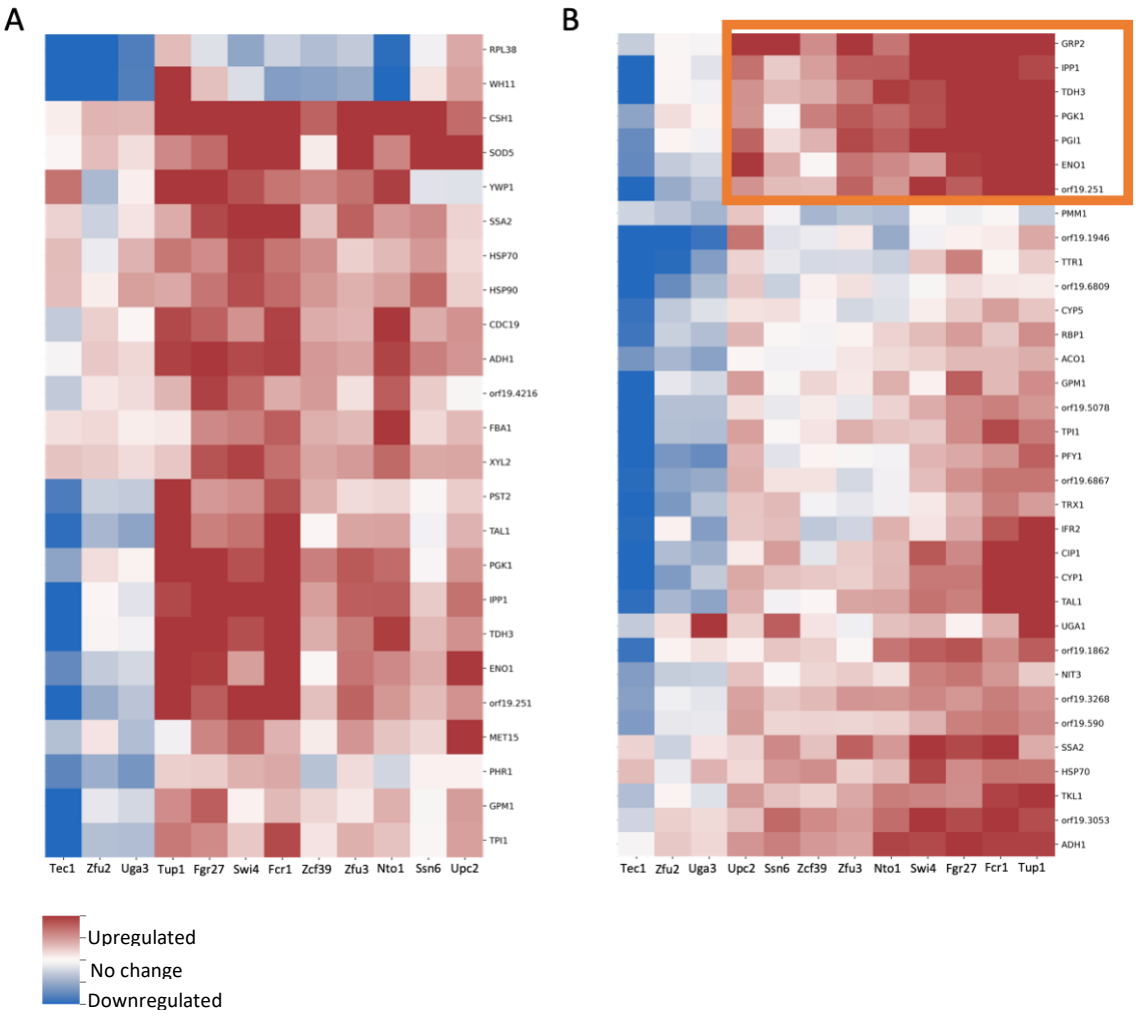

Figure 2.5 Functional enrichment reveals important enriched gene sets. A) Hyphal cell wall target genes, B) Biofilm matrix heatmap of target genes.

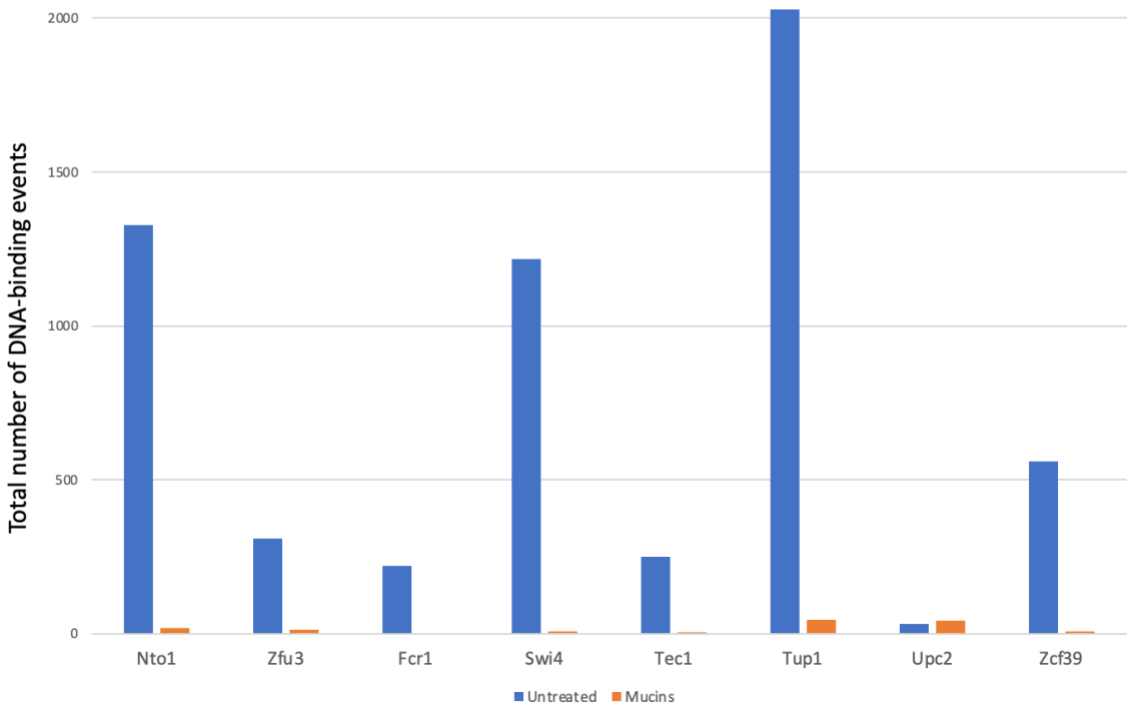

Figure 2.6 Mucin disrupts transcriptional regulator DNA-binding events. CUT&RUN results show that the eight identified regulators bind to hundreds of loci throughout the genome when the biofilms are in the absence of mucins. Intriguingly, when biofilms are grown in the presence of mucins, there appears to be a drastic disruption of binding events and transcription factors are bound to significantly fewer regions compared to the untreated condition.

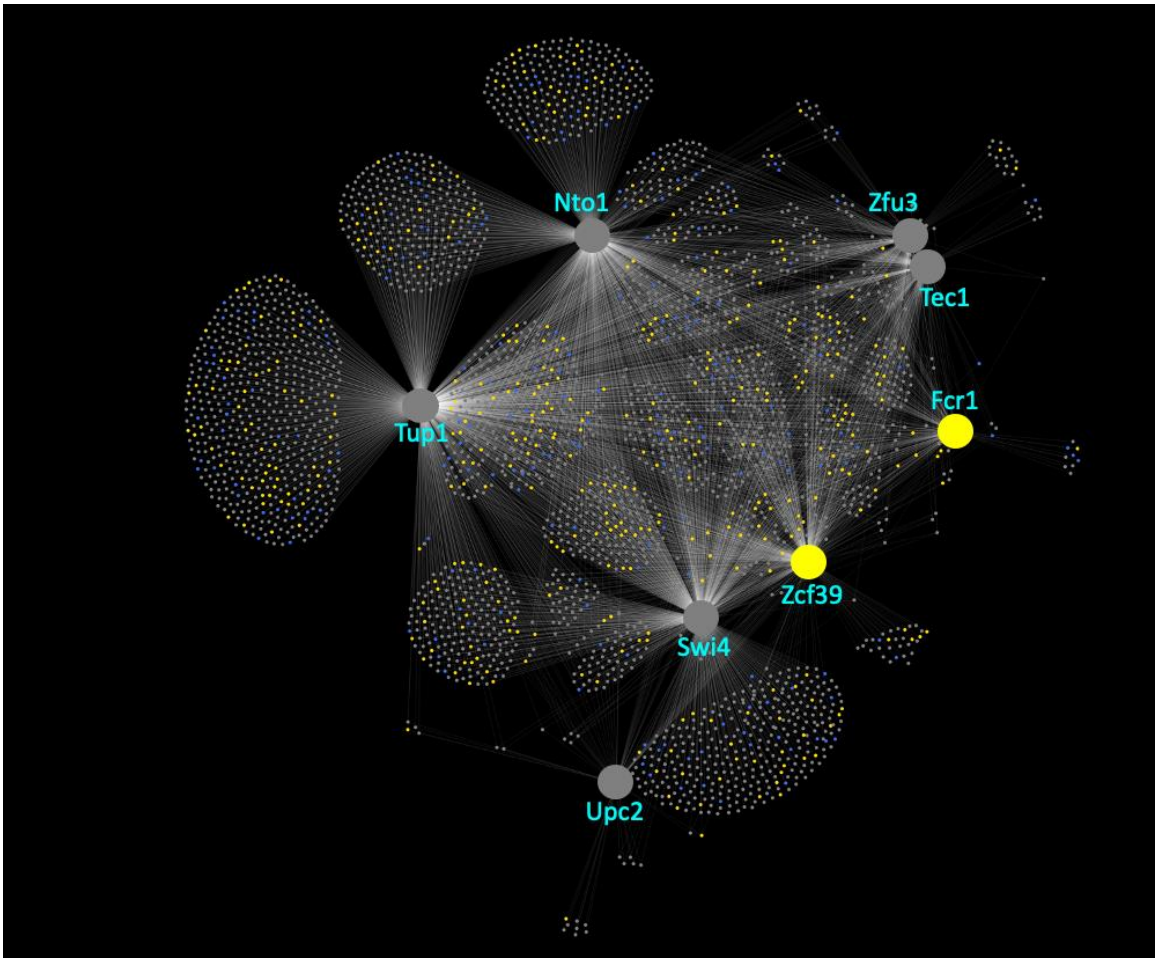

Figure 2.7 The complete transcriptional network controlling *C. albicans* interactions with mucin. The network consists of 3,186 genes, where 412 are downregulated (yellow circles), 214 are upregulated (blue circles), and 2,560 are not differentially expressed (grey circles) in the presence versus absence of mucin.

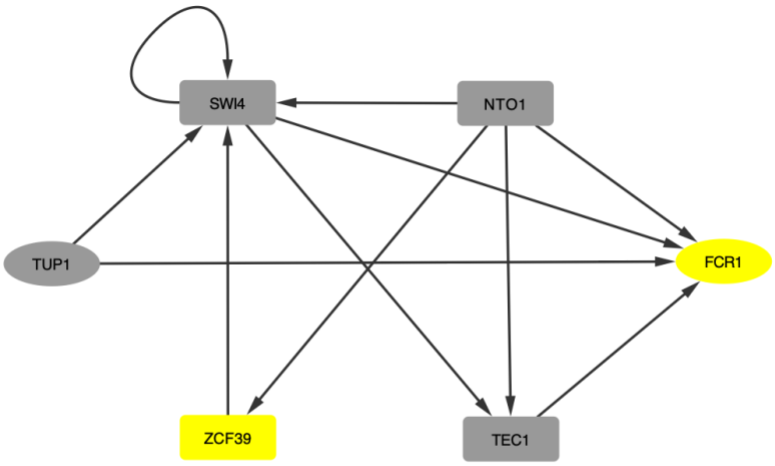

Figure 2.8 Core circuitry of transcriptional interactions among biofilm regulators. Nto1 directly binds upstream of SWI4, FCR1, TEC1, and ZCF39. Swi4 binds to and regulates FCR1 while TEC1 binds to its own intergenic regulatory region and autoregulates itself. Tup1 binds to and regulates SWI4 and TEC1. Zcf39 only regulates SWI4 and Tec1 only regulates FCR1. Regulators Zfu3 and Upc2 have no direct binding interactions with the other regulators therefore are excluded from the core circuitry. Oval shapes indicate the defective biofilm formers and rectangles indicate the enhanced biofilm formers. Grey ovals/rectangles indicate no change in expression and yellow ovals/rectangles represent differentially downregulated regulators.

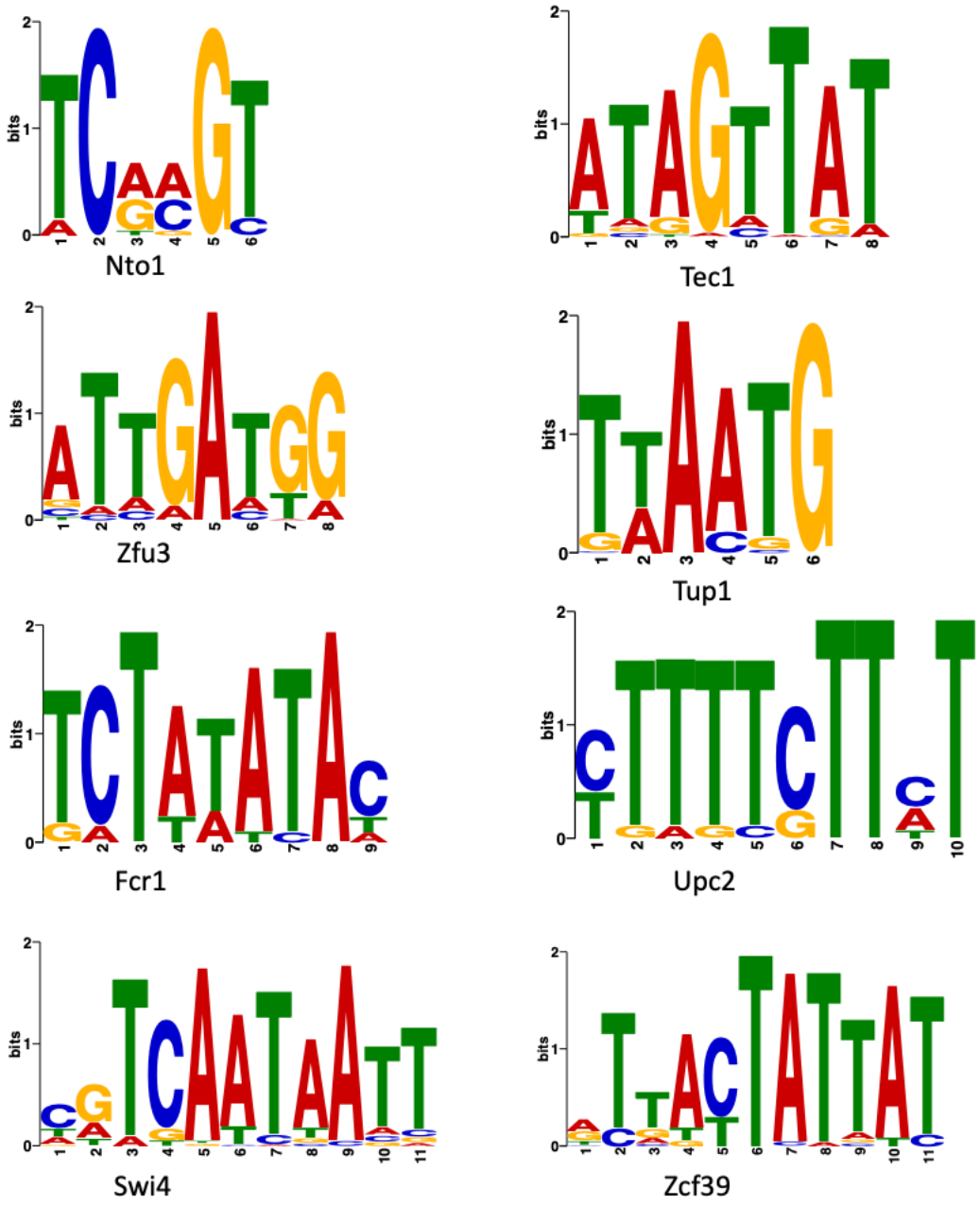

Figure 2.8 Newly identified *C. albicans* motifs. Motifs were identified using MEME-ChIP (STREME).

Chapter 3

Epithelial Infection with *Candida albicans* Elicits a Multi-system Response in Planarians

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Keywords: planarians; *Candida albicans*; infection; host-pathogen interactions; innate immune system; stem cells; animal models

Abstract

Candida albicans is one of the most common fungal pathogens of humans. Prior work introduced the planarian *Schmidtea mediterranea* as a new model system to study the host response to fungal infection at the organismal level. In the current study, we analyzed host-pathogen changes that occurred *in situ* during early infection with *C. albicans*. We found that the transcription factor Bcr1 and its downstream adhesin Als3 are required for *C. albicans* to adhere to and colonize the planarian epithelial surface, and that adherence of *C. albicans* triggers a multi-system host response that is mediated by the Dectin signaling pathway. This infection response is characterized by two peaks of stem cell divisions and transcriptional changes in differentiated tissues including the nervous and the excretory systems. This response bears some resemblance to a

wound-like response to physical injury; however, it takes place without visible tissue damage and it engages a distinct set of progenitor cells. Overall, we identified two *C. albicans* proteins that mediate epithelial infection of planarians and a comprehensive host response facilitated by diverse tissues to effectively clear the infection.

Introduction

The innate immune system is the first line of defense against invading pathogens (Espinosa and Rivera, 2016; Peiris et al., 2014). The complex interactions between the pathogen and the host is a constantly evolving arms race, where the pathogen aims to invade and proliferate in the host, while the host aims to defend against infection. Both the host and pathogen responses to one another are known to be influenced by the physical presence of the infecting microorganism on specific host tissues, by the production of pathogen-associated virulence factors, and by the extent of host tissue damaged during the infection. Nonetheless, the underlying molecular mechanisms associated with these host-pathogen responses are largely unknown.

Candida albicans is an opportunistic human fungal pathogen that can cause both superficial mucosal and life-threatening systemic infections, particularly in immunocompromised individuals (Filler, 2013; Pellon et al., 2020). *C. albicans* efficiently adapts to the dynamic host environment by quickly responding to host environmental cues, such as contact with epithelial cells, temperature, pH, nutrients, and iron levels (Brunke and Hube, 2014). *C. albicans* possesses a number of virulence traits that allow this pathogen to evade host innate immune defenses, such as the ability to change morphologies from the round budding yeast-form to the elongated hyphal form (Braun and Johnson, 1997; Mitchell, 1998; Saville et al., 2003). The yeast to hyphal transition is critical for *C. albicans* to invade and damage host tissues and to disseminate into the bloodstream (Wilson et al., 2016). Additionally, *C. albicans* can adhere to and colonize host tissues by forming recalcitrant biofilms on mucosal surfaces that provide physical protection from the host innate immune response (Nobile and Johnson, 2015; Valle Arevalo and Nobile, 2020).

C. albicans uses two main strategies to invade and damage host epithelial cells. The first involves inducing endocytosis by host cells, while the second involves active physical penetration of host cells by *C. albicans* hyphal cells (Filler, 2013; Filler et al., 1995; Pellon et al., 2020; Wachtler et al., 2012). Adhering to epithelial cells and forming hyphae is the first step for *C. albicans* to invade host cells via either induced endocytosis or active physical penetration strategies (Wilson et al., 2016). For induced endocytosis, the process is mediated by fungal cell surface proteins (e.g. Ssa1 and Als3) that bind to cadherins on host cell surfaces (Filler, 2013; Moreno-Ruiz et al., 2009; Nobile et al., 2006; Phan et al., 2007; Sun et al., 2010). Active physical penetration of host cells by *C. albicans* cells, on the other hand, is a fungal-driven process that relies on the ability of hyphal cells to penetrate the epithelial cells without host activity (Wilson et al., 2016). Overall, the molecular mechanisms mediating both strategies are not fully understood.

To date, the analysis of host-pathogen interactions, including our understanding of pathogen invasion strategies, has been possible through the use of *in vitro*, *ex vivo*, and *in vivo* infection models. Work in *C. albicans* has led to the identification of several molecular players and signaling pathways involved in the host-pathogen response to

acute *C. albicans* infections (Pellon et al., 2020). Nevertheless, the host response to infection likely goes beyond these localized interactions with the pathogen and may involve complex long-range intercellular communication between host tissues and organs to orchestrate an effective response against the invading pathogen (Godinho-Silva et al., 2019). Visualizing the complexity of the host response in real-time and integrating the contribution of different organs and systems has been challenging with currently available animal models (Bergeron et al., 2017; Chamilos et al., 2007; Glittenberg et al., 2011; Gratacap et al., 2017; Mallick et al., 2016; Mylonakis, 2008; Mylonakis et al., 2007; Peterson and Pukkila-Worley, 2018; Pukkila-Worley et al., 2009; Segal and Frenkel, 2018).

Previously, we introduced the planarian *Schmidtea mediterranea* as an *in vivo* infection model to study host-pathogen interactions with *C. albicans* (Maciel et al., 2019). We found that planarians can be conveniently infected by adding *C. albicans* cells directly into the media where planarians live, meaning that infection does not require invasive procedures, such as injections. This “passive” infection method allows for the spatiotemporal tracing of *C. albicans* throughout the course of an infection, beginning with its initial interaction with the planarian epithelial surface. We showed that *C. albicans* forms hyphae after adhering to the planarian epithelial surface and that the aggressive invasiveness of *C. albicans* hyphal cells on planarian tissues is counteracted by the planarian innate immune response that eventually completely clears the infection in about a week (Maciel et al., 2019). Aside from prompting an innate immune response, infection of planarians with *C. albicans* triggers a mitotic response that is mediated by pluripotent adult stem cells known as neoblasts, the only somatic cell population in planarians with the capacity to divide (Maciel et al., 2019). This finding is intriguing because fungal infections have also been shown to activate mammalian stem cell proliferation, which is believed to potentiate the host innate immune response to infection (Megías et al., 2016; Megías et al., 2012; Yáñez et al., 2011; Yáñez et al., 2009; Yang et al., 2012). These findings suggest the possibility of evolutionary conservation of the host stem cell and innate immune responses between mammals and planarians. Nonetheless, the underlying mechanisms used by planarians to effectively clear *C. albicans* infections remain unclear.

In the present study, we show that infection of planarians with *C. albicans* triggers systemic waves of neoblast proliferation that can be detected within six hours post-infection. These increases in neoblast proliferation are preceded by the adherence of *C. albicans* cells to the host epithelial cell surface. Furthermore, the presence of *C. albicans* elicits a reaction in planarians that resembles a regenerative response, including an increase in expression of the early growth response gene *Smed-egr2 (egr2)*, and an immediate wound response via the ERK signaling pathway (Owlarn et al., 2017; Wenemoser et al., 2012; Wurtzel et al., 2015). We also found that the host response is mediated by the *C. albicans* transcriptional regulator Bcr1 and its downstream cell surface protein Als3, which are key players in fungal cell adherence. Finally, we also observed that upon infection of planarians with *C. albicans*, there is an increase in expression of components of the Dectin signaling pathway within the host innate immune system, and an increase in the expression of host markers of neoblast subpopulations as well as markers of the nervous and excretory systems. Together, our findings demonstrate that the initial adherence of fungal cells to the epithelial surface of the planarian host elicits a multi-system response in the host involving the innate, excretory, and nervous systems, as well as neoblast function.

Materials and Methods

Planarian culture

The planarian colony used for all assays was CIW4, an asexual *Schmidtea mediterranea* strain. The colony was maintained as previously described (Oviedo et al., 2008b).

Microorganisms

The *Candida albicans* wildtype strain SN250, a derivative of the clinical isolate SC5314, was used as the isogenic wildtype strain along with the *bcr1* mutant strain (TF137) (Homann et al., 2009); the *als1* mutant strain and the *als3* mutant strain (Nobile et al., 2008); and the triple *sap1/sap2/sap3* mutant strain and the triple *sap4/sap5/sap6* mutant strain (Felk et al., 2002). All *C. albicans* strains were grown from cryogenic stocks on yeast extract peptone dextrose (YPD) agar for 48 hours at 30°C. Single colonies were then inoculated into liquid YPD media and grown for 12-6 hours at 30°C for use in the infection assays.

Infection assays

Ten animals were kept in 6mL wells with 4mL of planarian water (media) containing 25 million *C. albicans* cells per milliliter as previously described (Maciel et al., 2019). The animals were kept in the infected media until collected for specific experiments at various timepoints. The infection assay took place at 25°C, the preferred temperature for planarians, for the duration of the experiments.

C. albicans CFU measurements

Planarians were collected at the indicated timepoints during the infection and rinsed with planarian media. The animals were homogenized in 500µL of planarian media and diluted in 10mL of planarian media. After homogenization, 300µL of the homogenate was plated onto YPD media agar plates containing a cocktail of broad-spectrum antibiotics. The colonies were counted to obtain CFUs after being incubated at 30°C for 48 hours.

Protein extractions

Animals were washed thoroughly prior to being placed in 1.5mL centrifuge tubes. Approximately 150µL of 1X RIPA Buffer (Cell Signaling Technologies, 9806) containing protease inhibitors (Complete Mini Protease Inhibitor Cocktail (Roche, 04693124001); Halt Phosphatase Inhibitor cocktail (Thermo Scientific, 1862495), 1mM PMSF, 1mM DTT) was added to each tube. Samples were placed on ice and homogenized using a motorized pestle (Fisher,12-141-361) for 10 seconds, followed by a 45-minute incubation. Samples were then centrifuged at 20,817 g for 20 minutes at 4ºC. Approximately 100µL of supernatant was transferred into a clean tube and placed on ice. Protein concentrations were determined using a Bradford protein assay (VWR, E530-1L). In a 1.5mL tube, 50µg of supernatant was mixed with 6X Laemmli buffer (6%SDS,9% β-mercaptoethanol,4.8% glycerol, 0.03% bromophenol blue, 375mM Tris-HCl) and was heated to 94ºC for 10 minutes to denature and reduce the proteins.

Western blots

Protein lysate mixtures were loaded onto a 15% SDS-PAGE gel along with a molecular weight marker (Thermo Scientific, 26619). Samples were transferred to a methanol-activated PVDF membrane (Bio-Rad,162-0175) for 2 hours at 55 V in a 1X Tris-glycine transfer buffer (25mM Tris base, 192mM glycine,10%(v/v) methanol) on ice. The membrane was blocked with 5% BSA in TBST (20mM Tris-base, pH 7.6, 140mM NaCl, 0.1%Tween-20) for 2 hours at room temperature and incubated with the primary antibody dilutions for 16 hours at 4ºC on a rocker. The primary antibodies used were anti-actin (1:3000; Developmental Studies Hybridoma Bank, JLA20), anti-pERK (1:400; a gift from the Bartscherer lab) (Owlarn et al., 2017). The primary antibody was removed and the membrane was washed with TBST four times (5 minutes each wash), before the addition of the secondary antibody: goat-anti-rabbit HRP IgG antibody (1:10,000; Abcam, ab7097) for anti-ERK, goat-anti-mouse HRP IgG antibody (1:10000; Invitrogen, G-21040) for anti-actin. Secondary antibody dilutions were incubated for 1 hour at room temperature with 5% non-fat milk in TBST-SDS (20mM Tris-base, pH 7.6, 140mM NaCl, 0.2%Tween-20, 0.01% SDS). The membrane was washed with TBST four times (5 minutes each wash), followed by two 5-minute washes with 1X PBS. The addition of the HRP chemiluminescence substrate (Millipore, WBLUF0100A) allowed for the detection of a signal. For the removal of primary antibody pERK, blots were stripped for 15 minutes (Thermo Scientific, 21059) and washed with 1XPBS four times (5 minutes each wash) before blocking for actin. Band intensities were quantified by computing the area under the curve using imageJ software bundled with Java (Schneider et al., 2012). pERK activity was then normalized to actin. Blots were imaged using a ChemiDoc gel imaging system (Bio-Rad).

Whole-mount immunofluorescence

Non-infected and infected planarians were sacrificed with 5.7% 12N HCL solution for 5 min and fixed using Carnoys solution for 2h on ice. After the fixation, animals were stored in methanol at -20°C and then bleached overnight in a 6% H_2O_2 solution. Animals were then rehydrated in dilutions of methanol:PBSTx and stained as previously described (Ziman et al., 2020). The primary antibody used was α-H3p, 1:250 (Millipore Cat# 05–817R). The secondary antibodies used were goat anti-rabbit Alexa568, 1:800 (Invitrogen Cat# 11036) and HRP-conjugated goat anti-rabbit antibody (Millipore Cat# 12-348).

To stain *C. albicans* cells, infected animals were sacrificed in 10% NAC diluted in PBS. The planarians were then fixed in 4% formaldehyde in PBTx and permeabilized in 1% SDS. The animals were bleached in 3% H_2O_2 in 1X PBS. The primary antibody used was anti-*Candida*, 1:500 (ThermoFisher Cat# PA1-27158). The secondary antibody used was goat anti-rabbit Alexa568, 1:800 (Invitrogen Cat# 11036).

RNAi treatments

Double-stranded RNA (dsRNA) was synthesized as previously described (Oviedo et al., 2008a). The dsRNA was applied via microinjections in three consecutive days with a fourth injection a week after the last injection. The animals were starved for at least one week prior to the dsRNA injections.

Whole-mount fluorescence *in situ* hybridizations

Riboprobes were made using T3 and T7 polymerases and a digoxigenin-labeled ribonucleotide mix (Roche, Cat.# 11277073910) using PCR templates, as described previously (Pearson et al., 2009). *In situ* hybridization with and without fluorescence was performed as described previously (King and Newmark, 2013).

Imaging and data processing

Area measurements and cell counts were calculated using imageJ software bundled with Java (Schneider et al., 2012). The Nikon AZ100 Multizoom microscope was used to collect the digital images and the images were processed using NIS Elements AR3.2 software (Nikon). Photoshop software (Adobe) was used to adjust contrast and brightness.

Gene expression analyses

RNA was extracted using TRIzol (Thermo Fisher Scientific). qRT-PCR reactions were performed using SYBR Green Master Mix in a 7500 Fast Real Time PCR cycler (Applied Biosystems). The TATA-box-binding protein domain gene was used as the internal control. Each experiment was performed in triplicate for each timepoint. qRT-PCR was performed as previously described (Peiris et al., 2012).

Cell-free filter sterilized planarian media

The media from six hpi infection assays was removed from the planarian wells and filter sterilized using a Corning vacuum system with 0.22µm pore-size 13.6cm² PES Membrane (Cat. # 431153). Uninfected animals were then inoculated with the cell-free filter sterilized media using the infection assay described above.

Statistical analyses

Two-way ANOVA or t-test statistics were performed as indicated in the figure legends, and data are shown as the mean ± SEM or fold change ± SEM unless otherwise noted. Logrank statistical tests were performed for the survival curves as indicated in the figure legends. All statistics were performed by pooling biological replicates from each technical replicate using Prism7, Graphpad software Inc. (http://www.graphpad.com).

Results

Adherence of C. albicans to the planarian epithelial surface initiates an early wound-like response

To assess the host response to *C. albicans* infection, planarians were infected with a lethal concentration (25 X10^6 cells/mL) of *C. albicans* cells via soaking as previously described (Maciel et al., 2019). Our prior results demonstrated that planarians responded to fungal infection with an increase in mitotic activity; however, it was unclear when during the infection this fungal-induced proliferative response was occurring (Maciel et al., 2019). To address this, we infected planarians with *C. albicans* and analyzed host mitotic activity temporally over the course of 48 hours (**Figure 1A-B**; see **Supplemental Figure 1** for descriptions of the spatial orientations of the planarian whole mounts). We observed no host behavioral or macroscopic differences within the first 48

hours post-infection (hpi) between the uninfected and experimental groups. We did, however, detect two host mitotic peaks occurring at six and 24 hpi, which were preceded by decreased mitotic activity (**Figure 1B**). The largest burst in mitotic activity (~150%) was observed six hpi, and was spatially increased throughout the planarian body, suggesting a system-wide neoblast response (**Figure 1A-B**).

To determine if the mitotic response was due to the adherence or penetration of *C. albicans* cells through the epithelial surface of planarians, we focused on early timepoints of one, three, and six hpi. These experiments revealed that as early as one hpi, *C. albicans* cells began to adhere to the planarian epithelial surface and that by six hpi, the fungal cells were readily clustered throughout the epithelial surface (**Figure 1C**). The increased presence of *C. albicans* cells on the planarians during these early timepoints was determined by macerating planarians and measuring colony forming units (CFUs) after plating the homogenized slurry on agar plates. We found that in the first six hpi, the number of *C. albicans* cells increased by two orders of magnitude (i.e. ~10 CFUs at one hpi compared to ~1,000 CFUs at six hpi) (**Figure 1D**). To discern if the increase in *C. albicans* cells during this early infection was limited to the superficial adherence of *C. albicans* cells to the host epithelial surface or to penetration of *C. albicans* cells into host tissues, we performed immunostaining on transverse cross sections using an antibody specific for *C. albicans*. We found that *C. albicans* cells were adhered to the epithelial surface of the planarians, while no *C. albicans* antibody signal was detected in deep tissues even after six hpi (**Figure 1E and Supplemental Figure 2**). This finding is consistent with previous results demonstrating that *C. albicans* cells attach to the host epithelial surface during early timepoints of infection (Maciel et al., 2019).

In response to physical injury, planarians have been shown to elicit a regenerative mitotic response occurring six hours post-injury (Saló and Baguñà, 1984; Wenemoser and Reddien, 2010). Due to the apparent overlap between injury stimuli (i.e. the presence of penetrating *C. albicans* hyphal cells) and the mitotic response observed during the early infection period (six hpi), we hypothesized that invading *C. albicans* hyphal cells could trigger a wound response in planarians during early infection.

In response to injury in planarians, the planarian extracellular regulated kinase (ERK) becomes activated via phosphorylation (pERK), and is among the earliest events essential for regeneration initiation, occurring ~15 minutes post-injury (Owlarn et al., 2017). We, therefore, measured pERK protein levels at early infection timepoints using a western blot and found that there was a gradual increase in pERK levels, as early as 30 minutes post-infection (~threefold increase), which increased to about fourfold by six hpi (**Figure 1F**). We also measured the post-infection expression levels of *Smed-runt1 (runt1)* and *Smed-egr2* (*egr2*), two early wound response genes in planarians (Owlarn et al., 2017; Sandmann et al., 2011; Wenemoser et al., 2012; Wurtzel et al., 2015). We observed an increase in the expression of *egr2* (~twofold) over the first 48 hpi (**Figure 1G**). Intriguingly, *runt1* was either slightly reduced or showed no changes in expression over the first 48 hpi (**Figure 1G**). Overall, these results suggest that the adherence of *C. albicans* cells to the epithelial surfaces of planarians shortly after infection triggers a regenerative wound-like response in the host.

Als3 and Bcr1 are required for C. albicans to adhere to and colonize the planarian epithelial surface

Next, we sought to test if the dynamics of infection in planarians can be modulated by genetically disrupting *C. albicans* virulence factors known to contribute to disease progression in the host (Mayer et al., 2013). Based on our observations that *C. albicans* adheres to planarians in order to invade them, we decided to focus our attention on *C. albicans* virulence factors associated with adherence and biofilm formation. The agglutinin-like sequence (Als) family of cell surface glycoproteins are important *C. albicans* adhesins that mediate cell-cell and cell-substrate adherence (Hoyer and Cota, 2016). Two major Als proteins that are known to be important for adherence and biofilm formation are Als1 and Als3 (Nobile et al., 2006; Nobile et al., 2008). In addition to these two adhesins, a known master regulator of *C. albicans* biofilm formation that controls the expression of *ALS1* and *ALS3* is Bcr1 (Nobile et al., 2006; Nobile et al., 2012; Nobile and Mitchell, 2005). We, therefore, assessed the ability of the *als1*, *als3*, and *bcr1* mutant strains to infect planarians. In addition to adherence, *C. albicans* also produces secreted aspartyl proteases (Saps) that are important virulence factors in causing host cell damage (Mayer et al., 2013; Naglik et al., 2003). We, therefore, also assessed the ability of two triple deletion *SAP* mutant strains (encompassing the major *SAPs* involved in virulence), the *sap1/sap2/sap3* and the *sap4/sap5/sap6* mutant strains, to infect planarians.

To our surprise, we found that all *C. albicans* mutant and wildtype strains were capable of adhering to the planarian epithelial surface. Qualitatively, it appeared that the *als3* and *bcr1* mutant strains displayed less adherence six hpi (**Figure 2A**). Consistent with this observation, our results revealed an increase in the survival of planarians when they were infected with lethal doses of the *als3* and *bcr1* mutant strains compared to the wildtype strain (**Figure 2B**). Additionally, we discovered that when the *C. albicans* wildtype strain came into contact with planarians, there was a dramatic increase in the gene expression levels of *ALS1, ALS3,* and *BCR1* (**Figure 2C**). Overall, these results suggest that fungal cell adherence mediated by the adhesin Als3 and the transcription factor Bcr1 is important for *C. albicans* to colonize the epithelial surface of planarians during an infection.

Als3 and Bcr1 are important players in the planarian mitotic response to C. albicans infection

Given the importance of *C. albicans* Als3 and Bcr1 for colonization of the planarian epithelial surface, we next wanted to know whether Als3 and Bcr1 play roles in the mitotic response of planarians. We found that six hpi, all *C. albicans* mutant and wildtype strains were capable of mounting a mitotic response in planarians; however, infection with the *als3* and *bcr1* mutant strains displayed significantly less mitotic proliferation in planarians than that of the wildtype strain (**Figure 3A-B**). These findings indicate that Als3 and Bcr1 are involved in mediating the planarian mitotic response.

Since secreted aspartyl proteases (Saps) are important *C. albicans* virulence factors, we hypothesized that Saps may play roles in the planarian host response to *C. albicans* infection. To address this, we exposed planarians to cell-free filter sterilized media obtained from six hpi infection assays and measured planarian mitotic activity. We

found no difference in the mitotic activity between uninfected animals and the group exposed to filter sterilized media from the infection assays, suggesting that the increase in cell proliferation upon *C. albicans* infection requires the physical presence of the pathogen (**Figure 3C-D**). Altogether, these findings suggest that adherence of *C. albicans* cells to the planarian epithelial surface, rather than fungal secreted proteases, induces the host mitotic response during the early hours post-infection.

The planarian Dectin signaling pathway modulates the early mitotic response to and the clearance of the fungal infection

In order to understand how *C. albicans* cells interact with the planarian host to trigger hyper-proliferation, we decided to explore the initial molecular response mediated by the planarian innate immune system upon infection. Our previous work showed that the expression of genes in the host Dectin signaling pathway are generally upregulated one day post-infection with *C. albicans* (Maciel et al., 2019). To expand on these initial findings, we screened, by qRT-PCR, an expanded list of host genes encoding components of the Dectin signaling pathway, including the gene encoding the upstream SYK adapter protein *Smed-syk (syk)* and downstream gene components *Smed-card* (*card*), *Smed-bcl* (*bcl*), *Smed-malt1* (*malt1*), *Smed-tab1* (*tab1*), and *Smed-tak* (*tak*) during early infection with *C. albicans* (Wagener et al., 2018). We found that as early as three hpi there was a significant increase in the expression of all genes assayed of the Dectin signaling pathway, and that the upregulation of each gene was generally sustained for 48 hpi (**Figure 4A**). Notably, the upstream gene *syk* displayed an increase in expression (~threefold) in the first 6-12 hpi. We confirmed this upregulation in expression using fluorescent *in situ* hybridization (FISH) with a probe against *syk*, which revealed *syk* expression throughout the animal with enrichment in the digestive system including the pharynx of uninfected planarians (**Figure 4B**). In the infected planarians, we observed that *syk* expression became more prominent with the appearance of punctate foci readily visible in the main intestinal branches close to the brain at six hpi (observed in 6 out of 8 animals).

To assess the role of the Dectin signaling pathway in the planarian innate immune response, we disrupted *syk* function with RNA-interference (RNAi) and evaluated the planarian mitotic response and ability to clear the *C. albicans* infection. Intriguingly, downregulation of *syk* by RNAi led to a slight increase (13%) in mitotic activity when compared to the infected control at six hpi (**Figure 4C-D**). Loss of *syk* function by RNAi resulted in planarians having a reduced capacity to clear the infection, even after 12 days post-infection (**Figure 4E-F**). Taken together, these findings suggest that the Dectin signaling pathway is an important mediator of the innate immune response in planarians that modulates the early mitotic response to fungal infection and is required for proper clearance of the infection.

C. albicans infection triggers a heterogenous transcriptional response across planarian neoblast subpopulations

Recent work suggested a model in which transcriptomic changes occur in planarian neoblasts as they adopt new cellular identities necessary to support cellular

turnover and tissue regeneration (Zeng et al., 2018). To gain a deeper understanding of how neoblast dynamics are affected in response to *C. albicans* infection, we screened, by qRT-PCR, planarian genes encoding neoblast markers for various subpopulations of progenitor cells. We measured changes in gene expression for six different neoblast lineages at different timepoints post-infection, and our results revealed that transcriptomic changes were not homogenous throughout the neoblast subpopulations. We discovered an upregulation in the expression of several neoblast subclasses detected as early as three hpi and this upregulation was maintained for the next 48 hpi (**Figure 5A**). The neoblast lineages measured represent twelve neoblast clusters of cells (NB1-NB12) and are ordered based on *Smed-piwi-1 (piwi-1)* transcript levels; thus, NB1 expresses higher levels of *piwi-1* relative to NB12 (Zeng et al., 2018). We found that in the first 24 hpi there was a reduction in the expression of *Smed-tspan-1 (tspan-1)*, which defines the NB2 subpopulation that contains the pluripotent clonogenic neoblasts (cNeoblasts) (**Figure 5A**) (Wagner et al., 2011; Zeng et al., 2018). The expression of *tspan-1* was slightly increased at 48 hpi. We also found that expression of the *Smed-lmo3 (lmo3)* marker (NB3) was consistently downregulated throughout the first 48 hpi. Overall, we detected the highest expression levels associated with markers of the neoblast subpopulations NB9, NB11, NB4, NB7, and NB5. The absolute highest expression levels were detected with the marker *Smed-pou2-3* (*pou2-3*) (NB9), which gradually increased after three hpi and peaked more than eightfold at 48 hpi (**Figure 5A**). High expression levels were also detected for markers *Smed-ston2* (*ston2*) and *Smed-pdch11 (pdch11)* (NB11 and NB4, respectively). Of note, *Smed-pj-1b (pj-1b)* of the NB4 neoblast subpopulation was the only marker whose expression increased dramatically while the other eight NB4 markers remained close to the levels of the control (**Figure 5B**). Altogether, these results suggest that infection with *C. albicans* triggers a heterogenous transcriptional response across planarian neoblast subpopulations.

C. albicans infection modulates transcription within neuronal clusters and the excretory system of planarians

To further examine how increased gene expression levels of the NB9 and NB11 neoblast subpopulations impact the planarian response to infection with *C. albicans*, we extended our analysis to include markers of downstream planarian progenitor cells that lead to differentiated cells in the excretory and nervous systems (Rink et al., 2011; Ross et al., 2017; Zeng et al., 2018). The early progenitors of the nervous (NB11) and excretory (NB9) systems were upregulated during infection with *C. albicans* (**Figure 5A**), suggesting that the planarian infection response goes beyond neoblast subpopulations to also involve post-mitotic committed cells.

The planarian central nervous system is comprised of distinct neural subtypes, labeled by the synthesis of neurotransmitters that they produce (Ross et al., 2017). These subtypes include dopaminergic, octopaminergic, GABAergic, serotonergic, and cholinergic neurons, which all have specific markers (*Smed-th (th), Smed-tbh (tbh), Smed-gad (gad), Smed-tph (tph),* and *Smed-chat (chat)*, respectively). We wanted to understand how the increase in expression of NB11 upon *C. albicans* infection would impact the different neuronal subpopulations. To explore this idea, we measured the expression levels, by qRT-PCR, of these six neural markers at both 12 and 24 hpi, which

were the timepoints where the expression of NB9 and NB11 were highest (**Figure 5A**). We found that the expression levels for two neuron subtypes, *tbh* and *chat* were increased when compared to uninfected animals, with *chat* displaying the highest levels of upregulation (**Figure 6A**). We confirmed our qRT-PCR results by performing FISH using a *chat* probe, which is expressed along the brain and the ventral cords in uninfected planarians. The *chat* signal was found to be increased in the brain of infected planarians starting at three hpi (**Figure 6B**). We also found that the increase in expression of *chat* and the downstream adapter gene *syk* of the Dectin signaling pathway can be detected as early as 15-30 minutes post-infection and remains elevated for the first three hpi (**Figure 6C**). The expression levels of both *chat* and *syk* were observed to increase at 30 minutes and continued to increase in the later timepoints of infection.

To determine how the planarian excretory system is impacted by the increase in NB9 expression, we measured markers of the protonephridia, and more specifically, markers of the flame cells that contribute to electrolyte balance and mucus production, among other excretory functions (Rink et al., 2011). We observed an increase in expression of the markers of collecting ducts *Smed-slc9a-3 (slc9a-3),* distal tubules *Smed-slc4a-6 (slc4a-6)*, and proximal tubules *Smed-slc6a-13 (slc6a-13)* during the first 24 hpi (**Figure 6D**). We validated these results by performing FISH using a *Smed-inx10 (inx10)* probe (Oviedo and Levin, 2007)*,* which confirmed a steady increase in *inx10* expression throughout the planarian body at 24 hpi (**Figure 6E**). Overall, these findings suggest that both the nervous and excretory systems contribute to the host response during fungal infection.

Discussion

Here, we show that the planarian infection model is an advantageous animal model system to study host-pathogen interactions, particularly at early stages of epithelial infection with *C. albicans*. Our results demonstrate that the superficial adherence of *C. albicans* to the planarian epithelial surface triggers a multi-system host response involving stem cells and differentiated tissues in the planarian **(Figure 7)**. These findings underscore the advantages of analyzing host-pathogen interactions *in situ* as a powerful paradigm to identify crosstalk between tissues during fungal infection. Planarians are thus a unique model system to study the host response to infection at the organismal level, a feature that is largely missing from or challenging to achieve with existing animal models of infection.

Our results reveal that part of the host response to *C. albicans* epithelial infection involves a stereotypical pattern of neoblast division. Two systemic peaks of neoblast division occur at six and 24 hpi without visible signs of host tissue damage. This is intriguing because physiological increases in neoblast division generally occur via metabolic inputs such as nutrient availability through feeding or in response to tissue injury (Peiris et al., 2012; Saló and Baguñà, 1984; Wenemoser and Reddien, 2010). The metabolic-induced neoblast hyperproliferation is systemic and tends to peak around 6-12 hours post-feeding to gradually return to pre-feeding levels by 48 hours (Baguñà, 1976; Kang and Sánchez Alvarado, 2009). The amputation-induced neoblast division takes place as biphasic peaks that occur first systemically at six hours post-amputation and then localized near the injury site between 48-72 hours following wounding (Wenemoser

and Reddien, 2010). Our findings suggest that similar to amputation, fungal infection triggers neoblast proliferation by six hpi and it is preceded by ERK phosphorylation and the overexpression of the early wound response gene *egr2* (Owlarn et al., 2017; Wenemoser and Reddien, 2010). However, the neoblast response to *C. albicans* infection does not involve an increase in the expression of *runt1*, which is important for regeneration, and is not triggered by major tissue damage as observed by amputation or the resulting distention of the intestine that occurs after feeding. Furthermore, the second mitotic peak observed after fungal infection is systemic and occurs roughly 18 hours after the initial peak. Another striking difference with the regeneration response is that cell death does not precede the neoblast increase in proliferation during the early stages of infection (**Supplemental Figure 3**). Therefore, the mitotic response to fungal infection differs in time and location with respect to other stimuli such as feeding and amputation. We speculate that *C. albicans* infection activates an infection-specific neoblast proliferative response.

The neoblast hyper-proliferative response is preceded by the adherence of *C. albicans* to the planarian epithelial surface. This response is dependent on the *C. albicans* transcription factor Bcr1, which is a major regulator of adherence, and its downstream GPI-anchored protein Als3, which is an important adhesin. Although the precise molecular mechanisms leading to hyper-proliferation in planarians in response to infection with *C. albicans* are unclear, it seems likely that the ability of *C. albicans* to adhere to the planarian epithelial surface is a critical factor in inducing this response. Given that Bcr1 controls the expression of numerous genes encoding cell wall proteins, and is a major regulator of biofilm formation (Nobile et al., 2006; Nobile et al., 2012; Nobile and Mitchell, 2005), it seems likely that the ability to colonize and form biofilms on the planarian epithelial surface are important fungal processes in initiating this hyper-proliferative host response. Als3 is also required for biofilm formation in *C. albicans* and is a major adhesin expressed on the surface of hyphal cells. Thus, the ability to form adhesive hyphae that can actively penetrate host epithelial cells is another factor that likely mediates this host response (Wachtler et al., 2011). Interestingly, Als3 is known to bind to mammalian host ligands, such as E-cadherin on epithelial cells and N-cadherin on endothelial cells (Phan et al., 2005; Phan et al., 2007), which induces engulfment of fungal cells into the mammalian host via a clathrin-dependent mechanism. Given the importance of Als3 in triggering hyper-proliferation in planarians, it is possible that the process of host cell engulfment of *C. albicans* cells is also involved in initiating this host response. Taken together, it seems likely that both invasion mechanisms of induced endocytosis as well as active penetration of *C. albicans* cells are complementary mechanisms that induce hyper-proliferation of the planarian host.

Adherence of *C. albicans* cells to the planarian epithelial surface is followed by a generalized overexpression of host genes associated with the Dectin signaling pathway. In our previous work (Maciel et al., 2019), we also observed an overexpression of components of the Dectin signaling pathway (*syk* and *tak*) at later timepoints of the infection, suggesting that there is a consistent innate immune response in planarians during fungal infection. A key component of the Dectin signaling pathway is the adaptor protein SYK, and our results show a persistent overexpression of the *syk* gene throughout infection with *C. albicans*. Unexpectedly, functional disruption of *syk* by RNAi resulted in a slight increase in neoblast proliferation upon *C. albicans* infection. It remains unclear why this apparent increase in neoblast proliferation is accompanied by a delayed clearance of the infection. It is possible that this is due to redundancy with

other pathways or to an aberrant neoblast response to infection. Nonetheless, these findings suggest that *syk* is required for planarians to clear the *C. albicans* infection in a timely manner. Overall, although Dectin signaling is activated in planarians upon fungal infection, the canonical Dectin-1 C-type lectin pattern-recognition receptor has not yet been identified in planarians and planarian species are known to have a number of C-type lectin-like proteins (Gao et al., 2017; Shagin et al., 2002). Thus, additional experiments will be needed to identify potential pattern-recognition receptors in planarians, and to understand the molecular cascade and the evolutionary conservation of the Dectin signaling pathway in planarians with other animals, including humans and other vertebrates.

As part of the host response to *C. albicans* infection, we observed a differential expression in planarian markers of neoblast subpopulations. This finding suggests that not all neoblasts are engaged in the response to fungal infection and opens up the possibility of an infection-specific neoblast response. Recent work classified neoblast subpopulations based on the content of the marker *piwi-1* (Zeng et al., 2018). This study found that neoblasts with high *piwi-1* content (*piwi-1*^{high}) include the clonogenic neoblasts (cNeoblasts) that together are key players in the early regenerative response (Zeng et al., 2018). However, we found that the expression of markers for neoblast subpopulations with the highest content of *piwi-1* (e.g., NB1, NB2, and NB3) remained relatively low or did not change relative to the uninfected group in the initial 48 hpi. Instead, there was a dramatic increase in the expression of markers for neoblasts with reduced levels of *piwi-1* (i.e., NB4, NB5, NB7, NB9, and NB11) upon *C. albicans* infection. From these neoblasts subclasses NB9, NB11, and NB4 displayed the highest expression levels, greater than eightfold in the initial 48 hpi. These three neoblast subpopulations contain less than 5% of *piwi-1* positive cells (Zeng et al., 2018). This finding raises the possibility that the active cycling of neoblasts with low *piwi-1* content could be responsible for the two mitotic peaks we observed during infection with *C. albicans*. We propose that *C. albicans* infection elicits a heterogenous neoblast response that is distinct from that observed during regeneration.

Our findings indicate that the host response to *C. albicans* infection involves transcriptional changes in distinct differentiated planarian tissues. The upregulation in the expression of genes associated with the excretory system is detected as early as 12 hpi. The increased expression of genes within the excretory system may lead to an enhanced secretion of mucus, which contains antimicrobial components to aid in the elimination of the pathogen. The mucus barrier in planarians contains an extensive array of antimicrobial peptides, zymogens, and proteases that aid in the innate immune response to infection (Bocchinfuso et al., 2012). Likewise, the dramatic increase in the expression of the marker of cholinergic neurons (*chat*) in the initial 24 hpi, suggests that this neuronal type may be important in the innate immune response to *C. albicans* infection. This is consistent with the idea that certain neuronal groups can act as immunocompetent cells, which is an ancient function conserved between pre-bilaterians and mammals (Godinho-Silva et al., 2019; Klimovich et al., 2020). Indeed, murine nociceptive neurons are critical for the innate immune response to cutaneous infection with *C. albicans* (Kashem et al., 2015). Together, the increase in expression of markers of the neural and the excretory systems in planarians suggests the existence of long-range organismal communication that is likely needed to orchestrate defense against invading pathogens. It is tempting to speculate that the adherence of *C. albicans* cells to the planarian epithelial surface may trigger neural cues that activate the secretion of

mucus with antimicrobial properties and the innate immune response through the Dectin signaling pathway. Future experiments will address the precise interplay between neoblasts, differentiated tissues and the molecular cascade orchestrated by the planarian innate immune system to defend against invading pathogens **(Figure 7)**.

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Conflict of Interest Statement

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

Author Contributions

E.I.M., A.V.A, B.Z., C.J.N., and N.J.O. conceived, designed, and interpreted experiments. E.I.M., A.V.A, and B.Z. performed all experiments, acquired and analyzed data. E.I.M., A.V.A, B.Z., C.J.N., and N.J.O wrote the manuscript. All authors read the manuscript, provided comments and approved the final version.

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Contributions to the Field

Candida albicans is one of the most common fungal pathogens of humans. Here, we analyzed host-pathogen changes to fungal infection using an invertebrate planarian infection model. On the pathogen side, we found that the *C. albicans* transcription factor Bcr1 and its downstream adhesin Als3 are required for fungal colonization of the planarian epithelial surface. On the host side, we found that this colonization triggers a multi-system host response in planarians that is mediated by the Dectin signaling pathway.

Figures

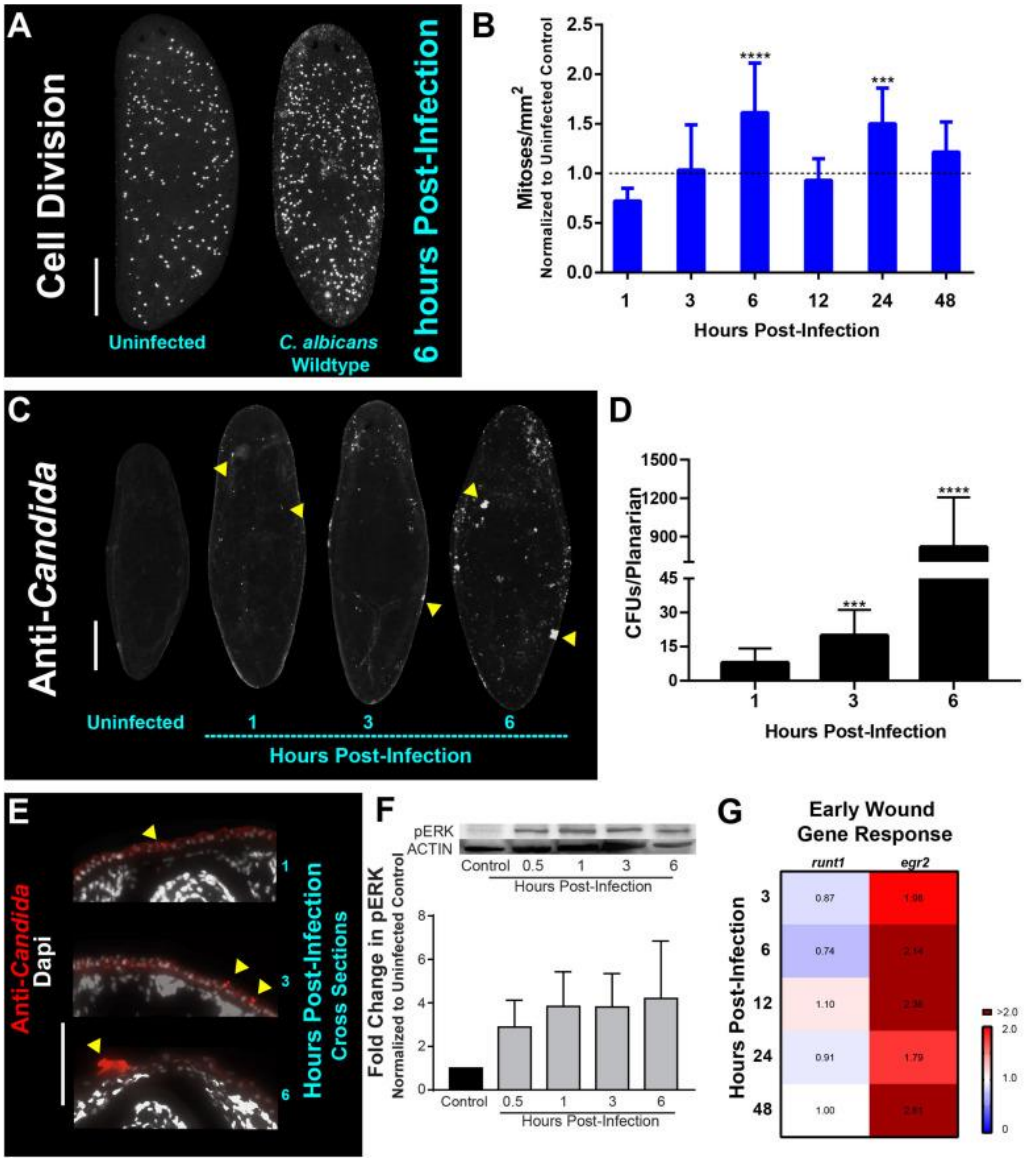


Figure 1. *C. albicans* infection leads to an early wound and mitotic response in planarians. A) Whole-mount immunostaining with anti-phospho-histone H3 (ser10) antibody for planarians, which labels mitotic cells (white foci) at six hours post-infection with the *C. albicans* wildtype strain. B) Number of mitoses in response to *C. albicans* cells between one to 48 hours normalized to an uninfected planarian. C) Whole-mount anti-*Candida* antibody stain at one, three, and six hours post-infection (white foci). Yellow triangles indicate notable *C. albicans* aggregates. D) *C. albicans* colony forming units (CFUs) normalized per planarian. Ten animals per infection timepoint were used. E) Transverse cross section images from the dorsal side of the planarian body showing the adherence of the *C. albicans* wildtype strain (red signal) at one, three, and six hours post-infection. Yellow triangles indicate notable *C. albicans* aggregates. F) Western blot of planarian phosphorylated-ERK (p-ERK) and quantification at different timepoints of a *C. albicans* infection. β-tubulin was used as an internal control. G) Gene expression levels represented in a heat map of wound-induced response planarian genes *runt1* and *egr2* at the different infection timepoints. Gene expression is represented by fold change normalized to an uninfected control. Color scale depicts red as upregulation and blue as downregulation; burgundy demonstrates upregulation greater than twofold. Data were obtained in triplicate per experiment for at least two biological and technical replicates. *C. albicans* infections were performed using 25 million cells/mL. All graphs represent mean ±SEM. Statistical comparisons are against the uninfected control. Scale bar is 200μm. Two-way ANOVA, *P<0.01; **P<0.005; ****P<0.0001.

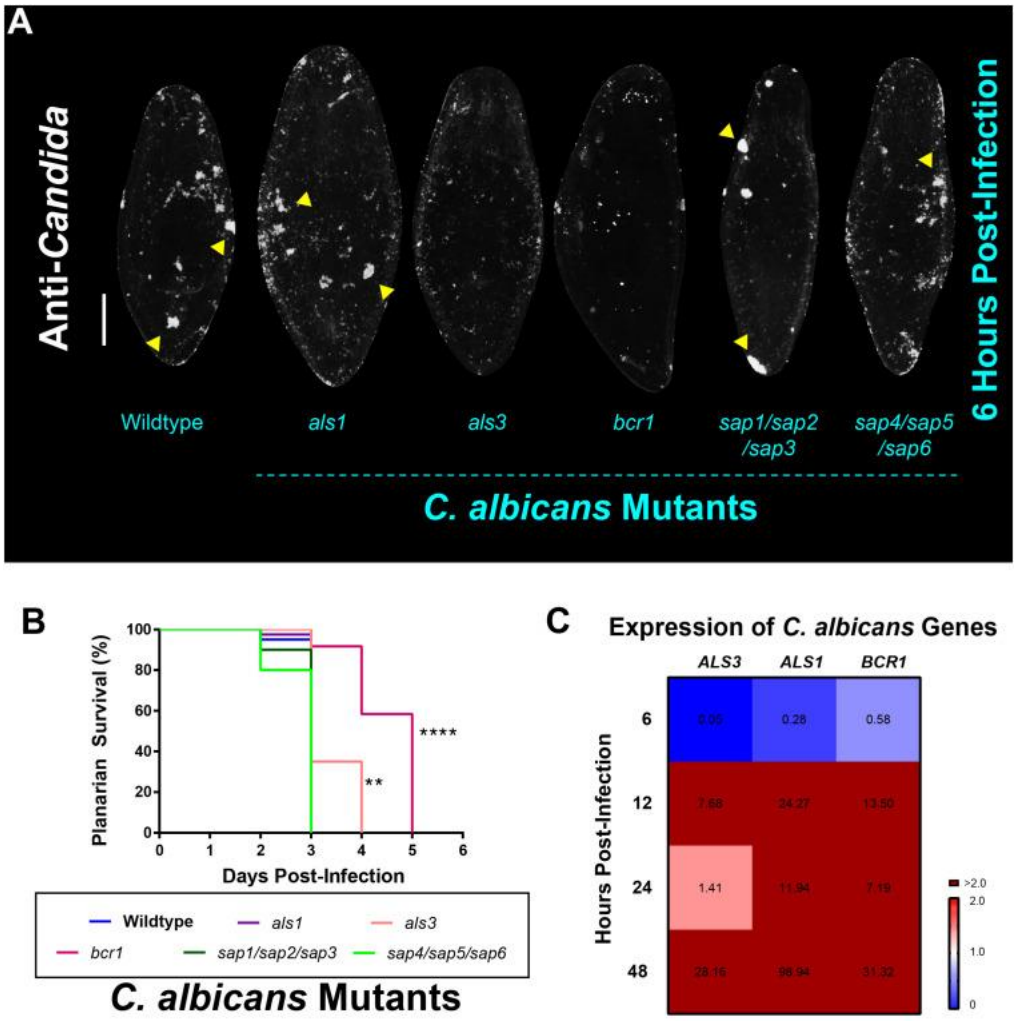


Figure 2. *C. albicans* Als3 and Bcr1 are important for virulence in planarians. (A) Whole-mount anti-*Candida* antibody stain of *C. albicans* mutant strains six hours post-infection. All mutant strains are capable of adhering to the planarian epithelial surface. Yellow triangles indicate notable *C. albicans* aggregates. (B) Planarian survival after infection with 25 million cells/mL of the different *C. albicans* mutant strains at the third day of infection. Logrank test, **P<0.005, ****P<0.0001. (C) Gene expression levels represented in a heat map of *C. albicans* genes *ALS3, ALS1,* and *BCR1* at the different infection timepoints. Gene expression is represented by fold change normalized to an uninfected control. Color scale depicts red as upregulation and blue as downregulation; burgundy demonstrates upregulation greater than twofold. Data were obtained in triplicate per experiment for at least two biological and technical replicates. Scale bar is 200μm.

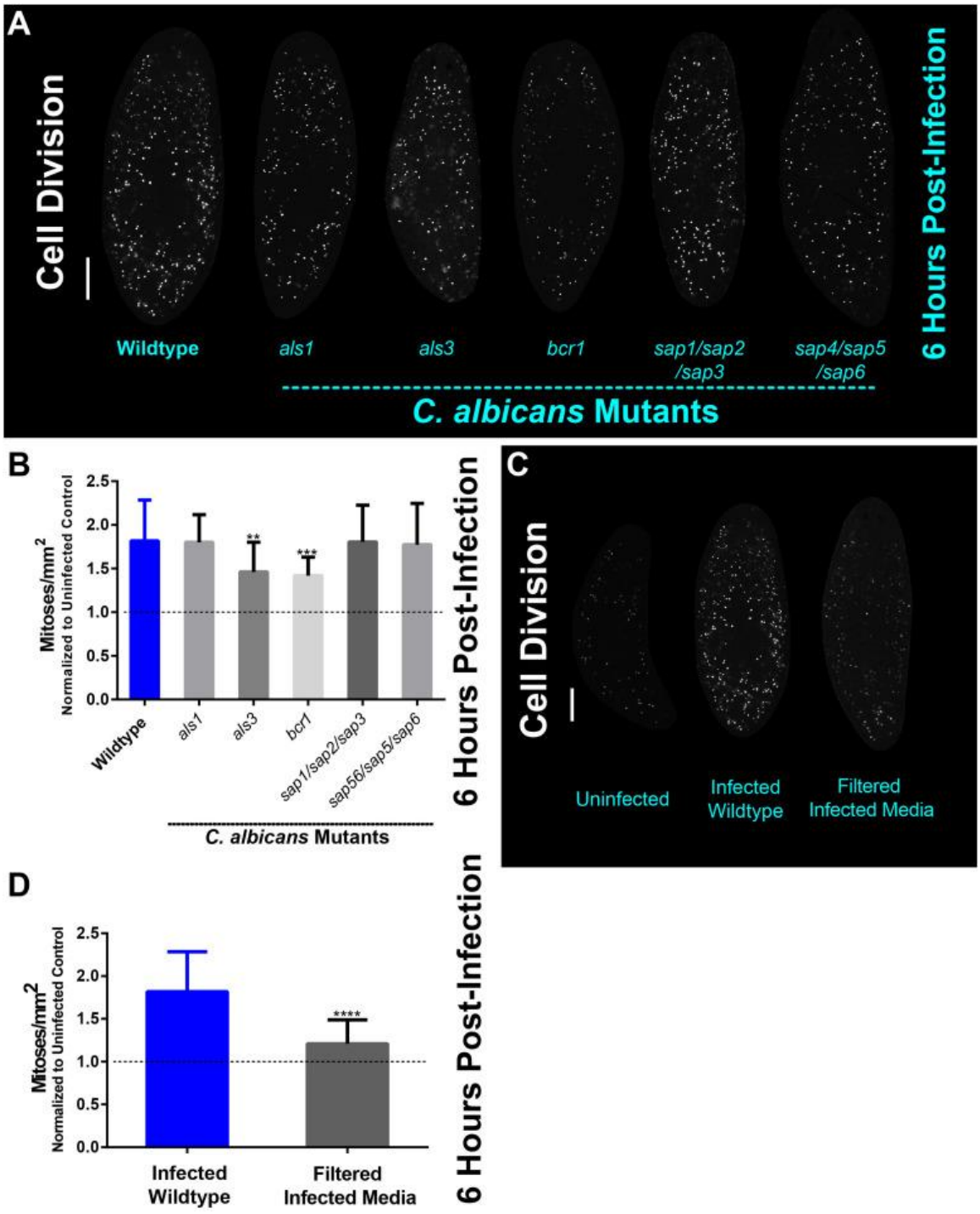


Figure 3. The planarian mitotic response decreases in infections with less virulent *C. albicans als3* and *bcr1* mutant strains. (A) Whole-mount immunostaining with anti-phospho-histone H3 (ser10) antibody for planarians, which labels mitotic cells (white foci) at six hours post-infection with the *C. albicans* mutant strains indicated. (B) Number of mitotic cells in response to *C. albicans* mutant strains normalized to uninfected

planarians at six hours post-infection. C) Whole-mount immunostaining with anti-phospho-histone H3 (ser10) antibody for planarians, which labels mitotic cells (white foci) at six hours post-infection for animals that were soaked in filter sterilized cell-free infected planarian media. (D) Whole mount immunostaining with anti-phospho-histone H3 (ser10) antibody, which labels mitotic cells (white foci) at six hours post-infection normalized to an uninfected control. *C. albicans* infections were performed using 25 million cells/mL. Graphs represent mean ±SEM. All statistical comparisons are against the wildtype strain unless noted with bars. Scale bar is 200μm. Two-way ANOVA, **P<0.005; ***P<0.001; ****P<0.0001.

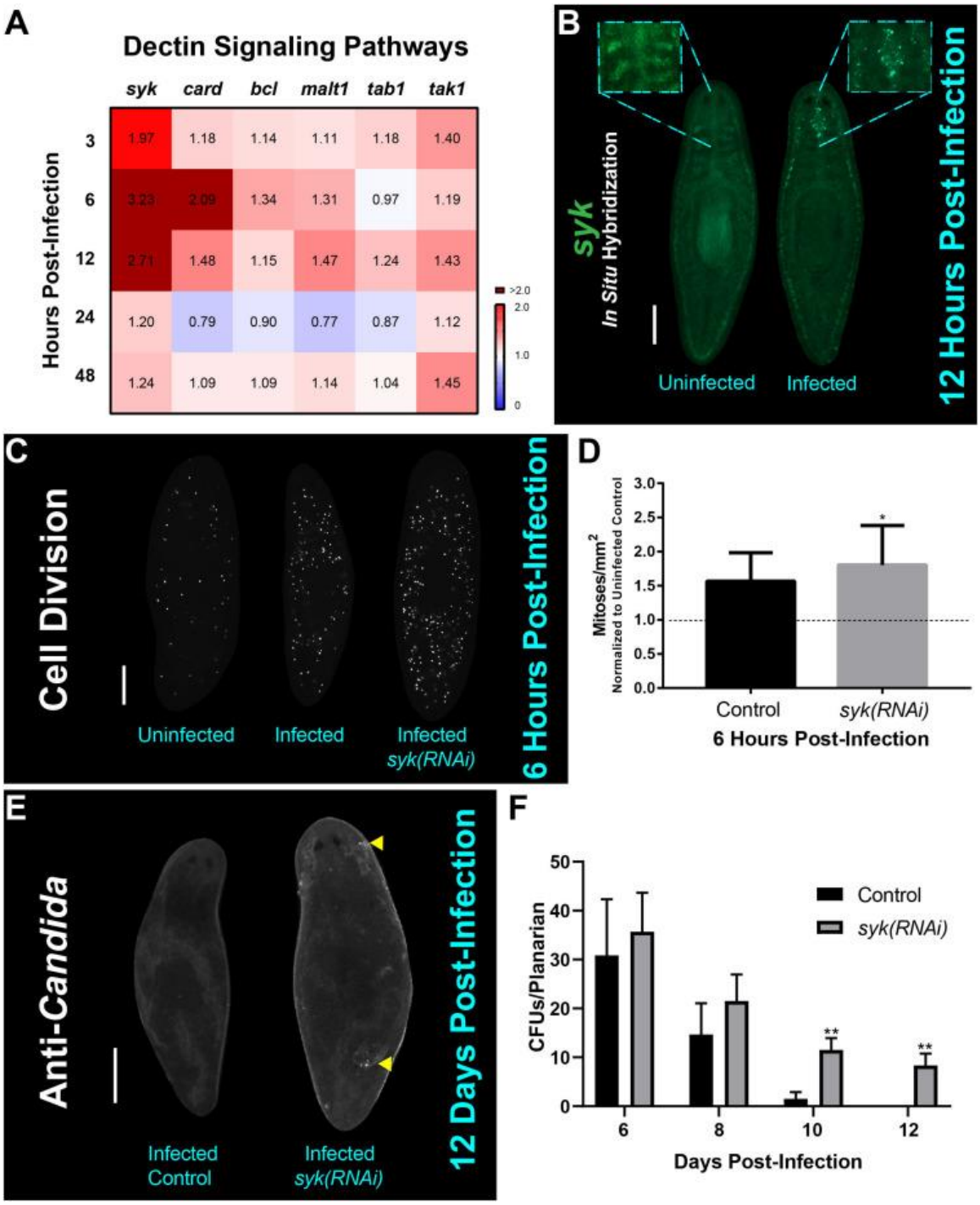
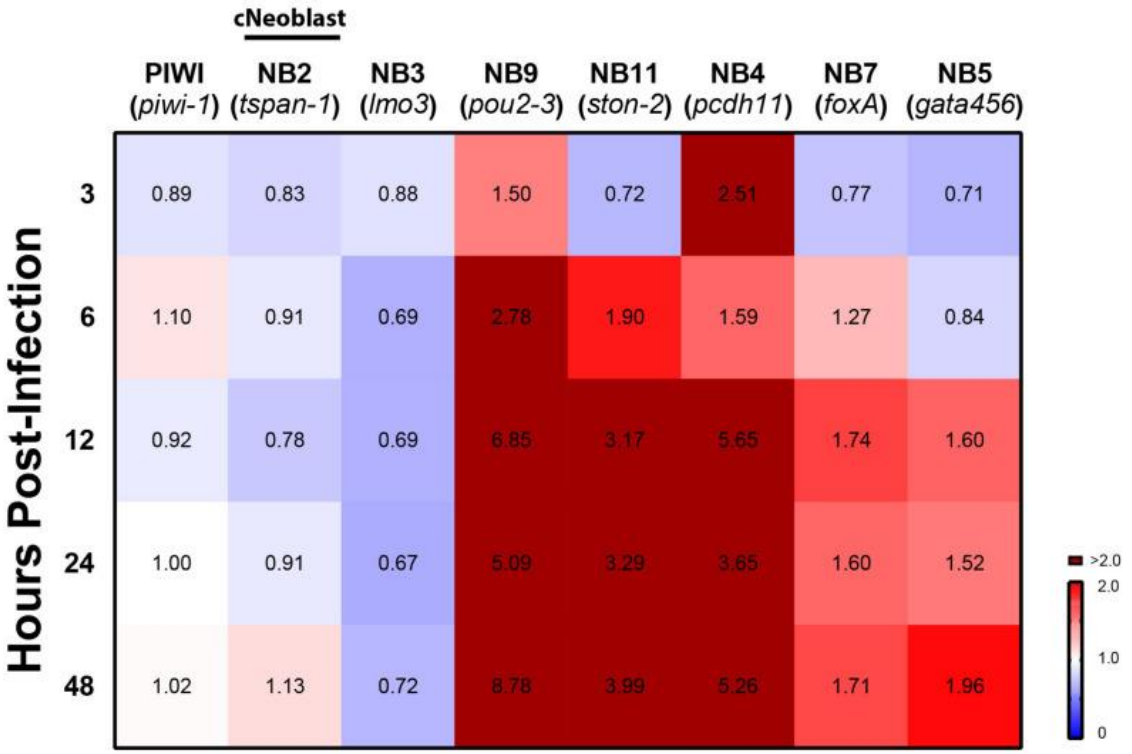


Figure 4. The Dectin signaling pathway plays a role in the host response to and clearance of *C. albicans* infection. (A) Gene expression levels for Dectin signaling pathway homologs at early and late timepoints of *C. albicans* infection using 25 million *C. albicans* cells/mL. Gene expression is represented in a heat map displaying fold change normalized to an uninfected control. Color scale depicts red for upregulation and

blue for downregulation; burgundy demonstrates upregulation over twofold. Data were obtained in triplicate per experiment for at least two biological and technical replicates. (B) Fluorescent *in situ* hybridization showing expression of *syk* in an uninfected and twelve-hour infected animal. The boxed regions are magnifications of representative uninfected and infected animals. This experiment was replicated two times using five animals per experiment. Scale bar is 200μm. Images are representative of eight animals for at least two biological and technical replicates. (C) Whole mount immunostaining with anti-phospho-histone H3 (ser10) antibody, which labels mitotic cells (white foci) at six hours post-infection. (D) Number of mitoses of control infected animal versus *syk(RNAi)* infected animals normalized to an uninfected control at six hours post-infection. (E) Representative images of whole mount anti-*Candida* antibody stain at twelve days post-infection (white foci) of a control animal and *syk(RNAi)* animal. Yellow triangles indicate notable *C. albicans* aggregates. (F) Number of *C. albicans* colony forming units (CFUs) normalized per planarian of infected control versus infected *syk(RNAi)* planarians throughout the course of infection. This experiment was replicated two times using five animals per experiment. 25 million *C. albicans* cells/mL was used for the infections. All graphs represent mean ±SEM. Scale bar is 200μm. T-test, *P<0.01; **P<0.001.

A


Expresssion of Neoblast Markers
cNeoblast
PIWI (piwi-1)
NB2 (tspan-1)
NB3 (lmo3)
NB9 (pou2-3)
NB11 (ston-2)
NB4 (pcdh11)
NB7 (foxA)
NB5 (gata456)
Hours Post-Infection
3
6
12
24
48
0.89 0.83 0.88 1.50 0.72 2.51 0.77 0.71
1.10 0.91 0.69 2.78 1.90 1.59 1.27 0.84
0.92 0.78 0.69 6.85 3.17 5.65 1.74 1.60
1.00 0.91 0.67 5.09 3.29 3.65 1.60 1.52
1.02 1.13 0.72 8.78 3.99 5.26 1.71 1.96
>2.0
2.0
1.0
0

B

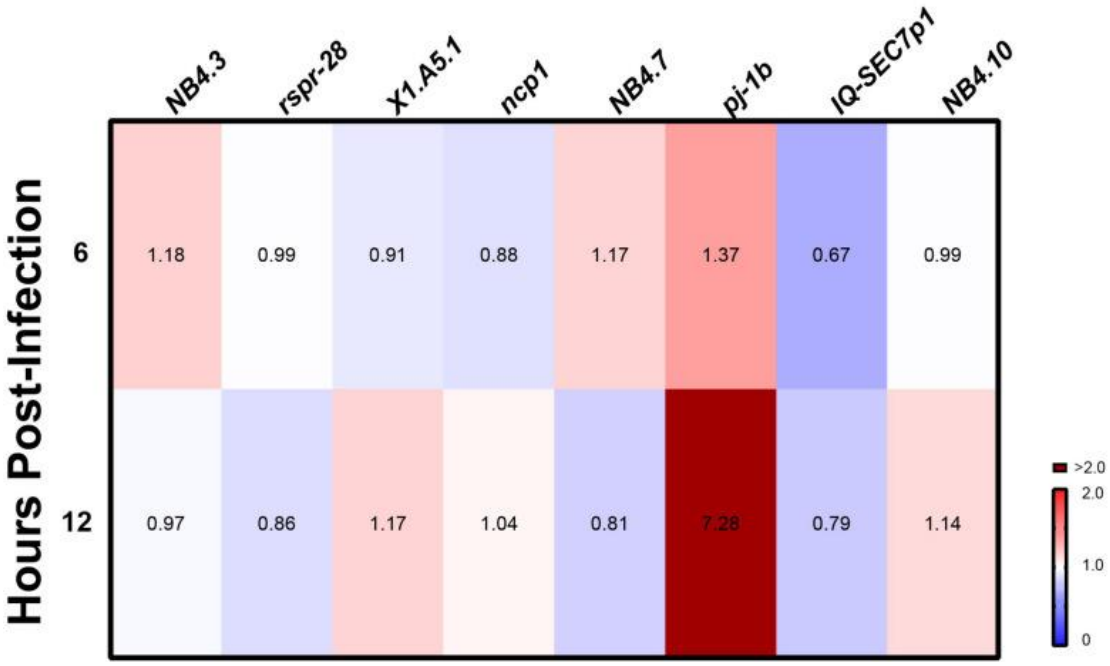
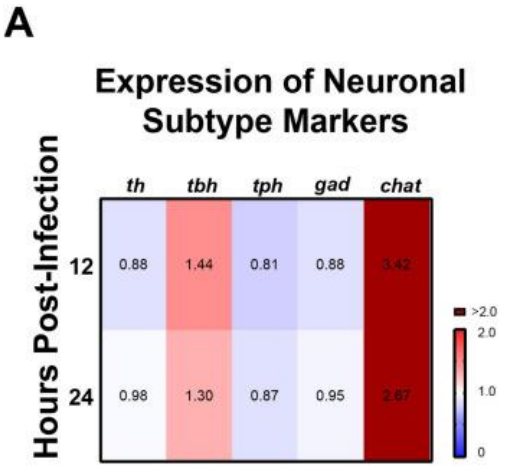
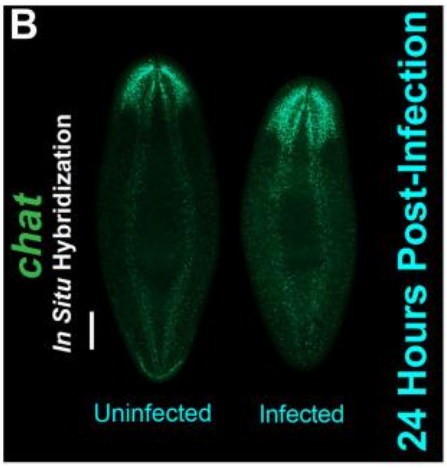
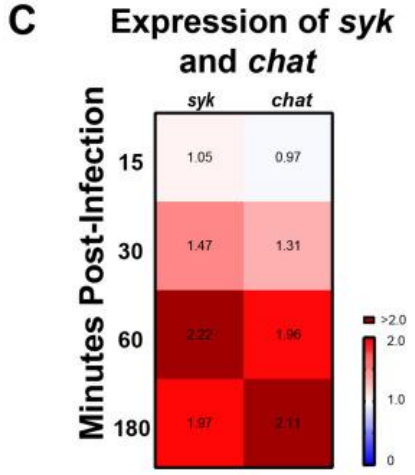
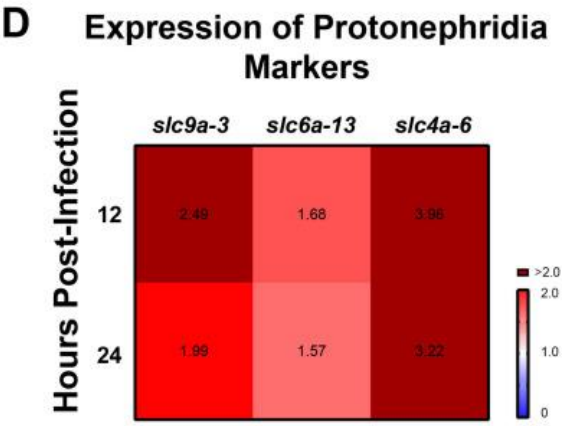

Expression of NB4 Markers
NB4.3
rspr-28
X1.A5.1
ncp1
NB4.7
pj-1b
IQ-SEC7p1
NB4.10
Hours Post-Infection
6
12
1.18 0.99 0.91 0.88 1.17 1.37 0.67 0.99
0.97 0.86 1.17 1.04 0.81 7.28 0.79 1.14
>2.0
2.0
1.0
0

Figure 5. Expression of planarian neoblast subclasses throughout infection with *C. albicans*. (A) Gene expression levels of neoblast markers and subclass markers of clonal neoblasts over the course of *C. albicans* infection. Expression levels are represented in a heat map, where the color scale depicts red as upregulation and blue as downregulation; burgundy is upregulation greater that twofold. Gene expression is represented by fold change normalized to an uninfected control. Gene expression values represent triplicate samples for at least two biological and technical replicates. B) Gene expression of eight other markers for NB4 (Zeng et al., 2018).


A
Expression of Neuronal Subtype Markers
th
tbh
tph
gad
chat
Hours Post-Infection
12
24
0.88
1.44
0.81
0.88
3.42
0.98
1.30
0.87
0.95
2.67
>2.0
2.0
1.0
0


B
chat
In Situ Hybridization
Uninfected
Infected
24 Hours Post-Infection


C
Expression of syk and chat
syk
chat
Minutes Post-Infection
15
30
60
180
1.05
0.97
1.47
1.31
2.22
1.96
1.97
2.11
>2.0
2.0
1.0
0


D
Expression of Protonephridia Markers
slc9a-3
slc6a-13
slc4a-6
Hours Post-Infection
12
24
2.49
1.68
3.96
1.99
1.57
3.22
>2.0
2.0
1.0
0

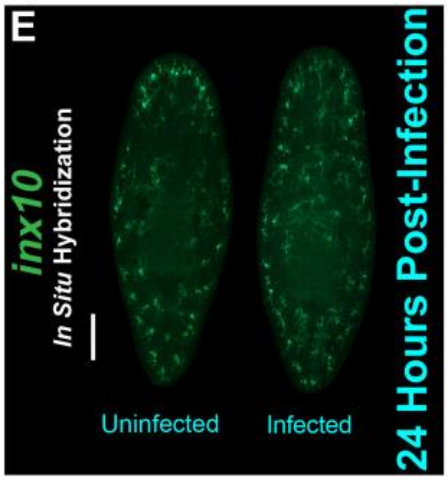

E
inx10
In Situ Hybridization
Uninfected
Infected
24 Hours Post-Infection

Figure 6. Neurons, protonephridia, and specific mesenchymal neoblast markers are upregulated in planarians during infection with *C. albicans*. A) Expression of five different neuron subtypes. Gene expression levels throughout the course of the *C. albicans* infection are represented in the heat map, where the color scale depicts red as upregulation and blue as downregulation; burgundy is upregulation greater than twofold. Gene expression is represented by fold change normalized to an uninfected control. B) Fluorescent *in situ* hybridization showing expression of *chat* in an uninfected and 24-hour infected planarian. Scale bar is 200µm. Images are representative of eight animals for at least two biological and technical replicates. C) Gene expression levels of *syk* and *chat* throughout the course of the *C. albicans* infection (15-180 minutes). Expression levels are represented in a heat map, where the color scale depicts red as upregulation and blue as downregulation; burgundy is upregulation greater than twofold. Gene expression is represented by fold change normalized to an uninfected control. Gene expression values represent triplicate samples for at least two biological and technical replicates. D) Expression of protonephridia structure markers: *slc9a-3* (collecting ducts), *slc4a-6* (distal tubules), and *slc6a-13* (proximal tubules). Gene expression levels throughout the course of the *C. albicans* infection are represented in the heat map, where the color scale depicts red as upregulation and blue as downregulation; burgundy is upregulation greater than twofold. Gene expression is represented by fold change normalized to an uninfected control. E) Fluorescent *in situ* hybridization showing expression of *inx10* in an uninfected and 24-hour infected animal. Scale bar is 200µm. Images are representative of eight animals for at least two biological and technical replicates.

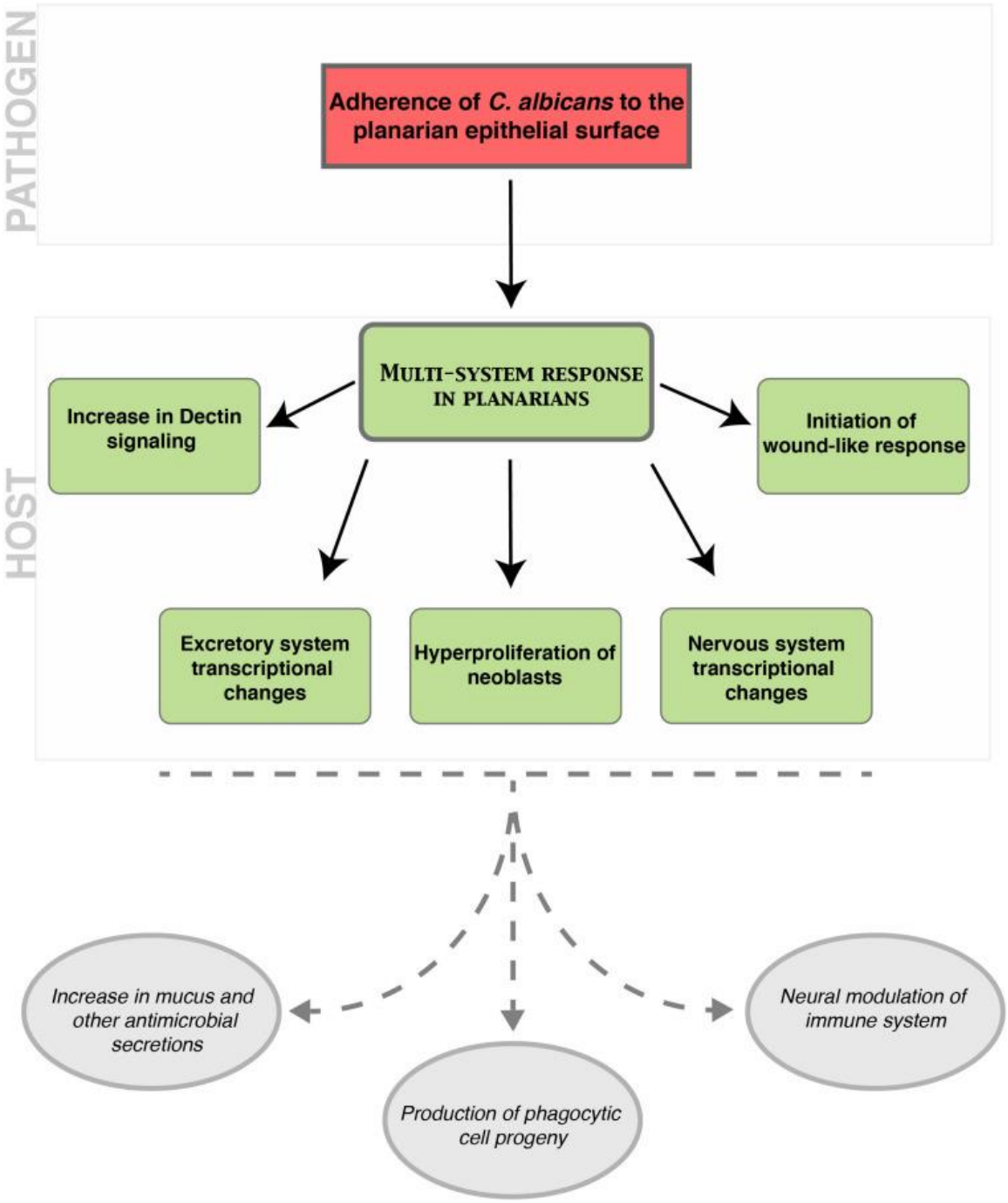
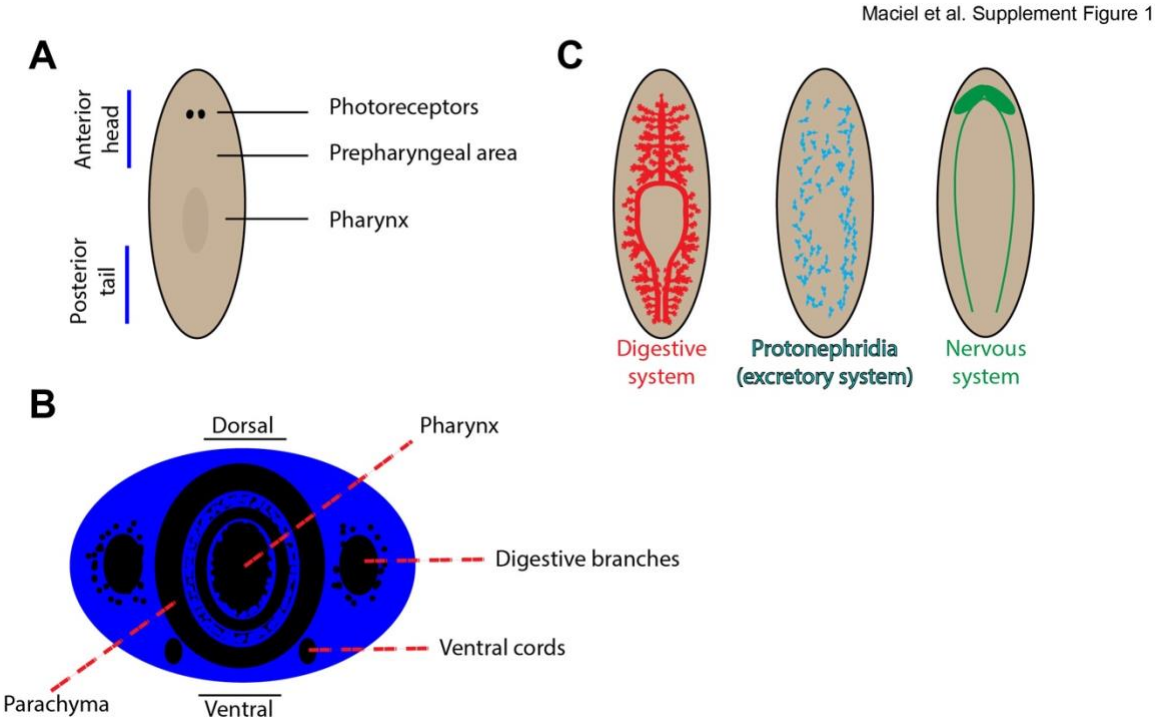


Figure 7. Model of the cascade of host-pathogen interactions that occur following an epithelial infection of planarians with *C. albicans*. The top red box indicates the role of *C. albicans* in initiating a multi-system response in planarians during an infection. The middle green boxes indicate the planarian responses to *C. albicans* during an infection including an increase in components of the Dectin signaling pathway, the initiation of a

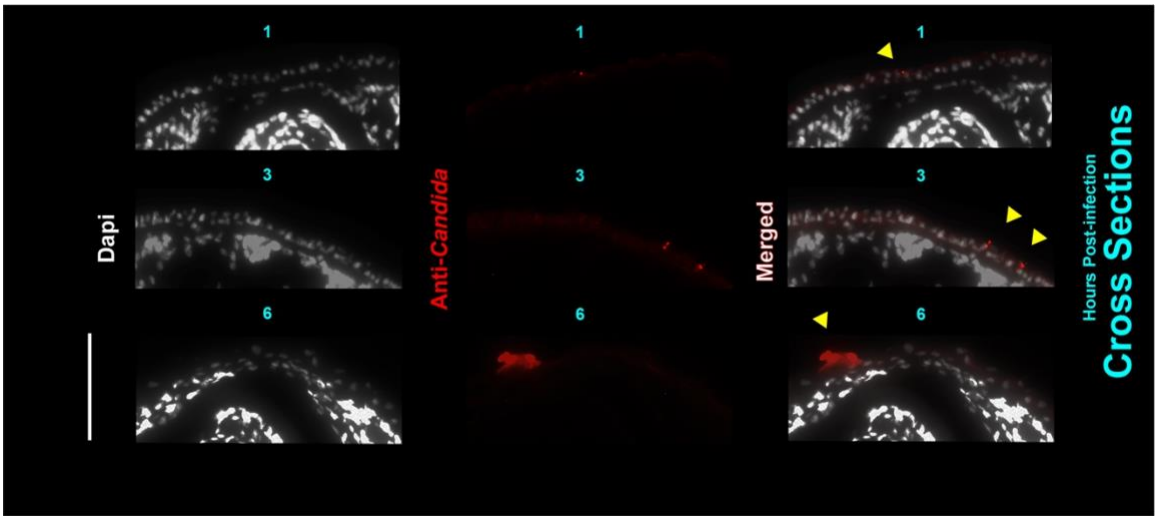
wound-like response, the hyperproliferation of neoblasts, and transcriptional changes in the excretory and nervous systems. The bottom grey ovals represent inferred host outcomes of the planarian multi-system response to the *C. albicans* infection. We propose that an increase in mucus and other antimicrobial secretions, the production of phagocytic cell progeny, and neural modulation of the immune system are downstream outcomes of this host-pathogen interaction.

Supplemental Figures



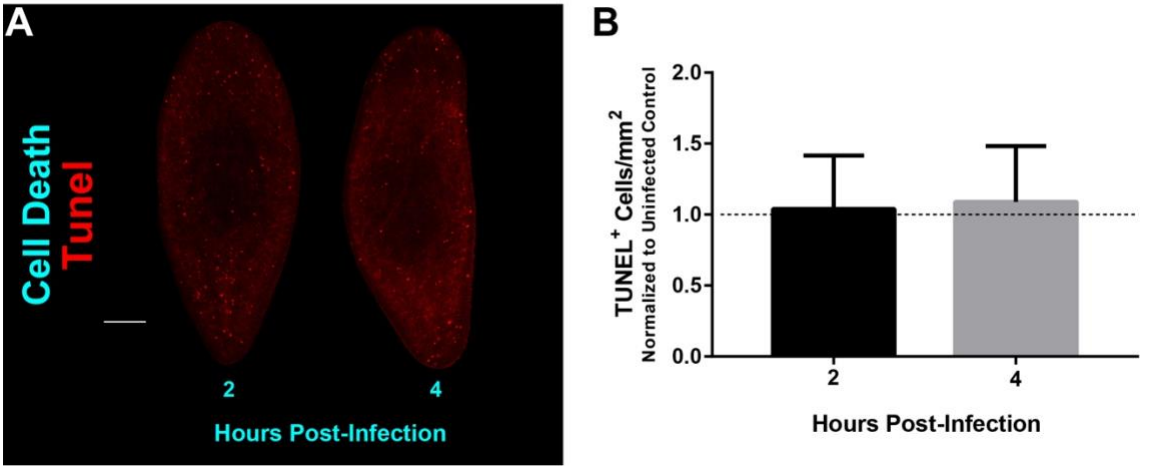
Supplement Figure 1. Orientation and basic anatomy of the planarian body. (A) Demonstrates the orientation the planarians in the whole-mount images. (B) Depiction of a transverse cross section of a planarian in the orientation found in the images throughout the figures. (C) Depiction of the digestive system, nervous system, and protonephridia of planarians.

Maciel et al. Supplement Figure 2



Supplement Figure 2. Cross section images of the planarian body during early infection with *C. albicans*. Individual transverse cross section images are displayed from the dorsal side of the planarian body showing staining with the DAPI nuclear marker (white signal), anti-*Candida* antibody (red signal), and merged images, at one, three, and six hours post-infection. Yellow triangles indicate notable *C. albicans* aggregates.

Maciel et al. Supplement Figure 3



Supplement Figure 3. Cell death occurring during the early stages of infection with *C. albicans*. (A) TUNEL straining (red foci) was performed in uninfected planarians to compare animals infected at two and four hours post-infection using 25 million cells/mL of *C. albicans*. (B) Levels of TUNEL+ cells in the planarian tissue two and four hours post-infection normalized to an uninfected control. Cell death experiments consisted of two biological replicates using four animals each. All graphs represent mean ±SEM. Scale bar is 200μm.

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Chapter 4

Conclusions and Future Directions

4.1 Conclusions

The common opportunistic fungal pathogen and commensal of humans, *C. albicans,* is the most prevalent fungal microorganism isolated from clinical settings (Nobile & Johnson, 2015). Although *C. albicans* typically resides as a commensal on the skin, and in the vaginal, gastrointestinal, and urogenital tracts of humans, it can also become a pathogen and cause superficial and life-threating systemic infections (Hall & Noverr, 2017; Nobile & Johnson, 2015). *C. albicans* has many virulence traits that contribute to its pathogenicity including biofilm formation, which aids in its survival in harsh host environments. *C. albicans* is typically in close contact with the mucosal epithelia in the human host, where it interacts with mucus, specifically with mucin glycoproteins of mucus (Kavanaugh et al., 2014). Mucins are known to interact with microorganisms, including *C. albicans,* and they have important antimicrobial properties (Valle Arevalo & Nobile, 2020). Since *C. albicans* is in a constant interaction with the human host, it is important to understand the currently unknown underlying mechanisms involved in how the pathogen interacts with host mucins.

In chapter one, we review the known and hypothesized interactions of microorganisms with host mucins with an emphasis on *C. albicans.* Mucus provides necessary physical and biological protection against pathogens, and mucins found in mucus are at the forefront of providing this crucial protection. We review what is known about the interactions of several bacterial pathogens, including *P. aeruginosa, E. coli, S. aureus, and A. muciniphila*, with mucins. Recent studies have shown that mucins inhibit bacterial virulence traits of these and other bacterial pathogens, including quorum sensing, toxin secretion, and biofilm formation. As for *C. albicans*, recent work has established that mucins suppress important virulence traits, such as the production of secreted proteases, surface adherence, filamentation, and biofilm formation. In this chapter, we also discuss the properties, functions, and structures of mucins along with the types of mucins that are found in the human body and how these components contribute to their interactions with pathogenic microorganisms.

In chapter two, we present the results from a high-throughput genetic screen using *in vitro* biofilm assays to identify the transcriptional regulators involved in controlling downstream target genes in the presence of mucins. Using the genetic screen in combination with genome-wide approaches, RNA-seq and CUT&RUN, we identified twelve master transcriptional regulators that when deleted, led to the formation of enhanced biofilms and defective biofilms in the presence of mucins. These include transcriptional regulators: Tup1, Ssn6, Fcr1, Tec1*,* Nto1, Fgr27, Zfu2, Zfu3, Swi4, Uga3, Zcf39, and Upc2. With the current CUT&RUN data we have, along with the RNA-seq data, we identified that the regulatory network is composed of eight master transcriptional regulators and ~3,000 downstream target genes. Taken together, this network offers new insights into how *C. albicans* interacts with the host and forms biofilms on host mucosal surfaces.

In chapter three, we introduce the planarian *S. mediterranea* as a newly established invertebrate model system to study the host response to fungal infections. We observed host-pathogen changes that occurred *in situ* at early infection timepoints and that the transcription factor Bcr1 and its downstream target, Als3, a cell surface protein,

were required for the ability of *C. albicans* to adhere to and colonize the planarian epithelial surface. Adherence of *C. albicans* to planarians furthermore activated a multi-system host response that is regulated by the Dectin signaling pathway. Overall, the host response to *C. albicans* resembles a wound-like response, eliciting neoblast proliferation as early as 6 h post-infection and involves the host innate, excretory, and nervous systems.

Together, these three chapters add to our current understanding and knowledge of how *C. albicans* mechanistically interacts with the host. The identified master regulators and downstream target genes could be potential targets of interest for fungal therapeutics. These findings can ultimately lead to reducing the burden these infections have in clinical settings. Additionally, the new planarian model system to study fungal epithelial infections can be used to study mucosal epithelium infections in an invertebrate system, significantly reducing cost compared to mammalian *in vivo* models.

4.2 Future directions

In this dissertation we unraveled the known and predicted interactions *C. albicans* has with host mucins, identified the transcriptional network controlling these interactions, and introduced the planarian model system for studying fungal infections of the host epithelium. Below I describe some future directions of interest that have come out of this work.

Part of this dissertation includes findings of the complete transcriptional program (the regulators and their target genes) that mediate the interactions of *C. albicans* with host mucins. The discovery of this network will allow us to understand how this interaction evolved in microbes and may be applicable to other species of microbes that also invade and commensally exist in their hosts. These experiments have not been performed before for any microbe in association with mucins, and so the network that was discovered here could pave the path for future work on other mucin-interaction networks. It may also shed new light on how microbes evolved to be more resistant to chemical and physical perturbations in the host. Bacteria and archaea, for example, are also major biofilm-forming microorganisms that colonize mammalian mucosal surfaces (Bang et al., 2014; van Wolferen et al., 2018; von Rosenvinge et al., 2013), and there may be some conserved overlap between the *C. albicans* mucins interaction network with that of other microorganisms including other *Candida* species.

With this newly discovered network governing the interactions of *C. albicans* with host mucins, future work could elucidate the molecular mechanisms of this interaction. For example, this work could lead to the discovery of a *C. albicans* receptor required for directly binding to a specific structural component of mucins. With this information, we could begin to develop structural analogs to interfere with the ability of the receptor to bind to mucins such that *C. albicans* would no longer be able to attach to mucins and would be incapable of causing an infection at the mucosal surface. Given that there is a large degree of variability in patients' susceptibilities to *C. albicans* mucosal infections, our work could provide new insight into why certain patients are more prone to *C. albicans* infections than others. For example, a specific structural feature of mucins may be critical for *C. albicans* to bind to the mucosal layer and cause disease, and variability in the human population for this structural feature may alter the way *C. albicans* interacts with it.

Additionally, it would be important to determine the signaling cascade(s) responsible for signaling the mucins master regulators. We know from our findings that many genes involved in adherence and filamentation are inhibited during biofilm formation in the presence of mucins. We also know that many signaling pathways in *C. albicans* are connected and therefore we anticipate that signaling pathways regulating adherence, filamentation and biofilm formation are interwoven. Since research on bacterial pathogens and mucins have shown that mucins are able to interfere with bacterial quorum sensing (Rutherford & Bassler, 2012), it is probable that something similar may be occurring in *C. albicans*. One signaling cascade that is important during *C. albicans'* virulence is the Ras/cAMP/PKA signaling pathway (Huang et al., 2019; Inglis & Sherlock, 2013), and this signaling pathway may be one involved in the activation of the mucin master regulators.

It would also be important to find the target genes that mediate the interactions with host mucins. We have identified the master regulators, which control over 3,000 downstream target genes. Next, performing knockout studies on prioritized target genes would help recognize the roles these genes play during biofilm formation in the presence of mucins. Our results indicate that the target genes of interest would include genes involved in adherence, filamentation and biofilm formation as well as uncharacterized genes. These target genes could be potential targets in the development of new antifungal therapeutics.

Our findings indicated that Tec1 is the only mucin master regulator that is common to the classical biofilm network master regulators. Tec1 controls hundreds of target genes in the classical biofilm network and once *TEC1* is deleted, in the presence of mucins, we see a flipped gene expression profile of the classical biofilm network master regulators and target genes. This indicates that Tec1 is responsible for certain “rewiring” events occurring when the cells are in the presence of mucins. Based on these findings, it would be interesting to see if deletion of *TEC1* leads to the classical biofilm master regulators binding to new target genes in the presence of mucins. Using a *tec1*Δ/Δ strain, we can GFP-tag the classical biofilm master regulators and perform genome-wide studies to identify potentially new target genes that are exclusive to mucin-mediated interactions. These new findings could reveal the full transcriptional machinery involved in controlling *C. albicans* interactions with host mucins.

4.3 References

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