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Fox, Emily P
Nobile, Clarissa J

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A sticky situation

Untangling the transcriptional network controlling biofilm development in *Candida albicans*

Emily P. Fox^{1,2} and Clarissa J. Nobile^{1,*}

¹Department of Microbiology and Immunology; University of California-San Francisco; San Francisco, CA USA; ²Tetrad Program; Department of Biochemistry and Biophysics; University of California-San Francisco; San Francisco, CA USA

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Candida albicans is a commensal microorganism of the human microbiome; it is also the most prevalent fungal pathogen of humans. Many infections caused by *C. albicans* are a direct consequence of its proclivity to form biofilms—resilient, surface-associated communities of cells where individual cells acquire specialized properties that are distinct from those observed in suspension cultures. We recently identified the transcriptional network that orchestrates the formation of biofilms in *C. albicans*. These results set the stage for understanding how biofilms are formed and, once formed, how the specialized properties of biofilms are elaborated. This information will provide new insight for understanding biofilms in more detail and may lead to improvements in preventing and treating biofilm-based infections in the future.

Introduction

Candida albicans is a natural component of the human microbiome, but it is also the major fungal pathogen of humans. This fungus asymptomatically colonizes many areas of the human body, especially the gastrointestinal and genitourinary tracts of healthy individuals. Alterations in host immunity, stress, resident microbiota and other factors can lead to *C. albicans* overgrowth, causing a wide range of infections from superficial mucosal to hematogenously disseminated candidiasis.¹ Candida infections are especially serious in immunocompromised individuals, such as AIDS patients, patients undergoing anticancer therapies, and transplantation patients receiving immunosuppression therapy, as well as immunocompetent patients with implanted medical devices.²⁻⁴

To date, most studies of *C. albicans* have been performed in suspension cultures; however, the medical impact of *C. albicans* (like that of many other microorganisms) depends on its ability to form surface-associated communities called biofilms.^{5,6} Biofilms are notorious for forming on various implanted medical devices, including catheters, pacemakers, heart valves, dentures and prosthetic joints, which provide a surface and sanctuary for biofilm

growth.^{7,8} The human health consequences of device-associated infections can be severe and often life-threatening.⁶

Estimates by the NIH in PA-03-047 indicate that biofilms in general (including both bacterial and fungal biofilms) are responsible for over 80% of all microbial infections (<http://grants.nih.gov/grants/guide/pa-files/PA-03-047.html>). For both structural and physiological reasons, biofilms are inherently resistant to antimicrobial therapy and host immune defenses. Biofilms cause many types of infections, ranging from superficial mucosal infections to severe disseminated bloodstream infections. These infections are most frequently seeded from biofilms formed on mucosal surfaces or implanted medical devices, such as catheters. Over five million central venous catheters are placed each year in the United States alone.⁸ Currently—even with recent improved clinical approaches—infection occurs in up to 54% of these catheters, and many of these result in life-threatening, systemic infections. With an estimated 100,000 deaths and \$6.5 billion in excess expenditure annually in the United States alone, these infections have enormous health and economic consequences.

C. albicans, the major fungal biofilm-former, causes device-associated infections with remarkably high mortality rates (approaching 40%).⁸⁻¹¹ *C. albicans* biofilms are resistant to standard antifungal drugs; not only do biofilms provide physical protection from the drugs, but also cells in biofilms become intrinsically resistant to drugs due to their altered metabolic states and their constitutive upregulation of drug pumps. These and other characteristics of *C. albicans* are not observed in suspension cultures, the growth state for which most *C. albicans* research has been performed in.

C. albicans biofilm formation in vitro can be broken down into four stages:^{10,12-16} (1) attachment and colonization of round yeast cells to a surface; (2) growth and proliferation of yeast cells creating a basal layer of anchoring cells; (3) growth of pseudohyphae (oval yeast cells joined end to end) and hyphae (long cylindrical cells) accompanying the production of the extracellular matrix and; (4) dispersal of cells from the biofilm to find new sites to colonize. These stages are illustrated in **Figure 1**.

Recent studies suggest that these characteristics of biofilm formation also apply in vivo. For example, in *C. albicans* biofilms from denture stomatitis patients, yeast cells, hyphae and extracellular matrix were observed.¹⁷ In addition, biofilms formed in two animal catheter models include yeast cells attached to the

*Correspondence to: Clarissa J. Nobile; Email: Clarissa.Nobile@ucsf.edu
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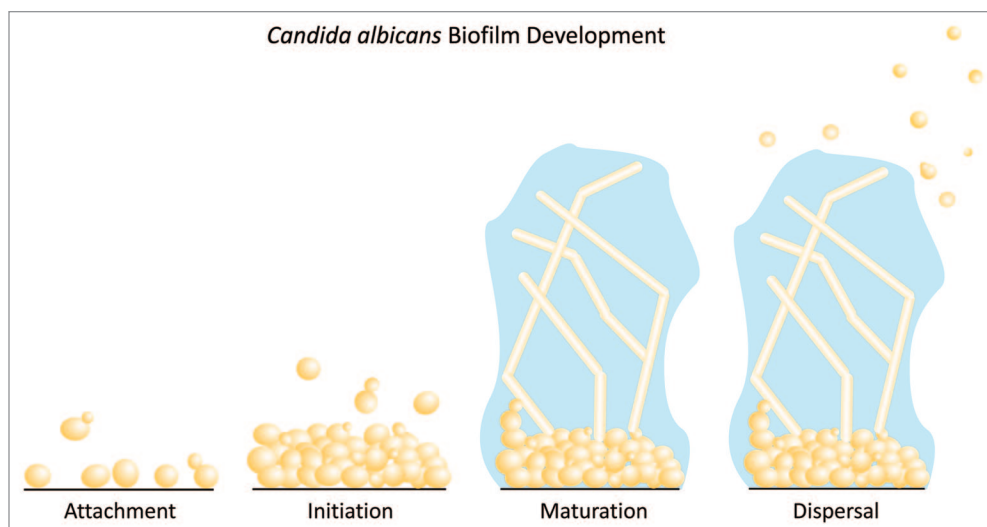


Figure 1. Schematic of *C. albicans* biofilm development over time. **Attachment:** pioneering yeast cells adhere to a substrate, such as a polystyrene plate, an implanted medical device, or a host surface. **Initiation:** biofilm development begins with cell-cell adhesion and proliferation. **Maturation:** hyphal cells form and the biofilm is encased in a secreted, extracellular matrix. **Dispersal:** cells bud off of the biofilm and are dispersed to seed new locations.

surface, along with long hyphae and extracellular matrix material found throughout these biofilms.^{18,19} Thus, the in vitro studies of *C. albicans* biofilms appear to correlate reasonably well with the situation in vivo, although differences between the in vitro and in vivo situations have certainly been observed.

Analysis so far has shown that hyphal formation and adherence are pivotal for *C. albicans* biofilm formation. However, many important features of *C. albicans* biofilms—the extracellular matrix, resistance to antifungal agents, the integration of the distinctive cell types into the mature biofilm, cell dispersal from a biofilm to seed new sites, and the communication between the cells within a biofilm—have yet to be worked out in detail. In addition, the interplay between the transcriptional programs that orchestrate biofilm formation and those that control other aspects of *C. albicans* biology is not understood. Finally, it is likely that many important aspects of *C. albicans* biofilm formation have simply not yet been discovered.

The Transcriptional Network Governing *C. albicans* Biofilm Formation

We recently identified the transcriptional network that orchestrates the development of *C. albicans* biofilms. This network consists of six master transcription regulators (Efg1, Tec1, Bcr1, Ndt80, Rob1 and Brg1) and approximately 1,000 target genes, whose expression is controlled by these regulators (Fig. 2A).²⁰ The six master regulators were identified by screening a library of ~165 transcription factor mutants²¹ for biofilm formation under standard in vitro biofilm conditions,¹⁵ looking for mutants that were altered in normal biofilm formation. Six deletion mutants that cause defects in biofilm formation were identified, three are novel (Rob1, Brg1 and Ndt80) and three were previously known to play roles in biofilm development (Bcr1, Tec1 and Efg1). All six transcription factor gene deletion strains had biofilm defects both in vitro and in vivo.

To begin to understand how the six transcription factors (Efg1, Tec1, Bcr1, Ndt80, Rob1 and Brg1) form a transcriptional network, we performed full genome chromatin immunoprecipitation (ChIP-chip) and gene expression microarray experiments under biofilm conditions. From these techniques, we determined the position across the genome to which each of the six transcription factors are bound and regulated during biofilm development. Taken as a whole, the data revealed that these six transcription regulators are arranged in a complex transcriptional circuit, where they control each other's expression, summarized in Figure 2B. To truly understand how biofilm formation is orchestrated and how it responds to external cues, future studies will need to experimentally dissect the circuit further.

Three aspects of the biofilm transcriptional network gave us clues as to its functional organization. First, the regulators were highly interconnected in that they were able to directly regulate one another. Second, we validated a “top down” approach and used the identified regulators to reveal downstream target genes that were important for normal biofilm formation. Lastly, we showed that the biofilm transcriptional program was composed of target genes that were regulated either directly (bound by a biofilm regulator) or indirectly (not bound, but differentially regulated in wild-type biofilms compared with biofilms formed by regulator deletion mutant strains). By integrating this information, we proposed a hierarchical cascade model to explain the indirect regulation of the target genes within the biofilm network. We will now discuss each of these aspects of the biofilm network and the hierarchical cascade model in detail.

Interconnected Regulators

We used a top-down approach in which we screened a knockout library of 165 known or suspected transcription regulators for in vitro biofilm formation. Our approach identified six transcription

regulator deletion mutants that were deficient in biofilm formation after 48 h of growth. As biofilms are complex structures composed of multiple cell types, it was entirely possible that these transcription regulators could work independently to control unique attributes of biofilms (such as drug resistance, adherence or cell morphology); however, this was not what we found. Instead, it seemed that biofilm development was controlled by an interconnected network of transcriptional regulators. Taken together, our ChIP-chip and RT-qPCR experiments indicated that each regulator binds and positively regulates the promoters of most of the other regulators (Fig. 2B). ChIP-chip and gene expression microarray data indicated that each regulator controls expression of a set of target genes: some genes in common with the other regulators, and some target genes unique to each regulator (Fig. 2A). This may suggest that some regulators control particular aspects of biofilm development, or that they regulate processes unrelated to biofilms. In addition, some regulators may respond to key inputs (such as time, temperature, nutrient availability, chemical messengers, etc.) to govern response to these stimuli while in the biofilm state.

Our recent work has uncovered a complex network of regulation that implicates six master regulators of biofilm development. However, these six are certainly not the only transcriptional regulators involved. Indeed, 52 additional regulators were bound by at least one of the six master regulators in our data set. Of these 52 regulators, deletion mutants were obtained for 34 of them, with no biofilm phenotype detected. The other 15 were not tested: seven were not attempted and eight we could not obtain deletion mutants for (implying they may be essential genes). Our work focused on mature biofilms that were grown for 48 h, and studies by other groups have identified transcriptional regulators of additional stages of biofilm development, or under different environmental conditions. For example, *GCN4*, *TYE7* and *ACE2* deletion mutants are all defective in biofilm formation.²²⁻²⁵ The transcriptional regulator Zap1 has been shown to control formation of the extracellular matrix that

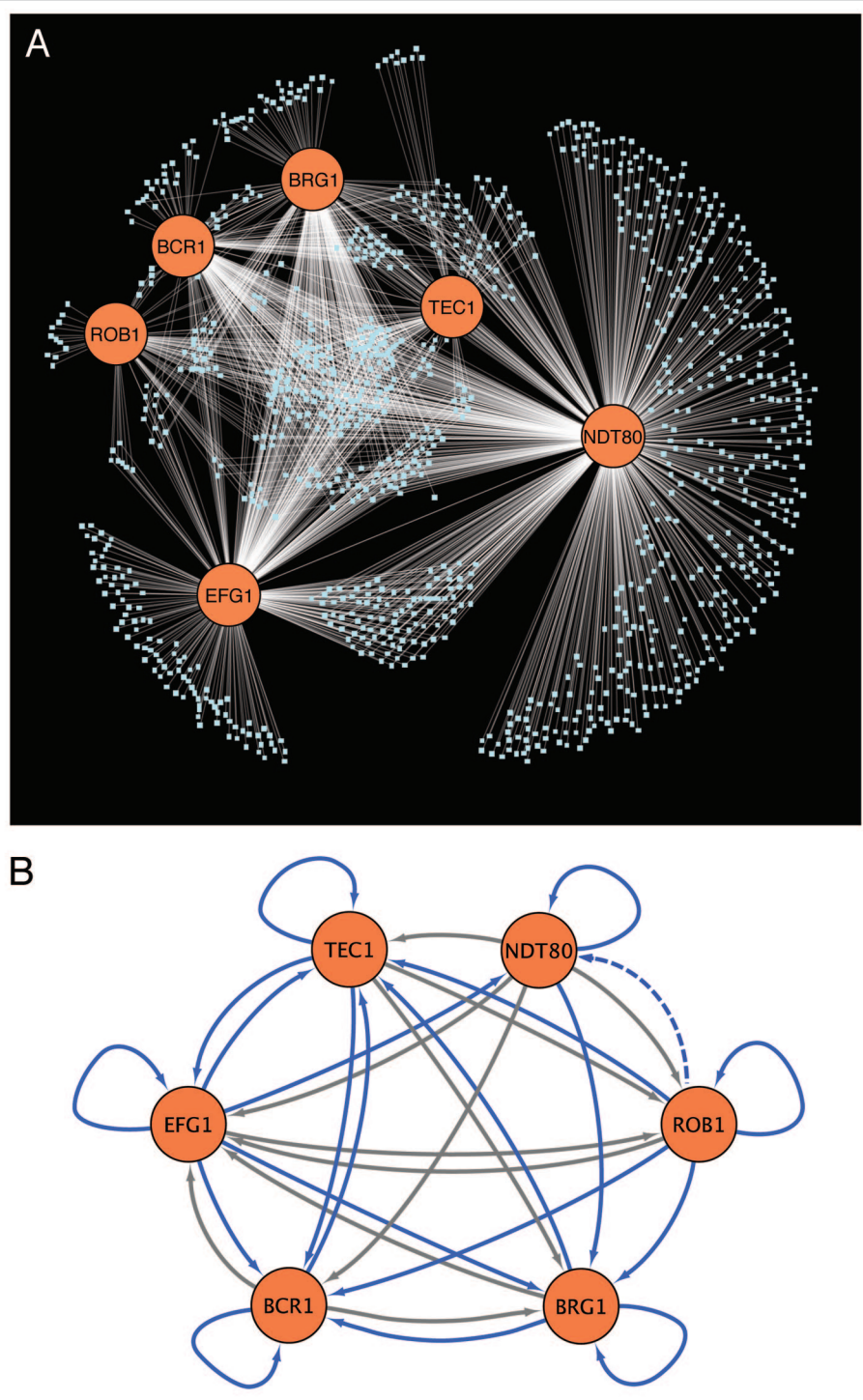


Figure 2. The transcriptional network governing biofilm development (adapted from Nobile et al., 2012).²⁰ (A) Six transcriptional regulators (Rob1, Bcr1, Brg1, Tec1, Ndt80 and Efg1, represented by orange circles) are required for normal biofilm formation. ChIP-chip identified the binding sites of each regulator. The target genes whose promoters are bound by each regulator are represented by blue squares, with white lines connecting each regulator to its targets. (B) Map of the binding and regulatory interactions between the transcriptional regulators. Gray arrows indicate direct binding interactions (determined by ChIP-chip). Blue arrows indicate both binding and regulatory interactions (determined by ChIP-chip, gene expression profiling, or qPCR). Solid lines indicate a binding interaction, whereas the dotted line represents a regulatory interaction only.

encases biofilms.²⁶ Adherence to a substrate is a key part of biofilm initiation, and 31 adherence regulators were identified in one study,²³ and another study found Ahr1 also regulates adherence.²⁷ Mature biofilms disperse cells, which can seed infection in new sites; Nrg1 was identified as a regulator of this process.¹⁶

The promoter regions of many of these additional biofilm transcriptional regulators were bound by at least one of the six biofilm regulators identified in our study, indicating crosstalk between regulation of adherence, mature biofilm formation, matrix secretion and biofilm dispersion. All regulators currently known to be involved in biofilm formation based on the presence of a biofilm phenotype in a mutant of the regulator are listed in Table 1. We also indicate whether those regulators are bound by any of the six core biofilm regulators, and thus how they may fit in within the biofilm network. Strikingly, of the 40 regulators that are required for either adherence or dispersal, the promoters of 20 of them are bound by at least one of the six regulators identified in our study. Our recently described network of six master regulators of biofilm development is highly interconnected and inclusion of the additional regulators described here illustrates that the transcriptional regulation of biofilm formation is even more complex than we initially estimated. Although the network is elaborate, it is still possible to extract meaningful information regarding the regulators and their downstream targets: which regulators control other regulators, which processes are controlled by which regulators, and which downstream target genes play important roles in biofilm development.

The complexity of the transcriptional network controlling biofilm development in *C. albicans* is not unusual. Indeed, other complex regulatory networks control diverse processes in many eukaryotic organisms. For example, human metabolism genes, mammalian stem cell differentiation and *Arabidopsis thaliana* circadian clock rhythms are all controlled by multiple transcriptional regulators with many downstream targets.²⁸⁻³² In addition, *S. cerevisiae* uses complex transcriptional circuits to control pseudohyphal growth and response to osmotic stress.^{33,34} The advantages of complex regulatory networks are unclear, but there are several hypotheses. One idea is that complex networks are able to sense and respond to multiple environmental inputs. Additionally, a regulator shared in common between one or more networks may be capable of initiating efficient responses within the various networks in response to shared stimuli. For example, Tec1 in *C. albicans* regulates both biofilm development and hyphal formation in response to environmental signals.³⁵ Another possibility is that increasing network complexity correlates with the ability to “fine-tune” gene regulation, which has been proposed previously for other networks, such as the ribosomal protein gene network.³⁶ While the significance of the architecture of these networks is yet to be understood, their existence is an emerging theme in biology and they seem to govern a multitude of biological processes.

Identifying Relevant Biofilm Targets

To validate that our approach is a useful tool for identifying functionally important target genes in biofilm development, we

analyzed a subset of our target genes for roles in biofilm formation. We identified 4,775 target genes that are bound by at least one regulator or that are differentially regulated when comparing at least one regulator deletion mutant strain to a wild-type strain. We used microarrays to compare gene expression in wild-type biofilms to that in each of six deletion regulator mutants. Eight target genes (*ORF19.3337*, *ALS1*, *TPO4*, *ORF19.4000*, *EHT1*, *HYR1*, *HWP1* and *CAN2*) were expressed at lower levels in all six of the biofilm regulator mutants compared with the reference strain and seven of these genes were also expressed at higher levels in biofilms compared with cells in suspension. Each of the eight target genes were also bound in their upstream promoter regions by at least one of the six biofilm regulators.

We created deletion mutant strains for each of these eight genes and also created strains overexpressing each of the eight genes in the genetic background of each regulator deletion mutant strain. We then analyzed the resulting 56 strains for their abilities to form biofilms. Three deletion mutant strains had biofilm defects: *als1Δ/Δ* and *hwp1Δ/Δ* (both previously identified),³⁷⁻³⁹ as well as *can2Δ/Δ*, which we newly identified. These three proteins are all predicted to be cell-surface localized. Hwp1 is a hyphal-specific protein whereas Als1 is expressed in both yeast and hyphae.⁴⁰⁻⁴² Als1 and Hwp1 play roles in cell-cell adhesion as well as cell-surface adhesion, which are both critical for biofilm formation. The Can2 (Orf19.111) protein sequence has 81% identity to Can1 (Orf19.97), a confirmed lysine/arginine/histidine permease. If Can2 is a functional amino acid permease, it may be supporting biofilm growth by providing amino acid substrates for metabolic pathways. Alternatively, Can2 may be involved in drug and toxic substance transport or nutrient sensing and signaling pathways, as amino acid permeases have been previously implicated in these functions.^{43,44} In *C. albicans*, arginine can activate filamentation and arginine biosynthesis promotes filamentation as a method for *C. albicans* to escape macrophages.⁴⁵ If Can2 facilitates arginine uptake, it may also contribute to filamentation. Notably, *CAN2* expression was found to be upregulated in wild-type biofilms formed in the rat central venous catheter biofilm model,⁴⁶ whereas the gene encoding the confirmed *C. albicans* amino acid permease, *CAN1*, was not differentially regulated in this model. This indicates that Can2 plays a distinct role to that of Can1 in the mammalian host environment. Interestingly, Gcn4 (another transcription regulator important for normal biofilm growth²⁴) is a regulator of amino acid biosynthesis during starvation,⁴⁷ suggestive of the need for amino acid acquisition during mature biofilm development. It is unclear whether Gcn4 regulates *CAN2*, but if so, this could explain the similar biofilm defective phenotypes observed in these two deletion mutants.

All but two of the eight top target gene candidates (*ORF19.3337* and *HYR1*) were able to partially rescue biofilm formation when overexpressed in at least one of the six regulator mutant strain backgrounds. The *bcr1Δ/Δ* mutant and the *tec1Δ/Δ* mutant were both rescued by overexpression of each of the six successful target genes. *BCR1* expression is known to be dependent on Tec1, so this may explain why these mutant strain

Table 1. Known transcriptional regulators involved in biofilm formation

ORF	Name	Biofilm-relevant mutant phenotype	Promoter bound? *	References
ORF19.6124	ACE2	Defective in cell separation and adherence	No	23, 25
ORF19.2331	ADA2	Defective in adherence	No	23
ORF19.7381	AHR1	Defective in adherence	Yes	27
ORF19.4766	ARG81	Defective in adherence	No	23
ORF19.723	BCR1	Biofilm biomass reduced, defective in adherence	Yes	15
ORF19.4056	BRG1	Biofilm biomass reduced	Yes	20
ORF19.4670	CAS5	Defective in adherence	Yes	23
ORF19.2356	CRZ2	Defective in adherence	Yes	23
ORF19.3127	CZF1	Defective in adherence	Yes	23
ORF19.3252	DAL81	Defective in adherence	No	23
ORF19.610	EFG1	Biofilm biomass reduced, unable to filament	Yes	20, 49
ORF19.3193	FCR3	Defective in adherence	Yes	23
ORF19.6680	FGR27	Defective in adherence	No	23
ORF19.1358	GCN4	Biofilm biomass reduced	Yes	24
ORF19.4225	LEU3	Defective in adherence	No	23
ORF19.5312	MET4	Defective in adherence	No	23
ORF19.2119	NDT80	Biofilm biomass reduced	Yes	20
ORF19.2012	NOT3	Defective in adherence	No	20
ORF19.7150	NRG1	Overexpression mutant has increased number of dispersed cells	Yes	50
ORF19.4998	ROB1	Biofilm biomass reduced	Yes	20
ORF19.5871	SNF5	Defective in adherence	No	20
ORF19.7319	SUC1	Defective in adherence	No	20
ORF19.798	TAF14	Defective in adherence	No	23
ORF19.5908	TEC1	Biofilm biomass reduced	Yes	15, 20
ORF19.4062	TRY2	Defective in adherence	No	23
ORF19.1971	TRY3	Defective in adherence	No	23
ORF19.5975	TRY4	Defective in adherence	Yes	23
ORF19.3434	TRY5	Defective in adherence	Yes	23
ORF19.6824	TRY6	Defective in adherence	Yes	23
ORF19.4941	TYE7	Hyperfilamentous	Yes	22
ORF19.7317	UGA33	Defective in adherence	No	23
ORF19.1035	WAR1	Defective in adherence	No	23
ORF19.3794	ZAP1	Overproduction of matrix, decrease in adherence gene expression	Yes	23, 26
ORF19.4767	ZCF28	Defective in adherence	No	23
ORF19.5924	ZCF31	Defective in adherence	Yes	23
ORF19.6182	ZCF34	Defective in adherence	No	23
ORF19.7583	ZCF39	Defective in adherence	No	23
ORF19.1718	ZCF8	Defective in adherence	Yes	23
ORF19.6781	ZFU2	Defective in adherence	No	23
ORF19.3187	ZNC1	Defective in adherence	No	23

*"Promoter bound" indicates that the gene is bound by any of the six biofilm regulators discussed in Nobile et al., 2012.²⁰

backgrounds are similarly rescued.¹⁵ Some of the rescues in the *bcr1Δ/Δ* and *tec1Δ/Δ* backgrounds appeared similar to the wild-type by microscopy: they were thick and contained both yeast and hyphal cells. However, the rescue was considered partial, as they were generally more delicate than wild-type biofilms. The partial rescues in the other regulator mutant backgrounds were

thicker than the original mutants, but were composed mostly of yeast cells.

We do not yet fully understand the roles of each of these downstream target proteins in biofilm formation. However, we have formed hypotheses based on previous work in *C. albicans* and homology to proteins in *Saccharomyces cerevisiae*. As Als1

and Hwp1 are both adhesin proteins, it is likely that their ability to rescue the mutant phenotypes (when they are overexpressed) is mediated by enhancing adhesion between biofilm cells. Very little is known about the other four genes whose overexpression resulted in a partial rescue of biofilm formation. For example, Orf19.4000 is a predicted homeodomain transcription factor, but is otherwise uncharacterized. If Orf19.4000 indeed regulates a set of target genes, then some of these genes may compensate for, or overlap with, the gene sets that are miss-regulated by the lack of Bcr1 and Tec1 in a *bcr1Δ/Δ* and *tec1Δ/Δ* deletion mutant, respectively. *TPO4* encodes a putative transporter with similarity to both polyamine and major facilitator superfamily drug transporters. Polyamine levels are carefully regulated in *Escherichia coli* and *S. cerevisiae*, as well as in higher eukaryotes,^{48,49} as they are essential for normal cell growth. Thus, one possibility is that both Tpo4 and Can2 are involved in transporting small molecules that are important for biofilm formation. These small molecules may be nutrients or signaling molecules. For example, farnesol, a known quorum sensing molecule produced by *C. albicans*, has been shown to have a slight inhibitory effect on biofilm formation.^{50,51} There are certain to be other, yet to be identified, secreted signaling molecules that affect biofilm formation, and transporters such as Tpo4 and Can2 may be the receptors of those signals. We also found that rescue was mediated by overexpression of *EHT1*, which encodes a putative alcohol acyl transferase, containing a predicted hydrolase catalytic domain. The *S. cerevisiae* homolog, Eht1, plays a role in fatty acid synthesis and localizes to lipid particles.⁵² Lipid vesicles often support membrane growth and rearrangement, which are an integral part of polarized growth, thus one possibility is that Eht1 is involved in this process. Supporting this, *EHT1* is induced in response to α pheromone, which results in mating projection formation, another form of polarized growth in *C. albicans*.⁵³

Using our genome-wide approaches, we began with a large and unbiased list of target genes that were potentially involved in biofilm formation. To prioritize these target genes for further study, we initially focused on the eight genes that are positively regulated by all six of the biofilm regulators. Remarkably, our work revealed that six out of eight target genes play functional roles in biofilm formation, validating our approach.

Direct Vs. Indirect Targets of Biofilm Formation

In our analysis, ChIP-chip binding data provided a list of target genes bound by each of the six transcriptional regulators. Gene expression microarray data comparing wild-type biofilms to deletion mutants of each regulator provided a list of target genes whose transcription is affected by the loss of that regulator. Of the genes that changed expression in the absence of each regulator, only a fraction of the promoters of those genes were bound by that regulator. Ndt80 bound 273 out of 999 genes (27%), Brg1 bound 130 out of 1,753 genes (7%), Efg1 bound 276 out of 2,947 genes (9%), Bcr1 bound 46 out of 409 genes (11%), Tec1 bound 40 out of 459 genes (9%) and Rob1 bound 46 out of 2,150 genes (2%). Thus, it is clear that the regulators must control some target genes directly (meaning they bind directly to the promoters

of those genes) and indirectly (meaning transcript levels change when the regulator is deleted, but the promoter is not bound). Thus, in the regulator deletion strains, changes in expression of the indirect target genes cannot be explained by loss of binding of the regulator at the promoters of those genes.

We proposed a solution to this dilemma, which we named “the hierarchical cascade model”²⁰ (Fig. 3). To illustrate this model, we will now discuss the eight target genes mentioned above in the section, “Identifying Relevant Biofilm Targets.” These eight target genes are positively regulated by all six regulators, yet they are not direct targets of all six. Each target gene, however, is a direct target of at least one of the regulators, and we found that each indirect target’s expression change can be explained by a cascade of regulation from each indirectly regulating regulator to at least one directly regulating regulator. For example, Tec1 binds and directly regulates three out of the eight target genes. Tec1 also directly regulates *EFG1*, which directly regulates three additional target genes, as well as regulating *NDT80*. Ndt80 then directly regulates the remaining two target genes. Therefore, a cascade of direct regulation, from regulator to regulator, explains expression changes in the *tec1Δ/Δ* strain of both the three direct target genes, as well as the five indirect target genes. We show that this hierarchical cascade can explain differential regulation of all eight target genes in the absence of any of the six regulators, even without the occurrence of a direct binding event by the initial regulator.

Our analysis of direct and indirect regulation of the eight target genes outlined above can be extrapolated to explain indirect regulation of a large portion of the total genes controlled by each regulator. Differential regulation of indirect target genes of any one regulator may be explained by regulation of the other five regulators. Indeed, if we consider binding by all of the regulators, 33% of Ndt80s targets, 25% of Brg1’s targets, 21% of Efg1’s targets, 42% of Bcr1’s targets, 36% of Tec1’s targets and 23% of Rob1’s targets can be explained by this hierarchical cascade model. Additionally, each regulator directly regulates many other transcriptional regulators other than the core six, and thus these additional regulators may explain an even larger proportion of the indirect targets. In other systems, the frequency of overlap observed between ChIP-chip data and gene expression data are also within the range of 20–50%.⁵⁴⁻⁵⁷ We propose that a hierarchical cascade of regulation via multiple transcriptional regulators may account for these observations in other systems, and may be a hallmark of complex regulatory circuits.

Conclusions

By screening a library of transcription factor mutants, we identified the complete or at least near-complete transcriptional network that orchestrates the formation of biofilms in *C. albicans*. From this network, we are now beginning to understand the importance of a small subset of the over 1,000 downstream targets of our six core biofilm transcriptional regulators. We are also beginning to connect this network with biofilm regulators identified in other labs. Future work in this field will focus on dissecting out the roles in biofilm formation of the many other target proteins of the biofilm regulatory network. We believe that this

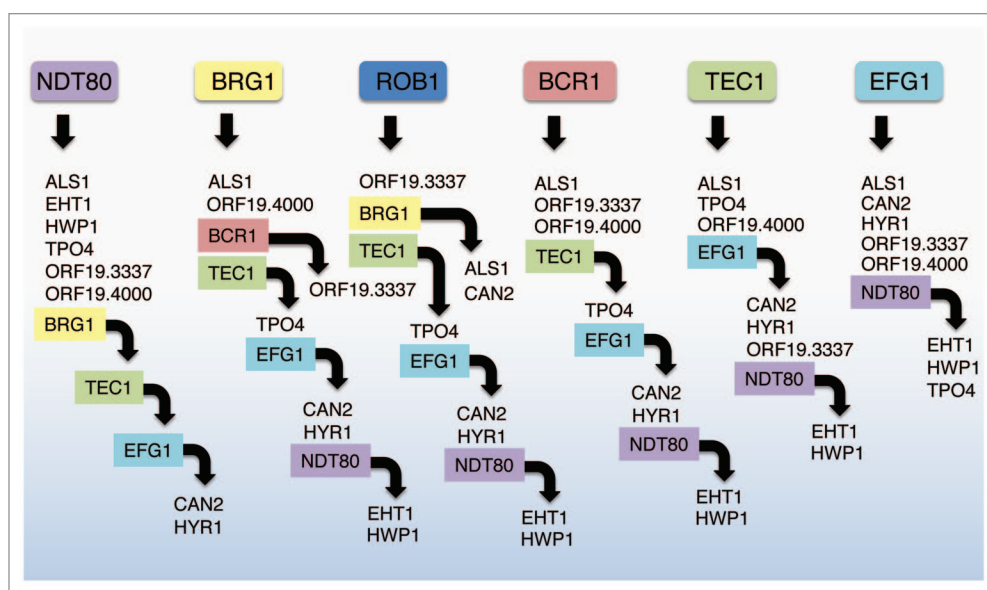


Figure 3. Hierarchical cascade model (adapted from Nobile et al., 2012, ref. 20). Eight target genes (*ALS1*, *EHT1*, *HWP1*, *TPO4*, *ORF19.3337*, *ORF19.4000*, *CAN2* and *HYR1*) were identified as being downregulated in the deletion mutant strain of each of the six transcription regulator mutants. Thus, each regulator positively regulates each of these eight target genes; however, binding of each regulator to the promoter of each target gene was not detected. The model shows a cascade of binding events that could explain regulation of all eight target genes by each of the six regulators. For example, Efg1 directly binds and regulates *ALS1*, *CAN2*, *HYR1*, *ORF19.3337* and *ORF19.4000*. Efg1 also binds and regulates *NDT80*, which in turn binds and regulates the remaining three target genes: *EHT1*, *HWP1* and *TPO4*. The black arrows indicate direct binding in each instance.

information will provide new insight for understanding biofilms in more detail and may lead to improvements in preventing and treating biofilm-based infections in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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