12-2012

Characterization Of Genes And Pathways Controlling Biofilm Formation In Saccharomyces Cerevisiae

Neha Sarode
nsarode@utk.edu

To the Graduate Council:

I am submitting herewith a dissertation written by Neha Sarode entitled "Characterization Of Genes And Pathways Controlling Biofilm Formation In Saccharomyces Cerevisiae." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.

Todd B. Reynolds, Major Professor

We have read this dissertation and recommend its acceptance:

Jeffery Becker, Albrecht Von Arnim, Andreas Nebenfuhr

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Characterization Of Genes and Pathways Controlling Biofilm Formation In *Saccharomyces cerevisiae*

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Neha Sarode

December 2012

Dedication

This dissertation is dedicated to my family. A deep feeling of gratitude for my parents Mr. D. N. Sarode and Mrs. Vrushali Sarode, who gave me the opportunity of an education and freedom to pursue my dreams wherever they took me. I won't be where I am without them. To my sister Shweta Ghevde, brother-in-law Shailendra Ghevde and my dearest niece Pavitra for their unconditional love and support throughout these years.

I also dedicate this work to all my dear friends here in Knoxville, who are like second family and never let me miss my family back home.

Finally, I would also like to dedicate this work and give special thanks to my dear husband Dr. Ambrish Roy for being my unwavering rock for the past 8 years (and counting). You are my best friend and my toughest critic, and I am thankful for that.

Acknowledgement

First and foremost, I would like to thank my advisor Dr. Todd B. Reynolds. This thesis was an impossible dream without his immense patience and constant guidance. He taught me everything I know about biofilms, encouraged me when I was struggling and most important of all stood by me when I thought I was all alone and lost. He transformed me into a better scientist and a stronger person and I will be indebted to him all my life for his kindness.

I would also like to thank my committee members Dr. Jeffery Becker, Dr. Albrecht Von Arnim and Dr. Andreas Nebenführ for their critical review, suggestions and guidance during my work. Dr. Becker, is a strict mentor who didn't hesitate to discipline me when required but also encouraged and appreciated my work when I deserved it. Both Dr. Arnim and Dr. Nebenführ, showed interest in my work, encouraged me and never hesitated to point out my shortcomings from the viewpoint of someone who is not as well acquainted with the yeast model system.

I owe my deepest gratitude to all past and present members of Reynolds lab and Becker lab. My work would be incomplete without their help, suggestions and critique.

Finally I would like to give my heartfelt thanks to the faculty and staff of Genome Science and Technology (GST) and Microbiology department.

Abstract

Biofilms are a mode of growth where aggregated cells adhere to a foreign surface and grow as a complex community. Biofilms have found wide utility in commercial industries, however infections caused by biofilms in clinical settings are a major cause of concern. Understanding molecular details of biofilm formation could help in exploitation or elimination efforts.

We utilize *Saccharomyces cerevisiae* as a model system to study biofilm formation. *S. cerevisiae* strain belonging to genetic background Σ [sigma] 1278b is capable of forming biofilms, on low density (0.3%) agar media. When grown at 25°[degree] C for 5 days, it develops into an elaborate floral shaped biofilm. The biofilm can be structurally differentiated into a central wrinkly part called hub, and a peripheral smooth part called rim. A flocculin family surface protein Flo11p, known to be essential for the phenotypes of adhesion and invasive growth, is also important for biofilm formation. We identified that certain vacuolar protein sorting (VPS) proteins don't affect Flo11p expression and yet were defective in biofilm formation. Thus showing that the phenotypes requiring Flo11p (invasive growth and adhesion) are genetically separable from the phenotype of biofilm formation.

We propose a model showing the existence of a putative biofilm pathway involving endosomal Multivesicular body (MVB) pathway, which affects biofilm formation without causing any defects in Flo11p expression or localization. We further identified that the cell wall integrity (CWI) pathway is partially involved in the biofilm pathway, and supposedly affects biofilm formation by causing defects in cell wall structure.

Although there is no detectable difference in Flo11p expression levels between the rim and hub cells within the biofilm, they are very distinct in appearance and also manifest differences in adhesion. What molecular markers contribute to these differences, however is not yet known. Using RNA-Seq, a high throughput sequencing method, differential expression levels of genes between the rim and hub was obtained. Analysis of the genes revealed the presence of a carbohydrate, named chitosan, in the hub. Further tests showed that though chitosan is not essential for biofilm formation, it plays a protective role against cell wall stressing agents in biofilms.

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Introduction to biofilms

1.1 Background

Unicellular microorganisms are known to collaborate and form multi-cellular communities, called biofilms, in natural settings. Biofilms are the preferred mode of growth for many microbes as they allow colonization of surfaces that otherwise will not support their growth, opportunity to develop multi-species synergistic interactions [1] and horizontal gene transfer within a community [2]. Biofilms have been found in varied environments, for example in locations with high temperature or acidity [3, 4], on river beds [5], on plant surfaces [6], and there are also reports describing approximately 3 billion years old fossil records interpreted as biofilms in South Africa [7]. Such successful inhabitation and ubiquitous existence of biofilms is a testament to their success.

Biofilms have been used to our advantage, e.g. for example in bioremediation [8], waste-water treatment and wine production. They however, can also be a source of nuisance. Biofilms formed by pathogenic microbes are a significant source of nosocomial (hospital acquired) infections, where they infect immunocompromised patients (*i.e.* elderly people, premature babies, HIV positive patients, and cancer chemotherapy and leukemia patients), often by colonizing implanted medical devices, and then spreading to other tissues through bloodstream infections [9]. Efficient elimination of biofilm-based infections necessitates in-depth knowledge of the mechanisms governing biofilm formation and architecture.

1.2 Characteristics of a biofilm

A biofilm can be described as a complex community of cells aggregated on a surface that often produces extracellular products for its protection. A proposed model for stages of biofilm formation is as follows (Figure 1.1) – (a) Adherence: this is the initial step where floating planktonic cells adhere to a foreign surface; (b) Initiation: once adhered to a surface, the cells start to actively divide and form aggregates; (c) Proliferation: at this stage the cells begin phenotypic switching and production of extracellular matrix (ECM); (d) Maturation: at this stage the biofilm is fully formed and is actively producing ECM and dispersing cell aggregates to repeat the cycle [10-12].

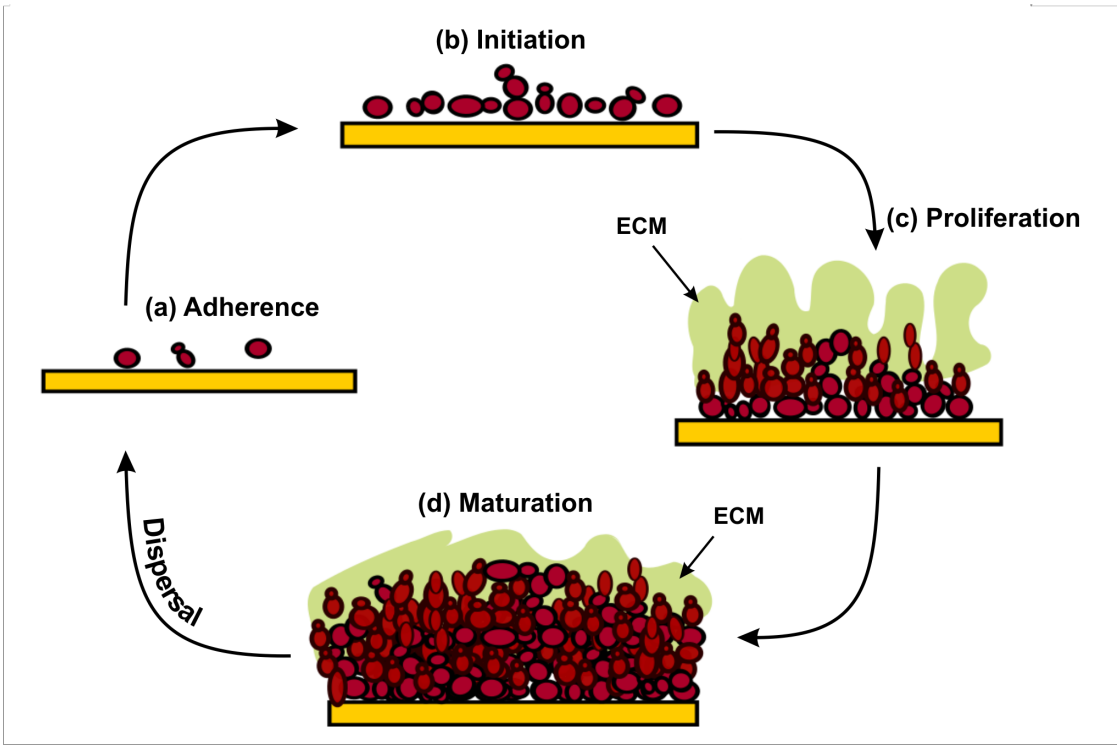

Figure 1.1: Stages of biofilm formation

ECM, also known as extracellular polysaccharide substance (EPS), is a slimy extracellular matrix mainly composed of polysaccharide, that is essential for both structural support and protection of the biofilm [12]. Its composition is as diverse as its residents, and is known to mainly consist of carbohydrates, adhesin proteins and in some cases other components like extracellular DNA, secreted enzymes etc. [13]. A mature biofilm has aggregates of cells called microcolonies, and also consists of structures called water channels interspersed between the microcolonies. These channels represent a primitive circulatory system, distributing nutrients and oxygen throughout the biofilm structure [12]. Another common feature contributing to the architectural complexity of a biofilm is that of differentiation within a biofilm by varying cell types. For example, in *Bacillus subtilis* biofilms, spore forming, motile and matrix producing cells localize to distinct regions within the biofilm [14]. Similarly, biofilms formed by fungi, like those belonging to major pathogenic group *Candida spp.* exhibit what is called a 'phenotypic switch' [15-17]. Phenotypic switch involves a reversible change in phenotypic forms or cell types (e.g. yeast to hyphae), and has a significant role in response to environmental stimuli and virulence [18, 19]. Mixed species biofilms are also observed in nature, however it is beyond the scope of this study and therefore will not be discussed in this dissertation report.

1.3 Fungal biofilms

Many pathogenic fungal species including *Candida* [20], *Aspergillus* [21], and *Cryptococcus* [22], produce biofilms and cause significant clinical and economic problems. Fungal biofilm infections are a major cause of mortality and morbidity in hospitals [23-27]. *Candida* spp. infections, termed candidiasis, are the fourth leading cause of hospital-acquired bloodstream infections in the United States [28, 29]. Among *Candida* spp., *C. albicans* is the most common source of infections. In fact, the mortality rate associated with systemic and superficial infections by *C. albicans* is about 40% [30, 31]. *Candida* spp. infections are generally associated with indwelling medical devices like catheters, heart valves, artificial joints etc. This association is thought to be related to the formation of biofilms by *Candida* on these devices. Biofilm-based infections are tenacious, and often the only way to resolve it is by removal of the implant followed by long-term antifungal treatment. If the biofilm is not completely eliminated, it continues to disseminate cells causing persistent infections [32, 33].

The already difficult problem of biofilm elimination is made worse by the drug resistance associated with biofilms [33-42]. Clinical resistance is defined as 'persistence or progression of an infection despite appropriate antimicrobial therapy' [43]. Numerous factors are considered to be responsible for fungal biofilms drug resistance, including alteration of growth rate, presence of ECM, expression of resistance genes, etc. [44, 45]. *C. albicans* biofilm drug resistance will be used as an example to elaborate the case in point.

In *C. albicans,* antifungal resistance is directly correlated to biofilm growth and maturity [46]. In fact, growth in a biofilm leads to differential upregulation of genes that contribute to drug resistance. For example, β-glucan present in the ECM of *C. albicans* biofilms was shown to possess drug-sequestering properties that enhance resistance of biofilms to some antifungals [47, 48]. Additionally, upregulation of genes encoding multidrug efflux pumps (*e.g.* Candida drug resistance, *CDR*) and multidrug resistance (*MDR*) genes, is also a major resistance mechanism [49, 50].

The increasing frequency of biofilm infections in clinical settings and the difficulty faced in treating them effectively, creates a palpable need to get detailed understanding of biofilm formation in fungi, to aid in identification of new drug targets. One approach is to study a close relative like *Saccharomyces cerevisiae* that is genetically more tractable.

1.3.1 *Saccharomyces cerevisiae* as a model system to study biofilm formation

S. cerevisiae, commonly known as baker's yeast, belongs to the phylum Ascomycota. It was the first eukaryote to be completely sequenced, annotated and made publicly available [51, 52]. It is not a pathogenic fungus *per se,* but is a relative of the fungal pathogen *Candida spp* [53-55]. Its utility as a model system is bolstered by the wealth of genetic and phenotypic data available on it. In addition to being a model for other fungi, it is also a model for higher eukaryotes, including humans, making it an ideal surrogate system to study some aspects of biology for its pathogenic relatives or humans [56-58].

S. cerevisiae belonging to strain background Σ1278 are capable of forming biofilms that we term mats, on semi-solid agar (0.3%) media [59, 60]. When grown at 25°C for 5 days, strains of the Σ1278 background develop an elaborate pattern that resembles a floral or wheel shape. The mat can be structurally differentiated into a central hub and peripheral rim (Figure 1.2). The hub has a characteristic wrinkled appearance, while the rim in contrast appears smooth. Spoke-like structures resembling water channels, a hallmark feature of biofilms, radiate from the central hub towards the edge of the growing mat. Cells of both rim and the hub are in yeast form, although pseudohyphal forms are also observed near the water channels.

1.3.2 Extracellular matrix (ECM)

A hallmark characteristic of a biofilm is the presence of ECM, a carbohydrate rich matrix that envelops in the mature biofilm. Though its composition can vary considerably depending on the

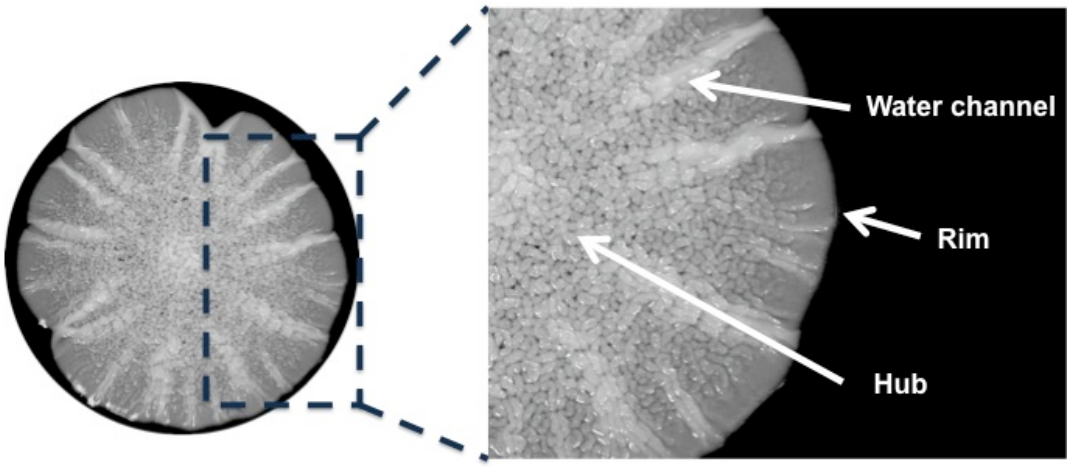

Figure 1.2: Structure of a mat formed by S. cerevisiae

composite species, it is known to have a significant impact on the architecture, virulence and drug resistance of a biofilm [13]. Common components found in ECM include water, exopolysaccharides, lipids, extracellular DNA and proteins [61-66].

ECM functions [67] include:

(a) Protection: decreased permeability of ECM is believed to exclude drugs and other harmful molecules from reaching the biofilm cells [68]. Additionally its ability to retain water also protects biofilms from desiccation [66].

(b) Nutrient reservoir: ECM retains and distributes nutrients, oxygen and waste products via conduits, in the form of water channels, throughout the biofilm structure [12].

(c) Genetic information exchange: ECM aggregates the biofilm cells together facilitating horizontal gene transfer in many bacterial biofilms. Horizontal gene transfer is a major factor for transfer of drug resistance genes within a biofilm community [2, 67].

Although ECM formed by biofilms of pathogenic fungi are the subject of many studies, there are also reports suggesting presence of ECM in its non-pathogenic, biofilm-forming relative *S. cerevisiae* [69-71]. Working on a simpler model system like *S. cerevisiae* could help to understand the basic molecular mechanisms of ECM assembly and its composition. Considering the importance of ECM in biofilm reinforcement, a deeper understanding of ECM could help in designing new drugs that can efficiently dismantle or eliminate the biofilm.

1.3.3 Fungal cell wall

Some of the drug discovery effort against *Candida* is targeted towards the fungal cell wall. This is because it is the primary defense of the cell and also harbors many essential virulence factors. Additionally, many components of the fungal cell wall are absent in mammalian cells, thus discerning details of the fungal cell wall architecture and/or synthesis could aid in identifying attractive drug targets that promise no or minimal host damage.

The cell wall is the outermost layer and an essential organelle of the cell. The interior of the cell (cytoplasm) is separated from the cell wall by a semi-permeable lipid bilayer called the plasma membrane. The plasma membrane structure is a highly selective barrier, interspersed with proteins, which efficiently control the ingress and egress of molecules across it.

The cell wall composes about 10-25% of the cell mass depending on the growth conditions. Its major functions [72] include:

(a) Protection: Cell wall is the primary protective barrier of the cell against environmental stresses e.g. temperature variations, mechanical forces, pH change etc.
(b) Maintaining osmotic balance: The cells are under constant mechanical and turgor pressure since the internal osmolarity of the cell is higher than the outside. Without a cell wall barrier, water coming into the cell to achieve osmolytic balance would rupture the plasma membrane. The cell wall thus protects the cell from osmotic shock.
(c) Maintaining cell shape: Fungi can exist in hyphal, pseudohyphal or yeast forms depending on the conditions. Each shape is important and is also shown to be crucial for response to stimulus and virulence [73]. The cell wall is a strong yet elastic structure that creates and maintains the cell shape during morphogenesis.

Though numerous models have been proposed describing the organization of the fungal cell wall, the exact details of its structure are still not completely clear. This is because most methods currently used to analyze the cell wall and its composition involve harsh treatments that destroy it or fix it (i.e. alkaline hydrolysis, freeze drying, embedding), and it has not been possible to find methods that give higher resolution in living cells. Additionally, there are differences in the cell wall compositions between species, and results from one cannot necessarily be extrapolated to the other.

With those limitations in mind, a commonly accepted general model of the *S. cerevisiae* cell wall is shown in Figure 1.3. The cell wall is a multi-layered structure, where the innermost layers are composed of polysaccharides, namely chitin and β-glucans. Chitin is a polysaccharide of N-acetylglucosamine (NAG). The β-glucans form a complex three-dimensional network in the core of the cell wall. This network consists mainly of linear β-1,3-glucan and branched β-1,6-glucan which in turn is linked to β-1,3-glucan via β-1,4-glucan. The β-glucan polysaccharide is considered to be the central load bearing layer of the wall. It also acts as a matrix that supports attachment of various glycoprotein adhesins [72].

The outer edge of the wall is a framework made of cell wall glycoproteins. One class of glycoproteins is modified by addition of a glycosylphosphatidylinositol anchor (GPI) to the extreme C-terminus, and these are referred to as GPI-anchored proteins. These proteins are targeted to the plasma membrane by the secretory pathway. They either remain attached to the outer leaflet of the plasma membrane by their GPI-anchor or are released by cleavage of the GPI-anchor, and are then covalently re-attached to β-1,6-glucan or chitin in the cell wall by their GPI-

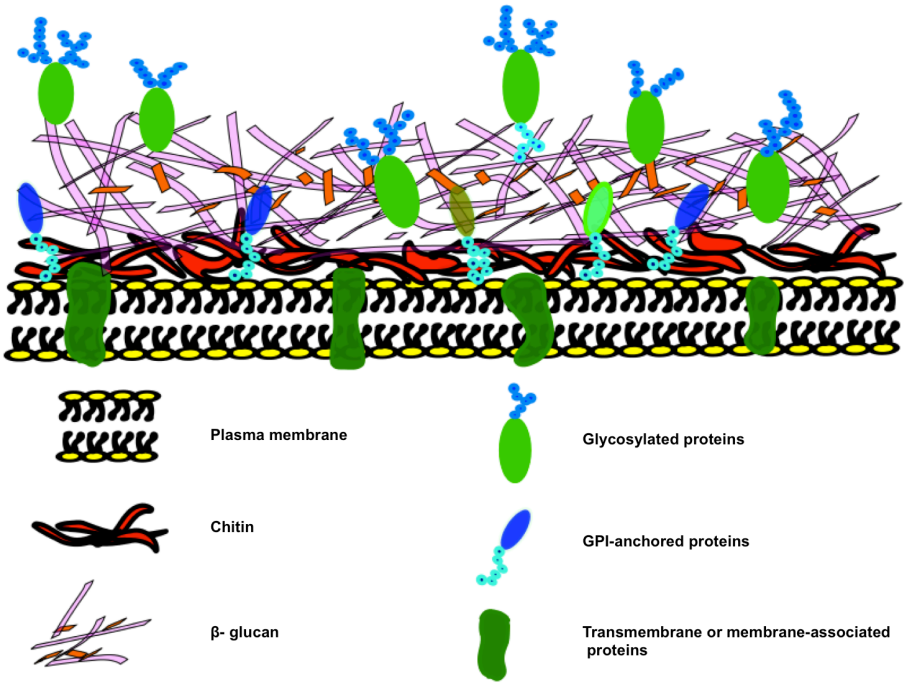

Figure 1.3: Model depicting cell wall architecture

remnant [74]. The other class is Pir (**P**roteins with **i**nternal **r**epeats) proteins, and they are covalently linked directly to β-1,3-glucan chains [75].

The cell wall can thus be described as being composed mainly of a complex network of polysaccharides acting as scaffolds for proteins. Since the cell wall is a dynamic structure whose composition is constantly altered depending on age, environmental condition or life stage, the cell expends substantial metabolic energy in its maintenance. In fact, a genomic scale screen carried out by de Groot *et al* showed that over 1200 genes (~23% of the genes in the genome) were directly or indirectly involved in cell wall formation and its regulation [76]. Cell wall integrity (CWI) [77], Multivesicular body (MVB) [78] and RIM101 [79] pathways are three pathways that affect the cell wall and are described below.

1.3.3.1 Cell wall integrity (CWI) pathway

Although cell wall maintenance and response to stress involves 'cross talk' between many pathways [80], the cell wall integrity (CWI) pathway [77, 81] is the principal signaling cascade responsible for the maintenance of the cell wall . The purpose of the CWI pathway is to detect and respond to cell wall requirements, for example, a stimulus to divide or environmental cell wall damage. Any defect in CWI signaling leads to increased sensitivity to cell wall stress such

as high temperature (37°C) and chemical stressors e.g. SDS, caffeine, Congo Red etc. The main steps of the CWI cascade are shown in Figure 1.4, and are described below.

Wsc1p, Wsc2p Wsc3p and Mid2p are the four transmembrane protein sensors that detect and transmit the signal to downstream components [77, 82-84]. All sensors are single pass proteins that share some structural domain features as follows – (a) a small C-terminal cytoplasmic domain; (b) a periplasmic central serine/threonine rich domain that is highly O-mannosylated; and (c) an external N-terminal domain. Among the aforementioned sensors, Wsc1p and Mid2p are the major sensors since a *wsc1Δ mid2Δ* double mutant requires osmotic support at all growth temperatures [84]. The activated sensors recruit a Guanine Exchange Factor (GEF) to the plasma membrane via phosphatidylinositol (PI)-4,5-bisphosphate (PIP_2) signaling [85].

The cytoplasmic domain of Wsc1p and Mid2p interact with the N-terminal domain of the GEF named Rom2p [86]. Rom2p is a regulator of **R**as-**ho**mologous (Rho) family GTPase named Rho1p. Rho1p is a member of the 5 Rho family (Rho1p − Rho5p) in yeast, and its deletion is lethal for the cell. It is called the 'master regulator of the CWI signaling', since it is the hub of the cell wall sensory network, receiving inputs from sensors and in turn regulating a variety of outputs like cell wall biogenesis, actin organization and polarized secretion [77, 81]. Rom2p interacts with the GDP bound Rho1 through its Dbl homology (DH) domain, and activates it by catalyzing the nucleotide exchange activity [87].

There are multiple direct targets of Rho1p activation namely protein kinase C-1 (Pkc1), glucan synthase (GS), formin proteins (affecting actin cytoskeleton), secretory pathway component Sec3p and **S**uppressor of **K**re **n**ull 7 (Skn7p) transcription factor. Pkc1p is another essential protein whose disruption leads to multiple cell wall defects [88]. Upon activation by Rho1p [89], Pkc1p in turn activates the mitogen activated protein kinase (MAPK) cascade composed of MAPKKK (Bck1p), MAPKK (Mkk1/2), and MAPK (Mpk1/Slt2p). Loss of MAPK cascade components leads to growth defects at high temperature (37°C). The MAPK cascade ultimately leads to activation of the transcription factor Rlm1p, which regulates expression of about 25 genes involved in cell wall biogenesis or encoding cell wall proteins

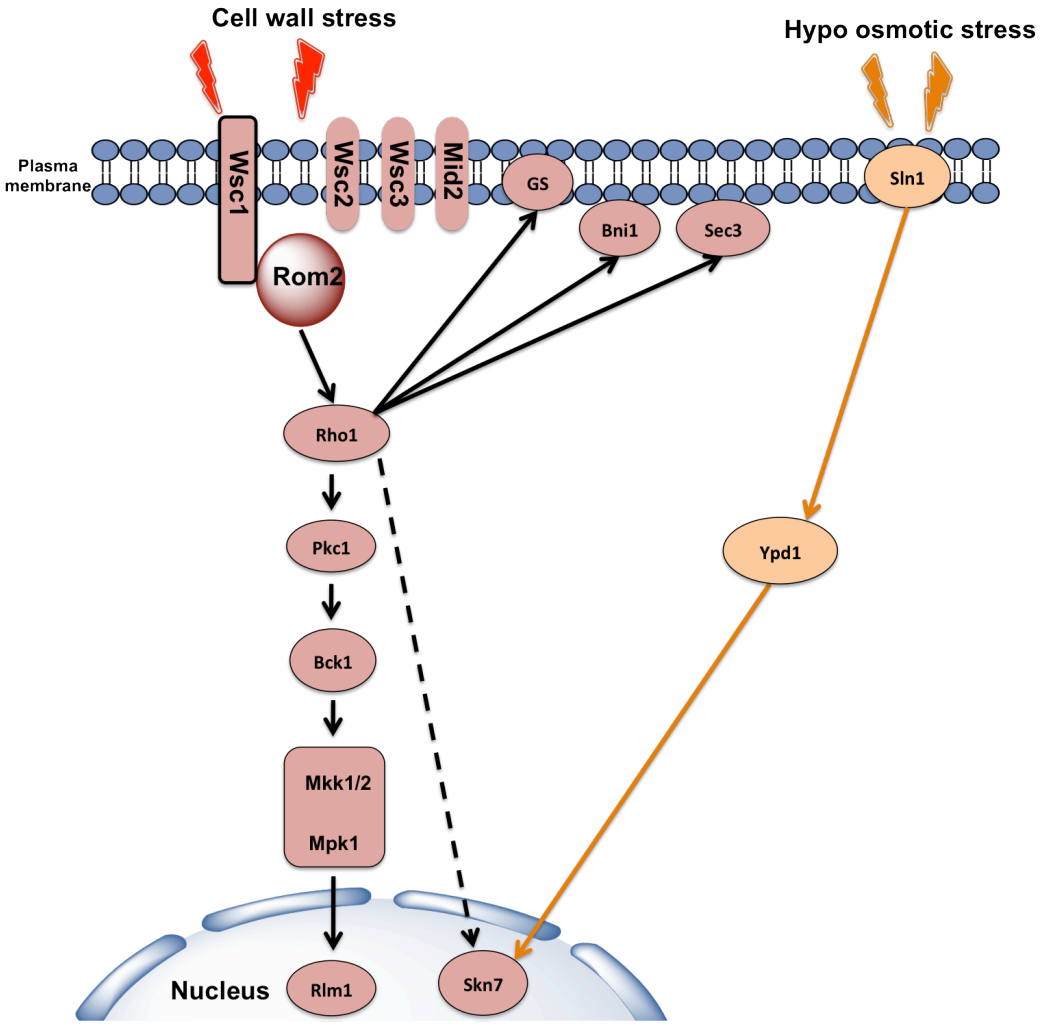

Figure 1.4: Model depicting CWI pathway with the hypo-osmotic stress sensing pathway Sln1 branch.

The Skn7p transcription factor is related to the bacterial two-component signal transduction pathways [90]. It is the target of both the CWI pathway (via Rho1p) and the osmoregulatory HOG pathway (via Sln1p). Upon activation, Skn7p regulates the expression of a score of cell wall genes that are required for cell wall maintenance.

1.3.3.2 Multivesicular body pathway (MVB)

The plasma membrane is embedded with numerous proteins that play important roles in multiple functions that affect the cell wall like membrane and small molecule transport, signal transduction, cell-cell interactions or cell-environment interactions. These include cell wall receptors like Wsc1p, and this protein is constantly endocytosed and recycled back to the plasma

membrane. Other types of receptors are degraded even as the cell wall is modified as a consequence of their activation, and the jobs of recycling or degradation are entrusted to the endosomal system [91].

The cell membrane proteins internalized to the endosome for degradation ultimately are delivered to the lumen of the vacuole. However, before being delivered to the vacuole the protein cargo first gets ubiquitinated and then internalized by the endosome into membrane invaginations called vesicles. The endosome structure with multiple such vesicles is called the multivesicular body (MVB), and the pathway from internalization of protein cargo to final fusion of the late endosome with the vacuole is called the MVB pathway [91].

Crucial components of the MVB pathway are endosomal sorting complex required for transport (ESCRT) complexes composed of a subset of vacuolar protein sorting (vps) proteins that are subclassified as class E vps proteins. Class E vps proteins are characterized based on their respective mutant phenotypes which are defined by the formation of an aberrant "prevacuolar" compartment within the endosome referred to as the Class E compartment [92]. All of the Class E vps mutants perturb the ubiquitin-dependent sorting of proteins by the MVB pathway [93, 94].

The steps of the MVB cascade (Figure 1.5) involve (a) Identification of the ubiquitinylated cargo by Vps27p and Hse1p; (b) Deformation of the endosomal membrane by the ESCRT-I complex (Vps37p, Vps28p, Vps23p) to allow subsequent steps of cargo intake; (c) Formation of invaginations by the ESCRT-II complex (Vps22p, Vps25p, Vps32p) leading to cargo protein engulfment and finally (d) Abscission by the ESCRT-III complex (Vps2p-Vps24p, Vps20p-Snf7p) to form intra-luminal vesicles containing the cargo. The complex is disassembled by the ATPase Vps4p. Fusion of the limiting membrane of the endosome with the vacuole ultimately leads to degradation of the intra-luminal vesicles and cargo. [95-97].

1.3.3.3 RIM101 pathway

MVB pathway components are also involved in a pH sensing signaling cascade named RIM101 pathway.

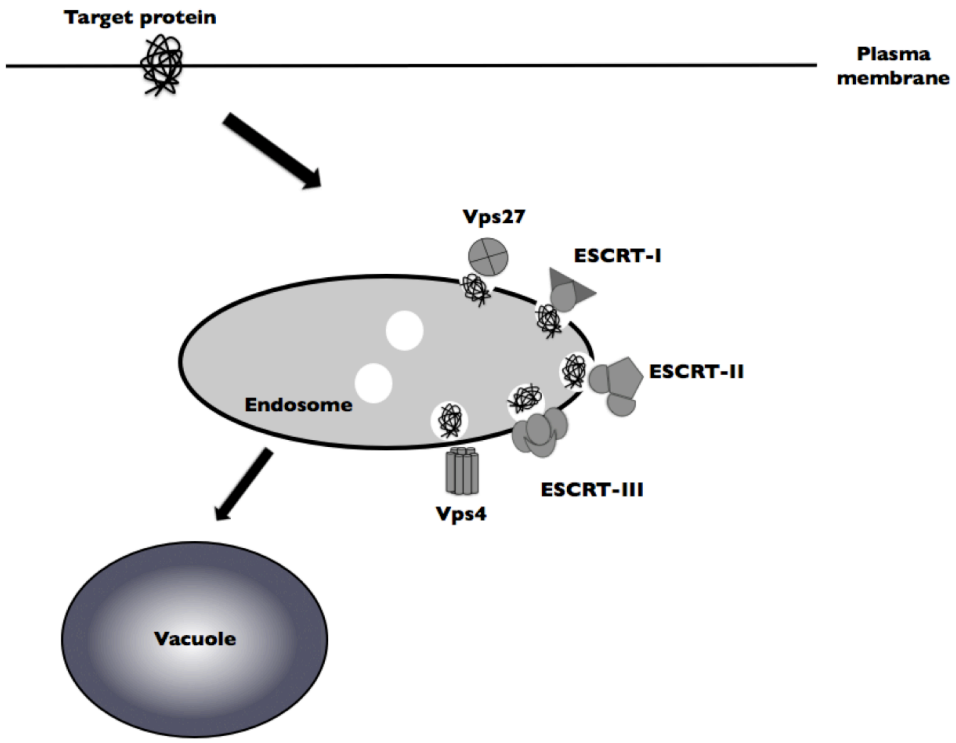

Figure 1.5: MVB pathway model

The steps of the pathway are as follows (Figure 1.6): pH change is sensed by plasma membrane receptors Rim21p and Dfg16p [99, 100]. Upon receptor activation, the β-arrestin-like protein Rim8p gets ubiquitinated, recruits the receptor to the endosome and interacts with the endosomal sorting complex required for transport I (ESCRT-I) complex protein Vps23p [101]. The ESCRT-I complex recruits the ESCRT-II complex and ESCRT-III Vps20p-Snf7p heterodimer sequentially [102]. Snf7 then interacts with Rim20p, a scaffolding protein that in turn interacts with a protease Rim13p, which cleaves the inhibitory C-terminal domain of Rim101p [103, 104]. Activated Rim101p then translocates to the nucleus and regulates alkaline pH response genes [105].

1.3.4 Fungal adhesins

As described in section 1.2, a key step for establishing a biofilm is the property of adhesion. Adherence to foreign surfaces like host tissues or indwelling medical devices (e.g. catheter,

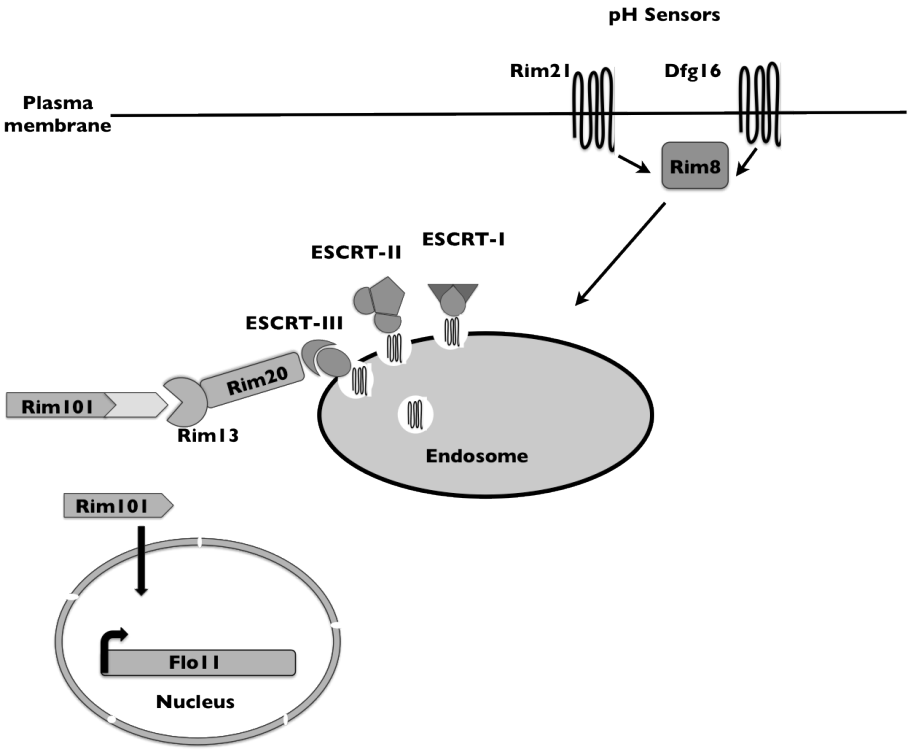

Figure 1.6: Model depicting Rim101 pathway

implants, etc.), allow fungi to establish biofilms and flourish on these otherwise inhospitable surfaces [106, 107]. The ability of the cells to adhere to each other is also crucial for mediation of cell-cell interactions. Cell aggregates formed by cell-to-cell adhesion, termed as flocculation, is advantageous in wine production where it allows convenient separation of the floating biofilms from its fermentation product [98].

In yeast, specialized proteins collectively referred to as adhesins or flocculins, confer this property of adhesion. Different fungal species possess different families of adhesin genes, like FLO (flocculation) in *Saccharomyces cerevisiae,* agglutinin-like sequence (ALS) in *Candida albicans* and epithelial adhesion (EPA) genes in *Candida glabrata*. Although these proteins are present on the cell wall, reports have emerged showing secreted adhesin proteins having a role in signal transduction as well [108, 109].

Cell wall associated adhesins are associated with the plasma membrane by a glycophosphatidylinositol (GPI) anchor sequence. They share common architectures that can be divided into three domains,

(i) Amino (N) terminal domain: this region possesses carbohydrate, protein, plastic, and/or calcium ion binding properties and protrudes from the cell surface.

(ii) Central stalk: this domain is rich in serine and threonine residues. It is of variable length and riddled with tandem repeats making it vulnerable to errors during replication, resulting in recombination based additions or deletions. These rearrangements are of significant functional relevance since they encourage adaptive evolution in adhesins. For example, Fidalgo *et al* [110] showed that rearrangements in this central tandem repeat region yielded more hydrophobic variants of Flo11p, which gave the yeast biofilms the ability to float and gain access to oxygen.

(iii) Carboxy (C) terminal domain: this region is covalently attached to the GPI anchor and is generally conserved within the gene families of adhesins e.g. FLO family members.

1.3.4.1 ALS adhesin family

Candida albicans is an opportunistic pathogen that is part of the natural flora, but turn pathogenic when the host's resistance is low, like in case of immune-compromised patients. The primary step for causing disease is its adhesion and invasion of mucosal or prosthetic surfaces. Once established, it can disseminate through bloodstream and invade other organs. *C. albicans* encodes adhesin proteins including Eap1p, Hwp1p and a family of proteins belonging to the ALS family. Adhesins belonging to agglutinin-like sequence (*ALS*) gene family, that encodes the cell surface adhesin proteins Als1p – Als7p and Als9p, are particularly important. Their role is not just limited to adhesion, but they are also considered to be important for host colonization, virulence, and biofilm formation [111, 112]. They are differentially expressed based on growth stage and environmental cues (growth media or host niches), and thus are considered to be an essential part of *C. albicans* host-site-specific response [113]. Among the *ALS* adhesin proteins Als1p and Als3p appear to be major players in virulence, since *als1Δ/als1Δ* and *als3Δ/als3Δ* exhibit reduced virulence in different infection models [114-116], while deletion of some other *ALS* proteins caused no major disruptions [117-119]. This suggests that the adhesin proteins can be multi-functional and have diverse effects on survival of pathogenic fungi within the host.

1.3.4.2 FLO adhesin family

In the *S. cerevisiae* genome, there are five genes encoding adhesins – *FLO1, FLO5, FLO9, FLO10* and *FLO11*. The *FLO* gene family is named so because some of its members (*FLO1*, *FLO5*, *FLO9, FLO10* and *FLO11*) encode proteins that are responsible for a phenotype called flocculation. Flocculation is a reversible, asexual, Ca^{2+} dependent and lectin-mediated adhesive phenotype, in which cells grown in liquid medium aggregate to form clumps or 'flocs'. Flocculation is a complex phenomenon where the type of FLO gene expressed, the ability and timing of flocculation and properties of the formed floc can vary considerably depending on the genetic background of the strain being used, environmental conditions and media conditions (Figure 1.7). Depending on the aforementioned conditions, the formed flocs separate from the bulk medium and either rise and float to the surface of the liquid or sediment to the bottom of the container [120]. This phenotype is of considerable importance in the brewing industry since it provides a cheap and effective way to separate the yeast cells form the final brewed product [98].

In the laboratory strain ∑1278b however, only *FLO11* gene is expressed, while the rest of the adhesin encoding genes of the FLO gene family are transcriptionally silenced [121]. The FLO11 promoter is almost 3kb in length and is one of the largest in the *S. cerevisiae* genome [122]. This is because, Flo11p expression is regulated by many pathways and factors including but not limited to the filamentous growth mitogen activated protein kinase (fMAPK), protein kinase A (PKA) cascades and RIM101 pathways [123-125]. Additionally, there is a population level heterogeneity in Flo11p expression due to epigenetic silencing, resulting in a mixture of cells with some expressing and some silenced for Flo11p [126].

Flo11p expression activation, as a response to nitrogen starvation stimulus, results in activation of pseudohyphal mode of growth. Pseudohypae are long branched chains of elongated cells resulting from unipolar budding. These pseudohyphae adhere to and penetrate into agar

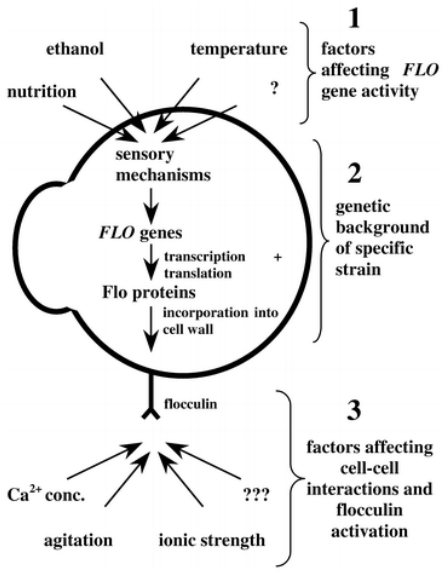

Figure 1.7: Factors affecting flocculation.
The factors can be divided into three categories according to their mode of action. It is to be noted that some factors can affect more than one mechanism. Figure from Verstrepen K. J. et al [98]

medium to forage for nutrients, resulting in a phenotype known as invasive growth [127]. Neutral-alkaline pH sensing RIM101 pathway (Figure 1.6) is essential for invasive growth since it regulates *FLO11* expression [128, 129].

1.4 Characterization of biofilm formation in *S. cerevisiae*

Mat formation in *S. cerevisiae* is driven by multiple environmental cues like pH and glucose level [60]. The cells forming the hub adhere strongly to each other and the agar surface, while the smooth rim can be separated easily from the hub using a simple assay we refer to as overlay adhesion assay (refer to Methods and materials in Chapter 2). Mat formation, adherence and invasive growth are Flo11p dependent phenotypes [59, 60]. However, there is no difference in Flo11p expression between the rim and hub cells [60]. Hence, details about factors responsible for the difference between rim and hub remain unknown. In chapter 2, we report the existence of a Flo11p-independent pathway that affects mat formation. Ours and other published reports provide evidence to suggest that mat or biofilm formation is a complex phenotype, requiring a myriad of factors in addition to adhesins [130, 131]. In chapter 3, we will provide evidence showing that a subset of the cell wall integrity pathway is part of this Flo11p-indepenent mechanism for a mat formation. Finally, in chapter 4 we will show that a factor that makes the

rim and hub cells distinct in a mat is the presence of a carbohydrate, named chitosan, in the hub, and this plays a role in resistance to cell wall stresses and antimicrobials.

1.5 Bibliography for Chapter 1

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Chapter 2

Vacuolar protein sorting genes regulate mat formation in *Saccharomyces cerevisiae* by Flo11p-dependent and –independent mechanisms

Disclosure

Chapter 2 was published in its entirety as Sarode N., Miracle B., Peng X., Ryan O., Reynolds TB., *Eukaryotic cell* 10(11):1516-26. Sarode N. created majority of the constructs and performed the assays for screening of mutants for mat formation, immunofluorescence assays, cell fractionation and Western blotting. Miracle B. performed the RT-PCR assays and helped create Flo11-HA [30, 1015]. Peng X., created some *VPS* deletion mutants. Ryan O., created the whole genome deletion library and carried out the preliminary screen for mat formation phenotype on library deletion mutants.

2.1 Background

Microbes exhibit "multicellular" behaviors such as swarming and the formation of colonies, fruiting bodies, and biofilms [1-5]. All of these behaviors depend on cells interacting with one another and the local environment. The baker's yeast *Saccharomyces cerevisiae* is able to grow in a number of different multicellular forms including pseudohyphae, floating biofilms on sherry wine, and biofilms on the surface of low density agar plates (referred to herein as mats) [6-8]. All of these growth forms are dependent on the presence of a glycosylphosphatidylinositol (GPI)-anchored cell surface adhesion protein called Flo11p, which is similar to fungal adhesins found in a number of different yeasts, including several pathogens [9, 10].

This chapter is focused on the Flo11p-dependent multicellular phenotypes of invasive growth and mat formation. During invasive growth, yeast grow as chains of cells that invade into the relatively dry surface of 2% agar plates made with yeast extract-peptone-dextrose (YPD) medium [7]. During mat formation, yeast grows as biofilms that spread over the wet surface of 0.3% agar YPD plates. As the mats mature, they generate two morphologically distinct regions. The central region of the mat is called the hub and consists of aggregates of cells that adhere to both the agar surface and one another and form channels and wrinkles that are hallmarks of biofilms. The outer region of the mat is called the rim, and it is smooth in appearance, and consists of a dividing, spreading population of cells that are not particularly adherent to one another or the agar surface [7, 11].

The regulation of Flo11p and its impact on the yeast multicellular behaviors such as invasive growth (which occurs in haploid yeast cells) and pseudohyphal growth (a related phenotype that occurs in diploid yeast cells) have been the subjects of numerous studies, many of which have been reviewed previously [9, 12]. Several of these mutations that perturb *FLO11* expression and affect invasive growth also disrupt mat formation, such as mutations in glucose sensing pathways and transcription factors that regulate inositol biosynthesis [11, 13].

In contrast, there are examples in the literature of mutations that cause defects in invasive growth but not mat formation, and vice versa. The *ste12*Δ mutation only has a very minor effect on mat formation, but quite a strong effect on invasive growth [7]. Conversely, a number of Hsp70-encoding genes such as *SSA1* and *SSA2* have strong defects in mat formation, but not invasive growth. These Hsp70 mutants also do not appear to affect Flo11 protein expression [14].

This chapter examines whether the Rim101p signal transduction cascade, which is known to control invasive growth and *FLO11* expression [15, 16], also regulates mat formation. The Rim101p signaling pathway is required for cells to respond to neutral or basic pH [17, 18], and is necessary for invasive growth. A model for the Rim101p pathway (Figure 2.1) is as follows: two plasma membrane receptors called Rim21p and Dfg16p [19] detect extracellular signals, such as neutral pH, and are recruited to the endosome by the ß-Arrestin-like protein Rim8p [20-23]. This event recruits several of the ESCRT (Endosomal Sorting Complex Required for Transport) complexes (I, II, and III), which are also required for proper protein sorting in the endosome. Snf7p of the ESCRT-III complex recruits the Rim13p protease via the Rim20p scaffolding protein, and Rim13p cleaves off the Rim101p C-terminal inhibitory domain to activate it.

The ESCRT complex subunits involved in Rim101p processing [20, 21] are part of a subset of vacuolar protein sorting (vps) components called Class E vps proteins. The original vps mutants were grouped into 6 classes (A through F) based on distinct vacuolar morphology defects [24]. About 13 vps mutants belong to Class E, and are characterized by the formation of an aberrant "prevacuolar" compartment within the endosome referred to as the Class E compartment [24]. All of the Class E vps mutants perturb the ubiquitin-dependent sorting of proteins through the endosome to the vacuole by a pathway referred to as the multivesicular body (MVB) pathway [25, 26]. However, only the ESCRT proteins affect Rim101p signaling [20, 21].

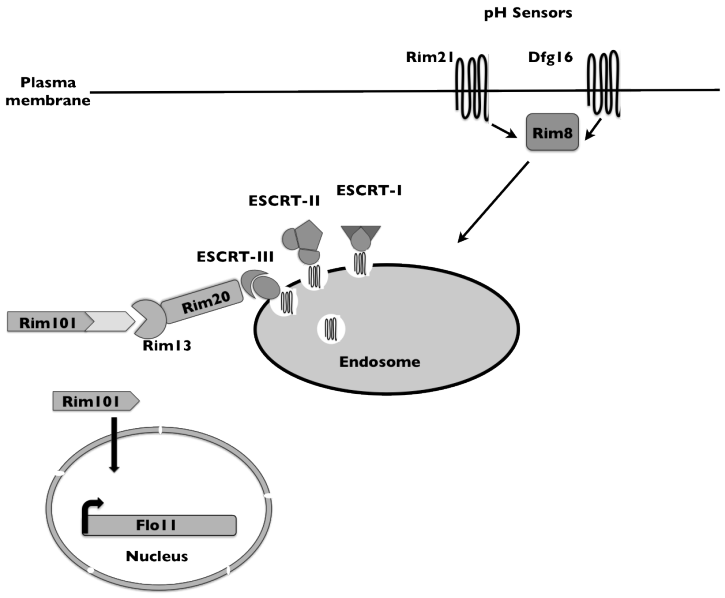

Figure 2.1 : Model for Rim101 pathway

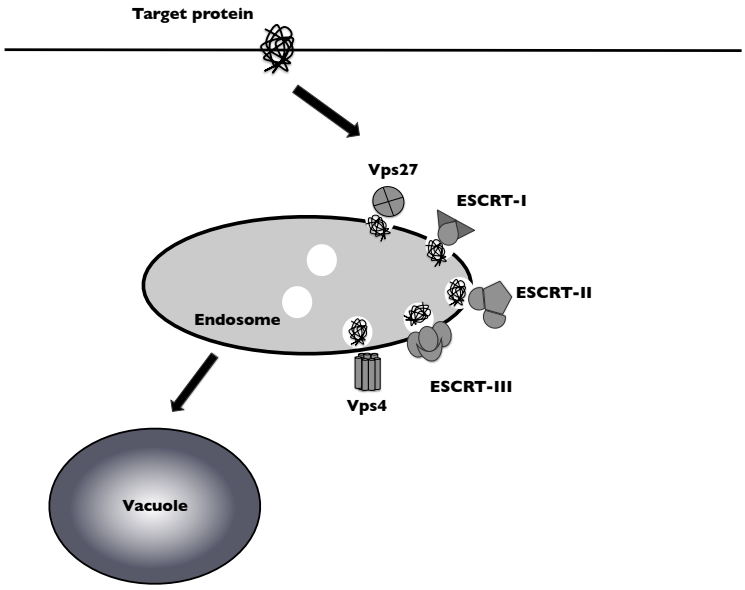

Figure 2.2 Model for Multivesicular body (MVB) pathway

The steps of the MVB cascade involve (a) Identification of the ubiquitinylated cargo by Vps27p and Hse1p; (b) Deformation of the endosomal membrane by the ESCRT-I complex (Vps37p, Vps28p, Vps23p) to allow subsequent steps of cargo intake; (c) Formation of invaginations by the ESCRT-II complex (Vps22p, Vps25p, Vps32p) leading to cargo protein engulfment and finally (d) Abscission by the ESCRT-III complex (Vps2p-Vps24p, Vps20p-Snf7p) to form intra-luminal vesicles containing the cargo. The complex is disassembled by the ATPase Vps4p. Fusion of the limiting membrane of the endosome with the vacuole ultimately leads to degradation of the intra-luminal vesicles and cargo (the MVB pathway is illustrated in Figure 2.2) [21, 27, 28].

This study reveals that several MVB mutants that are not part of ESCRT-I, II, or III affect mat formation, but not invasive growth, and can be used to genetically separate these phenotypes. The results presented in this chapter indicate the existence of two overlapping pathways that pass through the MVB and affect mat formation by *FLO11*-dependent and independent mechanisms. The first pathway is the Rim101p pathway, and it affects invasive growth and mat formation by controlling *FLO11* expression. The second pathway, henceforth called the biofilm pathway, requires the entire complement of class E vps components necessary for a properly functioning MVB and affects mat formation in a *FLO11*-independent manner.

2.2 Methods and materials

2.2.1 Strains, media, and growth conditions

All strains used in this study belong to yeast strain background ∑1278 (Appendix Table A-1) [7].

The strains found in appendix Table A-3 are from a whole genome deletion collection created in the ∑1278b background, by Owen Ryan and colleagues in the laboratory of Charles Boone, at the University of Toronto. A full characterization of the library and the phenotypes of all mutants regarding mat formation, invasive growth, and pseudohyphal growth will be published separately (Ryan et al, **submitted**). Mutants were generated by PCR-based gene disruption methods [11, 29]. Primers are listed in appendix Table A-2. The *RIM101-531* dominant active allele was also generated by PCR-based disruption of the C-terminal 95 codons of the *RIM101* gene (see Appendix Table A-2 for primers). Transformations were performed by the standard lithium acetate transformation method [30]. The yeast strain L6906 [31] carries a hemagglutinin (HA)-tagged form of *FLO11*, with the HA tag between amino acids 30 and 31 (*FLO11-HA*^{30}), and this was used for the immunofluorescence analyses. Primers PC675 and PC676 (see Appendix Table A-2) were used to insert an additional HA tag-encoding DNA sequence between codons encoding amino acids 1015 and 1016 of Flo11p (*FLO11-HA*^{30,1015}) [32] by the method of Schneider et al [33]. All strains were maintained on standard yeast extract-peptone-dextrose (YPD) media [30] and 250µg/ml G418 was used for selection of transformants, with the exception of the *RIM101-531* truncation, which was selected on minimal media lacking histidine [30]. Strains grown on low agar plates (YPD with 0.3% agar) [7] for 5 days at 25°C were used for overlay adhesion assays, immunofluorescence, and Western blotting.

2.2.2 Invasive growth assay and overlay adhesion assay

The invasive growth assay was performed as described previously [13]. The overlay adhesion assay was performed as described [11].

2.2.3 Real time reverse transcriptase polymerase chain reaction (rtRT-PCR)

Five days old mats were used to perform this assay. The cells from growing mats were collected from the surface of low agar YPD plates using a clean dry spatula and washed with ice-cold water. Total RNA was extracted as previously described [34]. Contaminating DNA was removed with the TurboDNA-free kit (Ambion) according the manufacturer's protocol. rtRT-PCR was

performed on an Bio-Rad iCycler real time PCR machine using the Verso SYBR green two step kit with random primers for the reverse transcription step according the manufacture's protocol. rtRT-PCR primers for *FLO11* and *ACT1* (reference gene) are listed in appendix Table A-2.

2.2.4 Immunofluorescence of Flo11-HA30 on the cell surface of cells from the rim and hub

The assay was performed as described in [11], where cells were taken from the rim of the growing mats.

2.2.5 Cell Fractionation

Fractionation of cells carrying Flo11-HA^{30,1015} was carried out as follows. Mats were grown on low agar YPD plates for 5 days at 25°C. Overlay adhesion assays were performed on the wild-type mats to separate rim and hub cells. Rim cells were washed into a microfuge tube off the plastic wrap with 1 ml of 50 mM Tris-HCl (pH 7.4) buffer. The adherent cells forming the central hub or the cells composing the entire mat from defective mutants were scraped from the agar using a clean dry spatula paying attention to bring a minimum carryover of agar during this process. The hub (wt) or mutant cells were then suspended in 1ml of 50mM TrisHCL (pH 7.4). Microfuge tubes containing cells from all of these separate samples were then taped onto a roller barrel and washed for 20 mins at 23°C. 20µl of sample was removed in a separate tube to be used for normalization calculations for loading SDS-PAGE gels (see normalization section below). The remaining cells from each sample were then pelleted and the supernatant was removed to a separate tube. This supernatant represents proteins shed from the cell wall (S), and proteins in this fraction were precipitated as described below (see protein precipitation section). The cell pellet was resuspended in 0.8 ml of 50mM TrisHCl (pH 7.4) and ruptured using glass beads in the presence of protease inhibitors (Protease Inhibitor Cocktail SE, EMD Chemicals Inc) by vortexing for 1 min and cooling on ice for 1min, and repeating this two-step cycle five times. The liquid above the glass bead layer was removed to a separate tube and centrifuged at ~13,000xg to pellet the cell wall and membranes. The supernatant (SF, representing the cytosolic or soluble fraction) was stored at -20°C. The membrane/cell wall pellet was resuspended in 100 µl of 50mM Tris-HCL (pH 7.4) + 2% Sodium dodecyl sulfate (SDS) and boiled for 5 min followed by centrifugation for 10mins. The supernatant containing membrane bound and non-covalently attached cell wall proteins was removed to a fresh tube to create the membrane/non-covalent fraction (M). The remaining cell wall pellet was boiled again for 10

mins in 100µl 50mM Tris-HCl (pH 7.4) + 2% SDS followed by centrifugation. This second membrane supernatant was then combined with the first (membrane fraction) to obtain the total membrane/non-covalent fraction (M). The final cell wall pellet was then resuspended in 100 µl of 50mM TrisHCl (pH 7.4) containing 2 units of ß-1,3-glucanase (Quantazyme, MP Biomedicals) and 0.3µl of β-mercaptoethanol and incubated for 2hrs at 30°C followed by centrifugation for 10min at 13,000 rpm. The supernatant from the Quantazyme treatment represents the fraction of proteins that are covalently attached to the cell wall (C). Proteins from both the membrane/non-covalent (M) and covalent fractions (C) were precipitated by adding 3 volumes of cold acetone and incubating at 4˚C overnight. The samples were then centrifuged and dried in a speedvac after which samples were resuspended in loading buffer. The fractions were analyzed by SDS-PAGE (4% stacking gel, 5% resolving gel) followed by Western blotting with an anti-HA antibody.

2.2.6 Normalization of fractionation samples for loading

10µl of cells from the washed mat samples were diluted into 490µl of de-flocculation buffer (50mM EDTA) and sonicated with a Misonix Microson XL2000 ultrasonic homogenizer sonicator for 5 pulses (~5 sec each) set on 4. The cells were then enumerated with a hemocytometer.

2.2.7 Precipitation of extracellular proteins from the mat

This method was adapted from Bensadoun A. *et al* [35]. Proteins from the extracellularly shed fraction (S) above were precipitated from the Tris-HCl buffer by first adding $1/100^{th}$ volume of 2% Sodium deoxycholate (DOC) and incubating for 30 mins at 4°C. $1/10^{th}$ volume of 100% Trichloroacetic acid (TCA) was then added for overnight precipitation at 4°C.

2.3 Results

2.3.1 Mutations in class E VPS mutants that block Rim101p processing disrupt invasive growth

The class E VPS genes that encode members of the ESCRT I, II, or III complexes were hypothesized to regulate haploid invasive growth because they affect Rim101p processing which is required for invasive growth and *FLO11* expression in *S. cerevisiae* [15, 16, 21]. These will henceforth be referred to as Class E-1 mutants. Their orthologs have also been shown to affect

filamentous growth in *C. albicans* by affecting Rim101p processing [21, 22]. In contrast, non-ESCRT-I, II, III class E VPS mutants, such as *vps27*Δ or *vps4*Δ, (henceforth referred to as Class E-2 mutants) do not affect Rim101p processing in *S. cerevisiae* or *C. albicans* [36], and were expected to not affect invasive growth in *S. cerevisiae*.

In the ∑1278b background of *S. cerevisiae*, Class E-1 mutants representing three ESCRT complexes, *vps28*Δ (ESCRT-I), *vp25*Δ (ESCRT-II), and *vps20*Δ (ESCRT-III), show a strong defect in invasive growth (Figure 2.3 A) while Class E-2 mutants such as *vps27*Δ and *vps4*Δ do not disrupt invasive growth.

Class E-1 mutants exhibit stronger invasive growth defects than the *rim101*Δ mutant itself or mutations in upstream Rim101p processing components such as *rim13*Δ or *rim9*Δ. While there is a thin layer of cells left behind in the *rim101*Δ*, rim13*Δ, and *rim9*Δ Rim101p pathway mutants, there are practically no cells left behind for the Class E-1 mutants (Figures 2.3 A&B).

2.3.2 Both Class E-1 and E-2 mutants perturb mat formation

Based on the invasive growth assays in Figure 2.3 it was predicted that the mutations that are known to perturb invasive growth (Class E-1 mutants) would perturb mat formation. In particular, it was hypothesized that the Class E-1 mutants would form defective biofilms that differ from the wild-type in three respects. (i) They would fail to form the wrinkles and channels that are hallmarks of the hub in the wild-type (Figure 2.3 C). (ii) They would not cover or spread over as large of a surface area of the agar plates as the wild-type. (iii) They would not adhere to the agar surface when tested for adhesion. In contrast, the mutations that did not perturb invasive growth (Class E-2 mutants, Figure 2.3 A) were predicted to have little to no effect on mat formation. However, when this was tested, it was discovered that both Class E-1 and Class E-2 mutants exhibit strong defects in mat formation that are similar to those of the Rim101p pathway mutants (Figure 2.3 C&D). Similar results have been found by Ryan et al in the laboratory of Charlie Boone at the University of Toronto while screening the ∑1278b whole genome deletion collection that they have generated (private communication). Biofilms formed by the *rim9*Δ and *rim13*Δ mutants resemble those of the *rim101*Δ mutant, while the *vps4*Δ mutant's biofilm resembles that of the *vps27*Δ strain (data not shown).

The Class E-1 and Class E-2 mutants spread poorly compared to wild-type, and they did not generate noticeable patterns on low agar. In addition, they all exhibited defects in adhesion to agar based on the overlay adhesion assay [11]. This assay is performed by laying a piece of

commercial plastic wrap on the agar over the growing cells and then removing it by lifting up on both sides. Cells that adhere to the agar surface stay behind, as seen for the wrinkled center (hub) of the wild-type mat (Figure 2.3 C). Cells that are not agar-adherent are removed as seen for the outer edge of the wild-type (rim). The entire cell population of the Rim101p pathway and Class E-1 mutants were removed by the plastic wrap (Figure 2.3D and data not shown), which is similar to what is seen for *flo11*Δ mutants [11]. The *vps27*Δ and *vps4*Δ mutants adhered slightly better to the agar surface than the other mutants (only *vps27*Δ is shown in Figure 2.3C), however the cells from these mutants that remained on the agar plate were poorly adherent compared to the hub cells from the wild-type.

2.3.3 *FLO11* expression is diminished in Class E-1 mutants but not the Class E-2 mutants

One reason for the difference between the Class E-1 and Class E-2 mutants might be that the Class E-2 mutants exhibit diminished *FLO11* gene expression by affecting some alternative pathway during mat formation, but not during invasive growth. In order to test this, both groups of mutants were compared for *FLO11* expression levels during mat formation by real time reverse transcriptase polymerase chain reaction (rtRT-PCR). These analyses revealed that the *vps28*Δ*, vps25*Δ, and *vps20*Δ mutants all expressed little *FLO11* compared to the wild-type (Figure 2.4). In contrast, *vps27*Δ and *vps4*Δ mutants expressed either higher or similar levels of *FLO11* compared to wild-type (Figure 2.4).

2.3.4 Two pathways act through the endosome to affect mat formation

A hypothesis to explain the differing phenotypes between the Class E-1 and Class E-2 mutants is that there are two distinct, but overlapping, pathways that affect mat formation and act through the endosome. One pathway is the Rim101p signal transduction cascade [18], which requires specific ESCRT-I, II, III components [21, 22], and is required for *FLO11* expression ([15], Figure 2.4,), and therefore affects both invasive growth and mat formation (Figure 2.3). The other pathway depends on a functional MVB pathway in general, but has little to no effect on *FLO11* expression and only affects mat formation, but not invasive growth (Figures 2.3 and 2.4).

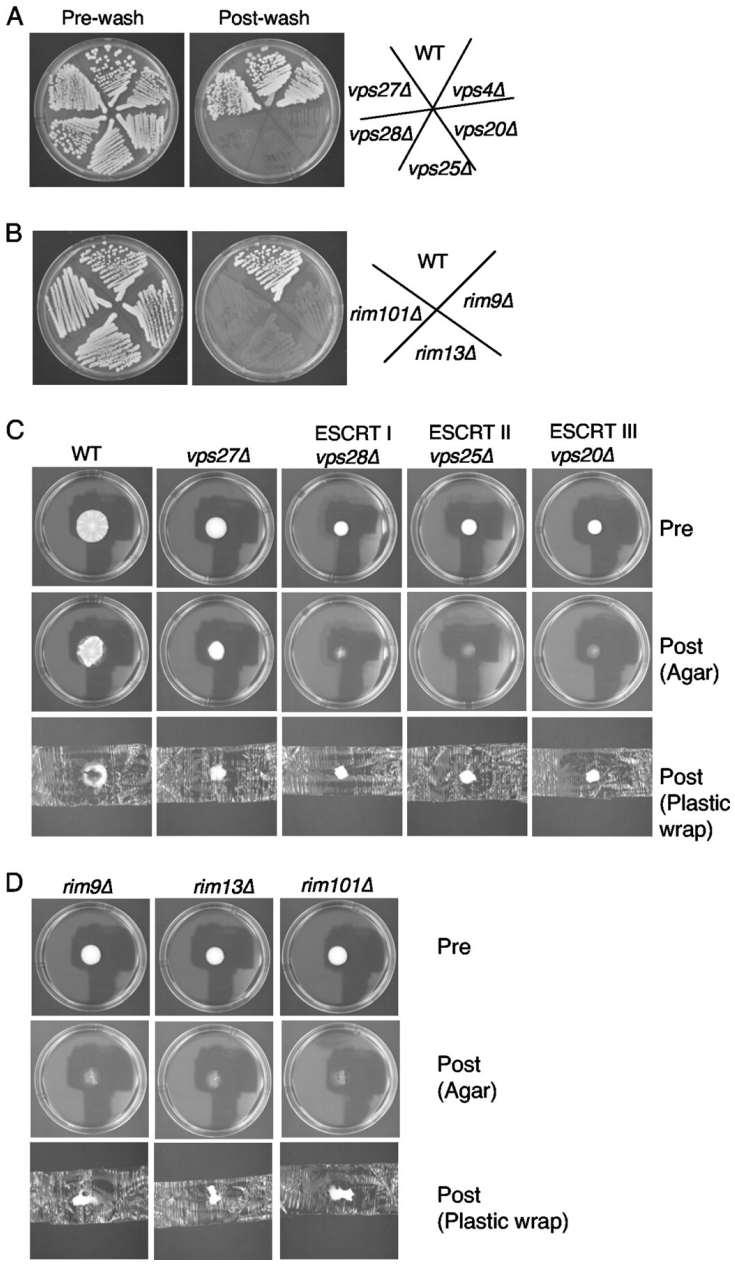

Figure 2.3 Class E-1 and E2 vps mutants have distinct effects on invasive growth and the mat formation.

Class E vps mutants that affect the Rim101p signaling pathway (class E-1) cause defects in mat formation and invasive growth, but class E vps mutants that do not affect the Rim101p pathway (class E-2) disrupt mat formation but not invasive growth. (A) The class E vps mutants vps27Δ, vps28Δ (ESCRT-I), vps25Δ (ESCRT-II), vps20Δ (ESCRT-III), and vps4Δ were subjected to the invasive growth assay (WT, wild type); (B) members of the Rim101p signaling pathway, rim101Δ, rim9Δ, and rim8Δ, were subjected to the invasive growth assay; (C and D) representative members of the class E-1 and E-2 vps mutants (C) and the Rim101p signal transduction pathway (D) were subjected to the overlay adhesion assay.

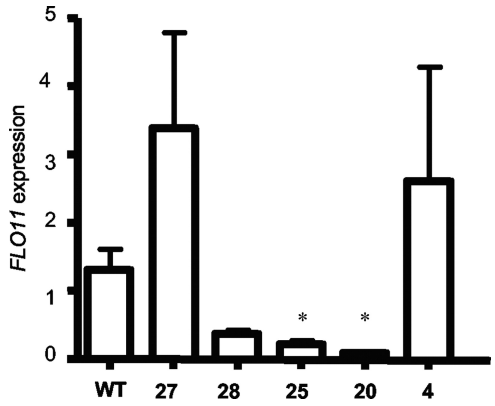

Figure 2.4: Effect of Class E-1 and E-2 vps mutants on FLO11 expression.
FLO11 expression is greatly diminished in class E-1 mutants known to affect Rim101p processing, but class E-2 mutants like vps27Δ and vps4Δ mutants do not show a decrease in FLO11 expression. Fold change in FLO11 expression was measured by rtRT-PCR, and ACT1 was used as a reference gene. WT, wild type; 27, vps27Δ; 28, vps28; 25, vps25Δ; 20, vps20Δ; 4, vps4Δ. *, $P < 0.05$ compared to wild type.

The above hypothesis suggests that the whole MVB pathway is required for mat formation, but it was possible that there was a unique role for Vps27p and Vps4p. This was tested by examining the invasive growth and mat formation phenotypes of a collection of vps mutants in the ∑1278b background. Analysis of 9 additional class E vps mutants (*did4*Δ*, snf8*Δ*, vps23*Δ*, vps24*Δ*, bro1*Δ*, snf7*Δ*, vps36*Δ*, vps37*Δ, and *mos10*Δ) reveals that they all have defects in mat formation, although the defects in the *vps37*Δ and *mos10*Δ mutants are less pronounced (Appendix Table A-3). Consistent with the above results, there is a correlation between ESCRT mutants known to perturb Rim101p signaling (Class E-1 mutants) and defects in both invasive growth and mat formation. The *vps37*Δ mutant is an exception to this, as it is defective for mat formation but not invasive growth. However, the *vps37*Δ mutant gave mixed results regarding its role in Rim101p processing [21, 37]. The *vps37*Δ mutant not withstanding, these results suggest that MVB trafficking is important for proper mat formation.

An alternative interpretation is that disruption of vacuolar function may be the root cause of the defect in mat formation. However, an additional 25 vps mutants were tested that do not belong to class E. Of these non-class E vps mutants, 11 have no defect in mat formation, and 8 cause only a partial defect leaving the mutants with less well-defined pattern formation, but a clear rim and hub by the overlay adhesion assay. Thus, 19 out of 25 non-class E vps mutants exhibit only a partial defect or no defect in mat formation (Appendix Table A-3). Only two genes represented among these 19 mutants, *VPS21* and *VPS62*, have strong homologs in *S. cerevisiae*, therefore for most of the 19 mutants, the lack of a strong defect in mat formation

cannot be accounted for by redundant gene functions. In addition, a *pep4*Δ mutant, which disrupts vacuolar protease activity [38, 39], is also wild-type for mat formation (data not shown).

Thus, there is a second pathway required for mat formation, which will be tentatively called the biofilm pathway, which is dependent on the MVB pathway and is hypothesized to act independently of the Rim101p pathway. If the biofilm pathway is really independent of Rim101p, then restoration of Rim101p transcription factor activity via a dominant allele of *RIM101* should bypass upstream defects in the Rim101p pathway, but not the biofilm pathway. The *RIM101-531* dominant allele encodes a truncated form of Rim101p missing the inhibitory C-terminal tail following amino acid 531. This truncated protein is active even when upstream components of the signal transduction pathway are disrupted, including both Class E- 1 (*i.e. vps28*Δ*, vps25*Δ*,* and *vps20*Δ) and non-MVB (*i.e. rim13*Δ) components [16].

Addition of the *RIM101-531* dominant active allele should have different predictable phenotypes in the non-MVB, Class E-1, and Class E-2 mutants. If *RIM101-531* is expressed in a *rim13*Δ strain (non-MVB), then this should restore *FLO11* expression, invasive growth, and mat formation since the *rim13*Δ mutant should only block Rim101p processing but not MVB trafficking. In contrast, the *RIM101-531* allele in the *vps25*Δ mutant (Class E-1) should suppress defects in *FLO11* expression and invasive growth, but not mat formation, since the *RIM101-531* allele will not restore MVB sorting. Finally, the *RIM101-531* allele should have no impact on the *vps27*Δ mutant (Class E-2). The *RIM101-531* allele was introduced into the *vps27*Δ*, vps25*Δ, and *rim13*Δ mutants by deleting the C-terminal 95 codons on the chromosome by homologous recombination (see materials and methods). The resulting double mutants were examined for invasive growth, *FLO11* expression, and mat formation. The *rim13*Δ *RIM101-531* double mutant was fully restored for invasive growth compared to the *rim13*Δ single mutant as was the *vps25*Δ *RIM101-531* mutant. The *vps27*Δ *RIM101-531* double mutant appears no different than the *vps27*Δ mutant (Figure 2.5A). Consistent with these results, *FLO11* gene expression in growing mats measured by rtRT-PCR is restored in the *vps25*Δ *RIM101-531* and *rim13*Δ *RIM101-531* double mutants and is not significantly different between *vps27*Δ and *vps27*Δ *RIM101-531* strains (Figure 2.5B).

These mutants also behave as predicted in the mat formation assay. The *rim13*Δ *RIM101-531* double mutant, although slightly reduced compared to the wild-type, is restored for mat

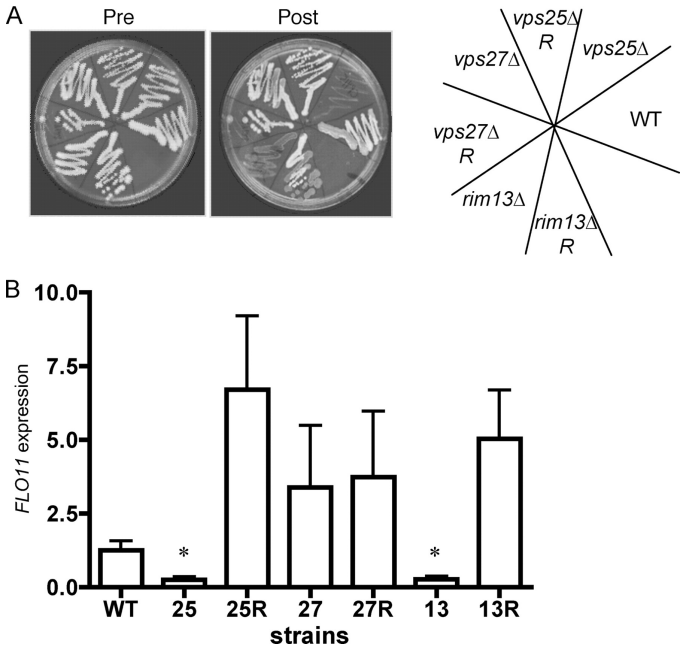

Figure 2.5: Effect of RIM101 suppressor mutant on invasive growth phenotype and FLO11 expression of Class E-1 and Class E-2 vps mutants.

The RIM101-531 allele suppresses invasive growth and FLO11 expression defects in the vps25Δ and rim13Δ mutants. (A) Strains carrying the RIM101-531 allele were subjected to the invasive growth assay. Mutants with a capital R are double mutants carrying the named mutation and the RIM101-531 allele. (B) Fold change in FLO11 expression was measured by rtRT-PCR, and ACT1 was used as a reference gene. WT, wild type; 25, vps25Δ; 25R, vps25Δ RIM101-531; 27, vps27Δ; 27R, vps27Δ RIM101-531; 13, rim13Δ; 13R, rim13Δ RIM101-531. *, $P < 0.05$ compared to wild type.

formation compared to the *rim13*Δ parent strain. It exhibits increased spreading on low agar with formation of patterns such as a clear hub, and it adheres similarly to wild-type in the overlay adhesion assay yielding a distinct hub and rim (Figure 2.6, plastic not shown). The *vps27*Δ mutant is unaffected by the introduction of *RIM101-531* allele. In contrast, the *vps25*Δ *RIM101-531* double mutant resembles a *vps27*Δ single mutant in the overlay adhesion assay (Figure 2.6). These results suggest that there exists a requirement for the MVB pathway in biofilms that is independent of Rim101 pathway.

2.3.5 Expression of Flo11p is diminished in Class E-1 mutants, but is similar to wild-type in Class E-2 mutants

The above experiments support the hypothesis that there is a biofilm signaling pathway that depends on functional MVB trafficking and is necessary for mat formation, but is independent of Rim101p signaling and *FLO11* expression. However, since the MVB pathway affects protein trafficking within the cell, it seemed possible that the mat formation defects were due to poor expression or mislocalization of Flo11p. In order to test this, the percentage of cells expressing

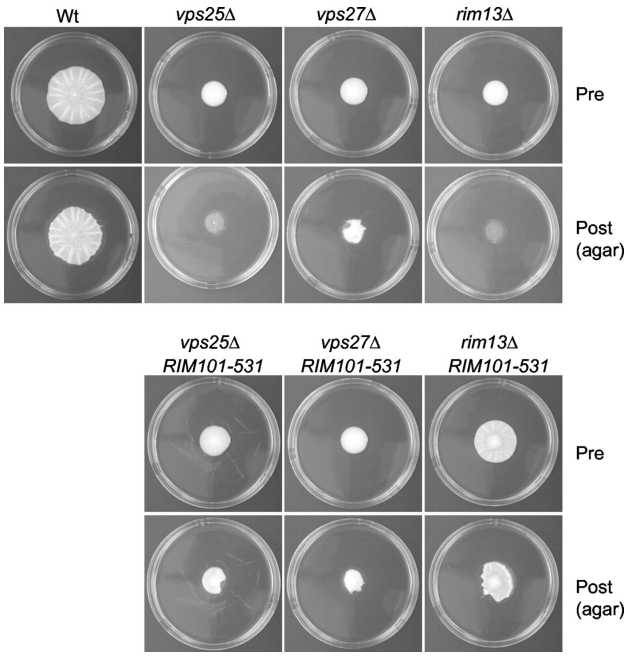

Figure 2.6: Effect of RIM101 suppressor mutant on overlay adhesion assay and mat formation phenotype of Class E-1 and Class E-2 vps mutants.

The RIM101-531 allele suppresses the mat formation defect in the rim13Δ mutant but not the vps25Δ or vps27Δ mutant. Pre, before the overlay adhesion assay; Post, agar after the overlay adhesion assay.

Flo11p on the cell surfaces within the mats of different strains were compared. Flo11p is expressed in a variegated manner in the ∑1278b strain such that only ~40-50% of the wild-type cells express the protein on the surface as assessed by immunofluorescence [11, 40]. Each of these strains carries on its chromosome an allele of *FLO11* encoding a protein with a hemagglutinin (HA) epitope tag located between amino acids 30 and 31 (*FLO11-HA*^{30}). Cells were collected from growing mats in the wild-type, *vps25*Δ, *vps27*Δ, and *rim13*Δ strains plus their respective *RIM101-531* double mutants and subjected to staining with anti-HA antibody to assess the percentages of cells expressing Flo11-HA^{30} on their cell surfaces. Consistent with *FLO11* gene expression results (Figure 2.5 B), the *vps25*Δ strain expressed little Flo11-HA^{30}, while the *vps25*Δ *RIM101-531* strain expressed wild-type levels of the protein (Figure 2.7). In contrast, the *vps27*Δ and *vps27*Δ *RIM101-531* strains were similar to wild-type. The *rim13*Δ mutant, like the *vps25*Δ strain, expressed much less Flo11-HA^{30} than wild-type, but the *rim13*Δ *RIM101-531* double mutant was restored for Flo11-HA^{30} expression.

2.3.6 Flo11p shedding and cell wall localization is not altered in the Class E- 2 mutants

Although Flo11-HA^{30} was clearly expressed on the cell surface of Class E-2 mutants, it was recently reported that Flo11p is shed outside the cell wall, and that this extracellular form is important for mat formation [32]. A separate report by another group indicated that Flo11p is not covalently attached to the cell wall, as are other canonical adhesins [41, 42], but is found in the membranes of yeast cells or is non-covalently associated with the cell wall [43]. Thus, it seemed possible that although no differences were seen in Flo11-HA^{30} expression between wild-type and *vps27*Δ strains based on immunofluorescence, the subcellular localization of Flo11-HA^{30} at the surface or outside the cells might be different. For example, perhaps the mutants shed all or most of their Flo11-HA^{30} or its association with the wall or membrane is altered.

In order to address the above concerns, cells were isolated from the growing mats and subjected to subcellular fractionation. Cells were collected from wild-type, *vps25*Δ (Class E-1),

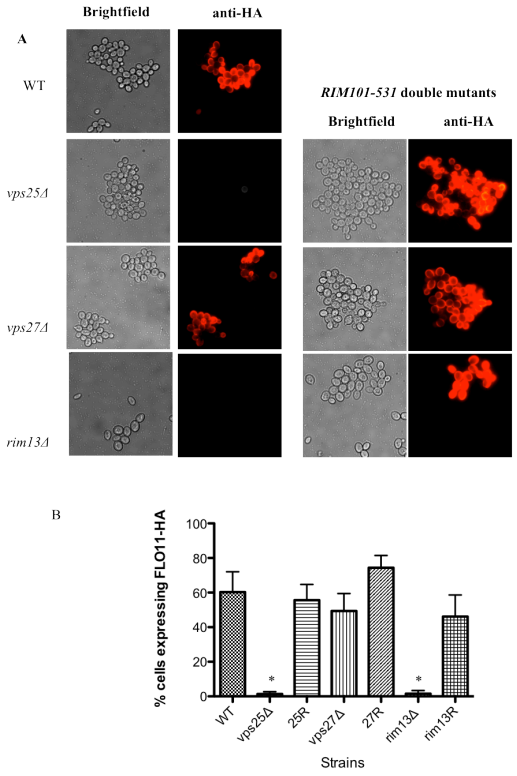

Figure 2.7: Immunofluorescence assay results of RIM101 suppressor mutant.
The RIM101-531 allele restores Flo11-HA30 expression in the vps25Δ and rim13Δ mutants. (A) Cells were subjected to secondary immunofluorescence with an anti-HA monoclonal primary antibody directed toward the HA tag in strains carrying Flo11-HA30. (B) Quantification of the percentage of cells expressing Flo11-HA30 from each strain. Wild type (WT); vps25Δ; vps25Δ RIM101-531 (25R); vps27Δ; vps27Δ RIM101-531 (27R); rim13Δ; rim13Δ RIM101-531 (13R). *, $P < 0.05$ compared to wild type.

*vps27*Δ (Class E-2), or *rim13*Δ (non-MVB) mutants. The overlay adhesion assay was used to purify separate populations of rim cells from the wild-type, and the hubs were scraped from the agar with a spatula. Whole mats from mat-defective vps mutants were collected by scraping from the agar surface. The cells were then fractionated (see Methods for more details) to obtain shed (S), membrane-associated (M) and covalently attached cell wall (C) fractions. Protein fractions were then analyzed by SDS-PAGE and Western blotting against Flo11-HA. Loading was normalized to the number of cells represented in each population from which proteins were extracted (see Methods for more details).

When this procedure was performed on the strains carrying Flo11-HA^{30}, it was found that the expected high molecular weight Flo11p band (>260 kDa) seen in [32] was seen only in the membrane fraction, and showed substantial degradation, even in the presence of protease inhibitors (data not shown). This version of Flo11-HA was tagged between amino acids 30 and 31 (Flo11-HA^{30}). Unlike it, the Flo11-HA used by Karunanithi, et al [32] was tagged at amino acid residue 1015 (Flo11-HA^{1015}). Therefore, another HA tag was added to *FLO11-HA^{30}* in the strain at residue 1015 to create doubly HA tagged Flo11-HA^{30,1015} strains, and the fractionation was repeated. In this case, a band was seen corresponding to Flo11p that ran at >260 kDa in the shed (S), membrane/non-covalent cell wall (M), and covalently attached (C) cell wall fractions (Figure 2.8, Flo11p band). These data indicate that Flo11p is both shed outside the cell wall and covalently attached to the cell wall, and is also found in the M fraction containing both membrane and non-covalently cell wall associated forms of the protein.

A very small molecular weight band is also present, and is found primarily in the M fraction (Figure 2.8, N-HA). Further analysis revealed this band to be ~17 kDa (Appendix Figure A-4, N-HA), although faint amounts of an ~37 kDa band were seen as well. It is suspected that the ~17 kDa N-HA band seen in the Western blots corresponds to the N-terminal 33 kDa myc-tagged band of Flo11p reported by Karunanithi et al [32], but may differ in size due to strain-associated differences in protease sites in Flo11p (see discussion).

It has been previously reported that the percentage of cells expressing Flo11-HA^{30} in the rim and hub is identical based on immunofluorescence data [11]. Consistent with these previous results, the Western blot analysis reveals no obvious or reproducible differences in the overall amounts or distribution of Flo11-HA^{30,1015} in the S, M, or C fractions of the rim or hub of the wild-type (Figure 2.8, rim and hub). This is despite the fact that there is a profound difference in

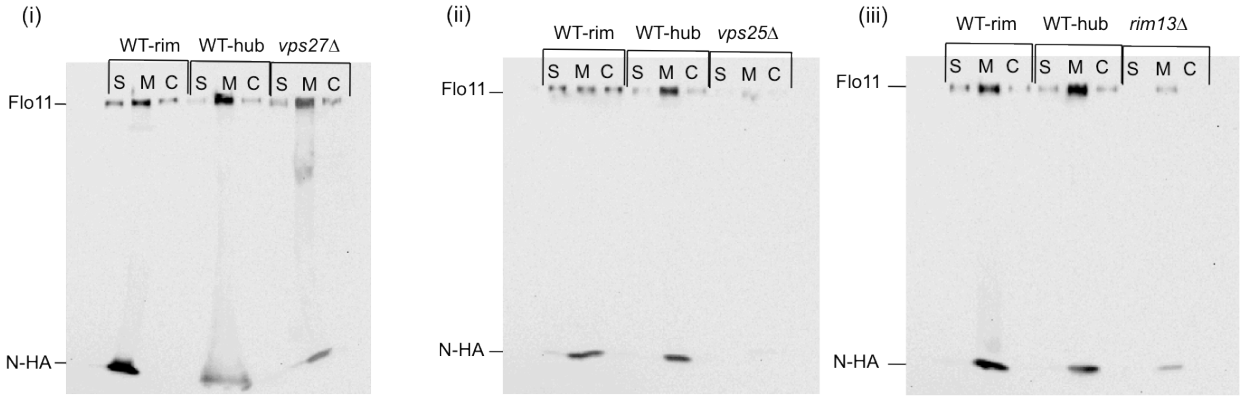

Figure 2.8: Western blot on fractionated rim and hub of wild type and representative Rim101 pathway, Class E-1 and E2 vps mutants.

Flo11p is both shed from the cell wall and covalently attached to it and is expressed and localized similarly in wild-type and vps27Δ strains. Western blotting was performed on fractionated samples from wild-type (i, ii and iii), vps27Δ (i), vps25Δ (ii), and rim13Δ (iii) strains carrying Flo11-HA30,1015. A high-molecular-mass Flo11p-HA30,1015 band (>260 kDa) was observed in wild-type and vps27Δ strains in all fractions, including shed (S), membrane bound/noncovalently cell wall associated (M), and covalently attached to cell wall (C) fractions. The vps25Δ and rim13Δ mutants show the absence of Flo11p-HA30,1015 in S and C fractions and considerably decreased signals in the M fraction. A small N-terminal fragment (17 kDa) referred to as N-HA was consistently observed in the M fraction.

the manner in which these cell populations adhere to agar in the overlay adhesion assay (Figure 2.3 C).

Finally, when Flo11-HA$^{30,\ 1015}$ expression and distribution is compared between the wild-type and mutant strains, there is a clear decrease in Flo11-HA$^{30,\ 1015}$ expression in all of thefractions in the *vps25*Δ and *rim13*Δ mutants, while there is no reproducible difference between wild-type and *vps27*Δ strains (Figure 2.8). Thus, these results are once again consistent with those from the rtRT-PCR and immunofluorescence experiments (Figures 2.5 and 2.7). Therefore, based on three different measures of *FLO11* gene or Flo11p protein expression (Figs 2.4, 2.5, 2.7 and 2.8) it appears that *vps27*Δ does not differ from wild-type in Flo11p expression, distribution, or shedding. Its failure to form a mat is likely attributable to some unidentified effector protein or molecule.

2.4 Discussion

FLO11 is clearly necessary for mat formation; however, it is not sufficient for this phenotype. Martineau *et al* [14] reported a similar finding in which they described several mutants in hsp70 homologues that exhibit defects in mat formation but not in Flo11p expression or invasive growth, although it was not known how these hsp70 homologs cause this defect. This chapter

reports that Class E-2 mutants cause defects in mat formation in a manner that is independent of Flo11p expression or localization. Thus, Class E-2 mutants, along with the hsp70 mutants reported previously, reveal that the phenotypes of invasive growth and mat formation can be clearly separated at the genetic level.

The differences in the expression of *FLO11* between the Class E-1 and Class E-2 mutants can be ascribed to the differing roles of these two types of mutants in processing of the Rim101p transcription factor. Class E-1 mutants, such as *vps28*Δ (ESCRT-I), *vps25*Δ (ESCRT-II), and *vps20*Δ (ESCRT-III), are necessary for Rim101p processing [21], which is in turn necessary for *FLO11* expression (Figures 2.5 and 2.7, and reference [15]). In contrast, the Class E-2 mutants, such as *vps27*Δ, do not affect Rim101p processing [21] and therefore do not cause diminished *FLO11* expression.

These data indicate that MVB sorting, the common process affected by both Class E-1 and E-2 mutants, is required for mat formation, but not invasive growth. This hypothesis is further strengthened by the fact that addition of a *RIM101-531* dominant active allele to the *vps25*Δ mutant could rescue this Class E-1 mutant's *FLO11* expression and invasive growth phenotypes, but not its mat formation defect (Figures 2.5 and 2.6). In fact, the *vps25*Δ *RIM101-531* double mutant strongly resembled the *vps27*Δ mutant in the overlay adhesion assay with its very slightly adhesive cells (Figure 2.6). Thus, even a constitutively active *RIM101-531* allele cannot rescue mat formation as long as MVB sorting is compromised. As a control, it was found that the *rim13*Δ mutant, which is defective for Rim101p processing, but not MVB function, was rescued for mat formation, invasive growth, and *FLO11* expression by the *RIM101-531* dominant active allele. Finally, the data supports a model suggesting that class E vps mutants cause mat formation defects by affecting MVB sorting rather than vacuolar function, as numerous non-class E vps mutants have little or no defect in mat formation (Appendix Table A-3).

Taken altogether, we present a model suggesting that there are two pathways passing through the endosome that affect mat formation (Figure 2.9). One pathway, the Rim101p pathway, affects *FLO11* expression, invasive growth, and mat formation, while the biofilm pathway, which is dependent on proper MVB sorting, is required for mat formation, but not *FLO11* expression or invasive growth.

It is suspected that the MVB mutations (Class E-1 or Class E-2) cause mislocalization of a component of the biofilm signaling pathway that is necessary for proper mat formation (Figure

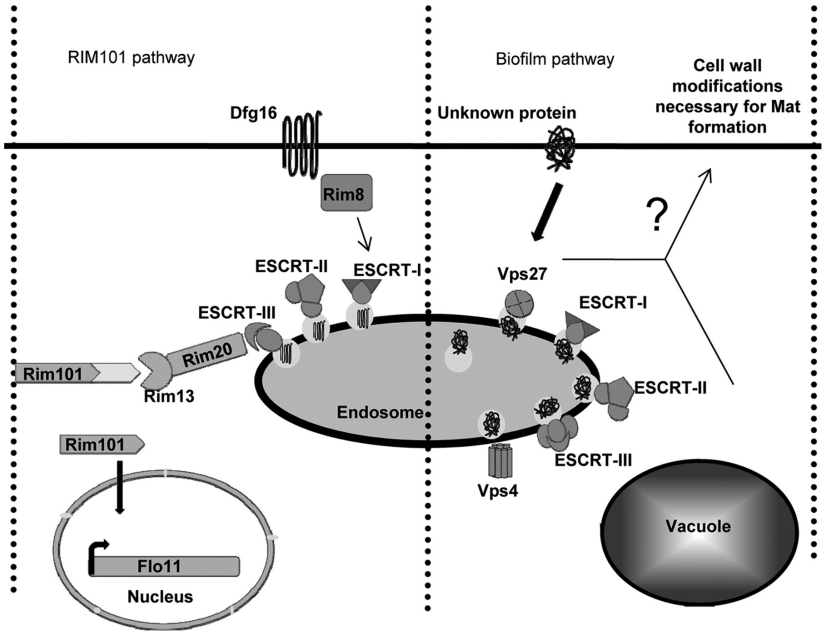

Figure 2.9: Model of pathways affecting mat formation. Two pathways affect mat formation through MVB.

One pathway is the well-characterized Rim101p pathway, which uses components of the ESCRT-I, -II, and -III complexes to transduce the signal to activate Rim101p and FLO11 expression, which are necessary for both invasive growth and mat formation. The second pathway is the putative biofilm pathway, which is hypothesized to have a component that must be properly sorted by the MVB in order to function. The biofilm pathway is not necessary for FLO11 expression or invasive growth but is necessary for mat formation, presumably by altering the cell wall in some unknown way.

2.9). It is further suspected that this pathway ultimately affects the cell wall in some unknown manner that strongly impacts mat formation in a Flo11p independent manner, but also has only a very modest effect on invasive growth. The future plan is to identify and characterize the components of the biofilm signaling pathway.

Based on the rtRT-PCR data (Figures 2.4 and 2.5 A) one might get the impression that the Class E-2 mutants such as *vps27*Δ and *vps4*Δ actually overexpress *FLO11*, and thus the biofilm pathway represses *FLO11*. However, when Flo11-HA is examined in these mutants (Figures 2.7 and 2.8), this does not appear to be the case. It is suspected that the higher expression of *FLO11* mRNA in the Class E-2 mutants may be misleading due to the size of wild-type mats compared to mutant mats and the fact that there are more glucose starved cells within wild-type mats that are no longer growing, thus giving a large hub population with diminished *FLO11* expression [11].

2.4.1 What is the functional form of Flo11p at the cell surface and shed extracellularly?

Karunanithi et al [32] recently showed that Flo11-HA^{1015} was proteolytically cleaved during its synthesis in a Kex2p-dependent manner and that this led to the release of a 33 kDa fragment that included the N-terminus of the protein [32].

Surprisingly, it was found that the N-terminus of Flo11-HA^{30} is localized at the cell surface based on immunofluorescence analysis (Figure 2.7). Thus, the form of Flo11p found in the cell wall of yeast is present with an intact N-terminus. However, this form of the protein was difficult to detect by Western blots, even with the addition of protease inhibitors, and was seen almost exclusively in the membrane fraction.

It is suspected that release of proteases from the vacuole during cell fractionation may result in degradation of this N-terminal tag in the covalent fraction, and perhaps some of the non-covalent fraction too, since a small N-terminal fragment accumulates in the membrane fractionation (Appendix Figure A-4). Since the shed (S) fraction was collected from intact cells, and it could not be detected by Western blotting the Flo11-HA^{30} form (data not shown), the N-terminus may be cleaved during shedding [32].

The 17 kDa fragment released from Flo11-HA^{30,1015} was primarily in the M fraction which contains cellular membranes and non-covalently attached cell wall proteins, although very faint amounts of it can be seen in the shed (S) fraction in some blots (Figure 2.10). No evidence was seen for it in the covalently attached cell wall fraction (C). This is consistent with the findings of Karunanithi et al [32], who found the 33 kDa fragment to be enriched in the cell pellet, which would contain mostly the membrane and non-covalently attached cell wall proteins.

2.4.2 Mats are biofilms

As a final point, the discovery by Karunanithi *et al* [32] of mucins such as Flo11p being shed extracellularly by *Saccharomyces* and the follow up discovery that Flo11p is shed extracellularly in the mat in both the rim and hub (Figure 2.8) suggest that Flo11p could itself be defined as part of an extracellular matrix (ECM). Flo11p greatly resembles the mucin proteins of mammals that make gel-like mucus layers. Thus, we believe that *S. cerevisiae* mats can rightly be described as biofilms that contain an ECM.

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Chapter 3

A subset of components of the cell wall integrity pathway are essential for biofilm formation in *Saccharomyces cerevisiae*

Disclosure

Chapter 3 is a manuscript under preparation. Sarode N. created all the constructs and performed all the assays in the chapter. *SKN7* plasmids with point mutations at D427, were kindly provided by Dr. Jan Fassler (The University of Iowa).

3.1 Introduction

Biofilms are the preferred modes of growth for the majority of microorganisms in nature. A biofilm is a community of cells that aggregate and colonize a foreign surface [1]. The major advantage of forming a biofilm is protection from the constant barrage of stresses that organisms are constantly exposed to in the environment [1]. Biofilms by pathogenic fungi like *Candida albicans* are a nuisance in clinical settings where they colonize invasive medical implants or establish infection in immunosuppressed patients [2, 3]. *Saccharomyces cerevisiae* is an attractive candidate to study genes important for biofilm formation since it is capable of forming an elaborate multicellular biofilm (hereafter referred to as a mat) on semisolid agar (0.3%). It grows into a wheel-like structure that can be structurally differentiated into a central wrinkled hub consisting of water channels, some of which resemble spokes of the wheel, all surrounded by a growing smooth rim [4].

The ability to form biofilms in fungi is largely dependent on various GPI-anchored adhesin proteins of the flocculin (FLO) family that are localized to the cell wall. However, reports have emerged recently showing the existence of cell wall factors independent of the FLO family that are important for biofilm formation [5, 6]. Flo11p is the only FLO protein expressed in mats formed by the *S. cerevisiae* ∑1278b background strain L6906 [7], and mat formation is dependent on Flo11p. However, mat formation is regulated by vacuolar protein sorting (vps) genes in both a Flo11p-dependent and –independent manner. The Flo11p-independent branch of the pathway (biofilm pathway) requires an intact and fully functional MultiVesicular Body (MVB) pathway traversing the endosome. Based on our results with the MVB pathway mutants, we previously hypothesized that MVB pathway mutants affect biofilm formation by mislocalizing an important component of the biofilm pathway leading to perturbation of the cell wall and ultimately to defects in biofilm formation.

There are a number of pathways that affect the cell wall, and one that has components affected by the MVB pathway is the cell wall integrity pathway (CWI). The CWI pathway consists of several signaling modules that include a family of single transmembrane domain sensors (Wsc1p is the main sensor for the wall), a Rho-type GTPase and its regulators (*i.e.* Rho1p and Rom2p), a protein kinase C homolog (Pkc1p), and a MAP kinase (MAPK) cascade (Bck1p-Mkk1/2p-Slt2p) [8]. Activation of the CWI pathway has pleiotropic effects on cell wall repair and biogenesis. The main function of the CWI pathway is maintenance of the highly

dynamic cell wall structure, by sensing signals (*i.e.* damage due to physical or environmental agents, hormones, signal to divide, etc), and relaying them downstream, leading to activation of appropriate genes leading to remodeling of the cell wall. For example, in addition to Pkc1p and the CWI-MAPK cascade, Rho1p regulates the Fks1p β-1,3-glucan synthase and the Skn7p transcription factor. In this chapter, data will be presented revealing that components of the CWI pathway, including the Wsc1p receptor, but excluding the CWI-MAPK cascade, disrupt mat formation, and therefore may comprise part or the entire biofilm pathway.

3.2 Methods and materials

3.2.1 Strains, media, and growth conditions

All strains used in this study belong to the yeast strain background ∑1278 [4] (Table A-5). The *wsc1Δ* and *skn7Δ* mutants were created by transforming in the KanMX6 disruption cassette amplified by PCR [9] from the genomes of the *wsc1*Δ and *skn7*Δ mutants pulled out from the respective mutants in a whole-genome deletion collection created in the Σ1278 background by Owen Ryan and colleagues in the laboratory of Charles Boone at the University of Toronto (*Ryan et al*, *Science*. in press). The GFP-His3Mx6 cassette from pFA6a-GFP-His3MX6 was subcloned just 5' to the stop codon of the *WSC1* gene using XbaI and HindIII restriction sites. Primers for PCR reactions are listed in Table A-6. Transformations were performed by the standard lithium acetate transformation method [10]. All strains were maintained on standard yeast extract-peptone-dextrose (YPD) media [10] containing 250μg/ml G418 or on minimal media lacking histidine [10]. Strains grown on low agar plates (YPD with 0.3% agar) [4] for 5 days at 25°C were used for overlay adhesion assays and immunofluorescence staining.

3.2.2 Overlay adhesion assay

The overlay adhesion assay was performed as described [11].

3.2.3 Immunofluorescence of Flo11-HA30 on the cell surface of cells from the rim and hub

The assay was performed as described in [11].

3.2.4 Western blotting

Precipitation of extracellular Flo11p from the mat, fractionation of cells, and western blotting was carried out as described in [6].

3.2.5 Site directed mutagenesis

The mutagenesis was performed using a primer-mediated PCR based method described in [12, 13], using primers listed in Table 2.

3.3 Results

3.3.1 Wsc1p affects mat formation in a Flo11p-independent manner:

Wsc1p is a sensor protein of the CWI pathway. It functions with other sensors (Mid2p, Wsc2p and Wsc3p) to sense cell wall damage or repair stimulus and activate the pathway. Any defect in Wsc1p signaling leads to increased sensitivity to cell wall perturbing factors like high temperature, calcofluor white, and caffeine [14-16]. As shown in Figure 3.1A, Wsc1p is also important for mat formation since *wsc1*Δ failed to form the typical patterned biofilm observed in the wild-type. However, it displays no defect in the Flo11p-dependent invasive growth phenotype (Figure 3.1B), its phenotypes being similar to the *vps27*Δ mutant [6].

In order to ascertain if the *wsc1*Δ mat formation defect is due to a defect in Flo11p localization, the percentage of cells expressing Flo11p on the cell wall were counted by immunofluorescence assay. The strain carries a HA epitope tag inserted between residue 30 and 31 of Flo11p (Flo11-HA^{30}) that can be stained using anti-HA antibody [6]. As can be seen in Figure 3.2A, there was no statistically significant difference between WT and *wsc1*Δ in the number of cells expressing Flo11-HA^{30} on the cell surface.

In addition to being expressed on the cell wall surface, Flo11p was recently also reported to be shed outside the cell [6, 17]. To verify that *wsc1*Δ did not lead to any defects in Flo11p shedding, *wsc1*Δ containing Flo11p tagged with an additional HA epitope tag at residue 1015 (Flo11-HA^{30,1015}) was used. The mat cells were subjected to subcellular fractionation to separate populations of Flo11p that was shed extracellularly (S), covalently attached to the wall (C), and found in the membrane (M) and these were analyzed by SDS-PAGE and Western blotting using an anti-HA antibody (Fig 2B). Consistent with the immunofluoresence data, there appeared to be no reproducible difference in Flo11-HA^{30,1015} levels between wild type and *wsc1*Δ.

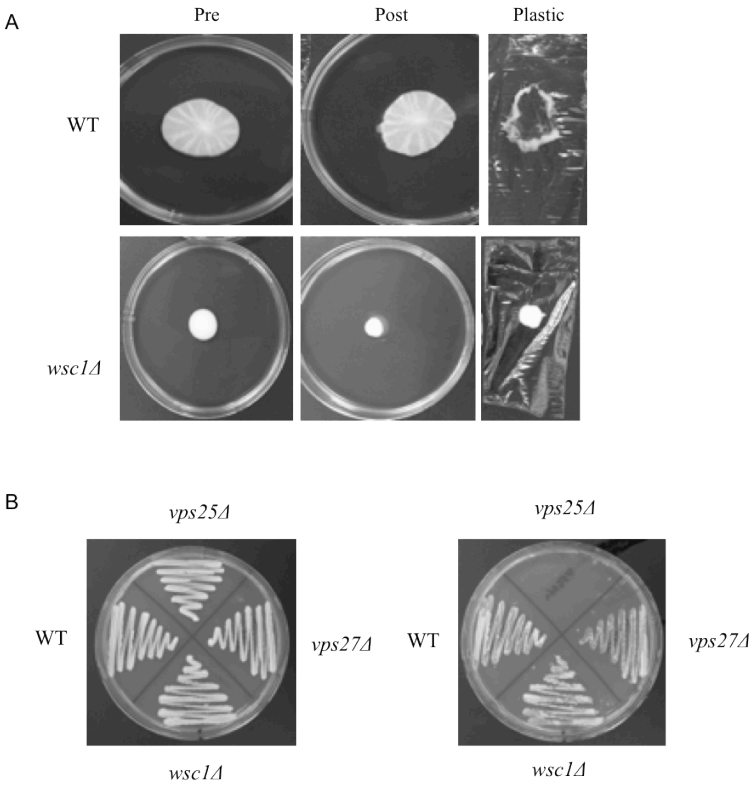

Figure 3.1: Wsc1p affects mat formation in a Flo11p-independent manner.
(a) Overlay adhesion assay performed on wild type (WT) and *wsc1Δ.* (b) Invasive wash assay performed on WT, *wsc1Δ, vps25Δ* (Flo11p-dependent) and *vps27Δ* (Flo11p-independent).

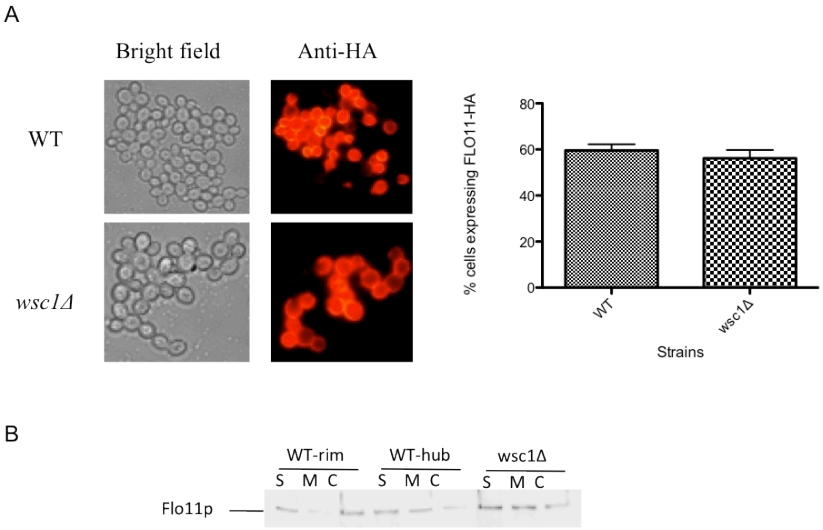

Figure 3.2: *wsc1Δ* shows no defect in Flo11-HA expression, localization and shedding
(A)Cells subjected to secondary immunofluorescence with anti-HA monoclonal primary against Flo11-HA30 and plot of the percentage of cells expressing Flo11-HA30 from each strain. (B)Western blot of shed (S), membrane bound (M) and covalently attached (C) fractions of the cell wall using anti-HA monoclonal antibody.

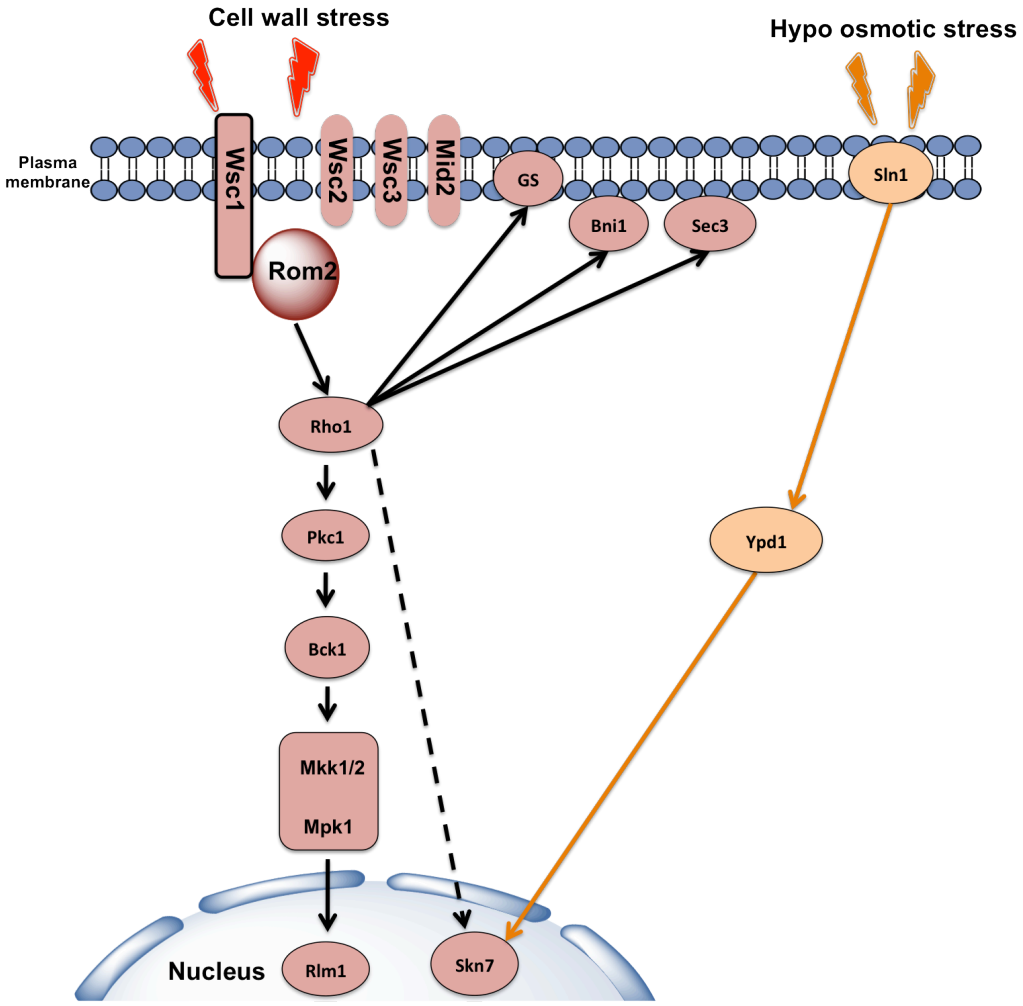

Figure 3.3: Model depicting CWI pathway with the hypo-osmotic stress sensing pathway Sln1 branch.

3.3.2 Cell wall integrity MAPK cascade is not essential for biofilm formation

Since Wsc1p is an important sensor of the CWI pathway, Figure 3.3, we wanted to determine what downstream components of the pathway are required for mat formation. Deletion mutants of different components were analyzed for their effects on biofilm formation. Loss of other sensors of the Wsc family (*i.e.* Wsc2p, Wsc3p) and Mid2p fail to cause any defect in biofilm formation, suggesting that Wsc1p is the major sensor of the CWI pathway for mat formation (data not shown). No mutants were generated for *PKC1* and *RHO1* since these genes are essential [18]. Deletion mutants were generated for all non-redundant components of the CWI-MAPK cascade including *mpk1*Δ, *bck1*Δ, and the downstream transcription factor *rlm1*Δ (Fig 3.3). None of these mutations led to a defect in mat formation (Fig 3.4).

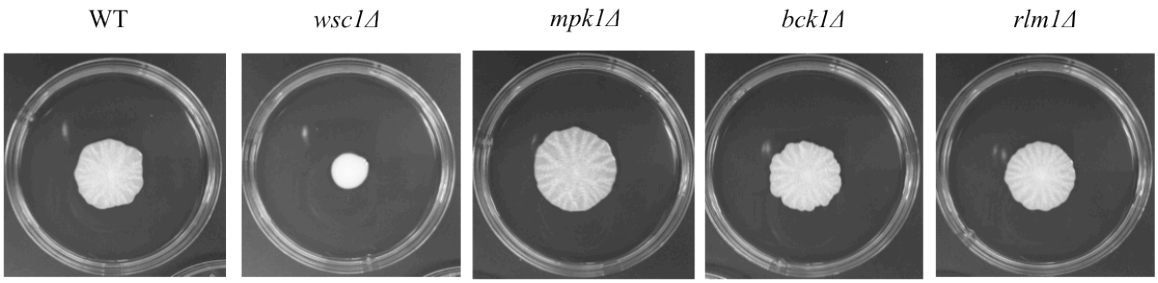

Figure 3.4: CWI pathway components downstream of *PKC1* including MAPK cascade and its effectors are not necessary for mat formation.
Mat formation phenotypes of wild type (WT), *wsc1Δ,* MAPK cascade genes (bck1*Δ, mpk1Δ*) and downstream transcription factor *rlm1Δ*.

3.3.3 Wsc1p-Rom2p interaction is essential for mat formation

The fact that the CWI-MAPK cascade is not involved in mat formation raised the question of which other canonical proteins are acting downstream of Wsc1p to affect mat formation. For example, the interaction between the sensor Wsc1p and the Rho1p guanylate exchange factor (GEF) Rom2p is the primary step activating the pathway. Therefore, we tested to see if a *rom2Δ* mutant is compromised for mat formation, and it is, in fact, defective (Figure 3.5). Therefore, we analyzed how known Wsc1p-Rom2p interactions affect mat formation.

Vay et al [19] used mutational analysis on the cytoplasmic tail of Wsc1p to identify the residues important for Wsc1p-Rom2p interactions. They identified residues Y303, S319-320, S322-323, L369, V371, N373, D375 to be crucial. If mutations that block this interaction also block mat formation, this will confirm that the Wsc1p-Rom2p interaction is important for mat formation. As Rom2p is a well-known activator of Rho1p [20], this will strongly implicate Rho1p, and establish a role for the primary upstream interaction of the CWI pathway (Figure 3.3).

The fusion gene of *WSC1* regulated by the *WSC1* promoter was subcloned into a vector such that it encoded Wsc1p with a green-fluorescent protein (GFP) tag on the C-terminal cytoplasmic tail. Transformation of the *wsc1*Δ mutant with *WSC1-GFP* (*wsc1*Δ::*WSC1-GFP*) led to rescue of mat formation (Figure 3.6A) and temperature sensitivity phenotypes (Figure 3.6 B), thus confirming it was fully functional .

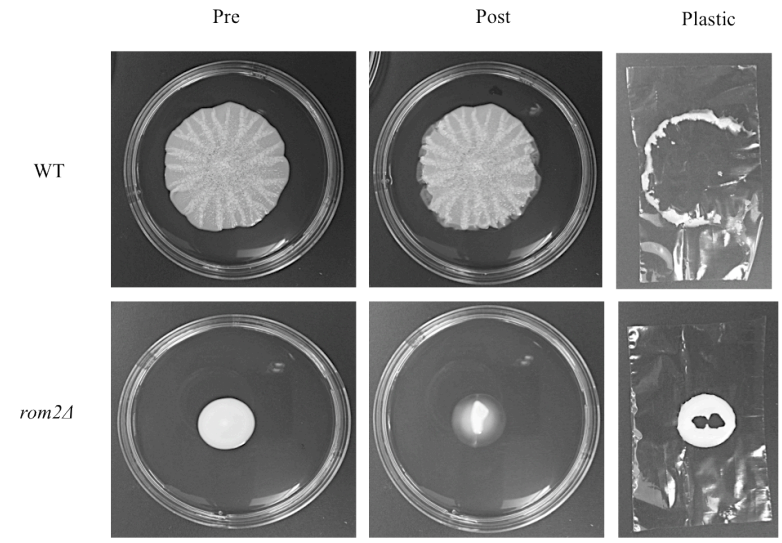

Figure 3.5: *rom2Δ* is defective in mat formation
Overlay adhesion assay performed on wild type (WT) and *rom2Δ*.

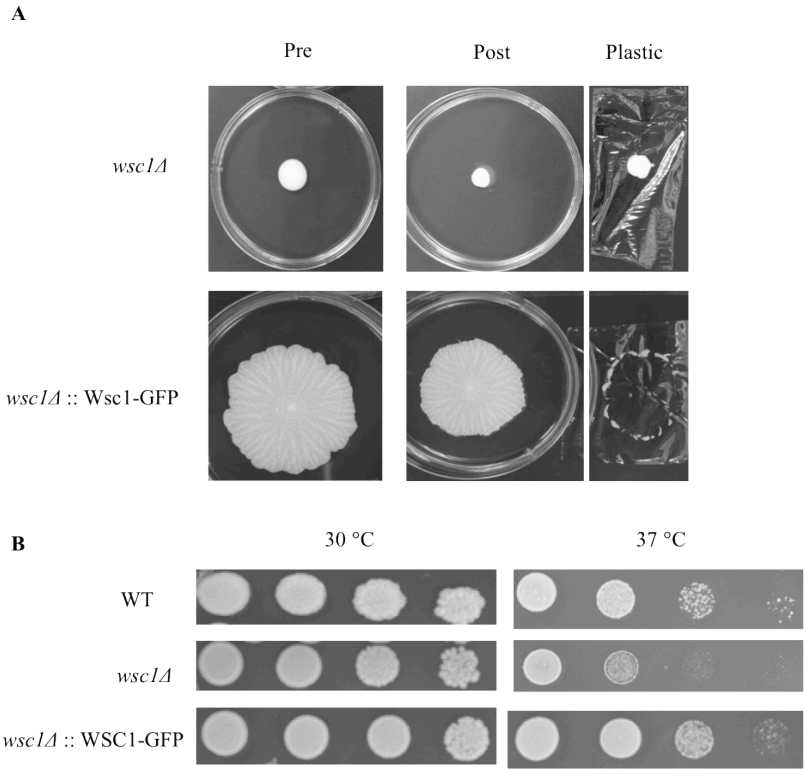

Figure 3.6: WSC1-GFP construct rescues (a) mat formation defect and (b) temperature sensitivity phenotypes of wsc1*Δ*.

The aforementioned amino acids that mediate Rom2p-Wsc1p interactions were mutated to alanine. Constructs were generated that carried a single point mutation - Y303A; combination double point mutations - S319A S320A and L369A V371A; combination triple point mutations Y303A L369A V371A and a complete truncation of the cytoplasmic tail (deletion of residue 301 - 378). No transformants could be obtained for point mutations in the terminal region of the cytoplasmic tail (N373, D375) either by themselves or in combination with any other point mutations, and the reason for this is unknown.

None of the point mutants fully complement the mat formation or temperature sensitive growth defects (Figure 3.7), however, the Y303A and S219A S220A mutants did complement both phenotypes better than the L369A, V371A or Y303A, L369A, V371A mutants. Thus, both mutant phenotypes appear to increase as the location of the mutations edges closer to the extreme C-terminus. In contrast to observations of Vay et al [19], we didn't observe any growth defect at 30°C in any of our mutants. This could be due to the fact that they carried out the mutations and complementation study in a *wsc1*Δ *mid2*Δ double mutant that exhibits severe lysis defect at all growth temperatures in absence of osmotic support.

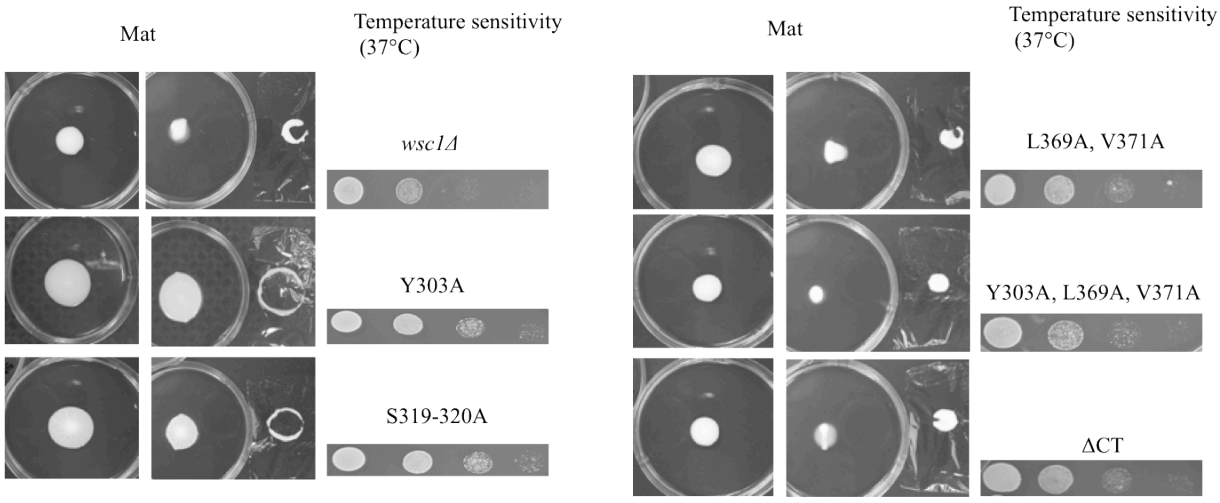

Figure 3.7: Wsc1p-Rom2p interaction is essential for mat formation.
Overlay adhesion assay performed on mats formed by *WSC1-GFP* point mutants, along with corresponding temperature sensitivity phenotype of every mutant.

3.3.4 Role of Skn7 in mat formation

The fact that the Wsc1p-Rom2p interaction is needed for mat formation but the CWI-MAPK is not, suggests that the biofilm pathway might be mediated via Rho1p through another downstream effector. One option is the Skn7p transcription factor, that acts downstream of Rho1p and parallel to the Pkc1p branch of the CWI pathway [18, 21] (Figure 3.3). Since the Pkc1p downstream effectors (*ie.* CWI-MAPK cascade) are non-essential for mat formation, we tested the *skn7*Δ mutant. This mutant is defective in mat formation (Figure 3.8A), but based on Flo11-HA^{30,1015} immunofluorescence (Figure 3.8B) and western blotting (Figure 3.8C), shows no defect in invasive growth or Flo11p localization, expression and shedding. Thus, like the *wsc1*Δ mutant, the *skn7Δ* mutant is defective in mat formation in a Flo11p-independent manner.

In addition to functioning downstream of the GTP-binding Rho1p in the CWI pathway, Skn7 has other distinct roles within the cell including oxidative stress response regulation and also acts downstream of the Sln1p histidine kinase as a response regulator [22, 23]. A conserved aspartic acid residue at position 427 in the receiver domain of Skn7p is known to be essential for its function in the Sln1p dependent pathway [22-25]. Mutating the aspartic acid to glutamic acid (D427E) generated a hyperactive form of Skn7p, while a mutation to aspargine (D427N) diminished its activity [23]. Plasmids containing Skn7p point mutants pCLM699 (Skn7D427N) and pCLM700 (Skn7D427E) were kindly provided by Dr. Jan S. Fassler [23]. If phosphorylation of the Skn7p conserved aspartic acid residue on the receiver domain plays a role in mat formation, then the hyperactive version of Skn7p (D427E) should rescue the mat formation defect of skn7Δ, while the inactive version (D427N) should fail to do so. Complementing the *skn7*Δ mutation with both the active and inactive mutant forms of Skn7p led to rescue of the mat formation defect of skn7Δ (Figure 3.9). This suggests that Skn7p is not acting downstream of Sln1p to control mat formation but rather is acting downstream of Rho1p. Thus, its activities in mat formation must be mediated by a D427 independent mechanism [21, 26].

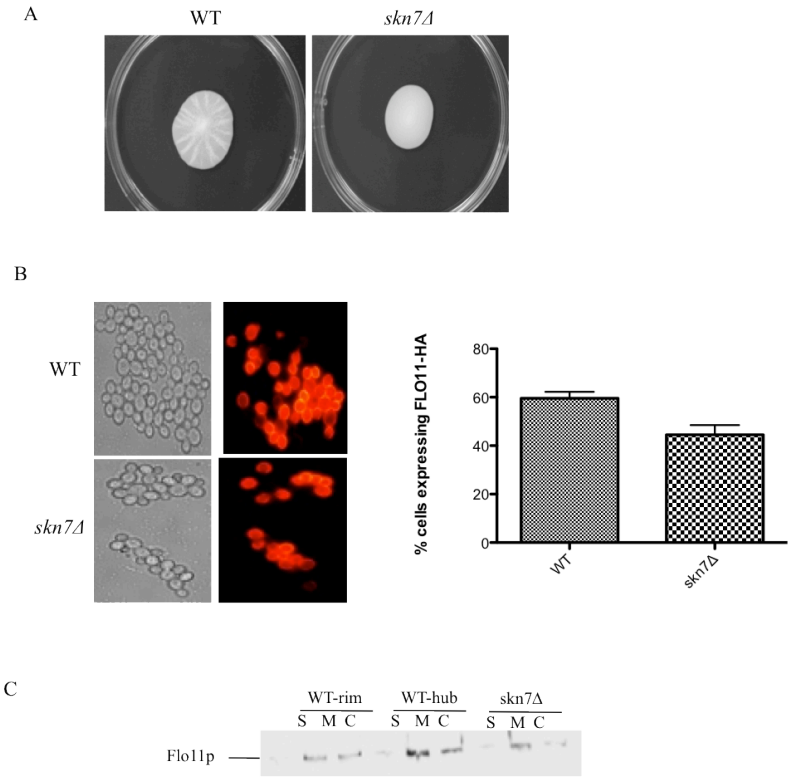

Figure 3.8: Skn7p affects mat formation in a Flo11p-independent manner.
(A) Overlay adhesion assay performed on wild type (WT) and *skn7Δ*. (B) Secondary immunofluorescence assay performed with anti-HA monoclonal primary against Flo11-HA30 and plot of the percentage of cells expressing Flo11-HA30 from each strain. (C) Western blot of shed (S), membrane bound (M) and covalently attached (C) fractions of the cell wall using anti-HA monoclonal antibody.

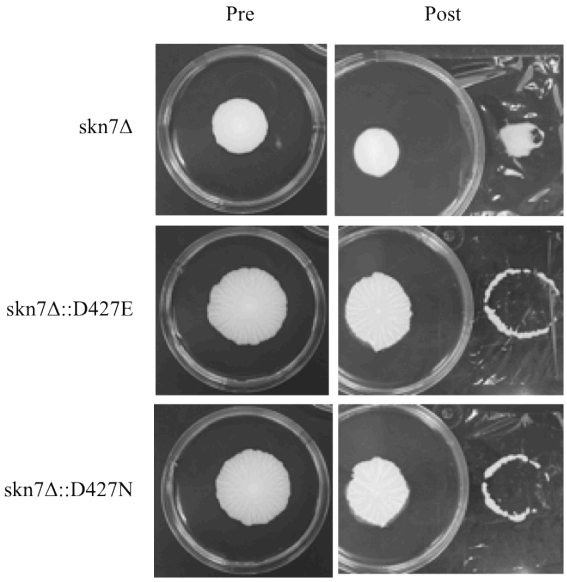

Figure 3.9: Sln1p-Skn7p branch is not essential for biofilm formation
Mat formation and overlay adhesion assay phenotype of Skn7D427 point mutants.

3.4 Discussion

In this chapter, we show that components of CWI pathway are required for mat formation in a manner that is independent of Flo11p and the canonical CWI-MAPK cascade. The involvement of the CWI pathway begins with Wsc1p, which is a sensory protein of the CWI pathway, whose activation has diverse effects on the cell wall. [27-29],

Our data clearly shows that Wsc1p-Rom2p interaction is essential for mat formation. This was shown by the fact that the *rom2*Δ and *wsc1*Δ mutants share the same phenotypes, and was confirmed by site directed mutagenesis studies showing that mutating the Wsc1p cytoplasmic tail residues, necessary for interaction with Rom2p, leads to defects in mat formation.

The observation that the CWI-MAPK cascade is not involved suggested that Wsc1p-Rom2p are acting through another pathway, and this unknown pathway likely branches out from the GTPase Rho1p, which is regulated by Rom2p. Rho1p is an essential GTPase at the center of a regulatory network having effectors that control cell wall biogenesis through polarization of actin cytoskeleton, activation of the transcription factor Skn7p and β-glucan synthesis.

Of these possibilities, mutant analysis implicates Skn7p. A *skn7Δ* mutant, like *wsc1Δ*, is defective in mat formation, and does so in a Flo11p-independent manner. The other possibilities were ruled out as follows. A *bni1*Δ mutant, which represents the Bni1p protein that acts downstream of Pkc1p independently of the CWI-MAPK cascade to affect the actin cytoskeleton is defective for mat formation, but also has defects in invasive growth, implicating Flo11p expression (data not shown). Neither of the *fks1*Δ and *fks2*Δ mutants had any defects in mat formation, and a double mutant is unviable. However, a *gas1*Δ mutant, which also affect β-1,3-glucan synthesis in the cell wall, is defective for mat formation, but in a Flo11p-dependent manner that affects invasive growth as well (data not shown). Thus, the phenotypes of the *skn7*Δ mutant suggest Skn7p is playing a role downstream of Wsc1p in mat formation.

However, Skn7p is regulated by both CWI pathway and high osmolarity glycerol (HOG) signaling pathways. The genes activated by Skn7p as a consequence of its activation through HOG pathway via its Sln1-Skn7 branch, are not identical to those activated as a result of cell wall stress through CWI pathway [22, 23]. This is because Skn7p is a modular transcription factor that can affect different sets of genes through different domains depending on which pathway activates it [21, 26].

The Sln1p histidine kinase activates Skn7p by phosphorylating the D427 residue, resulting in upregulation of certain target genes including *OCH1*, which encodes a Golgi complex glycosyltransferase [30]. To determine if Skn7p causes a defect in mat formation downstream of Sln1p, hyperactive (D427E) or inactive (D427N) point mutants of *SKN7*, that either overrespond or underrespond to Sln1p branch of the HOG pathway, respectively, were transformed into *skn7Δ*. Since both point mutants rescued the mat formation phenotype, it was shown that Skn7p does not act downstream of Sln1p to affect mat formation.

We recently showed that one Flo11p-indepenent mat formation pathway, referred to as biofilm pathway, involves the class E vacuolar protein sorting (vps) components of the MVB pathway. The proposed model of the biofilm pathway suggested that it would involve a cell wall sensory protein whose mislocalization in class E vps mutants results in defective mat formation.

It is possible that Wsc1p is this protein, and components of the CWI pathway, including Wsc1p, Rom2p, Rho1p, and Skn7p could be part of the Flo11p-independent biofilm pathway. Wsc1p localization depends on its recycling through a properly functioning endosomal MVB pathway, and a *vps27*Δ mutant, which disrupts MVB sorting, traps Wsc1p in an aberrant endosome known as the Class E compartment [29]. The *wsc1*Δ and *skn7*Δ mutants share very similar phenotypes with *vps27*Δ by affecting mat formation, but not invasive growth or Flo11p expression and localization. Thus, Wsc1p may be at the head of a biofilm pathway, but this has yet to be solidly supported.

3.4.1 Biofilm and CWI pathway have differential effects on mat formation in different ∑1278b strains

We have found that the CWI pathway affects mat formation in a manner that is independent of the CWI-MAPK cascade, and may be affected by endosomal sorting mutations. However, recently, Birkaya et al [31] found that in another ∑1278b strain, PC538, that the CWI-MAPK cascade affected mat formation, invasive growth, and *FLO11* expression, which is very different from our findings. In addition to the differences in the way the CWI-MAPK cascade affects mat formation, the PC538 strain also differs from TRY181 in expressing *FLO10* [31], having much more wrinkled mats, and being less affected by mutations in *VPS27* and other vps mutants (data not shown).

These phenotypic inconsistencies between the strains may be due to undefined genetic differences in the PC538 and TRY181 (derived from L6906). One possible difference that could

be contributing is that PC538 carries a *ste4*Δ mutation, which could affect other signaling pathways. However, there may be other differences as well. Unraveling the differences between these strains will be valuable in understanding how mat formation is regulated in yeast.

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Chapter 4

Chitosan synthesis in *Saccharomyces cerevisiae* biofilms protects cells from environmental stress.

Disclosure

Chapter 4 is a manuscript under preparation. Sarode N. created all the constructs and performed all the assays in the chapter. Gibbons J. from Dr. Antonis Rokas lab performed the RNA-Seq Illumina sequencing.

4.1 Introduction

Unicellular microorganisms frequently form biofilms. This multicellular mode of growth allows individual microbes to collaborate and form communities on foreign surfaces. Biofilms offer numerous advantages to the microbes within them including metabolic synergism and protection from stress, antimicrobials, toxins, and the ability to inhabit surfaces that otherwise will not support growth. The resistance exhibited by biofilms results from numerous factors including alteration of growth rates among cells in the community, presence of extracellular matrix (ECM), and increased expression of drug resistance pumps [1, 2]. Biofilm structure typically is characterized by extracellular matrix (ECM) secretion and structural differentiation of the cells [3-5].

Saccharomyces cerevisiae (Σ1278) is capable of forming a biofilm, which we term a mat, on semi-solid agar (0.3%) media [6, 7]. The wild type mat is structurally differentiated into a central hub and peripheral rim (Figure 4.1a and b). Spoke-like structures resembling water channels are also observed radiating from the central hub towards the mat edge. The cell mass forming the hub has a characteristic wrinkled appearance and on closer inspection appears to be composed of a network of intertwined water channels. The cells forming the wall of these channels remain firmly attached to the agar surface when the mat is subjected to the overlay adhesion assay (Figure 4.1c). In this assay, the mat is overlain with plastic wrap, which is then removed. Cells that adhere to the agar (*i.e.* the hub) stay firmly attached to the agar. In contrast, the periphery of the mat, called the rim, is smooth in appearance, and easily removed from the agar surface by the plastic wrap during the overlay adhesion assay (Figure 4.1c) [6-8]. Cells of both rim and the hub are in yeast form, although pseudohyphal forms are observed in and near the spokes.

Mat formation requires an adhesion protein called Flo11p [6, 7], that also affects other multicellular phenotypes. Surprisingly, in the mat there is no difference in Flo11p expression between the rim and hub cells [7]. This suggests that there are other molecules in the cell wall that differentiate between the rim and the hub. Reports have shown that there are genes and pathways that affect mat formation independently of Flo11p [8, 9]. These include the Ssa1-4

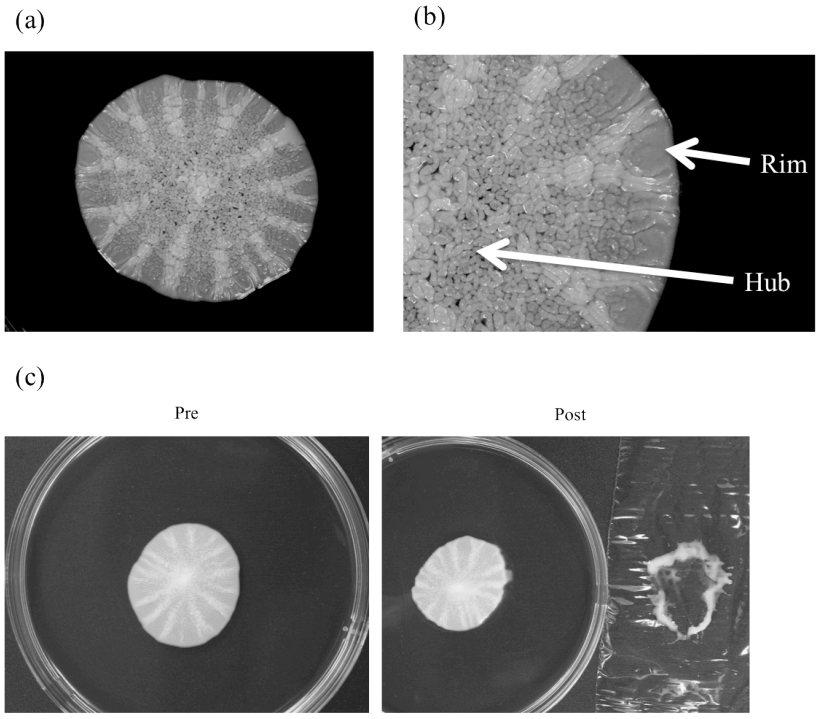

Figure 4.1: Structure of *S. cerevisiae* mat
(a) Floral pattern shape of the mat showing radiating spokes connecting the central hub to the smooth outer rim. (b) Close-up of the mat showing details of hub and rim. (c) Overlay adhesion assay performed on wild type mat.

Hsp70 proteins, multivesicular body (MVB) proteins, and components of the cell wall integrity (CWI) pathway (Sarode, et al, submitted). However, the cell wall target(s) of these pathways that are responsible for the difference between rim and hub remain unknown.

We utilized high-throughput sequencing of mRNA (RNA-Seq) to identify differences in transcriptional profiles between rim and hub cells within a mat. The goal was to identify genetic signatures that are specific to each population. In this report, we present data showing that a major difference between rim and hub cells is the presence of the carbohydrate chitosan in the hub. This is the first reported example of chitosan being expressed in vegetative cells of baker's yeast. We further show that the likely function of the chitosan in the hub cells is defense against environmental stresses.

4.2 Methods and materials

4.2.1 Strains, media, and growth conditions

All strains used in this study belong to the yeast strain background ∑1278 [6] (Appendix Table A-7). The *cda1Δ* mutant was created by transforming in the KanMX6 disruption cassette,

amplified by PCR from the genome of the *cda1*Δ mutant pulled out of whole-genome deletion collection. The knockout library was created in the Σ1278 background, by Owen Ryan and colleagues in the laboratory of Charles Boone, at the University of Toronto (Ryan O. *et al*, *Science* 2012, in press). The *cda2*Δ mutation was generated by replacing *CDA2* with the His3Mx6 cassette amplified from plasmid pFA6a-GFP-His3MX6 using PCR [7, 10]. Primers are listed in appendix Table A-8. Transformations were performed by the standard lithium acetate transformation method [11]. All strains were maintained on standard yeast extract-peptone-dextrose (YPD) media [11] containing 250µg/ml G418 in case of *cda1Δ* or on minimal media lacking histidine in case of *cda2Δ* and *cda1Δ cda2Δ* [11]. Strains grown on low agar plates (YPD with 0.3% agar) [6] for 5 days at 25°C were used for overlay adhesion assays and staining.

4.2.2 Overlay adhesion assay

The overlay adhesion assay was performed as described [7].

4.2.3 Illumina Library Preparation

The rim cells were separated from hub, from five-day-old mats growing on the surface of low-density agar plates, by performing overlay adhesion assay (Materials and methods). The hub cells were removed using a clean dry spatula. The cells were then washed with ice-cold water and total RNA was extracted as described in Kohrer K. *et al* [12]. Contaminating DNA was removed using the TurboDNA-free kit (Ambion) according the manufacturer's protocol and cleaned with the RNeasy kit (Qiagen) according to the manufacturer's instructions. cDNA libraries were constructed from mRNA within each sample and sequenced at the Vanderbilt University Genome Technology Core following Illumina specifications on an Illumina HiSeq 2000, as previously described [13, 14].

4.2.4 Eosin Y staining

Staining was performed as described in Baker et al [15]. Briefly, for staining of cells in liquid culture, cells grown overnight in 5ml YPD were diluted to optical density of $O.D_{600}$ 0.1, and then allowed to grow for 24hrs, 48hrs, 72hrs respectively. For staining cells from mats, cells were removed separately from hub and rim as described in [7], and washed with 1ml McIlvaine's buffer (MIB) [0.2 M Na_2HPO_4, 0.1 M Citric acid pH 5.8]. The cells were pelleted and resuspended in 500µl MIB. 30µl eosin Y (5mg/ml stock) was added to stain the cells and

incubated in dark for 10 min at room temperature. The excess dye was washed off with 1ml MIB buffer, the cells were pelleted and resuspended in 500µl MIB and examined under the fluorescein isothiocyanate (FITC) filter.

4.2.5 Calcofluor white staining

Cells were removed from rim and hub of mats as described in [7], washed and resuspended in 500µl sterile distilled water. Cell were then stained using 100µl (2 mg/ml) calcofluor white, washed and resuspended in 250µl sterile distilled water and examined under the 4',6-diamidino-2-phenylindole (DAPI) filter.

4.2.6 Cell wall stress assays

Rim cells were separated from the hub by overlay adhesion assay from five day old mats, using disinfected (100% ethanol) plastic wrap strips. Hub cells were removed using disinfected (100% ethanol) spatulas. The separated cells were suspended in 1ml sterile distilled water. 0.5 $O.D_{600}$ cells were removed to final volume of 500µl in sterile distilled water and serially diluted 10-fold. 10µl of each dilution (5µl in case of SDS containing plates) were then spotted onto YPD plates containing designated concentrations of cell wall stressing agents. All plates were incubated at 30°C for 48hrs.

4.2.7 Gene Ontology (GO) analysis

The normalized **r**eads **p**er **K**ilobase **p**er **m**illion (RPKM) values were transformed to $\log_2$ and the genes were then clustered using Cluster 3.0 [16]. The clustered genes were sorted according to their $\log_2$ RPKM values where values $\leq$ -1 and $\geq$ 1 were identified as downregulated and upregulated genes, respectively. It is to be noted, that for the analysis, the RPKM values for the wild-type rim and vps control strains were compared to those of wild-type hub. Hence, a normalized RPKM value of $\leq$ -1 in the column rRPKM(WTR/WTH) suggests that the particular gene was downregulated in the rim compared to the hub, whereas a normalized RPKM value of $\geq$ 1 in the same column suggests that the particular gene was upregulated in the rim compared to the hub. The list of gene ORF IDs were used as input for AmiGO GO Slimmer tool [17], and the number of genes in Biological process (GO:008150) category was plotted. The rRPKM values were used to as input for Microsoft's Fisher's exact test calculator (Available at: http://research.microsoft.com/en-us/um/redmond/projects/mscompbio/), to obtain P-values.

Bonferoni's correction calculations [18] were performed to determine if the obtained Fisher's p-values were statistically significant.

4.3 Results

4.3.1 RNA-Seq reveals sporulation genes are upregulated in the hub compared to the rim

RNA-Seq [19-23] was used to identify gene expression signatures that differ between the transcriptomes of rim and hub cells. Extracted RNA from wild-type rim and hub, along with two additional control strains defective in the mat formation (*vps25Δ* and *vps27Δ* [8]), were sequenced. The strains *vps25Δ* and *vps27Δ* lack vacuolar protein sorting components that are involved in the multivesicular body (MVB) sorting pathway [24-27]. These mutants were selected as controls because they both fail to form the typical pattern observed in the hub of wild-type mats through Flo11p-dependent (*vps25Δ*) and –independent (*vps27Δ*) mechanisms [8] (Appendix Figure A-9). In fact, the entire defective mat of each mutant resembles the rim.

The 50 bp read sets from each of the four samples were independently mapped to the *Saccharomyces cerevisiae* Sigma 1278 reference transcriptome, of which an average of 64% of reads were successfully mapped (*vps25Δ*: 66% (31,931,737 of 48,020,535); *vps27Δ*: 66% (33,039,913 of 49,729,266); wild type hub: 54% (16,961,043 of 31,409,990); wild type rim: 69% (20,459,737 of 29,796,180)). In all samples, 91% of mapped reads uniquely mapped to a single transcript. The nucleotide content of mapped reads represented an average 148x coverage of the *S. cerevisiae* Sigma 1278 reference transcriptome (*vps25Δ*: 185x; *vps27Δ*: 192x; wild type hub: 98x; wild type rim: 119x).

The genes were clustered using Cluster 3.0 [16] to identify genes that were differentially expressed by ≥2-fold in the hub compared to the rim and controls. Clustering revealed a total of 178 downregulated and 173 upregulated genes in the hub (Table A-10 and Table A-11). The Biological Process (BP) Gene Ontology [28] terms were assigned to the clustered genes using the AmiGO GO Slimmer tool [17], and BP categories with clusters of ≥5 genes (upregulated or downregulated) were selected for further analysis. Figure 4.2 shows the number of upregulated and downregulated genes in the hub, from the selected BP categories. Among the genes upregulated in the hub, 7 were classified under sporulation (GO:0043934) out of which 6 genes (Appendix Table A-12) had statistically significant Fisher's test p-values. This was a perplexing result, considering the fact that our strain is haploid, and hence incapable of sporulation. Among

these sporulation genes was *CDA2* (p-value $5.01e^{-11}$), which is considered to be sporulation specific gene involved in spore wall biogenesis [29].

CDA2 encodes a chitin deacetylase enzyme, Cda2p, which along with its homolog Cda1p, is involved in biosynthesis of chitosan (Figure 4.3a) [29]. Chitosan is a glucosamine polysaccharide (Figure 4.3c) that is made by removing the acetyl groups from chitin, a linear polysaccharide of β-1,4-linked N-acetyl glucosamine (poly-GlcNAc) residues (Figure 4.3b). Chitin is an essential component of the cell wall in all fungi, and plays an important role in maintaining cell wall integrity [30-32]. In *S. cerevisiae*, its deacetylated derivative chitosan (poly-GlcN, Figure 4.3c) was thought to be found exclusively in the ascospore wall and inter-spore bridges [33, 34] formed during sporulation in diploids. However, chitosan has been observed in cell walls of a few other fungi like *Cryptococcus neoformans*, *Aspergillus* spp., and *Rhizopus* spp., and has been shown to be essential for cell wall integrity and/or virulence in these particular fungi [15, 35, 36].

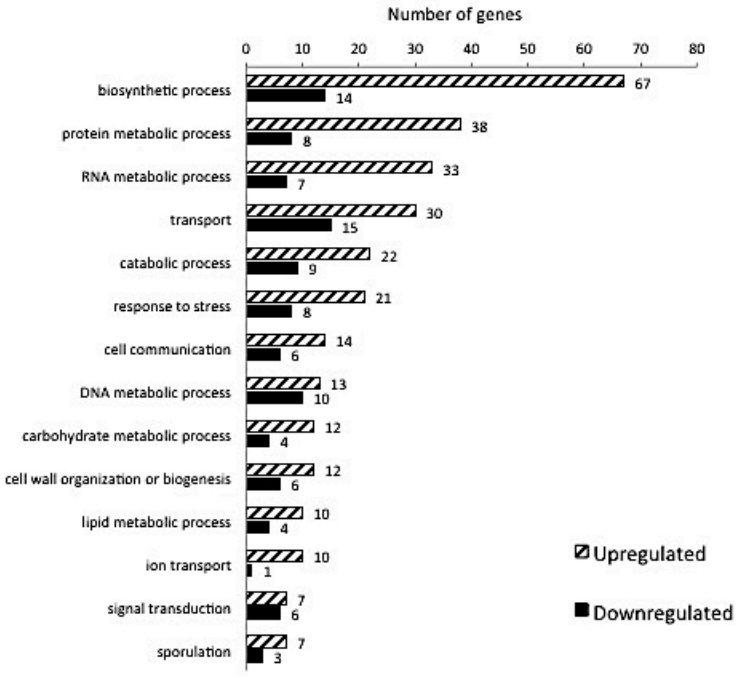

Figure 4.2: Distribution of hub genes in GO categories.
Biological processes of upregulated and downregulated genes in hub compared to expression level in rim.

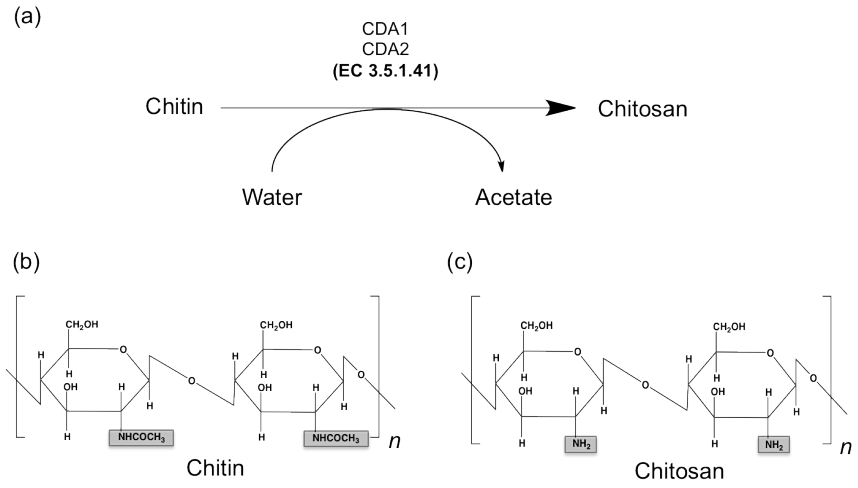

Figure 4.3: Chitosan biosynthesis and structure.
(a) Biosynthesis of chitosan in yeast involves deacetylation of chitin catalyzed by the enzymes *CDA1* and *CDA2*. Structures of polysaccharides (b) chitin and (c) chitosan. The acetyl group in chitin and the resultant deacetylated group are highlighted.

4.3.2 Chitosan is enriched in cells within the hub

These results suggest the possibility that a chitin deacetylase is expressed in the vegetative cells forming the biofilm and is enriched in the hub. If this is the case, then there should be a resulting enrichment of chitosan in the walls of cells in the hub. The cationic dye eosin Y binds chitosan and is used to stain chitosan in *C. neoformans* cells and *S. cerevisiae* spores [15, 37-39]. Therefore, we used eosin Y staining to determine if chitosan is present in the cell walls of yeast growing as mats.

Hub cells exhibited strong green fluorescence upon staining with eosin Y (Figure 4.4). In contrast, eosin Y showed much lower staining of rim cells. This was quantified by counting the number of cells that stained with eosin Y, and plotting that as a percentage of the total cells in the field of view (Figure 4.4b). It is to be noted that we observed only a few cells in the rim sample showing partial staining (restricted to the bud neck region) and some that stained like hub cells. We believe that these are either hub cells within the water channel that were carried over during overlay adhesion assay or are rim cells that are transforming into hub form of growth.

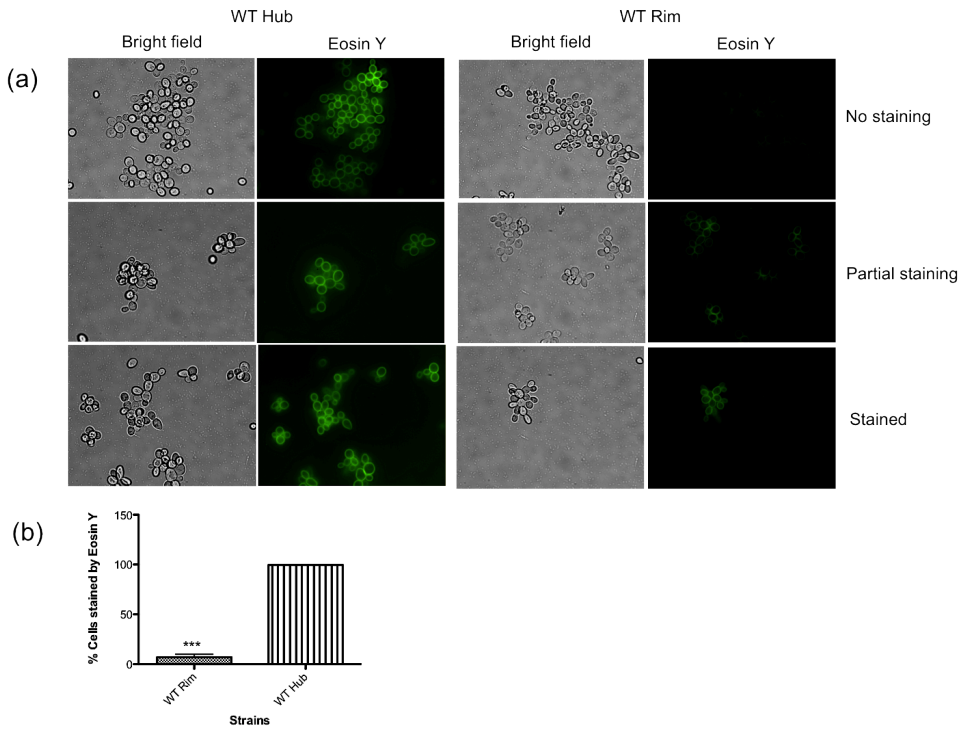

Figure 4.4: Enrichment of Chitosan in hub versus rim cells.
(a) Eosin Y staining of wild type rim and hub cells. In contrast to strong fluorescence of hub cells, the rim cells showed partial or complete loss by eosin Y staining (b) Plot of percentage of cells stained by eosin Y from rim and hub. *** = p-value < 0.0001.

Since mats were incubated for growth for 5 days, we wanted to verify if cell wall chitosan synthesis is a feature of cells within mats exclusively, or a function of long term growth. We stained vegetative cells grown in liquid culture and cells inoculated on low density agar, every 24hrs for 5 days (Figure 4.5a). We failed to observe any clear eosin Y staining signal in cells grown in both liquid culture and developing mats, for about 72hrs. After about 4 days, the number of dead cells increased in the culture, making it difficult to observe the staining, since dead cells absorb eosin Y and fluoresce very brightly. In contrast, the cells from the mat exhibited strong chitosan staining at 72hrs. It is to be noted that it is around 72hrs that the characteristic wrinkles and water channels begin to form in the hub [6].

Inoculating low agar plates with cells belonging to the lab strain background S288C, which is incapable of forming mats, failed to stain with eosin Y (Figure 4.5b). This suggests that chitosan synthesis is unique to cells capable of mat formation (i.e. mat specific).

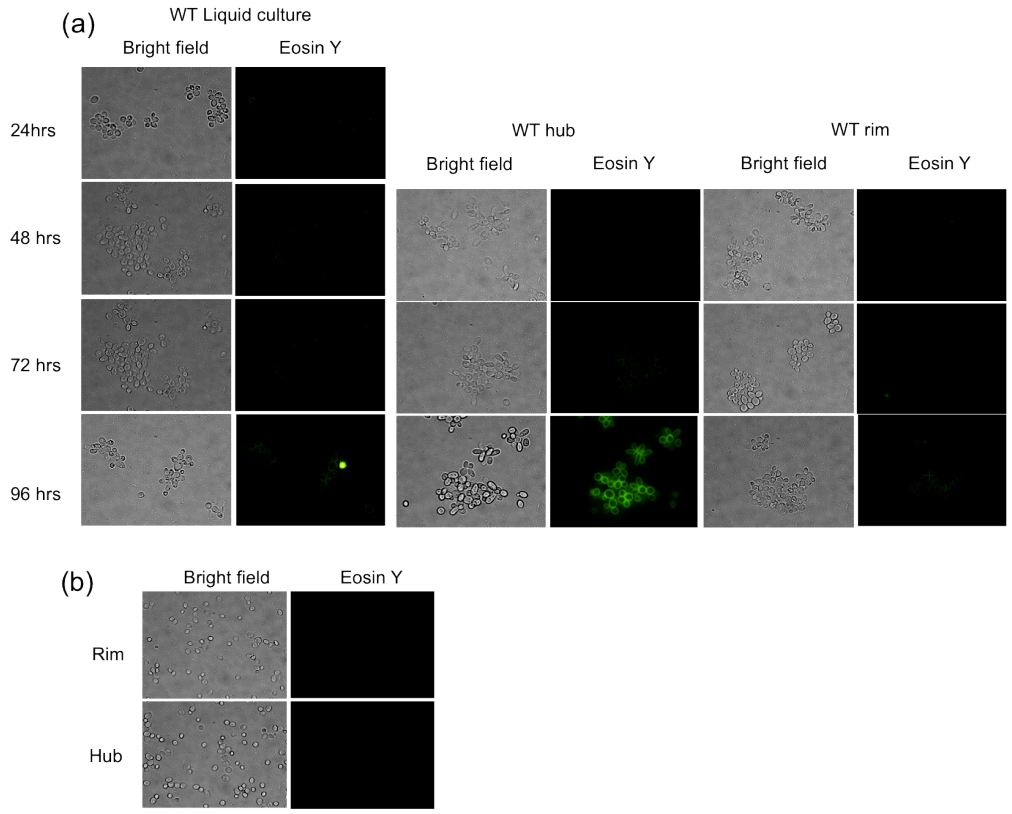

Figure 4.5: Chitosan presence is mat specific
Eosin Y staining of (a) ∑1278b background cells in liquid culture and mats grown for up to 4 days and (b) cells from S288C background that are defective for mat formation grown in mat inducing conditions.

4.3.3 Chitosan is not essential for mat formation

Both Cda1p and Cda2p regulate the biosynthesis of chitosan from chitin in *S. cerevisiae*. Therefore, *cda1Δ*, *cda2Δ* and *cda1Δcda2Δ* mutants were generated and tested for both mat formation and eosin Y staining. As seen in Figure 4.6, both single gene mutants (*cda1Δ* and *cda2Δ*) as well as the double mutant (*cda1Δcda2Δ*) displayed no defect in mat formation, suggesting that chitosan is not essential for this phenotype. However, there was a complete loss of staining by eosin Y in all mutants (Figure 4.7). In contrast, none of the mutants showed any defect in calcofluor white staining, indicating that chitin levels in the cells are not affected (Figure 4.8).

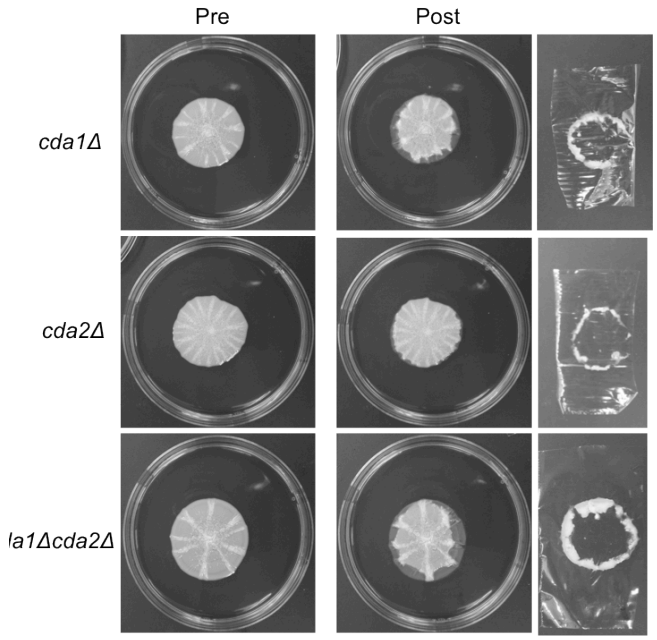

Figure 4.6: Chitosan is not essential for mat formation
5 days old mats of *cda1Δ, cda2Δ* single and *cda1Δ cda2Δ* double mutants on low density agar media show no defects for mat formation based on structure of phenotype in the overlay adhesion assay.

4.3.4 CDA mutants show increased sensitivity to cell wall stress

Many plant pathogenic fungi convert surface exposed cell wall chitin to chitosan to evade chitin-based immune responses of their host plants [40]. Additionally, studies in *Cryptococcus* spp. have suggested that chitosan is important for virulence and cell wall integrity [15, 35]. To determine if chitosan plays a protective role in mats, we further examined whether mutants defective in chitosan synthesis lack the ability to cope with certain cell wall stress conditions, and if the hub was less sensitive than the rim.

Sensitivity of WT, *cda1Δ*, *cda2Δ* and *cda1Δcda2Δ* mutants to SDS (0.08%), caffeine (3mg/ml), Calcofluor white (400μg/ml), and hydrogen peroxide (15mM) was determined by extracting cells from the rim or hub of the mat, and then spot diluting them onto regular YPD plates or YPD plates containing the corresponding testing agent (Figure 4.9). This result revealed that the rim cells were more sensitive to aforementioned cell wall perturbing agents than hub cells, and the loss of *CDA1*, *CDA2,* or both caused increased sensitivity throughout the mat.

Biofilm cells are more resistant to anti-infectives than their planktonic counterparts [41]. For example, *Candida albicans* biofilms produce extracellular β-1,3-glucan that protects them

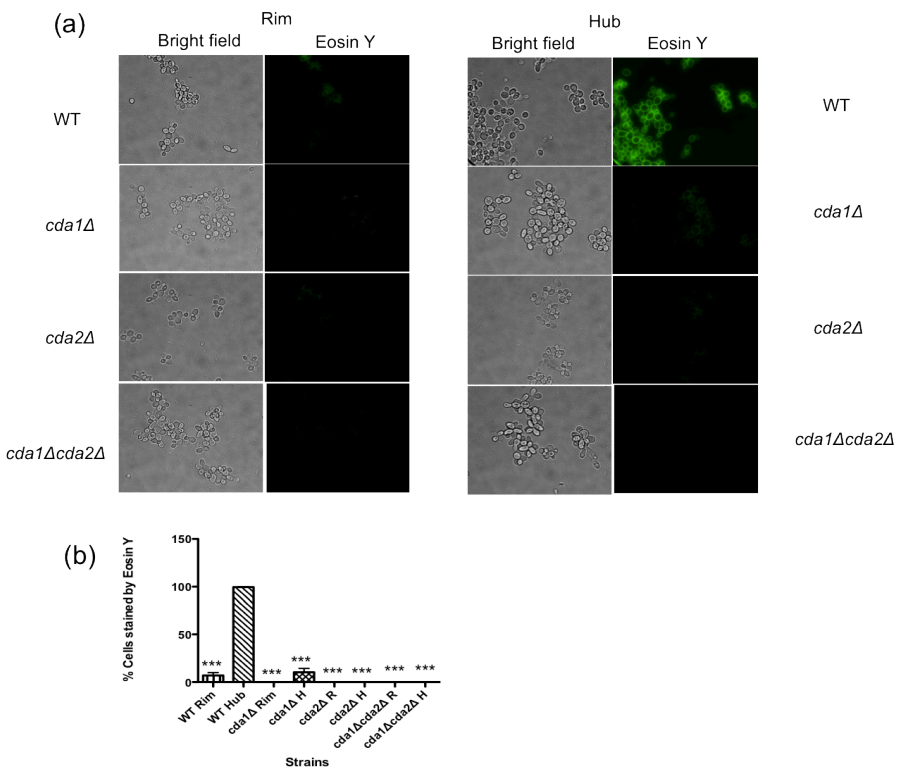

Figure 4.7: Loss of chitosan staining in *cda1Δ, cda2Δ* and *cda1Δ cda2Δ* mutant strains
(a) Loss of Eosin Y staining by both rim and hub of *cda1Δ, cda2Δ* and *cda1Δ cda2Δ* mutants. (b) Plot of percentage of cells stained by eosin Y in wild type and mutants. *** = p-value < 0.0001.

against fluconazole [42]. Therefore, we tested to see if chitosan would protect *S. cerevisiae* biofilm cells against the effects of fluconazole. This was indeed the case, as the wild-type hub was more resistant than the rim to fluconazole (20μg/ml), and the *cda1*Δ, *cda2*Δ, and *cda1*Δ *cda2*Δ mutants were all more sensitive than wild-type to this drug.

4.4 Discussion

Chitin is found in cell walls of fungi, shells of crustaceans and cuticles of insects, and is the second most abundant polysaccharide found in nature. Its applications in industry, however, have been limited, mainly due to its low solubility. In contrast, its deacetylated derivative chitosan, is gaining importance in both research and industry due to its desirable physicochemical properties like solubility in dilute acids, ability to bind to anionic compounds and its anti-microbial properties [43-46]. It is emerging as a versatile biopolymer with applications in cosmetics, wastewater treatment, digestive supplements, biomedical implant materials, and DNA-delivery

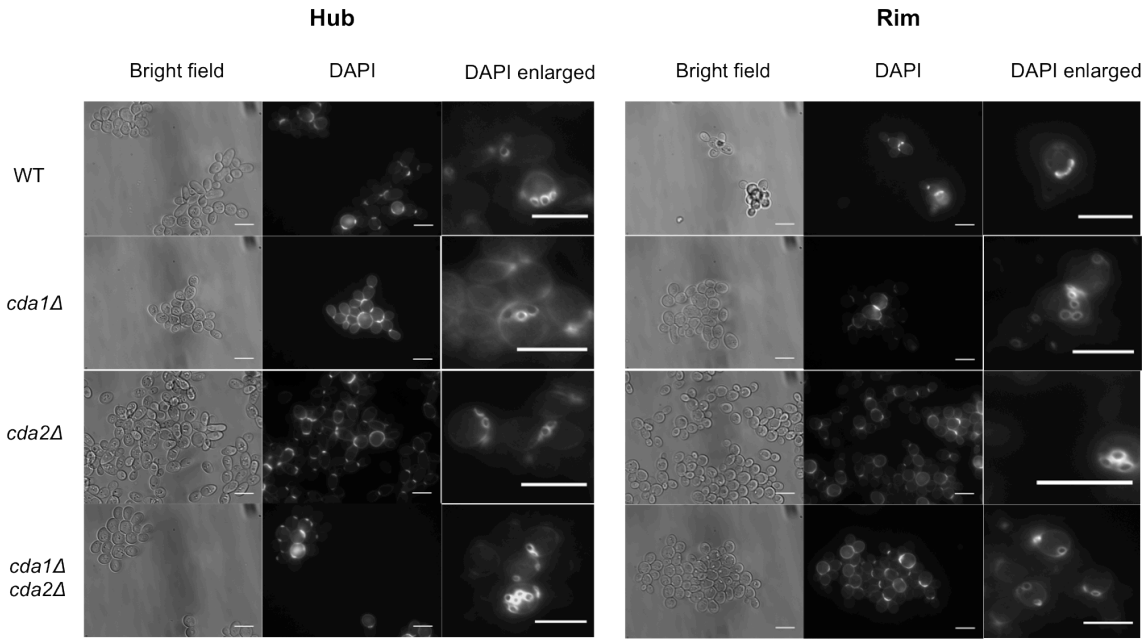

Figure 4.8: Chitin deacetylase mutations do not affect chitin staining
Calcofluor white staining of rim and hub from wild type, *cda1Δ, cda2Δ* and *cda1Δ cda2Δ* is similar. There is accumulation of calcofluor white in the bud necks of cells, while the higher magnification panel shows accumulation of chitin in bud scars. The white scale bar represents 20μm.

systems [47-51]. The major source of chitosan for industry has been as a downstream product of chitin extracted from shells of crustaceans. This is a laborious process that relies heavily on seafood that is a seasonal source. As a result, fungi (e.g. *Aspergillus spp* and *Mucor spp*) are being studied as cheaper and sustainable alternatives for chitosan extraction [43, 44, 52-55].

In the case of *S. cerevisiae,* chitosan research has been restricted to sporulation studies. The current upsurge of interest in chitosan extraction and its ever-expanding list of applications can be aided by the ease of genetic manipulation of *S. cerevisiae.* Using the deep sequencing method of RNA-Seq, we obtained a snapshot of the transcriptomic signature differences between the rim and hub cells of mats. Preliminary analysis revealed that certain sporulation-specific genes were upregulated in the hub compared to the rim. Although only 6 out of the 7 sporulation genes expression level changes were statistically significant, it was an interesting trend, given that haploids cannot sporulate. However, the presence of the chitin deacetylase gene (*CDA2*) suggested the possibility that biofilm hub cells synthesized chitosan. We supported our novel observation with strong genetic and eosin Y staining data to show the presence of chitosan in mat cells. It is to be noted that though eosin Y is widely used to stain chitosan, additional biochemical and analytical tests will need to be performed to confirm the presence of chitosan.

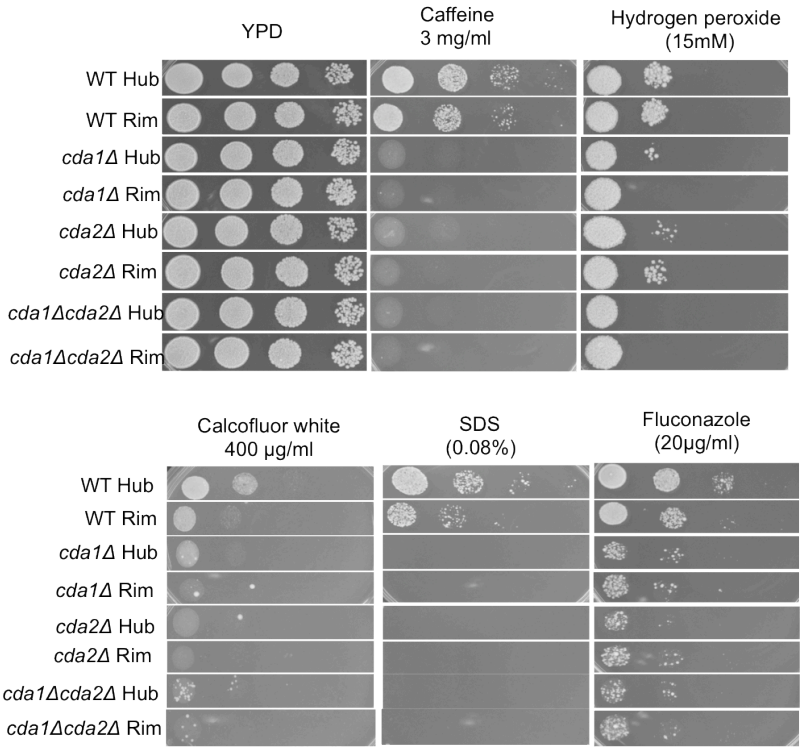

Figure 4.9: Abundance of chitosan correlates with resistance to antifungals and cell wall stress

Rim and hub cells from wild-type and mutant strains were resuspended and subjected to 10-fold spot dilution assays on YPD plates containing fluconazole or several different cell wall perturbing agents. All plates were incubated at 30°C for 48hrs.

Disruption of the *CDA*1 or *CDA2* gene, as well as both, causes loss of eosin Y staining, indicating these genes are required for chitosan synthesis (Figure 4.7). The loss of staining with each mutant alone may occur because the genes have an additive effect, and the level of chitosan synthesized in each single mutant is below the level of detection by Eosin Y. Loss of the chitin deacetylase enzymes did not affect accumulation of chitin in the bud neck and bud scars, as shown by calcofluor white staining (Figure 4.8), which indicates that eosin Y was not aberrantly staining chitin.

Although chitosan is found predominantly in the hub, it is not essential for mat formation, since single and double knockout mutants for *CDA1* and *CDA2* form wild-type mats (Figure 4.6). However, chitosan is essential as a protective barrier for the cells against natural stress conditions (Figure 4.9). The *cda1Δ, cda2Δ* single and double mutants exhibit a higher sensitivity to cell wall stressing agents. Additionally, the wild type hub is more resistant to these stressing agents than the rim, which correlates with the RNA-Seq (Appendix Table A-10) and eosin Y

staining data (Figure 4.4) showing higher levels of *CDA* gene expression and chitosan in hub forming cells of a mat.

The function of the spore wall, in both fungi and bacteria, is to assure the microbe's persistence through the hostile environment, until the return of favorable conditions. The *S. cerevisiae* spore wall is the protective barrier that makes the spores resilient and resistant to environmental insults including high temperature, osmotic shock, acids etc. [29, 56-58], and is composed of chitosan and another polymer named di-tyrosine [59, 60].

Biofilms are notoriously resistant to elimination methods [1, 2]. The presence of chitosan in the hub (Figure 4.3), taken together with the resistance it confers to cell wall perturbing agents (Figure 4.9), and the knowledge of its role in spore wall protection leads us to propose that chitosan is an important factor contributing to resistance in biofilms. In the biofilm, chitosan probably forms a barrier that protects the cells against the effects of the commonly used antifungal fluconazole (Figure 4.9).

In *S. cerevisiae*, chitosan possibly has a protective function as a barrier that shields cells from a number of environmental insults. Reduced levels of chitosan in the rim and the chitin deacetylase mutants, accompanied by their increased vulnerability to cell wall damaging agents, supports our hypothesis. This is the first report showing structural and genetic difference between rim and hub in a mat. Further research on the role of chitosan and other sporulation genes in a mat, could shed light on previously unexplored roles of these gene products in protection of biofilm forming cells.

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Chapter 5

Conclusions and future directions

5.1 Conclusions

Many microorganisms prefer to grow as biofilms in nature. A biofilm is a surface attached, community-based growth that provides advantages like protection from different stresses, opportunity for genetic exchange and regular dissemination to new colonizable locations [1]. Most human clinical infections involve microbial biofilms, making it essential to grasp the molecular details of biofilm formation in order to combat it effectively.

This dissertation details the approaches used to gain insight into detailed molecular mechanisms involved in fungal biofilm formation using *Saccharomyces cerevisiae* as a model system. It is well documented that *S. cerevisiae* and *Candida* spp. are more closely related compared to other fungal pathogens (Figure 5.1). Both *C. glabrata* and *C. albicans* form biofilms and are also the major source of nosocomial infections [2, 3], making *S. cerevisiae* a favorable genetic model system to study them. Additionally, *S. cerevisiae* is a well-characterized genetic tool with a vast literature and genetic resource. Reynolds *et al* [4] introduced *S. cerevisiae* as a model system for fungal biofilm research. Although *S. cerevisiae* and *Candida* spp. possess homologs for many genes, there are also reports of rewiring of homologous genes for new functions [5].

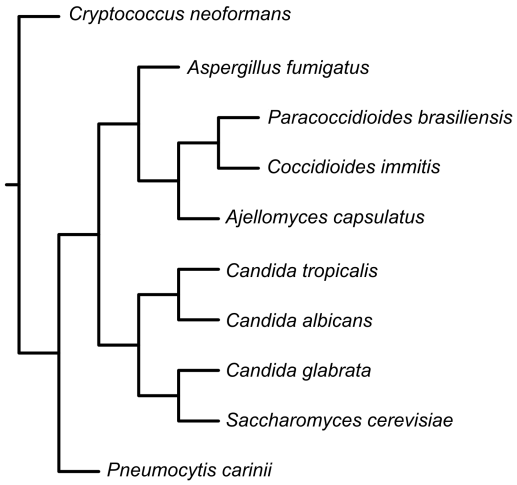

Figure 5.1: Phylogenetic tree showing relationship between selected fungal pathogens with *Saccharomyces cerevisiae*.
The tree was generated using NCBI taxonomy database [6, 7].

S. cerevisiae biofilms are also widely used in the brewing and wine industry, where their ability to ferment and flocculate is utilized [8]. Great efforts are dedicated into improving the fermentation process for e.g. improvement in fermentative flavor of the product, waste or harmful by-product elimination, increase in yield etc. [9, 10]. Genetic manipulation for strain improvement is the major approach used [11, 12], over isolation of new natural isolates, to achieve these goals. In recent years, *S. cerevisiae* biofilms are also gaining importance in the field of alternative clean fuel development for bioethanol production [13].

Hence, understanding *S. cerevisiae* biofilm formation is important not only due to its importance as a widely used genetic model system for clinical research, but also for its applications in food industry and alternative fuel research.

In this final part of the dissertation, the major findings will be summarized, including how these results contribute to current knowledge of fungal biofilms. Additionally, an outline for future experiments will be provided that will help explore new questions.

5.2 Vacuolar protein sorting genes regulate biofilm formation in *S. cerevisiae* by Flo11p-dependent and –independent mechanisms.

S. cerevisiae generates complex biofilms called mats on low-density (0.3%) agar plates. The mats can be morphologically divided into two regions: (i) hub, the interior region characterized by the presence of wrinkles and channels, and (ii) rim, the smooth periphery. Formation of mats depends on the adhesin Flo11p, which is also required for invasive growth, a phenotype in which the *S. cerevisiae* yeasts grow as chains of cells that dig into standard-density (2%) agar plates. In addition, it was also shown that mature Flo11p is covalently associated with the cell wall and shed into the extracellular matrix of the growing mat. Although both invasive growth and mat formation depend on Flo11p, mutations that perturb the multivesicular body (MVB) protein sorting pathway inhibit mat formation in a *FLO11*-independent manner. These mutants, represented by *vps27Δ*, disrupt mat formation but do not affect invasive growth, *FLO11* gene or protein product expression, or Flo11p localization. In contrast, an overlapping subset of MVB mutants (represented by ESCRT [endosomal sorting complex required for transport] complex genes such as *VPS25*) interrupt the Rim101p signal transduction cascade, which is required for *FLO11* expression, and thus block both invasive growth and mat formation.

5.2.2 Significance

Adhesion is the primary step of biofilm development. Specialized proteins, named adhesins, confer the property of adhesion to cells, and are thus important for biofilm formation. Besides their roles in biofilm adhesion, adhesins have also been shown to be essential for virulence in case of pathogenic fungi. As a result, the majority of research traditionally has been focused on studying adhesins and their role in biofilm formation.

In case of *S. cerevisiae* belonging to genetic background Σ1278b, Flo11p is the only adhesin protein expressed by the cells [14]. Essentially, it was shown that although Flo11p is important and required for adhesion and invasive growth, it is required but not sufficient for biofilm formation. Identification of a Flo11p-independent mechanism of biofilm formation directed attention towards cellular components other than adhesins, which are necessary for biofilm development. The proposed model suggested that mislocalization of an essential cell wall protein cargo in MVB pathway mutants also led to a defect in biofilm formation. Since the cell wall and its components are ideal targets for drug design, identification of new essential cell wall candidates could boost development of new drugs.

5.3 A subset of components of the cell wall integrity pathway are essential for biofilm formation in *S. cerevisiae*

Flo11p is the only FLO protein expressed in mats formed by the *S. cerevisiae* ∑1278b background strain L6906 [14], and is required for mat formation, invasive growth and adhesion. However, mat formation is regulated by vacuolar protein sorting (vps) genes in both a Flo11p-dependent and –independent manner (Chapter 2). The Flo11p-independent branch of the pathway (biofilm pathway) requires an intact and fully functional MultiVesicular Body (MVB) pathway traversing the endosome. Based on the results with the MVB pathway mutants, it was hypothesized that MVB pathway mutants affect biofilm formation by mislocalizing an important component of the biofilm pathway that leads to perturbation of the cell wall, ultimately leading to defect in biofilm formation. Among the pathways affecting the cell wall, the one that has known components affected by the MVB pathway is the cell wall integrity pathway (CWI) [15]. The main function of the CWI pathway is maintenance of the highly dynamic cell wall structure, by sensing signals (*i.e.* damage due to physical or environmental agents, hormones, signal to divide, etc), and relaying them downstream, leading to activation of appropriate genes and

consecutive remodeling of the cell wall (e.g. Fks1p, β-1,3-glucan synthase) and Skn7p (transcription factor). Genetic data revealed that components of the CWI pathway, including the Wsc1p receptor, but excluding the CWI-MAPK cascade, disrupt mat formation, and therefore may comprise part or the entire biofilm pathway (Chapter 3).

5.3.1. Significance

The cell wall and its components are absolutely crucial for cell survival. The cell wall is the interface through which the environmental cues are integrated into the cell to tender the appropriate response. Hence, it was not entirely surprising to find that components of the CWI pathway were essential for biofilm formation, which involves active cell-cell and cell-environment interaction. What was intriguing was that there was disparity in the role of individual components of the CWI pathway in mat formation. For example, although both Mid2p and Wsc1p are considered the major sensors for the CWI pathway, only Wsc1p caused a defect in mat formation. This suggests that there is a clear division of function between these sensors with no redundancy of function, even though they belong to the same pathway. Also, the MAPK cascade was found to be completely expendable for mat formation. Thus the pathway can be imagined as consisting of individual modules that could be shared with other pathways or not used, depending on the conditions.

5.4 Chitosan synthesis in *S. cerevisiae* biofilms protects cells from environmental stress

Although Flo11p is required for mat formation, it is similarly distributed on both rim and hub cells. Thus, the modifications in hub cells that distinguish them from rim cells are unknown. In order to elucidate this, high throughput mRNA sequencing (RNA-Seq) was used, and this led to the discovery that the glucosamine polymer chitosan is specifically generated in the hub of the matured mat. Additionally, chitosan biosynthesis was shown to be a characteristic property of cells capable of forming a mat on low-density agar medium and was conspicuously absent in cells grown in liquid culture and in cells incapable of generating a mat, belonging to S288C genetic background. Chitosan did not modify the adhesive properties of the hub cells, however it was shown protect the hub cells from environmental stresses such as antimicrobials.

5.4.1 Significance

Presence of chitosan in *S. cerevisiae* spore wall and interspore bridges is well documented. However, its presence in vegetative cells is a novel discovery. Additionally, identification of presence of chitosan as a differentiating factor between hub and rim cells that also correlates with the difference in sensitivity to cell wall stress agents, introduces chitosan as an important and overlooked defense mechanism in biofilm cells. Roles for chitosan in biofilm protection brings to attention a rather unexplored mode of defense that could be exploited for drug targeting. For example, the chitin specific dye calcofluor white is well known for its use as an antifungal and diagnostic agent [16, 17]; chitosan could provide an additional target for drug design and fungal diagnosis.

5.5 Future directions

5.5.1 Role of shed Flo11p in biofilm formation

Flo11p, also known as mucin-like protein (Muc1p), was reported as being shed outside the cell [18]. It appears that there are two forms of Flo11p, one is retained within the cell wall, while the other is cleaved and shed outside the cell. The membrane attached form is well characterized for its role in adhesion and invasive growth, however the function of the shed form remains to be determined. Karunanidhi *et al* [19] suggested that shed Flo11p could coat the cell surface and function as 'lubrication' of cells to glide onto agar surface. Additional studies could shed more light on its function in mats, the shed Flo11p could be speculated to have many possible functions like

(a) Part of ECM: ECM is a versatile component of a biofilm that contains variety of constituents like polysaccharides, DNA, proteins etc. Flo11p could function as part of a yet uncharacterized ECM in mats. Screening for mutants defective only in Flo11p shedding, and studying their effect on the mat phenotype could help determine whether shed Flo11p has any role in biofilm formation.

(b) Signaling molecule: Other mucin proteins like Msb2p, have been shown to exist as both integral membrane bound form and a secreted form in *S. cerevisiae*. The secreted Msb2 was reported to function in MAPK signaling [20, 21]. It will be interesting to determine whether Flo11p is also a signaling mucin, that either functions in some form of a feedback mechanism or in transmission of signal between cells of a biofilm.

(c) Interact with other proteins: Co-immunoprecipitation followed by mass spectrometer studies on shed Flo11p could help identify if the shed Flo11p binds to other proteins, if any. Since Flo11p has also been shown to have homotypic binding properties [22], it is also plausible that it binds to other membrane associated or shed flocculins or proteins.

5.5.2 Role of cell wall integrity pathway in biofilm formation

The cell wall integrity pathway is not a simple linear pathway but rather an interconnected network in which all players have not been identified as yet [5, 23]. For example, the role of Skn7p as a transcription factor in cell wall pathways is well documented, however the entire breadth of genes regulated by Skn7p in the context of biofilm formation is still not known. Identification of targets of Skn7p could shed light on additional factors that could be essential for cell wall biogenesis and in turn biofilm formation. RNA-Seq analysis to identify genes affected by *skn7Δ* will provide a list of genes that are directly and indirectly affected.

Screening and cataloging of genes in the whole genome knockout library for genes that cause defects in the biofilm formation, will be an extremely informative, although laborious, project. In fact, a preliminary screen for all cell wall related genes showing defects in mat formation was undertaken, but unfortunately wasn't followed through to the end. Following up on that work could help identify and categorize genes affecting the cell wall, and probably build a framework to unravel the cross talk circuitry involving CWI and other pathways affecting cell wall and biofilm formation.

5.5.3 RNA-Seq as a tool to paint the 'big-picture'

Using RNA-Seq, dubbed as a revolutionary tool for transcriptomics [24], was a fruitful endeavour. The expression data to identify differences between transcriptomes of hub and rim cells, and between wild-type and vps mutants (*vps25Δ* and *vps27Δ*) using RNA-Seq included an immense list of genes. Screening the genes to identify other interesting trends is another worthwhile undertaking that could be productive in terms of identifying previously unknown genes or unexpected modulation in expression levels of known genes and their effect on biofilm formation.

5.5.4 Chitosan analysis

Detection of chitosan in the mat cells unlocked an entirely new area for exploration. Chitosan research in *S. cerevisiae* is limited mainly to sporulation studies. Whether chitosan production in the cell wall of vegetative cells follows the same pathways as in sporulation, or whether there are any differences is an interesting question that can be addressed by screening the whole genome knockout library to identify genes that affect chitosan biosynthesis.

Chitosan is a versatile carbohydrate with commercial importance too. There are reports of using chitosan as a biomaterial for implant design [25], antibacterial agent [26] and dietary supplement [27]. Shellfish is the major source of chitosan for industries [28]. Considering that shellfish is also a very common cause of allergy in many individuals [29], alternative sources for chitosan (at least in case of implants and dietary supplement) is essential. Fungi are easy to grow in large numbers and manipulate genetically [30-35], and are being studied as an alternative source for chitosan. Introduction of *S. cerevisiae* into this list of fungal chitosan sources can be a boon to chitosan research, and its vast genetic and literature resources could be utilized to screen for different strains and/or mutants with higher chitosan content in their cell wall.

Whole genome knockout library screening is currently underway to identify possible transcription factors affecting chitosan production in *S. cerevisiae.* If genes affecting chitosan production are identified, they could be manipulated to generate mutants producing higher levels of chitosan. Additionally, extraction methods from fungi are already reported [30], but they are crude at best and the extract retains high levels of chitin (unpublished results). *S. cerevisiae* is a cheaper option to modify and improve the extraction protocol for future scaled-up industrial uses.

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Appendices

Table A-1: Strains used in chapter 2

Strain	Genotype	Reference
L6906	*MAT**a** ura3-52 his3::hisG FLO11::HA*$^{30, 1015}$	[14]
TRY181	*MAT**a** ura3-52 his3::hisG FLO11::HA*$^{30, 1015}$	This study
CPY74	*MAT**a** ura3-52 his3::hisG FLO11::HA*^{30} *vps4 :: kanMX6*	This study
CPY15	*MAT**a** ura3-52 his3::hisG FLO11:: HA*^{30} *vps25 :: kanMX6*	This study
NY70	*MAT**a** ura3-52 his3::hisG FLO11:: HA*$^{30, 1015}$ *vps25 :: kanMX6*	This study
CPY160	*MAT**a** ura3-52 his3::hisG FLO11:: HA*^{30} *vps28 :: kanMX6*	This study
CPY24	*MAT**a** ura3-52 his3::hisG FLO11:: HA*^{30} *vps27 :: kanMX6*	This study
NY64	*MAT**a** ura3-52 his3::hisG FLO11:: HA*$^{30, 1015}$ *vps27 :: kanMX6*	This study
CPY105	*MAT**a** ura3-52 his3::hisG FLO11:: HA*^{30} *rim13 :: kanMX6*	This study
NY62	*MAT**a** ura3-52 his3::hisG FLO11::HA*$^{30, 1015}$ *rim13 :: kanMX6*	This study
CPY115	*MAT**a** ura3-52 his3::hisG FLO11::HA*^{30} *rim101 :: kanMX6*	This study
NY78	*MAT**a** ura3-52 his3::hisG FLO11::HA*$^{30, 1015}$ *rim101 :: kanMX6*	This study
TRY120	*MAT**a** ura3-52 his3::hisG FLO11::HA*^{30} *vps27 :: kanMX6 RIM101-531*	This study
NY60	*MAT**a** ura3-52 his3::hisG FLO11::HA*$^{30, 1015}$ *vps27 :: kanMX6 RIM101-531*	This study
TRY118	*MAT**a** ura3-52 his3::hisG FLO11::HA*^{30} *vps25 :: kanMX6 RIM101-531*	This study
NY58	*MAT**a** ura3-52 his3::hisG FLO11::HA*$^{30, 1015}$ *vps25 :: kanMX6 RIM101-531*	This study
TRY124	*MAT**a** ura3-52 his3::hisG FLO11::HA*$^{30, 1015}$ *rim13 :: RIM101-531*	This study
NY82	*MAT**a** ura3-52 his3::hisG FLO11::HA*$^{30, 1015}$ *rim13 :: kanMX6 RIM101-531*	This study
CPY154	*MAT**a** ura3-52 his3::hisG FLO11:: HA*^{30} *vps20 :: kanMX6*	This study
CPY96	*MAT**a** ura3-52 his3::hisG FLO11:: HA*^{30} *rim9 :: kanMX6*	This study
CPY112	*MAT**a** ura3-52 his3::hisG FLO11:: HA*^{30} *rim101 :: kanMX6*	This study

Table A-2: Primers used in chapter 2

Primer	Purpose	Sequence
TRO369	Reverse primer used in conjunction with listed primers to confirm disruptions	GCACGTCAAGACTGTCAAGG
TRO394	Disrupt Vps4	CCAACTTCTACGCCAAGTATCCTA
TRO395	Disrupt Vps4	CAATCCTGAAAGTGAAGAATCCA
TRO396	Confirmvps4Δ	TAAGAGCAGTAAACCCGTTAGTGAC
TRO156	Disrupt Vps25	CAAATGATTACACCCCATGAA
TRO157	Disrupt Vps25	AAGGTTCAAGACTGGACCATG
TRO162	Confirmvps25Δ	TTTTAGATATTTGCGTTAGCTAAGG
TRO379	Disrupt Vps28	CGGATCCTTCTAAATTGAGAAGAG
TRO380	Disrupt Vps28	TGGATCAAAGATGATAGTCGCAG
TRO381	Confirmvps28Δ	TCCTTGCCGCCAATAATT
TRO266	Disrupt Vps27	CCGATTTTTTGGTAATATGTCAA
TRO267	Disrupt Vps27	AGCCAGGTGGTCAAAAAACA
TRO268	Confirmvps27Δ	ACAAAAGCAAACTGTTCGGAG
TRO503	Disrupt Rim13	AGTATCTTTGAACCGCGCAG
TRO504	Disrupt Rim13	GGATGGTCGTTCATTATTTTTGAG
TRO505	Confirmrim13Δ	CGTTACCTCCCACAAAACTTTTG
TRO482	Disrupt Rim101	GTCCAGCTCGGAGTTTCTAAA
TRO483	Disrupt Rim101	CGGGATCAACCGATCAAGATA
TRO484	Confirmrim101Δ	ACTTTTCTCTGCCCAGTGACA
TRO516	GenerateRIM101-531dominant allele	CAATGGCAGGTGGAACTTCATTGAAGCCTAACTGG GAATTTAGCCTGAACTGAGGCGCGCCACTTCTAAA
TRO517	GenerateRIM101-531dominant allele	TCTTCAATCGCCAGCTTACTCATGATAATATCATTA GTACAGCTTTTTTGGAATTCGAGCTCGTTTAAAC
TRO518	ConfirmRIM101-531	CCGCCTCTACAATCAAAGATACC
PC675	Insert HA tag between residues 1015 and 1016	GGATGCTCTCCAAAGACCCATTACAACTACTGTTCC ATGTTCAACCAGGGAACAAAAGCTGG
PC676	Insert HA tag between residues 1015 and 1016	GGTAGGTGAAGTGGTTGTTGATTCCGAGGCGGTTTC GCTTGGACTCTGTAGGGCGAATTGG
TRO621	Real-time PCR primers forFLO11	CACTTTTGAAGTTTATGCCACACAAG
TRO622	Real-time PCR primer for FLO11	CTTGCATATTGAGCGGCACTAC
TRO632	Real-time PCR primer for ACT1	CTCCACCACTGCTGAAAGAGAA
TRO636	Real-time PCR primer for ACT1	CCAAAGCGACGTAACATAGCTTT

Table A-3: Mat and invasive growth phenotypes of *vps* mutants

Mutant	Mat	Invasive growth	Class
*vps1*Δ	−	+	
*vps2*Δ/*did4*Δ	−	+	E
*vps3*Δ	−	+	
*vps4*Δ	−	+	E
*vps8*Δ	+	+	
*vps13*Δ	+	+	
*vps15*Δ	−	+	
*vps17*Δ	+	+	
*vps20*Δ	−	−	E
*vps21*Δ	+	±	
*vps22*Δ/*snf8*Δ	−	−	E
*vps23*Δ	−	−	E
*vps24*Δ	−	+	E
*vps25*Δ	−	−	E
*vps26*Δ/*pep4*Δ	±	+	
*vps27*Δ	−	+	E
*vps28*Δ	−	+	E
*vps30*Δ	+	+	
*vps31*Δ/*bro1*Δ	−	+	E
*vps32*Δ/*snf7*Δ	−	−	E
*vps34*Δ	−	−	
*vps35*Δ	±	+	
*vps36*Δ	−	−	E
*vps37*Δ	±	+	E
*vps38*Δ	−	+	
*vps39*Δ/*vam6*Δ	+	+	
*vps41*Δ	−	+	
*vps43*Δ/*vam7*Δ	±	+	
*vps44*Δ/*nhx1*Δ	−	+	
*vps46*Δ/*did2*Δ	+	+	
*vps51*Δ	+	+	
*vps52*Δ	±	+	
*vps53*Δ	±	+	
*vps64*Δ	+	±	
*vps66*Δ	±	+	
*vps68*Δ	+	+	
*vps54*Δ	±	+	
*vps60*Δ/*mos10*Δ	−	±	E
*vps62*Δ	+	+	

Figure A-4: Western blot gel of Flo11-HA^{30,1015}, shows the presence of a cleaved N-terminal HA-tagged band (~17 kDa).

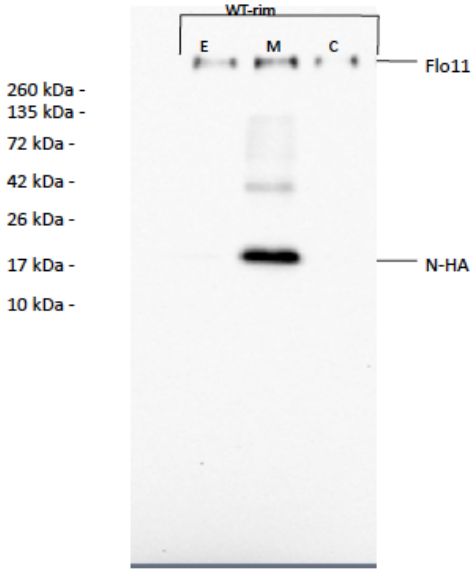

Table A-5: Yeast strains used in chapter 3

Strain	Genotype	Reference or source
TRY181	*MAT***a** *ura3-52 his3*::*hisG FLO11*::HA^{30,1015}	[36]
NY68	*MAT***a** *ura3-52 his3*::*hisG FLO11*::HA^{30,1015} *wsc1*::kanMX6	This study
NY78	*MAT***a** *ura3-52 his3*::*hisG FLO11*::HA^{30,1015} *skn7*::kanMX6	This study
NY270	*MAT***a** *ura3-52 his3*::*hisG FLO11*::HA^{30,1015} *rom2*::kanMX6	This study
NY87	*MAT***a** *ura3-52 his3*::*hisG FLO11*::HA^{30,1015} *wsc1*::WSC1-GFP-HIS3MX6	This study
NY236	*MAT***a** *ura3-52 his3*::*hisG FLO11*::HA^{30,1015} *wsc1Δ*::WSC1-GFP-HIS3MX6	This study
NY245	*MAT***a** *ura3-52 his3*::*hisG FLO11*::HA^{30,1015} *wsc1Δ*::WSC1-Y303A-GFP-HIS3MX6	This study
NY249	*MAT***a** *ura3-52 his3*::*hisG FLO11*::HA^{30,1015} *wsc1Δ*::WSC1-L369A-V371A-GFP-HIS3MX6	This study
NY251	*MAT***a** *ura3-52 his3*::*hisG FLO11*::HA^{30,1015} *wsc1Δ*::WSC1-S19A-S20A-GFP-HIS3MX6	This study
NY254	*MAT***a** *ura3-52 his3*::*hisG FLO11*::HA^{30,1015} *wsc1Δ*::WSC1-Y303A-L369A-V371AGFP-HIS3MX6	This study

Table A-6: Primers used in chapter 3

Name	Purpose	Sequence
TRO693	Disrupt *WSC1*	TTTTCGAAGCGAAAGCGAGA
TRO694	Disrupt *WSC1*	TTAATGTTCCTCGTTACTTCCAG
NSkn7F	Disrupt *SKN7*	CAAGATTGAAAGTGCTTCCAGG
NSkn7R	Disrupt *SKN7*	CGCATACTAAATTACTGTGTCTGT
TRO783	Insert GFP-HIS3MX6 from pFA6a-GFP-His3MX6	CAGGAGGGAAAAACAACGTTTTAACAGTGGTCAATCCAGACGAAGCTGAT
TRO784	Insert GFP-HIS3MX6 from pFA6a-GFP-His3MX6	AGACTTGCTTGGCAATAGTTTAAGAATATAATAATTTTTTTTGGGTTTCTTCA
TRO369	Reverse primer to confirm all disruptions	GCACGTCAAGACTGTCAAGG
NSO75	Create Y303A mutation in WSC1	GGAAGCCCAAGAGGCGATA
NSO76	Create Y303A mutation in WSC1	CTCTTGGGCTTCCTTTTCCAT
NSO79	Create S19A-S20A mutation in WSC1	CGCCGCTGCATTTTCATCTA
NSO80	Create S19A-S20A mutation in WSC1	GAAAATGCAGCGGCGTATAGTT
NSO85	Create S22A-S23A mutation in WSC1	CATTTGCAGCTAATCACGGGCCCT
NSO86	Create S22A-S23A mutation in WSC1	GTGATTAGCTGCAAATGAAGAGGCGT
NSO88	Create L369A-V371A mutation in WSC1	CAACGTTGCAACAGCGGTCAATCCA
NSO89	Create L369A-V371A mutation in WSC1	GATTGACCGCTGTTGCAACGTTGTTT
NSO90	Create N373A-D375A mutation in WSC1	GTCGCTCCAGCCGAAGCTGAT
NSO91	Create N373A-D375A mutation in WSC1	CTTCGGCTGGAGCGACCGCT
NSO77	Create WSC1 cytoplasmic tail truncation mutant	CAGGATGGAACGGATCCCCGGGT
NSO78	Create WSC1 cytoplasmic tail truncation mutant	CGGGGATCCGTTCCATCCTGTCTT

Table A-7: Yeast strains used in chapter 4

Strain	Genotype	Reference or source
TRY181	*MAT***a** *ura3-52 his3*::*hisG FLO11*::HA^{30,1015}	[36]
NY259	*MAT***a** *ura3-52 his3*::*hisG FLO11*::HA^{30,1015} *cda1*::kanMX6	This study
NY263	*MAT***a** *ura3-52 his3*::*hisG FLO11*::HA^{30,1015} *cda2*::his3MX6	This study
NY267	*MAT***a** *ura3-52 his3*::*hisG FLO11*::HA^{30,1015} *cda1*::kanMX6 *cda2*::his3MX6	This study

Table A-8: Primers used in chapter 4

Name	Purpose	Sequence
NSO99	Disrupt *CDA1*	CTAAGAGAGAGCAGGAAGTTGAAGA
NSO100	Disrupt *CDA1*	GCCAATTGTTATTTGCACTGA
NSO103	Confirm cda1Δ	CATGGCTATTGACAAGATAATCAGG
NSO101	Disrupt *CDA2*	AAACAAACTGCAAAAGAGTTGTTATTATTT CTACGGATCGGCAATTGAAACAGCTGAAGC TTCGTACGC
NSO102	Disrupt *CDA2*	TTTTCTTCAATTCCCTGAAAATTAGGACAA GAATTCTTTTATGTAATCAAGCATAGGCCA CTAGTGGATCTG
NSO104	Confirm cda2Δ	ATTGCAACGGCCTAAAGGAA
TRO369	Reverse primer to confirm all disruptions	GCACGTCAAGACTGTCAAGG

Figure A-9: Mat formation phenotype and overlay adhesion assay performed on *vps25Δ* and *vps27Δ*, which were used as control strains for RNA-Seq analysis.

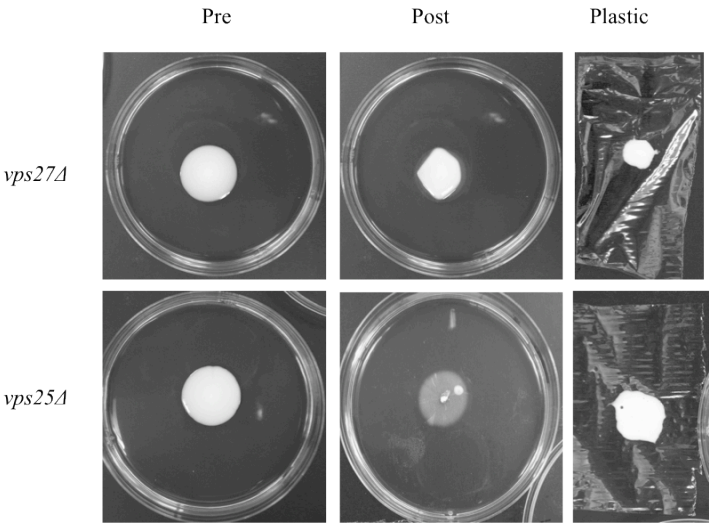

Table A-10 : Genes upregulated in the wild type biofilm hub compared to the rim

ORF	Gene	Ratio of RPKM of wild type (Hub/Rim)	Fisher's p-value	Significance	Function
YMR175W	SIP18	10617.65	2.087E-318	significant	Phospholipid-binding protein; expression is induced by osmotic stress
YEL009C	GCN4	2745.60	1.973E-319	significant	Basic leucine zipper (bZIP) transcriptional activator of amino acid biosynthetic genes in response to amino acid starvation; expression is tightly regulated at both the transcriptional and translational levels
YMR230W	RPS10B	920.85	2.060E-320	significant	Protein component of the small (40S) ribosomal subunit; nearly identical to Rps10Ap and has similarity to rat ribosomal protein S10
YHR193C	EGD2	630.76	2.506E-320	significant	Alpha subunit of the heteromeric nascent polypeptide-associated complex (NAC) involved in protein sorting and translocation, associated with cytoplasmic ribosomes
YER044C	ERG28	621.09	2.498E-320	significant	Endoplasmic reticulum membrane protein, may facilitate protein-protein interactions between the Erg26p dehydrogenase and the Erg27p 3-ketoreductase and/or tether these enzymes to the ER, also interacts with Erg6p
YNL067W	RPL9B	596.06	2.622E-320	significant	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl9Ap and has similarity to E. coli L6 and rat L9 ribosomal proteins

Table A-10 (Continued)

YCR097W	HMRA1	531.16	1.468E-320	significant	Silenced copy of a1 at HMR; homeobox corepressor that interacts with Alpha2p to repress haploid-specific gene transcription in diploid cells
YKL145W	RPT1	310.57	3.521E-320	significant	One of six ATPases of the 19S regulatory particle of the 26S proteasome involved in the degradation of ubiquitinated substrates; required for optimal CDC20 transcription; interacts with Rpn12p and Ubr1p; mutant has aneuploidy tolerance
YMR251W-A	HOR7	282.03	5.756E-320	significant	Protein of unknown function; overexpression suppresses Ca2+ sensitivity of mutants lacking inositol phosphorylceramide mannosyltransferases Csg1p and Csh1p; transcription is induced under hyperosmotic stress and repressed by alpha factor
YER148W	SPT15	272.28	1.534E-320	significant	TATA-binding protein, general transcription factor that interacts with other factors to form the preinitiation complex at promoters, essential for viability
YER159C	BUR6	270.74	8.576E-321	significant	Subunit of a heterodimeric NC2 transcription regulator complex with Ncb2p; complex binds to TBP and can repress transcription by preventing preinitiation complex assembly or stimulate activated transcription; homologous to human NC2alpha
YAL034C	FUN19	237.52	2.372E-320	significant	Non-essential protein of unknown function; expression induced in response to heat stress
YGR161C	RTS3	233.02	1.993E-320	significant	Putative component of the protein phosphatase type 2A complex
YOL052C-A	DDR2	220.61	1.167E-319	significant	Multistress response protein, expression is activated by a variety of xenobiotic agents and environmental or physiological stresses

Table A-10 (Continued)

YPR160W	GPH1	212.39	4.730E-320	significant	Non-essential glycogen phosphorylase required for the mobilization of glycogen, activity is regulated by cyclic AMP-mediated phosphorylation, expression is regulated by stress-response elements and by the HOG MAP kinase pathway
YBR196C	PGI1	189.36	1.770E-319	significant	Glycolytic enzyme phosphoglucose isomerase, catalyzes the interconversion of glucose-6-phosphate and fructose-6-phosphate; required for cell cycle progression and completion of the gluconeogenic events of sporulation
YFR032C-A	RPL29	188.48	8.972E-321	significant	Protein component of the large (60S) ribosomal subunit, has similarity to rat L29 ribosomal protein; not essential for translation, but required for proper joining of the large and small ribosomal subunits and for normal translation rate
YDR525W-A	SNA2	160.38	6.748E-321	significant	Protein of unknown function, has similarity to Pmp3p, which is involved in cation transport; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm in a punctate pattern
YPR010C-A	NA	156.13	5.080E-320	significant	Putative protein of unknown function; conserved among Saccharomyces sensu stricto species
YBL071C	NA	153.06	1.56E-232	significant	Dubious open reading frame, predicted protein contains a peroxisomal targeting signal
YHR052W-A	NA	142.83	1.790E-319	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps CUP1-1

Table A-10 (Continued)

YER091C	MET6	113.47	2.143E-320	significant	Cobalamin-independent methionine synthase, involved in methionine biosynthesis and regeneration; requires a minimum of two glutamates on the methyltetrahydrofolate substrate, similar to bacterial metE homologs
YCL048W-A	NA	111.84	1.028E-320	significant	Putative protein of unknown function
YML100W	TSL1	107.82	2.933E-320	significant	Large subunit of trehalose 6-phosphate synthase (Tps1p)/phosphatase (Tps2p) complex, which converts uridine-5'-diphosphoglucose and glucose 6-phosphate to trehalose, similar to Tps3p and may share function; mutant has aneuploidy tolerance
YBR121C	GRS1	86.01	1.408E-320	significant	Cytoplasmic and mitochondrial glycyl-tRNA synthase that ligates glycine to the cognate anticodon bearing tRNA; transcription termination factor that may interact with the 3'-end of pre-mRNA to promote 3'-end formation
YHL015W	RPS20	80.41	3.148E-320	significant	Protein component of the small (40S) ribosomal subunit; overproduction suppresses mutations affecting RNA polymerase III-dependent transcription; has similarity to E. coli S10 and rat S20 ribosomal proteins
YDR119W-A	NA	79.20	4.081E-320	significant	Putative protein of unknown function; may interact with respiratory chain complexes III (ubiquinol-cytochrome c reductase) or IV (cytochrome c oxidase)

Table A-10 (Continued)

YGR146C	ECL1	77.53	1.476E-320	significant	Protein of unknown function, affects chronological lifespan; induced by iron homeostasis transcription factor Aft2p; multicopy suppressor of temperature sensitive hsf1 mutant; induced by treatment with 8-methoxypsoralen and UVA irradiation
YDR098C	GRX3	76.22	5.869E-321	significant	Hydroperoxide and superoxide-radical responsive glutathione-dependent oxidoreductase; monothiol glutaredoxin subfamily member along with Grx4p and Grx5p; protects cells from oxidative damage
YPR158W	CUR1	73.78	4.19E-290	significant	prions; similar in sequence to Btn2p
YCR024C-B	NA	62.48	4.836E-320	significant	Putative protein of unknown function; identified by expression profiling and mass spectrometry
YFR031C-A	RPL2A	61.61	3.703E-320	significant	Protein component of the large (60S) ribosomal subunit, identical to Rpl2Bp and has similarity to E. coli L2 and rat L8 ribosomal proteins
YHR069C	RRP4	60.10	5.192E-321	significant	Exosome non-catalytic core component; involved in 3'-5' RNA processing and degradation in both the nucleus and the cytoplasm; predicted to contain RNA binding domains; has similarity to human hRrp4p (EXOSC2)
YOR316C	COT1	58.54	1.150E-320	significant	Vacuolar transporter that mediates zinc transport into the vacuole; overexpression confers resistance to cobalt and rhodium

Table A-10 (Continued)

YGL006W	PMC1	56.24	1.636E-320	significant	Vacuolar Ca2+ ATPase involved in depleting cytosol of Ca2+ ions; prevents growth inhibition by activation of calcineurin in the presence of elevated concentrations of calcium; similar to mammalian PMCA1a
YHR180W	NA	54.41	3.01E-138	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data
YNR034W-A	NA	52.73	1.145E-319	significant	Putative protein of unknown function; expression is regulated by Msn2p/Msn4p
YFR036W	CDC26	52.04	8.12E-97	significant	Subunit of the Anaphase-Promoting Complex/Cyclosome (APC/C), which is a ubiquitin-protein ligase required for degradation of anaphase inhibitors, including mitotic cyclins, during the metaphase/anaphase transition
YMR315W	NA	49.94	1.794E-320	significant	Protein with NADP(H) oxidoreductase activity; transcription is regulated by Stb5p in response to NADPH depletion induced by diamide; promoter contains a putative Stb5p binding site
YNL097C	PHO23	48.08	5.162E-321	significant	Probable component of the Rpd3 histone deacetylase complex, involved in transcriptional regulation of PHO5; C-terminus has similarity to human candidate tumor suppressor p33(ING1) and its isoform ING3
YLR287C-A	RPS30A	47.13	3.169E-320	significant	Protein component of the small (40S) ribosomal subunit; nearly identical to Rps30Bp and has similarity to rat S30 ribosomal protein

Table A-10 (Continued)

YPL249C-A	RPL36B	47.11	3.177E-320	significant	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl36Ap and has similarity to rat L36 ribosomal protein; binds to 5.8 S rRNA
YDR524C-B	NA	45.20	3.372E-320	significant	Putative protein of unknown function
YDR524C	AGE1	45.04	5.276E-321	significant	ADP-ribosylation factor (ARF) GTPase activating protein (GAP) effector, involved in the secretory and endocytic pathways; contains C2C2H2 cysteine/histidine motif
YER084W	NA	43.35	2.37E-223	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data
YLR410W	VIP1	43.10	1.224E-320	significant	Inositol hexakisphosphate (IP6) and inositol heptakisphosphate (IP7) kinase; IP7 production is important for phosphate signaling; involved in cortical actin cytoskeleton function, and invasive pseudohyphal growth analogous to S. pombe asp1
YHR213W-B	NA	43.09	4.41E-54	significant	Putative protein of unknown function; identified by gene-trapping, microarray-based expression analysis, and genome-wide homology searching
YDL159W	STE7	42.80	5.370E-321	significant	Signal transducing MAP kinase kinase involved in pheromone response, where it phosphorylates Fus3p, and in the pseudohyphal/invasive growth pathway, through phosphorylation of Kss1p; phosphorylated by Ste11p, degraded by ubiquitin pathway

Table A-10 (Continued)

YIL020C	HIS6	41.07	3.11E-174	significant	Phosphoribosyl-5-amino-1-phosphoribosyl-4-imidazolecarboxiamide isomerase, catalyzes the fourth step in histidine biosynthesis; mutations cause histidine auxotrophy and sensitivity to Cu, Co, and Ni salts
YNL162W	RPL42A	40.85	1.615E-320	significant	Protein component of the large (60S) ribosomal subunit, identical to Rpl42Bp and has similarity to rat L44 ribosomal protein
YPR108W	RPN7	39.13	1.614E-320	significant	Essential, non-ATPase regulatory subunit of the 26S proteasome, similar to another S. cerevisiae regulatory subunit, Rpn5p, as well as to mammalian proteasome subunits
YPL152W	RRD2	38.43	3.44E-232	significant	Activator of the phosphotyrosyl phosphatase activity of PP2A,peptidyl-prolyl cis/trans-isomerase; regulates G1 phase progression, the osmoresponse, microtubule dynamics; subunit of the Tap42p-Pph21p-Rrd2p complex
YOR142W	LSC1	37.65	1.465E-320	significant	Alpha subunit of succinyl-CoA ligase, which is a mitochondrial enzyme of the TCA cycle that catalyzes the nucleotide-dependent conversion of succinyl-CoA to succinate; phosphorylated
YOR008C	SLG1	36.84	1.040E-320	significant	Sensor-transducer of the stress-activated PKC1-MPK1 kinase pathway involved in maintenance of cell wall integrity; involved in organization of the actin cytoskeleton; secretory pathway Wsc1p is required for the arrest of secretion response

Table A-10 (Continued)

YHR086W	NAM8	35.08	0.00E+00	significant	RNA binding protein, component of the U1 snRNP protein; mutants are defective in meiotic recombination and in formation of viable spores, involved in the formation of DSBs through meiosis-specific splicing of MER2 pre-mRNA
YMR030W	RSF1	32.31	1.140E-320	significant	Protein required for respiratory growth; localized to both the nucleus and mitochondrion; may interact with transcription factors to mediate the transition to respiratory growth and activate transcription of nuclear and mitochondrial genes
YDR316W	OMS1	32.20	2.67E-239	significant	Protein integral to the mitochondrial membrane; has a conserved methyltransferase motif; multicopy suppressor of respiratory defects caused by OXA1 mutations
YBL068W	PRS4	31.32	4.61E-168	significant	5-phospho-ribosyl-1(alpha)-pyrophosphate synthetase, synthesizes PRPP, which is required for nucleotide, histidine, and tryptophan biosynthesis; one of five related enzymes, which are active as heteromultimeric complexes
YDR034C-A	NA	30.22	1.69E-07	significant	Putative protein of unknown function; contained within the solo Ty1 LTR element YDRWdelta7
YDL114W-A	NA	30.19	1.66E-03		Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; identified based on homology to hemiascomycetous yeasts

Table A-10 (Continued)

YKL096W-A	CWP2	29.92	1.590E-319	significant	Covalently linked cell wall mannoprotein, major constituent of the cell wall; plays a role in stabilizing the cell wall; involved in low pH resistance; precursor is GPI-anchored
YBL039C	URA7	29.64	7.85E-290	significant	Major CTP synthase isozyme (see also URA8), catalyzes the ATP-dependent transfer of the amide nitrogen from glutamine to UTP, forming CTP, the final step in de novo biosynthesis of pyrimidines; involved in phospholipid biosynthesis
YML054C	CYB2	28.42	3.913E-320	significant	Cytochrome b2 (L-lactate cytochrome-c oxidoreductase), component of the mitochondrial intermembrane space, required for lactate utilization; expression is repressed by glucose and anaerobic conditions
YFR009W	GCN20	27.72	5.128E-321	significant	Positive regulator of the Gcn2p kinase activity, forms a complex with Gcn1p; proposed to stimulate Gcn2p activation by an uncharged tRNA
YJL127C-B	NA	27.24	1.64E-10	significant	Putative protein of unknown function; identified based on homology to the filamentous fungus, Ashbya gossypii
YMR158W	MRPS8	26.11	2.98E-225	significant	Mitochondrial ribosomal protein of the small subunit
YDR363W-A	SEM1	24.30	3.05E-256	significant	Component of the lid subcomplex of the regulatory subunit of the 26S proteasome; involved in mRNA export mediated by the TREX-2 complex (Sac3p-Thp1p); ortholog of human DSS1

Table A-10 (Continued)

YDR169C	STB3	24.07	6.492E-321	significant	Ribosomal RNA processing element (RRPE)-binding protein involved in the glucose-induced transition from quiescence to growth; restricted to nucleus in quiescent cells, released into cytoplasm after glucose repletion; binds Sin3p
YLR154C-G	NA	22.60	1.960E-320	significant	Putative protein of unknown function identified by fungal homology comparisons and RT-PCR; this ORF is contained within RDN25-2 and RDN37-2
YHR073W	OSH3	21.96	5.414E-321	significant	Member of an oxysterol-binding protein family with seven members in S. cerevisiae; family members have overlapping, redundant functions in sterol metabolism and collectively perform a function essential for viability
YIL046W	MET30	21.36	3.026E-320	significant	F-box protein containing five copies of the WD40 motif, controls cell cycle function, sulfur metabolism, and methionine biosynthesis as part of the ubiquitin ligase complex; interacts with and regulates Met4p, localizes within the nucleus
YNL024C-A	KSH1	21.30	1.00E-93	significant	Essential protein suggested to function early in the secretory pathway; inviability is suppressed by overexpression of Golgi protein Tvp23p; ortholog of human Kish
YOR192C-C	NA	21.09	2.86E-21	significant	Putative protein of unknown function; identified by expression profiling and mass spectrometry

Table A-10 (Continued)

YOR072W-A	NA	18.76	7.92E-21	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps the uncharacterized ORF YOR072W; originally identified by fungal homology and RT-PCR
YHR139C	SPS100	18.31	7.183E-320	significant	Protein required for spore wall maturation; expressed during sporulation; may be a component of the spore wall; expression also induced in cells treated with the mycotoxin patulin
YPR036W-A	NA	18.17	8.064E-320	significant	Protein of unknown function; transcription is regulated by Pdr1p
YHR001W-A	QCR10	17.77	3.662E-320	significant	Subunit of the ubiqunol-cytochrome c oxidoreductase complex which includes Cobp, Rip1p, Cyt1p, Cor1p, Qcr2p, Qcr6p, Qcr7p, Qcr8p, Qcr9p, and Qcr10p and comprises part of the mitochondrial respiratory chain
YLR256W	HAP1	17.68	8.354E-321	significant	Zinc finger transcription factor involved in the complex regulation of gene expression in response to levels of heme and oxygen; the S288C sequence differs from other strain backgrounds due to a Ty1 insertion in the carboxy terminus
YJL062W-A	COA3	17.36	1.49E-18	significant	Mitochondrial inner membrane protein that participates in regulation of COX1 translation, Cox1p stabilization, and cytochrome oxidase assembly
YDR261C-D	NA	17.35	1.819E-319	significant	Retrotransposon TYA Gag and TYB Pol genes; in YDRCTY1-3 TYB is mutant and probably non-functional

Table A-10 (Continued)

YDR261C	EXG2	16.92	1.19E-144	significant	Exo-1,3-beta-glucanase, involved in cell wall beta-glucan assembly; may be anchored to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor
YLL006W	MMM1	16.39	5.51E-116	significant	ER integral membrane protein, component of the ERMES complex that links the ER to mitochondria and may promote inter-organellar calcium and phospholipid exchange as well as coordinating mitochondrial DNA replication and growth
YNL130C	CPT1	16.09	9.377E-321		Cholinephosphotransferase, required for phosphatidylcholine biosynthesis and for inositol-dependent regulation of EPT1 transcription
YCR028C-A	RIM1	14.23	3.020E-313	significant	Single-stranded DNA-binding protein essential for mitochondrial genome maintenance; involved in mitochondrial DNA replication
YBR191W	RPL21A	14.22	2.737E-320	significant	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl21Bp and has similarity to rat L21 ribosomal protein
YIR021W	MRS1	13.89	1.14E-188	significant	Protein required for the splicing of two mitochondrial group I introns (BI3 in COB and AI5beta in COX1); forms a splicing complex, containing four subunits of Mrs1p and two subunits of the BI3-encoded maturase, that binds to the BI3 RNA
YIL102C-A	NA	13.09	3.05E-24	significant	Putative protein of unknown function, identified based on comparisons of the genome sequences of six Saccharomyces species

Table A-10 (Continued)

YOL019W	NA	12.78	4.86E-118	significant	Protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cell periphery and vacuole
YGR240C	PFK1	12.37	6.524E-320	significant	Alpha subunit of heterooctameric phosphofructokinase involved in glycolysis, indispensable for anaerobic growth, activated by fructose-2,6-bisphosphate and AMP, mutation inhibits glucose induction of cell cycle-related genes
YOR161C	PNS1	11.40	8.443E-321	significant	Protein of unknown function; has similarity to Torpedo californica tCTL1p, which is postulated to be a choline transporter, neither null mutation nor overexpression affects choline transport
YDL247W-A	NA	11.12	5.52E-40	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; identified by sequence comparison with hemiascomycetous yeast species
YGL041C-B	NA	10.62	7.98E-08	significant	Putative protein of unknown function; identified by fungal homology and RT-PCR
YER172C	BRR2	10.50	5.691E-321	significant	RNA-dependent ATPase RNA helicase (DEIH box); required for disruption of U4/U6 base-pairing in native snRNPs to activate the spliceosome for catalysis; homologous to human U5-200kD
YOR376W-A	NA	10.09	6.12E-07	significant	Putative protein of unknown function; identified by fungal homology and RT-PCR

Table A-10 (Continued)

YKL165C	MCD4	10.02	6.739E-321	significant	Protein involved in glycosylphosphatidylinositol (GPI) anchor synthesis; multimembrane-spanning protein that localizes to the endoplasmic reticulum; highly conserved among eukaryotes
YML101C	CUE4	9.80	1.78E-108	significant	Protein of unknown function; has a CUE domain that binds ubiquitin, which may facilitate intramolecular monoubiquitination
YPL257W	NA	8.47	5.27E-20	significant	Putative protein of unknown function; homozygous diploid deletion strain exhibits low budding index; physically interacts with Hsp82p; YPL257W is not an essential gene
YAL019W	FUN30	8.46	2.01E-152	significant	Conserved member of the Snf2p family with ATP-dependent chromatin remodeling activity; has a role in silencing; potential Cdc28p substrate; authentic, non-tagged protein is detected in purified mitochondria in high-throughput studies
YMR013C	SEC59	8.36	3.38E-72	significant	Dolichol kinase, catalyzes the terminal step in dolichyl monophosphate (Dol-P) biosynthesis; required for viability and for normal rates of lipid intermediate synthesis and protein N-glycosylation
YPR169W	JIP5	8.33	8.779E-321	significant	Essential protein required for biogenesis of the large ribosomal subunit; interacts with proteins involved in RNA processing, ribosome biogenesis, ubiquitination and demethylation; similar to WDR55, a human WD repeat protein

Table A-10 (Continued)

YCL001W	RER1	8.17	2.99E-257	significant	Protein involved in retention of membrane proteins, including Sec12p, in the ER; localized to Golgi; functions as a retrieval receptor in returning membrane proteins to the ER
YHR007C	ERG11	7.92	5.045E-320	significant	Lanosterol 14-alpha-demethylase, catalyzes the C-14 demethylation of lanosterol to form 4,4"-dimethyl cholesta-8,14,24-triene-3-beta-ol in the ergosterol biosynthesis pathway; member of the cytochrome P450 family
YKL033W-A	NA	7.66	1.77E-17	significant	Putative protein of unknown function; similar to uncharacterized proteins from other fungi
YLR361C-A	NA	7.54	4.90E-02		Putative protein of unknown function
YER093C-A	AIM11	7.44	3.89E-16	significant	Protein of unknown function; null mutant is viable but shows increased loss of mitochondrial genome and synthetic interaction with prohibitin (phb1); contains an intron
YCL005W-A	VMA9	7.43	2.37E-23	significant	Vacuolar H+ ATPase subunit e of the V-ATPase V0 subcomplex; essential for vacuolar acidification; interacts with the V-ATPase assembly factor Vma21p in the ER; involved in V0 biogenesis
YGL188C	NA	7.38	9.49E-51	significant	Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data

Table A-10 (Continued)

YIL082W-A	NA	7.14	3.90E-187	significant	Retrotransposon TYA Gag and TYB Pol genes; transcribed/translated as one unit; polyprotein is processed to make a nucleocapsid-like protein (Gag), reverse transcriptase (RT), protease (PR), and integrase (IN); similar to retroviral genes
YLR262C-A	TMA7	7.11	1.66E-43	significant	Protein of unknown that associates with ribosomes; null mutant exhibits translation defects, altered polyribosome profiles, and resistance to the translation inhibitor anisomcyin
YHR072W-A	NOP10	6.91	4.69E-142	significant	Constituent of small nucleolar ribonucleoprotein particles containing H/ACA-type snoRNAs, which are required for pseudouridylation and processing of pre-18S rRNA
YER133W	GLC7	6.88	2.037E-320	significant	Type 1 serine/threonine protein phosphatase catalytic subunit, involved in many processes (eg: glycogen metabolism, sporulation, mitosis); accumulates at mating projections by interaction with Afr1p; interacts with many regulatory subunits
YHR079C	IRE1	6.81	8.23E-107	significant	Serine-threonine kinase and endoribonuclease; transmembrane protein that mediates the unfolded protein response (UPR) by regulating Hac1p synthesis through HAC1 mRNA splicing; Kar2p binds inactive Ire1p and releases from it upon ER stress

Table A-10 (Continued)

YIL047C	SYG1	6.40	6.393E-320	significant	Plasma membrane protein of unknown function; truncation and overexpression suppresses lethality of G-alpha protein deficiency
YEL076C	NA	6.32	2.58E-15	significant	Putative protein of unknown function
YBL008W	HIR1	5.80	6.13E-139	significant	Subunit of the HIR complex, a nucleosome assembly complex involved in regulation of histone gene transcription; contributes to nucleosome formation, heterochromatic gene silencing, and formation of functional kinetochores
YBR058C-A	TSC3	5.72	3.08E-72	significant	Protein that stimulates the activity of serine palmitoyltransferase (Lcb1p, Lcb2p) several-fold; involved in sphingolipid biosynthesis
YOR011W	AUS1	5.68	1.11E-285	significant	Transporter of the ATP-binding cassette family, involved in uptake of sterols and anaerobic growth
YHR039C-A	VMA10	5.32	7.05E-16	significant	Subunit G of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; involved in vacuolar acidification
YGL256W	ADH4	5.30	2.812E-320	significant	Alcohol dehydrogenase isoenzyme type IV, dimeric enzyme demonstrated to be zinc-dependent despite sequence similarity to iron-activated alcohol dehydrogenases; transcription is induced in response to zinc deficiency

Table A-10 (Continued)

YHR199C	AIM46	5.30	2.361E-320	significant	Putative protein of unknown function; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies; null mutant displays elevated frequency of mitochondrial genome loss
YML045W	NA	5.26	3.38E-133	significant	Retrotransposon TYA Gag and TYB Pol genes; transcribed/translated as one unit; polyprotein is processed to make a nucleocapsid-like protein (Gag), reverse transcriptase (RT), protease (PR), and integrase (IN); similar to retroviral genes
YHR126C	ANS1	5.08	7.68E-10	significant	Putative protein of unknown function; transcription dependent upon Azf1p
YHR143W-A	RPC10	5.06	5.91E-02		RNA polymerase subunit ABC10-alpha, found in RNA polymerase complexes I, II, and III
YFL012W	NA	5.00	5.07E-07	significant	Putative protein of unknown function; transcribed during sporulation; null mutant exhibits increased resistance to rapamycin
YBR182C	SMP1	4.96	1.33E-34	significant	Putative transcription factor involved in regulating the response to osmotic stress; member of the MADS-box family of transcription factors
YIL156W	UBP7	4.87	7.25E-197	significant	Ubiquitin-specific protease that cleaves ubiquitin-protein fusions
YLR437C	DIF1	4.86	9.49E-49	significant	Protein that regulates the nuclear localization of ribonucleotide reductase Rnr2p and Rnr4p subunits; phosphorylated by Dun1p in response to DNA damage and degraded; N-terminal half has similarity to S. pombe Spd1 protein

Table A-10 (Continued)

YHR005C-A	TIM10	4.76	3.31E-11	significant	Essential protein of the mitochondrial intermembrane space, forms a complex with Tim9p (TIM10 complex) that delivers hydrophobic proteins to the TIM22 complex for insertion into the inner membrane
YMR001C	CDC5	4.66	2.79E-149	significant	Polo-like kinase with multiple functions in mitosis and cytokinesis through substrate phosphorylation, also functions in adaptation to DNA damage during meiosis; has similarity to Xenopus Plx1 and S. pombe Plo1p; possible Cdc28p substrate
YER039C	HVG1	4.59	8.320E-321	significant	Protein of unknown function, has homology to Vrg4p
YDR320C-A	DAD4	4.58	7.93E-92	significant	Essential subunit of the Dam1 complex (aka DASH complex), couples kinetochores to the force produced by MT depolymerization thereby aiding in chromosome segregation; is transferred to the kinetochore prior to mitosis
YER074W	RPS24A	4.57	3.203E-320	significant	Protein component of the small (40S) ribosomal subunit; identical to Rps24Bp and has similarity to rat S24 ribosomal protein
YDR246W	TRS23	4.55	9.426E-321	significant	One of 10 subunits of the transport protein particle (TRAPP) complex of the cis-Golgi which mediates vesicle docking and fusion; involved in endoplasmic reticulum (ER) to Golgi membrane traffic; human homolog is TRAPPC4
YJR112W-A	NA	4.54	2.05E-65	significant	Putative protein of unknown function; identified based on homology to Ashbya gossypii

Table A-10 (Continued)

YDR182W	CDC1	4.49	6.333E-321	significant	Putative lipid phosphatase of the endoplasmic reticulum; shows Mn2+ dependence and may affect Ca2+ signaling; mutants display actin and general growth defects and pleiotropic defects in cell cycle progression and organelle distribution
YNL103W	MET4	4.48	9.313E-321	significant	Leucine-zipper transcriptional activator, responsible for the regulation of the sulfur amino acid pathway, requires different combinations of the auxiliary factors Cbf1p, Met28p, Met31p and Met32p
YMR294W-A	NA	4.47	3.20E-07	significant	Dubious open reading frame unlikely to encode a functional protein, substantially overlaps YMR295C; deletion causes sensitivity to unfolded protein response-inducing agents
YLR308W	CDA2	4.46	5.01E-11	significant	Chitin deacetylase, together with Cda1p involved in the biosynthesis ascospore wall component, chitosan; required for proper rigidity of the ascospore wall
YPL130W	SPO19	4.40	8.54E-11	significant	Meiosis-specific prospore protein; required to produce bending force necessary for proper assembly of the prospore membrane during sporulation; identified as a weak high-copy suppressor of the spo1-1 ts mutation
YPL119C-A	NA	4.40	1.42E-70	significant	Putative protein of unknown function; identified by expression profiling and mass spectrometry

Table A-10 (Continued)

YBR200W	BEM1	4.38	8.497E-321	significant	Protein containing SH3-domains, involved in establishing cell polarity and morphogenesis; functions as a scaffold protein for complexes that include Cdc24p, Ste5p, Ste20p, and Rsr1p
YNL042W	BOP3	4.35	1.95E-151	significant	Protein of unknown function, potential Cdc28p substrate; overproduction confers resistance to methylmercury
YOL159C	NA	4.22	6.57E-159	significant	Soluble protein of unknown function; deletion mutants are viable and have elevated levels of Ty1 retrotransposition and Ty1 cDNA
YOR381W	FRE3	4.12	3.18E-134	significant	Ferric reductase, reduces siderophore-bound iron prior to uptake by transporters; expression induced by low iron levels
YDR149C	NA	4.10	1.16E-61	significant	Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data; overlaps the verified gene NUM1; null mutation blocks anaerobic growth
YHR053C	CUP1-1	3.94	2.329E-319	significant	Metallothionein, binds copper and mediates resistance to high concentrations of copper and cadmium; locus is variably amplified in different strains, with two copies, CUP1-1 and CUP1-2, in the genomic sequence reference strain S288C
YHR055C	CUP1-2	3.94	2.329E-319	significant	Metallothionein, binds copper and mediates resistance to high concentrations of copper and cadmium; locus is variably amplified in different strains, with two copies, CUP1-1 and CUP1-2, in the genomic sequence reference strain S288C

Table A-10 (Continued)

YLR053C	NA	3.86	2.27E-173	significant	Putative protein of unknown function
YGR169C-A	NA	3.36	2.84E-09	significant	Putative protein of unknown function
YMR323W	ERR3	3.25	6.73E-20	significant	Protein of unknown function, has similarity to enolases
YPL281C	ERR2	3.25	6.73E-20	significant	Protein of unknown function, has similarity to enolases
YLR390W-A	CCW14	3.22	2.700E-320	significant	Covalently linked cell wall glycoprotein, present in the inner layer of the cell wall
YML009C	MRPL39	3.20	3.49E-26	significant	Mitochondrial ribosomal protein of the large subunit
YKL068W-A	NA	3.19	1.06E-123	significant	Putative protein of unknown function; identified by homology to Ashbya gossypii
YNL277W	MET2	3.17	7.410E-322	significant	L-homoserine-O-acetyltransferase, catalyzes the conversion of homoserine to O-acetyl homoserine which is the first step of the methionine biosynthetic pathway
YLR154W-F	NA	3.13	1.25E-50	significant	Dubious open reading frame unlikely to encode a protein; encoded within the the 35S rRNA gene on the opposite strand
YCR010C	ADY2	3.10	1.51E-206	significant	Acetate transporter required for normal sporulation; phosphorylated in mitochondria
YBR221W-A	NA	3.02	2.57E-01		Putative protein of unknown function; identified by expression profiling and mass spectrometry
YER180C	ISC10	2.96	2.15E-140	significant	Protein required for sporulation, transcript is induced 7.5 hours after induction of meiosis, expected to play significant role in the formation of reproductive cells

Table A-10 (Continued)

YHR136C	SPL2	2.91	4.86E-29	significant	Protein with similarity to cyclin-dependent kinase inhibitors; downregulates low-affinity phosphate transport during phosphate limitation; overproduction suppresses a plc1 null mutation; GFP-fusion protein localizes to the cytoplasm
YOR393W	ERR1	2.87	8.889E-320	significant	Protein of unknown function, has similarity to enolases
YIL057C	RGI2	2.77	8.347E-320	significant	Protein of unknown function involved in energy metabolism under respiratory conditions; expression induced under carbon limitation and repressed under high glucose
YGL088W	NA	2.77	9.00E-19	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps snR10, a snoRNA required for preRNA processing
YFR032C-B	NA	2.75	3.58E-01		Putative protein of unknown function; identified by gene-trapping, microarray-based expression analysis, and genome-wide homology searching
YPL038W	MET31	2.62	1.26E-53	significant	Zinc-finger DNA-binding protein, involved in transcriptional regulation of the methionine biosynthetic genes, similar to Met32p
YGR236C	SPG1	2.58	1.322E-319	significant	Protein required for survival at high temperature during stationary phase; not required for growth on nonfermentable carbon sources; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies

Table A-10 (Continued)

YBR233W-A	DAD3	2.58	1.77E-22	significant	Essential subunit of the Dam1 complex (aka DASH complex), couples kinetochores to the force produced by MT depolymerization thereby aiding in chromosome segregation; is transferred to the kinetochore prior to mitosis
YFR010W	UBP6	2.55	2.167E-320	significant	Ubiquitin-specific protease situated in the base subcomplex of the 26S proteasome, releases free ubiquitin from branched polyubiquitin chains; works in opposition to Hul5p polyubiquitin elongation activity; mutant has aneuploidy tolerance
YFR023W	PES4	2.53	1.37E-15	significant	Poly(A) binding protein, suppressor of DNA polymerase epsilon mutation, similar to Mip6p
YIR020C	NA	2.51	8.10E-01		Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data
YPL092W	SSU1	2.47	1.077E-319	significant	Plasma membrane sulfite pump involved in sulfite metabolism and required for efficient sulfite efflux; major facilitator superfamily protein
YER145C	FTR1	2.44	3.10E-242	significant	High affinity iron permease involved in the transport of iron across the plasma membrane; forms complex with Fet3p; expression is regulated by iron
YAL026C-A	NA	2.35	1.73E-86	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps the uncharacterized ORF YAL027W and the verified gene DRS2

Table A-10 (Continued)

YPR001W	CIT3	2.35	3.39E-185	significant	Dual specificity mitochondrial citrate and methylcitrate synthase; catalyzes the condensation of acetyl-CoA and oxaloacetate to form citrate and that of propionyl-CoA and oxaloacetate to form 2-methylcitrate
YPL187W	MF(ALPHA)1	2.34	3.49E-32	significant	Mating pheromone alpha-factor, made by alpha cells; interacts with mating type a cells to induce cell cycle arrest and other responses leading to mating; also encoded by MF(ALPHA)2, although MF(ALPHA)1 produces most alpha-factor
YOR073W	SGO1	2.24	2.68E-54	significant	Component of the spindle checkpoint, involved in sensing lack of tension on mitotic chromosomes; protects centromeric Rec8p at meiosis I; required for accurate chromosomal segregation at meiosis II and for mitotic chromosome stability
YOL154W	ZPS1	2.18	4.55E-65	significant	Putative GPI-anchored protein; transcription is induced under low-zinc conditions, as mediated by the Zap1p transcription factor, and at alkaline pH
YOR364W	NA	2.03	1.03E-07	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps the uncharacterized ORF YOR365C
YMR175W	SIP18	10617.65	2.087E-318	significant	Phospholipid-binding protein; expression is induced by osmotic stress

Table A-10 (Continued)

YEL009C	GCN4	2745.60	1.973E-319	significant	Basic leucine zipper (bZIP) transcriptional activator of amino acid biosynthetic genes in response to amino acid starvation; expression is tightly regulated at both the transcriptional and translational levels

Table A-11 : Genes downregulated in the wild type biofilm hub compared to the rim

ORF	Gene	Ratiof of RPKM of wild type (Hub/Rim)	Fisher's p-value	Significance	Function
YMR011W	HXT2	0.52	5.88E-65	significant	High-affinity glucose transporter of the major facilitator superfamily, expression is induced by low levels of glucose and repressed by high levels of glucose
YML043C	RRN11	0.52	4.93E-16	significant	Component of the core factor (CF) rDNA transcription factor complex; CF is required for transcription of 35S rRNA genes by RNA polymerase I and is composed of Rrn6p, Rrn7p, and Rrn11p
YNL231C	PDR16	0.50	7.08E-192	significant	Phosphatidylinositol transfer protein (PITP) controlled by the multiple drug resistance regulator Pdr1p, localizes to lipid particles and microsomes, controls levels of various lipids, may regulate lipid synthesis, homologous to Pdr17p
YMR318C	ADH6	0.50	8.85E-55	significant	NADPH-dependent medium chain alcohol dehydrogenase with broad substrate specificity; member of the cinnamyl family of alcohol dehydrogenases; may be involved in fusel alcohol synthesis or in aldehyde tolerance
YMR290W-A	NA	0.49	0.0360389		Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data; overlaps 5' end of essential HAS1 gene which encodes an ATP-dependent RNA helicase

Table A-11 (Continued)

YOL136C	PFK27	0.47	3.27E-36	significant	6-phosphofructo-2-kinase, catalyzes synthesis of fructose-2,6-bisphosphate; inhibited by phosphoenolpyruvate and sn-glycerol 3-phosphate, expression induced by glucose and sucrose, transcriptional regulation involves protein kinase A
YLR413W	NA	0.47	1.14E-136	significant	Putative protein of unknown function; YLR413W is not an essential gene
YJL052C-A	NA	0.47	0.631527		Putative protein of unknown function, identified based on comparison to related yeast species
YML007C-A	NA	0.47	0.0152419		Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to mitochondria
YHR092C	HXT4	0.46	3.34E-130	significant	High-affinity glucose transporter of the major facilitator superfamily, expression is induced by low levels of glucose and repressed by high levels of glucose
YBL043W	ECM13	0.46	3.28E-296	significant	Non-essential protein of unknown function; induced by treatment with 8-methoxypsoralen and UVA irradiation
YNL140C	NA	0.45	0.0527496		Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps the verified gene THO2/YNL139C
YIR020C-B	NA	0.45	0.555705		Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps verified ORF MRS1

Table A-11 (Continued)

YBR085W	AAC3	0.43	0.00029421		Mitochondrial inner membrane ADP/ATP translocator, exchanges cytosolic ADP for mitochondrially synthesized ATP; expressed under anaerobic conditions; similar to Pet9p and Aac1p; has roles in maintenance of viability and in respiration
YBR190W	NA	0.43	0.110157		Dubious open reading frame unlikely to encode a protein, based on experimental and comparative sequence data; partially overlaps the verified ribosomal protein gene RPL21A/YBR191W
YPL272C	NA	0.43	6.06E-83	significant	Putative protein of unknown function; gene expression induced in response to ketoconazole; YPL272C is not an essential gene
YLL025W	PAU17	0.43	1.88E-83	significant	Protein of unknown function, member of the seripauperin multigene family encoded mainly in subtelomeric regions; YLL025W is not an essential gene
YGR152C	RSR1	0.43	8.79E-27	significant	GTP-binding protein of the ras superfamily required for bud site selection, morphological changes in response to mating pheromone, and efficient cell fusion; localized to the plasma membrane; significantly similar to mammalian Rap GTPases
YLR180W	SAM1	0.41	5.40E-71	significant	S-adenosylmethionine synthetase, catalyzes transfer of the adenosyl group of ATP to the sulfur atom of methionine; one of two differentially regulated isozymes (Sam1p and Sam2p)

Table A-11 (Continued)

YIL011W	TIR3	0.40	2.51E-83	significant	Cell wall mannoprotein of the Srp1p/Tip1p family of serine-alanine-rich proteins; expressed under anaerobic conditions and required for anaerobic growth
YOR381W-A	NA	0.38	5.73E-106	significant	Putative protein of unknown function; identified by fungal homology and RT-PCR
YCR020C	PET18	0.37	0.650283		Protein of unknown function, has weak similarity to proteins involved in thiamin metabolism; expression is induced in the absence of thiamin
YNL042W-B	NA	0.36	3.11E-130	significant	Putative protein of unknown function
YDR509W	NA	0.35	0.0111517		Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data
YBR196C-A	NA	0.35	0.379892		Putative protein of unknown function; identified by fungal homology and RT-PCR
YOL159C-A	NA	0.35	8.21E-93	significant	Putative protein of unknown function; identified by sequence comparison with hemiascomycetous yeast species
YNL277W-A	NA	0.34	3.95E-283	significant	Putative protein of unknown function
YOR309C	NA	0.32	1.46E-25	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps the verified gene NOP58

Table A-11 (Continued)

YDR316W-B	NA	0.32	3.66E-08	significant	Retrotransposon TYA Gag and TYB Pol genes; transcribed/translated as one unit; polyprotein is processed to make a nucleocapsid-like protein (Gag), reverse transcriptase (RT), protease (PR), and integrase (IN); similar to retroviral genes
YER011W	TIR1	0.31	4.10E-320	significant	Cell wall mannoprotein of the Srp1p/Tip1p family of serine-alanine-rich proteins; expression is downregulated at acidic pH and induced by cold shock and anaerobiosis; abundance is increased in cells cultured without shaking
YOR277C	NA	0.27	1.75E-05		Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; almost completely overlaps the verified gene CAF20
YPL257W-A	NA	0.25	1.49E-152	significant	Retrotransposon TYA Gag gene co-transcribed with TYB Pol; translated as TYA or TYA-TYB polyprotein; Gag is a nucleocapsid protein that is the structural constituent of virus-like particles (VLPs); similar to retroviral Gag
YPL257W-B	NA	0.25	5.14E-29	significant	Retrotransposon TYA Gag and TYB Pol genes; transcribed/translated as one unit; polyprotein is processed to make a nucleocapsid-like protein (Gag), reverse transcriptase (RT), protease (PR), and integrase (IN); similar to retroviral genes

Table A-11 (Continued)

YKL138C-A	HSK3	0.24	1.22E-320	significant	Essential subunit of the Dam1 complex (aka DASH complex), couples kinetochores to the force produced by MT depolymerization thereby aiding in chromosome segregation; is transferred to the kinetochore prior to mitosis
YFR010W-A	NA	0.24	3.159E-320	significant	Dubious ORF unlikely to encode a protein, based on available experimental and comparative sequence data; completely overlaps the uncharacterized gene YFR011C; identified by expression profiling and mass spectrometry
YBR233W	PBP2	0.23	0.0389844		RNA binding protein with similarity to mammalian heterogeneous nuclear RNP K protein, involved in the regulation of telomere position effect and telomere length
YKL068W	NUP100	0.22	2.21E-70	significant	Subunit of the nuclear pore complex (NPC) that is localized to both sides of the pore; contains a repetitive GLFG motif that interacts with mRNA export factor Mex67p and with karyopherin Kap95p; homologous to Nup116p
YJL127W-A	NA	0.22	0.0023989		Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data
YOR218C	NA	0.22	2.97E-05		Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; open reading frame overlaps the verified gene RFC1/YOR217W

Table A-11 (Continued)

YCL001W-B	NA	0.22	1.44E-270	significant	Putative protein of unknown function; YCL001W-B gene has similarity to DOM34 and is present in a region duplicated between chromosomes XIV and III
YML009C-A	NA	0.21	1.17E-67	significant	Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data
YPL038W-A	NA	0.21	1.93E-79	significant	Putative protein of unknown function; identified by fungal homology and RT-PCR
YHR199C-A	NBL1	0.21	2.069E-320	significant	Subunit of the conserved chromosomal passenger complex (CPC; Ipl1p-Sli15p-Bir1p-Nbl1p), which regulates mitotic chromosome segregation; not required for the kinase activity of the complex; mediates the interaction of Sli15p and Bir1p
YJR112W	NNF1	0.20	4.59E-67	significant	Essential component of the MIND kinetochore complex (Mtw1p Including Nnf1p-Nsl1p-Dsn1p) which joins kinetochore subunits contacting DNA to those contacting microtubules; required for accurate chromosome segregation
YIL156W-A	NA	0.20	2.42E-220	significant	Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data
YGL188C-A	NA	0.20	8.48E-53	significant	Putative protein of unknown function

Table A-11 (Continued)

YMR294W	JNM1	0.20	4.41E-13	significant	Component of the yeast dynactin complex, consisting of Nip100p, Jnm1p, and Arp1p; required for proper nuclear migration and spindle partitioning during mitotic anaphase B
YER145C-A	NA	0.20	1.170E-320	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; overlaps the verified ORF LSM5/YER146W
YER189W	NA	0.19	7.05E-10	significant	Putative protein of unknown function
YPR158W-B	NA	0.18	9.11E-33	significant	Retrotransposon TYA Gag and TYB Pol genes; transcribed/translated as one unit; polyprotein is processed to make a nucleocapsid-like protein (Gag), reverse transcriptase (RT), protease (PR), and integrase (IN); similar to retroviral genes
YFL012W-A	NA	0.18	4.13E-09	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; overlaps the verified gene IES1/YFL013C
YDR320C	SWA2	0.18	9.09E-50	significant	Auxilin-like protein involved in vesicular transport; clathrin-binding protein required for uncoating of clathrin-coated vesicles
YHR143W	DSE2	0.17	1		Daughter cell-specific secreted protein with similarity to glucanases, degrades cell wall from the daughter side causing daughter to separate from mother; expression is repressed by cAMP

Table A-11 (Continued)

YNL057W	NA	0.17	3.95E-07	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data
YER074W-A	YOS1	0.17	4.968E-320	significant	Integral membrane protein required for ER to Golgi transport; localized to the Golgi, the ER, and COPII vesicles; interacts with Yip1p and Yif1p
YBR058C	UBP14	0.17	3.83E-59	significant	Ubiquitin-specific protease that specifically disassembles unanchored ubiquitin chains; involved in fructose-1,6-bisphosphatase (Fbp1p) degradation; similar to human isopeptidase T
YLR390W	ECM19	0.17	4.340E-320	significant	Putative protein of unknown function; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies
YLR361C	DCR2	0.16	0.0650825		Phosphoesterase involved in downregulation of the unfolded protein response, at least in part via dephosphorylation of Ire1p; dosage-dependent positive regulator of the G1/S phase transition through control of the timing of START
YGR169C	PUS6	0.15	8.73E-05		tRNA:pseudouridine synthase, catalyzes the conversion of uridine to pseudouridine at position 31 in cytoplasmic and mitochondrial tRNAs; mutation of Asp168 to Ala abolishes enzyme activity; not essential for viability
YNL103W-A	NA	0.14	1.268E-320	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps the verified gene MET4/YNL104C

Table A-11 (Continued)

YDL247W	MPH2	0.14	1.12E-60	significant	Alpha-glucoside permease, transports maltose, maltotriose, alpha-methylglucoside, and turanose; identical to Mph3p; encoded in a subtelomeric position in a region likely to have undergone duplication
YDR182W-A	NA	0.14	9.525E-321	significant	Putative protein of unknown function; identified by fungal homology and RT-PCR
YHR007C-A	NA	0.13	5.609E-320	significant	Putative protein of unknown function; identified by expression profiling and mass spectrometry
YML045W-A	NA	0.13	1.22E-192	significant	Retrotransposon TYA Gag gene co-transcribed with TYB Pol; translated as TYA or TYA-TYB polyprotein; Gag is a nucleocapsid protein that is the structural constituent of virus-like particles (VLPs); similar to retroviral Gag
YHR072W	ERG7	0.13	3.35E-104	significant	Lanosterol synthase, an essential enzyme that catalyzes the cyclization of squalene 2,3-epoxide, a step in ergosterol biosynthesis
YEL076C-A	NA	0.13	2.18E-26	significant	Putative protein of unknown function
YHR039C	MSC7	0.12	4.43E-38	significant	Protein of unknown function, green fluorescent protein (GFP)-fusion protein localizes to the endoplasmic reticulum; msc7 mutants are defective in directing meiotic recombination events to homologous chromatids
YDL114W	NA	0.10	0.0903137		Putative protein of unknown function with similarity to acyl-carrier-protein reductases; YDL114W is not an essential gene

Table A-11 (Continued)

YGR240C-A	NA	0.10	8.463E-320	significant	Putative protein of unknown function; identified by fungal homology and RT-PCR
YIL102C	NA	0.10	3.01E-19	significant	Putative protein of unknown function
YIL082W	NA	0.10	2.47E-237	significant	Retrotransposon TYA Gag gene co-transcribed with TYB Pol; translated as TYA or TYA-TYB polyprotein; Gag is a nucleocapsid protein that is the structural constituent of virus-like particles (VLPs); similar to retroviral Gag
YPR158W-A	NA	0.10	7.35E-115	significant	Retrotransposon TYA Gag gene co-transcribed with TYB Pol; translated as TYA or TYA-TYB polyprotein; Gag is a nucleocapsid protein that is the structural constituent of virus-like particles (VLPs); similar to retroviral Gag
YOR376W	NA	0.10	1.82E-05		Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; YOR376W is not an essential gene.
YHR005C	GPA1	0.09	2.40E-75	significant	GTP-binding alpha subunit of the heterotrimeric G protein that couples to pheromone receptors; negatively regulates the mating pathway by sequestering G(beta)gamma and by triggering an adaptive response; activates Vps34p at the endosome
YLR437C-A	NA	0.09	1.45E-55	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps the verified ORF CAR2/YLR438W

Table A-11 (Continued)

YMR158W-B	NA	0.09	7.40E-223	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; overlaps the verified gene ATG16/YMR159C
YLR262C	YPT6	0.09	8.44E-139	significant	Rab family GTPase, Ras-like GTP binding protein involved in the secretory pathway, required for fusion of endosome-derived vesicles with the late Golgi, maturation of the vacuolar carboxypeptidase Y; has similarity to the human GTPase, Rab6
YER093C	TSC11	0.09	0.977882		Subunit of TORC2 (Tor2p-Lst8p-Avo1-Avo2-Tsc11p-Bit61p), a membrane-associated complex that regulates actin cytoskeletal dynamics during polarized growth and cell wall integrity; involved in sphingolipid metabolism; contains a RasGEFN domain
YER172C-A	NA	0.09	7.613E-321	significant	Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data
YCL005W	LDB16	0.09	1.88E-66	significant	Protein of unknown function; null mutants have decreased net negative cell surface charge; GFP-fusion protein expression is induced in response to the DNA-damaging agent MMS; native protein is detected in purified mitochondria
YAL019W-A	NA	0.09	1.62E-200	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data

Table A-11 (Continued)

YMR013C-A	NA	0.08	1.19E-96	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; completely overlaps the verified ORF SEC59/YML013C
YBL008W-A	NA	0.08	4.01E-158	significant	Putative protein of unknown function; identified by fungal homology and RT-PCR
YHR139C-A	NA	0.08	1.153E-319	significant	Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data
YER133W-A	NA	0.08	3.470E-320	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps uncharacterized gene YER134C.
YIL046W-A	NA	0.08	1.787E-320	significant	Putative protein of unknown function; identified by expression profiling and mass spectrometry
YOR072W-B	NA	0.08	2.83E-19	significant	Putative protein of unknown function; identified by expression profiling and mass spectrometry
YOR161C-C	NA	0.07	1.308E-320	significant	Identified by gene-trapping, microarray-based expression analysis, and genome-wide homology searching
YHR213W	NA	0.07	2.48E-12	significant	Possible pseudogene; has similarity to Flo1p, which is a lectin-like protein involved in flocculation
YLR154C	RNH203	0.07	8.98E-257	significant	Ribonuclease H2 subunit, required for RNase H2 activity; related to human AGS3 that causes Aicardi-Goutieres syndrome

Table A-11 (Continued)

YKL033W	TTI1	0.07	3.87E-93	significant	Putative protein of unknown function; subunit of the ASTRA complex which is part of the chromatin remodeling machinery; similar to S. pombe Tti1p; detected in highly purified mitochondria in high-throughput studies
YCR028C	FEN2	0.06	2.381E-320	significant	Plasma membrane H+-pantothenate symporter; confers sensitivity to the antifungal agent fenpropimorph
YHR001W	OSH7	0.05	1.34E-281	significant	Member of an oxysterol-binding protein family with seven members in S. cerevisiae; family members have overlapping, redundant functions in sterol metabolism and collectively perform a function essential for viability
YPR036W	VMA13	0.05	7.061E-320	significant	Subunit H of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; serves as an activator or a structural stabilizer of the V-ATPase
YOR192C	THI72	0.05	2.12E-16	significant	Transporter of thiamine or related compound; shares sequence similarity with Thi7p
YDL159W-A	NA	0.05	1.29E-243	significant	Putative protein of unknown function; identified by sequence comparison with hemiascomycetous yeast species
YMR001C-A	NA	0.05	9.05E-235	significant	Putative protein of unknown function
YLL006W-A	NA	0.05	1.81E-142	significant	Putative protein of unknown function; identified by fungal homology and RT-PCR

Table A-11 (Continued)

YML101C-A	NA	0.04	2.19E-157	significant	Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data
YGL041C	NA	0.04	1.73E-16	significant	Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data
YLR410W-A	NA	0.04	9.861E-321	significant	Retrotransposon TYA Gag gene co-transcribed with TYB Pol; translated as TYA or TYA-TYB polyprotein; Gag is a nucleocapsid protein that is the structural constituent of virus-like particles (VLPs); similar to retroviral Gag
YOL019W-A	NA	0.04	6.29E-197	significant	Identified by gene-trapping, microarray-based expression analysis, and genome-wide homology searching
YML054C-A	NA	0.04	5.787E-320	significant	Putative protein of unknown function
YDR316W-A	NA	0.04	1.517E-320	significant	Retrotransposon TYA Gag gene co-transcribed with TYB Pol; translated as TYA or TYA-TYB polyprotein; Gag is a nucleocapsid protein that is the structural constituent of virus-like particles (VLPs); similar to retroviral Gag
YJL062W	LAS21	0.04	5.72E-58	significant	Integral plasma membrane protein involved in the synthesis of the glycosylphosphatidylinositol (GPI) core structure; mutations affect cell wall integrity

Table A-11 (Continued)

YNL024C	NA	0.04	2.90E-81	significant	Putative protein of unknown function with seven beta-strand methyltransferase motif; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; YNL024C is not an essential gene
YBR191W-A	NA	0.04	4.178E-320	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data
YLR256W-A	NA	0.04	1.436E-320	significant	Retrotransposon TYA Gag gene co-transcribed with TYB Pol; translated as TYA or TYA-TYB polyprotein; Gag is a nucleocapsid protein that is the structural constituent of virus-like particles (VLPs); similar to retroviral Gag
YDR363W	ESC2	0.04	8.36E-297	significant	Sumo-like domain protein; prevents accumulation of toxic intermediates during replication-associated recombinational repair; roles in silencing, lifespan, chromatid cohesion and the intra-S-phase DNA damage checkpoint; RENi family member
YNL130C-A	DGR1	0.04	1.169E-320	significant	Protein of unknown function; dgr1 null mutant is resistant to 2-deoxy-D-glucose
YHR086W-A	NA	0.04	6.842E-321	significant	Putative protein of unknown function; identified by fungal homology and RT-PCR
YIR021W-A	NA	0.03	1.93E-284	significant	Putative protein of unknown function; identified by expression profiling and mass spectrometry

Table A-11 (Continued)

YPR169W-A	NA	0.03	1.269E-320	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps two other dubious ORFs: YPR170C and YPR170W-B
YOR316C-A	NA	0.03	1.294E-320	significant	Putative protein of unknown function; identified by fungal homology and RT-PCR
YPR108W-A	NA	0.03	2.837E-320	significant	Putative protein of unknown function; identified by fungal homology and RT-PCR
YJL127C	SPT10	0.03	5.36E-16	significant	Putative histone acetylase with a role in transcriptional silencing, sequence-specific activator of histone genes, binds specifically and cooperatively to pairs of UAS elements in core histone promoters, functions at or near the TATA box
YHR073W-A	NA	0.03	9.950E-321	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps verified ORF YHR073W; identified by expression profiling and mass spectrometry
YMR030W-A	NA	0.03	8.379E-321	significant	Putative protein of unknown function
YBL068W-A	NA	0.03	5.58E-185	significant	Dubious open reading frame unlikely to encode a protein; identified by fungal homology and RT-PCR
YKL165C-A	NA	0.03	1.029E-320	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data

Table A-11 (Continued)

YNR034W	SOL1	0.03	1.116E-319	significant	Protein with a possible role in tRNA export; shows similarity to 6-phosphogluconolactonase non-catalytic domains but does not exhibit this enzymatic activity; homologous to Sol2p, Sol3p, and Sol4p
YIL020C-A	NA	0.03	5.55E-154	significant	Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data
YBL039C-A	NA	0.02	7.326E-321	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; completely overlaps the verified ORF URA7; identified by expression profiling and mass spectrometry
YPL152W-A	NA	0.02	1.26E-237	significant	Identified by gene-trapping, microarray-based expression analysis, and genome-wide homology searching
YNL162W-A	NA	0.02	2.095E-320	significant	Putative protein of unknown function; identified by homology
YFR009W-A	NA	0.02	9.654E-321	significant	Dubious ORF unlikely to encode a protein, based on available experimental and comparative sequence data; completely overlaps the verified gene YFR009W; identified by expression profiling and mass spectrometry
YDR169C-A	NA	0.02	8.641E-321	significant	Putative protein of unknown function; identified by fungal homology and RT-PCR
YLR287C	NA	0.02	3.544E-320	significant	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; YLR287C is not an essential gene

Table A-11 (Continued)

YIL025C	NA	0.02	0.256556		Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data
YHR052W	CIC1	0.02	6.180E-320	significant	Essential protein that interacts with proteasome components and has a potential role in proteasome substrate specificity; also copurifies with 66S pre-ribosomal particles
YFR036W-A	NA	0.02	2.50E-101	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps the verified gene RSC8
YER084W-A	NA	0.02	2.60E-124	significant	Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data
YHR180W-A	NA	0.02	4.61E-141	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps dubious ORF YHR180C-B and long terminal repeat YHRCsigma3
YHR069C-A	NA	0.02	6.590E-321	significant	Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data
YDR119W	VBA4	0.02	3.190E-320	significant	Protein of unknown function with proposed role as a basic amino acid permease based on phylogeny; GFP-fusion protein localizes to vacuolar membrane; physical interaction with Atg27p suggests a possible role in autophagy; non-essential gene

Table A-11 (Continued)

YHL015W-A	NA	0.02	5.678E-320	significant	Putative protein of unknown function
YPL249C	GYP5	0.01	3.868E-320	significant	GTPase-activating protein (GAP) for yeast Rab family members, involved in ER to Golgi trafficking; exhibits GAP activity toward Ypt1p that is stimulated by Gyl1p, also acts on Sec4p; interacts with Gyl1p, Rvs161p and Rvs167p
YBL071C-B	NA	0.01	5.55E-119	significant	Putative protein of unknown function; identified by gene-trapping, microarray-based expression analysis, and genome-wide homology searching
YOR142W-A	NA	0.01	2.772E-320	significant	Retrotransposon TYA Gag gene co-transcribed with TYB Pol; translated as TYA or TYA-TYB polyprotein; Gag is a nucleocapsid protein that is the structural constituent of virus-like particles (VLPs); similar to retroviral Gag
YKL096W	CWP1	0.01	2.349E-319	significant	Cell wall mannoprotein that localizes specifically to birth scars of daughter cells, linked to a beta-1,3- and beta-1,6-glucan heteropolymer through a phosphodiester bond; required for propionic acid resistance
YNL097C-B	NA	0.01	7.361E-321	significant	Putative protein of unknown function
YDR034C-C	NA	0.01	4.240E-320	significant	Retrotransposon TYA Gag gene co-transcribed with TYB Pol; translated as TYA or TYA-TYB polyprotein; Gag is a nucleocapsid protein that is the structural constituent of virus-like particles (VLPs); similar to retroviral Gag
YGR146C-A	NA	0.01	1.330E-320	significant	Putative protein of unknown function

Table A-11 (Continued)

YMR315W-A	NA	0.01	2.594E-320	significant	Putative protein of unknown function
YGL006W-A	NA	0.01	3.071E-320	significant	Putative protein of unknown function; identified by SAGE
YCL048W	SPS22	0.01	1.076E-320	significant	Protein of unknown function, redundant with Sps2p for the organization of the beta-glucan layer of the spore wall
YDR098C-A	NA	0.01	9.574E-321	significant	Retrotransposon TYA Gag gene co-transcribed with TYB Pol; translated as TYA or TYA-TYB polyprotein; Gag is a nucleocapsid protein that is the structural constituent of virus-like particles (VLPs); similar to retroviral Gag
YCR024C	SLM5	0.01	1.894E-320	significant	Mitochondrial asparaginyl-tRNA synthetase
YFR031C	SMC2	0.01	5.116E-320	significant	Subunit of the 155ondensing complex; essential SMC chromosomal ATPase family member that forms a complex with Smc4p to form the active ATPase; Smc2p/Smc4p complex binds DNA; required for clustering of tRNA genes at the nucleolus
YDR525W	API2	0.01	7.618E-321	significant	Dubious open reading frame, unlikely to encode a protein; not conserved in closely related Saccharomyces species; 26% of ORF overlaps the dubious ORF YDR524C-A; insertion mutation in a cdc34-2 mutant background causes altered bud morphology
YOR008C-A	NA	0.01	1.517E-320	significant	Putative protein of unknown function, includes a potential transmembrane domain; deletion results in slightly lengthened telomeres

Table A-11 (Continued)

YBR196C-B	NA	0.01	1.728E-319	significant	Putative protein of unknown function; identified by expression profiling and mass spectrometry
YML100W-A	NA	0.01	6.821E-320	significant	Putative protein of unknown function; identified by gene-trapping, microarray-based expression analysis, and genome-wide homology searching
YBR121C-A	NA	0.01	3.025E-320	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; completely contianed within the verified gene GRS1; identified by expression profiling and mass spectrometry
YER148W-A	NA	0.01	1.092E-320	significant	Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data
YER091C-A	NA	0.01	3.697E-320	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data
YPR010C	RPA135	0.01	4.459E-320	significant	RNA polymerase I second largest subunit A135
YAL034C-B	NA	0.00	2.576E-320	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data
YOL052C	SPE2	0.00	1.078E-319	significant	S-adenosylmethionine decarboxylase, required for the biosynthesis of spermidine and spermine; cells lacking Spe2p require spermine or spermidine for growth in the presence of oxygen but not when grown anaerobically

Table A-11 (Continued)

YGR161C-C	NA	0.00	2.789E-320	significant	Retrotransposon TYA Gag gene co-transcribed with TYB Pol; translated as TYA or TYA-TYB polyprotein; Gag is a nucleocapsid protein that is the structural constituent of virus-like particles (VLPs); similar to retroviral Gag
YMR251W	GTO3	0.00	7.541E-320	significant	Omega class glutathione transferase; putative cytosolic localization
YER159C-A	NA	0.00	1.279E-320	significant	Retrotransposon TYA Gag gene co-transcribed with TYB Pol; translated as TYA or TYA-TYB polyprotein; Gag is a nucleocapsid protein that is the structural constituent of virus-like particles (VLPs); similar to retroviral Gag
YCR097W-A	NA	0.00	1.309E-320	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; identified by homology to a hemiascomycetous yeast protein
YPR160W-A	NA	0.00	1.202E-319	significant	Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data; identified by expression profiling and mass spectrometry
YNL067W-B	NA	0.00	3.172E-320	significant	Putative protein of unknown function
YKL145W-A	NA	0.00	6.507E-320	significant	Dubious open reading frame, unlikely to encode a protein; completely overlaps the verified essential gene RPT1; identified by expression profiling and mass spectrometry

Table A-11 (Continued)

YDR261C-C	NA	0.00	2.957E-319	significant	Retrotransposon TYA Gag gene co-transcribed with TYB Pol; in YDRCTY1-3 TYB is mutant and probably non-functional
YER044C-A	MEI4	0.00	3.288E-320	significant	Meiosis-specific protein involved in double-strand break formation during meiotic recombination; required for chromosome synapsis and production of viable spores
YMR230W-A	NA	0.00	2.408E-320	significant	Putative protein of unknown function
YHR193C-A	NA	0.00	4.482E-320	significant	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; completely overlaps verified ORF MDM31
YFR032C	RRT5	0.00	1.578E-320	significant	Putative protein of unknown function; non-essential gene identified in a screen for mutants with increased levels of rDNA transcription; expressed at high levels during sporulation
YMR175W-A	NA	0.00	1.125E-318	significant	Putative protein of unknown function
YEL009C-A	NA	0.00	2.640E-319	significant	Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data
YDR524C-A	NA	0.00	4.991E-320	significant	Dubious open reading frame unlikely to encode a functional protein; identified by gene-trapping, microarray-based expression analysis, and genome-wide homology searching

Table A-12: Genes classified under GO category of sporulation, with significant Fisher's test p-value

SGD ID	ORF	RPKM[A]	p-value[B]
YLR308W*	CDA2	4.463236496	5.01E-11
YDR403W*	DIT1	1.673611999	6.07E-12
YER180C*	ISC10	2.961430546	2.15E-140
YER133W*	GLC7	6.87583955	2.03E-320
YPL130W*	SPO19	4.402923538	8.54E-11
YHR139C*	SPS100	18.3065963	7.18E-320
YOR338W	NA	1.065042244	0.112179

[A] Ratio of Normalized RPKM values of wild type hub and rim

[B] Fishers test P-value

* p-value Statistically significant

Vita

Neha Sarode was born in Mumbai, India. She completed her bachelors in Microbiology from Abasaheb Garware College, Pune followed by Masters in Bioinformatics from University of Pune, Maharashtra. She was accepted to the Ph.D. program in Genome Science and Technology at the University of Tennessee, Knoxville. She will receive her Doctor of Philosophy degree in Life Science.