A peer-reviewed version of this preprint was published in PeerJ on 28 September 2018.

Mancuso R, Chinnici J, Tsou C, Busarajan S, Munnangi R, Maddi A. 2018. Functions of *Candida albicans* cell wall glycosidases *Dfg5p* and *Dcw1p* in biofilm formation and HOG MAPK pathway. PeerJ 6:e5685 https://doi.org/10.7717/peerj.5685

Candida albicans cell wall glycosidases *DFG5* and *DCW1* are required for biofilm formation and Hog-1 signaling

Ryan Mancuso [1], **Jennifer Chinnici** [1], **Charlene Tsou** [1], **Sujay Busarajan** [1], **Abhiram Maddi** Corresp. [1]

[1] Periodontics & Endodontics, Oral Biology, State University of New York at Buffalo, Buffalo, New York, United States of America

Corresponding Author: Abhiram Maddi
Email address: amaddi@buffalo.edu

Background. *Candida albicans* is a commensal fungus that inhabits the oral mucosal surface and causes oral and systemic candidiasis. Oral candidiasis most commonly occurs in patients with AIDS, denture wearers and newborn children. Systemic candidiasis occurs mainly in immunocompromised patients and patients admitted to hospitals for prolonged periods. The *C. albicans* homologous genes, *DFG5* and *DCW1*, encode for two closely related cell wall proteins with putative glycosyltransferase enzyme activity and C-terminal GPI-anchors. Past studies have shown that individual *DFG5* and *DCW1* mutations are viable but simultaneous deletion of *DFG5* and *DCW1* in *C. albicans* results in lethality. However, the exact functions of these cell wall based enzymes, which represent ideal drug targets, are not understood.

Methods. *C. albicans DFG5/DCW1* heterologous and conditional double mutant strains, ES1 and ES195 respectively, were assessed for growth and biofilm formation in comparison to wild type and parental strains. Cell wall, osmotic and heat stress susceptibility of the mutant and control strains was assessed using agar spotting assays. Western Blot analysis of mutant strains and control strains was performed to assess Hog-1 phosphorylation status.

Results. Growth in planktonic cultures and biofilm formation was found to be affected in the *DFG5/DCW1* double mutants as compared to control strains. The mutant strains were also less resistant to cell wall, osmotic and heat stresses as compared to control strains. Hog-1 phosphorylation was affected in the mutant strains.

Conclusions. These data indicate that *Candida albicans DFG5* and *DCW1* play critical roles in biofilm formation and Hog-1 signaling pathway.

Title:

Candida albicans cell wall glycosidases *DFG5* and *DCW1* are required for biofilm formation and Hog-1 signaling

Authors:

Ryan Mancuso[o], Jennifer Chinnici[o], Charlene Tsou[o], Sujay Busarajan[o], Abhiram Maddi[o¶*]

Periodontics & Endodontics, School of Dental Medicine, SUNY University at Buffalo.

¶Oral Biology, School of Dental Medicine, SUNY University at Buffalo.

Corresponding Author:

Abhiram Maddi DDS, PhD.
Assistant Professor
Department of Periodontics & Endodontics
School of Dental Medicine
250B Squire Hall
University at Buffalo, Buffalo, NY
Email: amaddi@buffalo.edu
Phone: 716-8296182
Fax: 716-8296840

Abstract:

Background. *Candida albicans* is a commensal fungus that inhabits the oral mucosal surface and causes oral and systemic candidiasis. Oral candidiasis most commonly occurs in patients with AIDS, denture wearers and newborn children. Systemic candidiasis occurs mainly in immunocompromised patients and patients admitted to hospitals for prolonged periods. The *C. albicans* homologous genes, *DFG5* and *DCW1*, encode for two closely related cell wall proteins with putative glycosyltransferase enzyme activity and C-terminal GPI-anchors. Past studies have shown that individual *DF5* and *DCW1* mutations are viable but simultaneous deletion of *DFG5* and *DCW1* in *C. albicans* results in lethality. However, the exact functions of these cell wall based enzymes, which represent ideal drug targets, are not understood.

Methods. *C. albicans DFG5/DCW1* heterologous and conditional double mutant strains, ES1 and ES195 respectively, were assessed for growth and biofilm formation in camparison to wild type and parental strains. Cell wall, osmotic and heat stress susceptibility of the mutant and control strains was assessed using agar spotting assays. Western Blot analysis of mutant strains and control strains was performed to assess Hog-1 phosphorylation status.

Results. Growth in planktonic cultures and biofilm formation was found to be affected in the *DFG5/DCW1* double mutants as compared to control strains. The mutant strains were also less resistant to cell wall, osmotic and heat stresses as compared to control strains. Hog-1 phosphorylation was affected in the mutant strains.

Conclusions. These data indicate that *Candida albicans DFG5* and *DCW1* play critical roles in biofilm formation and Hog-1 signaling pathway.

INTRODUCTION:

A great majority of all fungal infections in humans are caused by *Candida albicans*, a dimorphic fungus that occurs in yeast and hyphae forms (Kim & Sudbery, 2011). Normally *C.*

albicans exists as a commensal in the human body and causes the disease candidiasis under certain conditions. The conditions that result in candidiasis include prolonged antibiotic treatment, immunosuppressive conditions that arise due to genetic disorders or drug therapy, HIV infection, medical and dental prostheses and dry mouth (Bondaryk, Kurzatkowski & Staniszewska, 2013; Cassone & Cauda, 2012). Oral mucosal candidiasis involving the mouth occurs in millions of people worldwide. Vaginal candidiasis occurs in 75% of the women at least once in their life time with a significant rate of recurrence. Candidemia resulting from the invasion of *C. albicans* into the bloodstream in human patients occurs in patients in intensive care. Candidemia has an alarming mortality rate of 35- 67% and is associated with extremely high treatment costs (Wilson *et al.,* 2002; Kett *et al.,* 2011). In most recent times, the cost of hospital treatment for a single episode of candidemia is estimated around $25,000-55,000 (Kett *et al.,* 2011). Candidemia leading to disseminated candidiasis also occurs in preterm neonates with an incidence rate of up to 10% resulting in significant impairment of neurodevelopment (Nguyen *et al.,* 2012). This signifies the impact of candida infections on public health and the economic burden on federal government.

Microbial biofilms have emerged as the major cause of pathogenic diseases in humans. *C. albicans* is the major fungal biofilm-former that causes medical device associated infections with extremely high mortality rates (approximately 40%) (Fox & Nobile, 2012). Also *C. albicans* biofilms are resistant to the currently available standard antifungal drugs. These biofilms function as physical barriers for protection from the drugs. Moreover cells within these biofilms become intrinsically resistant to drugs resulting from altered metabolic states and upregulation of efflux drug pumps (Fox & Nobile, 2012).

Unlike bacteria which are prokaryotes, fungi are eukaryotes and have a significant structural similarity to human cells. For this reason targeting the fungal cell has been relatively difficult as it results in toxicity to host cells. Several antifungal drugs, that are currently available to treat

mucosal and disseminated candidiasis, cause adverse toxicity in human patients. The main reason for high morbidity and significant hospital associated costs of treating systemic candidiasis is the rapid development of antifungal drug resistance among *Candida* species (Bondaryk, Kurzatkowski & Staniszewska, 2013; Fothergill *et al.,* 2014). Thus there is an urgent need for novel antifungal drugs and therapeutics which can overcome the frustrating problem of antifungal drug resistance.

Past studies in the yeast *Saccharomyces cerevisiae* have shown that *DFG5* and *DCW1* double deletion is lethal indicating that the cell wall proteins/enzymes encoded by these genes have a critical and redundant role in cell functions (Kitagaki *et al.,* 2002; Kitagaki, Ito & Shimoi, 2004). Similarly studies in *C. albicans* have shown that simultaneous deletion of the *DFG5* and *DCW1* genes is lethal indicating that these cell wall enzymes are critical for normal growth and survival (Spreghini *et al.,* 2003). Based on the observation that the expression of *HWP1*, a well-known cell wall gene expressed only in hyphae, was dependent upon the presence of Dfg5p, it has been thought to be involved in signal transduction mechanisms tied into the cell wall integrity pathway (Spreghini *et al.,* 2003). We have showed that *C. albicans* Dfg5p and Dcw1p are involved in cell wall protein cross-linking within the cell wall (Ao *et al.,* 2015). In a recent study in the yeast *Saccharomyces cerevisiae* it was observed that Hog-1 and Slt2 signaling pathways are affected in the *DFG5* deletion background (Nasution *et al.,* 2015). However, the role *DFG5* and *DCW1* in biofilm formation and Hog-1 signaling mechanisms has not been studied in the pathogenic fungus *C. albicans*.

As the single mutants were observed to be viable, heterologous and conditional mutants of *DFG5* and *DCW1* were developed in *C. albicans* to study the functions of these genes (Spreghini *et al.,* 2003). These include the ectopic *pMET3-DFG5* modulated *dfg5Δ/dcw1Δ* double mutant (ES195) and *dfg5Δ/DCW1::dcw1 (*ES1) mutant strains (Spreghini *et al.,* 2003). To our

knowledge this is the first study to determine the role of *Candida albicans DFG5* and *DCW1* in biofilm formation as well as in Hog-1 signaling mechanisms using these mutant strains.

MATERIALS & METHODS:

Strains and Growth Conditions

For WT, SC5314 strain of *Candida albicans* was used. In addition, parental WT strains BWP17 (URA- or uridine auxotroph) and reintegrated strain DAY185 (URA+) were used in some experiments. These strains along with the test strains, ES1 and ES195, were provided as a kind gift from Dr. Aaron Mitchell (Carnegie Mellon University, Pittsburgh, PA) and have also been deposited at the fungal genetics stock center (FGSC). The ES1 and ES195 strains have been previously described previously (Spreghini *et al.,* 2003). ES1 has a *dfg5Δ/dfg5Δ*::*dcw1Δ/DCW1* genotype. ES195 has a *dfg5Δ/dfg5Δ*::*dcw1Δ/dcw1Δ* genotype, but also contains an ectopic copy of the *DFG5* coding region with the upstream *MET3* regulatory elements. ES195 is viable when grown in the absence of methionine and cysteine (when the chimeric copy of *DFG5* is expressed), but stops growing when the chimeric gene is turned off by adding methionine and cysteine to the medium. The strains were cultured in Yeast Nitrogen Base (YNB) medium with ammonium sulfate and 2% glucose adjusted to pH 7. Synthetic complete supplement mixture (MP Biomedicals) was added as aminoacid supplement to YNB. 5mM Methionine and 2 mM cysteine were added to the medium for ES195 strain for conditional repression (85%) of the chimeric *MET3::DFG5* gene to generate a Dfg5p-deficient condition.

Candida albicans Biofilm Formation

Overnight cultures of WT, ES1, and ES195 strains were inoculated from frozen stocks in YNB and cultured overnight at 30°C with shaking at 225 RPM. The cell counts for the overnight

cultures were determined using a hemocytometer. The cells were transferred to fresh YNB (pH 7) supplemented with 20% FBS for a final concentration of $1x10^6$ cells/mL. To the tubes containing ES195, methionine and cysteine were added for a final concentration of 5mM and 2 mM respectively, to shut off the ectopic copy of *DFG5*. The cultures were transferred to 6 well polystyrene culture plates (Falcon, Corning, NY), 2ml per well, and incubated at 37°C statically for 24 hrs. After incubation, the media over the resulting biofilms was carefully removed and the biofilms were washed once with 1xPBS. They were then removed with a pipette and additional 1x PBS to pre-weighed microfuge tubes as described before (Li *et al.,* 2013). The samples were centrifuged to pellet the cells and remove most of the liquid to facilitate drying. The sample tubes were opened and placed in a desiccator jar with anhydrous calcium chloride used as the desiccant. Dry cell mass was quantified after ~3 days using and analytical weighing scale (Mettler Toledo).

Dual Species Biofilm Formation with Streptococcus gordonii

Overnight cultures of wild type, ES1, and ES195 *C. albicans* and Challis CH1 *S. gordonii* strains were inoculated from frozen stocks in a mixture of 50%TSBY and 50%YNB (TSBY/YNB) and grown in their respective growth conditions overnight. *C. albicans* overnight cultures were grown at 30°C with shaking at 225 rpm and the *S. gordonii* overnight culture was grown at 37°C statically in a candle jar. The concentrations of the *C. albicans* overnight cultures were determined via hemocytometer and the concentration of *S. gordonii* overnight culture was determined by measuring the optical density at 600nm (OD_{600}). The overnight cultures were then used to inoculate several different cultures with the starting concentration of $1x10^6$ cells/ml for each organism in a 6 ml total volume of TSBY/YNB supplemented with 20% FBS. Three dual species cultures were used: wild type *C. albicans* + *S. gordonii*, ES1 *C. albicans* + *S. gordonii*, and ES195 *C. albicans* + *S. gordonii*. A *S. gordonii* single culture was also used. Methionine and

cysteine were added to the ES195 *C. albicans* + *S. gordonii* culture, for a final concentration of 5mM and 2mM respectively, to turn off the ectopic copy of *DFG5*. All cultures were incubated for an hour at 37°C with shaking at 225 rpm. Each culture was transferred to uncoated 6-well polystyrene culture plates (2ml/well) and incubated statically for 24h at 37° C. After incubation, the media was carefully removed and the biofilms were washed once with 1x PBS (phosphate buffered saline). The biofilms were removed with additional 1xPBS and transferred to pre-weighed microfuge tubes. The samples were centrifuged to pellet the cells and remove most of the liquid to facilitate drying. The sample tubes were opened and placed in a desiccator jar with anhydrous calcium chloride used as the desiccant. Dry cell mass was determined after 3 days using an analytical weighing scale.

Cell Wall Stress Tests

To determine if the mutants were affected in the synthesis of the cell wall, growth tests in the presence of cell wall stress reagents were carried out as described previously [29]. Overnight cultures of WT, ES1, and ES195 strains were inoculated from frozen stocks in YNB and cultured overnight at 30° C with shaking at 225 RPM. The cell counts for the overnight cultures were determined using a hemocytometer. The cells were transferred to fresh YNB (pH 7) for a final concentration of $1x10^6$ cells/mL. To the tubes containing ES195, methionine and cysteine were added, for a final concentration of 5mM and 2 mM respectively, to shut off the ectopic copy of *DFG5*. Cultures were incubated at 30°C for 1 hour with shaking at 225 rpm. A 1:10 dilution series was made with each culture. 5 ul each of the undiluted, 1:10, 1:100, and 1:1000 dilution samples were spotted onto YNB pH7 plates containing one of the following cell wall stress agents: Calcofluor White (20 µg/ml), Caspofungin (0.25 µg/ml), Congo Red (1 µg/ml), 100 µg/ml SDS (100 µg/ml), or Sorbitol (1 M). Concentrations of stress agents were based on MIC values as described previously (Ao *et al.,* 2015; Nikolaou *et al.,* 2009; Heilmann *et al.,* 2013). In

addition, just for the 1M sorbitol experiments WT (SC5314), WT (BWP13), ES1 and ES195 were plated in YNB with 5mM methionine and 2 mM cysteine. Plates were incubated at 30°C for 48 hours. Spotting assays were also done under heat stress at 39°C. Images were taken at 24 and 48 hours. The ability of the ES1 and ES195 strains to grow in the presence of these cell wall stress agents was observed and compared with the growth of the WT strain.

Analysis of Hog-1 MAPK phosphorylation

Overnight cultures of WT (SC5314), WT parental (BWP17), ES1, and ES195 strains were inoculated from frozen stocks in YNB with complete supplement mixture (CSM) and cultured overnight at 30°C with shaking at 225 RPM. The cell counts for the overnight cultures were determined using a hemocytometer. The overnight cultures were added to fresh YNB for a total volume of 100ml for each strain. The cells were allowed to grow to about mid-log phase (around 5 x 10^7 cells/ml). To induce phosphorylation of Hog-1 and assess levels of activated protein, NaCl was added to each culture for a final concentration of 0.4M and allowed to incubate with shaking for 5 min. Control cultures (no NaCl added) were used to assess total Hog-1 levels. Methionine (5mM) and Cysteine (2mM) were added to ES195 to shut off the ectopic copy of *DFG5*. The cells were harvested by centrifugation at 5860 x g for 10 minutes and washed twice with cold 1xPBS. Cell pellets, approximately 300 ul, were transferred to microfuge tubes in order to pulverize them and prepare cell extracts. Two volumes of additional 1xPBS was added to each sample (600 ul) along with 1 volume of 0.5 mm zirconium oxide beads (300 ul) (Next Advance). Cells were then pulverized in a Bullet Blender Storm 24 (Next Advance) following the recommended settings for *C. albicans* (Speed 10 for 3 minutes). The samples were placed on ice for 5 minutes and subjected to one more cycle in the Bullet Blender. After pulverizing, the samples were centrifuged for 2 minutes at 12,000 x g. The supernatants, containing the cell extracts, were transferred to new tubes and subjected to a DC Protein Assay (BioRad) to

185 determine protein concentration. The cell extract protein (10µg) of non-induced cells were
186 subjected to SDS PAGE gel electrophoresis for protein separation. The protein gels were the
187 subjected to Western transfer to PVDF and then Western Blot analysis using anti-pHog-1
188 antibody (Cell Signaling Technology, Danvers, MA) for measuring phosphorylated Hog-1 was
189 performed as described previously (Adhikari & Cullen, 2014; Cullen, 2015). Anti-Rabbit HRP
190 conjugated secondary antibody was used to detect the primary antibody. The blot was then
191 stripped and then reprobed using the anti-Hog-1 antibody (Santacruz Biotechnology, Santacruz,
192 CA) for detecting total Hog-1. An ECL Clarity Kit (BioRad) was used with Image Lab 5.2.1
193 software and the Gel Doc XR+ (BioRad) to image the western blots and determine band
194 intensities for later analysis. Band intensities for phosphorylated Hog-1 in ES1 and ES195 and
195 WT(SC5314) strains were compared to that of the total Hog-1 for triplicate experiments on the
196 same blot using ECL analysis. Anti-pHog-1 blot for WT (SC5314) and WT parental (BWP13)
197 was run to determine if phosphorylation of Hog-1 was affected in the parental strain. Anti-
198 G6PDH (glucose-6-phosphate dehydrogenase) antibody (Sigma) was used for checking the levels
199 of the house keeping protein.

200 ***Statistical Analysis***

201 Statistical analysis was performed using Microsoft Excel on a Windows operating system. Each
202 experimental group had a triplicate of samples. Experimental groups were compared using
203 Student's t-test for two samples assuming equal variances. A p-Value of <0.01 was considered
204 significant.

205 **RESULTS:**

206 ***DFG5* and *DCW1* heterologous mutations lead to variable growth defects**

A 24 hour culture of the wild type and mutant strains revealed that the mutant strains ES1(*dfg5Δ/dfg5Δ*::*dcw1Δ/DCW1*) had a slight growth defect while ES195 (*dfg5Δ/dfg5Δ*::*dcw1Δ/dcw1Δ*/*MET3*::*DFG5*) with methionine and cysteine had a major growth defect. ES195 without methionine and cysteine grew normally and comparably to the wild type strains. This indicates that there is a variable rescue of growth when these genes are deleted with one copy of the other gene present. This data indicates that *DFG5* may be required for growth more than *DCW1* despite their redundant functions. On the other hand, the conditional repression (85%) of *DFG5* in ES195 leads to a severe defect, confirming that the simultaneous deletion of both *DFG5* and *DCW1* is lethal. This data also shows that the genetic background of the parental wild type strain (BWP17) and the reintegrated strain (DAY185) does not affect their growth rate in comparison to the wild type strain (SC5314) (**Figure 1**).

***DFG5* and *DCW1* deletion affects *Candida albicans* monospecies biofilm formation**

Both mutant strains, ES1 and ES195 produce significantly less biofilm, when cultured as a monospecies of *Candida albicans*, as compared to WT (**Figure 2**). The growth of the biofilms for either mutants is not statistically different from one another, however it is statistically different as compared to WT. In general, the mutants do not grow as well as WT upon exposure to cell wall stress agents or heat shock. ES195 typically has a more severe phenotype than ES1. This data suggests that *DFG5* and *DCW1* deletion affects biofilm formation of *Candida albicans*.

DFG5* and *DCW1* deletion leads to significantly reduced dual species biofilm formation with *Streptococcus gordonii

As part of the complex oral plaque biofilm, *Candida albicans* interacts with other microorganisms including bacteria. These bacteria include *Streptococci*. In this experiment we have utilized *Streptococcus gordonii* for culturing dual species biofilms with *Candida albicans*

mutant strains ES1, ES195 as well as WT. Interestingly, ES1 and ES195 strains made significantly reduced dual species biofilms with *Streptococcus gordonii* as compared to WT (**Figure 3**). This shows that *Candida albicans DFG5* and *DCW1* play a significant role in dual species biofilms with bacteria.

***DFG5* and *DCW1* deletion mutants are affected by osmotic stress**

The ability to withstand osmotic stress is critical for cell survival. Osmotic stress response is regulated by canonical Hog-1 pathway. The response to osmotic stress can be tested by growing cells in the presence of 1M sorbitol. In this experiment we compared the growth of WT, WT parental (BWP17), ES1 and ES195 in the presence or absence of 1M sorbitol. All strains were also incubated with 5mM methionine and 2mM cysteine for conditional repression of DFG5 in the ES195 mutant and also determine if the addition of these aminoacids caused any differences in growth between the control and mutant strains. After 48 h culture on YNB agar plates, WT, BWP17 and ES1 strains appeared to grow normally in the presence or absence of 1M sorbitol, indicating that they were not affected by osmotic stress (**Figure 4**). However, the ES195 strain was unable to grow in the presence of sorbitol while it grew slowly under control conditions. This indicates that the simultaneous deletion of *DFG5* and *DCW1* affects the ability to overcome osmotic stress. This experiment also clearly shows that the parental WT strain BWP17 grows similar to WT (SC5314) strain and thus is not affected by its parental genetic background. Hence for further spotting assays we utilized only the WT (SC5314) strain.

***DFG5* and *DCW1* deletion mutants are affected by cell wall stress and temperature stress at pH7**

The oral cavity has a pH of 7 generally. In this experiment we performed spotting assays to test the ability of ES1 and ES195 mutant strains in withstanding cell wall stress and temperature

stress at pH7. The mutant strains were severely affected by various cell wall stress agents including calcoflour white, caspofungin, congo red and SDS (**Figure 5A**). The mutant strains were also affected by temperature stress when cultured at 39ºC (**Figure 5B**). These data show that *Candida albicans DFG5* and *DCW1* play a significant role in cell wall integrity and also aid in withstanding temperature stress.

***DFG5 and DCW1* mutations result in shut-off of Hog-1 MAPK phosphorylation**

We first wanted to examine if the parental wild type strains had any defect in Hog-1 phosphorylation to rule out the possibility of side effects resulting from their genetic background. Hog-1 phosphorylation analysis of parental wild type (BWP17), reintegrated strain (DAY185) and WT (SC5314) using Western Blot and ECL analyses revealed that Hog-1 phosphorylation was not affected in these strains (**Figure 6A & 6B; Supplemental Figure S1**). This indicates that the genetic manipulation of the parental strains did not affect Hog-1 signaling pathway. Here we were only concerned about Hog-1 phosphorylation hence we did not check the whole Hog-1 levels for the parental and wild type strains. Western Blot analysis and ECL analysis was then performed for the WT (SC5314), ES1 and ES195 strains for determining relative intensity of phosphorylated-Hog-1 versus whole Hog-1. This experiment revealed that there was reduced or defective Hog-1 phosphorylation in ES1 and ES195 strains as compared to WT (**Figure 7A & 7B; Supplemental Figures S2 & S3**). These results indicate that *DFG5* and *DCW1* are required for Hog-1 MAPK phosphorylation and thus affect the Hog-1 signaling pathway.

DISCUSSION:

The only organelle that is unique to the fungal cell as compared to the human host is the cell wall. The cell wall has several critical functions. It deals with environmental stresses like changes in osmotic pressure, pH and temperature to protect cell integrity (Chaffin, 2008; Free,

2013). It is now understood that several cell wall associated proteins play a role in cell signaling pathways in response to stress. A transcriptional upregulation of genes involved in maintaining cell wall integrity occurs in response to signal transduction (Chaffin, 2008; Dichtl, Samantaray & Wagener, 2016). MAPK signaling pathways are among the various signaling pathways that regulate cell wall biogenesis and integrity. Most importantly, the cell wall plays a critical role in disease pathogenesis as well as in protecting the pathogen from the host immune system (Cullen & Edgerton, 2016).

The cell wall is a complex structure made of carbohydrates and cell wall mannoproteins. The carbohydrates form an extracellular matrix in which the mannoproteins are cross-linked. The cell wall proteins play important roles in cell physiology as well as in disease pathogenesis. The extracellular matrix is also needed for biofilm formation. Biofilm formation is an important virulence factor for pathogenic fungi in causing local and systemic disease (Costa-Orlandi *et al.,* 2017). In *C. albicans*, various genes are involved in adhesion, extracellular matrix formation, quorum sensing and morphogenesis of biofilms (Fox & Nobile, 2012; Finkel & Mitchell, 2011). Moreover, in *C. albicans* the yeast and hyphae forms have been found to have unique roles in biofilm formation (Finkel & Mitchell, 2011).

The cell wall enzymes Dfg5 and Dcw1 are predicted mannosidases/glycosyl hydrolases (*gh-76* family). They have been implicated in the cross-linking of cell wall proteins in the cell wall. There are three known ways of cross-linking cell wall proteins in the cell wall matrix of *C. albicans*: 1) by a possible Dfg5p/Dcw1p-mediated cross-linking of N-linked outer chain mannan to the cell wall glucans described in this application, 2) by cross-linking the GPI anchor to the cell wall glucans through an alkaline-sensitive linkage, and 3) PIR (proteins with internal repeats) can be cross-linked into the cell walls by a linkage between a glutamine residue in the PIR repeat and β-1,3-glucan (Free, 2013; Xie & Lipke, 2010). This redundancy in cell wall protein cross-linking is thought to help ensure the formation of a functional cell wall and

maintain its integrity. However, their exact enzymatic functions of Dfg5 and Dcw1 have not been demonstrated experimentally.

In this study we examined the functions of Dfg5 and Dcw1 in biofilm formation and Hog-1 signaling by using the double mutant strains, ES1 and ES195. Using the above *C. albicans* mutant strains we have previously confirmed that the Dfg5p and Dcw1p mannanases (cross-linking enzymes) function in cell wall biogenesis in *C. albicans* (Ao *et al.,* 2015). We have also showed previously that the *DFG5/DCW1* conditional mutants have a cell separation growth phenotype. The mutants are hypersensitive to cell wall stress reagents and to treatment with lyticase (β-glucanase), indicating that the mutant cell walls are weaker than wild type cell walls (Ao *et al.,* 2015). The *DFG5* and *DCW1* mutants produced cell walls containing reduced levels of cell wall proteins and released cell wall proteins into the growth medium. A carbohydrate analysis of the *DFG5/DCW1* mutant cell walls showed that the mannose levels were significantly reduced, indicating a reduced incorporation of cell wall proteins in the wall (Ao *et al.,* 2015). These characteristics are similar to our observations of the *Neurospora crassa dfg5/dcw1* double mutants, and demonstrate that *DFG5* and *DCW1* in *C. albicans* function in cross-linking cell wall proteins into the cell wall. However, the substrates of these enzymes and their exact mechanisms of cross-linking cell wall proteins into the cell wall are yet to be determined. But structural studies of the α-mannanases (GH-76 enzymes), have revealed that these enzymes are mainly involved in catalysis and could be potential targets for anti-fungal drug development (Thompson *et al.,* 2015).

We hypothesized that the lethality of the Δ*DFG5/ΔDCW1* in yeast and *Candida albicans* may be a manifestation of additional roles that Dfg5p and Dcw1p play in signal transduction pathways. This has been demonstrated especially in the yeast, *Saccharomyces cerevisiae*. Recent studies have shown that the Hog1 and Slt2 signaling pathways are activated in the Δ*dfg5* mutant of *Saccharomyces cerevisiae* (Nasution *et al.,* 2015). It was also demonstrated in the same study

that the expression of genes related to these signaling pathways was altered in the Δ*dfg5* mutant of *Saccharomyces cerevisiae*, by using RNA sequencing analysis (Nasution *et al.,* 2015). Our preliminary studies have focused on *C. albicans DFG5* and *DCW1* functions at pH 7 which is commonly present in the oral cavity. Our data indicates that heterologous mutations of *DFG5* and *DCW1* result in significantly reduced germ tube formation (**Figure 4**). Also these mutants have defective growth patterns under various cell wall stress conditions but exhibit elevated heat tolerance, data which is concurrent with studies in yeast (**Figure 5A**). However, in contrast to the yeast study described above, our experiments in *C. albicans* indicate that simultaneous mutations of *DFG5* and *DCW1* result in inactivation of Hog-1 phosphorylation (**Figure 7A & 7B**).

Little is known about signaling activity by GPI-anchored cell wall proteins in fungi. But, studies of human neutrophils have indicated that GPI-anchored proteins can be directly involved in Ca^{2+} signaling (Hiscox *et al.,* 2002). Other examples of signaling via GPI-anchored proteins have been shown to occur in the activation of resting B-lymphocytes by GPI-anchored mIgD receptors (Chaturvedi *et al.,* 2002). In neutrophils, the FcγRIIIB receptor is a GPI-anchored protein, whose cross-linking to the transmembrane FcγRIIA results in the activation of downstream MAPK signaling pathways (Futosi *et al.,* 2013). Signaling by GPI-anchored proteins is believed to be due to their close association with lipid rafts (Lakhan, Subharanjak & De, 2009). Moreover, *C. albicans* defective in MAPK signaling is avirulent in a mouse model of candidiasis (Guhad *et al.,* 1998).

SUMMARY & CONCLUSIONS:

We conclude that Dfg5 and Dcw1 function in cell wall biogenesis as well as in activating the Hog-1 MAPK pathway as depicted in our working model (**Figure 8**). Located in the cell wall and having enzymatic functions, Dfg5 and Dcw1 are readily accessible to therapeutic agents and are ideal targets for novel antifungal drugs. Thus it is not only necessary but novel to investigate the

signaling functions of these GPI-anchored cell wall enzymes. Our future studies will focus on the identifying the components of the Dfg5p/Dcw1p dependent Hog-1 signaling pathway.

ACKNOWLEDGEMENTS:

We would like to thank Dr. Paul Cullen, Associate Professor, Biological Sciences, University at Buffalo, for kindly providing us the antibodies against Hog-1 and pHog-1. We would also like to thank Mr. Jason Chwirut, University at Buffalo for preparing the figures in this manuscript.

REFERENCES:

Adhikari H, Cullen PJ. 2014. Metabolic respiration induces AMPK- and Ire1p-dependent activation of the p38-Type HOG MAPK pathway. *PLoS Genet.* 10(10):e1004734. DOI: 10.1371/journal.

Ao J, Chinnici JL, Maddi A, Free SJ. 2015. The N-linked outer chain mannans and the dfg5p and dcw1p endo-α-1,6-mannanases are needed for incorporation of *Candida albicans* glycoproteins into the cell wall. *Eukaryot Cell.* 14:792-803. DOI:10.1128/EC.00032-15.

Bondaryk M, Kurzątkowski W, Staniszewska M. 2013. Antifungal agents commonly used in the superficial and mucosal candidiasis treatment: mode of action and resistance development. *Postepy Dermatol Alergol.* 30:293-301. DOI: 10.5114/pdia.2013.38358.

Cassone A, Cauda R. 2012. Candida and candidiasis in HIV-infected patients: where commensalism, opportunistic behavior and frank pathogenicity lose their borders. *AIDS*. 26: 1457-72. DOI: 10.1097/QAD.0b013e3283536ba8.

Chaffin WL. 2008. *Candida albicans* cell wall proteins. *Microbiol Mol Biol Rev.* 72, 495-544. DOI:10.1128/MMBR.00032-07.

Chaturvedi A, Siddiqui Z, Bayiroglu F, Rao KV. 2002. A GPI-linked isoform of the IgD receptor regulates resting B cell activation. *Nat Immunol.* 10:951-7 (2002). DOI:10.1038/ni839.

Costa-Orlandi CB, Sardi JCO, Pitangui NS, de Oliveira HC, Scorzoni L, Galeane MC, Medina-Alarcón KP, Melo WCMA, Marcelino MY, Braz JD, Fusco-Almeida AM and Mendes-Giannini MS. 2017. Fungal biofilms and polymicrobial diseases. *J. Fungi* 3, 22.

Cullen PJ. 2015. Evaluating the activity of the filamentous growth mitogen-activated protein kinase pathway in yeast. *Cold Spring Harb Protoc.* (3):276-83. DOI:10.1101/pdb.prot085092.

Cullen PJ, Edgerton M. 2016. Unmasking fungal pathogens by studying MAPK-dependent cell wall regulation in *Candida albicans*. *Virulence.* 7:502-505. DOI: 10.1080/21505594.2016.1177695.

Dichtl K, Samantaray S, Wagener J. 2016. Cell wall integrity signalling in human pathogenic fungi. *Cell Microbiol.* 18:1228-1238. DOI:10.1111/cmi.12612.

Finkel JS, Mitchell, AP. 2011. Genetic control of *Candida albicans* biofilm development. *Nat Rev Microbiol.* 9:109-18. DOI: 10.1038/nrmicro2475.

Fothergill AW, Sutton DA, McCarthy DI, Wiederhold NP. 2014. Impact of new antifungal breakpoints on antifungal resistance in *Candida* species. *J Clin Microbiol.* 52: 994-7. DOI:10.1128/JCM.03044-13

388 Fox EP, Nobile CJ. 2012. A sticky situation: untangling the transcriptional network controlling
389 biofilm development in *Candida albicans*. *Transcription* 3: 315-22. DOI:10.4161/trns.22281.

390 Free SJ. 2013. Fungal cell wall organization and biosynthesis. *Adv Genet.* 81:33-82.
391 DOI:10.1016/B978-0-12-407677-8.00002-6.

392 Futosi K, Fodor S, Mócsai A. 2013. Neutrophil cell surface receptors and their intracellular signal
393 transduction pathways. *Int Immunopharmacol.* 17:638-50. DOI:10.1016/j.intimp.2013.06.034.

394 Guhad FA, Jensen HE, Aalbaek B, Csank C, Mohamed O, Harcus D, Thomas DY, Whiteway M,
395 Hau J. 1998. Mitogen-activated protein kinase-defective Candida albicans is avirulent in a novel
396 model of localized murine candidiasis. *FEMS Microbiol Lett.* 166(1):135-9 (1998).

397 Heilmann CJ, Sorgo AG, Mohammadi S, Sosinska GJ, de Koster CG, Brul S, de Koning LJ, Klis
398 FM. 2013. Surface stress induces a conserved cell wall stress response in the pathogenic fungus
399 Candida albicans. *Eukaryot Cell.* 12(2):254-64. DOI: 10.1128/EC.00278-12.

400 Hiscox S, Hallett MB, Morgan BP, van den Berg CW. 2002. GPI-anchored GFP signals Ca2+ but
401 is homogeneously distributed on the cell surface. *Biochem Biophys Res Commun*. 293:714-21.
402 DOI:10.1016/S0006-291X(02)00280-2.

Kett DH, Azoulay E, Echeverria PM, Vincent JL. 2011. Extended Prevalence of Infection in ICU Study (EPIC II) Group of Investigators. Candida bloodstream infections in intensive care units: analysis of the extended prevalence of infection in intensive care unit study. *Crit Care Med.* 39:665-70. doi: 10.1097/CCM.0b013e318206c1ca.

403 Kim J, Sudbery P. 2011. *Candida albicans*, a major human fungal pathogen. *J Microbiol.* 49:171-
404 7. DOI: 10.1007/s12275-011-1064-7.

405 Kitagaki H, Wu H, Shimoi H, Ito K. 2002. Two homologous genes, DCW1 (YKL046c) and
406 DFG5, are essential for cell growth and encode glycosylphosphatidylinositol (GPI)-anchored
407 membrane proteins required for cell wall biogenesis in *Saccharomyces cerevisiae*. *Mol*
408 *Microbiol.* 46:1011-22.

409 Kitagaki H, Ito K, Shimoi H. 2004. A temperature-sensitive dcw1 mutant of *Saccharomyces*
410 *cerevisiae* is cell cycle arrested with small buds which have aberrant cell walls. *Eukaryot Cell.*
411 3:1297-306. DOI:10.1128/EC.3.5.1297-1306.2004.

412 Lakhan SE, Sabharanjak S, De A. 2009. Endocytosis of glycosylphosphatidylinositol-anchored
413 proteins. *J Biomed Sci.* 16:93. DOI:10.1186/1423-0127-16-93.

414 Li R, Kumar R, Tati S, Puri S, & Edgerton M. 2013. *Candida albicans* flu1-mediated efflux of
415 salivary histatin 5 reduces its cytosolic concentration and fungicidal activity. *Antimicrobial*
416 *Agents and Chemotherapy* 57, 1832–1839.

417 Nasution O, Lee J, Srinivasa K, Choi IG, Lee YM, Kim E, Choi W, Kim W. 2015. Loss of Dfg5
418 glycosylphosphatidylinositol-anchored membrane protein confers enhanced heat tolerance in
419 *Saccharomyces cerevisiae*. *Environ Microbiol.* 17: 2721-34. DOI:10.1111/1462-2920.12649.

420 Nguyen KA, Zmeter G, Claris O, Kassai B. 2012. Epidemiology of invasive Candida infection in
421 a neonatal intensive care unit in France. *Acta Paediatr*.101: e137-9. DOI:10.1111/j.1651-
422 2227.2011.02514.x.

Nikolaou E, Agrafioti I, Stumpf M, Quinn J, Stansfield I, Brown AJ. 2009. Phylogenetic diversity of stress signalling pathways in fungi. *BMC Evol Biol.* 9:44. DOI: 10.1186/1471-2148-9-44.

Spreghini E, Davis DA, Subaran R, Kim M, Mitchell, AP. 2003. Roles of *Candida albicans* Dfg5p and Dcw1p cell surface proteins in growth and hypha formation. *Eukaryot Cell.* 2:746-55.

Thompson AJ, Speciale G, Iglesias-Fernández J, Hakki Z, Belz T, Cartmell A, Spears RJ, Chandler E, Temple MJ, Stepper J, Gilbert HJ, Rovira C, Williams SJ and Davies GJ. 2015. Evidence for a boat conformation at the transition state of GH76 α-1,6-mannanases--key enzymes in bacterial and fungal mannoprotein metabolism. *Angew Chem Int Ed Engl.* 54:5378-82 (2015). DOI:10.1002/anie.201410502.

Wilson LS, Reyes CM, Stolpman M, Speckman J, Allen K, Beney J. 2002. The direct cost and incidence of systemic fungal infections. *Value of Health*. 5:26-34. DOI:10.1046/j.1524-4733.2002.51108.x.

Xie X, Lipke PN. On the evolution of fungal and yeast cell walls. 2010. *Yeast.* 27:479-88. DOI: 10.1002/yea.1787.

Younes S, Bahnan W, Dimassi HI, Khalaf RA. 2011. The *Candida albicans* Hwp2 is necessary for proper adhesion, biofilm formation and oxidative stress tolerance. *Microbiol Res*. 166: 430–436. DOI:10.1016/j.micres.2010.08.004.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26526v1 | CC BY 4.0 Open Access | rec: 15 Feb 2018, publ: 15 Feb 2018

Figure 1

DFG5 and *DCW1* double mutants have growth defects

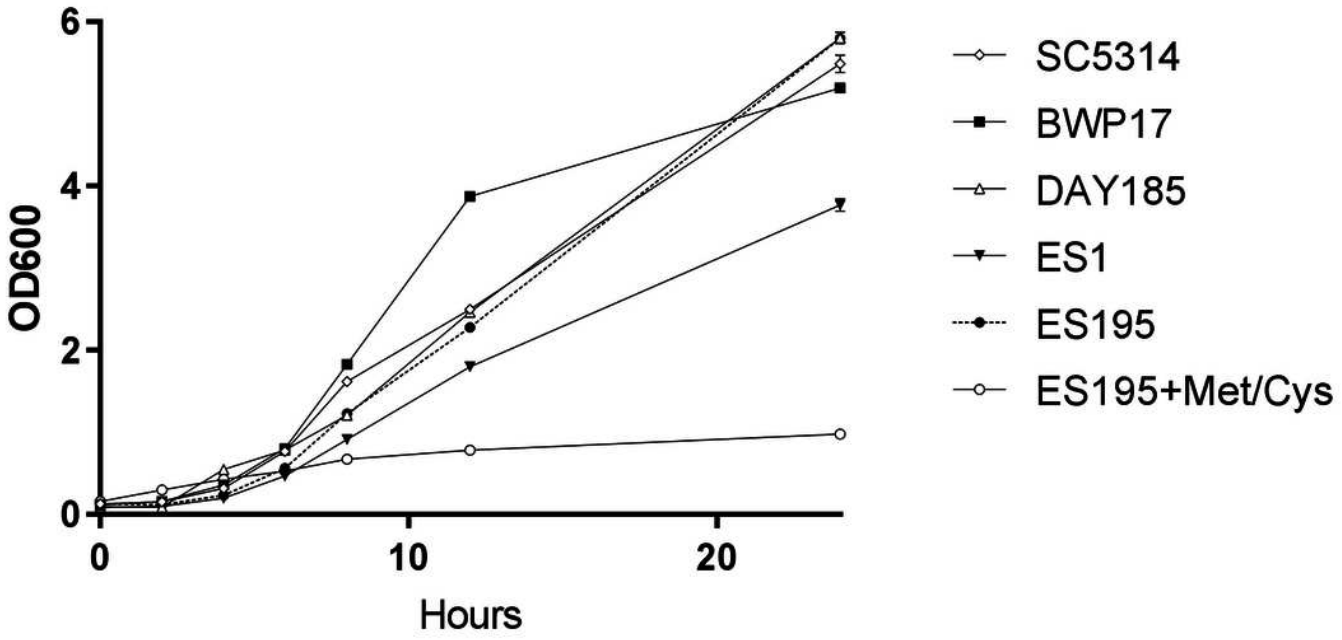

Figure 2

DFG5 and*DCW1* are required for biofilm formation in *Candida albicans.*

Biofilms were cultured over 24 hours followed by desiccation and determination of dry weight. Statistical analysis was done by Student's t-test ($p<0.01$).

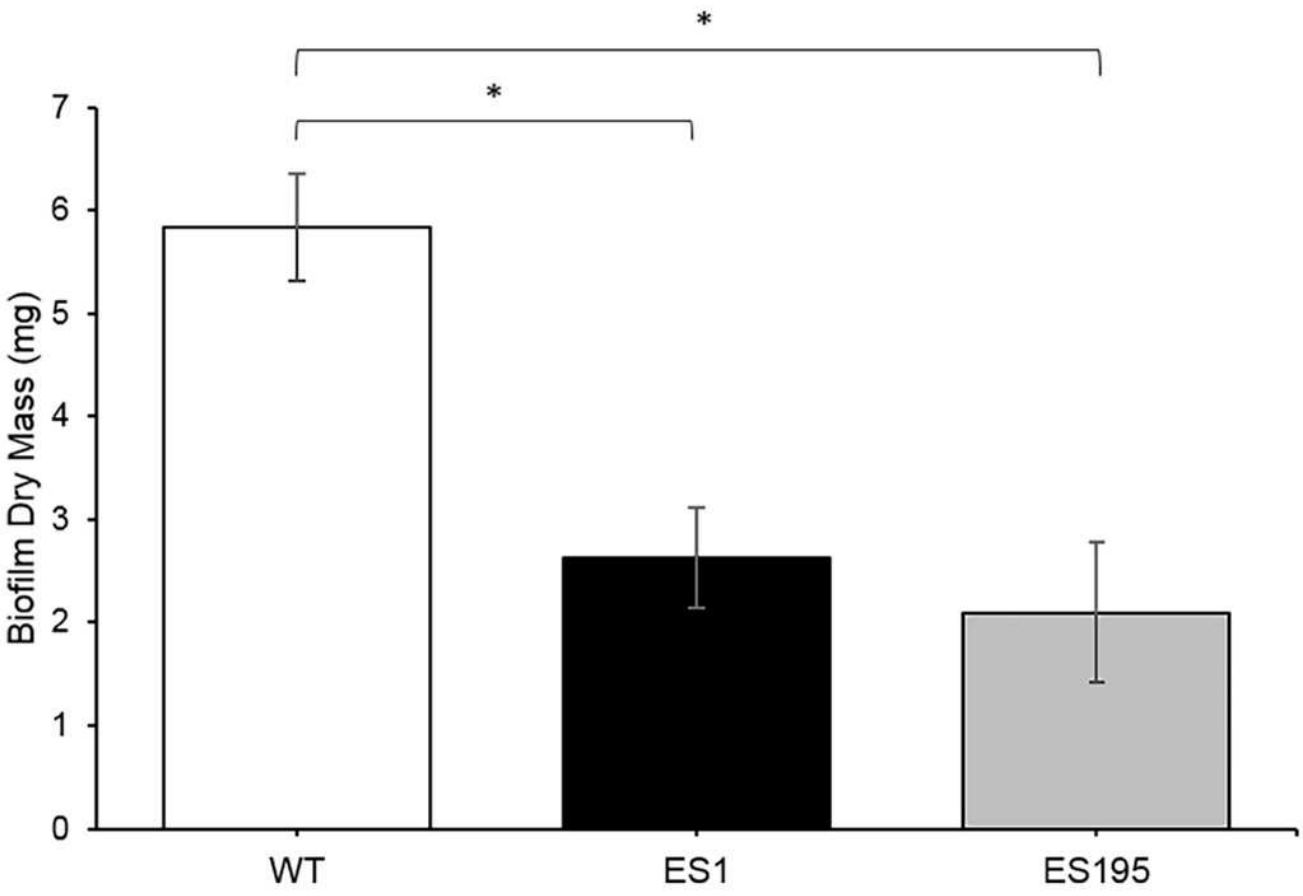

Figure 3

DFG5 and*DCW1* are required for dual species biofilm formation by *Candida albicans* and *Streptococcus gordonii.*

Biofilms were cultured over 24 hours followed by desiccation and determination of dry weight. Statistical analysis was done by Student's t-test ($p<0.01$).

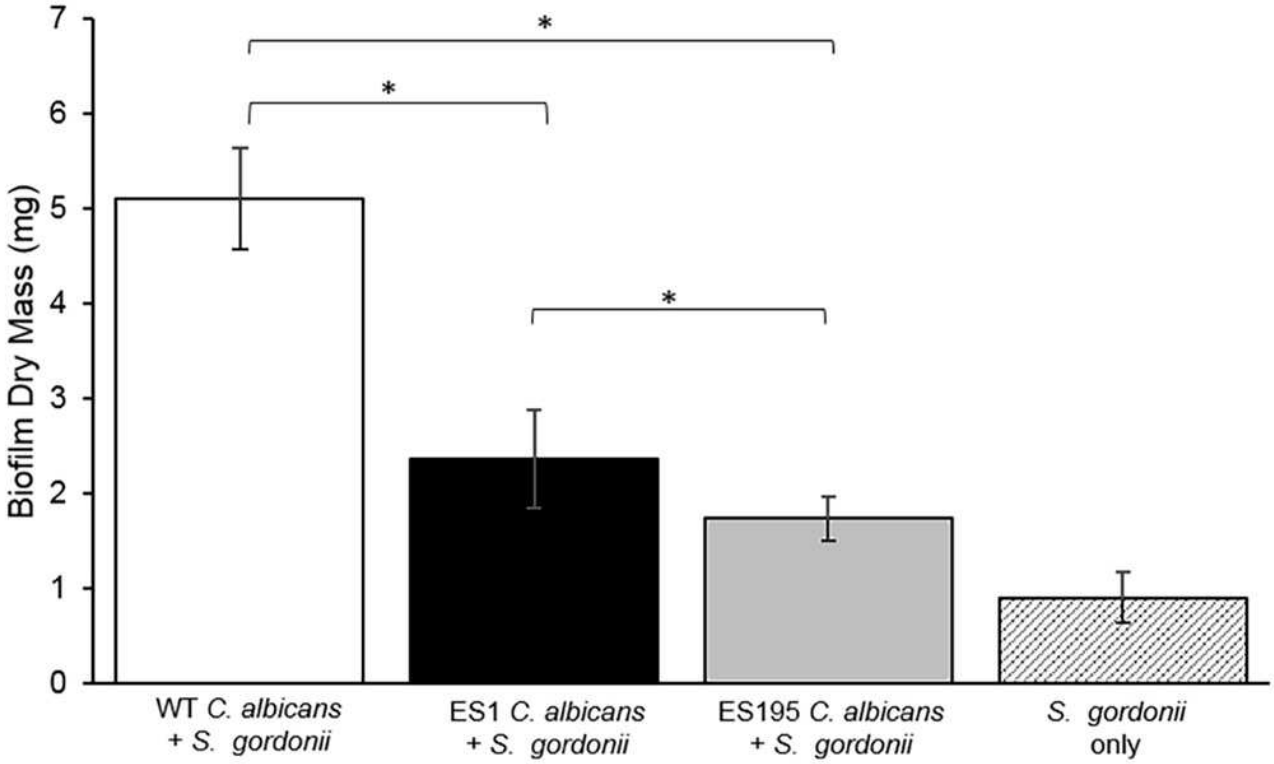

Figure 4

DFG5 and*DCW1* mutants are affected by osmoticstress

Control and mutant strains were spotted on YNB agar prepared with 2mM Cysteine, 5mM Methionine and 1M sorbitol. The plates were incubated at 30 degrees for 48 hours.

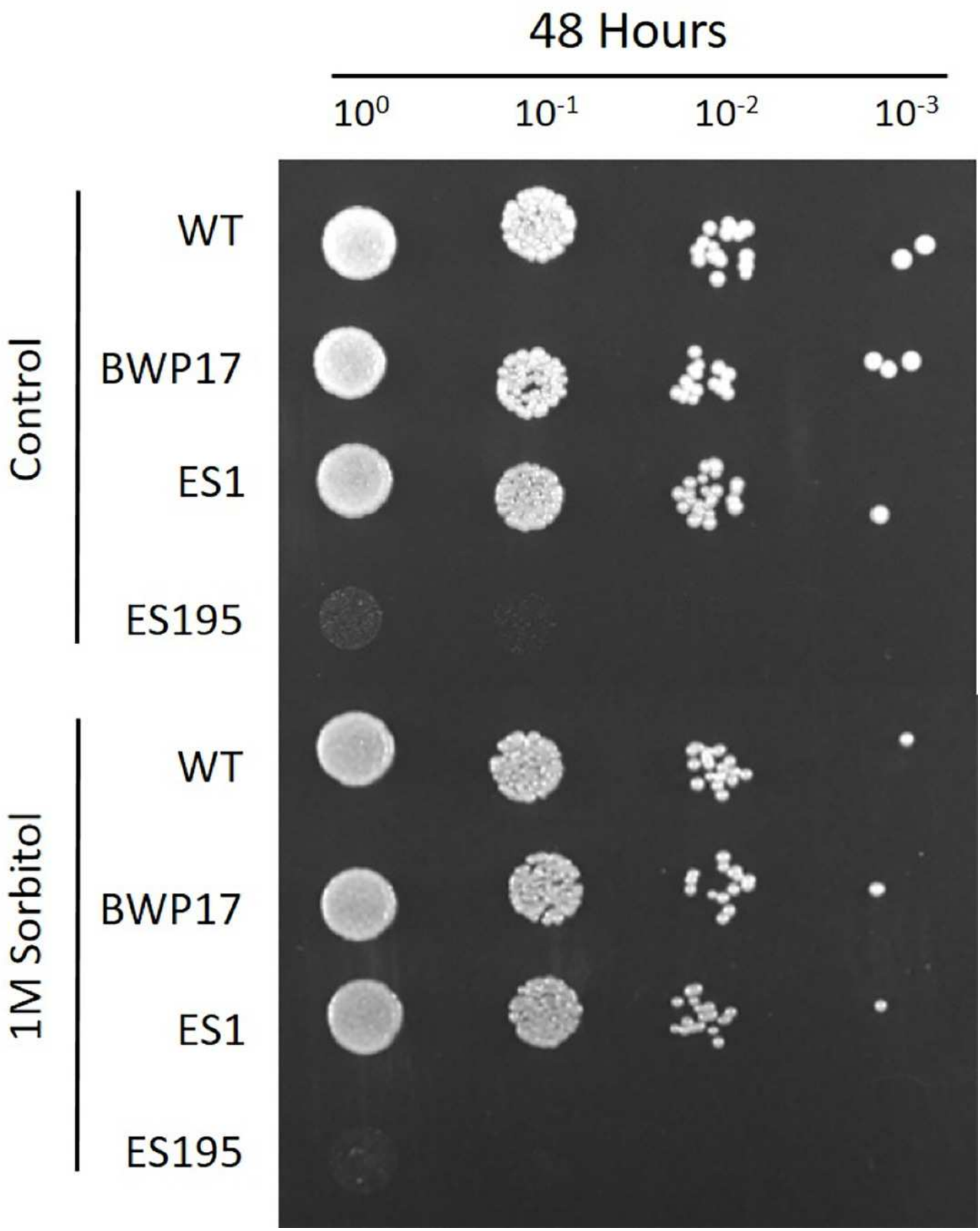

Figure 5

DFG5 and *DCW1* mutations increase susceptibility to cell wall stress and heat stress.

(A) Strains were spotted on YNB agar containing various cell wall stress causing agents. (B) Strains were spotted on YNB agar and incubated at high temperature.

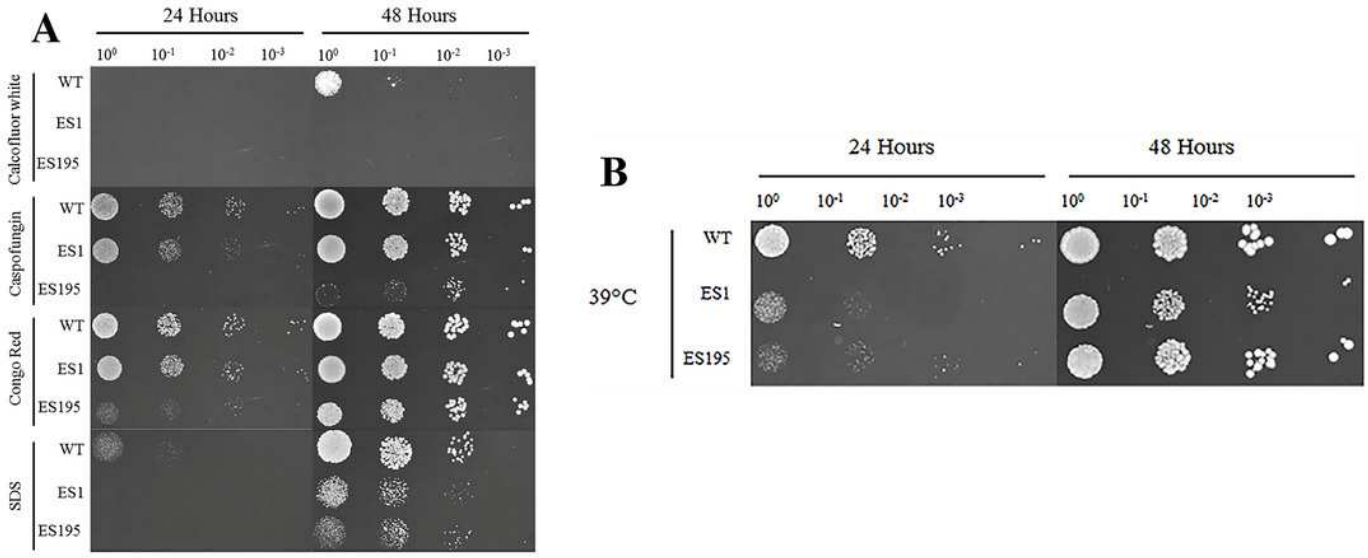

Figure 6

Hog-1 phosphorylation is not affected in wild type and parental strains

(A) Western blot analysis using anti-P-Hog-1 and anti-G6PD (**B)**Electrochemiluminiscence (ECL) analysis of P-Hog-1.

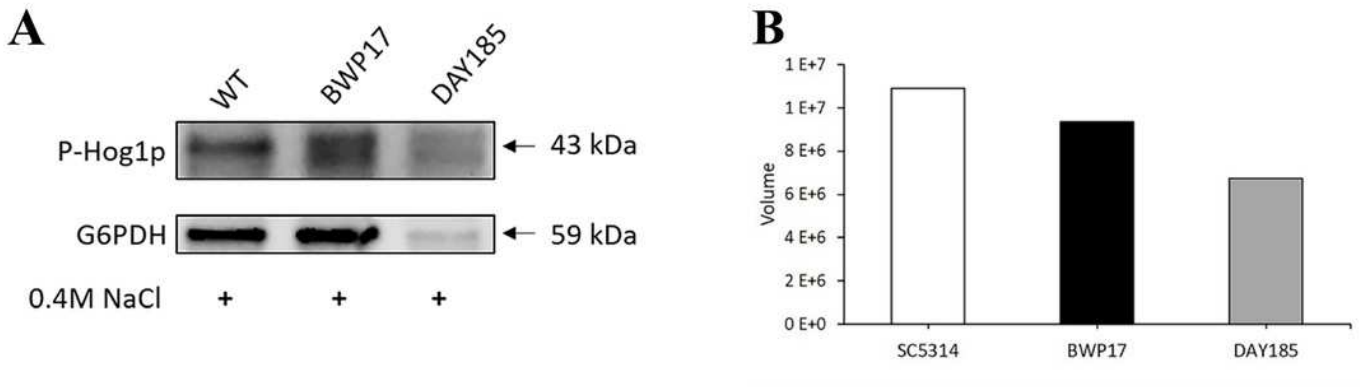

Figure 7

DFG5 and *DCW1* together are required for Hog-1 phosphorylation.

A. Western blot analysis using anti-P-Hog-1, whole Hog-1 and G6PD antibodies **B.** Relative intensity of P-Hog-1 to whole Hog-1 using ECL analysis.

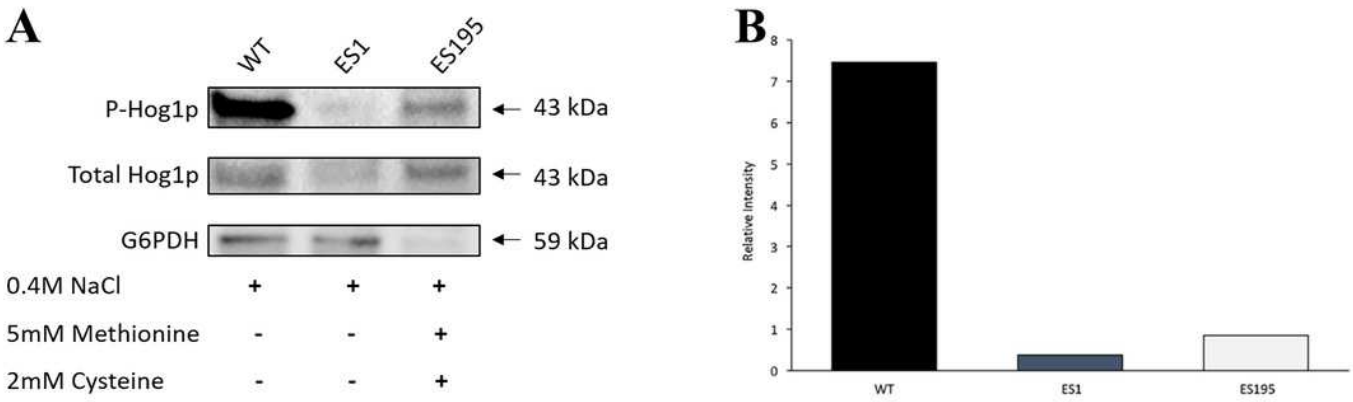

Figure 8

Dfg5 and Dcw1 cell wallenzymes, function in cell wall biogenesis as well as Hog-1 signaling.

The ligands and the downstream components involved in Dfg5 dependent Hog-1 signaling as well as the cell wall protein substrates for Dfg5 and Dcw1 remain unknown.