Stony Brook University

An Examination of *Candida albicans* Mannosylation Mutants and their CW Structures

A Thesis Presented

by

Frank August Mindlin

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Master of Science

in

Biochemistry and Cell Biology

Stony Brook University

December 2014

Stony Brook University

The Graduate School

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Abstract of the Thesis

An Examination of *Candida albicans* Mannosylation Mutants and their CW Structures

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Candida albicans is the most important human pathogenic fungus. Due to rising rates of infection and limited treatment options, it has become increasingly vital to identify the structures of the cell wall (CW) that can serve as ligands for anti-fungal drug recognition. Recently, a number of studies have focused their attention to the outer portion of the CW, which is comprised of glycoproteins modified with asparagine (N)-linked or serine or threonine (O)-linked mannan. Current models have shown N-linked mannan to be one of the key structures responsible for immune-cell recognition. Here I performed experiments toward testing the role of mannan in immune cell recognition, by constructing *C. albicans* mutants that are predicted to lack these structures.

The *S. cerevisiae mnn10* and *mnn11* mutants are defective in the α-1,6-mannosyltransferases that build the backbone of the mannan outer chain. The *MNN10* and *MNN11* genes are primarily responsible for α-1,6-linked mannose polymerizing activity of the

outer chain in *S. cerevisiae* and several other yeast species [4,5**]**. To test the model that *mnn10* and *mnn11* mutants are lacking critical components of the N-linked mannan ligand for immune recognition of *C. albicans*, I constructed *C. albicans* strains deleted for *MNN10* and its homologous gene *MNN11*. Future analyses of these *C. albicans* mannosyltransferase mutant strains in mice may provide insight into development of more specific anti-fungal therapies.

Dedication Page

This thesis is dedicated to everyone who has devoted their life to something they truly enjoy.

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List of Abbreviations

bp – base pairs

C. albicans – Candida albicans

CSP – Cell Surface Protein

CW - Calcofluor White

CWP – CW Proteins

EFG – Enhanced Filamentous Growth

ER – Endoplasmic Reticulum

GPI – Glycophosphatidylinositol

HygB – Hygromycin B

MNN – Mannosyltransferase or gene

NGL – Neoglycolipid

ORF – Open Reading Frame

PIR – Protein with Internal Repeats

PKC – Protein Kinase C

S. cerevisiae – Saccharomyces cerevisiae

WT – Wild Type

Introduction

About Candida albicans

Candida albicans is the number one human pathogenic fungus, causing infections that range from common yeast infection of mucosal membranes to life threatening systemic infections. *C. albicans* is traditionally regarded as a benign organism that occupies the oral cavity and gastrointestinal tract. Sudden physiological fluctuations in response to diet, stress, or infection can cause the yeast to exhibit pathogenic and virulent properties [39]. A number of recent studies have focused on the prevalence of *C. albicans* in hospitals and other medical settings where it has leading roles in nosocomial infections [24, 33, 61, 63]. Often regarded as an epidemic byproduct of advances in medical technology, *Candida* infections incur high medical and prevention costs [71]. Hence, it is vital to understand the mechanism of infection, and furthermore, prevention, in order to offset the increasingly high costs and the increasing mortality and morbidity rates of these infections.

Despite the abundance of information that exists about the *Candida albicans* signaling cascades post macrophage recognition, little is known about the fungal CW ligands that cause the initial macrophage interactions in the host. The CW and CW proteins (CWP) maintain the fungal structure and mediate interactions with the environment. For *Candida*, the structure of the CW involves a complex set of interactions between proteins and sugars, including fibrillar polysaccharides, that ultimately form 3 visually distinct layers that compromise the CW (Figure 1) [2].

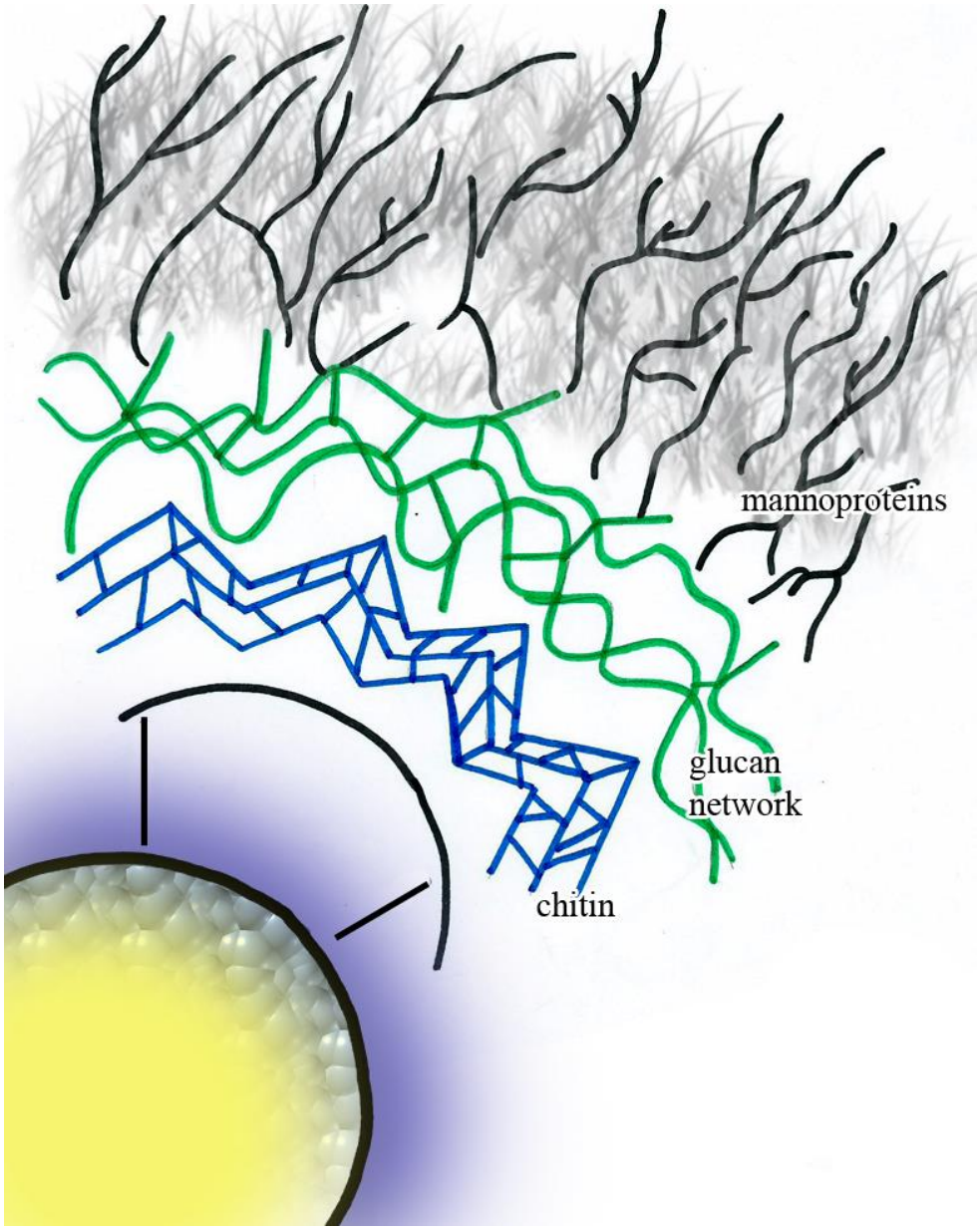

Figure 1 A schematic view of the *C. albicans* CW. A rigid chitin layer is located directly above the plasma membrane and below the - β-1,6 and β-1,3 glucan network and outermost mannoprotein layer. The CW contributes to the virulence of *Candida* and contains numerous proteins and sugars that act as ligands for phagocytic cells of the immune system [31].

CW Structure and Function

The outer protein coat is composed of mannan, a term used to describe mannose covalently bound to proteins (mannoproteins) via Asn (N-glycosydic bonds) and Ser/Thr (O-glycosydic bonds) [13]. Some of the mannan is also covalently linked to underlying β-glucans. The β-glucans are further linked to proteins via glycophosphatidylinositol (GPI) anchors (The fatty acid and inositol components of the anchor are usually lost before CW association) [13, 37, 74]. The outer layer is primarily composed of glycosylated proteins that contain mostly mannose, and that is heavily phosphorylated, resulting in the negative charge of the CW [21, 60]. A number of active carbohydrate-based enzymes also constitute parts of the external protein coat, the roles of which have been defined in other texts [13, 17].

CW components are very dynamic [74]. For instance, CWP abundance is cell-cycle dependent and also differentially localized in discrete parts of the cell, such as the hyphal wall or particular regions of the bud. This lack of glycoprotein uniformity in some surface regions of *C. albicans* increases the complexity of identifying and isolating the basal structure needed for immune cell recognition [44]. The middle skeletal layer, which is a matrix of β-(1,6)-glucan and β-(1,3)-glucan, has been found to be covalently linked to the outermost protein layer, which is primarily responsible for interactions with the host organism if the *Candida* is found internally [41, 60]. The innermost layer contains small levels of chitin, localized primarily just above the plasma membrane. This chitin is rigid and serves to gird the cell, which is under extreme osmotic pressure [1]. The β-(1,3)-glucan contains attachment sites that covalently bind β-(1,6)-glucan as well as chitin. This creates regions of nonsubstituted residues of glucose that are further strengthened by proteins with internal repeats (PIR), and hydrogen bonds that create a

continuous network around the CW [23, 41]. Due to the complexity of the CW structure, as reinforced by a multitude of proteins arranged in discrete patterns, the CW of *Candida albicans* becomes increasingly more difficult to understand and target with antifungal drugs.

Virulence - Mannoproteins

The virulence properties of *C. albicans* allow it to invade body cavities while evading detection. The complex structure of the CW and accompanying CWP further complicate our ability to target fungal cells for destruction. Simultaneously, this complexity of the CW increases the virulent properties of the fungus. The N-linked protein side chains and tightly-packed disulfide bridges seen in the CW reduce its permeability [41]. The ability of mannoproteins to regulate wall porosity has been regarded as a vital determinant of *C. albicans* virulence [41, 78].

An earlier study assessed the relationship between mannoproteins and porosity by treating *S. cerevisiae* cells with protease, and a follow-up analysis demonstrated the erosion of CW material along with a decrease in overall CW thickness and rigidity [70,78, 81]. The protease acted in conjunction with zymolyase to achieve the CW erosion and partial lysis [81]. Subsequent introduction of glucanase resulted in the breakdown of glucan structures in the CW, though they were not affected by glucanase prior to introduction of protease. This finding indicated that the outer-most mannan layer of the CW severely limited the ability of enzymes to permeate other CW components, and its absence evidently increased CW porosity and permeability. A parallel analysis observed that after introduction of protease, addition of horseradish peroxidase was able to cause membrane lysis, though the small basic molecule was unable to reach the membrane in the unaltered cell with abundant mannose [81]. This study not

only demonstrated the importance of mannoproteins in protection from injury, but also highlighted the benefit of targeting mannoproteins to increase cell porosity and sensitivity to enzymes (and drugs). The virulence of *C. albicans* can thus be partially explained by the abundant mannose layer that extends beyond the glucan layer, which effectively shields the cell.

Virulence - Morphology

Studying the virulence of *C. albicans* becomes particularly difficult when assessing the grand scope of morphologies that are native to this fungus. This makes detection, identification, and targeted treatment more challenging. These morphologies range from spherical-like yeast cells to more complex and elongated structures that include germ tubes, pseudohyphae, and true filamentous hyphae [15, 52]. The yeast morphology has been linked with the spread of cells throughout the human body, whereas the filamentous structures have been closely linked to pathogenicity due to a wider array of surface proteins that are visible [52, 75]. The difference in pathogenicity between the different morphologies has resulted in some difficulty in studying and elucidating the virulence factors that are attributed to avoidance by macrophage recognition.

An earlier study that focused on Dectin-1 highlighted the morphological and virulent differences between *C. albicans* CWs [75]. Dectin-1 is a transmembrane receptor protein located on phagocytes. One of the receptor's targets is β-glucan, which it subsequently engulfs, a process which actives the NF-KB and cytokine inflammatory pathways. In yeast cells, Dectin-1 was observed to bind to β-glucan and result in anti-inflammatory responses, despite the presence of a thick, outer mannan layer [75**,** 81]. This binding by Dectin-1 has been linked to the presence of bud scars on yeast *C. albicans*, which are byproducts of budding and leave exposed areas of β-glucan. Despite the relatively small amount of scarring per cell, the study has shown that it is

enough to initiate Dectin-1 binding, and that the exposed glucan contributes to the reduced virulence of yeast [75]. The study also found that the filamentous morphology of *C. albicans* was unreactive with Dectin-1, which could have two possible explanations. First, the filamentous morphology could be unreactive to Dectin-1 because cells are not actively dividing during this stage, and hence no β-glucan is exposed. The second possible explanation was that the presence of filaments was inhibiting Dectin-1 signaling. The second possibility was ruled out when zymosan was directly introduced to Dectin-1 and no inhibition was recorded, leading the group to believe that filaments do not actively inhibit Dectin-1, and the absence of exposed β-glucan is a more likely explanation for the lack of reactivity [75, 76**]**. By strategically enclosing the β-glucan with a thick mannan layer, filamentous *C. albicans* may be able to avoid detection in tissue, underscoring the complexity the multitude of virulence factors.

C. albicans requires the ability to achieve both yeast and filamentous morphologies for virulence [52**].** An earlier study illustrated how the absence of a transcription factor (Spt3p) resulted in a sole filamentous morphology in the *C. albicans* model and the lack of dimorphism prevented virulence [46, 75]. This finding was further complicated by the fact that surface proteins were altered during filamentous growth, resulting in the expression of different virulence factors in yeast and filamentous forms [27, 45].

Understanding the filamentation pathways in *C. albicans* is vital due to the direct relationship between filamentous morphology and virulence. Efg1 (Elongation Factor G) is a key transcription factor in *C. albicans*, which acts on the downstream effector Als1p, a Cell Surface Protein (CSP) that is related to virulence [26]. A Northern-blot analysis demonstrated the need for a functioning Efg1 transcription factor to express Als1p. The same study found that double *cph1/cph1 efg1/efg1 Candida* mutants are avirulent in multiple mouse models, even in response

to serum that would accelerate filament and hyphae growth in WT [26, 49]. The reduction in virulence has been attributed to the inability of *cph1 efg1* double mutants to form hyphae that are required for destruction by macrophage after fungal ingestion in the murine model, thus highlighting the importance of the filamentous morphology for fungal survival in the host, tissue invasion, and foraging functions [49].

Parallel Kinases between S. cerevisiae and C. albicans

The CW of *C. albicans* and *S. cerevisiae* share similar properties [40]. Furthermore, similarities between *C. albicans* and *S. cerevisiae* have resulted in comparing the parallel kinase cascades in order to understand more about the filament regulation pathways in yeast that lead to adherence and, consequently, virulence [26, 36, 43, 49, 69,70]. In *Saccharomyces cerevisiae*, Flo11p's primary function is to act as the downstream effector of filamentation, a homolog of which has been found to be Als1p, a CSP, in *Candida albicans* [26]. Flo11p has also been found to induce growth post-vegetation and, when overexpressed, to direct invasive filamentation and flocculation (in *S. cerevisiae*) [32]. Heterologous complementation identified Als1p in *C. albicans* to be the homolog of AGα1p (a cell surface glycoprotein) and to be functionally similar to Flo11p in *S. cerevisiae* [26, 32]. Als1 is characterized by central regions rich in Ser and Thr tandem repeats and a unique N and C-terminus that is similar to the Agα1p [26].

The ability of Als1 to regulate adherence to endothelial cells was studied by comparing adherence and flocculation abilities of double mutants and Als1-complemented mutants with WT and single knock-out strains [26]. Als1 double knockouts showed reduced adherence to endothelial cells by 35%, whereas single knockouts and complemented mutants did not show reduced adherence or flocculation. Furthermore, over-expression of Als1p in *C. albicans*

illustrated greater adhesion and virulence properties, and also resulted in enhanced flocculation in such mutants when compared to WT cells [26]. Immunofluorescence with monoclonal antibodies further identified Als1 to be a CSP and consequently demonstrated its role in filamentation and as a downstream effector of the EGF1 pathway [26].

Hygromycin (hyg)B Sensitivity

Aminoglycoside sensitivity in yeast has held particular interest in a number of scientific studies because of its ability to detect glycosylation defects, which can be studied and targeted to treat infections [6, 9, 18, 64, 67, 68]. Aminoglycosides are a class of antibiotics that bind to various specific locations of the 30S subunit of the ribosome and inhibit protein synthesis. They have gained popularity within the scientific community as other classes and types of antibiotics have shown to produce resistant strains of bacteria that cannot be combated in traditional ways [9]. The single binding site of HygB presents itself as an efficient tool to target a bacterial infection, given the diversity of virulence factors that are located within the CW of a pathogen. Sensitivity to HygB can also be used to identify *mnn* mutants that have defects within the glycosylation pathway [7, 18]. The defect in the glycosylation pathway can be used to further understand how *mnn* mutants avoid macrophage detection [64].

Mannoproteins – Overview

Mannoproteins, which constitute the outer layers of the *C. albicans* CW, are important for fungal survival, adherence, and immune recognition. Some mannoproteins are attached to

glucans and can be released when digested with β-glucanases [3, 12]. Glycans on mannoproteins are initiated with reactions in the ER and are elongated and extended in the Golgi [70, 72].

GPI anchors are responsible for linking the CWP, including large mannoproteins, to the outermost layers of the CW [13]. These structurally large mannoproteins contain long sugar (carbohydrate) chains that are added post-translationally during glycosylation within the ER and Golgi, and constitute the largest group of glycoproteins within the cell that are also heavily responsible for maintaining cell shape and structure [13]. Furthermore, the multitude of carbohydrate branches (though not present in every mannoprotein) have been speculated (and shown) to be at least partially responsible for virulence and immune cell recognition of *C. albicans in vivo* [13, 50].

N-linked mannans are amide linked between asparagine residues of a protein, whereas O-linked mannans are characterized by the addition of mannan via an ester bond to serine or threonine residues (Figures 2 and 3) [72]. The N-linked species are also distinguishable by having a higher molecular weight compared to O-linked species, and on average contain fewer α-1,3-linked mannoses [55]. N-linked glycans are synthesized in the ER, by a set of highly conserved reactions that result in the formation of a precursor oligosaccharide from dolichol-linked Glc-NAc sugar and consequent sugar attachment [19].

Once attached, these glycans promote folding and transport of glycoproteins to the Golgi, where glycans are further extended by a series of reactions catalyzed by mannosyltransferases [19]. First, a long α-1,6-linked mannose backbone is formed [70], which is then modified with α-1,2 and α-1,3 linked mannose side chains, as well as phosphomannan (Figure 3)[16, 28, 36]. O-linked mannans do not contain as many mannose – they consist of about 5, compared to over 200 mannose residues. The *PMT* gene family transferases in the ER catalyze the addition of 4

residues, which are then elongated by 1 or 2 mannoses in the Golgi (Figure 2) [72].

N-linked mannan has been observed to play a large role in immune cell recognition and stimulation due to the variety of glycosidic bonds present throughout the branches; the role of O-linked mannan in recognition is still disputed [28, 29]. Due to the changing morphology of *C. albicans* as a byproduct of varying environmental factors (temperature, pH), it has been found that mannoprotein structures also change depending upon the morphology [12, 25]. Temperature had a significant effect on mannosylation pathways in *C. albicans* [73]. A study by Trinel et al found that an increase in environmental temperature had no direct effect on β-1,2-mannosylation of psospholipomannan (a glycolipid) but expression of β-1,2-oligomannosides was reduced [73]. Another study found that when *C. albicans* cells mature from spherical yeast to longer hyphal forms, for example, the amount of β-1,2- capped branches remains the same, while the amount of β-1,2-linked mannan oligosaccharides decreases [66]. Through NMR and spectral analysis, a study further identified the presence of α-1,6 and β-1,2-linked mannan in *C. albicans* yeast and hyphal forms, which was related to prior findings of monoclonal antibodies targeting the β-linked mannan [66,73].

The presence of β-linked mannan side-chains raises the question of whether or not these β-linked mannoses are involved in immune cell recognition due to their continued presence in the filamentous (virulent) stage of the fungi. It should be noted that both yeast and hyphal forms of *C. albicans* are virulent. The filamentous form, however, is thought to be advantageous for the fungi when in contact with tissues and immune cells [81].

C. albicans mnn mutants contain truncated N-linked oligosaccharides as well as glycosylation defects. In a separate analysis of *S. cerevisiae*, these defects have been linked to the inability of N-linked oligosaccharide to properly elongate due to the presence of a side chain

mannose that terminates elongation, particularly in the *mnn10* mutant [4]. Spectroscopy analysis has previously confirmed that the addition of an extra branch to the oligosaccharide acts as a termination step for further elongation, while simultaneously allowing the addition of smaller α-1,3 branches. This was identified in *S. cerevisiae* and in a small number of substrates [4]. The *mnn8* and *mnn10* mutants have their outer chains reduced to fewer than 20 mannose units, whereas WT cells contain three to four times as much mannose [4]. When deleted in *S. cerevisiae*, the *MNN10* and *MNN11* genes, which encode α-1,6 mannosyltransferase, have demonstrated increased sensitivity to cytohelicase when compared to WT, and further brought into question the role that *mnn* mutants can have in immune cell [5]. Similarly, *S. cerevisiae mnn10* and *mnn11* mutants have increased sensitivity to HygB, thus underscoring the composition changes within the CW that may provide clues for how these mutants interact with macrophages and stimulate inflammatory responses [5,7]. Moreover, a significant number of *C. albicans* mannosyltransferases are not found in humans and would thus provide excellent targets for immune cell recognition and drug targeting [55].

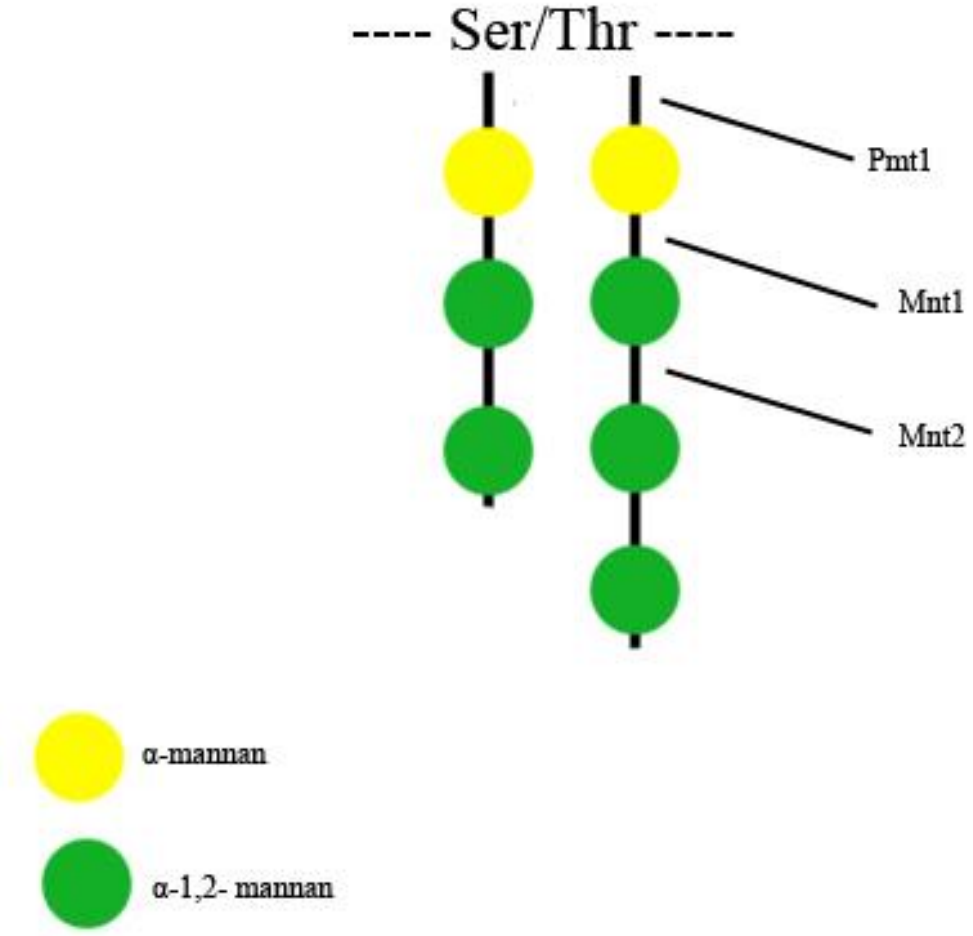

Figure 2 O-linked mannan is depicted above. Pmt1-6 add the initial α-mannose in the ER. Mnt1 and Mnt2 sequentially add α-1,2-mannoses. O-linked mannan chains are shorter than N-linked mannan chains [57].

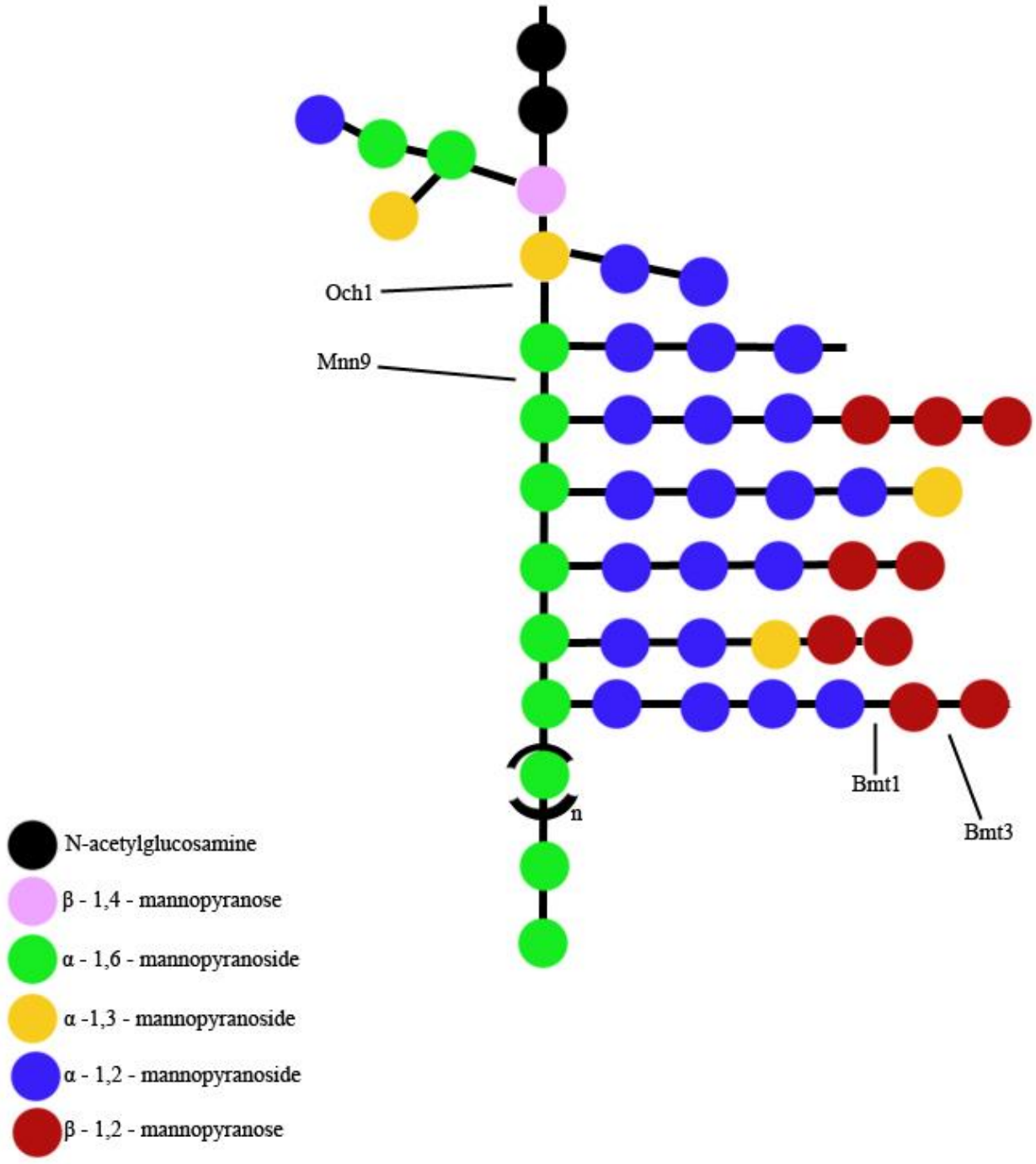
---Asn - X - Ser/Thr ---
Och1
Mnn9
Bmt1
Bmt3
n
N-acetylglucosamine
β - 1,4 - mannopyranose
α - 1,6 - mannopyranoside
α -1,3 - mannopyranoside
α - 1,2 - mannopyranoside
β - 1,2 - mannopyranose

Figure 3 Model for N- linked mannan structure. During N-mannosylation, Och1 adds the initial α-1,6-mannose to the core oligosaccharide that is attached in the endoplasmic reticulum (ER). Mnn9 adds an additional α-1,6-mannose. Other protein families (*MNN*, *MNT*) then proceed to extend the α-1,6-backbone with α-1,2 and α-1,3 linked branched residues. "n" indicates a number of consecutive α-1,6-mannoses in the backbone. The figure above has been adapted and edited from a publication by Murciano et al [57].

Objectives

The aim of this thesis was to assess the role of *mnn10* and *mnn11 Candida albicans* mutants in CW biosynthesis, immune cell recognition, and virulence. Toward this aim, I constructed plasmids designed to delete the chromosomal *MNN11* genes in *C. albicans*. Further implications of identifying this basal structure of recognition may result in more effective means of treatment, particularly because chitin and β-(1,3)-glucan are not found in mammalian cells and can thus be efficiently targeted.

The presence of mannose in the external layers of *Candida albicans* and *Saccharomyces cerevisiae* has resulted in myriad studies that assess its role as a ligand for the macrophages involved in the initial cell-host interactions [13, 38, 60]. Conservation of structure and function between the mannosyltransferase genes in *C. albicans* and *S. cerevisiae* have proven to be useful in understanding the effects that MTase mutants have on CW morphology among the species [5, 68]. One earlier study, for example, utilized a novel red fluorescent biomarker to quantitatively assay mutations that interfere with mannan, chitin, and glycan synthesis on recognition between *Candida albicans* by the macrophages [38].

Fungal-host interactions between *C. albicans* and macrophages were further followed

throughout phagocytosis using model murine assays, which ultimately identified that recognition by the macrophage requires CW N-linked mannan [38]. The recognition is based upon identification and disruption of the genes in the mannosyltransferase pathway responsible for the construction of the mannan layer of the CW.

To identify the structure requirement needed for immune macrophage recognition of *Candida albicans,* future studies will aim to identify the minimal structural requirement of the N-linked mannan by genetically disrupting the sequential steps involved in its biosynthetic pathway.

A model that compares the N-linked glycosylation biochemical pathway in the Golgi of humans and *C. albicans* can be located Figure 4 [53].

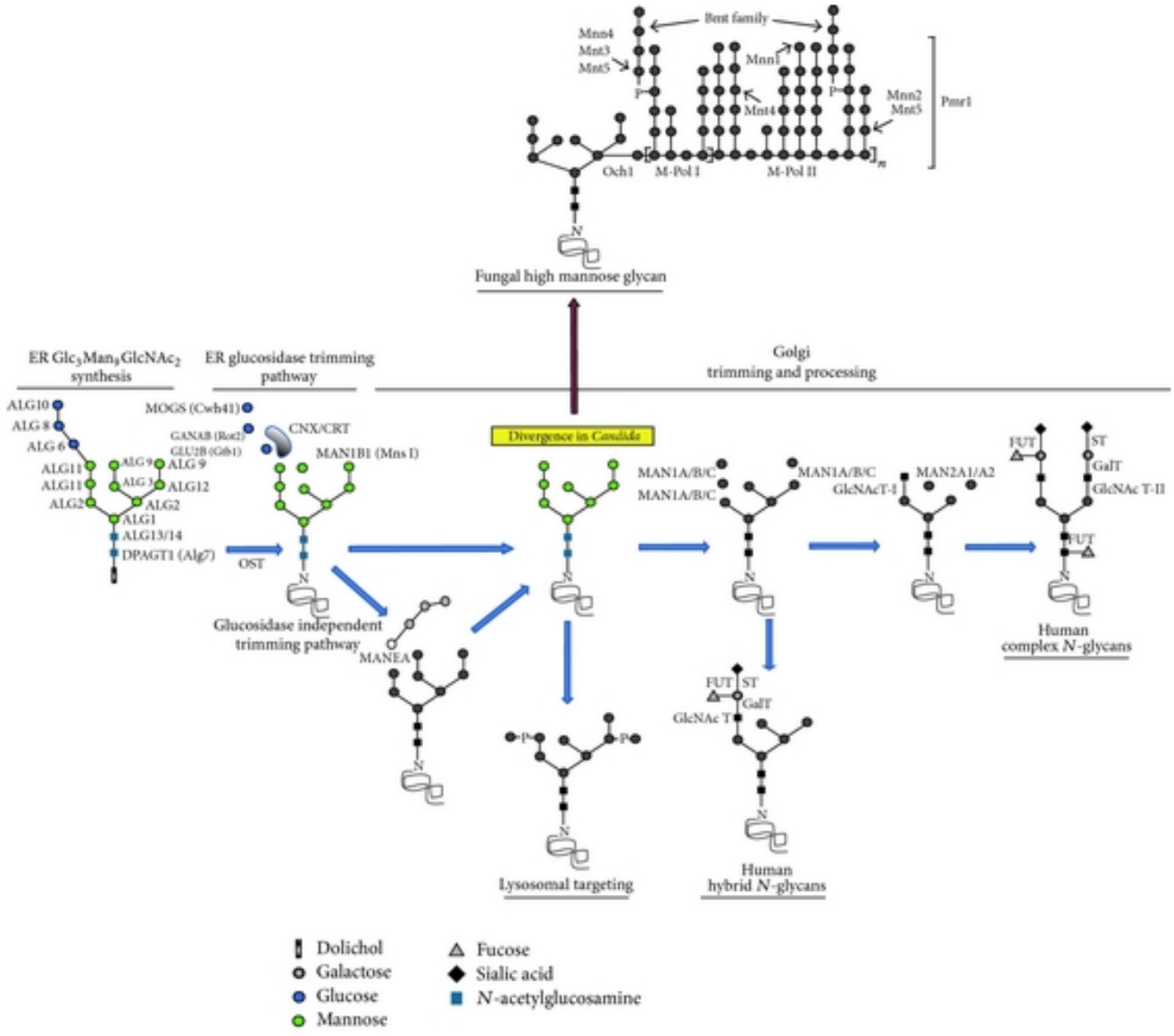

Figure 4 Comparison of the N-linked glycosylation pathways in humans and *Candida albicans*. The image above has been published in International Journal of Microbiology, 2014, Iván Martínez-Duncker et al [53].

Materials and Methods

Plasmid Preparation

Constructing *C. albicans* deletion strains is more complicated than *S. cerevisiae* for several reasons. First, *C. albicans* requires a much longer region of homology for homologous recombination - whereas *S. cerevisiae* requires a short sequence of approximately forty-five base pairs (bp) of homology - *C. albicans* requires at least 100 bp [80]. The second complication is that *C. albicans* is an obligate diploid, which requires deletion of two copies of a gene.

PCR is a valuable tool for the creation of knockout strains of *C. albicans*. Many modules have been developed for PCR- based gene targeting in *C. albicans* [30, 79]. These modules are based on the same principals and methods originally developed in *S. cerevisiae* [9]. The general approach involves PCR amplification of a selectable marker gene such that it is flanked with sequences homologous to the chromosomal locus that is the target for deletion. Homologous recombination allows replacement of the gene of interest with a selectable marker.

Creating a C. albicans Deletion Strain

Creating a strain in *C. albicans* in which both alleles of a given gene are deleted involves replacing each allele with a different selectable marker, or alternatively using a system that allows "recycling of a marker" by some scheme that allows pop out of the selectable marker, after its integration, so that it can be subsequently reused. To create heterozygous and homozygous *mnn11* mutants, several markers were used, including Ca*ARG4*, *HIS1* and SAT^R (Figure 5, 6). The *ARG4* and *HIS1* markers were amplified using pFA-based *Candida albicans* plasmid collection [30]. The SAT^R encodes a dominant nourseothricin resistance protein [35]. By

designing primers that contain sequences with "tails" of homology to the *Candida albicans MNN11*, each of these marker genes was amplified with homology to the 3' and 5' ends of *MNN11*. Two approaches were used to create these knockout constructs utilizing the plasmids pAdh – *MNN11*, pAdh – *mnn11Δ*::*ARG4*, pAdh – mnn11Δ::HIS1, pAdh – mnn11Δ::*SAT*^{R} (Refer to Table 1).

The *mnn11Δ::HIS1* construct was created by using sets of nested primers (Figure 5). The first set of primers contained the S1 and S2 primer sequence and anneal to the 3' and 5' ends, respectively, for the *HIS1* marker gene in the pFA-HIS1 plasmid. The S1 and S2 primers were extended with a tail of fifty bp of homology to *C. albicans MNN11*. A second set of primers that anneal to the ends of the first set of primers and contain the next fifty bp of homology on their tail were used to amplify products that thus contained a total of 100 bp of homology to the 5' and 3' region of *MNN11***.** Finally, a third set of nested primers with fifty bp of homology were annealed to the second set, and a tail with the next set of fifty bp of homology was added to *C. albicans MNN11*. With this method, a knockout construct marked with *HIS1* was made, that contained 150 bp of homology to *MNN11* on the 3' and 5' ends within the ORF.

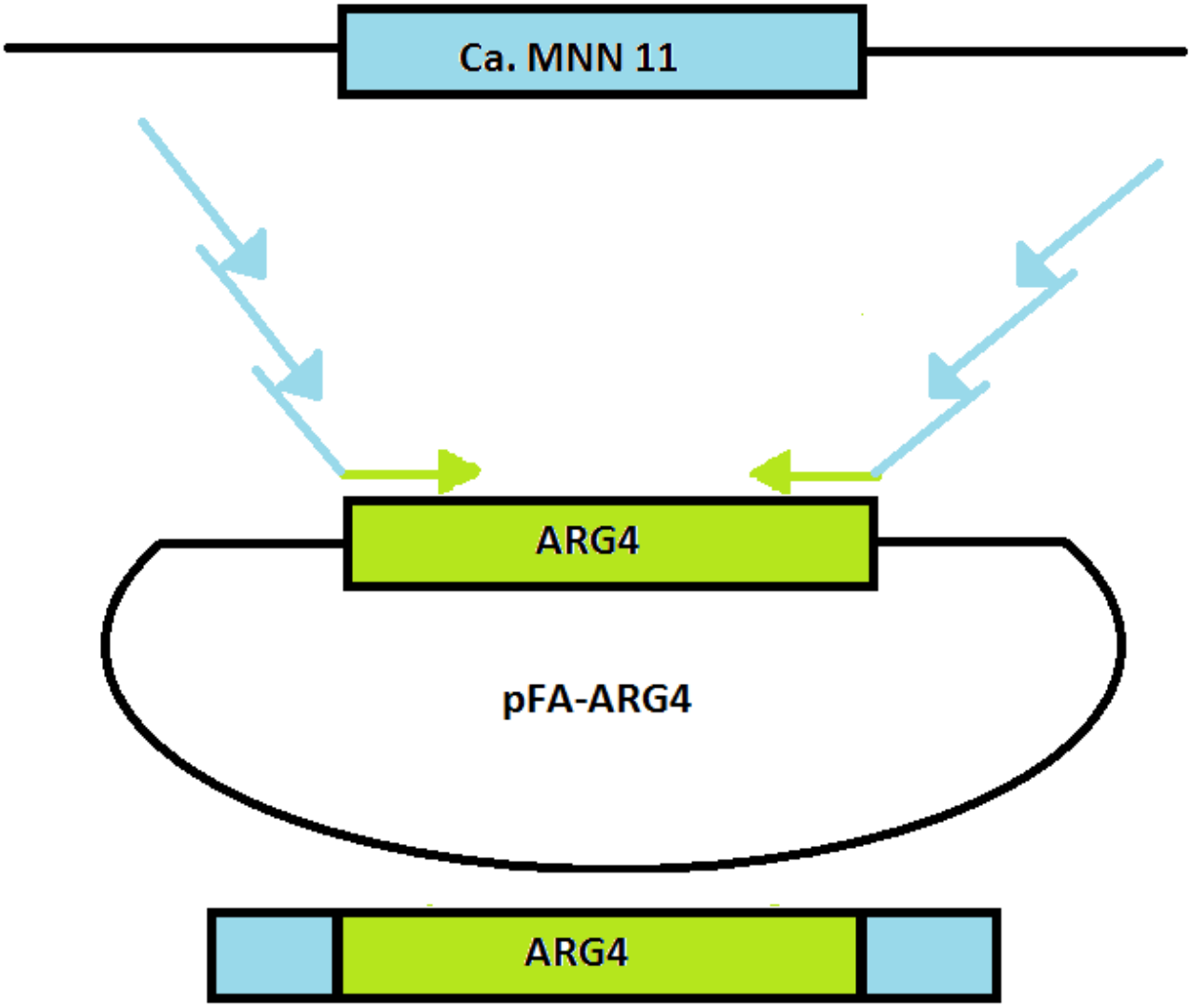

Figure 5 The image above depicts the strategy for using successive rounds of PCR to produce *ARG4* flanked with sequences homologous to the 5' and 3' end of *MNN11*. The fragment was introduced into the *C. albicans* strain *BWP17* (WT) to construct an *mnn11::ARG4* deletion. pFA-ARG4 was used as the template for *ARG4* amplification. A similar strategy was used to amplify *HIS1* and SAT^{R}, flanked by *MNN11* homologous ends, using different templates.

Knockout constructs for *ARG4* and *SAT* could not be amplified in the second or third rounds of PCR. Thus, for *ARG4* and *SAT*^{R}, plasmids were constructed for the knockout construct (Table 1). This was accomplished by performing co-transformations in *Saccharomyces* in which pAdh-*MNN11* was cut with restriction enzyme *Xho*I - which only cuts within the Ca*MNN11* gene (Figure 6) - to allow in vivo "gap repair" by homologous recombination with a PCR fragment of *ARG4* or *SAT*, which also contains fifty bp of homology to Ca*MNN11*.

Prototrophic colonies were selected on appropriate media and patched out. Three colonies for each co-transformation were selected and a rapid DNA isolation was performed to recover the plasmids from yeast. DNA from these colonies was transformed into *E. coli* strain DH5α by electroporation and three of the resulting colonies from each of these transformations were selected, prepared and characterized.

Plasmids were confirmed via restriction analysis, as well as PCR. The resulting knockout constructs from these plasmids contained the marker gene (*ARG4* or *SAT*^{R}*)* with 305 bp of flanking homology to the 3' end and 150 bp of homology to the 5'end of the Ca*MNN11* open reading frame (ORF). Each knockout fragment was isolated from the plasmid by restriction enzyme digest or by amplification with PCR, and transformed for integration into *Candida albicans* strain *BWP17* .The genotype of each of the heterozygous *mnn11Δ* strains were confirmed by PCR (R. Jones and N. Dean personal communication). Once heterozygotes were obtained, these same plasmids were used to construct *mnn11Δ :: ARG4 mnn11Δ ::SAT* homozygotes (R. Jones and N. Dean personal communication) whose genotypes were confirmed by PCR.

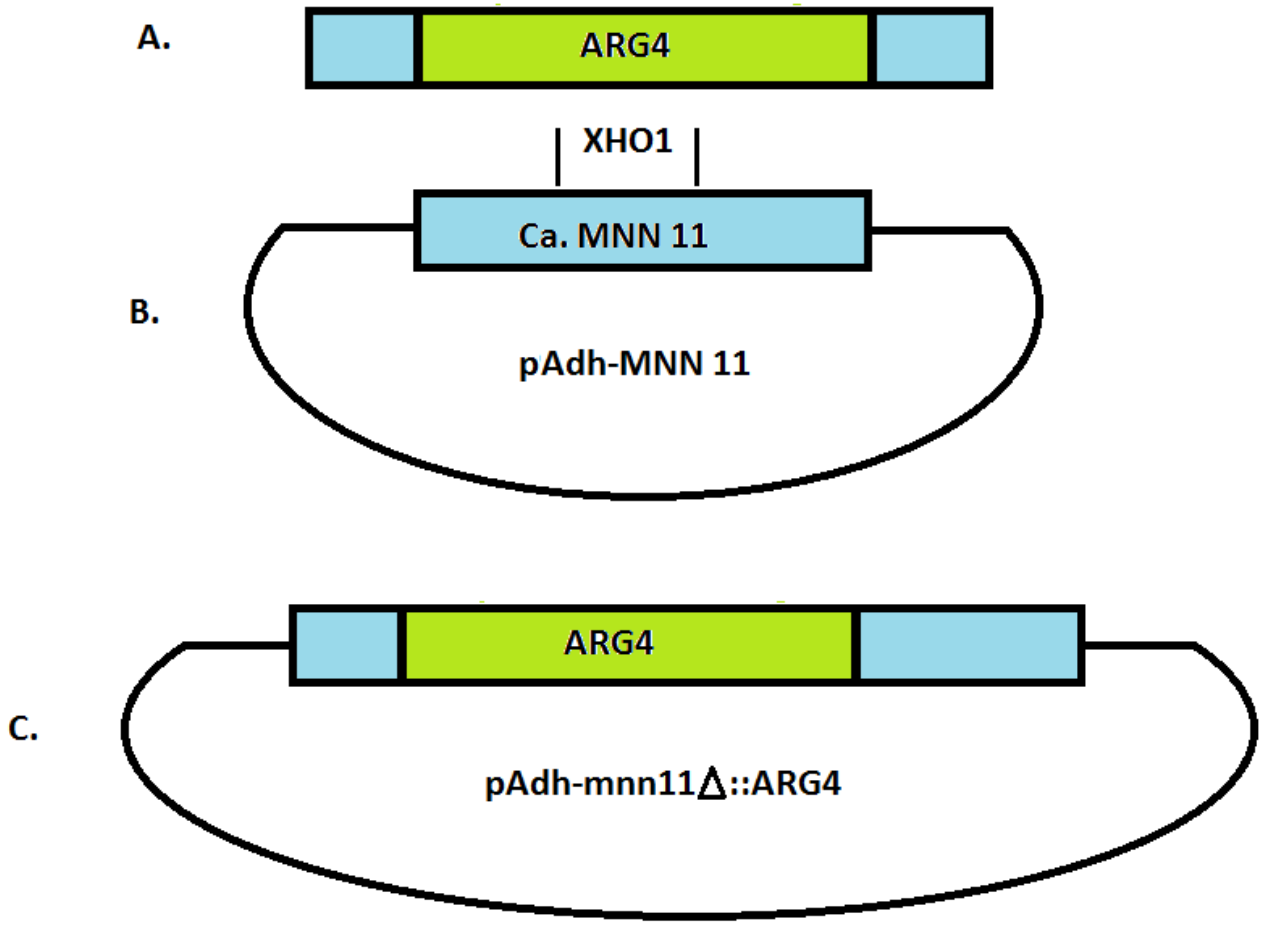

Figure 6 The diagram above illustrates the plasmid construction used in making mnn1Δ knockout cassettes. Panel A represents the PCR fragment with 50 to 100 base pairs of homology of Ca. *MNN11* on the 3' and 5' ends. Panel B depicts the pAdh-*MNN11* plasmid used for co-transformations in *S. cerevisiae*, in which homologous recombination between PCR fragment and pADH-*MNN11*, linearized with *XhoI* leads to the *mnn11Δ::ARG4* plasmid-borne deleted allele. Panel C depicts the completed pAdh-*mnn11* Δ::*ARG4* as an example of the plasmids with the same knockout construct.

Table 1 Strains and accompanying genotypes. Strains depicted with an asterisk (*) were used as parental strains for construction of *mnn11* heterozygotes.

Strains	**Genome**
SN148 *	*arg4Δ/arg4 Δleu2Δ/leu2Δ his1Δ/his1Δ ura3Δ::limm434/ura3Δ:limm434 iro1Δ::limm434/iro1Δ::imm434*
BWP17*	*ura3Δ::limm434/ura3Δ::limm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*
SKY68	*Bwp17 mnn10Δ::ARG4/mnn10Δ::HIS1*
FMY1	SN148 *MNN11 mnn11Δ*::*ARG4*

Results/Discussion

CW Integrity via HygB

Deletion of *MNN10* and *MNN11* has been previously studied in *S. cerevisiae* to assess the role of the α-1,6-mannosyltransferase in CW structures [2, 5, 19, 43]. Both of these *S. cerevisiae* mutants display hypersensitivity to HygB, have severely truncated N-linked glycans, and display several other phenotypes indicative of severe CW defects [3, 19, 54, 62]. Results from these studies have concluded that *S. cerevisiae mnn10* and *mnn11* mutants exhibit sensitivity to HygB, highlighted particularly by reduced cell growth. Assessing the integrity of *Candida* mutants *mnn10* and *mnn11* CWs via HygB sensitivity tests would have provided excellent insight into structural mannan mutations within the CW.

It has been postulated that the aminoglycoside sensitivity in mutants that is not present in WT *S. cerevisiae* is a byproduct of changes in the composition of the CW, which allows HygB to penetrate the cell and inhibit protein synthesis [5, 8, 18, 19]. The CW of *Candida albicans* consists of multiple layers of polysaccharides, which are linked to the CW mannoproteins and ultimately form a highly elastic three-dimensional network interconnected through hydrogen and covalent bonds [12, 17]. An earlier study had confirmed the similarities in HygB sensitivity and post-encounter morphology between *mnn9* mutants of *S. cerevisiae* and *C. albicans*, while another confirmed HygB sensitivity between *Kluyveromyces lactis* and *Saccharomyces cerevisiae* mutants [5, 68].

MNN10, MNN11, and HygB Sensitivity

Due to the presence of mannan in the outer-most layer of the CW, assessing its role as a potential ligand in immune cell recognition has been of particular interest. Both *S. cerevisiae* and *C. albicans* that contain mutations in the *mnn9* gene have demonstrated increased sensitivity to HygB [2, 68]. Multiple chemical analysis studies have confirmed structural mannan similarities between *Candida albicans* and *Saccharomyces cerevisiae*; thus, some parallels can be drawn between the two species in order to further comprehend CW compositions and related aminoglycoside sensitivities [59]. However, it should be noted that *C. albicans* has much more redundancy in the MTase gene family in comparison to *S. cerevisiae*, hence complicating the ability to draw parallels between CW compositions and drug sensitivity (personal correspondence with N. Dean).

The *S. cerevisiae mnn* mutants are characterized by defective glycosylation pathways which result in shorter oligosaccharide backbones and a deficiency in carbohydrates bound to the branches [2]. The structures of many *S. cerevisiae mnn* mutants were derived from *MNN2*, the mutant of which lacks the side chains and 1,2 MTase originally associated with WT cells. Via rabbit serum that bound to the α-1,6 chain it was possible to further isolate *S. cerevisiae mnn* mutants such as *mnn7*, *mnn9*, and *mnn10*, the latter of which contains an abnormally short oligosaccharide of approximately 10 mannose units and hence, fails to agglutinate [2]. Similarly, it was found the *mnn1* mutant lacks α-1,3 mannoses side chain on the backbone, while *mnn3* contains considerably shortened N and O-linked oligosaccharides [2].

It has been reported that *mnn10 S. cerevisiae* mutants are sensitive to HygB, whereas WT cells are not, and simultaneously exhibit various growth and behavioral defects [5, 19]. Analysis of the mutant *C. albicans* also exhibited morphological changes in *mnn9* mutants including

agglutination and a diminished growth rate [68]. The formation of hyphae in *C. albicans*, which has been noted to contribute to the mannan-based virulence, was also reduced and observed in clump-like forms [68]. *S. cerevisiae* with mutant *mnn9* was also unable to efficiently polarize and form buds, indicative of morphological changes in CW composition [19].

Changes in CW composition associated with the side chain and backbone variability [40] seen in *S. cerevisiae mnn*10 and *mnn*11 mutants can thus help explain the aminoglycoside sensitivity. When *mnn9* mutants were grown on HygB-rich media there was a complete inhibition of growth, whereas WT cells were able to grow in HygB of up to three times that amount [68].

Further evidence of changes in CW composition of *S. cerevisiae mnn* mutants was observed when mutants were grown on media containing ^{14}C-labeled glucose, which was incorporated into the β-glucan side chains. Isolation and separation of CW components demonstrated a significant decrease in the amount of mannan present in the outer-most layer [68]. Surprisingly, the β-(1,3)- glucan layers were observed to contain more slightly more glucose than the WT layer – this occurrence has been proposed to be a compensatory mechanism in lieu of a depleting mannan layer [68]. Such mechanisms have also been observed elsewhere and are under current investigation to assess how they may affect virulence [41, 68, 81].

An investigation of cytohelicase sensitivity in *S. cerevisiae* in *mnn*10 and *mnn*11 mutants also highlights the morphological mannose changes that occur in mutant *C. albicans*. When *mnn10*, *mnn11*, and *mnn10 mnn11* mutants of *S. cerevisiae* were combined with cytohelicase - a β-(1,3)-D-glucanase - cells demonstrated reduced growth and lysis [5, 9]. WT *S. cerevisiae* cells were not affected by cytohelicase, a finding that was attributed to the presence of a rich mannan layer in WT cells that prevented the glucan layer from hydrolysis and thus maintained the

integrity of the CW. The increased sensitivity to an enzyme that affects the middle layer of the CW demonstrated that significant morphological changes have had to occur in the *mnn* mutants to make the CW susceptible to enzymatic degradation [5].

Understanding the pathways through which *mnn* mutants demonstrate increased sensitivity to aminoglycosides, cytohelicases, and other potential agents can highlight weaknesses within the CW of *C. albicans* and serve as an asset in antifungal production.

Virulence of O-linked Oligosaccharides

While N-linked oligosaccharides have been at the primary focus for understanding immune cell recognition, the virulence of O-linked oligosaccharides has also been assessed [29]. The O-linked oligosaccharides have been shown to react with antibodies in multiple studies, and can thus serve as a tool for understanding how some *mnn* mutants avoid macrophage recognition. O-linked oligosaccharides consist of shorter branches (but usually many more per protein), which at one point were thought to diminish their ability to contain recognition sites for mammalian immune cells [29]. The situation, however, is a lot more complicated. One study was able to bind the O-linked oligomannosides to 4-hexadecylananine carrier molecule to form neoglycolipids (NGL), seroconversions of which resulted in increased antibody count [34]. When assessed in other models, such a neoantigen was found to reflect the antigenenicity one would except from a natural antigen, underscoring the accuracy of the procedure [34]. By studying the rising ELISA-NGL signals, which showed drastic changes following seroconversion, as well as analyzing thin layer chromatography which contained 6 mannose residues, the group detected the presence of antibodies in response to the O-linked mannans [34].

Similarly, another study also assessed the virulence of O-linked oligomannosides in

human tissue [22]. Via autoradiography, the presence of specific antibodies (particularly IgG and IgM) was detected in human tissue following introduction of mannan, after which the concentrations of the antibodies were more closely analyzed immunosorbent assays [22]. The study raised further questions about the nature of the produced antigens, as well as why an eighth of the test subjects failed to produce detectable levels of antigens. This, however, could essentially be an issue of methodology of introducing the mannan to tissue rather than a consequence of some biochemical nature of the O-linked mannan [22, 28]. Nonetheless, such studies are continuously updated and redone to introduce new methods of facilitating antigenic response and assess the interactions between mannans and macrophages that facilitate immune reactions.

Other Vital CW Components – Glucan

While the mannoproteins have garnered plenty of attention due to their outer-most location in the CW, it becomes increasingly more important to understand the role of chitin and glucans in maintaining not only cell shape and structure, but how these elements ultimately combine to create a network that is responsible for the virulence of *Candida albicans*. The β-(1,3)-glucan is found between the mannan and chitin layer (which has been traditionally thought to be responsible for maintaining the rigidity of the CW) [28, 31]. β-(1,6)- glucans form by CWP being linked by GPI to the skeletal glucan polysaccharides – however, this theory has been challenged because β-(1,6)-glucan has been found to contain non-reactive termini, which brings into question how sugars and myriad of proteins can be attached via GPI [31, 41]. A recently discovered β-(2,3)-glucan linkage in *C. albicans* seems to be a more likely candidate for the GPI linkage between CWP and skeletal oligosaccharides due to higher reactivity, and initial data has

concluded a sufficient amount of β-(2,3)- glucan to be present in the CW to bind proteins and sugars [31, 51]. Unlike the chitin layer, which fluctuates in percent composition as *C. albicans* undergoes morphological changes, the percentage of β-(1,3)-glucan remains relatively stable within the CW, highlighting its structural importance in both yeast and hyphae forms [31]. Its stability can prove to be an important factor in anti-fungal recognition because β-(1,3)-glucan is not found in mammalian cells and can hence be efficiently targeted.

While this thesis focuses on the role of mannan in virulence and immune recognition, another contradictory model is that glucan is key for recognition by macrophages. Introduction of hyphal glucan to human PBMCs (Peripheral Blood Mononuclear Cells**)** has shown to produce more pronounced pro-inflammatory responses when compared to yeast glucan, highlighting the weakness of earlier studies that primarily focused on measuring yeast glucan macrophage stimulation [51]. While yeast and hyphal forms of *C. albicans* contain relatively the same amount of β-1,3-glucan, the glucan is more exposed in the yeast morphology due to the presence of bud scars which lack mannan [31]. More recent studies report that introducing hyphal glucan stimulated macrophage activation and secretion of IL-1β several times greater than yeast glucan, indicating different downstream signaling that occurs upon recognition. The variations between hyphal and yeast glucan are still being assessed, with structural differences so far being attributed to the necessity of glucan to support hyphae development which consequently leads to differences in recognition [31, 51]. This finding pushes forth the idea that hyphal glucan, not just mannan, can be and is responsible for immune recognition within the host, and highlights the importance of further studying and assessing the CW components of *C. albicans* [51].

Other Vital CW Components – Chitin

Chitin, which is found under the glucan and mannan layers except during the presence of budding scars, is a polymer vital for cell structure and support in *C. albicans* [28]. It is synthesized via chitin synthases Chs1, Chs2, Chs3 and Chs8 by polymerizing GlcNAc and is deposited into the lateral walls [28, 47]. Chs3 primarily synthesizes short chitin microfibrils that constitute over half of the total chitin present in *C. albicans* CWs [28]. The production is regulated through complex signaling cascades that involve Ca^{2+}/Calcineurin and Protein Kinase C (PKC), details of which are still being studied [47]. The chitin is primarily regulated by the Ca^{2+}/calcineurin signaling pathway, which further interacts with the PKC cascade and Wsc transmembrane family of proteins in response to stress by signaling downstream and activating Rho1p (as studied in *Saccharomyces cerevisiae*) [56, 58]. It has been found that cultivating *C. albicans* cells in Ca^{2+} rich media increased the content of chitin within the cells, but had no measurable impact on glucan or mannan levels [47].

The fluidity of chitin content within the cell has been shown to be useful not only in cellular growth but also avoidance of antifungals, which can pose a challenge to healthcare professionals. In murine models it has been shown that *C. albicans* with increased chitin content was less susceptible to caspofungin, an antifungal, resulting in death within approximately 13 days of injection [47]. However, the presence of chitin on its own has not demonstrated the ability to stimulate production of cytokines within mammals [28]. Nonetheless, several studies have come forth to show that in some cases chitin can indeed stimulate an immune reaction, through only under controlled conditions [42, 65]. Allosamidin has been linked with chitinase inhibition, which may make it a tempting to use in a clinical setting if a method can be developed for delivering it past the outermost CW layers [20].

Conclusion

Candida albicans infections are particularly dangerous in immunocompromised individuals, but even in healthy adults the infection can prove to be expensive and difficult to diagnose and treat effectively. Difficulties with current treatments are often due to the anti-fungal's inability to accurately target the infection. Hence, it becomes ever more so important to understand the complexities of the disease-causing pathogen, to increase the efficiency of treatment while reducing unwanted side effects. Understanding the structure, particularly of the CW, of *Candida albicans* may shed light on the recognition mechanisms of macrophages for this pathogen and pave the way for more effective treatments. Developing a complete and thorough picture of the roles of the CW and nearby components (in both *C. albicans* and *S. cerevisiae*) can demonstrate how cellular constituents work together to achieve cell growth, durability, and virulence [48].

Insight can be obtained from *mnn* mutants that have defects in glycosylation pathways that result in an irregular outer-most CW layer. The WT mannan layer is rich in carbohydrates, and mutations within this layer have been linked to increased sensitivity to aminoglycosides that would otherwise have no effect on the organism [49, 55]. This indicates that weaknesses within the mannan-network can be utilized to more efficiently introduce anti-fungal drugs that would otherwise not be able to penetrate the cell. By understanding how *mnn* mutants can be targeted, drugs can be developed to more efficiently target mannoproteins, even in WT *C. albicans.* Similarly, due to its outer-most location and presence of carbohydrates, the mannan layer is hypothesized to contain ligands that are recognized by macrophages [38]. Changes to this layer have resulted in reduced macrophage recognition, emphasizing the lack of ligands recognized by immune cells. Other studies have also underscored the ability to target fungi (such as *S.*

cerevisiae) by unmasking the β-glucan layer (via drugs such as caspofungin), exposure to which results in a pro-inflammatory response [77]. The mannan layer thus also helps the fungi avoid immune system detection by burying the reactive β-glucan layer, and understanding how to effectively target the mannan layer with anti-fungals can result in a more robust immune response in the host.

The integrity of *mnn10* and *mnn11* mutant CWs should be assessed via HygB, as done in experiments utilizing *Campylobacter jejuni* [10]. Furthermore, components of the CW can also be isolated for study, such as using 2-mercaptoethanol, which can be used to extract glycoproteins [11]. Future experiments regarding *C. albicans* will involve assessing, via two methods, the minimal structures required for immune cell recognition by macrophages.

Phagocytosis assays using bone marrow murine macrophages (BALB/c mice) should be carried out to assess whether or not *mnn10* and *mnn11 C. albicans* mutant strains can still be recognized by macrophages. This can be accomplished via well-established published protocols [14, 38].

Via the competition assays, *mnn10* and *mnn11* mutants will be effectively compared against WT via competition assays to assess which mutation (if any) plays a more detrimental role in disabling the ligand structure of the CW that is vital for macrophage recognition. Understanding the role of the ligand (or ligands) may shed light upon which structures can increase or decrease the virulence of *Candida albicans* and provide further insight into how the ligands can be effectively enhanced to increase macrophage recognition and ultimately help fight infection.

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