Current Topics in Microbiology and Immunology

Jean-Paul Latgé *Editor*

The Fungal Cell Wall

An Armour and a Weapon for Human Fungal Pathogens

Springer

Current Topics in Microbiology and Immunology

Volume 425

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2019 Impact Factor: 3.095., 5-Year Impact Factor: 3.895

2019 Eigenfaktor Score: 0.00081, Article Influence Score: 1.363

2019 Cite Score: 6.0, SNIP: 1.023, h5-Index: 43

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Jean-Paul Latgé
Editor

The Fungal Cell Wall

An Armour and a Weapon for Human Fungal Pathogens

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ISSN 0070-217X ISSN 2196-9965 (electronic)
Current Topics in Microbiology and Immunology
ISBN 978-3-030-49927-3 ISBN 978-3-030-49928-0 (eBook)
https://doi.org/10.1007/978-3-030-49928-0

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To Anne Beauvais who inspired and supported me for so many years

Preface

The importance of the fungal cell wall has been recognized for centuries. Historically, chitin was first discovered and analysed in mushrooms by H. Braconnot in 1811 after a potassium hydroxide treatment, a chemical treatment which remains to date the most appropriate to investigate the composition of the cell wall of yeast and moulds. The second-most abundant on earth after cellulose and discovered 30 years before cellulose, chitin is present in all kingdoms (Chromista, Animalia, Protozoa and Fungi) except plants and has been at the origin of the separation of plants from the other kingdoms. Another major component of the cell wall is the polysaccharide β-1,3 glucans which has been discovered by Mangin at the end of the nineteenth century. It has been the topic of much structural research in the first half of the last century favoured by the Beer/Whisky Scottish industry but also recognized at the same time as one of the first potent immunostimulant.

In the early times, the study of the fungal cell walls has been only devoted to the analysis of the cell wall polymers. Two pioneers (with very different characters!) did open the mystery box of the cell wall synthesis: a student of F. Leloir, E. Cabib who has identified biochemically the role of the nucleotide sugars in the synthesis of chitin and glucans and S. Bartnicki Garcia who was the first one to identify organelles responsible for this synthesis and has launched the role of cell biology in the understanding of cell wall synthesis. This was 50 years ago. Then came the revolution of molecular biology and the identification of many genes since 1/3 to 1/4th of the total genome is now considered to be devoted to the construction of the cell wall. Too many genes indeed to be studied by a too low number of cell wall scientists to be able to reach a full picture of the cell wall synthesis metabolism which remains today poorly understood.

For a cell wall scientist, the question often arises: how did you fall into the "magic potion" of the cell wall research and spend your life to the study of the fungal cell wall. One reason has been for me the love of natural beauties: looking at swimming Entomophthorales protoplasts in an insect hemocoel look like scuba diving below a flight of Manta rays in an Indian ocean. For other scientists, the fungal cell wall was a way to obtain an international recognition: Toll like receptors or dectin1 would not have been identified without the use of β1,3 glucans, a major

component of the fungal cell wall. More down to earth for many other scientists, it has just been the desire to answer biological and mycological questions since the cell wall is a unique and essential organelle protecting the fungus against external stress; a role which has also attracted the diagnostic and drug industry.

The fungal cell wall is a fascinating topic but progresses in the area have been desperately slow. We hope that the reading of these chapters will stimulate the interest of many new young scientists. Dragging more people to this field is today an important challenge. A multi-facetted experimentalist approach of the cell wall with new biochemistry, immunology or cell biology technologies will certainly be exciting incentives.

I thank all the contributors to this Current topic for their patience during my editing and Anne for her support and help in the reviewing of the different chapters.

Heraklion, Crete, Greece Jean-Paul Latgé

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Synthetic Oligosaccharides Mimicking Fungal Cell Wall Polysaccharides

Vadim B. Krylov and Nikolay E. Nifantiev

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Abstract The cell wall of pathogenic fungi is highly important for the development of fungal infections and is the first cellular component to interact with the host immune system. The fungal cell wall is mainly built up of different polysaccharides representing ligands for pattern recognition receptors (PRRs) on immune cells and antibodies. Purified fungal polysaccharides are not easily available; in addition, they are structurally heterogenic and have wide molecular weight distribution that limits the possibility to use natural polysaccharides to assess the structure of their active determinants. The synthetic oligosaccharides of definite structure representing distinct polysaccharide fragments are indispensable tools for a variety of biological investigations and represent an advantageous alternative to natural polysaccharides. The attachment of a spacer group to these oligosaccharides permits their efficient transformation into immunogenic glycoconjugates as well as their immobilization on plates or microbeads. Herein, we summarize current information on synthetic availability of the variety of oligosaccharides related to main types of fungal cell wall components: galactomannan, α- and β-mannan, α- and β-(1 → 3)-glucan, chitin, chitosan, and others. These data are supplemented with published results of biochemical and immunological applications of synthetic oligosaccharides as

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Current Topics in Microbiology and Immunology (2020) 425: 1–16
https://doi.org/10.1007/82_2019_187
© Springer Nature Switzerland AG 2019
Published Online: 25 December 2019

molecular probes especially as the components of thematic glycoarrays suitable for characterization of anti-polysaccharide antibodies and cellular lectins or PRRs.

1 Introduction

Nowadays, the prevalence of fungal infections, especially severe invasive mycoses, is increasing all over the world and emerges as a challenge for modern medicine (Brown et al. 2012). The mortality rate associated with invasive fungal infections often exceeds 50%, which is connected both with delays in diagnostic and limited number of available anti-fungal drugs. Difficulties in disease management also arise from the poorly understood mechanism of interaction between the host immune system and fungal pathogens and especially the mechanism of fungal adaptation to host conditions allowing escape from the host defense reactions.

The fungal cell wall is the first component of a pathogen in contact with the immune system. The cell wall of most fungi contains a structural skeleton composed of chitin and branched β-(1 → 3)-glucan. This rigid central core is decorated with amorphous polysaccharides in which composition varies with the fungal species. Figure 1 is a schematic representation of the cell wall of different fungal species although the cellular localization of the different components of the cell wall is not clearly demonstrated for most polysaccharides. Moreover, the covalent and noncovalent forces holding together the different components remain often hypothetical.

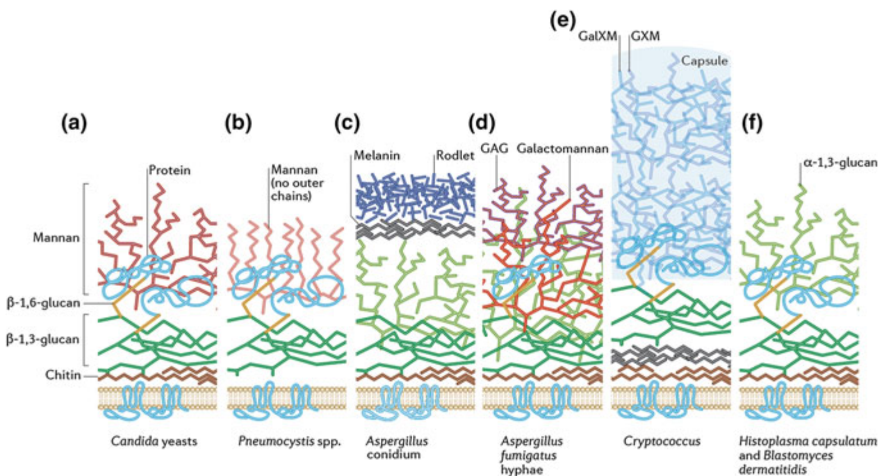

Fig. 1 Schematic view of cell wall composition of main human fungal pathogens. Reprinted by permission from Springer Nature: Nature Reviews Microbiology, vol. 14, Interactions of fungal pathogens with phagocytes, Erwig L.P., Gow N.A.R., pp. 163–176, Copyright, 2016

It is more and more obvious that polysaccharides play a major role in the immune interaction of the host with the pathogen (Hall and Gow 2013; Erwig and Gow 2016; Snarr et al. 2017). Moreover, cell wall polysaccharide antigenic markers of invasive fungal infections used in their diagnosis (Nelson et al. 1990; Tamura and Finkelman 2005; Cuenca-Estrella et al. 2012; Ullmann et al. 2018). In spite of the obvious importance of fungal cell wall polysaccharides for immune response modulation and for fungal infections diagnostic, little is known about the exact structure of their antigenic or immunogenic epitopes. This knowledge is essential for the understanding of the cellular immune response against the cell wall and for the development of diagnostic tests based on the use of circulating antigens antibodies.

To date, only the role of β-(1 → 3)-glucan and its associated receptor (Dectin 1) has been precisely analyzed (Brown 2006).

Precise quantitative analysis of carbohydrate-binding properties of anti-fungal lectins and antibodies could be performed using arrays composed of structurally defined glycans representing all the major carbohydrate structures present in the cell wall of fungal pathogens. The idea of using a comprehensive carbohydrate array for a wide range of glycobiology purposes has been proposed early by the Consortium for Functional Glycomics (CFG, http://www.functionalglycomics.org) since 2001 (Blixt et al. 2004; Raman et al. 2006). A number of thematic glycoarrays were designed and successfully applied to analyze viral and bacterial infections, cancer process, autoimmune disorders, etc. For example, microarrays composed of human glycans were used to survey the host specificity of different strains of influenza viruses (Stevens et al. 2006). Arrays were used for the analysis of glycan-specific antibodies against tumors in patient sera (Wang et al. 2008). An array composed of synthetic glycans related to cell wall polysaccharides from the human pathogen *Mycobacterium tuberculosis* and other mycobacteria was developed and applied for screening of specificity of immune system receptors (Zheng et al. 2017).

Various formats of arrays in which carbohydrate ligands are attached to microbeads, microtiter plate, chip, or glass slide have been used to study glycan–protein interactions. The immobilization techniques, though widely varying, can be divided into two main categories—noncovalent and covalent binding (Park et al. 2013) (Fig. 2). An example of noncovalent binding is attachment of lipid-conjugated oligosaccharides (neoglycolipids, NGLs) to nitrocellulose surface via hydrophobic interactions which is widely used to prepare glycan microarrays (Li and Feizi 2018) (Fig. 2a). Covalent and site-specific immobilization requires both introduction of a reactive functional group into the glycan and chemical derivatization of the surface (*e.g.* attachment of amine-linked sugars to the N-hydroxysuccinimide ester-coated surface, Fig. 2b) (Blixt et al. 2004).

Another immobilization strategy is based on a very strong biotin–streptavidin interaction ($K_d \sim 10^{-15}$ M) which, being formally noncovalent, is energetically closer to covalent binding. The attachment of biotin-linked sugars to the streptavidin-coated surface (Fig. 2c) has strong advantages. First of all, the amount of ligand is defined by the binding capacity of the surface which is well

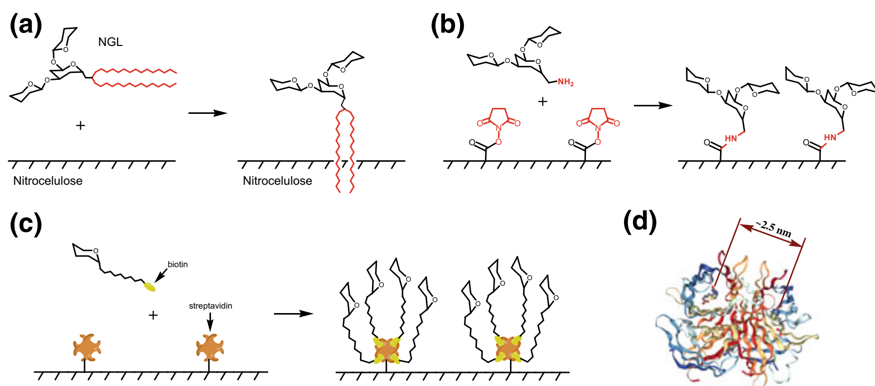

Fig. 2 Strategies of glycan immobilization on a surface. **a** noncovalent attachment of lipid-conjugated glycans to nitrocellulose; **b** covalent attachment of amine-linked sugars to the NHS ester-coated surface; **c** attachment via biotin–streptavidin interaction; and **d** structure of biotin–streptavidin complex (PDB: 6j6j), the distance between biotin-binding sites is shown

documented. Thus, the molar density of the coating glycan is constant and does not depend on its molecular weight, charge, and hydrophilic/hydrophobic properties. Another advantage is the standardized distance between binding sites. The streptavidin molecule is composed of four biotin-binding subunits with the average distance between biotin molecules of about 2.5 nm. For a standard commercial microtiter plate with the biotin-binding capacity of 5 pmol per well, the calculated average distance between biotin-binding streptavidin subunits is 6.1 nm assuming their uniform distribution. This exceeds the size of the immobilized oligosaccharide molecules allowing the study of the interaction properties of individual carbohydrate ligands (Galanina et al. 2003).

The value of carbohydrate arrays as a powerful and easy tool for investigation of protein–carbohydrate interactions is well recognized. However, there are no publications reporting a comprehensive glycoarray covering all the polysaccharide types of the fungal cell wall. In this review, we summarize the currently available information on synthetic availability of the variety of oligosaccharides related to major fungal cell wall polysaccharides.

2 Thematic Glycoarrays

Due to their insolubility and their complex heterogenic structure, pure fungal polysaccharides are not easy to obtain. This complicates the possibility to use them as probes to investigate their immune role. An alternative to natural polysaccharides is to use synthetic oligosaccharides of definite structure representing distinct polysaccharide fragments. For over 15 years, our laboratory has been performing

systematic synthesis of oligosaccharides related to the main types of fungal cell wall polysaccharides: galactomannan, α- and β-mannans, α- and β-(1 → 3)-glucans, chitin, galactosaminogalactan, galactoxylomannan, and others. The use of such oligosaccharides is illustrated with examples of biochemical and immunological applications of the use of synthetic oligosaccharides as molecular probes suitable for the characterization of anti-polysaccharide antibodies and cellular lectins.

2.1 Galactomannan

A specific carbohydrate antigen produced by *Aspergillus* and *Penicillium* species is a galactomannan (Latgé et al. 1994). It is a complex heteropolysaccharide built up of mannopyranose and galactofuranose monosaccharide residues. Latgé et al. (1994) proposed its structure representing α-(1 → 2)-/α-(1 → 6)-linked poly-D-mannoside backbone bearing β-(1 → 5)-linked oligogalactofuranoside side chains attached to some of the mannose units via either β-(1 → 3)- or β-(1 → 6)-bonds. Shibata et al. (Kudoh et al. 2015; Krylov et al. 2018a) revealed the presence of additional structural elements: β-(1 → 6)-linkage within the oligogalactofuranoside side chain and in addition to β-(1 → 3)- or β-(1 → 6)-bonds, β-(1 → 2)-attachment of the galactofuranoside side chain to the mannan backbone (Fig. 3a).

The first synthesized oligosaccharide fragments of the galactomannan corresponding to the homo-galactofuranosyl chains of variable length were either naïve or biotinylated (Veeneman et al. 1987; Zuurmond et al. 1990; Cattiaux et al. 2011). The first synthesis of a heterosaccharide fragment containing both galactofuranosyl and mannopyranosyl residues was then undertaken (Fu et al. 2005). Recently, galactomannan-related oligosaccharides (Argunov et al. 2015, 2016; Krylov et al. 2018a) have been synthesized employing an alternative synthetic strategy based on a new reaction in carbohydrate chemistry, namely pyranoside-*into*-furanoside rearrangement (Krylov et al. 2014, 2016; Gerbst et al. 2019), and controlled O(5) → O(6) benzoyl migration (Argunov et al. 2016). A library of 13 synthetic oligosaccharides (shown in Fig. 3B) was prepared in the form of aminopropyl glycosides allowing their further modification, conjugation, and application in different fields of glycoscience.

The synthetic galactomannan fragments (**10** on Fig. 3b) and immunogens prepared thereof were used for generation of monoclonal antibodies (mAb) and characterization of their specificity (Matveev et al. 2018). Thus, 7B8 and 8G4 mAbs, obtained by immunization of mice with BSA-conjugate of the synthetic pentasaccharide, efficiently recognized natural galactomannan of *A. fumigatus.*

The glycoarray formed from biotinylated oligosaccharides related to galactomannan (Fig. 3b) was used to establish fine carbohydrate specificity of anti-galactomannan poly- and monoclonal antibodies.

The galactomannan glycoarray was employed to reinvestigate the carbohydrate specificity of the EB-A2 monoclonal antibody used in the commercial Aspergillus sandwich immune assay. It was shown that EB-A2 could recognize

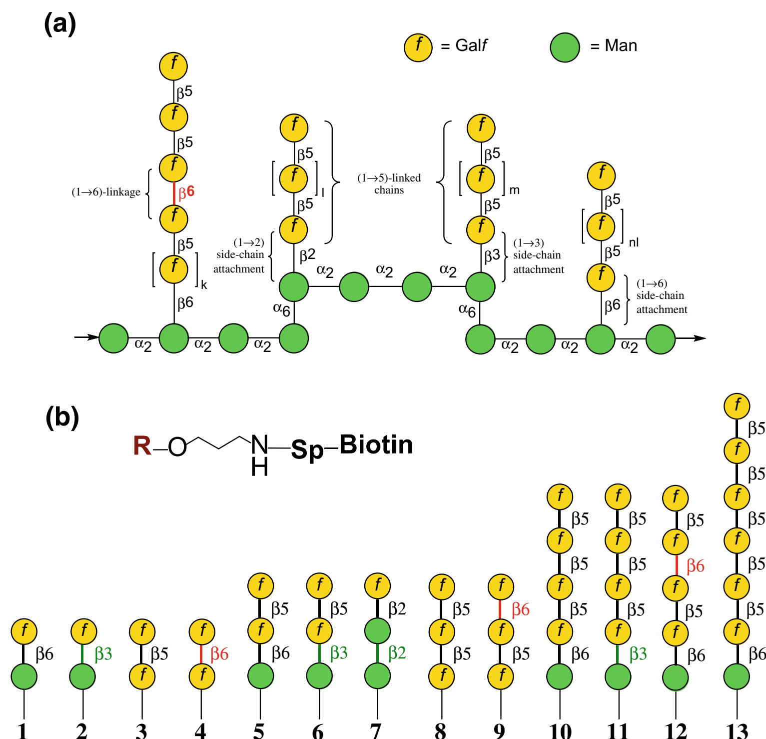

Fig. 3 **a** Tentative structure of galactomannan summarizing the structural features reported by Latgé (Latgé et al. 1994) and Shibata (Kudoh et al. 2015); **b** synthetic oligosaccharides prepared as aminopropyl glycosides. The carbohydrate sequences are represented according to symbol carbohydrate nomenclature (Neelamegham et al. 2019)

oligosaccharides sequences containing only disaccharide Galf-β-(1 → 5)-Galf fragment (Krylov et al. 2019), but not the tetrasaccharide one as had been reported previously (Stynen et al. 1992). This result could explain the occurrence of false-positive signals due to the presence of such epitope not only in *A. fumigatus* galactomannan but also in other bacteria and non-Aspergillus fungi. Monoclonal antibodies 7B8 and 8G4 recognize longer oligosaccharides sequences (Matveev et al. 2018) containing β-(1 → 5)-linked trigalactofuranoside (**8** on Fig. 3b) and pentasaccharide β-D-Galf-[(1 → 5)-β-D-Galf]$_3$-(1 → 6)-α-D-Manp (**10** on Fig. 3b) respectively.

Assaying of polyclonal rabbit antibodies obtained using different preparations of *Aspergillus* species as immunogens and purified on affine sorbent revealed different specificity profiles of anti-galactomannan antibodies. In all the cases, the smallest carbohydrate fragment recognized by the pAb consisted of two β-(1 → 5)-linked galactofuranosyl residues (Krylov et al. 2018c, b).

Synthetic oligosaccharides were also used to investigate the immunobiological activity of the galactomannan. Its action was studied using RAW 264.7 cell line murine macrophages and human PBMCs. Significant immunomodulating efficacy of the galactomannan-related oligosaccharides was established by a proliferation/cytotoxicity assay, phagocytosis and inductive cytokines, and growth factors release (Paulovičová et al. 2017). Oligosaccharides with Galf(1 → 5)Galf blocks was shown to induce the secretion of cytokines and chemokines by immune cells (Wong et al. 2020).

2.2 α- and β-Mannan

The outer layer of the cell wall of *Candida* species consists of mannoproteins. Their mannan moieties are important for host–fungal interactions and virulence. The mannan has a comb-like structure with an α-(1 → 6)-linked backbone bearing different types of oligomannoside side chains depending on the fungal species (Shibata et al. 2012) (Fig. 4a). Some oligosaccharides have a phosphodiester linkage which can be selectively cleaved by treatment with a weak acid solution, releasing the "acid-labile" fraction of the mannan. Early studies have shown that these mannoside fragments are responsible for the serotyping of *Candida* species and are so-called antigenic factors (Fig. 4a) (Suzuki 1997). Numerous studies have also shown the essential immunological role of these mannans (Netea et al. 2008).

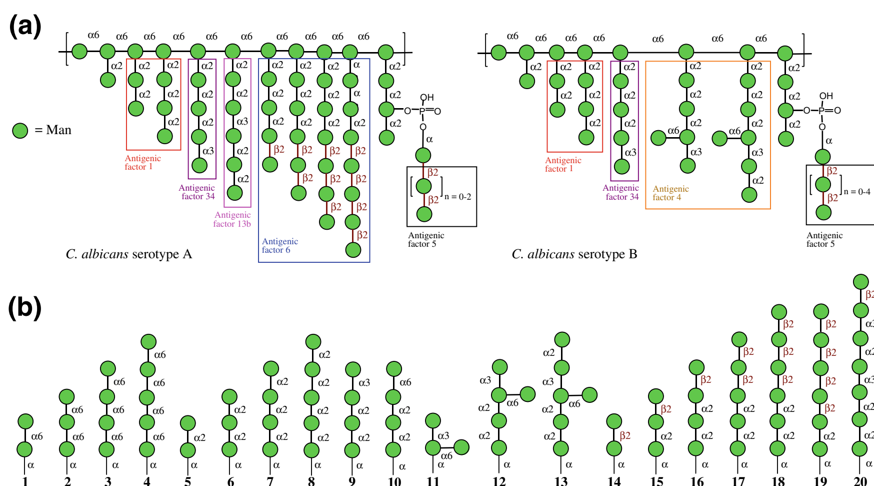

Fig. 4 **a** Structure of the cell wall mannan of *C. albicans* serotypes A and B with marked structures of main antigenic factors; **b** synthesized oligosaccharides related to mannan fragments. All synthetic ligands were prepared as aminopropyl glycosides. The carbohydrate sequences are represented according to symbol carbohydrate nomenclature (Neelamegham et al. 2019)

The synthesis of oligosaccharides related to fragments of yeast cell wall mannans was described in a monograph published in 2009 (Collot et al. 2009). The synthetic works published afterward were considered in a review by Karelin et al. in 2017 (Karelin et al. 2017). The systematic syntheses of oligosaccharides related to *Candida* mannan have been performed in our laboratory since 2007 and allow construction of a representative library of aminopropyl glycosides corresponding to main antigenic factors of yeast pathogens (Fig. 4b). For example, linear α-(1 → 6)-linked oligosaccharides **1–4** represent the mannan backbone, α-(1 → 2)-linked oligosaccharides **5–8** correspond to antigenic factor 1 (Karelin et al. 2007), and branched oligosaccharides **11–13** are related to antigenic factor 4 (Karelin et al. 2010; Argunov et al. 2011).

Preparation of β-linked oligomannosides is a special problem due to the lack of efficient and reliable corresponding synthetic methods. This task commonly requires application of original approaches. Chemo-enzymatic synthesis was developed for preparation of disaccharide ligand **14** with a β-mannoside linkage. It included chemical cleavage and enzymatic dephosphorylation of biotechnologically available phosphomannan followed by its chemical derivatization (Karelin et al. 2019). The strategy based on direct β-mannosylation with conformationally rigid 4,6-O-benzylidene protected mannosyl donors was applied for the synthesis of ligands **15**–**20** related to antigenic factors 5 and 6 (Karelin et al. 2015, 2016).

Synthetic oligosaccharides and corresponding conjugates mimicking *C. albicans* mannan were employed to study the interactions of immune cells with *Candida*. BSA-based conjugates of synthetic oligomannosides effectively induced humoral and cell-mediated immunity. The immunomodulating activity of the conjugates was evaluated based on the induction of pro-inflammatory cytokines. Mice immunization with BSA-conjugates resulted in the production of polyclonal antibodies against synthetic oligosaccharides that were capable of recognizing branched α-oligomannoside structures on the cell wall of *C. albicans* yeast and hyphae (Paulovičová et al. 2013a). Additionally, the anti-sera obtained after mice immunization with BSA-mannooligosaccharides conjugates were able to enhance phagocytosis of *C. albicans* cells by polymorphonuclear leukocytes (Paulovicová et al. 2010; Paulovičová et al. 2013b). It is also worth to mention that the collected databases of NMR-spectral characteristics for synthesized oligosaccharides can be used for the development of computer-assisted method for structural analysis of fungal mannans as it was applied for bacterial polysaccharides including vicinally branched ones (Lipkind et al. 1992).

2.3 α- and β-(1 → 3)-Glucans

Although the glucans (and especially β-(1 → 3)-D-glucan with their associated Dectin1 receptor) have been shown to be essential in the pathogenic life of different fungi, their immunological role has been insufficiently analyzed. The main restriction for the use of glucans from natural sources arises from their limited

solubility. Accordingly, the soluble oligosaccharides related to specified glucan fragments and conjugates thereof are highly demanded mimetics of such polysaccharides.

The synthetic approaches to β-(1 → 3)-D-glucan-related oligosaccharides are relatively well elaborated (Tsvetkov et al. 2015). Syntheses of linear (Yashunsky et al. 2016a) and branched (Yashunsky et al. 2016b) 3-aminopropyl glycosides of β-(1 → 3)-D-glucooligosaccharides were performed. β-(1 → 6)-D-Glucotetraoside and the corresponding biotinylated derivative was synthesized from monosaccharide building blocks via successive glycosylation with a monosaccharide donor (Yashunsky et al. 2018).

The synthesis of α-(1 → 3)-glucooligosaccharides with a predefined structure required special methods of 1,2-*cis*-glycosylation. Strategies based on remote participation of acyl protecting groups were developed (Komarova et al. 2014, 2016) and successfully applied (Komarova et al. 2015, 2018) to obtain oligo-α-(1 → 3)-D-glucosides containing from three to eleven glucose units (Fig. 5). An alternative synthetic approach to the assembly of oligosaccharides related to α-(1 → 3)-glucans (Wang et al. 2019) and α-(1 → 4)-glucans (Wang et al. 2018) was suggested in Codée's group. However, to the best of our knowledge, the results of their biochemical and immunological application studies have not been published yet.

Synthetically prepared oligosaccharides were used to generate mono- and polyclonal antibodies. For example, mouse monoclonal antibodies 3G11 and 5H5 were generated using synthetic nona-β-(1 → 3)-D-glucoside conjugated with the bovine serum albumin (G9-BSA) (Matveev et al. 2019). The glycoarray which included linear and branched synthetic oligosaccharide demonstrated that 5H5 mAbs recognized the linear triglucoside fragment, while 3G11 mAbs could bind the pentasaccharide. In addition, anti-*C. albicans* activity of 3G11 and 5H5 mAbs was demonstrated in vivo and in vitro experiments. Active immunization by synthetically prepared G9-BSA also had revealed effective induction of specific humoral responses against *C. albicans* infection (Paulovičová et al. 2016).

Immunization of mice with the BSA-conjugate of penta-α-(1 → 3)-D-glucoside led to generation of antibodies that recognized α-glucan on *A. fumigatus* cell surface

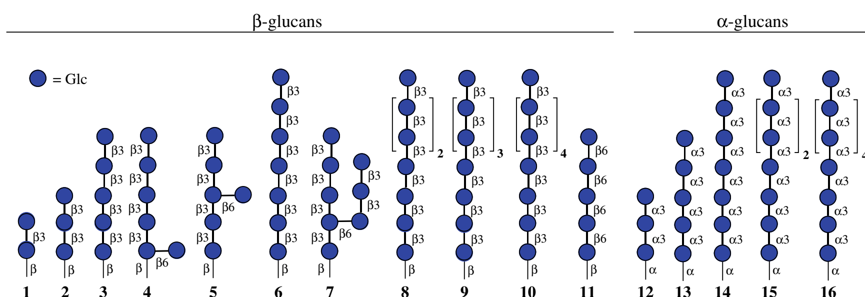

Fig. 5 Oligosaccharides related to α- and β-glucans

(Komarova et al. 2015). Additionally, synthetic biotinylated oligo-α-glucosides loaded on a streptavidin-coated plate were able to recognize human anti-α-glucan antibodies in sera of patients with aspergillosis and induced cytokine responses upon stimulation of human peripheral blood mononuclear cells (Komarova et al. 2018).

2.4 Polysaccharides Composed of 2-Deoxy-2-Aminosugars

In this section, a group of cell wall polysaccharides and corresponding oligosaccharide fragments composed of amino sugars is considered. The most common among them is chitin, an insoluble linear polymer, built up of β-(1 → 4)-linked *N*-acetylglucosamine units. This polysaccharide forms the inner layer of the fungal cell wall and is responsible for its rigidity (Erwig and Gow 2016). Deacetylated chitin, termed chitosan, is produced by many fungal species during their life cycle and plays regulatory and signaling roles. Chitin blocks the recognition of *C. albicans* by human PBMCs and murine macrophages, leading to significant reductions in cytokine production (Mora-Montes et al. 2011). Galactosaminogalactan (GAG) is the polysaccharide exposed on the outer surface of *A. fumigatus* cells. It acts as an immunosuppressor helping the fungal pathogen to survive in the host medium (Fontaine et al. 2011). Structurally, GAG represents a linear heterogeneous polysaccharide composed of α-(1 → 4)-linked galactose and N-acetylgalactosamine units. Both monosacharides are randomly distributed along the chain with the percentage of galactose ranging from 15 to 60%. GAG inhibits pro-inflammatory Th17 and Th1 responses in human peripheral blood mononuclear cells by inducing the expression of the anti-inflammatory interleukin-1 receptor antagonist (IL-1Ra), which blocks IL-1β signaling (Gresnigt et al. 2014). Additionally, immunosuppressive GAG activity associated with diminishing neutrophil infiltrates was observed in in vivo experiments (Fontaine et al. 2011).

The matter of a special interest is β-(1 → 6)–linked poly-*N*-acetyl-D-glucosamine (PNAG). Originally, it was isolated from the polysaccharide capsule of *Staphylococcus aureus* and developed as a promising target for antibacterial therapy and prophylaxis (Gening et al. 2007, 2010). To the best of our knowledge, this polysaccharide was not isolated from fungal species, though immunofluorescence labeling with specific monoclonal antibodies detected PNAG in such fungal species as *C. albicans*, *A. flavus*, *Fusarium solani*, and *C. neoformans*, additionally anti-PNAG antibodies have protective effect against these fungi (Cywes-Bentley et al. 2013).

Due to its poor solubility, using natural chitin for immunobiological studies is difficult. Synthetic chitooligosaccharides do not have these disadvantages. Thus, spacer-armed chitotriose, chitopentaose, and chitoheptaose and their biotinylated derivatives were prepared using a properly protected monosaccharide acceptor and a disaccharide donor (Yudina et al. 2015, 2016). The synthetic scheme developed for synthesis of GAG-related oligosaccharides involved the use of phenyl

2-azido-2-deoxy-1-seleno-α-D-galactosides (Mironov et al. 2004) allowing highly α-stereoselective formation of the glycoside bond (Khatuntseva et al. 2016). The synthesis of the GAG-related biotinylated oligo-α-(1 → 4)-D-galactosamines comprising from 2 to 6 monosaccharide units and of N-acetylated derivatives of above glycoconjugates was performed. Obtained series of GAG mimetics was used to investigate the epitopes recognized by anti-GAG monoclonal antibodies and of antibodies in blood sera of patients with allergic bronchopulmonary and chronic pulmonary aspergilloses (Kazakova et al. 2020) (Fig. 6).

3 Conclusions

The elucidation of the immune response modulation by fungal cell wall components is a highly important but a very complex task. The purification of standardized individual cell wall polysaccharides is almost impossible due to high variability and sensitivity of their structure to minor changes in cultivation conditions. Moreover, natural polysaccharides are highly heterogenic which can result in a high variability between the immune response against different strains.

The approach based on the use of synthetically prepared fragments related to fungal cell wall glycans is the most appropriate to elucidate the complex immune response against the fungal cell wall. In this review, we summarized recent works on synthesis of oligosaccharides related to polysaccharides of the fungal cell wall and a few examples of their potential to investigate their interactions with the host. All synthetic molecules were prepared in spacer-armed form which made possible their biotinylation and controlled site-specific immobilization on streptavidin-coated microtiter plates. The oligosaccharide ligands were combined to thematic glycoarrays used to determine fine specificity of antibodies and innate immune

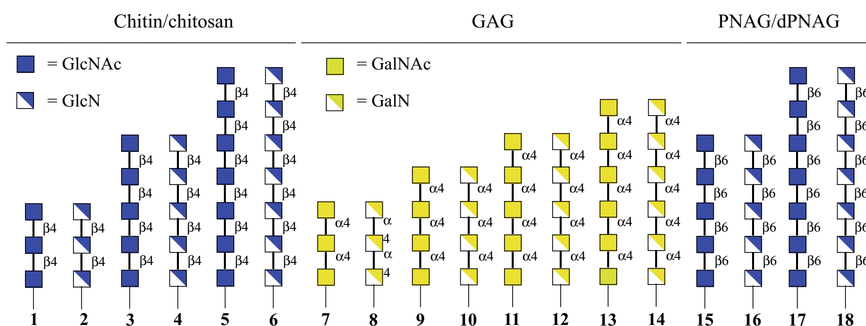

Fig. 6 Synthetic oligosaccharides related to polysaccharides composed of N-acetylated and non-acetylated aminosugars: chitin/chitosan; galactosaminogalactan (GAG); and β-(1 → 6)–linked poly-*N*-acetyl-D-glucosamine (PNAG) and its deacetylated derivative (dPNAG). The carbohydrate sequences are represented according to symbol carbohydrate nomenclature (Neelamegham et al. 2019)

system receptors. The data obtained with such glycoarrays have obvious practical importance for development of new diagnostic kits and vaccines for treatment and prophylaxis of fungal infections.

Acknowledgements This work was supported by the Russian Science Foundation (grant 19-73-30017). We thank Professor J.-P. Latgé and Dr. A. G. Gerbst for critical reading this manuscript and valuable comments.

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Aspergillus fumigatus DHN-Melanin

Georgios Chamilos and Agostinho Carvalho

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Abstract Dihydroxynaphthalene melanin (DHN-melanin) is an integral component of the conidial cell wall surface, which has a central role in the pathogenicity of the major human airborne fungal pathogen *Aspergillus fumigatus*. Although the biosynthetic pathway for *A. fumigatus* DHN-melanin production has been well characterized, the molecular interactions of DHN-melanin with the immune system have been incompletely understood. Recent studies demonstrated that apart from concealing immunostimulatory cell wall polysaccharides, calcium sequestration by DHN-melanin inhibits essential host effector pathways regulating phagosome biogenesis and prevents *A. fumigatus* conidia killing by phagocytes. From the host perspective, DHN-melanin is specifically recognized by a C-type lectin receptor

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Current Topics in Microbiology and Immunology (2020) 425: 17–28
https://doi.org/10.1007/82_2020_205
© Springer Nature Switzerland AG 2020
Published Online: 9 May 2020

(MelLeC) present in murine endothelia and in human myeloid cells. Furthermore, DHN-melanin activates platelets and facilitates opsonophagocytosis by macrophages via binding to soluble pattern recognition receptors. Dissecting the dynamics of DHN-melanin organization on the fungal cell wall and the molecular interplay with the immune system will lead to a better understanding of *A. fumigatus* pathophysiology.

1 Introduction

Melanins have evolved over 500 million years across all animal kingdoms, yet their biological functions remain enigmatic (Riley 1997; Burkhart and Burkhart 2005). Melanin pigments are ubiquitous light-absorbing polymers of various chemical composition, undetermined structure, and unique physicochemical properties (Riley 1997; Fogarty and Tobin 1996; Smith and Casadevall 2019; Solano 2014). Apart from conferring protection against UV light, mechanical, chemical, and thermal stress, melanins act as powerful cation chelators and anti-oxidants, bind and inactivate microbicidal peptides and antimicrobial drugs, inhibit activation of enzymes, and confer tolerance to dehydration (Riley 1997; Fogarty and Tobin 1996; Smith and Casadevall 2019; Solano 2014). Therefore, melanins might have evolved to confer protection against environmental stressors, including microbial predators (Burkhart and Burkhart 2005).

In infectious diseases, melanins serve as virulence factors that confer protection to microbial pathogens upon interactions with the host immune system (Smith and Casadevall 2019; Langfelder et al. 2003; Solano 2014). On the host perspective, melanins exert important immunomodulatory actions. Of interest, in invertebrates, melanins serve as antimicrobial effectors. In particular, activation of innate pattern recognition receptors (PRRs) by microbial ligands triggers the prophenoloxidase pathway of melanization, which acts as a primitive complement system of vertebrates (Burkhart and Burkhart 2005).This melanization response pathway results in the encapsulation of larger organisms, including parasites, and killing via the production of toxic-free radicals, quinine intermediate compounds, and other antimicrobial effectors. Notably, fungal melanin seems to activate the alternative complement pathway in mammalian serum (Rosas et al. 2002; Pinto et al. 2018).

In fungi, cell wall melanin has a central role in fungal survival and virulence during interaction with the host immune response (Kuo and Alexander 1967; Smith and Casadevall 2019; Perez-Cuesta et al. 2019). Fungal melanins are negatively charged, hydrophobic pigments of high molecular weight produced by oxidative polymerization of phenolic or indolic compounds, including catechol, 1,8-dihydroxynaphthalene (DHN) or 3,4-dihydroxyphenylalanine (DOPA). Most Ascomycota, including *Aspergillus fumigatus* synthesize DHN-melanin, whereas few species including *A. nidulans* are able to produce melanin through L-DOPA.

Furthermore, some fungi including *A. fumigatus* produce pyomelanins, resulting from the breakdown of aromatic amino acids (Heinekamp et al. 2013). This review will discuss recent advances in understanding biology and interactions of *A. fumigatus* cell wall DHN-melanin with the immune system.

A. Topology, gene regulation, and biosynthesis of melanin on *A. fumigatus* cell wall

Topology of DHN-melanin in dormant conidia. Melanin—dihydroxynaphthalene (DHN-melanin) is an integral component of *A. fumigatus* cell wall that accounts for the brownish-gray color of conidia (Tsai et al. 1998; Tsai et al. 1999; Langfelder et al. 1998). In particular, the outer layer of the *A. fumigatus* conidium is mainly composed of a hydrophobic rodlet protein layer (Aimanianda et al. 2009). DHN-melanin is located below the rodlet layer with melanin patches exposed on the surface, which account for the echinulate surface of dormant conidia of *A. fumigatus*in electron microscopy studies (Tsai et al. 1998; Bayry et al. 2014). Both melanin and rodlet layer have been traditionally regarded as immunologically inert molecules that conceal fungal pattern recognition molecular patterns (PAMPs) and prevent activation of PRRs (Aimanianda et al. 2009; Bayry et al. 2014).

The molecular interactions of DHN-melanin on *A. fumigatus*cell wall have not been precisely characterized. Atomic force microscopy studies suggest that DHN-melanin physically interacts with hydrophobins, since pigmentless *A. fumigatus* mutants lack the surface rodlet protein layer (Bayry et al. 2014; Pihet et al. 2009). Accordingly, genetic or chemical removal of the rodlet layer results in higher surface exposure of DHN-melanin and in potent inhibition of host immune responses in macrophages (Akoumianaki et al. 2016). Of interest, in *Cryptococcus*, melanin is organized in spherical structures termed melanin granules, cell wall micro domains of 200 nM size, which are further composed of nanospheres of 30 nM diameter that resemble mammalian melanosomes (Camacho et al. 2019). This model of cell wall melanin organization allows for physical separation of the melanin layer during cell division. Additionally, there is evidence of release of these melanin nanospheres in exosomes (Smith and Casadevall 2019). It is tempting to speculate that a similar model of cell wall organization and melanin release is relevant to *A. fumigatus*. New biophysical approaches such as solid-NMR studies could provide essential clues on molecular interactions of melanin with other cell wall components of *A. fumigatus* (Kang et al. 2018).

DHN-melanin surface removal during cell wall remodeling. The fate of cell wall melanin during *A. fumigatus* germination remains enigmatic. Of interest, although present on early germinating conidia, DHN-melanin is no longer recognized by the specialized host receptor MelLeC within 4 h of conidial swelling (Stappers et al. 2018). Similarly, activation of physiological immune responses, which are specifically inhibited by DHN-melanin, occurs within 2–4 h following phagocytosis of *A. fumigatus* conidia by monocytes/macrophages (Kyrmizi et al. 2018). Therefore, removal of DHN-melanin from the conidial surface results in its

functional inactivation. Importantly, the mechanism of removal or alteration of DHN-melanin from the conidial surface during germination is not understood. It is only known that (i) *A. fumigatus* is able to degrade DOPA-melanins of exogenous sources via uncharacterized enzymatic mechanisms (Luther and Lipke 1980); (ii) non-enzymatic degradation of different melanin pigments, including *A. fumigatus* DHN-melanin can also be achieved within few min of exposure to hydrogen peroxide in vitro (Akoumianaki et al. 2016; Korytowski and Sarna 1990). Whether this is a physiologically relevant mechanism upon activation of NADPH oxidase complex mediated ROS production inside the phagosome remains to be tested.

Biosynthesis of DHN-melanin and cell wall deposition. In *A. fumigatus*, the polyketide-derived DHN-melanin is synthesized by six enzymes encoded by a gene cluster located on the second chromosome (Tsai et al. 1999) (Fig. 1).The polyketide synthase PKS or ALB1 (for "albino 1") is responsible for the first biosynthetic step in *A. fumigatus*, resulting in the biosynthesis of the heptaketide naphthopyrone YWA1 from the substrates acetyl-CoA and malonyl-CoA. A series of subsequent enzymatic reactions carried out by Ayg1, Arp2, Arp1, and Arp2 through hydrolysis of YWA1 to produce 1,3,6,8-tetrahydroxynapthalene (T4HN) by Ayg1, followed by serial reduction and dehydration reactions to produce scytalone, 1,3,8-trihydroxynapthalene (THN), and vermelone. Vermelone is next dehydrated to form 1,8-DHN (DHN), which is then oxidized and polymerized by the copper oxidase Abr1 and the laccase Abr2 to form mature melanin (Tsai et al. 1999; Upadhyay et al. 2013).

Recent seminal studies have revealed important information on spatial and temporal regulation of biosynthesis of *A. fumigatus* DHN-melanin. Notably, the enzymes that function prior to the vermelone production, also called as early enzymes in DHN-melanin biosynthesis (Alb1/Ayg1/Arp1/Arp2), are post-translationally modified via palmitoylation for endosomal sorting and subsequently recruited to the secretory pathway via a non-canonical mechanisms that involves sorting nexins (Upadhyay 2016a, b). In contrast, the late enzymes are (Abr1/Abr2) located to the cell wall surface via trafficking through the classical secretory pathway. This mechanism of trafficking of enzymes implicated in *A. fumigatus* melanin biosynthesis likely ensures tightly regulated production and

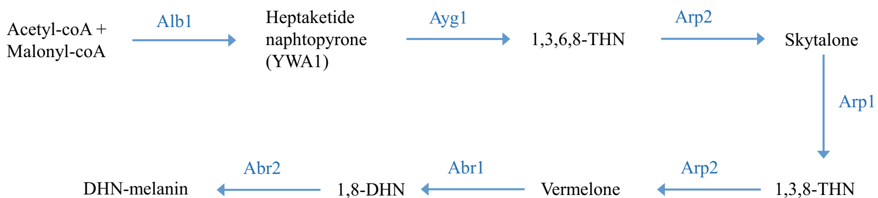

Fig. 1 Biosynthetic pathway of DHN-melanin in *A. fumigatus*. The initial step in melanin biosynthesis is regulated by pksP or alb1 gene. Adapted from Tsai et al. (1998)

has striking similarities with the compartmentalization of synthesis of mammalian melanins in melanosomes (Upadhyay 2016a, b).

Gene regulation of DHN-melanin production. The transcriptional control of DHN-melanin production is incompletely understood. Multiple pathways activated by G protein-coupled receptors (GPCRs) converge to activation of transcriptional regulators of pksP gene cluster control conidium formation, development, and melanin biosynthesis in *A. fumigatus*. The cAMP pathway activates the gene cluster of DHN-melanin biosynthesis via protein kinase catalytic unit 1 (PKaC1). MAPK signaling pathways, including MpkB and MpkA, which have antagonistic interactions, activate the master transcriptional regulator RlmA that binds directly to the pksP promoter (Manfiolli et al. 2019).

B. Biological properties and pathogenetic role of melanin

In vivo *virulence studies*. Virulence testing performed via the intravenous route in immunocompetent mice confirmed the marked decrease in pathogenicity of alb1 mutant as compared to the wild-type *A. fumigatus* strain (Jahn et al. 1997; Tsai et al. 1998; Langfelder et al. 1998). The alb1 mutant also is completely avirulent following pulmonary infection of mice immunosuppressed with cyclophosphamide (Akoumianaki et al. 2016). Of interest, infection of *Drosophila melanogasterToll*-deficient mutants via the oral or thoracic route with alb1 mutant resulted in attenuated pathogenicity, implying that the role of *A. fumigatus* melanin in virulence is evolutionarily conserved (Lionakis et al. 2005). In contrast, mutation in abr2 laccase encoding gene did not affect the virulence of *A. fumigatus* (Sugareva et al. 2006). Notably, the contribution of other genes of DHN-melanin biosynthesis in virulence has not been tested in vivo. In contrast, in the invertebrate experimental system *Galleria mellonela*, mutants in the biosynthetic pathway of DHN-melanin generated in two different genetic backgrounds displayed increased virulence (Jackson et al. 2009). This paradoxical increase in virulence might be related to an exuberant host immune response by *Galleria* phagocytes coupled with a robust melanization reaction; however, this hypothesis has not been explored mechanistically.

Interactions of melanin with professional phagocytes cells. In view of the central role of myeloid cells in immunity against *A. fumigatus*, the pathogenetic role of melanin has been predominantly tested during interactions with monocytes/ macrophages and neutrophils (Tsai et al. 1998; Langfelder et al. 1998; Jahn et al. 1997, 2000, 2002). These studies revealed the heightened susceptibility of melanin-deficient (abino) mutants to killing by macrophages and neutrophils in comparison with the wild-type isogenic conidia. Because the enhanced susceptibility of pksP mutant was accompanied by a tenfold increase in ROS as compared to the wild-type strain, it was concluded that melanin somehow compromises the ability of phagocytic cells to activate ROS production, which is consistent with the ROS scavenging properties of melanin pigments. Follow up studies suggested that

DHN-melanin has a direct inhibitory action on phagosome acidification and phagolysosomal fusion in monocytes/macrophages possibly via disruption of V-ATPase assembly to the phagosome, which correlated with the increased resistance of melanized conidia to killing by phagocytes (Thywißen et al. 2011; Heinekamp et al. 2013). However, a mechanistic explanation on the ability of DHN-melanin to inhibit phagosome responses remained elusive.

Targeting of LC3 associate phagocytosis (LAP) by melanin. In recent years, the molecular machinery regulating phagosome maturation has been characterized. Seminal work demonstrated that activation of certain PRR triggers recruitment of components of the autophagy pathway to the phagosome and promotes phagolysosomal fusion (Sanjuan et al. 2007). This specialized autophagic pathway termed LC3-associated phagocytosis (LAP) requires certain members of the autophagic machinery, activation of NADPH oxidase, and is regulated by Rubicon (Martinez et al. 2015). LAP has an important role in killing of conidia by mononuclear phagocytes and in physiological immunity against *A. fumigatus* (Kyrmizi et al. 2018; Akoumianaki et al. 2016; Martinez et al. 2015). *A. fumigatus* conidia selectively trigger the activation of LAP during intracellular germination via the Dectin-1 signaling pathway (Kyrmizi et al. 2018). Of interest, melanin targets the LAP pathway by interrupting the assembly of NADPH oxidase complex on the phagosome (Akoumianaki et al. 2016). This response is important for fungal pathogenicity as albino conidia recover full virulence in the setting of conditional inactivation of LAP in myeloid cells. Importantly, melanin inhibits a calcium/calmodulin signaling pathway regulating the activation of LAP (Kyrmizi et al. 2018). Specifically, calcium scavenging by melanin inside the phagosome lumen inhibits the recruitment of calmodulin to the phagosome, abrogating Rubicon-induced activation of NADPH oxidase and LAP. Whether this melanin calcium binding inhibitory action interferes with specialized calcium channel activation is currently unknown. Because calcium/calmodulin signaling is an upstream regulator of phagosome acidification (Kyrmizi et al. 2018), this mechanism explains the effect of melanin on other downstream phagosome responses, including V-ATPase recruitment for phagosome acidification. Recent phagosome proteomics studies indicate that melanin impacts on major host effector mechanisms including iron homeostasis, mTOR signaling and metabolism, and endosomal trafficking (Schmidt et al. 2018). Whether these differences in phagosome response of albino and melanized conidia are related to inhibition of calcium signaling, other immune effector pathways, or an effect of concealing of PAMPs remains to be elucidated. Importantly, all the aforementioned virulence properties of *A. fumigatus* DHN-melanin have been observed with DOPA-melanin of fungal or synthetic origin arguing for a lack of specificity of the immune role of fungal melanin (Akoumianaki et al. 2016).

Host recognition of DHN-melanin. A novelC-type lectin receptor, termed melanin-sensing C-type lectin receptor (MelLec), specifically recognizes DHN-melanin of *A. fumigatus* and other melanized fungi (Stappers et al. 2018). MelLec recognizes the naphthalene-diol unit of 1,8-dihydroxynaphthalene and has an important role in immunity against *A. fumigatus*. Of interest, in mice, MelLec is expressed in endothelia and a sub-population of epithelial cells and is absent from myeloid cells. In contrast, in humans, MelLec is expressed in myeloid cells and hypo-functional polymorphisms result in attenuated cytokine responses and increased risk for invasive aspergillosis in stem cell transplant recipients. Apart from MelLec, other innate immune mechanisms are also activated by *A. fumigatus* melanin. Of interest, melanin triggers the activation of platelets following *A. fumigatus* infection (Rambach et al. 2015), and these innate immune cells participate in antifungal host defense.

Finally, DHN-melanin activates PI3K/Akt signaling pathway to inhibit apoptosis during interactions with macrophages and epithelia (Volling et al. 2011; Amin et al. 2014). This important virulence mechanism could be used as a Trojan horse by the fungus to establish prolonged intracellular persistence. In addition, because PI3K/Akt signaling is an upstream activator of mTOR, a major regulator of cell metabolism, it would be interesting to explore the effect of melanin on shaping the metabolism of host immune cells.

2 Conclusions and Unresolved Questions on Biology of *A. fumigatus* melanin

Once considered an immunological inert cell wall component that confers resistance to mechanical and osmotic stress, DHN-melanin has been recently evolved as a major player of host-fungal interplay (Table 1). It has become apparent that DHN-melanin targets essential phagosome biogenesis pathways to block phagolysosome formation parallel with activation of anti-apoptotic pathways in immune cells to promote fungal survival within the host. At the same time, innate sensing of melanin via soluble and cell type specific PRRs mounts effective antifungal immune responses to counteract the inhibitory actions of melanin (Wong et al. 2018). Furthermore, metabolic rewiring during fungal-macrophage interplay could be regarded as an alternative immune effector pathway to control fungal disease. The full spectrum of interactions of melanin with host immune and non-immune cells has just started to be characterized. Many unresolved questions on the biological properties of *Aspergillus fumigatus* DHN-melanin as compared to other fungal melanins are presented in Table 2.

Table 1 Functional assessment of mutations in melanin biosynthetic pathway in comparison with the wild-type isogenic strain of *A. fumigatus* (NT = not tested)

Gene	Conidial color	Conidial hydrophobicity (AFM)	Rodlet layer on surface	Activation of PI3K/Akt signaling	Phagocytosis	Phagosome acidification	Killing of conidia	Activation of MelLeC
Alb1 (pksP)	White (colorless)	↓↓↓	↓↓↓	↓↓↓	↑↑↑	↑↑↑	↑↑↑	No
Ayg1	Yellow	↓↓↓	↓↓↓	↓↓↓	NT	↑	NT	Yes
Arp2	Pink	↓↓↓	↓↓↓	↓	NT	↑	NT	Yes
Arp1	Pink	↓↓↓	↓↓↓	N/A	NT	NT	NT	Yes
Abr1	Brown	↓↓↓	↓↓↓	N/A	NT	NT	NT	Yes
Abr2	Brown	↓	↓	N/A	NT	↑	NT	Yes
References	Tsai et al. (1999)	Bayry et al. (2014)	Bayry et al. (2014)	Volling et al. (2011)	Jahn et al. (2002)	Thywißen et al. (2011)	Jahn et al. (2002), Akoumianaki et al. (2016)	Stappers et al. (2018)

Gene	Cytokine responses	ROS production	Activation of LAP	Activation of platelets	Virulence in mammalian models	Virulence in *Drosophila* model	Virulence in *Galleria* model
Alb1 (pksP)	↑↑↑	↑↑↑	↑↑↑	↓↓↓	↓↓↓	↓↓↓	↑↑↑
Ayg1	↑↑↑	NT	NT	NT	NT	NT	↑↑↑
Arp2	↑↑↑	NT	NT	NT	NT	NT	↑↑↑
Arp1	↑ to wild-type level	NT	NT	NT	NT	NT	↑↑↑
Abr1	WT levels	NT	NT	NT	NT	NT	↑↑↑
Abr2	WT levels	NT	NT	NT	Virulent	NT	↑↑↑
References	Bayry et al. (2014)	Jahn et al. (2000, 2002)	Akoumianaki et al. (2016)	Rambach et al. (2015)	Tsai et al. (1998), Akoumianaki et al. (2016), Sugareva et al. (2006)	Lionakis et al. (2005)	Jackson et al. (2009)

Table 2 Intriguing questions to the role of the melanin in host-fungal interplay

1. Apart from monocytes/macrophages, the molecular interactions of melanin with other immune and non-immune cells are essentially unknown
2. A direct interaction of melanin with divalent cation channels regulating calcium signaling has not been formally tested experimentally
3. The biological significance of metal binding by melanin has not been explored apart from calcium
4. The potential function of melanin degradation or release, during either intracellular or extracellular life cycle of the fungus, in the form of exosomes is elusive
5. The requirement of surface exposure of melanin to retain its biological activity requires clarification
6. The potential role of other secondary metabolites produced by pksP cluster in virulence has not been considered
7. The importance of molecular interactions of melanin with other cell wall components has not been evaluated comprehensively
8. A potential function of melanin on cell metabolism apart from the phagolysosome requires systematic studies
9. The role of other melanins such as DOPA-melanin or pyomelanin has not been properly investigated and compared to DHN-melanin

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Hydrophobin Rodlets on the Fungal Cell Wall

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Abstract The conidia of airborne fungi are protected by a hydrophobic protein layer that coats the cell wall polysaccharides and renders the spores resistant to wetting and desiccation. A similar layer is presented on the outer surface of the aerial hyphae of some fungi. This layer serves multiple purposes, including facilitating spore dispersal, mediating the growth of hyphae into the air from moist environments, aiding host interactions in symbiotic relationships and increasing infectivity in pathogenic fungi. The layer consists of tightly packed, fibrillar structures termed "rodlets", which are approximately 10 nm in diameter, hundreds of nanometres long and grouped in fascicles. Rodlets are an extremely stable protein structure, being resistant to detergents, denaturants and alcohols and requiring strong acids for depolymerisation. They are produced through the self-assembly of small, surface-active proteins that belong to the hydrophobin

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Current Topics in Microbiology and Immunology (2020) 425: 29–51
https://doi.org/10.1007/82_2019_186
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Published Online: 25 December 2019

protein family. These small proteins are expressed by all filamentous fungi and are characterised by a high proportion of hydrophobic residues and the presence of eight cysteine residues. Rodlets are a form of the functional amyloid fibril, where the hydrophobin monomers are held together in the rodlets by intermolecular hydrogen bonds that contribute to a stable β-sheet core.

1 The Discovery of the Hydrophobic Rodlet Layer on Conidia

The structure of fungal conidia allows fungi to survive and persist in harsh environments that can be challenging in terms of extremes of pH, UV and osmotic pressure. *A. fumigatus* spores can survive storage in liquid nitrogen for up to 18 years and can survive 60 years when lyophilised (Kwon-Chung and Sugui 2013). While all fungal cells are surrounded by a protective cell wall composed of structural polysaccharides, the conidia have additional layers on the surface of the cell wall which confer unique protective properties and which modulate interactions with the environment and the host during infection. Most fungi use air dispersal to disseminate conidia, and the outermost surface of these so-called dry spores is comprised of a protein layer that is extremely hydrophobic. This layer lies on the outside of the conidial melanin layer, which in turn covers the cell wall carbohydrate structures (van de Veerdonk et al. 2017). It prevents wetting of the spores and hence spores remain light and are able to travel long distances on air currents (Beever and Dempsey 1978). The hydrophobic coating also facilitates the dispersal of conidia on the surface of water droplets (Whiteford and Spanu 2001). It is estimated that humans inhale between 100 and 1000 spores per day, depending on the season and environment (Latgé 1999). *Cladosporium*, *Alternaria*, *Penicillium* and *Aspergillus* species contribute the majority of airborne spores present in outdoor places, and most indoor outbreaks of nosocomial fungal disease are caused by airborne conidia from outside (Shams-Ghahfarokhi et al. 2014). *Aspergillus fumigatus* spores are more hydrophobic than conidia of other *Aspergillus* species and can disperse readily in the environment, making *A. fumigatus* conidia the most commonly found spores in the air (Kwon-Chung and Sugui 2013).

The hydrophobic outer protein layer of the conidia is composed of tightly packed fibrillar structures known as rodlets (Fig. 1a). The rodlet layer was first reported following electron microscopy studies of conidia of *Penicillium megasporum* (Hess et al. 1968). Rodlets have subsequently been characterised on the spores of *Cladosporium* (Latge et al. 1988), *Aspergillus* (Ghiorse and Edwards 1973), *Neurospora* species (Beever et al. 1979a; Hallett and Beever 1981) and many others (references in (Cole 1973; Cole et al. 1979; Gardner et al. 1983; Gerin et al. 1994; Lugones et al. 1996)). The rodlet morphology is also visible on vegetative hyphae of *Schizophyllum commune* (Wessels et al. 1972) and on aerial and submerged conidia of the entomopathogenic fungus *Beauveria bassiana* (Bidochka et al. 1995).

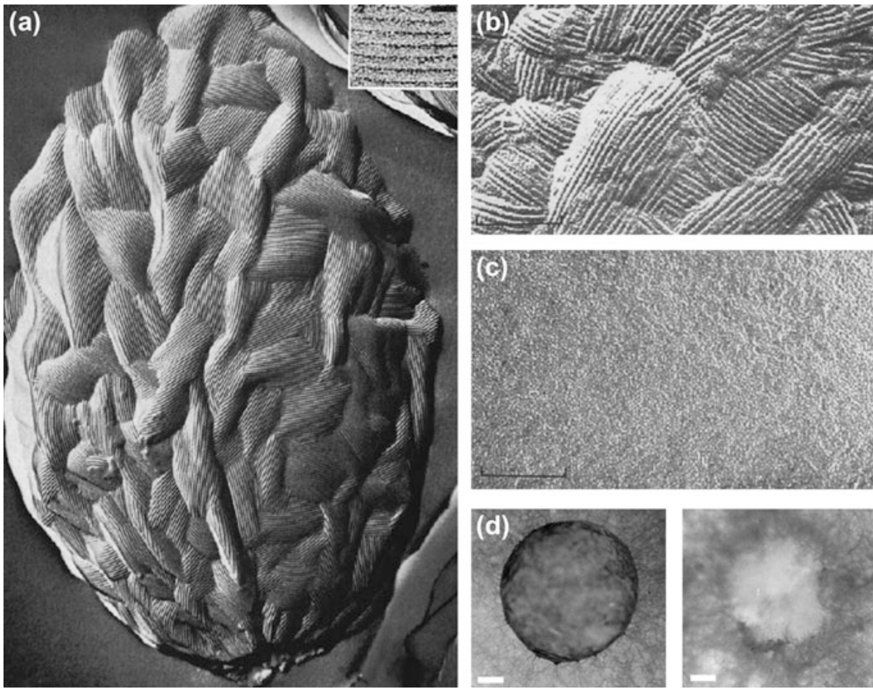


Fig. 1 **a** Freeze-fracture image of a *Penicillium* conidium, showing the patchwork covering of rodlets, grouped into fascicles; magnification *x18,200*. Inset shows higher magnification of rodlets, bar = 20 nm. Image from (Cole et al. 1979) by kind permission of the publisher. Surface carbon–platinum replicas of **b** wild type and **c** ΔRodA mutant conidia of *A. fumigatus* show that in the absence of RodA, no surface rodlet layer is present. Image reproduced with the permission from (Thau et al. 1994), bar = 100 nm. **d** Hydrophobin rodlets are responsible for the hydrophobicity of fungal aerial surfaces. A water droplet remains on the surface of aerial hyphae of wild-type *Schizophyllum commune*, while droplets immediately soak into the mycelium of the ΔSC3 strain. Image reproduced with the permission from (Wosten et al. 1999)

Morphological information regarding the rodlet layer on the surface of conidia has been obtained from freeze-etch electron microscopy investigations (Beever et al. 1979a; Hess et al. 1968; Hess and Stocks 1969) and, more recently, from atomic force microscopy using rodlets assembled in vitro on hydrophobic surfaces (Gunning et al. 1998) and on intact conidia (Dague et al. 2008; Valsecchi et al. 2019b). Freeze-etch studies indicate that, for most species, the rod-like structures are grouped into bundles or fascicles that form the component blocks of a patchwork-like covering over the entire surface of the spore (Hallett and Beever 1981). Within the fascicles, small numbers of rodlets lie almost parallel (Fig. 1b). The fascicles abut neighbouring fascicles of differing orientation and some may overlap, with interdigitation of the rodlets from adjacent bundles (Cole 1973; Cole et al. 1979). There are variations in the number of rodlets within the fascicles and in the length of rodlets, and some of these characteristics may be species-specific

(Hess et al. 1968). For example, in ten *Penicillium* species studied in detail (*P. brevicompactum, P. camemberti, P. chrysogenum, P. claviforme, P. cyclopium, P. cylindrosporum, P. digitatum, P. herquei, P. megasporum, and P. rugulosum*) rodlets are approximately 5 nm wide, with rodlet-to-rodlet spacing of 10 nm. Fascicles range from 10 to 250 nm in width, with rodlets up to 600 nm in length (Hess et al. 1968). The variation in observed fascicle width on a single spore is considerable, e.g. 3–30 nm for *P. chrysogenum* and 10–250 nm for *P. herquei*. Rodlet length also varies with average length observed for *P. chrysogenum* of 90 nm and for *P. rugulosum* 300 nm (Hess et al. 1968). Rodlets from *A. fumigatus*, *A. melleus*, *A. awamori*, *A. nidulans*, *A. ustus* and *A. wentii* range from 30 to greater than 300 nm (Hess and Stocks 1969; Thau et al. 1994). Rodlets from *N. crassa* are approximately 10 nm wide and range from 35 to 240 nm in length (Dempsey and Beever 1979). Rodlets composed of the hydrophobin MPG1 on *Magnaporthe oryzae* spores appear 5–7 nm in diameter (Kershaw et al. 1998). Early microscopy studies reported the observation that the rodlets were composed of chains of subunits (Cole et al. 1979). A detailed examination of the aerial spores and mycelium of *Micropolyspora* revealed the presence of rodlets of 6–8 nm diameter and fibres of 3–4 nm diameter. A two-stranded helical substructure was observed within the rodlets, suggesting that rodlets may be composed of two fibres (Takeo 1976).

2 Hydrophobins Are the Proteins that Make up the Rodlet Layer

The proteinaceous nature of the rodlet layer was established in the late 1970s through chemical analysis, as was its resistance to enzymatic treatment and hot SDS treatment (Cole et al. 1979). However, the nature of the proteins that form the rodlet layer was only revealed when Wessels and colleagues reported their identification of a family of small, hydrophobic proteins with eight conserved cysteine residues that were secreted into the culture medium and also accumulated in the walls of emerging hyphae (Wessels et al. 1991). The layer of rodlets conferred hydrophobicity on hydrophilic surfaces and on the surface of the *thn* mutant that lacks the ability to form aerial hyphae due to the lack of expression of the *SC3* gene. They gave these proteins the name "hydrophobins" and postulated that hydrophobins were involved in the emergence of aerial structures in *S. commune* and might play a general morphogenetic role in fungi. The hydrophobin SC3 was identified as the protein that forms the hydrophobic rodlet coating on the aerial hyphae of *S. commune* (Wessels et al. 1991; Wosten et al. 1994). The sequence similarity between *S. commune* hydrophobins and the rodlet-forming protein in *A. nidulans*, RodA, and the similar poor aerial dispersal of conidia formed by rodletless mutants of *A. nidulans* and *N. crassa* were noted soon after (Stringer et al. 1991) (Fig. 1c). Together, these studies revealed that the characteristic hydrophobicity of the fungal conidial surface was due to the rodlet layer, which was composed of a polymeric

form of a single hydrophobin protein (Fig. 1d). This new understanding of hydrophobin structure and properties in *S. commune, A. nidulans* and *N. crassa* led to a rapid identification of similar hydrophobins in other fungi. The *MPG1* gene was shown to encode a hydrophobin in *Magnaporthe oryzae*, and this hydrophobin MPG1 was demonstrated to play a role in the production of infectious structures (Talbot et al. 1993, 1996). The gene encoding RodA, the rodlet-forming hydrophobin in *A. fumigatus,* was cloned by homology with the *RODA* gene from *A. nidulans* (Thau et al. 1994). The protein product of the rodlet-forming gene in *N. crassa* was purified by Templeton and colleagues in 1995 (Templeton et al. 1995). It is named EAS for the "easily wettable" character of the spores produced by the *EAS* mutant strain of *N. crassa* (Beever and Dempsey 1978). Subsequently, genes encoding members of the hydrophobin protein family have been identified in all of the genomes of filamentous fungi sequenced to date (Linder et al. 2005; Littlejohn et al. 2012; Seidl-Seiboth et al. 2011).

3 Generic Properties of Class I Hydrophobin Rodlets

The ubiquitous presence of rodlets in the cell walls of filamentous fungi suggests that conferring hydrophobicity on aerial surfaces is likely a generic and vital property of these structures (de Vries et al. 1993). Hydrophobin rodlets are not covalently attached to the fungal surface, although the association of rodlets with the conidia is stronger in the presence of melanin and conversely, melanin assembly appears enhanced by the presence of rodlets (Valsecchi et al. 2019a). Bundles of rodlets may be shed from conidia when cells are passed through a cell fractionator (Cole 1973) or when conidia are subjected to shaking (Beever et al. 1979b) or sonication (Templeton et al. 1995). De Vries and colleagues showed that the polymeric hydrophobin structures could be extracted from mycelia with formic acid and solubilised and dissociated to a monomeric form by treatment with trifluoroacetic acid in the absence of reducing agent. This revealed that the hydrophobin rodlets are not stabilized by intermolecular disulphide bridges (de Vries et al. 1993). The conidial surface is rendered hydrophilic by genetic or chemical removal of the rodlet layer, and conidia that lack a hydrophobin rodlet layer are aggregation-prone (Beever et al. 1979a; Thau et al. 1994). Upon conidial germination, the ordered rodlet structure disappears, digested by conidial proteases (Valsecchi et al. 2019b). The rodlet layer is completely absent from the conidial surface after 5–6 h from the start of conidial swelling and germination (Fig. 2). Although the melanin and hydrophobin rodlet layers form a robust shell on the spore surface, resistant to a wide range of physicochemical insults, these layers do not appear to control water influx since the germination of the conidium of the parent strain and the RodA mutant occurs at the same speed (Valsecchi et al. 2019a).

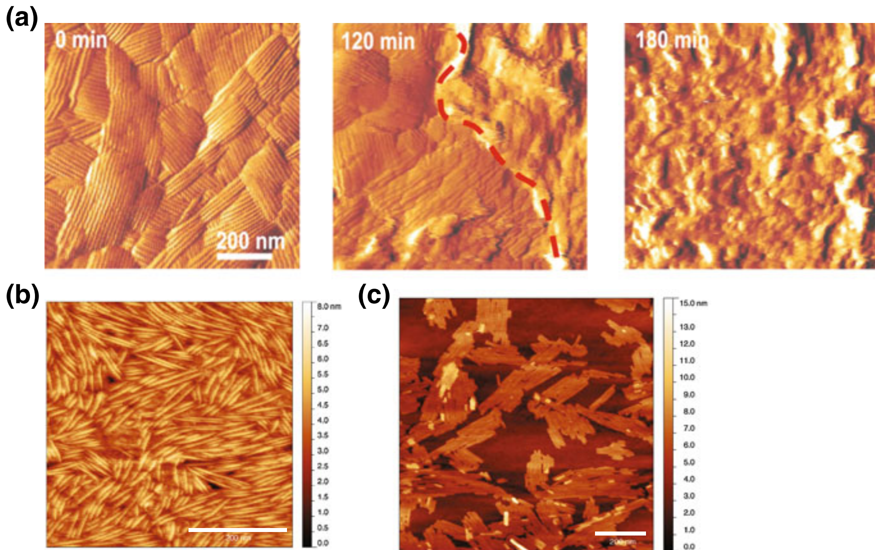


Fig. 2 **a** High-resolution atomic force microscopy (AFM) imaging of the surface of germinating conidia of *A. fumigatus* shows the intact rodlet layer at 0 min, gradual loss of rodlet morphology and emergence of amorphous material at 120 min and the complete absence of rodlets and exposure of cell wall polysaccharides at 180 min. Image reproduced with the permission from (Dague et al. 2008). Recombinant class I hydrophobin proteins spontaneously self-assemble into rodlets when deposited from solution onto highly oriented pyrolytic graphite. **b** Recombinant MPG1 hydrophobin (Pham et al. 2016) and **c** $EAS_{\Delta 15}$ hydrophobin (Kwan et al. 2008), a truncated form of EAS from *N. crassa*, display the characteristic rodlet morphology when self-assembled. Scale bar = 200 nm in all images

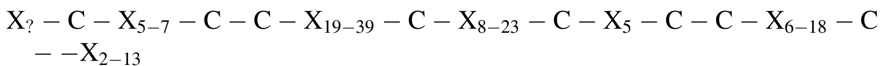
4 Sequence and Structural Analysis of Hydrophobins

The hydrophobin family consists of more than 1,000 small proteins (~5–20 kDa) that are characterised by the presence of a high proportion of hydrophobic amino acids and eight cysteine residues that occur in a distinctive pattern with two pairs of vicinal cysteines (Gandier et al. 2017; Linder et al. 2005). Filamentous fungi express multiple hydrophobin proteins at different stages of the life cycle to mediate transitions between hydrophobic and hydrophilic environments and while all of those characterised are surface-active and self-assemble into amphipathic layers, not all hydrophobins form layers that are composed of rodlets (Linder et al. 2005; Wosten and de Vocht 2000; Wosten and Scholtmeijer 2015; Zampieri et al. 2010).

Early sequence analysis of hydrophobins revealed that they could be divided into two categories, with distinct and characteristic hydrophobicity plots and patterns of inter-cysteine spacing (Wosten et al. 1994). These classes corresponded to hydrophobins that self-assembled into SDS-resistant rodlets (class I) and hydrophobins that formed less regular and less stable assemblies (class II). Class I rodlets require strong acids such as formic, trifluoroacetic or hydrofluoric acid to be solubilised to

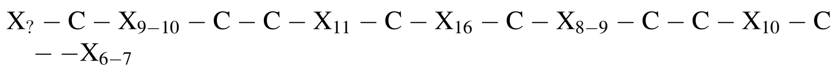
the monomeric protein form, while class II hydrophobin layers are depolymerised by treatment with hot alcohols and detergents (Linder et al. 2005). As more members of the hydrophobin family have been sequenced and identified, this classification has been adjusted to reflect that some hydrophobin members have sequence and predicted structural characteristics that are intermediate between representative members of the two major hydrophobin classes (Jensen et al. 2010; Littlejohn et al. 2012) (Fig. 3a). Additionally, some basidiomycete hydrophobins, e.g. the *S. commune* hydrophobin SC16, form a distinct class I subdivision, different in monomer structure from both ascomycete class I hydrophobins and from a mixed group of class I hydrophobins that display little sequence or loop length conservation (Gandier et al. 2017). Rodlet formation by SC16 occurs at an air–water interface, and the rodlets have a class I-like morphology and amyloid structure (Gandier et al. 2017). In fungi where multiple hydrophobins of different classes have been identified and studied, the different hydrophobins may have specific properties suited to different stages and challenges of the life cycle and environments or they may complement each other (Grunbacher et al. 2014; Lacroix et al. 2008; Moonjely et al. 2018; Valsecchi et al. 2017; Linder et al. 2005). For example, of the six hydrophobins present in the conidia of *A. nidulans*, all contribute to the hydrophobicity of the structures and at least three are able to form rodlets, yet only RodA is present in rodlet form on the wild-type spore surface (Grunbacher et al. 2014). Currently, the consensus for classification is based on the following criteria (Littlejohn et al. 2012):

Class I:



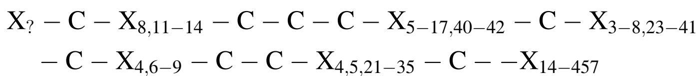
$$X_{?} - C - X_{5-7} - C - C - X_{19-39} - C - X_{8-23} - C - X_{5} - C - C - X_{6-18} - C - -X_{2-13}$$

Class II:



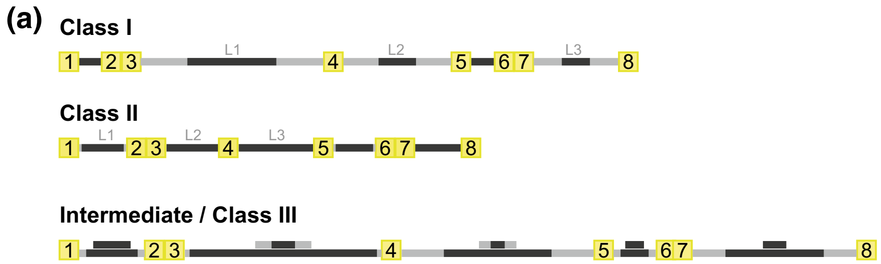
$$X_{?} - C - X_{9-10} - C - C - X_{11} - C - X_{16} - C - X_{8-9} - C - C - X_{10} - C - -X_{6-7}$$

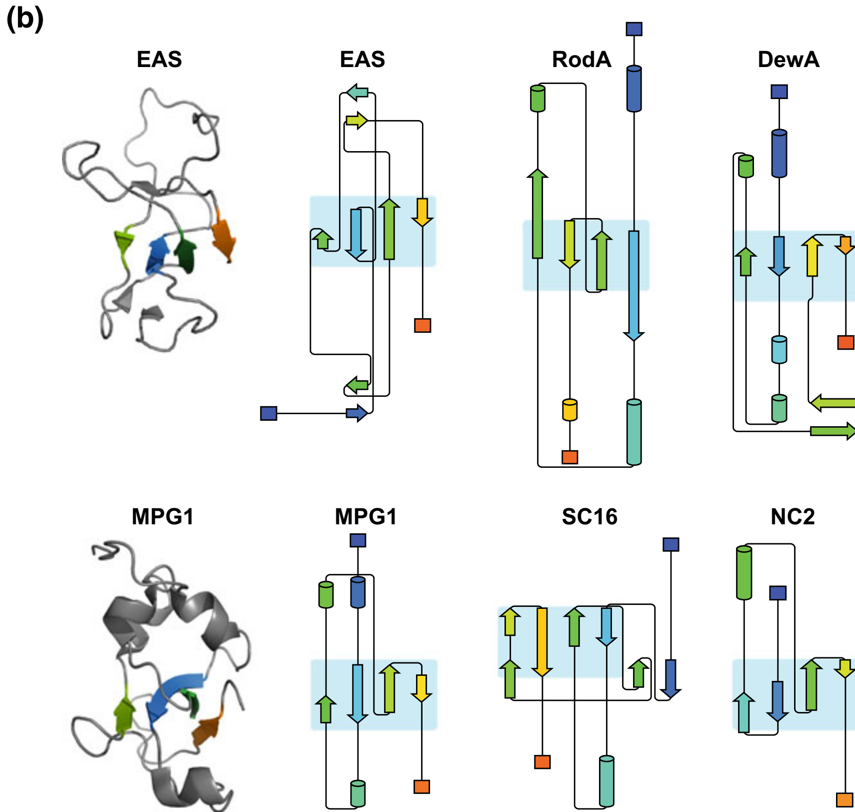
Intermediate/Class III:



$$X_{?} - C - X_{8,11-14} - C - C - C - X_{5-17,40-42} - C - X_{3-8,23-41} - C - X_{4,6-9} - C - C - X_{4,5,21-35} - C - -X_{14-457}$$

Structural understanding of hydrophobins has advanced rapidly since the crystal structures of the class II hydrophobins HFBI and HFBII, from *Trichoderma reesei*, were first reported (Hakanpaa et al. 2004, 2006). HFBI and its close homologue HFBII have small β-barrel structures that are tightly constrained by the presence of the four disulphide bridges and have two short loops (L1 and L2) and a slightly longer loop (L3), with an α-helix in L2 on the periphery of the barrel. The first





structure of the monomeric form of a class I hydrophobin was determined in 2006, when solution NMR spectroscopy revealed that EAS had a similar small β-barrel core structure but is significantly different in the loop regions (Kwan et al. 2006) (Fig. 3b). L2 in EAS contains β-sheet structure but L1 and L3 lack regular, stable elements of secondary structure and are flexible. The NMR structures of other class I hydrophobins, DewA, MPG1, RodA and SC16 reveal that while all of them contain a half β-barrel structure that is characteristic of hydrophobins, there are significant differences in the structures of the loop regions (Morris et al. 2013;

◀**Fig. 3** **a** Schematic representation of the differences and variations in inter-cysteine length that underlie the classification of hydrophobins into three classes. The positions of the eight conserved cysteine residues are highlighted in yellow. The lengths of the N- and C-terminal regions that lie before cysteine 1, and after cysteine 8, respectively, have been omitted for clarity. Along the amino acid sequence, the lengths of the polypeptide chains between the cysteine residues vary from that shown in black (shortest) to the combined length illustrated in black and grey (longest). In the intermediate/class III hydrophobins, two distinct sets of spacings are observed, as indicated by the two sets of black and/or grey bars. The positions of the loops 1, 2 and 3 are indicated by L1, L2 and L3. **b** Cartoon representations of the secondary structure elements of the monomeric forms of the class I hydrophobins EAS and MPG1 and topology diagrams indicating the four β-strands that make up the conserved β-barrel in EAS, RodA, DewA, MPG1, SC16 and the class II hydrophobin NC2. The corresponding four strands are coloured light green, blue, dark green and orange in the three-dimensional structures of EAS and MPG1, for comparison. Topology diagrams were produced with Pro-origami (Stivala et al. 2011) and representations of EAS and MPG1 structures with the PyMOL Molecular Graphics System, Version 2.3.0 Schrödinger, LLC, using PDB codes 2FMC (EAS), 2LSH (DewA), 4AOG (NC2), 6GCJ (RodA), 2NBH (SC16) and 2N4O (MPG1)

Pham et al. 2016, 2018; Pille et al. 2015; Ren et al. 2013; Valsecchi et al. 2019b). Helical elements are accommodated in the N-terminal regions and L1 and L2 regions but the extent and orientation of these elements is different in the individual proteins (Fig. 3b). The sequence analysis and structure of SC16 from *S. commune* reported by Gandier and colleagues (Gandier et al. 2017), combined with mutational analysis of Vmh2 from *Pleurotus ostreatus* (Pennacchio et al. 2018), suggest a further subdivision within basidiomycete hydrophobins, based on structure and mechanism of rodlet assembly. All of the hydrophobins display a large area of exposed hydrophobicity on the protein surface, unusual for soluble proteins (Sunde et al. 2008). The stable display of this relatively extensive hydrophobic surface area on a monomeric protein in solution is likely possible because of the presence of the four disulphides, which prevent folding of the polypeptide to bury the hydrophobic regions (Gandier et al. 2017; Wosten and Scholtmeijer 2015; Ren et al. 2014). Intact disulphides are required for function and structured self-assembly in most hydrophobins (Sallada et al. 2018; Valsecchi et al. 2019b). Additionally, in some members of the class I hydrophobins, the flexibility of the inter-cysteine loops may be critical in preventing unwanted self-assembly in solution (De Simone et al. 2012).

5 The Mechanism of Rodlet Assembly

Notably, Wessels and colleagues described in 1993 that the hydrophobin SC3 aggregates into rodlets when heated or exposed to bubbles and suggested that the aggregation of the protein was likely to occur at gas–liquid interfaces (de Vries et al. 1993). In a follow-up study, this group demonstrated that purified monomeric SC3 protein could be induced to self-assemble into structures with a rodlet morphology similar to that observed on the surface of aerial hyphae and that the process

occurred at the surface of air vesicles (Wosten et al. 1993). Biophysical studies of the process of self-assembly of purified SC3 demonstrated that self-assembly into the rodlet form is associated with an overall increase in β-sheet structure, with the involvement of a helical intermediate when assembly occurs on a hydrophobic surface (de Vocht et al. 1998).

New insight into the structure of the rodlet form, and a breakthrough in understanding the self-assembly process, came from two papers that almost concurrently reported that hydrophobin rodlets have an amyloid substructure, i.e. the ordered secondary structure within the rodlets consists of β-sheet structure in which the constituent β-strands lie at right angles to the rodlet long axis. Butko et al. provided spectroscopic evidence that interfacial assembly of SC3 generated an amyloid-like structure (Butko et al. 2001), and Mackay and colleagues showed that the rodlet form of EAS is a functional amyloid (Mackay et al. 2001). This appears to be the case for all rodlet-forming hydrophobins characterised to date (Gandier et al. 2017). Studies of recombinantly produced hydrophobins, namely RodA, RodB and RodC from *A. fumigatus* and MPG1, DewA and $EAS_{\Delta15}$, a truncated version of EAS (Kwan et al. 2008) have demonstrated that all of these hydrophobins apart from RodC can be triggered to assemble in vitro into a fibrillar form that is high in β-sheet content (Pham et al. 2018). However, the rodlets produced in vitro may differ in some details, for example in lateral packing, from those assembled in situ on spores, since the exact nature of the interacting surface provided by the cell wall is not replicated and the kinetics of formation may be different. While the underlying structural core is the same, morphological differences are commonly observed between amyloid fibrils assembled in vitro and those present in the biological context (Sunde et al. 2008; Kollmer et al. 2019).

The obvious difference between representative members of the two major classes is the presence of relatively long loops of varied sequences in class I hydrophobins, which are absent in class II members. This led to the hypothesis that the loops would play a key role in the assembly or structure of the rodlet form (Kwan et al. 2006). However, deletion analysis of L1, the long loop between cysteines 3 and 4 in EAS demonstrated that this loop could be dramatically shortened without compromising the ability of the protein to form rodlets Kwan et al. (2006, 2008). Instead, the mutational analysis demonstrated that in EAS, the L3 loop between cysteines 7 and 8 contains the key amyloidogenic segment (Macindoe et al. 2012). Subsequent solid-state NMR analysis has confirmed that this region forms the ordered β-sheet core of the EAS rodlet functional amyloid (Morris et al. 2012). Other class I hydrophobins may accommodate the amyloidogenic segments in other inter-cysteine regions that are sufficiently flexible to allow for the formation of intermolecular β-sheet structures. The amyloid-forming segment(s) of other class I hydrophobins have been suggested to be located in L1, L2 or L3 (Gandier et al. 2017; Morris et al. 2013; Pennacchio et al. 2018; Pham et al. 2016, 2018; Niu et al. 2014) and RodA is predicted to have two amyloidogenic segments, in L2 and L3 (Valsecchi et al. 2019b).

The majority of the class I hydrophobins that have been characterised require a hydrophilic–hydrophobic interface to trigger self-assembly (Lo et al. 2014; Pham et al. 2016). In solution and in the absence of an interface, the monomeric form of class I hydrophobins is prevented from self-assembling, possibly because the amyloid-forming regions are shielded due to intramolecular interactions and/or because the loops are too dynamic for intermolecular interactions to be favoured (De Simone et al. 2012; Kwan et al. 2006). Certain hydrophobins, including DewA and SC3, form dimers in the solution that are off-pathway for rodlet assembly and may represent an assembly control mechanism (Morris et al. 2013; Wang et al. 2004). SC3 has been shown to exist in three different conformations: a soluble, predominantly dimeric state in solution and α-helical and β-sheet states at a hydrophilic–hydrophobic interface (Wang et al. 2002). The SC3 dimers dissociate when the protein binds to a hydrophobic surface (Wang et al. 2004) and molecular dynamic simulations suggest that the conversion from the α-helical to a stable β-sheet conformation occurs when the monomers assemble into the rodlet state (Fan et al. 2006). SC3 has also been demonstrated to form nanorods in solution, with self-assembly promoted by low ionic strength and more alkaline pH, suggesting that the self-assembly of individual hydrophobins may be tailored to the environmental conditions present when they are expressed and functional (Zykwinska et al. 2014a).

In general, the relatively large, surface-exposed hydrophobic regions of these proteins result in their recruitment to hydrophilic–hydrophobic interfaces, and alignment there, to maximise hydrophobic interactions. The high surface activity of hydrophobins is reflected in the reduction in contact angle made by hydrophobin-containing solutions, compared to water droplets, on hydrophobic surfaces (Morris et al. 2011; Wosten et al. 1993). Interface properties are critical for recruitment and alignment and additives that reduce the surface tension of a solution can be seen to inhibit class I hydrophobin assembly (Morris et al. 2011). When class I hydrophobins are recruited to the interface, they undergo a conformational transition that exposes the amyloidogenic regions, allowing intermolecular interactions to occur and the stable amyloid core to develop (Macindoe et al. 2012). The very extensive hydrogen bonding between monomers that occurs along the rodlet length is likely responsible for the resistance of these structures to most denaturants. While EAS has a single amyloidogenic region in the L3 loop, RodA from *A. fumigatus* appears to have two amyloidogenic regions and the involvement of both of these in β-sheet-rich rodlet structures may be the reason for the extreme stability of RodA rodlets, which require hydrofluoric acid treatment to achieve complete depolymerisation (Valsecchi et al. 2019b). The model for class I hydrophobin self-assembly to form functional amyloid rodlets is illustrated in Fig. 4. While class II hydrophobins also assemble at hydrophilic–hydrophobic interfaces, they do not form stable intermolecular β-sheet structure, and hence, class II assemblies are readily depolymerized (Wosten and de Vocht 2000). The assemblies formed by class III hydrophobins have not yet been investigated experimentally.

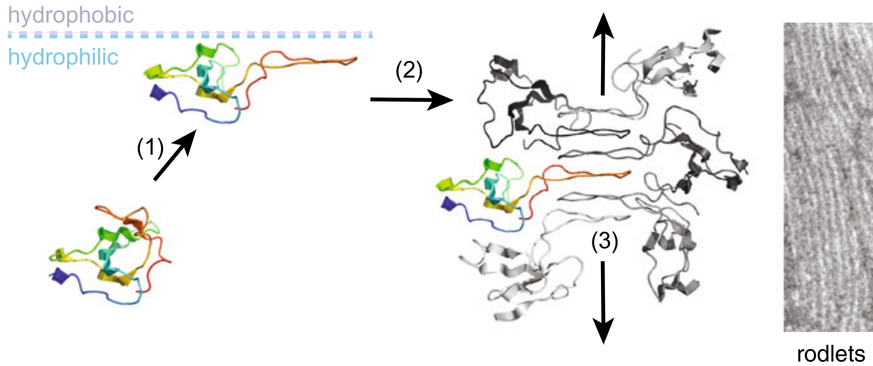


Fig. 4 Schematic representation of the assembly of monomeric forms of class I hydrophobins into the rodlet structure, based on biophysical characterisation of the process in $EAS_{\Delta 15}$ (Kwan et al. 2008; Macindoe et al. 2012; Morris et al. 2013). (1) Recruitment of the protein to the hydrophilic–hydrophobic interface, followed by (2) alignment and conformational change to expose the amyloidogenic segment(s) and (3) formation of intermolecular β-sheet in the rodlet form. Cartoon representations prepared using the PyMOL Molecular Graphics System, Version 2.3.0 Schrödinger, LLC, with PDB code 2K6A for the $EAS_{\Delta 15}$ monomer and rodlet form as in (Macindoe et al. 2012)

6 Unique Biological Roles of Rodlet Functional Amyloids

In addition to providing a hydrophobic coating on fungal structures, some rodlet-forming hydrophobins contribute to other properties that are characteristic of particular fungi, including aiding symbiotic relationships or increasing the pathogenicity of the fungus towards its host.

Hydrophobin rodlets are one type of a number of functional amyloid assemblies now recognised as being applied for diverse biological purposes by organisms from bacteria to mammals. In bacteria, filamentous fungi and yeasts, functional amyloids assembled from different proteins have roles that range from stabilising biofilms, altering surface properties, facilitating interactions, regulating heterokaryon incompatibility, modulating transcription, environmental adaptation and acting as cytotoxins (Shanmugam et al. 2019). Viral functional amyloids can inhibit host cell responses and cell death (Pham et al. 2019). Although fungal hydrophobin rodlets and certain bacterial and viral functional amyloids modulate the host response to infection, they do not themselves cause disease but may be considered pathogenicity factors (Bayry et al. 2012). In mammals, functional amyloids have been shown to be involved in pigment biosynthesis and sequestration, haemostatic control, hormone storage and release and signal transduction (Chuang et al. 2018).

Fungal functional amyloids take several different forms, involving the self-assembly of different types of protein which display no sequence or structural similarity. They include hydrophobin rodlets, prion aggregates in yeast such as those formed by sup35p and ure2p in *S. cerevisiae* and the HET-s prion filaments of

Podospora anserina. The formation of these amyloids is temporally and spatially regulated and tightly controlled to confer the desired biological function. While hydrophobin rodlet assembly follows a nucleation-dependent polymerisation mechanism like other amyloids, the formation of the nucleus is localised to a hydrophobic–hydrophilic interface (Pham et al. 2016). Disaggregation of rodlets occurs prior to germination, likely through protease digestion. Prion assembly and disassembly in yeast have been shown to be under chaperone control (Chuang et al. 2018).

This tight regulation of assembly and disassembly of functional amyloids contrasts with the formation and deposition of disease-associated amyloid fibrils, such as amyloid-β in Alzheimer's disease, prions in the spongiform encephalopathies and transthyretin in familial amyloidotic polyneuropathy (Pham et al. 2014). Disease-associated amyloid fibrils form as a consequence of protein misfolding and aggregation, are generally stable and resistant to clearance and are associated with cell death and tissue and organ disruption.

6.1 Hydrophobins in Symbiotic Relationships with Plants and in Lichens

Fungi from the *Tricholoma* genus form ectomycorrhizal symbiotic relationships with the roots of woodland trees. This relationship is mutualistic, allowing the fungus access to carbohydrates and growth factors and providing the tree with increased nutrient and water supply (Sammer et al. 2016). Species from *Tricholoma* utilise hydrophobins to aid the communication and maintain the relationship between host and fungus. Initial research identified a single class I hydrophobin gene, hyd1 within the *T. terreum* species (Mankel et al. 2002). Hyd1 forms hydrophobin rodlets that exist at the interface between the host tree and *T. terreum,* and it was therefore proposed to aid in host compatibility (Mankel et al. 2002). 43 hydrophobin gene sequences have been identified in the *Tricholoma* genus, with nine hydrophobin genes identified in *T. vaccinum* that are expressed at different stages of the life cycle. These nine hydrophobins were shown to be involved in aerial hyphae and fruiting body formation as well as host recognition and the response to metal stress (Sammer et al. 2016).

A similar advantageous role for hydrophobins is observed in lichens, where hydrophobins prevent waterlogging within airspaces. A lichen is a composite organism generated by a symbiotic relationship between a fungus and algae or cyanobacteria (Dyer 2002). Lichens have the ability to survive dramatic changes in wettability, and this has been attributed to the presence of hydrophobins. Multiple hydrophobins have been identified from two taxonomically unrelated fungi *Dictyonema glabratum* and *Xanthoria parietina* (Scherrer et al. 2000, 2002; Trembley et al. 2002). The hydrophobins create a hydrophobic coating on the hyphae that is in contact with the algae and maintain gas-filled spaces despite the

lichen being water-saturated (Honegger 1991). This ensures appropriate gas exchange, and therefore, photosynthesis can occur even in conditions of high water content (Dyer 2002). This function is similar to that attributed to the hydrophobin SC4 from *S. commune*. SC4 lines gas channels in fruiting bodies and prevents them from filling with water, therefore maximising gas exchange (van Wetter et al. 2000).

6.2 The Contribution of Hydrophobins to Plant and Insect Infections

MPG1, from *M. oryzae*, was the first hydrophobin to be demonstrated to perform a pathogenic role in a fungal disease. *M. oryzae* causes rice blast disease; responsible for destroying up to one-third of the annual global rice crop (Fernandez and Orth 2018). MPG1 self-assembles at the spore–rice leaf interface, aiding in the detection of the surface as well as attachment of the spore to the hydrophobic leaf (Talbot et al. 1996). Knockout of *mpg1* results in loss of the rodlet layer on spores and reduced appressorium formation (Beckerman and Ebbole 1996). The appressorium allows the fungus to puncture through the infection surface; therefore, a reduction in appressorium formation decreases the pathogenicity of the fungus. MPG1 has also been linked to the activity of the enzyme cutinase 2 produced by *M. oryzae* (Skamnioti and Gurr 2008). Cutinase enzymes can degrade the robust cutin coating on plant leaves. MPG1 stimulates cutinase 2 activation, which subsequently activates the appressorium formation and leads to penetration of the rice leaf (Pham et al. 2016; Skamnioti and Gurr 2009). *M. oryzae* also contains a class II hydrophobin MHP1. MHP1 is similar to other class II hydrophobins in that it does not form fibrillar rodlets but it has been shown to have a potential regulatory role in MPG1 rodlet formation (Pham et al. 2016).

The temporal and spatial expression of the four class I hydrophobins produced by the tomato pathogen *Cladosporium fulvum* suggests that they perform distinct roles in the formation of aerial hyphae, conidia and germ tubes and during infection (Lacroix et al. 2008; Whiteford and Spanu 2001).

Beauveria bassiana, an entomogenous pathogen, utilises two hydrophobins to create a rodlet layer on spores (Zhang et al. 2011). *B. bassiana* contains two class I hydrophobins Hyd1 and Hyd2, and initially, Hyd2 was proposed as the main component of the rodlet layer on spores (Cho et al. 2007; Holder and Keyhani 2005). However, it has been shown that both Hyd1 and Hyd2 contribute to the rodlet spore coat: Hyd2 is proposed to have a regulatory role and forms the boundary of the rodlet nanodomains, initiating the nucleation of Hyd1 rodlet formation (Zhang et al. 2011). This rodlet coat ultimately aids the attachment of a spore to the insect cuticle and therefore subsequent hyphal penetration. This discovery highlighted that the rodlets coating fungal spores are not necessarily composed of a single type of hydrophobin (Zhang et al. 2011).

6.3 *The Role of RodA and Other Hydrophobins in* A. Fumigatus

Hydrophobins in the human pathogen *A. fumigatus* have a pathogenic role in addition to aiding in spore dispersal and reversing wettability. *A. fumigatus* is an opportunistic pathogen that can infect the human lung and subsequently spread to other organs including the brain and kidneys (Latge 1999). The spores are deposited into the alveoli of the lungs where they invade the epithelial cell wall lining and germinate, leading to invasive aspergillosis, a condition that occurs predominantly in immunocompromised patients. Patients with pre-existing pulmonary disease are also more susceptible to allergic bronchopulmonary aspergillosis and the development of aspergilloma (Lee and Sheppard 2016). *A. fumigatus* also causes aspergillosis in a wide range of agricultural, domestic and wild animals and birds (Seyedmousavi et al. 2018).

The hydrophobin rodlet layer plays an important role in evasion of the host immune system by *A. fumigatus* since, along with the melanin layer, it immunosilences the conidium (Aimanianda et al. 2009; Thau et al. 1994; Beauvais and Latge 2018; Girardin et al. 1999; Paris et al. 2003; Valsecchi et al. 2017). In dormant conidia, the hydrophobin coating shields the carbohydrate moieties of the cell wall from detection by the dectin-1 receptor, which recognises β-1,3-glucan, the dectin-2 receptor, which recognises galactomannan, and Toll-like receptors. The presence of the rodlet layer thus prevents activation and maturation of dendritic cells, macrophages or neutrophils and prevents cytokine induction in response to the presence of conidia. Hence, even though most people breathe in several hundreds of *A. fumigatus* spores daily, and these are small enough to reach the alveoli in the lungs, they do not result in activation of the innate immune defenses. Complement components such as the major complement protein C3, acute phase proteins such as surfactants, and the pentraxin-related protein PTX3, act as soluble mediators that interact with dormant *A. fumigatus* conidia and result in spore ingestion by macrophages (Wong and Aimanianda 2017). The rodlet layer hides the spore from immune detection until appropriate germination conditions are met. At this point, the rodlet layer degrades and germination can commence. In immunocompromised patients, the macrophages fail to kill the conidia, and as a result, filamentous hyphae can form and invade the lung tissue, resulting in a necrotizing pneumonia and further dissemination to other tissues (Aimanianda et al. 2009; Dague et al. 2008; Lee and Sheppard 2016).

A. fumigatus contains multiple hydrophobins (Rod A–G); however, only RodA is well characterised (Valsecchi et al. 2017). Knockout of *RODA* reduces the dispersal of spores and results in hydrophilic spores that lack the rodlet layer, instead of displaying an amorphous surface (Thau et al. 1994; Valsecchi et al. 2019a). Chemical or genetic removal of the RodA rodlet layer generates spores that can activate the immune system, releasing cytokines, chemokines and reactive oxygen intermediates (Aimanianda et al. 2009). A decrease in surface RodA rodlets also results in an increase in phagocytosis of *A. fumigatus* spores (Dagenais et al. 2010).

RodA has been proposed to form a multilayer on spores compared to the observed monolayer on spores of other fungi (Zykwinska et al. 2014b). Similar to other hydrophobins, the rodlets are organised into nanodomains that vary in length (120–220 nm) and width (60–100 nm) but are composed of at least three rodlets. An analysis of atomic force microscopy images was used to elucidate the approximate height of ~5 nm for the RodA layer (Zykwinska et al. 2014b). Previous research into hydrophobins has shown that EAS and SC3 organise into monolayers of 2–2.5 nm in height (Zykwinska et al. 2014a; Yang et al. 2013). From this observation, it was proposed that RodA forms a bilayer structure with two monolayers stacked on top of each other. The first layer of the bilayer is attached to the fungal cell wall via its hydrophobic side, interacting with hydrophobic melanin, glycoproteins and polysaccharides. Pre-assembled oligomers from the surrounding growth environment interact with their hydrophilic side with the monolayer already assembled and therefore create a bilayer that renders the spore hydrophobic (Zykwinska et al. 2014b).

Recent attempts to characterise the remaining five hydrophobins in *A. fumigatus* suggest that not all are active in aiding fungal infection. The class I hydrophobins RodA–C are very similar in sequence but RodD–G have limited similarity (Valsecchi et al. 2017). RodD and RodE are controversial as hydrophobins as RodD lacks a cysteine, and RodE has three additional cysteines, while the presence of eight cysteines is a defining feature of hydrophobins. RodA, B and C are present in the conidial cell wall of *A. fumigatus*. However, only RodA forms rodlets on the conidium and is necessary for dispersal, hydrophobicity and immune evasion (Paris et al. 2003). When *A. fumigatus* grows in a biofilm, the *RODB* gene is highly expressed but the protein is not detectable in the biofilm by immunofluorescence (Valsecchi et al. 2017). RodB is able to form rodlets in vitro (Pham et al. 2018) but it does not appear to substitute for this function in ΔRodA mutants. The *RODB* gene is also highly expressed in vivo in a mouse invasive model of aspergillosis but the significance of this is unclear and despite being expressed, the role of the remaining hydrophobins RodC–G in *A. fumigatus* remains unknown (Valsecchi et al. 2017).

7 Conclusions

The rodlet covering formed by class I hydrophobin proteins on the outside of the cell wall of conidia and on aerial hyphae provides a protective coat on these fungal surfaces. Building on the recognition of the distinct morphology of the rodlet layers provided by imaging techniques, structural and mechanistic studies have revealed that all class I hydrophobins share a β-barrel structure, stabilized by a characteristic pattern of four disulphide bridges, and that rodlets are functional amyloid structures. The class I hydrophobins generally have longer inter-cysteine sequences, and a diversity of regular secondary structure elements, something that is lacking in class II hydrophobins but which is consistent with the class I ability to form inter-molecular interactions within the rodlet β-sheet-rich scaffold. The role of certain

rodlets is well understood, where they have been demonstrated to increase the attachment of fungi to host surfaces, to support the generation of infectious structures and to shield conidia from detection by host defenses, thereby contributing to infectivity and pathogenicity. However, a full picture of the roles of the multiple different rodlet-forming hydrophobins that are present in some fungal genomes remains to be elucidated. Additionally, understanding of the process by which class I hydrophobins localise to the outer surface of the fungal cell wall, for self-assembly into the unique and remarkable rodlet structure, could identify ways to manipulate the interaction of fungi with different environments.

Acknowledgements The authors acknowledge funding from the Australian Research Council over many years, for research into hydrophobin proteins, which has contributed to this work (DP0879121, DP120100756 and DP150104227) and support from the French-Australian Science and Technology (FAST) Program (FR110012). SRB is supported by the Australian Government in the form of a Research Training Program Scholarship. The authors wish to thank Chi Pham, Victor Lo, Vanessa Morris, Qin Ren, Jennifer Lai, Ingrid Macindoe and all other researchers and students working in the Sunde and Kwan Laboratories who have contributed to hydrophobin projects. We acknowledge the facilities and the scientific and technical assistance of Sydney Microscopy and Microanalysis at the Australian Centre for Microscopy and Microanalysis at the University of Sydney. We thank Chiara Neto for use of atomic force microscopy facilities.

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α- and β-1,3-Glucan Synthesis and Remodeling

Johannes Wagener, Kristina Striegler, and Nikola Wagener

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Abstract Glucans are characteristic and major constituents of fungal cell walls. Depending on the species, different glucan polysaccharides can be found. These differ in the linkage of the D-glucose monomers which can be either in α- or β-conformation and form 1,3, 1,4 or 1,6 *O*-glycosidic bonds. The linkages and polymer lengths define the physical properties of the glucan macromolecules, which may form a scaffold for other cell wall structures and influence the rigidity and elasticity of the wall. β-1,3-glucan is essential for the viability of many fungal pathogens. Therefore, the β-1,3-glucan synthase complex represents an excellent and primary target structure for antifungal drugs. Fungal cell wall β-glucan is also an important pathogen-associated molecular pattern (PAMP). To hide from innate immunity, many fungal pathogens depend on the synthesis of cell wall α-glucan,

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Current Topics in Microbiology and Immunology (2020) 425: 53–82
https://doi.org/10.1007/82_2020_200
© Springer Nature Switzerland AG 2020
Published Online: 20 March 2020

which functions as a stealth molecule to mask the β-glucans itself or links other masking structures to the cell wall. Here, we review the current knowledge about the biosynthetic machineries that synthesize β-1,3-glucan, β-1,6-glucan, and α-1,3-glucan. We summarize the discovery of the synthases, major regulatory traits, and the impact of glucan synthesis deficiencies on the fungal organisms. Despite all efforts, many aspects of glucan synthesis remain yet unresolved, keeping research directed toward cell wall biogenesis an exciting and continuously challenging topic.

1 Introduction

Glucans are polymers of D-glucose and represent the major constituent of many fungal cell walls. Several different kinds of glucan polymers are found in the walls of fungi. They differ in their *O*-glycosidic bonds between the D-glucose monomers which can adopt either α- or β-conformation and are formed between the carbon atoms 1,3, 1,4 or 1,6 of the pyranose moieties. Depending on the fungal species, the abundance of the individual glucans as well as their importance for the cell wall integrity and fungal physiology varies significantly. The cell walls of the better characterized *Ascomycota* such as *Saccharomycetes* (e.g., *Saccharomyces* and *Candida*) and *Eurotiomycetes* (e.g., *Aspergillus*, *Penicillium,* and *Neurospora*) typically consist of a significant amount of glucans. The walls of the opportunistic fungal pathogen *Candida albicans* contain 54–60% glucans (Ruiz-Herrera et al. 2006). Baker's yeast has approximately 60% glucans in its cell wall (Klis 1994), *Schizosaccharomyces pombe* 76–84% (Magnelli et al. 2005), and molds in the genus *Aspergillus* 60–85% glucans (Gastebois et al. 2009). In contrast, in other fungal species cell wall glucans are much less abundant. For example, cell walls of the molds in the lineage *Mucoromycotina* (e.g., *Rhizopus oryzae* and *Phycomyces blakesleeanus*) only contain 3–4% glucans (Mélida et al. 2015).

While β-glucans are characteristic for essentially all true fungi, α-glucans occur only in cell walls of certain fungal species. Consequently, certain enzymes that are essential for the synthesis of cell wall α-glucan are typically missing in lineages and species void of α-glucans in their cell walls, such as the baker's yeast *Saccharomyces cerevisiae*, the *Candida* species, *Fusarium graminearum,* and *Ustilago maydis* (Damveld et al. 2005a). Together with chitin, β-glucans build the scaffold for further cell wall structures. For example, galactomannan, a cell wall polymer typically—but not exclusively—found in *Aspergillus* species, is covalently bound to the cell wall β-1,3-glucan (Fontaine et al. 2000). Similarly, mannoproteins in Candida species are covalently bound to the β-1,3-glucan scaffold via short β-1,6-glucan linkers (Kapteyn et al. 1995). These are only some examples of the known cell wall constituents whose linkage to the cell wall will ultimately depend on the β-1,3-glucan scaffold.

Considering the large portion of β-1,3-glucan present in the cell walls of many fungal species and its role as scaffold for other constituents, it is conceivable that the lack thereof may have a devastating effect on growth and viability. In fact,

β-1,3-glucan biosynthesis is currently one of the most important targets for antifungal drugs. The echinocandin class of antifungals (e.g., caspofungin, anidulafungin, and micafungin) specifically inhibits β-1,3-glucan synthesis and is broadly applied in the clinics to treat invasive fungal infections (chapter "Cell wall-modifying antifungal drugs" by David S. Perlin). Notably, the impact of β-1,3-glucan synthesis inhibition clearly depends on the fungal species and obviously reflects the importance of this cell wall polymer in the respective organism. While yeasts such as *S. cerevisiae* and *Candida* spp. typically cannot live without β-1,3-glucan (Douglas et al. 1997; Inoue et al. 1995; Katiyar et al. 2012; Mazur et al. 1995; Mio et al. 1997), *Aspergillus fumigatus* can survive, but is heavily inhibited in growth and suffering from cell lysis (Dichtl et al. 2015). These observations are based on yeast and mold mutants, but are also in line with the activity of the echinocandin antifungals against these important pathogens. While Echinocandins are considered fungicidal against *Candida* species, they are fungistatic against *Aspergillus* species (Perlin 2015).

Being a characteristic and often essential structure in fungi, β-1,3-glucan is a well-conserved pathogen-associated molecular pattern (PAMP). Animals, which inherently lack this structure, developed specific pattern recognition receptors (PRR), such as the human C-type lectins Dectin-1, Langerin, and the collectins SP-A and SP-D, that detect β-1,3- (Dectin-1, Langerin) or β-1,6- (SP-A, SP-D) glucans and trigger an innate immune response (reviewed in Goyal et al. 2018). Variants of these PRRs are conserved from invertebrates to humans and play a major role in the defense of these organisms against fungal infections (chapter "PAMPs of the fungal cell wall and mammalian PRRs" by Remi Hatinguais, Janet A. Willment, and Gordon D. Brown) (Legentil et al. 2015). β-1,3-/β-1,6-glucan typically triggers activation of the host's defense mechanisms. However, inhibition of β-1,3-glucan synthesis has repeatedly and surprisingly been reported to reinforce—and not dampen—the antifungal activity of the innate immune system by paradoxically increasing the exposure of β-1,3-glucans at the cell surface of the fungi (Beyda et al. 2015; Lamaris et al. 2008; Wheeler et al. 2008). Taken together, this suggests that inhibition of β-1,3-glucan synthesis fosters exposure of PAMPs which then boosts activation of the immune defense. It is not clear whether fungal cell wall α-glucans are specifically detected by any PRRs. It was proposed that α-1,4-glucan of *Mycobacterium tuberculosis* is a ligand of DC-SIGN (Geurtsen et al. 2009), but α-1,4-glucan is only a minor constituent of the cell walls of α-1,3-glucan-positive fungi. In marked contrast to β-1,3-glucan, α-1,3-glucan is rather a virulence factor than a ligand for innate immunity. There is overwhelming evidence that α-1,3-glucan serves as a stealth polymer itself or as an important linker for other masking structures in various human, animal, and plant pathogenic fungi such as *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Blastomyces dermatitidis*, and *Magnaporthe grisea* (Beauvais et al. 2013; Fujikawa et al. 2012; Hogan and Klein 1994; Rappleye et al. 2004, 2007; Reese and Doering 2003; San-Blas and Vernet 1977).

2 Biosynthesis of Cell Wall Glucans

Glucans are highly abundant and essential structures of many fungal cell walls. Synthesis of the major fungal cell wall polymers β-1,3-glucan and α-1,3-glucan relies on two specialized and not directly related enzymatic complexes. The proteins representing the presumed catalytic cores of these two complexes were named as *the* β-1,3-glucan synthase or *the* α-1,3-glucan synthase, respectively. However, it should be emphasized that the enzymatic activities of these enzymes most likely rely on a concerted interaction with multiple other enzymes. In addition, it is controversial whether *the* presumed β-1,3-glucan, and α-1,3-glucan synthase enzymes really contain the catalytic domains that synthesize the eponymous glucan polymers.

2.1 β-1,3-Glucan Synthesis

β-1,3-glucans are characteristic for fungal cell walls, but are also found in other eukaryotes such as plants (callose) and protozoa and in certain bacteria. β-1,3-glucan chains have a helical structure that can present either as single-stranded or three-stranded helices (Bohn and BeMiller 1995; Kopecká and Kreger 1986; Laroche and Michaud 2007). This structure confers a remarkable elasticity to the polymer chain (Klis et al. 2006) and forms an optimal basis for the cell wall of a living organism. Besides plain linear β-1,3-glucan chains, modifications thereof are found in fungal cell walls. These present as branched polymers where branches are formed by β-1,6 linkages to the core chain (Klis et al. 2006; Latgé 2007; Lesage and Bussey 2006). The branches restrict the ability to form hydrogen bonds between linear segments of the β-1,3-glucan chains and, therefore, increase the elasticity of the cell wall even further (Morris et al. 1986; Rees and Scott 1971). By modifying these interconnections of the β-1,3-glucan polymers, the cell can vary the properties of the wall according to its needs.

β-1,3-glucan is synthesized by successive addition of glucose molecules generated by hydrolysis from cytoplasmic UDP-glucose [Fig. 1; (Frost et al. 1994)]. One of the direct products of the reaction, UDP, functions as a competitive inhibitor of β-1,3-glucan synthesis (López-Romero and Ruiz-Herrera 1978; Shematek et al. 1980). During in vitro experiments with plasma membranes isolated from *S. cerevisiae*, the nucleoside triphosphates ATP or GTP, glycerol, and bovine serum albumin were required for maximal activity of β-1,3-glucan synthase (Shematek et al. 1980). The glucose entity is added to the non-reducing end of the polysaccharide chain with a resulting product length of 60 residues in average (Shematek et al. 1980). However, determination of the degree of glucan polymerization in vivo had resulted in values of approximately 1500 glucose residues per glucan molecule (Fleet and Manners 1976; Manners et al. 1973). This discrepancy is probably explained by the fact that glucan polymers isolated from baker's yeast cell walls

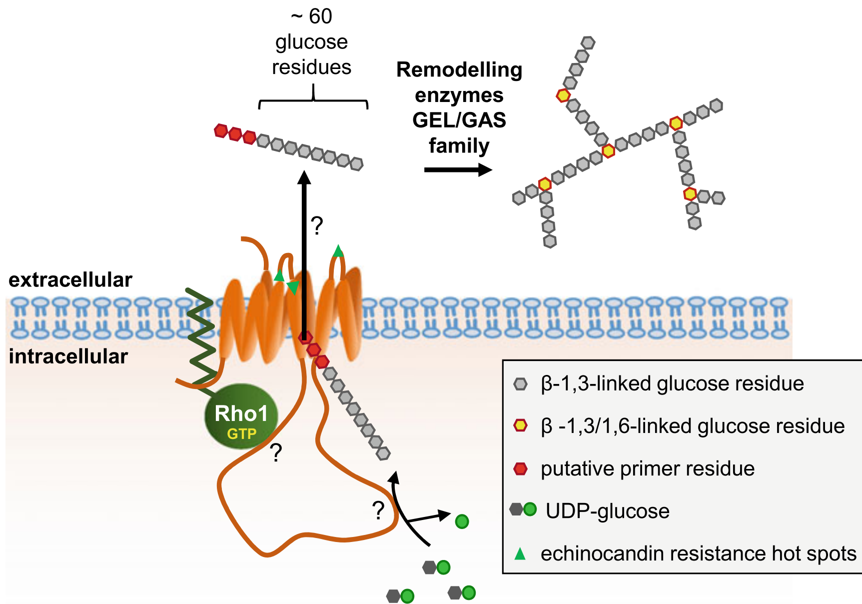

Fig. 1 Fungal β-1,3-glucan synthesis. Two subunits of the fungal β-1,3-glucan synthase complex have been identified, *the* β-1,3-glucan synthase (encoded by *fks* genes) and the Rho GTPase Rho1. The β-1,3-glucan synthase is a membrane protein approx. 1800 amino acids in length. 13 transmembrane helices (TM) span the plasma membrane. The large cytosolic loop between TM6 and TM7 is highly conserved and contains the presumed catalytic site for glucan synthesis and a domain required for interaction with regulating components. Rho1, which is essential for β-1,3-glucan synthesis, might interact with this loop. Some studies suggest a primer structure to initiate β-1,3-glucan synthesis. Cytosolic UDP-glucose is converted into UDP and the elongating β-1,3-glucan polymer. Amino acid exchange at three hot spot regions confers echinocandin resistance. These hot spots are located in the extracellular parts of the protein. Possibly, the β-1,3-glucan synthase forms a pore-like structure to pass the synthesized β-1,3-glucan chains to the outside. Various remodeling enzymes elongate and modify the linear glucan chain to build the cell wall matrix

undergo extensive remodeling upon leaving the β-1,3-glucan synthase and arrival in the extracellular space. This involves a bunch of conserved enzymes that show glucanase or glucanosyltransferase activity which elongate and branch the β-1,3-glucan chains via β-1,3- or β-1,6-glycosidic bonds (also discussed in chapter "Glucanases and Chitinases" by César Roncero and Carlos R. Vázquez de Aldana). For example, proteins of the Gas family have been shown to remodel β-1,3-glucans by cleaving β-1,3-glucan polymers internally and transferring the new reducing end to the non-reducing end of other β-1,3-glucan molecules and thereby elongate existing chains (Mouyna et al. 2000). Since Gas1 is anchored to the membrane only by a glycosylphosphatidylinositol (GPI) residue (Nuoffer et al. 1991), it might have been lost during isolation of glucan synthase, a membrane embedded protein, for in vitro experiments. Another enzyme, named Bgl2, was identified in *S. cerevisiae* to introduce branches into the glucan chains. It cooperates with Gas1 and increases

branching efficiency in vitro (Aimanianda et al. 2017). In vivo, *BGL2* deletion does not affect cell growth. However, parallel deletion of *GAS1* causes a synthetically sick phenotype (Aimanianda et al. 2017) which indicates that both enzymes fulfill overlapping roles. A homolog from *A. fumigatus*, Gel4, was recombinantly expressed and showed similar results to recombinant Gas1 from *S. cerevisiae* during in vitro branching assays (Aimanianda et al. 2017).

Shematek et al. believed β-1,3-glucan polymers to be synthesized de novo in *S. cerevisiae* since experiments with ^{14}C-labeled UDP-glucose showed the presence of the labeled substrate at the reducing end of the chain (Shematek et al. 1980). In agreement, Quigley and Selitrennikoff could not identify a primer structure for β-1,3-glucan synthesis in *Neurospora crassa* either (Quigley and Selitrennikoff 1987). The lacking requirement for a primer would distinguish this mechanism from that of other glycosyltransferases, which require acceptor molecules such as proteins, lipids, or polysaccharides for the transfer of sugar units (Paulson and Colley 1989). However, these findings do not exclude the possibility that the polysaccharide is formed by transfer to a primer synthesized from ^{14}C UDP-glucose itself by other enzymes in the plasma membrane. Experiments with *C. albicans* showed the inaccessibility of the reducing end of β-1,3-glucan because reducing treatment of the resulting glucan synthase product did not lead to transformation from glucose into sorbitol. This suggests synthesis of β-1,3-glucan on a primer molecule of so far unknown identity (Paulson and Colley 1989).

While the biochemistry and properties of β-1,3-glucan synthesis could be analyzed to some extent with crude cell extracts, the genes encoding the actual β-1,3-glucan synthase remained unknown for a long time. The first gene involved in β-1,3-glucan synthesis (*FKS1*) was identified in 1993 in *S. cerevisiae* in several parallel approaches conducted by independent research groups. One approach was designed to identify alleles in *S. cerevisiae* conferring hypersensitivity to the immunosuppressant tacrolimus (FK506) and cyclosporin A which inhibit the calmodulin-dependent protein phosphatase calcineurin. In this screen, an allele was identified and termed *fks1-1* (FK506 supersensitive) and calcineurin subunit deletion in this background turned out to be lethal (Parent et al. 1993). Sequencing of the gene from a genomic plasmid library that complemented the *fks1-1* mutation revealed a so far uncharacterized ORF that encodes a protein of about 1800 amino acids to be responsible for the observed effects (Eng et al. 1994). Douglas et al. could show that the gene *ETG1*, whose mutation can confer resistance to echinocandin antifungals, is identical to *FKS1* (Douglas et al. 1994). While screening for mutants that rely on the calcineurin-dependent signalling pathway, the *CND1* gene was identified. This gene was also finally found to be identical to *FKS1* (Garrett-Engele et al. 1995). The same allele was described to encode an enzyme that rescues calcofluor white hypersensitivity of the *cwh53-1* mutant (Ram et al. 1995), complements hypersensitivity to chitin synthesis inhibition (el-Sherbeini and Clemas 1995), confers susceptibility to papulacandin B (Castro et al. 1995), and co-purifies with β-1,3-glucan (Inoue et al. 1995). A second β-1,3-glucan synthase with high homology (88% identity) to Fks1, named Fks2, was identified in *S. cerevisiae* by Mazur et al. and Inoue et al. in 1995. This enzyme was shown to be functionally redundant with Fks1 as disruption of only

one of these two orthologues allows survival of the yeast cell; however, lack of both genes is lethal (Inoue et al. 1995; Mazur et al. 1995). Fks2 expression depends on Ca^{2+} in a calcineurin-dependent manner. Since calcineurin is the target of FK506, this explains why the *fks1-1* mutant exhibited FK506 hypersensitivity in the previously mentioned screens. The fact that Fks2 expression is also strongly induced in response to pheromones in a calcineurin-dependent manner, suggests a role for Fks2 in cell wall synthesis during the mating process (Mazur et al. 1995). A third β-1,3-glucan synthase homolog, Fks3, was identified in *S. cerevisiae* by homology search (Mazur et al. 1995) and functionally characterized in more detail by Ishihara and colleagues (Ishihara et al. 2007). Despite its high homology (56%) to Fks1 and Fks2 which clearly complement each others function in vivo, endogenous Fks3 is not sufficient to rescue a *fks1 fks2* double deletion. Further investigation of Fks3 function by phenotypic analysis revealed abnormal spore wall formation in the single *fks3* mutant that could be complemented neither by Fks1 nor Fks2 (Ishihara et al. 2007). Membrane extracts from cells overexpressing hemagglutinin-tagged Fks3 under the *FKS1* promoter with a temperature-sensitive *fks1* mutant protein and *FKS2* deletion in the background showed hardly any glucan synthase activity in vitro. The protein seemed to be lost from the enriched glucan synthase complex. However, in the same experiment also wild-type Fks1 expressed from a plasmid failed to restore full activity and the fact that in the same strain overexpression of hemagglutinin-tagged Fks3 under the *FKS1* promoter could restore growth at restrictive temperature points in the direction that Fks3 can take over glucan synthase function under specific conditions (Ishihara et al. 2007). All three homologs in *S. cerevisiae* are differentially expressed: Fks1 constitutes the main β-1,3-glucan synthase required under normal growth conditions, while Fks2 and Fks3 both seem to be involved in formation of spore walls with the specialty of Fks3 to stabilize active Rho1, the regulating enzyme in the β-1,3-glucan synthase complex (Ishihara et al. 2007; see below). How this stabilization is achieved remains to be elucidated, but one could speculate that an increased binding affinity between Fks3 and active Rho1 might confer elevated stress resistance that cannot be achieved simply by expression of constitutively active Rho1 (Ishihara et al. 2007).

Similar to the three β-1,3-glucan synthases of *S. cerevisiae*, the synthases encoded in other fungal species have a length of about 1600–1900 amino acids. Like baker's yeast, many other fungi harbor several β-1,3-glucan synthase genes. For example, *Phycomyces blakesleeanus* (*Mucoromycotina* lineage), despite the low amount of glucan in its cell wall, and the two pathogenic yeasts *C. albicans* and *C. glabrata* encode three β-1,3-glucan synthase homologs, and the fission yeast *S. pombe* even four. Interestingly, the importance of the individual genes for the respective species differs greatly. Partial redundancy of the synthases is observed in *C. glabrata*. Very similar to the situation in *S. cerevisiae*, *C. glabrata* deleted for *FKS1* or *FKS2* is viable, but Δ*fks1*Δ*fks2* is not (Katiyar et al. 2012). In contrast, viability of *C. albicans* strictly depends on one of the three glucan synthases, Gsc1/Fks1, while the other two synthase homologs are dispensable (Douglas et al. 1997; Mio et al. 1997; Suwunnakorn et al. 2018). *S. pombe* non-redundantly depends on three of the four β-1,3-glucan synthase homologs, namely Bgs1, Bgs3, and Bgs4

(Cortés et al. 2005; Liu et al. 1999, 2000, 2002; Martín et al. 2003). This indicates that each of the three β-1,3-glucan synthase homologs must exert a specific and essential function which cannot be compensated by the others [summarized in (García Cortés et al. 2016)]. Other species like *Cryptococcus neoformans*, *Aspergillus fumigatus,* and *Magnaporthe oryzae* encode only one β-1,3-glucan synthase. Similar to other characterized yeasts, *C. neoformans* is not viable without its β-1,3-glucan synthase (Thompson et al. 1999). β-1,3-glucan was initially thought to be essential for viability of all fungi. However, it became clear that the importance of β-1,3-glucan synthesis merely depends on the individual species and cannot be generalized. In contrast to all other characterized species, *A. fumigatus* can survive upon deletion of the only β-1,3-glucan synthase encoding gene, but shows severe growth defects with cell lysis and a retention of galactosaminogalactan. To compensate the lack of β-1,3-glucan, *Aspergillus* drastically increases the cell wall chitin and essentially depends on the formation of septa (Dichtl et al. 2015; Loiko and Wagener 2017). The compensatory upregulation of cell wall chitin depends on calcineurin signaling but also on other pathways and appears to be a uniform reaction in fungi upon β-1,3-glucan synthesis inhibition because similar observations were made in many different fungal species upon treatment with echinocandins (Fortwendel et al. 2009; Stevens et al. 2006; Walker et al. 2008; reviewed and discussed in Wagener and Loiko 2017). The lack of β-1,3-glucan results in the absence of an important scaffold (Fontaine et al. 2000; Kang et al. 2018). As a consequence, galactomannan, a cell wall polymer characteristic for *Aspergilli*, cannot be linked to the cell wall and is released to the supernatant (Dichtl et al. 2015). Possibly, other fungi which have only minor amounts of β-1,3-glucan in their cell walls (e.g., *Mucorales*; Mélida et al. 2015) and show no in vitro susceptibility to echinocandins (Almyroudis et al. 2007; Vitale et al. 2012) might have even less or no growth defects upon complete inactivation of their β-1,3-glucan synthases.

It was repeatedly questioned whether the *FKS* genes encode indeed the catalytic subunit of the β-1,3-glucan synthase. In vitro glucan synthase assays were performed with membrane fractions from *S. cerevisiae fks1* mutants showing a strongly reduced β-1,3-glucan synthase activity in the mutants compared to wild type (Douglas et al. 1994). Due to high impurity of membrane preparations, this observation could not exclude the possibility that Fks1 is not the enzyme itself but merely a factor required for synthesis. Evidence came from three studies supporting the assumption the catalytic center of the complex is encoded by *FKS1*. In the first study, an antibody raised against the Fks1 homolog Fks2, also recognizing native Fks1, was used to deplete membrane extracts of cells expressing either Fks1 or Fks2 specifically of β-1,3-glucan synthase activity. This resulted in association of β-1,3-glucan synthase activity with the respective precipitate (Mazur and Baginsky 1996) and demonstrates that Fks1 or Fks2 must be present in a protein complex with β-1,3-glucan synthase activity. Further evidence for the *FKS* gene products being part of the β-1,3-glucan synthase complex came from Inoue et al. They generated antibodies against a purified cell fraction with β-1,3-glucan synthase activity. The same antibodies were subsequently able to detect specifically a

200 kDa protein in western blot analysis that was identified by HPLC and subsequent peptide sequencing as Fks1 (Inoue et al. 1995). Finally, Schimoler-O'Rourke et al. were able to photo cross-link a radiolabeled UDP-glucose analog, the β-1,3-glucan synthase substrate, to the Fks1 homolog FKS-1 in *Neurospora crassa*. This demonstrated the direct interaction of FKS with UDP-glucose and strongly supports its catalytic role in β-1,3-glucan synthesis (Schimoler-O'Rourke et al. 2003). Final clarification of the synthase activity will be obtained by reconstituting the system in vitro. These findings in yeast and *N. crassa* were also supported by a study that aimed on identifying the β-1,3-glucan synthase of *A. fumigatus* (Beauvais et al. 2001). In this work, co-localization of Fks1 with newly synthesized β-1,3-glucan at the apex of the germ tube was observed. In addition, product entrapment of the synthase complex allowed the identification of Fks1, Rho1, a membrane H^+-ATPase and a homolog of a bacterial ABC glucan transporter which indicates the functional conservation of Fks1 (Beauvais et al. 2001).

The only known regulatory subunit of the synthase complex, the Rho GTPase Rho1, was identified by further analysis of the co-factors relevant for β-1,3-glucan synthesis. In an extensive in vitro study, Mg^{2+}-dependent ATP hydrolysis was found to be required for β-1,3-glucan synthesis activity while GTP stimulated the reaction by a hydrolysis-independent mechanism (Shematek and Cabib 1980). This suggested that GTP itself acts as a stimulating factor for the β-1,3-glucan synthase. ATP, on the other hand, would be most likely required to phosphorylate a so far unknown activator of the β-1,3-glucan synthase. Separation of cell membrane extracts into a membrane bound and a soluble phase revealed that both contain components that are crucial for β-1,3-glucan synthesis. The previous observation that small amounts of GTP stimulate glucan synthase activity (Shematek and Cabib 1980) and the phenotypic consistency of cell wall mutants with Rho GTPase mutants resulted in them being tested for their role in glucan synthesis. Finally, Rho1 could be confirmed as the activator of the glucan synthase complex (Drgonová et al. 1996). The physical interaction between Fks1 and Rho1 was proven by co-purification of both proteins (Qadota et al. 1996). Rho1 is a GTP binding protein which itself cannot be labeled by UDP-glucose (Mazur and Baginsky 1996) indicating its sole regulatory role during β-1,3-glucan synthesis. Rho1 is able to stimulate β-1,3-glucan synthesis in its de-ribosylated state while ribosylation of Rho1 strongly inhibits Fks activity. This observation was made in vitro with extracts from Δ*fks1* and Δ*fks2* mutants where only one β-1,3-glucan synthase was present (Mazur and Baginsky 1996) demonstrating both, Fks1 and Fks2, can be regulated by Rho1. However, it cannot be excluded that in vivo under certain conditions any of the β-1,3-glucan synthases is additionally regulated by other Rho GTPases than Rho1. The strict requirement of Rho1 for β-1,3-glucan synthase activity establishes a connection between β-1,3-glucan synthesis and the cell wall integrity pathway in which Rho1 functions as signal transducer (see 2.4) (Mazur and Baginsky 1996). The regulatory function of Rho1 appears to be strongly conserved in fungi. For example, in 1996, it was shown that overexpression of *rho1* in *S. pombe* can partially complement a temperature-sensitive β-1,3-glucan synthase mutant (Arellano et al. 1996). Expression of constitutively

active *rho1* alleles resulted in strikingly increased β-1,3-glucan synthase activity and thicker cell walls (Arellano et al. 1996). Repression of *rho1* in *A. fumigatus* results in cell lysis (Dichtl et al. 2012). And like in *S. cerevisiae*, Rho1 of *A. fumigatus* could be co-purified with the β-1,3-glucan synthase Fks1 which is again in agreement with direct interaction of both proteins in the β-1,3-glucan synthase complex (Beauvais et al. 2001).

The β-1,3-glucan synthase is a plasma membrane-integrated enzyme with multiple predicted transmembrane domains (Douglas et al. 1994; Johnson and Edlind 2012; Klis et al. 2006; Lesage and Bussey 2006). Sequence alignments of β-1,3-glucan synthase proteins from various fungi show the highest conservation of the protein in its central domain while N- and C-terminal regions are rather divergent (Johnson and Edlind 2012). The differences in the N- and C-terminal regions are probably linked to their different functional roles and cell cycle specificities observed in various different species. Topology predictions based on different algorithms resulted in varying prognoses concerning the number and positions of the transmembrane domains in yeast Fks1 (Johnson and Edlind 2012). To further investigate the topology of Fks1, a hemagglutinin (HA)-Suc2-His4C fusion assay (Kim et al. 2003) was performed. This assay employed fusions of Fks1 fragments, chosen to end in predicted loop regions between transmembrane domains, to a C-terminal reporter. If the His4C part, which harbors an enzymatic function essential for histidine biosynthesis, is localized to the cytosolic side, the cell is able to grow on medium without histidine. In contrast, when the C-terminus is localized to the extracellular space, the Suc2 moiety will become glycosylated in the endoplasmic reticulum. The glycosylation can be monitored by degradation of glycosyl residues resulting in a migration shift on SDS-PAGE. Based on this elegant assay, Johnson and Edlind could propose an updated topology model for Fks1 which differs in some aspects from the in silico prediction. They could confirm the presence of a soluble cytosolic N-terminus and six subsequent N-terminal helices. The short C-terminus protrudes into the extracellular space preceded by seven C-terminal transmembrane helices (four predicted C-terminal helices could not be experimentally confirmed). N- and C-terminal membrane-integrated domains of the protein are separated by a large and highly conserved cytosolic loop. Interestingly, a previous study by Okado et al. (2010) mapped and correlated certain mutations within the *FKS1* gene to specific phenotypes. It was shown that mutations in the soluble cytoplasmic N-terminus result only in slightly decreased glucan synthase activity in vitro, but cells have a strong defect in incorporation of glucan into the budding daughter cell wall (Okada et al. 2010). Probably, this region is involved in proper targeting or regulation of the enzyme, but solid data supporting these hypotheses are missing. A region within the central cytosolic loop close to the N-terminal transmembrane domains was identified to influence cell morphology and polarity (Okada et al. 2010). Since mutation in the more N-terminal region of the central loop only partially reduces the synthase activity in vitro, it was proposed that this part of the protein is required for interaction with a cell polarity regulating factor (Okada et al. 2010). The nature of this factor, however, remains to be identified.

In baker's yeast, Fks1 expression depends on the cell cycle (Mazur et al. 1995). In agreement with this expression profile and growth defect, one of the β-1,3-glucan synthases in *S. pombe*, Bgs1, was shown to be also involved in cell polarization (Cortés et al. 2002). The catalytic site of Fks1 seems to be located in the more C-terminal part of the central cytosolic loop as mutations in this region strongly decrease the in vitro glucan synthase activity (Okada et al. 2010). Based on the updated topology model of Johnson and Edlind, it was possible to map mutations conferring either resistance or hypersensitivity to echinocandin glucan synthase inhibitors in close proximity to the central loop, importantly, at the external side of the plasma membrane. Of note, some mutations confer specific resistance to particular echinocandins suggesting interaction sites for echinocandin-specific side chains (Johnson et al. 2011). However, all of them are in relatively close proximity to the membrane which is in agreement with the fact, that echinocandins carry a lipid tail that is crucial for their antifungal activity and probably anchor the molecule within the outer membrane leaflet (Denning 2003). Due to the final extracellular localization of β-1,3-glucan, it was suggested that during synthesis the growing, polymer enters the cell wall space guided through the membrane by a channel formed by the transmembrane domains of the enzyme. However, evidence for this hypothesis is still missing despite all studies trying to dissect β-1,3-glucan synthase function.

2.2 β-1,3;1,4- and β-1,6-Glucan Synthesis

Another cell wall glucan which is typically found in yeasts is β-1,6-glucan. Although it represents only approx. 12% of the entire cell wall polysaccharides in baker's yeast (Magnelli et al. 2002), inhibition of β-1,6-glucan synthesis results in a severe growth defect (Roemer et al. 1993). β-1,6-glucan was shown to cross-link chitin to β-1,3-glucans (Kollár et al. 1995) and cell wall anchored mannoproteins to chitin as well as to β-1,3-glucans (Kapteyn et al. 1996, 1997). This important role in anchoring mannoproteins to the yeast cell wall is probably the reason for the strong phenotype observed upon lack of β-1,6-glucan. Analysis of β-1,6-glucan synthesis in vitro employing either membrane preparations or whole permeabilized cells of *S. cerevisiae* revealed that the membrane fraction is sufficient for β-1,3-glucan synthesis, but not for synthesis of β-1,6-glucan (Aimanianda et al. 2009). Several players involved in β-1,6-glucan synthesis have been identified, mainly via screening approaches. One screen was performed to identify mutants with increased resistance to K1 killer toxin (Al-Aidroos and Bussey 1978; Boone et al. 1990). This toxin specifically binds to β-1,6-glucan and thereby permeabilizes the cell membrane to ion passage (Martinac et al. 1990; Pagé et al. 2003). The identified alleles were called killer toxin resistant (KRE) and some of them were investigated in more detail. *KRE5* encodes a putative glycosyl transferase that localizes to the ER (Boone et al. 1990; Meaden et al. 1990) and a *kre5-ts2* mutant was unable to synthesize β-1,6-glucan in an assay with permeabilized yeast cells (Aimanianda et al. 2009).

Kre6, another factor for β-1,6-glucan synthesis identified in these screens (Roemer and Bussey 1991), is a Golgi membrane protein which has a homolog, Skn1, that can complement the Kre6 function (Roemer et al. 1994). Besides this, a cytoplasmic factor involved in this pathway named Kre11 was identified, but again its precise role in β-1,6-glucan synthesis remains elusive (Brown et al. 1993). Only two known players of β-1,6-glucan synthesis localize to the plasma membrane or periplasmic space, the actual site where β-1,6-glucan synthesis takes place (Montijn et al. 1999). Kre9 is a soluble protein and its deletion reduces β-1,6-glucan to 20% of the original amount (Shahinian and Bussey 2000). Also, a *kre9* deletion mutant was tested in an in vitro assay and did not show any β-1,6-glucan synthase activity (Aimanianda et al. 2009). Kre1 is a protein, predicted to be GPI-anchored to the plasma membrane that was proposed for being the actual receptor of the K1 killer toxin (Breinig et al. 2002, 2004). Its deletion resulted in 40% decrease of β-1,6-glucan and in a reduction of the β-1,6-glucan polymer length (Boone et al. 1990). However, it remains to be elucidated whether one of these proteins functions as a β-1,6-glucan synthase or this enzyme remains to be identified. In contrast to yeasts, filamentous fungi like *N. crassa* and *A. fumigatus* completely lack β-1,6-glucan in their cell walls (Fontaine et al. 2000; Maddi and Free 2010). Instead, another polysaccharide species, β-1,3;1,4-glucan, was identified in *A. fumigatus* which comprises approx. 10% of the total β-glucan (Fontaine et al. 2000). So far, only one component was identified to be involved in β-1,3;1,4-glucan biosynthesis. *A. fumigatus* Tft1, a predicted glycosyltransferase, was shown to be essential for β-1,3;1,4-glucan synthesis as knock out of the encoding gene results in complete loss of β-1,3;1,4-glucan. However, the role of β-1,3;1,4-glucan for survival of *A. fumigatus* is unclear as neither depletion nor overexpression of the protein resulted in any growth phenotype (Samar et al. 2015). Surprisingly, deletion of a functional homolog of AfTft1 in *A. nidulans, celA,* that is required for β-1,3;1,4-glucan synthesis was reported to result in a strong growth phenotype reminiscent to other cell wall biosynthesis mutants (Guerriero et al. 2017).

2.3 α-1,3-Glucan Synthesis

Cell wall α-glucans have been identified in many filamentous fungi and some yeasts, including the *Ascomycota Schizosaccharomyces pombe*, *Aspergillus* species, *Histoplasma capsulatum*, *Blastomyces dermatitidis, Paracoccidioides brasiliensis* as well as the plant pathogenic fungi *Magnaporte grisea* and *Fusarium oxysporum f. sp. lycopersici* and in the basidomycetous fungus *Cryptococcus neoformans* (Bull 1970; Fujikawa et al. 2012; Hogan and Klein 1994; Horisberger et al. 1972; Kanetsuna et al. 1969; Manners and Meyer 1977; Reese and Doering 2003; San-Blas et al. 1978; Schoffelmeer et al. 1999). In *Aspergillus* species, α-glucans represent approximately 35–46% of the total cell wall carbohydrates (Gastebois et al. 2009). The fission yeast *S. pombe* contains 18–28% α-glucan in its cell wall

(Manners and Meyer 1977). In contrast and as stated above, cell wall α-glucans are absent from the baker's yeast *Saccharomyces cerevisiae*, all *Candida* species, *Fusarium graminearum,* and *Ustilago maydis* (Damveld et al. 2005a).

The chemical structure of cell wall α-glucans have been studied in the fission yeast *S. pombe*, *H. capsulatum*, *Aspergillus* species, and *P. brasiliensis*. Cell wall α-glucans in these fungi actually represent linear polymers consisting of glucose with α-1,3 and α-1,4-glycosidic bonds. α-1,3-glycosidic lined glucans represent the vast majority of these polymers. Grün et al. showed that cell wall α-glucan of *S. pombe* is a linear polysaccharide without branching points composed of approximately 260 glucose residues (Grün et al. 2005). Approximately 90% of these α-glucan polymers are linked by α-1,3-glycosidic bonds and the remaining 10% are linked by α-1,4-glycosidic bonds (Grün et al. 2005). A detailed analysis of these polymers revealed that the α-1,4-glycosidic bonds are not randomly distributed along the α-glucan chain. Instead, they occur at the reducing end of an α-1,3-glucan chain approximately 120 glucose residues in length. Two of these chains are interconnected to form one linear α-glucan polysaccharide macromolecule, having short α-1,4-glucan chains as a linker in between (Grün et al. 2005). A similar architecture was found for cell wall α-glucan from *A. wentii*, *A. nidulans,* and *A. fumigatus* even though the structure was somewhat different. The α-1,3-glucan chains were approximately 200 glucose residues in length and the linearly assembled α-glucan polysaccharide macromolecule consisted of multiple such chains interconnected by short α-1,4-glucan chains (Choma et al. 2013; Latgé 2010; Miyazawa et al. 2018). The average number of interconnected α-1,3-glucan chains per macromolecule varied and were determined to be twenty-five in *A. wentii* and approximately five in *A. nidulans* (Choma et al. 2013; Miyazawa et al. 2018). Apparently, the number of interconnected α-1,3-glucan chains largely depends on the enzymatic properties and expression rates of the individual α-1,3-glucan synthases (Miyazawa et al. 2018). In contrast to α-glucan from *S. pombe*, the reducing ends of α-glucan from *A. wentii* are terminated by α-1,3-glucan and not by α-1,4-glucan (Choma et al. 2013; Grün et al. 2005). Cell wall α-glucan of *P. brasiliensis* might differ significantly from α-glucan of *S. pombe* and *Aspergilli*. It was shown that the macromolecules have about 97% α-1,3-glucan, with some α-1,4-glucan branching points (Sorais et al. 2010).

Of essential importance for cell wall α-glucan synthesis in fungi are the α-1,3-glucan synthases. These enzymes are found in all α-1,3-glucan-positive fungi and were proposed to contain the catalytic domain(s) that synthesize α-glucan chains. The number of α-1,3-glucan synthases encoded in the genomes of different fungal species varies greatly. *S. pombe* and *A. niger* encode five α-1,3-glucan synthases, *A. fumigatus* three, *A. nidulans* two and *C. neoformans* and *H. capsulatum* only one. α-1,3-glucan synthase null mutants have been characterized in *A. nidulans*, *A. fumigatus*, *C. neoformans,* and *H. capsulatum* (He et al. 2014; Henry et al. 2012; Marion et al. 2006; Miyazawa et al. 2018; Reese et al. 2007; Yoshimi et al. 2013). The cell walls of respective single (*H. capsulatum*, *C. neoformans*), double (*A. nidulans*) or triple deletion mutants (*A. fumigatus*) were essentially free of α-1,3-glucan which clearly demonstrates the essential roles of α-1,3-glucan

synthases for α-1,3-glucan synthesis. Comprehensive analyses of the deletion mutants allowed deciphering the role of α-1,3-glucan synthesis for individual fungal species. *Aspergilli* deleted for all encoded α-1,3-glucan synthases hardly show any growth defects on solid medium (Henry et al. 2012; Yoshimi et al. 2013). Prominent phenotypes due to the lack of α-1,3-glucan synthesis in the cell wall of *Aspergillus* spp. appear to be an altered extracellular matrix and a significantly reduced amount of conidial and hyphal adhesion in liquid media (Fontaine et al. 2010; Yoshimi et al. 2013). Importantly, the virulence of the triple α-1,3-glucan synthase mutant of *A. fumigatus* is highly reduced in a murine model of aspergillosis (Beauvais et al. 2013) and the fungus can be early detected by immune cells and more efficiently engulfed by the macrophages of the host compared to wild type strain infections (Beauvais et al. 2013). Besides this, the susceptibility to certain cell wall stress conditions of such mutants is slightly altered (Damveld et al. 2005a; He et al. 2014; Valsecchi et al. 2019). A recent analysis of the cell wall architecture of *A. fumigatus* highlights the structural and functional versatilities of cell wall α-glucan in this mold (Kang et al. 2018), even though the evident minor growth defects of the α-1,3-glucan synthase deletion mutants argue against a major role in cell wall integrity (Henry et al. 2012; Yoshimi et al. 2013). The cell wall α-1,3-glucan synthase of the fungus *C. neoformans* is important for anchoring the polysaccharide capsule of this pathogen (Reese and Doering 2003). Deletion of the α-1,3-glucan synthase of *H. capsulatum* results in several in vitro growth defects, an altered cell wall structure and reduced ability to colonize murine lungs (Rappleye et al. 2004, 2007). In marked contrast to *Aspergillus*, *Cryptococcus* and *Histoplasma*, the fission yeast (*S. pombe*) cannot live upon deletion of its main α-1,3-glucan synthase *ags1* (Hochstenbach et al. 1998; Katayama et al. 1999). Mutants lacking the less important α-1,3-glucan synthases *mok11-14* show no morphological changes or affectations of viability but defects in sporulation (García et al. 2006).

The overall protein structure of the different α-1,3-glucan synthases encoded in the so far characterized species appears to be very similar (Fig. 2). With the exception of Mok14 of *S. pombe* (SpMok14), all α-1,3-glucan synthases have a length of about 2400 amino acids. SpMok14 is significantly shorter (approx. 1300 amino acids). The N-termini of the synthases harbor a secretory signal peptide which is readily predicted by bioinformatic algorithms such as SignalP 5.0 [Sec/SPI; (Almagro Armenteros et al. 2019)]. This is followed by an extracellular domain with similarity to α-amylases [PF00128; (El-Gebali et al. 2019)]. This domain is lacking in SpMok14. After approximately 1100 amino acids, a transmembrane domain is predicted [TMHMM Server v. 2.0; (Krogh et al. 2001)]. The following intracellular protein part shares homology with a starch synthase catalytic domain (PF08323). In case of three α-1,3-glucan synthases of *S. pombe* (Ags1, Mok12, and Mok14) and the α-1,3-glucan synthase of *C. neoformans*, an additional homology with glycosyl transferases (group 1; PF00534) can be found. Apparently, this domain is also present in the other α-1,3-glucan synthases but is currently simply not detected by the PF00534 hidden Markov model. The PF08323 (starch synthase catalytic domain) pattern combined with the PF00534 (glycosyl transferase group 1) pattern next to it encompass approximately 500 amino acids and are

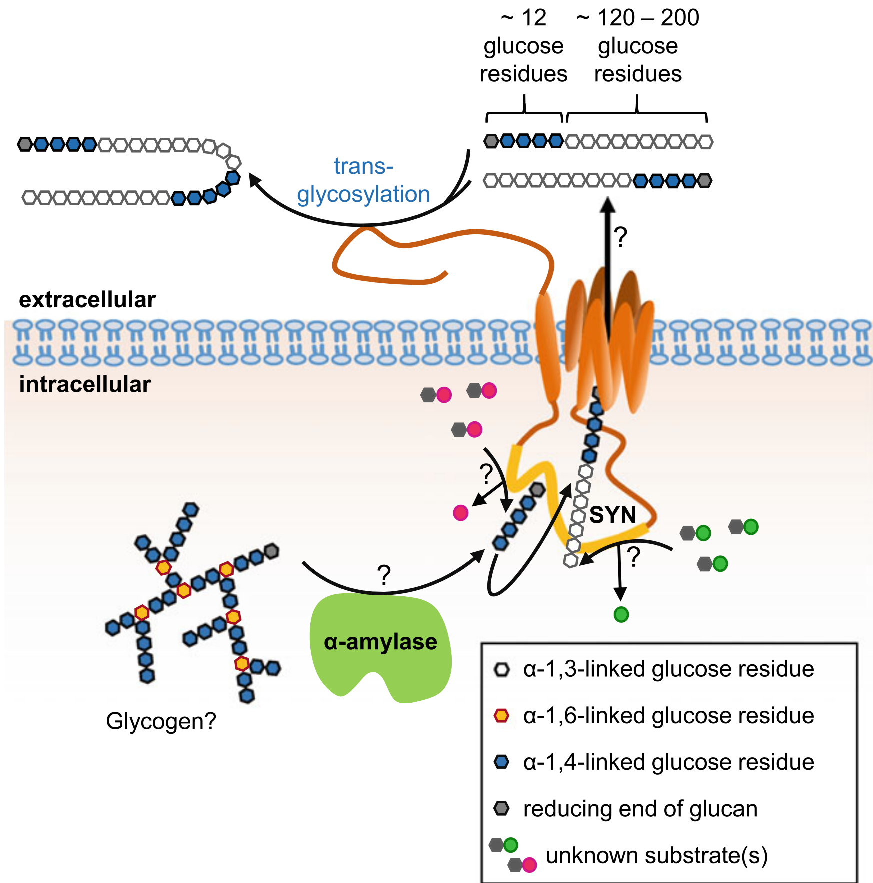

Fig. 2 Fungal α-1,3-glucan synthesis. The α-1,3-glucan synthase is a membrane protein approx. 2400 amino acids in length. The N-terminal part of the protein is approx. 1000 amino acids in length and facing the extracellular side. This domain is involved in linking the exported α-glucan chains to form longer polysaccharide macromolecules. The extracellular part is followed by a transmembrane domain and an intracellular part (approx. 1000 amino acids) which harbors the synthase (SYN) domain. It is controversial whether this part of the protein synthesizes α-1,4-glucan, α-1,3-glucan or both. At least in some fungal species, an intracellular α-amylase is required for synthesis of α-1,4-glucan which probably functions as a primer and is required for efficient α-1,3-glucan synthesis. The substrate(s) for α-1,4-glucan and α-1,3-glucan synthesis are currently not known. The C-terminal multiple membrane-spanning part of the α-1,3-glucan might form a pore-like structure to pass the synthesized α-glucan chains to the outside

commonly found in α-1,4-glucan-synthesizing enzymes such as glycogen and starch synthases. This domain is followed by approximately 400 amino acids that do not share much homology between the different α-1,3-glucan synthases. The respective intracellular part of the protein was denoted as the SYN (synthase) domain (Vos et al. 2007). The final C-terminal approximately 400 amino acids

harbor a multiple membrane-spanning domain. It was proposed that this part of the α-1,3-glucan synthases forms a pore-like structure to pass the α-glucan chains to the outside (Hochstenbach et al. 1998). But experiments that clearly prove this are still lacking.

In consideration of the conserved structure of α-1,3-glucan synthases with an extracellular and intracellular part, it was suggested that the intracellular part of the protein harbors the synthase domain that catalyzes the α-1,3-glucan chain synthesis (Hochstenbach et al. 1998). However, it must be noted that neither any biochemical synthesis activity of the intracellular protein part has been shown in vitro, nor was the substrate for α-glucan synthesis clearly identified. UDP-glucose was proposed to play some direct or indirect role (Hochstenbach et al. 1998; Marion et al. 2006). According to a model proposed by Grün et al., the α-1,3-glucan synthase might not assemble the α-1,3-glucan polymers directly. Instead, a short α-1,4-glucan oligomer ($\sim$12 glucose residues) is required as a primer to initiate α-1,3-glucan synthesis (Grün et al. 2005). The origin of such putative primers is not really clear. Vos et al. suggested that the intracellular SYN domain of the α-1,3-glucan synthase might synthesize α-1,4-glucan by itself (Vos et al. 2007). Indeed, the SYN domain shares significant homology with well-characterized α-1,4-glucan-synthesizing enzymes such as glycogen and starch synthases.

Vos et al. backed their hypothesis with a bunch of experimental results. Overexpression of the α-1,3-glucan synthase Ags1 in *S. pombe* (SpAgs1) results in increased accumulation of α-1,4-glucan (Vos et al. 2007). Importantly, results of this study are based on an iodine vapor staining assay and the assumption that iodine specifically stains α-1,4-glucan (and not other constituents of *S. pombe*). The α-1,3-glucan content was not quantified in this study. Overexpression of a mutant SpAgs1 which lacks the extracellular α-amylase-like domain (ΔTGL) also caused accumulation of α-1,4-glucan while overexpression of mutant SpAgs1 which either lacks the SYN domain or which harbors point mutations in a conserved Glu-X_7-Glu motif in the SYN domain did not result in accumulation of iodine-stainable material (Vos et al. 2007). The red-brownish color shade of the SpAgs1-overexpressing strains after iodine staining allowed Vos et al. to estimate the length of the α-1,4-glucan chains to be between 10 and 40 residues (Vos et al. 2007). This is in agreement with the finding that the α-1,3-glucan polymers are linked by short α-1,4-glucan chains approximately 12 glucose residues in length, which are overproduced upon overexpression of SpAgs1 (Vos et al. 2007).

In a recent study performed by Miyazawa et al., the effects of α-1,3-glucan synthase overexpression were investigated in *A. nidulans* (Miyazawa et al. 2018). This species encodes two α-1,3-glucan synthases (AgsA and AgsB). Overexpression of any of these α-1,3-glucan synthases increased the overall α-glucan amount in the cell wall, including α-1,3-glucan and not specifically α-1,4-glucan. Notably, the lengths of the α-1,3-glucan chains in the α-glucan polysaccharide macromolecules (approx. 200 glucose residues) remained the same (Miyazawa et al. 2018). Taken together, the results of Vos et al. and Miyazawa et al. both support a model where α-glucan synthesis at large depends on the intracellular SYN domain. However, the observation of Miyazawa et al. that

overexpression of the synthases increases cell wall α-1,3- and α-1,4-glucan equally (Miyazawa et al. 2018) somehow challenges the specific conclusion of Vos et al. that the SYN domain synthesizes α-1,4-glucan chains (Vos et al. 2007). If the hypothesis of Vos et al. holds true, it raises the question whether the SYN domain can additionally synthesize α-1,3-glucan chains or whether another unknown enzyme is required to exert this function. Of course, based on the current knowledge and because enzymatic domains that synthesize α-1,3-glucan in fungi are not defined yet, it could also be the other way around.

A possible alternative origin of α-1,4-glucan could involve intracellular α-1,4-amylases. Deletion of the α-1,4-amylase gene *AMY1* in *H. capsulatum* resulted in a mutant with greatly reduced and mislocalized α-1,3-glucan based on immunostaining experiments (Marion et al. 2006). Expression of a homologous protein of *Paracoccidioides brasiliensis* in this mutant restored the quantities and surface localization of α-1,3-glucan (Camacho et al. 2012). Homologs of these α-1,4-amylases were also studied in *Aspergillus*. The number of close homologs varies among the different *Aspergillus* species. While *A. fumigatus* and *A. nidulans* encode only one close homologue (AFUA_1G15150 and AN3309/*amyG*, respectively), *A. niger* and *A. oryzae* encode two (An01g13610/*amyD*, An09g03110/*emyE* and AO090005001193, AO090003001497, respectively). So far, a null mutant has only been characterized in *A. nidulans*. He et al. showed that in *A. nidulans* deletion of *amyG* resulted in a significant decrease of immunostainable α-1,3-glucan (He et al. 2014). In agreement with this microscopy-based result, cell wall analysis of this Δ*amyG* mutant demonstrated that the amount of glucose in the alkali-soluble cell wall fraction (which primarily represents α-1,3-glucan) is drastically reduced to approximately 13% of wild type. However, it is noteworthy that *A. nidulans* mutants that lack the genes encoding the α-1,3-glucan synthases (*agsA* and *agsB*) were found in the same study to exhibit even much less glucose in this fraction (reduced to approx. 1% of wild type) (He et al. 2014). In agreement, very similar results were obtained in a recent independent study in *A. nidulans* with respect to α-glucan-stainability and cell wall composition (Miyazawa et al. 2018). The observation that some mislocalized α-1,3-glucan was still stainable in the *H. capsulatum* Δ*amy1* mutant (Camacho et al. 2012; Marion et al. 2006) and the finding that a significant amount of glucose (i.e., cell wall α-glucan) was still present in the alkali-soluble fraction of the *A. nidulans* Δ*amyG* mutant (He et al. 2014; Miyazawa et al. 2018) suggests that the intracellular α-1,4-amylase contributes to but is not essential for α-1,3-glucan synthesis *per se*. The substrate used by the intracellular α-1,4-amylases to produce the presumed α-1,4-glucan primers for α-1,3-glucan synthesis as well as the enzymes important for the substrate synthesis remain unknown. Interestingly, fission yeast encodes many putative α-amylases, but none of them is a close homologue of *H. capsulatum* Amy1 or *A. nidulans* AmyG. This raises the question which enzyme could be responsible for this apparently important function in α-1,3-glucan synthesis in *S. pombe*.

Upon arrival of the α-glucan chains outside of the cell, the extracellular domain of the α-1,3-glucan synthases comes into play. The function of this domain was studied based on a temperature-sensitive *S. pombe* mutant (*ags1-1^{ts}*). This mutant

harbors a mutation in SpAgs1 that results in an amino acid exchange at position 696 (G696S, positioned in the extracellular domain but outside the α-amylase domain). The *ags1-1^ts^* mutant accumulates much less α-glucan in its cell wall and shows cell lysis at restrictive temperatures (Hochstenbach et al. 1998). Expression of SpAgs1 proteins with mutations in the SYN domain that diminish the ability of the enzyme to synthesize α-glucan chains can complement the temperature sensitivity of the *ags1-1^ts^* mutant. This demonstrates that the growth defect of G696S mutation is most likely linked to the function of the extracellular domain (Vos et al. 2007). Interestingly, the cell wall α-glucan in the *ags1-1^ts^* mutant is not only reduced but the polysaccharide macromolecules also had half the size compared to those of the wild type (Pereira et al. 2000). The lengths of the α-1,3-glucan chains remained similar to those of the wild type and kept some short α-1,4-glucan chains at the reducing end. The authors, therefore, concluded that the extracellular domain links the exported α-glucan chains to form longer polysaccharide macromolecules (Pereira et al. 2000).

2.4 Regulation of Glucan Synthesis

Several signaling pathways have an impact on cell wall biogenesis. Best characterized is the regulatory role of the cell wall integrity (CWI) signaling pathway. Core components of this pathway exist in all fungal species that were characterized so far. Detailed insights regarding its functionality and its crosstalk with other signaling pathways were obtained in baker's yeast (reviewed in Levin 2005, 2011). Several aspects of CWI signaling were also studied *S. pombe* (Pérez and Cansado 2010) and in many other fungal model organisms (e.g., Richthammer et al. 2012; Rodicio et al. 2008) and, in recent years, extensively in many pathogens due to its role in virulence (Chen et al. 2019a, b; Dichtl et al. 2016). The CWI pathway comprises specialized cell wall stress sensors at the cell surface, guanine nucleotide exchange factors, Rho GTPases, GTPase-activating proteins, several kinases and phosphatases, and transcription factors. The cell wall stress sensors are highly glycosylated and membrane-anchored mechanosensors that detect specific alterations in the cell wall. In *S. cerevisiae*, two types of cell wall stress sensors were identified (Wsc1-3; Mid2 and Mtl1) which are also conserved in other fungal species (Dichtl et al. 2012; Rodicio et al. 2008). Upon activation, both sensor types activate guanine nucleotide exchange factors (in *S. cerevisiae*: Rom1 and Rom2) and trigger GDP/GTP exchange at Rho GTPases (Kanno et al. 2015; Levin 2011; Richthammer et al. 2012; Samantaray et al. 2013). In *S. cerevisiae*, clearly the most important Rho GTPase in this context is Rho1 (*Sc*Rho1). In other fungal species, the Rho2 GTPases appear to play an additional key role (Kwon et al. 2011; Pérez and Cansado 2010; Richthammer et al. 2012). Importantly, especially Rho1 is also a central player in multiple other pathways, for example, it is involved in regulation of exocytosis and of the actin cytoskeleton (Levin 2011). A major downstream effector of Rho1 is protein kinase C (in *S. cerevisiae*: Pkc1). This kinase activates a

conserved MAP kinase module (in *S. cerevisiae*: Bck1 (MAPKKK), Mkk1 and Mkk2 (MAPKK), and Mpk1 (a.k.a. Slt2; MAPK), and its homologous pseudokinase Mlp1), but also multiple additional effectors (Dichtl et al. 2016; Levin 2011; Pérez and Cansado 2010). The central MAP kinases (in *S. cerevisiae*: Mpk1, and Mlp1) regulate the transcription of multiple enzymes involved in cell wall biosynthesis and stress response (Fujioka et al. 2007; Levin 2011; Park et al. 2016). The transcription factors regulated by Mpk1 and Mlp1 in *S. cerevisiae* are Rlm1 (*Sc*Rlm1; by Mpk1) and the SBF transcription factor Swi4/Swi6 (by Mpk1 and Mlp1).

While the interaction between the CWI pathway and cell wall biogenesis is well established, the exact regulatory steps and relevance of individual players on a molecular level remain only partially resolved. Regulation of β- and α-glucan synthesis probably occurs at multiple levels. These involve regulation on a transcriptional level and most likely direct regulation of the enzymatic activities of the synthases by protein–protein interactions.

2.4.1 Regulation of β-1,3-Glucan Synthesis

It is generally assumed that the Rho GTPase Rho1 is an essential part of the β-1,3-glucan synthase complex (extensively discussed 2.1). This function appears to be strongly conserved in other characterized fungal species and thereby strongly links the CWI pathway with β-1,3-glucan synthesis (Arellano et al. 1996; Beauvais et al. 2001).

Transcriptional regulation of β-1,3-glucan synthases may also occur via the CWI pathway. At least in *S. cerevisiae*, expression of one of the three β-1,3-glucan synthases, *fks2*, can be induced via the CWI MAP kinase Mpk1 and the downstream SBF transcription factor Swi4/Swi6 (Kim and Levin 2010, 2011). The relevance of the CWI pathway for transcriptional regulation of β-1,3-glucan synthases in other fungal species, e.g., *Aspergillus*, is controversial (Fujioka et al. 2007; Rocha et al. 2016). However, yet another key signaling pathway was identified to control expression of β-1,3-glucan synthases. This is the calcium-calcineurin signaling pathway (Liu et al. 2015). It was shown that the calcineurin inhibitor FK506 can effectively block *FKS2* expression in *S. cerevisiae* (Johnson and Edlind 2012; Mazur et al. 1995). Blocking expression of *FKS2* with FK506 was actually used to identify the first synthase gene (*FKS1*) in baker's yeast (Douglas 2001) (see also 2.1). Induction of *FKS2* expression required the transcription factor Crz1 (Stathopoulos and Cyert 1997). A similar calcineurin-dependency of β-1,3-glucan synthase expression appears to be conserved in other fungal species, e.g., *Candida glabrata* and *A. fumigatus* (Cramer et al. 2008; Katiyar et al. 2012; Kontoyiannis et al. 2003; Steinbach et al. 2007).

Besides this, significant regulation of cell wall synthesis also occurs on a spatio-temporal level by targeted secretion and localization of biosynthetic and regulatory enzymes. β-1,3-glucan synthases specifically localize at sites undergoing cell wall remodeling such as hyphal tips in case of filamentous fungi, budding sites

in case of yeasts and, in some species, at sites of septum formation. For example, the β-1,3-glucan synthase of *N. crassa* (FKS-1) specifically accumulates in certain macrovesicles in the Spitzenkörper and in the plasma membrane of hyphal tips (Sánchez-León and Riquelme 2015). In contrast to *N. crassa* where the β-1,3-glucan synthase is not found at sites of newly formed septa (Sánchez-León and Riquelme 2015), certain β-1,3-glucan synthases of yeasts (*S. pombe*, *S. cerevisiae*) are specifically localized and essentially required at these sites (Cortés et al. 2007; Onishi et al. 2013).

2.4.2 Regulation of α-1,3-Glucan Synthesis

Similar to the β-1,3-glucan synthases, specific and distinct localization patterns were also found for α-1,3-glucan synthases Ags1 and Ags2 by immunostaining in *A. fumigatus* (Beauvais et al. 2005). This suggests that the mechanisms that determine the localization of these enzymes have a significant impact on the regulation of α-1,3-glucan synthesis.

Direct interactions of Rho1 orthologs and α-1,3-glucan synthases were not reported. However, it was shown that Rho2 of *S. pombe* has an impact on cell wall α-glucan synthesis. Overexpression of Rho2 is lethal and also increases cell wall α-glucan but rather not β-1,3-glucan (Calonge et al. 2000; Villar-Tajadura et al. 2008). Both phenotypes depend on the protein kinase C homologue Pck2 (but not on Pck1) as deletion of *pck2* suppressed lethality and the increase of cell wall α-glucan (Calonge et al. 2000). This suggests that Rho2 does not regulate α-1,3-glucan synthases directly by protein–protein interaction. In fact, it was shown that the α-1,3-glucan synthase promoters are conserved targets of *Sc*Rlm1-like transcription factors. The orthologs of *Sc*Rlm1 in *A. niger*, *A. nidulans* and *A. fumigatus*, all were named RlmA, are targets of the CWI pathways of these fungi and control expression of several α-1,3-glucan synthase genes (Damveld et al. 2005a, b; Fiedler et al. 2014; Fujioka et al. 2007; Hagen et al. 2007; Katayama et al. 2015; Meyer et al. 2007; Rocha et al. 2016). Thus, regulation of α-1,3-glucan synthesis in these fungi may occur primarily on a transcriptional level via the Rho GTPase Rho2, the protein kinase C, followed by the downstream MAP kinase module which in turn activates the conserved transcription factor RlmA that binds to α-1,3-glucan synthase promoters.

3 Concluding Remarks

Due to its absence in mammals, the fungal cell wall is an established target for antifungal therapies and diagnosis of fungal infections. The echinocandins, which effectively inhibit the β-1,3-glucan synthesis, represent one of the few available antifungal drug classes suitable to treat life-threatening invasive fungal infections. Ibrexafungerp, the first clinical candidate of a novel class of antifungals, the

triterpene glycoside enfumafungin-derived "fungerps" which also target the β-1,3-glucan synthase, recently entered phase III clinical trials (Davis et al. 2019). These drugs also act by non-competitive inhibition of β-1,3-glucan synthase activity, but apparently bind to a region distinct from that bound by echinocandins which renders them active against echinocandin-resistant strains (Onishi et al. 2000). Serological tests have been developed and are routinely used for diagnosis of invasive fungal infections which apply fungal cell wall β-glucan as a biomarker. The role of β-glucans as central PAMPs for innate immune defense against fungal infections as well as the function of α-glucans as fungal virulence factor just became clear in the recent years. Multiple enzymes essential for α- and for β-glucan synthesis have been identified and characterized in fungi in the last three decades and great efforts have been made to elucidate the mechanisms of cell wall glucan synthesis. Still, many key questions remain unanswered. For example, there are conflicting results regarding the requirements and origin of primers for α- and β-1,3-glucan synthesis initiations. Furthermore, the biosynthesis of β-1,6-glucan, which is essential for linkage of mannoproteins in the cell wall of yeasts, remains mostly unresolved. There is still skepticism whether *the* β-1,3-glucan synthase as well as *the* α-1,3-glucan synthase enzymes really harbor the catalytic domains that synthesize the eponymous glucan polymers. The regulatory traits of cell wall biogenesis are just at the beginning of understanding. When and how does Rho1 interact with *the* β-1,3-glucan synthase? Are the α-1,3-glucan synthases only regulated on a transcriptional level? In which way is β- and α-glucan synthesis regulated on a spatio-temporal level? All these questions are of fundamental importance to understand fungal cell wall biogenesis. In consideration of the many medical implications of cell wall glucans, answers to all the open questions regarding cell wall glucan synthesis will contribute to improving existing and developing new therapeutic concepts.

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Chitin: A "Hidden Figure" in the Fungal Cell Wall

Hannah E. Brown, Shannon K. Esher, and J. Andrew Alspaugh

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Abstract Chitin and chitosan are two related polysaccharides that provide important structural stability to fungal cell walls. Often embedded deeply within the cell wall structure, these molecules anchor other components at the cell surface. Chitin-directed organization of the cell wall layers allows the fungal cell to effectively monitor and interact with the external environment. For fungal pathogens, this interaction includes maintaining cellular strategies to avoid excessive detection by the host innate immune system. In turn, mammalian and plant hosts have developed their own strategies to process fungal chitin, resulting in chitin fragments of varying molecular size. The size-dependent differences in the immune activation behaviors of variably sized chitin molecules help to explain how chitin and related

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Current Topics in Microbiology and Immunology (2020) 425: 83–111
https://doi.org/10.1007/82_2019_184
© Springer Nature Switzerland AG 2019
Published Online: 6 December 2019

chitooligomers can both inhibit and activate host immunity. Moreover, chitin and chitosan have recently been exploited for many biomedical applications, including targeted drug delivery and vaccine development.

1 Introduction

Chitin is one of the most common polysaccharides in nature, second only to cellulose in abundance. Found in all fungal species, as well as many insects and invertebrates, chitin has a simple primary structure. Chitin is a homopolymer composed of β-(1-4)-linked *N*-acetylglucosamine (GlcNAc) subunits (Latgé and Calderone 2005). Interestingly, chitin and cellulose are very similar in structure, differing only in the alkyl group side chain of the monosaccharide subunits that compose these two important polymers. In many fungi, chitin synthesis is accomplished by a family of related chitin synthases (Niño-vega et al. 2004; Roncero 2002). Although there is some degree of functional redundancy among these enzymes, fungal chitin synthases can be divided into distinct functional classes, with each class responsible for the production of distinct chitin subspecies at distinct locations in the cell. Accordingly, many of these enzymes are specifically localized to regions of the fungal cell corresponding to the site of action for individual types of chitin (Treitschke et al. 2010; Weber et al. 2006).

Chitin makes up about 1–15% of the fungal cell mass with yeasts containing the lowest percentage of overall chitin and filamentous fungi containing a higher concentration of chitin in their cell walls. In the model ascomycete yeast *Saccharomyces cerevisiae*, chitin comprises 1–2% of the cell wall mass (Lesage and Bussey 2006). The cell walls of other yeasts such as *Candida albicans* are composed of 2–5% chitin. The filamentous fungi contain higher percentages of overall chitin, comprising 4% of the biomass of the cell wall in *Neurospora crassa* and 7–15% *Aspergillus fumigatus* (Gastebois et al. 2009; Maddi and Free 2010). Interestingly, the budding yeast *Schizosaccharomyces pombe* does not contain any measurable chitin in its vegetative cells, but chitin is present in the conidia (Magnelli et al. 2005; Matsuo et al. 2004).

Although the primary structure of chitin is that of a simple, linear polysaccharide, the higher structural features of this molecule provide chitin with many of its most interesting biological properties (Fig. 1a). The chitin homopolymer forms antiparallel beta-sheets that are stabilized by intramolecular hydrogen bonds (Fig. 1b). In this way, the molecule becomes exceptionally rigid, with greater intrinsic strength than many other structural elements found in nature, including bone. The rigidity and resilience of these chitin microfibrils allow them to serve as a structural backbone for the fungal cell wall (Fig. 1c), as well as a rigid component of the exoskeleton of higher organisms.

In addition to its intrinsic molecular strength, chitin also exists in different polymer lengths. The size of individual chitin molecules is primarily determined by processes occurring after its production, rather than as a function of its biosynthesis.

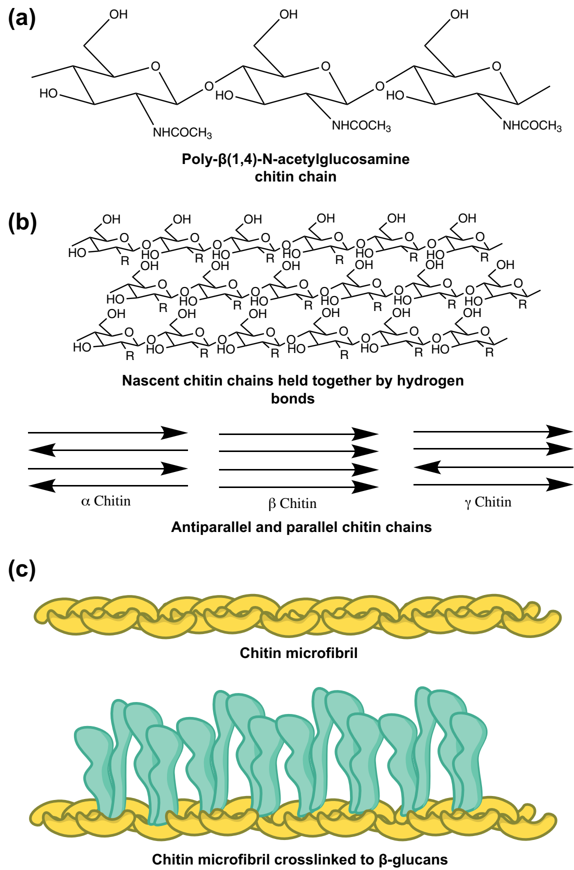

Fig. 1 **a** Chitin begins as a poly-β-(1,4)-*N*-acetylglucosamine chain that folds to form **b** nascent chains held together by hydrogen bonds. This folding can occur in an antiparallel (α and γ) or parallel (β) manner. **c** Chitin microfibrils are crosslinked to β-(1,3)-glucan to form the inner cell wall architecture [Adapted from Aranaz et al. (2009), Lenardon et al. (2010a)]

For example, chitinases are mammalian enzymes that interact with exogenously produced chitin to cleave large chitin molecules into smaller forms (Vega and Kalkum 2012). Recent studies have indicated that different sized chitin molecules have distinct biological features, including differential activation of host immune cells. Chitin fragments at their smallest measure less than 2 μm in length and at their largest can be up to 100 μm. This large size range has been subdivided further into categories of fragment sizes that correspond to differences in immune activation responses (Da Silva et al. 2009).

Additional post-synthetic modifications of chitin also result in changes in its biological functions. For example, varying degrees of chitin deacetylation result in its conversion to chitosan or deacetylated chitin. This process is regulated by a family of related enzymes known as chitin deacetylases (Baker et al. 2011; Upadhya et al. 2018). Like the chitin synthases, the different chitin deacetylases are localized at specific regions of the fungal cell, perhaps directing the formation of

different types of chitosan required at specific cellular sites. Together, chitin and chitosan form an important structural layer of the fungal cell wall, responsible for its physical integrity as well as helping to direct its interaction with the environment.

2 Chitin and Chitosan and the Fungal Cell Architecture

The external architecture of the fungal cell is complex, layered, and dynamic. Composed of a plasma membrane, a cell wall, a multitude of surface proteins, and a variably present external layer of polysaccharides, the fungal cell exterior has evolved intricate and adaptive mechanisms to protect the integrity of the cell. The most internal of these structures is the phospholipid bilayer that comprises the fungal plasma membrane. Importantly, this organelle maintains a distinct asymmetry between the inner and outer leaflets of the lipid bilayer. The enrichment of distinctly charged and sized phospholipids in each leaflet allows for transmembrane proteins to localize to specific microdomains within the membrane and to sense and internalize extracellular cues (Brown et al. 2018; Curto et al. 2014; van Meer 2011). The most negatively charged and bulky phospholipids are often directed to the cytosolic leaflet in order to hide them from the external environment, including from host immune cells that might recognize these lipids as molecular patterns for immune activation (Sheetz and Singer 1974; Shor et al. 2016). This asymmetry also allows for the cell membrane to maintain a precise curvature and to provide a scaffolding base for the remaining layers of the fungal cell surface.

The next, more external layer of the fungal cell is the cell wall, a complex and well-ordered structure composed of a backbone of polysaccharides, including chitin/chitosan and α/β-glucans, (galacto)-mannans, and glycosylated proteins (Fig. 2a). The cell wall carbohydrates are maintained in a distinct and ordered manner to direct structural stability, environmental sensing, and immune avoidance. Chitin and chitosan occupy the deepest layer of the cell wall (Figs. 1 and 2a) (Gow et al. 2017; Latgé 2007). Chitin molecules fold together to form nascent GlcNAc chains and orient themselves in either a parallel or antiparallel manner relative to other chains, classified as either α-, β- or γ-chitin polymorphs accordingly (Fig. 1b) (Minke and Blackwell 1978; Sugiyama et al. 1999). Intramolecular hydrogen bonds form along the GlcNAc polymeric chain, adding tremendous structural stability to this molecule (Fig. 1b). The tensile strength and integrity of chitin have been best studied in crab exoskeletons (Ifuku et al. 2009; Yen et al. 2009) and fleshy fungi such as mushrooms (Ifuku et al. 2011), as well as in the cell walls of pathogenic fungal species (Lopez-Romero and Ruiz-Herrera 1986). One study investigating chitin nanofibers extracted from crustaceans concluded that the strength of isolated exoskeletons was directly correlated with high chitin content (Mushi et al. 2014). Similarly, the structural stability of the fungal cell wall provided by the inner layers of chitin and chitosan allows it to perform a vast array of functions ranging from shielding the cell from extracellular stress to housing essential proteins embedded in the cell wall and membrane.

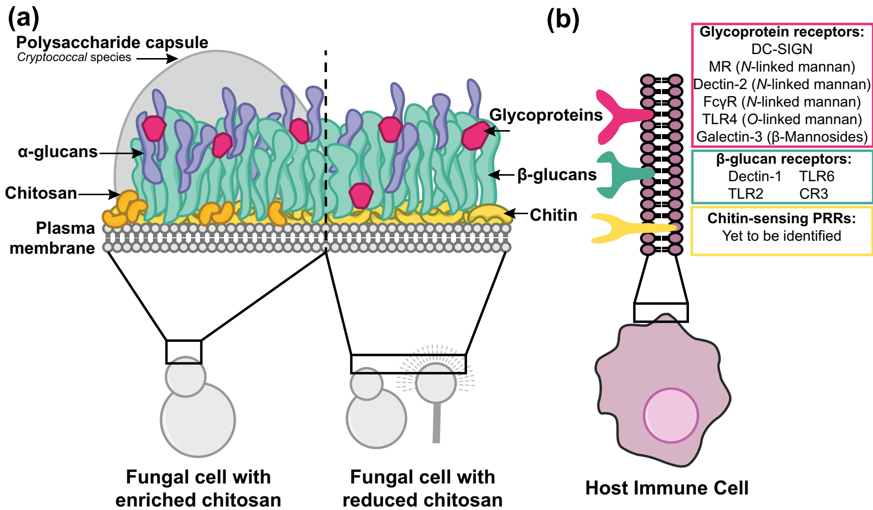

Fig. 2 **a** Outer cell wall components (α-glucans and various glycoproteins) build upon the foundation of chitin (and chitosan) and β-glucan crosslinking. **b** Host immune cell receptors have evolved to recognize various fungal cell wall components [Adapted from Esher et al. (2018), Perez-Garcia et al. (2011)]

Although fungal chitin is a relatively simple homopolymer, it possesses diversity in structure and function through variations in the size and structure of the microfibrils (Fig. 1c) as well as in its polymer length and degree of acetylation (Da Silva et al. 2009; Muszkieta et al. 2014). Sufficiently deacetylated forms of chitin (i.e., chitosan) are chemically distinct from the parent molecule. Although fully deacetylated forms of chitosan can be derived through ex vivo chemical reactions (He et al. 2016), most biological forms of chitosan in fungi consist of a glucosamine backbone with different degrees of deacetylation. Following chitin synthesis by chitin synthase enzymes, chitin deacetylases remove approximately 70–80% of the acetyl groups to form chitosan (Klis et al. 2006). Chitosan is therefore structurally similar to chitin, working to maintain cell barrier functions and integrity during vegetative growth.

In most fungal species, chitin is less abundant than other cell wall carbohydrates such as the α- and β-glucans. Additionally, chitosan is present in very low concentrations in the cell walls of many fungal species, especially some of the more frequently studied model ascomycete yeasts such as *C. albicans* in which approximately only 5% of the cell wall chitin will eventually be enzymatically converted into chitosan (Gharieb et al. 2015). However, other fungi, including many basidiomycetes and zygomycetes, possess much higher relative levels of chitosan. For example, in the basidiomycete fungus *Cryptococcus neoformans*, the relatively high chitosan levels help to direct cell wall integrity, bud separation, melanin production, and pigment retention, all of which are essential for cell survival, especially in the

context of an infected host (Baker et al. 2007). Accordingly, *C. neoformans* chitosan-deficient mutants were less virulent than isogenic wild-type strains in a murine model of cryptococcal infection (Baker et al. 2011; Upadhya et al. 2018). In addition to being required for full virulence in a mouse model, chitosan was also demonstrated to be important for cryptococcal infection persistence and retained fungal viability in mouse lungs (Baker et al. 2011).

Zygomycete fungi, such as *Mucor rouxii*, *Cunninghamella elegans*, and various *Rhizopus* species, also produce relatively high levels of chitosan compared to other fungal species, using this molecule in similar ways to strengthen the cell wall. In fact, in many zygomycete fungi, chitosan concentrations exceed that of chitin by three-fold, and it is thought to protect against hydrolysis by chitin-targeting mammalian chitinases (Gharieb et al. 2015; Gholizadeh Aghdam 2010; Klis et al. 2006). This wide range of chitosan concentrations implies that, for some fungal species, chitosan plays an important role in either cell viability or pathogenesis.

The deep layer of chitooligomers within the fungal cell wall creates a three-dimensional web-like structure on which the more superficially localized glucans are chemically crosslinked (Bowman and Free 2006). In *S. cerevisiae,* β-(1,3)-glucans are covalently attached to chitin (Figs. 1c and 2a), anchoring this more peripheral layer of the cell wall in a structured manner (Klis et al. 2006). Similarly in the filamentous fungus *A. fumigatus*, chitin covalently binds β-(1,3)-glucans (Chai et al. 2011; Muszkieta et al. 2019). In *C. neoformans*, chitin and chitin-derived structures have also been implicated in localizing the polysaccharide capsule and melanin to the cell wall (Fig. 2a). Rodrigues et al. (2008) demonstrated that the chitooligomer-binding lectin wheat germ agglutinin (WGA) bound to structures linking the cell wall to the polysaccharide capsule (Rodrigues et al. 2008). They further demonstrated that chitinase treatment caused the release of glucuronoxylomannan (GXM), the major component of the cryptococcal capsule (Rodrigues et al. 2008). Furthermore, *C. neoformans* strains lacking chitosan have a "leaky" melanin phenotype, indicating that they have defects in retaining their melanin or melanin-producing enzymes (Baker et al. 2007; Banks et al. 2005; Walton et al. 2005).

3 Chitin Synthases

Fungal chitin is synthesized from its monosaccharide precursor by a family of related chitin synthase (Chs) enzymes. Fungal genomes typically contain multiple genes encoding chitin synthases, and the number of *CHS* genes in a given species roughly correlates with the amount of chitin present in the cell wall. For example, *S. pombe* has only one *CHS* gene in its genome (*SpCHS1*), and its cell wall contains unmeasurably low amounts of chitin. In this species, chitin is only present during sporulation (Arellano et al. 2000). *S. cerevisiae* contains three *CHS* genes, and approximately 2% of its cell wall is composed of chitin. In contrast, filamentous ascomycetes and fungi in the Basidiomycota and Mucoromycota tend to have an

expanded *CHS* gene family (Free 2013; Mélida et al. 2015). Thirty-eight *CHS* genes have been identified in *Allomyces macrogynus*, a member of the Blastocladiomycota, an early emerging phylum among the fungi, and twenty-six have been identified in *Rhizopus oryzae* of the filamentous fungal class Mucoromycotina (Gonçalves et al. 2016). These fungal species also tend to have higher chitin and chitosan concentrations in their cell walls (Liu et al. 2017; Ruiz-Herrera and Ortiz-Castellanos 2010).

Many classification schemes have been developed to organize chitin synthases into functional classes based on predicted structural features, subcellular patterns of localization, and function inferred by loss-of-function mutations (Bowen et al. 1992; Roncero 2002; Nino-Vega 2004; Goncalves 2016). However, there is no consensus regarding Chs classification or consistency in organizational nomenclature. For example, the phylogenetic classification of fungal Chs proteins by Niño-Vega divides the enzymes into two families and seven classes. In contrast, a more recent study proposed a different classification scheme using a phylogenetic comparison of predicted *CHS* genes from over 130 fungal genomes (Gonçalves et al. 2016). These investigators divided the predicted fungal Chs proteins into three divisions and several subclasses. We chose to follow the Niño-Vega classification since it is limited to fungal genes.

Fungal Family I chitin synthases. The Chs proteins in fungal Family I contain a conserved catalytic domain and six C-terminal transmembrane domains (Fig. 3) (Gonçalves et al. 2016). Class I *CHS* genes appear to encode mostly redundant functions or enzymes expressed at low levels, as mutants in this class typically display negligible changes in Chs activity or cell wall chitin levels. One exception is the *S. cerevisiae CHS1* gene that plays a role in cellular repair after cytokinesis (Cabib et al. 1992).

The Class II *CHS* genes are present in most fungal species. In *S. cerevisiae* and *C. albicans*, the *ScCHS2* and *CaCHS1* genes are important for the primary septum formation in cell division (Munro et al. 2001a, b; Shaw et al. 1991). In contrast, mutations in many Class II *CHS* genes in filamentous fungi have less notable reductions in total cell Chs activity or morphological consequences (Roncero 2002).

The Class III *CHS* genes in fungal Family I appear to have been lost among many ascomycete yeasts, but this family is expanded in filamentous ascomycetes such as *Aspergillus* and *Neurospora* species (Mellado et al. 1996; Rogg et al. 2011). Consistent with the predominant hyphal morphogenesis among these fungal species, mutants in genes encoding Class III chitin synthases tend to have defects in polarized growth and hyphal tip extension, resulting in colonies of reduced size with tip-splitting defects (Mellado et al. 1996).

Fungal Family II chitin synthases. The Chs proteins in fungal Family II tend to be broadly present in diverse fungal species, and they are divided into several classes. Although the most active enzymes may differ between individual fungi, many of these Chs proteins play major roles in growth and morphogenesis, as assessed by loss-of-function mutants. The Class IV proteins are widely distributed among many fungal species. These include the Chs3 proteins in the ascomycetous yeasts *S. cerevisiae* and *C. albicans*. Although not essential for survival, *ScCHS3* and *CaCHS3* are the major chitin synthases in these species, and loss-of-function

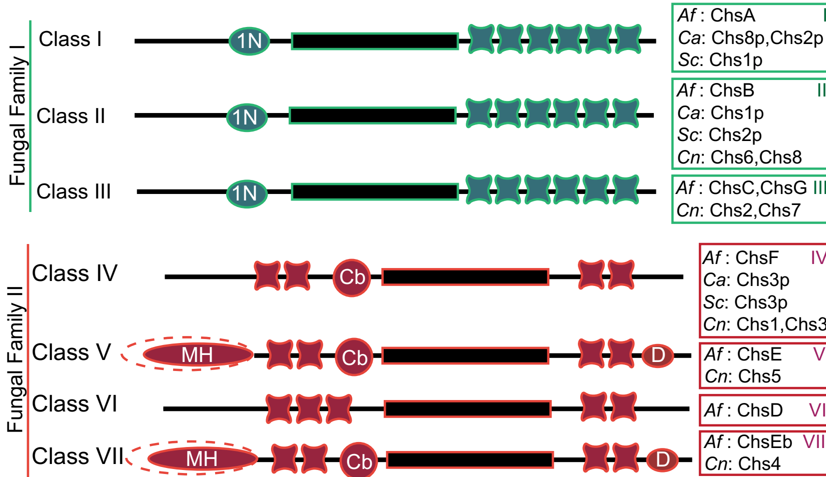

Fig. 3 Two families of fungal chitin synthases. Family I (teal) consists of Classes I–III Chs enzymes, which have conserved chitin synthase domains (rectangles), 6 C-terminal transmembrane domains (molar structures), and a chitin synthase N-terminal domain (1 N). Family II (burgundy) Chs proteins consist of Classes IV–VII and also have these same conserved chitin synthase domains (rectangles) and varying numbers of transmembrane domains on both the N and C terminus. The Class V and Class VII enzymes have myosin head domains (MH) and DEK C-terminal domains. Classes IV, V, and VII have cytochrome b domains (Cb). Examples from each of the big four fungal species [*Aspergillus fumigatus* (Af), *Candida albicans* (Ca), *Saccharomyces cerevisiae* (Sc), and *Cryptococcus neoformans* (Cn)] are listed to the right of each class. The domain prediction and structure analysis were adapted from (Gonçalves et al. 2016), and the fungal-specific Chs examples were adapted from (Rogg et al. 2012)

mutations result in very reduced chitin levels (Bulawa et al. 1995; Shaw et al. 1991; Valdivieso et al. 1991). Interestingly, the closest homologs by DNA sequence in filamentous fungi tend to result in less severe phenotypic alterations when mutated (Munro and Gow 2001).

In contrast, the Class V Chs proteins are present primarily in filamentous fungi, providing the bulk of chitin synthase activity in these species. Accordingly, loss-of-function mutations in Class V *CHS* genes have implicated these proteins in cell wall assembly, septum formation, and virulence (Amnuaykanjanasin and Epstein 2003; Gonçalves et al. 2016; Munro et al. 2004). Additionally, the Class V Chs proteins have predicted myosin-like domains fused to the end of the N-terminus. Mutational analysis has demonstrated that this domain is involved in actin-directed subcellular localization of these proteins to regions of active chitin synthesis, often in a cell cycle-dependent manner (Treitschke et al. 2010; Weber et al. 2006).

The proteins in both Family II Classes IV and V contain similar structural features, including catalytic domains, one or two transmembrane domains, and a cytochrome-b-like domain in the N-terminus (Fig. 3). Despite the similarity in structure between enzymes in these two subclasses, it appears that, in general, the

main chitin synthases in yeast-like fungi group together in phylogenetic Class IV, whereas Class V enzymes provide the main chitin synthase activity for fungi that grow predominantly as molds. Interestingly, the fungal Chs Class III (Family I), as well as Classes V, VI, and VII (Family II) are only present in filamentous fungi, perhaps related to the complex morphological transitions in these fungal species that require precise cytoskeletal and cell wall coordination (Muszkieta et al. 2014).

Other less widely conserved fungal *CHS*-like genes include those in Classes VI and VII (Niño-vega et al. 2004) as well as virus-like *CHS* genes such as *Ectocarpis siliculosus* virus-like CHS (ESV) and the *Chlorovirus*-like CHS (CV) genes (Fig. 3) (Gonçalves et al. 2016). No function has yet been assigned to the ESV genes, but the CV genes have been associated with host interaction and pathogenicity of plant fungal pathogens such as *Fusarium graminearum* (Zhang et al. 2016).

4 Chitin in Fungal Cell Replication and Stress Response

In addition to its role in anchoring an orderly array of fungal cell wall polysaccharides, chitin has been shown to aid in the cellular response to environmental stress and to assist the cell in replication (Gottlieb et al. 1991; Rodrigues et al. 2008; Segal et al. 1988). Chitin synthesis is localized at specific subcellular locations during the cell cycle. During yeast cell budding and early cell growth, chitin is enriched at the vulnerable bud tip, presumably to protect the emerging nascent cell. Following budding, chitin is more evenly distributed throughout the entire daughter cell wall during the period of rapid, isotropic cell growth (Sheu et al. 2000). Following complete cell division, chitin remains enriched at the site of the prior mother–bud interface, forming a permanent bud scar. Subsequently, enhanced chitin synthesis, via the activity of chitin synthase (Chs) enzymes, polarizes once again at the site of bud emergence as a new round of replication begins (Fischer et al. 2008). In the model yeast *S. cerevisiae*, researchers have demonstrated that this polarization of chitin synthesis is directed by the regulated localization of specific chitin synthases, such as that seen with relocation of Chs3p to the bud neck region to aid in cytokinesis (Bulik et al. 2003). Chs3p is maintained at a steady-state level within internal chitosome reservoirs and at the plasma membrane, from which it is trafficked by clathrin-dependent mechanisms involving additional Chs and septin proteins for recycling and relocalization to the cell surface (Chuang and Schekman 1996; DeMarini et al. 1997; Valdivia et al. 2002; Ziman et al. 1996). In addition, phosphorylation has been shown to play a role in the proper localization of these enzymes throughout the cell cycle in both *S. cerevisiae* and *C. albicans* (Lenardon et al. 2010a; Martínez-Rucobo et al. 2009; Teh et al. 2009).

This dynamic pattern of highly localized and regulated chitin synthesis is similar to the pattern of cyclical actin localization and delocalization that occurs during the yeast cell cycle. Similar to chitin, actin localizes in a highly polarized manner at the site of imminent bud formation and daughter cell emergence. While the daughter

cell symmetrically increases in size during the period of isotropic growth, both actin and chitin are more diffusely distributed throughout the cell surface. As the attached daughter cell reaches maturity, both actin and chitin are enriched at the site of cell separation (Munro et al. 2001a, b).

This process is similarly directed in fungi that grow predominantly as hyphae and in which chitin synthases and actin localization are concentrated at the apical tip and at the septa (Sánchez-León et al. 2011). The CHS-1 chitin synthase in the filamentous fungus *Neurospora crassa* is enriched at regions of active cell wall synthesis, such as the apical tip, developing septum, and the Spitzenkorper. In this species, and likely in other fungi that predominantly grow as hyphae, actin microfilaments help to direct specific chitin synthases to areas of the cell that require higher levels of chitin. Accordingly, a fluorescently tagged *N. crassa* CHS-1 fusion protein [CHS-1-green fluorescent protein (GFP)] was mislocalized by actin inhibitors and not by microtubule inhibitors (Sánchez-León et al. 2011).

Similarly, in the filamentous ascomycete fungus *Aspergillus nidulans*, the Class V chitin synthase CsmA contains a myosin-like domain that is required for proper protein localization and function. Given the interaction of myosin proteins and actin, these studies further support actin-mediated subcellular localization of specific chitin synthase activities during various stages of fungal development (Takeshita et al. 2005, 2015). Filamentous actin tracks are also involved in the polarized secretion of chitin synthases in the plant fungal pathogen *Ustilago maydis*, directing its proper hyphal development (Schuster et al. 2012).

In the opportunistic pathogen and filamentous fungus *A. fumigatus,* a relatively large group of eight fungal chitin synthases offer functional redundancy and flexibility in the cellular adaptation to stress. For example, strains with combinations of mutations of all Family 1 *CHS* chitin synthase genes only demonstrated growth defects due to loss of CHSG activity. However, virulence in animal models of invasive aspergillosis was retained even in the absence of all Family I genes. Hyphal morphology and conidiation were more severely affected by mutation of Family II *CHS* genes, especially *CSGA* (Muszkieta et al. 2014).

In many pathogenic fungi including *A. fumigatus*, *C. albicans*, and *C. neoformans*, chitin synthase gene expression and activity, as well as overall chitin production, while always required for basal growth, are increased in response to external stimuli that induce cell wall stress. These stresses include, but are not limited to, lytic enzyme activity, antifungal agents, and respiratory bursts within host immune cells (Lenardon et al. 2010b). For example, in *C. albicans*, the stress caused by treatment with cell wall-targeting drugs such as echinocandins leads to an increase in the activity of its four chitin synthases, as well as an overall increase in chitin levels. In fact, this increase in chitin provides protection against echinocandin treatment; elevated chitin in the cell wall *of C. albicans* reduced its susceptibility to caspofungin (Walker et al. 2008). Caspofungin treatment of *A. fumigatus* triggers a similar increase in the activity of its eight chitin synthases, as well as overall chitin levels (Fortwendel et al. 2009). *C. neoformans*, which has inherently low susceptibility to echinocandins, induces eight chitin synthases to generate high levels of chitin in response to increased temperature and a range of cell wall destabilizers

(Congo Red, caffeine, and SDS) (Banks et al. 2005). Increased cell wall chitin content resulting from enhanced chitin synthase activity protects these pathogenic fungi from external stresses.

Chitin's role in modifying the cell wall as a means of responding to stress has also been observed in non-pathogenic yeast including *S. cerevisiae,* in which its three chitin synthases become highly active in stressful growth environments (Bulik et al. 2003). In fact, chitin synthase activity, such as that due to the Chs3 protein in *S. cerevisiae*, can be induced either by the presence of extracellular stress or by adding additional glucosamine as a substrate for chitin production. Interestingly, the increase in chitin synthase activity in response to glucosamine was not associated with increased *CHS3* gene transcription nor increased Chs3 protein levels, suggesting that this enzyme has the ability to rapidly increase its functional capacity in response to the needs of the cell (Bulik et al. 2003). Alternatively, this could be explained by the increase in expression of other *CHS* genes and/or production of these enzymes as has been shown in *C. albicans*. In the absence of *C. albicans* Chitin Synthases 1 and 3 (Chs1 and Chs3), this organism is able to survive intense cell wall stress through the increased activity of the Chs2 and Chs8 enzymes, which are regulatory activators of chitin synthesis pathways (Walker et al. 2008).

5 How the Host Responds to Chitin

As one of the most external features of the fungal cell, the cell wall acts as an important immunological interface between fungal pathogens and the infected host. Many investigators have therefore studied the role of individual cell wall components on the initiation of an immune response by various host cells, especially those of the innate immune system. Many cell wall epitopes act as pathogen-associated molecular patterns (PAMPs) (Fig. 2b). PAMPs are common patterns displayed on microbes that are innately recognized by host cells through surface pattern recognition receptors (PRRs). This interaction leads to a host cell response, resulting in defense against potential environmental threats without the necessity of a prior encounter.

Many investigators have studied how the immune system is regulated in response to chitin exposure. Interestingly, these studies often present somewhat conflicting results. For example, introducing chitin to the host through either an intranasal or intraperitoneal route of infection results in priming of the immune system, suggesting that many of the host immune cells, such as alveolar macrophages and NK cells, are "preactivated" to elicit a response due to the immune-modulatory effects of chitin and its derivatives (Muzzarelli 2010; Rizzetto et al. 2016). Once these cells are primed by chitin exposure, they more readily secrete IFN-γ, IL-12, and TNF-α in response to other inflammatory stimuli (Shibata et al. 1997a, b) (Muzzarelli 2010; Ozdemir et al. 2006; Strong et al. 2002). Furthermore, when chitin and chitosan are administered as immune adjuvants, they

elicit enhanced Th1 immune responses reminiscent of those induced by well-known adjuvants, such as heat-killed *Mycobacterium bovis* (Shibata et al. 2000).

Similarly, chitin microparticles were able to alter the Th2-mediated allergic responses in models of ovalbumin-induced asthma and *A. fumigatus*-induced allergic sensitivity (Muzzarelli 2010; Ozdemir et al. 2006; Strong et al. 2002). Other groups have also shown that chitin elicits a robust Th1 immune response through the induction of IL-1, increasing both antibody levels and anti-tumor activities; however, these specific results may have been confounded by impure chitin preparations (Nishimura et al. 1986a, b). Chitin has also been shown to elicit a direct allergic response in the airways characterized by increases in tissue eosinophils and basophils, as well as elevated expression of the Th2 cytokine, IL-4 (Reese et al. 2007; Van Dyken et al. 2011). Furthermore, Da Silva et al. (2008) discovered that IL-17, a pro-inflammatory cytokine, was increased in expression and activity in murine lung macrophages that had been exposed to chitin. This IL-17 elevation was further shown to be dependent on Toll-like receptor 2 (TLR2) in that TLR2-deficient mice did not demonstrate a pulmonary inflammatory response to chitin exposure (Da Silva et al. 2008). In contrast, "ultra-purified" chitin has the ability to inhibit T-cell proliferation and induce the selective secretion of the anti-inflammatory cytokine IL-10 in a *C. albicans* model of infection, potentially acting as a signal to dampen immune activation during the clearing phase of a systemic fungal infection (Wagener et al. 2014). In this study, chitin was prepared in a pyrogen-free and microbiologically sterile manner with a purity of over 98% as analyzed by HPLC in an attempt to remove potentially confounding additional cell wall components from these assays. Therefore, a large body of investigators demonstrates varying degrees of immune activation by chitin and its associated biomolecules.

However, most recent analyses of these data suggest that there are important factors to be considered in evaluating studies exploring chitin as a regulator of immunity. First, the specific chemical forms of chitin, including polymer size and degree of acetylation, might dramatically affect interactions with host cells (Bueter et al. 2011; Da Silva et al. 2009, 2008). Additionally, chitin is often chemically associated with proteins and other polysaccharides when purified from different species. For example, fungal cell wall chitin is often covalently linked to beta-glucans (Fig. 1c). Therefore, varying degrees of chemically pure chitin derived from different biological sources have limited the interpretation of some studies exploring immune activation by chitin. Additionally, mammalian chitinases and chitotriosidases, enzymes that degrade and modify chitin molecules, might also affect the host response to chitin-bearing organisms.

6 Chitin Receptor

Although chitin is very abundant in nature, the identity of a singular and unique chitin-detecting PRR has not yet been established. Over the past few decades, several mammalian cell surface receptors have been shown to have a strong

association with chitin and to control various cellular responses to this molecule. For example, to explore mechanisms for how chitin is so readily phagocytosed by macrophages once it encounters the host immune system (Bueter et al. 2011), investigators searched for mammalian surface receptors that might directly interact with chitin as candidate chitin-sensing PRRs (Fig. 2b). Cell surface proteins that demonstrated in vitro chitin binding included Galectin-3, a lectin that is known to bind β-galactosides (Seetharaman et al. 1998), and NKR-P1, an activating receptor on natural killer (NK) cells (Semenuk et al. 2001). Furthermore, a secreted C-type lectin receptor, RegIIIγ, found in the Paneth cells of the gastrointestinal tract and known to bind to peptidoglycan, was identified as a candidate mediator of the chitin immunological response (Cash et al. 2006). Peptidoglycan and chitin are chemically related polysaccharides that both contain *N*-acetylglucosamine (Xu et al. 2008), providing some rationale for common recognition by RegIIIγ. More recently, a transmembrane receptor, FIBCD1, has been shown to bind chitin with a high affinity in a calcium- and acetylation-dependent manner, suggesting that this receptor would not recognize or bind to chitosan, the deacetylated form of chitin (Schlosser et al. 2009). If confirmed, this observation would suggest distinct mechanisms for recognition of chitin and chitosan by the host immune system, a potentially very important factor for immune interactions with the host by fungi possessing chitosan-rich cell walls.

Other well-established pattern recognition receptors, such as Dectin-1, have also been predicted to regulate host immune activation in response to chitin exposure (Mora-Montes et al. 2011). In these studies, the investigators prepared ultra-purified chitin in a pyrogen-free and microbiologically sterile manner with a purity of over 98% as analyzed by HPLC. This chitin preparation blocked the cytokine response in human peripheral blood mononuclear cells to *C. albicans* cells in a Dectin-1-dependent manner. Interestingly, Dectin-1 did not directly bind chitin in these assays. Moreover, other PRRs, such as TLR2, TLR4, and Mincle (macrophage-inducible C-type lectin), also did not interact with chitin in these assays. These results suggest a model in which chitin, though not typically exposed on the surface of *C. albicans*, was able to inhibit the ability of this fungal pathogen to engage and activate host innate immunity in a Dectin-1-dependent manner but without directly binding to common PRRs.

The observation that chitin might actually act to block immune activation by fungal cells was extended by studies in a *C. albicans* model of infection in which chitin inhibited T-cell proliferation and elicited the selective secretion of IL-10, a key anti-inflammatory cytokine (Wagener et al. 2014). In these same studies, chitin reduced inflammation caused by LPS exposure in vivo, leading the investigators to propose that chitin exposure by dying fungal cells might be one mechanism by which the host turns off immune response signals and resolves immune activation following challenge with a pathogen (Wagener et al. 2014). This study also found that digested chitin, prevalent in later stages of this interaction, was recognized by the mannose receptor (MR), resulting in uptake and further intracellular stimulation of TLR9 and NOD2 (Wagener et al. 2014). Although none of these receptors/mediators have been shown to directly bind to chitin in the setting of an infection,

these studies provide evidence that chitin significantly contributes to the varied aspects of host innate immune cell activation.

7 Chitin and Chitosan Immunostimulation

Chitin and chitosan, as previously mentioned, can have immunostimulatory effects on varying parts of the immune system. Because of this, many fungal organisms have developed ways to shield these molecules from host immune cells and avoid recognition. In *C. neoformans*, the polysaccharide capsule serves to mask these immunogenic cell wall components, and capsule-deficient mutants are highly attenuated in murine models of cryptococcal infection (Fig. 2a). However, strains with mutations in the alkaline-responsive Rim signaling pathway were found to be paradoxically hypervirulent in murine models of infection, despite the loss of surface polysaccharide capsule (O'Meara et al. 2010, 2013). This observation was explained by studies that demonstrated that the *rim101*Δ mutant cell wall is highly disorganized with increased exposure of chitin and chitosan. This poorly ordered cell wall directed an excessive immune reaction characterized by enhanced Th1- and Th17-mediated inflammation, with host damage primarily due to immune pathology (O'Meara et al. 2013; Ost et al. 2017). Relatedly, the *C. neoformans* *mar1*Δ mutant has increased cell wall chitooligomer exposure due to cell trafficking defects and reduced glucan and mannan content in the cell wall. Like the *rim* pathway mutants, the *mar1*Δ mutant, a strain with cell wall enzyme trafficking defects, was found to hyper-activate macrophages in vitro (Esher et al. 2018). Furthermore, *C. neoformans* mutant strains with high levels of chitooligomer exposure stimulated macrophage responses in a TLR2/MyD88- and Dectin-1/Card9-dependent manner (Esher et al. 2018; Ost et al. 2017). These studies highlight the importance of strict organization within the fungal cell wall in host–pathogen interactions, demonstrating how the more superficial layers can serve as an immunological shield, preventing immune recognition of the deeper and more immunogenic cell wall components.

Studies in human keratinocytes indicated that chitin was able to elicit activation of these skin cells in a manner characterized by the induction of TLR4, both at the transcript and protein level (Koller et al. 2011). Blocking TLR2 in the keratinocytes inhibited this induction. However, no direct binding of chitin by TLR2 or TLR4 has been demonstrated. While these results did not reveal a direct chitin-binding PRR, they did extend growing observations of the roles of chitooligomers as immune modulators.

Glycoproteins in yeast species such as *Candida albicans* are glycosylated with mannose chains, and these mannoproteins account for 30–50% of the cell wall mass (Brown and Catley 1992). In filamentous fungi, glycoproteins constitute between 15 and 30% of the cell wall dry weight and can be glycosylated with both galactose and mannose chains, resulting in galactomannan proteins that can directly interact

with the immune system (Bowman and Free 2006; Bowman et al. 2006). These glycosylated proteins can include chitin-modifying enzymes such as chitin deacetylases in *C. neoformans*, as well as adhesins in the human commensal fungus *C. albicans* (Huang et al. 2002; Rieg et al. 1998). Although the complex, branching carbohydrate structures attached to these proteins are hypothesized to aid in immune avoidance for fungal pathogens, glycosylated proteins on the fungal cell surface can also serve as immunodominant epitopes for a host immune response. In fact, glycoproteins have been used as the basis for fungal vaccine strategies (Schmidt et al. 2012; Specht et al. 2017). Cell wall proteins, including all modified versions of these highly abundant glycoproteins, work in tandem with chitin within the cell wall to protect the cell shape and size, guard from extracellular stress, mediate molecule absorption, regulate signal transmission, and participate in regenerating the cell wall itself.

In some filamentous fungi, such as *A. fumigatus*, there is an even more external layer rich in galactosaminogalactan (GAG) that is released by the mycelium. Production of GAG is especially important for filamentous fungi because this antigenic polysaccharide is released during infection and favors in vivo proliferation of the fungus through promoting immunosuppressive effects (Beauvais et al. 2007; Fontaine et al. 2011; Loussert et al. 2010). GAG and related cell surface carbohydrates may therefore serve to shield filamentous fungi from immune recognition, especially those with higher cell wall chitin content (Gastebois et al. 2009; Maddi and Free 2010).

The immunomodulatory roles of chitosan have also been studied. The deacetylated state of chitosan allows this molecule to interact with different components of the host immune system compared to chitin. For example, in *C. neoformans*, chitosan stimulates the NLRP3 inflammasome in a phagocytosis-dependent manner (Bueter et al. 2011, 2014). This is a chitosan-specific type of immunostimulation as chitin does not activate the inflammasome to the same extent (Bueter et al. 2011, 2014). This activation of the inflammasome by chitosan elicits specific cytokine responses that would not be accomplished through chitin alone, indicating that these molecules work together to differentially stimulate the host immune system.

8 Size-Dependent Immune Response

One of the most important observations regarding the role of chitin as an immune regulatory molecule is the relationship between the size of the chitin particles and the immunological response that is subsequently initiated. This relationship was originally identified through observation of the divergent immune responses that are elicited between small, easily phagocytosed chitin particles and larger, non-phagocytosable chitin (Shibata et al. 1997). Prior to this observation, it was

known that foreign structures, such as yeast cells, zymosan, or chitin/chitosan, were internalized by macrophages through initial binding to mannose receptors on the plasma membrane of the immune cells (Warr 1980). However, the size- and phagocytosis-dependent differences in immune cell activation were not fully appreciated until investigators demonstrated that small chitin and chitosan particles actually primed alveolar macrophages to initiate a significantly more robust immune response and oxidative burst compared to larger chitin particles (Shibata et al. 1997). This size-dependent activation of the immune system by various macromolecules is similarly observed in the detection of hyaluronic acid by TLR4. Hyaluronan fragments are produced following tissue injury and are subsequently and quickly cleared in order to avoid further damaging inflammation. These molecules were found to stimulate chemokine production by macrophages in a size-dependent and TLR4-dependent manner, similar to what has been reported for chitin fragments (Jiang et al. 2005).

More recent studies have further investigated the chitin size-dependent activation of inflammation. Chitin particles of varying sizes differentially stimulate IL-17 production by macrophages in a TLR2- and MyD88-dependent manner (Da Silva et al. 2008). In these studies, the investigators defined big chitin (BC) as those molecules ranging in length from 70 to 100 μm, with intermediate chitin (IC) fragments ranging from 40 to 70 μm, small chitin (SC) being smaller than 40 μm, and super small chitin (SSC) fragments being approximately 2 μm or smaller (Da Silva et al. 2009). When exposed to intermediate chitin (IC) and small chitin (SC), murine lung macrophages displayed a robust immune activation phenotype, characterized by increased TNF-α production. In contrast, exposure of these cells to BC or SSC molecules did not result in measurable activation, suggesting that chitin is recognized as a size-dependent PAMP (Da Silva et al. 2009). Similar to prior studies, this macrophage activation response was dependent on TLR2 and Dectin-1. Additionally, both IC and SC resulted in activation of downstream immune effectors such as NFkB and spleen tyrosine kinase (Syk), as well as an increase in p65 nuclear staining. These events are commonly observed after macrophage activation of TNF-α pathways by TLR2 stimulation. Interesting, SC, but not IC, also elicited an anti-inflammatory IL-10 cytokine response, which was inhibited through blockages of Dectin-1 and phagocytosis (Da Silva et al. 2009). Another dissimilarity between the response to SC and IC is that SC immune activation was dependent on the mannose receptor (Da Silva et al. 2009).

These results began to clarify prior observations of the differential effects of chitin in different models of immune activation: Perhaps, some of these effects were dependent on the size and form of the chitin molecules introduced into the system. Since the molecular size of chitin is dependent on post-synthesis cleavage events, these studies also emphasized the potential importance of chitin-modifying enzymes in the interaction of fungi with their environment.

Chitosan also displays a similar size-dependent immune activation. The studies mentioned previously, that identified a chitosan-specific activation of the inflammasome, also found that smaller chitosan molecules stimulated macrophages better

than larger ones, inducing more IL-1β cleavage and release (Bueter et al. 2011, 2014). This size-dependent immune activation could be explained by the ease at which macrophages can engulf smaller chitosan molecules compared to larger ones.

9 Mammalian Chitinases

While humans and other mammalian hosts do not actively make chitin, they do produce chitin-degrading enzymes or chitinases. As mentioned previously, these enzymes are crucial to the processing and cleavage of chitin encountered by the immune system and may play a role in the immune response and ultimate pathogen clearance (Elias et al. 2005; Gorzelanny et al. 2010; Zhu et al. 2004). Interestingly, the induction of chitinase activity occurs as a part of a generalized inflammatory response. However, the presence of these enzymes and their roles in chitin processing provide further support for chitooligomers as important molecules encountered in the external environment (Vega and Kalkum 2012).

Chitinases are members of the glycosyl hydrolase 18 family, and there are six known mammalian chitinase enzyme homologs (Boot et al. 2005; Kzhyshkowska et al. 2007). The two most well-studied are chitotriosidase (CHIT1) and acidic mammalian chitinase (AMCase) (Boot et al. 2001; Renkema et al. 1997), which are the only mammalian chitinases known to be catalytically active. The remaining four enzymes are chitinase homologs, but these proteins contain amino acid substitutions in their active sites, potentially explaining their lack of enzymatic activity. They have been annotated as chitinase-3-like protein 1 (CHI3Li, also referred to as YKL-40, Hcgp39, or GP39), stabilin-1 interacting chitinase-like protein (SI-CLP), chitinase-3-like protein 2 (YKL-39), and oviductin (Kzhyshkowska et al. 2007).

The first identified, enzymatically active chitinase, CHIT1, was identified in the plasma of patients with Gaucher’s disease, a genetic disorder that results in the cellular accumulation of glucosylceramide (Hollak et al. 1994). Following the successful cloning and characterization of CHIT1, a second chitinase, AMCase, was identified (Boot et al. 2001). Although very similar to CHIT1 in both structure and function, it is mostly active in acidic environments such as the stomach (Boot et al. 2001).

Shortly following the discovery of CHIT1, this enzyme was detected as both a marker of *A. fumigatus* infections and of macrophage activation (Overdijk et al. 1996; Renkema et al. 1997), suggesting that CHIT1 could be used as a diagnostic biomarker for severe mycoses. However, further studies revealed that additional stimuli, including IFN-γ, TNF-α, and LPS, can stimulate CHIT1 activity. Therefore, CHIT1 activity is better viewed as a non-specific marker of inflammation, rather than a specific biomarker for individual fungal infections (Malaguarnera et al. 2005).

Subsequent studies have further explored the role of chitinases in many inflammatory conditions. For example, single-nucleotide polymorphisms (SNPs) in the CHIT1 gene were found to lead to decreased expression of chitinase and increased susceptibility to allergic conditions such as asthma, as well as infections

due to the filarial parasite *Wuchereria bancrofti* (Bierbaum et al. 2006; Sibylle Bierbaum et al. 2005; Choi et al. 2001). AMCase activity was also similarly associated with allergic diseases such as asthma, rhinosinusitis, and nasal polyposis (Boot et al. 2001, 2005; Ramanathan et al. 2006). Further studies revealed that CHIT1 is highly expressed by macrophages, and CHIT1 mRNA levels are increased when macrophages are treated with phorbol 12-myristate 13-acetate (PMA), a stimulus that induces macrophage differentiation and priming (Boot et al. 2005; Pham et al. 2007; van Eijk et al. 2005).

The discovery that chitinase expression and activation were so tightly linked with innate immune activation led many to hypothesize that chitin sensing and processing by the immune system might be an innate mechanism for defense against invasive fungal infections (IFIs). One study using a murine inhalational model of *C. neoformans* infection demonstrated increased AMCase activity in the airways (Vicencio et al. 2008). The induction of this acidic chitinase in a tissue that is generally maintained at a nearly neutral pH suggests that this enzyme may be secreted for activity within acidic microenvironments in the lung, including regions of local hypoxia on the acidic phagolysosome. In a different study, rat lungs were inoculated with zymosan, a chitin-containing extract of the cell walls of *S. cerevisiae*. In this model, host CHIT1 activity was increased, suggesting an active role for cell wall material in inducing chitinases (Korolenko et al. 2000). Another study demonstrated that overexpression of chitinases in transgenic tobacco resulted in the relative resistance of these plants to fungal infections (Jach et al. 1995), further supporting a link between chitinases and chitin processing with host immunity to mycoses.

In studies investigating the immune response to degraded chitooligomers in *C. neoformans* (Gorzelanny et al. 2010), investigators identified that host-derived chitinases were responsible for cleaving chitin through a process of “processivity,” resulting in a robust macrophage response due to the creation of small and diffusible chitooligomer fragments. Furthermore, this processivity and immune activation led to a further increase in chitinase production, specifically mammalian CHIT1 (Gorzelanny et al. 2010). This work supported prior studies characterizing chitinase activity as a primary effector in the size-dependent immune activation by chitin. In a positive feedback loop, specific recognition of IC and SC by the immune system is enhanced by the host chitinase response, and in turn, the chitinase response is maintained by continuous exposure to cleaved chitin molecules.

Chitosanases are enzymes that catalyze the hydrolytic degradation of chitosan, but they are not found in mammals and seem to be only present in soil microorganisms (bacteria and fungi) and plants (Li et al. 2008; Rodríguez-Martín et al. 2010). Glucosaminidases can also convert chitosan into glucosamine, but these enzymes have only been isolated from fungal species (Ike et al. 2006). Chitinases can also function as chitosan-degrading enzymes, but this is dependent on the degree of acetylation. Chitinases need GlcNAc in the −1 position in order to catalytically cleave the substrate (Somashekar and Joseph 1996). Therefore, the role of chitosanases on mammalian–fungal interactions remains unclear.

10 Biomedical Applications of Chitin and Chitosan

Due to the importance of chitin synthesis on fungal growth and pathogenesis, targeting this process has been proposed as an effective way to treat infections and clear fungal disease. To date, there have been no chitin synthase (Chs) inhibitors approved for clinical trials. However, compounds targeting Class I Chs proteins in vitro have been developed, and these include the competitive enzyme inhibitors nikkomycin Z and other polyoxins. However, when first tested in vivo, these compounds failed to target other classes of chitin synthase enzymes, and they did not inhibit fungal growth when tested in a *C. albicans* model of infection (Munro 1995). Since those original studies, researchers have tried to expand the breadth of therapeutically promising Chs inhibitors. One drug that seemed promising, RO-09-3143, was fungistatic against wild-type *C. albicans* and fungicidal against strains with a mutation in the *CHS2* chitin synthase gene. This finding suggested a complicated mechanism of chitin synthesis with both overlapping and distinct functions for various Chs proteins (Munro et al. 2001a, b; Sudoh et al. 2000).

Given the compensatory increases in fungal cell wall chitin content in response to various cell stresses, other investigators have proposed using Chs inhibitors in combination with other antifungals, especially glucan synthase inhibitors. Targeting pathogens such as *C. albicans* and *A. fumigatus* with both glucan synthase and Chs inhibitors proved to be significantly more effective at fungal killing than treatment with either inhibitor alone (Fortwendel et al. 2009; Walker et al. 2008). In *C. albicans*, the compensatory increase in cell wall chitin as a response to echinocandins was inhibited by pre-treatment with the Chs inhibitor nikkomycin Z. Accordingly, strong antifungal synergy was observed in this species using nikkomycin Z in combination with glucan synthase inhibition (Walker et al. 2008). In contrast, *A. fumigatus* cell wall chitin was not decreased by treatment with nikkomycin Z alone, suggesting that this compound is not a highly effective inhibitor of the complex array of chitin synthases in this species. However, the combination of nikkomycin Z and caspofungin still resulted in synergistic antifungal activity (Fortwendel et al. 2009). In *C. neoformans*, no synergy was observed using nikkomycin Z and caspofungin in combination, despite a conserved compensatory chitin response to echinocandin therapy (Pianalto et al. 2019). However, in contrast to *C. albicans* and *A. fumigatus*, neither drug demonstrates striking primary anticryptococcal activity. The development of new antifungal compounds with different mechanisms of Chs inhibition would likely be a very attractive addition to current combination antifungal strategies. Given the central role for Class IV and V Chs enzymes in the growth and development of diverse fungal species, these classes of enzymes would be especially exciting new targets.

In addition to their roles as potential targets for antifungal therapy, chitin and chitosan have proven to be useful in novel vaccine design platforms. Mucosal vaccines that used chitosan as an adjuvant for human challenge studies with influenza antigens resulted in enhanced serum antibody production compared to historical, non-adjuvanted controls (Read et al. 2005). Additionally, chitosan

mucosal delivery systems for a detoxified diphtheria toxoid vaccination induced potent Th2-mediated immune responses in human subjects (McNeela et al. 2004).

In addition to using chitooligomers to shape immune responses, investigators have also used chitin-modifying enzymes as vaccine immunogens themselves. These studies build upon prior observations that mannosylated surface proteins, such as chitin deacetylases, often act as immunodominant epitopes after fungal exposure, resulting in measurable serum antibodies against these proteins (Huang et al. 2002). For example, investigators used a prime-boost strategy of intramuscular immunization with *C. neoformans* chitin deacetylase antigens (Cda1 and Cda2) in the context of glucan particles derived from *S. cerevisiae*. Preimmunization with these cryptococcal proteins provided protection from subsequent challenges with either *C. neoformans* or *Cryptococcus gattii* (Specht et al. 2017). A different vaccine strategy used a live attenuated a strain of *C. neoformans* that lacks all three chitin deacetylases (Cda1, Cda2, and Cda3). Infection with this hypovirulent mutant strain protected mice against subsequence challenges with the virulent wild-type isolate (Upadhya et al. 2018). These studies were especially compelling since *C. neoformans* infections do not typically induce secondary immunity against subsequent infections. Therefore, determining the mechanisms by which this type of mutant strain provides an immunizing effect will substantially inform the direction of new investigations promoting antifungal immunity.

11 Conclusions

Chitin is one of the most common molecules in nature, found in the majority of fungi, as well as in many insect and invertebrate species. Although it is a relatively simple homopolymer of *N*-acetylglucosamine, chitin and its deacetylated partner chitosan serve as the structural backbone of the fungal cell wall, acting as a matrix onto which the outer polysaccharide and glycoprotein layers are linked. These "hidden figures" within the fungal cell wall provide the architectural strength to ensure cell integrity in the face of stress, while also allowing the cell to minimize detection by the host immune system. Chitin synthesis is tightly regulated and intimately involved in growth during the cell cycle, as well as the response to cell stress, and the localization of chitin synthase enzymes is dynamic and highly organized. The interaction between chitin and the immune system is complex, and the outcome depends on many factors, including the type and size of chitin encountered. While no single PRR has been directly characterized as a chitin receptor, several mammalian cell surface receptors have been demonstrated to play a role in the host response to chitin. These responses include the production of chitin-degrading chitinase enzymes. Finally, in addition to their role the fungal cell wall and lifecycle, chitin and chitosan have a large number of potential biomedical applications, including serving as biosensors, diagnostic tools, drug delivery

devices, vaccine adjuvants, and in enhancers of wound healing. These findings, combined with recent efforts in chitin synthase classification and targeting, will help elucidate the many ways in which this biopolymer directs fungal physiology and environmental adaptation.

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Control of Actin and Calcium for Chitin Synthase Delivery to the Hyphal Tip of *Aspergillus*

Norio Takeshita

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Abstract Filamentous fungi are covered by a cell wall consisting mainly of chitin and glucan. The synthesis of chitin, a β-1,4-linked homopolymer of *N*-acetylglucosamine, is essential for hyphal morphogenesis. Fungal chitin synthases are integral membrane proteins that have been classified into seven classes. ChsB, a class III chitin synthase, is known to play a key role in hyphal tip growth and has been used here as a model to understand the cell biology of cell wall biosynthesis in *Aspergillus nidulans*. Chitin synthases are transported on secretory vesicles to the plasma membrane for new cell wall synthesis. Super-resolution localization imaging as a powerful biophysical approach indicated dynamics of the Spitzenkörper where spatiotemporally regulated exocytosis and cell extension, whereas high-speed pulse-chase imaging has revealed ChsB transport mechanism mediated by kinesin-1 and myosin-5. In addition, live imaging analysis showed correlations among intracellular Ca^{2+} levels, actin assembly, and exocytosis in

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Current Topics in Microbiology and Immunology (2020) 425: 113–129
https://doi.org/10.1007/82_2019_193
© Springer Nature Switzerland AG 2019
Published Online: 24 January 2020

growing hyphal tips. This suggests that pulsed Ca^{2+} influxes coordinate the temporal control of actin assembly and exocytosis, which results in stepwise cell extension. It is getting clear that turgor pressure and cell wall pressure are involved in the activation of Ca^{2+} channels for Ca^{2+} oscillation and cell extension. Here the cell wall synthesis and tip growth meet again.

1 Introduction

Filamentous fungi grow as highly polarized tubular cells called hyphae that extend the cell body at one end in a process called 'tip growth.' Cell extension sites are maintained at hyphal tips, where simultaneous actin assembly, exocytosis, and tip extension occur (Fischer et al. 2008; Riquelme et al. 2011; Takeshita et al. 2014; Riquelme et al. 2018). Several filamentous fungi that extend cells in this manner are excellent systems for analyzing this process (Lopez-Franco et al. 1994). Some filamentous fungi are pathogenic to animals and plants and invade host cells via hyphal growth (Perez-Nadales et al. 2014). Others have uses in biotechnology and food production such as enzyme production and fermentation, respectively, as they secrete large amounts of enzymes (Kobayashi et al. 2007; Punt et al. 2002). Both the pathogenicity and enzyme secretory ability of fungi are closely associated with hyphal growth. Thus, understanding polarized growth in filamentous fungi can provide insights that are important to medicine, agriculture, and biotechnology.

2 Chitin Biosynthesis

The cell wall not only imparts physical strength to the cell but also plays a role in transmitting information about the natural or artificial environmental conditions to the inside of the cell. The cell walls of *Aspergillus fumigatus* are composed of β-(1,3)-glucan, chitin, β-(1,3)-/β-(1,4)-glucan, ∝-(1,3)-glucan, galactomannan (GM), galactosaminogalactan (GAG), and proteins (Latge et al. 2017). Most cell wall proteins are modified by *N*-glycan, *O*-glycan, and/or a glycosylphosphatidylinositol anchor. These components are complexly intertwined to form the three-dimensional structure of cell walls (Fig. 1a). During hyphal tip growth, various glycan synthases, including β-(1,3)-glucan synthases, chitin synthases, and ∝-(1,3)-glucan synthases, are transported to the tips by secretory vesicles. The transported glycan synthases generate the corresponding glycans, which then penetrate into the interstices of the cell wall skeleton, where they act like cement. Thus, the hyphae grow by forming a complicated three-dimensional structure.

Chitin, a β-(1,4)-linked polymer of *N*-acetylglucosamine (GlcNAc), is a major skeletal component of the cell wall of *A. fumigatus* and gives the wall mechanical rigidity. The higher amount of chitin (>10%) present in the walls of *Aspergillus* sp. as compared to yeast (Blumenthal and Roseman 1957; Johnston 1965) indicates

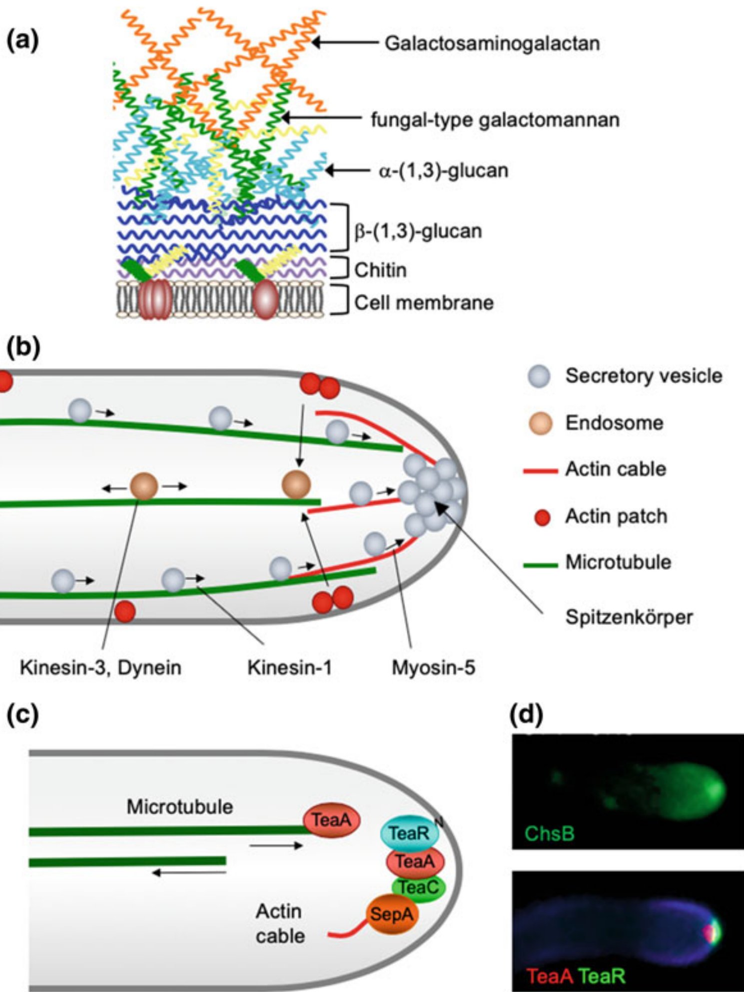

Fig. 1 Scheme of tip growth in *A. nidulans* hyphae. **a** Schematic representation of structural organization of the cell surface of *Aspergillus.* The different polysaccharides have their roles; glucans are the most abundant compounds in the fungal cell walls and an amorphous gel-like matrix, chitin as a cell wall skeleton. **b** Secretory vesicle trafficking via the microtubule and actin cytoskeleton depending on kinesin-1 and myosin-5, respectively. Before fusion with the plasma membrane, secretion vesicles accumulate at Spitzenkörper. **c** Scheme of the function of cell end markers in *A. nidulans*. **d** Localization of GFP-ChsB at Spitzenkörper (upper). Localization of cell end markers mRFP1-TeaA and GFP-TeaR at the hyphal tip (lower)

that the synthesis of chitin is essential for hyphal morphogenesis (Rogg et al. 2012). Chitin is biosynthesized by several chitin synthases localized at the plasma membrane; these are responsible for the sequential synthesis of GlcNAc using UDP-GlcNAc as a sugar donor.

Fungal chitin synthases are integral membrane proteins that have been classified into seven classes and three divisions according to their structural properties (Lenardon et al. 2010a, b; Gonçalves et al. 2016). *Aspergillus fumigatus* and *Aspergillus nidulans* have eight different chitin synthases (ChsA-ChsD, ChsF, ChsG, CsmA, and CmsB in *A. nidulans*) (Horiuchi 2009; de Groot et al. 2009). Among these, ChsB, a class III chitin synthase, is known to play a key role in hyphal tip growth, maintenance of cell wall integrity, and development (Yanai et al. 1994; Borgia et al. 1996; Fukuda et al. 2009). The *chsB* disruptant hyphae have enlarged tips, a high degree of branching, and disorganized lateral walls (Borgia et al. 1996). Class III chitin synthases are important for hyphal morphology, cell wall integrity, and pathogenicity in other filamentous fungi as well (Rogg et al. 2012; Muszkieta et al. 2014). Meanwhile, ChsA and ChsC are required for the formation of the septum and a normal conidiophore (Motoyama et al. 1994; Fujiwara et al. 2000; Ichinomiya et al. 2005). CsmA and CsmB, that are widely distributed in filamentous fungi and dimorphic yeasts but lacking in *S. cerevisiae* and *S. pombe,* have myosin motor-like domains (Fujiwara et al. 1997). The myosin motor-like domains bind to actin filaments, suggesting a direct link between the actin cytoskeleton and polarized cell wall synthesis (Takeshita et al. 2005, 2006). ChsB has been used as a model to understand the vesicles trafficking in chitin synthesis.

3 Transport of Chitin Synthase

Polarized growth of fungal hyphae is sustained by the continuous delivery of vesicles loaded with biomolecules to the hyphal tips (Rittenour et al. 2009; Sudvery 2008; Schuster et al. 2016; Takeshita 2016; Riquelme et al. 2018; Zhou et al. 2018). Vesicle trafficking supplies the required proteins and lipids via actin, as well as microtubule cytoskeletons and their corresponding motor proteins (Fig. 1b) (Taheri-Talesh et al. 2008; Steinberg 2011; Egan et al. 2012; Penalva et al. 2017; Renshaw et al. 2016). Microtubules serve as tracks of secretory vesicles for long-distance transport to hyphal tips and are important for rapid hyphal growth (Horio and Oakley 2005; Seiler et al. 1997). Actin cables formed from the hyphal tip in the retrograde direction are involved in exocytosis and secretory vesicle accumulation before exocytosis (Berepiki et al. 2011; Bergs et al. 2016). These vesicles accumulate at the apices prior to fusion with the membrane. They form a structure called Spitzenkörper (Harris 2009), which is thought to act as a vesicle supply center, a site where cargo for the hyphal tip is sorted (Riquelme and Sánchez-León 2014).

Besides their role as tracks for vesicle traffic, microtubules are necessary to maintain the direction of hyphal growth (Riquelme et al. 1998). Polar organization of the actin cytoskeleton is mediated mainly by microtubule-dependent positioning of polarity marker proteins (Fig. 1c). One polarity marker in *Aspergillus nidulans* (TeaA) is specifically delivered to the apex by growing microtubules, and it is anchored to the apical membrane by direct interaction with another polarity marker

(TeaR) at the plasma membrane (Fig. 1c) (Fischer et al. 2008; Takeshita et al. 2008). Their interdependent interaction at the apical membrane initiates the recruitment of additional components including the formin which polymerizes actin cables for targeted cargo delivery (Higashitsuji et al. 2009). Defective polarity markers result in hyphae that are curved or zigzagged instead of straight (Takeshita et al. 2008).

In *A. nidulans*, microtubules extend all the way to the hyphal tip, whereas actin cables are found mostly near the hyphal tip (Bergs et al. 2016). Vesicles containing components of the growth machinery are transported along microtubules from posterior sites to the apical region, transferred to actin cables, and finally delivered to the apical cortex of the hypha (Egan et al. 2012; Fischer et al. 2008; Pantazopoulou et al. 2014; Taheri-Talesh et al. 2008; Takeshita et al. 2014). These secretory vesicles (SVs) are released from the trans-Golgi network after maturation (Pantazopoulou et al. 2014; Pinar et al. 2015). Since gene deletion of kinesin-1 or myosin-5 decreases the amount of SVs at the hyphal tips, resulting in growth retardation, SVs are believed to be transported along microtubules by kinesin-1 and further along actin filaments by myosin-5 to the hyphal tip for exocytosis (Pantazopoulou et al. 2014; Seiler et al. 1997; Taheri-Talesh et al. 2012). However, localization analysis reported that kinesin-1 diffuses in the cytoplasm and myosin-5 accumulates at the hyphal tip (Requena et al. 2001; Taheri-Talesh et al. 2012). SV transport was not directly observed, probably due to the small size and fast motion. Early endosomes (EEs) are easier to track, so their bi-directional transport along microtubules by kinesin-3 and dynein has been thoroughly studied (Abenza et al. 2009, 2010; Egan et al. 2012; Lenz et al. 2006; Schuster et al. 2011).

Chitin synthases are thought to be transported on SVs to the plasma membrane for new cell wall synthesis (Fig. 1d), where they are subsequently internalized by endocytosis and transported on EEs for degradation in vacuoles, or recycled back to the plasma membrane (Sacristan et al. 2012). Actin patches are peripheral punctate structures, where the endocytic machinery is probably located (Araujo-Bazán et al. 2008). Kinesin-1 is required for transport of ChsB to the subapical region. However, mechanistic details could not be resolved due to high background fluorescence near the hyphal tip, insufficient time resolution to resolve fast motions, and the inability to distinguish between SVs and EEs (Takeshita et al. 2015).

An essential role for chitin synthase phosphorylation in the polarized biosynthesis of fungal cell walls is demonstrated in the polymorphic human pathogen *Candida albicans* (Lenardon et al. 2010a, b). Class III chitin synthase (Chs3) is localized at the tips of growing buds and hyphae, and at the septum. A phospho-proteome analysis of *C. albicans* revealed that Chs3 is phosphorylated. Mutation of this site showed the phosphorylation is required for the correct localization and function of Chs3.

4 Super-resolution Imaging and Cluster Analysis of Chitin Synthase

ChsB localizes to hyphal tips and concentrates at the Spitzenkörper in *A. nidulans* (Fukuda et al. 2009). In recent study, super-resolution localization imaging and high-speed pulse-chase imaging as a powerful biophysical approach have been used to analyze ChsB transport and dynamics of the Spitzenkörper (Zhou et al. 2018). The resolution of conventional light microscopy techniques is limited to around 250–300 nm due to light diffraction. Super-resolution microscopy techniques, such as STORM, PALM, etc., have overcome the diffraction limit, resulting in lateral image resolution as high as 20 nm, providing a powerful tool to investigate protein localization in high detail (Sahl and Moerner 2013).

To quantitatively analyze the spatio-temporal development of the Spitzenkörper with very high resolution, hyphae expressing ChsB was imaged as a fusion protein with mEosFPthermo (Wiedenmann et al. 2011). The thermostable monomeric green fluorescent chromophore can be permanently photoconverted to red with near-UV irradiation (Nienhaus et al. 2005, 2006). That is widely employed for fluorescence imaging, pulse-chase experiments, and super-resolution photoactivation localization microscopy (PALM) (Betzig et al. 2006; Hess et al. 2006). PALM uses photoswitchable fluorophores to achieve temporal control of the emission through conversion between fluorescent 'on/red' and 'off/green' states. When sample excitation is a sufficient low intensity, only a random sparse fluorophore subset will be in the 'on/red' state at any time, allowing these molecules to be imaged individually, precisely localized. Such strategy leads to the construction of a super-resolution image.

Single-molecular imaging-based localization microscopy revealed a pronounced fluorescent cluster of mEos-ChsB at the hyphal apex, representing the Spitzenkörper, and multiple speckles mostly near the plasma membrane (Fig. 2a) (Zhou et al. 2018). ChsB accumulation at the hyphal tip was classified by cluster analysis, where more than 10 molecules within 50 nm are defined as a cluster (Fig. 2b). Cluster images of 2.5 s time intervals were generated for a total period of 120 s with a moving window binning technique (500 frames binning with 50 frames shift) (Fig. 2c) (Ishitsuka et al. 2015). Each cluster is shown in different colors. The cluster areas and numbers of ChsB molecules within each cluster were calculated over the time course of the experiment (Fig. 2d). The green cluster of 0.1 μm^2 containing ~100 molecules is visible from 7.5–60 s. It grows via fusion with the blue cluster and evolves into the pink, crescent-shaped cluster of ~0.2 μm^2 containing ~200 molecules, visible from 62.5–80 s. Subsequently, this cluster breaks up into two smaller ones (~0.05–0.1 μm^2, ~50–100 molecules), depicted in light green and light blue. The shape change of the cluster from globular to crescent reflects the transition from vesicle accumulation prior to exocytosis to vesicle fusion with the apical plasma membrane during exocytosis.

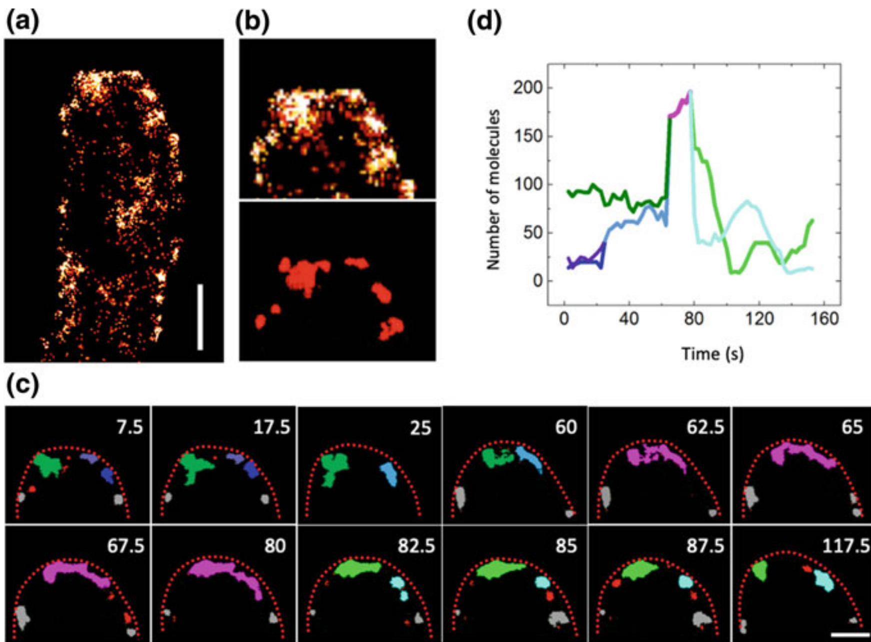

Fig. 2 Super-resolution imaging of Spitzenkörper dynamics. **a** Localization image of a hypha with mEosFP-ChsB clusters (constructed with 500 frames for 25 s). Scale bars; 1 µm. **b** Top: image of the hyphal tip. Each dot indicates single molecule. Bottom: ChsB accumulations classified by cluster analysis; more than ten molecules within 50 nm are defined as a cluster. **c** Sequence of ChsB cluster images (clusters in different colors) rendered from images reconstructed by time-lapse PALM (2.5 s interval by moving window binning for 120 s). Scale bars; 300 nm. **d** Time courses of number of ChsB molecules. Lines are drawn in colors corresponding to the clusters in (**c**) (modified Zhou et al. 2018)

5 Pulse-Chase Analysis of mEosFP-ChsB After Photoconversion

High-speed pulse-chase imaging of mEos-ChsB after photoconversion was employed to monitor its transport (Zhou et al. 2018). After photobleaching all red-emitting molecules with a 561 nm laser, a spot ~5 µm behind the hyphal tip was irradiated for 1 s with a tightly focused 405 nm laser beam to locally photoconvert mEos-ChsB to its red-emitting form (Fig. 3a). In Fig. 3b, image '0' shows the red fluorescence excited by the 405 nm laser, marking the local photoconversion spot. Then, the 561 nm laser was again switched on (image '1'), and images were acquired for 15–30 s with a dwell time of 50 ms. A large red-emitting spot appeared at the site of photoconversion, which gradually faded and dispersed due to vesicle transport away from the photoconversion region. By taking advantage of the low background in this pulse-chase imaging scheme, both anterograde (from back to tip) and retrograde (from tip to back) vesicle movements are easily observed in a kymograph along the axis of the hypha (Fig. 3c). The typical linear vesicle displacements were occasionally interrupted by brief stops, and there were also some

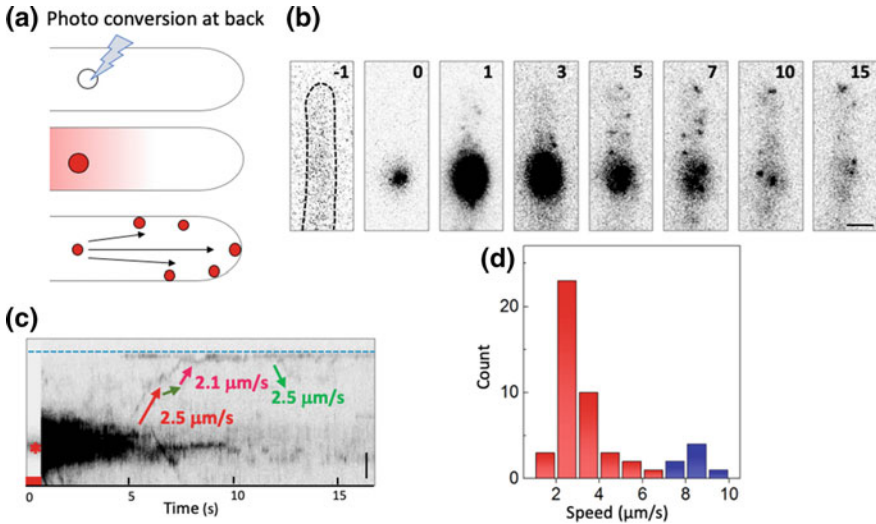

Fig. 3 High-speed pulse-chase analysis of mEos-ChsB transport. **a** Sequence of pulse-chase analysis of mEosFP-ChsB after photoconversion at back of hyphae. **b** Images of mEosFP-ChsB prior to photoconversion (−1), with 405-nm light applied at the spot marked by the dashed line for 1 s (0) and after photoconversion for 15 s. **c** Kymograph calculated from panel (**b**); arrows indicate anterograde and retrograde transport. The blue dashed line and the red asterisk mark the positions of the hyphal tip and the photoconversion locus, respectively; the red square indicates the photoconversion interval. **d** Speed distribution of anterograde transport. Slow anterograde (red) and fast anterograde (blue) transport (mean ± SD; n = 42, and 7, respectively) (modified Zhou et al. 2018)

immobile spots. We further noticed that the fluorescence from the hyphal tip stayed constant beyond ~5 s after photoconversion.

The slopes of the lines in the kymograph encode the speed of ChsB vesicle movement. From observations of a large number of hyphae, we noticed that most displacements occurred at speeds of 2–4 µm s^{-1}; however, there were also clearly faster processes with speeds of 7–10 µm s^{-1}. Accordingly, the speed histogram of anterograde movements appears to consist of two sub-distributions (Fig. 3d), a predominant distribution associated with slow transport centered on 3.0 ± 1.0 µm s^{-1} and a smaller distribution representing fast transport centered on 8.3 ± 0.7 µm s^{-1}. By comparison with the transport of EE and SV markers, the slow transport and fast transport were unambiguously assigned to ChsB associated with EEs and SVs, respectively. In fungi, EEs are 4–5 times larger than SVs (Gibeaux et al. 2013; Lin et al. 2016). Therefore, the slower transport of EEs is probably caused by the size of the cargo. Of note, in cultured mammalian cells, the speeds of kinesin-1 and kinesin-3 are similar, ~1–2 µm s^{-1} (Hammond et al. 2009; Tanenbaum et al. 2014). Comparative analysis using motor protein deletion mutants allowed us to assign the fast movements (7–10 µm s^{-1}) to transport of secretory vesicles by kinesin-1, and the slower ones (2–7 µm s^{-1}) to transport by kinesin-3 on early endosomes (Zhou et al. 2018). These results show how motor proteins ensure the supply of vesicles to the hyphal tip, where temporally regulated exocytosis results in stepwise tip extension.

6 Oscillation of Fungal Tip Growth

Time-lapse super-resolution PALM (photoactivation localization microscopy) analysis revealed that membrane-associated polarity marker TeaR in *A. nidulans* transiently assembles (approximately 120 nm) at the hyphal tip membrane and

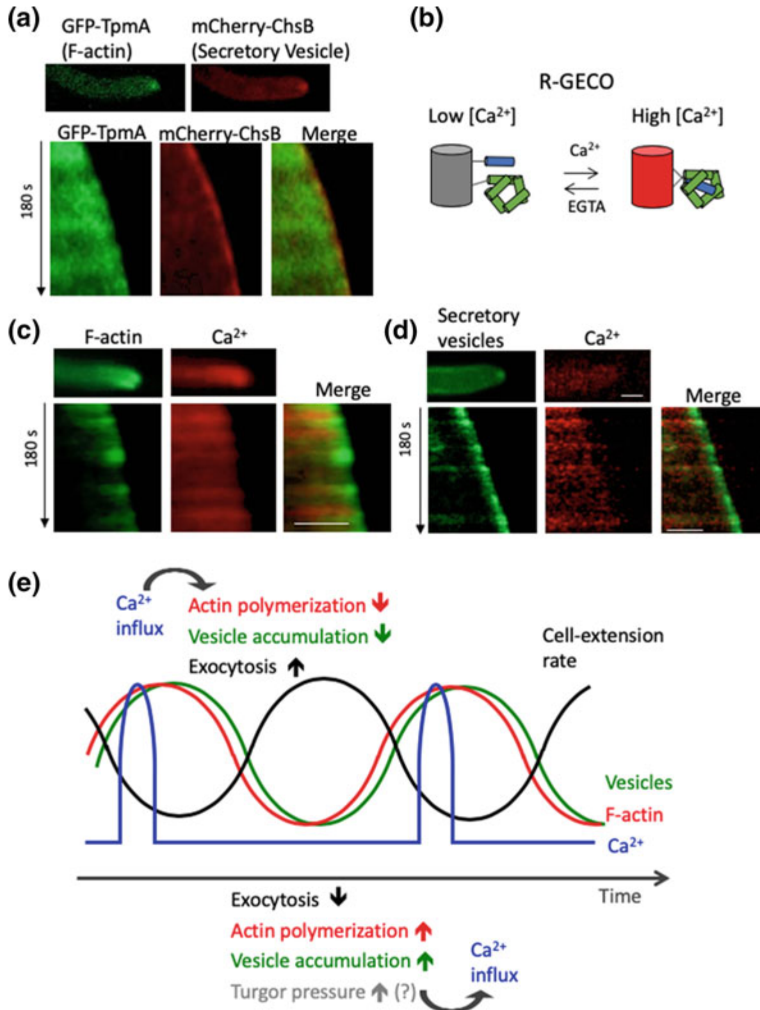

Fig. 4 Pulses of Ca^{2+} coordinate actin assembly and exocytosis. **a** Fluorescence image of F-actin and SV visualized by GFP-TpmA and mCherry-ChsB, respectively, in the growing hypha. Kymographs of F-actin (green) and SV (red) along the growth axis in the growing hypha. Total 180 s. **b** Scheme of the Ca^{2+} biosensor, R-GECO. **c**, **d** Fluorescence images and kymographs along the growth axis of F-actin (GFP-TpmA) (**c**) or secretory vesicles (GFP-BglA) (**d**) and Ca^{2+} (R-GECO). Total 180 s. Scale bar: 2 µm. (e) Scheme of oscillation in fungal tip growth coordinated by Ca^{2+} influx (modified Takeshita et al. 2017)

disperses along the membrane after exocytosis, which inserts a new membrane that results in local membrane extension (Ishitsuka et al. 2015). These findings gave rise to a 'transient polarity assembly model' to explain how fungal tip cells extend through repeated cycles of TeaR assembly/disassembly, actin polymerization, and exocytosis, rather than by constant elongation (Ishitsuka et al. 2015; Takeshita 2016). The findings of colocalization studies further support the notion that TeaR clusters represent zones of exocytosis and are prerequisite for apical membrane extension. In line with this model, recent work on *Neurospora crassa* has identified bursts of exocytotic events at various sites within the apical membrane rather than a persistent exocytosis site (Riquelme et al. 2014).

F-actin and secretory vesicles (SV) were visualized by fluorescence of GFP-tagged tropomyosin (TpmA) and mCherry-tagged ChsB, respectively (Fig. 4a) (Takeshita et al. 2017). Prominent signals were visible at the hyphal tip, and time-resolved recording and frame analysis by kymographs revealed that the signal intensity oscillated. The mean interval of the intensity of F-actin peaks was 29 ± 8 s, whereas the mean interval of peaks of SV was comparable to the one of F-actin, 30 ± 7 s. The temporal relationship between the presence of F-actin and SV was calculated as the normalized cross-correlation of their signal intensities, revealing the central peak is 0.50, indicative of a positive correlation between the signals of F-actin (green) and SV (red) (1 and -1 represent perfect positive and negative correlations, respectively). There were a few second delays in the signals of SV in comparison to the signals of F-actin, indicating that SV accumulate during actin polymerization phases and SV are depleted due to exocytosis during actin depolymerization.

7 Ca^{2+} Oscillation

Intracellular Ca^{2+} levels regulate actin assembly and vesicle fusion (Janmey 1994; Schneggenburger and Neher 2005). The red-fluorescent Ca^{2+} biosensor R-GECO was produced in *A. nidulans* (Fig. 4b) (Takeshita et al. 2017). Pulses of the R-GECO signal were observed: The mean interval between peaks was 26 ± 7 s. The R-GECO signal appeared as a tip-high gradient and diffused backwards. Such R-GECO pulses continued for multiple times before they disappeared (max. $n = 8$ in 180 s), probably due to a limited turnover of R-GECO. The fluorescence of R-GECO could not be detected in media without CaCl2 or with 1 μM CaCl2 + 10 mM EGTA, indicating that the increase of the intracellular Ca^{2+} level is induced by the influx of Ca^{2+} to the cells at hyphal tips.

The signals of R-GECO and GFP-TpmA (F-actin) or BglA-GFP (∝-glycosidase, secreted protein as a marker for SV) showed oscillation in kymographs along the growth axis indicated temporal changes of signal intensities (Fig. 4c, d). The signal intensities of F-actin (green) and the Ca^{2+} concentration (red) at the tip indicated that oscillations of peak intensities had similar periods (29 ± 8 and 26 ± 7 s, respectively) and were synchronized. Normalized cross-correlation analysis yielded

a positive correlation between the concentrations of F-actin (green) and Ca^{2+} (red), with the central peak at 0.40 and indicated that the peaks of Ca^{2+} appeared a few seconds earlier than those of F-actin. These results are in agreement with the notion that Ca^{2+} influx at the growing hypha induces actin depolymerization. Hyphae producing R-GECO and BglA-GFP (SV) also showed oscillations of the GFP intensity at the tip and R-GECO pulses (Fig. 4d), with similar periods (30 ± 7 and 26 ± 7 s, respectively), which were synchronized. Normalized cross-correlation analysis yielded a positive correlation with a central peak value of 0.43 and a few seconds delay of BglA-GFP, indicating that Ca^{2+} influx affords exocytosis mediated by fusion of SV with the plasma membrane as well as actin depolymerization.

The oscillation of Ca^{2+} levels at the hyphal tips of filamentous fungi suggested the stepwise extension of hyphal tips (Kim et al. 2012). Indeed, critical correlations were shown between intracellular Ca^{2+} levels, actin polymerization, exocytosis, and cell extension at fungal tips (Takeshita et al. 2017). Thus, the pulsed Ca^{2+} influx coordinates the temporally controlled actin polymerization and exocytosis that drive stepwise cell extension (Fig. 4e). Several Ca^{2+} channels, pumps, and transporters, such as the plasma membrane, ER, Golgi, mitochondria, and vacuoles function in fungal organelles (Zelter et al. 2004). The Ca^{2+} channels at the plasma membrane of *Saccharomyces cerevisiae*, Mid1 and Cch1p, share a single pathway that responds to environmental stressors and ensures cellular Ca^{2+} homeostasis (Iida et al. 1994; Locke et al. 2000; Paidhungat and Garrett 1997). Deletion of the orthologs *midA* and *cchA* from *A. nidulans* causes defective polarized growth and cell wall synthesis (Wang et al. 2012). Proper tip growth and the oscillation of F-actin, secretory vesicles, and growth rates require Ca^{2+} channels (Takeshita et al. 2017). The oscillatory model explains how transient Ca^{2+} influx depolymerizes F-actin at the cortex, stimulates secretory vesicles to fuse with the plasma membrane, and extends the cell tip faster. After Ca^{2+} diffusion, F-actin and secretory vesicles accumulate at hyphal tips.

The key event appears to be the activation of Ca^{2+} channels. One attractive notion is that the Ca^{2+} channels could be stretch-activated. Cells gradually build up turgor pressure against the membrane and the cell wall during slow growth phases, and Ca^{2+} channels might be activated when membrane tension exceeds a threshold. The entry of Ca^{2+} into the cell promotes exocytosis and leads to cell extension, which in turn decreases turgor pressure and inactivates the channels. Indeed, the ortholog Mid1 of *S. cerevisiae* is stretch-activated (Kanzaki et al. 1999). The next items to address would be missing links between turgor pressure and cell wall extension.

8 Biological Meaning of Oscillations

Relationships between cellular responses and receptor stimuli are encoded by the spatial and temporal dynamics of downstream signaling networks (Kholodenko 2006). Positive feedback, alone or in combination with negative feedback, can

trigger oscillations, for example, the Ca^{2+} oscillations that arise from Ca^{2+}-induced Ca^{2+} release (Goldbeter 2002). The shape of oscillations is characterized by their amplitude and phase. The frequency modulation of Ca^{2+} oscillations provides an efficient means to differentiate intracellular biological responses (Smedler and Uhlen 2014).

The oscillation of cortical F-actin presumably follows that of Ca^{2+} and correlates with the oscillations of vesicle secretion. These coordinated steps result in growth oscillation (Takeshita 2018). A feedback cycle might be efficient for all these steps to continuously proceed. In addition, oscillatory cell growth allows cells to respond more rapidly and frequently to internal and external cues such as chemical or mechanical environmental signals. Indeed, Ca^{2+} influx by Ca^{2+} channels is involved in the control of directional hyphal growth in *C. albicans* (Brand et al. 2007). The influx of Ca^{2+} promotes Cdc42 GTPase trafficking and amplifies Cdc42-mediated directional growth signals (Brand et al. 2014). Stepwise growth coordinated by a transient Ca^{2+} influx might link growth with chemotropism and chemotaxis. Cell–cell fusion is essential for colony development in *N. crassa* (Herzog et al. 2015). Before growing partners fuse in a tip-to-tip manner, the cells coordinately switch between two physiological stages via the oscillatory recruitment of a MAP kinase (MAK-2) and a protein of unknown molecular function (SO) to the apical plasma membrane of growing fusion tips (Fleissner et al. 2009; Serrano et al. 2017). The oscillation of signaling, which is probably related to signal sending and signal receiving, allows cells to coordinate their behavior and achieve efficient cell fusion (Goryachev et al. 2012).

9 Conclusion and Perspective

The dynamic responses to external and internal signals are fundamental to the increased understanding of chemotropism, cell–cell fusion, microbial interaction, and the fungal penetration of plant and animal cells. The pulsed Ca^{2+} influx coordinates the temporally controlled actin polymerization and exocytosis that drive stepwise cell extension of filamentous fungi. Besides them, to understand the hyphal tip growth, we need to pay attention to the balance between turgor pressure and cell wall pressure, which is regulated by cell wall synthesis, degradation, and maturation. The filamentous fungus elongates hyphae by tip growth while forming a cell wall comprising a complex three-dimensional structure. Understanding the cell wall biosynthesis is very important in understanding the interaction with other microorganisms and the infection mechanism of filamentous fungi to plants and animals. Recent studies have identified many glycosyltransferase genes involved in biosynthesis of constituent polysaccharides. Next open questions are the formation mechanism of the polysaccharide structure which changes according to the environmental changes and the cell wall formation mechanism at the time of differentiation, also how all the chitin and glucan synthases and cell wall relating proteins are trafficking coordinated in the formation of the cell wall skeletons.

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Glucanases and Chitinases

César Roncero and Carlos R. Vázquez de Aldana

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Abstract In many yeast and fungi, β-(1,3)-glucan and chitin are essential components of the cell wall, an important structure that surrounds cells and which is responsible for their mechanical protection and necessary for maintaining the cellular shape. In addition, the cell wall is a dynamic structure that needs to be remodelled along with the different phases of the fungal life cycle or in response to extracellular stimuli. Since β-(1,3)-glucan and chitin perform a central structural role in the assembly of the cell wall, it has been postulated that β-(1,3)-glucanases and chitinases should perform an important function in cell wall softening and remodelling. This review focusses on fungal glucanases and chitinases and their role during fungal morphogenesis.

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Current Topics in Microbiology and Immunology (2020) 425: 131–166
https://doi.org/10.1007/82_2019_185
© Springer Nature Switzerland AG 2019
Published Online: 6 December 2019

1 The Fungal Cell Wall and Its Components

The cell wall is the outer structure that surrounds the fungal cell and is essential for the mechanical protection of the cell and to maintain the cellular shape. In spite of their rigidity and mechanical properties, fungal cell walls are highly dynamic structures required to maintain cell viability, and their composition and structure changes in response to environmental growth conditions and extracellular stresses. In addition, it needs to be remodelled during diverse morphogenetic processes, such as cell growth, cytokinesis or the development of specialised types of fungal cells (reviewed in Latgé 2007; Levin 2011; Free 2013; Gow et al. 2017; Hopke et al. 2018). During fungal infections, the cell wall is the interface of the pathogen that interacts with host cells, therefore playing a key role in fungal pathogenicity and virulence (reviewed in Erwig and Gow 2016; Geoghegan et al. 2017; Latgé et al. 2017). The cell wall provides adherent properties that are vital for an invasion of the host tissues and protects against the defensive machinery of the host (reviewed in Lipke 2018).

Most fungal cell walls have a similar multi-layered structure, with an innermost layer built of a matrix of long chains of structural polysaccharides to which a variety of proteins and other superficial components are added to form the outer layers, which are more heterogeneous and sometimes species-specific (reviewed in Latgé 2007; Orlean 2012; Free 2013; Klis et al. 2014; Latgé and Beauvais 2014; Gow et al. 2017). The inner layer in most fungi is composed of branched β-(1,3)-glucan, with both β-(1,6)-glucan and some chitin being connected to the non-reducing ends (NREs) of the glucan filament, whereas the outer layers are more varied and contain many glycoproteins that are covalently attached to the glucan network (reviewed in Klis et al. 2001, 2002; Lesage and Bussey 2006; Latgé 2007; Cabib and Arroyo 2013). Fungal cells contain a large collection of carbohydrate active enzymes required for the assembly and modification of this structure along the life cycle and also in response to environmental stresses. These include multigene families of chitin and glucan synthases as well as remodelling enzymes such as glycohydrolases (glucanases, chitinases) and transglycosidases.

1.1 Glucans

The main glucan of the inner layer is β-(1,3)-glucan, composed of glucose monomers linked by β-(1,3) bonds, although there are also β-(1,6), α-(1,3) and α-(1,4)-glucans. β-(1,3)-glucan is usually the most abundant glucan, accounting for 65–90% of the total cell wall β-glucans. Depending on the organism, β-(1,3)-glucan represents between 25–30% of the total cell wall in *Aspergillus fumigatus* to 50–55% in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Lipke and Ovalle 1998; Gastebois et al. 2009; Orlean 2012). This polymer is mainly composed of a β-(1,3)-glucan backbone periodically branched with β-(1,6)-linked β-(1,3)-glucan side

chains. The frequency of β-(1,6) branches varies depending on the organism (reviewed in Latgé 2007; Orlean 2012; Gow et al. 2017).

The fungal cell walls also contain a lower proportion of β-(1,6)-glucan, ranging from 5 to 12% in *S. cerevisiae* to 21% in *Candida albicans* (Manners et al. 1973; Klis et al. 1997; Lipke and Ovalle 1998; Lesage and Bussey 2006; Orlean 2012). The structure and degree of branching are highly variable depending on the fungi. *S. pombe* is the organism that contains a higher degree of branched β-(1,6)-glucan (Magnelli et al. 2005), whereas linear β-(1,6)-glucan is absent in *A. fumigatus*. β-(1,6)-glucan acts as a glue to covalently bind β-(1,3)-glucan, chitin and glycoproteins (Kollar et al. 1995; Kapteyn et al. 1996; Kollar et al. 1997; Fontaine et al. 2000; Iorio et al. 2008). β-(1,6)-glucan is also important for the assembly of cell wall proteins (CWPs) to the β-(1,3)-glucan–chitin skeleton, acting as a linker that attaches the proteins via a glycosylphosphatidylinositol (GPI) remnants (Klis et al. 2001).

α-(1,3)-glucan is also important in the organisation of the cell wall of yeast and moulds human pathogens (*Cryptococcus neoformans* and *A. fumigatus*), as well as in *S. pombe,* but is absent from budding yeasts like *S. cerevisiae* or *C. albicans*. The fraction of this polymer is variable, ranging from 28 to 46% (reviewed in Yoshimi et al. 2017). α-(1,3)-glucan is crucial for the virulence of several fungal pathogens. Thus, it has been shown that the absence of α-(1,3)-glucan results in the loss of the surface capsule and prevents fungal pathogenesis in *C. neoformans* (Reese et al. 2007), and that deletion of the three α-(1,3)-glucan synthase genes in *A. fumigatus* produces extensive structural modifications of the conidial cell wall and decreased virulence (Beauvais et al. 2013).

1.2 Chitin

Chitin is an *N*-acetylglucosamine (GlcNAc) polymer in which the monomers are linked by β-(1,4) bonds. The microfibrils are stabilised through hydrogen bonds, resulting in a huge tensile strength (Gow et al. 2017). Chitin is present in the cell wall of all fungal species except *S. pombe*, although the proportion varies greatly. In budding yeast, it only represents around 1–3% of the cell wall, whereas it is around 10–20% in *A. fumigatus* and *Neurospora crassa* (Kapteyn et al. 1997; Bowman and Free 2006; Lesage and Bussey 2006).

Even though chitin is a minor component of the cell wall in budding yeast, it is essential for cell survival since it plays an important role in cell division (reviewed in Cabib and Arroyo 2013). A fraction of the chitin is attached to β-(1,3)-glucan, and another fraction is free (Kollar et al. 1997). In yeasts, chitin is attached to β-(1,6)-glucan through a β-(1,3)-linked glucose branch and to the NRE terminal glucose of β-(1,3)-glucan. A detailed analysis of the binding status of chitin in budding yeasts has shown differences depending on the cell wall position (Cabib and Durán 2005). Thus, most of the bound chitin at the bud neck is linked to β-(1,3)-glucan, in lateral walls the attachment to β-(1,6)-glucan predominates, and most of the chitin in the primary septum is free.

2 Fungal Glucanases

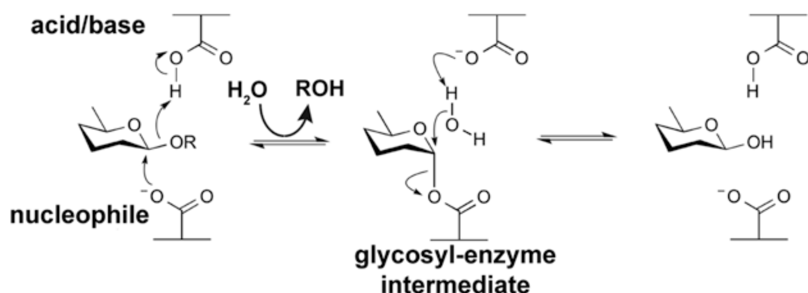
2.1 General Features of Glucan-Hydrolysing Enzymes

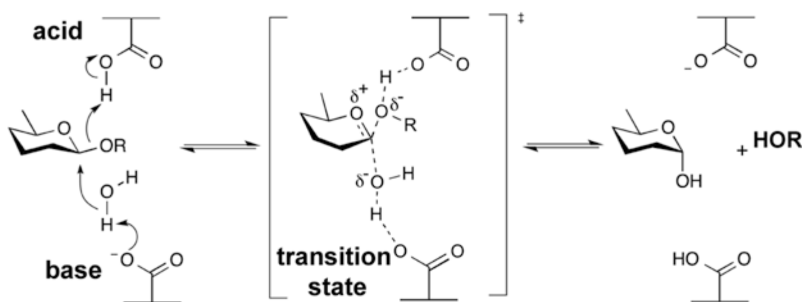
Glucan-hydrolysing enzymes, also known as glycoside hydrolases (GH) or glycosidases, are enzymes that catalyse the hydrolytic cleavage of glycosidic bonds, leading to the formation of a sugar hemiacetal. Glucan-hydrolysing enzymes are present in many different organisms, including fungi, bacteria, archaea, algae, molluscs and higher plants (reviewed in Pitson et al. 1993; Martin et al. 2007). These enzymes catalyse the hydrolysis of glycosidic bonds via general acid catalysis, and they require a proton donor and nucleophile/base for activity, which are provided by two amino acids, typically glutamate or aspartate. Two reaction mechanisms are found for these enzymes, leading to the retention or inversion of the anomeric configuration (Davies and Henrissat 1995) (Fig. 1a). In both mechanisms, the position of the proton donor is identical, but the nucleophilic base is in close proximity to the sugar anomeric carbon in retaining enzymes, while it is more distant in inverting enzymes which must accommodate a water molecule between the base and the sugar (McCarter and Withers 1994).

Hydrolysis with the retention of configuration is normally achieved via two steps, double-displacement mechanism involving a covalent glycosyl enzyme intermediate. In the first step, the nucleophile attacks the anomeric centre to displace the aglycone and forms a glycosyl enzyme intermediate. In the second step, the glycosyl enzyme is hydrolysed by water. Hydrolysis with the inversion of the anomeric configuration occurs in one step by a single-displacement mechanism involving oxocarbenium ion-like transition states.

2.1.1 Modes of Action of Fungal Glucanases

GHs can be classified in many different ways. Glucanases have been systematically classified by the type of glycosidic linkages that they hydrolyse and their action pattern. Two major groups are recognised: exo- and endo-hydrolases. The terms *exo*- and *endo*- refer to the ability of a GH to cleave the substrate at the end (usually the NRE) or within the middle of a chain (Fig. 1b) (Davies and Henrissat 1995). Exo-hydrolases cleave glucose residues from the NRE, generating glucose as the only product, while endo-hydrolases cleave internal linkages at apparently random sites, releasing smaller oligosaccharides as products. In the context of the cell wall, some fungal glucanases can also function as glucosyltransferases (Fontaine et al. 1997a; Mouyna et al. 1998). Glucanases may cleave a single glucan chain several times before being freed from their substrate for further hydrolysis or can function by a multi-chain single attack strategy, where another glucan chain is attacked soon after the initial glucan chain is cleaved, as has been described for exo-β-(1,3)-glucanase in *A. fumigatus* (Fontaine et al. 1997a).





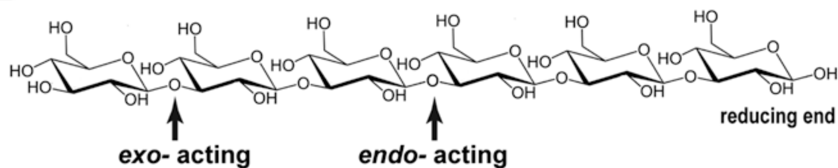


Fig. 1 Mechanism of action of glucanases. **a** Glycoside hydrolases catalyse the hydrolysis of glycosidic bonds via general acid catalysis. Two reaction mechanisms are most commonly found for these enzymes, leading to the retention or the inversion of the anomeric configuration. Hydrolysis with retention of configuration is achieved via two steps, double-displacement mechanism involving a covalent glycosyl enzyme intermediate. Hydrolysis with inversion of the anomeric configuration occurs in one step by a single-displacement mechanism. **b** Exo-hydrolases hydrolyse the glucan chain by cleaving glucose residues from the non-reducing end, generating glucose as the only hydrolysis product. Endo-hydrolases cleave internal linkages along the polysaccharide chain, releasing smaller oligosaccharides as hydrolysis products

Another classification of GHs is based on EC codes assigned by the Enzyme Commission number. This is a numerical classification of enzymes based solely on the chemical reactions that they catalyse. All of the enzymes that catalyse the same reaction receive the same EC number, and when an enzyme can catalyse different reactions, it can bear more than one EC number. For instance, glycoside hydrolases belong to the EC 3.2.1-class, and this includes enzymes hydrolysing *O*- and *S*-glucosyl compounds.

2.1.2 Sequence-Based Classification of Fungal Glucanases: CAZy Database

A classification of glycoside hydrolases based on sequence and structural data was developed several years ago by Henrissat (1991), which uses algorithmic methods to assign sequences to various families. GHs have been classified into more than 160 families (Bourne and Henrissat 2001), with a classification that is permanently updated and available through the carbohydrate active enzyme (CAZy, http://www.cazy.org/) database (Lombard et al. 2014) and the associated CAZypedia (CAZypedia_Consortium 2018). Each GH family contains proteins that are related by sequence and/or structure, which indicates a similar mechanism of action and a similar geometry around the glycosidic bond (Henrissat et al. 1995). GH families are grouped into larger groups, termed “clans” (Davies and Henrissat 1995; Henrissat and Bairoch 1996). Therefore, this classification reveals the possible phylogenetic relationship between different families based on structural features rather than a common mechanism of action. For example, enzymes with glucan exo-β-(1,3)-glucosidase activity (E.C.3.2.1.58) are found in families 3, 5, 16, 17 and 55, while those with glucan endo-β-(1,3)-glucosidase activity (E.C.3.2.1.39) are present in families 17 and 55, but also in families 16, 64, 81, 128, 152, 157 and 158.

2.2 Function of Fungal α- and β-Glucanases

The fungal cell wall is a complex network in which abundant branching and extensive cross-linking between chitin, glucan and other wall components exist (Cabib et al. 2001; Klis et al. 2002). In spite of its mechanical strength, the cell wall is a highly dynamic structure that is constantly being remodelled during the life cycle of fungi, such as during cell expansion and cell separation in yeasts, and during spore germination, hyphal branching or septum formation in filamentous fungi (reviewed in Adams 2004; Roncero and Sánchez 2010; Mouyna et al. 2013). In addition, environmental signals, such as nutrients or pH, can modulate cell wall composition and architecture, as shown in *C. albicans* (Ene et al. 2015; Sherrington et al. 2017).

Glucan and chitin biosynthetic enzymes generate long, linear chains of β-(1,3)-glucose or β-(1,4)-*N*-acetylglucosamine, respectively. Therefore, branching and cross-linking of the different components and cell wall remodelling during

morphogenetic processes depend on the activity of additional enzymatic activities associated with the fungal cell wall. Many fungal cell wall-associated proteins have been characterised to date, and they have different catalytic activities, such as glucanase or chitinase activity and some of them also function as transglycosidases. These enzymes therefore contribute to the remodelling of cell wall components during growth and morphogenesis, breaking and re-forming bonds within and between polymers.

Yeast and fungi contain a wide range of endo- and exo-glucanases belonging to different CAZy families. In most cases, they are multigene families which code for redundant proteins with similar catalytic activity, making the analysis of the function of each individual gene difficult. In some cases, proteins from the same family have different spatial or temporal regulation, either because they have different localisation signals or because they are differentially expressed, suggesting that the proteins perform their function at specific regions of the cell or at particular moments. Most of the fungal β-glucanases are secreted proteins that contain a signal sequence for secretion and either remain associated with the cell wall or are released to the surrounding medium where they exert their function. However, there are also examples of glucanases that lack a signal sequence and are present in the cytoplasm of cells, either as diffuse cytoplasmic proteins such as the *S. pombe* exo-glucanase SpExg3 or associated with the endocytic machinery, as is the case of the endo-glucanase SpEng2 (Dueñas-Santero et al. 2010; Encinar del Dedo et al. 2014).

This chapter is focused on hydrolase activities that are associated with the cell wall and have a defined role in fungal morphogenesis. In both saprophytic and mycoparasitic fungi, β-glucanases have an important nutritional role, where they are part of the biochemical machinery required for cell wall degradation. For example, mycoparasitic *Trichoderma* species secrete β-(1,3) and β-(1,6)-glucanases that efficiently hydrolyse other fungal cell walls (de la Cruz et al. 1995; Kim et al. 2002). These enzymes might also serve to degrade callose during plant parasitic attack (Moy et al. 2002). These enzymes are not discussed here (see Martin et al. 2007 for additional discussion).

2.2.1 Family GH5 Exo-Glucanases

Family GH5 is one of the largest groups of hydrolases with different substrate specificities such as β-glucosidase (EC 3.2.1.21), glucan β-(1,3)-glucosidase (EC 3.2.1.58), endo β-(1,4)-glucanase/cellulase (EC 3.2.1.4) or endo-β-(1,3)-glucanase/ laminarinase (EC 3.2.1.39). They are widely distributed across Archaea, bacteria and eukaryotes, notably fungi and plants, although there are no known human enzymes, and they have been classified into 51 different subfamilies (Aspeborg et al. 2012). We will focus on proteins belonging to Subfamily GH5_9, which includes a group of cell wall modifying enzymes present in yeast and fungi with glucan (1,3)-β-glucosidase (EC 3.2.1.58), endo-β-(1,6)-glucanase (EC 3.2.1.75) or β-glucosidase (EC 3.2.1.21) activity, and that are thought to function in fungal cell

wall modification. All of them contain a GH5 domain accompanied by different localisation signals: some contain a signal sequence, some contain a GPI-anchor at the C-terminus or a single transmembrane domain (TM) for membrane association and others appear to be intracellular.

Members of this subfamily are present in most yeast and fungi, often as a family of redundant enzymes. *S. cerevisiae* contains three related exo-β-(1,3)-glucanases known as ScExg1, ScExg2 and ScSsg1. According to the InterPro Database (Mitchell et al. 2019), the three contain a GH5 domain after a signal sequence but have different functions in the life cycle. ScExg1 is an extracellular protein while ScExg2 is bound to the membrane via a GPI-anchor (Fig. 2a and Table 1) (Vázquez de Aldana et al. 1991; Larriba et al. 1995). *SSG1* (also known as *SPR1*) codes for a sporulation-specific exo-β-(1,3)-glucanase present in sporulating diploids, and its deletion produces a delay in the formation of mature asci and reduces spore thermoresistance (Muthukumar et al. 1993; San Segundo et al. 1993).

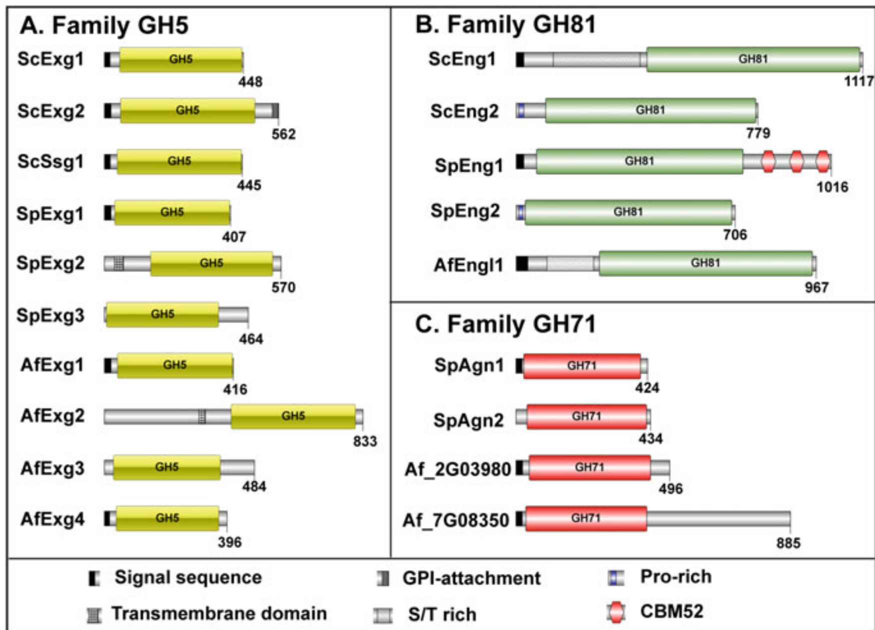


Fig. 2 Domain organisation of GH5, GH81 and GH71 glucanases. **a** Schematic representation of the domain organisation of GH5 hydrolases from *S. cerevisiae, S. pombe* and *A. fumigatus*. Protein domains were identified using the InterPro database (http://www.ebi.ac.uk/interpro/) and are drawn to scale. Proteins in this family have a Glycoside hydrolase family 5 domain (IPR001547) and different localisation signals. Length of the protein is indicated at the C-terminus. **b** GH81 proteins contain a glycosyl hydrolase family 81 domain (IPR040451 and IPR040720), preceded by a signal sequence or a Pro-rich region. In *S. pombe*, SpEng1 has three repeats of the carbohydrate-binding module family CBM52 (IPR018909) at the C-terminus of the protein. **c** α-glucanases contain a Glycoside hydrolase family 71 domain (IPR005197). Only two of the *A. fumigatus* GH71 α-glucanases (AFUA_2G03980 and AFUA_7G08350) are shown

In contrast, no growth phenotype for the single *exg1*Δ and *exg2*Δ mutants has been observed (Vázquez de Aldana et al. 1991; Larriba et al. 1995), and the triple *ssg1*Δ *exg1*Δ *exg2*Δ mutant is similar to the *ssg1*Δ mutant in sporulation (San Segundo et al. 1993).

C. albicans also contains three GH5 proteins, known as CaXog1, CaExg2 and CaSpr1. CaXog1 is similar in organisation to ScExg1, whereas CaExg2 and CaSpr1 contain a putative GPI-attachment site. *xog1*Δ null mutants have no growth or filamentation defects, indicating that the enzyme is not essential in *C. albicans*, although the mutant is slightly more sensitive to glucan synthesis inhibitors and more resistant to chitin biosynthesis inhibitors than the parental strain (González et al. 1997).

S. pombe contains three orthologues that have been characterised (Dueñas-Santero et al. 2010). SpExg1 is a secreted protein; SpExg2 has a TM domain, and it remains associated with the membrane; and SpExg3 is a cytoplasmic protein (Fig. 2a and Table 1). The phenotype of the triple *exg1*Δ *exg2*Δ *exg3*Δ mutant is similar to the parental strain, but overexpression of SpExg2 produces an increase in cell wall material at the poles and septum (Dueñas-Santero et al. 2010).

The *A. fumigatus* genome contains four GH5 proteins (AfExg1 to AfExg4) with different predicted localisations (Mouyna et al. 2013) (Fig. 2a and Table 1) although their function is unknown, since none of the genes has been deleted.

Characterisation of the biochemical activity of the proteins indicated that ScExg1 and CaXog1 are able to degrade β-(1,3)-glucans (Vázquez de Aldana et al. 1991; Chambers et al. 1993). However, *S. cerevisiae exg1*Δ mutants were found to be more sensitive to the K1 killer toxin while *EXG1* overexpression led to resistance (Jiang et al. 1995). Increased resistance to this toxin often correlates with low levels of β-(1,6)-glucan, which is the receptor molecule (Shahinian and Bussey 2000). Furthermore, *EXG1* overexpression also resulted in a modest reduction in the cell wall β-(1,6)-glucan while disruption of the gene led to a small increase in this component. Therefore, it was suggested that ScExg1 may have a functional role in cell wall glucan metabolism and might function in vivo as a β-(1,6)-glucanase (Jiang et al. 1995). Interestingly, recombinant ScExg1 is able to cleave β-(1,3)-linkages as well as β-(1,6)-linkages (Suzuki et al. 2001). The activity of CaXog1 was also characterised and has marked specificity for β-(1,3)-linkages, and it also acts as a transglycosylase in the presence of high concentrations of laminari-oligosaccharides (Stubbs et al. 1999). Analysis of the enzymatic activity of the *S. pombe* proteins shows that SpExg1 and SpExg3 are only active on β-(1,6)-glucans with an endo-hydrolytic mode of action while no enzymatic activity was detected for SpExg2 (Dueñas-Santero et al. 2010). The *exg1*$^{+}$ gene has periodic expression during the cell cycle, with a maximum coincident with the septation process and SpExg1 localises to the septum region, suggesting a possible function in the assembly or remodelling of this structure.

Table 1 Cell wall glucanases and chitinases present in yeast and fungi

Glucanases				
Family	*S. cerevisiae*	*C. albicans*	*S. pombe*	*A. fumigatus*
GH3	–	Bgl22 (CR_09420C_A) Ngs1 (CR_00190W_A)	SPBC1683.04	Exg12 (AFUA_1G05770) Exg13 (AFUA_7G06140) Exg14 (AFUA_7G00240) Exg15 (AFUA_1G17410) Exg16 (AFUA_8G02100) Exg17 (AFUA_6G14490) Exg18 (AFUA_5G07190) Exg19 (AFUA_6G11910) Exg20 (AFUA_6G08700) Exg21 (AFUA_6G03570)
GH5	Exg1 (YLR300w) Exg2 (YDR261c) Ssg1/Spr1 (YOR190w)	Xog1 (C1_02990C_A) Exg2 (C1_02630C_A) Spr1 (C2_06840W_A)	Exg1 (SPBC1105.05) Exg2 (SPAC12B10.11) Exg3 (SPBC2D10.05)	Exg1 (AFUA_1G03600) Exg2 (AFUA_6G09250) Exg3 (AFUA_7G05610) Exg4 (AFUA_2G09350)
GH16	–	–	SPBC21B10.07	Eng2 (AFUA_2G14360) Eng3 (AFUA_1G05290) Eng4 (AFUA_5G02280) Eng5 (AFUA_4G13360) Eng6 (AFUA_6G14540) Eng7 (AFUA_3G03080) Eng8 (AFUA_5G14030)
GH55	–	–	–	Exg5 (AFUA_6G11980) Exg6 (AFUA_6G13270) Exg7 (AFUA_3G07520) Exg8 (AFUA_1G14450) Exg9 (AFUA_2G00430) Exg10 (AFUA_4G03350)
GH71	–	–	Agn1 (SPAC14C4.09) Agn2 (SPBC646.06c)	AFUA_2G03980 AFUA_5G03940 AFUA_7G08510 AFUA_1G03340 AFUA_1G00650 AFUA_8G06030 AFUA_7G08350
GH81	Eng1/Dse4 (YNR067c) Eng2/Acf2 (YLR144c)	Eng1 (C1_03680W_A) Eng2 (C1_03680W_A)	Eng1 (SPAC821.09) Eng2 (SPAC23D3.10c)	Engl1 (AFUA_1G04260)
GH132 G. I	Sun4/Scw3 (YNL2411w) Uth1 (YKR042w) Sim1 (YIL123w) Nca3 (YJL116c)	Sun41 (C6_00820W_A) Sun42 (C1_13940W_A)	Psu1 (SPAC1002.13c) SPBC2G2.17c	Sun1 (AFUA_7G05450)
G. II	YMR244w	C3_04450C_A	Adg3 (SPCC18.01c)	Sun2 (AFUA_1G13940)

Table 1 Cell wall glucanases and chitinases present in yeast and fungi

Chitinases (Family GH18)			
	Class A	Class B	Class C
S. cerevisiae	Cts2 (YDR371 W)	Cts1 (YLR286C)	
C. albicans	Cht4 (C2_02010C_A)	Cht1 (CR_00180C_A) Cht2 (C5_04130C_A) Cht3 (CR_10110W_A)	
U. maydis	Cts1 (UMAG_10419) Cts3 (UMAG_06190)	Cts2 (UMAG_02758)	
N. crassa	Ncgh18-2 (NCU03209) Ncgh18-3 (NCU03026) Ncgh18-4 (NCU04883) Ncgh18-5 (NCU04554) Ncgh18-7 (NCU06020)	Ncgh18-1 (NCU04500) Ncgh18-10 (NCU01393) Ncgh18-11 (NCU12033) Ncchit-1 (NCU02184)	Ncgh18-6 (NCU05317) Ncgh18-8 (NCU07484) Ncgh18-9 (NCU07035)
A. fumigatus	AFUA_1G02800 AFUA_8G01410 AFUA_7G08490 AFUA_3G11280 AFUA_1G00310 AFUA_3G07160	AFUA_5G03960 AFUA_5G06840 AFUA_6G13720 AFUA_5G01400 AFUA_6G09310 AFUA_6G09780	Chi1 (AFUA_5G03760) Chi2 (AFUA_8G00700) Chi3 (AFUA_7G05140) Chi4 (AFUA_5G03530) Chi5 (AFUA_3G07110)

The common name of the protein and the systematic name of the gene are indicated. Group I (G. I) and Group II (G. II) glucanases are indicated in family GH132

2.2.2 Family GH81 Endo-Glucanases

Family GH81 contains proteins with endo-β-(1,3)-glucanase (EC 3.2.1.39) activity. Two orthologues are present in *S. cerevisiae*, *S. pombe* and *C. albicans*, known as Eng1 and Eng2, while a single member is present in *A. fumigatus* (*ENGL1*) (Table 1). AfEngl1 was initially isolated as a glycosylated protein from *A. fumigatus* autolysates (Fontaine et al. 1997a) and represents 10–15% of the β-(1,3)-glucanase activity of *A. fumigatus* cell walls. The active site of the enzyme recognises five glucose units linked by a β-(1,3) bond. The activity of ScEng1, SpEng1 and SpEng2 was also characterised, and they have the same enzymatic activity as that described for AfEngl1 (Baladrón et al. 2002; Martín-Cuadrado et al. 2008b).

GH81 endo-glucanases have different localisations. Eng1 proteins in *S. cerevisiae*, *C. albicans* and *A. fumigatus* have a signal sequence followed by a Ser/Thr-rich region and the C-terminal GH81 domain, suggesting that they are secreted proteins (Fig. 2b). In *S. pombe,* SpEng1 contains a C-terminal carbohydrate-binding module (CBM) consisting of three repeats of 50 amino acids, each belonging to family CBM52, which are essential for catalytic activity against insoluble substrates and for in vivo localisation (Martín-Cuadrado et al. 2003, 2008a). Eng2 proteins lack any secretion signal and only contain the GH81 domain. They are cytoplasmic enzymes containing a Pro-rich region near the N-terminus.

Their function has been analysed in yeast and fungi. Deletion of *ENG1* in *S. cerevisiae* results in the formation of clumps of cells, suggesting that ScEng1, like

the ScCts1 chitinase, is involved in the dissolution of the mother–daughter septum during cell separation (Baladrón et al. 2002); a similar function has been described for CaEng1 (Esteban et al. 2005). CaEng1 partially complements the separation defect of the *S. cerevisiae eng1*Δ mutant, suggesting a close functional relationship between them. ScEng1 asymmetrically localises to the daughter side of the septum, since *ENG1* is one of the daughter-specific genes regulated by the ScAce2 transcription factor (Colman-Lerner et al. 2001; Baladrón et al. 2002).

In *S. pombe*, the absence of SpEng1 produces defects in cell separation, since mutant cells fail to degrade the primary septum (Martín-Cuadrado et al. 2003). The septum in fission yeast is different to that of budding yeast, and the primary septum is mainly composed of linear β-(1,3)-glucan instead of chitin (reviewed in Sipiczki 2007; García-Cortés et al. 2016). Thus, SpEng1 has the same function as chitinase ScCts1 in *S. cerevisiae*, which provides the main catalytic activity required for primary septum dissolution. SpEng1 is secreted to the septum, forming a ring that surrounds it; its localisation requires a functional exocyst and septins, as well as CBM52 (Martín-Cuadrado et al. 2005, 2008a).

The gene encoding a GH81 endo-β-(1,3)-glucanase in *A. fumigatus* was cloned and named *ENGL1* (Mouyna et al. 2002). Initial studies indicated that this endo-β-(1,3)-glucanase has no relevant morphogenetic function, since the Δ*engl1* deletion mutant has no apparent phenotype, consistent with the fact that *A. fumigatus* is a filamentous fungus that does not require cell separation. However, recent studies have shown that AfEngl1 is expressed in resting conidia and during germination, and that AfEngl1, together with four other endo-β-(1,3)-glucanases from Family GH16, contributes separation of the conidia (Mouyna et al. 2016) (see 2.2.7. Family GH16 glucanases).

The function of the cytoplasmic Eng2 has been characterised in *S. pombe.* SpEng2 has also endo-β-(1,3)-glucanase (Martín-Cuadrado et al. 2008b) and performs its function after sporulation, being required for degradation of the ascus wall and for spore release (Encinar del Dedo et al. 2009). Later, it was shown that SpEng2 plays an additional function during vegetative growth, which is unrelated to its enzymatic activity, suggesting that it is a new "moonlighting protein" (reviewed in Gancedo and Flores 2008; Royle 2011). Depletion of SpEng2 causes profound defects in endocytic uptake, which are not due to the absence of the glucanase activity but require the N-terminal Pro-rich region (Encinar del Dedo et al. 2014). It was proposed that SpEng2 is a component of a novel endocytic module, which probably couples the endocytic coat to the actin module during endocytosis. Interestingly, its orthologue in *S. cerevisiae* might have a similar function, since it is also known as ScAcf2 (Assembly Complimenting Factor 2), and it was originally identified as a factor required for the polymerisation of cortical actin patches in semi-permeabilised cells (Lechler and Li 1997).

2.2.3 Family GH71 α-Glucanases

α-(1,3)-glucanases (mutanases, EC3.2.1.59) hydrolyse α-(1,3)-glucan and these enzymes are grouped into two GH families, GH71 glucanases present in fungi and

GH87 enzymes from bacteria (for a review, see Suyotha et al. 2016). Blast searches and phylogenetic analyses showed a high number of proteins containing GH71 domains in Basidiomycota and Pezizomycotina, but not in Saccharomycotina (Sipiczki et al. 2014). The lack of GH71 proteins in Saccharomycotina species, such as *S. cerevisiae* or *C. albicans,* is associated with the lack of α-(1,3)-glucan in the cell walls of these yeasts.

Fungal α-(1,3)-glucanases are divided into two subgroups based on the physiological roles. The first group is α-(1,3)-glucanases from *Trichoderma,* a mycoparasitic fungi that secrete many cell wall degrading enzymes with a nutritional role, hydrolysing extracellular carbohydrates for assimilation as carbon sources and will not be discussed further here. The typical example of the second group are the SpAgn1 and SpAgn2 α-(1,3)-glucanases from *S. pombe* (Fig. 2c and Table 1). SpAgn1 is only active against (1,3)-α-glucan, and the main reaction products are oligosaccharides with a degree of polymerisation of 2–7, and the same substrate specificity and mechanism of action was described for SpAgn2 (Dekker et al. 2004, 2007), indicating that both enzymes are α-(1,3)-glucanases with an endo-hydrolytic mode of action.

SpAgn1 and SpAgn2 are paralogues involved in different cellular processes. The deletion of $agn1^{+}$ results in the formation of clumps of cells that remain attached by cell wall material, an indication that it is involved in the hydrolysis of the cell wall α-(1,3)-glucan that surrounds the septum (the septum edging) (Dekker et al. 2004). SpAgn1 contains a signal sequence (Fig. 2c), and its correct localisation to the septum region requires the exocyst and septins (Martín-Cuadrado et al. 2005). Therefore, the complementary action of the β-glucanase SpEng1 and the α-glucanase SpAgn1 is necessary for the efficient degradation of the primary septum and the α- and β-glucans of the septum edging, allowing the two daughter cells to become two independent entities (Martín-Cuadrado et al. 2003; Dekker et al. 2004; García et al. 2005).

SpAgn2 is required for the endolysis of ascus cell wall, since the *agn2*Δ mutant has a defect in the release of the ascospores (Dekker et al. 2007). Therefore, the SpEng2 β-glucanase and the SpAgn2 α-glucanase form another pair of complementary enzymes with similar functions during sporulation. The fact that the deletion of either of these enzymes results in the same phenotype indicates that α-(1,3)-glucan and β-(1,3)-glucan from the diploid cell wall must be hydrolysed for ascospores to be efficiently released by the concerted action of SpAgn2 and SpEng2.

Paracoccidioides brasiliensis is a human systemic pathogen with clinical relevance in the Latin America area. The outermost layer of the cell wall is composed of α-(1,3)-glucan, and it is believed that it plays a protective role against host defence mechanisms (San-Blas et al. 1977). A single gene belonging to GH71 family has been identified and termed *AGN1*. Its transcription is sharply increased under growth conditions that induce α-(1,3)-glucan synthesis, suggesting a role in α-(1,3)-glucan remodelling (Villalobos-Duno et al. 2013). Interestingly, expression of *AGN1* in *S. pombe* was able to complement the separation defect of the *agn1*Δ mutant, indicating that it might have a similar function and be involved in the remodelling of the *P. brasiliensis* cell wall.

In *A. nidulans,* the α-(1,3)-glucanase *mutA* (AN7349) was identified as a gene that is differentially expressed during sexual development (Wei et al. 2001). In this organism, α-(1,3)-glucan is considered the main reserve material accumulated during vegetative growth and consumed during sexual development. However, the Δ*mutA* strain shows reduced degradation of α-(1,3)-glucan but it is able to form cleistothecia, probably because of genetic redundancy within this family of proteins. A similar function has been proposed for the *A. niger agnB* gene (van Munster et al. 2015). In *A. fumigatus*, seven proteins belonging to family GH71 are present (Fig. 2c and Table 1). All of them contain a signal sequence for secretion, but their function is currently unknown.

2.2.4 Family GH132 Exo-Glucanases

Family GH132 contains a group of fungal proteins that were initially annotated as β-glucosidases based on their sequence similarity to the BglA β-glucosidase of *Candida wickerhamii* (Skory and Freer 1995). Their biochemical activity was completely unknown until AfSun1 and CaSun41 were purified and characterised (Gastebois et al. 2013). Both are active against β-(1,3)-glucans but not against p-nitrophenyl-glucose (pNPG), confirming that they are not β-glucosidases. AfSun1 and CaSun41 act as exo-β-(1,3)-glucanases that degrade substrates from laminaribiose to insoluble β-(1,3)-glucans. Interestingly, a minor transferase activity was also detected. Based on these results, this group of proteins, which are also known as SUN proteins, were included in family GH132 in the CAZy database.

The SUN family was originally identified in *S. cerevisiae,* and it is composed of four paralogous genes: *SIM1*, *UTH1*, *NCA3* and *SUN4.* The proteins share a common structure that includes a signal peptide, a low complexity region rich in serine and threonine, and a conserved SUN domain characterised by a C-X5-C-X3-C-X24-C motif predicted to bind iron based on similarities to Fe-S proteins, although there are no experimental evidences indicating that iron is required for enzymatic activity (Fig. 3a) (Bandara et al. 1998). Phylogenetic analysis showed that they can be classified into two groups. Proteins from Group I have the described structure, whereas members of Group II have a shorter N-terminal region and different spacing between the third and fourth cysteines of the SUN domain (Firon et al. 2007).

SUN proteins were first described to be involved in different cellular processes in *S. cerevisiae*, such as yeast-ageing, resistance to various stresses, prolonged replicative lifespan or mitochondrial function (Pelissier et al. 1995; Bandara et al. 1998; Camougrand et al. 2004). Indeed, some proteins have a dual localisation in the cell wall and in the mitochondrial membrane (Velours et al. 2002). *S. cerevisiae* contains four GH132 proteins from Group I encoded by *SUN4/SCW3, UTH1, SIM1* and *NCA3* and a Group II protein (Ymr244w) (Table 1 and Fig. 3a). ScSun4 (ScScw3) was initially identified as a cell wall protein released from intact cells by dithiothreitol (Cappellaro et al. 1998), and it was shown to function during cell separation since the *sun4*Δ strain often possesses more than one bud, and daughter

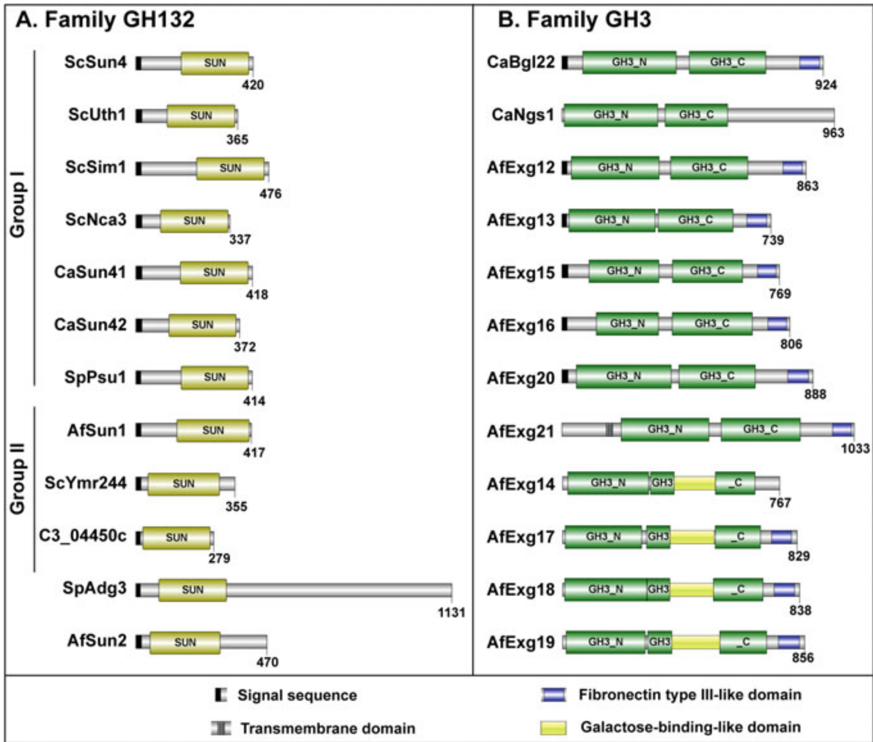


Fig. 3 Domain organisation of GH132 and GH3 glucanases. **a** GH132 glucanases or SUN proteins have been classified into two groups. Proteins from group I have a common structure that include a signal secretion peptide, a low complexity region rich in serine and threonine residues and a conserved C-terminal SUN domain (IPR005556) containing a C-X5-C-X3-C-X24-C motif. Members of group II have a shorter N-terminal region and a different spacing between the third and fourth cysteines of the SUN domain. **b** Schematic representation of the domain organisation of GH3 glucanases from *C. albicans* (CaBgl22 and CaNgs1) and *A. fumigatus* (AfExg12 to AfExg21). The GH3 is composed of two independent signatures in the InterPro database, the Glycoside hydrolase family 3, N-terminal domain (IPR001764) and the Glycoside hydrolase family 3, C-terminal domain (IPR002772). Most of the proteins contain a Fibronectin type III-like domain (IPR026891) near the C-terminus. The four *A. fumigatus* proteins that lack a secretion signal (AfExg14, AfExg17, AfExg18 and AfExg19) contain an insertion in the C-terminal GH3 domain that corresponds to a galactose-binding-like domain(IPR040502)

cells remain attached to the mother cell (Mouassite et al. 2000). More recently, ScSun4 was shown to localise to the daughter side of the bud neck together with ScDse2 and ScEgt2 and was involved in the separation of the daughter from the mother cell (Kuznetsov et al. 2016). ScUth1 may also affect the function of the cell wall, since the β-glucan and chitin composition of the *uth1*Δ mutant is different from that of the wild-type, and it is more resistant to zymolyase treatment (Ritch et al. 2010). Little is known about the function of the other three members of SUN family.

In *C. albicans*, there are three SUN proteins known as CaSun41, CaSun42 and C3_04450C_A (Table 1 and Fig. 3a). Deletion of *CaSUN41* also results in a separation defect, whereas the double deletion of *CaSUN41* and *CaSUN42* is synthetically lethal due to the lysis of mother cells after septation, and this phenotype is rescued by osmotic protection (Firon et al. 2007). Cell wall defects seen in this double mutant are mainly localised in the region surrounding the septa in mother yeast cells and subapical hyphal compartments. CaSun41 is also important for cell attachment to a substrate and for biofilm formation. The *sun41*Δ strain forms aberrant hyphae and has decreased virulence (Hiller et al. 2007; Norice et al. 2007). All of these observations led to the proposal that *C. albicans* SUN proteins could be involved in cell wall remodelling linked to the maintenance of cell integrity during cell division (Firon et al. 2007).

In *S. pombe*, there are three proteins containing a SUN domain, SpPsu1, SpAdg3 and SPBC2G2.17c. Deletion of the Group I gene *psu1*$^+$ results in cell wall defects during cell separation, generating swollen cells that eventually undergo lysis (Omi et al. 1999). SpAdg3 is one of the genes whose transcription is regulated by SpAce2, and the protein is required for efficient cell separation (Alonso-Nuñez et al. 2005).

A. fumigatus contains two *SUN* proteins, the Group I AfSun1 and Group II AfSun2 (Gastebois et al. 2013). The expression of *AfSUN1* is strongly induced during conidial swelling and mycelial growth, whereas *AfSUN2* is not expressed. Accordingly, the deletion of *AfSUN1* produces defects during hyphal growth and conidiation. Hyphal cells lacking AfSun1 have swollen hyphae, leaky tips and a double cell wall, indicating that this protein is also involved in cell wall biogenesis in *A. fumigatus*, like in yeast. Furthermore, the Δ*Afsun1* had intrahyphal hyphae and many Woronin bodies in the septal region, an indication of difficulties in the completion/closure of the septum that suggests a role of *Af*Sun1 during septation. In contrast, the deletion of *AfSUN2* had no apparent phenotype. In spite of the initial reports on the role of SUN proteins in various cellular process not cell wall associated, there is increasing evidence suggesting that SUN proteins play indeed a role in cell wall biogenesis, septum integrity and cell separation in yeast and fungi (Mouassite et al. 2000; Ritch et al. 2010; Gastebois et al. 2013).

2.2.5 Family GH3 Exo-Glucanases

Family GH3 contains exo-hydrolases that act on different substrates and display a variety enzymatic activities, such as β-glucosidase (EC 3.2.1.21), glucan β-(1,3)-glucosidase (EC 3.2.1.58), glucan β-(1,4)-glucosidase (EC 3.2.1.74) or exo-β-(1,3)-(1,4)-glucanase (EC 3.2.1.-) among others. In filamentous fungi, there is a large degree of redundancy and several proteins are present in the genome (from 5 to 11). In contrast, *C. albicans* contains two GH3 proteins (CaBgl22 and CaNgs1), *S. pombe* only one (SPBC1683.04) and no orthologues are present in *S. cerevisiae* (Fig. 3b and Table 1).

There is little information about the function of these proteins in fungal cells. The only protein whose enzymatic activity has been characterised is the

A. fumigatus glucanase known as ExoGII (encoded by *EXG12*). ExoGII was isolated from cell wall autolysates and characterised as a protein with activity against β-(1,3)-glucan, β-(1,6)-glucan and also acting on pNPG (Fontaine et al. 1997b). In addition to ExoGII, *A. fumigatus* contains another nine proteins from this family, named AfExg13 to AfExg21 (Table 1) (Mouyna et al. 2002). All of them contain the N-terminal and C-terminal glycoside hydrolase family 3 signatures (Fig. 3b), but a phylogenetic tree constructed with GH3 fungal proteins showed the existence of two main groups (Mouyna et al. 2013). The first group is composed of the five proteins that contain a signal sequence (AfExg12, AfExg13, AfExg15, AfExg16 and AfExg20) and AfExg21, which contains a putative TM domain. The second phylogenetic group contains the remaining proteins that lack any secretion signal or TM domains, and the C-terminal GH3 signature is separated into two fragments by a galactose-binding-like domain (Fig. 3b). In addition, all of them, except AfEgx14, contain an additional Fibronectin type III-like domain (FnIII) at the C-terminus. Fibronectin (FN) is a protein able to bind simultaneously to cell surface receptors, collagen, proteoglycans and other FN molecules (Maurer et al. 2015), although the function of the Fibronectin-like domain in GH3 proteins is unknown.

C. albicans contains two GH3 proteins, known as CaBgl22 and CaNgs1 (Fig. 3b). Deletion of *BGL22* is viable, and its function is not known. CaNgs1 has a modular structure slightly different, since it lacks a secretion signal and only contains the N-terminal domain of GH3 proteins. Recent work might shed light on the function of cytoplasmic GH3 proteins. CaNgs1 has been identified as a GlcNAc sensor and transducer important for GlcNAc-induced hyphal development (Su et al. 2016). The N-terminal GH3 domain acts as an *N*-acetyl glucosaminidase that can bind GlcNAc, and this binding activates its C-terminal domain necessary for down-regulation of the Nrg1 repressor.

The *S. pombe* SPBC1683.04 protein also contains the GH3 domain and the C-terminal FnIII domain but lacks a secretion signal. Little is known about its function, although the mutant has been identified in a large-scale screen searching for morphological abnormalities during sexual reproduction (Dudin et al. 2017). The null mutant forms a mating projection with a longer tip than normal and shows incomplete cell wall disassembly at the site of cell–cell fusion, suggesting that it might function in cell wall reorganisation during conjugation.

2.2.6 Family GH55 Exo-Glucanases

Family GH55 contains proteins with exo-β-(1,3)-glucanase (EC 3.2.1.58) and endo-β-(1,3)-glucanase (EC 3.2.1.39) activity present in bacteria and filamentous fungi, but are absent in the genome of *S. cerevisiae*, *C. albicans* or *S. pombe*. *A. fumigatus* contains six GH55 proteins that have been named AfExg5 to AfExg10 (Table 1) (Mouyna et al. 2013).

Similar to other GH families, the *A. fumigatus* proteins have different localisation signals (Fig. 4a). All of them contain two repeats of a domain similar to the pectate lyase 3 domain, although this is only inferred by sequence similarity and

might not reflect a similar function. Indeed, the only enzymatic activity characterised in *A. fumigatus* corresponds to AfExg6, and it has been shown that it is the exo-β-(1,3)-glucanase isolated several years ago from cell wall autolysates and named ExoGI (Fontaine et al. 1997b). This enzyme exclusively hydrolyses β-(1,3)-glucans and has a minimal substrate size of four glucose residues. Recently, it has been shown that GH55 family proteins are essential for the proper organisation and structuring of the conidial cell wall, and conidia maturation, normal dissemination and germination (Millet et al. 2019). A sextuple mutant lacking *AfEXG5* to *AfEXG10* has defects in conidial cell wall maturation that result in abnormal shape, becoming ovoid and unable to separate.

Interestingly, the function of a GH55 glucanase in *N. crassa* has been described and it is similar to that mentioned for *A. fumigatus*. A proteomic analysis of the conidial cell wall identified the *CGL-1* β-(1,3)-glucanase (NCU07523) and the *NAG-1* exo-chitinase (NCU10852) as components of cell wall (Ao et al. 2016). Analysis of the phenotype of the deletion mutants of both genes showed that the conidia failed to properly separate. These results suggest that the exo-chitinase and the glucanase are required for the remodelling of the conidial cell wall, which is necessary for the conidia to separate from one another and facilitate dispersal.

More recently, the activity of *H. capsulatum* Exg8 was characterised, and it acts as an exo-glucanase that produces mono-, di- and trisaccharides from laminarin, suggesting a minimal substrate size similar to that of AfExg6 (Garfoot et al. 2017).

2.2.7 Family GH16 Endo-Glucanases

Family GH16 is a large family in the CAZy database that contains proteins active on β-(1,4) or β-(1,3) glycosidic bonds in various glucans and galactans. A wide variety of enzymatic activities are included in this family, such as endo-β-(1,3)-glucanase (EC 3.2.1.39), endo-β(1,3) (4)-glucanase (EC 3.2.1.6), xyloglucan xyloglucosyltransferase (EC 2.4.1.207), keratan-sulphate endo-β(1,4)-galactosidase (EC 3.2.1.103), licheninase (EC 3.2.1.73), β-agarase (EC 3.2.1.81) or xyloglucanase (EC 3.2.1.151).

Even though GH16 proteins have diverged significantly in their primary sequences, they all feature a common fold (a classical sandwich-like β-jelly roll) and a common catalytic motif, justifying their inclusion in the same CAZy family. However, phylogenetic analysis indicates that this family could be divided into different subfamilies (Mertz et al. 2009). In the Conserved Domain Database (CDD, https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) (Marchler-Bauer et al. 2017), it contains six different subfamilies: *GH16_lichenases* (cd02175), *GH16_kappa_carrageenases* (cd02177), *GH16_XETs* (Xyloglucan endotransglycosylase, cd02176), *GH16_β_agarases* (cd02178), *GH16_fungal_CRH1_transglycosilase* (cd02183) and *GH16_laminarinase_like* (cd08023).

Fungal endo-β-(1,3)-glucanases are included in subfamily *GH16_laminarinase_like*, and are mainly present in filamentous fungi, with numbers ranging from one in *Magnaporthe grisea* to eight in *A. fumigatus*. *S. pombe* is the only yeast that possesses a GH16 glucanase (SPBC21B10.07), since they are absent in the genome of *S. cerevisiae* or *C. albicans*. The seven GH16 glucanases in *A. fumigatus* have been named AfEng2 to AfEng8 (Mouyna et al. 2013) (Table 1, Fig. 4b). All of them seem to be extracellular proteins, since they contain either a signal peptide or a TM domain in the middle of the protein, suggesting that they localise to the plasma membrane with the active domain pointing towards the cell wall.

The biochemical activity of AfEng2 was characterised by its expression in *Pichia pastoris* (Hartl et al. 2011). The recombinant protein acts on β-(1,3)-glucans and lichenan but not on β-(1,6) substrates. AfEng2 preferentially acts on soluble polymers like laminarin and shorter β-(1,3) oligosaccharides and showed a slight transferase activity. Deletion of the *ENG2* gene showed that the mutant has no phenotype, and it is similar to the parental strain, suggesting that AfEng2 alone does not play an essential role in fungal morphogenesis (Hartl et al. 2011). However, a deletion of four of the GH16 genes (*ENG2* to *ENG5*) in combination with a deletion of the endo-β-(1,3)-glucanase Engl1 (Family GH81) resulted in a defect in conidial

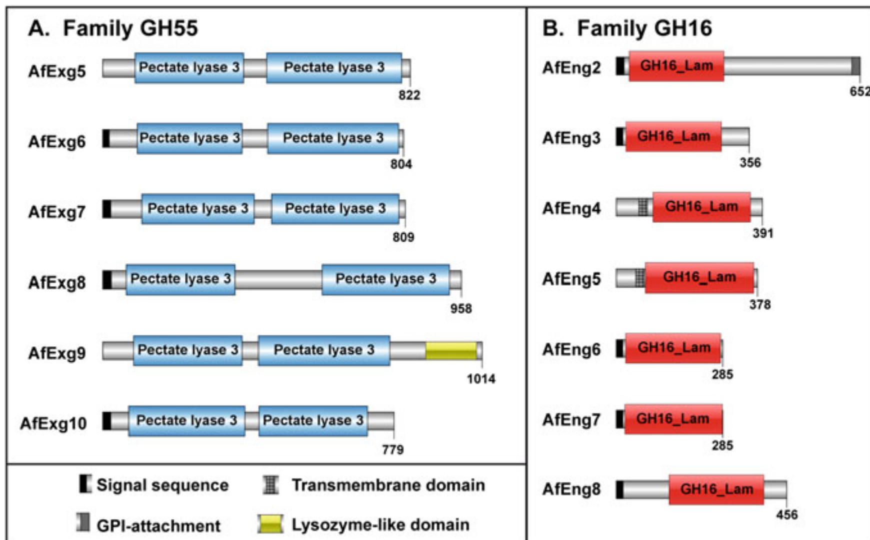


Fig. 4 Domain organisation of GH55 and GH16 glucanases. **a** *A. fumigatus* exo-β-(1,3)-glucanases belonging to family GH55 share a common structural organisation, containing two repeats of the pectate lyase superfamily domain (IPR024535). Four of them contain a signal sequence, whereas AfExg5 and AfExg9 lack this localisation signal. AfExg9 is the largest of all of them and contains a C-terminal Lysozyme-like domain (IPR023346). **b** Schematic representation of the domain organisation of GH16 endo-β-(1,3)-glucanases from *A. fumigatus* (AfEng2 to AfEng8). The proteins contain a Glycoside hydrolase family 16 domain (IPR000757) accompanied by different localisation signals

morphogenesis (Mouyna et al. 2016). The quintuple mutant Δ*eng1,2,3,4,5* formed linear chains of conidia that were unable to separate, but the germination rate was not affected. Thus, endo β-(1,3)-glucanases from families GH16 and GH81 are essential for proper conidial cell wall assembly and for conidia separation after conidiation is complete in a filamentous fungus.

3 Fungal Chitinases

3.1 Organising the Action: From Chitin to N-*Acetylglucosamine*

The breakdown of chitin in the fungal cell wall is promoted by a number of chitinolytic enzymes including lytic polysaccharide monooxygenases (LPMOs), chitinases and *N*-acetylglucosaminidases (NAGAs), all of them working in a coordinated manner for efficient degradation (Langner and Göhre 2016). These enzymes will act co-ordinately on chitin following the simple scheme depicted in Fig. 5. LPMOs and endo-acting chitinases produce strand breaks at random positions within the chitin chains, this cleavage generating free reducing and

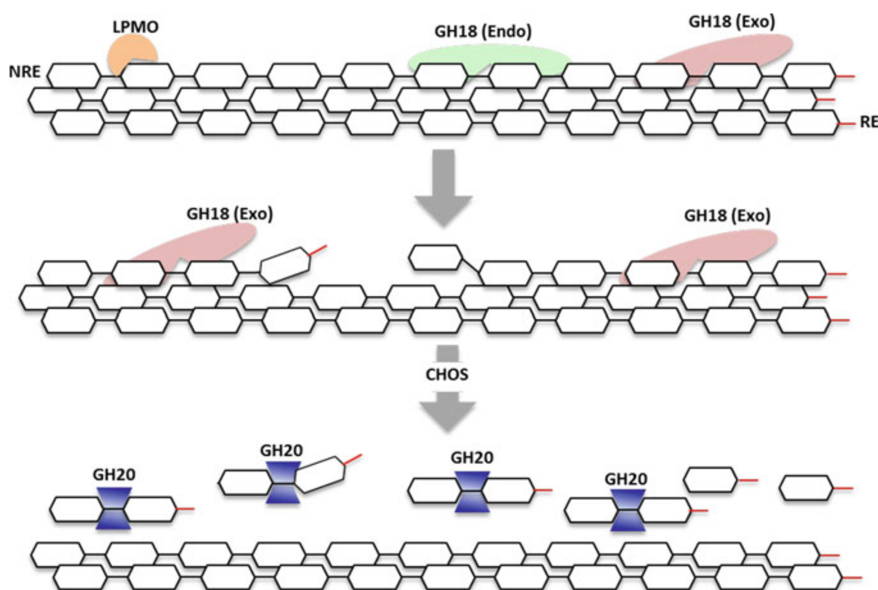


Fig. 5 Hierarchical machinery involved in chitin degradation. Chitin chains are fragmented by LPMOs and endo-chitinases to serve as substrates for the action of exo-chitinases that released Chito-oligosaccharides, which are further processed to *N*-Acetyl glucosamine monomers by the action of *N*-Acetylglucosaminidases belonging to family GH20

non-reducing ends. The free ends will serve as targets for processive exo-acting chitinases, which release short chito-oligosaccharides (CHOS), mainly $(GlcNAc)_2$ and $(GlcNAc)_3$, from either the reducing or non-reducing end, leading to the complete decomposition of the strand (Horn et al. 2006). CHOS, mainly $(GlcNAc)_2$, are the preferred substrates for *N*-acetylglucosaminidases, which catalyse the further degradation to GlcNAc from NRE (Chen et al. 2015).

These three types of enzymes belong to different CAZy groups. LPMO enzymes have only been recently described and are classified as auxiliary enzymes belonging to A9-15 classes (Levasseur et al. 2013), and they have been found in most fungal groups (CAZy, http://www.cazy.org/). Their mechanism of action is still not fully understood, but accumulating evidence shows that it involves binding of a copper ion that will enable the transport of an electron to molecular oxygen, thereby creating a superoxo-intermediate which then initiates the cleavage of the polymer (reviewed in Courtade and Aachmann 2019). These types of enzymes could have an important biotechnological interest, but their role in fungal biology is unknown; therefore, they are out of the scope of this review.

Chitinases, in contrast, have been studied for years, and their mechanism of action is well known. All fungal chitinases belong to CAZy family GH18 and use a substrate-assisted retaining mechanism (van Aalten et al. 2001) in which a conserved glutamate of the catalytic diad (D–E) serves as the general acid/base. During hydrolysis, the anomeric configuration of the substrate is retained. Their processivity separates them in endo-acting and processive (exo-acting) chitinases, a characteristic that directly depends on the tridimensional architecture of the binding cleft of the enzyme, which is organised in a rather open conformation in the non-processive, endo-acting chitinases clearly different from the "tunnel-shaped" conformation attained by the processive enzymes.

N-acetylglucosaminidases belong to family GH20, and their mode of action is similar to that of GH18 proteins previously described (Chen et al. 2015).

3.2 Structure and Diversity Between Fungal Chitinases

The chitinases, although they share a unique catalytic domain, are a rather complex group of enzymes that include proteins with a very different organisation. Some proteins are secreted by means of a signal peptide while others lack a canonical sequence and are secreted by alternative and poorly studied secretion mechanisms. Some of them are expected to be integral membrane proteins due to the presence in their sequence of TM domains. Most of them are glycosylated, and some are also modified by the addition of GPI modules. Moreover, some chitinases contain different CBMs that can be arranged in multiple copies (Tzelepis et al. 2012). On top of that, the number of chitinase genes presented in each fungal family differs radically, from a single gene in *S. pombe* to more than 30 genes in different phytopathogenic species of the *Trichoderma* genus (Karlsson and Stenlid 2008).

Based on this complexity, the classification of chitinases is difficult and the most comprehensive classification is based exclusively on their GH18 catalytic domains, which are relatively well conserved. Based on this, they are classified into two big clusters (reviewed in Karlsson and Stenlid 2008). Cluster A includes the enzymes classified as exo-chitinases and can be subdivided into 6 groups, A-II to A-V and C-I and C-II. Groups A-II to A-V showed the highest degree of amino acid conservation, whereas C-I and C-II displayed the lowest levels of amino acid conservation. The catalytic domain of the cluster B showed intermediate levels of conservation, although the catalytic domain of sub-classes B-V can be considered as one of the most conserved. All of the enzymes belonging to cluster B display endo-chitinase activity and many have been linked to morphogenetic processes (see below). Interestingly, not all chitinase groups are uniformly distributed among fungi and the subgroups A-II, C-I and C-II appear to be unique for filamentous ascomycetes. Moreover, subgroups C-I and C-II have been identified as the most likely targets for the observed expansion of chitinases between filamentous fungi. These C-subgroups of chitinases share extensive homology with the α-toxin of the yeast killer system from *K. lactis* (Butler et al. 1991), which facilitates internalisation of the catalytic component of this toxin, the gamma subunit, by the permeabilisation of the cell wall and membrane of the susceptible strains (Jablonowski et al. 2001). This has led to the suggestion that C-I and C-II chitinases would be involved in competitive fungal–fungal interactions.

The data presented above clearly reflect a very significant expansion in size of the chitinase gene families included in GH18, suggesting that this gene family has been important for species fitness during evolution, probably by facilitating adaptation to specific natural environments. It is difficult to know exactly how many chitinase genes were present in the original fungal ancestor, although it has been estimated that 5 chitinase genes were already present in this extant ancestral species (Karlsson and Stenlid 2008) whose origin can be placed around 800 million years ago, after the divergence of the Chytridiomycetes (Stajich et al. 2009). From this original number, gene expansion took place to produce an average number of 12–15 different genes. Figure 6 shows a schematic representation of the diversity of fungal chitinases using *N. crassa* as a model (Tzelepis et al. 2012). However, other expansion processes should have occurred between the filamentous ascomycetes, mostly through the C-I and C-II subgroups. This expansion has a maximum between mycoparasitic fungi, probably associated with a specific role of these new enzymes in this lifestyle. Interestingly, not only has the expansion phenomena been found, but also significant contractions as well. Between filamentous ascomycetes, the contraction of the subgroup C-II observed in human pathogenic species is noteworthy, as this led to the absence of this type of enzyme in organisms like *Coccidioides immitis* or *H. capsulatum* (Karlsson and Stenlid 2008), a clear indication for the irrelevance of this type of enzyme for the human pathogenic lifestyle. It is also relevant the reduced number of chitinase genes present in fungi living as yeast, independently of their phylogenetic relationship. With the exception of *S. pombe*, having a strong contraction to only a single group B chitinase gene, these yeast-like fungi contain typically between 2 and 3 chitinase genes, belonging

always to clusters A and B. This reduced number of genes has made them very suitable models for the study of the biological function of these enzymes (see below).

3.3 The Biological Roles of Fungal Chitinases

As explained above, the problems with the establishment of a coherent classification of fungal chitinases are translated into the identification of the biological function of these proteins. Gene redundancy has made extremely difficult to create fungal strains in which complete families of chitinases have been eliminated; therefore, most of our knowledge is based on the characterisation of single and double mutants complemented with more general studies of gene expression. In order to approach this chapter, we will refer as study model to the filamentous fungi *N. crassa,* which contains an intermediate number chitinase genes (Tzelepis et al. 2012) and to several yeast-like fungi, for what multiple gene deletion strains have been extensively characterised. In general, chitinases are believed to participate in at least 4 clear biological functions:

1. **Nutrition**. Chitinases are used for fungi to thrive in nature using chitinous substrates. This role is achieved by the coordinated action of LPMOs, chitinases and NAGA enzymes to produce single carbohydrates as carbon source (Langner and Göhre 2016). This function correlates with the fact that the expression of several chitinase genes is strongly regulated by the carbon source and induced upon growth in chitinous-based media. In our referred model, *N. crassa*, the expression of type A *gh18-7* and *gh18-5* and type C *gh18-9* and *gh18-8* chitinases has been shown to be under nutritional control; these are expected to perform nutritional functions, although individual mutants in the *gh18-5* and *gh18-8* genes did not show apparent phenotypes (Tzelepis et al. 2012). Moreover, *N*-acetyl hexosaminidases *Nag1* and *Nag2* are essential for growth in chitin or chitobiose in *T. atroviride* (Brunner et al. 2003), and a clear indication that full degradation of the chitinous material is required to become an effective carbon source.
2. **Cellular Antagonism**. Fungal cells normally compete in nature in specific environments, and cellular antagonism has been described between them. Moreover, some fungi have also been described as mycoparasites. Considering that most fungi contain high levels of chitinous material in their cell wall, it is not surprising that chitinase production would affect interaction between fungal cells. Accordingly, the *N. crassa* exo-chitinases *gh18-7*, *gh18-3*, *gh18-4*, *gh18-6* and *gh18-8* and endo-chitinases *gh18-11* and *chit-1* are induced during competitive interaction with other fungi (Tzelepis et al. 2012). In other mycoparasitic fungi like *T. atroviridae,* the chitinases *ech42* and *ech37* are induced in the presence of fungal cell walls, but the induction of *ech42* also depends on the *N*-acetyl hexosaminidase *Nag1*, linking this induction to the presence of free

GlcNAc and thus linking the *N*-acetyl hexosaminidase activity to mycoparasitism (Brunner et al. 2003). In other fungi, the induction of these types of enzymes depends on the biological origin of the cell walls used as inductor, which in fact indicates a crosstalk between competitor fungi (Tzelepis et al. 2014). The nature of this interaction is not fully understood, but it probably relies in the different nature of the fungal cell walls and in the capacity of the different chitinases for their degradation and for the associated release of molecular inductors. It is generally believed that class C exo-chitinases have an important role in cellular antagonism and mycoparasitism because of their similarity with *K. lactis* toxin, although their expression patterns are rather complex and probably their action involved self and non-self cell wall degradation (Tzelepis et al. 2014).

It should be noticed the close relationship between the cellular antagonism and the cellular nutritional program; therefore, it is not surprising to find that the expression of multiple chitinase enzymes potentially involved in fungal competition is also regulated through carbon catabolite repression.

3. **Autolysis**. Autolysis is the natural process of self-digestion of aged hyphal cultures. It has considerable interest from an application point of view as a way of increasing product yields from fungal cells or as a mechanism of promoting cell death during fungal infections. However, autolysis is also extremely important in nature cycles as a way of releasing nutrients from death cells to provide nutrients for other cells present in the same environment. In this respect, autolysis would have an important nutritional impact affecting fungal growth under nutritional depletion but also for fungal competition. Therefore, this function is intimately linked to previous ones and the chitinase enzymes involved in fungal autolysis are those described in previous epigraphs, whose production is nutritionally regulated.
4. **Morphogenesis**. Fungal cells are able to grow based on a delicate balance between the strength and plasticity of their cell walls; therefore, it has been assumed for many years that a balance between synthesis and degradation of structural polymers as chitin is required for fungal cell expansion (Riquelme et al. 2018). Accordingly, it has been proposed that a discrete action of chitinases at the hyphal tip favours fungal cell expansion and growth; similarly, yeast cells need to separate after cytokinesis; therefore, chitinases are expected to act at the latest step of the yeast cell cycle. While these models are conceptually very sound, only the latter has been experimentally confirmed to show that chitinases are required for cell separation in different cellular systems (Kuranda and Robbins 1991). A direct involvement of chitinases in hyphal growth is still under debate due to the absence of conclusive experimental evidence showing that the absence of chitinases affects hyphal development.

An additional aspect of fungal morphogenesis is fungal sexual and asexual development, where chitinases could exert also defined functions beyond indirect effects through the nutritional program. In yeast, the role of some chitinases during

sporulation has been established, but in filamentous fungi the evidence for a function of some chitinases in spore development is scarce and therefore still under debate.

In conclusion, while some roles of chitinases in fungal morphogenesis are well established, these roles are not general and will require additional evidence. We will discuss the different aspects of these roles in more detail in Sect. 3.4.

An important issue common to the biological functions associated with chitinases is the accessibility of these enzymes to the different substrates, which would dictate whether they could have deleterious or positive effects by participating in self and non-self cell wall degradation (Gruber and Seidl-Seiboth 2012). It is assumed that most chitinases are secreted either by conventional or unconventional routes depending on the presence of signal peptides on their sequence. Secretion to the periplasmic space will facilitate their interaction with its own cell wall and its further participation in fungal cell wall morphogenesis (see below). This interaction would be facilitated by additional modules that retain these enzymes in the periplasmic space, like TM domains or GPI-anchor sequences. Moreover, some chitinases had also CBMs that would favour their interaction with high molecular weight polysaccharides (Fig. 6). However, most chitinases lack these domains and the major pool of these enzymes are located in the culture supernatant, strongly suggesting that they act from the outer side of the cell wall. How can chitinases discriminate the own cell wall from those of potential fungal competitors? Although the discrimination mechanisms are not fully understood, it was suggested that the accessibility of chitin within the fungal cell wall could be a major determinant in these processes (reviewed in Gow et al. 2017). In healthy hyphae, accessibility may be limited by the protection conferred by the outermost face of the cell walls, formed by distinct proteins. This hypothesis is supported by the fact that a chitinolytic mixture from autolytic extracts of *T. atroviride* spores does not inhibit the germination of its own spores but strongly affects spore germination of other fungi like *T. reesei*, *N. crassa* and *A. niger* (Hartl et al. 2012). Accordingly, the induction of chitinase production in fungi by cell wall extracts depends on the biological origin of the cell walls (Tzelepis et al. 2014), which will be necessarily linked to the capacity of its enzymes to degrade the foreign cell wall and produce the appropriated inductor molecules. The increased expression of these enzymes will favour fungal competition, not only by killing competitor cells (cellular antagonism), but also by degrading their cell walls, providing additional nutritional resources (nutrition).

3.4 The Role of Chitinases in Fungal Morphogenesis: From Yeasts to Filamentous Cells

As explained above, the number of chitinase-encoding genes is rather variable due to a strong genetic expansion in some fungi, especially filamentous fungi.

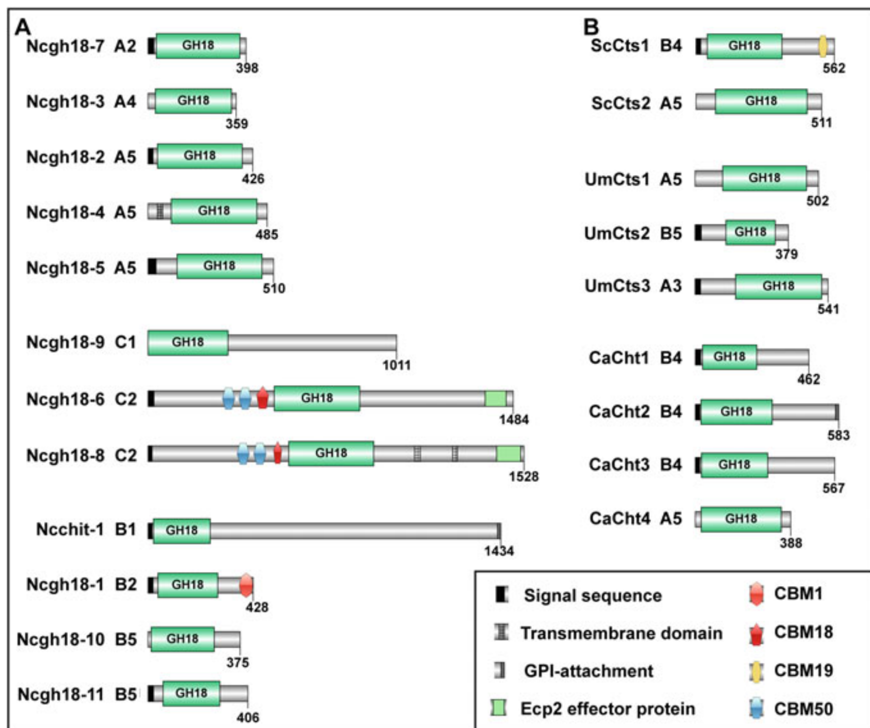


Fig. 6 Structural diversity of GH18 chitinases in *Neurospora crassa* (a) and in several yeast-like fungi (b). The domains present in each protein are indicated as well as the chitinase class to what each protein belong (based on Karlsson and Stenlid 2008). All the proteins contain a glycoside hydrolase family 18 catalytic domain (IPR001223) accompanied by different localisation signals and/or CBM modules: CBM1 (IPR000254), CBM18 (IPR036861), CBM19 (IPR005089) or CBM50 (IPR036779). Ncgh18-6 and Ncgh18-8 also contain an Ecp2 effector protein domain (IPR029226) near the C-terminus

Therefore, the number of multiple mutants characterised is limited, which compromises the interpretation of the physiological data due to the possible redundancy between similar genes. Accordingly, most of our knowledge about the role of chitinases in fungal morphogenesis comes from studies of fungi with a limited repertoire of chitinase genes, mostly yeasts, independent of their capacity to form filaments under some conditions.

The first report linking chitinase activity to morphogenesis was obtained by Cabib's group, showing that *S. cerevisiae* yeast cells were lysed in acidic media in the absence of the chitin synthase *CHS1*, a lysis that was prevented by the chemical inhibition of the chitinase activity with allosamidin (Cabib et al. 1989). Later it was shown that deletion of the *CTS1* gene encoding a class B endo-chitinase activity prevents cell separation (Kuranda and Robbins 1991) and that ScChs1 synthase activity counterbalances ScCts1 chitinase activity during cell separation, acting as a

repair activity (Cabib et al. 1992). In agreement with these results, a deleterious effect of chitinase activity has also been shown in mutants defective in the synthesis of the chitin ring (Gómez et al. 2009). Very similar results have been described for the chitinase encoded by the *CHT3* from *C. albicans,* including a functional heterologous complementation (Dünkler et al. 2005). In *U. maydis,* a double deletion *cts1*Δ *cts2*Δ also produced a cellular separation defect (Langner et al. 2015). Interestingly, *S. cerevisiae* and *U. maydis* contain a unique endo-chitinase-encoding gene, but *C. albicans* contains three *CHT1*, *CHT2* and *CHT3* although only *CHT3* appears to encode a biological functional endo-chitinase. Based on this evidence, it can be concluded that endo-chitinases have an important morphogenetic role in yeasts. Their expression is cell cycle regulated, and it is dependent on the Ace2 transcription factor, which promotes their specific expression in daughter cells (Colman-Lerner et al. 2001; Kelly et al. 2004). This localised expression promotes the specific secretion to the septum zone only from the daughter side leading to a limited degradation of the cell wall, which eventually produces cell separation without cell lysis (Ufano et al. 2004). In this degradative effort, the action of chitinases is complemented by the action of specific endo-glucanases as described above in this same chapter (2.2.2. Family GH81). The mechanisms that direct this differential secretion as well that those involved in the limited action of these enzymes are out of the scope of this review.

The class B chitinase group is one of the best conserved in GH18, and these chitinases are present in most fungi in variable numbers; therefore, it has been tempting to speculate with a general function of this type of enzymes along with fungal kingdom. This would be consistent with the hyphal tip localisation of the type B chitinase ChiA in A. *nidulans* (Yamazaki et al. 2008). Surprisingly, individual mutants of different class B chitinases did not show apparent morphological phenotypes. In *N. crassa,* Δ*gh18-10* and Δ*chi-1* mutants have normal morphology but show delayed growth under different experimental conditions. While the effect of the Δ*chi-1* mutation has been tentatively interpreted as a consequence of a reduced cell wall plasticity at the hyphal tip, the effect of the absence of *gh18-10* is more difficult to interpret. The phenotype of this Δ*gh18-10* mutant has been explained by a nutritional role of this chitinase, but also by a potential role of this enzyme at the ER as a deglycosylating activity (Tzelepis et al. 2012). More importantly, an *A. fumigatus* strain lacking the 5 class B endo-chitinases present in this fungi has been constructed to show that, despite a significant reduction in chitinase activity, its physiology is very similar to the control in the multiple aspects tested (Alcazar-Fuoli et al. 2011). Additional individual mutants generated in other filamentous fungi also lacked morphogenetic phenotypes. Moreover, a triple *cts1*Δ *cts2*Δ *cts3*Δ mutant of *U. maydis* formed normal infective filaments (Langner et al. 2015) and the strictly filamentous fungi *Ashbya gossypii* lacks any endo-chitinase-encoding gene (Dünkler et al. 2008). In this context, it is also relevant that the addition of allosamidin, a potent inhibitor of chitinase activity, has no effect on filamentous fungi but strongly affects the growth of yeast cells (Sakuda et al. 2013). Altogether, the multiple evidences from several systems argue against any role of endo-chitinases in the morphogenesis of the fungal hyphae, although

this hypothesis can always be questioned by the potential functional redundancy with other type of chitinases.

Fungal cells contain also a varied panoply of exo-chitinases belonging to classes A and C that could have an impact on cell morphogenesis. Therefore, multiple individual mutants have been constructed in order to characterise their phenotypes. In general, it can be stated that none of the mutants obtained in different fungi showed significant morphogenetic alterations, although the growth of some mutant strains is affected, probably because of nutritional reasons. The strong genetic redundancy between these classes of enzymes had been frequently invoked to explain the absence of clear phenotypes; however, in yeast-like fungi, which contain a single class A exo-chitinase, the deletion of these genes did not promote either apparent phenotypes, arguing against a general role for this type of enzymes in hyphal morphogenesis.

It should be noted, however, that some individual exo-chitinase mutants showed some alterations in spore formation. The *A. gossypii cts2*Δ mutant produces spores with aberrant cell walls, a defect that can be suppressed by the heterologous expression of equivalent exo-chitinases from *S. cerevisiae* or *C. albicans*. Moreover, a quadruple chitinase mutant in *C. neoformans* is defective in sexual development but not in vegetative growth (Baker et al. 2009) and some individual mutants of *N. crassa* also showed altered patterns of perithecial formation (Tzelepis et al. 2012). However, the importance of nutrition in sporulation made extremely difficult to discriminate between a direct defect of these mutants on cellular morphogenesis or an indirect effect through nutritional signalling.

In conclusion, despite extensive efforts along the years, the role of chitinases in fungal morphogenesis is unclear beyond yeast cells and their interest as potential antifungal targets has diminished over the years.

3.5 The Far Side of Chitinases in Fungal Cells

We have centred until now on the role of chitinases in fungal biology, but it should be noted that chitinases are ubiquitous in nature and are also produced by plants and animal cells. In this case, foreign chitinases would degrade fungal cell walls potentially acting as true antifungal agents. The mechanism involved in this antifungal response is well studied in plant, and in general, plant chitinases do not act directly lysing fungal cells but promoting limited degradation of chitin to produce chitin-derived inductors that trigger strong cellular responses in plant cells including Ca^{2+} spiking, ROS production, activation of the MAPK cascade, up-regulation of defence gene expression, callose deposition and molecular flux via plasmodesmata (Langner and Göhre 2016). Similarly, animal cells are also infected by fungi and the recognition of these pathogens through chitin-derived molecules is critical to trigger effective responses.

4 Concluding Remarks

In these pages, we have highlighted the enormous repertoire of glycosyl hydrolases in fungal cells. This large repertoire is the consequence of multiple gene expansion processes and unveils the importance of these enzymes for the adaptive fitness of the different fungal species to specific environments. The genetic expansion of the different families has produced an important degree of genetic redundancy that had made it difficult to establish the real importance of these enzymes in the physiology of the fungi, and convincing data on the role of these enzymes on fungal morphogenesis had been reported for yeast cells in most cases. Figure 7 is an attempt to summarise the known functions of glucanases and chitinases described in this review in the life cycle of yeast and fungi. The proposed functions are mainly based on the phenotypes reported for mutants lacking a either a single gene or the whole family of redundant genes. It is interesting to note that in both yeast and fungi, these hydrolases are preferentially involved in cell separation (mother–daughter cells in yeasts and conidium in fungi) rather than for plasticizing the cell wall, as it was suggested in the past. Perhaps the high degree of redundancy, not only in the number of genes belonging to a single a family, but also the redundant glycosylase and transglycosylase activities present in the cells makes it difficult to find clear phenotypes associated with cell wall softening. Another interesting difference between yeast and fungi is that fungi contain a large number of hydrolases that are

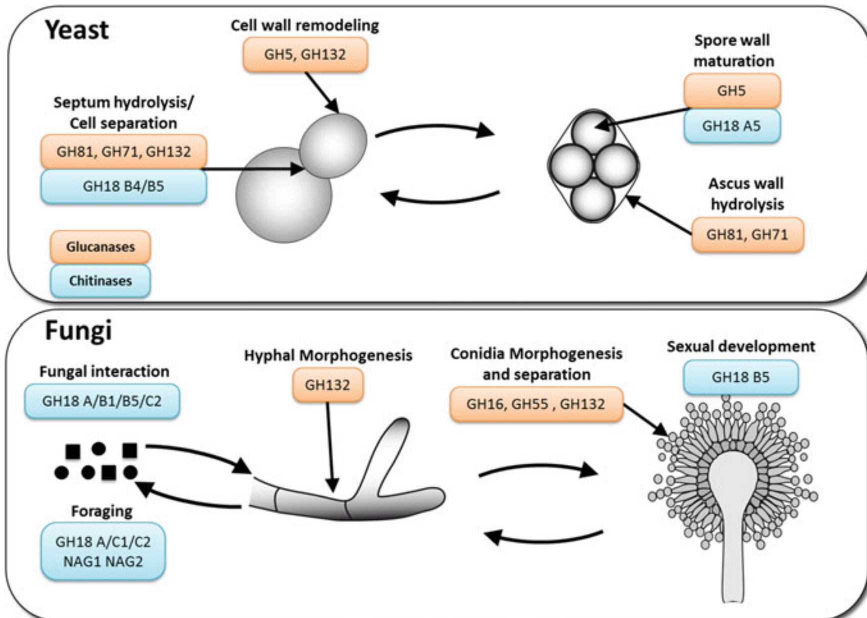


Fig. 7 A general overview of the function of glucanases and chitinases in yeast and moulds

secreted to catabolise complex polysaccharides for nutrition (which is not the case for yeast). This expansion of the different chitinase families probably reflects the general requirement of moulds for thriving in complex natural environments where competence for nutrients is higher.

The importance of these enzymes goes beyond the limits of the cells, since the wall of the fungal cells is the interface through which they interact with the environment and a direct target for the host in fungal pathogenesis. Accordingly, the production of glycosyl hydrolases from the host side favours the recognition of the pathogen and the triggering of the appropriate cellular responses to control it.

Acknowledgements This work was supported by grants from the Spanish Government to CR (BFU2017-84508-P) and CRV (BIO2015-70195-C2-1-R) and from Junta de Castilla y León to CR (SA116G19). The IBFG is supported by Programa "Escalera de Excelencia" from Junta de Castilla y León (CLU-2017-03) and University of Salamanca. All Spanish funding is co-sponsored by the European Union FEDER programme.

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GPI Anchored Proteins in *Aspergillus fumigatus* and Cell Wall Morphogenesis

Marketa Samalova, Paul Carr, Mike Bromley, Michael Blatzer, Maryse Moya-Nilges, Jean-Paul Latgé, and Isabelle Mouyna

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Abstract Glycosylphosphatidylinositol (GPI) anchored proteins are a class of proteins attached to the extracellular leaflet of the plasma membrane via a post-translational modification, the glycolipid anchor. GPI anchored proteins are expressed in all eukaryotes, from fungi to plants and animals. They display very diverse functions ranging from enzymatic activity, signaling, cell adhesion, cell wall metabolism, and immune response. In this review, we investigated for the first time

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Electronic supplementary material The online version of this chapter (https://doi.org/10.1007/82_2020_207) contains supplementary material, which is available to authorized users.

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Current Topics in Microbiology and Immunology (2020) 425: 167–186
https://doi.org/10.1007/82_2020_207
© Springer Nature Switzerland AG 2020
Published Online: 17 May 2020

an exhaustive list of all the GPI anchored proteins present in the *Aspergillus fumigatus* genome. An *A. fumigatus* mutant library of all the genes that encode in silico identified GPI anchored proteins has been constructed and the phenotypic analysis of all these mutants has been characterized including their growth, conidial viability or morphology, adhesion and the ability to form biofilms. We showed the presence of different fungal categories of GPI anchored proteins in the *A. fumigatus* genome associated to their role in cell wall remodeling, adhesion, and biofilm formation.

1 Introduction

The fungal cell wall is composed of polysaccharides and glycoproteins. The main central core of this cell wall is very similar in all fungal species but the nature of the carbohydrates and the degree and type of bridges between polysaccharides vary from one species to another. Synthases responsible for the biogeneration of linear polysaccharides are transmembrane proteins acting alone or in protein complexes (Latgé et al. 2017). The neosynthesized polysaccharides are extruded through the plasma membrane via as yet, undefined mechanisms. They are modified in the periplasmic space by remodeling enzymes. Many of the cell wall associated proteins responsible for the remodeling of these polysaccharides are anchored to the plasma membrane by a **g**lycosylphosphatidyl**I**nositol (GPI) anchor and designed as GPI anchored proteins.

The role of GPI anchored proteins has been previously investigated in *Saccharomyces cerevisiae* and *Candida albicans* (Caro et al. 1997; Plaine et al. 2008). In silico analysis suggested that *C. albicans* possesses 115 putative GPI anchored proteins, almost twice the number reported for *S. cerevisiae*. Moreover, it has been shown previously that some of the GPI anchored proteins play a major enzymatic role in cell wall morphogenesis like, for example, the elongation of β-(1–3)-glucans in yeasts and molds (Popolo and Vai 1999; Mouyna et al. 2000a; Gastebois et al. 2010a), whereas in yeast, it was also mentioned that these proteins are covalently bound to the cell wall polysaccharide (Caro et al. 1997; Kapteyn et al. 2000; Frieman et al. 2002). Herein, we describe our in silico analysis to provide comprehensive role of the cohort of genes that encode GPI anchored proteins in *A. fumigatus* genome. To aid our understanding of the role of these GPI proteins in the construction of the cell wall, we have generated and characterized null mutants for all of the genes we identified in this study.

2 Identification of putative GPI anchored proteins in the *A. fumigatus* genome

The identification of putative GPI anchored proteins in the *A. fumigatus* genome (AF293; http://fungi.ensembl.org/Aspergillusfumigatus/Info/Index) has been undertaken using the prediction programs PredGPI (http://gpcr.biocomp.unibo.it/

predgpi/proteome.htm) and big PI (http://mendel.imp.ac.at/sat/gpi/gpi_server.html) (Eisenhaber et al. 2004). In total, 86 proteins have been identified and predicted as being GPI anchored (see Table 1).

3 Comparative genomic analysis

By performing BLAST analysis (https://www.yeastgenome.org/blast-fungal and https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) with these proteins, we were able to show that all had orthologues in a second *A. fumigatus* isolate A1163. Orthologues of only 28 proteins (32.5%) were commons to the yeasts *S. cerevisiae* and *C. albicans* and filamentous fungi and a further 38 proteins (44%) were restricted to filamentous fungal species. Interestingly, 20 GPI anchored proteins (23.5%) were found exclusively in the genomes of the *Aspergilli* (Table 1).

4 Functions of GPI anchored proteins

Of the GPI anchored proteins that we have identified, the role of 34 proteins has been previously characterized either in *A. fumigatus* or in other fungi. In the following section, we describe their known roles.

(a) GPI anchored common to yeast and filamentous fungi acting on cell wall morphogenesis

Among the GPI anchored proteins previously described, several enzymes, *GEL*, *BGT2*, *DFG*, *SUN,* and *CRH*, have been well studied and shown to have functions associated with remodeling cell wall polysaccharides. The GPI anchors on these proteins result in them being co-localized with other cell membrane proteins that have direct roles in cell wall biogenesis and hence allow them to modify neosynthesized polysaccharides. The most extensively studied of these enzymes belong to the *GEL* family (GH72 in the CaZy database http://www.cazy.org/ which describes families of structurally related catalytic and carbohydrate-binding modules). Seven members of this family are encoded in the *A. fumigatus* genome, whereas *S. cerevisiae* (GAS) and *C. albicans* (PHR) have five members each (Rolli et al. 2011; Popolo et al. 2017). *GEL/GAS/PHR* family enzymes are responsible for the elongation of β-(1,3)-glucans, which is an essential activity given that deletion of *GEL4* in *A. fumigatus* is lethal (Hartland et al. 1996; Mouyna et al. 2000a, b; Gastebois et al. 2010a). It was recently shown that some members of this family have a dual activity that allows them not only to elongate but also to branch the neo elongated β-(1,3)-glucan (Aimanianda et al. 2017). This branching activity is only seen in enzymes that have the carbohydrate-binding module, CBM43, and loss of this motif abolishes β-(1,3)-glucan branching (Aimanianda et al. 2017).

Table 1 List of predictive GPI anchored proteins

AFUB number	AFUA.number	Gene name or function	Phenotype	Fungi	References
AFUB_018250	AFUA_2G01170	GEL1 §	no	Yeast and Filamentous	Hartland et al. (1996); Mouyna et al. (2000a, b)
AFUB_077400	AFUA_6G11390	GEL2 §	Conidia, Growth, S-D	Yeast and Filamentous	Mouyna et al. (2005)
AFUB_028470	AFUA_2G12850	GEL3	no phenotype	Yeast and Filamentous	Gastebois et al. (2010a
AFUB_022370	AFUA_2G05340	GEL4	Growth, S-D	Yeast and Filamentous	Gastebois et al. (2010a)
AFUB_084480	AFUA_8G02130	GEL5	no	Yeast and Filamentous	Gastebois et al. (2010a)
AFUB_036000	AFUA_3G13200	GEL6	no	Yeast and Filamentous	Gastebois et al. (2010a)
AFUB_078410	AFUA_6G12410	GEL7	no	Yeast and Filamentous	Gastebois et al. (2010a)
AFUB_048180	AFUA_3G00270	BGT2	no	Yeast and Filamentous	Gastebois et al. (2010b); Millet et al. (2018)
AFUB_002130	AFUA_1G01730	DFG1	no	Yeast and Filamentous	Muszkieta et al. (2019)
AFUB_017760	AFUA_2G00680	DFG2	no	Yeast and Filamentous	Muszkieta et al. (2019)
AFUB_048110	AFUA_3G00340	DFG3	Growth, S-D	Yeast and Filamentous	Muszkieta et al. (2019)
AFUB_047740	AFUA_3G00700	DFG4	no	Yeast and Filamentous	Muszkieta et al. (2019)
AFUB_101170	AFUA_4G00620	DFG5	no	Yeast and Filamentous	Muszkieta et al. (2019)
AFUB_100440	AFUA_4G02710	DFG7	no	Yeast and Filamentous	Muszkieta et al. (2019)
AFUB_013430	AFUA_1G13940	SUN2	no	Yeast and Filamentous	Gastebois et al. (2013)
AFUB_095070	AFUA_6G03230	CRH1 §	no	Yeast and Filamentous	Fang et al. (2019)
AFUB_020180	AFUA_2G03120	CRH2 §	no	Yeast and Filamentous	Fang et al. (2019)
AFUB_074470	AFUA_6G08510	CRH4	no	Yeast and Filamentous	Fang et al. (2019)
AFUB_015530	AFUA_1G16190	CRH5	no	Yeast and Filamentous	Fang et al. (2019)
AFUB_029980	AFUA_2G14360	ENG2	no	Yeast and Filamentous	Hartl et al. (2011)
AFUB_034540	AFUA_3G14680	PBL3	no	Yeast and Filamentous	Shen et al. (2004)
AFUB_052270	AFUA_5G03760	Chitinase A1	no	Yeast and Filamentous	Alcazar-Fuoli et al. (2011)
AFUB_063890	AFUA_4G06820	Ecm33 §	Conidia,virulence	Yeast and Filamentous	Chabane et al. (2006)

(continued)

Table 1 (continued)

AFUB number	AFUA.number	Gene name or function	Phenotype	Fungi	References
AFUB_076480	AFUA_6G10430	CDA6	no	Yeast and Filamentous	Mouyna et al (2020)
AFUB_092930	AFUA_6G05350	OPSB	no	Yeast and Filamentous	
AFUB_064130	AFUA_4G07040	CTSD	no	Yeast and Filamentous	Vickers et al. (2007)
AFUB_042000	AFUA_3G07050		no	Yeast and Filamentous	
AFUB_056560	AFUA_5G09020		no	Yeast and Filamentous	
AFUB_020300	AFUA_2G03230	AmylaseA	no	Filamentous fungi***	
AFUB_047500	AFUA_3G00900	Amylase	conidia, Growth,conidiation	Filamentous fungi***	
AFUB_000660	AFUA_6G14090	CFEMA	no	Filamentous Fungi	Vaknin et al. (2014)
AFUB_076620	AFUA_6G10580	CFEMB	no	Filamentous Fungi	Vaknin et al. (20201414)
AFUB_072620	AFUA_6G06690	CFEMC	no	Filamentous Fungi	Vaknin et al. (2014)
AFUB_057130	AFUA_5G09580	RODA #	Conidia,virulence	Filamentous Fungi	Aimanianda et al. (2009); Valsecchi et al. (2017a)
AFUB_016640	AFUA_1G17250	RODB #	no	Filamentous Fungi	Valsecchi et al. (2017a)
AFUB_042020	AFUA_3G07030	Glutaminase	no	Filamentous Fungi	
AFUB_081470	AFUA_8G06030	α(1-3) glucanase	no	Filamentous Fungi	
AFUB_097010	AFUA_6G00500	chitosanase	no	Filamentous Fungi	
AFUB_003980	AFUA_1G03570	PhoA §	no	Filamentous Fungi	Bernard et al. (2002)
AFUB_022180	AFUA_2G05150	AfMP2	Biofilm	Filamentous Fungi	Woo et al. (2018)
AFUB_099880	AFUA_4G03240	AFMP1	no	Filamentous Fungi	Woo et al. (2018)
AFUB_006180	AFUA_1G05790		Biofilm	Filamentous Fungi	
AFUB_087030	AFUA_7G00450		Biofilm	Filamentous Fungi	
AFUB_001030	AFUA_6G13710		no	Filamentous Fungi	
AFUB_004040	AFUA_1G03630		no	Filamentous Fungi	
AFUB_008960	AFUA_1G09510		no	Filamentous Fungi	

(continued)

Table 1 (continued)

AFUB number	AFUA.number	Gene name or function	Phenotype	Fungi	References
AFUB_009040	AFUA_1G09590		no	Filamentous Fungi	
AFUB_009100	AFUA_1G09650		no	Filamentous Fungi	
AFUB_018780	AFUA_2G01710		no	Filamentous Fungi	
AFUB_035550	AFUA_3G13640		no	Filamentous Fungi	
AFUB_036090	AFUA_3G13110		no	Filamentous Fungi	
AFUB_044890	AFUA_3G03370		no	Filamentous Fungi	
AFUB_047260	AFUA_3G01150		no	Filamentous Fungi	
AFUB_047510	AFUA_3G00880		no	Filamentous Fungi	
AFUB_050450	AFUA_5G01920		no	Filamentous Fungi	
AFUB_056330	AFUA_5G08800		no	Filamentous Fungi	
AFUB_057570	AFUA_5G09960		no	Filamentous Fungi	
AFUB_057610	AFUA_5G10010		no	Filamentous Fungi	
AFUB_069330	AFUA_4G12370		no	Filamentous Fungi	
AFUB_082130	AFUA_8G05410		no	Filamentous Fungi	
AFUB_083170	AFUA_8G04370		no	Filamentous Fungi	
AFUB_084140	AFUA_8G02450		no	Filamentous Fungi	
AFUB_085740	AFUA_8G00830		no	Filamentous Fungi	
AFUB_088990	AFUA_7G02440		no	Filamentous Fungi	
AFUB_089500	AFUA_7G03970		no	Filamentous Fungi	
AFUB_095500	AFUA_6G02800		no	Filamentous Fungi	
AFUB_010650	AFUA_1G11220		Conidia, S-D	Aspergillus	
AFUB_066710	AFUA_4G09600		conidia, conidiation	Aspergillus	
AFUB_096850	AFUA_6G00620		Conidia	Aspergillus	
AFUB_099690	AFUA_4G03360		Conidia	Aspergillus	
AFUB_018220	AFUA_2G01140		Conidia	Aspergillus	

(continued)

Table 1 (continued)

AFUB number	AFUA.number	Gene name or function	Phenotype	Fungi	References
AFUB_040120	AFUA_3G08990	CSPA	Conidia, adhesion	Aspergillus	Levdansly et al. (2010); Valsecchi et al. (2017b)
AFUB_019530	AFUA_2G02440		no	Aspergillus	
AFUB_031860	AFUA_2G16180		no	Aspergillus	
AFUB_044000	AFUA_3G03960		no	Aspergillus	
AFUB_084580	AFUA_8G02030		no	Aspergillus	
AFUB_087170	AFUA_7G00580		no	Aspergillus	
AFUB_087560	AFUA_7G00970		no	Aspergillus	
AFUB_000740	AFUA_6G14010		no	Aspergillus	
AFUB_082630	AFUA_8G04860		no	Aspergillus	
AFUB_030420	AFUA_2G14780		no	Aspergillus*	
AFUB_037960	AFUA_3G11190		no	Aspergillus*	
AFUB_089000	AFUA_7G02460		no	Aspergillus*	
AFUB_016760	AFUA_1G17390		no	Aspergillus*	
AFUB_066570	AFUA_4G09450		no	Aspergillus*	
AFUB_084830	AFUA_8G01770		Conidia, Growth, S-D	Aspergillus*	Mouyna et al. (2020) in preparation

List of the putative GPI-anchored proteins identified by the two softwares in the A. fumigatus genome including the corresponding AFUB and AFUA number (http://fungi.ensembl.org/Aspergillus_fumigatus/Info/Index), the gene name when identified, the phenotype of the mutant and their presence in the other genomes. Yeast and Filamentous: Proteins which are present in *C. albicans*, *S. cerevisiae*, *A. fumigatus* and others filamentous fungi; Filamentous Fungi: proteins present in filamentous fungi and not in the yeast genome; Filamentous Fungi***: these proteins are not present in the *S. cerevisiae* and C. albicans genome but they are present in the S. pombe and C. neoformans genome. Aspergillus: proteins only present in *Aspergillus* species; *Aspergillus**: proteins only present in few species of Aspergillus like *A. clavatus*, *A. lentulus*, *A. thermomutatus*, and the *A. turcosus* species; S-D: sensitivity to drugs. The GPI mutant library was screened for the growth on different media (Malt or Minimal medium), or Minimal medium (MM) including calcofluor white (40mg/ml), or congo red (50mg/ml) after 48h at 37°C, conidial morphology, conidial viability as described by (Millet et al. 2018), adhesion (104 conidia were incubated at 37°C on MM medium + 0.01% tween 20 on plates TPP for 24h) as described by Fontaine et al., (2010) and the ability to form biofilm on agar plates on MM medium after 22h of growth at 37° as described by (Beauvais et al., 2007). NB: no=no phenotype; S-D: higher sensitivity to drugs; Conidia: mutants which are affected in their conidia (shape, linear chains); Conidiation: mutants which are affected in conidiation. # RODA and RODB predicted to be GPI in silico but proved biochemically to be non GPI. [§] Proteins proved to be GPI biochemically

The GH17 family in *A. fumigatus* contains five members (*BGT1–3, SCW4 and SCW11)*; however, *BGT2* is the only member of this family that is GPI anchored. Bgt1 transfers the donor β-(1,3)-glucan on the non-reducing end of the chain (Mouyna et al. 1998), whereas Bgt2 preferentially transfers within the β-(1,3)-glucan chain (Gastebois et al. 2010b). No phenotype has been associated to the deletion of *BGT2* alone in *A. fumigatus* or its ortholog *BGL2* in the yeast *S. cerevisiae* (Cappellaro et al. 1998). However, Millet et al. (2018) and Sestak et al. (2004) showed that in *A. fumigatus* and *S. cerevisiae,* the non-GPI-members of the

GH17 family, especially Scw4, Scw11, and Bgt3 and Scw4, Scw10, and Scw11, are important for cell wall integrity. The enzymatic activity of Scw4, Scw11, and Bgt3 is still unknown but the analysis of the quintuple null mutant showed that Scw4, Scw11, and Bgt3 have antagonistic and distinct functions to Bgt2 and Bgt1.

Recently, it has been shown in *A. fumigatus* that the *DFG* family (GH76 CaZy family) is involved in the covalent binding of Galactomannan (GM) to the β-(1,3)-glucan–chitin core of the cell wall. This family contains seven members in *A. fumigatus*, all of which are GPI anchored proteins, except *DFG6* (Muszkieta et al. 2019). The single mutant Dfg3 is playing the major role in the association of the GM to the glucan core. However, the phenotype defect was enhanced in the septuple *DFG* deleted mutant, such as highly reduced growth with hyper-branched hyphae and higher sensitivity to drugs, showing that Dfgs have additional activities on structural properties of the cell wall (Muszkieta et al. 2019). In both, *S. cerevisiae* and *C. albicans*, although single knockouts of *DFG5* and *DCW1* are viable, a double knockout is synthetically lethal (Kitagaki et al. 2002; Spreghini et al. 2003). Interestingly as yeasts do not have galactomannan in their cell wall, the biochemical function of these remodeling enzymes remains to be discovered.

The *SUN* family *in A. fumigatus* (also known as the GH132 CaZy family) comprises two members, *SUN1* and *SUN2* which is the only one predicted to be GPI anchored in *A. fumigatus*. They are so called as they encode a SUN domain originally identified in the yeast proteins *SIM1*, *UTH1*, *NCA3,* and *SUN4*. The SUN domain is closely related, at the sequence level, to a β-glucosidase of *Candida wickerhamii;* however, the yeast proteins have no detectable β-glucosidase activity. The deletion of *SUN2*, which is most closely related to the uncharacterized protein YMR244W in *S. cerevisiae*, did not induce any morphological alterations. In contrast, the deletion of the *SUN1* genes in yeasts and molds has been shown to exhibit defects in septum closure (Hiller et al. 2007; Norice et al. 2007; Firon et al. 2007; Gastebois et al. 2013) However, the baker's yeast *SUN1* and their ortholog in *C. albicans SUN41/SUN42,* which encodes an exo β-(1,3)-glucanase but are not a GPI anchored protein, play a role in cell wall morphogenesis. Inactivation of *SUN1* genes and orthologs leads to a defect in the separation of daughter cells from mother cells, and simultaneous inactivation of *SUN41* and *SUN42* is lethal in the absence of osmotic protection. Like for *A. fumigatus*, cell wall defects seen in this double mutant are mainly localized in the region surrounding the septa in mother yeast cells and subapical hyphal compartments. The role taken by each SUN protein remains unknown as well as the role of the GPI anchor in the function of *A. fumigatus SUN2* in the cell.

The *CRH* (for Congo Red Hypersensitivity) GH16 CaZy family has been associated to glucan/chitin linkage in yeast *S. cerevisiae* (Rodríguez-Peña et al. 2000; Cabib et al. 2008; Blanco et al. 2012; Arroyo et al. 2016). In *A. fumigatus*, five members are present in the genome (four proteins being GPI anchored proteins). The phenotype of the quintuple mutant is very weak and not associated to congo red resistance. Congo red toxicity is pleiopropic with this molecule acting not only on cell wall biosynthesis but also in oxido-reduction pathways. Moreover, the biochemical function of the Crh proteins has not been demonstrated and there is

not a definite proof that these genes could be essential for the establishment of chitin–glucan linkages (Fang et al. 2019).

Members of the *SPS2* family (which are not assigned to a CaZy family) play an essential role in the formation of the ascospore cell wall in *S. cerevisiae* (Coluccio et al. 2004), whereas the ortholog in *A. fumigatus*, *ECM33*, is important for conidial morphogenesis and virulence (Chabane et al. 2006). However, its enzymatic function remains unknown.

Three GPI anchored proteins, CFEM (A-C), containing fungal-specific CFEM domains (Common in Fungal Extracellular Membrane) are characterized by spaced cysteine residues (Kulkarni et al. 2003). Most CFEM-containing cell wall proteins studied to date have been shown to be involved in host-pathogen interactions and virulence. In *C. albicans*, deletion of the three GPI anchored-CFEM-encoding genes in the genome (Rbt5/Rbt51/Csa1) results in an increased sensitivity to cell wall damaging agents and a reduced ability to form a biofilm (Pérez et al. 2006, 2011). In contrast, in *A. fumigatus*, (Vaknin et al. 2014) showed that these proteins, even though their respective mutants display a higher sensitivity to congo red and calcofluor white than their parental strain, did not play any role in cell wall morphogenesis or virulence.

Finally, no phenotype has been associated to the endo β-(1,3)-glucanase *ENG2* (Hartl et al. 2011) or the chitinase A1 (Alcazar-Fuoli et al. 2011) and the chitin deacetylase *CDA6* (Mouyna et al. 2020), which are the only GPI members in their respective family. However, the sequential deletion of *ENG2–5* belonging to the GH16 family altogether with *ENG1* (GH81) showed conidiogenesis defects, with linear chains of conidia unable to separate while the germination rate was not affected (Mouyna et al. 2016).

(b) GPI anchored proteins only found in filamentous fungi which are associated to cell wall structures

In addition to the GPI anchored proteins common to yeast and filamentous fungi which have been shown to be biochemically associated to cell wall construction, other GPI anchored proteins identified in silico are present only in the cell wall of filamentous fungi and are involved in adhesion and biofilm formation (Table 1).

The outer layer of the conidium is composed of melanin covered by a rodlet layer that confers hydrophobic properties to *A. fumigatus* conidia. This rodlet layer is exclusively composed of hydrophobins, which are low molecular weight proteins rich in cysteins residues. This rodlet layer masks conidial recognition by the human innate immune system (Aimanianda et al. 2009). Recently, (Valsecchi et al. 2017a) showed that seven hydrophobins (RodA–RodG) are present in the genome of *A. fumigatus*. RodA and RodB were identified as putative GPI anchored protein based on our in silico analysis. However, two lines of evidence indicate that the proteins are probably not GPI anchored: the predicted ω cleavage site which is the amino acid immediately upstream of the putative site of GPI anchor addition (the omega site) is located between Cys-residues C7 and C8, which would disrupt a conserved disulfide bridge that is important to stabilize the structure of the proteins; moreover,

it has been shown that the C-terminus of RodA extracted from conidia corresponds to that of the full-length protein (Pille et al. 2015; Valsecchi et al. 2017a).

It has been shown by Levdansky et al. (2010) that deletion of *CSPA*, a repeat rich GPI anchored protein only found in *Aspergillus* sp., is involved in reduced adhesion and increase speed of conidial germination. Moreover, Valsecchi et al. (2017b) showed that conidia of the *CSPA* mutant tended to stay grouped together in long chains and adhered also between themselves. This gene has been shown to be regulated by the Myb1 transcription factor (Valsecchi et al. 2017b).

5 Investigating the role of newly identified GPI anchored proteins in cell wall morphogenesis

Most of the previously analyzed GPI proteins were associated somehow to cell wall construction and fungal morphogenesis. These results suggested that all GPI anchored proteins may have essential functions in fungal growth some of them being undefined and this was at the basis of the study of the GPI proteins in *A.fumigatus*. In order to investigate exhaustively the role of the GPI anchored proteins, an *A. fumigatus* mutant library of all the genes identified in silico were constructed following the procedures outlined in Zhao et al. (2019) and Furukawa et al. (2020) using the oligonucleotide primers described in Supplementary Table 1 and screened for growth, conidiation, and biofilm formation.

From the screening analysis, three categories of GPI anchored protein null mutants were identified: proteins found in yeast and filamentous fungi, proteins found exclusively in filamentous fungi, and proteins found exclusively in *Aspergillus* species. Ten of the 57 new mutants (the previously published mutants are not counted) showed a distinct phenotype from the parental strain including conidial morphology, growth, sensitivity to congo red and calcofluor white, adhesion or biofilm formation (Table 1).

(a) Proteins found in Yeast and filamentous fungi

28 proteins are present in yeast and filamentous fungi genome, 23 being already described previously (see above) and 38 proteins are present exclusively in filamentous fungi genome.

- **Proteins with putative enzymatic functions**

Secreted proteases have always attracted attention as potential mediators of fungal invasion, conidophore development, or adhesion (Monod et al. 2002). We did not observe any distinct growth phenotype after the deletion of the aspartic proteases *CTSD* (AFUA_4G07040) (Vickers et al. 2007) and *OPSB* (AFUA_6G05350). Phospholipases (Plbs) activity which can destabilize host membranes are also considered to be virulence factors for pathogenic fungi like *C. albicans* (Leidich et al. 1998). In *A. fumigatus*, the mutant resulting from the deletion of the

phospholipase *PLB3* (AFUA_3G14680) (Shen et al. 2004) is not affected. Similarly, phosphatase plays a major role in the fungal life. In *A. fumigatus*, the acid phosphatase PhoA (AFUA_1G03570) which is specific to filamentous fungi (Bernard et al. 2002) are not directly associated to growth (data not shown). Moreover, the two genes encoding a putative chitosanase and a putative α-(1–3)-glucanase (respectively AFUA_6G00500 and AFUA_8G06030) which were predicted as GPI anchored proteins specific to filamentous fungi, do not play a role in the cell wall remodeling in *A. fumigatus* since the corresponding deleted mutant behaved like the parental strain (data not shown). However, non-GPI anchored homologs of these proteins (three for chitosanases and eight for α-(1–3)-glucanases) are present in the *A. fumigatus* genome and could be involved in compensatory mechanisms after the deletion of the GPI gene of the family.

The GPI anchored protein encoded by AFUA_3G00900, is a putative amylase. The null mutant exhibits a twofold decrease in conidiation, a slight reduction in radial growth and increased resistance to congo red (data not shown). The protein encoded by this gene belongs to the GH13 family. This CAZYme family is a large family containing various hydrolyzing and transglycosylating enzymes, mostly acting on α-(1,4)- or α-(1,6)-glycosidic linkages, which can be involved in starch degradation or in the synthesis or modification of alpha-glucan in the fungal cell wall (Morita et al. 2006; Yuan et al. 2008). In addition to AFUA_300900, four other GH13 proteins are present in the *A. fumigatus* genome: AFUA_2G03230, another GPI anchored protein specific to filamentous fungi (Table 1), AFUA_2G00710, AFUA_4G10130, and AFUA_2G13460. In contrast to AFUA_3G00900, we saw no phenotype associated with the deletion of AFUA_2G03230. The phylogenetic tree of the GH13 family of *A. fumigatus* showed two distinct groups, the first group (with AFUA_2G00710 AFUA_4G10130) associated to proteins involved in starch degradation like AmyA and AmyB in *A. niger* (Korman et al. 1990) and the second group (AFUA_3G00900, AFUA_2G03230 and AFUA_2G13460) associated to proteins with transferase activities like AgtA and AgtB in *A. niger* and Aah3 in *S. pombe* (Morita et al. 2006; van der Kaaij et al. 2007b; Yuan et al. 2008) (Fig. 1). In *A. niger*, both enzymes showed transglycosylation activity on donor substrates with alpha-(1,4)-glycosidic bonds and at least five anhydroglucose units. The enzymes, designated AgtA and AgtB, produced new alpha-(1,4)-glycosidic bonds (van der Kaaij et al. 2007b). In *S. pombe*, disruption of *AAH3* encoding a GPI anchored protein resulted in hypersensitivity toward cell wall-degrading enzymes and an aberrant cell shape, indicating that normal cell wall biosynthesis was affected (Morita et al. 2006). Disruption of *AgtA* in *A. niger* also affected cell wall stability. The protein sequence of AFUA_3G00900 and AFUA_2G13460 is very closely related to AgtA and AgtB of *A. niger* (between 50 and 60% of identity) and notably the catalytic conserved domain characteristics of transferase activities of this GH13 families (van der Kaaij et al. 2007a) suggest they may be also transferases in *A. fumigatus*.

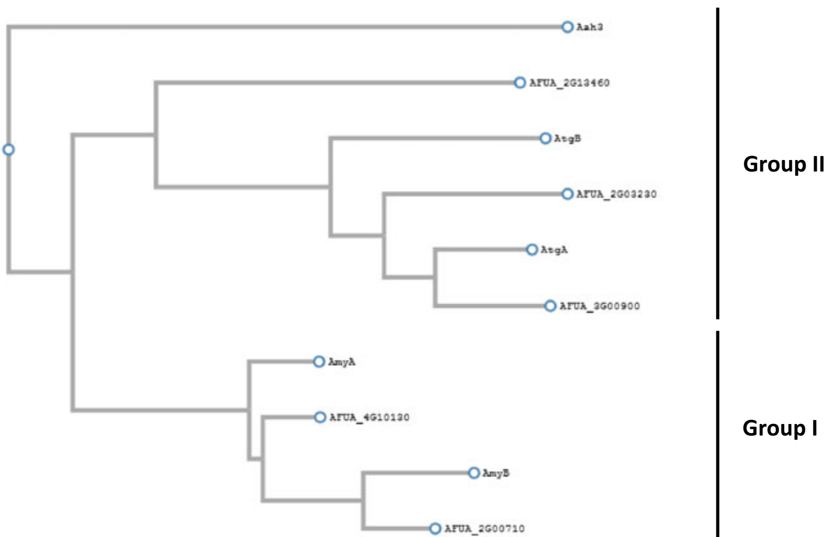

Fig. 1 Phylogeny of the GH13 family of *A. fumigatus*, AtgA-B and AmyA-B of *A. niger* and aah3 of *S. pombe*. Sequence alignment and phylogenetic reconstructions have been done using clustalW (https://www.genome.jp/tools-bin/clustalw). The tree was constructed using FastTree v2. 1.8 with default parameters

- **Proteins with unknown function**

Most of the proteins exclusively present in filamentous fungi genome display unknown functions (25 on the 38 identified).

Three null mutants corresponding to the genes (AFUA_2G05150, AFUA_7G00450, and AFUA_1G05790) showed a twofold reduced ability to form biofilm (Fig. 2a). AFUA_2G05150 is annotated as the cell wall galactomanno-protein Mp2. In contrast, the AFUA_4G03240 null mutant (also a GPI anchored protein) annotated as the galactomannoprotein Mp1 did not show any difference in biofilm formation in our study. Mp1 and Mp2 are homologous to *Penicillium marneffei* Mp1, a cell surface antigenic cell wall mannoprotein and a virulence factor (Cao et al. 1998; Woo et al. 2016). *A. fumigatus* Mp1 and Mp2 have been shown to be also immunogenic (Yuen et al. 2001; Woo et al. 2002; Chong et al. 2004). We constructed the double mutant Δ*mp*1/Δ*mp*2 but we did not observe additional decreases in biofilm formation or reduction in adhesion in comparison to the single mutant Δ*mp*2 (data not shown). Recently, (Woo et al. 2018) identified two distantly others homologs in *A. fumigatus*, Mp3 and Mp4, containing also one lipid-binding domain and showed that Mp4 was involved in virulence.

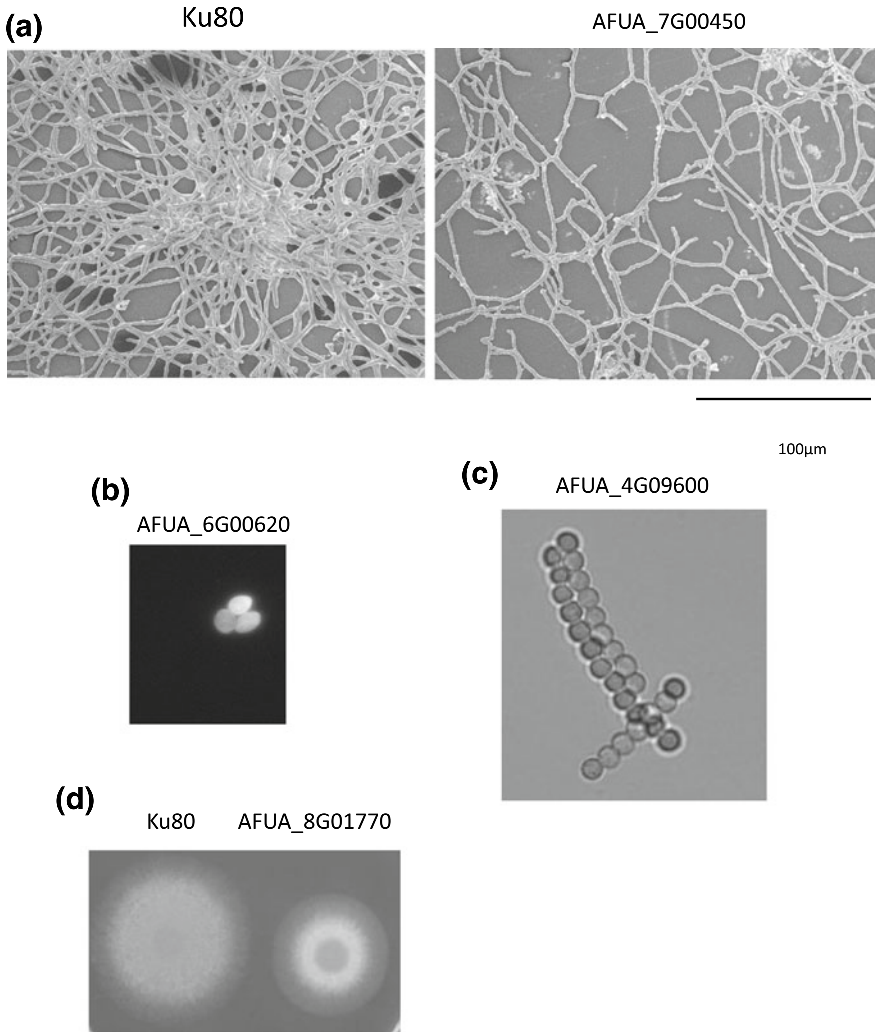

Fig. 2 Phenotype analysis of some GPI anchored protein mutants: **a** SEM of the AFUA_1G05790 deletion mutant involved in biofilm formation compared to the parental strain Ku80. **b** Light microscopy of the shape of conidia after deletion of AFUA_6G00620 gene (63x). **c** Light microscopy of the linear chains of conidia after the deletion of AFUA_4G09600 gene. **d** Growth on Malt medium of the AFUA_8G01770 deletion mutant after 48 h at 37 °C in comparison to the parental strain

(b) **Proteins found exclusively in *Aspergillus* species**

For the deletion of AFUA_2G01140, AFUA_4G03360, AFUA_6G00620, and AFUA_1G11220 which encode proteins of unknown function, we observed that the shape of 5% of the conidia were ovoids (an example is given in Fig. 2b). In the case

of AFUA_1G11220, the deletion of this gene was also associated with a twofold increase in congo red and calcofluor white sensitivity (data not shown). This modification of the morphology of the conidia and of the sensitivity to cell wall drugs suggests that the proteins encoded by these genes could be involved in the construction of the conidial cell wall.

Deletion of AFUA_4G09600, a protein containing several repetitions of amino acid motif GGPSGNDGGN and VKDAYTDDHSV also found only in *Aspergillus* sps, is correlated to a threefold reduction in conidiation compared to the parental strain (data not shown). We also observed linear chains of conidia in this mutant (Fig. 2c). This phenotype is reminiscent of the *CSPA* null mutant phenotype (Valsecchi et al. 2017b).

Six GPI proteins (AFUA_2G14780, AFUA_3G11190, AFUA_7G02460, AFUA_1G17390, AFUA_4G09450, AFUA_8G01770) are only present in the *Aspergillus* species close phylogenetically of *A. fumigatus* (*A. clavatus*, *A. lentulus*, *A. thermomutatus,* and *A. turcosus* (Table 1). No significant homology or domain has been found with any known proteins. Only the deletion of AFUA_8G01700 showed a distinct phenotype from the parental strain, reduced growth, higher sensitivity to drugs and reduced adhesion (Mouyna et al. 2020, manuscript in preparation) (Fig. 2d).

6 Discussion and Conclusion

Even if we try to dress an exhaustive list of all the GPI anchored proteins present in the *A. fumigatus* genome using different algorithms, some proteins could have been wrongly identified as GPI proteins (RodA and RodB) or missed. For example, the conidial surface protein CcpA has been shown to be GPI anchored (Voltersen et al. 2018) while it was not identified using the prediction softwares. Only few proteins have been demonstrated biochemically to be GPI anchored proteins after cleavage of the anchor by a phospholipase C releasing the protein in the Triton X-114 fraction and recognized by a cross-reacting determinant antibody. A proteomic analysis identified biochemically Gel1 and Gel2, Crh1, Crh2, Ecm33, PhoA as GPI anchored proteins (Bruneau et al. 2001). All of these proteins were identified in our bioinformatics predictions.

The localization of GPI anchored proteins has been also controversial. In the yeast *S. cerevisiae*, and *Candida* (Kapteyn et al. 2000; Frieman et al. 2002), it has been demonstrated that many GPI proteins (called GPI anchored cell wall proteins or GPI-CWPs) arrive at the plasma membrane but are then liberated. A remnant of the GPI anchor reacts with β1,6 glucan resulting in cross-linking of the GPI-CWP into the cell wall (Van der Vaart et al. 1997) suggesting that there are two terminal fates for GPI proteins—residence at the plasma membrane (GPI anchored plasma membrane proteins or GPI-PMPs) and residence at the cell wall (GPI-CWPs) (Lu et al. 1994). Moreover, based on in silico analysis of GPI anchored proteins in *S. cerevisiae*, Caro et al. (1997) proposed that a signal of two basic amino acids in the four amino acids upstream of the ω site acts to retain the protein at the plasma

membrane. In the absence of this retention signal, the proteins are mobilized to the cell wall. Using fusions of the GPI signal sequences from *S. cerevisiae* to alpha-galactosidase, (Hamada et al. 1998) found a good correlation between presence or absence of the dibasic motif and partitioning of the fusion protein to the plasma membrane or cell wall. Analysis of various point mutations in specific GPI anchor signal sequences also supported the importance of the dibasic motif in GPI anchored protein localization. In contrast, in *A. fumigatus*, the structural cell wall composition did not reveal the presence of β(1–6)glucan (Fontaine et al. 2000). Moreover, no proteins have been shown to be covalently attached to the cell wall after their release from the membrane (Bernard et al. 2002). In addition, none of the FLO, CWP or TIR family proteins identified in the *S. cerevisiae* genome (Caro et al. 1997) and predicted to be associated to the cell wall, have been found in the *A. fumigatus* genome.

The different categories of GPI anchored proteins found in *A. fumigatus* and their function are summarized in Fig. 3. The first category of proteins is highly conserved in all fungi (yeast as well as filamentous fungi) and is essential in cell wall morphogenesis. Indeed, the structural core of the cell wall between yeasts and molds is conserved. Most of them belong to multigenic families of proteins. Their analysis showed that most of the time, one or two genes in a family are responsible for the phenotype observed (Gastebois et al. 2010a; Millet et al. 2018; Muszkieta et al. 2019). Accordingly, all proteins in the same family are unlikely to have a shared function, which supports the redundancy of genes already observed in the *Aspergillus* genome. In the second category, we identified and characterized proteins present only in filamentous fungi, which are mostly involved in biofilm formation, adhesion, and virulence process. However, 60% of the proteins belonging to this category did not present any domain or identity with previously annotated

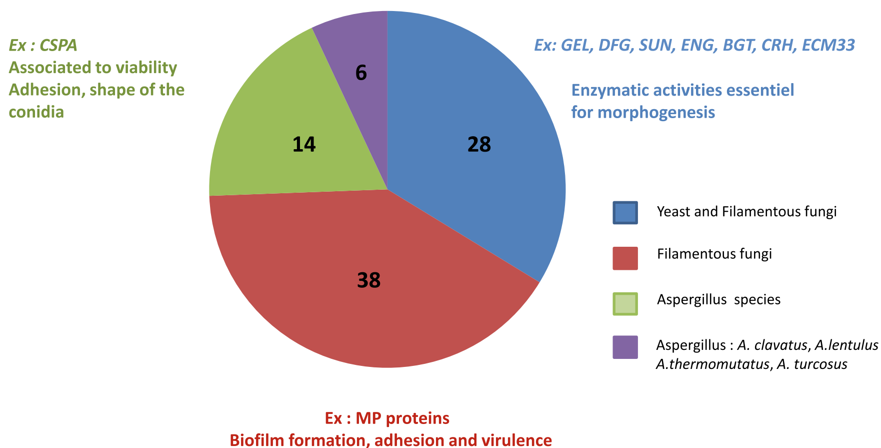

Fig. 3 Different fungal categories of GPI anchored proteins, which show an association between their putative role (cell wall remodeling, adhesion, biofilm or virulence) and their category

proteins or a distinct phenotype associated to their gene mutation. Finally, the third category of proteins is only present in *Aspergillus* species, or even in few related species of *Aspergillus*. These proteins seem to be mostly associated with the formation of the conidial stage but again their function is unknown. This review suggests that other non-GPI-bound transglycosidases are important for the remodeling of cell wall construction and remain to be discovered.

Acknowledgements This research was funded by l'Agence Nationale pour la Recherche (AfuInf ANR-16-CE92-0039), la Fondation pour la Recherche Médicale (DEQ 20150331722 LATGE Equipe FRM 2015) and the AIC (Action Incitative Ciblée) grant of Pasteur Institute. This work was also supported by the Wellcome trust grant 208396/Z/17/Z to MB.

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PAMPs of the Fungal Cell Wall and Mammalian PRRs

Remi Hatinguais, Janet A. Willment, and Gordon D. Brown

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Abstract Fungi are opportunistic pathogens that infect immunocompromised patients and are responsible for an estimated 1.5 million deaths every year. The antifungal innate immune response is mediated through the recognition of pathogen-associated molecular patterns (PAMPs) by the host's pattern recognition receptors (PRRs). PRRs are immune receptors that ensure the internalisation and the killing of fungal pathogens. They also mount the inflammatory response, which

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Current Topics in Microbiology and Immunology (2020) 425: 187–223
https://doi.org/10.1007/82_2020_201
© Springer Nature Switzerland AG 2020
Published Online: 17 March 2020

contributes to initiate and polarise the adaptive response, controlled by lymphocytes. Both the innate and adaptive immune responses are required to control fungal infections. The immune recognition of fungal pathogen primarily occurs at the interface between the membrane of innate immune cells and the fungal cell wall, which contains a number of PAMPs. This chapter will focus on describing the main mammalian PRRs that have been shown to bind to PAMPs from the fungal cell wall of the four main fungal pathogens: *Candida albicans, Aspergillus fumigatus, Cryptococcus neoformans* and *Pneumocystis jirovecii*. We will describe these receptors, their functions and ligands to provide the reader with an overview of how the immune system recognises fungal pathogens and responds to them.

1 General Introduction

Fungi are ubiquitous microbes found in the soil, plants and the microbiota of animals. These microorganisms are responsible for a broad range of infection types in humans from benign superficial colonisation of the skin and mucosa to deadly invasive infections. The main fungi responsible for profound infections are the opportunistic pathogens *Candida albicans, Aspergillus fumigatus, Cryptococcus neoformans*, and *Pneumocystis jirovecii* (Brown et al. 2012). The mortality rate of these infections ranges from 20 to 95% despite the existence of antifungal drugs (Brown et al. 2012). Invasive infections are often the result of an underlying immunological disorder. Primary immune deficiencies (genetic disorders resulting in a broad or narrow defect of the immune system) can predispose patients to a more or less broad range of bacterial, viral, parasitic and/or fungal infections (Lanternier et al. 2013). Immunodeficiencies can also be secondary to another pathology (e.g. cancer, diabetes, AIDS) or to a medical treatment (corticosteroids, chemotherapy, graft rejection prevention, etc.). Thus, fungal infections constitute a major burden for public health as they affect a significant number of people and are associated with a high mortality rate in patients with comorbidities (Brown et al. 2012). Understanding the antifungal immune response and how it is impacted by comorbidities and their respective treatments are crucial to predict, prevent and treat these infections.

In this chapter, we will focus on the immune recognition of fungal pathogen by innate immune cells. The innate immune response is a critical step of antifungal response in order to eliminate the pathogens and to initiate and polarise the $CD4^+$ T helper (Th) adaptive response. It is commonly accepted that Th1 and Th17 adaptive immune responses are protective against fungal pathogens (Romani 2011). Th2 responses favour the production of antibodies, which only have a minor antifungal effect while promoting the development of fungal allergic sensitisation (Romani 2011). Th2 responses have only shown to be protective during *Pneumocystis* spp. infection (Myers et al. 2013). Finally, regulatory $CD4^+$ T cells (Treg) act as a double-edge sword since their immunosuppressive properties prevent an immune response against commensal fungi of the microbiota but can also repress the normal

immune response during invasive infection (Romani 2011). The respective functions of the different Th subsets will not be further discussed but have been recently reviewed (Speakman et al. 2020).

2 Innate Immune Recognition

While T cells require the presentation of microbial antigens by Antigen-Presenting Cells (APCs), cells involved in the innate immune response recognise pathogens either directly or after opsonisation. Pathogen-Associated Molecular Patters (PAMPs) constitute the molecular immunogenic signatures of pathogens, and they are usually essential structural components absent from the host and are directly recognised by the host cells through binding to cellular or soluble expressed Pattern Recognition Receptors (PRRs) (Janeway 1989). Far from being completely separate components, these different effectors collaborate tightly in order to provide fully protective host immunity.

Soluble proteins such as collectins, pentraxins and proteins from the complement system can function as opsonins to facilitate pathogen engulfment by phagocytes and/or as direct fungicidal effectors through recruitment of the complement pathway components (Wong and Aimanianda 2017). Even though some of these soluble factors have been shown to bind to specific components of the fungal cell wall, they will not be further discussed as this chapter will focus on cell membrane expressed PRRs. The recognition of *A. fumigatus* conidia by the soluble PRRs has been reviewed in (Wong and Aimanianda 2017). The functions of the complement and collectins in antifungal immunity have been in reviewed (Speth et al. 2008; Brummer and Stevens 2010).

Most cell types, including leukocytes, express at least one type of the cellular PRRs. There are four main types of cellular PRRs: Toll-Like Receptors (TLRs), C-type Lectin-like Receptors (CLRs), NOD-Like Receptors (NLRs) and RIG-I-Like Receptors (RLRs) (Brubaker et al. 2015). NLRs have been shown to have a major role in the antifungal response; however, their activation seems to be due to the triggering of other PRRs rather than by direct recognition of the pathogen (Gross et al. 2009) and will not be discussed here. Also not further mentioned in this chapter are RLRs, required for immunity against *C. albicans* (Jaeger et al. 2015), although currently the only known ligand for these receptors is viral RNA (Brubaker et al. 2015).

TLRs and CLRs have been extensively studied in the context of fungal infection. In addition, other receptors such as integrins and scavenger receptors also function as PRRs. While some PRRs exert a redundant function during fungal infection, an efficient antifungal immunity requires the cooperation of PRRs. Collaborative triggering of these receptors induces the activation of signalling cascades resulting in engulfment of the pathogen by professional phagocytes, initiation of inflammation and secretion of cytokines and chemokines required for activation and polarisation of the adaptive response.

3 Fungal Cell Wall

The fungal cell wall is an essential element of the fungus and, thanks to its dynamic remodelling, provides protection against exogenous stresses. This armour constitutes a major virulence factor against the host's fungicidal capacity, allows the formation of hyphae and ensures invasion through tissue barriers (Gow et al. 2017). Therefore, the cell wall composition is directly affected by environmental conditions (temperature, pH, nutrients, oxidants, etc.) and the fungal morphology. Indeed, dimorphic fungi can have a fundamentally different cell wall composition, as it is the case with *A. fumigatus* (see below). The biology and structure of the fungal cell wall have been reviewed elsewhere (Erwig and Gow 2016; Gow et al. 2017) and will only be briefly presented here with regard to the four main fungal pathogens.

The fungal cell wall can be divided into two main structures: the inner and outer cell walls. The inner cell wall is composed of chitin (polymers of N-acetylglucosamine) and linear β-glucans (β-1,3-glucans with or without β-1,6-glucans branches) (Erwig and Gow 2016). It is relatively well conserved among all fungi genera and is protected from immune recognition by the outer cell wall. The composition of the outer cell wall is more variable across different fungi, and it contains polysaccharides, proteins and in some cases pigments.

In *C. albicans,* the outer cell wall contains proteins heavily decorated with O-linked, N-linked and phosphorylated mannans, which prevent the immune recognition of the inner cell wall components (Gantner et al. 2005). This layer is thinner at the level of the bud scar resulting from the division of the yeast, rendering PAMPs from the inner cell wall accessible for immune recognition. The fungal morphology (yeast or hyphae) affects the type of proteins found in the outer cell wall and also influences the structure of the inner wall. Yeasts contain a linear form of β-glucan, whereas in hyphae this polysaccharide adopts a cyclic configuration, which directly affects the immune recognition (Lowman et al. 2014).

The infecting form of the *Aspergillus* genus is the conidium, a spore released in the air that enters the lungs during normal breathing (Kosmidis and Denning 2015). Conidia are covered by a hydrophobic layer composed of immunologically inert proteins from the hydrophobin family (Aimanianda et al. 2009). This so-called rodlet layer covers a coat of pigments, melanins, whose exact nature depends on the species: DOPA-melanin, dihydroxynaphthalene-melanin (DHN-melanin) and pyomelanin (Smith and Casadevall 2019). DHN-melanin is the most abundant pigment of the conidia surface of *A. fumigatus* (Smith and Casadevall 2019). When conidia germinate, both the rodlet and the melanin layers are removed, uncovering α-1,3-glucans and β-glucans (Latge et al. 2017). These carbohydrates are also exposed at the surface of hyphae, although partially masked by galactosaminoglycans and galactomannans from the outer cell wall (Erwig and Gow 2016).

The cell wall of *Cryptococcus* spp. is composed of chitin, β-glucans and α-glucans (Erwig and Gow 2016), and DOPA-melanin, which is located within the inner part of the cell wall (Smith and Casadevall 2019). Remarkably, the cell wall

of *Cryptococcus* spp. is covered by a very thick layer of glucuronoxylomannans (GXM) and galactoxylomannans (GalXMs) that constitute the core of these pathogens' capsule (Erwig and Gow 2016). The capsule also contains other saccharides (sialic acid and hyaluronic acid) and mannoproteins (Kwon-Chung et al. 2014). In vivo, *C. neoformans* can form Titan cells, up to 100 μm in size compared to 5–7 μm for yeast cells (Erwig and Gow 2016). These large cells are too big to be internalised by immune cells and contain more chitin and mannose than the yeast form (Mukaremera et al. 2018). The precise effects of these changes in the cell wall composition on immune response are still unclear but it has been proposed that the increased amount of chitin in Titan cells promotes a Th2 adaptive response (Wiesner et al. 2015; Mukaremera et al. 2018).

P. jirovecii exhibits a peculiar life cycle, reminiscent of some parasites, as it occurs obligatory in the mammalian host and specifically within the lungs. The cell cycle of this parasite is still not completely understood, and it is proposed that mating of the trophic form (spore) produces an ascus (also called cyst) which will undergo meiosis and release eight immature trophic forms (ascospores) (Skalski et al. 2015). It is believed that the trophic form is also able to undergo binary scission. Both the trophic forms and asci produce DOPA-melanin and contain high levels of mannosylated glycoproteins, including glycoprotein A (GpA) (Icenhour et al. 2003; Skalski et al. 2015). β-glucans are present in the inner cell wall of asci but absent from the trophic form (Skalski et al. 2015). Chitin has been reported to be produced by trophic forms and asci of rodent-infecting *P. carinii* (Walker et al. 1990), whether *P. jirovecii* is also able to produce this polymer is still unknown.

Hence, the composition of the fungal cell wall is diverse, even though some of its constituents are widely shared across fungal genera (*i.e.* β-glucan, chitin). Many of these components are actually PAMPs and constitute the cracks in the fungal armour. Recognition of the fungal cell wall PAMPs by PRRs is a critical step in antifungal immunity, activating the effectors of the innate immune response and shaping the adaptive response.

4 Toll-like Receptors

The TLR family consists of 10 proteins in human and 12 in mouse, expressed in many cell types including epithelial cells and innate immune cells (Kawai and Akira 2010). TLRs recognise a wide range of bacterial, viral, fungal and parasitic PAMPs and also have endogenous ligands through their typical leucine-rich repeat domain (Kawai and Akira 2010). Binding to a ligand induces the dimerization of the receptors and the initiation of an intracellular signalling cascade through their Toll/IL-1 Receptor (TIR) domain. Although we will focus on TLRs that recognise fungal cell wall PAMPs (TLR2, TLR4 and TLR9), it is notable that fungal nucleic acids also constitute ligands for the intracellular receptors TLR3 and TLR9 that respectively recognise RNA and DNA (Miyazato et al. 2009; Carvalho et al. 2012).

4.1 Signalling Pathways of TLRs

Triggering of TLRs can activate two different main signalling cascades: the Myeloid Differentiation factor 88 (MyD88) pathway and the TIR-domain-containing adapter-inducing Interferon-β (TRIF) pathway (Fig. 1) (Kawai and Akira 2010). The MyD88 pathway is induced by TLR2 and TLR4 from the plasma membrane, whereas TLR9 is not expressed at the cell surface and activates MyD88 from the endosome (Gay et al. 2014). For reasons which are still unclear, TLR4 is also able to signal through TRIF from the endosome once it has been internalised.

Upon ligand binding and dimerization, TLRs intracellularly recruit Mal (Myd88 adapter-like, also known as TIRAP) and MyD88. This adaptor molecule activates the IL-1 Receptor-Associated Kinases IRAK4 and IRAK1 that phosphorylate TNF Receptor-Associated Factor 6 (TRAF6) (Gay et al. 2014). Phosphorylation of TRAF6 allows the dissociation of this protein from the receptor and activation of the TGF-Activated Kinase 1 (TAK1 complex) (Gay et al. 2014). Through its kinase activity, TAK1 activates two different signalling cascades: the Nuclear Factor κB (NFκB) and the Mitogen-Activated Protein Kinase (MAPK) pathways. Typically, the canonical NFκB transcription factor comprises two subunits: RelA (p65) and p50. Its activation is repressed by cytoplasmic protein chaperon Inhibitor of NFκB (IκB) (Mitchell et al. 2016). TAK1 activates the IκB kinase (IKK) complex that causes the phosphorylation and degradation of IκB. Released NFκB translocates into the nucleus and induces the transcription of pro-inflammatory cytokines such as TNFα, IL-6 and IL-12 (Mitchell et al. 2001).

TAK1 is a MAPK Kinase Kinase (MAPKKK), it phosphorylates MAPKKs, which results in the activation of Erk, Jnk and p38 MAPKs (Kawai and Akira 2010). MAPKs contribute to the activation of transcription factor Activator Protein-1 (AP-1), which induces transcription of pro-inflammatory cytokines and enzymes involved in the production of Reactive Nitrogen Species (RNS) such as inducible Nitric Oxide Synthase (iNOS) (Arthur and Ley 2013).

The TRIF pathway is independent from MyD88 but requires the adaptor protein TRIF-Related Adaptator Molecule (TRAM) to be activated by TLRs (Kawai and Akira 2010). Besides, the activation of NFκB, TRIF also interacts with Interferon-Regulatory Factor (IRF) 3 and 7 that then induces the production of type I interferons. Of note, TLR9-mediated activation of TRAF6 also activates IRF7 (Gay et al. 2014). The role of type I interferons in antifungal immunity is not completely understood but several studies have shown a protective effect of these cytokines during infection by *C. albicans* and *C. neoformans* (Biondo et al. 2008, 2011).

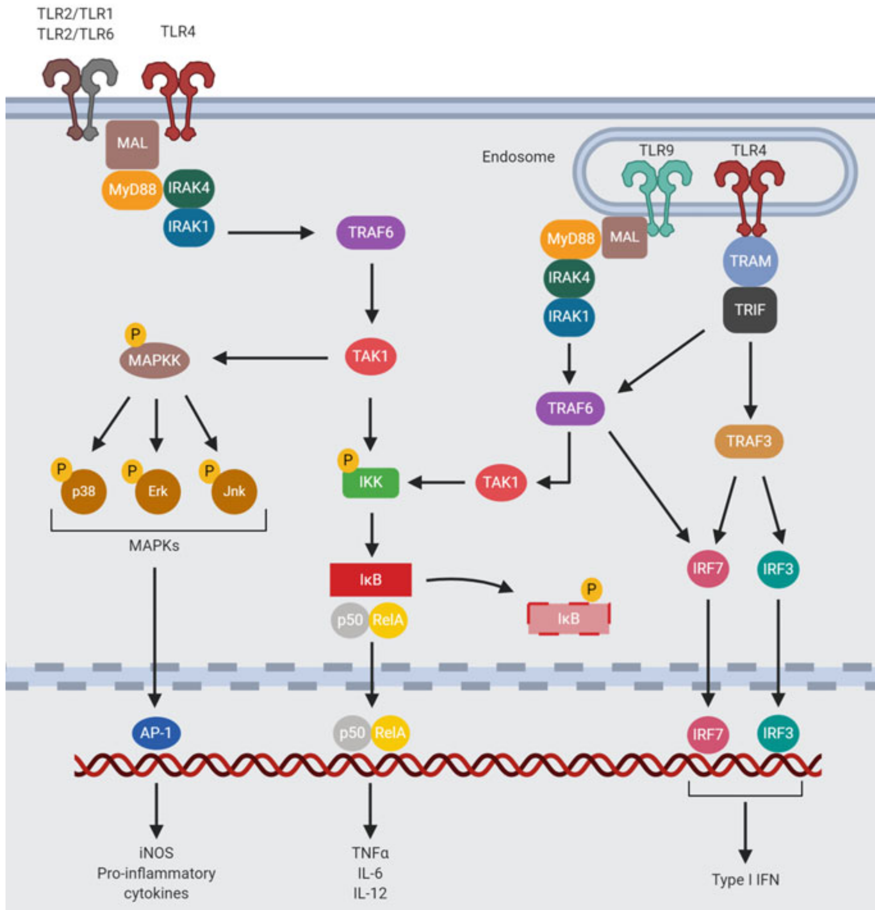

Fig. 1 Signalling pathways triggered by TLR activation. At the plasma membrane, dimerization of TLR2 with TLR1 or TLR6, homodimerization of TLR4, and TLR9 dimers in the endosome activate the MyD88-dependent pathway resulting in TRAF6-mediated activation of TAK1. TAK1 induces the MAPK cascade by phosphorylating MAPKKs that activate in turn p38, Erk and Jnk. This pathway results in the activation of transcription factors such as AP-1 that induces transcription of genes coding for iNOS and pro-inflammatory cytokines. TAK1 also activates IKK, which causes the degradation of chaperone IκB by phosphorylation and the subsequent release of NFκB, which then translocates into the nucleus and is responsible for transcription of pro-inflammatory cytokine-encoding genes. The TRIF pathway is induced by the recruitment of TRAM to internalised TLR4. TRIF activates TRAF6 and TRAF3, which contribute to the TAK1-mediated NFκB activation pathway and also activation of IRF3 and IRF7 that then induce the transcription of type I interferons

4.2 TLRs and Fungal Infections

The overall role of TLRs in antifungal immunity has been debated since their discovery. For instance, several authors disagreed about the respective roles of TLR2 and TLR4 during *C. albicans* systemic infections. A study eventually suggested that the contribution of TLR4 to inflammation was dependent on the strain of the pathogen studied (Netea et al. 2010). An explanation for this observation is the strain-dependent relative proportions of the different cell wall components (Cavalieri et al. 2017), which adds a layer of complexity in deciphering the role of PRRs during fungal infections.

In addition, there are major differences in the functions of TLRs between human and mice. While deficiency for MyD88 in mice is associated with increased susceptibility to a wide range of pathogens, including fungi (Bellocchio et al. 2004; von Bernuth et al. 2008), patients with a deficiency for this protein (or IRAK4) are not more susceptible to fungal infections (Picard et al. 2003; von Bernuth et al. 2008). On the other hand, single nucleotides polymorphisms (SNPs) in genes encoding for TLRs have been associated with increased risk to develop some fungal infections, reviewed in (Campos et al. 2019). The biological consequences of these SNPs are not always understood, even though the effects on fungal infection susceptibility suggest an overall non-redundant role for TLRs in antifungal immunity.

4.2.1 TLR2

TLR2 forms heterodimers with TLR1 or TLR6 to transduce signals, it recognises fungal pathogens mainly through binding of mannosylated PAMPs (Oliveira-Nascimento et al. 2012). While the first fungal ligand of TLR2 identified was phosphomannan, present at the cell wall surface of *C. albicans* yeasts (Jouault et al. 2003), this receptor also recognises mannosylated cell wall components from other fungi. More recently, chitin was identified as a ligand for TLR2 (Wagener et al. 2014; Fuchs et al. 2018). Chitin requires a minimum of 6 N-acetylglucosamine moieties to induce a pro-inflammatory response through TLR2-TLR1 dimers (Fuchs et al. 2018). There is some controversy about TLR2 binding chitin, as Becker and colleagues could not identify any specific interaction with PRRs and only found immunoglobulins to interact with this polymer (Becker et al. 2016).

TLR2 is a major receptor for anti-*Candida* immunity at the mucosal level. In mice, TLR2 deficiency has been associated with increased susceptibility to colonisation of the gastrointestinal (GI) (Prieto et al. 2016) and reproductive (Miro et al. 2018) tracts, and animals exhibit a local excessive inflammation in response to the pathogen (Miro et al. 2018). During systemic infection, TLR2 contributes to IL-10 production by APCs, which favours the polarisation of Th cells into Treg (Netea et al. 2004). Tregs regulate inflammation, and this might be the reason why some authors have reported an increased resistance of TLR2-deficient mice compared to wild-type (WT) animals (Bellocchio et al. 2004; Netea et al. 2010, 2004).

A human polymorphism of *TLR2* has been associated with recurrent vulvovaginal candidiasis (RVVC) (Rosentul et al. 2014).

TLR2 recognises α-1,3-glucans from the cell wall of *A. fumigatus* (Stephen-Victor et al. 2017) and the deletion of this receptor leads to increased susceptibility to pulmonary infection in immunocompromised mice (Bellocchio et al. 2004; Balloy et al. 2005). While the dimerization of TLR2 with either TLR1 or TLR6 does not have obvious consequences on intracellular signalling pathways in vitro (Farhat et al. 2008), these receptors appear to be non-redundant in vivo as it was shown that TLR6-deficient mice are more prone to airway hyper-responsiveness during an *A. fumigatus* allergic sensitisation model (Moreira et al. 2011). In humans, SNPs in either *TLR1* or *TLR6*, but not in *TLR2,* are associated with risks of invasive aspergillosis (Kesh et al. 2005).

The binding of TLR2 to purified GXMs from the capsule of *C. neoformans* (Fonseca et al. 2010) requires collaboration with CD14 (Yauch et al. 2004). Although the TLR signalling molecule MyD88 is required in mouse for protection against *C. neoformans*, individual TLRs or CD14 knock-out mice are not as susceptible, showing receptor collaboration is necessary for full immunity (Yauch et al. 2004).

The role of TLR2 in anti-*Pneumocystis* immune response is not well understood yet. Mice studies using *P. murina*, the species responsible for mouse infection, showed that this pathogen is able to activate TLR2 but the ligand is unknown (Zhang et al. 2006). Deficiency in TLR2 increases the susceptibility to *P. murina* (Wang et al. 2008). However, only alveolar macrophages but not epithelial cells appear to require TLR2 to mount an inflammatory response (Zhang et al. 2006; Bello-Irizarry et al. 2012).

4.2.2 TLR4

TLR4 collaborates with CD14 to bind O-linked mannans and mannosylated proteins from *C. albicans* cell wall but the precise biologically active structure of these PAMPs is still unknown (Tada et al. 2002; Netea et al. 2006; Pietrella et al. 2006). In vitro, the inflammatory response mounted by murine macrophages and human monocytes against *C. albicans* yeasts requires TLR4, while this receptor appears to play only a minor role against hyphal forms (Tada et al. 2002; van der Graaf et al. 2005). The role of TLR4 is not only affected by the fungal strain used (Netea et al. 2010) but also depends on the host cell type studied: TLR4 deficiency partially impairs the macrophage anti-*C. albicans* reponse, whereas it does affect neutrophils. To date, there is no human polymorphism of *TLR4* associated with increased susceptibility to candidiasis (Rosentul et al. 2014).

TLR4 recognises the mannans from *A. fumigatus* conidia and swollen conidia but its ligand is thought to be masked at the surface of hyphae (Mambula et al. 2002; Netea et al. 2003). TLR4 is required to ensure protection in immunosuppressed mice (Bellocchio et al. 2004), although it is dispensable in immunocompetent mice

(Dubourdeau et al. 2006). Several SNPs of this receptor have been associated with increased susceptibility to invasive aspergillosis in patients following allogenic hematopoietic-stem cell transplantation regardless of whether the mutation was carried by the donor or the recipient (Bochud et al. 2008; Koldehoff et al. 2013).

TLR4 is activated by GXMs from the capsule of *C. neoformans* (Shoham et al. 2001; Monari et al. 2005). Yet, GXMs do not induce the secretion of pro-inflammatory cytokines, even though NFκB translocates into the nucleus (Shoham et al. 2001). The role of TLR4 during cryptococcal infection seems to be only minor in mouse models (Yauch et al. 2004; Nakamura et al. 2006).

There is no known *Pneumocystis* spp. ligand for TLR4. The reports regarding the function of TLR4 on the response of alveolar macrophages towards *P. murina* in vitro are contradictory (Ding et al. 2005; Zhang et al. 2006), and it does not affect the response of alveolar epithelial cells (Bello-Irizarry et al. 2012). During infection by *P. murina*, the absence of TLR4 only has a minor effect on inflammation and does not affect the fungal burden (Ding et al. 2005).

4.2.3 TLR9

TLR9 is an intracellular receptor that can be trafficked to the phagosome where it recognises its ligand(s). In addition to recognising fungal DNA (Miyazato et al. 2009), TLR9 recognises chitin (Wagener et al. 2014). Internalisation of chitin by the mannose receptor (MR) allows the binding of this PAMP to TLR9, which then induces IL-10 secretion (Wagener et al. 2014). It has been proposed that the effects of chitin on the inflammatory response depend on the size and the concentration of this polysaccharide. Low concentrations of chitin and chitin particles of small size (less than 40 μm) induce IL-10 through TLR9 and the MR (Da Silva et al. 2009; Wagener et al. 2014). In contrast, high concentrations of chitin and chitin particles of intermediate size (40–70 μm) mainly induce the pro-inflammatory cytokine TNFα in a TLR2- and Dectin-1-dependent manner (Da Silva et al. 2009; Wagener et al. 2014).

TLR9 negatively regulates pro-inflammatory cytokine secretion by murine macrophages and human blood leukocytes in response to *C. albicans* (van de Veerdonk et al. 2009; Kasperkovitz et al. 2011), and it also decreases their fungicidal abilities (Kasperkovitz et al. 2011). In vivo, TLR9 seems to be redundant as the deletion of this receptor does not increase the susceptibility to systemic candidiasis (van de Veerdonk et al. 2009). On the contrary, some authors have reported enhanced control of infection by TLR9-deficient animals compared to the WT (Bellocchio et al. 2004).

TLR9 is a major PRR for anti-*A. fumigatus* immune response. The phagocytosis of *A. fumigatus* conidia induces the activation of TLR9, which contributes to activate the Nuclear Factor of Activated T cells (NFAT) (Herbst et al. 2015). NFAT induces the production of pro-inflammatory cytokines and chemokines required for neutrophils recruitment in the lungs (Herbst et al. 2015). TLR9 exerts a protective effect against invasive aspergillosis in immunocompromised mice but favours the

development of fungal sensitisation (Ramaprakash et al. 2009). In human, this receptor also protects against invasive infection and TLR9 SNPs have been associated with higher susceptibility to fungal allergic conditions (Carvalho et al. 2008; Overton et al. 2017).

TLR9 is involved in mounting the inflammatory response against *C. neoformans*, activation of this PRR induces IL-12 and induces the recruitment of leukocytes in the lungs (Nakamura et al. 2008; Wang et al. 2011, Qiu et al. 2012). TLR9-deficient mice are more susceptible to infection than WT counterparts (Nakamura et al. 2008; Wang et al. 2011) but the role of this receptor in humans is unexplored. TLR9 has not been studied in the context of *P. jirovecii* infection.

5 C-Type Lectins-like Receptors (CLRs)

Proteins containing a C-Type Lectin-like Domain (CTLD) constitute a superfamily of transmembrane or secreted glycoproteins, divided into 17 groups (grouped from I to XVII) (Zelensky and Gready 2005). The CTLD has a conserved structure and is responsible for binding ligand(s). To be noted, in addition to the participation in the antifungal immune response, some CLRs can bind ligands from bacteria, viruses, parasites as well as self-ligands or Damage-Associated Molecular Patterns (DAMPs) from the mammalian host and exert physiological or pathological roles (Brown et al. 2018).

The transmembrane expressed CLRs involved in antifungal immunity belong to three different groups of CTLD-containing proteins. The MR is a type I-transmembrane protein that belongs to group VI, while CLRs from the groups II (DC-SIGN, MCL, Dectin-2, Mincle, Langerin, CD23) and V (Dectin-1, MelLec) are type II-transmembrane receptors (Zelensky and Gready 2005).

5.1 Signalling Through the CLRs

CLRs primarily signal through two main pathways: the spleen tyrosine kinase pathway (Syk) and the Ras-Raf pathway. Upon binding of their respective ligands, Dectin-1, Dectin-2, MCL, Mincle, CD23 and MR recruit Syk either directly or after coupling with Fc receptor γ chain (FcRγ) (Sancho and Reis e Sousa 2012). Two CLRs are known for activating the Ras-Raf pathway: DC-SIGN and Dectin-1. The signalling cascades induced by MelLec and Langerin are not known.

5.1.1 Syk pathway

Activation of Syk by Dectin-1, Dectin-2, MCL, Mincle, CD23 and MR leads to the activation of several transcription factors such as NFκB, AP-1 and NFAT (Fig. 2)

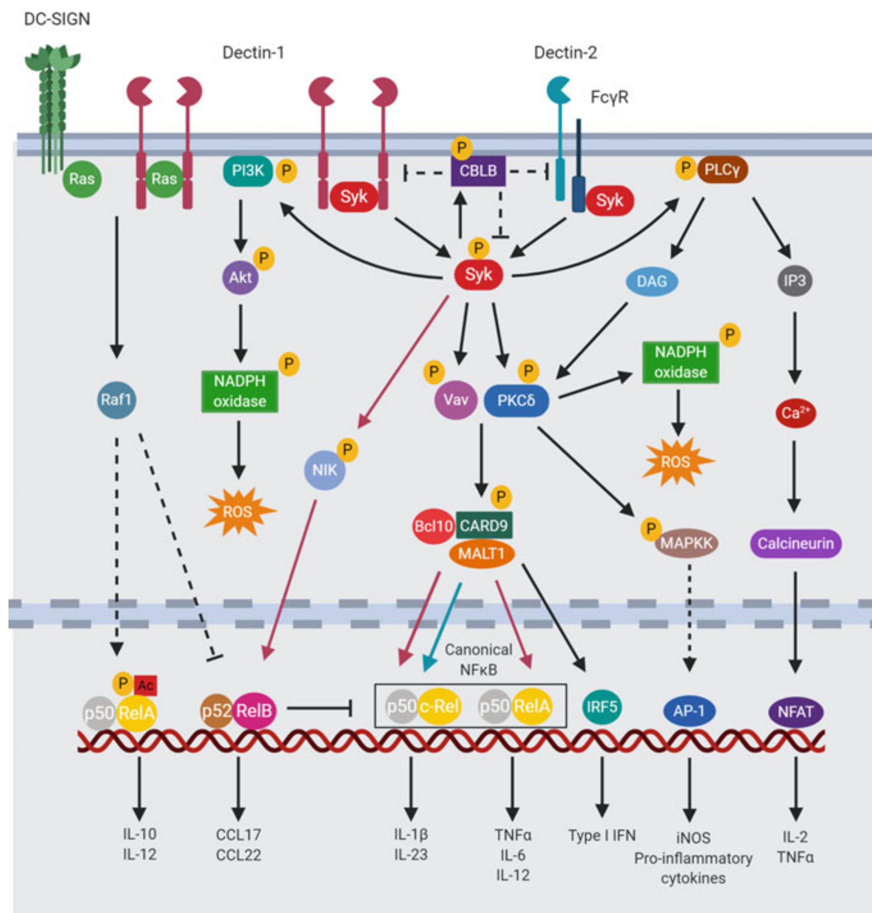

Fig. 2 Signalling pathways triggered by CLR activation in a DC. CLRs associate with Syk, binding to the hemITAM of Dectin-1 or through their association with FcRγ (complexed with Dectin-2, Mincle, MR or MCL). Syk phosphorylates a wide range of targets, including PI3K that induces Akt-mediated NADPH oxidase-mediated ROS production. Syk-mediated activation of PLCγ induces the production of DAG and IP3 through the degradation of PIP2. IP3 induces the release of calcium from the endoplasmic reticulum, which activates calcineurin and results in translocation of transcription factor NFAT, responsible for the production of IL-2. Activation for PKCδ by Syk induces the NADPH oxidase-mediated ROS production and the activation of AP-1 through the MAPK pathway. Syk activates Vav and PKCδ, that induce the formation of the CARD9-Bcl10-MALT1 (CBM) complex Dectin-1 induces activation of both RelA and c-Rel canonical NFκB (red arrows), while Dectin-2 only activates c-Rel through MALT1 (blue arrow), which is responsible for the induction of the transcription of Th17-polarising cytokines IL-1β and IL23. Dectin-1 also induces the activation of NIK which activates the non-canonical NFκB RelB, which limits the action of the canonical NFκB. The CBM complex also induces the translocation of IRF5 into the nucleus, which induces the transcription of type I interferon. CBLB regulates the activation of the Syk pathway by targeting Dectin-1, Dectin-2 and Syk to the proteasome for degradation. DC-SIGN and Dectin-1 are able to trigger the Ras-Raf1 pathway that results in post-translational modifications of RelA and inhibition of RelB. Acetylation of RelA increases the production of IL-10, IL-12 and IL-23

(Rogers et al. 2005; Sato et al. 2006; Strasser et al. 2012). Dectin-2, MCL, Mincle, MR and CD23 are associated with FcRγ at the cell membrane (Robinson et al. 2009; Kerscher et al. 2016; Rajaram et al. 2017; Guo et al. 2018). The binding of their respective ligands by these CLRs induces the phosphorylation of an amino acid consensus sequence—called Immunereceptor Tyrosine-based Activation Motif (ITAM)—located in the cytoplasmic tail of FcRγ and that is responsible for Syk recruitment and activation (Sancho and Reis e Sousa 2012). In contrast, Dectin-1 does not require FcRγ: this receptor possesses an incomplete ITAM motif (hemITAM) in its cytoplasmic tail that is able to recruit Syk in a SH2-dependent manner (Rogers et al. 2005; Deng et al. 2015). The activation of Syk by Dectin-1 involves the clustering of this receptor at the point of contact with the pathogen, which is termed the *phagocytic synapse* (Goodridge et al. 2011).

Through phosphorylation of Vav proteins and Protein Kinase Cδ (PKCδ), Syk induces the formation of the CBM (CARD9-Bcl10-MALT1) complex (Gross et al. 2006; Lanternier et al. 2013; Roth et al. 2016). The CBM complex activates IRF5, which mediates the transcription of type I interferons (del Fresno et al. 2013). The CBM also activates the canonical NFκB family members RelA and c-Rel (Gringhuis et al. 2009; Gringhuis et al. 2011). NFκB then translocates to the nucleus and induces high-level transcription of Th1- and Th17-polarising cytokines: IL-12, and IL-1β and IL-23, respectively (Gringhuis et al. 2009; 2011). The CBM complex also activates the NFκB-inducing kinase (NIK), which activates the non-canonical NFκB RelB that represses the activity of canonical NFκB and induces the transcription of Treg-attracting chemokines (Gringhuis et al. 2011).

PKCδ also activates the NADPH oxidase and thus induces Reactive Oxygen Species (ROS) generation in macrophages and neutrophils (Li et al. 2016). ROS production and fungal killing by neutrophils require PKCδ activation (Li et al. 2016), while in monocytes the NADPH oxidase is also activated by another Syk-mediated pathway involving the Phosphatidylinositol-3 Kinase (PI3K) and Akt (Shah et al. 2009; Camilli et al. 2018). Even though PKCδ also contributes to ROS production in macrophages, fungal killing depends on the activation of CARD9 and is not affected by the absence of PKCδ, suggesting that macrophages mostly rely on non-oxidative mechanisms to clear fungi (Li et al. 2016).

Syk also activates the Phospholipase Cγ (PLCγ), which produces inositol triphosphate (IP3) and diacylglycerol (DAG) from phosphoinositide-bisphosphate (PIP2) (Lowell 2011). PLCγ is required to induce the MAPK pathway (Tassi et al. 2009; Xu et al. 2009), a known result of DAG production (Lowell 2011). On the other hand, IP3 is responsible for the release of calcium from intracellular stocks in the endoplasmic reticulum, which then activates calcineurin that subsequently activates NFAT (Lowell 2011). This transcription factor is involved in the transcription of IL-2, required for T cell activation and proliferation. The major role of this signalling pathway was further demonstrated by showing that PLCγ-deficient DCs were unable to activate T cells in vitro (Tassi et al. 2009).

The Syk pathway exerts a self-regulating control in time through the activation of ubiquitin ligase Casitas B Lymphoma B (CBLB). The activation of CBLB induces the ubiquitinylation of Dectin-1, Dectin-2 and Syk, resulting in the

degradation of these proteins in the proteasome and thus reducing the antifungal action of macrophages and DCs but not neutrophils (Wirnsberger et al. 2016; Xiao et al. 2016). Inhibition of CBLB in mice has been shown to protect animals infected with a lethal inoculum of *C. albicans* (Wirnsberger et al. 2016), highlighting the major role of this regulation pathway and its potential therapeutic applications.

5.1.2 Ras-Raf Pathway

Dectin-1 and DC-SIGN are both able to activate the small guanosine triphosphatase (GTPase) Ras (Gringhuis et al. 2007; Gringhuis et al. 2009) that in turn activates Raf-1 (den Dunnen et al. 2009). Raf-1 modulates the immune response by inducing post-translational modification (phosphorylation and acetylation) of the NFκB RelA subunit (den Dunnen et al. 2009). Acetylation of NFκB promotes the transcription of IL-10 (Gringhuis et al. 2007). On the other hand, phosphorylation of RelA represses the nuclear translocation of RelB into the nucleus (Gringhuis et al. 2009). Thus, by counterbalancing the Syk-dependent activation of the non-canonical NFκB, the Ras-Raf-1 pathway contributes to amplify the synthesis of IL-1β, IL-12 and IL-23 that are required for Th1 and Th17 polarisation (Gringhuis et al. 2009).

5.2 CLRs and Fungal Infections

CLRs orchestrate the antifungal immune response via collaboration with other PRRs, such as TLRs. For instance, stimulation with only Dectin-1 specific ligands does not induce potent inflammation (Gantner et al. 2003), except in the case of binding to a non-phagocytosed ligand (Hernanz-Falcon et al. 2009). Yet, when a TLR is simultaneously triggered, Dectin-1-mediated activation of Syk induces the degradation of IκB, which potentiates the signal initiated by TLRs and results in a synergic effect on the secretion of pro-inflammatory cytokines (Gantner et al. 2003; Dennehy et al. 2008). CLRs also collaborate with each other to mount the inflammatory response to fungal pathogens (Thompson et al. 2019) and to polarise the adaptive immune response. Through activation of all three members of the NFκB family (RelA, RelB and c-Rel), Dectin-1 promotes both the Th1 and Th17 polarisation of the adaptive immune response and exerts a broad antifungal effect (Gringhuis et al. 2009, 2011). In contrast, Dectin-2 has been reported to favour the differentiation of Th17 rather than Th1 (Robinson et al. 2009). Secretion of Th17-polarising cytokines IL-1β and IL-23 following CLR activation seems to be completely dependent on c-Rel, which is selectively activated by Dectin-2 for unclear reasons (Gringhuis et al. 2011).

The major function of the CLR family in the antifungal immunity lies in the activation of CARD9, whose deficiency predisposes patients to fungal but not to other types of infection (Drummond et al. 2016). SNPs in the gene encoding for this protein impair fungal killing by granulocytes and the polarisation of Th17 cells

(Drewniak et al. 2013). In mouse, CARD9 deficiency increases the susceptibility to chronic mucocutaneous candidiasis (CMC) and to central nervous system (CNS) infection by *C. albicans* and by *A. fumigatus* (Glocker et al. 2009; Jhingran et al. 2012; Drummond et al. 2019). In the brain, deficiency for CARD9 prevents neutrophil recruitment and fungal clearance (Drummond et al. 2019). Interestingly, mice deficient for a single CLR did not recapitulate this phenotype, suggesting a redundant role for these receptors and stressing the importance of PRRs collaboration (Drummond et al. 2019). CARD9 SNPs in patients predispose them to CNS infection by *C. albicans* and mucocutaneous infections by a range of fungal pathogens, including *Candida* spp. (Drummond et al. 2016). The other members of the CBM complex, Bcl10 and MALT1, have also been associated with increased risk of CMC in human but not in mouse (Drummond et al. 2016); however, these deficiencies are less specific to antifungal immunity as they also predispose humans to infection by viral and bacterial pathogens (Drummond et al. 2016).

5.2.1 Dectin-1 (CLEC7A)

Dectin-1 binds β-1,3-glucans and β-1,6-glucans (Brown 2006; Brown and Gordon 2001) and is predominantly expressed by leukocytes, including some subtypes of T cells (Brown 2006). Binding of Dectin-1 to particulate β-glucan induces a rearrangement of the cell membrane and the clustering of the receptor at the phagocytic synapse and internalisation of the particle (Goodridge et al. 2011). Interestingly, the ability of Dectin-1 to engulf its ligand has a significant impact on the cellular response: frustrated phagocytosis, the inability to internalise a particle due to its size or pharmacological inhibition, induces a more potent Dectin-1-mediated inflammation than smaller sized, phagocytosable particles (Hernanz-Falcon et al. 2009). It is proposed that this phenomenon is due to the lack of Dectin-1 signal attenuation by internalisation of this receptor (Hernanz-Falcon et al. 2009). As already mentioned above, when the ligand is phagocytosable, triggering of Dectin-1 alone does not induce secretion of pro-inflammatory cytokines, yet costimulation with a TLR agonist potentiates the activation of NFκB and the production of cytokines (Brown et al. 2003; Gantner et al. 2003).

Another phenomenon regulated by the pathogen size is the release of neutrophil extracellular traps (NETs) (Branzk et al. 2014; Papayannopoulos 2018). Dectin-1 mediated uptake of the pathogen induces the delivery of neutrophil elastase into the phagolysosome (Branzk et al. 2014). When neutrophils encounter hyphae too large to be engulfed, elastase is delivered into the nucleus instead and induces the release of NETs (Branzk et al. 2014), which have been shown to contribute in vitro to the killing of *C. albicans* but not of *A. fumigatus* (Urban et al. 2006; Gazendam et al. 2016).

After uptake of the pathogen by APCs, Dectin-1 controls the acidification and the maturation of the phagosome (Mansour et al. 2013) and recruits MHC-II to facilitate the presentation of fungal antigens to T cells by macrophages and DCs (Ma et al. 2012).

In addition of the protective effects of Dectin-1 activation on innate and adaptive immunity, β-glucan-mediated triggering of Dectin-1 is able to imprint mononuclear phagocytes in a metabolic and epigenetic manner, rendering them more responsive to a secondary stimulation (Ifrim et al. 2013; Cheng et al. 2014; Saeed et al. 2014). This phenomenon, termed *trained immunity*, is independent of the presence of lymphocytes and has been shown to provide a long-term non-specific immunity (Quintin et al. 2012). The benefits of trained immunity during fungal infection still have to be demonstrated in human.

In *C. albicans*, β-glucans are exposed at the level of the bud scar on the yeast. Hyphae are protected from Dectin-1 recognition in vitro due to the mannan layer (Gantner et al. 2005) but β-glucans appear to be exposed in vivo a few days after infection (Wheeler et al. 2008). As Dectin-1 induces a more potent inflammatory response when its ligand is too large to be phagocytosed, it is appealing to suggest that Dectin-1 response supports discrimination between yeast/conidia and larger hyphal structures. Another factor that might be able to participate in the ability of host cells to differentiate between yeasts and hyphae is their respective cell wall composition. Indeed, β-glucans from *C. albicans* hyphae possess 2,3-linkages and adopt a cyclic conformation. As a result, they are more potent inducers of pro-inflammatory cytokines than branched linear β-glucans of *C. albicans* yeast (Lowman et al. 2014). Thus, more studies are required to fully establish how yeasts and hyphae are differentially recognised by the host. In vivo, Dectin-1 is required to control systemic infection by *C. albicans* and the absence of this receptor leads to decreased secretion of pro-inflammatory cytokines and consequently impaired recruitment of leukocytes to infected tissues (Taylor et al. 2007). Importantly, while the pathogen strain does not affect the role of Dectin-1 in vitro, it has been shown that the importance of Dectin-1 in vivo is directly affected by the *C. albicans* strain (Marakalala et al. 2013). Dectin-1 is a major PRR to control oral and genital mucosal infection by *C. albicans*; it is also required to control the colonisation of the GI tract after during systemic infection but not during gavage of animals (Taylor et al. 2007; Vautier et al. 2012). Dectin-1 deficiency does not affect $CD8^+$ cells but impairs the activation of $CD4^+$ T cells, which are required to control GI tract infection (Drummond et al. 2016). Several SNPs in human *CLEC7A* have been associated with increased risk of RVVC and oropharyngeal candidiasis (Campos et al. 2019).

In *A. fumigatus*, exposure of β-glucan is stage-specific: this PAMP is masked on the resting conidia but is uncovered when the spores start to swell and germinate (Steele et al. 2005); it is also accessible on hyphae (Gersuk et al. 2006). Thus, recognition of *A. fumigatus* spores by Dectin-1 occurs once β-glucan becomes exposed by conidia swelling extracellularly or when in the phagosome (Faro-Trindade et al. 2012). Dectin-1 is required in vivo to mount the inflammatory response against *A. fumigatus* and to recruit neutrophils to prevent lungs colonisation by the fungus (Werner et al. 2009). Several SNPs in the human gene encoding for this receptor are associated with higher susceptibility to invasive aspergillosis in patients undergoing chemotherapy or hematopoietic stem cell transplantation (Sainz et al. 2012).

Dectin-1 is thought to be involved in *C. neoformans* uptake in collaboration with Dectin-2 (Lim et al. 2018). However, deficiency for Dectin-1 does not increase mice susceptibility to infection by this fungus (Nakamura et al. 2007) and its role in humans has not been explored.

Recognition of *Pneumocystis* spp. by Dectin-1 induces the internalisation and killing in alveolar macrophages and induces the production of ROS (Steele et al. 2003; Saijo et al. 2007). However, Dectin-1 does not affect the secretion of TNFα and IL-12 in response to *Pneumocystis* spp., instead the release of these cytokines requires signalling through MyD88 and suggests involvement of TLRs (Saijo et al. 2007). Dectin-1 deficiency does not impair the clearance of *P. carinii* in mouse but it delays the elimination of the fungus from the airways (Saijo et al. 2007).

5.2.2 MelLec (CLEC1A)

Melanin-sensing C-type lectin (MelLec) binds DHN-melanin that covers the spores under the rodlet layer of *A. fumigatus* (Stappers et al. 2018). MelLec is not able to bind to DOPA-melanin, produced by other fungal pathogens, including *Cryptococcus* spp. (Stappers et al. 2018; Smith and Casadevall 2019). As of today, the role of MelLec has only been studied in the context of *A. fumigatus* infection. In mouse, MelLec has been shown to be expressed by endothelial cells in several tissues and also by an epithelial cell subpopulation in the lungs and liver but not by leukocytes (Stappers et al. 2018). Following challenge with *A. fumigatus* conidia, MelLec is required for early leukocytes recruitment in the lungs of mice but its signalling pathway is still unknown (Stappers et al. 2018). MelLec has been shown to inhibit Th17 polarisation in a rat heart transplantation model (Thebault et al. 2009) but whether it can affect the adaptive immune response to fungi remains to be determined.

In humans, this receptor is expressed by endothelial cells and leukocytes, including monocytes, dendritic cells and granulocytes, but not by lymphocytes (Sattler et al. 2012). A SNP in the human gene has been associated with higher risk to develop invasive aspergillosis following hematopoietic stem cell transplantation if the donor carries the mutation, independently of the allele carried by the recipient (Stappers et al. 2018).

5.2.3 Dectin-2 (CLEC6A)

Dectin-2 recognises α-1,2-mannobiose structures (disaccharide constituted of 2 mannoses) present at the surface of the fungal cell wall (McGreal et al. 2006; Feinberg et al. 2017). This receptor is constitutively expressed by monocytes, dendritic cells and macrophages, but expression in neutrophils occurs only after stimulation by pro-inflammatory cytokines (Taylor et al. 2014). A splicing variant of Dectin-2, devoid of most the transmembrane and the intracellular domains (isoform β), thus unable to induce the signalling cascade following triggering has

been identified (Gavino et al. 2005). Interestingly, during the encounter between macrophages and *C. albicans* there is a shift in expression of the full-length Dectin-2 (isoform α) towards the spliced isoform β, which might constitute a post-transcriptional mechanism to regulate the antifungal immune response (Munoz et al. 2019).

This receptor is able to bind to *C. albicans* yeast and hyphae and requires cooperation with FcRγ to transduce signals (Sato et al. 2006; Saijo et al. 2010). Several studies have reported a preferential binding to hyphae even though it is still unclear whether this phenotype is due to a difference in the nature or the proportion of ligand(s) (Sato et al. 2006; Robinson et al. 2009; Saijo et al. 2010). The role of Dectin-2 seems to be quite minor during disseminated candidiasis as this CLR is poorly involved in the internalisation and the killing of the yeast (Ifrim et al. 2016) and does not improve mouse survival in the short term (Robinson et al. 2009; Ifrim et al. 2016). However, when the infection is prolonged, Dectin-2 deficiency impairs the clearance of the fungal burden in the kidneys and the survival of the animals (Saijo et al. 2010; Ifrim et al. 2016). This phenotype might be explained by the major role that Dectin-2 plays in polarising the T cell response towards Th17 response (Robinson et al. 2009; Saijo et al. 2010), yet there are no known Dectin-2 human polymorphisms associated with an increased susceptibility to candidiasis.

Dectin-2 has been shown to be involved in the anti-*A. fumigatus* immune response; however, its ligand seems to be masked by the rodlet layer (Carrion Sde et al. 2013). Thus, Dectin-2 might only be able to interact with *A. fumigatus* during swelling and germination as the outer layers are lost (Sun et al. 2014; Loures et al. 2015). Activation of Dectin-2 contributes directly to the elimination of the fungus by increasing the ability of cells to produce ROS (Taylor et al. 2014) and secrete pro-inflammatory cytokines (Sun et al. 2014; Loures et al. 2015).

The exact component of *C. neoformans* recognised by Dectin-2 has not been clearly identified and only *C. neoformans* lysate but not the whole yeast has been reported to activate Dectin-2, suggesting that the ligand is masked by the capsule (Nakamura et al. 2015). This hypothesis is supported by another study showing that Dectin-2 is able to bind a strain of *C. neoformans* devoid of the capsule (McGreal et al. 2006). Nevertheless, several authors have reported a role for Dectin-2 in the internalisation of *C. neoformans* in collaboration with Dectin-1 (Walsh et al. 2017; Lim et al. 2018). The Th17-polarising function of Dectin-2 was confirmed in vivo as mice devoid for Dectin-2 exhibit an aberrant Th2 response when challenged with *C. neoformans*, although they have similar clearance of fungus from the airways (Nakamura et al. 2015). A human SNP of *CLEC6A* has been associated with increased risk of pulmonary cryptococcosis (Hu et al. 2015).

It has been shown that Dectin-2 is able to bind GpA from the cell wall of *Pneumocystis* spp. (Kottom et al. 2018, 2019). In vitro, deficiency for Dectin-2 impairs the inflammatory response mounted by alveolar macrophages incubated with *P. murina* cell wall extract (Kottom et al. 2018). However, the absence of this receptor appears not to affect the fungal clearance by mice (Kottom et al. 2018).

5.2.4 MCL (CLEC4D, CLECSF8, Dectin-3)

The Macrophage C-type Lectin (MCL) is expressed by neutrophils, monocytes and monocytes-derived macrophages and DCs (Graham et al. 2012; Kerscher et al. 2016). Some cell types, including alveolar macrophages, do not express MCL at basal level but surface expression of this receptor is upregulated upon stimulation with microbial components (Kerscher et al. 2016). Interestingly, MCL and Mincle, another group II CLR, require each other and FcRγ for surface expression in mouse (Kerscher et al. 2016). However, in human, it appears that MCL is not required for Mincle expression (Wevers et al. 2014).

MCL can bind α-mannans in the cell wall of *C. albicans* hyphae and does so more efficiently as a heterodimer with Dectin-2 (Zhu et al. 2013). MCL-deficient mice are less resistant to low-dose infection with *C. albicans* than WT animals (Zhu et al. 2013). On the other hand, in the case of infection with a high-dose inoculum, this receptor might only have a minor role (Graham et al. 2012). Thus, the in vivo function of MCL is not fully resolved and its role in human antifungal response is unexplored.

The role of MCL during cryptococcal infection also remains unclear as two studies from the same group have successively obtained contradictory results on the ability of this receptor to trigger uptake of the *C. neoformans* and anti-cryptococcal activity by DCs (Hole et al. 2016; Campuzano et al. 2017). However, MCL deficiency does not affect mice survival upon *C. neoformans* infection (Hole et al. 2016; Campuzano et al. 2017). MCL ligand on *C. neoformans* is not known, and there is no data on the role of this receptor in anti-*A. fumigatus* or anti-*P. jirovecii* immune response.

5.2.5 Mincle (CLEC4E, CLECSF9)

Macrophage-inducible C-type lectin (Mincle) is a CLR expressed by monocytes, macrophages, DCs and neutrophils. This receptor requires FcRγ and MCL to be expressed at the cell surface, and its expression is upregulated when cells are stimulated with microbial compounds (Kerscher et al. 2016).

Mincle binds *C. albicans* (Bugarcic et al. 2008) and participates in the uptake and killing by murine macrophages (Haider et al. 2019). In mouse, Mincle improves the clearance of *C. albicans* in the kidney but has no effect on the overall mouse survival following infection (Wells et al. 2008; Thompson et al. 2019). Mincle represses Dectin-1-dependent secretion of IL-2 and induction of Th1 cells in vitro (Wevers et al. 2014). Nevertheless, Mincle deficiency does not affect the mortality rate of mice in a systemic *C. albicans* infection model (Thompson et al. 2019). The role of Mincle in humans is not completely understood as one study showed that Mincle does not bind to *C. albicans* and another suggested that Mincle expression induces the phagocytosis and killing of this fungus by neutrophils but not by monocytes (Vijayan et al. 2012). Thus, more studies are required to decipher the full role of this receptor in candidiasis.

Regarding the role of Mincle in aspergillosis, this receptor has only been studied in a mouse model of fungal keratitis, an infection of the cornea that can result in blindness (Yu et al. 2018). Mincle contributes towards the inflammatory response against *A. fumigatus* in the cornea and impairment of this receptor worsens the infection (Yu et al. 2018).

GpA recognition by Mincle allows the binding of *Pneumocystis* spp. by macrophages (Kottom et al. 2017, 2019). In mouse, deficiency for Mincle has only a minor effect on the mortality of animals during the early stages of infection and it partially impairs the fungal burden (Kottom et al. 2017). Compared to WT animals, Mincle-deficient mice exhibit higher levels of pro-inflammatory cytokines (TNFα, IL-1β) and anti-inflammatory cytokines (IL-1RA) in the lungs following infection but decreased expression of IL-17 (Kottom et al. 2017). Whether Mincle plays a role in humans or not remains to be determined. Mincle has not been studied in the context of infection by *C. neoformans*.

5.2.6 DC-SIGN (CD209, Mouse SIGNR1 to SIGNR8)

The human Dendritic Cell-Specific intercellular adhesion ICAM-3-Grabbing Non-integrin (DC-SIGN) protein has 8 murine homologues: SIGNR1–8 (SIGN-Related) (Powlesland et al. 2006). SIGNR5 has been proposed to be the closest homologue based on its genomic localisation and its high-level expression in splenic DCs (Park et al. 2001). Nevertheless, SIGNR3 was shown to be the homologue with the closest biochemical properties: similar affinity for mannosyl- and fucosyl-glycans, induction of endocytosis upon binding and ligand release at endosomal pH (Powlesland et al. 2006). DC-SIGN possesses a single CRD and is present as tetramer at the cell surface of DCs (Mitchell et al. 2001). The DC-SIGN family of receptors binds mannose and mannosylated proteins present at the surface of fungi (Takahara et al. 2012).

SIGNR1 is able to bind *C. albicans* but has poor phagocytic capacities (Taylor et al. 2004). Upon binding to *C. albicans*, DC-SIGN contributes to potentiate the production of TNFα induced by TLR2 activation (Takahara et al. 2012a, b). Interestingly, TLR2 and DC-SIGN appear to associate at the cell membrane, but whether this affects the recognition of pathogens by either one is not determined (Takahara et al. 2012b). The role of DC-SIGN in vivo or in human candidiasis is not deciphered.

DC-SIGN mediates the binding and internalisation of *A. fumigatus* through the recognition of galactomannans (Serrano-Gomez et al. 2005). A DC-SIGN over-expression model suggested that this receptor contributes to the production of IL-10 and IL-12 (Li et al. 2018). The effects of this receptor on Th polarisation remain to be determined. Nevertheless, several human *DC-SIGN* SNPs have been associated with increased risk of developing invasive aspergillosis in patients undergoing chemotherapy or hematopoietic stem-cell transplantation (Sainz et al. 2012).

DC-SIGN binds mannoproteins from the *C. neoformans* cell wall and GpA from *Pneumocystis* spp. (Mansour et al. 2006; Kottom et al. 2019). However, the role of

DC-SIGN has not been further characterised and more studies are required to determine the role of this receptor during the infection by these two pathogens.

5.2.7 Mannose Receptor (CD206)

The Mannose Receptor (MR) was the first membrane receptor whose binding to yeasts was shown to be inhibited by mannans (Warr 1980). The MR is expressed by macrophages, DCs, and some subsets of endothelial and epithelial cells (Martinez-Pomares 2012); some have also reported expression in human monocytes (Smeekens 2011). The mannose receptor is a multidomain receptor that comprises 8 CRDs organised in an extended linear conformation and responsible for the binding of mannose and mannosylated sugars and proteins (Martinez-Pomares 2012; Lam et al. 2007). MR also possesses two non-CRD cysteine-rich domains that mainly bind endogenous ligands (Martinez-Pomares 2012). Due to the spatial arrangement of its CRDs, MR recognises linear and short (2 to 6) mannosylated saccharides; in comparison, the tetrameric organisation of DC-SIGN allows the latter to bind to complex sugars (Frison et al. 2003). MR also recognises and induces the internalisation of chitin particles, which is required for this compound to exert anti-inflammatory effects (Wagener et al. 2014). Interestingly, Dectin-1 activation induces the cleavage of membrane-bound MR, which releases a soluble form of the receptor's extracellular domains (Gazi et al. 2011) in serum and alveolar fluid. The soluble MR can bind to extracellular pathogens, but its functions are poorly understood (Fraser et al. 2000).

The precise role of MR during fungal infections is still unclear. Several authors have reported that MR is unable to induce the internalisation of *C. albicans* (Lee et al. 2003; Heinsbroek et al. 2008). Instead, it was proposed that *C. albicans* uptake is mediated by Dectin-1 and that the MR is recruited to the phagosome (Heinsbroek et al. 2008), suggesting that these PRRs collaborate sequentially. MR signals through the FcRγ and collaborates with TLR2 and Dectin-1 to polarise T cells towards a protective Th17 response in mouse (van de Veerdonk et al. 2009; Rajaram et al. 2017). In spite of this, MR-deficient mice are not more susceptible to *C. albicans* infection than WT animals, nor do their immune cells have altered candidacidal abilities (Lee et al. 2003).

In vitro, MR contributes to initiate the secretion of TNFα and IL-1β by human corneal epithelial cells when infected with *A. fumigatus* (Wang et al. 2016). In these cells, triggering of MR increases the expression of Dectin-1, which is also required to mount the anti-*A. fumigatus* immune response in the cornea (Xu et al. 2015).

The ability of MR to induce the internalisation of *C. neoformans* is not resolved (Mansour et al. 2006). Binding of mannoproteins from *C. neoformans* cell wall by MR is essential to induce an efficient adaptive immune response (Mansour et al. 2006; Dan et al. 2008), even though the effects of this receptor on Th polarisation remain to be determined. The deficiency of the MR increases susceptibility to *C. neoformans* infection in mouse (Dan et al. 2008) but there are no reports of

increased susceptibility infection due to MR polymorphisms. MR has not been explored in the immune response against *P. jirovecii*.

5.2.8 Langerin (CD207)

Langerin is expressed by Langerhans cells (LC) found in the skin and the mucosa; it is also expressed by minor populations of DCs in the lungs, dermis, gut and lymphoid tissues (Takahara et al. 2002; De Jesus et al. 2014; Bigley et al. 2015; Patel et al. 2017). Langerin is a homotrimeric protein that binds mannose structures and β-glucans and targets them to the Birbeck granules - the endosomal compartments of LCs.

Langerin can interact with *C. albicans*, but not *C. neoformans,* through interaction with β-glucans and mannans (de Jong et al. 2010; Tateno et al. 2010). It is able to mediate uptake of *Candida* spp. and β-glucan preparations (*i.e.* zymosan) (de Jong et al. 2010). LCs are required to induce Th17 but not Th1 adaptive responses at the skin level in response to *C. albicans* (Igyarto et al. 2011). In addition, Langerin$^+$ DCs are necessary to initiate the adaptive responses against *C. albicans* at the level of the skin and the oral cavity (Igyarto et al. 2011; Sparber et al. 2018). However, in the studies mentioned above, Langerin was used as cell marker and no direct role for Langerin in the recognition of *C. albicans* or initiation of the adaptive response has been demonstrated. Other functions of Langerin in antifungal immunity are still unexplored.

5.3 CD23 (FcεRII)

The low-affinity IgE Fc receptor (CD23) is expressed by lymphocytes, granulocytes, DCs, monocytes, macrophages and some epithelial cells (Acharya et al. 2010; Palaniyandi et al. 2011). This CLR can be either membrane bound as trimer (Kilmon et al. 2004) or released as a secreted monomer (McCloskey et al. 2007) or trimer (Beavil et al. 1995). In addition to the binding of immunoglobulins, it was recently shown that CD23 is able to interact directly with β-glucan and α-mannans (Guo et al. 2018).

Consistently with its ability to induce NO synthase upon activation, CD23 was reported to be involved in the killing, but not in the uptake of *C. albicans* by macrophages (Guo et al. 2018). Moreover, mice deficient for CD23 were more susceptible to *C. albicans* and *A. fumigatus* infection but not to *C. neoformans* challenge (Guo et al. 2018). More studies are needed to determine whether it can affect the adaptive response or if this receptor is involved in the anti-*P. jirovecii* immunity.

6 Other PRRs

6.1 CR3 (CD11b/CD18, Mac-1)

Complement receptor 3 (CR3) is composed of two integrins, CD11b and CD18; it is expressed by granulocytes, monocytes, macrophages and DCs (Erdei et al. 2019). CR3 is able to bind GXMs and β-glucans from fungal pathogens (Ross et al. 1987; Dong and Murphy 1997). Although Dectin-1 has been shown to have a major role in the phagocytosis and initiation of inflammation by mononuclear phagocytes in response to β-glucan, CR3 is the main receptor for this PAMP in neutrophils (van Bruggen et al. 2009), whereas it has only a minor role in mononuclear phagocytes (Li et al. 2011). It is believed that this difference lies in the difference of expression of these PRRs by these different cell types (van Bruggen et al. 2009). Actually, in neutrophils, CR3 and Dectin-1 collaborate tightly to respond to fungal pathogens. Integrins are often expressed as inactive receptors at the cell surface and need a signal from another receptor (PRR, cytokine receptor, etc.) to acquire ligand-binding properties through conformational modifications (Hynes 2002). Triggering of Dectin-1 induces the activation of CR3 through the Syk-Vav pathway, and this step is required for neutrophils to acquire their fungicidal abilities (Li et al. 2011). The interactions of CR3 with complement-opsonized pathogens can also mediate internalisation of the opsonized target and influence Th cell polarisation (Gresnigt et al. 2013) but we will only discuss its functions as a PRR.

Upon recognition of β-glucan, CR3 is able to mediate internalisation of pathogens and the production of ROS by neutrophils (van Bruggen et al. 2009; Li et al. 2011). CR3 is involved in the killing of non-opsonized *C. albicans* yeasts and *A. fumigatus* conidia through iron sequestration (Gazendam et al. 2014, Gazendam et al. 2016). In contrast, the killing of *A. fumigatus* hyphae seems to occur by the production of NETs and ROS (Clark et al. 2018). CR3 signals through the Syk pathway and activates the NADPH oxidase mainly through activation of PKCδ (Li et al. 2016) but the PI3K-Akt pathway has also been reported to be involved (Bose et al. 2014). Activation of the MAPK signalling cascade potentiates the signal induced by Dectin-1 and contributes to an increased secretion of pro-inflammatory cytokines (Huang et al. 2015). There is no known role for CR3 in the immune response of *C. neoformans* or *P. jirovecii*.

Deficiency for CD18 has been shown to be a major risk factor for susceptibility to invasive fungal infection (Lanternier et al. 2013). This immunodeficiency is called leukocyte adhesion deficiency (LAD) type I, and cells from LAD patients are less responsive to zymosan (Ross et al. 1987). However, reasons underlying susceptibility to fungal infections might also link with the decreased ability of neutrophils from these patients to leave the bloodstream and access the infected tissues (Hanna and Etzioni 2012).

6.2 CD14

CD14 is a membrane-bound protein expressed at the surface of myeloid cells, and it can also be secreted as a soluble form (Wu et al. 2019). This protein has been extensively Characterised for its functions as a co-receptor for TLR2 and TLR4 (Wu et al. 2019), and this collaboration has been shown to be important for activation of these receptors in response to fungal pathogens, such as *A. fumigatus* and a minor role for *C. neoformans* (Wang et al. 2001; Tada et al. 2002; Yauch et al. 2004). Through its ability to bind LPS and to activate NFAT in a PLCγ-dependent manner (Wu et al. 2019), CD14 is also a PRR in itself. This receptor has been shown to bind GXMs from *C. neoformans* independently of TLR4 (Shoham et al. 2001) but whether this binding leads to the TLR-independent activation of CD14 remains unknown. There is no other report of CD14 functioning directly as a PRR in fungal infections, and it is proposed to function as a co-receptor only.

6.3 Scavenger Receptors

The scavenger receptors constitute a "supergroup" of receptors defined by their ability to bind common ligands, including low-density lipoproteins, rather than a common structure (Zani et al. 2015). In addition to endogenous ligands, a few scavenger receptors also bind β-glucans (Zani et al. 2015).

CD5 has been shown to activate the MAPK pathway and induce the secretion of pro-inflammatory cytokines in response to zymosan (Vera et al. 2009). CD36 and SCARF1 act as TLR2 co-receptors, mediate the uptake of *C. neoformans* and exert a protective role in vivo against this pathogen (Means et al. 2009).

LOX-1 is a scavenger receptor belonging to the CLRs superfamily (Zelensky and Gready 2005); it is expressed on endothelial cells and in the corneal epithelium (Li et al. 2015; He et al. 2016). This receptor has been shown to participate in antifungal immune response in *A. fumigatus* keratitis. In response to this fungus, LOX-1 contributes to induce the production of pro-inflammatory cytokines and ROS (Gao et al. 2016; He et al. 2016). Nevertheless, whether its functions are linked to the binding of a fungal ligand remains to be confirmed.

6.4 Ephrin Type-A Receptor 2 (EphA2)

EphA2 is a tyrosine-kinase transmembrane receptor expressed on endothelial cells, epithelial cells and some subsets of leukocytes, including neutrophils, monocytes and DCs (Funk and Orr 2013). Ephrin receptors contribute to intercellular communication by recognising membrane-bound ligands. They regulate endothelial

permeability, inflammation, embryogenesis and carcinogenesis (Darling and Lamb 2019).

In addition, EphA2 binds β-glucans and can induce the internalisation of *C. albicans* by epithelial cells and neutrophils (Swidergall et al. 2018, 2019). It activates the MAPK pathway in an FcRγ-dependent manner and induces the secretion of pro-inflammatory cytokines and antifungal peptides (Swidergall et al. 2019). Deficiency for EphA2 impairs the recruitment of leukocytes to the oral mucosa and the oxidative killing of *C. albicans* by neutrophils (Swidergall et al. 2018, 2019). The role of EphA2 in oral infection by *C. albicans* might be limited to the early stages of infection as fungal clearance is only delayed in mice deficient for this receptor (Swidergall et al. 2019).

EphA2 is able to induce the binding and internalisation of *C. neoformans* to endothelial cells in vitro (Aaron et al. 2018). In an in vitro blood–brain barrier model, silencing of EphA2 has been shown to impair barrier crossing by *C. neoformans* (Aaron et al. 2018), highlighting its potential interest for therapeutic application in the prevention of cryptococcal meningitis.

7 Conclusion

The innate immune response to fungal pathogens is required to prevent invasive infections and initiate the adaptive immune response. As described in this chapter, many PRRs are activated by PAMPs from the fungal cell wall and have been more or less extensively characterised. During fungal infections, the interplay between the fungus and immune cells is highly dynamic and the interaction between the pathogen and the host primarily occurs at the level of the fungal cell wall whose structure alters as the morphology and composition change. The identity and role(s) of different PRRs involved in the immune response are governed not only by the fungus encountered but also by the fungal morphotype, the localisation of infection and the host cell type considered. PRR collaboration is essential to ensure an efficient antifungal immune response that also requires modulation to avoid deleterious excessive inflammation that can lead to organ failure in the case of severe sepsis (Annane et al. 2005).

The fungal cell wall constitutes the armour which provides protection against external aggression from the host immune response. It also constitutes a weak point whose recognition leads to the initiation of the immune response and the subsequent elimination of the pathogen. Fungi are able to escape and avoid immune recognition by altering the composition of their cell wall to mask or shed major PAMPs, such as β-glucans (Ballou et al. 2016; Hopke et al. 2018). Other fungal immune evasion strategies include the activation of the immunosuppressive components of immunity and secretion of immunomodulatory proteins (Marcos et al. 2016).

A great body of knowledge has been accumulated for Dectin-1 and the TLRs, notably thanks to epidemiological studies that have linked SNPs, in their genes or

those of their signalling intermediates, to altered susceptibility to infections (Lanternier et al. 2013). On the other hand, the role of several PRRs, such as MelLec and the scavenger receptors, is not completely understood and needs to be studied further to fully appreciate their contribution to the antifungal immune response. In addition, it is still unclear why different PRRs have different biological functions while they activate the same signalling cascades. Finally, we need to expand our understanding of these immune receptors, and how they collaborate in mounting an efficient immune response, to develop more effective therapeutic strategies and vaccines against fungal pathogens to alleviate their burden on human health.

Acknowledgements We thank the Wellcome Trust (102705) and the Medical Research Council Centre for Medical Mycology and the University of Exeter (MR/N006364/2) for funding.

Statement All figures were made using BioRender.com.

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Exopolysaccharides and Biofilms

François Le Mauff

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Abstract During infection, many fungal pathogens form biofilms within tissues or on biomedical devices. The growth of fungi within biofilms increases dramatically their resistance to both immune defences and antifungal therapies. In the last twenty years, studies have begun to shed light on many of the steps involved in biofilm synthesis and composition, revealing new antifungal strategies. This chapter will focus on the biofilm exopolysaccharides produced by *A. fumigatus* and *C. albicans,* the two main causes of human fungal infections. We will review the current state of

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Current Topics in Microbiology and Immunology (2020) 425: 225–254
https://doi.org/10.1007/82_2020_199
© Springer Nature Switzerland AG 2020
Published Online: 19 February 2020

our understanding of the structure, biosynthesis, and role of exopolysaccharides in biofilm development and function with a view to identifying future strategies for prophylaxis and treatment of these devastating infections.

1 Introduction

As with most scientific discoveries, biofilms were discovered by a technology breakthrough: The development of powerful microscopy lenses that allowed microorganisms to be observed for the first time. During the seventeenth century, Antony Van Leeuwenhoek used his unique single lens microscope to scrutinize diverse samples such as rainwater, the surface of molding meat or the surface of his own teeth (Fred 1933). The description of all his observations revealed the presence of microbial communities (Fred 1933). Three hundred years later, in the late 1960s, several reports flourished suggesting the existence of a bacterial "glycocalyx" made of secreted carbohydrates extracellularly modified for adherence purposes (Costerton et al. 1978). In 1978, this new microbiological concept was formalized into what we now know as biofilms (Donlan and Costerton 2002). Bacterial biofilms and their importance for viability in the environment were first to become recognized (Hall-Stoodley et al. 2004), and it was only in the 2000s that fungal biofilms were recognized as a major health concern (Harding et al. 2009).

Over the past 10–20 years, the composition, architecture, and synthesis of fungal biofilms have begun to be investigated in detail. While these adherent multicellular communities of microorganisms embedded in an extracellular matrix are as diverse as the microorganisms themselves (Mitchell et al. 2016), their strategy to build effective adherent biofilms is remarkably similar. This process consists of the secretion of copious amounts of polysaccharides, enzymes, and structural proteins to form the biofilm core to which lipids, DNA, melanin, and metabolites, can be added to refine the biofilm structure and function (Reichhardt et al. 2016). Once assembled, biofilms can be pictured as a microbial fortress. In the same way, a fortress' fortifications provide a safe environment for its inhabitants, the adhesive properties of biofilms allow organisms to colonize surfaces and surround them with an defensive layer protecting them from biotic and abiotic stresses (Stewart and Franklin 2008). Furthermore, the network formed by the biofilm allows differential efficiency of water and nutrient distribution creating diverse microenvironments from which physiologically heterogeneous populations arise (Flemming and Wingender 2010; Stewart and Franklin 2008). From quiescent persister cells to metabolically active cells secreting exopolysaccharides that can engage or deactivate defense mechanisms of specific aggressors, this population diversity is responsible for most of the biofilm-related resistance mechanisms (Mah and O'toole 2001; Snarr et al. 2017b; Ramage et al. 2009).

The biofilm's ability to adhere and grow on diverse substrata and to enhance resistance mechanisms granted them the title of "most successful form of life on Earth" (Flemming and Ridgway 2009). Unfortunately, medical device surfaces and human tissues are one of the surfaces upon which biofilms form, leading to the development of infections that are often resistant to conventional antimicrobial therapy (Boisvert et al. 2016; Rasmussen and Givskov 2006). Biofilms constitute a source of recurrent infections with high mortality rates, in part due to the increasing use of medical devices such as catheters and prostheses (Desai et al. 2014; Lagree and Mitchell 2017).

In the environment, dozens of thousands of fungi species exist (Blackwell 2011). However, only a few are medically relevant and form biofilms including: *Candida* sp. (Hawser and Douglas 1994), *Aspergillus* sp. (van de Veerdonk et al. 2017), *Cryptococcus* sp. (Martinez and Casadevall 2015), *Rhizopus* sp. (Singh et al. 2011), *Histoplasma* sp. (Pitangui et al. 2012), *Coccidioides* sp. (Davis et al. 2002), *Trichosporon* sp. (Di Bonaventura et al. 2006), and *Pneumocystis* sp. (Cushion et al. 2009). This chapter will focus on the biofilm and biofilm exopolysaccharides produced by the two main causes of human fungal infections, *C. albicans* and *A. fumigatus* which represent more than 85% of the fungal infections (Sanguinetti and Posteraro 2016). The biofilm composition and the polysaccharide synthesis pathways will be presented and the strategies by which fungi use these structures for adherence, invasion, and resistance to antifungals will be reviewed. Finally, currently available and potential therapies against biofilm infections will be discussed.

2 Biofilm Composition, Structure and Synthesis

2.1 Aspergillus fumigatus *Biofilm*

Aspergilli are saprophytic filamentous molds that play an essential role in recycling carbon and nitrogen in the environment (Latgé 1999). Due to their efficient dissemination strategy via the production of airborne conidia, *Aspergilli* are one of the most ubiquitous fungi and can be found everywhere on the planet from the polar circles to the most arid deserts (Abdel-Azeem et al. 2016). As a consequence, all humans inhale *Aspergilli* conidia on a daily basis (Latgé 2001). However, despite the existence of 339 *Aspergilli* species, only 40 are reported as pathogenic (Gugnani 2003; Abdel-Azeem et al. 2016), and more than 80% of the aspergillosis are caused by one single species, *Aspergillus fumigatus* (Singh and Paterson 2005; Morgan et al. 2005). The remaining aspergillosis cases are being due to *A. terreus*, *A. flavus*, *A. niger,* and *A. nidulans* (Dagenais and Keller 2009). These species are also able to form biofilms but will not be the focus of this chapter (Gutierrez-Correa et al. 2012; Al-Gabr et al. 2013; Priegnitz et al. 2012; El-Ganiny et al. 2010).

As with almost all fungal infections, preexisting health conditions are required to develop aspergillosis (Badiee and Hashemizadeh 2014). Preexisting lung conditions, such as cavitary lung disease, asthma, or cystic fibrosis, can lead to chronic aspergillosis. In this condition, *Aspergillus* is contained by the immune system at the site of infection, but is not cleared (Denning 2001). In contrast, in severely immunocompromised patients hyphae can invade and destroy lung tissue, a condition known as invasive aspergillosis (Tekaia and Latge 2005). In both conditions, a biofilm is formed in vivo by *A. fumigatus* and is thought to contribute to virulence (Kaur and Singh 2014; Reichhardt et al. 2019). The architecture of the biofilm in the chronic and invasive aspergillosis differs. During chronic infections, *A. fumigatus* form aspergillomas, balls of agglutinated hyphae covered in a thick layer of extracellular matrix. Only the hyphae at the periphery of the fungal ball are viable with hyphae present at the center dying from starvation. In invasive disease, all the hyphae are viable and spread into the tissue, surrounding themselves with a thin layer of extracellular matrix (Loussert et al. 2010; van de Veerdonk et al. 2017). Immunolocalization of polysaccharides within these biofilms revealed the presence of galactomannans (GM) and galactosaminogalactan (GAG) in both types of biofilms, while α-glucans were observed only in aspergillomas (Loussert et al. 2010). In vitro analysis of *A. fumigatus* biofilms by solid-state NMR spectroscopy revealed that these exopolysaccharides comprise 43% of the extracellular matrix, accompanied by proteins (40%), lipids (14%), and nucleic acid (3%) (Reichhardt et al. 2015). It is important to note that these in vitro grown biofilms differ from those generated in vivo (Müller et al. 2011). As electron microscopy and immunolabeling studies have demonstrated, hyphae tend to agglutinate in a monolayer format in vitro while in vivo they grow in three dimensions and contain higher levels of GAG and GM in their extracellular matrix (Müller et al. 2011).

2.1.1 α-glucans

α-glucans are a major component of *A. fumigatus* hyphae and conidia cell wall (Fontaine et al. 2000). However, their role in biofilm remains unclear. The synthesis of these polymers of α-(1-3)-glucan is mediated by three genes in *A. fumigatus*: *ags1*, *ags2,* and *ags3* (Henry et al. 2012) (Fig. 1). The deletion of these three genes is required to abolish the synthesis of the polymer and results in conidia with reduced viability and attenuated virulence (Beauvais et al. 2013). While no studies have directly examined the role of α-glucans in biofilm formation or adhesion, the overexpression of homologous *ags* genes in *A. nidulans* causes the formation of biofilms as adhesive as those of *A. fumigatus,* suggesting a role for α-glucans in adhesion (He et al. 2018). These findings are supported by studies in *A. oryzae* in which disruption of *ags* genes was associated with reduced hyphal aggregation (Miyazawa et al. 2019), a finding consistent with the observation that α-glucans mediate aggregation of swollen conidia in *A. fumigatus* (Fontaine et al. 2010).

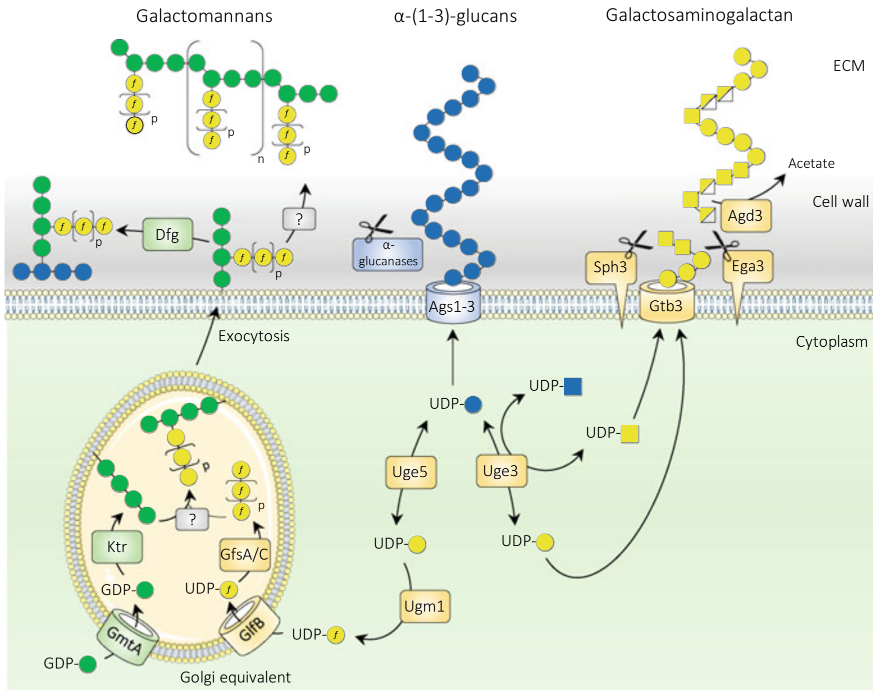

Fig. 1 *A. fumigatus* exopolysaccharides synthesis and secretion pathway. The polysaccharides are represented following the symbol nomenclature for glycan (Varki et al. 2015). Blue circle: Glucose, Yellow circle: Galactose, Green circle: Mannose, Blue square: N-acetylglucosamine, Yellow square: N-acetylgalactosamine, Half yellow square: Galactosamine. The enzymes involved in the different pathways are represented under a cylinder shape for the transporter/transferase or under a square shape for any other proteins and including a scissor cartoon for the glycoside hydrolases. *UDP* Uracil diphosphate; *GDP* Guanosine diphosphate; *ECM* Extracellular matrix

Collectively, these studies suggest the hypothesis that α-glucans are the "glue" that holds hyphae together within aspergillomas and in vitro biofilms, but are absent from the extracellular matrix of the more dispersed hyphae formed during invasive aspergillosis (Beauvais and Latgé 2015).

2.1.2 Galactomannans

Galactomannans are one of the two polysaccharides found in all types of biofilms made by *A. fumigatus* (Loussert et al. 2010; Beauvais and Latgé 2015). However, as with the α-glucans, little is known about their role in biofilm structure. GM plays an important structural role within the cell wall where it is complexed with chitin and β-(1,3)-glucans (Latge and Beauvais 2014). The chitin-glucan-GM complex is involved in conidiation, conidia germination, polarized growth, and cell wall

permeability (Muszkieta et al. 2019; Henry et al. 2019). Many of the enzymes governing GM synthesis have been identified, although several gaps in the pathway remain (Muszkieta et al. 2019; Henry et al. 2019) (Fig. 1). GM synthesis is initiated with the synthesis of UDP-galactofuranose (Gal$_f$) through the successive actions of *uge5* and *ugm1* (also called *glfA*) (Lee et al. 2014; Oppenheimer et al. 2010). Uge5 has been shown to be a UDP-4-glucose epimerase generating UDP-Gal$_p$ from UDP-Glc$_p$ specifically for galactomannan synthesis (Lee et al. 2014). Ugm1 is a UDP-galactopyranose mutase converting the UDP-Gal$_p$ produced by Uge5 to UDP-Gal$_f$ (Oppenheimer et al. 2010). UDP-Gal$_f$ is then transported into the Golgi by *glfB* (Engel et al. 2009), where *gfsA* and *gfsC* assemble chains of 4 to 5 β-(1,5) linked Gal$_f$ residues, which comprise the short side chain of GM (Katafuchi et al. 2017; Oka 2018; Komachi et al. 2013) (Fig. 1). Less is known about the synthesis of the α-(1,6) repeats of tetra-α-(1,2)-mannose backbone and the linkage of the Gal$_f$ side chains to this polymer. Synthesis of the mannan backbone requires the GDP-Man transporter *gmtA* and members of the *ktr* family to synthesize α-(1,2)-mannose (Engel et al. 2012; Henry et al. 2019) (Fig. 1). The enzymes responsible for the insertion of the α-(1,6) linkage in the polymer every four mannose residues and mediating the substitution of the mannose with the β-(1,5)-Gal$_f$ side chains in β-(1,3) or in β-(1,6) remain unknown (Latge et al. 1994) (Fig. 1). Once outside the cell, GM is found associated to the plasma membrane by a GPI anchor (Costachel et al. 2005; Li et al. 2018) and linked to β-(1,3)-glucans through the transglycosylation activity of the Dfg proteins (Muszkieta et al. 2019). GM is also found free in the cell wall and is secreted, but it is unknown if these GM molecules are specifically targeted to these compartments or simply by-products of cell wall synthesis (Fig. 1). Under specific culture conditions, longer Gal$_f$ side chains bearing additional β-(1,5)-Gal$_f$ chains linked in β-(1,6) to the galactose residue at the non-reducing end of the lateral chains have been observed (Kudoh et al. 2015). It has been speculated that the presence of these long galactan side chains is related to the low abundance of extracellular galactofuranosidases synthesized under these conditions (Kudoh et al. 2015).

Several mutants with alterations in the GM biosynthesis pathway display alterations in biofilm formation and adhesion. The *Δdfg* (Muszkieta et al. 2019) or β-(1,3)-glucan-deficient *Δfks*1 mutant (Dichtl et al. 2015), which lack cell wall-associated GM exhibited increased cell wall-associated GAG. It has been suggested that in the absence of GM, the cell wall retains the GAG which overwise would have been secreted into the extracellular matrix (Dichtl et al. 2015). The *Δuge*5, *Δugm*1, or in the *ΔugtA* (*glfB* homologous in *A. nidulans*) mutants which lack the β-(1,5)-Gal$_f$ side chains have all been reported to be more adherent likely due to a increased secretion of GAG compensating for the absence of the Gal$_f$ (Lee et al. 2014; Lamarre et al. 2009; Afroz et al. 2011). The *Δugm*1 and *ΔugtA* mutants also exhibit an altered cell surface in which the smooth amorphous layer typical of the wild type was covered by an organized material (Lamarre et al. 2009; Afroz et al. 2011). These adherence phenotypes may be explained by the redirection of UDP-Gal$_p$ toward other metabolic pathways such as GAG (Lee et al. 2014) or α-glucans synthesis since in an *A. niger* Δ*ugmA* mutant the overexpression of *agsA*

(*ags*1 homolog) was observed (Arentshorst et al. 2019). Alternately, the increased adherence of strains lacking the β-(1,5)-Gal_f may stem from unmasking of the mannose backbone, a hypothesis supported by the fact that mannosidase treatment can reduce adherence of these mutants (Lamarre et al. 2009). A similar role for galactose in inhibiting adhesion was reported in yeast, in which cell surface galactose inhibits the co-flocculation of *Schizosaccharomyces pombe* and *Pediococcus damnosus* (Peng et al. 2001).

2.1.3 Galactosaminogalactan

Like GM, galactosaminogalactan is found in all types of biofilms made by *A. fumigatus* (Loussert et al. 2010). Although this polymer is mostly found in the extracellular matrix or culture supernatant, its synthesis begins in early germination (Paulussen et al. 2017). GAG is the key element in the formation of adherent biofilms. It exerts multiple immunomodulatory functions, and its synthesis has been correlated to the degree of virulence of the strain (Sheppard 2011; Speth et al. 2019; Lee et al. 2012), suggesting it is a true polysaccharide virulence factor (Briard et al. 2016; Fontaine et al. 2011).

GAG was first discovered in 1960 in *Aspergillus parasiticus* and termed galactosaminoglycan since the authors only found galactosamine residues in the polymer (Distler and Roseman 1960). The finding of this new polysaccharide triggered the reports of other species producing a galactosamine-containing polymer including other *Aspergilli* (Gorin and Eveleigh 1970; Leal and Ruperez 1978; Bardalaye and Nordin 1976), *Physarum polyphalum, Cordyceps ophioglossoides, Bipolaris sorokiniana, Peacilomyces,* and *Penicillium frequentans* (Farr et al. 1977; Yamada et al. 1984; Pringle 1981; Takagi and Kadowaki 1985; Guerrero et al. 1988; Bartnicki-Garcia 1968). With the successive discoveries of GAG in the different species, the common features of the polymer were discovered. The linkage in α-(1,4) between the galactosamine was established as the norm, and acetylation of galactosamine has been observed but with different ratios across the different species. In addition, the presence of α-(1,4)-galactose residues were found in most of the polymers, hence the modern name of galactosaminogalactan (Bardalaye and Nordin 1976; Ruperez and Leal 1981; Guerrero et al. 1988).

In *A. fumigatus*, a polymer of N-acetylgalactosamine (GalNAc) was first reported in 2000 while describing the organization of the alkali-insoluble fraction of the cell wall (Fontaine et al. 2000). *A. fumigatus* galactosaminogalactan was initially reported to be composed of GalNAc and galactopyranose (Gal_p) linked in α-(1,4) (Fontaine et al. 2011), and the presence of deacetylated GalN residues confirmed in a subsequent publication (Lee et al. 2016). The distribution and the ratio of Gal_p to GalNAc in the polymer appear to be quite heterogeneous between different fractions of GAG and are thought to vary between secreted and cell wall-associated forms (Fontaine et al. 2000, 2011).

GAG synthesis is thought to be mediated by the products of a 5-gene cluster comprising *gtb*3 (AfuA_3g07860), *agd*3 (AfuA_3g07870), *ega*3 (AfuA_3g07890), *sph*3 (AfuA_3g07900), and *uge*3 (AfuA_3g07910) (Lee et al. 2016) (Fig. 1). GAG synthesis begins with the activity of the UDP-4-Glucose epimerase Uge3. Uge3 was the first enzyme of the GAG cluster to have been identified and characterized (Gravelat et al. 2013; Lee et al. 2014) from a comparative transcriptomic study of two regulatory mutant strains with impaired adhesion, *ΔstuA* and *ΔmedA*. Uge3 was found to mediate the interconversion of UDP-Glc to UDP-Gal, and UDP-N-acetylglucosamine (UDP-GlcNAc) to UDP-GalNAc (Fig. 1). Deletion of *uge*3 totally abrogated GAG synthesis without impacting the GM galactose content (Lee et al. 2014), resulting in a defect in biofilm formation, reduced virulence, and increased inflammatory response due to β-(1,3)-glucan exposure (Gravelat et al. 2010, 2013). Further studies revealed the presence of two other *uge genes* in the *A. fumigatus* genome, *uge*4 and *uge*5, with redundant UDP-4-glucose epimerase activities but distinct polysaccharide synthesis roles. Uge 5 activity was required to supply UDP-Gal for GM synthesis and had minimal effects on the GAG pathway while deletion of *uge*4 had no effect on the quantity of GM or GAG produced or any other biosynthetic pathways tested (Lee et al. 2014).

Following nucleotide sugar interconversion, their polymerization into GAG is hypothesized to take place at the plasma membrane through the action of Gtb3, a putative membrane glycosyltransferase (Fig. 1). No studies of this protein have been performed to date to confirm this gene annotation, and the mechanism of GAG polymerization remains unknown (Latge et al. 2017). However, in the 5-gene cluster model of GAG biosynthesis, Gtb3 would need to accept both UDP-Gal and UDP-GalNAc for polymerization of the heteropolysaccharide, a rare characteristic only shared by a limited amount of glycosyltransferases (Rini and Esko 2017; Zhang et al. 2016; Narimatsu 2006). Alternately, it is possible that a second glycosyltransferase is involved in GAG synthesis and remains to be discovered.

Following synthesis of the nascent polymer, GAG maturation then continues extracellularly where the enzymes Sph3 and Agd3 can act on GalNAc-rich regions of the polymer (Le Mauff et al. 2019; Lee et al. 2016) (Fig. 1). Sph3 is a type II integral membrane protein can cleave GAG and is the sole member of the glycoside hydrolase (GH) family 135 (Bamford et al. 2015). Sph3 is a highly specific α-(1,4)-N-acetylgalactosaminidase with a retaining endolytic mechanism requiring a sequence of at least seven GalNAc residues (Le Mauff et al. 2019). Despite this hydrolytic activity, Sph3 is essential for GAG synthesis (Bamford et al. 2015). The mechanisms underlying this requirement of Sph3 for GAG synthesis and biofilm formation remain unclear but may involve controlling polymer length to facilitate export through the cell wall.

Agd3 is the enzyme at the center of the GAG-mediated biofilm adherence (Ostapska et al. 2018). Agd3 is a polysaccharide deacetylase converting GalNAc residues to GalN rendering the polymer cationic and adherent at a physiological pH (Lee et al. 2016) (Fig. 1). Deletion of *agd*3 resulted in a strain that produces fully acetylated GAG that cannot adhere to the hyphal wall and is shed into the culture supernatants. As a result, the Δ*agd*3 mutant is unable to form adherent biofilm and

exhibits exposure of the β-(1,3)-glucans at the surface of the hyphae (Lee et al. 2016). Detailed studies of Agd3 structure, mechanism of action, and impact on GAG composition have not yet been performed.

The structure and the function of the final enzyme encoded by this gene cluster, Ega3, have recently been reported. Ega3 is a transmembrane protein belonging to the GH114 family that is a highly specific endo-α-(1,4)-galactosaminidase (Bamford et al. 2019) (Fig. 1). Ega3 exhibits no activity against α-(1,4)-GalNAc oligosaccharides, substrates of Sph3, demonstrating the non-redundancy of the two GH present in the 5-gene cluster (Le Mauff et al. 2019; Bamford et al. 2019). To date, no deletion mutant has been reported, so the role of Ega3 in biofilm formation remains unknown. Upregulation of *ega*3 during biofilm formation has been reported, suggesting that it likely plays an important role in GAG biosynthesis and function (Muszkieta et al. 2013).

The factors governing the expression of the GAG biosynthetic gene cluster remain poorly understood. To date, 4 putative transcription factors have been shown to impact GAG synthesis: the two developmental regulators MedA and StuA, (Gravelat et al. 2010, 2013) as well as SomA and PtaB, which regulate the expression of MedA and StuA (Lin et al. 2015; Zhang et al. 2018). Whether these proteins directly or indirectly regulate, expression of the GAG cluster genes is unknown although a recent study reported that the histone acetyltransferase GncE was also involved in the synthesis of GAG through a downregulation of *ptaB* and of *uge*3 (Lin et al. 2019). Interestingly, in all these studies, a defect in the condition pathway was observed in parallel to the defect in GAG synthesis and biofilm formation suggesting that these two processes are intrinsically linked.

2.2 Candida albicans *Biofilm*

Candida species are the main causal agents of fungal infections worldwide (Zarnowski et al. 2016). These yeasts constitute the fourth most common origin of nosocomial bloodstream infections and are thought to cause more than 400,000 life-threatening infections annually (Brown et al. 2012; Yapar 2014). Despite the existence of more than 200 Candida species, five of these account for 90% of human infections: *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei* (Pappas et al. 2016). Recently, a sixth strain, *Candida auri*s, has emerged as a new multi-drug resistant fungal pathogen (de Cassia Orlandi Sardi et al. 2018; Vallabhaneni et al. 2019). However, this chapter will focus only on the main etiological agent of candidemia, *C. albicans*, as biofilms formed by this genre are extraordinarily diverse (Cavalheiro and Teixeira; 2018; Jeffery-Smith et al. 2018).

The formation and composition of *C. albicans* biofilm have been the subject of several studies and reviews (Cavalheiro and Teixeira 2018; Seneviratne et al. 2017; Jadhav and Karuppayil 2017; Soll and Daniels 2016; Mitchell et al. 2016). *C.*

albicans biofilm formation occurs in three distinct steps (Seneviratne et al. 2008). During the early phase (up to 11 h), yeast cells adhere to surfaces and initiate differentiation into hyphae. In the next 19 h, during the intermediate stage, cells complete their differentiation and exopolysaccharides secretion begins. Finally, the maturation takes place, biofilm becomes thicker by accumulation of extracellular matrix in which a dense network of hyphae is embedded (Seneviratne et al. 2008). The formation and dispersion of yeast cells from these mature biofilms reinitiate the cycle of infection and biofilm formation (Uppuluri et al. 2010). The development of methods for the large-scale production of biofilm matrix has enabled detailed characterization of the *C. albicans* biofilm matrix (Zarnowski et al. 2016). The matrix is composed of proteins (55%), polysaccharides (25%), lipids (15%), and nucleic acid (5%) (Zarnowski et al. 2014). *C. albicans* can also integrate molecules secreted by the host into its biofilm upon infection (Nett et al. 2015).

2.2.1 Exopolysaccharides

Compositional analysis of the *C. albicans* matrix has revealed the presence of polysaccharides largely composed of four monosaccharides: arabinose (47.9%), mannose (20%), glucose (12.5%), and xylose (12.6%), split differentially into two fractions of distinct molecular weights (Zarnowski et al. 2014) (Fig. 2).

High Molecular Weight Polysaccharides

The high molecular weight polymer fraction of the biofilm matrix has received the most attention. The mannose and glucose contents of this fraction originate from mannans and glucans (Zarnowski et al. 2014). Mannans are the most abundant polymers in *C. albicans* biofilms, comprising about 87% of the biofilm polysaccharide fraction and help mediate adhesion to surfaces (Shibata et al. 2012). These polymers are made of an α-(1,6)-mannan chain that is heavily substituted in α-(1,2) by short chains of three to four (1,2)-mannoses (Zarnowski et al. 2014) (Fig. 2). This structure is similar to the structure of N-glycans present in the cell wall of *C. albicans* (Graus et al. 2018). Synthesis of biofilm mannans is linked to the synthesis pathway of these N-glycans as both tunicamycin treatment and deletion of *mnn* and *alg* genes alter the mannan fractions of biofilms (Mitchell et al. 2015). However, the average length of the mannan biofilm is much longer than those within the cell wall counterpart (Pierce et al. 2017; Zarnowski et al. 2014). Furthermore, biofilm mannans are not covalently linked to proteins, as it is the case in the cell wall.

The glucan fraction contains two polymers: β-(1,3)-glucans and β-(1,6)-glucans (Fig. 2). Despite the importance of the β-(1,3)-glucans in antifungal resistance, this polymer represents only 0.1% of the polysaccharide biofilm fraction (Zarnowski et al. 2014). Despite this observation, β-(1,3)-glucans are crucial for biofilm formation. Deletion of the biofilm specific β-(1,3)-glucan CAZymes *bgl*2, *phr*1, and

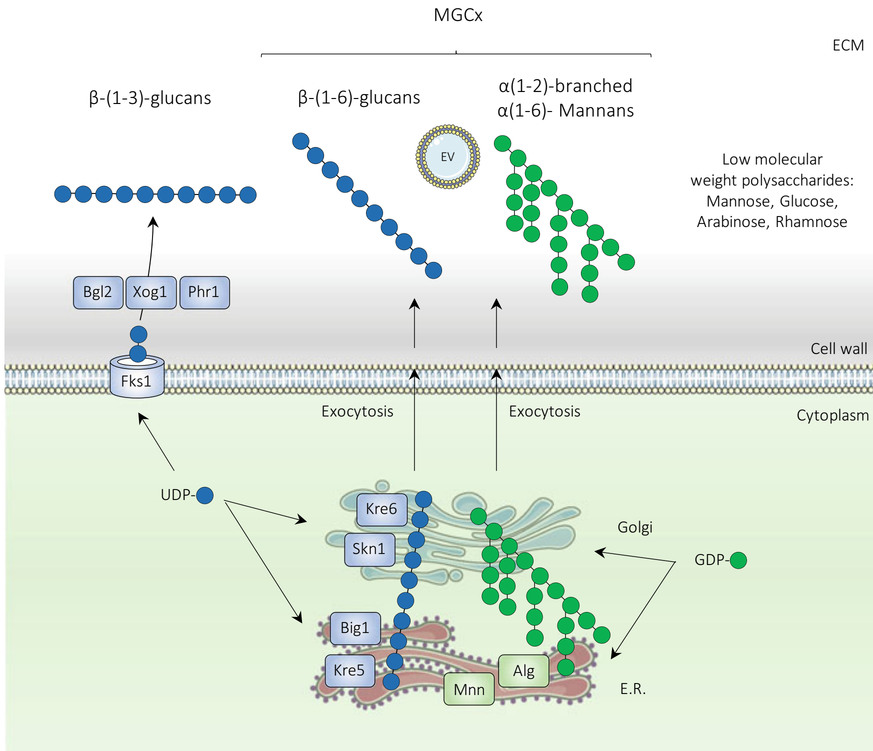

Fig. 2 *C. albicans* exopolysaccharides synthesis and secretion pathway. The polysaccharides are represented following the symbol nomenclature for glycan (Varki et al. 2015). Blue circle: Glucose, Green circle: Mannose. The enzymes involved in the different pathways are represented under a cylinder shape for the transporter/transferase or under a square shape for any other proteins. *E.R.* Endoplasmic Reticulum; *UDP* Uracil diphosphate; *GDP* Guanosine diphosphate; *EV* Extracellular vesicles; *ECM* Extracellular matrix; *MGCx* mannan–glucan complex

*xog*1, coding for 2 glucan transferases and an exoglucanase, respectively, resulted in altered biofilm formation, thickness, and increased antifungal sensitivity (Taff et al. 2012). Furthermore, the conditional deletion of *fks*1, coding for β-(1,3)-glucan glucan synthase, resulted in decreased biofilm-associated β-(1,3)-glucans, mannans, and β-(1,6)-glucans (Mitchell et al. 2015) suggesting that β-(1,3)-glucans play an important anchoring role in the biofilm matrix. Little is known about the β-(1,6)-glucans which constitute the remaining 13% of the biofilm polysaccharide. Their synthesis is governed by the genes *big*1 and *skn*1, *kre*5-9 *and* 6 (Umeyama et al. 2006; Han et al. 2019). Deletion of these genes results in a dramatic decrease of the amount of β-(1,6)-glucans in the biofilm, thereby influencing the amount of mannans and β-(1,3)-glucans as well as the presence of proteins required for adhesion (Mitchell et al. 2015).

As indicated by the mutant studies detailed above, normal biofilm formation by *C. albicans* requires the coordinated participation of mannans, β-(1,3)-glucans and β-(1,6)-glucans. Extracellular polysaccharide interactions are integral to this process since mixed mutant biofilm complementation can support the formation of biofilms with similar properties to those formed by wild type organisms (Mitchell et al. 2015). Together these polysaccharides are forming the mannan–glucan complex, MGCx, which possesses structural features found unique to biofilms (Zarnowski et al. 2014; Mitchell et al. 2015). Recent studies of this complex revealed that extracellular vesicles secreted through the ESCRT machinery are involved in the biosynthesis of biofilm MGCx. Analysis of the biochemical composition of the extracellular vesicles revealed a polysaccharide content with a similar proportion of glucose and mannose as was found in the biofilm matrix, as well as the presence of enzymes involved in matrix assembly and maturation (Zarnowski et al. 2018).

Low Molecular Weight Polysaccharides

This fraction represents more than 60% of the biofilm polysaccharides and exhibits a more diverse composition with arabinose, mannose, glucose, and xylose. However, little is known about the composition of polymers contained within this fraction (Zarnowski et al. 2014).

2.2.2 Proteins

A recent proteomic investigation identified 565 proteins encompassing more than 16 different metabolic pathways within the *C. albicans* biofilm matrix (Zarnowski et al. 2014). The majority of these proteins are involved in one of three general pathways: carbohydrate metabolism (177 proteins), amino acid metabolism (136 proteins), and energy-related metabolism (Pierce et al. 2017). In addition, studies of *C. albicans* biofilms formed in vivo have demonstrated that inflammatory proteins derived from leukocytes and erythrocytes can be encased into the biofilm matrix (Zarnowski et al. 2014; Nett et al. 2015).

In contrast to *A. fumigatus*, in which a polysaccharide functions as the dominant adhesin, *C. albicans* relies on a variety of cell wall proteins to adhere to abiotic surfaces and host cell surfaces (Wang et al. 2012). Over 35 proteins have been implicated in mediating fungal adherence at different stages of the biofilm life cycle (exhaustively reviewed in (Araujo et al. 2017). These proteins can be classified into two groups, transcription factors required for biofilm maturation and GPI-anchored cell surface proteins directly mediating cell-to-cell and cell-to-surface adhesion (Araujo et al. 2017; Richard and Plaine 2007). Among these proteins, the eight members of the Agglutinin like sequence (Als) protein family have been the most studied and are capable of recognizing and interacting with a wide range of host constituents during infection (Araujo et al. 2017; Sheppard et al. 2004). Of these

proteins, Als1, 3 and 5 have been found to play the most important role in mediating fungal adhesion in partnership with Hyphal wall protein 1, Hwp1 (Nobile et al. 2008; Staab et al. 1999; Modrzewska and Kurnatowski 2015).

2.2.3 Lipids

Neutral glycerolipids make up the majority of the lipid component of the biofilm. Some of these glycerolipids originate from extracellular vesicles that were recently identified to be transporting oligosaccharides and proteins involved in the synthesis of the MGCx (Zarnowski et al. 2014, 2018). The role of lipids in biofilm formation and structure is unknown but has been hypothesized to function largely as carriers of other structural elements.

2.2.4 Nucleic Acids

Despite the low amount nucleic acid present in the biofilm (5%), extracellular DNA plays a predominant role in biofilm formation, structural integrity, and maintenance and has been found to enhance resistance to antifungals (Martins et al. 2010).

2.2.5 Biofilm Regulation

The genetic network allowing the formation of biofilm by *C. albicans* has been well elucidated through the study of a comprehensive library of transcription factor knockouts. Screening studies of this library have identified six master regulators of biofilm formation in *C. albicans*: *bcr*1, *tec*1, *efg*1, *ndt*80, *rob*1, and *brg*1 (Nobile et al. 2012). An exhaustive RNA sequencing of the regions bound by these six transcription factors they regulate revealed a tightly interwoven genetic intermediate network of 23 genes that together govern the expression of over a thousand target genes (Nobile et al. 2012). The complete list of the genes involved at the different stage of *C. albicans* biofilm has been reviewed in detail elsewhere (Nobile and Johnson 2015).

2.3 Other Relevant Human Fungal Pathogens Biofilms

2.3.1 Cryptococcus sp.

Cryptococcus neoformans and *gatii* are the two main ethological agents of cryptococcosis. These organisms can infect the lungs and/or the central nervous system (Chang et al. 2006; Bratton et al. 2012). As with *A. fumigatus* and *C. albicans*, an important virulence factor is the secretion of exopolysaccharides:

glucuronoxylomannan, GXM, and galactoxylomannan, GalXM (McClelland et al. 2006; Doering 2009; Denham et al. 2018). These polymers form a capsule which surrounds and protects the fungal cell (Casadevall et al. 2018a; Araujo et al. 2016) and are also shed copiously to form biofilms (Martinez and Casadevall 2015). GalXM is an α-(1-6)-galactan alternately branched in C-3 by a chain of β-(1-4)-Gal-α-(1-3)-mannose-α-(1-3)-mannose substituted by 0–3 β-xyloses (Vaishnav et al. 1998). GXM is composed of an α-(1-3)-mannan backbone among which one out three residues is substituted by glucuronic acid in β-(1-2). On this backbone, differential substitutions by β-(1-2) and or β-(1-4)-xylopyranoses residues occur, defining the serotype of *Cryptococcus* strains (Cherniak et al. 1998). GXM contributes to cryptococcal adhesion in two ways. The anionic charge of the GlcA residues permits the aggregation of the GXM through divalent cationic ions (Nimrichter et al. 2007), likely in the same fashion that pectin auto-assembles into an "egg-box" conformation under calcium influence (Peaucelle et al. 2012; Wang et al. 2020). In addition, variations in xylose substitutions have also been linked to strain differences in the ability to form robust biofilms (Martinez and Casadevall 2007). *C. neoformans* serotypes D and A form stronger biofilms than serotypes B and C which are typically produced by *C. gatii* (Martinez and Casadevall 2005; Chen et al. 2008). In addition to the GXM and GalXM, glucose, ribose, and fucose have also been found in the biofilm matrix of *Cryptococci* but the molecule(s) containing these sugars remain undefined (Martinez and Casadevall 2015).

2.3.2 Mucorales

Infection with members of the Mucorales is commonly seen in patients with impaired immune system, high iron states or diabetes (Danion et al. 2015). The most prevalent agents of such infection are *Rhizopus arrhizus*, more widely reported as *R. oryzae* in the literature, and *R. microsporus* (Walther et al. 2019; Dolatabadi et al. 2014). The exopolysaccharides produced by these species are incompletely characterized, but contain mannose, fucose, glucuronic acid, and minor amount of glucose and galactose (Mélida et al. 2015). Based on the comparative analysis of the exopolysaccharides produced by different species of *Mucor* and *Rhizopus*, two structures have been proposed in the literature: a mucoran mostly made of glucuronic acid and mannose (Bartnicki-Garcia and Lindberg 1972), and a fucomannan constituted of mannose and fucose (Miyazaki et al. 1979). A more recent study of the cell wall polysaccharides of *R. oryzae* corroborated the likely presence of fucomannan, but failed to identify mannose linked in C-3, calling into question the presence of mucoran within the fungal cell wall (Mélida et al. 2015). Further studies are required to better understand the formation of biofilms by these important pathogens.

3 Biofilm, a Shelter from the Environment

In addition to mediating adhesion, biofilms provide a microenvironment that protects the fungi from external stresses such as host immune defences and antifungals (Boles et al. 2004).

3.1 Biofilm and Exopolysaccharide-Related Resistance Mechanisms to the Immune System

Although many biofilm-associated fungal exopolysaccharides can be recognized by the immune system (Snarr et al. 2017b; Patin et al. 2019), exopolysaccharides can also serve to protect the fungi from host immune responses. One strategy used by fungi to tip the scales in favor of immune evasion consists in concealing the most immunogenic epitopes with other less immuno-reactive exopolysaccharides (Steele et al. 2005; Beaussart et al. 2015). In *A. fumigatus* and *C. albicans,* one of the most immunogenic exopolysaccharides is the β-glucans, located in the cell wall of *A. fumigatus* (Fesel and Zuccaro 2016) and in the cell wall and biofilms of *C. albicans* (Ene et al. 2015; Gulati and Nobile 2016). β-glucans are recognized by C-type lectin receptor Dectin-1 leading to a wide range of antifungal responses including regulation of leukocyte phagocytosis, phagolysosomes recruitment and maturation, production of reactive oxygen species, activation of autophagy, and induction of pro- and anti-inflammatory cytokine secretion (Drummond and Brown 2011).

In *A. fumigatus*, β-glucans are hidden by rodlet proteins in resting conidia. β-glucan concealment is therefore required during germination and hyphal growth (Beauvais et al. 2013). During early conidia swelling and germination, α-glucans are thought to serve to conceal β-glucans. Consistent with this hypothesis, the α-glucan-deficient triple Δags mutant is more easily phagocytosed and killed by mouse alveolar macrophages (Beauvais et al. 2013). Concealing β-glucans with α-glucans exploits both the fact that no mammalian receptors have been identified to date for this polymer (Snarr et al. 2017b), and that α-glucans fail to induce differentiation of T cells due to their inability to induce large amounts of T-cell polarizing cytokines (Stephen-Victor et al. 2017). In growing hyphae, β-glucans are concealed by GAG (Gravelat et al. 2013). Although no immune receptor for GAG has yet to be identified, GAG mediates many immunosuppressive effects during infection, including the induction of neutrophil apoptosis, and IL-1 receptor antagonist secretion (Rambach et al. 2015; Eisinger et al. 2018; Gresnigt et al. 2014). GAG also serves to protect hyphae from neutrophil extracellular traps (NET) killing via a potential electrostatic repulsion of the cationic antimicrobial peptides present within the NETs by GAG and thus protecting *A. fumigatus* from their effects (Lee et al. 2015).

In *C. albicans*, the mannans within the MGCx serve to mask the glucans from the immune system (Mora-Montes et al. 2010; Graus et al. 2018). The presence of

the MGCx has been demonstrated to inhibit the formation of NETs and the production of ROS which would normally kill the *C. albicans* (May et al. 2016; Kernien et al. 2017; Xie et al. 2012; Urban et al. 2006).

3.2 Biofilm and Exopolysaccharide-Related Resistance Mechanisms to Antifungals

Five families of antifungals are today available: the polyenes, the azoles, the echinocandins, the pyrimidines analogues, and the allylamines (Denning and Hope 2010). Despite this diversity of therapeutic options, fungal infections remain a threat to human health. Biofilms contribute to the failure of antifungal therapy as successful eradication of biofilm-associated fungi requires concentration of antimicrobials that are higher than the ones required to kill planktonic cells, and are usually toxics to the host (Rasmussen and Givskov 2006). The resistance mechanisms best linked to fungal biofilms center around prevention of antifungals reaching their intracellular targets (Van Acker et al. 2014).

3.2.1 Drug Penetration and Sequestration

The biofilm matrix provides an important physical barrier limiting the penetration of antifungals to fungal cells within the biofilm either through limiting the penetration of antifungals or binding and sequestering these molecules (Nett and Andes 2017).

Candida β-(1,3)-glucans can mediate the sequestration of amphotericin B, azoles and flucytosine (Müller 2014; Mitchell et al. 2013; Nett et al. 2007). Β-(1,3)-glucans interact physically with antifungal such as amphotericin B (Vediyappan et al. 2010), and the deletion of genes involved in the synthesis or modification of the β-(1,3)-glucans such as *fks*1, *blg*2, *phr*1, or *xog*1 impairs the ability of the biofilm to protect the cells from antifungals (Nett et al. 2010; Taff et al. 2012). However, the low quantity of β-(1,3)-glucans in *C. albicans* biofilm to about 0.1% (Zarnowski et al. 2014) suggests that other molecules may be involved in this process. Indeed, two other molecules were reported to mediate antifungal sequestration: Extracellular DNA for which role in resistance to amphotericin B was established (Martins et al. 2012; Panariello et al. 2019), and MGCx for which a role in resistance to fluconazole was demonstrated (Dominguez et al. 2018).

In *A. fumigatus*, cationic hyphal-associated GAG has been reported to limit the uptake and antifungal effects of posaconazole (Snarr et al. 2017a). GAG-mediated enhancement of resistance to amphotericin B and caspofungin was also reported; however, the effects on intracellular penetration of these drugs were not studied (Snarr et al. 2017a). Similar effects on caspofungin and amphotericin B resistance have been reported with extracellular DNA (Rajendran et al. 2013).

3.2.2 Efflux Pumps

A common mechanism of drug resistance is the upregulation of efflux pumps such as ATP binding cassette (ABC) or major facilitator superfamily (MFS) transporters, in order to prevent antifungals from reaching effective intracellular concentrations (Goffeau 2008; Sanglard and Odds 2002). These efflux pumps have been found to be overexpressed by cells in *C. albicans* and *A. fumigatus* biofilms (Ramage et al. 2002; Cannon et al. 2009). However, several reports demonstrated that these proteins play a role largely during the establishment of the biofilm in order to protect cells while the extracellular matrix is being synthesized and secreted (Ramage et al. 2002; Mukherjee et al. 2003; Watamoto et al. 2011).

3.2.3 Persister Cells

When fungal cells adhere to a substrate, they can switch to a dormant-like phenotype that is associated with multi-drug resistance. These cells are called persister cells and are found exclusively in biofilms (Borghi et al. 2016; LaFleur et al. 2006). The existence of such cells have been shown in *A. fumigatus* (Beauvais and Muller 2009), and in *C. albicans* biofilms (LaFleur et al. 2006). Although the relevance of metabolic reprogramming in mediating persister cell resistance to antifungals is somewhat controversial (Denega et al. 2019), metabolic changes in the cells surviving antifungal treatments have been documented (Li et al. 2015). Persister cells also exhibit increased resistance to reactive oxygen species (ROS) by multiple mechanisms including limiting their production, direct detoxification, and inhibition of the metabolic cascade resulting from ROS accumulation (Van Acker et al. 2014; Li et al. 2015; Wuyts et al. 2018).

4 How Do We Fight Back?

Given the importance of biofilms in fungal infections, new therapeutics that target these structures are crucially needed. There is an urgent need for effective approaches preventing biofilm formation and innovative treatments to eradicate existing biofilms.

4.1 Inhibitors of Biofilm

Inhibition of fungal adhesion to surfaces is one approach to prevent biofilm formation. The study of biofilm development has identified *A. fumigatus* GAG the *C. albicans* Als proteins, especially Als3, as key adhesins involved in biofilm

formation of these fungi. Thus, the inhibition of their synthesis or function represents potential therapeutic opportunities.

The adhesive properties of GAG require deacetylation by the carbohydrate esterase type 4 (CE4) Agd3 (Lee et al. 2016). The extracellular localization and the fundamental role of this enzyme in virulence make Agd3 a promising therapeutic target (Lee et al. 2016). The use of an N-acetylhexosamine polymer deacetylated by a CE4 for adherence is not unique (Ostapska et al. 2018) and may have originated in bacteria (Sheppard and Howell 2016). *Pseudomonas aeruginosa* Pel polymer and the PelA deacetylase (Jennings et al. 2015), *Listeria monocytogenes* galactose substituted poly-N-acetylmannosamine and the PssB deacetylase (Köseoğlu et al. 2015), and *Staphylococcus aureus*, *Bordetella bronchiseptica*, *Streptococcus pneumoniae*, *Escherichia coli* with their PNAG and associated IcaB, BpsB, PgdA, and PgaB deacetylases, respectively, (Cerca et al. 2007; Little et al. 2015; Vollmer and Tomasz 2002; Itoh et al. 2008), are only a few of the bacterial species that produce these types of adhesive polymers. Attempts to develop inhibitors of PNAG deacetylases to inhibit bacterial biofilm adhesion have been reported (DiFrancesco et al. 2018). GlcNAc derivatives bearing metal chelating group were developed and reported a partial mixed inhibition mode in the low micromolar range (DiFrancesco et al. 2018). However, no inhibition of biofilm formation was demonstrated with these compounds to date. The development of Agd3-inhibitors has not been reported to date.

Inhibition of Als protein function has been studied in the context of vaccination with Als3 N-terminal protein (Fidel and Cutler 2011; Schmidt et al. 2012; Casadevall and Pirofski 2018; Edwards Jr et al. 2018). Anti-Als3 scFv3 antibody was found to inhibit the adhesion of *C. albicans* to cells (Laforce-Nesbitt et al. 2008), and the recombinant Als3 vaccine has recently shown promise in a clinical trial for the prevention of vulvovaginal candidiasis (Edwards Jr et al. 2018). Other efforts to prevent Candida biofilm have focused on the prevention of the morphologic transition from yeast to hyphae (Raut et al. 2013, 2014; Morales et al. 2013) or the modification of surfaces to prevent fungal adherence. Two strategies have been reported to prevent biofilm formation at the surface of medical device: "lock therapies" in which the lumen of catheters devices are filled with high concentration of antifungals or ethanol (Cavalheiro and Teixeira 2018), or the "coating" of the device with antifungals or other antimicrobial compounds (Ceresa et al. 2015, 2016; Sroisiri and Boonyanit 2010). Each of these approaches holds promise, but further pre-clinical and clinical studies are required to move these approaches to clinical use.

4.2 Disruption of Biofilm

Studies of biofilm development have also yielded several promising strategies for the treatment of established biofilms. Since the late 2000s, enzymes targeting structural elements of biofilms have been reported able to disrupt biofilms and to

potentiate antifungals. β-(1,3)-glucanase was the first enzyme used to disrupt *C. albicans* biofilms and was reported to increase the activity of neutrophils and efficiency of antifungals in vivo and in vitro (Nett et al. 2007; Tan et al. 2017, 2018). DNAase has also been found to exhibit activity against both *C. albicans* and *A. fumigatus* biofilms (Rajendran et al. 2013; Martins et al. 2010) and enhanced the activity of both caspofungin and the amphotericin B, but not fluconazole (Martins et al. 2012).

A second enzyme-based strategy has been reported in which the glycoside hydrolases Sph3 and Ega3 from the GAG biosynthetic pathway were repurposed as anti-GAG therapeutics (Le Mauff et al. 2019; Bamford et al. 2019). Recombinant hydrolase domains from these enzymes are able to degrade GAG and disrupt biofilms (Le Mauff et al. 2019; Bamford et al. 2019). Hydrolase treatment was able to attenuate the virulence of *A. fumigatus* in a mouse model of invasive disease (Snarr et al. 2017a). In addition, glycoside hydrolase treatment improved antifungal efficiency by improving intracellular uptake of these agents in vitro (Snarr et al. 2017a).

5 Conclusion

Fungal biofilms infections represent a great medical challenge. Over the last two decades, the compositions, structures, and roles of individual biofilm components have been elucidated; however, many more challenges remain. While our understanding of *C. albicans* biofilms has advanced greatly, *A. fumigatus* biofilms are not as well understood and the synthesis and regulation of GAG have not been completely elucidated. Nonetheless, the advances in our understanding of fungal biofilms are beginning to provide the first tools in the fight against these infections. Such advances may also improve our abilities to fight polymicrobial biofilm-related infections. Understanding mechanisms by which fungi build and maintain biofilms will be invaluable in identifying future therapeutic and diagnostic targets for these important diseases.

Acknowledgements I would like to thank Dr. Don Sheppard for is mentorship and review of the book chapter. I am also grateful to Josée Chabot, Ira Lacdao, Yazan Abu Yousef, and Ansh Goyal for their review of the different sections of the chapter.

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Cell Wall-Modifying Antifungal Drugs

David S. Perlin

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Abstract Antifungal therapy is a critical component of patient management for invasive fungal diseases. Yet, therapeutic choices are limited as only a few drug classes are available to treat systemic disease, and some infecting strains are resistant to one or more drug classes. The ideal antifungal inhibits a fungal-specific essential target not present in human cells to avoid off-target toxicities. The fungal cell wall is an ideal drug target because its integrity is critical to cell survival and a majority of biosynthetic enzymes and wall components is unique to fungi. Among currently approved antifungal agents and those in clinical development, drugs targeting biosynthetic enzymes of the cell wall show safe and efficacious antifungal properties, which validates the cell wall as a target. The echinocandins, which inhibit β-1,3-glucan synthase, are recommended as first-line therapy for *Candida* infections. Newer cell wall-active drugs in clinical development encompass next-generation glucan synthase inhibitors including a novel echinocandin and an

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Current Topics in Microbiology and Immunology (2020) 425: 255–275
https://doi.org/10.1007/82_2019_188
© Springer Nature Switzerland AG 2019
Published Online: 25 December 2019

enfumafungin, an inhibitor of Gwt1, a key component of GPI anchor protein biosynthesis, and a classic inhibitor of chitin biosynthesis. As the cell wall is rich in potential drug discovery targets, it is primed to help deliver the next generation of antifungal drugs.

1 Introduction

Fungal infections are a major global health problem with significant morbidity and mortality (Brown et al. 2012). Invasive fungal infections cause life-threatening meningitis, pneumonia, asthma, and mucosal diseases like oral and vaginal thrush. Most serious invasive fungal infections are a consequence of underlying health problems such as AIDS, cancer, stem cell or organ transplantation, and corticosteroid therapies with *Cryptococcus*, *Candida,* and *Aspergillus species* accounting for most deaths (Brown et al. 2012). In all cases, the clinical management of invasive fungal diseases requires effective antifungal therapy. In contract to bacterial infections, treatment options for fungi are limited. Current antifungal drugs target, either directly or indirectly, the plasma membrane (azoles, polyenes), nucleic acid biosynthesis (flucytosine), or cell wall (echinocandins). The paltry number of targets represented and limited chemical classes available is problematic especially as organisms with either acquired and inherent resistance to approved drugs are increasingly encountered (Perlin et al. 2017). Yet, after decades of paltry drug development, we are entering a renaissance for antifungal drugs, as new drug candidates representing novel targets and chemical matter are in clinical development (Perfect 2017). The cell wall has long been considered an ideal target for the antifungal drug, and now, the nearly two-decade success of echinocandins as safe and efficacious drugs for primary therapy and prophylaxis has validated its importance, leading to next-generation products. The cell wall is a target-rich structure that is ripe for new discovery.

2 Fungal Cell Wall as a Prime Antifungal Target

The cell wall is an essential structure that ensures the integrity of the fungal cell by maintaining its rigidity and shape. Nearly 90% of the fungal cell wall is composed of polysaccharides that are absent in humans (Latge 2007). Yet, far from static, it is a highly dynamic structure that undergoes extensive remodeling during cell growth, division, and maturation (Ene et al. 2015). The biological importance of the fungal cell wall can be appreciated by the fact that nearly 20% of the yeast genome is committed to the biosynthesis of the cell wall (Gow et al. 2017). These genes include the carbohydrate-active enzymes (CAZymes) [http://www.cazy.org]) involved with the synthesis of glucans and chitin, cell wall remodeling glycohydrolases (e.g., glucanases, chitinases) and transglycosidases. Many of the core

structural components are conserved across fungal genera, while other components are species-specific (Gow et al. 2017). Maintaining the structural integrity of the cell wall is of the utmost importance. The cell wall contains an integrated network of environmental sensors allowing fungi to resist stress, osmotic pressure, or toxic molecules (Latge 2010). Many of the critical cellular pathways have been elucidated that modulate and protect the cell in response to cell wall stresses (Ene et al. 2015). The biosynthetic enzymes critical to forming and remodeling the cell wall are unique to fungi and are not represented in humans. Hence, they serve as ideal targets for pathogen-specific antifungal therapy. It is well established that genetic or chemical modulation of major cell wall components, which alter the wall's structural integrity, results in cell collapse and death. It is also imperative to understand that the cell wall is the primary interface with its local environment within the human host. As such, it plays a critical role in immune recognition and surveillance, as it has many antigenic features that are important for health and disease (Hall and Gow 2013).

Structurally, the cell walls of prominent fungal pathogens including the *Candida spp.*, *Aspergillus spp.*, *Pneumocystis*, *Cryptococcus,* and endemic fungi have important structural similarities that include a prominent backbone layer of the glucose polymer β-1,3-glucan, as well as chitin and mannoproteins. Chitin is far less prevalent in the wall, but it forms covalent cross-links with the β-glucan scaffold contributing to the strength of the cell wall. The presence of β-1,3-glucan among yeasts and molds as an essential scaffold component is a nearly universal feature. Nevertheless, the cell walls of yeasts and molds are not the same, as their organization of associated structural components varies. Mannoproteins (30–40%) form the outer lamella of the cell wall and are represented by glycosylphosphatidylinositol (GPI)-modified proteins. They form covalent linkages to β-1,6-glucan while other mannoproteins are linked to β-1,3-glucan. Cell wall proteins are often mannosylated via *O*- and *N*-linkages that contribute to pathogenicity and cell wall dynamics (Hall and Gow 2013). The outer layer is more variable among the fungi and accounts for many of the phenotypic differences and diverse host interactions. The composition and form of the cell wall often vary during growth and development. Yeasts such as *Candida* and *Pneumocystis jiroveci* have an outer cell wall comprising highly mannosylated glycoproteins that covers the inner wall. In *A. fumigatus*, α-1,3-glucan (not found in yeasts) galactomannan, and galactosaminogalactan are found at conidia stage, along with an outer layer of hydrophobins and melanin (Latge 2007 #675). In *C. neoformans*, a glutinous capsule of glucuronoxylomannan and galactoxylomannan occludes the polysaccharides (Gow 2017). Collectively, the core structural carbohydrate elements, cross-linked polymers, and proteins create a highly stable yet dynamic structure. The wide array of fungal-specific biosynthetic and remodeling enzymes provides ideal targets for antifungal drug development and has been exploited in the past decades. They now comprise the specific targets for some of our most important antifungal drugs.

3 Inhibitors of Glucan Synthase

1,3-β-D-Glucan synthase remains an attractive target for antifungal drug action because it is present in many pathogenic fungi, which affords broad antifungal spectrum. Also, since there is no mammalian counterpart, it is presumed that compounds selectively inhibiting glucan synthase have little or no mechanism-based toxicity. The β-1,3-D-glucan synthase is a multi-subunit enzyme complex that catalyzes the transfer of sugar moieties from activated donor molecules to specific acceptor molecules forming glycosidic bonds (Orlean 1982). Our understanding of glucan synthase has come from genetic and biochemical studies in yeast (Douglas et al. 1994; Sawistowska-Schroder et al. 1984) and more recently from studies of *FKS*-resistant mutants from *Candida* spp (Park et al. 2005; Garcia-Effron et al. 2009a, b). The enzyme complex has at least two subunits, Fks and Rho. Fks is the catalytic subunit and is encoded by three related genes, *FKS1*, *FKS2*, and *FKS3*. *FKS1* is essential in *C. albicans* but in *C. glabrata and S. cerevisiae,* genetic disruptants are viable due to the paralog *FKS2*. These *FKS2* genes are calcineurin dependent and down regulated by FK506 (Eng et al. 1994). In *Aspergillus,* there is a single *FKS1* gene. Rho1p, a GTP-binding protein, found to copurify with Fks1p in preparations of the enzyme purified by product entrapment, helps regulates the activity of glucan synthase (Mazur and Baginsky 1996), most likely through Pkc1p (Sekiya-Kawasaki et al. 2002), although the precise nature of the activation remains unclear. The three known chemical classes of natural product inhibitors of 1,3-β-D-glucan synthesis include the lipopeptides (echinocandins, arborcandins), the glycolipid papulacandins, and the terpenoids (e.g., enfumafungins) All glucan synthase inhibitors are non-competitive with their biosynthetic substrate UDP-glucose (Douglas 2001). The echinocandins have enjoyed the most clinical success, while an enfumafungin is looming and is in late-stage clinical development. Finally, poacic acid is a natural plant metabolite found in the lignocellulosic hydrolysates of grasses that has antifungal activity, and it targets β-1,3-glucan synthesis by a mechanism non-overlapping with echinocandins (Lee et al. 2018). It is not yet in clinical development.

3.1 Echinocandins

The clinical success of the echinocandins, which can only be administered parentally, has validated β-1,3-glucan synthase (GS) as an antifungal target. The echinocandins are semi-synthetic cyclic hexapeptides with an amide-linked fatty acyl side chain (Zambias et al. 1992). The early echinocandins showed potent in vitro inhibition of glucan synthase with a pronounced affect only on V_{max} (Sawistowska-Schroder et al. 1984) and in vivo antifungal activity in murine infection models of *C. albicans* (Bartizal et al. 1992) and *Pneumocystis jiroveci* (Schmatz et al. 1991). They target and inhibit β-1-3-D-glucan synthase, and as such,

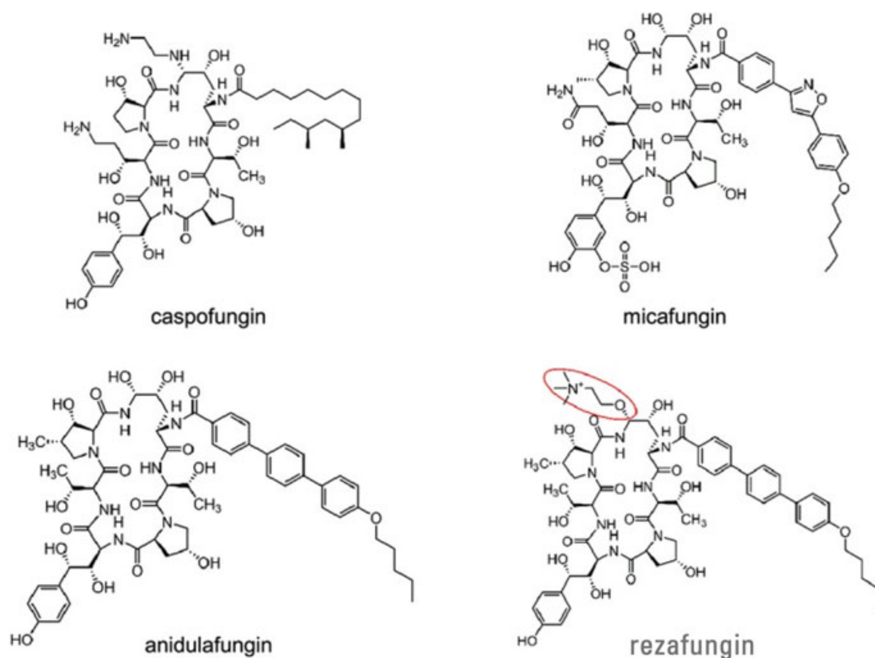


Fig. 1 Chemical structure for echinocandin drugs. All drugs are FDA approved except rezafungin, which is in late-stage clinical development. The red circle indicates the choline ether modification, which imparts its strong stability and half-life

they are broadly active against diverse *Candida* species, against which they are fungicidal (Bartizal et al. 1997). In *Aspergillus*, echinocandins slow growth and cause lysis of some growing tips resulting in altered the hyphal morphology (Kurtz et al. 1994b). In vitro, the cells form rosette-type structures. Echinocandin-induced alteration of the cell wall architecture enhances its immunoreactive properties in *Candida* and *Aspergillus*, especially at low drug levels (Wheeler and Fink 2006; Hohl et al. 2008). Echinocandin class drugs share a closely related overall chemical structure but vary in the hexapeptide ring and the amide-linked side chains (Fig. 1). Given their current two-decade long clinical history of safe and efficacious therapy, echinocandins are now the IDSA recommended preferred antifungal agent for treatment of candidiasis among high-risk patient populations (Pappas et al. 2016).

3.2 Early Echinocandin History

Papulacandins A–E, isolated from the fermentation broths of *Papularia sphaerosperma,* inhibited β-(1,3)-D-glucan synthase and were first reported in 1977 (Traxler et al. 1977). They contained a benzannulated spiroketal unit, which is the

signature for many bioactive natural products including the current echinocandin drugs. They showed narrow spectrum being highly high specific against yeasts but largely inactive against filamentous fungi. Studies with a series of papulacandin derivatives demonstrated that the fatty acid chain and the galactose residue were not required for activity at the target site, but the long fatty acid tail was essential for biological activity (Zambias et al. 1992). The echinocandins were introduced as a broader activity cyclic peptide antifungal, which showed lysis of actively growing *C. albicans* (Cassone et al. 1981; Kurtz et al. 1994a). They inhibit glucan synthase activity by reducing the V_{max} of the enzyme (Sawistowska-Schroder et al. 1984; Garcia-Effron et al. 2009b). Cilofungin, a semi-synthetic analog of echinocandin B, inhibited β-(1,3)-glucan synthase resulting in severe modifications of the cell wall and cytoplasmic membrane of sensitive organisms. It was the first clinically applied member of the echinocandin family (Taft and Selitrennikoff 1990). Ultimately, cilofungin was supplanted by the current echinocandins caspofungin, micafungin, and anidulafungin.

In 2001, the US Food and Drug Administration approved caspofungin for salvage therapy for patients with invasive aspergillosis refractory to conventional therapy. Subsequently, all three echinocandin drugs, caspofungin, micafungin, and anidulafungin, were approved for the treatment of esophageal and invasive candidiasis, including candidemia, empirical therapy in febrile neutropenic patients and prophylaxis in patients undergoing hematopoietic stem cell transplantation. The echinocandins are largely inactive against *Zygomycetes, Cryptococcus* species, or *Fusarium* species, which may reflect a reduced importance of β-(1,3)-glucan and/or compensatory mechanisms to stabilize the cell wall. They are highly effective against azole-resistant yeasts owing to their separate mechanism of action and the fact that they are not substrates for multidrug transporters (Niimi et al. 2006). Furthermore, echinocandins show activity against *Candida* biofilms (Bachmann et al. 2002) albeit culture condition-dependent (Kucharikova et al. 2011). Despite a common mechanism of action, the three echinocandins vary in basic pharmacodynamic properties including metabolism, half-life, drug–drug interactions, and pharmacodynamic targets (Lepak et al. 2015; Nett and Andes 2016). Echinocandin drugs are only administered intravenous (IV) due to their high molecular weights, low solubility, and poor absorption by the gastrointestinal tract. Fungicidal efficacy correlates with the ratio of AUC:MIC, although there is also a concentration-dependent (C_{max}) killing (Nett and Andes 2016). All echinocandin drugs exhibit a high degree of binding to plasma proteins (>99%) and distribute minimally to the brain, gastrointestinal tract, and eye. The echinocandins have an excellent therapeutic index with a low potential for toxicity or drug–drug interactions (Chen et al. 2011).

3.3 Echinocandin Drug Resistance

Echinocandin therapy is highly efficacious, but increasingly echinocandin drug resistance is a threat to successful clinical management. Among *C. albicans* and other *Candida* species, the frequency of resistance is relatively low at 1–3%, but this is not true for *C. glabrata*, where resistance is more severe, often presenting as multidrug resistance (Ostrosky-Zeichner 2013; Perlin et al. 2017). Echinocandin resistance among *C. glabrata* isolates ranges from 3 to 5% in population-based studies (Perlin 2015). Yet, some centers report rates of 10–15% (Alexander et al. 2013; Farmakiotis et al. 2014). Echinocandin resistance always arises de novo during therapy and is associated with repeated or chronic drug exposure, although resistance can follow brief drug exposure (Lewis et al. 2013). Colonization of *C. glabrata* within the gastrointestinal (GI) tract and intraabdominal abscesses represents major internal reservoirs for infection. The global resistance problem is expected to grow more severe as expanding numbers of patients are exposed to antifungal prophylaxis, drugs like caspofungin are now generic and cheaper, and new echinocandins are looming.

3.4 FKS *Mechanism of Resistance*

Clinical resistance resulting in breakthrough infections involves modification of Fks subunits of glucan synthase (Park et al. 2005). Unlike azoles, echinocandins are not substrates for multidrug transporters (Niimi et al. 2006). Resistance conferring amino acid substitutions in Fks subunits induces elevated MIC values (Arendrup and Perlin 2014) and reduces the sensitivity of glucan synthase (IC_{50}) to drug by 100- to >3000-fold (Garcia-Effron et al. 2009a). For most *Candida* spp., mutations occur in two highly conserved "hot-spot" regions of *FKS1*. These limited regions encompass residues (*C. albicans*) Phe641-Pro649 and Arg1361(Arendrup and Perlin 2014). Amino acid changes at Ser641 and Ser645 (*C. albicans*) are the most prevalent (~90%) causing the most pronounced resistance phenotype (Arendrup and Perlin 2014). In *C. glabrata*, mutations conferring resistance occur at conserved positions in both *FKS1* and *FKS2* with S629P, F625S, D632E (FKS1) and S663P,F, Y and F659S,V (FKS2) being the most prominent (Pham et al. 2014). Nearly 19 years after the FDA-approved caspofungin as a first-in-class echinocandin, mutations in *FKS* conferring reduced sensitivity to drug are still the only mechanism associated with *Candida* clinical failures (Shields et al. 2012; Perlin 2015; Shields et al. 2012), and clinical breakpoints (CLSI and EUCAST) reflect this underlying mechanism (Pfaller et al. 2011). Recently, a new mechanism independent of *FKS* mutation has been elucidated in *Aspergillus* involving changes in the lipid microenvironment of glucan synthases rendering it insensitive to echinocandin drugs. This mechanism is also relevant for drug tolerance in *Candida* species (Healey et al. 2012) and may explain the long-standing phenomenon of paradoxical growth at high drug levels.

3.5 Drug Resistance Emergence: Tolerance and Escape

Exposure of fungal cells to echinocandins leads to cell wall deformation resulting in osmotic instability and death to most cells. Yet, in a typical infection ($\sim 10^9$ cells), a subset ($\sim 10^{4-5}$) of cells survive and show drug tolerance over a wide range of drug exposures. In vivo, this response is observed as drug stasis in target organs (Slater et al. 2011). Ultimately, such cells have the potential to ultimately "escape" drug action and form genetically stable *FKS*-resistant mutants resulting in clinical failure. Drug resistance occurs following a progression of stages involving (1) cell stress, (2) tolerance, and (3) drug (phenotypic) escape (Healey and Perlin 2018). Tolerance reflects a repertoire of adaptive response mechanisms that stabilize the cell wall against drug stress. Much of what is known about the genetics of cell wall biosynthesis/remodeling comes from studies in *S. cerevisiae* (Lesage and Bussey 2006). Once the cell has sensed environmental changes, signaling cascades activate transcriptional regulators, which modulate the expression of specific target genes (Rosenwald et al. 2016). Among the different signaling pathways, the mitogen-activated protein kinase (MAPK) cascades are well studied (Rispail et al. 2009) consisting of a conserved cascade of kinases. For each cascade, a single MAPK moves into the nucleus after being phosphorylated altering gene expression. The major physiological activities assigned to MAPKs in fungi are cell wall biosynthesis, osmoregulation, mating, and virulence. Sensor–transducer proteins Slg1/Wsc1, Wsc2, Wsc3, Mid2, and Mtl1 detect damage and signal to Rom1/2 and Rho1, which activates protein kinase Pkc1 causing a kinase cascade including Bck1 (a MAPKKK), Mkk1/2 (MAPKKs), and Slt2/Mpk1. Slt2 phosphorylates several transcription factors, including Rlm1 and Swi4/6 turning on the expression of genes encoding proteins responsible for the synthesis of the cell wall including FKS2/GSC2 (Levin 2011). An important adaptive response is the upregulation of chitin biosynthesis genes, *CHS*, and mobilization of chitin from internal stores to stabilize the cell wall (Valdivia and Schekman 2003). In addition, the high-affinity calcium uptake system signals some genes responsible for cell wall biosynthesis and plasma membrane via the calcineurin-regulated transcription factor, Crz1, as well as HSP90 and its client proteins (Cowen and Steinbach 2008). Collectively, these adaptive cellular pathways have the ability to stabilize cells in the presence of "fungicidal" levels of echinocandins.

3.6 Echinocandins: The Next Generation

Rezafungin (formerly CD101) is a novel semi-synthetic echinocandin with broad-spectrum activity and demonstrated high potency in vitro (Arendrup et al. 2018b) and in vivo activity against *Candida* spp., *Aspergillus* spp., *Pneumocystis* spp., *Trichophyton mentagrophytes, Trichophyton rubrum and Microsporum gypseum.* Rezafungin and comparator echinocandins (micafungin, caspofungin and

anidulafungin) antifungal agents were evaluated for drug susceptibility by the Clinical and Laboratory Standards Institute (CLSI) method against invasive fungal isolates, including 531 *Candida* species and 56 *Aspergillus* spp., collected worldwide Pfaller et al. 2017a, b). Rezafungin at ≤ 0.12 µg/ml inhibited 95% of all *C. glabrata* isolates and 100% of *C. albicans, C. dubliniensis, C. tropicalis,* and *C. krusei* isolates. Typical of an echinocandins, Rezafungin, showed a high MIC of ≤ 4 µg/ml against *C. parapsilosis*. Rezafungin was comparable in activity to the three echinocandin drugs against all *Candida* species except C. *krusei*, which was fourfold less active with caspofungin. All isolates of *A. fumigatus* were inhibited by Rezafungin at ≤ 0.03 µg/ml (minimum effective concentration [MEC]), and all four agents were highly active against *A. fumigatus* with MEC for 90% of isolates at 0.015–0.03 µg/ml. Similarly, a large multicenter evaluation of 2018 clinical *Candida* spp isolates for drug susceptibility according to the European Committee on Antimicrobial Susceptibility Testing (*EUCAST*) (E.Def 7.3.1) methodology found that Rezafungin MICs (geometric MIC (GM-MIC) were lowest for *C. albicans* (0.016, 0.002–0.125 µg/ml) and highest for *C. parapsilosis* (1.657, 0.063 $\rightarrow$ 4 µg/ml); MICs for all other species were within these values (GM-MICs 0.048–0.055 µg/ml) (Arendrup et al. 2018b).

Like other echinocandins, it is an IV-only drug that targets and inhibits glucan synthase (Zhao et al. 2016). It is under late-stage clinical investigation for targeted therapy of candidemia and invasive candidiasis (Phase 3 ReSTORE), as well as prophylaxis (Phase 3 ReSPECT) for invasive fungal infections, including *Candida, Aspergillus,* and *Pneumocystis.* The FDA has designated rezafungin as a Qualified Infectious Disease Product (QIDP) with Fast Track status and orphan drug designation. Rezafungin is a chemical analog of anidulafungin (Fig. 1) in which the hemiaminal is replaced with a choline aminal ether that imparts greater stability and solubility to the product parent compound (Krishnan et al. 2017). This chemical stability imparts important pharmacokinetic and taxological advantages (Ong et al. 2016; Lepak et al. 2018), including a terminal half-life of approximately 130 h in humans that allows for protracted interval dosing (Sandison et al. 2017). It has demonstrable in vitro and in vivo potency that is either equivalent to or an improvement upon that of comparator echinocandins (Krishnan et al. 2017). Its chemical properties facilitate greater penetration of rezafungin into infected lesions in an intrabdominal abscess model relative to micafungin (Zhao et al. 2017). Rezafungin is subject to the same basic *FKS* resistance mechanisms as other echinocandins. But because it can be dosed at a much higher level without toxicity, it can overcome weaker phenotypes caused by certain *FKS* mutations (Bader et al. 2018) and may help prevent resistance emergence (Zhao et al. 2016) (Fig. 2).

3.7 Enfumafungin

Like the echinocandins, enfumafungins are potent inhibitors of glucan biosynthesis. Chemically, they are a hemiacetal triterpene glycoside (Fig. 3) produced by

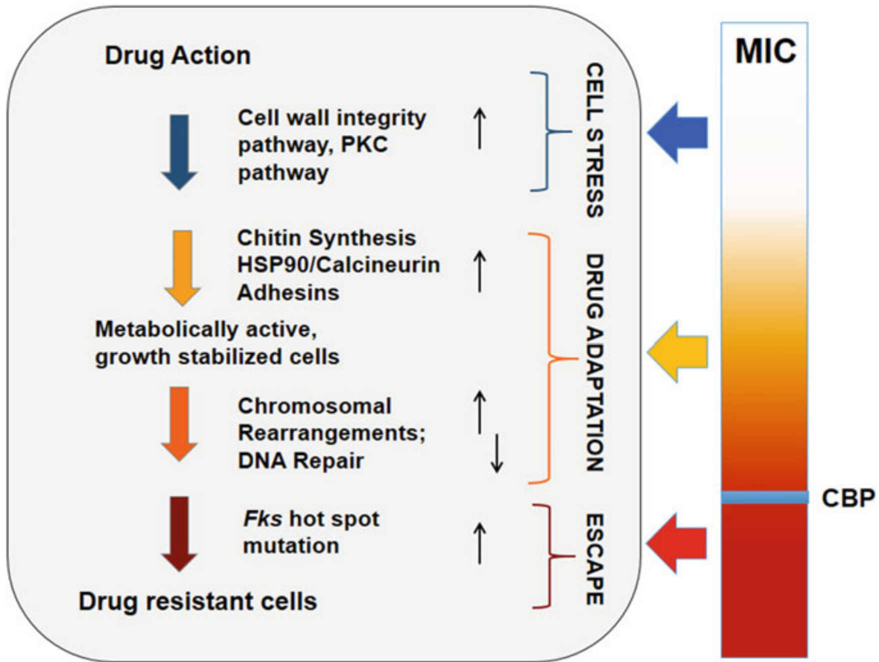
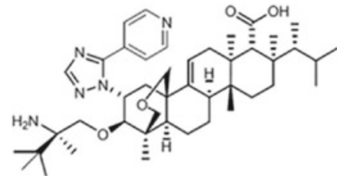


Fig. 2 Evolution of echinocandin resistance. Multistep cellular processes influencing the ability of a fungal cell to adapt to echinocandin drug exposure resulting in the formation of phenotypic *FKS* escape mutants that are refractory to drug and cause clinical failures (Healey and Perlin 2018)

Fig. 3 Structures of Ibrexafungerp, a semi-synthetic enfumafungin derivative



Hormonema spp. associated with living leaves of *Juniperus communis* (Pelaez et al. 2000). The antifungal mode of action of enfumafungin and other antifungal triterpenoid glycosides was determined to be the inhibition of (l,3)-β-D-glucan synthase (Onishi et al. 2000). This potent class of glucan synthase inhibitors with favorable solubility characteristics which has resulted in Ibrexafungerp (formerly MK3118 and SCY-078) is a semi-synthetic derivative of the enfumafungin being developed as a new class of β 1,3-glucan synthesis inhibitors with both oral and intravenous antifungal treatment for *Candida* and *Aspergillus* species fungal infections.

Using both CLSI and EUCAST methodologies for antifungal susceptibility testing, it was broadly active against all *Candida* and *Aspergillus* species with MIC values of ≤ 1 μg/ml and ≤ 0.015 μg/ml against clinical isolates of 7 *Candida* spp. and 40 *Aspergillus* spp., respectively. It showed similar potency to caspofungin against *C. albicans*, *C. tropicalis, C. parapsilosis,* and *C. krusei*, but it was eightfold more potent than caspofungin against *C. glabrata* strains (Pfaller et al. 2017a, b; Lamoth and Alexander 2015; Schell et al. 2017). Ibrexafungerp has both IV and oral formulations, which distinguishes it from IV-only echinocandins. It is currently in development for the treatment of fungal infections caused primarily by *Candida* and *Aspergillus* species. It has demonstrated broad spectrum of antifungal activity, in vitro and in vivo, against multidrug-resistant pathogens, including azole- and certain echinocandin-resistant strains with prominent *fks* mutations (Jimenez-Ortigosa et al. 2014, 2017). The FDA has granted QIPD and Fast Track designations for the formulations of SCY-078 for the indications of invasive candidiasis (IC) (including *candidemia*), invasive aspergillosis (IA), and VVC, and has granted Orphan Drug Designation for the IC and IA indications. It has in vivo activity against *Aspergillus* and *Candida* (Ghannoum et al. 2018; Lepak et al. 2015), including against multidrug-resistant (MDR) species *Candida auris* (Ghannoum et al. 2019). Ibrexafungerp is currently in Phase 3 clinical development for the treatment of multiple serious and life-threatening invasive fungal infections caused by *Candida*, *Aspergillus,* and *Pneumocystis* species, as well as efficacy against vulvovaginal candidiasis (VVC) (Larkin et al. 2019). Ibrexafungerp, like other enfumafungins, is not in the echinocandin chemical class. Accordingly, it is highly active against strains of *C albicans* and *C. glabrata* with prominent fks hot-spot 1 mutations conferring echinocandin resistance (e.g., S645F in *C. albicans*.) However, it showed markedly less susceptibility against prominent N-terminal hot-spot 1 mutations (e.g., S641F) that also confer echinocandin resistance suggesting that there may be shared but non-identical binding domain for Ibrexafungerp and echinocandins for inhibition of glucan synthase. Further support for this view has come from detailed in vitro selection of resistant mutants in *C. glabrata* in which mutations common to echinocandin resistance were found, but new mutations outside the hot-spot regions were identified. The mutations conferred both elevated MIC values and several log-fold decreases in sensitivity to drug in glucan synthases kinetic inhibition studies (Jimenez-Ortigosa et al. 2017).

4 Inhibitors of Glycosylphosphatidylinositol

Glycosylphosphatidylinositol (GPI) forms a lipid anchor for many cell-surface proteins. It is a protein posttranslational modification with a glycolipid and found widely in eukaryotes. (Kinoshita 2016). After GPI attachment to proteins, the GPI anchor is remodeled, which regulates the trafficking and localization of GPI-anchored proteins. In fungi, GPIs are transferred to selected glycoproteins that are then transported to the plasma membrane, where they remain anchored to the

outer surface of the plasma membrane via the GPI or they become cross-linked to the cell wall. The GPI anchor is assembled on a phosphatidylinositol lipid in the endoplasmic reticulum where it is covalently attached to a protein's carboxyl terminus. The GPI backbone consists of phosphatidylinositol, glycans comprising glucosamine and mannoses (n = 3), and a terminal phosphoethanolamine, which is amide-bonded to the carboxyl terminus of the protein during GPI attachment. The lipid moiety of the GPI anchor can be a 1-alkyl-2-acyl phosphatidylinositol, diacyl phosphatidylinositol, or inositol-phosphoceramide (Kinoshita 2016). GPI biosynthesis is essential for fungal growth. The GPI proteins are secreted to the cell surface, where they may remain bound to the plasma membrane or, more often, cross-linked to β-1,6-glucan polymers of the cell wall. There are important functional differences in the GPI anchor process that distinguish fungi from mammalian cells, including Gwt1-dependent acylation of inositol and Mcd4-mediated ethanolamine phosphate (EtNP) addition to mannose 1 (Man1) of the GPI core despite the presence of human homologs Pig-W and Pig-N (Pittet and Conzelmann 2007). These properties make this process in fungi desirable as an antifungal target. Utilizing a chemical genomics-based screening platform, novel inhibitors of Gwt1 and Mcd4 were identified with potent antifungal properties (Mann et al. 2015).

Gwt1p is a membrane protein predicted to have 13 predicted transmembrane domains with the amino and carboxy termini facing luminal and cytoplasmic sides of the endoplasmic reticulum (ER) (Fig. 4). Inositol acylation is believed to occur on the luminal side of the ER membrane (Sagane et al. 2011). Utilizing a chemical genomics-based fitness screening platform for *C. albicans*, Mann et al. 2015 identified novel inhibitors of Gwt1 and a second enzyme in the glycosylphosphatidylinositol (GPI) cell wall anchor pathway, Mcd4 (Mann et al. 2015). In 2011, E1210, 3-(3-{4-[(pyridin-2-yloxy)methyl]benzyl}isoxazol-5-yl)pyridin-2-amine, discovered by the Tsukuba Research Laboratories of Eisai Co., Ltd., was described as a new first-in-class, broad-spectrum antifungal with a novel mechanism of action involving inhibition of fungal GPI biosynthesis. It showed efficacy in murine infection models of

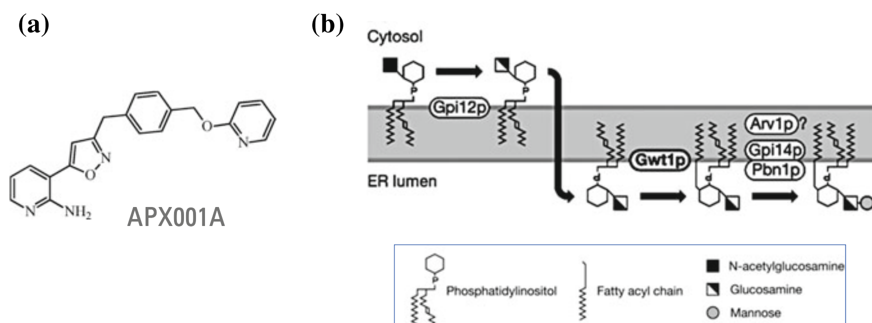


Fig. 4 Inhibition of GPI-anchor protein biosynthesis. **a** Chemical structure of APX001A. **b** Putative GPI-anchor protein biosynthetic pathway and role of Gwt1 (Adapted from Sagane et al. 2011.)

oropharyngeal and disseminated candidiasis, pulmonary aspergillosis, and disseminated fusariosis (Hata et al. 2011).

APX001 is a first-in-class, broad-spectrum antifungal agent for the treatment of invasive fungal infections. It is the N-phosphonooxymethyl prodrug of APX001A (2-amino-1-((phosphonooxy)methyl)-3-(3-((4-((2-pyridinyloxy)methyl) phenyl)methyl)-5-isoxazolyl) -pyridinium) (Fig. 4) (Matsukura 2013); it has received FDA's Orphan Drug designation for six different fungal infections and is classified as a Qualified Infectious Disease Product in the USA. APX001A is active against major pathogenic fungi including *Candida* species (except *C. krusei*), *Cryptococcus neoformans,* Aspergillus species, and other molds like *Fusarium* and *Scedosporium* (Kinoshita 2016). In an in vitro susceptibility study of 1706 contemporary clinical fungal isolates collected in 2017 from 68 medical centers worldwide using the CLSI reference broth microdilution method, APX001A was more potent than conventional azole and echinocandin antifungal agents (Pfaller et al. 2019). It displayed highly potent $MIC_{50/90}$ values of 0.008/0.06 μg/ml against *Candida* species that were 1–2 log orders more sensitive than existing antifungal agents. APX001A was $\geq$eightfold more active than fluconazole against *C. neoformans* var. *grubii* and showed comparable activity to the echinocandins with Aspergillus spp. APX001A was also highly active against uncommon species of *Candida* and rare molds, including 11 isolates of *Scedosporium* spp. Finally, APX001A demonstrated potent in vitro activity against recent fungal isolates, including echinocandin- and fluconazole-resistant strains (Pfaller et al. 2019). In a related study, Arendrup et al. (2018a) reported that APX001A was highly active against most *Candida* species including drug-resistant *Candida auris* and Candida *glabrata*, although it was less active on *C.* krusei and *C. norvegensis.* Some non-wild type isolates were fluconazole resistant, and a correlation was observed between APX001A and fluconazole MICs across all species except C. *guilliermondii* and *C. auris*. This raises the prospect that APX001A may be a substrate for drug efflux transporters induced in the azole-resistant strains. Azole resistance in *C. auris* correlates with specific amino acid mutations in Erg 11 (Healey et al. 2018), and as such, APX001A is active in vivo against drug-resistant *C. auris* (Hager et al. 2018). APX001 shows wide tissue distribution in rats and monkeys including the brain (Mansbach et al. 2017). Importantly, treatment with APX001 leads to a significant reduction in brain CFU in both a rabbit model of hematogenous *C. albicans* meningoencephalitis and a mouse disseminated *C. auris* model (Hager et al. 2018). APX001A has good in vitro activity against the mold form of *Coccidioides* with a potent MEC_{90} of 8 ng/ml (Viriyakosol et al. 2019). Finally, APX001 is highly effective in murine models of invasive pulmonary aspergillosis (Gebremariam et al. 2019). APX001 is being developed as both an IV and oral formulation, which facilitates IV treatment within the hospital setting and step-down oral treatment after patient discharge. In recently completed Phase 1 studies, both formulations were well tolerated and showed favorable safety profile in Phase 1 studies with a low proclivity for clinically relevant drug–drug interactions. A Phase 2 study is being conducted in patients with candidemia, as well as an additional Phase 1b study in patients who are neutropenic (e.g., acute leukemia

patients receiving chemotherapy) and at risk of fungal infections. Finally, the nature of resistance resulting in therapeutic failures is unknown at this stage. Mutations in GWT1 can lead to higher MIC values, and *C. krusei* is inherently resistant to drug suggesting that over-expression of certain drug pumps may be a factor for emerging resistance.

5 Inhibitors of Chitin Synthesis

Chitin is an essential structural component of fungal cell walls and septa, and it is synthesized by multiple chitin biosynthetic enzymes. As chitin is not found in human cells, it has been a long-standing attractive target for antifungal therapy (Ruiz-Herrera and San-Blas 2003). Chitin synthesis is catalyzed by the transfer of GlcNAc from UDP-GlcNAc to chitin by chitin synthase. In *Candida albicans*, chitin is synthesized by a family of four isoenzymes, which comprise three separate classes of chitin synthase enzymes, Chs1 (class II), Chs3 (class IV), Chs2 and Chs8 (class I) (Lenardon et al. 2010). The two class I enzymes, Chs2 and Chs8, are responsible for the majority of chitin synthase activity (Preechasuth et al. 2015). Class I enzymes reinforce cell wall integrity during early polarized growth.

Polyoxins and Nikkomycins are two structurally related groups of secondary metabolites that are selective inhibitors of chitin synthetase. The polyoxins (polyoxin A, B, D) and nikkomycins (nikkomycin X, Z) described in the 1960s and 1970s are peptide–nucleoside compounds produced by *Streptomyces cacaoi* and *Streptomyces tendae*, respectively (Zhang and Miller 1999). They are structural similarity to UDP-N-acetylglucosamine (Fig. 5), and the precursor substrate for chitin and both has comparable in vitro potency against isolated chitin synthetases from a variety of fungi.

They are competitive inhibitors of Chs2, Chs1, and Chs3 (Cabib 1991; Gaughran et al. 1994). In yeasts, nikkomycin Z enters the cell via distinct peptide transport systems (McCarthy et al. 1985). Extensive efforts over the decades, neither polyoxins nor nikkomycins, have been developed to treat common *Candida*

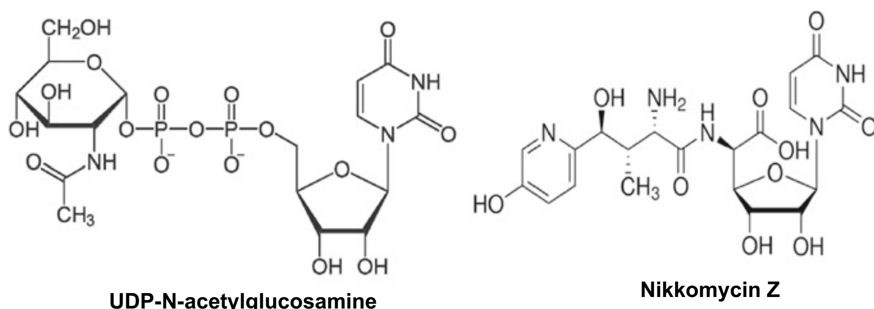


Fig. 5 Chemical structure for UDP-N-acetylglucosamine and Nikkomycin Z

and *Aspergillus* invasive infections. This most likely reflects the complexity of the chitin synthase enzymes and the dynamic and resilient nature of the cell wall stress responses. However, recently Nikkomycin Z has been advanced as a first-in-class antifungal drug for Coccidioidomycosis (Valley Fever). In ongoing multi-dose human safety Phase I trials, nikkomycin Z has thus far showed little or no toxicity. This agent also had previously demonstrated in vivo activity in mouse models of histoplasmosis (Goldberg et al. 2000) and blastomycosis (Clemons and Stevens 1997). The US Food and Drug Administration (FDA) granted nikkomycin Z as a "qualifying infectious disease product" (QIDP). Resistance to nikkomycin can occur through a defect in the one of the transporters for dipeptides (Yadan et al. 1984), as well as in Gig1, which may play a role GlcNAc metabolism (Gunasekera et al. 2010).

6 Conclusion and Perspective

The clinical success of the echinocandin drugs has validated the importance of the cell wall as a prominent target for safe and efficacious antifungal therapy. In particular, direct inhibition of β-(1,3)-glucan synthase has emerged as an important focal point for current drug development with a new long-acting echinocandin and an orally available enfumafungin nearing clinical approval. The current echinocandin drugs are limited by spectrum, delivery by IV-only route, limited tissue distribution, and emergence of drug resistance. These limitations are substantially addressed by these newer agents. The new Gwt1 GPI anchor protein inhibitor also overcomes many of the limitations of echinocandins with high potency and activity against a range of fungi including *Aspergillus*, other molds, and *Cryptococcus*. Finally, after decades of investment, nikkomycin Z with its potent inhibition of chitin biosynthesis is poised to have a significant impact on difficult to treat endemic fungi. Other important targets in the cell wall ripe for discovery include β-1,6-glucan synthase or other enzymes involved in the interconnections of cell wall glucans, which have lead inhibitors but have not been developed (Kitamura et al. 2009).

The importance of the wall to the fungal cell is also reflected by the fact that numerous stress-response pathways have evolved to maintain its integrity. These pathways, which rapidly sense wall insult and injury, play a critical role in adaptation of the cell to cell wall-active drugs. This drug-adapted tolerance state predisposes the cell for genetically induced breakthrough resulting in clinical drug resistance. Intervening in the tolerance pathways may be an important adjunct for cell wall-directed therapies to prevent downstream drug resistance. The complexity of the cell wall and its biosynthetic machinery provides a fertile landscape for drug discovery as numerous essential targets abound. Yet, equally important, the cell wall houses numerous non-essential targets that are critical for host recognition and targeting these virulence components may be a highly productive route for new antifungals that can be used for therapy and prophylaxis. The lesson of current

cancer immunotherapy is that non-essential virulence targets can be easily modified with a new generation of biologics (e.g., antibodies) and cell therapy.

Finally, it is time to consider the use of combination therapy involving multiple cell wall targets to address difficult to treat infections, including certain molds. Combined inhibition of β-(1,3)-glucan synthase by echinocandins and chitin synthase with nikkomycin Z has a profound impact on cellular morphology that significantly impact viability and pathogenicity (Cheung and Hui 2017).

Acknowledgements This work was supported by grants from the US National Institutes of Health (AI109025) and Astellas (Reference Center for Molecular Evaluation of Drug) to D.S.P.

Disclosures

Dr. Perlin serves on scientific advisory boards and/or receives grant support from Merck, Astellas, Synexus, Cidara, and Amplyx.

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Mitochondrial Control of Fungal Cell Walls: Models and Relevance in Fungal Pathogens

Barbara Koch and Ana Traven

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Abstract Proper structure and function of the fungal cell wall are controlled by metabolic processes, as well as an interplay between a range of cellular organelles. Somewhat surprisingly, mitochondrial function has been shown to be important for proper cell wall biogenesis and integrity. Mitochondria also play a role in the susceptibility of fungi to cell wall-targeting drugs. This is true in a range of fungal species, including important human fungal pathogens. The biochemical mechanisms that explain the roles of mitochondria in cell wall biology have remained elusive, but studies to date strongly support the idea that mitochondrial control over cellular lipid homeostasis is at the core of these processes. Excitingly, recent evidence suggests that the mitochondria–lipid linkages drive resistance to the echinocandin drug caspofungin, a clinically important therapeutic that targets cell wall biosynthesis. Here, we review the state of affairs in mitochondria–fungal cell wall research and

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Current Topics in Microbiology and Immunology (2020) 425: 277–296
https://doi.org/10.1007/82_2019_183
© Springer Nature Switzerland AG 2019
Published Online: 6 December 2019

propose models that could be tested in future studies. Elucidating the mechanisms that drive fungal cell wall integrity through mitochondrial functions holds promise for developing new strategies to combat fungal infections, including the possibility to potentiate the effects of antifungal drugs and curb drug resistance.

1 Introduction

It is estimated that around 300 fungal species are pathogens of humans (Hawksworth and Lücking 2017), with *Candida, Aspergillus, Cryptococcus* and *Pneumocystis* being the most common causes of deadly infections (Brown et al. 2012). Serious fungal infections primarily occur in immunocompromised individuals, for example upon cancer chemotherapy-associated neutropenia or with long-term corticosteroid therapy. Apart from this, hospital procedures, such as central venous catheters and surgeries that break down mucosal barriers also increase the risk of contracting a fungal infection (Perlroth et al. 2007).

Most human fungal pathogens are opportunistic and are either environmental or human commensal species. In their natural habitats, fungi have evolved to be exceptionally well adapted to rapid changes in nutrient supply. This serves them well in the situation of infection, as the availability of many nutrients is limited in the human host (Miramón and Lorenz 2017). The possibility to quickly adjust their metabolism to the conditions they are facing helps fungi survive in many different host niches. Consistent with this, mitochondria have been shown to be important for virulence of human fungal pathogens, reviewed by Calderone et al. (2015) and Shingu-Vazquez and Traven (2011). Mitochondria play several functions in fungal virulence, including in the biology of cell walls, which is the topic that we review here.

The mechanisms that control cell wall biogenesis, remodeling and integrity have been the focus of intense research because this structure maintains viability of the fungal cell and further promotes host cell attachment, invasion, immune recognition and the response of fungi to a major class of antifungal drugs, the echinocandin inhibitors of β-1,3 glucan synthase. The inner wall structure is composed of chitin, β-1,3 glucan and β-1,6 glucan (Gow et al. 2017; Latgé 2007). While the inner core is very similar in different fungal species, the outer layer varies and consists of mannosylated glycoproteins. The composition and structure of the cell wall determine the recognition of fungi by the host's immune system. Mannan, β-glucan, chitin and other cell wall polysaccharides serve as pathogen-associated molecular patterns (PAMPs), which are detected by monocytes, macrophages and neutrophils in the circulation and in infected tissues via membrane-localized or soluble pattern recognition receptors (PRRs), including TLR2, TLR4 and Dectin-1 (Netea et al. 2015). Recognition of PAMPs promotes phagocytosis and, ideally, killing of the fungal cell. As such, the fungal cell wall "reveals" the fungus as an invader to the host. In recent years, it has become apparent that fungi have developed mechanisms to facilitate evasion of the immune system by concealing ("*masking*") their cell wall components in response to stimuli such as non-fermentable carbon source and low oxygen (Ballou et al. 2017; Lopes et al.

2018; Pradhan et al. 2018). These results indicate that the structure of the cell wall and its organization respond to the metabolic state of the fungal cell. The nature of the environmental changes causing cell wall rearrangements, chiefly fermentable versus non-fermentable carbon source and the levels of oxygen (Ballou et al. 2017; Ene et al. 2012; Lopes et al. 2018; Pradhan et al. 2018) strongly points to the involvement of mitochondria in this process. Indeed, several lines of evidence show that mitochondrial defects trigger fungal cell wall dysfunction (Dagley et al. 2011; Qu et al. 2012; She et al. 2015, 2013; Sun et al. 2013). In this review, we will summarize the current state of research and explore the models for how mitochondria might influence the fungal cell wall. Of the major human fungal pathogens, this topic has been most intensively studied in *Candida albicans*, which is why this organism will be the predominant model of choice in this review. It is reasonable to expect that much of our understanding of mitochondrial roles in cell wall biology will be broadly applicable across fungal species.

2 Metabolic Aspects of Cell Wall Biogenesis and Links with Mitochondrial Functions

Cell wall biogenesis is a metabolically challenging process, which requires the biosynthesis and remodeling of complex carbohydrates, a regulated interplay of several organelles, intracellular transport and trafficking processes and the function of multiple signaling pathways that respond to cell wall dysfunction. The building blocks of the cell wall are directly linked to metabolism; for example, glucan is a polymer of glucose, chitin is a polymer of *N*-acetyl-glucosamine, and the synthesis of glycosyl-phosphatidylinositol (GPI) anchors present in many cell wall proteins integrates carbohydrate and phospholipid metabolism. The main synthesizing enzymes for chitin and glucan are found in the phospholipid environment of the plasma membrane (meaning that the lipid environment could control their activity), while mannosylation of glycoproteins occurs in endoplasmic reticulum (ER) and the Golgi via the action of membrane enzymes, and GPI anchors for cell wall proteins are attached in the ER. A key pathway to regulate cellular integrity and cell wall maintenance is the protein kinase C (PKC) pathway, which is conserved in most fungal species including human fungal pathogens (Rispail et al. 2009). Furthermore, the Ca^{2+}/calcineurin pathway, the HOG pathway and the RIM101 pathway all contribute to cell wall adaptation in response to environmental conditions and signals (Du and Huang 2016; Román et al. 2019; Yu et al. 2015).

Mitochondria are metabolic hubs, and every eukaryotic cell contains several of them. Mitochondria are best known for their role in oxidative phosphorylation as energy providers, and are home for other major metabolic activities such as the citric acid cycle. Mitochondria are also the location of the biosynthesis of certain amino acids, iron–sulfur clusters and heme. Beyond that, mitochondria are intensively involved in phospholipid metabolism as the majority of phosphatidylethanolamine (PE) for cellular membranes is synthesized in the mitochondria. Mitochondrial morphology is associated with correct organelle function. Under standard growth

conditions, fungal mitochondria most commonly display a network of tubular organelles. This structure is maintained via dynamic fusion and fission events, and further to this, dynamic changes to mitochondrial morphology can occur in response to stress, such as stress-induced fragmentation (Fannjiang et al. 2004).

It is also now appreciated that mitochondria do not exist in isolation within cells. Instead, they are highly interconnected via so-called *membrane contact sites*, which are often mediated by protein tethers and enable the functional connections between mitochondrial membranes and other membrane structures in the cell, including the plasma membrane, the ER, the vacuole, peroxisomes and also lipid droplets (Lackner 2019; Scorrano et al. 2019). The contacts of mitochondria with other organelles are important for the transfer of molecules, such as phospholipids, and further serve to coordinate mitochondrial dynamics, division and inheritance (Jeong et al. 2017; Kawano et al. 2018; Lackner et al. 2013). Multiple mitochondrial tethers have been described in fungi, including ER-mitochondria encounter structure (ERMES) (Kornmann et al. 2009), vacuole and mitochondrial patch (vCLAMP) (Elbaz-Alon et al. 2014) and mitochondria-ER-cortex anchor (MECA) (Lackner et al. 2013), reviewed by Lackner (2019).

Mitochondrial activity influences the structure and organization of the fungal cell wall, but the biochemical mechanisms behind this remain unclear. Substantial research efforts are focused on solving the link between mitochondrial function, mitochondrial morphology and cell wall structure. This interest is also fueled by the demand for new antifungal drugs, as the mitochondrion would be an attractive drug target. Even though mitochondrial factors are generally conserved between fungi and humans, it is possible to identify fungal-selective inhibitors, as exemplified by the cytochrome bc1 inhibitor Inz-1 (Vincent et al. 2016). Additionally, there are mitochondrial proteins that play important roles in the biology of fungal pathogens, but lack structural homolog in mammalian mitochondria or do not exist at all in mammals. Examples are the ER-mitochondria tether ERMES characterized in *C. albicans* and *A. fumigatus* (Becker et al. 2010; Geiβel et al. 2017; Tucey et al. 2016) and the *C. albicans* Goa1 protein needed for assembly of respiratory complex I in the mitochondrial inner membrane (Bambach et al. 2009).

One of the early reports showing that mitochondrial function is connected to cell wall biogenesis came from Lussier et al. (1997). In order to identify genes involved in cell wall assembly, the authors screened transposon-mutagenized baker's yeast *Saccharomyces cerevisiae* for altered sensitivity to calcofluor white and discovered mitochondria-related genes, such as the cytochrome c oxidase assembly factor *COX11*, the phosphatidylglycerol synthase *PGS1* (also known as *PEL1*) and *IFM1*, which encodes the mitochondrial translation initiation factor. Deletion of these genes resulted in hypersensitivity to calcofluor white and in some cases led to additional phenotypes indicative of aberrant cell wall structure, such as sensitivity to zymolyase and in the case of the *pgs1* mutant reduced N-acetylglucosamine (chitin) (it should be noted that for the *pgs1,* mutant the transposon insertion was in the promoter region, rather than the coding sequence) (Lussier et al. 1997). A few years later, Page et al. identified 17 deletion mutants of *S. cerevisiae* with impaired respiration and ATP metabolism that showed increased resistance to the K1 killer toxin (Pagé et al. 2003).

The K1 pore-forming toxin is produced by K1 killer yeast strains and requires the presence of β-1,6-glucan and O-mannosylation on the cell wall to cause cellular damage (Hutchins and Bussey 1983). Mitochondrial mutants of several *Candida* species and *S. cerevisiae* are hypersensitive to the echinocandins (Chamilos et al. 2006; Dagley et al. 2011; Sarinová et al. 2007) and can also be hypersensitive to cell wall inhibitors such as congo red and calcofluor white (Dagley et al. 2011; Qu et al. 2012; She et al. 2016, 2015, 2013; Sun et al. 2013). Links between mitochondria and the cell wall have also been made in *C. neoformans* through Vps45, an SM (Sec1/Mun18) protein found to have partial mitochondrial localization and impact on mitochondrial and cell wall functions (Caza et al. 2018). Recent work in *A. fumigatus* demonstrated a link between respiratory complex I activity and the ability of cells to grow at high concentrations of the echinocandin caspofungin (the so-called *paradoxical effect*) (Aruanno et al. 2019). This suggests links between mitochondria respiration and cell wall stress responses in this pathogenic species.

3 Mitochondrial Phospholipid Metabolism and Cell Wall Structure

Mitochondria display a complex phospholipid membrane system consisting of an outer and an inner mitochondrial membrane. These two membranes differ in structure and lipid composition, which creates a specific environment for optimal membrane and enzyme function. Mitochondria are able to synthesize some of their phospholipids on their own, but they are also dependent on phospholipid supply from the ER; for review see Tatsuta et al.(2014). The capacity of mitochondria to synthesize their own lipids is restricted to phosphatidylglycerol (PG), cardiolipin (CL) and phosphatidylethanolamines (PE). Enzymes synthesizing phosphatidylcholine (PC), phosphatidic acid (PA), phosphatidylinositol (PI) and phosphatidylserine (PS) are missing in mitochondria. Therefore, these organelles are strictly dependent on the import of these lipids. In *S. cerevisiae*, mitochondria represent the major source of cellular PE, although different routes for PE synthesis exist in various organelles such as the Golgi apparatus, ER or lipid droplets. For PE synthesis in mitochondria, PS is imported from the ER and the mitochondrial phosphatidylserine decarboxylase Psd1p decarboxylates PS to form PE, which is then redistributed within the cell (Birner et al. 2001; Clancey et al. 1993). While we can assume this to be conserved, whether mitochondria-derived PE predominates in other fungal species remains to be directly determined.

The contribution of mitochondria to phospholipid homeostasis of the cell has been implicated in the regulation of cell wall integrity. Deletion of *PGS1*, the mitochondrial phosphatidylglycerol phosphate synthase, deprives the cell of CL and PG. Loss of PG and CL results in hypersensitivity to cell wall perturbing agents such as caffeine and calcofluor white and reduces the β-1,6 and β-1,3 glucan levels in *S. cerevisiae* (Zhong et al. 2005). In the *pgs1* deletion mutant, chitin is mislocalized from bud scars to being along the cell walls and its levels increased

threefold (Zhong et al. 2005). Upregulation of chitin levels to fortify the wall is a typical response to cell wall dysfunction, and therefore, this phenotype further underscores a role for *PGS1* in cell wall integrity in *S. cerevisiae*. The reduction in glucan levels in the *pgs1* deletion mutant was attributed to decrease glucan synthase activity and dysfunction of cell wall integrity pathway activation (Zhong et al. 2007). The function of CL in cell wall integrity is also shown by the fact that inactivation of the cardiolipin synthase gene *CRD1* in *S. cerevisiae* causes hyper-susceptibility to caspofungin, which belongs to the echinocandin family of antifungal compounds (Sarinová et al. 2007). CL is only found in mitochondrial membranes (mainly in the inner membrane), posing the question of what its roles in cell wall biogenesis might be. Although speculative at the moment, the answer could be related to signaling for cell wall integrity. In mammalian cells, CL has been shown to play important signaling functions at mitochondria (Dudek 2017), and CL has also been implicated in cell cycle control signaling in *S. cerevisiae* under conditions of respiratory deficiency (Chen et al. 2010a, b).

Deletion of *PGS1* not only compromises phospholipid homeostasis in mitochondria, but also results in loss of the mitochondrial genome (mtDNA) and therefore leads to respiratory deficient cells. In itself, loss of mtDNA introduces a drastic change in cell wall composition compared to wild type cells, mainly decreasing the levels of glucans (Zhong et al. 2005). Whereas the links between mitochondrial function and cell wall integrity are undoubtable, the relationship between mitochondrial membrane lipids and loss of mtDNA complicates the mechanistic interpretation of the *pgs1* mutant data—is the loss of cell wall glucan resulting from changes in lipid composition or from respiratory deficiency? This consideration is pertinent to other mitochondrial mutants as well, as many mitochondrial mutations have indirect effects on mtDNA stability, and mitochondrial membrane lipids are intimately linked with the function and assembly of the respiratory chain.

In addition to PG and CL, PS and PE are also important for proper cell wall biogenesis. The phosphatidylserine synthase Cho1 catalyzes the production of PS from cytidyldiphosphate-diacylglycerol (CDP-DAG) and serine in the ER. PS travels from the ER to mitochondria, where it serves as a substrate for mitochondrial Psd1 to form PE. Deletion of *cho1* in *C. albicans* leads to cell wall integrity deficiencies, which result in abnormalities of the ultrastructure of the cell wall, higher levels of chitin and hypersensitivity to SDS and caspofungin (Chen et al. 2010a, b). The *cho1* mutant has a drastic reduction in PS and also displays lower levels of PE (due to PS being a PE precursor) (Chen et al. 2010a, b). A role for PE in cell wall integrity in *C. albicans* has been demonstrated directly by the phenotypes of the mutant that lacks mitochondrial *PSD1*, and the double mutant of *PSD1* and the backup phosphatidylserine decarboxylase *PSD2*. These mutants are hyper-susceptible to caspofungin and their reduced growth can be rescued by osmotic stabilization (Chen et al. 2010a, b).

4 Mitochondrial Morphology, Cell Wall Structure and Links to Phospholipids

The lipid composition of mitochondrial membranes is connected to mitochondrial morphology, as mutants with impaired mitochondrial lipid homeostasis can display a disrupted structure of mitochondria (Dagley et al. 2011; Kornmann et al. 2009). Several mitochondrial morphology mutants in *S. cerevisiae* show hypersensitivity toward caspofungin and calcofluor white. A high proportion of these mutants is connected to phospholipid homeostasis, including *SAM37, MDM10, MDM35, MDM31* and *UPS2* (Dagley et al. 2011). Deletions of *MDM10, MDM35, MDM31* and *UPS2* result in altered levels of PE, and for *MDM10* and *MDM31* also in CL (Kornmann et al. 2009; Osman et al. 2009; Tamura et al. 2009). Mdm10 is located at the outer mitochondrial membrane as a subunit of the ERMES mitochondria-ER tether, as well as a subunit of the sorting and assembly machinery (SAM) involved in the biogenesis of mitochondrial outer membrane proteins (Kornmann et al. 2009; Meisinger et al. 2004). Loss of the SAM subunit Sam37 in *S. cerevisiae* results in a 50% slower conversion of PS to PE and PC in mitochondria (Dagley et al. 2011), showing a role for Sam37 in phospholipid homeostasis. Mdm31 is in the inner mitochondrial membrane and shows genetic interactions with ERMES (Dimmer et al. 2005), and Mdm35, found in the mitochondrial intermembrane space, also contributes to phospholipid homeostasis (Osman et al. 2009; Tamura et al. 2009).

The functions of ERMES and SAM in cell wall functions have been further explored in *C. albicans*. Deletion of the *C. albicans SAM37* gene results in a cell wall characterized by increased thickness, although it displays the same relative levels of mannan, β-1,6 and β-1,3 glucans as the wild type (Qu et al. 2012). The *sam37* deletion strain is also avirulent in the mouse bloodstream infection model (Qu et al. 2012). It should also be mentioned that deletion of *Sam37* results in loss of mtDNA (Qu et al. 2012). As *C. albicans* is considered a so-called petite-negative organism, only cells which are able to maintain their mtDNA will survive. Therefore, *sam37* deletion mutants are slow-growing and only viable because a proportion of cells kept their mtDNA and respiratory proficiency (Qu et al. 2012). Conditional inactivation of the ERMES subunit *MMM1* in *C. albicans* led to a rapid change in mitochondrial morphology and loss of tubular structure (Tucey et al. 2016). Hyphae of the *mmm1* mutant are of normal morphology with a tendency to form shorter filaments, and the *mmm1* mutant showed reduced exposed β-1,3 glucan on the cell wall possibly due to slower hyphal extension (Tucey et al. 2016). Further indication of a role for ERMES in cell wall integrity is that *C. albicans* mutants in the core subunits *MMM1*, *MDM10* and *MDM12* display higher susceptibility to caspofungin (Koch et al. 2017). Deletion of the *C. albicans* gene encoding the mitochondrial GTPase Gem1, which is associated with ERMES in *S. cerevisiae*, also resulted in loss of tubular mitochondrial morphology, cell wall stress susceptibility and delayed activation of the cell wall stress signaling pathway orchestrated by the kinase Cek1 (Koch et al. 2017). These data suggest links between mitochondrial morphology and cell wall signaling in *C. albicans*.

5 Models for How Mitochondrial Morphology and Lipid Homeostasis Might Impinge on Cell Wall Integrity

The mechanisms by which mitochondria influence cell wall biogenesis via their roles in phospholipid homeostasis and organelle morphology are not solved yet. Some possibilities can be proposed (Fig. 1). It has recently been shown that proteins associated with cell wall biogenesis are also located in mitochondria. Recent proteome characterization in *S. cerevisiae* of highly purified mitochondria using stringent criteria identified about 200 proteins that were not yet annotated as mitochondrial proteins (Vögtle et al. 2017). Among them were the factors involved in cell wall biogenesis such as Bgl2 (an endo-β-1,3 glucanase involved in cell wall maintenance) and the glucanase Exg2, as well as β-1,3 glucanosyltransferases from the GAS family (Gas3, Gas5) for which the precise roles in cell wall integrity remain to be understood (Vögtle et al. 2017). Rho1, the GTP-binding protein that regulates the activity of the β-1,3 glucan synthase Fks1 was also found to be localized in mitochondria (Reinders et al. 2006; Renvoisé et al. 2014; Zahedi et al. 2006), and surprisingly so was Fks1 itself, although with low coverage (Sickmann

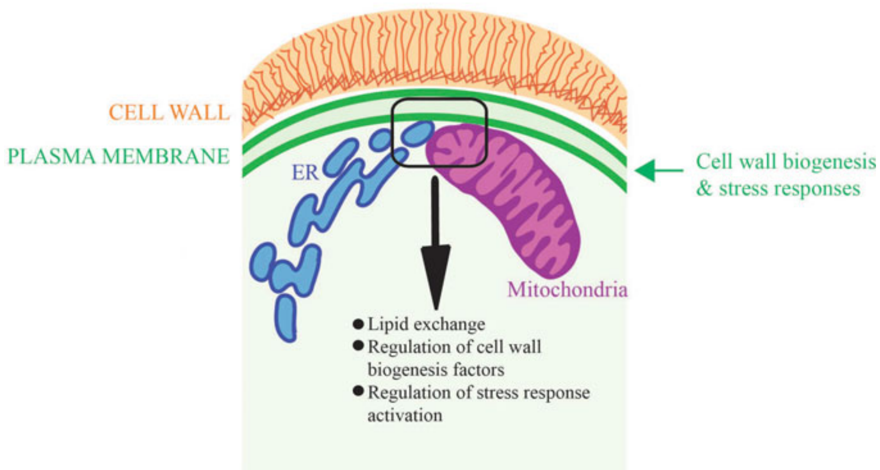

Fig. 1 Model for how mitochondrial morphology and lipids homeostasis might regulate cell wall integrity. Cell wall biogenesis enzymes and various regulators of signaling are localized in the plasma membrane. As such, the plasma membrane is the location of cell wall biogenesis and it is important in signal transduction mechanisms responding to cell wall stress. Physical closeness mediated by membrane contact sites between mitochondria, the ER and the plasma membrane (see black box) might play a role in establishing a microenvironment and lipid composition that promotes proper cell wall biogenesis and stress responses. In baker's yeast *S. cerevisiae,* multiple protein tethers have been described, which mediate membrane-to-membrane contacts with mitochondria and bring them in proximity to other organelles, reviewed by Lackner (2019). Membrane contact sites do not allow for membrane fusion, but the membranes are close, from <80 nm to being significantly further apart (approximately 300 nm, such as in the case of the plasma membrane tethering protein Num1 of the MECA complex) (Ping et al. 2016) and reviewed by Scorrano et al. (2019). Disruption of mitochondrial morphology would be expected to impair contacts with other organelles, which could in turn impact on the membrane-localized processes that are important in cell wall biology (biosynthesis, integrity and stress responses).

et al. 2003; Vögtle et al. 2017). Mitochondrial localization of these cell wall biogenesis factors or regulators is surprising, and it should be kept in mind that these findings come from "omics" studies and future research will need to clarify if this is a case of "localization" or "association" with mitochondria. Mitochondria are known to be tethered to the plasma membrane via the MECA tether (Lackner et al. 2013). These membrane contact sites essentially bring mitochondria in physical proximity to the location of cell wall biogenesis—the plasma membrane (Fig. 1). By fluorescence microscopy, the MECA complex can be observed all around cell periphery of yeast cells (*S. cerevisiae*), including at the mother-bud neck (Kraft and Lackner 2017). To our knowledge, the MECA complex has not been visualized in filamentous hyphal species, but it is worth noting that, for example in *Neurospora*, mitochondria are found in high numbers at the growing hyphal tip (where cell wall synthesis occurs) (Levina and Lew 2006). Fks1 is an integral plasma membrane protein, and its regulator Rho1 also localizes to the membrane. It is possible that their mitochondrial association is a result of their co-localization to membrane contact sites between the mitochondrial outer membrane and the plasma membrane. A speculative model might be that the lipid composition of mitochondrial membranes, dynamic regulation of mitochondrial morphology and the impact of these processes on mitochondria–plasma membrane contacts might play a role in controlling the activity of these cell wall factors. Rho1 is an important regulator of the PKC-dependent cell wall integrity pathway, and roles of mitochondrial PG and CL have been proposed in PKC pathway activation in *S. cerevisiae* (Zhong et al. 2007). Indeed, cell wall stress signaling fundamentally originates at the plasma membrane and in *C. albicans* inactivation of the mitochondrial GTPase Gem1 delayed Cek1 pathway activation in response to cell wall stress (Koch et al. 2017).

The membrane linkages between mitochondria, the ER and the plasma membrane could further facilitate the exchange of lipids and regulate the mitochondria–lipids–cell wall linkages. We have previously proposed that mitochondrial effects on phospholipid homeostasis might impact on the activity of glucan or chitin synthases, which are integral plasma membrane enzymes (Dagley et al. 2011; Shingu-Vazquez and Traven 2011). A recent example from *Aspergillus fumigatus* showed an indirect way for mitochondria to influence the lipid composition of the plasma membrane and with that the susceptibility of glucan synthase FKS1 to caspofungin (Satish et al. 2019). Lipid profiling by mass spectrometry showed that the growth with caspofungin triggers an increase in dihydrosphingosine and phytosphingosine in lipid fractions containing Fks1, and the authors postulated that these lipid changes trigger a distinct conformation of Fks1 and limit the interaction with caspofungin (Satish et al. 2019). This effect of caspofungin on the lipid microenvironment of Fks1 was linked to caspofungin-induced mitochondrial ROS production (Satish et al. 2019). How exactly mitochondrial ROS leads to an altered sphingolipid composition still needs to be investigated, but the proximity of mitochondria, the ER and the plasma membrane places the relevant organelles together. In *S. cerevisiae,* mitochondria-derived ROS activates TORC2 signaling regulating the early steps of sphingolipid biosynthesis (Niles et al. 2014; Satish et al. 2019), and sphingolipid homeostasis more generally has been connected to

mitochondrial functions, including the mitochondria–nucleus signaling pathway called the retrograde response that responds to mitochondrial dysfunction (Spincemaille et al. 2014). Similar mechanisms might be involved in the caspofungin–mitochondria–sphingolipids process described in *A. fumigatus*.

Mitochondrial lipid functions, particularly in PE synthesis, could further impact on cell wall integrity via effects on GPI anchors. These anchors are added to proteins in the ER via the transaminidase enzyme complex. This concept is supported by phenotypes of the *sam37* mutant in *S. cerevisiae*, which shows slower conversion of PS to PE and lower protein levels of the GPI-anchored β-1,3 glucanosyltraferase Gas1 (Dagley et al. 2011). This can be partially rescued by exogenous ethanolamine, which can serve as a precursor for PE synthesis via the non-mitochondrial Kennedy pathway (Dagley et al. 2011).

6 Mitochondrial Respiration and Cell Wall Structure

Yeasts use two different pathways to meet their energy demands, namely respiration and fermentation. *S. cerevisiae* uses a combination of fermentation and respiration with a strong preference toward fermentation even when oxygen levels are high; whereas, *C. albicans*, *C. parapsilosis*, *C. tropicalis* and other related species continue respiration even in the presence of high levels of fermentable sugar (Veiga et al. 2000). *C. albicans* and related species possess the electron transport complexes I-V located in the inner mitochondrial membrane and utilize the canonical electron transport chain (ETC). They also harbor alternative oxidases (Aox1 and Aox2) permitting respiration even when the classical ETC is compromised (Aoki and Ito-Kuwa 1984). *C. glabrata*, similarly to *S. cerevisiae,* does not possess a complex I (Koszul et al. 2003).

Fungal mutants deficient in mitochondrial respiration tend to exhibit hypersensitivity to cell wall-targeting drugs, indicating that their cell wall structure and organization are altered (Fig. 2). The contribution of complex I of the ETC toward cell wall integrity is one of the best studied, especially the roles of the *C. albicans* proteins Goa1, Nuo1, Nuo2 and Ndh51. The function of Goa1 likely lies in regulating the activity of complex I; whereas, Nuo1 and Nuo2 assist in the assembly of this complex, and NDH51 is a complex I subunit. What makes these proteins of special interest is that only Ndh51 is highly conserved in mammals, while GOA1 is found only in the CTG clade of *Candida* spp., which includes most of the human pathogenic *Candida* species (Bambach et al. 2009). Nuo1 is found in fungi, algae and plants, and Nuo2 is fungal specific (She et al. 2015). Inactivation of the genes *GOA1, NUO1* or *NUO2* results in reduced energy production, which is mainly attributed to reduced function of complex I (She et al. 2015). Loss of any one of these genes also results in increased ROS levels and, in case of *GOA1*, increased sensitivity to congo red and calcofluor white (Li et al. 2016; She et al. 2013). A transcriptomic analysis of *C. albicans* deletion strains of the genes *GOA1, NUO1, NUO2* and *NDH51* confirmed that genes involved in cell wall biogenesis and integrity were differentially regulated in all four mutants and showed that the loss of

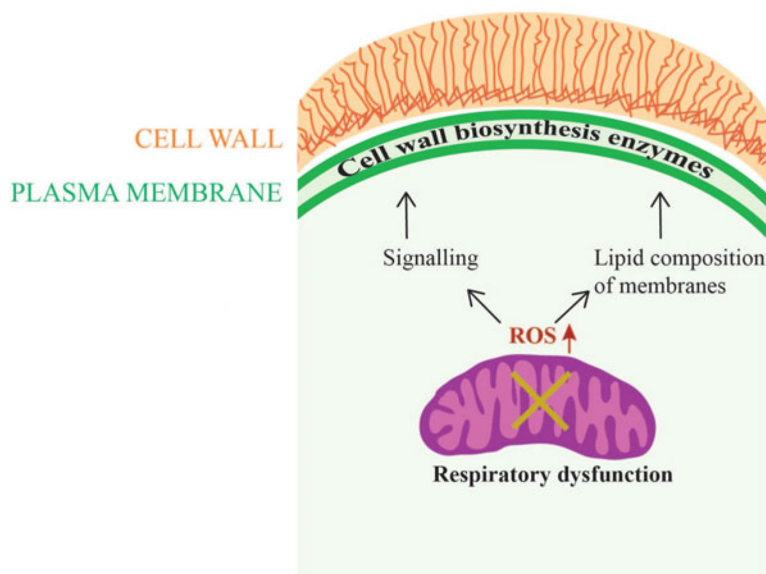

Fig. 2 ROS induced by respiratory blocks impact on cell wall structure and drug resistance. Genetic or biochemical inhibition of mitochondrial respiration triggers production of ROS. Moreover, caspofugin has been shown to induce mitochondrial ROS production in *A. fumigatus* (Satish et al. 2019). Intriguingly, mitochondrial ROS induction changes the sphingolipid composition of the glucan synthase environment in the membrane, triggering caspofungin resistance. ROS could further act as a signaling molecule for cell wall integrity.

GOA1, NUO1 or *NUO2* had more effect on cell wall-related pathways than the loss of *NDH51* (She et al. 2015). The absence of these three factors in mammalian cells and their effect on mitochondria and cell wall integrity would make them acceptable targets for new antifungal drugs. Therefore, several studies have been conducted with more detailed analyses of the consequences of their loss on the cell wall.

A transcriptomics study showed that 86 genes involved in cell wall biogenesis, integrity and adhesion including *FKS1*, *PHR1* and *PHR3* and the β-1,2 mannosyltransferase *BMT3* are downregulated in the *goa1* mutant (She et al. 2013; Sun et al. 2013). This accounts for about 5.4% of all downregulated genes (She et al. 2013). Of note, although the genes are related to cell wall functions, not all of the 86 genes encode cell wall proteins or biogenesis/remodeling factors, as genes encoding factors related to the cell membrane, signaling, stress response and various proteins found to be antigenic are also included. Downregulation of these genes does not come without consequences for the cell wall. In the *goa1* mutant, the outer cell wall surface comprising N-linked mannans is less fibrillar, fibrils are shorter, and the inner cell wall is more electron transparent, but its diameter is similar to wild type cells (She et al. 2016). The overall hexose content of the cell wall in the *goa1* mutant is reduced; however, β-1,3 glucan and chitin are less affected (She et al. 2016).

N-linked mannan/mannoproteins are important immunogenic moieties, and they confer a net negative charge to the cell wall. While a wild type *C. albicans* cell wall comprises high, intermediate and low molecular weight mannans, the *goa1* mutant lacks the high and intermediate weight mannans suggesting that either less of mannan is synthesized or that mannan instability is occurring (She et al. 2016). Furthermore, a reduction in β-mannosyl but not α-mannosyl linkages is observed (She et al. 2016). While the glucan content is not affected in the *goa1* mutant, its structure could be. Compared to the wild type strain, in the *goa1* mutant the frequency of β-1, 6 glucan side chains is increased (two times higher), but the chains display reduced length (She et al. 2016).

Considering the cell wall changes of the *goa1* mutant, it appears that loss of a functional complex I of the ETC, which goes hand in hand with a loss of energetic capacity and metabolic flexibility of cells, appears to have very specific effects on the cell wall (She et al. 2016). In agreement with the observed cell wall changes, the *goa1* mutant is phagocytosed less by mouse macrophages and neutrophils than wild type *C. albicans*, but is killed as much (She et al. 2013). The *goa1* mutant has additionally shown to be non-responsive to hypoxia in terms of changes to the cell wall that result in masking of β-glucan, which was not occurring in the mutant (Pradhan et al. 2018). This provides a further mechanism by which mitochondrial respiration would determine cell surface architecture in infection niches (where hypoxia occurs), thereby influencing how the immune system detects *C. albicans*. A detailed analysis of the cell walls of the *nuo1* and *nuo2* null mutant strains has still to be conducted, but it is known that these strains are avirulent in a mouse model and have a similar transcriptional pattern to the *goa1* mutant (She et al. 2015).

Proteomic investigations of the *goa1*, *nuo1* and *nuo2* deletion mutants showed that even though downregulation of the β-1,2 mannosyltransferases was observable on a transcriptional level and phenotypic alterations in N-linked mannans were measurable after the inactivation of *GOA1*, there were no alterations in β-1,2 mannosyltransferase protein amounts (She et al. 2018). Nevertheless, protein levels of the alpha 1,3 mannosyltransferase Mnn15 were reduced, as were the levels of proteins involved in phospholipid and ergosterol biosynthesis, which were even more affected in the *nuo1* and *nuo2* mutants than in the *goa1* mutant (She et al. 2018). These links between complex I, respiration and lipid homeostasis suggest again that it might not always be possible to uncouple the various functions of mitochondria with respect to cell wall biogenesis and integrity.

Respiration can also be inhibited chemically. In the context of therapy, however, standard respiratory inhibitors, such as cyanide or antimycin A, cannot be used as they are cytotoxic. To test the inhibition of respiration with a compound that is well tolerated by humans, Duvenage et al. used the nitric oxide (NO) donor sodium nitroprusside (SNP) to test the effects of respiration inhibition in *C. albicans*. SNP is a widely used medication to lower blood pressure and might have the potential to be repurposed as a drug against *C. albicans*. Nitric oxide (NO) released by SNP transiently inhibits complex IV of the ETC. Duvenage et al. (2019) also investigated the effects of inhibition of the alternative oxidases Aox1 and Aox2 on the cell wall of *C. albicans* using salicylhydroxamic acid (SHAM). In contrast to the effects resulting

from a loss of function of complex I, which modifies the cell wall in a way that leads to less recognition of *C. albicans* by immune cells, transient inhibition of complex IV by NO and additional inhibition of the alternative oxidases by SHAM, results in an increased recognition by macrophages (Duvenage et al. 2019). Similar to loss of function of complex I, treatment with SNP in combination with SHAM leads to hypersensitivity to congo red and calcofluor white, indicating differences in cell wall structure, but investigation of the cell wall revealed that the relative levels of chitin, glucan and mannan were not affected (Duvenage et al. 2019). Only the outer cell wall exhibited a reduction in thickness (Duvenage et al. 2019). Transcriptional analyses did indeed identify downregulation of several genes involved in chitin synthesis and organization. In contrast, genes in mannan and glucan biosynthesis and organization were upregulated after exposure to SNP and SHAM. As there were no observable changes in relative cell wall composition, differential expression of these genes likely leads to cell wall rearrangements (Duvenage et al. 2019). In line with this assumption, inhibition of complex IV by SNP leads to exposure of the normally hidden chitin layer of the cell wall (Duvenage et al. 2019). In addition to chitin, β-1,3 glucan was also more exposed on the cell surface as it was recognized by the receptor Dectin-1. All of these changes are in accordance with the increased recognition by macrophages in treated *C. albicans* cells.

Intriguingly, SNP and SHAM treatment led to caspofungin resistance and an accumulation of lipid droplets (Duvenage et al. 2019). This effect is dependent on the presence of the transcription factor Upc2, as deletion of *UPC2* abolishes the resistance to caspofungin of SNP and SHAM-treated cells (Duvenage et al. 2019). This again provides a link between respiration and lipid homeostasis, as Upc2 is a regulator of ergosterol biosynthesis. As discussed above, mitochondrial ROS stress was shown to trigger alterations to the plasma membrane lipids surrounding glucan synthase Fks1, the target of caspofungin, and causes resistance to this drug in *A. fumigatus* (Satish et al. 2019). Inhibition of complex IV and the alternative respiratory pathway leads to changes in lipid metabolism as can be judged by the appearance of lipid droplets on a microscopic level, on a transcriptional level downregulation of *CHO1* and *CHO2* (indicating effects on phospholipid metabolism), downregulation of the expression of ergosterol and linoleic acid biosynthesis genes, and caspofungin resistance depended on *UPC2* on a genetic level (Duvenage et al. 2019). It is possible that changes in the lipid microenvironment of Fks1 are also the reason for caspofungin resistance in *C. albicans*. It is not known whether these changes in lipid metabolism are also driven by increased ROS stress that might be a result of inhibition of complex IV and the alternative respiratory pathway.

Even though inhibition of complex IV by SNP caused increased macrophage recognition due to exposure of chitin and β-1,3 glucan, virulence was increased in a systemic mouse model, and the reason for this is rather surprising (Duvenage et al. 2019). SNP causes rapid activation of filamentation, while this effect is not observable with SHAM-treated cells (Duvenage et al. 2019). The development of hyphae is a key virulence trait of *C. albicans.* Several studies have indicated that exogenous and endogenous nitric oxide levels influence the morphological switch from yeast to hyphal growth of *C. albicans*. For example, exposure to SNP induced

germination in several *C. albicans* wild type strains (Abaitua et al. 1999). It was also shown that a deletion strain of *YHB1*, the main NO detoxifying enzyme in *C. albicans*, results in hyper-filamentation possibly due to elevated endogenous NO levels (Hromatka et al. 2005). We have recently shown that the restriction of endogenous NO by scavengers inhibits the ability of *C. albicans* to filament (Koch et al. 2018). In the same study, we demonstrated that the compound mdivi-1 triggers reprogramming of metabolism-related genes including a reduction in the expression of genes important for mitochondrial respiration and lowers the levels of endogenous NO (Koch et al. 2018). Collectively, these studies highlight the complex interactions between mitochondrial activity, cell wall changes, immune interactions and fungal cell morphology, which ultimately conspire to drive virulence of *C. albicans*.

7 Summary and Future Directions

It is clear that metabolic pathways and mitochondrial functions regulate cell wall structure of fungal pathogens. The challenge now is to understand the mechanistic aspects and how these processes could be targeted therapeutically. For example, while several fungal mitochondrial mutants display increased susceptibility to cell wall inhibition and the echinocandin drugs, two recent studies, one in *C. albicans* and another one in *A. fumigatus*, showed the opposite—that mitochondrial dysfunction can in some cases trigger echinocandin resistance (Duvenage et al. 2019; Satish et al. 2019). Complex changes in cell wall structure have been reported as a result of mitochondrial dysfunction, making it difficult to predict how a certain mitochondrial mutation will affect cell wall structure and drug susceptibility. Several years ago, in 2011, we wrote a review proposing that roles in ensuring proper cellular lipid homeostasis underscore the functions of mitochondria in cell wall biogenesis and drug susceptibility (Shingu-Vazquez and Traven 2011). This prediction still holds—a leitmotiv in the studies that we discussed here is that the changes in lipid pathways are common in mitochondrial mutants that show cell wall phenotypes. Furthermore, a change in the lipid environment of glucan synthase induced by mitochondrial dysfunction has now been directly implicated in resistance to the echinocandin caspofungin, with potential clinical relevance (Satish et al. 2019). It is interesting that it has been suggested that ROS production in mitochondria impacts on lipid homeostasis and caspofungin susceptibility (Satish et al. 2019). Blocks in the respiratory chain are known inducers of ROS, and therefore, lipid homeostasis defects could underlie the cell wall phenotypes observed in mitochondrial respiration mutants more broadly. In addition, we propose that, when thinking about mitochondria–cell wall–lipid functional networks, we should keep in mind the physical connections between membranes in eukaryotic cells. Mitochondria are physically tethered to several other organelles, including the plasma membrane which is a critical site for cell wall biogenesis and cell wall stress responses. While our model in Fig. 1 on how the membrane contact sites of

mitochondria might promote cell wall biosynthesis and stress responses are speculative at the moment, we hope it will promote future research of the roles of mitochondria in cell wall biology.

As discussed in this review, one key issue with understanding how exactly mitochondria impact on the cell wall is the pleiotropic nature of mitochondrial mutations. One experimental solution is to use conditional repressible mutants, in which some of the direct and indirect effects of mitochondrial mutations might be temporally separable. An example of this strategy from our own work is with the ERMES complex in *C. albicans*, for which conditional inactivation revealed that a mitochondrial morphology defect precedes fitness and lipid phenotypes (Tucey et al. 2016). This allowed us to more directly link mitochondrial morphology to virulence-related pathways (Tucey et al. 2016). We also note that in fungal and mammalian species mitochondria act as a signaling platform, for example through ROS production, but also through localization of important factors to mitochondria under specific circumstances. The data showing that in *S. cerevisiae* several cell wall biogenesis factors and regulators such as Rho1 localize to mitochondria are intriguing, but it should be noted that these localization data come from high-throughput studies, and it needs to be shown whether this is a true localization or association with mitochondria. More directed approaches for testing mitochondrial association of these cell wall regulators in fungal pathogens under various conditions should be done, and then roles in signaling studied were warranted.

Acknowledgements Work in the Traven laboratory on mitochondrial functions in *C. albicans* has been supported by grants from the National Health and Medical Research Council (APP1023973 and APP1101562) (to A.T.). B. K. was supported by the Erwin Schroedinger Fellowship from the Austrian Science Fund.

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Impact of the Environment upon the *Candida albicans* Cell Wall and Resultant Effects upon Immune Surveillance

Delma S. Childers, Gabriela M. Avelar, Judith M. Bain, Daniel E. Larcombe, Arnab Pradhan, Susan Budge, Helen Heaney, and Alistair J. P. Brown

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Abstract The fungal cell wall is an essential organelle that maintains cellular morphology and protects the fungus from environmental insults. For fungal pathogens such as *Candida albicans,* it provides a degree of protection against attack by host immune defences. However, the cell wall also presents key epitopes that trigger host immunity and attractive targets for antifungal drugs. Rather than

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Current Topics in Microbiology and Immunology (2020) 425: 297–330
https://doi.org/10.1007/82_2019_182
© Springer Nature Switzerland AG 2019
Published Online: 29 November 2019

being a rigid shield, it has become clear that the fungal cell wall is an elastic organelle that permits rapid changes in cell volume and the transit of large liposomal particles such as extracellular vesicles. The fungal cell wall is also flexible in that it adapts to local environmental inputs, thereby enhancing the fitness of the fungus in these microenvironments. Recent evidence indicates that this cell wall adaptation affects host-fungus interactions by altering the exposure of major cell wall epitopes that are recognised by innate immune cells. Therefore, we discuss the impact of environmental adaptation upon fungal cell wall structure, and how this affects immune recognition, focussing on *C. albicans* and drawing parallels with other fungal pathogens.

1 Introduction

The ascomycete fungus, *Candida albicans*, is carried as a relatively harmless commensal by most healthy individuals in their oral cavity, or urogenital and gastrointestinal tracts. In general, the local epithelial barriers, innate immune defences and microbiota limit the colonisation and outgrowth by *C. albicans* cells. However, the perturbation of any of these local defences often leads to local mucosal infection (*thrush*) (Sobel 2007; Hertel et al. 2016). Most women suffer at least one episode of vaginitis in their lifetime, and oral thrush is common in babies, the elderly, diabetics and HIV patients. *C. albicans* is the most common cause of fungal mucosal infections (Denning et al. 2018). In neutropenic patients, whose immune defences are severely compromised, *C. albicans* can cause systemic infections of the blood and internal organs (Perlroth et al. 2007; Gouba and Drancourt 2015). Despite the availability of several classes of the antifungal drug, including azoles, polyenes, echinocandins and flucytosine (Odds et al. 2003), these systemic infections display about 40% mortality (Brown et al. 2012; Kullberg and Arendrup 2015). This, combined with the emergence of resistance to the current antifungal drugs in clinical use, means that there is a clear need for the development of new, more effective antifungals (Brown et al. 2012).

From a clinical perspective, the fungal cell wall represents an attractive target for the development of new antifungal drugs (Odds et al. 2003; Gow et al. 2017). This is because human cells lack a cell wall, whereas the cell wall is essential for the viability of fungal pathogens such as *C. albicans* (Douglas et al. 1997; Munro and Gow 2001; Munro et al. 2001). Therefore, drugs that target cell wall biosynthesis or function are less likely to perturb human cells. The *C. albicans* cell wall also represents the first point of direct contact with the host, and cell wall molecules are exploited as key recognition targets by our immune defences. For this reason, the cell wall is also an attractive target for the development of vaccines and immunotherapeutics that might prevent or combat *Candida* infections. In addition, structural distinctions between the cell walls of pathogenic fungal species (Erwig and Gow 2016) represent a point of leverage for the development of the novel diagnostics that are required to accelerate the diagnosis, and thereby improve the

prognoses of life-threatening systemic infections (Brown et al. 2012). Therefore, a comprehensive understanding of the structure and function of the fungal cell wall is vital for the elaboration of the new antifungal drugs, immunotherapies, diagnostics and vaccines that ultimately will improve patient outcomes.

From the perspective of the fungus, the cell wall is a vital organelle that requires significant metabolic and energetic investment to construct. (The wall comprises about 30% of the dry weight of a yeast cell (Nguyen et al. 1998). The cell wall provides protection against environmental insults (Gow et al. 2017). It maintains cell shape and osmotic integrity, asserting the cellular morphology driven by the regulatory apparatus that establishes the balance between isotropic and polarised growth, generating morphogenetic transitions between yeast, pseudohyphal and hyphal growth forms in response to the environmental conditions (Sudbery 2011). Yet the cell wall is an elastic, not a rigid structure, which permits the transit of large liposomes and extracellular vesicles (Vargas et al. 2015; Walker et al. 2018), as well as rapid changes in cell volume in response to osmotic challenges (Ene et al. 2015). Furthermore, rather than being a relatively inert shield, the cell wall responds to local inputs as the fungus adapts to environmental change (Sosinska et al. 2008; Ene et al. 2012; Hall 2015). Therefore, the fungal cell wall is a remarkable organelle that is simultaneously robust but elastic, and stable but flexible.

In this chapter, we focus on the cell wall of the major pathogen, *C. albicans*. We review the structure of the *C. albicans* cell wall, its stability and elasticity; how the cell wall responds to environmental challenges, whether natural or therapeutic; and how changes in the *C. albicans* cell wall affect host-fungus interactions. We then discuss parallels with other fungal pathogens before suggesting key questions for the future.

2 Structure and Synthesis of the *C. Albicans* Cell Wall

Significant differences exist between the cell walls of the major fungal pathogens of humans (Gow et al. 2017; Erwig and Gow 2016). Nevertheless, some of the macromolecular building blocks that comprise the cell wall are conserved across most of these fungal species. These consist of β-1,3- and β-1,6-glucan, chitin and mannoproteins. Additionally, some fungal cell walls contain melanin, chitosan and β-1,4-glucan (Gow et al. 2017; Shepherd 1987; Klis et al. 2001; Bowman and Free 2006). A combination of microscopy, biochemistry and molecular genetics has shown clearly that the *C. albicans* cell wall comprises two main layers: an inner layer of chitin and glucan cross-linked together and an outer layer of mannan fibrils that are covalently attached to this inner layer via their anchoring mannoproteins (Fig. 1).

Chitin is a linear homopolymer of β-1,4-linked *N*-acetylglucosamine, which forms antiparallel chains linked by intrachain hydrogen bonds. Chitin accounts for only about 2–3% of the dry weight of the *C. albicans* yeast cell wall. Yet it is a strong fibrous structural component of the inner layer that contributes significantly

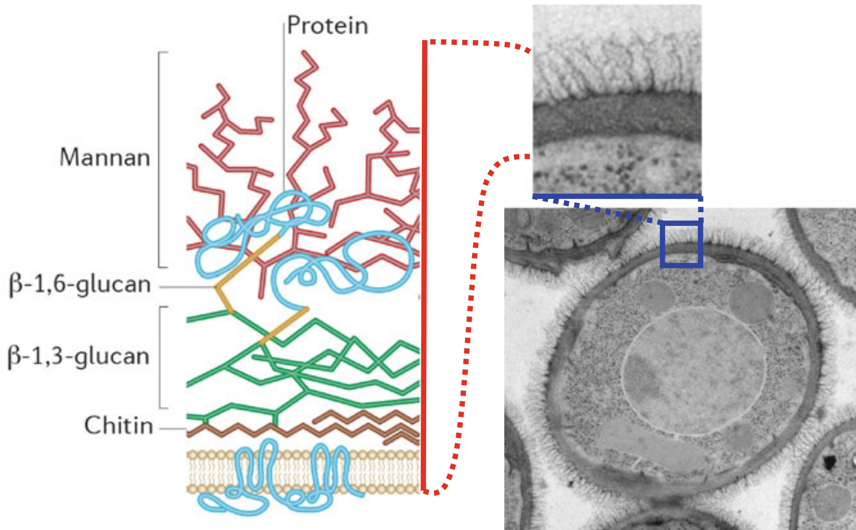


Fig. 1 Architecture of the *C. albicans*cell wall. The model of the cell wall structure illustrates the organisation of chitin and glucan in the inner cell wall and the *N*-mannan fibrils of the outer cell wall, which are linked to the inner cell wall via the GPI-anchored proteins from which these fibrils radiate. GPI-proteins are attached to β-1,6-glucan which, in turn, is linked to β-1,3-glucan, whereas Pir proteins are linked directly to β-1,3-glucan. The cartoon, which is taken with permission from (Erwig and Gow 2016), is compared with a transmission electron micrograph of the *C. albicans* cell wall (upper panel), which is an expanded region (blue box) from a micrograph of a complete cell (lower panel). The diameters of inner and outer layers of the cell wall are each about 0.14 μm across (Pradhan et al. 2018)

to the overall integrity of the cell wall. *C. albicans* mutants with impaired chitin synthesis present with a disordered cell wall architecture and display osmotic instability (Lenardon et al. 2007; Lenardon et al. 2010). In *C. albicans*, a small fraction of chitin (less than 5%) is deacetylated to chitosan by one or more chitin deacetylases, making chitin fibrils more elastic and protecting them from the action of hostile chitinases (Lenardon et al. 2010).

In *C. albicans* chitin is synthesised by a family of four chitin synthases, representing three different classes of chitin synthase that generate chitin microfibrils of different lengths (Lenardon et al. 2007). Together, these enzymes engineer the chitin skeleton in the fungal cell wall and septum. Chs1 is an essential Class II enzyme that is required for the synthesis of the primary septum. Chs3 is a Class IV enzyme, which is usually located in the tip of buds and hyphal cells and synthesises the majority of chitin found in the fungal cell wall and septum. Chs2 and Chs8 also contribute to cell wall integrity during normal growth and stress conditions. These Class I enzymes account for most of the chitin synthase activity that is measurable in vitro, and indeed the deletion of *CHS2* alone reduces in vitro chitin synthase activity by 80–91% (Munro and Gow 2001; Lenardon et al. 2010; Staniszewska et al. 2013; Preechasuth et al. 2015).

β-Glucan is the major structural polysaccharide of the *C. albicans* cell wall, accounting for 50–60% of the dry weight of the yeast cell wall (Shepherd 1987; Klis et al. 2001). β-Glucan is composed of chains of glucose residues linked via β-1,3- or β-1,6 linkages. β-1,3-Glucan fibrils represent the main structural component of the *C. albicans* cell wall, and chitin, β-1,6-glucan and mannoproteins are covalently attached to this β-1,3-glucan network in the inner layer of the wall. β-1,3-Glucan is synthesised at the plasma membrane and extruded into extracellular space by the beta-1,3-glucan synthase complex, which consists of catalytic subunits encoded by *GSC1/FKS1* and *GSC2/FKS2*, and a small regulatory GTPase encoded by *RHO1* (Mio et al. 1997; Kondoh et al. 1997). β-1,3-Glucan synthase is essential for fungal viability and is the target of echinocandin drugs, such as caspofungin (Douglas et al. 1997).

β-1,6-Glucan is less abundant than β-1,3-glucan. Branched β-1,6-glucan structures are crosslinked to β-1,3-glucan in the inner layer of the *C. albicans* cell wall, providing an additional platform for the covalent anchoring of some cell wall mannoproteins (Kapteyn et al. 2000). A number of genes are involved in β-1,6-glucan biosynthesis, including *KRE5*, *KRE6*, *KRE9*, *BIG1* and *SKN1*. Although it is not clear where β-1,6-glucan synthesis occurs, it does involve enzymes localised in the endoplasmic reticulum and the Golgi apparatus (Umeyama et al. 2006).

The mannoproteins in the *C. albicans* cell wall are frequently heavily decorated with *N*- and/or *O*-linked oligosaccharides (Klis et al. 2001; Chaffin 2008). Together with phospholipomannans, these represent up to 30–40% of the dry weight of the cell wall. The *O*-mannans are relatively short linear carbohydrate polymers comprised of two to six α-1,2-linked mannose units. Their synthesis requires the activities of *PMR1*, the *PMT* gene family, *MNT1* and *MNT2* (Buurman et al. 1998; Bates et al. 2005; Munro et al. 2005; Timpel et al. 1998). The addition of the first mannose residue to the polypeptide chain is catalysed by *O*-mannosyltransferases (encoded by *PMT* genes), whilst Mnt1 and Mnt2 are responsible for the addition of the first and second α-1,2-mannose units into the α-mannose backbone. The resultant *O*-linked oligosaccharides are thought to promote a rod-like conformation to the serine-threonine-rich repeats to which they are generally attached (Gatti et al. 1994).

The outer layer of the *C. albicans* cell wall is composed of highly branched *N*-linked oligosaccharide structures that are covalently linked to asparagine residues in the mannoproteins. These *N*-mannans contain a *N*-glycan core, to which are attached long branched chains with an α-1,6-mannose backbone and side chains of oligomannosides linked via α-1,2 or α-1,3 bonds (Klis et al. 2001). The synthesis of the *N*-linked oligosaccharide core structure occurs in the endoplasmic reticulum and involves the sequential addition of sugar residues by glycosyltransferases, encoded by asparagine-linked glycosylation (*ALG*) genes. The mannosyltransferase Och1 catalyses the addition of the first alpha-1,6-mannose (Bates et al. 2006), and the branched oligosaccharide structure is then added to the nascent protein by the oligosaccharyltransferase complex. After the initial glycosylation, the mannoprotein is further modified in the ER and Golgi apparatus. Golgi resident enzymes, encoded by members of the *KTR/KRE/MNT* and *MNN* gene families, process and elongate the *N*-linked as well as *O*-linked oligosaccharides (Mora-Montes et al. 2010;

Hall et al. 2013; Hall and Gow 2013). Phosphomannan is a β-1,2-mannose moiety linked to the branched *N*-glycan via a phosphodiester bond. A similar moiety can be linked to lipid domains creating the phospholipomannans. Different enzymes from the *MNN* and *BMT* families participate in the synthesis of phosphomannan and phospholipomannans (Hall and Gow 2013; Hobson et al. 2004; Murciano et al. 2011).

There are two main classes of cell wall mannoprotein in *C. albicans* based on the nature of their linkage to cell wall polymers. GPI-anchored proteins, which are the most abundant class of cell wall mannoprotein, are covalently attached via their carboxy-terminal glycosylphosphatidylinositol (GPI) anchor to β-1,6-glucan which, in turn, is linked to β-1,3-glucan (Kapteyn et al. 2000). Pir proteins (proteins with internal repeats) are less abundant, and these are covalently linked directly to β-1,3-glucan (Kapteyn et al. 2000; Toh-e et al. 1993).

Cell wall proteins provide anchors for the mannan outer layer of the cell wall. They contribute to the structural integrity of the cell wall, and some are cell wall remodelling enzymes responsible for generating essential covalent linkages between cell wall components (Ene et al. 2015; Pardini et al. 2006; Popolo et al. 2017). Transglycosylases from the GH72 family catalyse glucan remodelling, and their inactivation affects growth, morphology and virulence. For example, *PHR1* and *PHR2* (pH-responsive genes 1 and 2) encode members of this family, and they catalyse the pH-regulated cross-links between β-1,6- and β-1,3-glucans. *PHR1* plays a crucial role in the formation of the hyphal cell wall and in pathogenesis (Popolo et al. 2017; Fonzi 1999). The GPI-anchored yapsin-like aspartic proteases Sap9 and Sap10 have functions in cell surface integrity and cell separation during budding, whilst the *CRH* family of chitin-glucanosyltransferases (Crh11, Crh12, Utr2) are involved in formation of linkages between β-1,3-glucan and chitin (Pardini et al. 2006). The degree of cross-linking between components of the cell wall is important for its organisation and integrity, as this determines its elasticity, resistance and porosity. This is evidenced by the deletion or over-expression of genes encoding cell wall remodelling enzymes, which result in altered sensitivity to cell wall disrupting agents, such as Congo Red, Calcofluor White, SDS and high Ca^{2+} concentrations (Ene et al. 2015; Pardini et al. 2006; Popolo et al. 2017).

Besides its structural role, the cell wall promotes *C. albicans* pathogenicity, for example, through adhesion, invasion and damage. Adhesins are important not only for fungal colonisation but also for biofilm formation and interactions with other microbes. Most of the known adhesins are GPI anchored proteins, and many are members of multigene families such as the *ALS* and *HWP* gene families (Staab et al. 1999; Hoyer et al. 2008). Some adhesion genes, such as *ALS3* and *HWP1*, are expressed during hypha formation, which is why this morphotype is particularly adherent. The HWP adhesin family is required for adhesion to host cell proteins, biofilm formation, cell-cell aggregation and mating (Staab et al. 1999; Hofs et al. 2016). *HWP1*, *HWP2* and *RBT1* expressions are induced not only during hypha formation but also during mating of opaque cells. Another member of this family, *EAP1,* is expressed in both yeast and hyphal cells and is differentially regulated during yeast phenotypic switching (Hofs et al. 2016; Gow and Hube 2012).

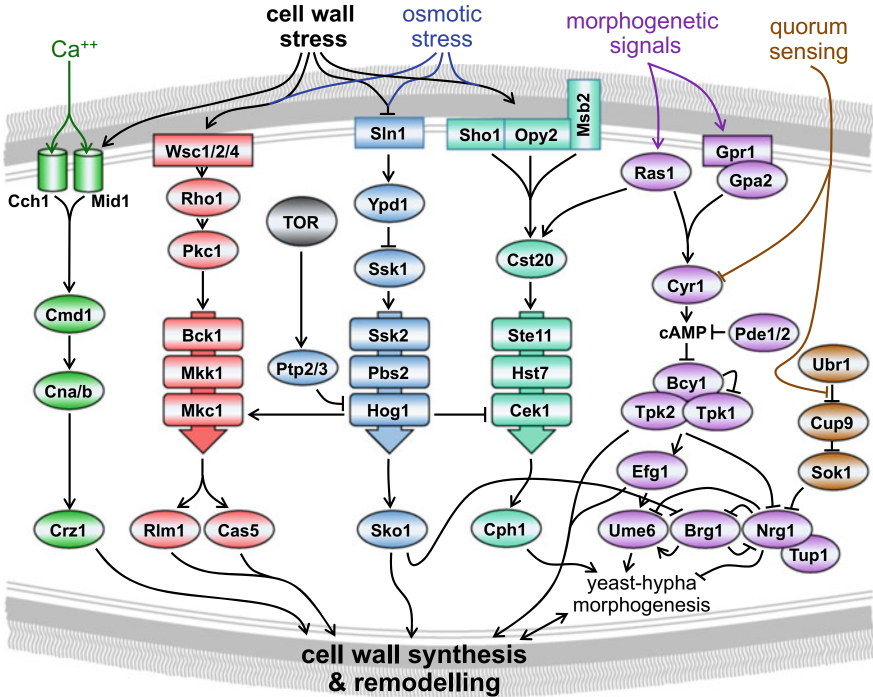
Als3 acts both as an adhesin and an invasin as it binds to a host receptor on epithelial or endothelial cells to induce endocytosis (Phan et al. 2007). Als3 also enables iron acquisition by binding transferrin and has effects on host cell damage and cytokine induction (Almeida et al. 2008).

The general structure of the *C. albicans* cell wall has been reasonably well understood for some time (Gow et al. 2017; Shepherd 1987; Klis et al. 2001). However, recent technological advances are extending our knowledge of this field. For example, atomic force microscopy is providing direct information about cell wall structure and elasticity (Dague et al. 2010). Also, super-resolution microscopy has shown that phosphomannans, which are negatively charged, are critical for glucan masking at the cell surface (Graus et al. 2018). Furthermore, high-resolution electron tomography has permitted the development of the first scale model of *C. albicans* cell wall architecture (Megan Lenardon, Prashant Sood and Neil Gow, personal communication). This deeper understanding of *C. albicans* cell wall biosynthesis, structure and organisation is helping the development of new therapies and diagnostics.

In the past, the fungal cell wall was often portrayed as a rigid shield-like structure in which, for example, chitin was compared to the steel in reinforced concrete. However, it has become clear that the cell wall is actually a surprisingly elastic structure. *C. albicans* releases extracellular vesicles which carry diverse cargo (including enzymes, toxins and nucleic acids, for example) that are believed to function in cell-to-cell communication, metabolism and pathogenesis (Vargas et al. 2015; Deatherage and Cookson 2012; Joffe et al. 2016). These extracellular vesicles vary in size from 50 to 850 nm, and yet these membrane-bound compartments are able to transit through the cell wall into the surrounding milieu (Vargas et al. 2015). Another study has demonstrated that large liposomal particles can gain entry to the cell by traversing the *C. albicans* cell wall. Transmission electron microscopy has revealed that Ambisome particles of 60–80 nm, which are much larger than the predicted pore size of the cell wall (approximately 6 nm), are able to transit through the cell wall whilst both particle and cell wall retain their integrity (Walker et al. 2018). The remarkable elasticity of the *C. albicans* cell wall is further reflected in the ability of *C. albicans* cells to undergo rapid and dramatic changes in volume in response to acute hyper-osmotic stress (Ene et al. 2015). Therefore, the cross-linked polymers of the *C. albicans* cell wall have evolved to provide an elastic and flexible structure, not a rigid shield.

3 Cell Wall Remodelling in Response to Damage

The *C. albicans* cell wall is a dynamic structure that changes in response to morphogenetic triggers, other environmental inputs, genetic perturbation and antifungal treatment. Transcriptomic, proteomic and biochemical studies from a number of research groups have revealed condition-specific programmes of cell wall protein expression (Chaffin 2008) and carbohydrate content or synthesis (Hall 2015).



A complex network of signalling pathways regulates this cell wall adaptation. These pathways include the cell wall integrity pathway, high osmolarity glycerol (Hog1) mitogen-activated protein kinase (MAPK) signalling, the calcineurin-calmodulin pathway, the protein kinase A (PKA) pathway, the Cek1 MAPK pathway, mitochondrial reactive oxygen species (ROS) signalling, casein kinase I (Yck2, Yck3) and the heat shock transcription factor (Hsf1)-Hsp90 auto-regulatory circuit (Munro et al. 2007; Blankenship et al. 2010; Galan-Diez et al. 2010; Leach et al. 2011; Znaidi et al. 2018; Pradhan et al. 2018) (Fig. 2). This complexity probably reflects the absolute requirement to retain cell wall homoeostasis in the face of a diverse range of environmental inputs and challenges.

This complex regulatory network presents an issue for antifungal therapy because, following exposure to agents that compromise cell wall integrity, the network provides mechanisms for compensatory changes to the fungal cell wall. The major classes of antifungal drug impose major insults on the cell wall by targeting the synthesis or structural integrity of the cell wall and plasma membrane. Echinocandins, such as Caspofungin, target β-glucan biosynthesis via the catalytic subunit of glucan synthase, Fks1. The inhibition of Fks1, and hence β-glucan synthesis, by Caspofungin induces compensatory activities in the form of increased chitin synthesis and deposition in the cell wall (Walker et al. 2008). This elevation in cell wall chitin then protects cells against further Caspofungin treatment both

◂**Fig. 2 A complex network of signalling pathways regulates cell wall synthesis and remodelling in *C. albicans***. Cell wall remodelling depends on the cell integrity pathway (red) (Paravicini et al. 1996; Navarro-Garcia et al. 1998). Cell wall damage is thought to be detected by Wsc1/2/4, which activates protein kinase C (Pkc1) via Rho1. This leads to activation of the Mkc1 MAPK module which triggers cell wall remodelling via the transcription factor Rlm1 but primarily via Cas5 (Dichtl et al. 2016; Bruno et al. 2006; Delgado-Silva et al. 2014). The Hog1 pathway (blue) also contributes to the control of cell wall synthesis and remodelling (Munro et al. 2007). Cell wall or osmotic stress down-regulates Sln1, which leads to the activation of the Hog1 MAPK module via the Ypd1 and Ssk1 phosphorelay (San Jose et al. 1996; Smith et al. 2004; Cheetham et al. 2007; Day et al. 2017). Hog1 then modulates cell wall largely via the transcription factor Sko1, which also represses Brg1 (Su et al. 2013). Hog1 is down-regulated by the phosphatases Ptp2/3, which are activated by TOR signalling (grey) (Su et al. 2013). Hog1 also activates Mkc1 signalling and represses the Cek1 pathway (dark green) (Monge et al. 2006). Msb2 acts in concert with Sho1 to activate the Cek1 pathway in response to osmotic stress or cell wall damage, and Opy2 also contributes to Cek1 activation via Cst20 (Leberer et al. 1996; Roman et al. 2009; Cantero and Ernst 2011; Herrero de Dios et al. 2013). Cek1 activates Cph1 which is thought to contribute to cell wall remodelling during hyphal development. Morphogenesis is also activated by cAMP-PKA signalling (purple), which leads to cell wall remodelling. In response to a variety of environmental inputs, the Gpr1-Gpa2 and Ras modules activate adenyl cyclase (Cyr1), which leads to cAMP accumulation and inactivation of the PKA regulatory subunit Bcy1 (Noble et al. 2017). This leads to activation of the PKA catalytic subunits (Tpk1, Tpk2) which stimulates a network of transcription factors (Efg1, Ume6, Brg1) and releases Nrg1-Tup1-mediated repression to activate hypha-specific genes, hyphal development and cell wall remodelling (Staniszewska et al. 2013; Ebanks et al. 2006; Castillo et al. 2008; Gil-Bona et al. 2015; Fanning et al. 2012). This pathway is repressed by quorum sensing (brown), which inhibits adenyl cyclase (Cyr1), and also stabilises the repressor Nrg1 via Ubr1, Cup9 and Sok1 (Lindsay et al. 2012; Lu et al. 2014a, b). Calcium (Ca^{++})—camodulin (Cmd1)—calcineurin (Cna/b) signalling (lime green) also plays an important role in cell wall remodelling (Munro et al. 2007; Sanglard et al. 2003; Sato et al. 2004). Activation of this pathway, possibly via the stretch-activated channel Mid1, leads to the up-regulation of the transcription factor Crz1, which promotes cell wall remodelling (Santos and de Larrinoa 2005; Karababa et al. 2006)

in vitro and in vivo, thereby compromising the efficacy of the antifungal drug (Lee et al. 2012).

Azole drugs, such as fluconazole, target lanosterol 14-α-demethylase (Erg11) on the ergosterol biosynthesis pathway. This induces significant changes in plasma membrane rigidity and integrity. Although azoles do not appear to affect the cell wall directly, proteomics and cell wall sensitivity assays have shown that fluconazole treatment indirectly perturbs the integrity of the cell wall (Sorgo et al. 2011).

Clearly, genetic perturbation of cell wall components can significantly affect cell wall architecture. The loss of β-glucan synthase (Fks1) is lethal to *C. albicans* (Douglas et al. 1997; Mio et al. 1997). However, only one of the four chitin synthase genes (*CHS1*) is essential for viability (Munro et al. 2001; Gow et al. 1994), due to compensatory changes in chitin synthesis rescuing the loss of other *CHS* genes (Lenardon et al. 2007). In general, cell wall mannoproteins themselves are rarely essential for viability, but the inactivation of some specific GPI-anchored proteins can perturb integrity of the *C. albicans* cell wall (Albrecht et al. 2006; Plaine et al. 2008). Mutations with more general effects upon the localisation or

mannosylation of GPI anchored cell wall proteins also affect the sensitivity of *C. albicans* to cell wall stresses (Buurman et al. 1998; Bates et al. 2005; Munro et al. 2005; Bates et al. 2006; Richard et al. 2002; Moreno-Ruiz et al. 2009). This type of approach, involving the analysis of cellular responses to the disruption of cell wall genes or to cell wall perturbing agents, has helped to elucidate the roles of specific proteins or protein families in virulence-related phenotypes, such as adhesion and biofilm formation. In the context of this article, it has also highlighted key mechanisms underlying cell wall maintenance and homoeostasis.

The cell wall integrity signalling pathway drives the main compensatory changes in the cell wall that are initiated in response to antifungal drugs, other cell wall stresses, and genetic insults. This pathway has been evolutionarily conserved across those fungi investigated and has been extensively studied in the model yeast, *Saccharomyces cerevisiae*. The cell wall integrity pathway responds to the activation of cell wall stress sensors by up-regulating the expression of cell wall synthesis genes via a highly conserved MAPK signalling cascade (Fig. 2). In *S. cerevisiae*, signalling via the cell wall integrity pathway is initiated by the membrane proteins Wsc1, Wsc2, Wsc3, Mid2 and Mtl1, which act as cell integrity sensors (Levin 2011). Upon loss of cell wall integrity, these sensors interact with Rom2 to activate Rho1, which then activates protein kinase C (Pkc1). Pkc1 signals to a MAPK module comprising Bck1, which activates MKK1/2, which phosphorylate and activate the MAPK, Slt2. Slt2 then activates the transcription factors Rlm1 and Swi4/6, which induce the expression of genes that include the cell wall synthesis machinery (Levin 2011).

C. albicans has homologues for many components of the cell wall integrity pathway (Paravicini et al. 1996; Navarro-Garcia et al. 1998; Dichtl et al. 2016). Mutations in many affect the virulence of *C. albicans*, as well as its cell wall integrity, which suggests a key role for the cell wall integrity pathway in host niches. Furthermore, some components of this PKC-MAPK module in *C. albicans* have broader roles than their homologues in *S. cerevisiae*. For example, Mkc1, the *C. albicans* homologue of the *S. cerevisiae* MAPK Slt2, has an expanded role in regulating cellular morphogenesis under certain conditions (Navarro-Garcia et al. 1998). In addition, in *C. albicans,* Cas5 (rather than Rlm1) appears to be the transcription factor that plays the major role in controlling key gene outputs of the cell wall integrity pathway (Bruno et al. 2006). The cell wall integrity pathway also engages in cross-talk with other important signalling pathways that include the cAMP-PKA, target of rapamycin (TOR) and Hog1 pathways, which help to coordinate the response to specific stressors (Fuchs and Mylonakis 2009; Garcia et al. 2017) (Fig. 2). It is worth noting that the cell wall integrity pathway also regulates important virulence traits in other fungi, for example, capsule synthesis in *Cryptococcus neoformans* (Donlin et al. 2014) and drug resistance and virulence in *Aspergillus fumigatus* (Valiante et al. 2015).

4 Cell Wall Remodelling in Response to Environmental Change

The ability of the *C. albicans* cell wall to remodel itself in response to sub-lethal concentrations of cell wall damaging agents (above) reflects the fact that cell wall remodelling is simply an important component of the normal adaptive responses of this fungus to environmental change. Yeast-hypha morphogenesis is one of the most studied adaptive responses of *C. albicans* because of the importance of this reversible morphological transition for host-fungus interactions and virulence (Lo et al. 1997; Saville et al. 2003; Mukaremera et al. 2017). A range of environmental stimuli trigger hyphal development, including temperatures above 36 °C, neutral pH, serum, bacterial peptidoglycan, high CO_2 levels, release from quorum sensing and nutrient starvation. The resultant yeast-to-hypha transition is accompanied by shifts in the carbohydrate and proteomic content of the cell wall (Staniszewska et al. 2013; Ebanks et al. 2006; Castillo et al. 2008; Gil-Bona et al. 2015). The cell walls of *C. albicans* hyphae can have up to twofold less mannan, threefold more glucan and five times more chitin than the walls of yeast cells (Staniszewska et al. 2013). Furthermore, changes in glucan structure are associated with hypha formation (Lowman et al. 2014). These changes in cell wall structure attenuate Dectin-1-mediated recognition of hyphae by innate immune cells, which compound the physical challenges associated with the phagocytosis of mycelia (Lowman et al. 2014; Gantner et al. 2005; Bain et al. 2014; Hopke et al. 2018).

Carbon source availability differs significantly between host niches. For example, glucose concentrations are about 0.06–0.1% in the bloodstream, but are essentially zero in the colon (Barelle et al. 2006), whilst significant amounts of short-chain fatty acids, such as lactate, are present in the vagina and colon (Owen and Katz 1999; Louis et al. 2007). Changes in carbon source have been found to exert major effects on the architecture and content of the *C. albicans* cell wall. Although the relative amounts of chitin, glucan and mannan remain similar, *C. albicans* cells grown on lactate, rather than glucose, have a thinner and less elastic cell wall (Ene et al. 2015; Ene et al. 2012). These changes in cell wall architecture correlate with changes in the cell wall proteome and secretome. In particular, the levels of certain chitinases increase (Cht1, Cht3), as do the cell wall remodelling enzymes Pga4, Phr1, Phr2, Pir1 and Xog1 (Ene et al. 2012).

The availability of essential micronutrients such as iron and zinc also varies between host niches, and this is exacerbated by the host's attempts to deprive invading pathogens of these micronutrients via nutritional immunity (Weinberg 1975; Hood and Skaar 2012; Potrykus et al. 2013). Therefore, the ability to scavenge iron and zinc is critical for fungal pathogenicity and tissue invasion (Ramanan and Wang 2000; Citiulo et al. 2012). In *C. albicans*, adaptation to iron starvation triggers changes in the expression of genes encoding cell wall proteins, biosynthetic enzymes and cross-linking enzymes (e.g. Als2, Bgl2, Cht2, Mnt4, Phr2, Pir1, Scw11) (Lan et al. 2004) and the elevation of Hwp1 and Rbt5 in the cell wall proteome (Sosinska et al. 2008). Recent data from our laboratory has shown

that iron limitation is also accompanied by significant changes in cell wall architecture (Pradhan et al. 2019). Changes in zinc availability also affect the *C. albicans* cell wall. Adaptation to zinc deprivation yields more adherent *C. albicans* cells that expose less mannan, but more chitin at their cell surface (Malavia et al. 2017). Also, zinc mobilisation is linked to PKA signalling (Kjellerup et al. 2018), which influences cell wall remodelling (Munro et al. 2007).

Host niches also vary significantly in their ambient pH. For example, the bloodstream is maintained at around pH 7.4, whereas the vaginal mucosa varies from pH 4 to pH 5 (Sobel 2007; O'Hanlon et al. 2013), and the major compartments of the gastrointestinal tract range from pH 2 to pH 7.5 (Evans et al. 1988; Fallingborg 1999; Koziolek et al. 2015). This type of change in ambient pH has a significant effect upon the *C. albicans* cell wall. When cells are exposed to low pH, the chitin content of the cell wall increases and the mannan fibrils in the outer layer of the cell wall become shorter and more disorganised (Sherrington et al. 2017). The expression of cell wall protein genes is also affected by changes in ambient pH. For example, exposure to alkaline pHs leads to the up-regulation of genes encoding cell wall biosynthetic enzymes (Kre6, Ecm38), modifying enzymes (Cht2, Crh1, Phr1), adhesins (Als3, Hwp1) and other cell wall mannoproteins (Hyr1, Rbt1, Rbt4) (Saporito-Irwin et al. 1995; Hoyer et al. 1998; Bensen et al. 2004). Growth at an alkaline pH also induces the expression of cell wall and secreted proteins that play important roles in host-fungus interactions, such as the zincophore Pra1 and the candidalysin precursor, Ece1 (Citiulo et al. 2012; Bensen et al. 2004; Moyes et al. 2016).

Oxygen levels vary dramatically between host niches, approaching zero in the human colon and in some fungal lesions (Savage 1977; Grahl et al. 2012; Lopes et al. 2018). Adaptation to hypoxia drives changes to the architecture of the *C albicans* cell wall (Fig. 3), yielding a thinner inner glucan-chitin layer and thinner mannan outer layer (Pradhan et al. 2018). Hypoxia up-regulates *ECM33,* which is important for cell wall biogenesis and integrity (Martinez-Lopez et al. 2006), and *ALG2,* which encodes a putative mannosyltransferase (Setiadi et al. 2006). There is also an increase in the abundance of specific GPI-anchored proteins in the cell wall proteome (Hwp1, Pir1, Rbt5) (Sosinska et al. 2008). Hypoxic regulation of cell wall changes depends largely upon a combination of mitochondrial, PKA and Efg1 signalling (Pradhan et al. 2018; Setiadi et al. 2006).

Changes in ambient temperature also affect the *C. albicans* cell wall. When *C. albicans* yeast cells grow at 42 °C, their chitin content increases relative to cells grown at 30 °C (Heilmann et al. 2013). Furthermore, the levels of the cell wall β-glucan glycosidases, Phr1 and Phr2, and chitin transglycosidases, Crh11 and Utr2, increase during growth at 42 °C (Heilmann et al. 2013). In addition, the temperature has an effect on the branched *N*-mannan composition of the cell wall: there is a decrease in β-1,2-linked mannose and an increase in α-1,3-linked mannose during growth at higher temperatures (Okawa and Goto 2006). Unsurprisingly, the perturbation of thermal regulatory processes in *C. albicans* also affects the cell wall. For example, depletion of the molecular chaperone Hsp90, which regulates

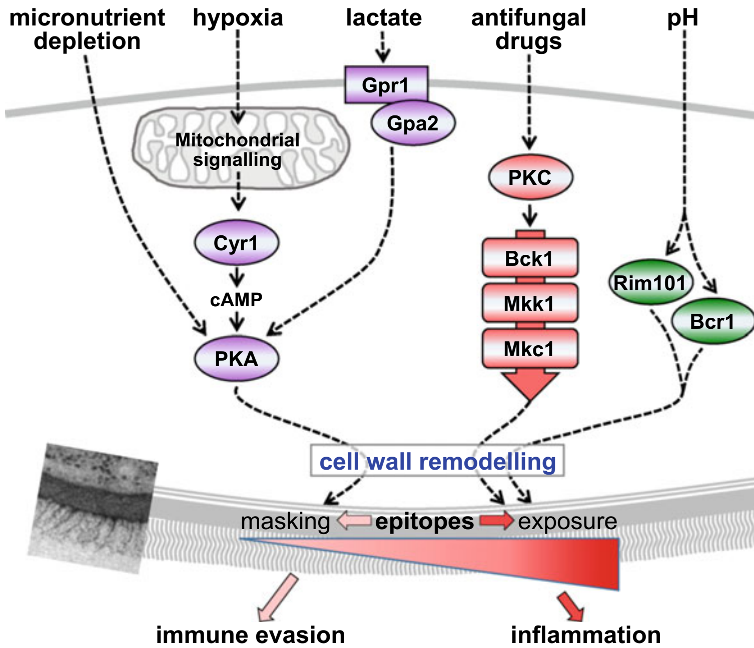


Fig. 3 Environmental factors modulate the exposure of cell surface epitopes to promote immune evasion or inflammation. A number of factors have been shown to influence the exposure of key epitopes (PAMPs) on the *C. albicans* cell surface. Caspofungin treatment leads to β-glucan exposure via the cell integrity (Mkc1) pathway (red) (Wheeler and Fink 2006; Wheeler et al. 2008). In contrast, host-derived lactate triggers β-glucan masking via Gpr1-Gpa2 and PKA signalling (purple) (Pradhan et al. 2018; Ballou et al. 2016). Hypoxia also initiates β-glucan masking, but this is mediated by mitochondrial signalling (grey), which then activates the PKA pathway (purple) (Pradhan et al. 2018). Micronutrient depletion leads to morphological changes that coincide with elevated chitin exposure at the cell surface (Malavia et al. 2017). This might be transduced via PKA signalling (Kjellerup et al. 2018). Growth in acidic pHs leads to increased β-glucan and chitin exposure (Sherrington et al. 2017). The increase in chitin exposure is mediated by Bcr1 and Rim101 signalling (Sherrington et al. 2017)

the transcription factor Hsf1, affects the chitin content of the cell wall and leads to an increase in the thickness of both the inner and outer layers of the wall (Leach et al. 2012).

As discussed above, exposure to antifungal drugs or to cell wall stresses triggers cell wall remodelling. Other types of environmental stress also affect the cell wall. *C. albicans* is exposed to oxidative stress during phagocytic attack, and the cell wall provides the first line of defence against the oxidative damage caused by reactive oxygen species (ROS). ROS-detoxifying enzymes such as superoxide dismutases (Sod4, Sod5) and catalase (Cat1) are found at the cell surface (Crowe et al. 2003; Frohner et al. 2009). Furthermore, the peroxidase Tsa1 is localised to the hyphal cell wall (Urban et al. 2005). All of these are up-regulated upon

encountering oxidative stress (Frohner et al. 2009; Urban et al. 2005; Enjalbert et al. 2006). Oxidative stress also influences cell wall architecture by inducing elongation of β-1,2-linked mannose side chains (Koyama et al. 2009).

Changes in osmolarity drive changes in cell volume. Under these circumstances, the elasticity of the cell wall underlies the ability of *C. albicans* cells to adjust their volume without incurring fatal rupturing of the wall or plasma membrane (Ene et al. 2015). This cell wall elasticity is dependent on the expression of the CHR family of transglycosylases (Chr11, Chr12, Utr2) (Ene et al. 2015). Adaptation to osmotic shock is dependent on signalling through the MAP kinases Hog1 and Mkc1, both of which regulate cell wall synthesis and remodelling (Navarro-Garcia et al. 1998; San Jose et al. 1996; Smith et al. 2004; Herrero-de-Dios et al. 2014) (Fig. 2).

Quorum sensing also influences cell wall biogenesis by modulating yeast-hypha morphogenesis and PKA signalling at high cell densities (Fig. 2). *C. albicans* generates farnesol, which accumulates at high cell densities. Farnesol attenuates the activity of adenyl cyclase, thereby down-regulating PKA activity (Lindsay et al. 2012). Farnesol also inhibits hyphal development by blocking Ubr1-mediated protein degradation of Nrg1, which represses hyphal development (Lu et al. 2014a; Murad et al. 2001).

Clearly, the cell wall is a flexible organelle that responds to local environmental inputs. These adaptive changes in cell wall structure and organisation directly affect the fitness of the fungus in these microenvironments. However, they also affect the fitness of the fungus indirectly in these microenvironments by influencing host-fungus interactions (below).

5 The Cell Wall in Immune Surveillance

As mentioned above, the cell wall is the first point of direct contact between *C. albicans* cells and innate immune cells. The cell wall polymers chitin, β-glucan and mannan are present on diverse fungal pathogens (Erwig and Gow 2016). The immune system has evolved to recognise these cell wall polymers as key epitopes, or pathogen-associated molecular patterns (PAMPs) (Netea et al. 2008). Professional phagocytes (including neutrophils, macrophages and dendritic cells) and non-professional phagocytes (such as epithelial and endothelial cells) express an array of fungal-sensing receptors, or pattern recognition receptors (PRRs) (Brown and Gordon 2001; Dambuza and Brown 2015). These host receptors detect *C. albicans* PAMPs, many of which are located at the cell surface, and this recognition elicits innate immune responses (Brown and Netea 2007; Netea et al. 2015; Lionakis and Levitz 2018) (Fig. 4).

Chitin is located in the inner layer of the *C. albicans* cell wall, in relatively low abundance compared to the other main cell wall components. Consequently, most of the chitin in the lateral cell wall is largely shielded by the outer layer of mannan fibrils. Nevertheless, chitin is exposed at the cell surface in bud/birth scars and at sites of cell wall damage and does act as a PAMP (Netea et al. 2008). Chitin is

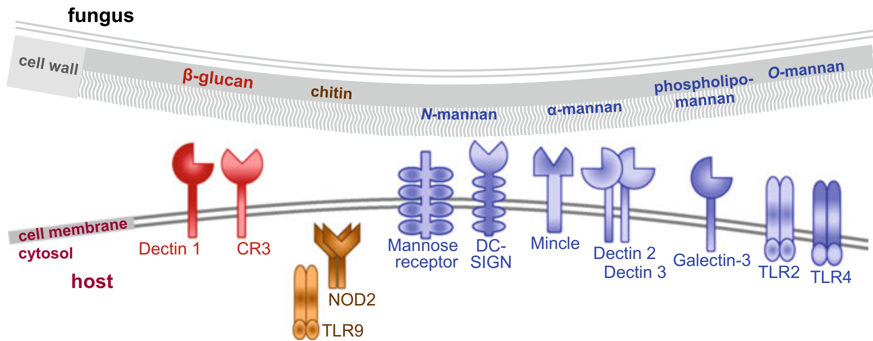


Fig. 4 Host receptors that recognise fungal cell wall components. A range of CLRs, TLRs and other PRRs in host cells recognise PAMPs such as glucan (red), chitin (brown) and mannan (blue) in the *C. albicans* cell wall. This figure is adapted from (Netea et al. 2015) using information described in the text

thought to undergo degradation into small particles (<1 um) during the inactivation of fungal cells by neutrophils and macrophages and by chitinase digestion (Wagener et al. 2014). These chitin particles are recognised by the mannose receptor (MR) and, thereafter, intracellularly by NOD2 and TLR9, eliciting an anti-inflammatory programme that includes elevated IL-10 expression and the dampening of TNF-α levels (Wagener et al. 2014). Furthermore, *C. albicans* chitin suppresses the generation of nitric oxide by macrophages and shifts macrophage polarisation from a pro-inflammatory M1 state towards anti-inflammatory M2 activation (Wagener et al. 2017).

β-Glucan is highly immunogenic and the recognition of this PAMP is critical for antifungal immunity. Whilst most β-glucan is buried in the inner layer of the *C. albicans* cell wall and masked by mannan fibrils (Graus et al. 2018; Wheeler and Fink 2006), some β-glucan is exposed at bud scars and at small puncta over the lateral cell surface (Gantner et al. 2005) Bain et al. unpublished). β-Glucan recognition occurs predominantly through the C-type lectin receptor (CLR), Dectin-1 (Brown and Gordon 2001). Dectin-1-mediated recognition of β-glucan promotes the formation of a phagocytic synapse, which activates pro-inflammatory signalling through Syk/CARD9, driving the respiratory burst and the release of cytokines such as TNF-α, IL-6 and IL-12 (Goodridge et al. 2011). In addition, the recognition of β-glucan by Dectin-1 triggers phagocytosis, phagosome maturation and ultimately clearance of the offending fungal cell (Mansour et al. 2013).

The critical importance of Dectin-1 in anti-*Candida* immunity is highlighted by the susceptibility of Dectin1 knockout mice to lethal infection (Taylor et al. 2007), as well as by the association of a genetic polymorphism in human Dectin-1, which disrupts β-glucan recognition by phagocytes and abrogates cytokine expression, with familial recurrent vulvovaginal candidiasis (Ferwerda et al. 2009). Furthermore, elevated β-glucan exposure on *C. albicans* cells correlates with their reduced fitness in the gastrointestinal tract (Sem et al. 2016), probably because this

exposure enhances Dectin-1-mediated clearance of the fungal cells from the gut. In addition to activating phagocytosis and pro-inflammatory functions, Dectin-1-mediated sensing of *C. albicans* β-glucan enables "trained immunity" via epigenetic reprogramming of monocyte metabolism to drive enhanced protection against secondary infections (Saeed et al. 2014). Also, a link to adaptive immunity was demonstrated in a study, showing that Dectin-1 expressed on dendritic cells controls CD4+ T cell-mediated gut immunity in mice (Drummond et al. 2016).

These observations illustrate the central importance of Dectin-1 in anti-*Candida* innate and adaptive immune defences. However, additional receptors contribute to the recognition of β-glucan. CR3 (Mac1, CD11b/CD18) is an integrin expressed on several myeloid and lymphoid cell types with affinity for a variety of ligands including iC3b-opsonised target cells (Xia et al. 1999). The I-domain of the CD11b subunit can bind β-glucan (Thornton et al. 1996) and contributes, along with Dectin-1, to the recognition of *C. albicans* hyphae by macrophages (Maxson et al. 2018). CR3 is also important for the recognition of *C. albicans* by neutrophils, which leads to activation and fungal killing (O'Brien and Reichner 2016). Other β-glucan receptors include the glycosphingolipid lactosylceramide, the scavenger receptors SCARF and CD36 and CD23 (Jimenez-Lucho et al. 1990; Means et al. 2009; Guo et al. 2018).

As stated above, the frond-like mannan fibrils that decorate the outer cell wall limit the exposure of β-glucan to immune recognition (Graus et al. 2018). Nevertheless, the mannan fibrils themselves contain molecular signatures that potentiate host immune responses (Netea et al. 2008). *N*-mannan is detected by the mannose receptor, which promotes the oxidative burst and Th1/Th17 responses to control *C. albicans* infection (van de Veerdonk et al. 2009). DC-SIGN (SIGN-R in mice), which is expressed by dendritic cells, also binds fungal *N*-mannan. This leads to interactions with plasma membrane "pickets", such as CD44, that connect the *N*-mannan-DC-SIGN synapse to the cytoskeleton, thereby stabilising phagocytic binding to the target *C. albicans* cells (Te Riet et al. 2017).

The PRRs Dectin-2 and Dectin-3 (MCL, ClecSF8) recognise hyphal α-mannan (McGreal et al. 2006; Saijo et al. 2010; Zhu et al. 2013). Indeed, heterodimerisation of Dectin-2 with Dectin-3 drives a more potent NFkB response than either of these receptors alone (Zhu et al. 2013). The Mincle (macrophage inducible Ca^{++}-dependent lectin) receptor also recognises α-mannan in the *C. albicans* cell wall to drive TNF-α production, thereby promoting protection against systemic infection in mice (Lionakis and Levitz 2018; Wells et al. 2008). In humans, Mincle expression on monocytes is non-phagocytic, but drives pro-inflammatory responses, whereas Mincle expression on neutrophils mediates phagocytosis and killing of *C. albicans* (Vijayan et al. 2012). *C. albicans* α-mannans are also recognised by CD23, resulting in NFkB activation (Guo et al. 2018).

Mannose-binding lectin (MBL) is a secreted circulatory PRR that supports opsono-phagocytosis, and mice that lack MBL succumb to lethal *C. albicans* infections (Held et al. 2008). Gut epithelial cells secrete MBL upon sensing *C. albicans* to regulate gut homoeostasis and control infection (Choteau et al. 2016). Galectin-3, which is expressed in the cytoplasm of host cells and in body

fluids (Dong et al. 2018), has direct fungicidal activity against *C. albicans* cells (Kohatsu et al. 2006). Galectin-3 binds fungal β-1,2 mannoside residues, which are found in phospholipomannan and occasional side branch caps of *N*-mannan chains in the outer wall of *C. albicans* (Roman et al. 2016). Meanwhile, the PRR Langerin recognises mannan and β-glucan and is the dominant receptor on Langerhans cells, which are specialised dendritic cells that are positioned within the epidermis to sample *Candida* species during gut colonisation (de Jong et al. 2010; De Jesus et al. 2015).

The role of Toll-Like Receptors (TLRs) in mammalian antifungal defences was initially suggested by a *Drosophila melanogaster* study that revealed the regulation of drosomycin by the Toll pathway (Lemaitre et al. 1996). Subsequently, TLR2 and TLR4 were shown to modulate cytokine production during candidiasis (Netea et al. 2002). These TLR receptors recognise phospholipomannan and *O*-linked mannan in the *C. albicans* cell wall, respectively (Tada et al. 2002; Jouault et al. 2003; Netea et al. 2006).

Host receptors do not act efficiently in isolation. Instead, sensing of fungal targets is best achieved by collaboration between PRRs and the multi-valent engagement of multiple PAMPs on the cell surface. The inflammatory programme is maximised by co-stimulation of TLR and CLR and activation of MyD88 and Syk/CARD9 pathways, respectively. For example, Dectin-1 and TLR2 cooperate to drive TNF-α production following recognition of *C. albicans* β-glucan (Brown et al. 2003; Gantner et al. 2003). Dectin-1 also mediates cooperative signalling with CR3 and SIGN-R1 (Taylor et al. 2004; Huang et al. 2015) and, as mentioned above, the paired engagement of Dectin-2 and Dectin-3 synergistically boosts inflammatory responses (Zhu et al. 2013). Our understanding of fungal recognition, combinatorial signalling and effector function is limited, and this is further complicated by the context of immune cells involved, their activation status and the nature of the fungal target encountered.

6 The Cell Wall in Immune Evasion

Most studies of fungal immunology have focussed on the immune cell—the receptors and their ligands, mechanisms of intracellular and cytokine signalling and phagocytosis, for example. Less attention has been paid to the fungus and in particular to the impact of fungal adaptation upon PAMP exposure. Indeed, most fungal immunology studies have examined fungal cells that were grown under standardised, but non-physiological conditions in vitro. Yet, as described above, *C. albicans* remodels its cell wall in response to environmental change. It is therefore unsurprising that the conditions under which *C. albicans* is grown significantly affect PAMP exposure, and thereby, the outcome of host-fungus interactions (Hopke et al. 2018). It is becoming clear that, in reality, *C. albicans* is a moving target for the immune system.

Early indications that *C. albicans* is a moving immunological target arose from Wheeler's work showing that dynamic morphogenetic changes during infection affect the degree of β-glucan exposure on the fungal cells (Wheeler et al. 2008). In part, this effect appears to be mediated by the damage that neutrophil extracellular traps cause to the fungal cell surface in situ, and the subsequent fungal cell wall remodelling and repair, which is largely mediated by Hog1-dependent processes (Hopke et al. 2016).

The paradigm of the moving immunological target was clearly demonstrated by the observation that exposing *C. albicans* cells to physiological levels of lactate (a metabolite generated in the vagina and gut by host cells and the microbiota) triggers β-glucan masking at the fungal cell surface (Ballou et al. 2016). *C. albicans* cells detect extracellular lactate via the receptor Gpr1, which signals through Gpa2, PKA, Crz1 and Ace2, leading to reduced β-glucan exposure at the cell surface (Pradhan et al. 2018; Ballou et al. 2016) (Fig. 3). This results in decreased macrophage phagocytosis, lower rates of neutrophil recruitment to sites of infection, and decreased production of the pro-inflammatory cytokines TNF-α and MIP-1 (Ballou et al. 2016). This work, together with the correlative studies of Sem and co-workers (Sem et al. 2016), suggests that *C. albicans* exploits local environmental signals to evade immune recognition and thereby enhance its fitness in certain host niches.

More recently, *C. albicans* has been shown to trigger β-glucan masking and immune evasion in response to hypoxia (Pradhan et al. 2018; Lopes et al. 2018). During the development of a lesion, oxygen concentrations are lower through the combined activities of the infecting *C. albicans* cells and the neutrophils in immune infiltrates that form in an attempt to clear these fungal cells. The resultant hypoxic microenvironment activates β-glucan masking by the *C. albicans* cells, thereby protecting them from clearance by the surrounding neutrophils (Lopes et al. 2018). The hypoxic signal is transduced via the mitochondrion, which leads to PKA-mediated β-glucan masking (Pradhan et al. 2018) (Fig. 3). More recently, we have shown that iron depletion also promotes β-glucan masking in *C. albicans* (Pradhan et al. 2019). Iron depletion is highly relevant to systemic infection as the fungus becomes exposed to iron-limiting conditions in tissues as a consequence of the nutritional immunity imposed by immune infiltrates around fungal lesions (Potrykus et al. 2013). Clearly, *C. albicans* is able to exploit the local signals in certain host niches to evade immune recognition.

Other host niches appear to trigger PAMP exposure and inflammation, rather than PAMP masking and immune evasion. *C. albicans* cells that are exposed to the relatively low ambient pH of the human vagina tend to expose higher levels of β-glucan and chitin at their surface than cells grown at neutral pH of the bloodstream, for example (Sherrington et al. 2017). The elevated chitin exposure appears to be mediated by a reduction in chitinase (Cht2) expression via Bcr1 and Rim101 signalling (Fig. 3). The exposed fungal cells are phagocytosed more efficiently by macrophages and neutrophils, they stimulate increased production of pro-inflammatory cytokines, and they recruit immune cells more efficiently to infection sites (Sherrington et al. 2017). These observations appear to resonate with

the inflammatory behaviour of *C. albicans* during vulvovaginal candidiasis (Hall and Noverr 2017).

Artificial environmental inputs, such as antifungal drugs, also convert *C. albicans* into a moving immunological target. Exposure to sub-inhibitory concentrations of caspofungin increases β-glucan exposure in *C. albicans* to sufficient levels to elicit a potent TNF-α response from macrophages (Wheeler and Fink 2006). This caspofungin-mediated β-glucan exposure is relevant in vivo during infection (Wheeler et al. 2008). A heightened immune response to *C. albicans* can also be caused by mannan grazing by *Bacteroidetes* (a Gram-negative member of the gut microbiota), possibly via trimming of the outer fibrillar layer of the cell wall to reveal the underlying β-glucan (Cuskin et al. 2015).

7 Parallels with Other Fungal Pathogens

C. albicans is not the only fungal pathogen to evade host immune responses by masking a major PAMP in their cell wall. *A. fumigatus*, *C. neoformans*, *Histoplasma capsulatum* and other dimorphic fungal pathogens have evolved effective mechanisms to avoid Dectin-1-mediated immune responses. These fungal pathogens mask PAMPs via two major mechanisms: firstly, by physically masking the PAMP with non-stimulatory cell wall molecules; or secondly, by hydrolase-mediated remodelling of the exposed PAMP.

A. fumigatus is the most common cause of invasive mould infections in immunocompromised patients (Brown et al. 2012). The initial host-pathogen interaction, and an important stage for immune evasion, occurs between conidia, lung epithelial cells, and resident alveolar macrophages. The *A. fumigatus* cell wall contains pro-inflammatory PAMPs, such as galactomannans and β-glucan, which stimulate robust antifungal immune responses and clearance mechanisms (Luther et al. 2007; Heinekamp et al. 2015; Stappers et al. 2018). *A. fumigatus* PAMP exposure peaks with conidial swelling and early hyphal germination, but is masked in mature hyphae and ungerminated conidia (Hohl et al. 2005). These ungerminated conidia mask their cell wall PAMPs under a rodlet layer composed of DHN-melanin and the hydrophobic RodA protein (Aimanianda et al. 2009).

RodA masks Dectin-1- and Dectin-2-mediated detection of *A. fumigatus* conidial PAMPs, and this promotes early immune evasion and fungal survival in the host (Carrion Sde et al. 2013). DHN-melanin plays an important role in preventing phagosomal acidification, thereby enhancing virulence (Langfelder et al. 1998; Thywissen et al. 2011). However, DHN-melanin is also a PAMP and the ligand for the newly characterised host PRR, MelLec (Stappers et al. 2018). Sensing of DHN-melanin by MelLec is important for the control of systemic *A. fumigatus* infection and MelLec polymorphisms are associated with increased risk of aspergillosis in certain cohorts of transplant patients (Stappers et al. 2018).

The protective rodlet layer is lost during *A. fumigatus* germination to reveal the underlying PAMPs. However, *A. fumigatus* hyphae synthesise a cell wall

polysaccharide, galactosaminogalactan, which masks β-glucan whilst mediating adherence to host cells (Gravelat et al. 2013). *A. fumigatus* mutants with defects in galactosaminogalactan biosynthesis display increased β-glucan exposure, they are attenuated in their virulence, and they induce hyper-inflammation in mice (Gravelat et al. 2013).

C. neoformans is another environmentally prevalent human fungal pathogen that causes disease in immunocompromised patients (Brown et al. 2012; Voelz and May 2010). Despite its clinical significance, relatively little is known about how the immune system recognises *C. neoformans* (Heung 2017). In addition to the conserved carbohydrate polymers that typically form fungal cell walls (e.g. chitin, β-glucans, and mannans (Erwig and Gow 2016), *C. neoformans* possesses a unique polysaccharide capsule, primarily composed of glucuronoxylomannan, which masks its cell wall PAMPs. Glucuronoxylomannan is recognised by the receptor TLR4, but TLR4 engagement is not sufficient to induce TNF-α or influence mouse susceptibility to cryptococcosis (Shoham et al. 2001; Yauch et al. 2004). Interestingly, the collectin SP-D binds to glucuronoxylomannan in vitro, and its interaction with *C. neoformans* cells facilitates fungal protection from macrophage killing (Geunes-Boyer et al. 2009). This suggests a possible proactive immune evasion role for *C. neoformans* capsule beyond simply passively shielding cell wall PAMPs (Geunes-Boyer et al. 2009).

Acapsular *C. neoformans* mutants are avirulent and are phagocytosed more efficiently than encapsulated cells (Geunes-Boyer et al. 2009), which is likely due to the unmasking of the underlying immune-stimulatory PAMPs. These appear to include mannoprotein moieties recognised by the Mannose Receptor (Mansour et al. 2002), as mice lacking the Mannose Receptor are more susceptible to infection than wild-type mice (Dan et al. 2008). Other major receptors, such as Dectin-1, Dectin-2, and Dectin-3, are not essential for in vivo defences against cryptococcosis (Nakamura et al. 2007; Nakamura et al. 2015; Campuzano et al. 2017).

The virulence of dimorphic fungal pathogens, such as *H. capsulatum, Blastomyces dermatitidis* and *Paracoccidioides brasiliensis*, has been linked to α-1,3-glucan in their cell walls. α-1,3-Glucan blocks the recognition of cell wall β-1,3-glucan via Dectin-1 by physically masking β-1,3-glucan (Rappleye et al. 2007). Consequently, *P. brasiliensis* and *B. dermatitidis* mutants with low α-1,3-glucan production display decreased virulence in mouse models of infection (San-Blas et al. 1977; Hogan and Klein 1994). However, this association between *H. capsulatum* virulence and α-1,3-glucan is dependent on strain chemotype. *H. capsulatum* strains of chemotype II require α-1,3-glucan for virulence (Edwards et al. 2011) and the inactivation of α-1,3-glucan synthesis attenuates *H. capsulatum* virulence in mice (Rappleye et al. 2004).

In addition to physically masking β-1,3-glucan with α-1,3-glucan, *H. capsulatum* also enzymatically reduces β-glucan exposure in its cell wall (Garfoot and Rappleye 2016). This is achieved by expressing Eng1, an endoglucanase that hydrolyses β- (Sobel 2007; Denning et al. 2018) -glycosyl linkages. Eng1 decreases β-glucan exposure at the cell surface, thereby reducing Dectin-1-mediated recognition of

H. capsulatum cells, and enhancing the virulence of *H. capsulatum* (Garfoot and Rappleye 2016). Therefore the combined effects of physical masking (via α-1,3-glucan) and enzymatic trimming (via Eng1) provide *H. capsulatum* with effective PAMP masking mechanisms (Fig. 3). No doubt these contribute to the inability of innate immune cells to control *H. capsulatum* infection, with macrophages ultimately serving as a reservoir for disseminated infection (Garfoot and Rappleye 2016). Interestingly, Eng1 homologues exist in other important fungal pathogens, including *C. albicans*, suggesting that PAMP trimming mechanisms might contribute to immune evasion in these fungi.

8 Conclusions and Outlook

To summarise, the fungal cell wall is a remarkable organelle that retains a high degree of elasticity and permeability, whilst retaining sufficient tensile strength and spatial integrity to preserve the morphology of the cell. In this way, the cell wall is able to protect the fungal cell against certain acute environmental stresses, whilst permitting communication with the host or local microbiota through the release of large extracellular vesicles. Furthermore, through a complex signalling network that regulates cellular adaptation and cell wall synthesis, the cell wall is responsive to a wide variety of environmental challenges. This cell wall remodelling allows a fungal pathogen to evade the potentially lethal effects of certain antifungal drugs or debilitating mutations, and of local cell wall stresses imposed by host niches.

However, the cell wall is also a point of fragility for a fungal pathogen, as it carries immuno-stimulatory epitopes that can trigger antifungal host defences. Therefore the cell wall has a major influence upon host-fungus interactions. Nevertheless, the ability to remodel the cell wall has provided fungal pathogens with the capacity to evolve effective immune evasion strategies that either mask or remove cell surface PAMPs. *C. albicans,* in particular, has “learned” to exploit a variety of host-derived signals to activate β-glucan masking and immune evasion, including lactate, iron deprivation and hypoxia.

A number of fascinating questions remain to be answered. For example, what is the exact nature and frequency of the covalent cross-links between the major cell wall polymers in the *C. albicans* cell wall? And how, together with the properties of these polymers, do these cross-links promote the remarkable elasticity and morphological stability of the cell wall? The development of monoclonal or recombinant antibodies that are specific for particular cross-links would permit the frequency and spatial distribution of these linkages to be analysed in situ on the *C. albicans* cell wall. This would be particularly interesting in the context of environmental or genetic changes that affect cell wall elasticity and/or morphology (e.g. Ene et al. 2012, 2015; Pardini et al. 2006; Fonzi 1999).

It would be fascinating to screen for host inputs that influence β-glucan exposure in *C. albicans* and thereby affect immune evasion. A number of specific host inputs have been identified already, but an unbiased screen of host signals has yet to be

reported. Then, given the complexity and diversity of host niches, it would be important to test combinations of inputs to establish which signals are most influential in particular niches, and to test PAMP exposure on cells isolated directly from these niches. For example, recent data (e.g. (Pericolini et al. 2018) suggests that lactate-mediated β-glucan masking might dominate over pH-mediated β-glucan exposure during vulvovaginal infection. But what signals dominate in the gastrointestinal tract, and how does this affect *C. albicans* colonisation of the colon, for example?

It is also important to understand exactly how do *C. albicans* cells mask β-glucan at their cell surface—by covering it with mannan or by trimming via an Eng1-like activity (Graus et al. 2018; Garfoot et al. 2016)? Does β-glucan masking attenuate *C. albicans*-phagocyte interactions by simply delaying phagocytic recognition, or does masking (also) reduce the dynamics of phagocytic uptake and/or phagolysosomal maturation?

These questions are not simply of academic interest. A better understanding of the intricacies of cell wall structure and biogenesis is likely to reveal new therapeutic targets that will compromise this essential organelle. Furthermore, a better understanding of the immune evasion strategies exploited by fungal pathogens might reveal ways in which PAMP masking might be blocked. This type of drug might provide a potential means of augmenting antifungal immunotherapies. These could potentially include, for example, specific fungal polysaccharides that provide immuno-amelioration for certain infectious or inflammatory diseases. Time will tell.

Acknowledgements This work was funded by a programme grant from the UK Medical Research Council [www.mrc.ac.uk: MR/M026663/1], and by PhD studentships from the University of Aberdeen to AP, DL. The work was also supported by the Medical Research Council Centre for Medical Mycology (MR/N006364/1) and by the Wellcome Trust [www.wellcome.ac.uk : 097377]. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

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Revisiting Old Questions and New Approaches to Investigate the Fungal Cell Wall Construction

Michael Blatzer, Anne Beauvais, Bernard Henrissat, and Jean-Paul Latgé

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Abstract The beginning of our understanding of the cell wall construction came from the work of talented biochemists in the 70–80's. Then came the era of sequencing. Paradoxically, the accumulation of fungal genomes complicated rather

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Current Topics in Microbiology and Immunology (2020) 425: 331–369
https://doi.org/10.1007/82_2020_209
© Springer Nature Switzerland AG 2020
Published Online: 17 May 2020

than solved the mystery of cell wall construction, by revealing the involvement of a much higher number of proteins than originally thought. The situation has become even more complicated since it is now recognized that the cell wall is an organelle whose composition continuously evolves with the changes in the environment or with the age of the fungal cell. The use of new and sophisticated technologies to observe cell wall construction at an almost atomic scale should improve our knowledge of the cell wall construction. This essay will present some of the major and still unresolved questions to understand the fungal cell wall biosynthesis and some of these exciting futurist approaches.

What is our current knowledge in the understanding of the fungal cell wall biosynthesis in 2020? Many contributors to the Current Topics in Microbiology and Immunology issue entitled “The fungal cell wall. An armor and a weapon for human fungal pathogens” have presented the state of the art of our knowledge on the structural organization, biosynthesis, and biological function of the cell wall of the human fungal pathogens. Here, we present some of the still unresolved but essential questions in the area. Questions are focused on human pathogenic fungi but will be comparatively assessed with plant cell wall and fungal models. We also hope that new methodologies presented at the end of this report will inspire the development of new approaches absolutely required to advance the fungal cell wall field.

1 Coupling Genome Analysis with Cell Wall Construction

Fungal diversity is enormous: around 120,000 fungal species are known and many more are expected to be discovered. The huge amount of data gathered with the increasing number of sequenced genomes offers the possibility to redefine fungal evolutionary relationships and to assess the metabolic diversity that confers fungi the remarkable adaptability to colonize virtually all environments. Fungi emerged as the third kingdom of life and are one of the oldest and also largest groups of living organisms; they play important roles in virtually all ecosystems. The fungal kingdom is extremely diverse regarding morphology, lifestyle, habitats, and complexity. The origin of all fungi might stem from a unicellular, flagellated eukaryvorous aquatic organism that evolved via unicellular parasites to terrestrial mycelial, multicellular and multinucleated forms of variable size (Naranjo-Ortiz and Gabaldón 2019). The fungal lifestyles are as diverse as their habitats, ranging from obligate parasites over obligate anaerobic forms to terrestrial symbionts, commensals, and pathogens.

In spite of their differences in lifestyle and morphology, fungi share one essential common feature which is their cell wall, characterized by the presence of a polysaccharide skeleton comprising β-1,3 glucan and chitin for most species. Besides enzymes that are required for the synthesis of the major cell wall

components, fungi also encode a huge collection of carbohydrate-active enzymes that degrade, modify, or build glycosidic bonds of different cell wall components. The cell wall is the first organelle of fungi interacting with the environment. In contrast to old "postulates," it is dynamic, actively remodeled according to growth stage or environmental conditions (Elhasi and Blomberg 2019). In the host, it has opposite functions since it is required to protect the fungus but also to activate immune responses (Gow et al. 2017; Latgé et al. 2017).

Unlike nucleic acids and proteins, the assembly of glycans is not template-based but it is dictated by the specificity of carbohydrate-processing enzymes. The enzymes that assemble, modify, and break down polysaccharides and glycoconjugates are classified in sequence-based families in the carbohydrate-active enzyme (CAZy) database (www.cazy.org), a continuously updated online resource available since 1998. Due to the link of the carbohydrate metabolism of an organism and its genome, the CAZy database has become an essential source for the analysis and recovery of unknown putative carbohydrate-active enzymes across all organisms. The huge number of sequenced genomes offers the possibility to compare the diverse equipment of carbohydrate-active enzymes among the different fungi that can cause disease in humans. However, a comprehensive picture of the complexity of carbohydrate-active enzymes (CAZymes) acting on the fungal cell wall is still missing, as well as a thorough comparison of the equipment of carbohydrate-active enzymes between different fungal pathogens. The CAZy database systematically groups the information on carbohydrate-active enzymes in the following five enzyme classes: glycoside hydrolases (GHs), glycosyltransferases (GTs), polysaccharide lyases (PLs), carbohydrates esterases (CEs), and redox auxiliary activities (AAs). Redox auxiliary enzymes have not been investigated yet for a role in cell wall synthesis or turnover but may be of interest since a growing number of publications associate cell wall, redox potentials, and mitochondria (Duvenage et al. 2019; Yu et al. 2016). The database also lists the various families of non-catalytic carbohydrate-binding modules (CBMs) that are frequently appended to the CAZymes. The association between the CAZy families of the major human pathogenic species and the cell wall composition of the different fungal classes is shown in Tables 1, 2, 3, 4, 5, 6, and 7 and was based on the analysis of the jgi mycocosm Web site (https://mycocosm.jgi.doe.gov/).

Other efforts are made by the JGI 1000 fungal genomes project, an international initiative that aims to sequence at least two reference genomes from the more than 500 recognized families of fungi. The JGI MycoCosm fungal genomics resource currently holds almost 1500 genomes that integrate several types of annotations such as Gene Ontology, PFAM domains, KEGG, KOG, and CAZy (Grigoriev et al. 2014). Compared to several thousand fungal species that are able to colonize plants (Knogge 1996) and cause diseases in plants, only about 300 fungal species are described to cause disease in humans with the most prominent being *Candida*, *Aspergillus*, *Cryptococcus*, *Pneumocystis,* Dermatophytes, and Zygomycetes (Brown et al. 2012). To date the curated CAZy database covers over 16,000 genomes, with only 1.6% eukaryotic genomes and human pathogenic fungi accounting for a tiny low percentage (<0.2%).

Table 1 Association between CAZYme families in the major fungal pathogens and model organisms and their cell wall composition

	Phylogeny	organism	median genome [Mb]	median protein count	total CAZYmes	CAZYmes %	GT	GH	PL	AA	CE	CBM	EXPN	chitin	chitosan	β1,3 glucan	α1,3 glucan	β1,6 glucan	mannan	galactofurane	fucoidan	glucuronan
							CAZY family															
Dikarya	Ascomycota, Pezizomycotina, Eurotiomycetes	*Aspergillus flavus*	36,81	12832	627	4,89	123	324	25	70	26	54	3	■		■	■		■	■		
		Aspergillus fumigatus	28,83	9544	537	5,63	105	278	15	35	23	73	5	■		■	■		■	■		
		Talaromyces marneffei	28,34	11841	487	4,11	98	230	6	37	19	89	6	■		■	■		■	■		
	Ascomycota, Pezizomycotina, Sordariomycetes	*Fusarium graminearum*	36,67	13334	587	4,40	105	256	22	73	42	79	7	■		■	■		■	■		
	Ascomycota, Taphrinomycotina, Pneumocystidomycetes	*Pneumocystis jirovecii*	8,18	3649	56	1,53	36	15	0	0	0	5	0			■			■			
	Ascomycota, Saccharomycotina, Saccharomycetes	*Saccharomyces cerevisiae*	11,88	5404	143	2,65	68	52	0	6	2	15	0	■		■		■	■			
		Candida albicans	14,68	6265	154	2,46	74	57	0	9	2	11	1	■		■		■	■			
	Basidiomycota, Agaricomycotina, Tremellomycetes	*Cryptococcus neoformans*	18,6	6941	202	2,91	68	90	4	16	4	14	5	■	■	■	■		■			
	Basidiomycota, Ustilagomycotina, Malasseziomycetes	*Malassezia globosa*	8,94	4286	103	2,40	43	30	1	18	4	2	4	■		■			■			
Fungi incertae sedis	Zoopagomycota, Entomorphthoromycotina, Enteromorphthoromycetes	*Conidiobolus coronatus*	39,9	10527	296	2,81	132	97	0	38	6	16	2	■		■			■			
	Mucoromycota, Mucoromycotina, Mucormycetes	*Rhizopus microsporus*	45,7	11496	302	2,63	117	92	4	11	23	34	12	■	■				■		■	■
		Lichtheimia corymbifera	42,42	11542	353	3,06	142	101	6	14	25	47	12	■	■				■		■	■
	Microsporida, Unikarionidae, Encephalitozoon	*Encephalitozoon cuniculi*	2,29	1915	11	0,57	8	2	0	0	1	0	0	■					■			

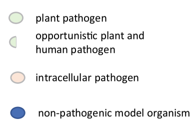

Table 2 Glycosyl hydrolase families in fungal species of different orders (including major human pathogens)

	A. flavus	*A. fumigatus*	*T. marneffei*	*F. graminearum*	*P. jirovecii*	*S. cerevisiae*	*C. albicans*	*C. neoformans*	*M. globosa*	*C. coronatus*	*R. microsporus*	*L. corymbifera*	*E. cuniculi*	
CAZYmestotal	627	537	487	587	56	143	154	202	103	296	302	353	11	
GH	324	278	230	256	15	52	57	90	30	97	92	101	2	**Involved in cell wall (putatively-)**
GH1	3	5	3	3	–	–	–	–	–	–	–	–	–	
GH2	8	6	6	10	–	–	1	–	–	–	–	–	–	
GH3	24	20	14	21	–	–	3	3	1	3	5	6	–	
GH5	15	14	8	14	–	5	5	12	6	8	5	6	–	β glycans
GH5_4	1	1	–	–	–	–	–	–	3	–	–	–	–	
GH5_5	2	4	1	2	–	–	–	–	1	–	–	–	–	
GH5_7	2	2	1	2	–	–	–	–	–	–	–	–	–	
GH5_9	2	2	2	1	–	3	3	6	–	–	1	1	–	
GH5_11	–	–	1	–	–	–	–	–	–	–	–	–	–	
GH5_12	1	1	1	2	–	1	1	3	–	6	2	2	–	
GH5_15	2	1	1	2	–	–	–	–	–	2	2	1	–	α glucans
GH5_16	1	1	–	1	–	–	–	–	–	–	–	–	–	
GH5_22	1	1	–	2	–	–	–	1	–	–	–	–	–	
GH5_23	1	–	–	1	–	–	–	–	–	–	–	–	–	
GH5_24	–	–	–	1	–	–	–	–	–	–	–	–	–	
GH5_27	1	1	–	–	–	–	–	–	–	–	–	1	–	
GH5_49	1	–	1	–	–	1	1	–	–	–	–	1	–	
GH5_50	–	–	–	–	–	–	–	1	2	–	–	–	–	
GH6	1	1	1	1	–	–	–	–	–	–	–	–	–	
GH7	3	4	2	2	–	–	–	–	–	–	–	–	–	
GH8	–	–	–	–	–	–	–	–	1	–	2	1	–	
GH9	–	–	–	–	–	–	–	1	–	–	3	4	–	
GH10	4	4	1	5	–	–	–	–	–	–	–	–	–	

(continued)

Table 2 (continued)

	A. flavus	*A. fumigatus*	*T. marneffei*	*F. graminearum*	*P. jirovecii*	*S. cerevisiae*	*C. albicans*	*C. neoformans*	*M. globosa*	*C. coronatus*	*R. microsporus*	*L. corymbifera*	*E. cuniculi*	
GH11	4	3	3	3	–	–	–	–	–	–	–	–	–	
GH12	5	4	3	4	–	–	–	–	–	–	–	1	–	
GH13	16	16	11	8	3	9	4	12	–	3	3	5	–	α glycans
GH13_1	3	5	3	1	–	–	–	5	–	–	1	2	–	
GH13_5	2	1	1	–	–	–	–	2	–	1	–	–	–	
GH13_8	1	1	1	1	2	1	1	1	–	1	1	1	–	
GH13_22	3	3	2	–	–	–	–	1	–	–	–	–	–	
GH13_25	1	1	1	1	1	1	1	1	–	1	1	2	–	
GH13_30	–	–	–	–	–	–	–	1	–	–	–	–	–	
GH13_40	6	5	3	5	–	7	2	1	–	–	–	–	–	
GH15	3	5	4	3	–	1	1	2	–	1	3	3	–	
GH16	13	14	15	24	1	5	7	13	7	17	8	6	–	β-1,3 glucan
GH17	5	5	4	6	–	4	5	1	–	6	2	4	–	
GH18	17	16	27	20	–	2	4	4	1	19	12	11	–	Chitin
GH19	–	–	–	–	–	–	–	–	–	–	–	–	1	Chitin
GH20	3	2	2	3	–	–	1	1	–	6	4	4	–	β glycan
GH23	–	1	–	–	–	–	–	1	–	–	–	–	–	
GH24	2	–	–	–	–	–	–	–	–	–	–	–	–	
GH25	1	3	1	–	–	–	–	–	–	2	–	1	–	
GH26	1	–	–	–	–	–	–	–	–	–	–	–	–	
GH27	3	4	2	2	–	–	–	–	–	–	1	2	1	
GH28	21	13	8	6	–	1	–	1	–	–	6	3	–	
GH29	–	–	–	1	–	–	–	–	–	–	–	1	–	
GH30	–	1	5	–	–	–	–	–	–	–	–	–	–	
GH30_3	–	1	2	–	–	–	–	–	–	–	–	–	–	
GH30_5	–	–	2	–	–	–	–	–	–	–	–	–	–	
GH30_7	–	–	8	–	–	–	–	–	–	–	–	–	–	

(continued)

Table 2 (continued)

	A. flavus	*A. fumigatus*	*T. marneffei*	*F. graminearum*	*P. jirovecii*	*S. cerevisiae*	*C. albicans*	*C. neoformans*	*M. globosa*	*C. coronatus*	*R. microsporus*	*L. corymbifera*	*E. cuniculi*	
GH31	10	7	1	8	1	1	3	3	2	4	3	5	–	
GH32	4	5	–	5	2	1	–	1	–	–	–	–	–	
GH33	1	1	–	1	–	–	–	1	–	–	–	–	–	
GH35	8	5	4	3	–	–	–	–	–	1	1	2	–	
GH36	3	3	1	3	–	–	–	1	–	–	4	3	–	
GH37	1	1	1	2	1	2	1	2	1	2	3	5	1	
GH38	1	1	1	1	–	1	1	1	–	2	2	3	–	
GH39	–	1	1	2	–	–	–	–	–	–	–	–	–	
GH43	21	18	7	18	–	–	–	–	1	–	–	2	–	
GH45	1	1	2	1	–	–	–	–	–	–	5	1	–	
GH46	6	6	7	–	–	–	–	–	–	–	2	3	–	Chitosan
GH47	–	–	1	10	1	3	2	3	3	11	8	6	–	
GH51	4	2	1	2	–	–	–	1	–	–	–	2	–	
GH53	2	1	1	1	–	–	–	–	–	–	–	–	–	
GH54	1	–	4	1	–	–	–	–	–	–	–	–	–	
GH55	3	8	7	3	–	–	–	–	3	–	–	–	–	β 1,3 glucan
GH62	2	2	2	1	–	–	–	–	–	–	–	–	–	
GH63	1	1	1	1	1	1	1	–	1	1	1	1	–	
GH64	–	–	3	2	–	–	–	–	–	–	–	–	–	β 1,3 glucan
GH65	1	1	1	–	–	1	1	–	–	–	–	–	–	
GH67	1	1	2	1	–	–	–	–	–	–	–	–	–	
GH71	10	9	7	–	–	–	–	4	–	–	–	–	–	β 1,3 glucan
GH72	8	7	5	3	1	5	5	1	–	5	1	2	–	
GH74	–	2	1	1	–	–	–	–	–	–	–	–	–	
GH75	4	3	1	1	–	–	–	–	–	–	–	–	–	Chitosan
GH76	10	7	6	7	1	2	3	–	–	–	–	–	–	
GH78	12	6	2	7	–	–	–	2	–	–	–	–	–	

(continued)

Table 2 (continued)

	A. flavus	*A. fumigatus*	*T. marneffei*	*F. graminearum*	*P. jirovecii*	*S. cerevisiae*	*C. albicans*	*C. neoformans*	*M. globosa*	*C. coronatus*	*R. microsporus*	*L. corymbifera*	*E. cuniculi*	
GH79	5	–	2	1	–	–	–	4	–	–	–	1	–	
GH81	1	1	1	1	1	2	2	–	–	1	1	1	–	β 1,3 glucan
GH85	–	–	–	–	–	–	–	–	1	–	–	–	–	
GH88	3	2	–	1	–	–	–	–	–	–	–	–	–	
GH89	1	1	2	–	–	–	1	1	–	–	–	–	–	
GH92	6	7	5	–	–	–	–	–	–	–	–	–	–	
GH93	3	3	2	2	–	–	–	–	–	–	–	–	–	
GH95	3	2	1	2	–	–	–	–	–	–	–	–	–	
GH105	4	3	4	3	–	–	–	1	–	–	–	3	–	
GH106	5	1	–	1	–	–	–	1	–	–	–	–	–	
GH114	1	1	1	2	–	–	–	1	–	–	–	–	–	
GH115	3	1	–	2	–	–	–	1	–	–	–	–	–	
GH125	1	1	1	3	–	–	1	–	–	–	2	1	–	
GH127	–	1	2	1	–	–	–	–	–	–	–	–	–	
GH128	2	2	6	4	–	–	–	7	2	–	–	–	–	
GH130	–	–	–	–	–	–	1	–	–	–	–	–	–	
GH131	3	2	–	1	–	–	–	–	–	–	–	–	–	
GH132	2	2	2	2	1	5	3	–	–	3	–	–	–	
GH133	1	1	1	1	1	1	1	1	–	2	1	2	–	
GH134	3	1	–	–	–	–	–	–	–	–	4	–	–	
GH135	4	4	–	–	–	–	–	–	–	–	–	–	–	
GH136	1	–	–	1	–	–	–	–	–	–	–	–	–	
GH139	–	–	1	–	–	–	–	–	–	–	–	–	–	
GH140	1	–	–	–	–	–	–	–	–	–	–	–	–	
GH141	–	–	–	1	–	–	–	–	–	–	–	–	–	
GH142	1	–	–	1	–	–	–	–	–	–	–	–	–	
GH145	–	–	–	1	–	–	–	–	–	–	–	–	–	

(continued)

Table 2 (continued)

	A. flavus	*A. fumigatus*	*T. marneffei*	*F. graminearum*	*P. jirovecii*	*S. cerevisiae*	*C. albicans*	*C. neoformans*	*M. globosa*	*C. coronatus*	*R. microsporus*	*L. corymbifera*	*E. cuniculi*	
GH146	–	–	–	1	–	–	–	–	–	–	–	–	–	
GH152	–	–	1	1	–	–	–	1	–	–	–	–	–	
GH154	3	2	1	2	–	–	–	1	–	–	–	–	–	
GH162	–	1	–	1	–	–	–	–	–	–	–	–	–	

Table 3 Glycosyl transferase families in fungal species of different orders (including major human pathogens)

	A. flavus	*A. fumigatus*	*T. marneffei*	*F. graminearum*	*P. jirovecii*	*S. cerevisiae*	*C. albicans*	*C. neoformans*	*M. globosa*	*C. coronatus*	*R. microsporus*	*L. corymbifera*	*E. cuniculi*	
CAZYmes total	627	537	487	587	56	143	154	202	103	296	302	353	11	**Cell wall-associated transferase activity**
GT	123	105	98	105	36	68	74	68	43	132	117	142	8	
GT1	9	9	6	16	2	3	3	5	3	50	9	10	–	
GT2	20	15	17	19	2	5	6	10	9	17	27	30	2	Chitin synthase
GT3	1	1	1	1	1	2	1	1	4	1	2	3	1	Glycogen synthase
GT4	11	8	5	5	3	3	3	5	–	3	5	7	–	
GT5	3	3	2	–	–	–	–	1	–	–	–	–	–	
GT8	5	4	4	6	2	3	3	6	1	2	3	3	–	GlcNAc transferase?
GT10	–	–	–	–	–	–	–	–	–	–	1	2	–	
GT14	–	–	–	–	–	–	–	–	–	1	–	–	–	
GT15	3	3	7	5	–	9	5	1	3	16	12	16	1	Mannosyl transferase
GT17	–	–	–	2	–	–	–	–	–	–	–	–	–	
GT20	6	7	7	3	2	4	3	3	3	2	7	8	2	
GT21	1	1	1	1	1	–	1	1	1	–	1	1	–	
GT22	4	4	4	4	2	4	4	3	3	3	5	5	–	
GT24	1	1	1	1	1	1	1	1	1	1	1	3	–	
GT25	6	3	2	–	–	–	–	–	–	–	–	–	–	
GT31	12	8	8	5	–	–	–	2	1	–	3	1	–	Galactofuranosyl transferase
GT32	10	6	8	5	2	4	3	7	1	10	2	3	–	Mannosyltransferase
GT33	1	1	1	1	2	1	1	1	1	1	1	1	1	GDP-Man: chitobiosyldiphosphodolichol β-mannosyltransferase?
GT34	2	3	3	2	–	2	2	–	–	–	1	3	–	
GT35	1	1	1	1	2	1	1	1	–	–	2	–	1	
GT39	3	3	3	3	4	7	5	3	3	5	6	8	–	
GT41	1	1	1	1	–	–	–	–	–	1	1	1	–	
GT47	–	–	–	–	–	–	–	1	–	–	1	1	–	
GT48	1	1	1	1	1	3	3	1	2	4	2	3	–	1,3 beta glucan synthase

(continued)

Table 3 (continued)

	A. flavus	*A. fumigatus*	*T. marneffei*	*F. graminearum*	*P. jirovecii*	*S. cerevisiae*	*C. albicans*	*C. neoformans*	*M. globosa*	*C. coronatus*	*R. microsporus*	*L. corymbifera*	*E. cuniculi*	
GT49	–	–	–	–	–	–	–	–	–	–	8	11	–	β-1,3-N-acetylglucosaminyltransferase?
GT50	1	1	1	1	1	1	1	2	1	1	1	1	–	
GT57	2	2	2	2	4	2	2	–	2	2	2	2	–	Dol-P-Glc: α-1,3 glucosyltransferase
GT58	1	1	1	1	1	1	1	1	–	1	1	1	–	
GT59	1	1	1	1	2	1	1	–	–	1	1	1	–	
GT62	3	3	3	3	–	3	4	–	–	–	2	5	–	
GT64	–	–	–	2	–	–	–	–	–	–	2	1	–	
GT66	1	1	1	1	1	1	1	1	1	1	1	1	–	
GT69	3	3	1	1	–	–	–	2	2	2	–	1	–	Mannosyltransferase
GT71	5	3	2	4	–	6	12	2	–	7	3	4	–	
GT76	1	2	2	1	–	1	1	1	1	–	1	1	–	
GT77	–	–	–	–	–	–	–	–	–	–	3	4	–	
GT90	4	5	1	6	–	–	–	6	–	–	–	6	–	
GT91	–	–	–	–	–	–	6	–	–	–	–	–	–	β-1,2-mannosyltransferase
Myosin_motor	2	2	2	2	–	–	–	1	1	5	9	–	–	

Table 4 Can auxiliary activities and polysaccharide lyases be of any role in fungal cell wall?

	A. flavus	*A. fumigatus*	*T. marneffei*	*F. graminearum*	*P. jirovecii*	*S. cerevisiae*	*C. albicans*	*C. neoformans*	*M. globosa*	*C. coronatus*	*R. microsporus*	*L. corymbifera*	*E. cuniculi*
CAZYmes total	627	537	487	587	56	143	154	202	103	296	302	353	11
Polysaccharide lyases (PL)	25	15	6	22	0	0	0	4	1	0	4	6	0
PL1	12	6	3	9	–	–	–	–	–	–	–	–	–
PL3	3	3	–	1	–	–	–	–	–	–	–	–	–
PL4	3	3	–	7	–	–	–	1	–	–	–	6	–
PL7	1	–	1	2	–	–	–	–	–	–	–	–	–
PL8	–	–	–	–	–	–	–	–	–	–	1	–	–
PL9	1	1	–	–	–	–	–	–	–	–	–	–	–
PL11	–	–	–	1	–	–	–	–	–	–	–	–	–
PL14	–	–	–	–	–	–	–	2	1	–	3	–	–
PL20	2	1	1	1	–	–	–	–	–	–	–	–	–
PL26	2	1	–	1	–	–	–	–	–	–	–	–	–
PL27	1	–	–	–	–	–	–	–	–	–	–	–	–
PL35	–	–	1	–	–	–	–	1	–	–	–	–	–

(continued)

Table 4 (continued)

	A. flavus	*A. fumigatus*	*T. marneffei*	*F. graminearum*	*P. jirovecii*	*S. cerevisiae*	*C. albicans*	*C. neoformans*	*M. globosa*	*C. coronatus*	*R. microsporus*	*L. corymbifera*	*E. cuniculi*
Auxiliary Activities (AA)	70	35	37	73	0	6	9	16	18	38	11	14	0
AA1	11	5	11	14	–	3	5	5	12	1	5	7	–
AA2	–	–	–	1	–	–	–	–	–	–	–	–	–
AA3	35	11	10	20	–	–	–	3	5	10	2	–	–
AA4	–	1	4	1	–	–	–	–	–	–	–	–	–
AA5	2	1	4	5	–	–	–	3	1	–	2	1	–
AA6	1	1	4	1	–	3	4	2	–	8	2	6	–
AA7	3	1	1	5	–	–	–	–	–	–	–	–	–
AA8	3	2	–	4	–	–	–	1	–	–	–	–	–
AA9	7	8	1	13	–	–	–	–	–	–	–	–	–
AA11	5	3	2	4	–	–	–	1	–	19	–	–	–
AA12	–	–	–	2	–	–	–	–	–	–	–	–	–
AA13	1	–	–	1	–	–	–	–	–	–	–	–	–
AA14	–	1	–	1	–	–	–	1	–	–	–	–	–
AA16	2	1	–	1	–	–	–	–	–	–	–	–	–

Table 5 Carbohydrate esterase families in the different human fungal species

		A. flavus	*A. fumigatus*	*T. marneffei*	*F. graminearum*	*P. jirovecii*	*S. cerevisiae*	*C. albicans*	*C. neoformans*	*M. globosa*	*C. coronatus*	*R. microsporus*	*L. corymbifera*	*E. cuniculi*
CAZYmes total		627	537	487	587	56	143	154	202	103	296	302	353	11
Carbohydrate exterase (CE)		26	23	19	42	–	2	2	4	4	6	23	25	1
CE1	Xylan	3	2	2	4	–	–	–	–	–	–	–	–	–
CE2		–	–	1	1	–	–	–	–	–	–	–	–	–
CE3		3	1	1	5	–	–	–	–	–	–	–	–	–
CE4	Chitin	2	7	4	7	–	2	1	4	3	4	18	17	1
CE5	Xylan	6	5	3	12	–	–	–	–	–	–	–	–	–
CE8		5	5	3	6	–	–	–	–	–	–	3	2	–
CE9		1	1	1	1	–	–	1	–	1	1	1	1	–
CE12		4	2	1	3	–	–	–	–	–	–	–	–	–
CE15		–	1	–	–	–	–	–	–	–	–	–	–	–
CE16		2	3	3	3	–	–	–	–	–	1	1	5	–
Distantly related to plant expansins (EXPN)		3	5	6	7	–	–	1	5	4	2	12	12	–

Table 6 Carbohydrate-binding modules in fungi

		A. flavus	*A. fumigatus*	*T. marneffei*	*F. graminearum*	*P. jirovecii*	*S. cerevisiae*	*C. albicans*	*C. neoformans*	*M. globosa*	*C. coronatus*	*R. microsporus*	*L. corymbifera*	*E. cuniculi*
CAZYmes total		627	537	487	587	56	143	154	202	103	296	302	353	11
CBM	Described binding in fungi	54	73	89	79	5	15	11	14	2	16	34	47	0
CBM1	Cellulose	5	16	17	12	–	–	–	–	–	–	2	2	–
CBM5	Cellulose	–	–	–	–	–	–	–	–	–	–	3	2	–
CBM6	Amorphous cellulose, xylan	–	–	–	1	–	–	–	–	–	–	–	–	–
CBM12	Chitin	–	–	–	–	–	–	–	–	–	–	1	2	–
CBM13	Xylan, N-acetylgalactosamine	2	–	–	2	–	–	–	5	–	–	16	22	–
CBM14	Chitin	2	1	–	–	–	–	–	–	–	5	–	–	–
CBM18	Chitin	5	11	19	26	–	2	1	1	–	1	1	1	–
CBM19	Chitin	1	1	2	–	–	1	–	–	–	–	–	–	–
CBM20	α 1,4/1,6 glucan	1	4	3	2	–	–	–	–	–	–	–	–	–
CBM21	α 1,4/1,6 glucan	1	1	2	2	–	4	2	2	–	1	5	5	–
CBM24	α-1,3-glucan	9	11	2	2	–	–	–	–	–	–	–	–	–
CBM32	Galactose, lactose, pectin	2	0	1	3	–	–	–	–	–	–	–	–	–
CBM35	Galactan, β mannan	3	3	2	3	–	–	–	–	–	–	–	–	–
CBM38	Inulin	–	1	–	–	–	–	–	–	–	–	–	–	–
CBM42	Arabinoxylan	1	–	4	1	–	–	–	–	–	–	–	–	–
CBM43	β-1,3 glucan	5	4	1	1	1	2	3	1	–	5	1	2	–
CBM48	α 1,4/1,6 glucan	3	2	3	2	3	6	5	3	1	2	2	3	–
CBM50	Chitin, chitopentaose	13	14	29	20	–	–	–	2	1	–	3	8	–
CBM52	β-1,3-Glucan	–	–	–	–	1	–	–	–	–	–	–	–	–
CBM63	Cellulose	0	1	–	3	–	–	–	–	–	2	–	–	–
CBM66	Fructan	–	2	4	–	–	–	–	–	–	–	–	–	–

Clearly, the CAZy database is an essential reference source for the analysis and recovery of genomic, structural, and biochemical information across organisms. Even though the CAZy database integrates mainly glycoside hydrolases allowing the fungus to grow on fresh or decaying plant material, the presence of cell wall-associated CAZymes common to many species has been also used to identify enzymes involved in morphogenetic processes. This is how the GPI-anchored CAZymes encoded by *GEL*, *DFG*, *SPS2, SUN,* and *CRH* were suspected to be cell wall-associated and shown later to play an active role in cell wall construction. The discovery of new cell wall enzymes remains, however, a very difficult challenge especially since the mechanisms of cell wall synthesis are complex and have been insufficiently clarified at the molecular level as seen in the following paragraphs describing the synthesis of the two major fungal polysaccharides: chitin and β-1,3 glucan (Latge and Beauvais 2014).

a. **Chitin—a fungal key polysaccharide**

The comparison of Microsporidia, Chytridiomycetes, and Zygomycetes with Ascomycetes and Basidomycetes (Table 1) suggests that the most recent fungi in the phylogeny appear to have the most complex cell wall composition. However, it is also interesting to observe that some of the Zygomycetes have a complex cell wall with an "algal" signature represented by fucoidan and glucuronan polysaccharides (Mélida et al. 2015), a feature perhaps due to the fact that they populate mainly the same habitat. Chitin, a polymer of β-1,4-linked N-acetylglucosamine, is the key component of the fungal cell wall, though not in terms of abundance in the cell wall. This polysaccharide is present in all fungal lineages and distinguishes the fungal cell wall from that of plants. Chitin indeed evolved soon after plants split from the other eukaryotes. Although it is a rigid molecule, it did not prevent the ancestral fungal cells to move since the first fungi had flagella. The cell wall of the most deeply branching fungal taxon, *Rozella* (Cryptomycota) is chitin-based. The group of early diverging fungi includes members of Chytridiomycetes and Zygomycetes are also chitin-rich. However, chitin is not necessary for all fungi since it is missing in *Pneumocystis* or *Schizoaccharomyces pombe*. Of note, the *S. pombe* genome harbors a class I chitin synthase gene. The proportion of chitin in fungal cell walls varies widely from 1 to 2% of cell dry weight in yeasts up to 40% in Zygomycetes (Bartnicki-Garcia and Lippman 1969).

Chitin biosynthesis is best studied in *Saccharomyces cerevisiae,* which encodes three chitin synthases belonging to the CAZy family GT2. The chitin synthases of the baker's yeast can be subclassed in classes I, II, IV of the total seven subclasses and the class IV Chs3 is responsible for the synthesis of 90% of the entire chitin content (Osmond et al. 1999). One of the limitations in the analysis of the CAZy family can be seen here: the other two GT2 members encoded by *S. cerevisiae* are not associated to chitin synthesis but are Alg5 and Dpm1 which are required in the assembly of the core glycolipid of GPI membrane anchor and in the synthesis of O- and N-linked mannans, respectively. In addition, the quantity and the class of chitin synthases genes encoded in a fungal genome can greatly vary, from a single gene to multiple copies of different classes. The phylogeny of the chitin synthase genes is

indeed quite complex with some of the candidate chitin synthases being found in organisms which do not have chitin, raising the question of a putative role of chitin synthases in other pathways than only chitin synthesis (Zakrzewski et al. 2014).

The construction of the cell wall chitin involves the cooperative and sequential activity of several CAZy families including synthases (GT2), chitinases (GH18, GH19), or remodeling enzymes such as chitin deacetylases (CE4). Some of these enzymes can also harbor a CBM, but there also exist several chitin-binding modules not appended to any catalytic domain. The chitin synthase activity to date has been shown exclusively in vitro with rudimentary biochemical experiments using NDP-sugars and crude membrane fractions. In each CAZy family involved, there are several proteins, but their precise role and function are rarely properly identified. For chitin hydrolases, a search in the CAZy database will recover mostly GH18 or GH19 enzymes sometimes appended to chitin-binding domains of families CBM5, 12, 14, 18, 19, or 50 (Table 7) generating diverse modular structures. The biological function of fungal chitinases is difficult to appreciate as there is no easy way to separate the chitinases which have a morphogenetic role in the construction/remodeling of the cell wall from those that have a simple nutritional role (Alcazar-Fuoli et al. 2011). Similar to obligate pathogens, *S. cerevisiae* has a limited number of chitinases which are obviously not focused on nutrient acquisition and have been shown to be exclusively involved in cell wall modifications. There are 16 putative chitinases encoded in the *Aspergillus fumigatus* genome (Table 2). All belong to family GH18, nine have just the catalytic GH18 module while the others are appended to CBM18, 19 or 50 modules. Delineating the role of these enzymes is arduous and involves multiple deletions of the corresponding hydrolase genes. In *A. fumigatus*, a quintuple chitinase-deleted mutant showed only a minor loss of chitinase activity and ongoing work suggests still more than 25% of chitinase activity in a decuple mutant (Blatzer and Latgé unpublished).

Also, it is often difficult to correlate the presence of a CAZyme(s) and the composition of the fungal cell wall which is often not accurately known. This is the case for chitin deacetylases (CE4, Table 5) which are found in all fungal phyla while only Zygomycetes or *Cryptococcus sp*. appear to contain their product chitosan, the deacetylated derivative of chitin, in the cell wall. In *Saccharomyces cerevisiae,* chitosan is only produced during sporulation in the ascospore cell wall (Christodoulidou et al. 1996). *A. fumigatus* harbors seven CE4 family members; one is coupled to two CBM18 chitin-binding modules while the other two members are not (Table 7). *Cryptococcus neoformans* harbors four carbohydrate esterases of the CE4 family and three members have been experimentally shown to contribute to chitosan production. *C. neoformans* chitosan-deficient strains show a budding defect and more importantly chitosan-deficient strains are rapidly cleared by the host in the lungs (Banks et al. 2005). Interestingly, the chitin deacetylase activity of Cda1 is critical for fungal virulence in a murine infection model (Upadhya et al. 2018). What would be the use of enzymes such as chitin deacetylases which are expressed during growth by some *Aspergillus* sp. when the fungal cell wall does not contain chitosan (Mouyna et al. 2020)? Is it possible that the presence of low amounts of glucosamine go unnoticed after classical very harsh hydrolysis

Table 7 An example of CAZYme organization and carbohydrate-binding modules in *A. fumigatus*

Chitin binding	***β glucan binding***	***Other mixed CBM modules***
CBM 18—CBM 18—CE 4	***4 x*** CBM 43—GH 72	AA 5/Subf 2—CBM 32
CBM 18—GH 16	***Cellulose binding***	CBM 35—GH 27
CBM 18—GH 18	***3 x*** AA 9—CBM 1	***2 x*** CBM 35—GH 43
CBM 18—CBM 18—GH 18	CBM 1—CE 5	CBM 38—GH 32—GH 32
CBM 18—CBM 50—GH 18	CBM 1—CE 16	CBM 48—GH 13/Subf 8
CBM 18—CBM 18—CBM 50—GH 18	***2 x*** CBM 1—GH 5/Subf 5	CBM 66—CBM 66
2 x CBM 18—CBM 50—CBM 50—GH 18	CBM 1—GH 5/Subf 7	***Single CBM only modules***
CBM 19—GH 18	CBM 1—GH 6	CBM 1 distantly related to plant expansins
2 x CBM 50—CBM 50	***2 x*** CBM 1—GH 7	CBM1
CBM 50—CBM 50—CBM 50	CBM 1—GH 10	CBM 14
α ***glucan binding***	CBM 1—GH 45	CBM 21
CBM 24—CBM 24	CBM 1—GH 62	CBM 48
CBM 24—CBM 24—GH 71	***Starch binding***	CBM 50
CBM 24—CBM 24—CBM 24—GH 71	CBM 20—GH 13/Subf 1	CBM 63 distantly related to plant expansins
CBM 24—CBM 24—CBM 24—CBM 24—GH 71	***3 x*** CBM 20—GH 15	

procedures used for cell wall analysis? Could the chitin be susceptible to post-synthetic modifications and contain some glucosamine oligosaccharides which would decrease the stiffness of the chitin fibrils and lead to a more plastic cell wall exoskeleton? These are open questions which are also relevant for other cell wall polysaccharides.

b. **β-1,3 glucan, another major cell wall structural polysaccharide**

β-1,3 glucans are the most abundant component of the cell walls of Ascomycetes and Basidiomycetes. With chitin, they contribute to the rigidity of the cell wall as fibrillar core and hold a central place in the cell wall organization and architecture. They are branched with β-1,6 connections and are covalently linked to other cell wall polysaccharides. Their synthesis is mediated by β-1,3 glucan synthases belonging to GT48 family, which use UDP-glucose as the only glucosyl donor. Deletion of the β-1,3 glucan synthase catalytic subunit (*FKS*) genes is lethal (*FKS1* deletion in *C. albicans*, or simultaneous deletion of *FKS1* and *FKS2* in *C. glabrata*) or lead to very impaired growth and abnormal morphology in *A. fumigatus* (Dichtl et al. 2015; Douglas et al. 1997; Katiyar et al. 2006). Of note, the microsporidian

Encephalitozoon cuniculi lacks a *FKS* ortholog, consistent with the lack of β-1,3 glucans. β-1,3 glucans have been also the only structural core polysaccharide for which a very specific pattern recognition receptor (PRR) ligand, dectin1, has been characterized (Taylor et al. 2007) and a homolog of the mammalian dectin-1 triggers immune responses in plants (Mélida et al. 2018).

The steady-state growth theory established in the 70's assumes the continuous secretion at the apex of an expansible mixture of wall polymers that is continuously removed at the base of the extension zone the rigid cell wall arising from interactions between the polymers (Bartnicki-Garcia et al. 2000; Bartnicki-García 1999). This suggests that β-1,3 glucanases are indeed only useful for cell separation as seen with yeast and more recently in *A. fumigatus* (Kuznetsov et al. 2016; Millet et al. 2019; Onwubiko et al. 2020; Wloka and Bi 2012). In *A. fumigatus*, five GH families have so far been identified to act as β-1,3 glucanases: GH3, GH5, GH16, GH81, and GH55. The latter family has been shown to be important during conidiation and proper spore maturation and spore dissemination (Millet et al. 2019). Also, the endoglucanase activity of the GH16 members together with that of the only GH81 member impact on conidial maturation (Mouyna et al. 2016). An extension of this morphogenetic role of glucanases during cell separation is that the septum insertion requires some degradation of the mature rigid cell wall for the centripetal growth required for septum formation in filamentous fungi. In addition, many of these hydrolases have now been recognized as true transglycosidases as shown by the GH72 family. The remodeling role of these glycoside hydrolases during cell wall expansion should be better assessed.

c. **Fungal life and CAZymes**

The CAZyme content appears to be co-evolving with the lifestyle of fungi. Among the selected human pathogenic fungi, *Encephalitozoon cuniculi* possesses not only the smallest genome but also the most reduced CAZyme portfolio with only 11 CAZymes belonging to three CAZy families (Table 1). Due to its peculiar obligate intracellular lifestyle, it is not known if this CAZyme set constitutes a very reduced set for the specialized environment of this pathogen. For example, as a basal fungus, it possesses a mitochondrial remnant without DNA (Han et al. 2019). Chitin and mannose have been identified as cell wall components of this pathogen. This is reflected in the CAZy makeup, which consists of only two glycoside hydrolase namely a class I chitinase from family GH19 and a trehalase of family GH37. Furthermore, eight glycosyltransferases (which make up more than 70% of its tiny CAZyme repertoire) are encoded in the genome of *E. cuniculi*. Family GT48 β-1,3 glucan synthase is missing from the *E. cuniculi* genome, in agreement with the absence of β-1,3 glucan in this organism. Two GT2 members are present with predicted chitin synthase and dolicholphosphate mannosyltransferase functions. Another α-mannosyltransferase (PIG-V) from the family GT76, a third KTR like GT15 glycolipid α-mannosyltransferase, and a GT4 family glycosyltransferase likely to participate to GPI anchor biosynthesis complete the mannosytransferase portfolio of *E. cuniculi* and confirm the essential role of the GPI pathway in fungal life (and cell wall construction?). Such in silico analysis would suggest to inspect

more deeply these glycolipid CAZymes whose role in the cell wall biosynthesis has been insufficiently investigated. Of the third CAZy class, the carbohydrate esterases, *E. cuninculi* possesses one single CE4 member, a polysaccharide deacetylase responsible for the production of chitosan, whose presence has not been investigated biochemically in this species.

At the other extreme of the spectrum of fungi, the opportunistic human, plant, and insect pathogenic *A. flavus* can be found. While *E. cuniculi* is very specialized regarding host and habitat and limited in its CAZyme makeup, *A. flavus* holds the highest number of CAZymes among aerobic fungi with over 620 proteins. *A. flavus* is able to infect intact plant tissues or insects or humans but is also able to survive as a saprotroph as permitted by its large CAZyme equipment which allows this species to acquire nutrients in diverse environments (St Leger et al. 2000). Indeed, opportunistic ascomycetous molds encode the largest number of glycoside hydrolases (Table 1).

Pneumocystis jirovecii, another obligate pathogen, also harbors limited CAZyme equipment with only 56 members (Table 1). This equipment is sufficient for the fungus to survive and proliferate in the human lung but it is noteworthy that it lacks the CAZymes that are required by rotting fungi or plant pathogens. *P. jirovecii* is the only fungal representative lacking chitinases, in agreement with the fact that its cell wall does not contain chitin and that chitin is also absent as carbon source in its habitat, the human lung. The genome of *P. jirovecii* encodes only 15 glycoside hydrolases (GH) members, a tiny number compared to plant pathogenic or plant biomass decaying fungi which encode over 250 glycoside hydrolases, a majority of which to decompose plant carbohydrates. Similarly, the commensal and opportunistic pathogen *Candida albicans*, which is also adapted to the human host, either on mucosal surfaces or in the gastrointestinal tract, also displays a reduced number of GHs with 52 glycoside hydrolase genes. The number of glycosyltransferases is also decreased in *P. jirovecii* with only 36 members. Interestingly, the family GT2 members in this species are not chitin synthases, as no chitin is present, but have been identified in silico as a dolicholphosphate mannosyl transferase and a UDP-ceramide-glucosyltransferase. Surprisingly, *P. jirovecii* appears to have no GT15 homolog while it has mannans in its cell wall. These results suggest that mannan synthesis in this species may be different from the other molds and yeasts.

Another difficulty in the analysis of the CAZyme genes is that the genomic survey most of the time does not take into accounts the relative and often huge differences seen in the composition occurring between the different morphotypes of the fungus (yeast vs mycelium in dimorphic or trimorphic fungi such as *Histoplasma* or *Paracoccidioides*, mycelium vs conidium in filamentous fungi). It is then unknown if a set of CAZymes are more important for the synthesis of one or the other morphotype.

If we take into consideration all the points mentioned above and especially the huge differences and variations between the cell walls of the different fungal groups (Table 1), it is clear that the *S. cerevisiae* model for cell wall biosynthesis is not a paradigm. Drastic changes occurred during evolution of the genes encoding the enzymes involved in cell wall synthesis, as revealed by the variable degrees of

similarity between the corresponding genes in the different fungal phyla. One example is the CRH proteins of the GH16 family, which are involved in the building of chitin–glucan linkages in *S. cerevisiae* (Blanco et al. 2012; Cabib et al. 2008), but not in filamentous fungi since the entire deletion of the CRH family (5 genes) in *A. fumigatus* does not change the morphogenesis of the cell wall [(Fang et al. 2019) Mouyna and Millet unpublished]. Does it mean that the dogma the construction of the cell wall requiring the covalent binding of glucan and chitin should be abandoned or that some modifying enzymes have not been identified yet? Moreover, orthologous genes with very similar sequences and even similar enzymatic function play a different role in the cell wall construction in *S. cerevisiae* and other fungi. The DFG family assigned to the GH76 family is essential in yeast and the analysis of the mutants suggested that the two members of this family have a regulatory role in hypha development (Kitagaki et al. 2002; Spreghini et al. 2003). In molds, this family is not essential and has been shown to play a role in the anchoring of galactomannan to the glucan–chitin core (Muszkieta et al. 2019). Significant discrepancies have been also found in the analysis of mannan synthesis in yeast and molds. In yeast, mannosyl transferases which belong to the GT32, GT34, GT62, and GT71 are responsible for the synthesis of the linear α-1,6 mannan chains and their branching (Stolz and Munro 2002). In *A. fumigatus*, the eleven orthologous genes of the yeast polymerase families do exist and are able to transfer mannose to mannan from GDP-mannose in vitro but are not associated to the synthesis of the cell wall mannan. In contrast, the homologs of the *KTR* genes of the GT15 family, which are not involved in mannan cell wall production in yeast, are essential for the synthesis of the cell wall mannan in *A. fumigatus* (Henry et al. 2019). To date, the enzymatic activity of these cell wall CAZymes has been mainly investigated in vitro using recombinant proteins. Do these enzymes play a similar role in situ in a cell wall environment with different acceptors and donors? Probably not. This field has been totally untouched to date. Associated to this problem is the poor understanding of the location of most of the cell wall proteins. This is very clear for the function assigned to GPI proteins in yeast and molds. In *S. cerevisiae,* it has been repeatedly suggested (but not fully demonstrated) that these proteins have a structural role in the cell wall and would be covalently bound to polysaccharides. In *A. fumigatus,* GPI proteins are not bound to polysaccharides but have an enzymatic function essential for the remodeling of the cell wall polysaccharides (Li et al. 2018).

2 Carbohydrate-Binding Domains

Carbohydrate-binding domains (CBMs) are independently folding protein modules that have carbohydrate-binding properties but no enzymatic activity. They are often appended to CAZymes and frequently attached to proteins of unknown function. Within the fungi, the most common CBMs belong to cellulose-binding (CBM1) or chitin-binding (CBM18 and CBM50/LysM domain) families (Tables 6, 7 and

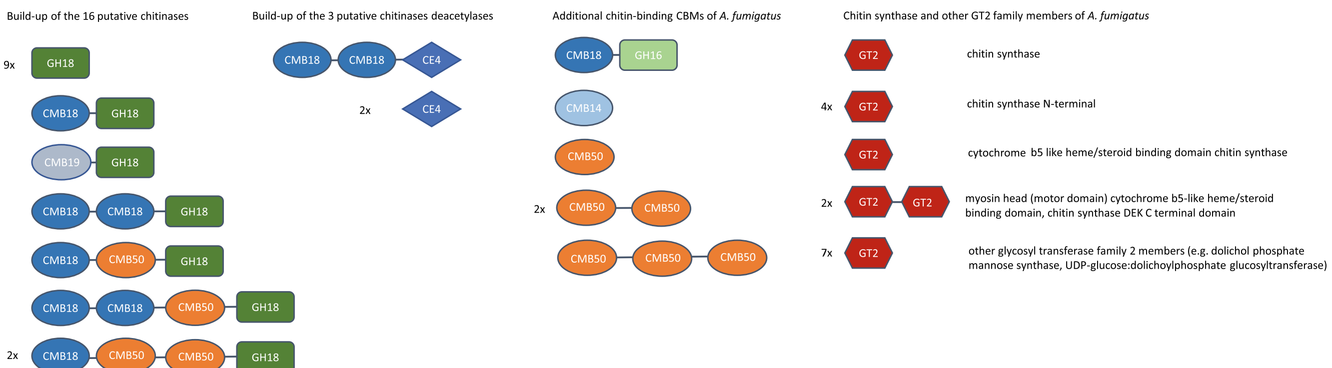

Fig. 1 An example of the organization of CBMs in chitin synthesis and chitin modifying CAZYmes in the *A. fumigatus* genome

Fig. 1). Cellulose-binding CBMs like those of family CBM1 and CBM63 are often attached to glycoside hydrolases, carbohydrate esterases, or auxiliary activity redox enzymes, and they serve to target their appended catalytic domain to the plant cell wall. Some of these CBM-containing proteins may not have an enzymatic function associated to plant degradation. This is the case of some CBM1 and CBM63 members such as AFUA_6G03280 or AFUA_5G08030 in *A. fumigatus*, which bear distant similarity with plant expansins. They are non-catalytic carbohydrate-binding proteins that facilitate cell wall expansion during plant growth (Chase et al. 2020).

While CBMs have been widely investigated for their role in complex polymer decomposition and biotechnological applications, in particular lignocellulose breakdown, CBM-containing proteins have not yet been extensively studied regarding their role in fungal cell wall deconstruction or remodeling (Pham et al. 2010).

Most fungal proteins with characterized CBMs are chitin-binding proteins. In addition to being a structural component of the cell wall and a natural food for saprotrophic fungi, chitin is a well-described pathogen-associated molecular pattern (PAMP) for pathogenic fungi regardless if the host is a plant or mammal. In mammals, chitin is able to activate a variety of innate and adaptive immune responses (Elieh Ali Komi et al. 2018). To overcome host immunity, fungal plant pathogens secrete effector molecules that can repress the host defense systems. A class of conserved secreted effectors are LysM effectors, that carry no recognizable protein domains other than the CBM50 motif (Kombrink and Thomma 2013). In *A. fumigatus*, eight putative LysM-domain proteins were identified including three putative chitinases (AFUA_5G03960, AFUA_5G06840, and AFUA_6G13720) and LdpA and B (AFUA_5G03980 and AFUA_1G15420) with multiple putative LysM domains but without catalytic domain typical of LysM domains found in plant pathogens (Muraosa et al. 2019). Single- and double-deletion mutants revealed that LdpA and LdpB have no significant defects on cell wall integrity or chitin content and virulence. Interestingly the commensal *C. albicans*, as well as the skin-associated *Malassezia globosa* or the intracellular pathogen *P. jirovecii* lack CBM50 or other chitin-binding effectors. These results suggest that LysM molecules are not essential for the biosynthesis of cell wall chitin in *Aspergillus* and that such molecules may not have the same function in plant and in mammal pathogens. Mammalian fungal pathogens, such as opportunistic pathogens, may use their LysM effector homologs in other processes rather than host colonization, such as survival in the environment. The absence of LysM effector genes in *C. albicans*, *M. globosa*, and *P. jirovecii*, which are among the few fungal species that are commensals of humans and animals and that do not occur free-living in the environment, seems to support this hypothesis (Kombrink and Thomma 2013).

The *A. fumigatus* genome encodes several proteins with more than one CBM (Fig. 1). The reasons for the presence of several CBM50 domains on a single polypeptide remain unknown.

Most of the proteins with a chitin-binding module are chitinases. Others are transglycosidases. Crh transglycosidases supposed to synthesize glucan–chitin

branched polymers in yeast but not in molds belong to two GH16 subfamilies GH16_18 and GH16_19 which possess or not different CBMs. When present, only chitin-binding CBM 18 and 50 are found in these Crh proteins in the absence of any β-1,3 glucan-binding CBM. This also points out the lack of understanding of the biochemical activity of transglycosidases in situ since the Crh enzymes are able to produce in vitro chitin–chitin or chitin—β-1,3 or even β-1,6 glucan cross-links without any characterization of the acceptor and donor molecules for these enzymes in situ (Blanco et al. 2012; Fang et al. 2019). No transglycosidases have been indeed identified yet with two different CBMs recognizing the two polysaccharides to be transglycosylated by the enzyme. Another question to rise is why only one of the fourteen GH16 members in *A. fumigatus* possesses a CMB18 chitin-binding module while the thirteen others are not associated with any other module. This would question the role of the CBM in the control of the activity of these enzymes.

In contrast to chitin-modifying enzymes and in spite of the important role of β-1,3 glucan enzymes, nearly all β-1,3 glucan CAZymes among human pathogens (Table 6) possess a CBM43-binding domain. However, the presence or absence of this CBM is essential in establishing the specificity of the CAZyme activity as seen with the GH72 proteins (Aimanianda et al. 2017). All members of the GH72 have a β-1,3 glucan elongating activity (Hartland et al. 1996; Mouyna et al. 2000) but some of the GH72 proteins which contain a CBM43 at their C terminus have in addition a β-1,3 glucan branching activity suggesting that linear elongation of β-1,3 glucan is a prerequisite and that the positioning of elongated β-1,3-glucan by the CBM43 is absolutely required for the subsequent branching activity of Gas/Gel proteins. In *P. jivocenii*, a CBM 52 [characterized in *Schizosaccharomyces pombe* (Martín-Cuadrado et al. 2008)] has been noticed.

Even though mannan, galactan, galacturonan, β-1,6 glucans or fucoidan must be transglycosylated to be covalently bound to the structural cell wall core of various fungal species, no CBMs able to bind these polysaccharides have been identified yet. Such observations suggest that there may be many unknown CBMs to discover and especially some with key acceptor function in cell wall remodelases.

3 Gene Clusters and Gene Families in Fungi

Bacterial cell wall and exopolysaccharides are produced and degraded by the products of gene clusters whose expression is highly coordinated (Pan et al. 2015; Christiansen et al. 2020; Pilhofer et al. 2008; Schmid et al. 2015). By contrast, even when the products of several genes cooperate for the biosynthesis of polysaccharides, the corresponding fungal genes do not reside on the same chromosome location. Fungal gene clusters do exist but they have been essentially described for the enzymes synthesizing secondary metabolites (www.jcvi.org/smurf/) where they need the cooperation of several enzymes but not for the synthesis of glycans. There seems to exist a single exception to this rule, namely the synthesis of the galactosaminogalactan (GAG) in *A. fumigatus* which was originally described as the first

virulence polysaccharide in the fungal kingdom (Fontaine et al. 2011). A transcriptomic analysis of *A. fumigatus* regulatory mutants deficient in GAG production identified a cluster of genes (AFUA_3G07860 to AFUA_3G07910) linked to GAG synthesis on chromosome 3 [(Bamford et al. 2015; Lee et al. 2014) Briard et al., submitted]. This cluster encoded five putative enzymes: (i) Uge3 (AFUA_3G07910) a bifunctional cytoplasmic uridine diphosphate (UDP)-glucose-4-epimerase that mediates the production of UDP-GalNAc and UDP-Gal, (ii) Agd3 a secreted protein required for the partial deacetylation of newly synthesized GAG polymer (AFUA_3G07890), (iii) Sph3 (AFUA_3G07890) a member of CAZy family GH135, with endo-α-1,4-*N*-acetylgalactosaminidase activity, (iv) Ega3 (AFUA_3G07890) a CAZy family GH114 endo-α-1,4-galactosaminidase specific for GalN–GalN linkages, and (v) Gt4c (AFUA_3G07860) which encodes a CAZy family GT4 glycosyltransferase whose catalytic domain is appended to a large domain featuring multiple transmembrane segments. Cell wall chitin and glucan polysaccharides are synthesized by synthases and composed of monomers. In contrast, GAG is an alternating polysaccharide requiring a close collaboration of enzymes which will add one monomer after another monomer of different nature. In addition, the presence of hydrolases of the GH114 and 135 families in this cluster suggest that GAG hydrolysis must be also concerted. The similarity between the exopolysaccharide deacetylase PelA of *Pseudomonas aeruginosa* and Agd3 of *A. fumigatus* as well the similarities with the exopolysaccharide synthesis analogous to the bacterial ICA/PGA systems has helped understanding the function of all members of the GAG cluster in *A. fumigatus* (Lee et al. 2016). However, the complexity of the GAG structure and the identification of a single glycosyltransferase in the cluster of *A. fumigatus* suggests that other undiscovered yet glycosyltransferases may be located somewhere else in the genome. The GAG cluster of *A. fumigatus* is conserved in other Aspergilli, but also in the Ascomycete *Botrytis cinerea*, *Phialocephala subalpina*, *Niesslia*, *Gloniopsis* sp., and the Basidiomycete *Trichosporon asahii*. However, the GH135 gene is missing from the cluster in *Sclerotinia sclerotiorum*, *Neurospora crassa*, *Clonostachys rosea*, and *Fusarium oxysporum*, while the GH135 and GT4 genes are missing in *Alternaria brassicicola*, suggesting that the colocalization of the GAG enzymes on the genome is not an absolute requirement, neither is the coupling of synthesis and hydrolysis. The chemical nature of all the fungal exopolysaccharides remains, however, to be investigated in all these fungi containing "GAG genes".

Other attempts have been made to identify phylogenetic clusters including chitin synthases, chitinases, and putative cell wall metabolism genes. Across six *Aspergillus* species, a cluster of five cell wall metabolic enzymes could be identified: a chitin synthase activator, a class V (or class i) myosin, a serine/threonine kinase, a type 2A protein phosphatase PP2A (with a WD40 domain), and a cell wall glucanase Scw11 (or beta-glucosidase Bgl2). The chitin synthase activator located in this syntenic block shares its highest identity to an ortholog of *S. cerevisiae*'s Chs4p/Skt5, a post-translational regulator of the Chs3 complex during vegetative growth. Interestingly, the chitin synthase activator and the class IV chitin synthase are close to each other in a head-to-head orientation, typical for secondary

metabolite clusters or functionally related genes (Pacheco-Arjona and Ramirez-Prado 2014). Another cluster with four other genes homologous to the ones of the GAG cluster of the chromosome 3 (AFUA_4G14090 for UDP glucose 4 epimerase, AFUA_4G14085 and AFUA_4G14080 for two Spherulin-like proteins and AFUA_4G14070 for a glycoside transferase) do not have any apparent function in GAG synthesis at least during growth in vitro. In contrast to the above-mentioned cluster, which is expressed in all environmental conditions, the genes of the second cluster in the chromosome 4 are mainly highly expressed in vivo (Latgé et al. unpublished), but have not been associated yet to the pathobiology of *A. fumigatus*. These data suggest that it may exist in the CAZy database more clusters homologous to the bacterial operons which are responsible for the synthesis of polysaccharides. Such clusters would be devoted to the synthesis of heterogenous polymer lacking repeating units.

One issue in cell wall biosynthesis is the total lack of understanding of the significance of the presence of multiple genes per CAZy family of cell wall-associated proteins. This is especially true for mold enzymes. Several examples are the GEL, KTR, DFG, or CHS families. In the GEL family, belonging to CAZy family GH72, even though all these enzymes display the same glucan elongation activity, mutants showed that Gel1 does not have any impact on the construction of the cell wall whereas Gel4 is essential in *A. fumigatus* (Gastebois et al. 2010). In the GT15 family of mannosyltransferases, two KTR enzymes KTR4 and 7 are essential for the synthesis of the cell wall mannan whereas the 3rd member of the family does not play a role in the synthesis of mannan (Henry et al. 2019). In the GH76 family of the Dfg proteins which have been shown to be involved in the transfer of the galactomannan to the branched β-1,3 glucan, the *DFG3* gene is essential whereas all the other genes do not have any (or a minute) role in the cell wall morphogenesis (Muszkieta et al. 2019). Finally, some of the eight chitin synthases of *A. fumigatus* do not seem to play any role in chitin biosynthesis whereas others are absolutely needed. However, even though some of the enzymes have a stronger impact in the function of interest, gene deletions of all members of the family have suggested that in several cases these enzymes work cooperatively (Muszkieta et al. 2014). Even though it has not been convincingly shown in the absence of localization or specific activity per individual protein, it is assumed that the presence of multiple genes per family enables a spatio-temporal expression of these activities for a definite fungal stage or under different nutritional or environmental conditions. Moreover, the characterization of cell wall-associated enzymatic activities is performed most of the time in vitro which is certainly not the most appropriate conditions to perceive the reality of the cell wall synthesis. Donor and acceptor molecules and the physico-chemical protein environment may be different in situ. Such differences could explain why the two *KTR* genes have been shown in vitro to have only an α-1,2 mannosyltransferase activity while the mannan of *A. fumigatus* is a mixture of α-1,6 an α-1,2 mannan. Obviously, efforts to mimic *in cellulo* enzyme activities should be better developed.

4 Uncertainties in the Structure of the Cell Wall

Most studies have suggested that the skeleton of the cell wall is a branched β-1,3 glucan bound to chitin. However, a recent solid NMR study of the *A. fumigatus* cell wall questions this conclusion and suggests that the α-1,3 glucans may be the central polymer around which are arranged the other polymers especially chitin (Kang et al. 2018). Could we then consider that β-1,3 glucans have a function of stabilizer [like callose in plants (De Storme and Geelen 2014)] and that the structure analyzed are not the reality due to the chemical modifications induced by the harsh treatments to isolate soluble individual components. However, these NMR findings do not provide a clear picture either, since mutants devoid of α-1,3 glucans do not have an altered growth or even morphogenetic issues (Henry et al. 2012). We still believe that the fibrillar aspect of the wall is due to chitin and β-1,3 glucan since electron microscopy suggests that α-1,3 glucans are amorphous. In any case, this provocative NMR study is very interesting and indicates that the structural organization of the fungal cell wall is far from fully understood. For example, the possible association of microfibrils of structural polysaccharides with an amorphous material made up of proteins and other types of polysaccharides have never been investigated in situ in living fungus.

In the past, it was considered that the cell wall was an inert skeletal structure whose modifications only associated with the extension of the fungal cell. Data are now accumulating showing that the cell wall is constantly rearranged and modified structurally and quantitatively depending on the external environment [(Beauvais and Latgé 2015; Beauvais et al. 2014) see APJ Brown chapter]. Characterizing these changes especially those occurring during growth in vivo on the polysaccharides exposed on the surface of the cell wall is essential to understand better the interactions between host and fungal pathogens (Valsecchi et al. 2019). Interestingly, the fungal carbohydrate ligands recognized by C-type lectins are poorly understood with the exception of the specific recognition by dectin1 of long β-1,3 glucan fibrils (although dectin1 is also able to recognize *Mycobacterium*, which lacks β-1,3 glucan). The ligands recognized by the other C-type lectins remain poorly characterized. Is this due to the poorly defined carbohydrate poly- or monomers used as ligands? Such recognition studies usually require soluble ligands and most of the studies are based on insufficiently purified molecules. The new availability of pure oligosaccharides representative of the cell wall polysaccharides, obtained by chemical synthesis, can now allow the construction of glycoarrays to analyze the recognition of ligands by C-type lectins (see Nifantiev chapter) but also to mimic fungal infection with oligosaccharide-conjugated particles (Mansour et al. 2013). If the use of such definite tools does not identify precisely the ligands for the lectins, it can suggest that the recognition is more based on the 3D structure of the polysaccharide than on a specific chemical composition of the polysaccharide. However, modeling the three-dimensional features of polysaccharides and carbohydrate polymers is still in its infancy (Perez et al. 2015). More work devoted to this area could help decipher the molecular bases of these host cell–fungal cell wall

polysaccharide interactions and further precise the binding capacity of the CBMs. Another important point already mentioned above is that the composition of the cell wall varies depending on the fungal morphotype considered (yeast, conidia, and mycelium). Unfortunately, the analysis of the role of the cell wall during infection is rarely based on the morphotype of the infective propagule or on a propagule which has been grown in a medium mimicking the in vivo situation. For example, in Chytridiomycetes or Zygomycetes, the presence of β-1,3-glucans is restricted to the sporangiospore wall or flagellate spores. In *A. fumigatus,* the mycelium is covered by GAG (which is absent from the conidium), which favors the infection while the conidium in contrast is covered and protected by melanin (absent from the hyphae) [(Akoumianaki et al. 2016); Briard et al. submitted]. In spite of the progresses made in the analysis of the fungal cell wall in the last 50 years, the structure of the fungal cell wall remains uncertain as suggested by all different putative schemas of the fungal cell wall, often contradictory, proposed by multiple authors (look at Google "images of fungal cell wall"). Even though it is often not rewarding in terms of publications, such studies should be implemented in the future.

5 Similar Questions and Common Problems in the Understanding of Cell Wall Biosynthesis in Plants and Fungi?

The plant and fungal cell walls face similar challenges: they have a composite architecture based on a crystalline core (cellulose or chitin) surrounded by a matrix of hydrated polysaccharides with aromatic material or proteins. If there are differences between plants and fungi, the majority of the questions asked in the analysis of the fungal cell wall have not been answered in plant cell wall growth. For example, what is exactly and what determines the length of a polysaccharide in a growing or aged cell wall? What determines the substitution patterns of a polysaccharide and what is the minimal branching level between polysaccharides to keep the viscoelastic property of the cell wall? What are the auxiliary proteins and cofactors in polysaccharide synthesis? How is the product influenced by the supply of activated precursors such as nucleotide sugars and how are nucleotide sugar transporters regulated? Activation of the cell wall-associated enzymes is thought to occur after their integration into the plasma membrane, although the exact mechanism is unknown. One possibility is that the activation is achieved through phosphorylation as it was shown for chitin synthases (Lenardon et al. 2010; Martínez-Rucobo et al. 2009). How are the sequential biosynthetic events in the addition of branched chains controlled and how are the sites of insertion defined? What are the hot spots important to convey biomechanical stability to the wall? In plants like in fungi, understanding the functional biochemical analysis of glycosyltransferases has been slow because they are membrane proteins which sometimes operate as part of large multimeric complexes. The glycosyltransferases have

been identified based on biochemical enrichment strategies, heterologous protein expression followed by in vitro activity assays, and/or the isolation of mutants or overexpression (rarely undertaken in fungi) followed by the analysis of the cell wall of the mutants. Substrate specificity for acceptor and donor in vitro and vivo, enzyme kinetics, and cellular localization (rarely undertaken) are usually poorly understood in both eukaryotic phyla. Because of this complexity, it has been impossible to reconstruct synthetically an entire biosynthetic machinery to produce a complex branched cell wall polysaccharide in vitro with a structure similar to that found in the native cell wall. Will it be an interest to strengthen cross-fertilization between these two kingdoms? Will we learn about the role of β-1,3 glucans in fungi if we engineer the expression of fungal *FKS* genes in *Arabidopsis* or Gram negative bacteria such as *Agrobacterium* producing the plant or bacterial β-1,3 glucans callose or curdlan (Pauly et al. 2019)?

A grand challenge in the cell wall field is to relate cell wall structure to the mechanics of cell walls and the action of cell wall-loosening agents (enzymes or biophysical changes) that induce wall stress relaxation and provoke water uptake needed for cell growth. Discussed in plants, the water entrance has not been really followed up in fungi. Aquaporins which are present in fungi are obviously not the unique element to regulate water entrance (Latgé et al. 2017). A deeper understanding of wall deformations will require more extensive experimental testing in combination with quantitative models of how the structural components of the cell wall are linked to one another and what kinds of polymer motions occur during rapid, reversible deformations versus the slow irreversible enlargement of the growing wall. Are these modifications only due to enzymes? In plants, the best-studied example of non-enzymatic degradation of the cell wall matrix is the family of expansins (Cosgrove 2016; Nikolaidis et al. 2014). Orthologous expansin genes have been found in *Aspergillus* (Table 4), but their deletion which has been initiated has not led yet to any modification of the fungal cell wall (Mouyna and Latgé, unpublished). Moreover, the role of an increase in the intracellular osmotic pressure in the modification of the elasticity of the cell wall structure has not been investigated.

To date, a number of publications on plant cell wall and fungal cell wall are equally numerous. Even though the studies on the plant and fungal cell walls target different objectives and are more focused on biotechnology in the case of plants and antifungals and immunology in human fungal pathogens, scientific questions are numerous and remain similar.

6 New Approaches to Understand Cell Wall Synthesis and Degradation

The invention of the microscope in the late 1600s was critical to fuel interest in biology. Since then, increasingly sophisticated microscopic tools have led to an ever-more refined picture of the cell especially on the role of membrane complexes which can accurately define the underlying molecular interactions through extensive studies in biophysics, membrane biology, and advanced imaging. However, these new technologies have not been applied to the study of the fungal cell wall and only very scarcely to plant or bacterial cell wall. Super-resolution microscopy techniques reach previously inaccessible spatial scales and the understanding of short-lived interactions (protein–protein, protein–lipid, and protein–carbohydrate interactions) that are not amenable to standard biochemical assays and have been previously inaccessible in live cells. Super-resolution microscopy, such as PALM, STORM, and STED, is able to accurately describe the nanoscale structure of living cells with a sub-100 nm resolution. The development of CryoEM and super-resolution microscopy which has been recently complemented by the development of new Titan Krios microscopes will allow a **"near-atomic" resolution of samples in a fully hydrated environment** (Atanasova et al. 2019; Gustafsson et al. 2016; Sahl et al. 2017; Sezgin 2017). All these high-resolution microscopy techniques should allow a better characterization of the membrane complexes responsible for the synthesis and remodeling of the polysaccharides and the insertion and secretion of the glycans in the membrane complex. The first example to go after will be the Fks1/Rho1 complex which has been known to be crucial for the synthesis of β-1,3 glucans. Many other questions should be investigated: for example, how do transglycosidases of family GH72 interact with the neosynthesized β-1,3 glucan and the Fks synthase complex? Or how do the different chitin synthases cooperate with each other and interact with the glucan synthase?

These new technological approaches would not only allow the **localization of the different CAZymes involved in fungal cell wall synthesis and turnover**, but they would enable studying (i) the timing of cell wall deposition, (ii) the variations of cell wall composition during cell aging, and (iii) the identification of the linkages truly responsible for the rigidity of the cell wall. Cell wall regeneration in protoplasts is an ancient tool used in the 70's (Moore and Peberdy 1976; Peberdy and Gibson 1971) which could be revisited now with the technology mentioned above. Protoplasts are the most appropriate cells since they do not possess a cell wall which would make the application of these technologies difficult. Multiple glucan chains are simultaneously synthesized by single plasma membrane-localized synthases and in contrast to the situation in plants (Li et al. 2014), we still do not know what is the length of the neosynthesized glucan and chitin fibrils in situ, how are assembled the polysaccharide molecules to form an elementary microfibril and how are all these neosynthesized microfibrils imbricated.

Coupling super-resolution approaches to click chemistry for the introduction of specific labeled molecules inside the neosynthesized polysaccharides has not been undertaken yet in the fungal field and very little in the plant or bacterial cell wall fields (DeMeester et al. 2019; Simon et al. 2018).

Preliminary data suggest that a new method called PIP for polysaccharide immunoprecipitation can be beneficial (Latgé unpublished). The idea behind PIP is close to that used in chromatin immunoprecipitation (ChIP). Insoluble polysaccharides and associated CAZymes in living cells are cross-linked by chemical fixation and made soluble after cleavage by appropriate glycoside hydrolases. The soluble molecules are immunoprecipitated with carbohydrate-specific antibodies or lectins and the associated protein could be sequenced. Enrichment of specific CAZyme proteins associated in vivo to the target polysaccharides should be an indication of their involvement in the polysaccharide synthesis. Product entrapment techniques (Inoue et al. 1995; Kang et al. 1984) which have been used in the past should be now revisited with these new technologies.

These methods could be also applied to the analysis of the mechanisms responsible for the **secretion of cell wall polymers**, which remains largely unknown to date although they have an essential role in biofilm formation. One still unanswered and very controversial question is the putative role of extracellular vesicles (and even their existence in the plant cell wall biosynthesis field) in the secretion of the polysaccharides [(Rodrigues and Casadevall 2018; Zarnowski et al. 2018) Rizzo et al., submitted]. In plants, the association between extracellular vesicles and the cell wall is not clear (Cui et al. 2020). It is however obvious that the secretion and transport pathways responsible for the passage of the cell wall polymers through the plasma membrane and their deposition in the cell wall space is different from the active secretory mechanisms responsible for the secretion of proteins. The composition of the glycan present in biofilms or capsules is different from the polysaccharides constitutive of the cell wall. In *Aspergillus*, the GAG present in the extracellular mycelial matrix plays a role in the adhesion of hyphae in the colony and to the host epithelial cells (Beaussart et al. 2015; Lee et al. 2015). The capsule of *C. neoformans* is rich in glucuronoxylomannan and glucuronoxylomannogalactan components which are also not present in the cell wall. The polymers in *C. albicans* biofilm are rich in α-1,6-mannan and β-1,6- and β-1,3-glucans, a composition different of the typical *Candida* cell wall (Zarnowski et al. 2018; Doering 2009; Fontaine et al. 2011; Zarnowski et al. 2014). All these polysaccharides must travel through the cell wall using (a) pathway(s) not defined to date. The type of transport may also depend on signals for this recognition which are also unknown. It has been recently shown that galactomannan in *Aspergillus* which is covalently bound to the cell wall is transported by a different mechanism than the one used for the membrane GPI-anchored galactomannan (Muszkieta et al. 2019). The passage of the polysaccharides through the cell wall will also depend on the porosity of the cell wall. The mechanisms responsible for the **permeability** (or impermeability) of the cell wall to external toxic or beneficial molecules remain poorly understood or even explored (Liu et al. 2019; Stirke et al. 2019). The use of

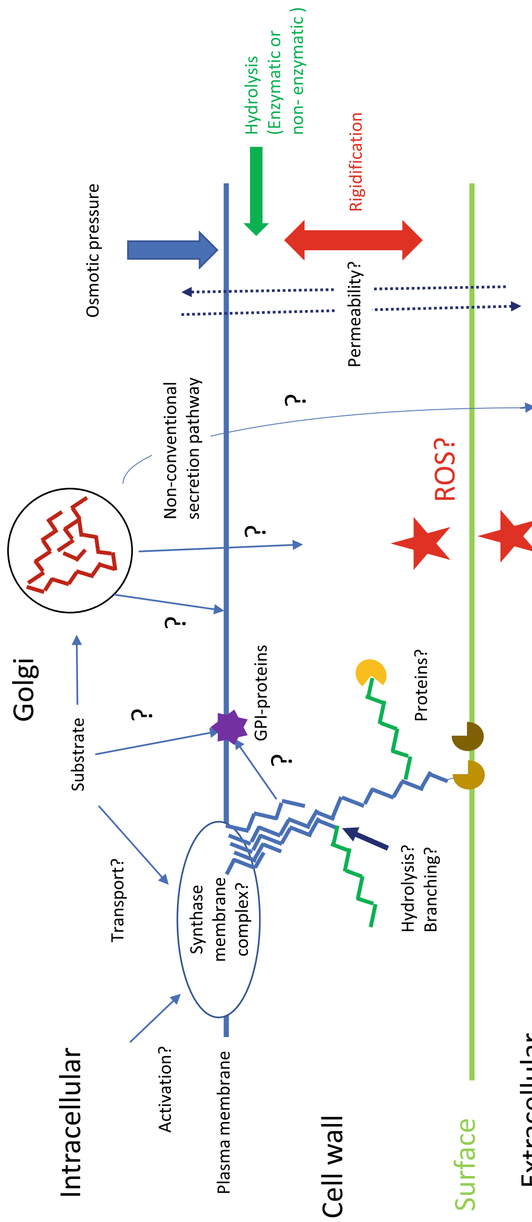

Fig. 2 Unanswered questions in fungal cell wall construction

nanoparticles bound to several ligands has been shown to be a useful strategy to analyze permeability [(Walker et al. 2018) Hua et al., unpublished].

Most questions are focused on cell wall synthesis but the **degradation of the cell wall** is an issue which is less addressed. Enzymatic degradation of the cell wall has been often mentioned as the only way for the fungal cell to soften the cell wall and make the cell wall viscoelastic to allow the fungus to expand. However, the essential role of glycoside hydrolases during yeast budding and conidium germination, or hyphal branching has not been fully demonstrated and the relationship between internal turgor pressure and hydrolases remains fuzzy (Lew 2011). The part taken by the internal osmotic pressure vs enzymatic hydrolysis has yet to be quantified by biophysical methods. Another intriguing question is the intra-phagosomal degradation of the cell wall polysaccharides. How are the cell wall components degraded and presented by antigen-presenting cells to select for polysaccharide-specific T-cells since human glycoside hydrolases have not been shown to be able to degrade enzymatically cell wall polymers (even though they are able to degrade chito-oligosaccharides or glycosaminoglycans and hyaluronan)? (Nakamura et al. 2019; Kumar and Zhang 2019). In the phagosome, cell wall polysaccharides can be degraded by the action of reactive oxygen radicals (ROS) but the rearrangements of the glycan polymers consecutively to the oxidation of the polysaccharides remain unknown. The role of ROS in the degradation of glycans has been shown especially inside the phagocytes (Baum and Cobb 2017; Duan and Kasper 2011; Velez et al. 2009). Such understanding is needed and would certainly help the understanding of the PAMP/PRR interaction and especially the identification of the ligands for C-type lectins and help defining better how glycan-specific T-cells emerge. Better identifying the links between glycobiology, fungal virulence, and immunology is certainly a way of the future to promote the study the fungal cell wall.

7 Conclusion

Fungal genome sequencing has made considerable progress over the last 20 years, but this progress translates only slowly in a better understanding of cell wall synthesis and turnover, due to the intrinsic experimental difficulties of characterizing a poorly understood composite structure and to the difficult characterization of dozens of membrane enzymes with overlapping substrates and products. Even though the study of the cell wall biosynthesis has been initiated half a century ago, major questions presented in Fig. 2 remain unsolved. It is clear that breakthrough in the field will require new innovative approaches and technologies to advance our understanding of cell wall construction. All the approaches presented in this essay are of course not exclusive and new ideas and concepts are certainly welcome to break codes and transcend ideas accepted to date in the fungal field.

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